Clostridioides difficile pathogenesis and control

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[Au: Thank you for writing this important Review for *Nature Reviews Microbiology*. All our articles are thoroughly and often heavily edited, taking into account clarity, language, scientific correctness, consistency and house style, to ensure they meet our high publication standards. The majority of changes have been made to bring the article in line with house style and to tighten up the narrative flow. These changes are just suggestions and you should feel free to discuss them with me. At places marked [Au:OK?], I'd be grateful if you could check especially carefully that the meaning of what you wrote has not been altered by the editing. I have also asked a few questions where I felt a bit more information or clarity was needed.]

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Abstract [Au: Just to note, that the abstract can use up to 150 words (currently it uses 115), so there is space for a bit more text here.]

Clostridioides difficile infection (CDI) continues to be a notable burden worldwide, both in terms of patient mortality and morbidity, and the economic costs associated with treatment, diagnosis

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and management. The epidemiology of *C. difficile* has changed markedly over the decades, with high CDI rates driven by clinical pressures exacerbated by the SARS-CoV2 pandemic, antibiotic resistance and selective pressures caused by antimicrobial use **[Au: The abstract should only mention topics covered in the review and antibiotic availability in different parts of the world is not discussed in the main text. As such, I suggest streamlining this sentence and instead adding sentences into the abstract on key topics of this review, for example, pathogenic mechanisms plus a bit more on the microbiota. I suggested some text but please feel free to amend this as you see fit, OK?]** . *C. difficile* is challenging to diagnose and treat as it forms spores and can ~~also~~ persist asymptomatically within the gut ~~without causing symptoms., and~~ Some strains express multiple virulence factors, including adhesins and toxins. The gut microbiota is crucially important in CDI, as a healthy microbiota is resistant to colonization with *C. difficile*. Dysbiosis, often caused by antimicrobial exposure, enables *C. difficile* spores to germinate and produce toxin, causing symptoms which can range from mild diarrhoea to fulminant colitis and death. This Review describes changes in epidemiology and effects on diagnosis, discusses recent breakthroughs in the understanding of pathogenesis and antibiotic resistance, and explores the role of microbiota dysbiosis in CDI and novel microbiota therapies in CDI treatment.

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[H1] Introduction

Clostridioides difficile (reclassified from '*Clostridium difficile*' in 2016 (ref.[1]) **[Au: Ref formatting adjusted for clarity as the citation follows a number.]**) is a Gram-positive, obligately anaerobic, spore-forming bacillus. This microorganism is ubiquitous and transmitted between hosts via the faecal–oral route. *C. difficile* produces toxins that can cause clinical symptoms,[2] and a spectrum of disease that ranges from mild, self-limiting diarrhoea through to fulminant disease, pseudomembranous colitis, sepsis, toxic megacolon and death **[Au: Edits for flow, OK?]** .[3,4] **[Au: In the introduction, it could be helpful to specify early that individuals can be asymptomatic carriers and perhaps the rate of asymptomatic carriage, if this is known (or include information on carrier rates in the epidemiology section)?]**

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C. difficile was first identified in 1935 (ref.[5]) and was determined to be the causative agent of pseudomembranous colitis in 1978 (ref.[6,7]). The incidence and epidemiology of *C. difficile* infection (CDI) has changed dramatically since this time, but *C. difficile* continues to cause notable **[Au: Journal style uses 'significant' in the context of statistical**

significance, so I have edited the text throughout to use alternative words in appropriate places.] economic burden worldwide, which is exacerbated by high rates of recurrent disease. Rates can vary, but approximately 30% of patients with CDI **[Au: Please note, we use patient-first language and the text has been edited accordingly throughout.]** are thought to go on to develop a recurrence **[Au: after resolution of symptoms or after treatment with antibiotics?]** , with most recurrent disease occurring within the first 8 (especially 4) weeks following resolution of symptoms **[Au: Edits OK? Please see previous comment.]** .[8] Approximately 50% of these patients **[Au: patients with a single occurrence of recurrent CDI? If so, we could say 'Approximately 50% of these patients…' for clarity.]** then go on to develop multiple recurrent infections.[9] Recurrent disease is associated with 33% higher all-cause mortality,[10] 2.5 times higher hospital admission rate, 4 times longer hospital stay,[11] and significantly higher costs than the initial CDI episode (mean total costs £12,710 versus £31,121; P<0.002).[12] *C. difficile* strains can differ in their propensity to spread and cause disease, with PCR ribotyping being the gold standard for identifying and tracking strains.[13] (See Box 2 for details of typing methods). Ribotype 027 (RT027) strains have been particularly epidemic, causing substantial **[Au:OK? Or we could use 'large' or 'huge' here. Nature Reviews style prefers to avoid emotive language when considering healthcare.]** problems in healthcare facilities worldwide.[14]

The major risk factors for primary CDI are advanced age, duration of hospitalisation and exposure to antibiotics.[15] A 2024 **[Au:OK?]** study identified older age, chronic kidney disease and recent hospitalisation, as independent risk factors for multiple recurrences of CDI.[9] Whilst healthcare-associated **[Au: To keep the text accessible, journal style avoids most two letter abbreviations.]** CDI drove many of the large outbreaks of the early 2000s,[14] the burden of community-associated CDI seems to be increasing.[16] This change might **[Au: Long sentence split for flow, OK?]** be driven by a different range of strains, latent reservoirs and transmission routes not yet fully understood.[17] Of note, asymptomatic carriers could be a reservoir of *C. difficile* in the nosocomial environment.[18] Reported rates of asymptomatic carriage vary hugely, from 3-21% of hospital admissions to as high at 50% of long term care facility residents.[19] Almost all antibiotics have been associated with increased risk of CDI, owing to the effects of antibiotics on the gut microbiota, which disrupt its resistance to *C. difficile* colonization[20] **[Au:OK? Please clarify, colonization by non-commensal organisms, or pathogens, or specifically *C. difficile*?]** . Exposure to third

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generation cephalosporins and fluroquinolones has been particularly implicated and is strongly associated with RT027 infection[21]; however, CDI risk is notably linked to the antibiotic resistance of infecting strains **[Au: Edits OK? For clarity and flow.]** .[22,23] Cumulative antibiotic exposure is probably **[Au: Please not, journal style prefers to avoid use of 'may' as it can be a bit ambiguous in meaning. If more certainly should be implied here, we could say 'Cumulative antibiotic exposure is probably the greatest contributor…']** ~~be~~ the greatest contributor to CDI risk, with each day of additional antibiotic exposure reported to increase the odds of CDI by 12.8% (odds ratio [OR], 1.128, $P < 0.0001$)[24]. The 'CDI paradox' is that although antibiotic exposure drives infection, first-line treatment for CDI is antibiotic therapy. [3,25] This paradox **[Au:OK? Journal style prefers to avoid a hanging 'This..']** has driven interest in microbiota restoration therapies as a treatment or adjunct to treatment for CDI.[26]

This Review explores the ~~recent~~ **[Au: Please could you be more specific here regarding the timeline of epidemiology that is discussed in your review (e.g. explores epidemiology of CDI from 2000s onwards)?]** epidemiology of CDI from 2000s onwards and how this is linked to changes in diagnostic guidelines. Whilst the disease-causing mechanisms of *C. difficile* have been established for some time, we discuss breakthroughs in certain aspects of *C. difficile* pathogenesis and crucially in the understanding of antimicrobial resistance mechanisms. Detailed discussion of CDI treatment and the development of novel treatment agents is outside the scope of this Review. Rather, we outline some of the increasing evidence of the role of microbiota dysbiosis in CDI and the emerging role of microbiota restoration in CDI treatment. We describe the two novel microbiota restoration therapies that have recently been approved by the FDA, and touch on other treatment strategies in development, which include anti-toxin antibodies and vaccines **[Au: Edits for flow, OK?]**.

[H1] Incidence and epidemiology

The epidemiology of *C. difficile* has changed substantially over the years. In the early 2000s, incidence was dominated worldwide by outbreaks of the epidemic RT027 **[Au: Ribotype is now defined at first use above. For style reasons, we will use the RT abbreviation with strain numbers but the full term when used alone.]** strains. [14] The huge clinical burden of these outbreaks drove research and guidelines to manage this dangerous pathogen.[3,15,21,25] In England, mandatory reporting was introduced for CDI, as well as a national *C. difficile*

ribotyping-based surveillance programme, and both approaches were associated with reduced prevalence of RT027, reduced CDI incidence and decreased CDI-related mortality **[Au: Edits OK?]** .[27] Worrying increases in incidence have occurred since the SARS-CoV-2 pandemic (**Box 1**), which highlights the importance of continued surveillance. Various factors influence the transmission of CDI within the hospital setting, such as rates of antimicrobial usage, sampling and testing rates, and community prevalence of CDI.[28] Community-associated-CDI might have different transmission routes,[17] potentially including food or animal sources.[29,30] However, transmission sources of *C. difficile* fall outside the scope of this Review. **[Au: Edits for style and flow, OK?]**

[H2] Epidemiology in Europe

The increase in *C. difficile* diversity across Europe is well described (**Figure 1**). A point-prevalence study conducted in 12 European countries/regions **[Au: Style edit]** in 2018 identified more than 60 distinct ribotypes in hospitalised patients **[Au: Only need one call-out to the figure in this paragraph.]** . The prevalence of hypervirulent RT027 and related RT181 remained high, but was localised to countries/regions in Eastern Europe.[31] A study describing the implementation of a sentinel surveillance system in Germany (2019–2021) captured high strain diversity (>50 ribotypes).[32] Moreover, **[Au: Long sentence split for flow, OK?]** hypervirulent RT027 prevalence across the study periods remained low (3.5%), a significant decline from its high occurrence (21.7%) a decade ago.[33] RT018 outbreaks were noted in previous years (2015-2017)-**[Au: Please is it possible to add more specific time context here?]**- in France and Germany,[34,35] but prevalence was very low (<1%), whereas epidemic RT078 emerged as the second most common ribotype~~s~~ in Germany in 2021 (7.8%).[32]

In England, where surveillance of CDI is mandatory, the prevalence of individual ribotypes reported in 2018–2023 have remained stable (**Figure 1**).[36] Worryingly, outbreaks of a new strain, RT955, which has RT027-like characteristics (high levels of transmission and mortality), was noted in two UK centres over the past 2 years and is currently being investigated.[37]

Epidemic RT027 and related strains remain stubbornly prevalent in certain regions. A study conducted in Greece between 2016 and 2019 identified RT027-related RT181 as the most prevalent (36%), followed by RT017 (10%) (**Figure 1**).[38]

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[H2] Epidemiology in the rest of the world

The most recent US Centre for Disease Control and Prevention **[Au:OK?]** annual report estimates the incidence rate of CDI to be 110.2 cases per 100,000 persons in 2021, a 10% increase compared with 2020, but still below the reported rate in 2019 (121.2 cases per 100,000 **[Au: should this be 100,000 persons to be consistent with the 2021 rate?]** persons).[39] A US national survey published in 2023 **[Au:OK? US national survey?]** reported the most common circulating ribotypes in 2020–2021 to be similar to those in Europe; RT014/–020 (14.0%), RT106 (10.3%), RT027 (10%), RT002 (8%) and RT078–126 (4.3%) **[Au: Edits OK?]** ,[40] and to those reported in Canada[41], where the prevalence of RT027 decreased since 2017 (15.4% versus 7.7%).

In Australia, RT014/–020 **[Au:OK? Above a hyphen is used to refer to RT14–020, which I edited to an en rule for style reasons. Should we use a solidus above instead? We should use one format consistently throughout. I won't edit the other instances just yet, until you let me know the preferred format.]** , RT002 and RT056 were the most common ribotypes in 2013–2018 (29.5%, 11.8% and 5.4%, respectively), while RT027 was rarely found (<1%).[42,43] In other parts of the world, the burden of CDI is less documented due to a lack of nationwide surveillance programmes. A 2024 **[Au:OK?]** review of studies conducted in South-East Asia and the Western Pacific reported that RT017, RT014/020, RT012 and RT002 were widespread.[44] RT017 is also the most prevalent type in countries/regions in West and East Asia~~the Far East~~ **[Au: Although this is the term used in the paper, please is it possible to use a different name for this region? I.e. East Asia, South-East Asia or Asia–Pacific (depending on the countries included in the study)? Far East is an outdated term.]** .[45] In Latin America, two reviews published this decade described historical data, with the most recent citing a small study published in 2018 showing that RT027 was the most prevalent ribotype in Mexico **[Au: Edits for clarity, OK?]** .[46,47] In Japan, RT018-related, RT014, RT002, RT369 and RT017 were reported to be the most common ribotypes, with little change in prevalence in the past two decades.[48]

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~~[H1]~~ Laboratory diagnosis of CDI

Accurate diagnosis of CDI must take into consideration the clinical symptoms of the patient, supported by laboratory diagnostic results. It is essential for understanding the

disease status of a patient, thereby ensuring optimal treatment and infection control precautions.[49] **[Au: I suggest moving this paragraph up, as it introduces some crucially important concepts and this initial paragraph is hard to follow without this knowledge.]** The use of different laboratory diagnostics for CDI remains contentious[50] and requires several considerations. First, no diagnostic test is infallible and CDI could be present even when diagnostic test results are negative.[51] Second, it is important to remember that people can carry the organism asymptomatically, so detection of the organism alone is not diagnostic of infection.[50,52] Key to detection of infection is the detection of two toxins, TcdA and TcdB which are produced by *C. difficile* and are key factors in toxin-mediated disease. [53,54] **[Au: Paragraphs combined.]** ~~D~~Accurate diagnosis can be challenging owing to asymptomatic carriage, however, and all testing **[Au: Specifically for these toxins? Or the presence of *C. difficile* itself? Please clarify testing in the context of this sentence. We could edit to 'any testing' to clarify if all tests are being referred to.]** should be limited to those patients with true diarrhoea **[Au: Edited for clarity, OK?]** .[18] Current guidance does not support testing of children aged ≤2 years old. [3,15,55] Indeed, diagnosis in all children is particularly problematic, in addition to individuals **[Au:OK? Or did you specifically mean children with IBD?]** with inflammatory bowel disease, as both groups have a higher rate of carriage than the general population **[Au:OK? Or 'than healthy adults'? The use of higher here requires a comparator.]** .[56,57]

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While most national and international guidelines now include the use of two or three-step testing algorithms,[3,15,55,58] adherence to these guidelines and the diagnostic tests used still varies.[59] Selection of assays for use is further complicated by the fact that the assays are designed to detect different things; *C. difficile* toxin, the microorganism itself or its DNA.[60] The following section describes the different tests available and their strengths and limitations.

~~[H2]~~ Detection of toxin

The gold-standard method for detection of free TcdB toxin **[Au:OK?]** from diluted fecal samples is the cell cytotoxicity neutralisation assay, although it is of limited use within a routine diagnostic laboratory owing to the long incubation time required to confirm a negative result **[Au: Edits for clarity and flow, OK?]** .[50] In addition, lack of consensus on

methodology, including how to dilute the fecal sample and which cell lines to use, lead to differing reported sensitivities. [61]

To reduce the time to diagnosis, rapid enzyme immunoassays (EIAs) were developed for detection of TcdA,[62] and subsequently for both TcdA and TcdB, after TcdA-negative–TcdB-positive strains were discovered.[63] The performance of these EIAs is variable and could be affected by strains with differential toxin genotypes.[60,64-68] A weakness of many studies assessing these EIAs **[Au:OK? If not, please clarify.]** is that they falsely inflate the predictive value of assays by selectively testing populations with unrealistically high prevalences.[60] In a low prevalence setting, some EIAs might have sensitivities below 50%.[60] New ultrasensitive *C. difficile* toxin assays have been developed, but are disappointingly not currently available **[Au:OK?]** commercially.[69-71]

~~[H2]~~ Detection of *C. difficile*

Culture of *C. difficile* alone cannot be diagnostic of CDI, as people can carry non-toxigenic strains of *C. difficile* in their gut.[72] Cytotoxigenic culture can identify those patients carrying *C. difficile* with pathogenic potential in their faeces,[73] but has several limitations. First, it can only assess the toxin-producing ability of the strain within a laboratory, which does not necessarily relate to *in vivo* production. Detection of free-toxin in the sample is associated with mortality, rather than detection of an isolate with toxin producing ability. [50] Second, the additional incubation steps required make the turn-around time longer for cytotoxigenic culture than for cell cytotoxicity neutralisation assays. Third, cytotoxigenic culture cannot differentiate between CDI and asymptomatic carriage, so it is also essential that this assay is limited to those with diarrhoeal fecal samples **[Au: All edits OK? For clarity or journal style.]**. [72]

Commercial assays to detect the microorganism via the presence of a cell-surface protein produced by *C. difficile* ***[Au:OK?]*** ,[74] glutamate dehydrogenase, do not seem to have the performance variability of toxin EIAs.[75] However, GDH assays cannot differentiate between toxigenic and non-toxigenic strains, so cannot be used alone to diagnose CDI.

Nucleic acid amplification test (NAAT) detection of toxigenic *C. difficile* is rapid and can be high throughput, depending on platform. Similar to cytotoxigenic culture, the main limitation of NAAT is its inability to detect free-toxin in the sample. In addition, NAAT can potentially detect dead cells, without gene expression **[Au: Edits for flow, OK?]** .

Unfortunately, a test to determine if toxin genes are being expressed and therefore provide detection of 'live' cells, such as a reverse transcriptase assay, is not currently available. Standalone NAAT therefore has the potential to overdiagnose CDI.[52] Patient samples that are NAAT-positive and toxin-positive are significantly associated with greater antibiotic exposure, higher bacterial load, more gut inflammation and presence of diarrhoea (all p <0.001) compared with patients that had NAAT-positive and toxin-negative samples **[Au: / edited to and for clarity and long sentence split for flow, OK?]** .[52] In addition, CDI attributable mortality is higher in patients with NAAT-positive and toxin-positive results **[Au: Please clarify, NAAT-positive and toxin positive? Or just toxin-positive alone?]** compared with those with NAAT-positive and toxin-negative results.[50,52,76,77] Although a 2024 meta-analysis of 26 studies found that all-cause mortality was reduced if patients with NAAT-positive and toxin−negative results were treated **[Au: Long sentence split for flow, OK?]** .[78] Diagnostic stewardship (the practice of ensuring that the right diagnostic tests are used for the right patients at the right time to improve patient care, optimize the use of health care resources, and limit the spread of antimicrobial resistance) **[Au: Please add a brief explanation of diagnostic stewardship for our non-clinically focused readers.]** has been used by some centres to ameliorate false positives from standalone NAAT.[79,80] A US study demonstrated a two-fold reduction in NAAT request rates following diagnostic stewardship and education; reported CDI rates also reduced.[80] However, with improved testing criteria, diagnostic stewardship runs the risk of leading to selective testing, which increases the chance of missing patients with CDI **[Au:OK? Edited for clarity.]** . This effect has been seen in European studies, where 23% of patients with CDI within hospitals and almost 50% of patients in the community, were undiagnosed, with younger patients most likely to have undiagnosed CDI due to lack of testing.[31,81,82] Thus, although increasing age is a known risk factor for CDI,[83] it should not be used as a criterion for testing.[82]

Some added value can be found with NAATs, in that those samples with a lower cycle threshold (therefore higher bacterial burden) could indicate poorer patient outcome **[Au: Edits OK? For passive language.]** .[84] In addition, some commercially available tests also include a presumptive identification of potential RT027, based on the presence of a truncated *tcdC* gene that is linked with this ribotype.[85] Of note, however, is that this test is not definitive, as several ribotypes contain this truncated gene **[Au: Edits for clarity, OK?]** .[36] One study reported that one such PCR assay (Cepheid GeneXpert C diff, USA) was more

sensitive than GDH EIAs for detecting certain PCR ribotypes, but sample numbers were extremely small and this finding has not been replicated.[65]

~~[H2]~~ The algorithmic approach

To improve performance of testing strategies, assays have been combined into algorithms.[3,15,50,55,58] The first assay usually detects the presence of *C. difficile* (such as via GDH EIA or NAAT), followed by detection of the clinically important toxin, although multiple approaches have been used to combine these tests. The pivotal study of algorithms also confirmed that mortality and severity of infection correlated with the presence of free-toxin in a patient fecal sample over just detection of toxin genes.[50]

~~[H2]~~ Novel technologies

Development in CDI diagnostics has been limited, since the algorithms were put into guidelines. Some promising ultrasensitive toxin tests were developed, with a limit of detection below that of the cell cytotoxicity neutralisation assay ~~CCNA~~ **[Au: Please expand this abbreviation.]** , although none are now commercially available.[71,86] Studies using ~~this technology~~ these ultrasensitive toxin tests **[Au: Please clarify, which technology?]** have shown that patients with CDI have higher median fecal toxin concentrations **[Au:OK?]** than asymptomatic carriers[87] and that even low levels of toxin could differentiate patients with CDI from those carrying the organism alone.[31]

Molecular detection of *C. difficile* is included in many multiplex gastro-pathogen panels, but as described earlier, detection of the microorganism alone is not sufficient to determine true CDI. For example, one 2024 study found that only 38% of samples positive by one panel also tested positive for toxin **[Au:OK?]** .[88]

~~[H2]~~ Adjunct tests

Laboratory tests measuring gut inflammation via markers released by polymorphonuclear leukocytes, such as lactoferrin and calprotectin, could offer additional information on the severity of infection in some patients.[89] A case–control study found higher fecal levels **[Au: circulating levels or fecal levels? Please clarify.]** of both calprotectin and lactoferrin in patients with CDI and in those with free-toxin in their faecal samples, compared with

control individuals.[90] This study provides further evidence **[Au: Edited for clarity and flow, OK?]** of the effect of toxin on the gut mucosa. Study data for the clinical utility of these markers in CDI diagnosis has, however, been conflicting and further research is needed.[91,92]

~~[H1]~~ Antibiotic resistance in *C. difficile*

Antimicrobial options for the treatment of CDI have always been limited. Metronidazole and vancomycin were the only available options until 2011–2012, when fidaxomicin was introduced.[58] However, the relationship between antimicrobials and *C. difficile* is complex, involving not only antimicrobials~~those~~ used to treat CDI but also those that carry an increased risk of eliciting CDI, and ~~antimicrobials that~~ those that *C. difficile* might also be incidentally exposed to antimicrobials in the gut **[Au: Edits OK? The previous wording needed clarification]** . Resistance to both treatment and non-treatment antibiotics can have implications for CDI transmission and control.

The emergence of ~~PCR~~ RT027 and its association with fluoroquinolone resistance in the early 2000s highlighted the need for regular surveillance of *C. difficile* strains in circulation **[Au:OK?]** .[93] ~~In recent years~~ Over the last decade **[Au: Please is it possible to add more specific time context here. E.g. Over the past X years/decade(s)]** , large-scale surveillance studies have provided valuable insights on rates and spread of resistance and emerging resistant ribotypes. A detailed examination of antimicrobial resistance mechanisms (**Table 1**) is beyond the scope of this Review, but has been covered elsewhere.[94] Evaluation of historical and contemporaneous UK isolates has shown that antimicrobial resistance has been a feature of *C. difficile* for a long time, yet it has increased in more recent isolates. [95]

[H2] Resistance to *C. difficile* treatment agents

[H3] Metronidazole. Metronidazole is a nitroimidazole and was the mainstay of treatment for CDI for many years. However, reports of decreased metronidazole efficacy and the advent of fidaxomicin led to m~~M~~etronidazole no longer being endorsed as a first line treatment for CDI in guidelines from North America (IDSA/SHEA), Europe (ESCMID) and the UK (NICE) **[Au: Edits OK?]** .[3,25,96] A 2021 study showed that clinical failures were associated with reduced *C. difficile* metronidazole susceptibility, despite previous evidence indicating no such

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association **[Au:OK? Edited for clarity and to edit out the author name. Journal style is to name colleagues only in a historical context.]** .[97] Nonetheless, anecdotal evidence indicates metronidazole is still used to treat CDI despite not being recommended in national guidance.

Rates of metronidazole resistance are generally low and were reported at 0.2% in a longitudinal Pan-European study,[98] with minimum inhibitory concentrations (MICs) **[Au: defined at first use.]** being highest in ~~PCR~~ RT027 and closely related ribotypes (for example, RT198), often in particular geographic locations.[31,98] Higher rates have been reported,[99] but local epidemiology and susceptibility testing methodology could account for variations. In 2024~~Recently~~, the UK reported an outbreak of RT955, which is closely related to RT027[37] **[Au: Does ref 99 support this statement about the outbreak too? Please add specific time context in place of 'recently']** . All isolates in the outbreak were resistant to metronidazole (European Committee on Antimicrobial Susceptibility Testing **[Au:OK?]** breakpoint R≥2mg/l)[100].

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The mechanisms behind metronidazole resistance are varied, complex and still emerging. As well as earlier observations of unstable metronidazole resistance,[101] the importance of media and conditions in detecting metronidazole resistance in some *C. difficile* isolates[102,103] has been described **[Au: Edit OK? The previous wording was a little hard to follow.]** . Two clear mechanisms have emerged to date: plasmid-mediated resistance and haem-dependent metronidazole. **[Au: Paragraphs merged to avoid the appearance of a 4th level of heading, which we cannot accommodate.]** Plasmid-mediated resistance was identified with the discovery of a high copy number plasmid (pCD-METRO) that conferred resistance to metronidazole in *C. difficile* isolates from a patient who had failed metronidazole treatment.[104] However, this plasmid is fairly rare as only 15 strains in >10,000 publicly available genomes were subsequently identified ***[Au: Edits OK? For clarity and names edited out for journal style.]*** [105] and the genetic mechanisms remain unclear **[Au: Paragraphs merged.]** The demonstration that haem was necessary for the reliable detection of metronidazole resistance in *C. difficile* provided evidence of haem-dependent metronidazole resistance.[106] Subsequent work described **[Au: Edits for clarity and style, OK?]** the genetic validation of haem-dependent metronidazole resistance and its association with fluoroquinolone-resistant epidemic *C. difficile*.[107] The existence of haem-dependent metronidazole resistance and the underlying mechanism explains the

considerable variation in earlier estimates of metronidazole resistance and underlines the need for a standardised media for *C. difficile* susceptibility testing.

[H3] Vancomycin. Vancomycin was first used as a treatment for CDI in the 1970s and remains a first line option in current guidance.[3,25] Rates of vancomycin resistance among *C. difficile* have been very low, despite ~85% of clinical *C. difficile* isolates showing molecular evidence of the inducible chromosomal operon, *vanG*.[108] *vanG* **[Au: Repetitive text omitted.]** produces D-ala-D-Ser rather than D-Ala-D-Ala, which results in decreased vancomycin binding affinity.[109] In laboratory-generated strains and in clinical isolates with elevated vancomycin MICs (4–8mg/l), mutations in the two component VanSR system resulted in constitutive *vanG* expression and decreased vancomycin killing.[110] The underlying pathway mechanisms following *in vitro* mutation generation have been described and are associated with notable fitness costs, possibly explaining the lack of clinically isolated *C. difficile* with high-level vancomycin resistance **[Au: All edits OK? For clarity, flow and to edit out the names.]** .[111]

Dissemination of vancomycin resistance genes on plasmid Tn1549 has been reported in several studies, yet, the relationship between phenotype and genotype is much less clear.[112-114] Vancomycin resistance in *C. difficile* has also been associated with *vanA, vanB* ,*vanW* and *vanZ,* but their true involvement is less well understood. A study of clinical isolates from Brazil showed elevated MICs in the presence of one or more *van* genes in five of seven isolates, but also demonstrated the presence of *van* genes in the two susceptible isolates.[115] More recently in 2024, **[Au:OK?]** associated reduced clinical outcomes (30 day sustained clinical cure and 14 day initial clinical cure) with elevated vancomycin MICs.[116] However, these isolates were largely RT027, which is itself associated with poorer outcomes.

Whether elevated vancomycin MICs of 4–8mg/l are clinically significant in the light of intestinal drug concentrations that are several hundred-fold higher than the breakpoint (S<2mg/l: R≥2mg/l) remains questionable. More work is clearly needed to understand the scope of vancomycin resistance and the underlying mechanisms **[Au: Edits for passive language.]** .

[H3] Fidaxomicin. Fidaxomicin was introduced in 2011 and is now a first line option for the treatment of CDI in both European and US guidelines, [3,25] but second line in the UK.[96] It is a macrolide antibiotic that interrupts transcription and protein synthesis by inhibiting

bacterial RNA polymerase. Fidaxomicin has a particular potency for the RNA polymerase of clostridia over other bacterial species, giving it a much narrower spectrum activity and lower disruptive effects on the gut microbiota than other CDI treatment antibiotics **[Au:OK? If not, please clarify the comparator.]** . Reported cases of fidaxomicin resistance in the literature are very uncommon.[117-119] [120] Two groups have **[Au:OK? Names edited out as per journal style.]** reported cases of resistance emerging following fidaxomicin treatment.[119,120] However, no clinical failures were associated with fidaxomicin resistance in these reports, probably due to extremely high intestinal levels of fidaxomicin (>1000mg/kg) that far exceed the MICs observed in these studies (resistant isolate MICS = 0.25->64mg/l). More recently in 2025 **[Au:OK?]** , *C. difficile* isolates with reduced fidaxomicin susceptibility (MICs 8-=32mg/l **[Au: should this equals sign instead be an en rule, to indicate a range?]**) were described in 6 of 108 fidaxomicin-treated patients (5.6%). This study included three patients with initially sensitive *C. difficile* isolates who went on to experience clinical failure of fidaxomicin treatment.[121]

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Fidaxomicin resistance in clinical *C. difficile* strains is associated with mutations in *rpoB* or *rpoC* that lead to amino acid substitutions.[118-120] Fidaxomicin-resistant *C. difficile* isolates harbouring *rpoB* mutations have also been associated with fitness costs in toxin production, growth and sporulation, [118,119] and could explain why fidaxomicin resistance is not more commonly noted. Given these reported fitness costs and high intestinal antibiotic levels, the clinical significance of fidaxomicin resistance requires further investigation, particularly in light of recent reports of clinical failures.[121]

[H2] Resistance to non-treatment antimicrobials

Prior antimicrobial treatment is a major risk factor for CDI, with broad spectrum antibiotics in particular carrying the greatest **[Au: Edited to remove the need for a comparator, OK?]** risk due to their profound effects on the gut microbiota. Moreover, resistance *in C. difficile* could also have implications for transmission of antimicrobial resistance to other bacteria that reside within the gut environment. In addition, resistance has profound effects on *C. difficile* transmission and incidence rates; for example, the prevalence of fluoroquinolone prescribing, combined with high levels of fluoroquinolone resistance, is thought to have been a key factor in the selection and epidemic spread of PCR RT027 **[Au: The highlighted sentence is quite convoluted and the meaning is not clear. I tried to**

split up and clarify, is this what you mean here?] . While particularly known for this association with RT027 epidemic spread **[Au: Please clarify, the association of fluoroquinolone resistance with RT027 epidemic spread?]** , fluoroquinolone resistance is found in many *C. difficile* ribotypes across a wide geographical area.[98] Similarly, resistance to clindamycin in *C. difficile* is widespread across many ribotypes[98] and geographical locations.[98] This antibiotic is known for its high propensity to predispose to CDI, and is therefore subject to formulary restrictions in many places.

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Third generation cephalosporins are also well known for their predisposition to CDI and *C. difficile* isolates are often phenotypically resistant in studies. However, in contrast to clindamycin and fluoroquinolones, relatively little research has been done on the underlying mechanisms until fairly recently. **[Au: Long sentence split for flow.]** The *C. difficile* genome has been reported to encode endogenous D-class β-lactamases active against various β-lactam antibiotics[122]. **[Au:OK? Additional clarity was required here.]** Furthermore, substitution mutations in the genes encoding penicillin binding protein have been described that are associated with increased cephalosporin MICs in *C. difficile* **[Au:OK? For clarity.]** [123], some of which coincided with fluoroquinolone resistance in epidemic lineages and could be a further factor in outbreaks of these ribotypes.[21,23] A new group of Zn^{2+}-binding penicillin binding proteins has also been described.[124] These enzymes are essential for mediating cell elongation and so are a likely driver of intrinsic cephalosporin resistance.[124]

Rifampicin has been considered as a possible treatment for CDI but there is widespread high level resistance among *C. difficile* strains, particularly among epidemic ribotypes such as RT027 (ref.[125]) and related ribotypes, with some evidence of this resistance arising during or after rifamycin treatment **[Au: Edits OK?]** .[126] Like fidaxomicin, rifamycins bind to RpoB but at a different site, with no overlap in resistance.[127] Tigecycline has also been suggested as a treatment for CDI and there is little evidence of resistance to this agent, despite tetracyline resistance being well-described in *C. difficile*. However, resistance to both chloramphenicol and tetracycline are carried on mobile elements[128,129] and present the possibility of transfer to other gut species. *C. difficile* is a spore forming microorganism and so has additional capacity for survival outside the body and onward transmission. Therefore, *C. difficile* has considerable potential as a reservoir for and

purveyor of antimicrobial resistance. This risk **[Au: Edits OK?]** further highlights the need for continued surveillance for antimicrobial resistance in this organism.

[H1] Pathogenicity and virulence factors

The ability of *C. difficile* to form spores helps to facilitate its survival in the environment and transmission[130]. In addition, *C. difficile* expresses multiple virulence factors, including cell surface proteins (adhesins) that mediate adherence to host epithelial cells and enable gut colonisation,[131] and toxin production that damages the epithelial barrier, leading to inflammation and diarrhoea.[132] These have recently been extensively discussed in other reviews[132,133] and will be summarised here. **[Au: Edits OK? Numbered list edited out for style reasons and text amended as spore formation in itself isn't a virulence factor that can be expressed.]**

[H2] Sporulation and biofilm formation

The ability to produce endospores (spores) is critical for *C. difficile* transmission in the aerobic environment (**Figure 2**, **Figure 3**). **[Au: Paragraphs merged, as very short paragraphs can look odd in the final layout.]** Similar to other sporulating *Firmicutes*, *C. difficile* sporulation is activated by phosphorylation of the conserved regulatory protein Spo0A. Regulation of this gene in *C. difficile* is not yet fully understood[130,134,135,] and does not seem to involve the conserved regulatory factors of other spore-formers.[136] However, **[Au: Long sentence split for clarity and flow, OK?]** both RstA and Spo0E orthologues have been indicated to play regulatory roles.[137,138]

The structure and morphology of *C. difficile* spores is similar to other bacterial species, particularly *Bacillus subtills*;[139,140] however, *C. difficile* spores show a more heterogeneous outer layer, known as the exosporium.[140-142] **[Au: Paragraphs merged.]** *C. difficile* spore resilience to adverse conditions increases the risk of host-to-host transmission. Following their ingestion, spores can colonise the large intestine of susceptible hosts (**Figure 2**). Spore germination in the gut occurs in response to specific signals. The presence of critical germinants (for example, the bile acid taurocholate)[143] and co-germinants (amino acids such as glycine[143] and Ca^{2+} ions[144]) are perceived by *C. difficile* via the pseudoprotease receptors CspC (germinant receptor) and CspA (co germinant receptor),[143,145-147]. CspC and CspA **[Au: Long sentence split for flow.]** integrate both bile

acid and glycine or Ca^{2+} signals[148] in a 'feedforward loop' to activate germination of neighboring spores.[149] Germination initiates the cell active growth phase, leading to multiplication of vegetative cells that can produce toxin and cause CDI. [145,147]

In vitro studies in a colorectal adenocarcinoma cell line (Caco-2 cells) **[Au:OK?]** , reported an increased adherence of spores to intestinal epithelial cells, where adherens junctions were damaged by *C. difficile* toxins. Spore adherence to the intestinal epithelium could contribute to the bacterium persistence in the colonic environment and recurrent infection. [150] **[Au: Paragraphs merged.]** Similarly, *in vitro* studies using a model reflective of human colonic conditions have shown that *C. difficile* spores can integrate in multi-community intestinal biofilms, potentially enabling the bacterium to remain in the colon and provide a reservoir for recurrent infections.[151] It has long been known that monocultures of *C. difficile* can form self-encased biofilms,[152] but that *C. difficile* can also contribute **[Au:OK? For clarity.]** towards multispecies biofilms at the mucosal layer.[153] [154] More recently in 2021 **[Au:OK?]** , mucosal dwelling *C. difficile* cells were shown to be composed of both spores and vegetative cells.[151] The interaction of *C. difficile* with other microorganisms in a multispecies biofilm can be antagonistic (*Lactobacillus rhamnosus, Bifidobacterium longum* and *Bifidobacterium breve*)[151] or synergistic (*Finegoldia magna,*[153] *Enterococcus faecalis*[155] and *C. paraputrificum*[151]). However, **[Au: Long sentence split for flow.]** the relationship is not always clearcut and interactions can differ in biofilm and sessile populations, or single and mixed species culture (*Clostridium scindens*[156,157]). When designing future therapies, this population of *C. difficile* cells encased in a self-produced extracellular matrix needs to be specifically targeted.

[H2] Cell surface proteins

C. difficile expresses several cell surface proteins that facilitate cell adhesion to host cells. These include the bacterial surface layer (S-layer), flagella[158], pilli[159] and 28 accessory cell wall proteins (CWPs)~~,~~ ~~a~~All ~~of t~~These proteins enable *C. difficile*~~have~~ **[Au: Long sentence split for flow.]** adhesion ~~properties~~ [131,160-162] and have been shown in animal models to support effective *C. difficile* colonisation of the colonic environment.[159,163]

The role of *C. difficile* S-layer in virulence is suggested by *in vitro* and *ex vivo* evidence of its adhesion to epithelium and Caco-2 cells.[164,165] Roles have also been proposed for S-layer protein A (S~~s~~lpA) in sporulation, resistance to innate immunity effectors~~,~~ and

toxin production.[166] Compared with wild-type strains, S-layer mutants show altered susceptibility to lysozyme[167] (a large molecule with antimicrobial properties) and avidocins[166], which indicates that these proteins might also be relevant in antibiotic resistance and highlights the potential of SslpA as drug target.[164,166,167] S-layer importance is underlined by the high metabolic cost required for its production and the poor growth of *C. difficile* isogenic mutants of *slpA* (the S-layer precursor gene) **[Au:OK? For clarity.]** .[161,162] A study in 2024 reported a **[Au: Edited for journal style, OK?]** *slpA* gene deletion mutant with impaired growth, toxin production, sporulation, motility and adhesion to human cells.[168]

CWPs make up approximately 5–20% of S-layer composition, and are responsible for additional functions, such as phase variation and biofilm formation~~particularly adhesion~~ **[Au: The S-layer was first mentioned at the top of this paragraph in the context of adhesion, but the text here says CWPs are responsible for additional functions, particularly adhesion. I.e., it reads as if the CWPs are responsible for additional functions to adhesion, particularly adhesion. Please double check the wording and amend as necessary.]** .[160,169] The expression of CWPs and their involvement in pathogenesis is suggested by the detection of antibodies to Cwp84 and Cwp66) in serum from patients with CDI **[Au:OK? If not, please clarify the patient group.]** .[170] [171] In 2022 **[Au:OK?]** , a Cwp66 deletion mutant was characterised,[172] demonstrating an association with increased tolerance to stresses including hydrogen peroxide, low pH and to certain antimicrobials, namely vancomycin. These observations suggest that CWPs could have a comprehensive role in *C. difficile* pathogenesis, regulating metabolism and supporting cell persistence via multiple pathways.

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[H2] Toxin production

CDI symptoms result from the action of two cytotoxins, TcdA and TcdB, encoded within the well conserved pathogenicity locus (PaLoc, 19.6 kb) of toxigenic strains. TcdA and TcdB are glucosyltransferases that inactivate Rho guanosine triphosphatases via glucosylation, thereby affecting the cellular cytoskeleton and impairing the intestinal barrier. Internalisation by intestinal epithelial cells **[Au: Are these toxins taken up by all host cells or are they specific for a cell type; e.g. intestinal epithelial cells?]** and the model of action of these toxins has recently been reviewed in detail.[132] Although these toxins share 63% homology, they bind to different host cell receptors[132] and have independent virulence

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potential. Glycoprotein 96 and members of the low-density lipoprotein receptor family **[Au:OK?]** , such as low-density lipoprotein receptor-related protein-1, have been suggested as TcdA receptors.[173,174] TcdB has been proposed to bind to a number of protein receptors including chondroitin sulfate proteoglycan 4 (CSGP4),[175] poliovirus receptor-like 3,[176] Frizzled receptors 1, 2, and 7, [175] [174,176] and tissue factor pathway inhibitor (TFPI).[177] Frizzled receptors and CSPG4 have been linked to TcdB-induced secretion of pro-inflammatory peptides and cytokines from neurons and pericytes.[178]

Hamster and mice studies have looked at the activity of each toxin and established that both TcdA and TcdB alone are able to cause symptomatic CDI, characterised by weight loss and diarrhoea. **[Au: Long sentence split for flow. Please add more specific time context than recent.]** ~~rRecent research has highlighted that~~ However, TcdB-only producing isogenic mutants cause higher virulence than~~compared to~~ those expressing only TcdA.[2,179,180] **[Au: Paragraphs merged.]** Further studies using organoids from mouse colonic tissue informed that TcdB-mediated damage can alter colonic stem cell function, inducing deep damage to the intestinal mucosa at a faster rate than normal cellular regeneration, and suggesting a correlation between high TcdB expression and disease severity.[180] Thus, TcdB neutralisation has long been seen as a potential therapeutic target. Advances have been made **[Au: Long sentence split for flow, OK?]** using de novo-designed mini-proteins in mice that are effective at neutralizing the major TcdB subtypes *in vivo*.[181]

~~**[Au: I suggest moving this small paragraph to the end of this subsection, so that the different toxins (TcdA, TcdB and CDT) are discussed first, before phase variation discussion.]** C. difficile has been shown to useemploy phase variation (high-frequency, reversible changes in gene expression) **[Au:OK? Term defined to keep the text accessible.]** to generate heterogenicity in both flagella and toxin gene expression.[182] Inversion of a flagellar switch sequence by the tyrosine recombinase RecV affects the expression of the sigma factor SigD, which induces the expression of both flagella and toxin genes, a process mediated by the transcription termination factor Rho.[183,184] Crucially, this heterogeneity has been shown to impact colonisation and virulence in animal models.[182,185]~~

Additionally, approximately 17–23 % of strains produce a *C. difficile* toxin (CDT), also called binary toxin.[186] CDT is an ADP-ribosyltransferase encoded by two genes, *cdtA*

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and *cdtB*, that are located on the binary toxin locus (CdtLoc, 6.2 kb). The positive response regulator gene, *cdtR*, located upstream of *cdtA* and *cdtB,*[187] has been associated with regulating TcdA and TcdB toxin production in RT027 strains, but not in RT078 strains, which suggests a strain-dependent genetic link between PaLoc and CdtLoc.[188] This link **[Au:OK?]** is further supported by reports that natural and lab-generated *cdtR* genetic variants with a 69-bp sequence deletion, can downregulate the expression of PaLoc genes and binary toxin genes, resulting in an avirulent phenotype.[189] As well as confirming the enteropathogenic effects of CDT alone, mutant studies have also indicated that the presence of CDT could increase the virulence of strains producing only TcdA.[2] Furthermore, purified CDT toxin has been shown *in vitro* to induce the formation of *C. difficile* microcolonies with a biofilm-like structure characterized by increased resistance to vancomycin, which could contribute to bacterial survival in the intestinal mucosal layer.[190]

[Au: New paragraph. The previous paragraph on CDT was too long and needed a break.] These results, together with the fact that CDT is often found in hypervirulent *C. difficile* strains, such as RT027 and RT078, support the role of this toxin in *C. difficile* pathogenesis. Nonetheless, the complexity of CDT **[Au: Edited for consistency, OK?.]** action is not yet fully elucidated. A 2024 study comparing a *cdtB*- mutant with the wild-type strain, suggested that CDT contributes to weight loss in mice, but that this effect is independent of activation of the inflammasome.[191] This mechanism requires further study, as it differs from the authors own *in vitro* observations. It also is at odd with **[Au: Long sentence split for flow, OK?]** previous reports[192] of a *C. difficile* mutant with restored *cdtB* function causing infection in a hamster model but not in a mouse model. These studies underline the effect of the host immune responses in CDI outcome.

[Au: I suggest moving this small paragraph to the end of this subsection, so that the different toxins (TcdA, TcdB and CDT) are discussed first, before phase variation discussion.] C. difficile has been shown to use phase variation (high-frequency, reversible changes in gene expression) **[Au:OK? Term defined to keep the text accessible.]** to generate heterogenicity in both flagella and toxin gene expression.[182] Inversion of a flagellar switch sequence by the tyrosine recombinase RecV affects the expression of the sigma factor SigD, which induces the expression of both flagella and toxin genes, a process mediated by the transcription termination factor Rho.[183,184] Crucially, this heterogeneity has been shown to impact colonisation and virulence in animal models.[182,185]

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[H2] Host response and clinical symptoms

Immune responses to *C. difficile* virulence factors are implicated in disease severity and clinical presentation of CDI. Asymptomatic carriers of the bacterium produce higher levels of serum IgG anti-TcdA and anti-TcdB **[Au: Antitoxin-A = anti-TcdA and antitoxin-B = anti-TcdB? The same terms should be used consistently throughout.]** relative to patients that developed symptomatic disease. Higher levels of anti-TcdA IgG antibodies and anti-TcdA and anti-TcdB IgM antibodies were also associated with a lower risk of recurrent CDI.[193,194] However, anti-TcdB immunity seems to be limited to short periods, with studies reporting 14 (ref.[194]) to 90-days[195] protection windows after the primary infection. These findings suggest that individuals with a stronger immune response to *C. difficile* toxins are less likely to develop symptomatic disease or multiple episodes of infection, but this protection might be limited in time.[193-195] However, when immunity is compromised the risk of bacterial exposure increases. *C. difficile* adhesion to the epithelium cells is then mediated by toxin activity and cell wall proteins, damaging the epithelium barrier and disrupting the tissue tight junction. The damaged epithelial layer allows red blood cells into the intestinal lumen, and permits **[Au:OK?]** microbial cells to disseminate extra-intestinally. These concerted actions promote an acute inflammatory response, accompanied by release of proinflammatory cytokines (such as IL-1β, TNF and IL-8) from epithelial cells and an infiltration of neutrophils that further damages the host tissue.[132,193,196] As a result, patients develop CDI, with symptoms varying from mild diarrhea to severe colitis with pseudomembrane, which can be fatal in some cases.[3,4,7,197]

[Au: Paragraph break. The previous paragraph was too long and needed splitting.]
The chain inflammatory response occurring in CDI can aggravate pathogen-induced damage in the intestine, thereby resulting in higher disease severity. Thus, strengthening innate pathways of host defense can reduce acute CDI symptoms and promote better outcomes. Innate lymphoid cells (ILCs) that reside in the intestine ~~and~~ can restore the integrity of the intestinal barrier **[Au:OK?]** following infection.[198,199] Transferring ILC1s and IFN-γ (aided by ILC3s and IL-22) into highly susceptible mice helped to preserve the integrity of the lumen and reduce mortality associated with CDI **[Au:OK?]** . Other animal studies have also supported the concept that innate immune responses mediated by ILCs (particularly IL-22) **[Au:OK?]** can help restrict the infiltration of microbial cells in the epithelium, which leads to

a faster recovery of weight and resolution of diarrhoea.[198] Importantly, *C. difficile* can also exploit the host inflammatory response. For example, *C. difficile* toxin in a mouse model mediates inflammation **[Au: Edits for flow, OK?]** , upregulating immune cell expression of aldose reductase enzymes. *C. difficile* can then utilise the host-derived sorbitol produced by these enzymes. [200] Storage of non-crystalline iron in membrane bound ferrosomes enables *C. difficile* to overcome nutritional deficits in a inflamed gut, where host-derived calprotectin mediates iron sequestration. [201]

Understanding the mechanisms by which we can strengthen the host immune response to CDI can offer new treatment options. For instance, eosinophils have been shown to have a protective role against CDT activity. Two CDT+ PCR RT027 strains induced host inflammation in mice by recruiting Toll-like receptor 2, which suppressed the protective activity of host eosinophils by indirectly inducing eosinophil apoptosis. This finding added clarity to the mechanism used by CDT **[Au:OK? For consistency.]** to enhance *C. difficile* virulence and evade host immune responses, and can offer a potential therapeutic target. [202]

[H1] The role of the gut microbiota **[Au: H1 headings should use 41 characters or less including spaces. Heading edited for length, OK? Please feel free to amend my suggestion but stick to the character count.]**

It has long been understood that the normal flora of the colon has an important role in providing colonization resistance against CDI, and that microbial dysbiosis is a key factor in susceptibility to the disease.

[H2] The gut microbiota in health and CDI **[Au:OK? Edited to make it a bit more descriptive. H2 headings can use up to 48 characters, including spaces.]**

The microbiome of the lower intestinal tract comprises a diverse community of microorganisms,[203] which have a crucial role in host immune regulation,[204-206] maintaining colonocyte homeostasis and epithelial barrier support,[207,208] metabolic regulation[209] and colonisation resistance against pathogen invasion.[20] The specific composition of the gut microbiota varies within and between individuals, with over 2,000 intestinal microbial species identified, of which over 90% belong to the phyla Firmicutes, Bacteroidota (formally

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Bacteroidetes), Pseudomonadota (formally Proteobacteria) and Actinobacteria.[210] A healthy gut is generally considered to be dominated by the Firmicutes and Bacteroidetes phyla, with functional redundancy ensuring stability of metabolic pathways.[211] The Firmicutes phyla is predominantly composed of genera such as *Clostridium*, *Lactobacillus*, *Bacillus*, *Ruminicococcus* and *Enterococcus*, and the Bacteroidetes phylum is mainly represented by *Bacteroidaceae, Prevotellaceae, Rikenellaceae* and *Porphyromonoadaceae.*[212]

A healthy microbiome is able to protect the host from pathogen invasion and expansion through a process known as colonisation resistance. Mechanisms of colonization resistance **[Au:OK? For clarity.]** can include competing with exogenous microorganisms for nutrients and space, metabolic mechanisms such as bile acid and short chain fatty acid metabolism, and active antagonism through antimicrobial proteins and bacteriocins.[213-215] Animal studies have demonstrated that the human commensal *Clostridium scindens* directly inhibited CDI through the conversion of primary bile acids to secondary bile acids, which inhibit spore germination and vegetative cell outgrowth.[215] Commensal *Paraclostridium bifermentans* ***[Au: Is this microorganism a commensal or a pathogen? Also please clarify the same for C. sardiniense and C. scindens.]*** co-infection reduced CDI disease severity (compared with *C. difficile* mono-colonised mice), whereas co-infection with the commensal butyrate-producer, *Clostridium sardiniense*, resulted in a more severe disease phenotype,[216] likely due to differential arginine deiminase fermentation pathways of these species. Gut microbiota mediated arginine and ornithine metabolism has also been implicated in the asymptomatic colonisation of *C. difficile*.[217] [216] The continuing development of tools, such as predictive models for systems analysis of *C. difficile* transcriptomic data, enables systems-level studies of virulence mechanisms,[218] and mechanistic aspects of colonisation resistance.[216]

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The loss of colonisation resistance is most commonly caused by antibiotic use, and is associated with disruptions to beneficial microorganisms, accompanied by a shift in dominant phyla to that of Proteobacteria.[219] This disruption to the symbiotic balance between the host and the microbiota is known as dysbiosis and is typically characterised by an overall reduction in diversity and abundance, accompanied by alterations in metabolic function.[220] Intestinal dysbiosis has been linked to a number of different disease states, including, inflammatory bowel disease and metabolic disorders,[221] and facilitates *C. difficile* colonisation of the intestinal tract, thereby leading to proliferation and disease. Dietary changes such as severe calorie restriction **[Au: Please could an example of such a dietary**

change be specified for context?] have also been shown to affect the structure and function of the microbiota, affecting colonization resistance against *C. difficile*.[222,223] Humanization of the gut microbiota of germ-free mice with the microbiota from severe calorie restricted individuals was associated with the enrichment of endogenous *C. difficile* [222] while diet-derived modulation of the microbiota was seen in mice fed two different diet formulations that altered the severity of C. difficile induced colitis. [223] Treatment of CDI with antibiotics further exacerbates this dysbiosis and can leave a patient at risk of disease recurrence. The restoration of homeostatic microbiota and their associated metabolites is essential for recovery and the prevention of recurrences (Figure 4). Understanding the critical components of the gut microbiome that prevent *C. difficile* expansion affords the potential to block *C. difficile* proliferation using bacteria-based biotherapeutics.[220]

~~[H2]~~ Fecal microbiota transplantation

The efficacy of microbiota restoration was first demonstrated using a fecal microbiota transplantation (FMT) for the treatment of recurrent CDI. FMT involves the transplant of minimally manipulated feces from a healthy donor to the colon of a recipient with recurrent CDI, which has been demonstrated to restore intestinal microbiota and metabolome homeostasis.[224,225] Stool preparations can be administered through colonoscopy, nasogastric delivery, enema or through an oral capsule.[226] FMT is recommended in the treatment of recurrent CDI following treatment with either fidaxomicin or vancomycin[3,25], but notable variation in efficacy rates has been reported **[Au:OK?]** .[227] The efficacy of FMT is dependent on the transplanted microbiota, as well as host-specific factors. These include the impact of diet on disease severity[223] and the role of host immunity on FMT success, with inflammatory environments promoting the survival of pathogens whilst inhibiting FMT engraftment.[228] A deeper understanding of the underlying mechanisms of FMT is needed to elucidate FMT efficacy.

Despite the success of FMT treatments, clear challenges have arisen with regards to standardised procedures for harvesting, screening and preparing donor stool. The need to screen and reject FMT donors adds considerable cost to this procedure.[229] Safety concerns have been raised regarding the potential transmission of pathogens, following the transfer of Shiga toxin-producing *Escherichia coli*[230] (an extended-spectrum beta-lactamase producing *E. coli*) **[Au:OK? Single use abbreviations edited out.]** that resulted in FMT

recipient fatalities.[231] With increasing evidence for the role of the gut microbiota in other disease states, the use of undefined microbial consortia could have unknown long-term health implications; thus, a shift has occurred **[Au:OK?]** towards more standardised, defined and well characterised microbial interventions. The recent US FDA regulatory approvals of fecal-derived RBX2660 (trade name Rebyota) and SER-109 (trade name Vowst) biotherapeutics (discussed in the next section) **[Au: Edits OK?]** for the prevention of CDI recurrence are therefore welcome advances to address the challenge of recurrent CDIs.

~~[H2]~~ Microbiota-derived therapeutics

Given the risks associated with the use of undefined bacterial communities such as FMT, much recent work has focused on standardized alternatives. These can be individual species (such as *C. scindens*, identified to be directly antagonistic to *C. difficile* germination[215] and vegetative cell growth[232]), or groups of microorganisms able to restore colonization resistance and gut diversity more widely. The most clinically promising biotherapeutics, such as the recently approved RBX2660 and SER-109 **[Au: The trade names were introduced in the previous section.]** have used complex groups of microorganisms derived from human gut microbial communities.

RBX2660 aims to deliver the effectiveness of FMT but instead using a standardized and regulated product, for the treatment for recurrent CDI. A Bayesian model was used to demonstrate a clinically meaningful treatment effect by RBX2660~~2500~~ **[Au: Is this a previous formulation or a typo? FYI, company names edited out for editorial reasons.]** across a randomized, double-blind, placebo-controlled, Phase III study, 2017-01 and from a Phase IIb study, 2014-01.[233,234] Adults who had one or more CDI recurrences with a positive stool assay for *C. difficile* (that is, either for a toxigenic strain or for toxin) and who were previously treated with standard-of-care antibiotics were randomly assigned 2:1 to a blinded, single-dose enema of RBX2660 (n=180) or placebo (n=87). The primary endpoint was treatment success, defined as the absence of CDI diarrhea within 8 weeks of study treatment. Treatment success rate was modelled to be 70.6% with RBX2660 versus 57.5% with placebo. The majority (>90%) of successfully treated patients in both study arms at 8 weeks had a sustained response up until 6 months. The size of the improvement in treatment success for RBX2660 versus placebo was 13.1% (95% CI: 2.3, 24.0).[234] RBX2660 was generally well tolerated. The incidence of treatment-emergent adverse events

was higher in RBX2660 recipients versus placebo recipients and was mostly driven by a higher incidence of mild gastrointestinal events (abdominal pain and diarrhea). No serious treatment-related adverse effects were reported. Of note, RBX2660 was evaluated in only a limited number of immunocompromised patients.[233]

SER-109 **[Au: Company name edited out for editorial reasons.]** is a live biotheraputic comprising an encapsulated mixture of purified *Firmicutes* spores, obtained from the feces of healthy humans. Thus, this biotherapeutic contains considerably fewer different bacteria than those present in RBX2660. The resilience of spores means they survive a purification process, including ethanol treatment, to reduce the risk that transmissible infectious microorganisms could contaminate SER-109. ECOSPOR III (SERES-012, NCT03183128) was a phase III multicentre, randomized, placebo-controlled study that enrolled 182 adults with ≥3 episodes of CDI within the previous 12 months (inclusive of the study entry episode).[235] All participants received standard of care oral antibiotic treatment (either vancomycin or fidaxomicin) and were stratified according to age (aged <65 or ≥65 years) and CDI antibiotic received, before randomization to SER-109 ($\sim 3\times10^7$ spore colony-forming units) or placebo, administered as four matching oral capsules once daily over 3 consecutive days. Given that both vancomycin or fidaxomicin can persist in feces after cessation of oral administration, 10 ounces of magnesium citrate was administered the night before SER-109 receipt to limit inactivation of the bacteria in this therapy. Notably, toxin testing was required at study entry and at suspected recurrence to ensure enrollment of patients with active CDI and accurate assessment of the endpoint. At 8 weeks post-treatment, 88% of SER-109 recipients were free from *C. difficile* recurrence compared with 60% in the placebo group (relative risk of recurrent CDI in SER-109 recipients, 0.32, 95% confidence interval [CI], 0.18 to 0.58; P<0.001). Notably, this reduction in risk of recurrence was maintained at 24 weeks, with the respective proportions of patients without recurrent CDI recurrence being 79% versus 53%.[236] Efficacy was confirmed across the stratified subgroups. SER-109 was generally well tolerated with no drug-related serious adverse events.

The superiority of SER-109 compared with placebo at preventing CDI recurrence was associated with clear changes in microbiome composition and concentrations of secondary bile acids in particular.[235] Engraftment of SER-109 bacteria was observed by week 1 and persisted through week 8. Numbers of engrafting SER-109 bacterial species

were higher among SER-109 versus placebo recipients through week 8. Following dosing with SER-109, declines were observed in proinflammatory Enterobacteriaceae and increases in Firmicutes (that can promote the synthesis of secondary bile acids). Greater increases in secondary bile acids from baseline occurred in SER-109 recipients compared with placebo recipients **[Au:OK?]** at all time-points through week 8.

Such regulated biotherapeutics, with clearly defined efficacy and safety parameters, will provide more certainty than is currently associated with FMT, and competitor products will likely become available for CDI treatment. However, the fairly high acquisition costs of biotherapeutics mean that cost-effectiveness data are needed to establish their respective uses in CDI treatment pathways.

[H2] Antitoxin antibodies

Bezlotoxumab was the first approved therapeutic *C. difficile* anti-Tcd~~toxin~~ B monoclonal antibody **[Au: anti-TcdB monoclonal antibody?]** , ~~intended~~ shown to successfully reduce the risk of CDI recurrence when used with standard of care antibiotics.[237] However, less than 10 years following its launch, production has been discontinued, which appears to be a commercial decision following only modest use **[Au: Is Bezlotoxumab effective? The text doesn't actually specify this.]** . A further novel monoclonal anti-Tcd~~toxin~~ B antibody**[Au: anti-TcdB monoclonal antibody?]** is now under development.[238] Animal studies support a possible role for *C. difficile*-specific colostrum-derived antibodies **[Au:OK?]** as an immunotherapeutic for the prevention or treatment of CDI.[239]

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[H2] Non-toxigenic *C. difficile*

Clinical proof of concept has been demonstrated for the use of a non-toxigenic *C. difficile* strain to block pathogenic strains and so reduce the rate of CDI recurrence when used as an adjunct to standard of care antibiotics.[240] However, despite this successful phase II clinical trial, there has been no further clinical development of this approach in the past decade. This lack of development **[Au:OK?]** possibly reflects the high cost of investigating new agents and noting multiple clinical development failures of novel CDI therapeutics, including tolevamer,[241] surotomycin,[242] cadazolid[243] and ridinilizole.[244]

[H2] Phage therapy

C. difficile-specific phages have been investigated for the possible development of new therapies for CDI, and indeed many *C. difficile*-specific phages have been identified.[245] However, clinical trials of phage-based therapy for CDI have not commenced and so its potential remains unknown.

[H2] Vaccines

Attempts to date to develop an efficacious vaccine to prevent CDI have not been successful, partly reflecting the low attack rate in the general public and so a need to target higher risk individuals, with very large clinical trials.[246,247] Achieving vaccine immunogenicity in older adults and immunocompromised individuals adds to the challenges here. In 2024 **[Au:OK?]** , a multivalent mRNA vaccine approach targeting the combined repetitive oligopeptide and receptor binding domains of TcdA and TcdB, and the metalloprotease virulence factor Pro-Pro endopeptidase 1 was shown to protect mice from lethal CDI,[248] but this vaccine remains to be progressed to human trials.

~~[H1]~~ Conclusions **[Au: Heading edited to fit with journal style.]**

Whilst careful antibiotic stewardship and optimal diagnosis and patient management has drastically reduced large CDI outbreaks in healthcare facilities, *C. difficile* continues to cause considerable mortality and morbidity worldwide. The epidemiology of CDI still varies considerably across countries/regions, which seems to be only partially explained by ascertainment bias. As such, we need to understand better what drives these geographical variances. Although our knowledge of *C. difficile* pathogenicity and mechanisms of antibiotic resistance has vastly improved in recent years, continued surveillance (including phenotypic susceptibility testing) is crucial in preventing outbreaks of novel epidemic and resistant ribotypes. A key aim is to understand the beneficial and harmful effects of diet on key components of the microbiome and so on colonization resistance and health outcomes, including CDI.

Although understanding of the mechanisms of microbiota-mediated colonization resistance is in its infancy, targeted microbiota restoration therapies and adjuncts to therapy show promise in improving patient outcomes. The recent advances in live biotherapeutic products, which are derived from the human gut microbiome, are welcome and represent laudable examples of therapeutics that have been designed to address the pathogenesis of CDI. Hitherto, the reliance on antibiotics to treat an often antibiotic-induced infection has been poignant. The relative effectiveness of antibiotic therapies is closely aligned with the extent of microbiome derangement they induce and so the risk of CDI recurrence. Ideally, new antibiotics to treat CDI should have very narrow spectra of activity, with long residual activity after dosing has ceased. Such attributes would limit microbiome derangement and provide a window of protection for when non-eradicated spores might germinate.

Our reliance on combinations of tests to identify who has CDI is imperfect. Better diagnostic options that have optimal sensitivity to detect *C. difficile*, but notably also with improved specificity for CDI itself, are needed. Such diagnostics need to be priced at a level where they are truly accessible and so will be used widely. Also, being able to identify who is at increased risk of CDI and/or CDI recurrence would allow both targeted use of prophylactic options and measures to reduce the risk of re-inducing CDI.

Lastly, the efforts to develop effective CDI vaccines need to be redoubled. A key challenge here is the size and cost of the clinical trials required to demonstrate that investigational vaccines are effective. The CDI attack rate is still fairly low among individuals deemed to be at increased risk of the infection. Thus, very large trials have been needed. Being able to identify who is at markedly increased risk of CDI would offer the chance of smaller trials to determine proof of concept and ultimate clinical effectiveness. The proportion of the populations of almost all developed countries/regions that are aged >65 years **[Au:OK? Journal style avoids the term elderly, preferring to use specific age thresholds or to use the term 'older adults'.]** are expected to increase markedly during the 21st century. So, CDI is likely to become a greater threat, emphasizing the potential healthcare and societal value of cost-effective CDI vaccines.

~~Whilst careful antibiotic stewardship and optimal diagnosis and patient management has drastically reduced large CDI outbreaks in healthcare facilities, *C. difficile* continues to cause significant mortality and morbidity worldwide~~ **[Au: The highlighted sentences in this paragraph**

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are the same as ones near the start of the conclusions; please rephrase this paragraph or delete.] ~~Our knowledge of *C. difficile* pathogenicity and mechanisms of antibiotic resistance has vastly improved in recent years, although continued surveillance (including phenotypic susceptibility testing) is crucial in preventing outbreaks of novel epidemic and resistant ribotypes. Although understanding of the mechanisms of microbiota-mediated colonization resistance is in its infancy, targeted microbiota restoration therapies / adjuncts to therapy show promise in improving patient outcomes.~~

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~~[H1]~~ **References** [Please ensure that references are cited sequentially in the following order: main text, tables, figure legends and then boxes. The numbered references should be listed at the end of the article in the format: 1. Author, A. B. & Author, B. C. Title of the article. Nat. Cell Biol. 6, 123–131 (2001). (with journal abbreviation italic, and volume bold). If there are six or more authors to a reference, only the first author should be listed followed by 'et al.'. For more details on reference format please consult the Guidelines to Authors.

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This phase III, double-blind, randomized, placebo-controlled clinical trial demonstrated that administration of SER-109 following standard-of-care antibiotic therapy was superior in preventing recurrence of symptoms than placebo.

~~[H1]~~ **Author contributions** [Au: Please amend this statement so that it fits with the following format— The authors contributed equally to all aspects of the article. OR All authors/X.X. researched data for the article. All authors/X.X. contributed substantially to discussion of the content. All authors/X.X. wrote the article. All authors/X.X. reviewed and/or edited the manuscript before submission.]

All authors contributed substantially to the discussion of content, researching data, writing and reviewing and editing this manuscript. CC led the structure, review and edit process.

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directly arises from the grant. In particular, grants that support data generation for distinct studies that are related in topic but that are not relevant for this specific publication should not be acknowledged. Therefore, for non-primary articles (reviews, perspectives etc), which report no new data, it is generally not best practice to acknowledge grant funding for projects just because they are related to the topic of the non-primary article.]

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~~[H1]~~ Competing interests [Competing interests: Please declare financial and non-financial competing interests for all authors. Please refer to our competing interest policy for more information. If none of the authors have competing interests, a negative statement must be included. The declarations made in this section must match those in the submission system.]

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The European Tissue Symposium and Tillotts. The other authors have no competing interests.

Table 1. A summary of antibiotic resistance characteristics of *C. difficile* to CDI treatment and non-CDI treatment agents. [Au: For later formatting reasons, display items and their associated reference citations must go in the following order after the reference list: Tables, figure legends, boxes. I moved up this table accordingly. Please use your reference management software to update the order of the reference list. Thanks!]

Antibiotic [Class]	Mode of action	Resistance mechanism	Genes involved	spread
CDI treatment antimicrobials				
Metronidazole [nitromidazole] [Au: Please note, table cells cannot be left empty. Please merge this cell with the one above if appropriate, or if not, add something (even if NA)]	Nucleic acid synthesis	Plasmid mediated	*pCD-METRO* [104]	Uncommon
		Haem-dependent	*PnimB*^{G} promotor variant leading to constitutive transcription of *nimB:* production of a haem binding flavoenzye that degrades nitroimidazoles [106,107]	Uncommon, however, variation in susceptibility testing methods may have led to underestimation.
Vancomycin [glycopeptide]	Bacterial cell wall synthesis	Not well understood	Possible *VanSR vanG, vanA, vanB, vanW vanZ* involvement possibly plasmid mediated *(Tn1549)*	Uncommon, some recent reports of elevated MICs
Fidaxomicin [macrolide]	RNA polymerase	Mutations in *RpoB and RpoC*	al1143Leu/Gly /Asp in *RpoB,*[118-120] Gln1149Pro and in *RpoC* leading to Arg89Gly. [120]	Very uncommon
Antimicrobials known to predispose to CDI				
Clindamycin [lincosamide]	Disruption of bacterial protein synthesis	Methylation of ribosome to prevent antibiotic binding	*erm*(B) on mobile elements such as Tn5398 or Tn6194[249]	widespread across many ribotypes and geographical locations[98]
			cfr (B), *cfr*(C) and *cfr*(E) (a new *cfr*-like gene)[250]	
Moxifloxacin Ciprofloxacin [fluroquinolones]	Inhibition of bacterial DNA gyrase, preventing	mutations in the quinolone resistance-	*gyrA* and/or *gyrB*[251,252]	particularly associated with RT027, but found in many

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	replication and transcription	determining regions (QRDR)	Thr82Ile is the most widely studied mutation	ribotypes across a wide geographical area.[98]
[Third generation cephalosporins]	interfere with bacterial cell wall synthesis	Mutations in binding proteins or acquisition of b-lactamases.	Endogenous D-class b-lactamases have been reported, [122,253] substitutions in Penicillin Binding Protein 1 and 3[23]	co-incided with fluoroquinolone resistance in epidemic lineages[23]
Other antimicrobials				
[Tetracyclines]	bind to the 30S ribosomal subunit, inhibiting bacterial protein synthesis	ribosomal protectant proteins	*tet*(M) on mobile element Tn96164 *tet*(44) on mobile element Tn6164	***[Au: Empty cell. Add 'NA' or 'None'?]*** *NA*
Rifampicin [rifamycins]	bind to RpoB (different site of action to fidaxomicin)	Mutations occur in the rifamycin resistance determining region (RRDR).	most common mutation described is Arg505Lys	widespread high level resistance, particularly among epidemic ribotypes such as RT027[98 125] some association with rifamycin treatment.[126]
Chloramphenicol	inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit	chloramphenicol acetyltransferase enzyme	the *cat*D gene on Tn*4453a* and Tn*4453b*	seen across many *C. difficile* ribotypes.[254]

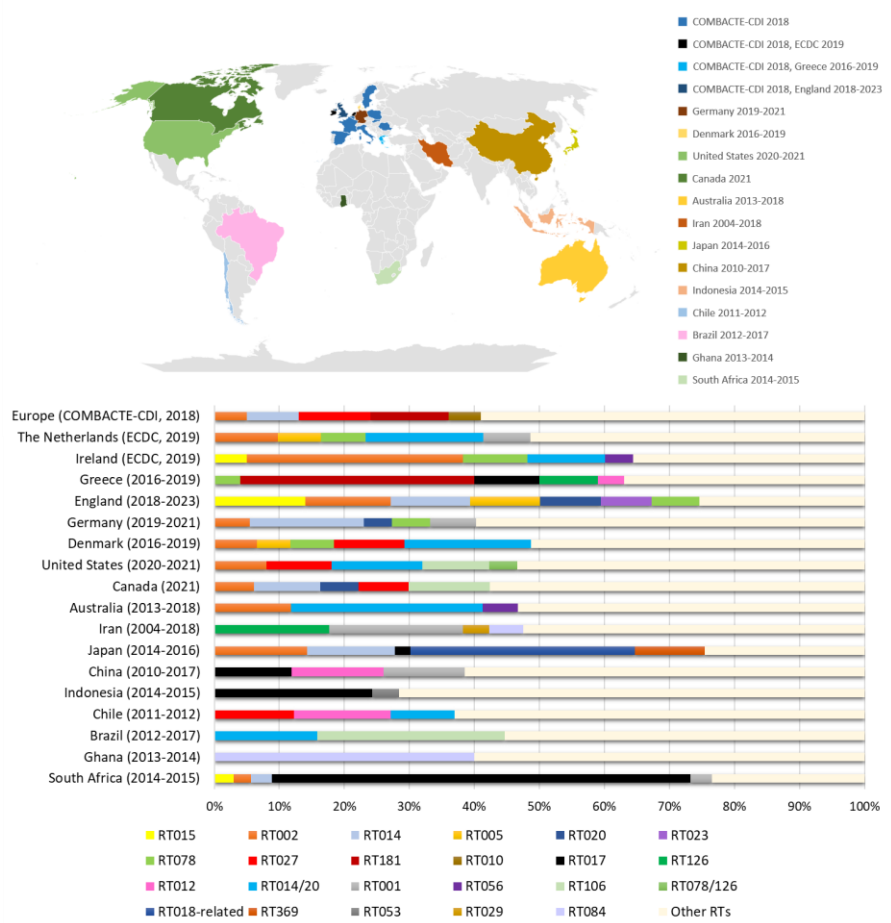

Figure 1: Most common *C. difficile* ribotypes reported according to published data. Stacked bar charts show the percentage prevalence of indicated ribotypes. Epidemic ribotypes, such as ribotype 027 (RT027), RT181 **[Au:OK?]** , RT001, RT078 and RT014/020 continued to circulate in Europe, RT014/20 and RT027 in the USA and Canada, and RT014/20 in Australia. RT027 was rarely found in Australia (<1%). While recent epidemiological data in other parts of the world are lacking, some historical data are shown, highlighting circulation of RT017 in Asia and South Africa. The range in years shown indicates the collection period of *C. difficile* isolates for each of the data sources. Data sources are as follows; Europe (n=198) 2018 point prevalence study across 12 European countries/regions,[31] Greece (n=221) the most recent surveillance information that included at least the year 2019 [38], Denmark (n=2692) the most recent surveillance information that included at least the year 2019,[255] The Netherlands (n=1082) a report from the European Centre for Disease Prevention and Control,[256] Ireland (n=581) a report from the European Centre for Disease Prevention and Control,[256] Germany (n=876)[32], England (n=31,435) report from The UK Health Security Agency,[36] United States (n=300) a US-based national surveillance study

[currently ref 32], Canada (n=392) report from the Canadian Nosocomial Infection Surveillance Program ,[41] Australia (n=1,523) surveillance report,[42] Iran (n=366) 14-year-long cross-sectional study[257] Due to the lack of surveillance programs in other parts of the world, historical data (pre-2018) were retrieved from published reviews, including a study in Japan (n=177),[48] China (n=319) and Indonesia (n=340),[45] Chile (n=81) and Brazil (n=38),[46] Ghana (n=15) and South Africa (n=269).[258]

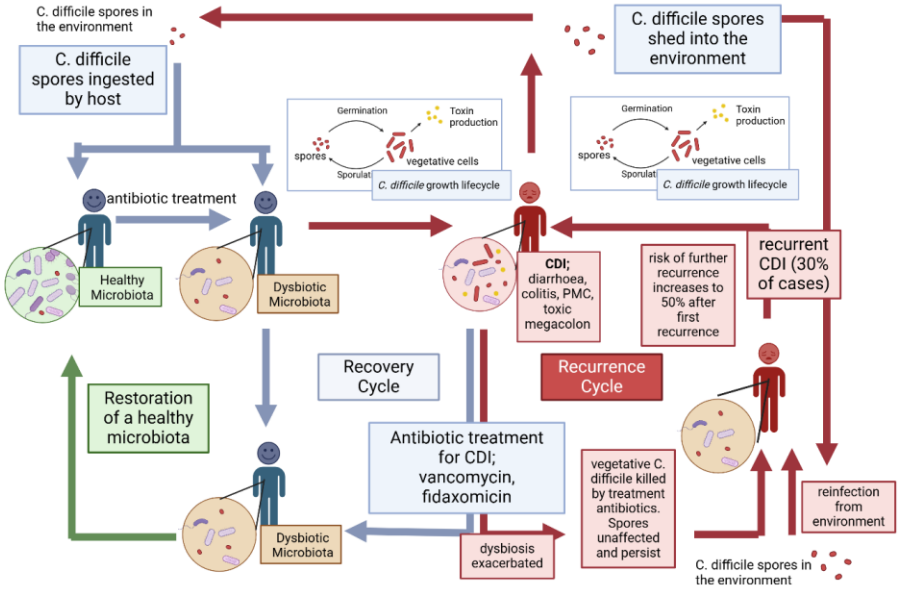

Figure 2: The *C. difficile* infection cycle. C. difficile spores are ingested from the environment. In a susceptible host (e.g., following antibiotic mediated disruption), spores are able to germinate and a proliferating vegetative population produces the toxins that mediate CDI. CDI treatment antimicrobials can further exacerbate dysbiosis, and both recrudescent spores within the gut, or a re-infection of spores from the environment can result in recurrent disease. Each incident of recurrence increases the chances of further recurrent disease, prolonging the recurrence cycle. Restoration of a healthy microbiota can restore colonization resistance, breaking the recurrence cycle and leading to recovery.

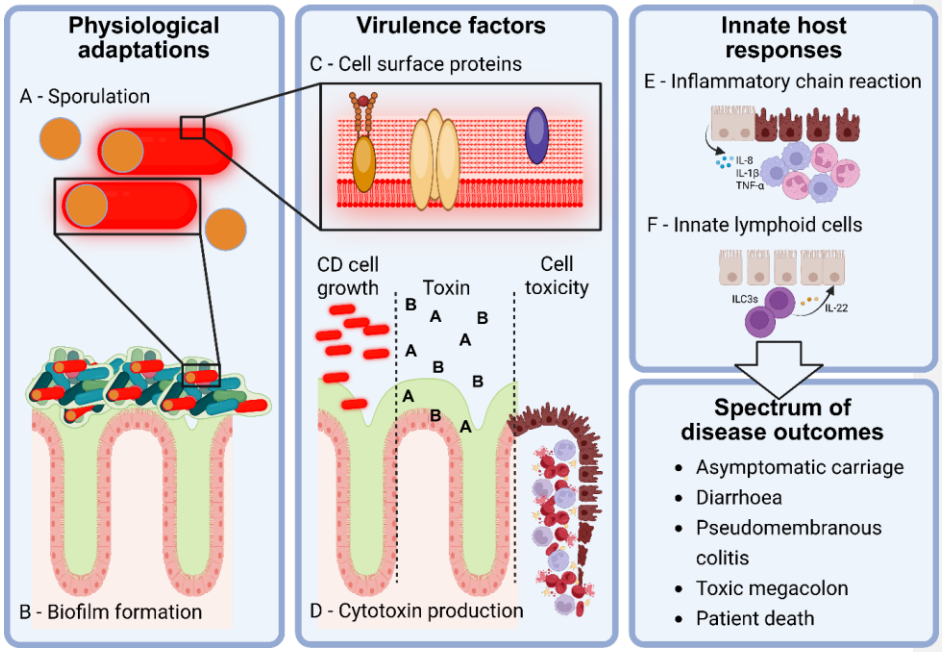

Figure 3: Summary of pathogenicity factors during CDI and recurrent disease. During the initial phase of disease, metabolically inactive spores survive the harsh digestive environment, where they germinate into metabolically active vegetative cells. Both spores and vegetative *C. difficile* cells can integrate into the mucosal biofilm to form a reservoir for recurrent infection. The *C. difficile* cell surface, characterised by the S-layer and cell wall proteins **[Au: Please can you add labels for the key cell surface components on the figure set?]** , have a crucial role in adhesion on host cells, biofilm formation, resistance to host antimicrobial factors and environmental sensing. The main factor that causes most of the symptoms associated with CDI is the production of cytotoxins, TcdA and TcdB. This cytotoxicity causes damage to the epithelial layer, which induces an inflammatory innate immune response that can enhance the damage, but innate lymphoid cells can limit this damage by helping to strengthen the epithelial layer. During the final phase of pathogenesis, spores can be expelled from the body to contaminate the environment and infect others, thus starting the infection cycle again. All these factors can give rise to a spectrum of disease outcomes experienced by patients with CDI.

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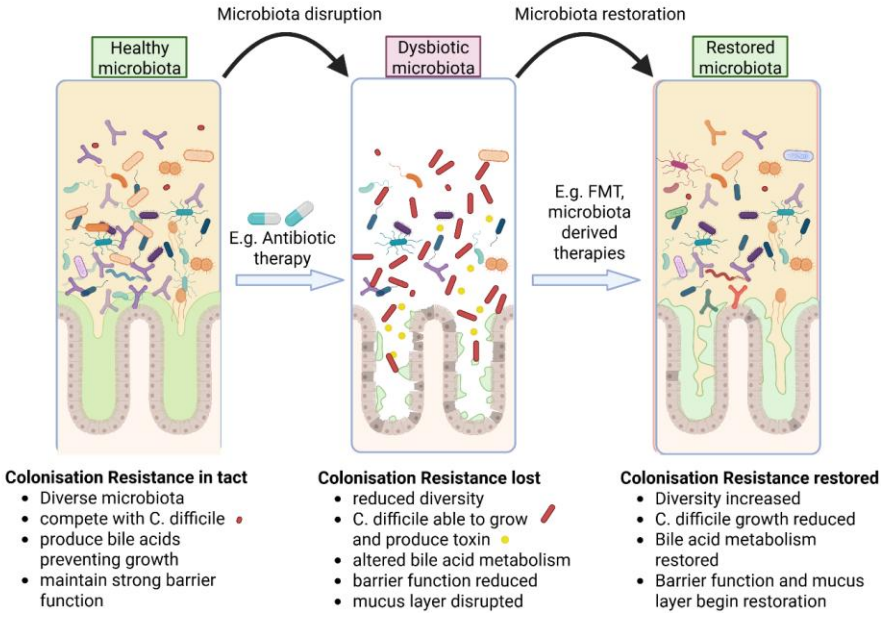

Figure 4: The microbiota in CDI. **[Au: I couldn't find a call-out for figure 4 in the main text. Please can you add one in an appropriate place?]** A healthy, diverse gut microbial community confers colonization resistance against CDI. Disruption to microbial communities (e.g. by antibiotic exposure) alters the structure and function of the microbial communities, allowing C. difficile germination and proliferation, leading to toxin production and resulting damage. Restoration of microbiota can be facilitated by FMT or microbiota derived therapies. Restoration of the metabolic and functional potential of the microbial communities can prevent further C. difficile growth and restore colonization resistance.

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Box 1 | Impact of the SARS CoV-2 pandemic on CDI incidence and epidemiology

A worrying increase in CDI incidence has been noted since the SARS-CoV2 pandemic and the emergence of novel ribotypes is potentially problematic.[259] In England, a 25% increase was reported in CDI incidence during **[Au:OK? If not, please clarify the 25% increase.]** 2021–2023 compared with pre-pandemic level. [36,260] Similar increases have been reported in Canada, Greece, Spain and Australia.[261-264] This picture is not universal, however. In Germany, a 50% decrease in the number of CDI cases was observed in 2021 compared with 2015, potentially associated with implementation of antimicrobial stewardship and hospital hygiene programmes.[265] Increased infection control measures might also have decreased CDI incidence in a Belgium hospital from 2020 to 2022. [266] In Spain, some studies reported an increase, whereas others noted a decrease or no change. [267] As resources were redirected to

deal with the pandemic, decreases in CDI could also be explained by lack of clinical suspicion and testing, leading to underdiagnosis.

The ECDC 2022–2023 survey of healthcare-associated infections in European hospitals reports a 10% increase in healthcare-associated CDI incidence compared with 2016–2017 (ref.[268]). An increase in healthcare-associated CDI in 2020 compared with 2019 and 2018 was also described (2.05 versus 1.50 and versus 1.70 cases per 10,000 patient-days, respectively).[256] However, the effect of the pandemic in the EU–EEA is not yet conclusive. Comparison between years should be made with caution, as few countries/regions reported surveillance data to the European Centre for Disease Prevention and Control **[Au:OK?]** in 2020; no formal data call were issued in 2020 and 2021 due to the changing national priorities in response to the SARS-CoV2 pandemic. [256] **[Au: Paragraph merged.]** Similarly, the impact of the SARS-CoV2 pandemic on CDI incidence in the USA is not yet fully conclusive, as some studies reported an increase, while others showed a stable or decreased incidence.[269] **[Au: Paragraphs merged.]** Continued surveillance of CDI is encouraged to further elucidate changes in CDI incidence.

Box 2 | Overview of typing methods used for *C. difficile* **[Au: I can't see a call-out to box 2 anywhere in the main text. Please can you add one in an appropriate location? Thanks!]**

Molecular characterisation of *C. difficile* strains is an important component of surveillance programmes, which enables tracking of epidemic spread caused by virulent types. PCR-ribotyping is widely used as the gold standard method for providing epidemiological data. This method is based on the amplification of the intergenic region between the 16S and 23S ribosomal RNA gene, generating a distinct banding pattern unique to a specific PCR ribotype.[13] Pulse-field gel electrophoresis (PFGE) and variable-number tandem-repeat analysis (MLVA) are alternative typing methods, but these are labour-intensive and not as widely used as PCR-ribotyping; however, PFGE is still used in North America.[270] While having lower resolution than other typing methods, toxinotyping provides clear information on the toxigenic status of *C. difficile* strains. This method relies on PCR amplification and restriction enzyme digestion of regions in the pathogenicity locus and correlates well with PCR-ribotyping. [270]

Multi locus sequence typing (MLST) schemes have also been developed to enable assignment of a sequence type based on the genetic variation of seven housekeeping genes and are useful tool for evolutionary studies.[271] While MLST correlates often, but not always, with PCR-ribotyping, none of those typing methods can ascertain transmission events. Whole genome sequencing (WGS) provides a higher level of resolution for identification of genetically related strains and for understanding taxonomic relationships. [272-274] Therefore, integration of WGS in surveillance programmes is currently being sought and has the potential to facilitate outbreak investigations and further understanding of transmission networks, as demonstrated by recent studies in healthcare settings[275,276]

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Clostridioides difficile infection (CDI) is challenging to diagnose and treat, and is associated with considerable mortality, morbidity and economic costs worldwide. In this Review, Chilton et al.

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discuss changes in global epidemiology, breakthroughs in pathogenesis and antibiotic resistance, the role of microbiota dysbiosis and the potential for microbiota-based therapeutics for CDI.