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REVIEW ARTICLE

Clostridium difficile, the Difficult "Kloster" Fuelled by Antibiotics

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Abstract
Clostridium difficile is normally present in low numbers in a healthy adult gastro-intestinal tract (GIT). Drastic changes in the microbial population, e.g., dysbiosis caused by extensive treatment with antibiotics, stimulates the growth of resistant strains and the onset of *C. difficile* infection (CDI). Symptoms of infection varies from mild diarrhea to colitis (associated with dehydration and bleeding), pseudomembranous colitis with yellow ulcerations in the mucosa of the colon, to fulminant colitis (perforation of the gut membrane), and multiple organ failure. Inflamed epithelial cells and damaged mucosal tissue predisposes the colon to other opportunistic pathogens such as *Clostridium perfringens, Staphylococcus aureus, Klebsiella oxytoca, Candida* spp., and *Salmonella* spp. This may lead to small intestinal bacterial overgrowth (SIBO), sepsis, toxic megacolon, and even colorectal cancer. Many stains of *C. difficile* are resistant to metronidazole and vancomycin. Vaccination may be an answer to CDI, but requires more research. Success in treatment with probiotics depends on the strains used. Oral or rectal fecal transplants are partly effective, as spores in the small intestine may germinate and colonize the colon. The effect of antibiotics on *C. difficile* and commensal gut microbiota is summarized and changes in gut physiology are discussed. The need to search for non-antibiotic methods in the treatment of CDI and *C. difficile*-associated disease (CDAD) is emphasized.

Introduction

Clostridium difficile infection (CDI) is contagious, as shown by spreading of one of the ribotypes (027) throughout the world (Fig. 1). Symptoms range from asymptomatic, severe abdominal pain, diarrhea to the development of a toxic megacolon [19]. *C. difficile*-associated colitis (CDAC) is characterized by an erythematous mucosa, friability, and bleeding. In more severe cases yellow plaques (small ulcerations) form and is described as pseudomembranous colitis, with clear lesions on the gut wall. Fulminant colitis, described as perforation of the gut membrane, develops in 3–8% of the patients and may resulst in multiple organ failure [19].

CDI is impelled by the uncontrolled growth of *C. difficile* in the large intestinal tract as a result of drastic changes in the gut microbiome [7, 8]. According to Durovic et al. [20], the most common route for transmission of *C. difficile*, based on the number of infection cases reported, is through healthcare settings (63%) and contact with symptomatic carriers (53%), followed by transfer between patients in hospitals (40%) and long-term care facilities (30%). Of interest is that 20% of the reports mentioned contact with asymptomatic carriers and exposure to livestock as a possible sources of infection [20]. CDI is almost always associated with antibiotic treatment and an estimated 61% of patients diagnosed with iriitable bowel disease (IBD) and exposed to antibiotics developed CDI [39]. Barc et al. [4] have shown that treatment with amoxicillin-clavulanic acid resulted in increased levels of Bacteroidetes and Enterobacteriaceae, with a simultaneous decline in the *Clostridium coccoides*-Eubacterium rectale group. Other studies have shown that 30 days of exposure to antibiotics increased the risk of developing CDI by a factor of 12.0 [45].

Long-term treatment with broad-spectrum antibiotics such as cefoperazone, clindamycin, vancomycin, ampicillin, amoxicillin, cephalosporins, and fluoroquinolones are almost always associated with the development of CDI. Patients treated with these antibiotics showed a drastic decline in Firmicutes, especially from the families Lachnospiraceae and Ruminococcaceae [2, 93, 94]. This usually co-insides with a depletion in secondary bile acids, e.g., deoxycholate

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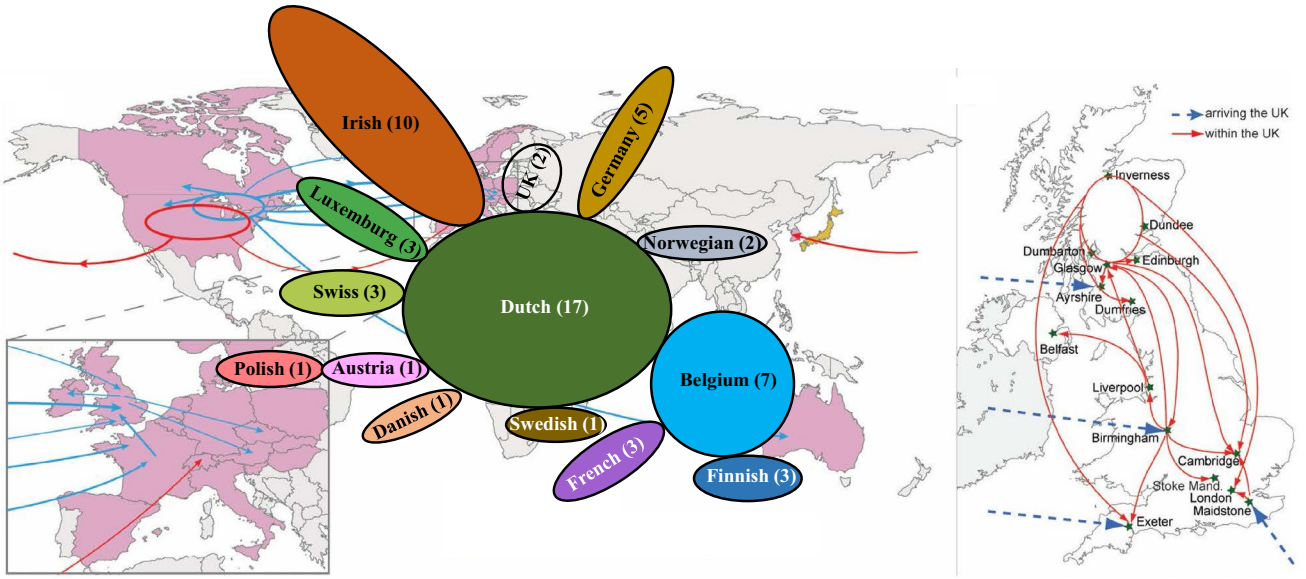

Fig. 1 Spreading of *C. difficile* ribotype 027 isolates in 14 European countries by 2010, as recorded with data generated by multilocus variable-number tandem-repeat analyses [26]. Numbers in brackets refer to the number of 027 isolates per country. Isolates in 10 of the countries were genetically related to isolates reported in The Netherlands. Isolates in France and Finland were genetically more related to isolates in Belgium and the Isolate from Poland closer related to the isolate from Austria. The spreading of ribotype 027 isolates from the USA to other continents is shown in the background. The background image was taken from He et al. [31]

(DCA), lithocholate (LCA), ursodeoxycholate (UDCA), hyodeoxycholate and muricholate [2, 82, 93]. These sudden changes in the GIT stimulates the adherence of *C. difficile* to epithelial cells and mucus, leading to high levels of colonization, especially in the colon [10]. The impact of antibiotics on 12 of the most prominent bacteria in the human GIT (*Bacteroides, Bifidobacterium, Clostridium, Enterococcus, Escherichia, Faecalibacterium, Fusobacterium, Lactobacillus, Prevotella, Staphylococcus, Streptococcus*, and *Veillonella*) is shown in Fig. 2.

Age Matters

Prevalence of *C. difficile* in the GIT of healthy adults may be as high as 17% [52, 70]. A small percentage of the adult population (approximately 4%) are asymptomatic carriers of *C. difficile* [69, 89]. This suggests that the microbiota of a healthy gut suppresses the growth of *C. difficile*, or the outgrowth of endospores, and that the immune system of healthy individuals is fully tuned into preventing an outbreak in the GIT. However, physiological decline over time renders elderly more prone to acquiring/developing CDI [34, 59]. In contrast, the majority of infants colonized with *C. difficile* are asymptomatic [40, 81], possibly due to the absence of toxin-binding receptors in the infant gut. Asymptomatic infants develop antibodies to *C. difficile* enterotoxin A (TcdA) and cytotoxin B (TcdB), which suggests that they may develop a life-long immune response [99]. Colonization of *C. difficile* almost always occurs in the presence of *Ruminococcus gnavus* and *Clostridium nexile*. Both species produce a trypsin-dependent antimicrobial substance active against *C. perfringens*, but with less of an effect on *C. difficile* [65].

Growth and Adhesion to the Gastro-Intestinal Tract

Clostridium difficile converts succinate to butyrate [16, 23, 24]. Butyrate supports the germination of endospores [97] and increases the production of intestinal antimicrobial peptides, stimulates mucin production, and decreases the permeability of epithelial cells by preventing the formation of tight junction proteins [80]. Furthermore, butyrate plays an important role in regulating the expression of host genes involved in inflammation, cell differentiation, and apoptosis [12, 27, 84].

Excess bile acids in the colon stimulates the germination of *C. difficile* endospores [86, 104]. Chenodeoxycholate (CDCA) inhibits spore germination and the outgrowth of vegetative cells [86]. Deoxycholate (DCA), on the other hand, stimulates spore germination, but inhibits the growth of *C. difficile* [85–87]. Primary bile acids (TCA, CA) increase in the GIT after a course of antibiotics with concomitant decline in secondary bile acids, potentially promoting CDI [88].

The ability of *C. difficile* to adhere to gut epithelial cells and mucus may be ascribed to the number of virulence factors, of which proteolytic enzymes (i.e., cysteine protease), adhesins (cell-wall protein Cwp66, the GroEL heat-shock

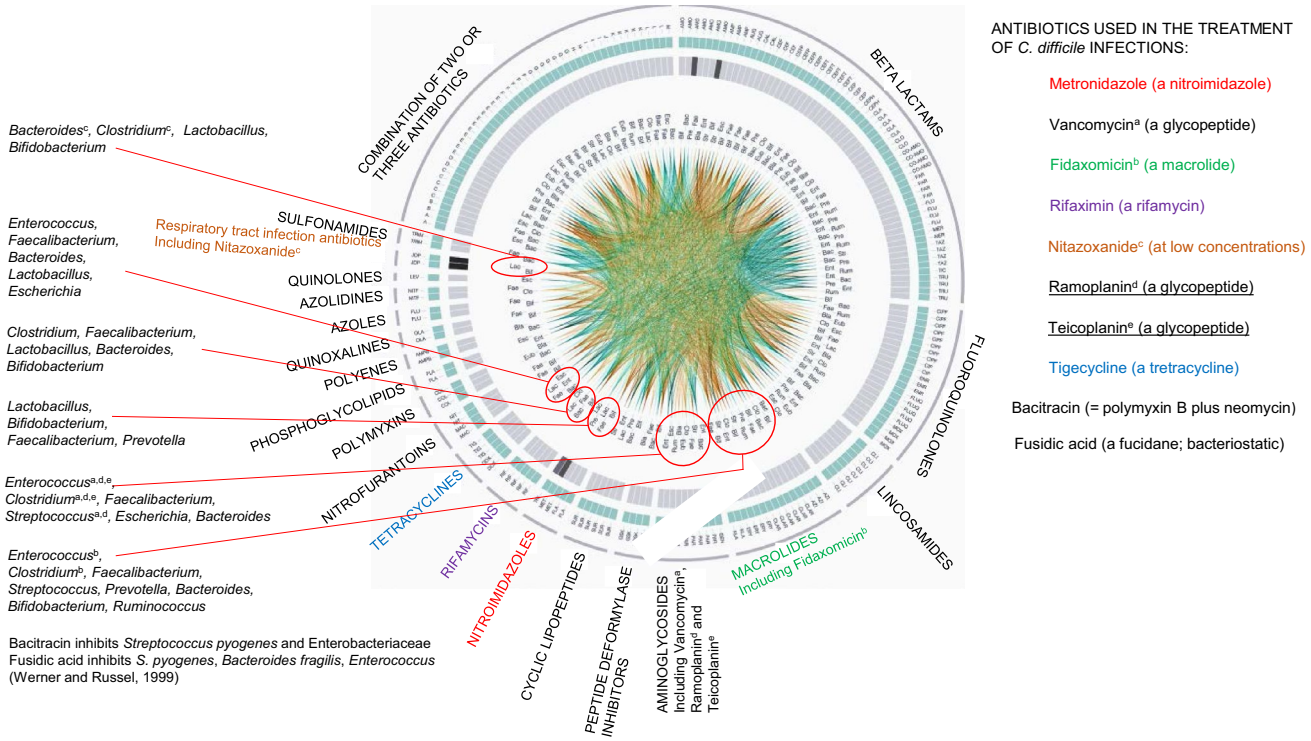

Fig. 2 The effect of nine antibiotics (metronidazole, vancomycin, fidaxomicin, rifaximin, nitazoxanide, ramoplanin, teicoplanin, tigecycline and bacitracin) and Fusidic acid on *C. difficile* and 11 commensal genera *Enterococcus, Lactobacillus, Ruminococcus, Faecalibacterium, Streptococcus, Eubacterium* and *Blautia* (within Firmicutes phylum), *Prevotella* and *Bacteroides* (Bacteroidetes), *Bifidobacterium* (Actinobacteria) and *Escherichia* (Proteobacteria) in the GIT. Modifed from Rojo et al. [79]. Data on vancomycin, fidaxomicin, nitazoxanide, ramoplanin and teicoplanin were from https://www.drugs.com/mmx/vancomycin-hydrochloride.html (accessed 22/03/2018) and http://antimicrobe.org/new/drugpopup/Teicoplanin.pdf (accessed 22/03/2018)

protein and a 68 kDa fibronectin binding protein), and flagella components FliC (flagellin) and FliD (flagellar cap protein) have been the best studied [32, 33, 92, 101]. Infection with *C. difficile* causes weakening of tight junctions in the epithelial barrier, which leads to drastic changes in permeability across the gut wall, including the translocation of bacteria and their products, and the infiltration of neutrophils into the lumen. Neutrophils, together with fibrin, form plaques, visible as pseudomembranes, on the colonic wall [28]. Enzymes produced by the activated neutrophils and the release of reactive oxygen species interact with the enterotoxins of *C. difficile* and destroy tissue cells. Transmigration of recruited neutrophils to the mucosa involves the expression of leukocytes and endothelial cell adhesion molecules, driven by the production of a wide range of chemoattractants and the activation of cytokines. IL-8 is the principle cytokine involved in migration. The activation of neutrophils and molecules released from immune and epithelial cells is a critical step in the inflammatory cascade following colonization by *C. difficile*. The molecular mechanism by which *C. difficile* induces the inflammatory process involves activation of NFκB, which appears to be required for transcription of the IL-8 gene [42, 51].

Toxins

The genes encoding toxins A and B (*tcdA* and *tcdB*) are located on the chromosome of *C. difficile*, within the 19.6-kilobase (kb) pathogenicity locus (PaLoc) along with the three accessory genes (*tcdC, tcdD* and *tcdE*) [55]. Toxins A and B share a 49% amino acid homology [71]. The N-terminal domain of both toxins possesses cytotoxic activity. The C-terminal domain of the two toxins bind to the epithelial cells [101]. The transmembrane domain facilitates the entry of the two toxins into the cytoplasm. The *tcdD* gene product up-regulates toxin transcription, whilst *tcdC* encodes a toxin gene repressor [101]. The protein encoded by *tcdE* lyses the cell wall and releases toxins A and B into the colonic lumen [91].

Toxin A binds to a trisaccharide, composed of α-Gal-(1,3)-β-gal-(1,4)-β-GlcNac [53, 54]. This implies that toxin A binds to human enterocytes via different oligosaccharides, or possibly by protein–protein interaction. The disaccharide β-Gal-(1,4)-β-GlcNac, present in humans, is most likely one of the receptors. Little is known about the receptors required by toxin B. After adhesion to the colonic cells, the toxins are translocated into target cells through receptor-mediated

endocytosis and start their destructive processes by inactivating guanosine triphosphate (GTP)-binding proteins of the proteins Rho, Rac, and Cdc42 involved in cell signaling [41, 44]. The inactivation of GTP-binding proteins is mediated by catalyzing the transfer of a glucose residue from UDP-glucose to GTP-binding Rho proteins [102]. Glucosylation of Rho GTPases leads to actin cytoskeleton disaggregation, increased membrane permeability, loss of barrier function, cell rounding, cytotoxicity, and ultimately cell death [100]. The toxins induce the formation of microtubule protrusions on the surface of intestinal epithelial cells, which supports the adherence of *C. difficile* [83]. Massive cellular immune responses generated by the toxins stimulate neutrophils to infiltrate the site of infection and upregulate the release of cytokines such as IL-8, IL-6, IL-1β, leukotrienes B4 and interferon γ [100]. This leads to pseudomembranous colitis, visualized with endoscopic examinations as raised, white or yellowish nodules of 2–10 mm in diameter on the surface of the colon [100]. Symptoms of CDI include mild or severe diarrhea, abdominal pain, fever, leucocytosis, and may develop hypoalbuminemia as albumin in the exudate from ulcers enters the colonic lumen. Acute diarrhea with hypoalbuminemia is a good indicator of CDI [100].

Karlsson et al. [46, 47] have shown that toxins produced by *C. difficile* may be regulated by amino acids in the colon. Cysteine, glycine, isoleucine, leucine, methionine, proline, threonine, tryptophan, and valine reduced toxin production 100-fold, whereas a combination of alanine, arginine, aspartic acid, histidine, lysine, phenylalanine, serine, and tyrosine had no effect on toxin production [47].

Where Does *C. difficile* Fit into the Human Microbiome?

The majority of bacteria in the GIT belongs to the Firmicutes and Bacteroidetes phyla, and to a lesser extend proteobacteria, actinobacteria, verrucomicrobia, and cyanobacteria [21, 61, 95]. Bacteroidetes and Proteobacteria are more prevalent in individuals older than 70 years. Sudden changes in the gut microbiome induce alterations to the protective mucus layer, resulting in changes of the mucosal immune system, loss in integrity of the epithelial barrier, and changes in peristalsis and the absorption of nutrients [3, 49, 66, 108]. In a state of dysbiosis, major changes are observed in the production of vitamins and ion absorption, and conversion of dietary polyphenolic compounds into active forms [22]. Inflamed epithelial cells and damaged mucosal tissue predisposes the GIT to *C. difficile* and other opportunistic pathogens, which gives rise to IBD, small intestinal bacterial overgrowth (SIBO), functional gastro-intestinal disorders (including IBS), and sometimes colorectal cancer [90]. Patients treated with antibiotics over an extended period run the risk of developing an overgrowth of *C. difficile*, the causative agent of 10–20% of all AAD [5, 6, 50], colitis, pseudomembranous colitis (PMC), toxic megacolon and sepsis [19]. Other pathogens associated with AAD, but to a much lesser extent, are *C. perfringens, Staphylococcus aureus, Klebsiella oxytoca, Candida* spp., and *Salmonella* spp. [36].

Treatment

Initial, mild, or moderate episodes of CDI are treated with oral metronidazole (500 mg three times per day for 10–14 days) [13]. Severe episodes of CDI are treated with oral vancomycin (125 mg four times per day for 10–14 days) [13]. Severe, complicated CDI is treated with increased levels of vancomycin (500 mg, orally, four times per day), plus metronidazole (500 mg every 8 h intravenously). Vancomycin may also be applied rectally [13]. Although metronidazole is the antibiotic of choice, failure rates of 22–38% have been reported [67, 68]. Treatment with metronidazole and vancomycin yielded the same success rates with mild CDI. In severe cases of CDI, the eradication of *C. difficile* was more successful with the administration of vancomycin (97% cure rate), compared to metronidazole with a 76% cure rate [78, 106]. Based on this report, vancomycin is the first line treatment for severe and mild CDI. Critical cases of CDI may require much more aggressive treatment, e.g., 2 g vancomycin per day [71].

Resistance to metronidazole was reported in a study on patients infected with ribotype 027. As many as 20% of patients successfully treated with metronidazole experienced a relapse of CDI [60, 107], labeling the treatment of CDI with antibiotics as a risk factor [40, 99]. Treatment of a first episode recurrent infection with a repeat course of either metronidazole or vancomycin for 10–14 days was successful in approximately 50% of patients [37, 58]. In a few critical cases, patients were treated with fidaxomicin, a macrocyclic antibiotic approved by the food and drug administration (FDA) in 2011. Rifaximin, nitazoxanide, ramoplanin, teicoplanin, and tigecycline have also been used to treat CDIs. Case studies using these antibiotics are, however, few and treatment is expensive, thus limiting their use. Bacitracin and fusidic acid have also been used in the treatment of CDI, but their efficacy has not been proved superior to vancomycin and metronidazole [103].

Alternative treatments include immunoglobulins, vaccination, novel antibiotics and probiotics. Glucose, sialic acid, and *N*-acetyl glucosamine are limiting growth factors and are considered important in the exclusion of *C. difficile* from the GIT [104]. The large intestine is lacking free glucose [9], but contains adequate amounts of amino acids. Thus, *C. difficile* compete against commensal microbiota for amino acids and, if not available, produce toxins. This explains why protein malnutrition, which is often the case amongst elderly people,

may be a risk factor for CDAD, especially when exposed to antibiotics.

More research is needed to find mechanisms that would neutralize the toxins produced by pathogens and avoid damage to the mucosa and epithelial cells. More in-depth studies on antibiotic-gut wall interactions need to be done. Erythromycin and a combination of amoxicillin and clavulanate (trade name Augmentin), for instance, increase gut motility and may exacerbate CDI. Further research on the effect of prolonged treatment with immune suppressants, H_2-receptor antagonists and proton-pump inhibitors (PPIs) need to be conducted. According to Thomson et al. [96], PPIs enhances the survival of *C. difficile* through the stomach, thereby supporting the hypothesis that PPI's may initiate CDI.

Probiotics and Fecal Transplants

Floch et al. [25] claimed relieve from AAD after treating patients with combinations of *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii*, and *Lactobacillus casei* DN-114001, *Lactobacillus delbrueckii* subsp. bulgaricus and *Streptococcus thermophilus*. Gorbach [30] reported an increase in IgA- and other immunoglobulin-secreting cells in the intestinal mucosa of patients that received *L. rhamnosus* GG. This resulted in enhanced immune response to *C. difficile* and its toxins and was in agreement with a previous study by Mack et al. [63], showing that *L. rhamnosus* GG may protect the gut barrier [64]. Littman and Pamer [62] and Van Baarlen et al. [98] have shown that *L. rhamnosus* GG lowered the levels of TNF-α, chemokine CCL20, IL-12, IL-2, IL-23, and IL-27 and, by doing so, prevented damage to the epithelial barrier. Soluble proteins p40 and p70 from *L. rhamnosus* GG inhibited cytokine-induced apoptosis and disruption of the epithelial barrier. These proteins are important in regulating the integrity of the epithelial barrier by maintaining tight junction and adhesion junction proteins.

Littman and Pamer [62] and Van Baarlen et al. [98] reported that peptidoglycan from *L. casei* decreased the secretion of IL-12 and IL-23 by dendritic cells involved in IBD. In another study [74], the administration of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* seemed to have a neutralizing effect on the toxins of *C. difficile*, as only 46% of patients that received the probiotic were toxin-positive, compared to 78% of patients in the placebo group. This suggested that many of the patients treated with the probiotic were asymptomatic carriers.

Lawley et al. [57] have shown that the colonization of *C. difficile* to epithelial cells could be prevented by administering a mixed culture of *Staphylococcus, Enterococcus, Lactobacillus, Anaerostipes, Bacteroidetes*, and *Enterorhabdus. Saccharomyces boulardii* upregulated the expression of antitoxin A secretory immunoglobulin A expression in animal models of CDAD and inhibited the binding of toxin A to epithelial cells [11, 75]. A mixed culture of non-toxigenic *C. difficile, Escherichia coli, Bifidobacterium bifidum*, and members of Lachnospiraceae prevented the colonization of *C. difficile* in germ-free mice [14, 77].

A meta-analysis study conducted by Lau and Chamberlain [56] has shown that probiotics are associated with a 60.5% reduction in the incidence of CDAD and that the use of a combination of strains (*Lactobacillus, Saccharomyces*, and several lactic acid bacteria) reduced the risk of CDAD by 63.7, 58.5, and 58.2%, respectively. The general reduction in CDAD reported for adults administerd probiotics was 59.5% and for children 65.9% [55]. The risk reduction for hospitalized patients was 61% [56].

Despite these positive reports, many research groups are less optimistic about using probiotics in the treatment of CDI. This opinion is supported in a review published by the Cochrane Group [73]. Whilst the authors concluded "moderate quality evidence suggests that probiotics are both safe and effective for preventing *C. difficile*-associated diarrhea", they were of the opinion that more research and case studies are required to provide sufficient evidence in support of probiotic therapy [35]. However, in a more recent systematic review and meta-analysis by the Cochrane Collaboration, a more firm conclusion on the general positive effects of probiotics in CDAD was made [29]. Isa and Moucari [38] stated that it is "difficult to draw any solid conclusion about the prophylactic use of probiotics in AAD", but advised patients with a history of AAD to take probiotics as a prophylactic measure and lower the risk of developing CDAD. Yet, the authors still warrants more research to be conducted on probiotics and CDAD. The UK Health Protection Agency good practice guidance for the management of *C. difficile* infection [17] does not support the use of probiotics in the prevention or treatment of CDI.

The World Gastroenterology Organization [105] is of the opinion that *S. boulardii* or *L. rhamnosus* GG may be used to treat AAD. The WGO also advocated the use of *L. casei* DN-114001 in the prevention of AAD and CDAD. In addition, bacteria such as *Lactobacillus* can have a direct antimicrobial activity by secretion of bacteriocins and other antimicrobial peptides [43]. Given the low cost of probiotics and that no negative effects have been reported in the treatment of CDI, the administering of probiotics to patients receiving antibiotics should be encouraged.

Bacteriocins have many beneficial properties which make them viable alternatives to antibiotics. These include their potency and high specific activity against pathogens, thereby causing less collateral damage to the gut microbiota [76]. Lantibiotics and thiopeptides are generally more active against Gram-positive strains [15].

Lactobacillus reuteri Lr1, isolated from healthy horses, adhered to buccal epithelial cells and aggregated with cells of *C. difficile* C6, isolated from the GIT of a horse that died

from severe colic. Adherence of *C. difficile* C6 to epithelial cells declined from 60 to 3% when challenged with *L. reuteri* Lr1 and the number of viable clostridia decreased ten-fold during dosage. *L. reuteri* Lr1 may thus be used to control *C. difficile* cell numbers in the GIT [18].

Oral or rectal fecal transplants from healthy individuals to patients with CDI may restore secondary bile acids and cell numbers of Lachnospiraceae [72]. Kassam et al. [48] reported the successful treatment of more than 90% of patients with recurrent CDI by using fecal transplants. The precise components of the fecal microbiome that provide resistance against *C. difficile* are not known, but the phyla Bacteroidetes and Firmicutes represents a critical component [1, 85]. The treatment is, however, only partly effective, as spores in the small intestine may still germinate and colonize the GIT. Collecting samples from the small intestinal tract of humans is difficult and most findings are extrapolated from studies conducted on murine models [58]. Nevertheless, data collected from murine studies are of value, as the bile acid composition in the murine and human intestinal tract is very similar.

Conclusions

Clostridium difficile lives up to its name, i.e., being a difficult spindle (Kloster) to control, especially in the GIT of patients that have been exposed to excessive doses of antibiotics and with a weakened immune system. In a healthy gut, cell growth, and the germination of endospores of *C. difficile* are repressed by commensal microorganisms, of which lactic acid bacteria forms the largest group. Although alternative options have been evaluated to treat CDI, best results are still obtained by treating patients with vancomycin and metronidazole. Most of the major bacterial groups in the GIT are negatively affected by antibiotics, resulting in dysbiosis. Furthermore, treatment with antibiotics may soon be ineffective, as many strains of *C. difficile* have developed resistance to most of the antibiotics currently in use. Apart from *S. boullardii*, only a few species of lactic acid bacteria, mainly *Lactobacillus* spp., have been experimented with in the treatment of CDAD. All of these studies have clearly shown that the antimicrobial effect against *C. difficile* is strain-specific. The challenge is thus to find strains that would colonize the GIT effectively and outcompete *C. difficile*. This requires an in-depth study on *C. difficile* receptors in the mucus and epithelial cells, and a better understanding of the cellular interactions between the competing strains, especially on a molecular level. Probiotics may never cure patients from CDI, but may prevent or control the adhesion of *C. difficile* to the GIT and make life of a difficult "Kloster" more difficult.

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Compliance with Ethical Standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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