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Overview of *Clostridium difficile* Infection: Life Cycle, Epidemiology, Antimicrobial Resistance and Treatment

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Additional information is available at the end of the chapter

Abstract

The use of antimicrobial agents and acquired resistances explains in part the emergence and spreading of epidemic strains of *Clostridium difficile*. Continued use of antimicrobial therapy still represents an acute danger in triggering the emergence and spreading of new resistant and multiresistant strains including against first-line antibiotics. We examine the pathway of peptidoglycan synthesis in this organism and associated resistances, as well as resistance to other classes of antibiotics. The life cycle of *C. difficile* involves growth, spore formation and germination. Spores endow the organism with a formidable capacity of persistence in the environment and in the host, resistance, dissemination and infectious potential. Highly resistant spores produced by antibiotic-resistant/multiresistant strains may be one of the most serious challenges we face in what concerns the containment of *C. difficile*. Finally, we review recent developments in the treatment and prevention of *C. difficile* infection.

Keywords: *Clostridium difficile* infection, antibiotics, epidemiology, drug resistance, spores, β-lactam antibiotics, fidaxomycin, *Clostridium scindens*

1. *Clostridium difficile* life cycle: antibiotic-resistant spores as infectious, persistence and dissemination vehicles

1.1. *Clostridium difficile* biology

The human gut is the home of a community of as many as 1000 species of commensal, beneficial and pathogenic microorganisms. Recent studies suggest that at least half of the bacterial

genera found in the gut produce resilient spores [1]. One of these organisms is *Clostridium difficile*, a Gram-positive spore-forming anaerobe. *C. difficile* was recently placed in the Peptostreptococcaceae family and renamed *Peptoclostridium difficile* [2] and later *Clostridioides difficile* [3], yet we use herein the still more familiar designation of *C. difficile*.

Although *C. difficile* (*Bacillus difficile* by that time) has been first described in 1935 by Hall and O'Toole as part of the bacterial flora of the meconium and faeces of infants [4], it was only in 1974 that three independent studies have implicated *C. difficile* has an important cause of disease in humans. These studies also showed that the organism produced a toxin that was highly lethal to mice, but the toxicity to humans was not demonstrated. In one of these studies, the causal effect of antibiotic exposure and gut disease was demonstrated. The study, conducted by Tedesco and co-authors, found a significant association between patients receiving clindamycin and the development of pseudomembranous colitis (PMC), although at this stage the etiology of this observation remained unknown [5]. In 1977, *C. difficile* was isolated from the faeces of hamsters with clindamycin-induced colitis, confirming this pathogen as the cause of antibiotic-induced disease in animals [6]. In 1978, two studies provided confirmation of the causal association of *C. difficile* infection (CDI) and antibiotic-associated PMC in humans [7, 8].

The original name of *C. difficile* reflects the difficulties in the isolation and growth of this bacterium in the laboratory. Nowadays, we face the problem of being unable to control the growth and spreading of this organism. In the last decade, epidemic strains, including those of ribotype 027 (RT027), have emerged that caused outbreaks associated with increased disease severity and higher recurrence, morbidity and mortality rates, and *C. difficile* is now considered the major causative agent of nosocomial diseases associated to antibiotic therapy in adults [9–11]. *C. difficile* causes close to 500,000 infections and 29,000 deaths each year in the United States alone, with about 20% of CDI leading to recurrence, and imposing an economic burden on the healthcare system estimated at over US$ 1 billion [12, 13] or an estimated €3 billion in Europe [14]. *C. difficile* is categorized as an urgent threat, the highest level of concern, by the CDC (Center for Disease Control and Prevention). Moreover, increased rates of community-associated *C. difficile* disease, affecting groups not previously at risk, such as children, healthy young adults and pregnant women, and zoonotic transmission are a raising concern [11].

1.2. The life cycle

C. difficile is an enteric pathogen that relies on the disturbance of the normal gut microbiota to expand in the gut and cause infection; individuals with a normal, balanced microbiota are usually resistant to infection by *C. difficile* [14–16] (see below). Unlike most of the commensals, *C. difficile* resists to a wide range of antibiotics (see below). Resistance to antibiotics such as erythromycin, chloramphenicol or tetracycline is largely mediated by transposons that are present in the *C. difficile* genome [17–19]. Individuals with a normal, balanced microbiota are usually resistant to infection by *C. difficile* (see subsequent text). Disease symptoms range from mild diarrhoea and abdominal pain to life-threatening inflammatory lesions such as PMC, toxin megacolon or bowel perforation, and in severe cases sepsis and death [16, 20, 21]. These symptoms are mainly caused by two potent proinflammatory cytotoxins, TcdA and TcdB, that following release from the bacterium, translocate to the cytosol of target host

cells and inactivate, by monoglucosylation, small GTP-binding proteins, including Rho, Rac and Cdc42. TcdA and TcdB cause actin condensation, disintegration of the cytoskeleton, cell rounding and eventually cell death [22]. These toxins are coded for by two genes, *tcdA* and *tcdB*, located in the pathogenicity locus (PaLoc) (**Figure 1A**). The PaLoc also contains three additional genes, *tcdE, tcdR* and *tcdC*. TcdE is thought to be a putative holin-like protein involved in toxin secretion; its impact on toxin secretion, however, is still under debate [23, 24]. TcdR is an RNA polymerase sigma factor that serves as the main positive regulator of expression of the PaLoc and activates its own expression from two tandem promoters [25, 26] (**Figure 1A**). Importantly, the expression of *tcdR* is also activated from a promoter responsive to σ^D, the main regulatory protein involved in the final stages of flagellar assembly [27] (see also below) (**Figure 1A**). TcdC is thought to be a TcdR-specific anti-sigma factor that negatively regulates TcdR-dependent transcription [23, 28, 29]. TcdC was also shown to bind DNA, which suggests an alternative function for this anti-sigma factor [30]. Some *C. difficile* strains, as those of RT027 and RT078, also produce a binary toxin known as CDT (*C. difficile* transferase) (**Figure 1B**). The CDTb component of CTD binds to the host cells and translocates CTDa, that ADP-ribosylates actin, inducing depolymerization of the actin cytoskeleton. Toxin-induced actin depolymerization also induces redistribution of microtubules and formation of long microtubule-based protrusions at the surface of the intestinal epithelia cells; these protrusions trap the bacteria in small compartments, increasing the adherence of *C. difficile* [31–33]. In mice, by inducing inflammation via a Toll-like receptor 2 (TLR2), CDT suppresses a protective colonic eosinophilic response [34].
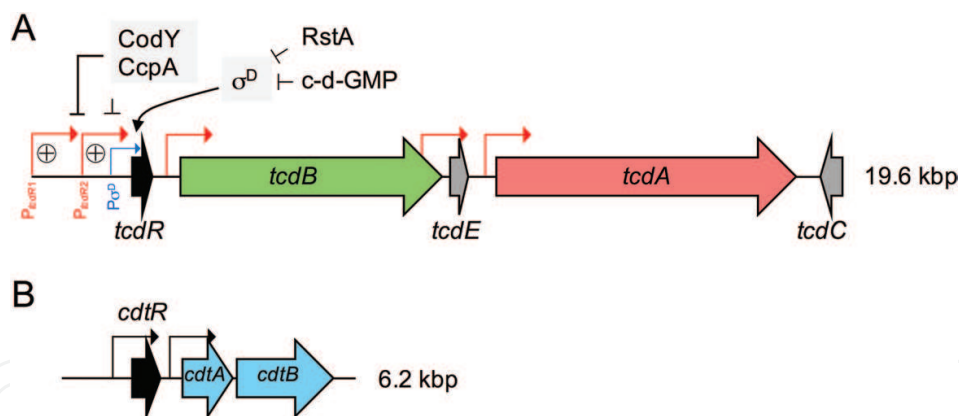
Figure 1. Schematic representation of the PaLoc (A) and CdtLoc (B) loci of *C. difficile*. A: This shows the genetic organization of the PaLoc in toxinogenic strains, which includes the *tcdR, tcdB, tcdE, tcdA* and *tcdC* genes. *tcdR* codes for an RNA polymerase sigma factor that controls expression of the *tcdB* and *tcdA* genes and possibly also of *tcdE*. The main promoters in the region are represented by broken arrows. The figure highlight the main regulatory circuits highlighted in the text. Transcription of the *tcdR* gene is governed by at least three promoters, two of which are auto-regulatory ('+' signs); a third promoter is under the control of σ^D, the regulatory protein governing the last stages in assembly of the flagellum. A promoter recognized by the housekeeping sigma factor σ^A, located downstream of the σ^D-type promoter has also been proposed [108]. Transcription of *tcdR* is directly repressed by CodY and CcpA but the latter also represses the expression of other PaLoc genes (not shown) [103, 108]. c-d-GMP also represses the expression of the PaLoc by inhibiting the production of σ^D. B: This shows the organization of the binary toxin locus or CdtLoc. *cdtR* codes for a response regulator required for full expression of the downstream genes, *cdtA* and *cdtB*, coding for the two components of the binary toxin CDT. The putative kinase that activates CdtR is unknown. The CDT toxin is only produced by some strains, including those of RT027 and RT078; in some strains, the locus is absent, whereas in others, such as 630Δerm, *cdtA* and *cdtB* are pseudogenized [33].

Since *C. difficile* is a strict anaerobe, its virulence potential is linked to the ability to form spores. Spores are resistant to heat, oxygen and other environmental insults, including commonly used ethanol-based disinfectants. When ingested, the spores are able to pass the gastric barrier and reach the intestine where they are thought to attach to the epithelial cells in order to achieve proper germination, which is induced by certain bile salts (see below) (**Figure 2**). Spore germination and outgrowth, in the absence of a competitive microbiota, will result in the establishment of a population of vegetative cells that will expand, produce the TcdA and TcdB toxins and eventually more spores (**Figure 2**). The toxins will cause damage to the colonic mucosa and eventually severe diarrhoea; shedding of the spores to the environment allows the infection of new hosts (**Figure 2**) [15, 22, 35, 36].

Spores are the vehicle for transmission as well as for environmental persistence. Mice exposed to spores exhibited recurrent infection with the same strain (disease relapse), but a *spo0A* mutant, lacking the key regulatory protein governing entry into sporulation and thus unable to form spores, was incapable of recolonization and host-host transmission [37]. Evidence suggests that *C. difficile* forms biofilms *in vivo* and *in vitro* and that the main virulence and persistence factors (toxins and spores) are produced within these structures [38–40]. In mice, infection with *C. difficile* spores followed by a 7-day period of treatment with clindamycin results in entry into a highly contagious period, during which high number of spores are shed [40]. For a RT027 strain, the 'supershedder' state remained for months, even after
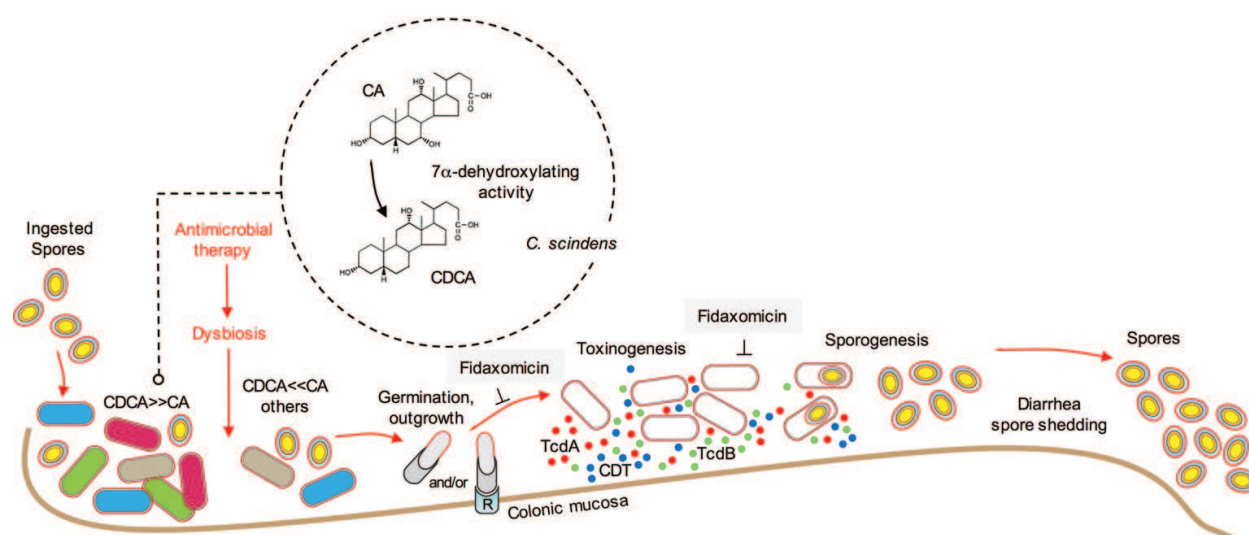
Figure 2. Schematic representation of the *C. difficile* infectious cycle. Spores are ingested and are able to pass the gastric barrier to reach the intestine. In the intestine, when the normal gut microbiota is disturbed, as during antibiotic treatment, the ratio of the bile salts derivatives cholate (CA) and chenodeoxycholate (CDCA) increases and the spores will germinate. *C. scindens*, for example, produces a 7α-dehydroxylating activity that converts CA into CDCA, blocking germination of *C. difficile* spores (insert). Spore germination, which may occur following recognition of a receptor (R) in the colon, and cell outgrowth will eventually produce a population of vegetative, actively growing cells, which will initiate toxinogenesis and sporogenesis. The two cytotoxins TcdA and TcdB will cause severe damage to the epithelium and are the main direct causes of the disease symptoms. The CDT binary toxin, present in some *C. difficile* strains, as those of RT027, is also represented. Other virulence factors (e.g. the flagellum, the S-layer) are not represented for simplicity. Shedding of the highly resistant spores will allow their accumulation into the environment and the infection of new hosts. Spores that remain in the host, in an unknown niche, are also the cause of disease recurrence.

cessation of antibiotic treatment, causing chronic intestinal disease [40]. During this period, the bacteria persisted as microcolonies and biofilm-like structures at the surface of the intestinal mucosa [40]. Bacteria within the biofilm are protected and therefore more resistant to antibiotics and oxygen [38]; conversely, spores may help shielding the biofilm. While an evolutionary link exists between sporulation and biofilm formation [41], a direct demonstration of the role of biofilms in disease and whether *C. difficile* persist in the intestine after antibiotic treatment as a biofilm, spores or both, is, however, lacking. Although the toxins, responsible for most of the disease symptoms, and spores (as a transmission/persistence factor), are the major *C. difficile* virulence factors [22, 37, 42], other virulence factors include the S-layer, fibronectin-binding proteins, flagella, fimbriae and the heat shock protein GroEL (for recent reviews, see [10, 11, 43]).

1.3. Spore formation

Spores are arguably the most resilient cellular form known to us; they are hard to eradicate and can accumulate and persist in the environment for long periods of time, without losing viability [16, 44–46]. The spore thus has a central role in the persistence of the organism in the environment, infection, recurrence and transmission of the disease [37].

Two classes of Firmicutes are able to produce endospores: the *Bacilli*, which includes the extensively studied model organism *Bacillus subtilis*, and the *Clostridia*, to which *C. difficile* belongs. The development of new tools that allow the genetic manipulation of *C. difficile*, together with transcriptomic and proteomic analysis, provided insight onto the composition and structure of the spore, and onto the spore differentiation process [47–54]. Sporulation proceeds through a series of well-defined morphological stages that culminates about 8 h after the onset of the process in the production of (usually) one dormant spore per cell (**Figure 3A**) [55–58]. The morphological steps of the sporulation process observed in *B. subtilis* are conserved in *C. difficile* [53]. Sporulation begins with a vegetative cell that contains two copies of the chromosome which becomes condensed to form a single filament stretching along the long axis of the cell. An asymmetric division then partitions the cell into a small forespore and a larger mother cell (**Figure 3A**). At this stage, the two cells lie side by side. Asymmetric division traps about 30% of one chromosome in the forespore, while the remaining of the chromosome is pumped into the forespore following division. Later, the mother cell engulfs the forespore to produce a free protoplast isolated from the external medium (**Figure 3A**). The engulfed forespore is separated from the mother cell cytoplasm by a system of two membranes that derive from the septal membranes. Next, the engulfed forespore is surrounded by two peptidoglycan (PG) layers, the primordial germ cell wall (PGCW) and the cortex, and by proteinaceous surface layers (see subsequent text). At this point, the spore becomes phase bright (**Figure 3B**) and develops full resistance to physical and chemical agents. At the end of the differentiation process, the mother cell lyses to release the mature spore. At the transcriptional level, the process is controlled by a cascade of cell type-specific alternative RNA polymerase sigma factors [55, 56, 59] (**Figure 3A**). Cell type-specific gene expression and single-cell analysis of transcription and protein localization have been monitored in *C. difficile* using oxygen-insensitive fluorescence-based reporters (reviewed in Ref. [60]).
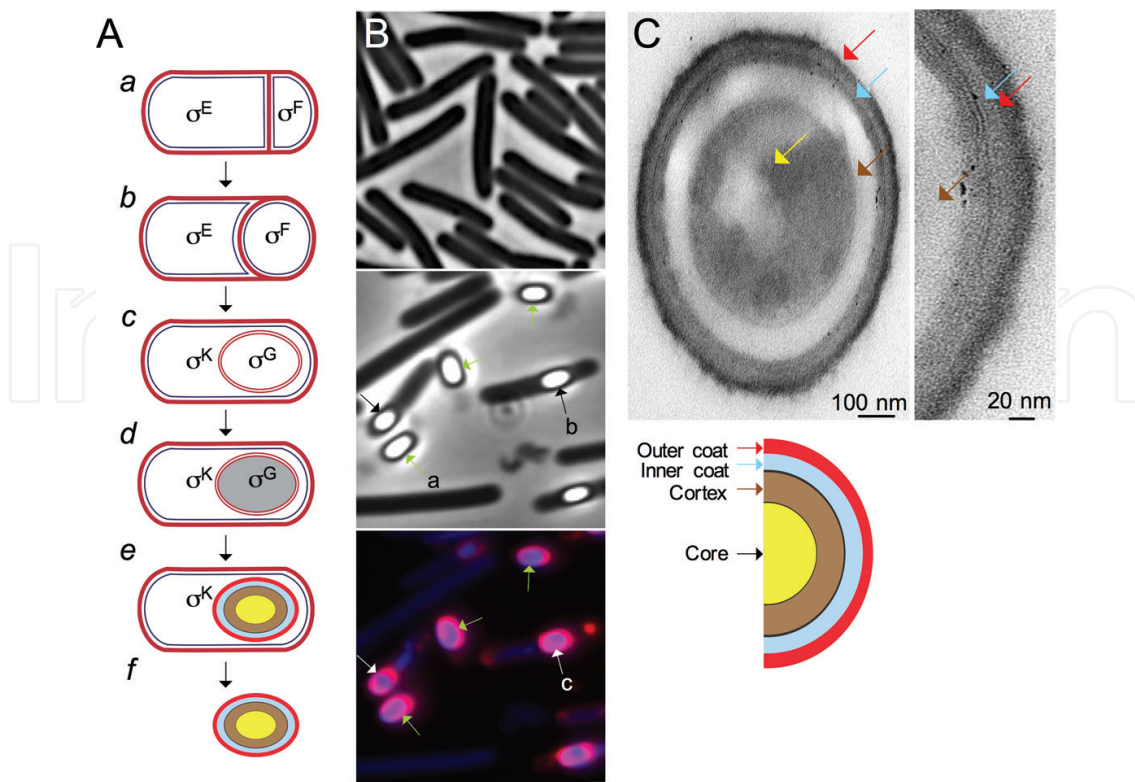
Figure 3. Sporulation in *C. difficile*. A: At the onset of the process, the rod-shaped cells divide asymmetrically to produce a larger mother cell and a smaller forespore (the future spore) (*a*). Asymmetric division involves PG synthesis within the septum. The mother cell then starts to engulf the forespore (*b*), eventually releasing it as a free protoplast inside its cytoplasm (*c*). PG polymerization contributes to the engulfment process. Following engulfment completion, the forespore is no longer in contact with the external medium and is separated from the mother cell by a system of two membranes that derive from the asymmetric division septum. Following engulfment completion, the forespore becomes visible as a phase dark body inside the mother cell (*d*). Synthesis of the primordial germ cell wall takes place from the forespore, whereas synthesis of the spore cortex PG layer is a function of the mother cell (see also insert in Figure 4). Development of full spore refractility coincides with the formation of cortex. Finally, the coat and exosporium are assembled (*e*). The spore is released into the environment through autolysis of the mother cell (*f*). B: Phase contrast microscopy and fluorescence microscopy of a *C. difficile* vegetative (top) and a sporulating culture (middle and bottom panels). For the sporulating culture, at a late stage in spore morphogenesis, the population consists of free spores (*a*) and refractile spores still inside the mother cell (*b*). The refractile spores exhibit most of the resistance properties of the released spores. In the bottom panel, the cells were stained with DAPI (a DNA dye) and the membrane dye FM4-64, which stains the developing spore and also the free spores (*c*). Scale bar, 1 μm. C: transmission electron microscopy (TEM) image of a thin cross section of a *C. difficile* spore. The main spore structures are labelled in the diagram. Note that an exosporium-like layer is not visible in the microscopy image, but its position, at the edge of the outer coat, is indicated in the diagram. The panel on the right shows a magnification of the spore surface. The diagram identifies the main structures or compartments normally seen by TEM. Scale bar, 0.2 μm.

1.4. Spore functional morphology

The basic endospore architecture is conserved across species. Transmission electron micros-copy (TEM) shows three main concentric compartments (the core, cortex and surface layers) (**Figure 3C**) [53]. The core contains the bacterial chromosome compacted by the action of small acid-soluble spore proteins (SASPs) that can bind to the DNA altering its conformation [61]. These proteins provide resistance to damaging factors such as dry heat, UV mutagen-esis, nucleases, chemicals and desiccation [62]. Immediately juxtaposed to the inner spore

membrane that delimits the core is the PGCW, whose composition is similar to the vegetative cell wall and serves as a primer for the assembly of the cell wall by the newly formed cells that result from spore germination [63]. The cortex is a more external layer formed by a specialized PG, essential for the maintenance of the dehydrated state of the spore core, spore mineralization, heat resistance and dormancy [46]. While the formation of the PGCW is controlled from the forespore, the assembly of the cortex is mainly a function of the mother cell [64, 65]. Surrounding the cortex is a proteinaceous coat layer. The coat consists of an inner layer and an electron-dense outer layer (**Figure 3C**). Enzymes constitute an important part of the identified coat-associated proteins and are responsible for the enzymatic activities present at the surface of spores and which contribute to protection against chemical and physical agents (such as organic solvents, oxidative agents and UV light) [46, 50, 64]. The coat also protects the cortex layer from the action of PG-breaking enzymes, and has an important role in the interaction of spores with germinants, abiotic and biotic surfaces [64, 65]. In some pathogens, an additional layer, termed exosporium, surrounds the coat [64, 66]. The exosporium contributes to spore protection, acts as a selective permeability barrier and modulates germination through the action of associated enzymes and interactions with host cells [64, 66]. In *C. difficile*, the presence of an exosporium-like structure remains a matter of controversy; several reports suggest that this layer is fragile and easily lost [50, 67], whereas other reports indicate that it is a stable layer which is only removed by proteases and/or sonication [68–71]. The morphology of the exosporium-like layer is strain dependent; some strains have an electron-dense, compact exosporium-like layer attached to the coat, whereas others have a hair-like exosporium-like layer [58, 71, 72]. *C. difficile* has three paralogs of a collagen-like glycoprotein, BclA, which in the pathogens *B. anthracis* and *B. cereus* forms the external hair-like nap of the exosporium [66]. In these organisms, BclA forms highly stable trimeric filaments that may contribute to spore rigidity; it mediates interactions with host cells and spore uptake, and an immune-modulatory mechanism that promotes spore persistence [66]. Spores of a *C. difficile bclA1* mutant germinate faster presumably because of increased accessibility of germinants to their receptors, but fail to colonize mice; thus, proper assembly of the spore surface is essential for colonization of the mouse gastrointestinal tract [73]. While sporulation by *C. difficile in vitro* is highly asynchronous and is completed after days of incubation, sporulation genes are quickly induced following inoculation of germ-free mice [74]. Further emphasizing the importance of the spore surface layers, the most highly expressed gene *in vivo* following inoculation of germ-free mice, *cdeM*, codes for a component of the spore surface layers, and an insertional *cdeM* mutant shows impaired colonization [70, 74].

1.5. Spore germination and outgrowth

To cause disease, the dormant *C. difficile* spores must germinate in the host gastrointestinal tract. With appropriate environmental stimuli, spores initiate germination leading to the resumption of vegetative growth if sufficient nutrients are present. *C. difficile* responds to unique germinants, such as bile salts [35, 75]. While the bile salt cholate (CA) induces spore germination, another primary bile salt, chenodeoxycholate (CDCA), has been identified as a potent inhibitor of the process [35, 76]. Bile salt levels are influenced by the commensal gut microbiota. *C. scindens*, for instance, has a bile acid 7α-dehydroxylating activity, which

converts CA into CDCA and has been implicated in resistance to infection by *C. difficile* [77] (see also the subsequent text) (**Figure 2**). Upon antibiotic administration, the disruption of the microbiota alters the metabolism of these two compounds and the CA concentration becomes higher than CDCA, triggering spore germination [78]. Germination induced by the CA derivative taurocholate (TA) is also enhanced in the presence of amino acids, such as glycine and histidine that act as co-germinants [35, 79]. In *C. difficile*, once germinants reach their receptors, located in the cortex region, the lytic enzymes that hydrolyse the cortex are activated [80–82]. Cortex hydrolysis allows the germ cell wall and the inner spore membrane to expand, and a massive release of calcium-dipicolinic acid from the core is observed [80, 83]. This leads to complete rehydration, enzyme activation, initiation of metabolism, macromolecular synthesis and spore outgrowth, a process during which the spore protoplast is converted into a vegetative cell [84, 85].

2. Antimicrobial therapy and the development of *C. difficile* infection

CDI is paradoxical as it occurs in the setting of antibiotic administration to treat other bacterial diseases. The disturbance of the indigenous gut microbiota by antibiotic administration is a key component, together with other risk factors, in the susceptibility to CDI.

Although the human gut microbiota is a complex ecosystem consisting of a vast number of bacteria, Archaea, viruses, yeast and fungi, the bacterial part of the microbiota is the most studied, mainly through metagenomic approaches, and is essentially formed, in healthy adults, by anaerobic bacteria belonging to the Bacteroidetes and Firmicutes phyla [86]. This ecosystem, which has coevolved with its human host, is essential to health, and is involved in many physiological functions, including metabolic transformations and regulation of immune responses [87, 88]. On the other hand, the disruption of the gut microbiota (or dysbiosis), as through antibiotic exposure, is associated with the pathogenesis of both intestinal and extra-intestinal disorders [89–91].

2.1. Effect of antibiotics on gut microbiota

Several studies in humans or in mouse models have shown that antibiotics radically alter the composition of the colonic microbiota, significantly decreasing the richness and diversity of the bacterial community, as well as its metabolic state. Dethlefsen and Relman demonstrated, by pyrosequencing of the *16S rRNA* gene, that the effect on the human gut microbiota of a 5-day treatment with ciprofloxacin was profound and rapid, with a loss of diversity and a shift in community composition occurring within 3–4 days of drug initiation [92]. In all three individuals involved in the study, the taxonomic composition of the community closely resembled its pre-treatment state by 4 weeks after the end of treatment, but several taxa failed to recover within 6 months [92]. In parallel, mouse models have shown similar long-term disturbance to the gut microbiota after the intake of antibiotics. Using pyrosequencing targeting the V6 hypervariable region of the *16S rRNA* gene, Antonopoulos and co-authors showed that an antibiotic cocktail of amoxicillin, metronidazole and bismuth induced a shift

in gut microbial community structure, with an increase in Proteobacteria and a decrease in the dominant baseline microbial communities of Bacteroidetes and Firmicutes [93]. Another study performed on mice and using the same technology targeting the V1-V3 of the *16S rRNA* gene showed that a single dose of the broad-spectrum antibiotic clindamycin markedly reduced the diversity of the intestinal microbiota for at least 28 days, with an enduring loss of ~90% of normal microbial taxa from the caecum [94]. The extensive duration of the impact of clindamycin is consistent with human studies demonstrating that *Bacteroidetes* species in the faecal microbiota are reduced within 2 years following clindamycin therapy [95]. Finally, an integrated multi-omics approach, addressing the total microbiota, active microbiota, metagenome, metatranscriptome, metametabolome and metaproteome, to evaluate the changes in the faecal microbiota of a single patient after β-lactam-treatment, showed that antibiotics significantly alter the gut microbial ecology and interactions with host metabolism [96].

2.2. Antibiotics, the gut metabolic state and susceptibility to CDI

CDI is one of the gastrointestinal diseases that occur in the setting of antibiotic administration. Indeed, antimicrobial therapy is one of the main risk factors for CDI, as alteration of the gut microbiota increases the susceptibility to CDI [94, 97].

This increased susceptibility is directly correlated with the metabolic state of the altered gut microbiota. High-throughput metabolomics studies, using proteomics and multiple mass spectrometry, performed on microbiome after antibiotic treatments have shown that antibiotics reduce the levels of most products of bacterial metabolism (such as secondary bile acids, glucose, free fatty acids and dipeptides), while promoting accumulation of their precursors (oligosaccharides, sugar alcohols and primary bile acids), reflecting the modified metabolic activity of the altered gut microbiome [98, 99].

Cumulative evidence indicates that antibiotic-mediated alteration of the gut microbiome, besides reducing competitive indigenous flora, converts the global metabolic profile to one that favours *C. difficile*, both spore germination and growth of the bacteria. Indeed, *in vitro* and *ex vivo* analyses demonstrate that *C. difficile* can exploit specific metabolites that become more abundant in the mouse gut after antibiotic treatment, including the primary bile acid CA for germination, and carbon sources such as mannitol, fructose, sorbitol, raffinose and stachyose for growth [98]. Infection by *C. difficile* results in the induction of genes involved in fermentation and carbohydrate transport and metabolism [74, 100].

It is interesting to note that an abundant metabolic product resulting from bacterial growth in the gut, butyrate, induces the differentiation of colonic regulatory T cells [101]. In addition, it is well documented that this compound can decrease intestinal permeability and enhance colonic defence barriers by increasing mucin production and antimicrobial peptide levels, thus preventing host from infection [102]. Therefore, the decrease or elimination of butyrate due to microbiota dysbiosis will impair the intestinal defence barrier and increase osmotic load in the intestinal lumen, contributing to CDI susceptibility or occurrence. On the other hand, the control of *C. difficile* toxin A and B genes expression seems to be dependent on the bacterium's nutritional environment. As mentioned above, the main positive regulator of toxins expression is RNA polymerase sigma factor TcdR, whose production is influenced by

various environmental signals. Regulatory proteins, such as CcpA, CodY, PrdR, SigL and Rex, and the Agr quorum-sensing system also play a role in controlling toxin gene expression in *C. difficile*, linking the metabolic and redox state of the cell to virulence [103].

The *agr1* locus is present in all sequenced strains of *C. difficile* and consists of two genes, *agrB1* and *agrD1*, that direct the production of a thiolactone, also known as the T1 signal, which accumulates extracellularly in a cell density-dependent manner [104, 105]. The T1 signal is required for *tcdA* and *tcdB* transcription, and when added to cultures, it is sufficient to cause premature expression of the toxin-encoding genes, suggesting a key role in regulating toxin production during growth [105]. At least some RT027 strains, such as R20191, have a second *agr* locus, termed *agr2*; in contrast to the *agr1* locus which carries only the genes for generation of the quorum signal, the *agr2* locus carries both the signal generation module and the genes required for signal detection (*agrC2*, coding for a sensor kinase) and transduction (*agrA2*, coding for a response regulator) [105, 106]. The *agr1* locus, however, seems essential for the generation of the T1 signal even in RT027 strains [105]. It is not known which two component systems are involved in T1 signal detection and transduction in strains lacking *agr2*. Importantly, *agrA* is required for complete expression of the toxin-encoding genes as well as the flagellar regulon, and an insertional mutant is impaired in colonization and infection in a mouse model [106]. In the *agrA2* mutant, decreased expression of the genes coding for three phosphodiesterases is likely to stimulate the degradation of c-di-GMP [106], may directly contribute to reduced expression of the gene coding for σ^D and, thus, reduced expression of *tcdA* and *tcdB*, in line with the observation that the flagellar regulon influences toxin production [107].

CcpA, in particular, binds directly to the regulatory region of several PaLoc genes, including (and with greater affinity) to *tcdR*, exerting glucose-dependent repression of toxin production; CodY, which binds branched-chain amino acids and GTP and represses the expression of many genes involved in responses to nutrient limitation, also binds directly to the *tcdR*-regulatory region [103] (**Figure 1A**).

Taking butyrate as an example, during infection, *C. difficile* appears to be able to utilize this compound [74], an activator of toxin synthesis, by a yet unknown molecular mechanism, and the genes involved in the metabolic pathway converting succinate or acetyl-CoA into butyrate are also regulated by CcpA, CodY and Rex. Thus, these metabolic regulators contribute to control the production of TcdA and TcdB by regulating the synthesis of butyrate (reviewed in Refs. [103, 108]). *C. difficile* Spo0A also contributes to the regulation of metabolism and, at least in some strains, to toxin production. Indeed, glucose uptake, glycolysis and butyrate production are downregulated in a *C. difficile spo0A* mutant [109]. That butyrate is produced by several bacteria of intestinal tract as well, suggesting that *C. difficile* turns on toxin synthesis when in the presence of other butyrate-producing species; this appears paradoxical considering that *C. difficile* is only able to colonize the colon when the normal microbiota is compromised. Discovering how the bacterium regulates butyrate metabolism and associated toxin production will likely unravel new ways of attenuating virulence, as suggested [103, 108]. In all, the direct or indirect control of the expression of the toxin-encoding genes by global metabolic regulators suggests that virulence is part of a strategy to enhance the availability of nutrient resources [103]. The link between the expression of *tcdR* and flagellar assembly, resulting from the σ^D-type promoter in the *tcdR*-regulatory region [27, 107] (**Figure 1A**), may also be viewed in this context. However,

while σ^D drives the production of the toxins, it is unknown whether toxin export coincides with flagellar assembly and motility. Elevated levels of c-di-GMP promote, in several bacteria and also in *C. difficile* [110], sessile growth in detriment of motility; in *C. difficile*, elevated levels of c-di-GMP reduce the expression of the gene coding for σ^D and thus also the expression of *tcdR* and of the PaLoc genes [111] (**Figure 1A**). Finding an appropriate niche may suppress motility, and intracellular TcdA and TcdB may be only exported at this stage; the overlap between toxin production and motility, however, requires further investigation.

The link between toxin production and spore differentiation is also unclear. It is unknown whether the population of cells that produces TcdA and TcdB coincides with the population that enters sporulation, or whether toxin producers and sporulating cells represent distinct populations. It is also unknown if and to what extent motility, spore formation and toxin production overlap. In any event, some degree of coordination exists between these processes, as emphasized by the recent discovery of regulatory protein RstA [112]. RstA represses transcription of the *sigD* gene, and thus it also curtails toxin production while positively controlling sporulation initiation, regardless of the presence of a functional *sigD* gene [112].

3. The *Clostridium difficile* peptidoglycan biosynthesis pathway

Peptidoglycan, one of the components present in the bacterial cell wall, is the target of some of the more effective antibiotics known. PG is required for cell division, cell elongation and also for spore differentiation (asymmetric division at the onset of sporulation, engulfment and synthesis of the PGCW and cortex) (**Figure 3**). We provide an overview of the PG-biosynthetic pathway in *C. difficile*, as deduced from genome information, and we integrate information on the structure and synthesis of the PG macromolecule with resistance mechanisms to antibiotics that target this pathway.

PG is a heterogeneous polymer of glycan chains cross-linked by short peptides and is the only common polymer of both Gram-negative and Gram-positive cell walls. While Gram-negative cell walls are composed by a thin layer of PG located between the cell membrane and the outer membrane which is composed mainly of lipopolysaccharides, the Gram-positive cell wall, in general, has a thick layer of PG decorated by accessory polymers, such as teichoic acids [113, 114]. Secondary cell wall polysaccharides are also present in *C. difficile* but will not be covered here [115]. PG is not only essential for the preservation of cell integrity, as it confers mechanical resistance against pressure, but also has an important role in the maintenance of cell shape and anchoring of proteins and other polymers on the cell surface [116, 117].

The polysaccharide chains that form the PG are composed of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNac) residues linked by β 1→4 bonds. The MurNac residue has a stem peptide linked to the carboxyl group. The chains are cross-linked through the formation of peptide bonds between these stem peptides of alternating strands [114, 117, 118]. These stem peptides have the sequence L-Ala, D-Glu, meso-diaminopimelate (mDAP), D-Ala and D-Ala in all Gram-negative, most cyanobacteria and *Bacillus* and *Clostridia* species. In several Gram-positive species, however, mDAP is substituted by L-Lys (e.g. in

Staphylococcus aureus) [116, 119]. The biosynthesis of PG is divided into three stages: (1) synthesis of the nucleotide precursors UDP-GlcNAc and UDP-MurNAc (cytoplasmic stage), (2) synthesis of lipid-linked intermediates (cytoplasmic/membrane stage) and (3) polymerization of the PG monomers (cell surface stage) [120, 121] (**Figure 4**).

Synthesis in the cytoplasm involves the action of six Mur ligases (MurA to MurF) that catalyse the formation of UDP-MurNac-pentapeptide from UDP-GlcNAc (**Figure 4**). In the process, UDP-GlcNac is converted to UDP-MurNAc by two sequential reactions catalysed by MurA and MurB. Next, the amino acids of the stem peptide are added sequentially to the UDP-MurNAc residue through the action of MurC, D, E and F. MurC is responsible for the addition of the first amino acid which corresponds to an L-Ala. MurD recognizes the UDP-MurNac-L-Ala and adds the second amino acid (D-Glu). MurE adds the third amino acid, either mDAP or L-Lys. Finally, MurF adds the fourth and fifth as a dipeptide D-Ala-D-Ala (D-Ala-D-Ser or D-Ala-D-Lac in some vancomycin-resistant organisms; see also subsequent text), leading to the formation of UDP-MurNAc-pentapeptide [116, 117, 119, 120]. Both the Alr racemase, involved in the formation of D-Ala from L-Ala, and the Ddl ligase, involved in the formation of D-Ala-D-Ala, are inhibited by D-cycloserine (see **Figure 4**).

The membrane stage starts with the transfer of the phospho-MurNAc-pentapeptide moiety from the soluble UDP-MurNAc-pentapeptide to the membrane receptor undecaprenyl pyrophosphate (C_{55}-P, also known as bactoprenol), yielding undecaprenyl-pyrophosphoryl-Mur-NAc-pentapeptide (or lipid I). This transfer reaction is catalysed by the integral membrane protein MraY. In a second step, MurG catalyses the formation of a β 1→4 bond between MurNAc and a GlcNAc moiety from a UDP-GlcNac molecule (**Figure 4**). *In vitro* selection experiments have shown that *murG* is the site of mutations conferring increased resistance to vancomycin (see also subsequent text) [122]. The MurG-catalysed reaction produces the PG monomer undecaprenyl-pyrophosphoryl-Mur-NAc-(pentapeptide)-GlcNAc (lipid II). Flippases, the partially redundant MurJ and Amj proteins in *B. subtilis*, then translocate lipid II to the *trans*-side of the membrane [117, 123, 124]. In *B. subtilis*, a sporulation-specific protein, SpoVB, produced in the mother cell under σ^E control (**Figure 3A** and **Figure 4**), most likely fulfils the same role during spore formation [125–127]. The MurJ homologue of *C. difficile* is coded for by *CD630_10680* while a second flippase (*CD630_34980*) may be the homologue of SpoVB (**Figure 4**); whether the latter is specifically involved in spore formation and thus whether *C. difficile* relies on a single flippase for growth is presently unknown.

During the cell surface stage, the glycan strands are polymerized and peptide bridges are created between adjacent strands. Polymerization occurs through a transglycosylation reaction between the C_1 from the MurNAc residue of the nascent strand and the C_4 from the GlcNAc residue of the lipid II-linked precursor. Cross-linking of the glycan strands generally occurs between the D-Ala at position 4 of the stem peptide and the mDAP (or L-Lys) at position 3 of a stem peptide from an adjacent strand. This reaction is catalysed by the transpeptidase domain present in all PBPs that are able to cleave the D-Ala-D-Ala bond of the stem peptide, releasing the last D-Ala residue, which energizes the transpeptidase reaction [117, 128]. The undecaprenyl-pyrophosphate is translocated back to the inner side of the membrane and recycled, to receive a new UDP-MurNAc-pentapeptide molecule [121, 128] (**Figure 4**). Until
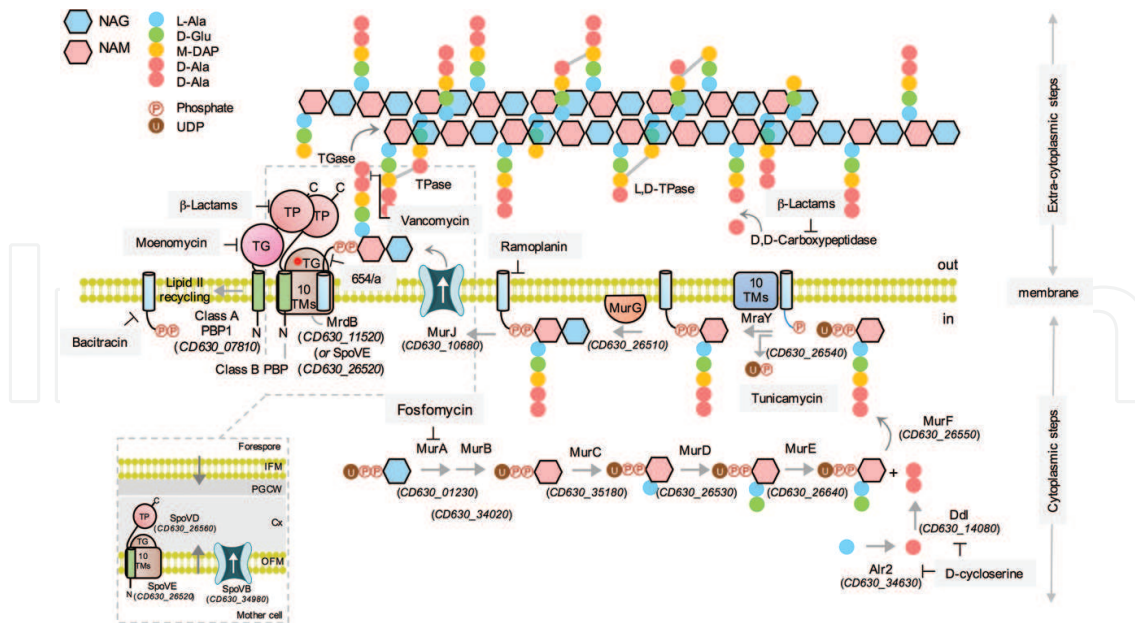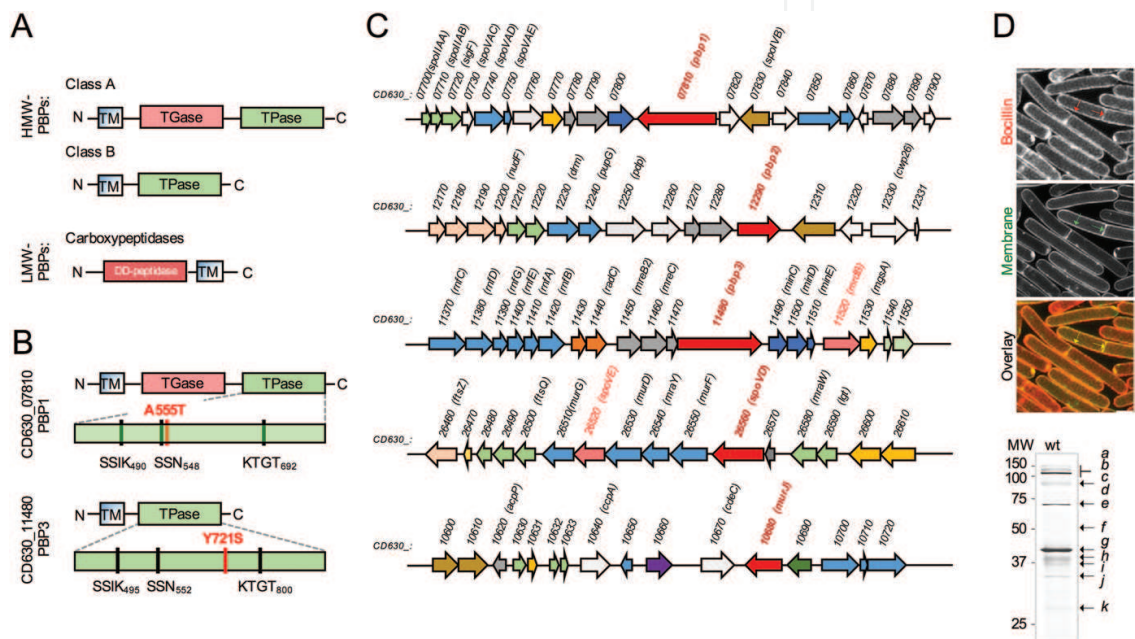
Figure 4. Overview of the *C. difficile* peptidoglycan biosynthetic pathway. The *C. difficile* counterparts of the genes known to intervene at the represented steps in the pathway are highlighted (the ORF code for strain 630Δerm is used). No cytoskeletal proteins are drawn for simplicity. The boxed complex, the core of which is formed by a SEDS-type transglycosylase (active site on the extracytoplasmic side of the membrane represented by a red dot) and a Class B PBP may function in elongation or division, depending on the functions of the represented proteins, which remain to be determined. Synthesis of the spore cortex PG follows a similar pathway but may involve sporulation-specific proteins, including a complex between a SEDS-type transglycosylase, SpoVE, and SpoVD, a Class B PBP (insert; IFM, inner forespore membrane; OFM, outer forespore membrane); SpoVB may be a mother cell-specific flippase. The direction of synthesis of the cortex (Cx) and the primordial germ cell wall (PGCW) is shown by arrows. Steps blocked by selected antibiotics are shown; the site of action of 654/a is also shown although this compound is a specific inhibitor of the SEDS protein RodA of *B. subtilis*. TM, transmembrane domain; TGase, transglycosylase domain; TPase, transpeptidase domain.

recently, the transglycosylation reaction has been almost exclusively attributed to the action of Class A PBPs (containing a transglycosylation domain; see subsequent text; see also **Figure 5A**). Some bacteria, however, lack Class A PBPs, whereas others are able to survive without their Class A enzymes [129–132]. The very recent demonstration that integral membrane proteins of the SEDS family (shape, elongation, division and sporulation) [133] have transglycosylase activity solved the paradox [134–136]. In fact, in *B. subtilis*, RodA (a SEDS protein) is a transglycosylase that associates with the complex responsible for PG synthesis during cell elongation [128, 134] (**Figure 4**). In *B. subtilis*, a RodA paralog, FtsW, has an equivalent function during cell division and a third protein, SpoVE, is specifically required for the synthesis of the spore cortex. SEDS proteins functionally cooperate with elongation- or division-specific Class B PBPs (transpeptidases). In *B. subtilis*, the direct interaction between SpoVE and SpoVD (a sporulation-specific transpeptidase) is essential for the formation of the spore cortex and spore heat resistance [125, 137] (see also **Figure 4**).

Homologues of all the enzymes involved in the cytoplasmic membrane and extracytoplasmic steps of PG biosynthesis are found in the *C. difficile* genome (**Figure 5**). Some important differences in the final structure of the PG do exist, however. Firstly, up to 93% of the GlcNAc residues are *N*-deacetylated while no modifications are found in MurNAc [115, 138, 139].

N-deacetylation of GlcNAc has been documented in other Gram-positive bacteria such as *B. subtilis*, *B. anthracis*, *Listeria monocytogenes* and *Streptococcus pneumoniae* and confers resistance to cleavage of the $\beta 1 \rightarrow 4$ bonds between MurNAc and GlcNAc by a muramidase (lysozyme), a first-line defence of the host innate immune response [140, 141]. *N*-deacetylation is achieved through the action of deacetylases such as PgdA from *S. pneumoniae* [140]. In *C. difficile*, complete resistance to lysozyme involves the extracytoplasmic sigma factor σ^V, which is induced by lysozyme, and leads to further PG deacetylation [139]. Importantly, disruption of the gene coding for σ^V (*csfV*) results in a strongly attenuated mutant in a hamster model of CDI. The *pdaV* gene (*CD630_1556*) codes for a protein with homology to PG

Figure 5. Class A and B penicillin-binding proteins of *C. difficile*. A: General structure of high- and low-molecular-weight (HMW and LMW) penicillin-binding proteins (PBP's). TM, Transmembrane domain; TGase, transglycosylase domain; TPase, transpeptidase domain; DD-Pep, DD-carboxypeptidase. B: The amino acid substitutions in PBP1 and PBP3 of *C. difficile* shown in red (A555T and Y721S) have been found in clones with increased resistance to imipenem, a carbapenem. The substitutions are indicated relative to the sequence found in a susceptible strain, such as the widely used laboratory strain 630Δerm. The groups of residues shown below the black lines form the TPase catalytic site, with the numbers indicating the position relative to the N-terminus of the protein. These mutations may confer resistance by decreasing the affinity of the enzyme for the antibiotic. The amino acid residues are represented in a single letter code. C: The genes coding for the four HMW Class A and B PBPs of *C. difficile* and the gene coding for the MurJ flippase and their genomic contexts. Note that the proteins most similar to the products of CD630_07810, CD630_12290 and CD630_11480 are PBP1, PBP2 and PBP3 of *B. subtilis*, respectively. CD630_26520 and CD630_26560 are labelled as *spoVE* and *spoVD*, as they occupy similar relative positions in the *dcw* cluster of *B. subtilis*. *B. subtilis* has two other SEDS proteins, FtsW and RodA that function specifically in cell division and cell elongation, respectively, while SpoVE is involved in the synthesis of the cortex PG during sporulation. Since *C. difficile* only has two SEDS proteins, it is likely that CD630_26520 supports cortex synthesis and either elongation or division. SpoVD is also specifically involved in the synthesis of the spore cortex in *B. subtilis* and possibly also in *C. difficile*. D: growing cells of *C. difficile* were labelled with Bocillin 650/665 Penicillin and with the membrane dye MTG and imaged by fluorescence microscopy; the bottom panel is the merge between the images collected in the red (Bocillin 650/665 Penicillin) and green (MTG/Membrane) channels. Note the labelling of both the division septa and the lateral wall of the cell. Scale bar, 1 μm. The bottom panel shows the SDS-PAGE and fluorimaging analysis of extracts prepared from the labelled cells. At least 10 bands are detected, although some may be stable proteolytic fragments of higher molecular weight species (bands *a-i*). Additional experiments are required to assign the bands detected to a specific PBP.

deacetylases and is itself under the control of σ^V; the expression of *pdaV* from an inducible promoter bypasses the requirement for *csfV* for lysozyme resistance and increased the level of *N*-deacetylated GlcNAc [139]. Thus, PdaV is a PG deacetylase, and complete resistance to lysozyme is required during infection. *C. difficile* codes for nine other putative PG deacetylases; of those, only *CD630_32570* was upregulated *in vivo* (as compared to growth *in vitro*) during infection in *C. difficile*-monoassociated mice [74]. Another characteristic of the *C. difficile* cell wall PG is that mDAP is found at the third position of the stem peptide. While most of the peptide bridges in bacteria are made between the D-Ala residue (fourth position) and the mDAP residue through the action of D-D-transpeptidases, approximately 73% of the cross-links in the *C. difficile* PG occur between mDAP residues of adjacent strands, through 3→3 bonds catalysed by L-D-transpeptidases [138, 142]. Two L-D-transpeptidase homologues, termed Ldt$_{CD1}$ (CD630_29630) and Ldt$_{CD2}$ (CD630_27130), were shown to participate in these reactions; a third homologue (CD630_30070) is likely to be involved because disruption of either *ldt* gene reduces but does not eliminate 3→3 cross-linking [138]. That β-lactams, but not carbapenems, inhibit D-D- but not L-D-transpeptidases may be part of the reason why *C. difficile* is tolerant to high concentrations of these antibiotics [115, 138, 142]. Nevertheless, while the L-D-transpeptidation pathway is insensitive to ampicillin, *C. difficile* is susceptible to this drug. This suggests that D-D-transpeptidation is important for the overall assembly of the PG [138], perhaps explaining the susceptibility of *C. difficile* not only to β-lactams. Thirdly, while most species have D-Ala-D-Ala at the end of the stem peptide, a significant fraction of the tetrapeptides ends with a Gly [138]. Intriguingly, while at least the prevalence of the L,D-transpeptidation pathway and the reduced presence of D-Ala-D-Ala-ending stem peptides could in principle contribute to vancomycin resistance, *C. difficile* is susceptible to this antibiotic. Moreover, *C. difficile* carries a *vanG*-type operon (*vanGCD*) most similar to that of *E. faecalis.* The **van** operon codes for enzymes that synthesize PG precursors ending in D-Ser or D-Lac and others that eliminate the natural (D-Ala) precursors; it thus confers vancomycin resistance [115, 142, 143]. The *van* operon of *C. difficile* is inducible by vancomycin and causes synthesis of UDP-MurNAc-pentapeptides ending in D-Ser [144]; yet, genetic ablation of the **van** operon in *C. difficile*, reduced the vancomycin MIC only slightly (from 1.5 to 0.75 mg/l) [143]. One possibility is that the incorporation of D-Ala-D-Ala into PG precursors is always favoured by the MurF enzyme [143]. Also noteworthy, deletion of the *dlt* operon, involved in D-alanylation of wall teichoic acids, also reduced the vancomycin MIC slightly (from 1 to 0.75 μg/ml for the strain used) [145]. Conversely, reduced susceptibility of *C. difficile* to vancomycin has been observed but the underlying mechanism is unknown [146, 147] (**Table 1**).

3.1. Penicillin-binding proteins

PBPs belong to a superfamily of acyl serine transferases that bind β-lactam antibiotics through a transpeptidase/carboxypeptidase domain which is thereby inactivated. These proteins can be divided as high-molecular-weight (HMW) PBPs and low-molecular-weight (LMW) PBPs [117, 128] (**Figure 5A**). The HMW PBPs are multimodular proteins responsible for the insertion of new molecules in the PG and cross-link formation. Generally, they contain an N-terminal cytoplasmic tail, a transmembrane anchor and two extracytoplasmic domains joined by a linker. One of the extracytoplasmic domains has transpeptidase activity responsible for the

Antibiotic	Mechanism of action	Relevance in CDI	Resistance frequency	Mechanism of resistance
Metronidazole	DNA damage after reduction of metronidazole inside the bacterial cell	Used in treatment of mild/moderate CDI	Rare; reduced susceptibility reported in frequent ribotypes	Multifactorial; 5-nitroimidazole reductase and modifications in multiple proteins involved in DNA repair, iron uptake and metronidazole reduction (putative)
Vancomycin	Inhibits cell wall synthesis by binding to the dipeptide D-Ala-D-Ala of peptidoglycan precursors	Used in treatment of severe and recurrent CDI	Rare	Mutations in *murG* (putative)
Fidaxomycin	Inhibition of RNA synthesis by binding to RNA polymerase (in a site distinct from rifamycins)	Used in treatment of severe and recurrent CDI	Rare	Mutations in *rpoB*, *rpoC* and *rarR* (reduced susceptibility in *in vitro* mutants)
MLS_B	Inhibition of protein synthesis by binding to 23S rRNA	Associated with high risk for CDI	High	Target protection by *ermB*, localized in the elements Tn5398, Tn9164 and Tn6215, or *cfr*, localized in Tn6218
Fluoroquinolones	Inhibition of DNA synthesis by binding to DNA gyrase and topoisomerase IV	Associated with high risk of CDI; resistance contributed to spread of the epidemic ribotype 027	High; associated with frequent and epidemic ribotypes	Target modification by mutations in *gyrA* and *gyrB*
Rifamycins	Inhibition of RNA synthesis by binding to RpoB	Used adjunctively for the treatment of recurrent CDI	Common; associated with frequent ribotypes	Target modification by mutations in *rpoB*
Tetracyclines	Inhibition of protein synthesis by binding to 30S ribosomal subunit	Resistance found in multiresistant isolates; resistance shared between human and swine isolates	Common	Target protection by *tetM*, carried by the elements Tn5398 or Tn916-like
Chloramphenicol	Inhibition of protein synthesis binding to the 50S ribosomal subunit	Resistance associated with prevalent ribotypes	Uncommon	Inactivation of the antibiotic by *catD*, carried by Tn4453 elements

CDI – *Clostridium difficile* infection.

Table 1. Summary of *Clostridium difficile* resistance to antibiotics and associated mechanisms.

cross-link of adjacent stem peptides. This domain is localized in the C-terminal and has three specific motifs that compose the active site: SXXK, (S/Y)XN and (K/H)(S/T)G (**Figure 5A**). The other extracytoplasmic domain is variable and allows sorting of these PBPs in two classes [128, 148]. Class A HMW PBPs are called bifunctional PBPs since they have a transglycosylase domain, which catalyses the polymerization of the new glycan strands. Transglycosylation can occur without a functional transpeptidase domain, but inactivation of the first impairs the transpeptidase activity [121, 149]. Class B HMW PBPs have instead another domain proposed to play a role in interactions with additional components of the PG synthesis machinery, such as a SEDS protein [121, 128, 149].

The LMW PBPs are DD peptidases that in the majority of the cases catalyse DD-carboxypeptidase reactions in the D-Ala-D-Ala motifs. Contrary to the HMW PBPs, these proteins are bound to the membrane through a C-terminal-located transmembrane domain or an amphipathic helix. The catalytic domain is localized in the N-terminal. The LMW PBPs play a role in regulating the degree of PG cross-linking since removal of the carboxy-terminal residue of the stem peptide prevents the cross-linking [117, 128, 144].

The number of PBPs and the proportion of the different types vary among different species and cell shapes. The rod-shaped spore-forming *B. subtilis* has 16 PBPs: four of Class A, six of Class B and six LMW PBPs. *S. aureus*, a coccus, has only four PBPs: one Class A, two Class B and one LMW. β-lactam resistance of methicillin-resistant strains of *S. aureus* (MRSA), however, stems from the acquisition of an extra, low-affinity PBP, PBP2a [117, 128, 134, 150]. It has been suggested that rod-shape species have more PBPs in order to be able to synthesize new PG not only during cell division, as in cocci, but also during cell elongation. Spore-forming species additionally have to synthesize the spore germ cell wall and cortex [150, 151].

C. difficile has nine PBPs identified of which only one is of Class A, three of Class B and five LMW PBPs (**Figure 5C** and **Table 2**). Note that the numbering of the *C. difficile* PBPs used herein is based on the nomenclature used in a recent study [152]. Strain M68, a recent representative of RT017 [153], has an additional Class B PBP, referred to as PBP5, which may have been recently acquired by horizontal gene transfer. Labelling of exponentially growing cells with a fluorescent derivative of penicillin (Bocillin 650/665 Penicillin) shows labelling of both the lateral wall of the cell and the division septum, as seen for several other rod-shaped bacteria (**Figure 5D**). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell extracts prepared from the labelled cells followed by fluorimaging reveals a collection of 11 labelled bands; although some of these bands may correspond to stable degradation products, the pattern is generally consistent with the genomic information (**Figure 5D**).

Like *S. aureus, C. difficile* has only one gene coding for a bifunctional PBP in a monocistronic operon (PBP1; *CD630_07810*). The genes coding for the three Class B PBPs are *CD630_12290* (PBP2), *CD630_11480* (PBP3) and *CD630_26560* (SpoVD). The gene coding for PBP2 is also monocistronic. The gene coding for PBP3 is the last gene of an operon that codes for the MreB2 and MreC components of the cell shape/elongation machinery suggesting constitutive production of the protein and a possible involvement in cell elongation. As in *B. subtilis*, *spoVD* is located within a region containing several other cell division and cell wall genes, the

Class	Gene	Protein	N° aa	kDa
A	CD630_07810	PBP1[†]	897	96.5
B	CD630_12290	PBP2[†]	554	62.6
B	CD630_11480	PBP3[†]	992	111.3
B	CD630_26560	SpoVD	659	73.2
LMW D-D-Carboxypeptidase	CD630_05150	-	409	45.5
LMW D-D-Carboxypeptidase	CD630_12910	DacF[*]	387	41.9
LMW D-D-Carboxypeptidase	CD630_16270	VanY[*]	268	31.7
LMW D-D-Carboxypeptidase	CD630_21410	-	397	44.5
LMW D-D-Carboxypeptidase	CD630_24980	DacF[*]	429	48.2

[†]Nomenclature according to Ref. [152].

[*]Nomenclature according to the NCBI (https://www.ncbi.nlm.nih.gov/gene).

Table 2. The penicillin-binding proteins of *Clostridium difficile*.

dcw cluster that also codes for the SEDS-type cortex-dedicated transglycosylase SpoVE. It is not known whether the SpoVD homologue of *C. difficile* is also cortex-specific, as in *B. subtilis*. The *dcw* gene *CD630_26520* codes for a SEDS protein and occupies the position homologous to that of *spoVE* in the *dcw* cluster of *B. subtilis* (**Figure 5C**). Since *C. difficile* only codes for two SEDS members, it is unclear whether *CD630-26500* is a functional homologue of *spoVE* and whether it accumulates a role in spore cortex synthesis with a vegetative function, in either division or elongation. Among the LMW PBPs, it is worth noting that the putative carboxy-peptidase coded for by *CD630_16270* is a homologue of proteins able to confer vancomycin resistance (see earlier text).

3.2. Bacterial shape and peptidoglycan synthesis

PG is responsible not only for resistance against physical and chemical stress but also for the maintenance of cell shape. In nature, a wide diversity of bacteria shape exists, ranging from spheres (*cocci*) to rods (*bacilli*), that has been historically used for the characterization and classification of species [154, 155]. Cell shape is defined by the different PG synthesis apparatus present in the bacteria that, through interaction with cytoskeletal elements, define the points where newly synthesized PG is inserted. Cell shape changes through the addition of antibiotics that inhibit specific complexes or by mutations that inactivate critical components of cytoskeleton or PG-biosynthetic machinery [156]. Rod-shaped bacteria have two distinct multi-protein complexes driving PG synthesis: the elongasome and divisome. The elongasome is responsible for the synthesis of PG in the lateral wall, whereas the divisome is

assembled for the synthesis of the septal PG during cell division. In general, cocci only synthesize new PG during cell division [151, 154, 157].

Among elongasome components are MreB, MreC, MreD, RodA, RodZ, a Class B PBP and/or a Class A PBP. MreB is an actin homologue that assembles into short filaments that move independently perpendicular to the long axis of the cell, and is the key element that spatially governs activity of the elongasome (reviewed in Refs. [149, 158, 159]). MreC and MreD are integral membrane proteins of unknown function and RodZ connects MreB with the synthesis machinery [104, 119, 135–138].

Two actin homologues, MreB2 (*CD630_10225*) and MreB (*CD630_01270*), are found in *C. difficile*. Interestingly, the last gene of the *mreBCD* operon codes for PBP3 suggesting the participation of this Class B PBP in lateral cell wall synthesis. Elongasome complexes include a SEDS-type transglycosylase that interacts directly with a class B PBP [149, 158]. In the case of *C. difficile*, the core of the elongasome may include PBP3 and the SEDS protein might be the RodA homologue MrdB (coded for by *CD630_11520*); this gene distances 1.9 kb from the *pbp3* gene, that is, within the genetic distance range identified by Meeske et al. for SEDS/Class B PBPs pairs [134]. The only Class A PBP codified by the genome, PBP1 (*CD630_07810*), may also be part of the elongasome. Remarkably, RodZ, thought to help linking MreB to the membrane and to extracytoplasmic complexes [158], is absent in *C. difficile*.

Among the divisome components in model organisms such as *B. subtilis* and *Escherichia coli* are FtsZ, FtsA, ZipA, DivIB, DivIC (also referred to as FtsQ and FtsB in *E. coli*), FtsL, FtsW, a Class B PBP and/or a Class A PBP. FtsZ is a tubulin homologue that organizes PG synthesis during cell division [121, 149]. FtsZ is tethered to the membrane by proteins such as FtsA and ZipA. In *E. coli* and in *B. subtilis*, the formation of the Z-ring at midcell relies on two main systems. In *E. coli*, the MinCD inhibitor oscillates from pole to pole through the action of MinE, causing the overall concentration of the inhibitor to be maximal at the poles and minimal at midcell, while in *B. subtilis*, from which MinE is absent (as is the case of most Gram-positive species), MinCD is sequestered at the poles [161]. In both organisms, nucleoid occlusion only allows polymerization in areas not occupied by the chromosome (reviewed in [161]). The conjugation of the two systems restricts Z-ring formation to midcell. FtsW is a cell division-specific SEDS glycosyltransferase [151, 160–162].

Benzamides or derivatives of the alkaloid berberine are among the compounds that block FtsZ function leading to filamentation [163–165]. Importantly, benzamides showed efficacy in a mice model of systemic *S. aureus* infection [163, 164].

The main components of the divisome complex are found in the *C. difficile* genome with a genetic organization similar to *B. subtilis*. In *B. subtilis*, however, *ftsZ* is co-transcribed with *ftsA* downstream from the *mur* genes and *divIB*, whereas *divIC* is organized in an operon upstream from *spoIIE* and *ftsL* is co-transcribed with *mraW* and *pbdB* (Class B PBP). In *C. difficile*, *ftsZ* (*CD630_26460*) is downstream from *divIB* (*CD630_26500*), but surprisingly, no *ftsA* gene is found in its vicinity or elsewhere in the genome. How FtsZ is tethered to the membrane in the absence of FtsA is unknown. DivIC (*CD630_34920*) is also upstream from *spoIIE*, required for proper division and cell type-specific activation of σ^F at the onset of sporulation

(see **Figure 3A**). No *ftsL* or *ftsW* genes were identified in the genome, however; as discussed above, it is possible that the *CD630_26520* gene participates both in cortex synthesis and in cell division (**Figure 4**). The absence of class B PBP and of a SEDS protein in the vicinity of these genes may indicate that PBP1 may play a crucial role also in cell division. Between the *pbp3* and *mrdB* genes is the *minCDE* operon. This genetic organization is reminiscent of *B. subtilis*, where the *mreBCD* and *minCD* genes are co-transcribed [156]. Remarkably, however, the presence of *minE* in *C. difficile* suggests that polar division is controlled through oscillation of a MinCDE complex. Finally, a gene cluster coding for three cell division proteins called MldA, B and C (midcell-localizing division proteins) can only be found in *C. difficile* and closely related species; mutants lacking MldA and MldB loose the rod-shape and daughter cells separation is inefficient [166].

In addition to the elongasome and divisome complexes, generally found in rods, spore-forming bacteria, like *B. subtilis*, usually contain a third PG-synthesizing complex that drives biogenesis of the spore cortex and the core of which is formed by SpoVE and SpoVD homologues, as discussed above [167, 168]. It is likely that such a complex also operates in *C. difficile*. Antibiotics such as bacitracin (which interferes with the dephosphorylation of C_{55}-isoprenyl pyrophosphate), fosfomycin (an inhibitor of MurA) and D-cycloserine (which inhibits the alanine racemase Alr and the D-Ala-D-Ala ligase Ddl) block PG synthesis during spore development [123, 169, 170].

Several modifications of the cortex PG, as shown by the work in *B. subtilis*, are functionally important. The cortex has a low percentage of cross-links because of the action of D-D-carboxypeptidases. Approximately 75% of the stem peptides are removed by the DacA, DacB, DacC and DacF enzymes, with DacB and DacF playing a more essential role since spores from mutant strains lacking these two proteins are unstable and show higher cross-linking, higher core water content and decreased heat resistance. *C. difficile* codes for two DacF-like proteins (CD630_12910 and CD630_24980) and mutants unable to produce these proteins may show the same type of alterations as suggested from the work in *C. perfringens* [171, 172]. Importantly, about 33% of the MurNAc residues are in the δ-lactam form, a modification that requires the concerted action of an amidase and a MurNAc deacetylase [173]. This modification allows the cortex PG to be degraded during germination, while the germ cell wall is maintained [174]. It is likely that the same modification is found in the cortex of *C. difficile* but its structure has not yet been reported.

4. Antibiotic resistance and the emergence and spreading of epidemic *Clostridium difficile* strains: historical perspectives and changing epidemiology

Virtually all antibiotics are associated with CDI, but the higher risk is linked to prolonged administration of broad-spectrum agents. Several studies using meta-analyses to examine the risk of CDI associated with the various antibiotic classes showed that the strongest and most consistent association was with clindamycin (variable odds ratio (OR): 2.86, 16.8 and 20.43),

cephalosporins, particularly those of the second and third generation (variable OR: 2.23, 3.20, 4.47 and 5.68) and fluoroquinolones (variable OR: 1.66, 5.50 and 5.65) [175–177]. In one of these studies, the association between CDI risk and fluoroquinolones was modest (OR: 1.66), but the authors argued that this was not surprising since this association was more specifically related to CDI caused by the fluoroquinolone-resistant epidemic strain [177–179]. This is consistent with the fact that ciprofloxacin causes a relatively low disruption of the anaerobic gut microflora [180]. Carbapenems also increase CDI risk consistently, although with a weak association when considered alone (OR, 1.84), but stronger when included in the group of cephalosporins/monobactams/carbapenems (OR, 5.68) [175, 177]. Tetracyclines are not associated with CDI risk (variable OR: 0.91 and 0.92) [175–177].

There are therefore two effects to consider in the association between infection and antibiotics that act synergistically. One is the effect of the antibiotic on microflora imbalance, and the other is the increased risk of CDI in a patient taking an antibiotic for which the infecting strain is resistant. Indeed, once antibiotic treatment starts, infection with a *C. difficile* strain that is resistant to the antibiotic is more likely while the antibiotic is being administered due to the presence of the antibiotic in the gut. When the antibiotic treatment stops, the levels of the antibiotic in the gut diminish rapidly, but the microbiota remains disturbed for a variable period of time, depending on the antibiotic. During this time, patients can be infected with either resistant or susceptible *C. difficile*. Accordingly, mounting evidence suggests that antimicrobial resistance in *C. difficile* is a key player in the epidemiology of CDI [181]. For clindamycin, the risk of CDI associated with a clindamycin-resistant strain is increased in patients receiving this antibiotic [182]. Regarding cephalosporins, to which *C. difficile* is intrinsically resistant, the use of this antibiotic has been identified as a CDI risk factor in hospitals for the last decades [181].

More recently, there was a rise in the fluoroquinolone-associated risk concomitantly with the emergence and geographical dispersion of a fluoroquinolone-resistant *C. difficile* strain, designated BI/NAP1/027, for restriction endonuclease analysis group BI, pulse-field gel electrophoresis-type NAP1 and polymerase chain reaction ribotype (RT) 027. In addition to the high-level resistance to fluoroquinolones, RT027 strains are characterized by the increased production of both the TcdA and TcdB toxins, the presence of the binary toxin CDT and the presence of a mutation in the gene coding for the anti-TcdR anti-sigma factor TcdC [183]. Whole-genome sequencing (WGS) and phylogenetic analysis showed that this strain emerged in North America in the early 2000s, soon after acquiring a fluoroquinolone resistance mutation in *gyrA*, causing higher rates and more severe cases of CDI, and then spread widely leading to severe healthcare outbreaks also in the UK, continental Europe and Australia [183–185]. The link between the emergence of this clone and the use of fluoroquinolones is unequivocal. Indeed, fluoroquinolones were one of the most frequently prescribed antibiotic classes in North America during the late 1990s and early 2000s, so that the selective pressure for the acquisition and maintenance of fluoroquinolone resistance within healthcare settings during this period would have been at its highest [186].

Molecular-based epidemiological studies show a constant changing in the epidemiology of CDI. While in early 2000s RT027 was responsible for CDI outbreaks of increased severity, a

study from 2008 analysing the epidemiology of *C. difficile* in Europe, involving a network of 106 laboratories in 34 countries, showed that RT027 accounted for only 5% of all *C. difficile* isolates, with a great diversity of ribotypes being observed [187]. Notably, in another recent multicenter study comprising 482 participating hospitals from 19 European countries, the epidemic strain RT027 was the most prevalent (19%), although distinct regional patterns of ribotype distribution were seen [188]. In parallel, other ribotypes, of reportedly increased virulence, have started to emerge.

More recently, a toxin A-negative, toxin B-positive *C. difficile* strain, from RT017, has emerged in several countries, sometimes to epidemic proportions. In a CDI surveillance study conducted in Poland between 2004 and 2006, RT017 accounted for approximately 40% of the *C. difficile* isolates studied, while in Bulgaria the occurrence of RT017 between 2008 and 2012 reached 28% [189, 190]. In the Netherlands, one hospital was affected by an outbreak caused by both RT027 and RT017 [185]. In Asia, RT017 strains are one of the most prevalent ribotypes, particularly in China, Korea and Thailand [191–193].

In Portugal, CDI surveillance based on a network of sentinel hospitals has been carried out since 2010, and showed that RT017 is one the most common ribotypes circulating in the country [194]. In particular, one RT017 clone was shown to be endemic in a hospital from 2012 until today, and a different RT017 clone has emerged in another hospital in the beginning of 2016 (our unpublished data). Despite belonging to different genetic lineages, based on multiple locus variable number tandem repeat analysis, both of these clones harbour several genetic determinants of antibiotic resistance such as *ermB*, *tetM*, and mutations in *rpoB* and *gyrA/gyrB*, which confer a multiresistant phenotype (see next section for details on antibiotic resistance and associated mechanisms). In addition, they were shown to be resistant to imipenem, the first antibiotic of the carbapenems class (highly resistant to β-lactamases and widely used against drug-resistant Gram-negative bacteria). Through WGS of the endemic clone, we have identified two mutations affecting the transpeptidase domain of two penicillin-binding protein genes (*pbp1* and *pbp3*; see **Figure 5A** and **B**). The mutations are therefore likely to be associated with imipenem resistance possibly by reducing the affinity of the drug to one or both proteins [152]. The emergence of resistance to carbapenems in multiresistant clones of *C. difficile* might lead to the fast spread of these strains in hospital settings, in an analogy with the initial spreading of the fluoroquinolone-resistant strains, and thus deserves urgent and continuous surveillance.

5. Overview of resistance to different classes of antibiotics and associated mechanisms in *Clostridium difficile*

As previously mentioned, antibiotics play a major role in the development of CDI. Through the disruption of the protective gut microbiota, antibiotics promote the conditions for, not only, the germination of the *C. difficile* spores (see earlier text) once the levels of antibiotic in the gut start to decrease, but also for the growth of antibiotic resistant *C. difficile* during the treatment, when there are still high levels of antibiotic in the gut. As such, *C. difficile* strains

that are resistant to several classes of antibiotics will have a selective advantage for the development of CDI [20]. Both the rates of resistance in *C. difficile* and the distribution of ribotypes vary extensively between countries. However, as a result of antibiotic-selective pressure, the most common ribotypes, which include the epidemic strains, are usually the ones presenting the highest rates of resistance [146, 188, 195, 196]. Antibiotic resistance in bacteria can be due to mutations in specific genes or due to the presence of genes acquired by horizontal gene transfer. The mechanisms of resistance are then divided into three main categories: (i) modifications of the antibiotic target, (ii) inactivation of the antibiotic and (iii) reduction of the intracellular concentration of the antibiotic [197].

5.1. Metronidazole, vancomycin and fidaxomicin

The surveillance of *C. difficile* susceptibility to the antibiotics used for CDI treatment, that is, metronidazole, vancomycin and fidaxomicin [198], is crucial. Resistance to metronidazole and vancomycin has been seldom observed but reduced susceptibility is now being reported more often [195]. In two recent studies, the MIC_{90} (the minimum concentration of metronidazole necessary to inhibit 90% of the isolates) of metronidazole was of 2 mg/L for both European and US strains, while for vancomycin it was of 2 and 4 mg/L, respectively [146, 199]. Reduced susceptibility to metronidazole has been observed in some of the most frequent RTs, including RT027, RT001 and RT106 [146, 200]. Heterogeneous and unstable resistance to metronidazole has also been described, in which cases the resistance was only observed in primary fresh isolates or after exposing the thawed isolates to low concentrations of the antibiotic, also depending on the methodology used; this indicates that these heteroresistant populations might go undetected during routine susceptibility testing [201]. Reduced susceptibility to vancomycin was also observed in two emergent RTs (RT018 and RT356) in the pan-European survey [146] and in the epidemic RT027, among US isolates [147]. Although the clinical impact of reduced susceptibility to metronidazole in CDI is still not clear, there is evidence of a correlation between reduced susceptibility and recurrent CDI, which is supported by the low concentration of metronidazole observed in the gut, unlike vancomycin which is detected at high concentrations [202, 203]. As discussed above, the mechanisms of resistance to metronidazole and vancomycin in *C. difficile* remain to be clarified (but see also subsequent text).

Metronidazole enters the bacterial cell by passive diffusion as an inactive prodrug. It is then reduced into its cytotoxic active form through the transfer of an electron to the nitro group of the drug, forming a nitroso-free radical, which interacts with DNA, inflicting DNA damage and inhibiting synthesis, ultimately leading to cell death [204, 205]. Some studies point to a multifactorial mechanism of metronidazole resistance in *C. difficile*, which includes (i) the activity of a putative 5-nitroimidazole reductase, which converts the nitro group of the prodrug into its non-toxic amine derivative; (ii) the elevated expression of proteins involved in DNA repair, such as the UvrABC excinuclease, exodeoxyribonuclease, endonuclease III, endonuclease IV and DNA mismatch repair protein; (iii) alterations in proteins related to iron uptake, such as an increased expression and mutations in the ferric uptake transcriptional regulator Fur [206, 207], which plays a central role in iron homeostasis by controlling the expression of a regulon that includes genes involved in the mitigation of oxidative stress and

in redox metabolism [207]; in *Helicobacter pylori*, point mutations in *fur* have been shown to increase metronidazole resistance by reducing the binding affinity of the modified Fur protein to the promoter region of the superoxide dismutase encoding gene *sodB* and hence rendering Fur unable to efficiently repress transcription of *sodB*; high levels of SodB, in turn, allow *H. pylori* to counteract the oxidative stress generated by the activated metronidazole [208–210]; and (iv) alterations in the metabolic pathway involving pyruvate-ferredoxin oxidoreductase (Pfo), responsible for metronidazole reduction; a reduced concentration of Pfo, for example, will likely lead to a less efficient activation of metronidazole inside the bacterial cell [211].

Vancomycin inhibits bacterial cell wall synthesis by binding to the D-Ala-D-Ala dipeptide of the peptidoglycan precursor before cross-linking of adjacent peptidoglycan strands. The *in vitro* selection of isolates with reduced susceptibility to vancomycin showed the acquisition of a mutation causing a P108L substitution in *murG*; since MurG converts lipid I to lipid II, a target of vancomycin, it is conceivable that alterations in this pathway might affect the activity of vancomycin (see earlier text; **Figure 4**). Other mutations found included a stop codon in an RNA/single-stranded DNA exonuclease (*CD630_36590*), a single amino acid deletion in the *sdaB*-encoded L-serine dehydrogenase, and a missense mutation causing a D244Y substitution in *rpoC*, coding for the β′subunit of RNA polymerase. How mutations in *murG*, *sdaB* and *rpoC* and *dlt* operon contribute to the reduced susceptibility to vancomycin is still unknown [122] (**Table 1**).

Fidaxomicin blocks an initial step in transcription by RNA polymerase. Binding of the RNA polymerase holoenzyme to a promoter results in the formation of a closed complex, which is isomerized to an open promoter complex through opening of the double-stranded DNA; the melted region, or transcription bubble, extends approximately from positions −12 to +2 relative to the transcription start site [212]. Fidaxomicin inhibits transcription initiation if added before the stable holoenzyme/promoter open complex is formed, in contrast with antibiotics that inhibit RNA elongation such as the rifamycins [212]. *In vitro* selection for resistance led to the identification of a mutation in the *rpoB* gene (coding for the β subunit of RNA polymerase) causing the single amino acid substitutions Q1074K or Q1073R [122, 213]. Substitutions in the β′subunit have also been linked to resistance (R337A) or reduced susceptibility to fidaxomicin (two simultaneous substitutions, Q781R and D1127E) [213, 214]. Another mutation associated with reduced susceptibility to fidaxomicin, selected *in vitro*, is a frameshift mutation in the *CD630_22120* gene coding for a homologue of the multidrug resistance-associated transcriptional regulator MarR; the role of this regulatory protein in fidaxomicin resistance is still unclear [122] (**Table 2**). Resistance to fidaxomicin has only been observed in one isolate with a MIC of 16 mg/L [215], being that usually all isolates are inhibited at concentrations of ≤1 mg/L [146, 199, 216].

5.2. Clindamycin

Clindamycin is one of the antibiotics associated with an increased risk of CDI [177], and resistance to this antibiotic is one of the most common in *C. difficile*. Clindamycin resistance is often found in >30% of the isolates worldwide and frequent RTs tend to exhibit higher rates of resistance. Furthermore, clindamycin resistance is frequently found in multidrug-resistant isolates

in association with resistance to fluoroquinolones and rifampicin [146, 192, 199, 216–219]. Resistance to clindamycin, a lincosamide that inhibits bacterial protein synthesis by binding to the bacterial 23S rRNA, is generally due to the presence of *ermB* (erythromycin resistance methylase gene). The rRNA adenine N-6-methyltransferase encoded by this gene methylates the adenine at position 2058 of 23S rRN, which prevents binding of the MLS$_B$ (macrolide, lincosamide and streptogramin B) antibiotics. This gene is found in mobile elements, such as the transposons Tn5398, which contains two copies of *ermB*, Tn6194 and Tn6215. These elements are horizontally transferred between *C. difficile* strains and also to and from other genera [220]. Even so, there is a significant proportion of clindamycin-resistant *C. difficile* strains, which are *ermB*-negative [218]. Recently, a *cfr*-like gene was found to confer resistance to multiple antibiotics in *C. difficile*, including clindamycin/erythromycin, linezolid and chloramphenicol/florfenicol. Like *ermB*, this gene is found in a transposon, Tn6218, and confers resistance through modification of the bacterial 23S rRNA at position A2503 [221, 222].

5.3. Fluoroquinolones

Fluoroquinolones play a major role in the paradigm of CDI. Resistance to this class of antibiotics, which inhibit bacterial DNA synthesis by binding to the type II topoisomerases DNA gyrase and topoisomerase IV [223], was associated with the worldwide spread of the epidemic RT027 in the 2000s and rapidly became a prominent risk factor for CDI, as previously described [224]. Since then, fluoroquinolones resistance has been reported worldwide with increasing rates and is frequently found in common and epidemic strains, such as those of RT027 (in which the resistance is almost ubiquitous), RT017 and RT018 [146, 192, 225]. The rates of resistance vary considerably between countries; however, this is likely due to differences in ribotype diversity, since the countries with higher diversity of ribotypes tend to exhibit lower rates of fluoroquinolones resistance and vice versa [146]. This same trend was observed in Portugal when comparing ribotype diversity and resistance rates between regions [194]. Fluoroquinolone resistance in *C. difficile*, as a result of antibiotics use selective pressure, is associated with well-known mutations in the quinolone-resistance determining region of DNA gyrase subunits *gyrA* and *gyrB*. Mutations causing a T82I substitution in GyrA are the most commonly identified in *C. difficile*, and are found in ribotypes such as RT027 and RT017 [194, 218, 226]. Importantly, resistance to fluoroquinolones is likely to be maintained even without antibiotic pressure, as it does not impose a fitness cost in *C. difficile* [227].

5.4. Rifamycins

Rifamycins are a class of antibiotics that inhibit bacterial RNA synthesis by binding to the β subunit of RNA polymerase (coded for by the *rpoB* gene) [228]. Two members of this class, rifampicin and rifaximin, have been used adjunctively for the treatment of recurrent CDI, despite not being recommended due to the absence of evidence supporting their efficacy (vs. monotherapy) [198, 229, 230]. In fact, high rates of rifampicin resistance have been identified in epidemic strains [231]. In a recent study, rifampicin resistance (13.4%) was reported in 17/22 European countries, and although it was observed in several RTs, it was mostly

associated with frequent RTs, such as RT027, RT018 and RT356 [146]. Also, in Portugal, all the RT017 strains, a predominant type [194], show high resistance to rifampicin (unpublished data). It has been demonstrated that *in vitro* susceptibility to rifampicin is predictive of rifaximin susceptibility [228] and the emergence of rifamycin resistance during a recurrent *C. difficile* infection following rifaximin treatment has been clearly demonstrated [232]. As in other bacteria, resistance to this class of antibiotics in *C. difficile* results from specific substitutions in, or near to, the residues in the β subunit of RNA polymerase that interact with rifamycins. The substitution R505K is the most frequently found in *C. difficile*, either alone or along with other substitutions, such as the frequently found H502N. Furthermore, the resistant isolates do not seem to have a clonal origin but rather appear to arise independently as the result of antibiotic pressure [218, 228].

5.5. Tetracyclines

Tetracyclines inhibit protein synthesis in bacteria by binding to the 30S subunit of the bacterial ribosome, thereby preventing the association of aminoacyl-tRNA [233]. Tetracycline resistance is a relatively common trait in *C. difficile*. The resistance rates vary widely between countries, with some presenting high rates but the majority having <10%-resistant isolates [234, 235]. The exposure to tetracyclines, however, does not seem to be associated with an increased risk of CDI [177]. Even so, resistance to tetracycline is usually found in multidrug-resistant isolates, such as those of RT012 and RT017 [218, 234, 235], and is, in most strains, associated with the presence of the *tetM* gene, which codes for a ribosome protection protein. This gene is carried by transposons Tn5397, which is found in RT012, and Tn916-like, which is found in RTs such as RT017 and RT078 [236]. TetM confers protection by binding to the ribosome in the proximity of the tetracycline-binding site and hence dislodging and preventing binding of the tetracyclines to their target [237]. Although less frequent, another *tet* gene, *tetW*, which also codes for a ribosomal protection protein, has been found in tetracycline-resistant *C. difficile* isolated from humans and animals that also harboured the *tetM* gene [218, 238]. Furthermore, the co-presence of *tetM* and *ermB* in a Tn916-like element presumably originated by the recombination of different elements has also been described [239].

Tetracyclines are the most used antibiotics for veterinary purposes [240]. Notably, the high homology between *C. difficile* RT078 isolates from human and swine has been emphasized by the common tetracycline resistance determinants found in both groups, strongly suggesting a zoonotic spread of *C. difficile* and resistance [241].

5.6. Chloramphenicol

Chloramphenicol inhibits bacterial protein synthesis by binding to the A-site of the 50S-ribosomal subunit [242]. Resistance to chloramphenicol is relatively uncommon in *C. difficile*, and is found in only 3.7% of the European isolates with a marked variation between countries that seem to reflect a localized association with specific RT, namely RT001, that apparently spread after acquiring resistance to chloramphenicol [146]. Resistance to this antibiotic is usually due to the presence of *catD*, which encodes a chloramphenicol acetyltransferase that catalyses the transfer of an acetyl group from acetyl-CoA to chloramphenicol,

thus rendering the antibiotic unable to bind to the ribosome [243], and is carried by a Tn4453 element [244]. Another gene, *cfr*, has been found to confer resistance to chloramphenicol and also to other classes of antibiotics in *C. difficile*, as already mentioned earlier (see section "*Clindamycin*").

5.7. Multidrug resistance in *C. difficile*

In 2005, 82 of 316 European *C. difficile* isolates were resistant to at least three antibiotics. The predominant phenotype among these strains, which mainly belonged to RT001, RT017 and RT012, was multiple resistances to clindamycin, erythromycin, moxifloxacin and rifampicin [218]. More recently, 27.5% of RT027 strains in the US were also multidrug resistant [225]. In China, 73.3% of the strains were multidrug resistant and mainly included strains from RT017, which is predominant in Asian countries, as discussed earlier [234]. Similarly, in 2012, 85.5% of Polish strains were multiresistant, being all strains from RT027, RT176 (related to RT027), RT012 and RT046 [217]. The recent pan-European study also associates specific RT with multidrug resistance, including RT027, RT001 and RT017, which are common RT in many countries, but also RT018 and RT356 that are predominant in Italy. Another highly resistant RT, RT356, also found in Italy, is resistant to rifampicin, moxifloxacin, clindamycin, imipenem and chloramphenicol [146]. Similarly, most of RT017 strains isolated in Portugal are resistant to moxifloxacin, clindamycin, erythromycin, rifampicin, tetracycline and imipenem (our unpublished data; see also earlier text and **Figure 3B**); it seems possible that the resistance to imipenem arose due to the intense use of carbapenems in Portugal in the last decade [245]. The recently described *cfr* gene in *C. difficile*, to which we alluded to earlier, is another concern since it provides multidrug resistance and the extent of its dissemination in *C. difficile* isolates is still unknown [221]. Notably, most of the ribotypes associated with multidrug resistance are epidemic and/or associated with increased CDI severity, which hints at multidrug resistance constituting a selective advantage for the spread and infective potential of these strains. Overall, these data indicate a clear association between prevalent RT and multidrug resistance, in line with the view that antibiotic pressure drives the evolution of these strains.

6. Novel strategies for treatment and prevention of *C. difficile* infection

Strategies to neutralize *C. difficile* target all different stages in the organism's life cycle. Chlorine-based disinfectants are used to reduce the load of spores in contaminated surfaces in healthcare facilities [246]. Triggering germination has been proposed as a method to enhance the killing of spores that accumulate in healthcare facilities, in combination with UV-C or disinfectants [247]. The recent demonstration of sporicidal activity of ceragenin CSA-13 is worth mentioning. Ceragenin CSA-13 is a cationic compound that mimics endogeneous antimicrobial peptides and shows a wide spectrum of bactericidal activity [248]. When incubated with *B. subtilis* spores, ceragenin CSA-13 appears to disrupt the inner spore membrane, causing the release of Ca^{2+}-dipicolinate from the core and loss of spore viability [248]. Although likely, activity against spores of *C. difficile* was not reported.

Antimicrobial stewardship is an important aspect in strategies designed to prevent and control CDI outbreaks, in addition to infection control and containment measures and environmental decontamination [229]. The importance of antibiotic stewardship practices is exemplified and stressed here by the identification of the *pbp1* and *pbp3* alleles in imipenem-resistant strains of *C. difficile* (see earlier; **Figure 5B**). Other reviews provide a detailed discussion on measures for the treatment and prevention of CDI [9, 229]. 'Breakthrough' approaches in the treatment and prevention of CDI fall into three main areas [9]: (i) antibiotic therapies, (ii) biotherapeutics and (iii) immunological therapies [9]. We review here some of the approaches in each of these broad areas, and also refer to the effect of diet in the control of *C. difficile*.

6.1. Antibiotics

As the broad-spectrum metronidazole and vancomycin have a considerable impact on the microbiota and show high recurrence rates, there has been an intense search for new anti-microbials of narrower spectrum. The narrow-spectrum antibiotic fidaxomicin, approved by the Food and Drug Administration (FDA) in 2011, is an example. It is a macrocyclic non-systemic antibiotic shown to be highly selective against *C. difficile* and superior to vancomycin in eliciting a sustained clinical response, that is, cure without disease recurrence [249]. Several aspects of fidaxomicin action may contribute to its efficacy. *In vitro*, the drug prevents toxin production [250], and when added to cultures at the onset of stationary phase, fidaxomicin also prevented entry into sporulation [251]. It also prevented the outgrowth of cells from germinated spores (it did not prevent the initiation of spore germination, however) [252]. The inhibition of spore outgrowth and of sporulation may be the main factors contributing to the efficacy of fidaxomicin in suppressing disease recurrence and transmission (**Figure 2**).

Other narrow spectrum antimicrobials are undergoing clinical trials (www.clinicaltrials.org). Surotomycin, also called CB-183,315, is a lipopeptide antibiotic structurally related to daptomycin which is currently in phase III clinical development. As is the case for daptomycin, Surotomycin may work by dissipating the membrane potential [253]. *In vitro* studies showed activity of Surotomycin against *C. difficile* isolates with elevated MICs for metronidazole, moxifloxacin and vancomycin but lack of activity against Enterobacteriaceae and species of the *Bacteroides fragilis* group [254, 255], suggesting that it may not cause significant disruption of the microbiota.

Another example is Cadazolid. This drug is primarily a protein synthesis inhibitor, but the molecule also includes a fluoroquinolone moiety that acts as a weak inhibitor of DNA synthesis at much higher concentrations of the drug [256]. The addition of Cadazolid to stationary phase cultures inhibits the production of the TcdA and TcdB toxins and spore formation, while in both the hamster and mouse models Cadazolid was as effective as vancomycin [257]. Trials suggest that Cadazolid may be as effective as vancomycin, but with lower recurrence rates [9].

Ridinilazole [2, 2'-bis(4-pyridyl)3H,3'H 5,5'-bibenzimidazole] was more active than fidaxomicin, metronidazole and vancomycin against *C. difficile*; it was less effective against other intestinal bacteria, including the *B. fragilis* group, and Gram-positive species of *Bifidobacteria*,

among others [258, 259]. Phase II trials are under way to investigate the efficacy of Ridinilazole in comparison to fidaxomicin and vancomycin. The mechanism of action of Ridinilazole has not been described. The addition of Ridinilazole to *C. difficile* cultures at concentrations below the MIC results in cell filamentation, however, suggesting that it may act as a cell division inhibitor [260].

CRS3123 (formerly REP3123) is a synthetic diaryldiamine that inhibits methionyl-tRNA synthetases and protein synthesis in Gram-positive bacteria [261]. *In vitro*, CRS3123 inhibits the growth of *C. difficile* and blocks toxin and toxin production when added to stationary phase cultures; CRS3123 demonstrated good potency against *C. difficile*, but was much less active against other bacteria of the normal intestinal flora [261–263]. It also proved superior to vancomycin in the hamster model of CDI [263]. Clinical trials to determine the efficacy and pharmacokinetics of CRS3123 have been completed but no results are yet posted.

The newly discovered SEDS-type transglycosylases, which appear as promising targets for new antibiotics, are worth noting. Screening of a library of actinomycete strains against a *B. subtilis* strain lacking all four Class A PBPs (transglycosylases/transpeptidases) led to the identification of a compound, 654/A, that caused large zones of inhibition on plates of the indicator strain (but not of the congenic WT) and morphological defects characteristic of mutants impaired in cell wall synthesis [136]. Consistent with the possibility that 654/A targets a SEDS transglycosylase, not only it acted synergistically with moenomycin, an inhibitor of the transglycosylase domain of Class A PBPs, but overproduction of the elongation-specific SEDS protein RodA mitigated its effects [136]. 654/A was also active against *S. aureus*. Activity against *C. difficile* was not reported.

6.2. Bacteriotherapies

Among the class of bacteriotherapies are the use of probiotics, spores of non-toxinogenic strains, faecal microbiota transplantation (FMT) and precise manipulation of the gut microbiota. While a number of probiotic formulations are under clinical testing, evidence for their efficacy in the treatment of CDI is presently controversial [9]. FMT, on the other hand, which relies on the disruption of dysbiosis in patients undergoing antibiotic treatment, has been used with a success rate of over 90% [9]. Difficulties associated with FMT, such as poor reproducibility, availability of the material and patient acceptance, could in principle be solved by the identification of the bacteria in faecal material responsible for the beneficial effect. The isolated strains could be produced and formulated under controlled conditions and used for therapeutic interventions. This quest is supported by several findings. Gut dysbiosis could be disrupted by a mixture of six intestinal bacteria (including S. *warneri*, *Enterococcus hirae*, *Lactobacillus reuteri*, and three novel species of *Anaerostipes*, *Bacteroidetes* and *Enterohabdus*), and this mix could clear infection by a RT027 strain in mice [40]. Also, a defined mixture of 17 gut-indigenous strains, enriched in Clostridia, isolated from the faeces of healthy humans, induced the differentiation of gut-regulatory T cells [264, 265]. A more defined intervention is illustrated by the finding that the administration of *C. scindens*, both in mice and in humans, enhances resistance to infection in a secondary bile acid-dependent manner [77]. While the depletion of the bile acid-hydroxylating activity of *C. scindens* caused by antibiotics, and the

decreased resistance to CDI clearly illustrates the link between antibiotics, bile acid metabolism and susceptibility to CDI, restoration of secondary bile metabolism may be a key mechanism for the success of FMT in treating recurrent CDI [266, 267]. While these interventions rely on the bacteria that are transferred to the compromised host, a very recent report indicates that the transfer of sterile filtrates from donor faeces to patients with a diagnostic of CDI is sufficient to eliminate symptoms [268]. The authors of this study suggest that bacterial components, metabolites or perhaps phages mimic or substitute for many of the effects of FMT [268].

Also in the category of the biotherapeutic approaches to control and prevent CDI is the oral administration of spores of non-toxinogenic strains. A strain, NTCD-M3, isolated at high frequency from hospitalized patients asymptomatically colonized, was found to lack the toxin-encoding genes [269]. Spores produced by NTCD-M3 were given orally to patients under metronidazole or vancomycin treatment for their first episode of CDI or first CDI recurrence [270, 271]. Colonization was a function of the number of spores given daily. Recurrence was lower in patients receiving spores relative to the control group and correlated with colonization. Colonization was transient and lost after 22 weeks, perhaps because of the recovery of the gut microbiota [270, 271]. Presumably, recurrence was prevented because NTCD-M3 outcompeted toxinogenic strains and prevented further colonization by the latter strains. The exact mechanism, however, is not known. So far, the transfer of the PaLoc from toxinogenic to non-toxinogenic strains has only been detected *in vitro* [272].

6.3. Vaccines

Antibodies directed against the receptor-binding domains of the TcdA and TcdB toxins prevent binding to their receptors and confer protective immunity against CDI in animal models and protection against recurrent disease in humans [10, 11]. Three vaccine candidates are currently under development for CDI, all of which involve parenteral delivery of toxoids. Sanofi has a toxoid vaccine composed of partially purified and formalin-inactivated TcdA and TcdB toxins [273, 274]. The Pfizer vaccine contains mutant forms of the TcdA and TcdB toxins with mutations thought to abrogate glucosyltransferase and auto-protease activities; residual activity of the toxins, however, required pre-incubation of the antigens with specific antibodies or formalin [275]. The Valneva vaccine contains a recombinant fusion protein between the receptor-binding domains of both TcdA and TcdB which induced neutralizing levels of serum antibodies to both toxins and reduced disease severity while conferring significant protection against a lethal dose of *C. difficile* spores in hamsters [276].

In an alternative strategy, *B. subtilis* spores were used as a delivery vehicle for the carboxy terminal repeat domains of TcdA and TcdB fused to surface-exposed spore coat proteins [277]. Oral immunization with spores displaying the TcdA repeat domain alone conferred protection against challenge with a *C. difficile* strain producing both toxins, and vaccinated animals survived reinfection. Mucosal immunization was required to generate secretory IgA and the local production of these neutralizing polymeric antibodies correlated with protection [277]. A trial to assess safety and immunogenicity of a spore-based vaccine (CDVAX) started on 1 January 2017.

6.4. Diet

Finally, recent work has highlighted a role for the diet in the prevention of CDI. Zn has a role in modulating the diversity of the microbiota: mice fed with a high Zn diet showed decreased microbiota diversity, as opposed to mice on a low Zn diet [278]. A Zn-binding protein, Calprotectin added to *C. difficile* cultures, prevented growth in a Zn-dependent manner (as a mutant deficient in Zn binding did not prevent growth), and Calprotectin-deficient mice showed decreased survival and increased disease severity following challenge with an RT027 strain [278].

Acknowledgements

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