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Integrating urban wastewater surveillance for *Clostridioides difficile* with Public Health monitoring, a pilot study

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RESUMO

Clostridioides difficile é uma bactéria anaeróbia capaz de colonizar o intestino humano. Esta produz esporos, facilitando a sua sobrevivência em ambientes adversos, e disseminação. A sintomatologia surge após uma disrupção da microbiota intestinal, habitualmente provocada pelo uso de antibióticos. Tal permite a proliferação da bactéria, evasão do sistema imunitário do hospedeiro e produção de toxinas, resultando na infeção oportunista. A infeção por *C. difficile* (CDI) é comummente associada a ambientes hospitalares, e indivíduos mais velhos, e/ou previamente internados, são mais suscetíveis. Porém, a incidência de CDI em populações antes consideradas de baixo risco tem vindo a aumentar, com intensificação do número de casos reportados na comunidade.

A transmissão desta bactéria ocorre por via fecal-oral, através do contacto entre indivíduos infetados ou suscetíveis, ou com superfícies contaminadas. Os sintomas despoletados incluem diarreia e dor abdominal, colite pseudomembranosa, e até morte. A CDI constitui a principal causa de diarreia associada a cuidados de saúde na Europa, exercendo uma pressão considerável sobre os sistemas de saúde. Em Portugal, a vigilância da CDI é realizada no Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA), juntamente com a Direção-Geral da Saúde, compreendendo uma rede de hospitais portugueses que enviam amostras de fezes de pacientes diagnosticados com CDI para a Unidade de Referência de Doenças Gastrintestinais, do INSA. No laboratório de referência, é realizada cultura anaeróbia, e posterior caracterização fenotípica e molecular dos isolados presuntivos de *C. difficile*.

O potencial patogénico desta bactéria prende-se principalmente com a produção de duas toxinas pró-inflamatórias, A e B. Algumas estirpes são ainda produtoras de uma toxina binária (CDT), frequentemente associadas a ribotipos hipervirulentos epidémicos. Embora a maioria das estirpes de *C. difficile* seja toxigénica, algumas não produzem toxinas, porém, podem adquirir e transmitir MGEs (elementos genéticos móveis), conferindo resistência antimicrobiana, e tornar-se uma ameaça.

A CDI é descrita como paradoxal, surgindo após administração de antibióticos para tratar outras infeções bacterianas. Assim, a terapia antimicrobiana constitui um fator de risco para o desenvolvimento da CDI, pois ao alterar o equilíbrio da microbiota intestinal, facilita a ação/proliferação da bactéria. Adicionalmente, o genoma de *C. difficile* destaca-se pela sua plasticidade genética, que permite adquirir resistência a múltiplos antibióticos. Tal limita as opções de tratamento disponíveis, criando desafios na abordagem da doença.

C. difficile pode também estar presente no intestino de animais e no ambiente, estabelecendo uma complexa rede de transmissão que deve ser investigada na perspetiva *One Health*. A menor prevalência de ribotipos hipervirulentos epidémicos, como o RT027 (associado a surtos hospitalares graves), face a uma maior incidência de ribotipos encontrados no meio ambiente, indicia uma alteração na epidemiologia da CDI. Dos perfis emergentes, destacam-se o RT106 e RT014/020, frequentemente associados a CDI adquirida na comunidade (CA-CDI). Tal sugere a existência de reservatórios fora do ambiente hospitalar.

O tratamento de águas residuais (AR) constitui uma medida crucial para a Saúde Pública, pois as estações de tratamento de águas residuais (ETARs) recebem afluentes que podem conter agentes patogénicos. Atualmente, *C. difficile* não está contemplado nos microrganismos monitorizados durante o tratamento, dada a associação a ambientes hospitalares. Porém, a recente mudança na epidemiologia, sugere que a monitorização de *C. difficile* em AR pode ser extremamente relevante, juntamente com a vigilância de CDI em humanos. Estudar as AR permitiria determinar se os esporos da bactéria conseguem resistir aos tratamentos, e consequentemente, contaminar o meio ambiente.

Este estudo pretende atualizar o conhecimento sobre a epidemiologia da CDI em Portugal, caracterizando estirpes de *C. difficile* obtidas de amostras provenientes de 17 hospitais, distribuídos por cinco regiões de Portugal (Norte, Centro, Área Metropolitana de Lisboa, Alentejo e Açores), da rede nacional de vigilância, e proceder à monitorização de *C. difficile* em AR, através da caracterização de estirpes de amostras recolhidas numa ETAR de Lisboa. Foram recuperados 384 isolados de *C. difficile* de amostras clínicas, e 140 isolados de *C. difficile* de amostras de AR. Destes, 99 foram obtidos de amostras de AR brutas (afluente) e 41 do fluxo de saída da linha líquida (efluente tratado) da ETAR. Foram ainda recolhidas três amostras de Água para Reutilização (ApR), nas quais não foi detetada *C. difficile*, sugerindo a eficácia do tratamento efetuado. Todos os isolados, clínicos e de AR, foram caracterizados quanto ao ribotipo e perfis de toxinas, e suscetibilidade antimicrobiana (TSA), nomeadamente à clindamicina, moxifloxacina, rifampicina, metronidazol e vancomicina.

Observou-se grande variabilidade genética, tendo sido identificados 92 ribotipos distintos entre os 384 isolados clínicos. Os seis mais prevalentes foram os ribotipos toxigénicos RT106 (14,1%), RT014/020 (10,2%), RT078/126 (4,9%), RT002 (4,4%), e RT012 e RT013 (ambos com 3,4%). Dois foram comuns às cinco regiões do país, o RT014/020 e RT106. Salienta-se na região Norte o mais prevalente, o hipervirulento RT027, com 22 (16,4%) isolados provenientes de um hospital, associados a um surto declarado em outubro de 2023, ainda em curso. De notar que o RT027 não era detetado nos hospitais portugueses da rede de vigilância desde 2021. Embora estatisticamente seja o segundo ribotipo mais prevalente, o RT027 não foi incluído na análise geral para evitar enviesamento dos resultados. A sequenciação total do genoma (WGS) de cinco isolados revelou mutações nos genes *gyrA* e *rpoB*, e a presença do gene *ermB*, conferindo resistência genotípica à moxifloxacina, rifampicina e clindamicina, respetivamente. Estes demonstraram ainda resistência fenotípica à gentamicina, e suscetibilidade reduzida à vancomicina, exibindo a mutação VanRCd Thr115Ala recentemente descrita, sendo a primeira descrição deste clone multirresistente em Portugal.

Como expectável, as estirpes toxigénicas foram mais prevalentes (90,1%) entre os 384 isolados clínicos. Após TSA, todos os isolados demonstraram suscetibilidade à vancomicina e metronidazol. A maior taxa de resistência foi observada para a clindamicina (21,4%), com os 82 isolados predominantemente pertencentes aos ribotipos toxigénicos RT012 (11,1%), RT106 (7,3%), RT078/126, e RT559 (6,1% cada), e não-toxigénicos RT010 e RT039 (4,9% cada). Os 60 (15,6%) isolados resistentes à moxifloxacina pertenciam maioritariamente a três ribotipos (todos toxigénicos): RT106 (25,0%), RT078/126 (13,3%) e RT559 (8,3%). Já a resistência à rifampicina foi detetada em três (6,5%) isolados, de ribotipos RT085 não toxigénico (4,0%), RT181 hipervirulento (4,0%) e RT284 toxigénico (4,0%).

A recuperação de *C. difficile* foi de 100,0% e de 87,5% entre as oito amostras de afluente e as oito amostras de efluente, respetivamente. É de salientar a utilização de duas técnicas de isolamento distintas, e em cada foram repicados, pelo menos, cinco isolados por amostra. A ribotipagem dos 140 isolados de AR revelou 33 perfis distintos. Entre os isolados de afluente, os ribotipos toxigénicos RT014/020 e RT106 (15,2% cada) foram os mais prevalentes, seguidos do hipervirulento RT078/126 (12,1%), e dos não-toxigénico RT009 e toxigénico RT011 (7,1% cada), em linha com os achados clínicos. No efluente tratado, os ribotipos mais comuns compreenderam o toxigénico RT430 (24,4%), os não-toxigénicos RT010 (17,1%) e RT009 (14,6%), e o toxigénico RT103 (12,2%). As estirpes toxigénicas foram mais prevalentes tanto no afluente (76,7%), como no efluente tratado (65,9%). Salienta-se que cinco dos perfis mais prevalentes em isolados clínicos foram identificados em isolados de AR: RT106, RT014/020, RT078/126, RT012 e RT013. Após TSA, o cenário foi semelhante ao encontrado nos isolados clínicos; assim, todos apresentaram suscetibilidade à vancomicina e metronidazol. A maior taxa de resistência observada nos 99 isolados do afluente foi registada para a

clindamicina (14,1%), com 14 isolados predominantemente pertencentes ao não-toxigénico RT084 (28,6%) e aos toxigénicos RT012 (21,4%) e RT739 (14,3%); seguida da moxifloxacina (9,1%), com nove isolados de três ribotipos (todos toxigénicos): RT078/126 (77,7%), RT106 e RT014/020 (11,1% cada). Apenas um isolado (1,0%) de RT009, não-toxigénico, foi resistente à rifampicina. No efluente tratado, apenas foi encontrada resistência à clindamicina, presente em cinco dos 41 isolados, todos de RT009, não-toxigénico.

Uma análise baseada em SNVs (*single-nucleotide variants*) após WGS de 31 isolados, clínicos e de AR, agrupados por ribotipo, revelou uma elevada similaridade genética entre isolados das duas fontes. A maioria dos grupos filogenéticos obtidos continha estirpes de ambas as fontes, existindo isolados clínicos e de AR sem distância entre si. Adicionalmente, uma análise *in silico* da resistência antimicrobiana revelou a presença de MGEs raramente descritos e nunca antes detetados em estirpes europeias de *C. difficile*, como um plasmídeo num isolado de afluente, e um MGE contendo o gene *ermB* num isolado clínico e noutro de efluente tratado. A presença destes elementos evidencia a troca genética ativa via transferência horizontal de genes que ocorre dentro do intestino do hospedeiro e na ETAR, constituindo um risco para a disseminação de resistências a antibióticos no meio ambiente.

Finalmente, a análise de isolados clínicos de *C. difficile* evidenciou a mudança na epidemiologia da CDI, com maior prevalência de ribotipos normalmente encontrados no meio ambiente. Adicionalmente, salientou a reemergência do hipervirulento RT027 em Portugal. Foi demonstrado que a vigilância de *C. difficile* em AR pode contribuir para o entendimento da epidemiologia de CA-CDI, estando presentes estirpes de *C. difficile* toxigénicas, e não-toxigénicas, com resistências a antimicrobianos passíveis, ou não, de ser usados no tratamento da CDI. Considerando a epidemiologia da CDI, e os resultados muito preliminares deste estudo relativamente às AR, torna-se necessária uma investigação mais profunda de reservatórios ambientais de *C. difficile*, no contexto *One Health*.

Palavras-chave: *Clostridioides difficile*; vigilância de CDI em Portugal; águas residuais urbanas; CA-CDI; suscetibilidade antimicrobiana

ABSTRACT

Clostridioides difficile is an anaerobic, spore-producing bacterium that colonizes the human gut. After a disruption of the colonic microbiota, often due to antibiotic use, symptoms are triggered. Infection by *C. difficile* (CDI) is a major cause of healthcare-associated diarrhea, raising concern in community settings. This study investigated CDI epidemiology in Portugal, analyzing clinical isolates from Portuguese hospitals and environmental isolates from a Portuguese wastewater treatment plant (WWTP). A total of 384 clinical isolates and 140 wastewater isolates (99 influent, 41 effluent) were recovered. No *C. difficile* was detected in water for reuse (ApR) samples, suggesting treatment efficacy. Among clinical samples, 92 ribotypes were identified, with RT106 and RT014/020 predominating. An outbreak of multidrug-resistant RT027 was reported, linked to the VanRCd Thr115Ala mutation in *vanR*, not previously described in Portugal. Antimicrobial susceptibility testing (AST) of clinical isolates revealed resistance to clindamycin (21.4%), moxifloxacin (15.6%) and rifampicin (6.5%), all susceptible to vancomycin and metronidazole. *C. difficile* was recovered in 100.0% of influent and 87.5% of effluent samples. Thirty-three ribotypes were uncovered, with RT014/020 and RT106 predominating, mirroring clinical findings. The AST profile of wastewater isolates was similar to clinical isolates, in both influent and effluent samples. Ribotypes commonly found in humans were prevalent in wastewater, indicating possible environmental transmission. Whole-genome sequencing-based analysis revealed high genetic similarity between clinical and wastewater isolates, with some differing by only one single-nucleotide variant. Novel mobile genetic elements were identified in both clinical and wastewater isolates, suggesting active horizontal gene transfer and emphasizing the environmental risk that *C. difficile* strains recovered from wastewater may pose. This study highlights the need for *C. difficile* monitoring in urban wastewaters to better understand the epidemiology of community-associated infections, and its findings reinforce the importance of considering *C. difficile* as a One Health issue, integrating human, environmental and animal health perspectives.

Keywords: *Clostridioides difficile*; CDI surveillance in Portugal; urban wastewaters; CA-CDI; antimicrobial susceptibility

Publications within the scope of this study

Isidro, J., Dionísio, F., Almeida S., Santos C., Santos M., Reis E., Oliveira R. J., Gomes J., Oleastro M. (2024) Prolonged hospital outbreak associated with multidrug resistant *Clostridioides difficile* RT027 clone exhibiting reduced susceptibility to vancomycin. European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE). Stockholm, Sweden. 20-22 Nov 2024. (Accepted for poster presentation)

Roseiro, I., Louro, M., Isidro, J., Dionísio, F., Oleastro M., Henriques O. A., Serrano M. (2024). Within host evolution of *Clostridioides difficile*. Evolving Microbes and Global Health Symposium. Oeiras, Portugal. 22-25 Oct 2024. (Accepted for oral presentation)

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A

ADP	Adenosine Diphosphate
AMR	Antimicrobial Resistance
ApR	Water for Reuse
AR	*Águas Residuais*
ARG	Antimicrobial Resistance Gene
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection

C

CA-CDI	Community-Acquired *Clostridioides difficile* Infection
CARD	Comprehensive Antibiotic Resistance Database
CDI	*Clostridioides difficile* Infection
CDT	*Clostridioides difficile* Transferase
CFU	Colony-Forming Unit
cgMLST	Core Genome Multilocus Sequence Typing
CLSI	Clinical and Laboratory Standards Institute
COMBACTE-CDI	Combatting Bacterial Resistance in Europe - *Clostridioides difficile* infections

D

D-Ala-D-ala	D-Alanyl-D-alanine Dipeptidase
D-Ala-D-ser	D-Alanyl-D-serine Ligase
DDI	*Departamento de Doenças Infecciosas*
DGH	*Departamento de Genética Humana*
DGS	*Direção-Geral da Saúde*
DNA	Deoxyribonucleic Acid
DSA	*Departamento de Saúde Ambiental*

E

ECDC	European Centre for Disease Prevention and Control
EIA	Enzyme Immunoassay
ETAR	*Estação de Tratamento de Águas Residuais*
EUCAST	European Committee on Antimicrobial Susceptibility Testing

G

GDH	Glutamate Dehydrogenase
GI	Genetic Island

H

HGT	Horizontal Gene Transfer

I

IGV	Integrative Genomics Viewer
INSA	*Instituto Nacional de Saúde Doutor* Ricardo Jorge
IS	Insertion Sequence

M

MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MLS_B	Macrolide-Lincosamide-Streptogramin B
MLST	Multilocus Sequence Typing

P

PBP	Penicillin-Binding Proteins
PCR	Polymerase Chain Reaction

Q

QC	Quality Control
QRDR	Quinolone-Resistance Determinant Region

R

RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RT	Ribotype

S

SNP	Single-Nucleotide Polymorphism
SNS	National Health Service
SNV	Single-Nucleotide Variant
ST	Sequence Type

T

TSA	*Testes de Suscetibilidade Antimicrobiana*

U

UAS	*Unidade de Água e Solo*
UK	United Kingdom
URGI	*Unidade de Referência de Doenças Gastrintestinais*
USA	United States of America
UTI	*Unidade de Tecnologia e Inovação*
UV	Ultraviolet

W

WGS	Whole-Genome Sequencing
WWTP	Wastewater Treatment Plant
WW	Wastewater

1. INTRODUCTION

1.1 *Clostridioides difficile* – Infection dynamics, environmental persistence and impact on healthcare facilities

Clostridioides difficile is an anaerobic Gram-positive bacillus able to colonize the human intestine asymptomatically, or effectively cause disease in susceptible individuals (Chisholm et al., 2022; Oleastro et al., 2014). This bacterium produces spores, enabling its survival in extreme environmental conditions that the vegetative form could not withstand. Spores are considered the infectious form of *C. difficile*, resisting heat, UV light, radiation, oxygenated environments, and acidic conditions, such as those found in the gastric juice. This resilience enables the pathogen's dissemination and persistence in the environment. After being ingested, when spores reach the duodenum, bile salts induce germination, and in the absence of competing microbiota, the vegetative cells colonize the intestine by adhering to epithelial cells (Al-Zahrani, 2023; Oleastro et al., 2014). These cells produce toxins that damage the intestinal mucosa, leading to severe diarrhea. Eventually, vegetative cells will produce spores again, shedded in feces, restarting the infection cycle by contaminating the environment and potentially infecting others. Notably, even after treatment, some spores may remain in the host's intestine, leading to recurrent infections when conditions become suitable for germination (Isidro et al., 2017).

Thus, infection by *Clostridioides difficile* (CDI) arises from a disruption of the colonic microbiota, allowing the growth of *C. difficile* and evasion of the host's immune system, triggering symptoms. Symptomatology fluctuates between mild symptoms and severe complications, depending on the patient's associated risk factors. Older individuals, actively or previously institutionalized, dealing with recurrent infections - and hence, exposed to regular antibiotic administration - present a greater risk of developing severe CDI. The most common clinical manifestations are diarrhea, abdominal pain, vomits, fatigue and loss of appetite, while severe ones comprise pseudomembranous colitis, septic shock and death, amongst others (Crispino, 2023; Oleastro et al., 2014).

C. difficile spores can be found in soil, water, animals, and on various surfaces. These can persist for months or even years in environments where the vegetative form would not survive, facilitating its spread within the community. Transmission occurs via the fecal-oral route through direct contact between individuals, and the spores' resistance to common cleaning practices facilitates the spread of infection in hospitals. *C. difficile* spores are resistant to many cleaning agents and alcohol-based disinfectants, allowing it to survive on contaminated surfaces, medical equipment, and even on the hands and uniforms of healthcare professionals. This increases the risk of transmission in healthcare settings (Khun et al., 2023). Thus, in order to reduce the load of *C. difficile* spores, hospitals tend to use chlorine-based disinfectants, such as an unbuffered 1:10 dilution of hypochlorite (Kenters et al., 2017). However, airborne hydrogen peroxide has also demonstrated great results, as it is a broad-spectrum disinfectant effective against pathogens responsible for nosocomial infections and comprises low toxicity and high compatibility with most surfaces, unlike bleach (Falagas et al., 2011).

CDI is usually related to hospitals or other healthcare facilities, comprising the main cause of healthcare-associated diarrhea in Europe (Davies et al., 2014). This infection entails such an unreasonable burden in healthcare systems, whether for Public Health or financially, that a quick and accurate diagnosis becomes urgent and essential. Therefore, the European Centre for Disease Prevention and Control (ECDC) encouraged the standardization of CDI surveillance in each country. In Portugal, this surveillance is coordinated by *Instituto Nacional de Saúde Doutor* Ricardo Jorge (INSA) and *Direção-Geral da Saúde* (DGS) since 2010, where a network of sentinel hospitals within the National Health Service (SNS) sends feces samples from patients positive for CDI to the reference laboratory to

test *C. difficile* antimicrobial susceptibility and perform its molecular characterization (ribotype determination and toxin genetic profile), and ultimately improve diagnostic (Isidro et al., 2017; Nazareth et al., 2022).

1.2 Virulence factors of *Clostridioides difficile*

The clinical manifestation of the infection caused by *Clostridioides difficile* is deeply associated with this pathogen's virulence factors. This bacterium's pathogenic potential is mainly related to the activity of two potent toxins, TcdA and TcdB, encoded by genes *tcdA* and *tcdB*, respectively, both located in the pathogenicity locus (PaLoc) (Buddle & Fagan, 2023). The PaLoc comprises a total of five genes *tcdR, tcdB, tcdE, tcdA* e *tcdC*, as presented in Figure 1.1:

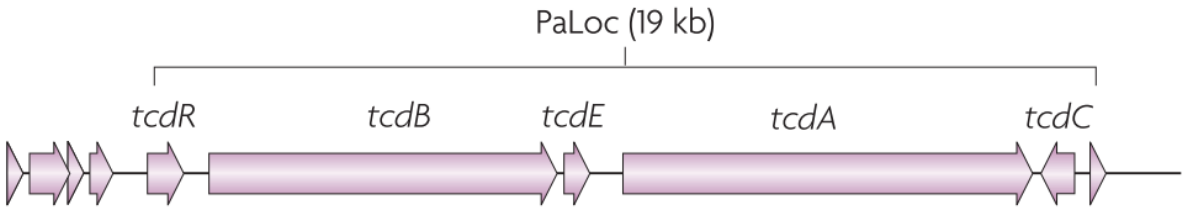

Figure 1.1. *Clostridioides difficile* pathogenicity locus (PaLoc) [adapted from Rupnik et al. (2009)].

TcdA and TcdB production is not only dependent on the strain of *C. difficile* at issue, but also on environmental factors, such as nutrient levels, temperature and subinhibitory levels of antibiotic (Rupnik et al., 2009). After being released by the bacterium, TcdA and TcdB are internalized by gut epithelial cells, and when reaching the cytosol, these toxins glycosylate GTP-binding proteins (such as small Rho proteins). The inactivation of such proteins leads to actin condensation, cytoskeleton disintegration and disruption of tight junctions, cell rounding and, eventually, cell death (Buddle & Fagan, 2023; Isidro et al., 2017). Symptoms are further exacerbated by the host's immune response, where multiple inflammatory mediators (cytokines) are released from intestinal epithelial cells, mast cells and macrophages, promoting an influx of inflammatory cells. Consequently, this results in epithelium destruction and loss of intestinal barrier function (Rupnik et al., 2009).

Considering genome proximity, as well as the resemblance in its sequence, it is believed that these two cytotoxins may have emerged from a gene duplication. However, literature indicates that, in addition to a broader tropism, TcdB presents higher cytoxicity when compared to TcdA (Hunt & Ballard, 2013). In Rupnik et al. (2009), clinical data and results obtained from a study performed in hamsters are compared, revealing TcdA-TcdB+ strains trigger the range of symptoms associated with CDI, whereas in TcdA+TcdB- strains, no virulence is demonstrated. Furthermore, this paper discloses that TcdB is considered to be more potent, being responsible for mucosal necrosis and loss of barrier function when testing the human colonic tissue, unlike TcdA.

Hunt & Ballard (2013) reveals that the expression of the majority of PaLoc genes occurs in latter stages of *C. difficile* growth, nevertheless, *tcdC* is the only factor expressed during the exponential phase. On the one hand, *tcdR* is transcribed during the stationary phase and encodes for a sigma factor of RNA polymerase, the main positive regulator of PaLoc expression. Besides activating its own expression, TcdR binds with promoting regions upstream of *tcdA* and *tcdB*, leading to its transcription. On the other hand, the TcdC protein negatively regulates this transcription, since it is an anti-sigma factor specific for TcdR. In other words, by preventing the bond between TcdR and RNA polymerase during the exponential phase, it suppresses the expression of toxins TcdA and TcdB. Additionally, *tcdR* transcription can also be repressed by the presence of high levels of glucose (Isidro et al., 2017).

Lastly, the *tcdE* gene encodes for a holin-like molecule (TcdE), which is believed to play a role in the secretion of toxins from the host's organism, however, its mechanism, as well as its impact in secretion itself, is still up for debate (Hunt & Ballard, 2013; Isidro et al., 2017).

In addition to toxins TcdA and TcdB, a small percentage of *C. difficile* strains also produce the binary toxin (CDT) (Hunt & Ballard, 2013). Epidemic virulent ribotypes are often among these strains, sparking interest in the potential role of this toxin in *C. difficile* virulence. However, it remains unclear whether CDT provides any significant advantage to the bacterium, and there is no consensus that the toxin enhances the virulence of the infecting strain. Therefore, its presence being linked to more severe clinical outcomes is highly debated (Berry et al., 2017; Hunt & Ballard, 2013). This toxin is composed of two proteins, CdtA and CdtB, encoded by genes *cdtA* and *cdtB*, respectively, thus composing the binary toxin locus (CdtLoc), alongside the regulator gene *cdtR* upstream, as presented in Figure 1.2:

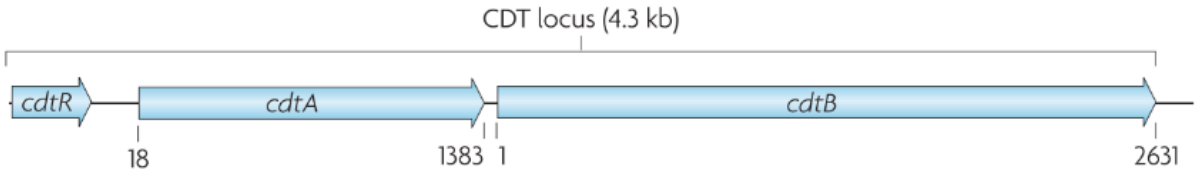

Figure 1.2. *Clostridioides difficile* binary toxin locus (CdtLoc) [adapted from Rupnik et al. (2009)].

CDT is mainly produced by strains positive for both the *tcdA* and *tcdB* genes. Less commonly, this toxin can also be present in strains that are TcdA+ and TcdB- (Alves et al., 2022), and it has also been reported in strains negative for the *tcdA* and *tcdB* genes (Eckert et al., 2014), indicating that CDT alone can cause symptomatic infection. Interestingly, both profiles were described in RT033 strains. Additionally, as for the PaLoc, there is variability in the CdtLoc across strains, as this locus may be fully intact, contain only remnants of *cdtA* and *cdtB* or be entirely absent (Hunt & Ballard, 2013).

Regarding its action mechanism, the binding component, CdtB, establishes a connection with the host cell, translocating the catalytic component, CdtA, to the cytosol. In the cytosol, CdtA ADP-ribosylates actin molecules, inducing depolymerization of the actin cytoskeleton, and consequently, the formation of long protrusions based in microtubules that end up trapping bacteria. Thus, this facilitates the bacterium's adhesion to the surface of gut epithelial cells, leading to increasingly virulent and recurrent strains (such as of ribotypes RT027 and RT078/126) (Alves et al., 2022; Isidro et al., 2017).

Although toxins are the main virulence factors of *Clostridioides difficile*, other determinants also play an active role in its pathogenicity. As previously mentioned, being a strict anaerobe, this pathogen's capacity to form spores resistant to oxygen and heat, amongst other environmental factors, enhances its virulence. Biofilm formation is another contributing factor, and despite information regarding this subject still being scarce, literature describes bacteria within biofilms as being more protected and with greater resistance to inauspicious factors, such as oxygen and the presence of antibiotics, when in the host's gut (Isidro et al., 2017). It is suggested that during infection, *C. difficile* persists on the surface of the intestinal mucosa in biofilm-like structures, which may act as a reservoir for the pathogen and contribute to CDI recurrence (Frost et al., 2021). However, further research is needed to better understand the role of biofilms in *C. difficile* virulence. Additionally, mutations in sporulation-related mechanisms have been shown to reduce biofilm formation (Buddle & Fagan, 2023). Moreover, proteins in the vegetative cells' surface layer (S-layer) are also fundamental for an improved adherence to the intestinal mucosa, and to further induce an inflammatory response as well (Rupnik et al., 2009).

1.3 Antimicrobial susceptibility of *Clostridioides difficile*

The gastrointestinal infection triggered by this strict anaerobe can be described as paradoxical, since it emerges when antibiotics are administered to treat other bacterial diseases. Antibiotics have a significant impact on the composition of the gut microbiota, both in terms of diversity of the bacterial community, and at a metabolic level. By reducing the products of bacterial metabolism and promoting the accumulation of precursors, such as primary bile acids, mannitol and fructose, antimicrobials end up offering ideal conditions for spore germination and growth of *C. difficile*. Therefore, it is understandable how antibiotic therapy constitutes one of the most concerning risk factors for CDI, enabling this bacterium's action as an opportunistic pathogen. Furthermore, the risk of CDI associated with the use of antibiotics implies two aspects, the effect of the antibiotic on the intestinal microbiota and the administration of an antibiotic to which a strain of *C. difficile* present in the host's organism is resistant. In this last scenario, the resistant strain has a selective advantage, thereby increasing the probability of infection even during the administration of such antibiotic. Nonetheless, after antimicrobial therapy, the host can be infected by a resistant, or susceptible, *C. difficile* strain, with no benefits for the resistant strain at this stage (Buddle & Fagan, 2023; Isidro et al., 2017).

Although all antibiotics are associated with CDI, there is a greater risk associated with prolonged administration of broad-spectrum agents, such as clindamycin, cephalosporins and fluoroquinolones. The aforementioned lead to a long-lasting dysbiosis, increasing the risk of CDI during therapy itself, as well as three months following its end, depending on the antibiotic. Typically, metronidazole is administered in mild-to-moderate CDI cases, and, vancomycin and fidaxomicin consist of first-line antibiotics administered in severe cases of CDI, since being the most effective against this pathogen (Isidro et al., 2017; Nazareth et al., 2022). Nevertheless, increasing recurrence rates associated with metronidazole have dethroned this antibiotic as a first-instance treatment for CDI, and vancomycin has recently replaced it, thus posing a risk, due to higher vancomycin selection pressures (Buddle & Fagan, 2023).

1.4 Antimicrobial resistance mechanisms in *Clostridioides difficile*

The *C. difficile* genome is characterized by its remarkable genetic plasticity, as approximately 11.0% is composed of mobile genetic elements (MGEs), including plasmids, bacteriophages, insertion sequence (IS) elements, as well as conjugative and mobilizable transposons (Mullany et al., 2015; Sebaihia et al., 2006). As a result, antibiotic resistance may arise from mutations in specific genes or from genes acquired by horizontal gene transfer (HGT), whilst the mechanisms that lead to such resistance comprehend modifications of the antibiotic target, inactivation of the antibiotic itself or reduction of intracellular concentration of antibiotic (Isidro et al., 2017).

Both fluoroquinolones and rifamycins are inhibited due to point mutations in target genes. On the one hand, the most common phenotype in *Clostridioides difficile* is resistance to fluoroquinolones, such as moxifloxacin. This class of antibiotics poses one of the greatest risk factors for CDI, since its association with the emergence of RT027. Point mutations occur in the quinolone-resistance determinant region (QRDR) of DNA gyrase subunits (*gyrA* and *gyrB* genes). On the other hand, the rifamycin group includes rifampicin, whose resistance has been highly reported in epidemic strains of *C. difficile*. This mechanism of resistance comprehends mutations in the *rpoB* gene encoding for the β-subunit of RNA polymerase, thus altering the antibiotic binding site (Isidro et al., 2017).

Another common form of resistance observed in *C. difficile* involves the macrolide-lincosamide-streptogramin B (MLS_B) family of antibiotics, which includes erythromycin and clindamycin. Such resistance is triggered by the presence of the erythromycin ribosomal methylase gene

B (*ermB*), obtained from transposons in the *C. difficile* genome. As the name suggests, *ermB* encodes for a methylase that modifies 23S ribosomal RNA (rRNA), lowering the affinity of the antibiotic binding site. However, *C. difficile* strains have been identified as erythromycin-resistant while lacking *ermB*, suggesting the existence of an alternative resistance mechanism (Buddle & Fagan, 2023; Isidro et al., 2017).

Although rare, both reduced susceptibility and resistance to metronidazole have been reported in *C. difficile*, most frequently amongst non-toxigenic strains, as those of RT010. It is known that there are multiple pathways of resistance to this antibiotic, however, the understanding of such mechanisms is still limited. One of these routes is plasmid-mediated resistance and the plasmid in question (pCD-METRO) is acquired via HGT. Boekhoud et al. (2020) affirms pCD-METRO is already internationally disseminated and can be found in epidemic strains, despite the specific genes involved in such resistance have yet to be determined. Unfortunately, the existence of multiple mechanisms of resistance to metronidazole demonstrates how antibiotics used for CDI treatment may impact *C. difficile* pathogenesis, in case of treatment failure or recurrence (Buddle & Fagan, 2023).

Resistance to vancomycin has not been reported yet, however, the number of *C. difficile* isolates with reduced susceptibility has been increasing over time. Despite limited information, studies have described multiple mechanisms that can lead to this scenario. In 2013, Peltier et al. explored a modification in terminal D-Ala-D-ala to D-Ala-D-ser as a source for reduced susceptibility to vancomycin, compromising the antibiotic binding site. In 2021, Pu et al. described the alteration in *C. difficile* susceptibility to vancomycin through the acquisition of a plasmid, pX18-498, suggesting the existence of multiple mechanisms of resistance. Furthermore, recent data has revealed multiple *van* gene clusters in *C. difficile* isolates associated with reduced susceptibility to vancomycin. These clusters are described as phenotypically silent, only expressed in the presence of vancomycin. Nevertheless, its expression is dependent on a two-component regulatory system: the *vanS* gene, which encodes a membrane sensor kinase, and the *vanR* gene, which encodes a cytoplasmic response regulator (Blau et al., 2023). Additionally, Wickramage et al. (2023) has identified a mutation (Thr115Ala substitution) in *vanR* associated with elevated minimum inhibitory concentrations (MICs), in clinical *C. difficile* isolates. When describing this mutation, Shen et al. (2020) suggests that this alteration enhances stability when interacting with *C. difficile* DNA, thereby improving the activation of resistance gene transcription.

Table 1.1 condenses the mechanisms of resistance, or reduced susceptibility, that have already been reported in *C. difficile* strains.

Table 1.1. Summary of *Clostridioides difficile* resistance to antibiotics and its associated mechanisms of action and resistance.

Antibiotic	Mechanism of action	Resistance frequency	Mechanism of resistance	Reference
MLS$_B$	Inhibition of protein synthesis by binding to 23S rRNA	High	Target protection by *ermB*, located in the transposons Tn5398, Tn9164 and Tn6215, or *cfr*, in Tn6218	Buddle & Fagan (2023); Isidro et al. (2017)

Antibiotic	Mechanism of action	Resistance frequency	Mechanism of resistance	Reference
Fluoroquinolones	Inhibition of DNA synthesis by binding to DNA gyrase and topoisomerase IV	High; associated with frequent and epidemic ribotypes	Target modification by mutations in *gyrA* and *gyrB*	Isidro et al. (2017)
Rifamycins	Inhibition of RNA synthesis by binding to RpoB	Common	Target modification by mutations in *rpoB*	Isidro et al. (2017)
Metronidazole	DNA damage after reduction of metronidazole inside the bacterial cell	Rare; reduced susceptibility reported in frequent ribotypes	Multifactorial; plasmid-mediated (pCD-METRO); 5-nitroimidazole reductase; modifications in multiple proteins involved in DNA repair, iron uptake and metronidazole reduction (putative)	Boekhoud et al. (2020); Isidro et al. (2017)
Vancomycin	Inhibits cell wall synthesis by binding to dipeptide D-Ala-D-Ala of peptidoglycan precursors	Rare	Mutations in terminal D-Ala-D-Ala; plasmid-mediated (pX18-498); Thr115Ala substitution in *vanR*	Peltier et al. (2012); Pu et al. (2021); Wickramage et al. (2023)
Fidaxomicin	Inhibition of RNA synthesis by binding to RNA polymerase (site distinct from rifamycins)	Rare	Mutations in *rpoB*, *rpoC* and *rarR* (reduced susceptibility in *in vitro* mutants)	Isidro et al. (2017)

Antibiotic	Mechanism of action	Resistance frequency	Mechanism of resistance	Reference
Tetracyclines	Inhibition of protein synthesis by binding to 30S ribosomal subunit	Common	Target protection by *tetM*, carried by the transposons Tn5398 or Tn916-like	Isidro et al. (2017)
Imipenem	Inhibition of cell wall synthesis by binding to penicillin-binding proteins (PBPs)	Uncommon	Mutations in the transpeptidase domain of two penicillin-binding protein genes (*pbp1* and *pbp3*)	Isidro et al. (2017)

Therefore, if *C. difficile* presents resistance to these many antibiotics, there will be a limitation in the current available treatment options for CDI. This pathogen's genome has shown to be extremely flexible and incredibly able to adapt, and by developing resistance to antibiotics, colonization, persistence and recurrence are promoted. Moreover, it is noteworthy that multidrug resistance is generally associated with epidemic and/or severe CDI causing *C. difficile* strains. Such resistance grants these strains a selective advantage over others in terms of dissemination and infectious potential, particularly in settings with high antibiotic pressure, as hospitals for instance.(Buddle & Fagan, 2023; Isidro et al., 2017).

1.5 Genotyping approaches in *Clostridioides difficile* epidemiology

When studying the epidemiology of *Clostridioides difficile* and attempting to control hospital outbreaks, genotyping becomes essential. To do so, the reference method is PCR-ribotyping, which analyzes the intergenic region between the 16S and 23S ribosomal RNA genes (Krutova, Kinross, et al., 2018). This technique identifies variations in the length of PCR-generated fragments, resolved by gel or capillary electrophoresis (Fawley et al., 2015), which are then compared with a database of known ribotypes (RTs). Ribotyping is widely used for CDI surveillance and to study *C. difficile* outbreaks due to its well-established technique and its high inter-laboratory reproducibility, however, it does have limitations in terms of its moderate discrimination power (Abad-Fau et al., 2023).

An alternative genotyping method is the sequence-based analysis Multilocus Sequence Typing (MLST), where several conserved regions of the genome are analyzed, and isolates are grouped into sequence types (STs). While MLST provides useful genetic discrimination, it does not offer the necessary resolution to accurately distinguish cases of direct transmission between patients, especially in hospital settings. There is a correlation between some RTs and STs (Seth-Smith et al., 2021), as both tend to cluster similarly in phylogenetic analyses, hence, MLST can be used as a tool for identification when ribotyping fails (Abad-Fau et al., 2023). Nevertheless, MLST, based on only a few loci, has limited discriminatory power when compared to more advanced methods. In contrast, cgMLST (core genome MLST) improves this resolution by analyzing thousands of loci in the genome of *C. difficile*, being able to distinguish between isolates, and thus, more effective in outbreak investigations (Abad-Fau et al., 2023; Seth-Smith et al., 2021).

Furthermore, whole-genome sequencing (WGS) offers the highest discriminatory power amongst all methods, allowing for the analysis of differences at single-nucleotide polymorphism (SNP) level. This technique is particularly useful not only for outbreak investigations regarding hospital-associated CDI, but also for the analysis of environmental *C. difficile* isolates, regarding the investigation of possible sources of community-acquired CDI (CA-CDI) causative strains (Seth-Smith et al., 2021). Additionally, WGS is commonly used for researching virulence factors and antimicrobial resistance (AMR) mechanisms, as for instance in Gargis et al. (2023) and Isidro et al. (2018). Therefore, this technique is slowly becoming the gold standard for epidemiological investigations (Seth-Smith et al., 2021).

1.6 The changing epidemiology of *Clostridioides difficile*

C. difficile can be found in the gut of humans and animals, as well as in the environment, establishing a complex transmission network of great significance for One Health. That being so, besides hospitals and other healthcare facilities, transmission can also occur in the community, and the incidence of CA-CDI has been increasing over the last decade (Chisholm et al., 2022; Khun et al., 2023). For instance, in the early 2000s, fluoroquinolones were the most commonly prescribed class of antibiotics in the United States of America (USA). The selective pressure from its use led to an increase in the prevalence of hypervirulent, resistant strains of the RT027 lineage. These strains demonstrate higher expression of toxins A and B due to a mutation in *tcdC*, in addition to producing binary toxin, and are also associated with more severe cases of CDI, with higher transmission rates and increased mortality. As a result, a major outbreak took place in North America and Europe (Oleastro et al., 2014). This scenario raised global concerns about CDI monitoring and established RT027 as one of the main causes of infection in the years that followed. However, recent changes in the epidemiology of *C. difficile* have been observed worldwide, with a decline in epidemic strains such as RT027 and an increase in the emergence of ribotypes linked to colonization in animals (pets, food chain) (Alves et al., 2023) and commonly found in the environment (Viprey et al., 2021). The emerging ribotypes are associated with cases of CA-CDI, and include RT106, and RT014/020 (Nazareth et al., 2022; Tickler et al., 2019).

Furthermore, there is an increase of CDI cases in individuals without any associated risk factors or contact with hospital environments, along with growing rates in asymptomatic colonization by *C. difficile* in the community. Hence, this scenario implies the existence of once unknown reservoirs, outside healthcare facilities, even though CDI is primarily regarded as a nosocomial disease associated with the elderly population (Cizek et al., 2022; Nazareth et al., 2022). On the one hand, One Health approaches have uncovered that *C. difficile* spores enable propagation, as well as the survival of this bacterium, in distinct environments, so much that strains have been isolated from food, soil, animals (such as swine), river water and municipal wastewaters (Cizek et al., 2022). For instance, resorting to a pig farm model, Alves et al. (2022) reassures the fundamental role of animal production units in the CDI panorama. By revealing significant prevalence of *C. difficile* in environmental samples (that comprise soil, manure and water), this study highlights the importance of the environment as a reservoir for toxigenic strains and potential source of CA-CDI. On the other hand, it has grown increasingly clear that healthy individuals can act as asymptomatic carriers, with their gastrointestinal tract serving as a reservoir for infectious *C. difficile* strains present in the environment. This poses a transmission risk to more vulnerable individuals within the community and suggests the emergence of novel risk factors associated with the rise of CA-CDI, such as the consumption of contaminated food or water (Baghani et al., 2020). Moreover, the growing number of outpatient antibiotic prescriptions also contributes to the development of CA-CDI, increasing susceptibility amongst individuals (Gupta & Khanna, 2014).

Thus, besides hospitals, the aforementioned reservoirs can play a major role in the transmission of CA-CDI, and within this transmission chain are Wastewater Treatment Plants (WWTPs), as illustrated in Figure 1.3:

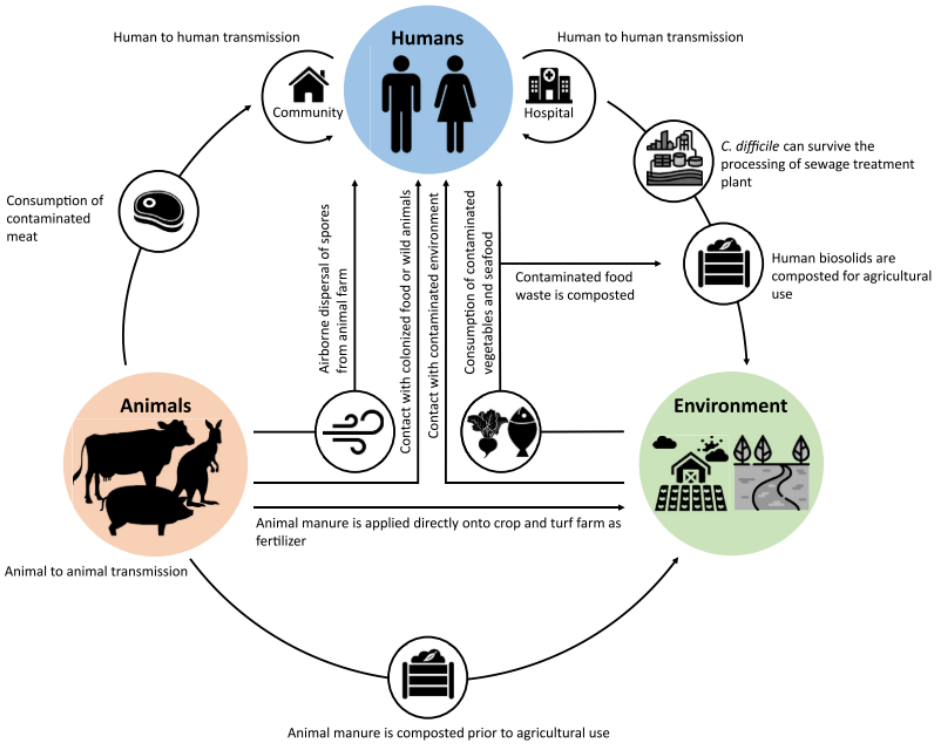

Figure 1.3. The complex transmission network of *Clostridioides difficile* [from Lim et al. (2020)].

1.7 *Clostridioides difficile* surveillance in wastewater

Wastewater treatment is not only an environmental protective measure, but also a Public Health measure. WWTPs manage influents containing high levels of organic waste, where pathogens may be present; therefore, stringent control measures and monitoring of microorganisms, such as fecal coliforms or enteric pathogens, are essential during treatment (Baghani et al., 2020). Currently, *C. difficile* is not among the microorganisms routinely monitored in wastewater in general, as it has been traditionally associated with hospital environments over the years. However, the recent shift in the epidemiology of CDI, along with the emergence of new potential environmental and animal reservoirs outside healthcare facilities, suggests that monitoring this pathogen in treated effluents could be highly valuable. The surveillance of *C. difficile* in urban wastewater could help determine whether bacterial spores can withstand treatment processes carried out at the WWTPs and, consequently, whether they may be released into the environment. As a result, spores could eventually contaminate retail fish or seafood, animals for consumption, or even fruits and vegetables, thereby, potentially facilitating colonization in both animals and humans (Baghani et al., 2020; Cizek et al., 2022).

Furthermore, alongside the existing surveillance of CDI in human populations, as the one implemented in Portugal, monitoring this pathogen in urban wastewater could contribute to our understanding of CA-CDI, although the extent to which environmental and animal contamination contributes to CDI is still uncertain (Nikaeen et al., 2015). Meanwhile, literature has already described a significant overlap amongst ribotypes found in human, environmental sources and animals. For

instance, known toxigenic, as well as hypervirulent, *C. difficile* strains have been isolated from Taiwan's river system, as described in Tsai et al. (2022), from river water in the Czech Republic (Cizek et al., 2022), as well as from a WWTP in Western Australia (Chisholm et al., 2022). In addition, as a hotspot for a wide range of resistant bacteria, WWTPs play a crucial role in the dissemination of antibiotic resistance genes (ARGs) via transposons or MGEs present in bacteria (Baghani et al., 2020). So, since the discharge of treated effluents can lead to the release of pathogens, as well as ARGs, and an evident shift in *C. difficile* epidemiology has been verified, shedding a light on this microorganism's presence in WWTPs becomes increasingly relevant.

There are several levels of treatment carried out at WWTPs to achieve the legal discharge requirements. These vary between preliminary (or pretreatment), primary, secondary, and tertiary level, as illustrated in Figure 1.4. Nonetheless, not all WWTPs perform every aforementioned treatment. Pretreatment aims to prepare wastewater for the following stages, by removing large solids or debris, such as cotton swabs, stones, sand or grease. In order to do so, this process comprises gradation, desanding and degreasing. The primary level of treatment consists of a primary decantation, as a result of sedimentation and flocculation processes. Regarding secondary treatment, it usually consists of a biological treatment that targets the removal of biodegradable organic matter, where a secondary decantation takes place, originating clear, treated water. When confronted with a more sensitive aquatic receiving environment, the removal of remaining suspended solids becomes necessary. This is accomplished with a tertiary treatment level, through a process of filtration. Furthermore, secondary effluent may require an additional step of disinfection to ensure that there is no risk for Public Health. Disinfection aims to eliminate remaining pathogens in effluents and can be carried out through chemical or physical processes, such as UV radiation. Additionally, WWTPs can also produce treated water for reuse purposes, such as irrigation of agricultural fields, public green spaces or golf courts, equipment cleaning in industry and even public street washing. To achieve this, the disinfection step is necessary. (Silva Messias, 2012).

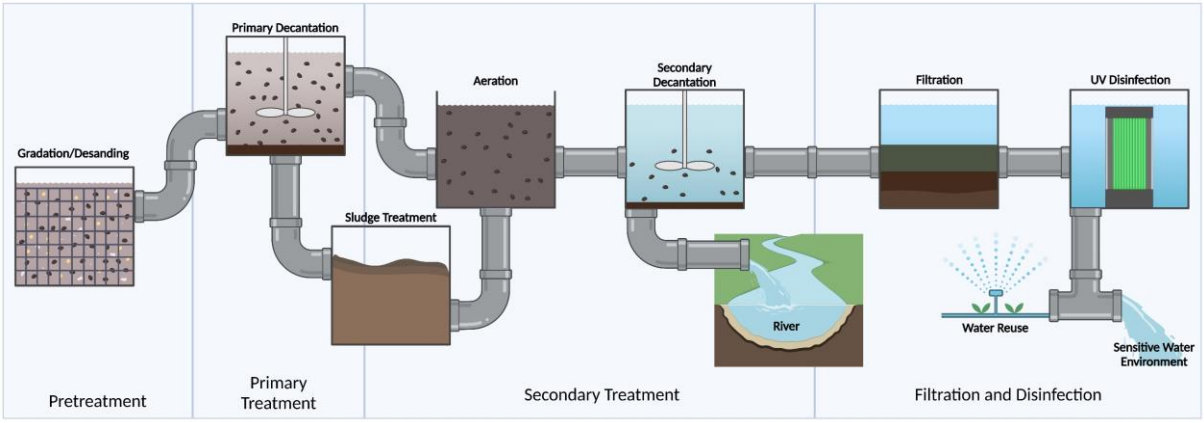

Figure 1.4. Example of the treatment stages in a wastewater treatment plant (focused on the effluent line; sludge treatment is not detailed) (created with BioRender.com).

2. AIMS AND OBJECTIVES

C. difficile is the leading cause of antibiotic-associated diarrhea, comprising a major concern worldwide, and CDI surveillance programmes note an epidemiological shift towards an increasing CA-CDI, reflecting a well-established intracommunity transmission network. However, information about the reservoirs outside healthcare facilities, is largely lacking, namely in Portugal. Therefore, the present dissertation aims to update the current knowledge on the epidemiology of CDI in Portugal, as well as shed light on how the surveillance of *C. difficile* in urban wastewaters can contribute to the current knowledge of possible reservoirs outside healthcare facilities, and to the understanding of CA-CDI epidemiology in the country. To better comprehend what is possibly being released into the environment, a characterization of potential *C. difficile* strains at the inlet and outlet of WWTPs is in order. Hence, the main objectives of this project comprise:

i) Identification and characterization of the phenotype and genotype of *Clostridioides difficile* strains isolated as part of the national surveillance network for infection by *C. difficile* (CDI), which includes several hospitals within the National Health Service of Portugal;
ii) Identification of the most prevalent ribotypes (RTs) in hospital settings in Portugal;
iii) Identification and characterization of strains isolated from the inlet and outlet of a Portuguese wastewater treatment plant (WWTP), using phenotypic and molecular techniques;
iv) Identification of the RTs in wastewaters with the potential to spread into the environment;
v) Comparison of the prevalence and diversity of RTs from hospital settings with those from wastewaters, along with an analysis of pathogenicity factors among these *C. difficile* strains.
vi) Analysis of antimicrobial susceptibility patterns and identification of resistance mechanisms, for both clinical and wastewater isolates, and further comparison;
vii) Identification of mobile genetic elements (MGEs), *in silico* antimicrobial resistance mechanisms and cluster analysis with human clinical strains and wastewater isolates, through a whole-genome sequence (WGS)-based approach.

3. MATERIALS AND METHODS

3.1 Clinical samples - Sampling

Under the scope of the national CDI surveillance programme, human fecal samples with positive enzyme immunoassay (EIA) test for the presence of *Clostridioides difficile* toxins A and B and/or positive EIA for glutamate dehydrogenase (GDH) were sent to *Unidade de Referência de Doenças Gastrintestinais* (URGI) of the *Departamento de Doenças Infecciosas* (DDI), at INSA. Each sample was accompanied by a brief questionnaire on the patient's clinical and epidemiological data, including age, sex and symptoms. Samples were received between September 2023 and May 2024.

3.2 Clinical samples - *Clostridioides difficile* isolation

Alcohol shock was performed by adding 1 mL of stool sample to 1 mL of absolute ethanol, for 1 h at room temperature, followed by homogenization. To obtain isolated colonies, 10 µL of the samples were inoculated onto ChromID® *C. difficile* agar (bioMérieux, Marcy l'Etoile, France) resorting to the four-quadrant streak method. Plates were then incubated for 48 h under anaerobic conditions generated using the anaerobic cultivation system Anoxomat® III Anaerobic Jar System (Anoxomat, Mart, Netherlands), at 37ºC. After incubation, and based on colony morphology, plates were assessed for the presence of *C. difficile*. From each presumably positive sample, one to two colonies were transferred onto Brucella blood agar supplemented with hemin and vitamin K1 (BD BBL™, Heidelberg, Germany) and incubated for 24 h under anaerobic conditions, at 37ºC. The steps followed are illustrated in Figure 3.1. Furthermore, species identification was carried out by MALDI-TOF (VITEK® MS, bioMérieux), when required.

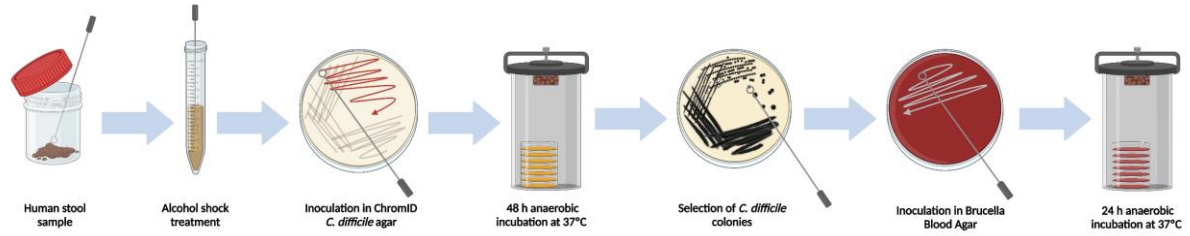

Figure 3.1. Flowchart of the *Clostridioides difficile* isolation protocol for clinical samples (created with BioRender.com).

3.3 Wastewater samples - Sampling

Urban wastewater samples were collected from raw wastewater (influent) and the outflow from the liquid line (treated effluent) of a WWTP located in the Lisbon Metropolitan Area, in a collaboration with *Departamento de Saúde Ambiental* (DSA), at INSA, and *Câmara Municipal de Lisboa*. Sampling was conducted over a five-month period (February, March, April, May and June of 2024) approximately every two weeks, resulting in a total of eight sampling events, in which the first five samplings only influent and effluent samples were collected, and in the last three samplings influent, effluent, as well as ApR (water for reuse) samples were collected. A total of 19 samples (of 1000 mL each) eight from both influent and effluent and three from ApR, were obtained and studied.

3.4 Wastewater samples – Water Filtration

Upon sampling, wastewater filtration was performed at *Unidade de Água e Solo* (UAS) of the *Departamento de Saúde Ambiental,* at INSA. For each sample, two 0.45-µm-pore cellulose membranes (EZ-Pak® Membrane Filter) were obtained by filtering 25 mL of the water matrix. The two membranes of each filtered sample were placed, individually, in 10 mL of *C. difficile* enrichment broth (BIOGERM® Laboratórios) and directly in ChromID® *C. difficile* agar (bioMérieux, Marcy l'Etoile, France), as observed in Figure 3.2. The enrichment broth was incubated under anaerobic conditions generated using the anaerobic cultivation system Anoxomat® III Anaerobic Jar System (Anoxomat, Mart, Netherlands) for seven days at 37°C, with atmosphere renewal every 48 h, and the plates were also anaerobically incubated for 48-72h at 37°C. Protocol was adapted from Chisholm et al. (2022).

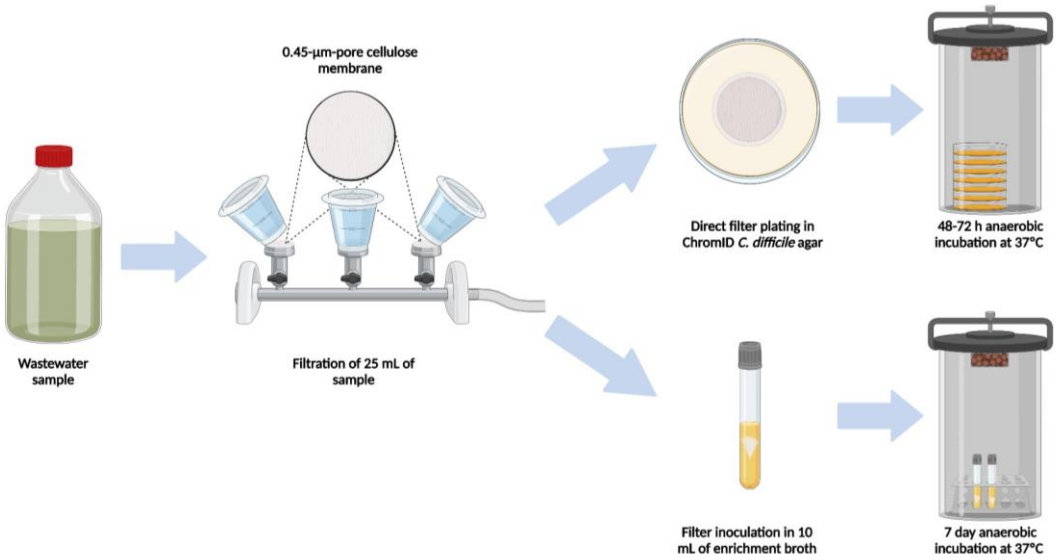

Figure 3.2. Flowchart of the wastewater filtration protocol, for influent, effluent and water for reuse samples (created with BioRender.com).

3.5 Wastewater samples - *Clostridioides difficile* isolation

Plates from influent samples where the filter was directly inoculated were too numerous to count. In order to obtain isolated colonies, around 10 µL of biomass from influent plates was first suspended in 0.5 mL of Brucella broth and then transferred onto two new ChromID® *C. difficile* agar (bioMérieux, Marcy l'Etoile, France) by using the four-quadrant streak method. In effluent and ApR plates where the filter was inoculated, suspected colonies for *C. difficile* were selected and directly transferred to ChromID® *C. difficile* agar (bioMérieux, Marcy l'Etoile, France). All plates were incubated in anaerobic conditions for 48 h, at 37 °C, as illustrated in Figure 3.3.

Regarding the enrichment broth from each analyzed sample (influent, effluent and ApR), alcohol shock was performed by mixing 2 mL of the enrichment mixture in 2 mL of absolute ethanol for 1 h at room temperature. This suspension was centrifuged at 3500 rpm for 10 minutes, the supernatant discarded, and the pellet plated onto a ChromID® *C. difficile* agar (bioMérieux, Marcy l'Etoile, France) plate using the four-quadrant streak method to obtain isolated colonies. Plates were also incubated anaerobically for 48h at 37°C, as demonstrated in Figure 3.4.

After incubation, and based on colony morphology, samples were assessed for the presence of *C. difficile*. From each wastewater sample, at least five presumptive positive colonies were subcultered onto Brucella blood agar supplemented with hemin and vitamin K1 (BD BBL™, Heidelberg, Germany) and incubated for 24 h under anaerobic conditions, at 37ºC. Species identification was carried out by MALDI-TOF (VITEK® MS, bioMérieux), when required.

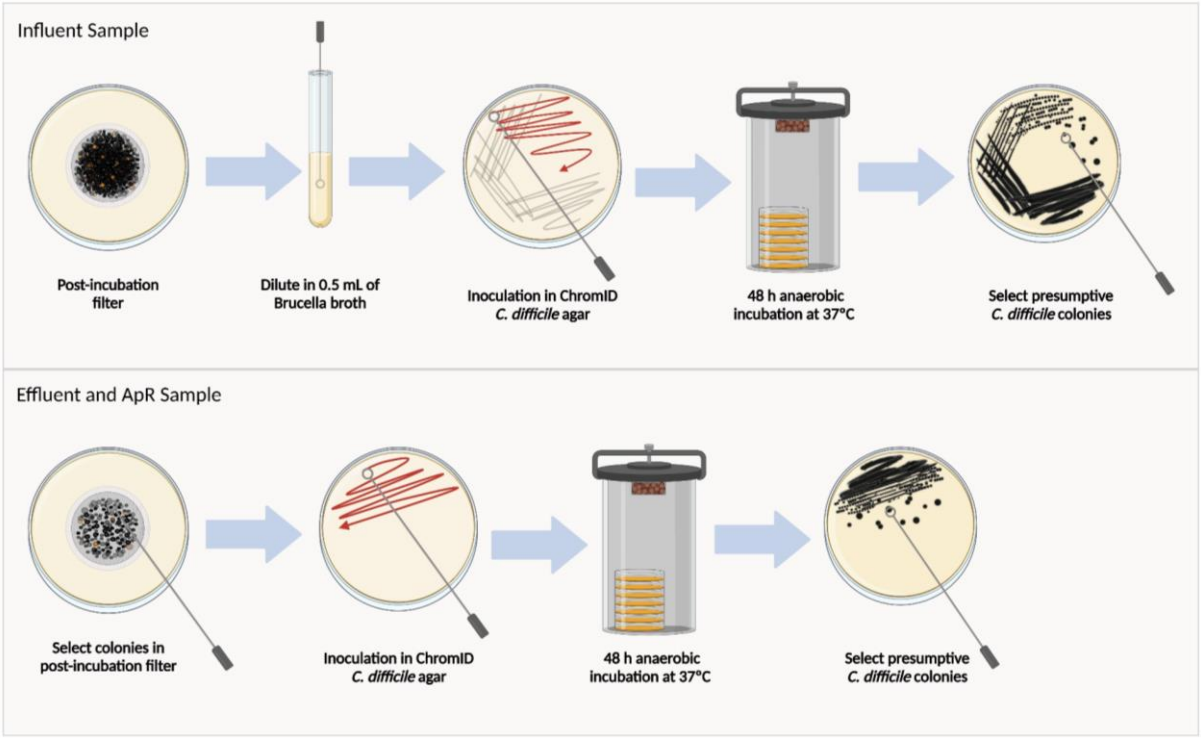

Figure 3.3. Flowchart of the *Clostridioides difficile* isolation protocol by direct plating for influent, treated effluent and water for reuse samples (created with BioRender.com).

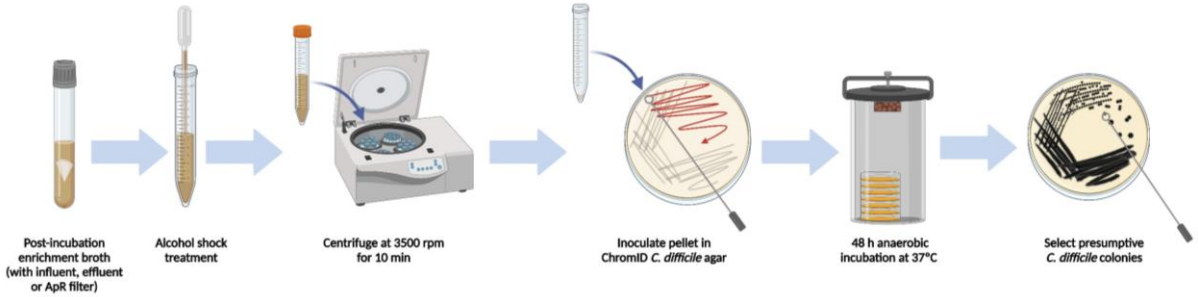

Figure 3.4. Flowchart of the *Clostridioides difficile* isolation protocol by enrichment broth for influent, treated effluent and water for reuse samples (created with BioRender.com).

3.6 Antimicrobial susceptibility testing (AST)

AST was performed for moxifloxacin, vancomycin, metronidazole, rifampicin and clindamycin by disk diffusion (Liofilchem®) or Etest® strips (bioMérieux) in all analyzed strains. Additionally, AST was performed for erythromycin, gentamicin, imipenem and tetracycline by Etest® strips (bioMérieux) in an outbreak-associated group of clinical strains. Overnight cultures of the *C. difficile* strains were

suspended in Brucella Broth to a density of 1.0 McFarland (~3x108 CFU/mL), and with a sterile cotton swab, the inoculum was spread onto plates of Brucella blood agar supplemented with hemin and vitamin K1 (BD BBL™, Heidelberg, Germany). To optimize the growth of *C. difficile*, this medium was previously reduced in an anaerobic atmosphere for 18-24 h. When the surface was completely dry, antibiotic disks and strips were placed in each corresponding plate, which were after incubated for 24 h under anaerobic conditions, at 37ºC. The aforementioned steps were accomplished within 30 minutes, in order to avoid the prolonged exposure of strains to aerobic atmosphere.

The diameters of inhibition zone considered for moxifloxacin (5 µg, ≥20 mm), vancomycin (5 µg, ≥19 mm) and metronidazole (5 µg, ≥23 mm) were based on Erikstrup et al. (2012), and for rifampicin (5 µg, ≥30 mm), this breakpoint was based on Cruz De Oliveira De Menezes (2017). For clindamycin, Etest® strips were initially used to categorize strains according to Freeman et al. (2015) (MIC ≥8 mg/L), and more recently, disk diffusion (2 µg) was performed, where resistant strains had an inhibition zone diameter ≤6 mm. MIC breakpoints were defined according to the latest European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (v 14.0, 2024) for vancomycin (>2 mg/L) and metronidazole (>2 mg/L), according to previous EUCAST guidelines (v 11.0, 2021) for moxifloxacin (>4 mg/L) and rifampicin (>0.004 mg/L), and according to the Clinical and Laboratory Standards Institute (CLSI) for imipenem (≥16 mg/L), erythromycin (≥8 mg/L), gentamicin (≥16 mg/L) and tetracycline (≥16 mg/L). AST was performed for both clinical and wastewater isolates. The reference *C. difficile* strain ATCC 700057 was included for quality control.

3.7 Molecular characterization – Toxin genetic profile and ribotyping

DNA was extracted from presumably positive colonies with 24 h of growth – from both clinical and wastewater isolates – using the NucliSENS® easyMAG® automated system (bioMérieux), according to manufacturer's instructions.

The extracted DNA was used for the amplification of the glutamate dehydrogenase (*gluD*) gene, enabling the identification of *C. difficile*, as well as for the amplification of toxin-encoding genes *tcdA*, *tcdB* and *cdtA*/*cdtB* to profile toxins, in a multiplex PCR using the primers listed in Table 3.1. Furthermore, the multiplex PCR was carried out using HotStarTaq Master Mix (QIAGEN), followed by agarose gel electrophoresis under the conditions described in Table 3.2.

The same extracted DNA was used as well for PCR ribotyping of isolates, where the 16S-23S intergenic spacer region was amplified, followed by a capillary gel electrophoresis-based approach according to Fawley et al. (2015), carried out by the *Unidade de Tecnologia e Inovação* (UTI) of the *Departamento de Genética Humana* (DGH), at INSA. The respective primers used are listed in Table 3.1, and the necessary conditions to perform PCR are described in Table 3.2. PCR-ribotyping was performed by using KAPA2G Robust HotStart ReadyMix (Kapa Biosystems) or HotStarTaq Plus Master Mix (QIAGEN), and employing a 16S rRNA primer labelled at the 5' end with 6-carboxyfluorescein (6-FAM). Moreover, the obtained RT profiles were identified by resorting to the Webribo database (https://webribo.ages.at/) or the European Union Reference Laboratory.

Table 3.1. Primers used in this study and respective sequences, gene targets and references.

PCR	Primer name	Primer sequence (5'-3')	Gene target	Reference
Multiplex	tcdA-F3345	GCATGATAAGGCAACTTCAGTGGTA	*tcdA*	Persson et al. (2008)
	tcdA-R3969	AGTTCCTCCTGCTCCATCAAATG		
	tcdB-F5670	CCAAARTGGAGTGTTACAAACAGGTG	*tcdB*	
	tcdB-R6079A	GCATTTCTCCATTCTCAGCAAAGTA		
	tcdB-R6079B	GCATTTCTCCGTTTTCAGCAAAGTA		
	cdtA-F739A	GGGAAGCACTATATTAAAGCAGAAGC	*cdtA*	
	cdtA-F739B	GGGAAACATTATATTAAAGCAGAAGC		
	cdtA-R958	CTGGGTTAGGATTATTTACTGGACCA		
	ctdB-F617	TTGACCCAAAGTTGATGTCTGATTG	*cdtB*	
	cdtB-R878	CGGATCTCTTGCTTCAGTCTTTATAG		
	Cdiff_gluDF1	GTCTTGGATGGTTGATGAGTAC	*gluD*	Paltansing et al. (2007)
	Cdiff_gluDR1	TTCCTAATTTAGCAGCAGCTTC		
Ribotyping	16S	FAM-GTGCGGCTGGATCACCTCCT	16S-23S intergenic spacer region	Bidet et al. (1999)
	23S	CCCTGCACCCTTAATAACTTGACC		

Table 3.2. Pairs of primers used in this study and respective concentration, PCR thermocycling and electrophoresis conditions.

Primer pair	Concentration (µM)	PCR annealing temperature	PCR product size (bp)	Electrophoresis conditions (agarose %; time; voltage)
tcdA-F3345/ tcdA-R3969	0.6	54ºC	629	2,5%; 40 min; 100 V
tcdB-F5670/ tcdB-R6079A, tcdB-R6079B	0.4		410	
	0.2			
cdtA-F739A, cdtA-F739B/ cdtA-R958	0.05		221	
	0.1			
ctdB-F617/ cdtB-R878	0.1		262	
Cdiff_gluDF1/ Cdiff_gluDR1	0.6		158	
16S/23S	0.2	57ºC	-	Capillary electrophoresis

3.8 Whole-genome sequencing

For each selected strain, from both clinical and wastewater samples, DNA was extracted using the Isolate II Genomic DNA kit (Bioline, UK) according to manufacturer's protocol, and further quantified using a fluorometric method, with the Qubit dsDNA HS Assay Kit (Invitrogen). Samples were delivered to the UTI, at INSA, where WGS was performed. DNA samples were then subjected to Nextera XT library preparation and paired-end sequencing on an Illumina NextSeq 2000 apparatus, with 150 bp read size.

3.9 Bioinformatics analysis

The bioinformatics analysis necessary for this project was performed by the *Unidade de Tecnologia e Inovação* (UTI), at INSA, and the obtained results were later delivered. Reads' quality control (QC), contamination check, multilocus sequence typing (MLST) and genome assembly was performed with INNUca v4.2.3 (https://github.com/B-UMMI/INNUca). For variant calling (detection of mutations), trimmed reads (after QC) were mapped against a reference genome (depending on the ribotype under analysis) using Snippy v4.5.1 (https://github.com/tseemann/snippy). The extraction of single-nucleotide variant (SNV) sites was done using snippy-core. Phylogenetic trees were inferred based on SNVs alignments with MEGA7 (Kumar et al., 2016) using the Maximum Likelihood method based on the General Time Reversible model, with 100 bootstraps. Phylogenetic trees were visualized using FigTree v1.4.4 (https://github.com/rambaut/figtree). Mean and pairwise distances were inferred with MEGA7 over the SNVs alignments.

For the identification of genetic determinants of resistance to antimicrobials, ABRicate (https://github.com/tseemann/abricate) was used to screen the genome assemblies against the Comprehensive Antibiotic Resistance Database (CARD) and ResFinder databases, using default parameters. Furthermore, the *C. difficile*-specific workflow integrated in the Clostyper pipeline (https://gitlab.com/FLI_Bioinfo/clostyper) was used to identify known mutations conferring resistance to antimicrobials, namely, mutations in *gyrA* and *gyrB* (fluoroquinolones) and *rpoB* (rifampicin).

The identification of mobile genetic elements, including phages, transposons, genetic islands and plasmids, relied on several approaches, both assembly-based and read-based, for cross confirmation:

i) the *C. difficile*-specific workflow integrated in the Clostyper pipeline (https://gitlab.com/FLI_Bioinfo/clostyper) was applied to screen the raw data against the Clostyper database (that includes determinants of resistance, phages, transposons and plasmids);
ii) a custom database [including the mobile elements from the Clostyper *C. difficile*-specific database and the genetic islands described by Roxas et al. (2020)] was built to screen the genome assemblies using ABRicate;
iii) Snippy was used to map the trimmed reads against the above mentioned databases and the presence/absence of each element was visually confirmed on Integrative Genomics Viewer (IGV);
iv) NCBI Blastn was used for confirmatory steps and screening of the identified elements against publicly available genomes.

3.10 Data treatment

Further sample data and strain characterization results, from both clinical and wastewater samples, were documented and analyzed using Microsoft Excel™ spreadsheets. All plots generated from the data on clinical isolates and wastewater isolates were also created using Microsoft Excel™. Additionally, the map created for clinical isolates was produced with QGIS 3.34.10.

4. RESULTS

4.1 *Clostridioides difficile* clinical samples

Between September 2023 and May 2024, a total of 384 *C. difficile* isolates were recovered from stool samples sent to URGI by 17 different Portuguese hospitals included in the national CDI surveillance programme, as illustrated in Figure 4.1.

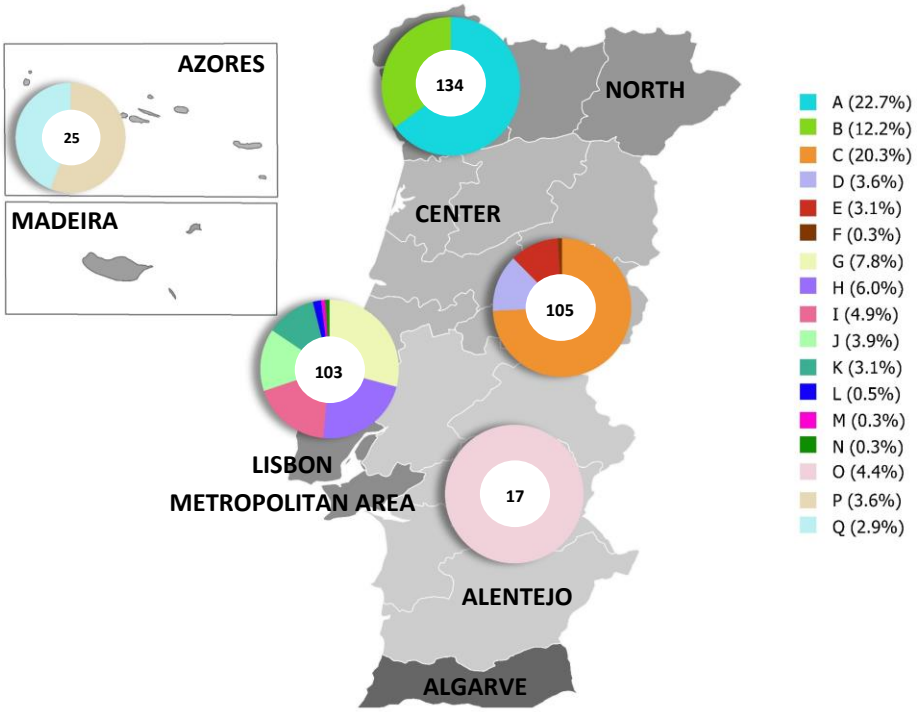

Figure 4.1. Distribution of the 384 *Clostridioides difficile* isolates recovered from clinical samples sent by 17 hospitals (identified from "A" to "Q") across four regions of mainland Portugal (North, Center, Lisbon Metropolitan Area and Alentejo) and Azores. The frequency of isolates obtained per region is inside the respective chart. The percentage of isolates recovered per each hospital is also presented.

The national surveillance program for CDI, implemented in line with the *European surveillance of Clostridioides (Clostridium) difficile infections* protocol, and coordinated by the *Direção-Geral da Saúde* (DGS) in collaboration with INSA, specifies that participating hospitals must send fecal samples testing positive for *Clostridioides difficile*. These samples are to be used for microbiological analysis, which includes bacterial isolation, as well as its phenotypical and genotypical characterization.

4.2 *Clostridioides difficile* clinical strains – Geographic distribution, toxin genetic profile and PCR-ribotyping

All 384 isolates recovered from the clinical samples were ribotyped, showing a considerable diversity of circulating profiles among strains collected from CDI cases, with 92 different profiles, 73 known and 19 unknown profiles, according to the Webribo platform. As displayed in Figure 4.2, when excluding RT027 (outbreak associated), the six most prevalent ribotypes were RT106 (14.1%), followed by RT014/020 (10.2%), RT078/126 (4.9%), RT002 (4.4%), and RT012 and RT013 (both with 3.4%). Fifty less common PCR-ribotypes accounted for 22.1% of the total.

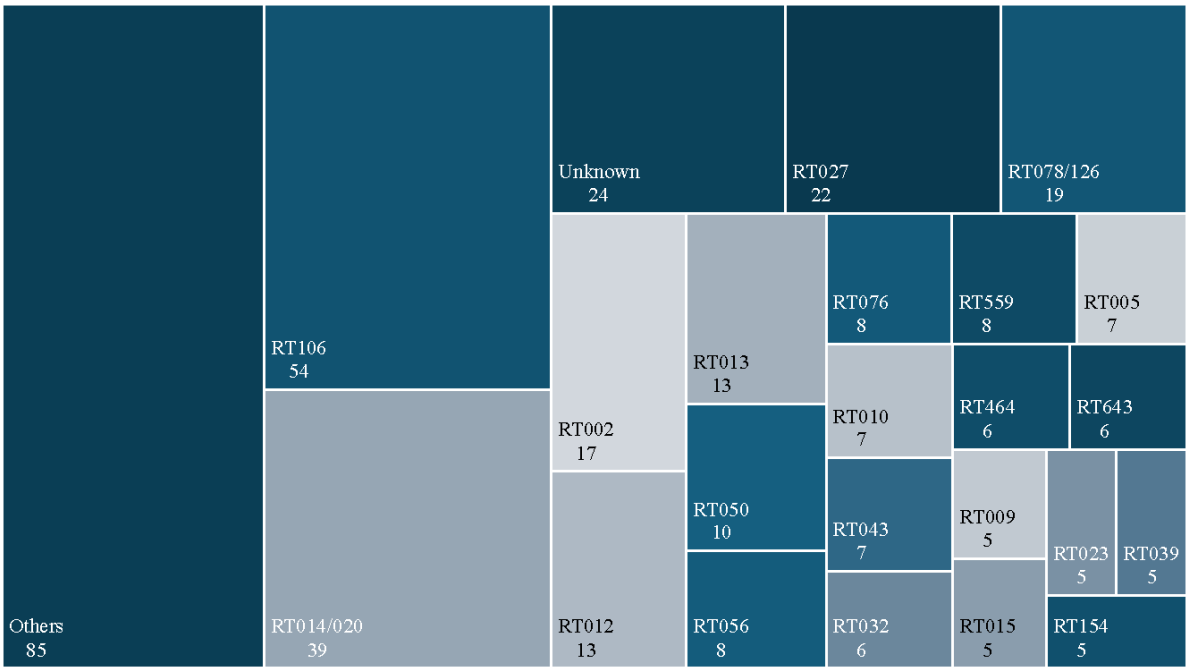

Figure 4.2. Ribotype distribution of 384 *Clostridioides difficile* clinical isolates obtained between September 2023 and May 2024. Frequency is below each ribotype. RT014 and RT020, as well as RT078 and RT126, were grouped, according to literature in general, since the profiles are practically identical. "Others" refers to 50 different ribotypes which include four or less isolates. "Unknown" refers to profiles that were not identified by Webribo.

The distribution of these ribotypes across the five regions of Portugal included in this study is shown in Figure 4.3. Out of the 73 known profiles, 36 RTs (of which 25 were represented by single isolates) emerged in only one of the five geographic regions: 13 RTs were found exclusively in the North, 10 in the Center, as well as in the Lisbon Metropolitan Area, one in Alentejo and two in the Azores. In contrast, two of the frequently found ribotypes were common to all five regions, namely RT014/020 and RT106. In the North region, the most prevalent ribotype was RT027, with 22 (16.4%) isolates originating from a single hospital (B), in the context of an outbreak. In the Center and Lisbon Metropolitan Area, the most prevalent ribotype was RT106, with 12 (11.4%) isolates from hospital C and 20 (19.4%) isolates from four different hospitals (H, I, J, and K), respectively. In the Alentejo region, RT643 was the most prominent, with seven (35.3%) isolates from hospital O, while in the Azores, RT050 and RT056 were the most common, each with six (24.0%) isolates from hospitals P and Q. Regarding ribotype diversity across regions, Alentejo showed the highest diversity ($p = 0.529$), displaying the higher number in the "Others" category, followed by the Azores ($p = 0.480$), the Lisbon Metropolitan Area ($p = 0.379$) and the Center ($p = 0.362$), with the North region showing the lowest

diversity (p = 0.299) (Figure 4.3). Even though an association of the most prevalent ribotype with a single hospital was not unique to the North of Portugal (it was also observed in the Center and Alentejo regions), the 22 isolates of the hypervirulent RT027 linked to hospital B were attributed to an outbreak (**see section 4.4 below**). Although this outbreak was declared in October 2023 (and is still ongoing), hospital B only integrated the national CDI surveillance program in January 2024. For this reason, RT027 was not included amongst the top six most prevalent ribotypes in this study, and when excluding this ribotype, RT014/020 becomes the most prevalent in the North region, with 18 (13.4%) isolates.

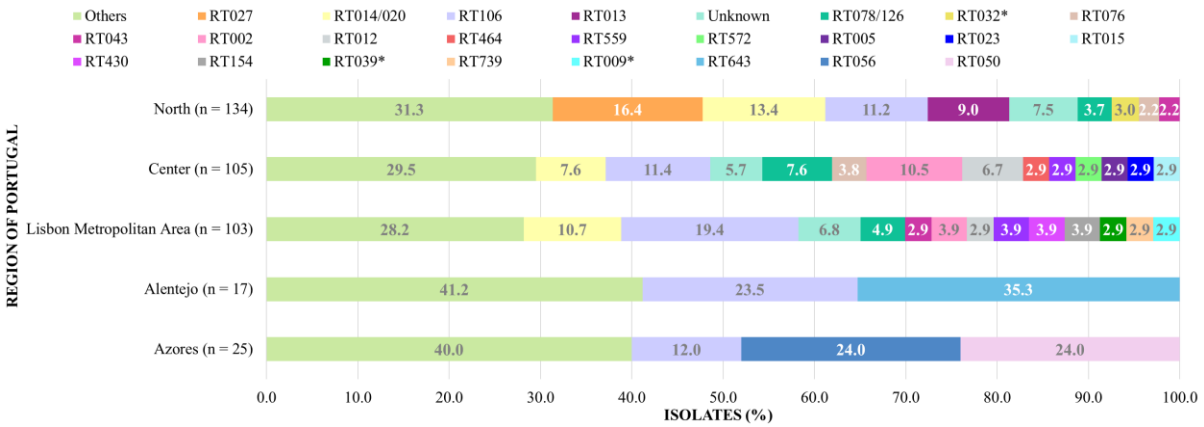

Figure 4.3. Distribution of clinical *Clostridioides difficile* ribotypes across the five regions of Portugal (North, Center, Lisbon Metropolitan Area, Alentejo and Azores) included in this study. Non-toxigenic strains are identified with "*". "Others" refers to 31, 25, 26, seven and nine different ribotypes with two or less isolates for the North, Center, Lisbon Metropolitan Area, Alentejo and Azores regions, respectively. "Unknown" refers to profiles that were not identified by Webribo.

Five out of six of the most frequent ribotypes, RT106, RT014/020, RT002, RT012 and RT013 were positive for the genes encoding the toxins A and B, *tcdA* and *tcdB*, respectively, and negative for the genes encoding the binary toxin, *cdtA*/*cdtB*, while the remaining, belonging to RT078/126, were positive for all the toxin genes (Table 4.1). Furthermore, this hypervirulent RT078/126 was detected in nine (52.9%) of the 17 hospitals included in the national CDI surveillance programme, from all regions (hospitals A, B, C, D, E, G, H, K and O). However, the majority of isolates (8/19; 42.1%) originated from the Center.

As expected, toxigenic strains isolated from cases of CDI were more prevalent, with 346 isolates (90.1%), compared to 38 (9.9%) non-toxigenic isolates. The most common toxin genetic profile among toxigenic isolates was *tcdA*+, *tcdB*+, *cdtA*-/*cdtB*-, found in 290 (75.5%) isolates (Table 4.1), while the hypervirulent profile (*tcdA*+, *tcdB*+, *cdtA*+/*cdtB*+) was found in 49 (12.7%) isolates. Less common profiles were also identified, such as *tcdA*+, *tcdB*-, *cdtA*-/*cdtB*- or *tcdA*-, *tcdB*+, *cdtA*-/*cdtB*-.

Table 4.1. Toxin gene content of the 384 *Clostridioides difficile* clinical isolates and respective ribotype diversity.

Toxin genetic profile	No. of isolates (%) (n = 384)	RTs (no.; %)
tcdA+, tcdB+, cdtA+/cdtB+	49 (12.7)	RT027 (22;44.9), RT078/126 (19; 38.8), RT023 (5; 10.2), Unknown (2;4.1), RT181 (1; 2.0)
tcdA+, tcdB+, cdtA-/cdtB-	290 (75.5)	RT106 (54;18.6), RT014/020 (39; 13.5), RT002 (17; 5.9), RT012 (13; 4.5), RT013 (13;4.5), Unknown (13; 4.5), RT050 (10; 3.4), Others (131; 45.2)
tcdA-, tcdB-, cdtA-/cdtB-	38 (9.9)	RT010 (6; 15.8), RT032 (6;15.8), RT009 (5;13.1), RT039 (5;13.1), Unknown (3;7.9), RT031 (2;5.3), RT035 (2;5.3), RT073 (2;5.3), RT085 (2;5.3), RT204 (2;5.3), RT051 (1;2.6), RT084 (1;2.6), RT199 (1;2.6)
tcdA-, tcdB+, cdtA-/cdtB-	6 (1.6)	Unknown (6;100.0)
tcdA+, tcdB-, cdtA-/cdtB-	1 (0.3)	RT010 (1;100.0)

"Others" refers to 50 different RTs which include eight or less isolates. "Unknown" refers to profiles that were not identified by Webribo.

4.3 *Clostridioides difficile* clinical strains - Antimicrobial susceptibility

The antimicrobial susceptibility testing was performed for moxifloxacin, vancomycin, metronidazole, rifampicin and clindamycin, for all 384 *C. difficile* isolates from clinical samples.

Overall, the highest rate of resistance was observed for clindamycin (82; 21.4%), followed by moxifloxacin (60; 15.6%) and rifampicin, with only 6.5% (corresponding to 25 isolates), as observed in Table 4.2. All studied isolates were susceptible to vancomycin and metronidazole.

Table 4.2. Antibiotic susceptibility of the 384 *Clostridioides difficile* clinical isolates to moxifloxacin, vancomycin, metronidazole, rifampicin and clindamycin.

Antibiotic	Resistant isolates, no. (%) (n = 384)
Clindamycin	82 (21.4)
Moxifloxacin	60 (15.6)
Rifampicin	25 (6.5)
Vancomycin	0
Metronidazole	0

Regarding RT diversity among resistant isolates, as shown in Figure 4.4, the most common ribotype across all tested antibiotics was RT027. All 22 outbreak-associated isolates were resistant to clindamycin, moxifloxacin, and rifampicin, significantly contributing to the overall resistance rates. Nevertheless, it is important to note that such isolates are associated with the previously mentioned outbreak. Hence, excluding the RT027, clindamycin exhibited the greatest RT diversity (p = 0.341), with isolates belonging to RT012 (9; 11.1%), RT106 (6; 7.3%), RT078/126 and RT559 (each 5; 6.1%), RT010 and RT039 (both 4; 4.9%), as well as RT014/020 and RT643 (both 3; 3.7%). Moxifloxacin followed (p = 0.200), with three predominant resistant ribotypes: RT106 (15; 25.0%), RT078/126 (8; 13.3%) and RT559 (5; 8.3%). Lastly, the three rifampicin-resistant isolates each displayed a unique ribotype — RT085 (4.0%), RT181 (4.0%), and RT284 (4.0%) — showing the lowest RT diversity (p = 0.160).

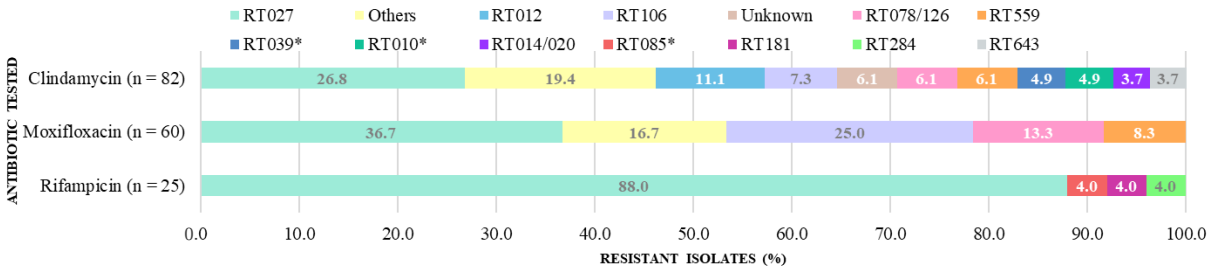

Figure 4.4. Distribution of *Clostridioides difficile* resistant clinical isolates according to ribotype profile. Non-toxigenic strains are identified with "*". "Others" refers to seven different ribotypes in moxifloxacin and 17 different ribotypes in clindamycin with two or less isolates. "Unknown" refers to profiles that were not identified by Webribo.

Regarding the toxin genetic profile of these resistant isolates, there was a higher prevalence of toxigenic resistant strains compared to non-toxigenic ones amongst antibiotics overall, as displayed in Figure 4.5. For clindamycin the most common toxin genetic profile of resistant isolates was *tcdA*+, *tcdB*+, *cdtA*-/*cdtB*- (43; 53.1%). Additionally, one toxigenic isolate of profile *tcdA*+, *tcdB*-, *cdtA*-/*cdtB*- also presented resistance towards clindamycin, representing 1.2%. In contrast, for both moxifloxacin and rifampicin, hypervirulent isolates were more frequently resistant, representing 50.8% (n = 27) and 92.0% (n = 23), respectively. However, this is due to the presence of the outbreak-associated RT027 strain. When these 22 isolates are excluded, the toxin genetic profile *tcdA*+, *tcdB*+, *cdtA*+/*cdtB*+ accounts for only 13.6% (n = 8) of moxifloxacin-resistant isolates and 4.0% (n = 1) of rifampicin-resistant isolates. This indicates that the *tcdA*+, *tcdB*+, *cdtA*-/*cdtB*- profile (27; 45.8%) becomes the most prevalent amongst moxifloxacin-resistant isolates. Moreover, each of the three rifampicin-resistant isolates exhibit a distinct toxin genetic profile.

When it comes to non-toxigenic strains, although they do not produce toxins, such strains can still carry resistance mechanisms that play a crucial role in CDI. Therefore, it is important to highlight these resistant isolates found amongst clinical strains. Overall, non-toxigenic strains represented 12.3% (n = 10) of isolates resistant to clindamycin, while only 3.4% (n = 2) and 4.0% (n = 1) of isolates resistant to moxifloxacin and rifampicin, respectively (Figure 4.5). Notably, of the 13 non-toxigenic resistant isolates, 76.9% (n = 10) were resistant to clindamycin, 15.4% (n = 2) to moxifloxacin and 7.7% (n = 1) to rifampicin.

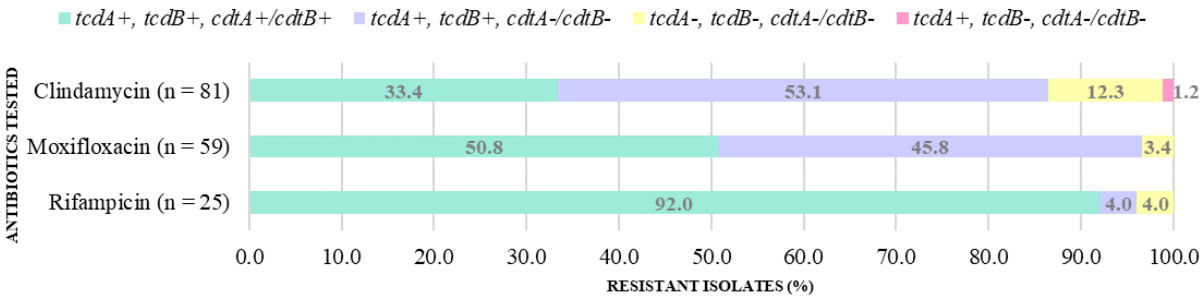

Figure 4.5. Distribution of *Clostridioides difficile* resistant clinical isolates according to toxin genetic profile.

Differences in the distribution of resistant isolates across the regions of Portugal, where the hospitals included in this study are located, were also identified. Overall, resistant isolates were more concentrated in the northern region of the country, consisting of 43.2% (n = 35) clindamycin-resistant isolates, 57.6% (n = 34) of moxifloxacin-resistant isolates and 92.0% (n = 23) of rifampicin-resistant isolates, as shown in Figure 4.6. However, it is important to note that 22 of these isolates consist of the outbreak-associated RT027 strain. When excluded, the number of resistant isolates in the North significantly decreases for clindamycin (13; 16.0%), moxifloxacin (12; 20.3%) and rifampicin (1; 4.0%). Therefore, amongst clinical isolates, clindamycin resistance was predominant in the Center region (24; 29.7%), moxifloxacin resistance was evenly distributed between the North and Center and rifampicin resistance was identified in three regions of mainland Portugal (North, Center, and the Lisbon Metropolitan Area), with only one isolate per region.

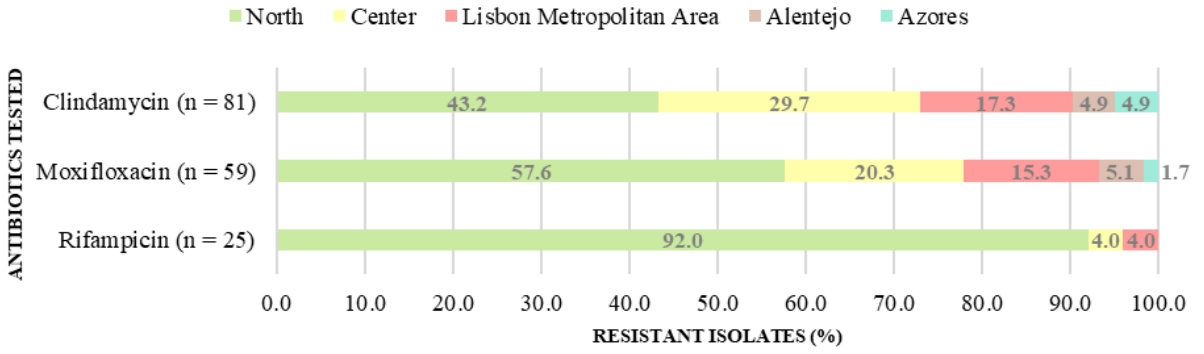

Figure 4.6. Distribution of *Clostridioides difficile* resistant clinical isolates according to the five regions of Portugal (North, Center, Lisbon Metropolitan Area, Alentejo and Azores) included in this study.

Resistance to more than one of the tested antibiotics was observed in both toxigenic and non-toxigenic strains, besides outbreak-associated RT027 isolates. Amongst toxigenic strains, two isolates of RT012 and RT369, as well as five RT559 isolates, were resistant to both clindamycin and moxifloxacin, while one RT284 isolate showed multiple resistance to clindamycin, moxifloxacin, and rifampicin. A hypervirulent RT181 isolate was resistant to both moxifloxacin and rifampicin, while three RT078/126 isolates were resistant to clindamycin and moxifloxacin. Regarding non-toxigenic strains, two isolates (both RT039) showed resistance to clindamycin and moxifloxacin, while one RT085 isolate was resistant to both clindamycin and rifampicin.

4.4 *Clostridioides difficile* clinical strains - RT027 outbreak

In order to better characterize the outbreak-associated isolates from RT027, all 22 were studied with an expanded antibiotic panel including MIC determination, and all exhibited multiple resistance - to moxifloxacin, rifampicin, clindamycin, erythromycin and gentamicin - and reduced susceptibility to vancomycin, as illustrated in Table 4.3.

Table 4.3. Antimicrobial susceptibility and determinants of resistance of the 22 outbreak-associated *Clostridioides difficile* RT027 isolates characterized in this study.

Antibiotic	R Breakpoint (mg/L)	MIC (mg/L)	Phenotype	Genetic determinant of resistance*
Moxifloxacin	>4[a]	>32	R	GyrA T82I
Vancomycin	>2[a]	1.5-2	Reduced susceptibility	VanRCd T115A
Metronidazole	>2[a]	2	S	NA
Rifampicin	>0.004[a]	>32	R	RpoB R505K
Clindamycin	>8[b]	>256	R	*ermB*
Imipenem	≥16[c]	4	S	NA
Gentamicin	≥16[d]	>256	R	-
Erythromycin	≥8[c]	>256	R	*ermB*
Tetracycline	≥16[c]	0.094	S	NA

*Determined by WGS. [a] Breakpoint defined by the EUCAST guidelines (European Committee on Antimicrobial Susceptibility Testing). [b] Breakpoint according to Freeman et al. (2015). [c] Breakpoint according to the Clinical and Laboratory Standards Institute (CLSI) interpretative values for anaerobes. [d] Breakpoint according to the Clinical and Laboratory Standards Institute (CLSI) interpretative values for *Staphylococcus* spp. R – resistant; S – susceptible; NA – not applicable

Given AST results, five RT027 isolates, collected in January and February 2024, were selected for WGS to confirm they belonged to one single clone, and thereby confirming the occurrence of an outbreak. An SNV-based analysis was conducted using the genomes, comparing them to the reference strain R20291 (RT027; acc. no. FN545816), as well as to the genomes of four strains of RT027, of sporadic cases of CDI, previously obtained in the context of CDI surveillance, in Portugal. The analysis revealed that the outbreak isolates formed a cluster, distant from the other strains, with four exhibiting no SNP differences between them, while one isolate differed by three SNPs from the others, as shown in Figure 4.7.

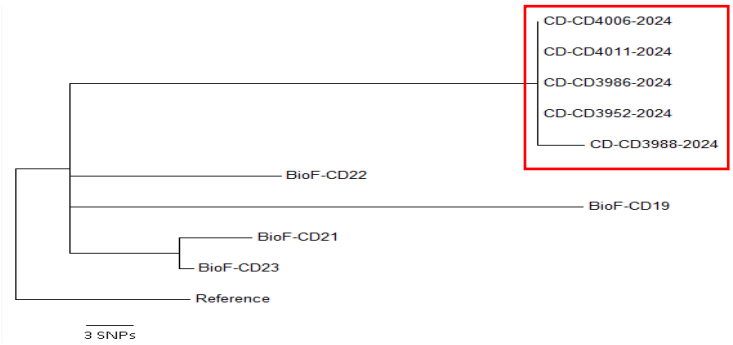

Figure 4.7. Maximum likelihood tree based on 86 single-nucleotide variant (SNV) positions of five RT027 *Clostridioides difficile* clinical outbreak-associated isolates and four isolates from sporadic cases of CDI occurred in Portugal, generated by mapping reads against a reference strain R20291 (RT027; acc. no. FN545816).

4.5 Wastewater samples

Wastewater (WW) samples were collected from both the entrance and exit of a WWTP in the Lisbon Metropolitan Area. Sampling took place between February and June 2024, with a total of eight sampling events. During the first five events, only influent and treated effluent samples were collected, while in the final three, samples from the influent, effluent, and ApR (water for reuse) were obtained. In total, 19 WW samples were collected and analyzed: eight from both the influent and effluent, and three from ApR. *C. difficile* was found in 15 (78.9%) of these samples. A total of 181 isolates were cultured, with 140 confirmed as *Clostridioides difficile*. The remaining isolates were identified as *Clostridium butyricum* or *Lactobacillus mucosae*. Overall *C. difficile* recovery was of 100.0% from influent, with a total of 99 isolates, and 87.5% from effluent, with a total of 41 isolates. In ApR samples, no *C. difficile* was recovered. Table 4.4 summarizes the characteristics of the WW samples collected for this study and the number of *C. difficile* isolates in each sample.

Table 4.4. Overview of the wastewater samples and respective prevalence of *Clostridioides difficile*, level of treatment applied to each sample and final treated effluent receiving body.

Sampling date	Sample	Sample ID	Number of *C. difficile* isolates	Treatment level	Final effluent purpose/ receiving body
08/02/2024	Influent	CDA1	16	Pretreatment	Tagus estuary
	Effluent	CDE1	10	Pretreatment; primary; secondary	
08/03/2024	Influent	CDA2	20	Pretreatment	
	Effluent	CDE2	5	Pretreatment; primary; secondary	
26/03/2024	Influent	CDA3	15	Pretreatment	
	Effluent	CDE3	5	Pretreatment; primary; secondary	
11/04/2024	Influent	CDA4	12	Pretreatment	
	Effluent	CDE4	2	Pretreatment; primary; secondary	
24/04/2024	Influent	CDA5	12	Pretreatment	
	Effluent	CDE5	10	Pretreatment; primary; secondary	
09/05/2024	Influent	CDA6	10	Pretreatment	
	Effluent	CDE6	0	Pretreatment; primary; secondary	
	ApR	CDR1	0	Pretreatment; primary; secondary; tertiary and disinfection*	Reuse
23/05/2024	Influent	CDA7	7	Pretreatment	Tagus estuary
	Effluent	CDE7	5	Pretreatment; primary; secondary	
	ApR	CDR2	0	Pretreatment; primary; secondary; tertiary and disinfection*	Reuse
07/06/2024	Influent	CDA8	7	Pretreatment	Tagus estuary
	Effluent	CDE8	4	Pretreatment; primary; secondary	
	ApR	CDR3	0	Pretreatment; primary; secondary; tertiary and disinfection*	Reuse

*Tertiary level of treatment and disinfection may consist either of ultrafiltration with addition of sodium hypochlorite or microfiltration with UV disinfection, depending on the line of treatment. ApR - water for reuse.

4.6 *Clostridioides difficile* wastewater strains - Toxin genetic profile and PCR-ribotyping

All 140 isolates were ribotyped, resulting in overall 25 known profiles and eight unknown profiles (six influent and two effluent isolates), according to the Webribo platform. Overall, a greater number of known RT profiles were identified in influent samples (22/25), compared to effluent samples (9/25), as displayed in Figure 4.8. Amongst influent samples, both RT014/020 and RT106 were the most prevalent ribotypes, accounting for 15.2% of isolates, followed by RT078/126 (12.1%), similar to the clinical samples. In addition, both RT009 and RT011 were found in 7.1% of isolates, with RT009 being non-toxigenic. For effluent samples, RT430 was the most common (24.4%), followed by RT010 (17.1%, non-toxigenic), RT009 (14.6%, non-toxigenic) and RT103 (12.2%). In Figure 4.8 it is further shown that six PCR-ribotype profiles were found in both influent and effluent samples: three toxigenic profiles (RT081, RT103, and RT104) and three non-toxigenic profiles (RT009, RT010, and RT084). Moreover, it is important to note that a higher number of isolates per sample did not always correspond to greater ribotype diversity. For example, all 16 isolates from sample CDA1 (p = 0.250) were grouped into just four ribotypes, while 12 isolates from sample CDA4 (p = 0.667) were spread across eight ribotypes.

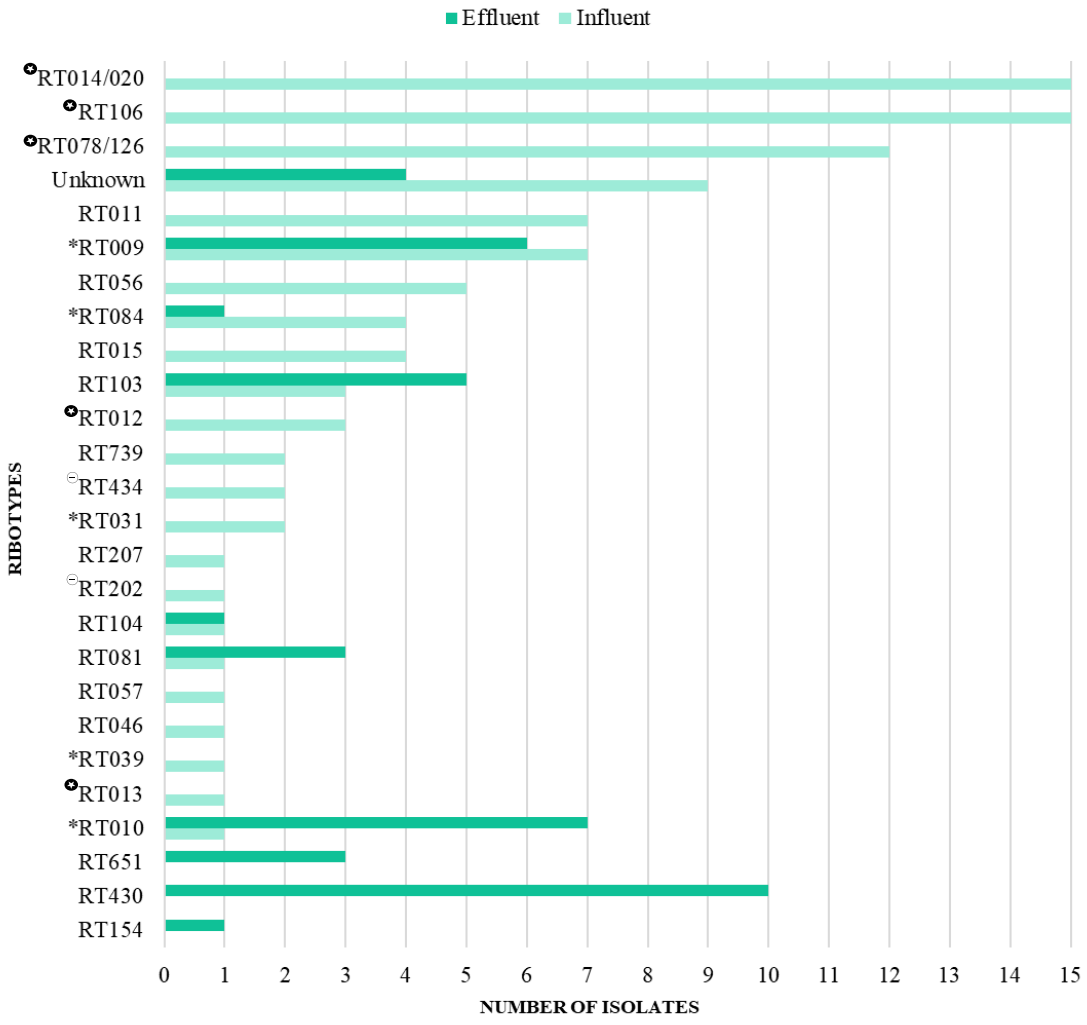

Figure 4.8. Distribution and diversity of ribotypes identified amongst the 140 *Clostridioides difficile* isolates from wastewater samples, collected between February and June 2024, according to sample type, in influent (n = 99) and in treated effluent (n = 41). Ribotypes identified with "⊖" have not been found in clinical samples in this study; while "✪" integrate the top six most prevalent clinical ribotypes, "*" refers to non-toxigenic (although RT011 is not marked, this toxin genetic profile was found in one isolate). "Unknown" refers to profiles that were not identified by Webribo.

Regarding influent samples, the two most frequent ribotypes, RT014/020 and RT106, were positive for *tcdA* and *tcdB* genes and negative for *cdtA*/*cdtB* genes, while isolates of the third most common ribotype, RT078/126, were positive for all toxin genes, similar to the clinical isolates. Isolates of RT009 were negative for all toxin genes, as well as one RT011 isolate, all remaining RT011 isolates were of genotype *tcdA*+, *tcdB*+, *cdtA*-/*cdtB*-, as indicated in Table 4.5. Regarding treated effluent samples, out of the top four ribotypes found, RT430 and RT103 were positive for *tcdA* and *tcdB* genes and negative for *cdtA*/*cdtB* genes, while RT009 and RT010 were both negative for all toxin genes (Table 4.5).

Toxigenic strains were more abundant than non-toxigenic strains in both influent (76.7%) and effluent (65.9%) samples. In influent samples, 76 isolates tested positive for toxin genes, with 12 also positive for the binary toxin. In effluent samples, 27 isolates were positive for *tcdA* and *tcdB* toxin genes, but negative for the binary toxin. The remaining 37 isolates tested negative for all toxin genes, being found in 23 isolates from influent and in 14 from effluent. As observed in Table 4.5, out of the three toxin profiles identified, *tcdA*+, *tcdB*+, *cdtA*-/*cdtB*- was the most common, in both influent (64.6%) and effluent (65.9%) isolates, similar to the clinical isolates.

Table 4.5. Toxin gene content of the 140 *Clostridioides difficile* isolates from wastewater samples, in both influent and treated effluent samples and respective ribotype diversity.

Toxin genetic profile (total no. of isolates)	Sample	No. of isolates (%)	RTs (no.; %)
tcdA+, tcdB+, cdtA+/cdtB+ (n = 12)	Influent	12 (100.0)	RT078/126 (12; 100.0)
	Effluent	0	NA
tcdA+, tcdB+, cdtA-/cdtB- (n = 91)	Influent	64 (70.3)	RT106 (15; 23.4), RT014/020 (15; 23.4), RT011 (6; 9.4), RT056 (5; 7.8), RT015 (4; 6.3), RT012 (3; 4.7), RT103 (3; 4.7), Others (13; 20.3)
	Effluent	27 (29.7)	RT430 (10; 37.0), RT103 (5; 18.5), RT081 (3; 11.1), RT651 (3; 11.1), RT104 (1; 3.7) RT154 (1; 3.7), Unknown (4; 14.8)
tcdA-, tcdB-, cdtA-/cdtB- (n = 37)	Influent	23 (62.2)	RT009 (7; 30.4), RT084 (4; 17.4), RT031 (2; 8.7), RT010 (1; 4.3), RT011 (1; 4.3), RT039 (1; 4.3), Unknown (7; 30.4)
	Effluent	14 (37.8)	RT009 (7; 50.0), RT010 (6; 42.9), RT084 (1; 7.1)

"Others" refers to 10 different RTs which include two or less isolates. "Unknown" refers to profiles that were not identified by Webribo, two different profiles in effluent and four different profiles in influent. NA - not applicable.

4.7 *Clostridioides difficile* wastewater strains – Strain diversity according to the isolation method employed

Each sample was subjected to two distinct isolation methods: direct plating of the filter and enrichment of the filter in broth followed by plating. The direct plating method allowed for a clear visualization of differences between the influent, treated effluent, and ApR samples. While the number of colonies in both influent and effluent samples was too numerous to count (Figure 4.9A), isolated colonies could still be picked from the effluent samples (Figure 4.9B). In contrast, the ApR samples showed between one to 10 colonies, as illustrated in Figure 4.9C. Using the enrichment broth method, isolated colonies were obtained from both the influent and effluent samples, but no growth was observed in the ApR samples. This is demonstrated in Figure 4.10.

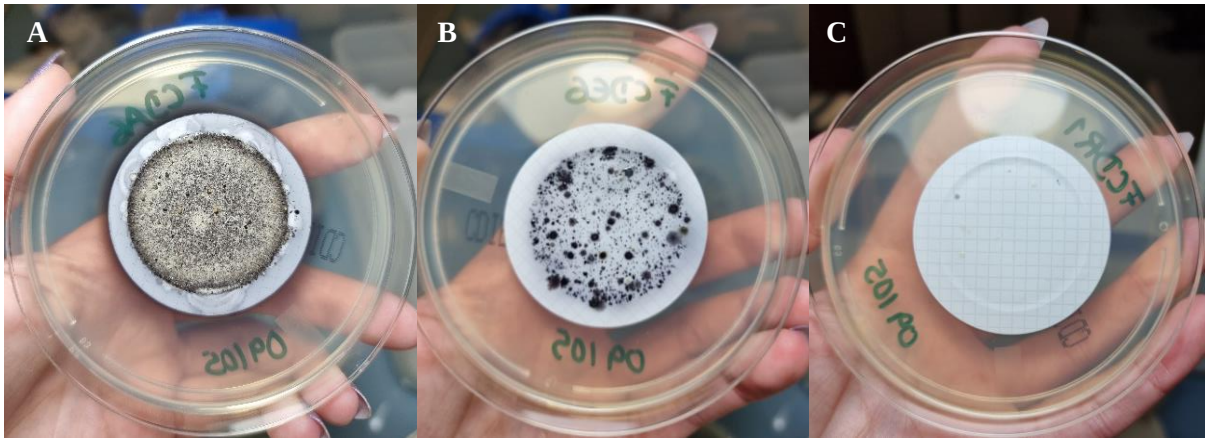

Figure 4.9. Plates in which the filter was directly inoculated after wastewater filtration with 48 h of growth, where **(A)** represents an influent sample, **(B)** a treated effluent sample and **(C)** a sample of water for reuse.

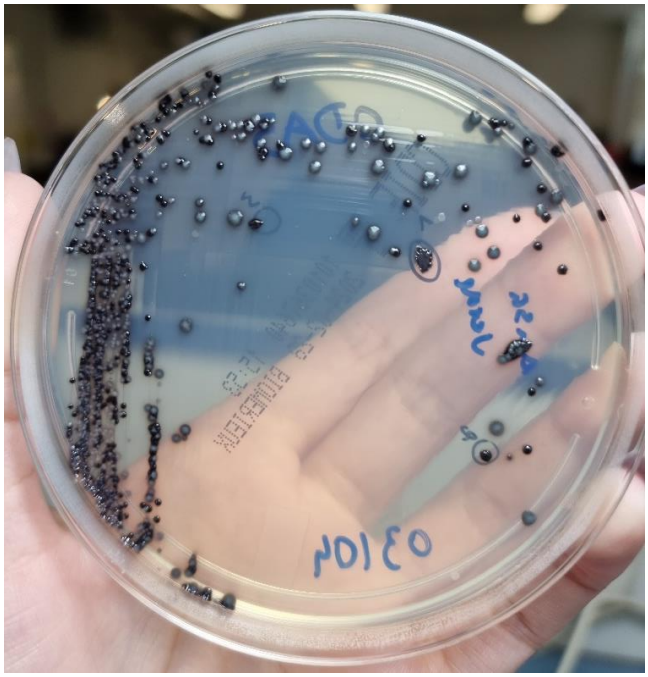

Figure 4.10. Plate in which the enrichment broth of an influent sample was inoculated, after a 48 h incubation. *Clostridioides difficile* colonies are marked with a circle.

Both methods successfully isolated *Clostridioides difficile*, as observed in Table 4.6. The direct plating isolation technique had a positivity rate of 100.0% in influent samples and 75.0% in effluent samples. The enrichment broth method had a positivity rate of 62.5% in influent samples and 3.8% in effluent samples. As previously mentioned, *C. difficile* was not found in any of the three ApR tested samples.

Table 4.6. Performance comparison between the two *Clostridioides difficile* isolation approaches used in this study for wastewater samples.

Sample ID	Presence of *C. difficile* with direct plating	Presence of *C. difficile* with enrichment broth
CDA1	**Positive**	**Positive**
CDE1	**Positive**	**Positive**
CDA2	**Positive**	**Positive**
CDE2	**Positive**	Negative
CDA3	**Positive**	**Positive**
CDE3	**Positive**	Negative
CDA4	**Positive**	**Positive**
CDE4	**Positive**	Negative
CDA5	**Positive**	**Positive**
CDE5	**Positive**	**Positive**
CDA6	**Positive**	Negative
CDE6	Negative	Negative
CDR1	Negative	Negative
CDA7	**Positive**	Negative
CDE7	Negative	**Positive**
CDR2	Negative	Negative
CDA8	**Positive**	Negative
CDE8	**Positive**	Negative
CDR3	Negative	Negative

CDA - influent samples; CDE – treated effluent samples; CDR – water for reuse samples.

While the enrichment broth method yielded fewer *C. difficile* isolates in influent (19.2%) and effluent (43.9%), it demonstrated higher ribotype diversity in both influent ($p = 0.421$) and effluent ($p = 0.333$) samples. In contrast, direct plating was more efficient in producing a larger number of *C. difficile* isolates but presented lower RT diversity in both influent ($p = 0.338$) and effluent ($p = 0.304$) samples. The aforementioned results are summarized in Table 4.7.

Table 4.7. Comparison between the number of *Clostridioides difficile* isolates and ribotype diversity, in both influent and treated effluent samples, according to the isolation techniques.

Isolation method	Sample (total no. of isolates)	*C. difficile* isolates (no.; %)	RTs (no.; %)
Direct plating	Influent (n = 99)	80 (80.8)	RT014/020 (13; 16.2), RT106 (11; 13.7), RT009 (7; 8.8), RT011 (7; 8.8), RT056 (5; 6.2), RT084 (4; 5.0), Unknown (9; 11.3), Others (24; 30.0)
	Effluent (n =41)	23 (56.1)	RT009 (5; 21.7), RT103 (5; 21.7), RT430 (5; 21.7), RT081 (3; 13.0), RT651 (3; 13.0), RT084 (1; 4.3), RT154 (1; 4.3)

Enrichment broth	Influent (n = 99)	19 (19.2)	RT078/126 (7; 36.8), RT106 (4; 21.1), RT015 (2;10.5), RT014/020 (2; 10.5), RT010 (1; 5.3), RT031 (1; 5.3), RT046 (1; 5.3), RT104 (1; 5.3)
	Effluent (n =41)	18 (43.9)	RT010 (7; 38.9), RT430 (5; 27.8), RT009 (1; 5.6), RT104 (1; 5.6), Unknown (4; 22.2)

"Others" refers to 15 different RTs which include three or less isolates. "Unknown" refers to six and two different profiles that were not identified by Webribo, in influent and treated effluent respectively.

The two methods isolated the same ribotype in four influent samples, more specifically, RT014/020 in sample CDA1, RT106 and RT078/126 in sample CDA2, RT031 and RT106 in sample CDA3 and RT015 in sample CDA5. This was not observed in any of the effluent samples. Additionally, the same ribotype was identified in both influent and effluent samples in two points only, April 24th (CDA5/CDE5) and June 7th (CDA8/CDE8), corresponding to RT009 and RT081, in that order. Interestingly, the RT009 influent isolate was obtained by direct plating and the effluent isolate by enrichment broth, while both RT081 influent and effluent isolates were obtained by direct plating.

4.8 *Clostridioides difficile* wastewater strains - Antimicrobial susceptibility

The antimicrobial susceptibility testing was performed for moxifloxacin, vancomycin, metronidazole, rifampicin and clindamycin for all 140 *C. difficile* isolates from WW samples. Overall, clindamycin showed the highest rate of resistance in influent isolates (14; 14.1%), followed by moxifloxacin (9; 9.1%) and rifampicin (1; 1.0%). Regarding the treated effluent, resistance was only found for clindamycin, representing 12.2% (n = 5) of the 41 effluent isolates analyzed. All studied isolates were susceptible to vancomycin and metronidazole, similar to clinical isolates, as illustrated in Table 4.8. Hence, resistant isolates were more frequent in influent samples (24 influent isolates compared to five effluent isolates).

Table 4.8. Antibiotic susceptibility of the 140 *Clostridioides difficile* isolates from wastewater samples, 99 from influent and 41 from treated effluent samples, to moxifloxacin, vancomycin, metronidazole, rifampicin and clindamycin.

Antibiotic	Sample (total no. of isolates)	Resistant isolates, no. (%)
Clindamycin	Influent (n = 99)	14 (14.1)
	Effluent (n = 41)	5 (12.2)
Moxifloxacin	Influent (n = 99)	9 (9.1)
	Effluent (n = 41)	0
Rifampicin	Influent (n = 99)	1 (1.0)
	Effluent (n = 41)	0
Vancomycin	Influent (n = 99)	0
	Effluent (n = 41)	0
Metronidazole	Influent (n = 99)	0
	Effluent (n = 41)	0

Regarding RT diversity amongst resistant influent isolates, as shown in Figure 4.11, clindamycin exhibited the greatest RT diversity (p = 0.500), with isolates spanning RT084 (4; 28.6%), RT012 (3; 21.4%), RT739 (2; 14.3%), as well as RT009, RT039, RT081, RT78/126 and RT430 (each 1; 7.1%). Moxifloxacin followed (p = 0.333), with three predominant ribotypes: RT078/126 (7; 77.7%), and RT106 and RT014/020 (both with 1;11.1%). Lastly, only one non-toxigenic RT009 isolate was found to be resistant to rifampicin. Notably, this same isolate was also resistant to clindamycin (Figure 4.11). This same distribution was similar to the one observed in the clinical isolates.

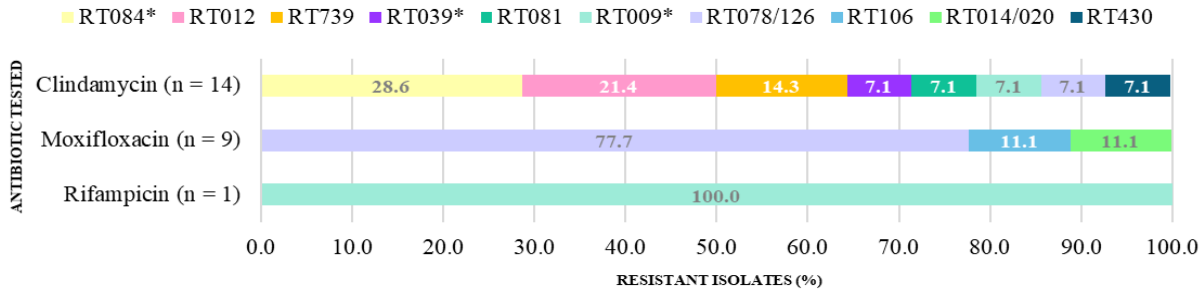

Figure 4.11. Distribution of *Clostridioides difficile* resistant isolates from wastewater samples found in influent samples according to ribotype profile. Non-toxigenic strains are identified with "*".

Regarding the toxin genetic profile of resistant influent isolates, there is a higher prevalence of toxigenic resistant strains compared to non-toxigenic ones amongst antibiotics overall, as displayed in Figure 4.12. The most common toxin genetic profiles of isolates resistant to clindamycin were *tcdA*+, *tcdB*+, *cdtA*-/*cdtB*- and *tcdA*-, *tcdB*-, *cdtA*-/*cdtB*- (both 6; 46.2%), with only one (7.6%) resistant hypervirulent isolate. While for moxifloxacin, hypervirulent isolates were more frequently resistant, representing 70.0% (n = 7), followed by toxigenic isolates negative for the CDT encoding gene (3; 30.0%). Lastly, the one rifampicin-resistant isolate was non-toxigenic. Once again it is important to note that, despite being non-toxigenic, these strains carry resistance mechanisms that play a crucial role in CDI, and as previously mentioned, this RT009 isolate was not only resistant to rifampicin, but also clindamycin-resistant.

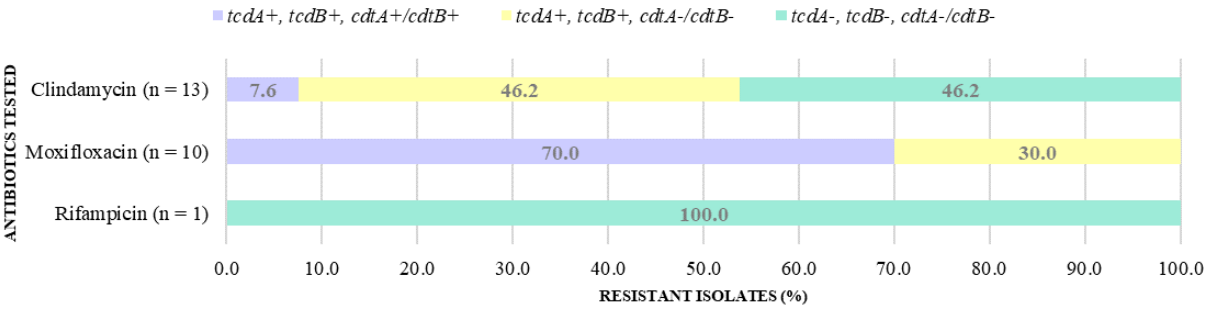

Figure 4.12. Distribution of *Clostridioides difficile* resistant isolates from wastewater samples found in influent samples according to toxin genetic profile.

Regarding RT and toxin genes profile diversity amongst resistant treated effluent isolates, all five clindamycin-resistant isolates were non-toxigenic, of RT009. In addition, such isolates were detected within the same sample, CDE2.

4.9 Whole-genome sequencing of *Clostridioides difficile* clinical and wastewater strains

In this study, 31 *C. difficile* isolates were considered for deeper genetic analysis by WGS, aiming to perform a comparison between the two sampling environments. The isolates were chosen based on sampling date, region and RT, all sharing a profile commonly associated with CDI in humans, with ribotypes ranging from RT106, RT078/126, RT430 and RT651. Seventeen strains were selected from human stool samples, as shown in Table 4.9, and 14 strains were selected from wastewater samples — 11 from influent and three from treated effluent — as detailed in Table 4.10.

Table 4.9. Internal identification of the 17 *Clostridioides difficile* isolates from clinical samples that were subjected to whole-genome sequencing, with the respective ribotypes, hospital of origin, corresponding Portugal region and sampling date.

Isolate ID	RT	Hospital	Region	Sampling date
CD3927	106	H	Lisbon Metropolitan Area	06/01/2024
CD3974	106	H	Lisbon Metropolitan Area	23/01/2024
CD4001	106	K	Lisbon Metropolitan Area	17/01/2024
CD4004	106	K	Lisbon Metropolitan Area	24/01/2024
CD4005	651	K	Lisbon Metropolitan Area	02/02/2024
CD4015	106	G	Lisbon Metropolitan Area	09/01/2024
CD4026	106	G	Lisbon Metropolitan Area	18/01/2024
CD4034	126	C	Center	12/01/2024
CD4054	126	B	North	01/03/2024
CD4095	106	G	Lisbon Metropolitan Area	04/03/2024
CD4096	430	G	Lisbon Metropolitan Area	07/03/2024
CD4097	106	G	Lisbon Metropolitan Area	20/03/2024
CD4109	106	I	Lisbon Metropolitan Area	24/03/2024
CD4110	106	I	Lisbon Metropolitan Area	20/03/2024
CD4125	126	H	Lisbon Metropolitan Area	31/03/2024
CD4132	126	A	North	06/04/2024
CD4149	126	D	Center	19/04/2024

Table 4.10. Internal identification of the 14 *Clostridioides difficile* isolates from wastewater samples that were subjected to whole-genome sequencing, with the respective ribotypes and corresponding isolation method and sampling date.

Isolate ID	RT	Isolation Method	Sample	Sampling date
CDA1_C2	126	Enrichment broth	Influent	08/02/2024
CDA2_C1	126	Enrichment broth	Influent	08/03/2024
CDA2_C3	106	Enrichment broth	Influent	08/03/2024
CDA2_C5	106	Enrichment broth	Influent	08/03/2024
FCDA2_C16	106	Direct plating	Influent	08/03/2024
FCDA2_C6	106	Direct plating	Influent	08/03/2024
FCDA2_C8	126	Direct plating	Influent	08/03/2024
CDA3_C3	106	Enrichment broth	Influent	26/03/2024
FCDA3_C1	106	Direct plating	Influent	26/03/2024
FCDA4_C11	126	Direct plating	Influent	11/04/2024
FCDA4_C6	106	Direct plating	Influent	11/04/2024
FCDE1_C2	651	Direct plating	Effluent	08/02/2024
FCDE3_C1	430	Direct plating	Effluent	26/03/2024
CDE7_C1	430	Enrichment broth	Effluent	23/05/2024

Regarding the 17 *C. difficile* isolates of RT106, WGS analysis confirmed the absence of the genes coding for the CDT toxin. In addition, an MLST analysis showed that 16 isolates shared the same sequence type, ST-42, while one wastewater isolate (FCDA4_C6) belonged to ST-28. After WGS, an SNV-based analysis was conducted using the genomes of the 17 strains of RT106, 10 from human samples and seven from wastewater samples (all from influent). These genomes were compared to a clinical isolate, used as reference (CD4001).

The analysis revealed that clinical strains exhibited distances ranging from one to 40 SNVs, while wastewater strains varied between nine and 297 SNVs, and overall, the distance across both groups ranged from three to 297 SNVs between isolates. Two main clusters are displayed in Figure 4.13A: one comprising the 16 isolates belonging to ST-42, with strains differing by one to 42 SNVs, and the other consisted of a single ST-28 wastewater isolate, which had a minimum distance of 282 SNVs from the remaining RT106 strains. Within the first cluster mentioned, two distinct subclusters are evident, both comprising clinical isolates along with wastewater isolates. In the first subcluster, the genetic proximity between an influent isolate (FCDA3_C1) and the clinical isolates CD4004 and CD4110 is particularly notable, with a distance of three and four SNVs, respectively. In contrast, in the second subcluster, the sole wastewater isolate does not exhibit such a close proximity to any of the three

neighboring clinical isolates, with a minimum distance of 12 SNVs. Overall, the SNV analysis supports a high genetic similarity among clinical and wastewater isolates.

Regarding the *in silico* AMR analysis, the moxifloxacin-resistant RT106 isolates (from wastewater) carried the previously described amino acid mutation T82I in GyrA, and all three clindamycin-resistant RT106 isolates (from human samples) harbored the erythromycin ribosomal methylase gene B (*ermB*). In two isolates, *ermB* was located in a Tn6189-like transposon, while in the other clinical isolate, it was found in a novel *ermB* mobile genetic element (MGE). It was previously described that *C. difficile* isolates from RT106 can harbor unique genetic islands that can confer higher virulence and/or antibiotic resistance (Roxas et al., 2020). All the analyzed RT106 isolates contained the GI1 (genetic island one), and none possessed the GI2. But seven isolates (including two from wastewater) also harbored genetic island three (GI3). Eight isolates (two from wastewater) carried the previously described plasmid pCD-ECE6, and an uncommonly described plasmid, pZ1323HCD0047-3, was identified in the wastewater isolate from ST-28. These findings are illustrated in Figure 4.13B.

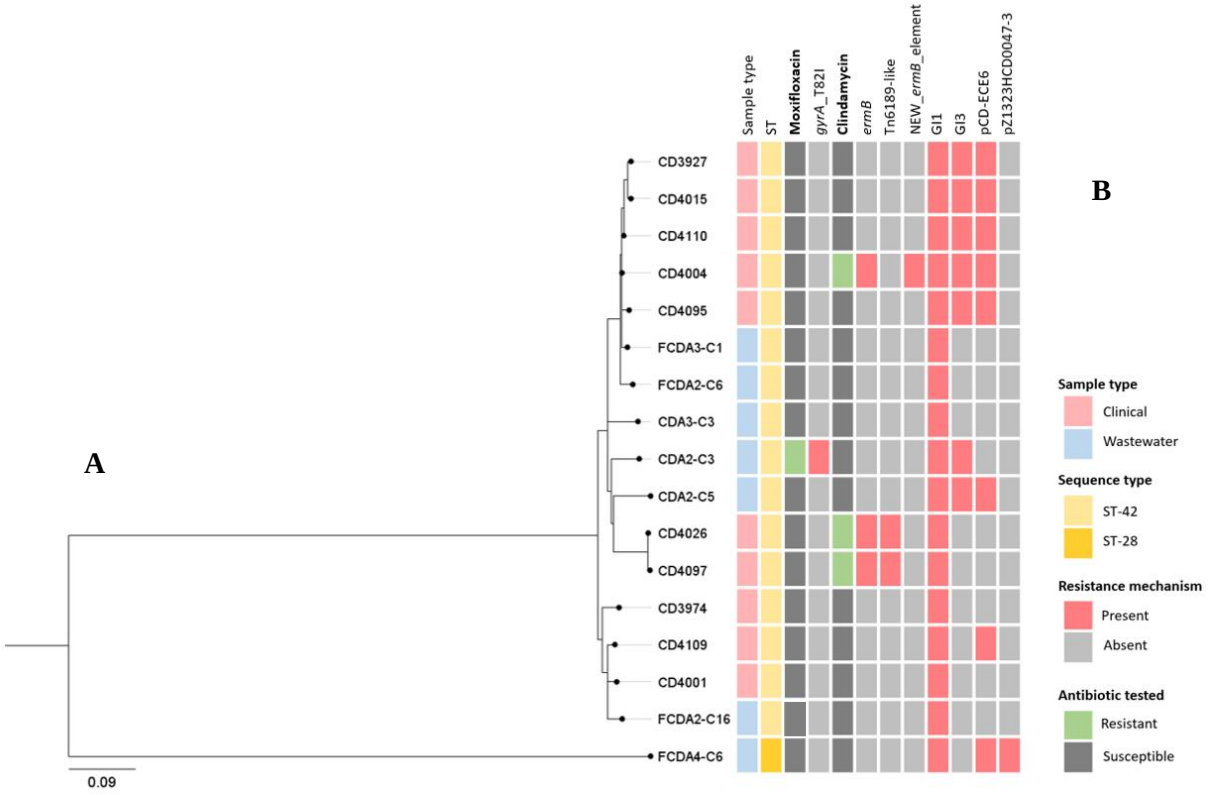

Figure 4.13. Phylogeny of *Clostridioides difficile* isolates of ribotype RT106, from both clinical and wastewater samples, and genetic determinants of antibiotic resistance based on WGS sequences. **(A)** Maximum likelihood tree based on 408 single-nucleotide variant positions of 17 RT106 *Clostridioides difficile* isolates, 10 from clinical samples and seven from wastewater samples, generated by mapping reads against the genome of clinical isolate CD4001, used as reference. **(B)** Heatmap exhibiting the type of sample, sequence type (ST) and distribution of antimicrobial resistance determinants, as well as the associated phenotypes, for each isolate.

Regarding the RT078/126 isolates, WGS analysis confirmed the presence of the genes coding for the CDT toxin in eight strains, while one (CD4054) was found to be contaminated hampering this analysis. The MLST analysis showed that seven isolates shared the same sequence type, ST-11, while for two isolates (one from each source) the ST was not possible to uncover. Upon WGS, a phylogenetic tree was generated based on SNV analysis, resorting to the genomes of the nine strains of RT078/126,

five from human samples and four from wastewater samples (all from influent as well). These genomes were compared to the wastewater isolate CDA1_C2, used as reference.

The analysis revealed that clinical isolates varied from an indistinguishable pair to a distance of 30 SNVs, while influent isolates had a pair with a distance of one SNV and a maximum distance of 27 SNVs among themselves. When analyzing the entire set, three isolates were indistinguishable, with the maximum distance between isolates reaching 31 SNVs. Three main clusters are shown in Figure 4.14A. The first cluster comprised five isolates (three from wastewater), with strains differing by one to 14 SNVs. Notably, a clinical isolate and a wastewater isolate were almost identical, distancing by one SNV only. The second cluster contained three strains (one from wastewater) that are indistinguishable from each other. The third cluster comprised a single clinical isolate that differed by 24 to 31 SNVs from the other strains. Overall, the SNV analysis supports a high genetic similarity among clinical and wastewater isolates.

All four moxifloxacin-resistant RT078/126 isolates (one from wastewater) carried the amino acid mutation T82I in GyrA. Similarly, both clindamycin-resistant RT078/126 isolates (one from each source) harbored the *ermB* gene, located in the Tn6194-like transposon, while all carried an additional transposon (Tn916). Furthermore, six strains also carried the *tetM* gene, responsible for tetracycline resistance (not tested in the present study), including two isolates from wastewater samples. These findings are illustrated in Figure 4.14B.

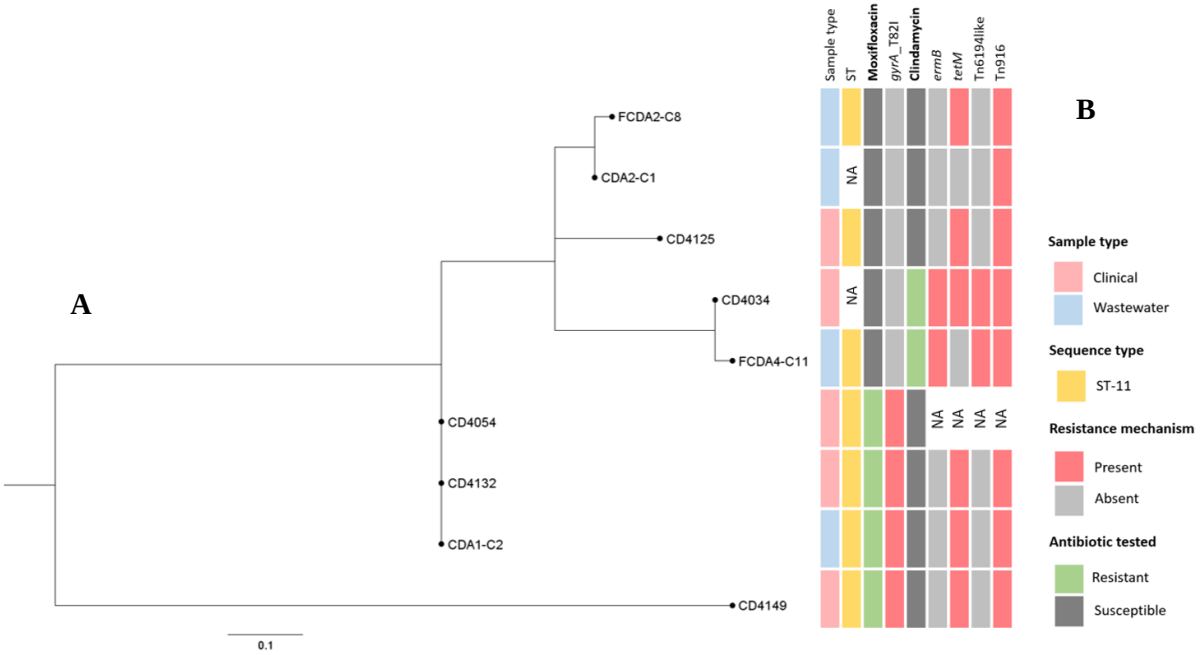

Figure 4.14. Phylogeny of *Clostridioides difficile* isolates of ribotype RT078/126, from both clinical and wastewater samples, and genetic determinants of antibiotic resistance based on WGS sequences. **(A)** Maximum likelihood tree based on 44 single-nucleotide variant positions of nine RT078/126 *Clostridioides difficile* isolates, five from clinical samples and four from wastewater samples, generated by mapping reads against the genome of wastewater isolate CDA1_C2, used as reference. **(B)** Heatmap exhibiting the type of sample, sequence type (ST) and distribution of antimicrobial resistance determinants, as well as the associated phenotypes, for each isolate. NA – not available

Regarding the remaining isolates from RT430 and RT651, they all shared the same sequence type, ST-239. As a result, the isolates from these two ribotypes were arranged into a single group, comprising two clinical strains and three wastewater strains (all from effluent). The WGS analysis confirmed the absence of the genes coding for the CDT toxin, as expected. The SNV-based analysis, using the clinical isolate CD4096 as reference, revealed that, regarding the RT430 isolates, only one

clinical isolate belonged to this ribotype, with distances of 10 and 18 SNVs from the two wastewater isolates sharing the same ribotype, which were eight SNVs apart from each other. The two RT651 isolates, one clinical and the other from influent, were separated by 12 SNVs. Two main clusters are illustrated in Figure 4.15A. The first contained three isolates, two of which were obtained from wastewater samples, with strains differing by seven to 17 SNVs. The second cluster comprised two isolates, one from each sample type, with a distance of seven SNVs between them, showing high genetic proximity.

Only one ST-239 isolate exhibited resistance upon AST: a clindamycin-resistant effluent isolate that harbored the *ermB* gene, located in the novel *ermB* MGE, similarly to the RT106 clinical isolate described above, as observed in Figure 4.15B.

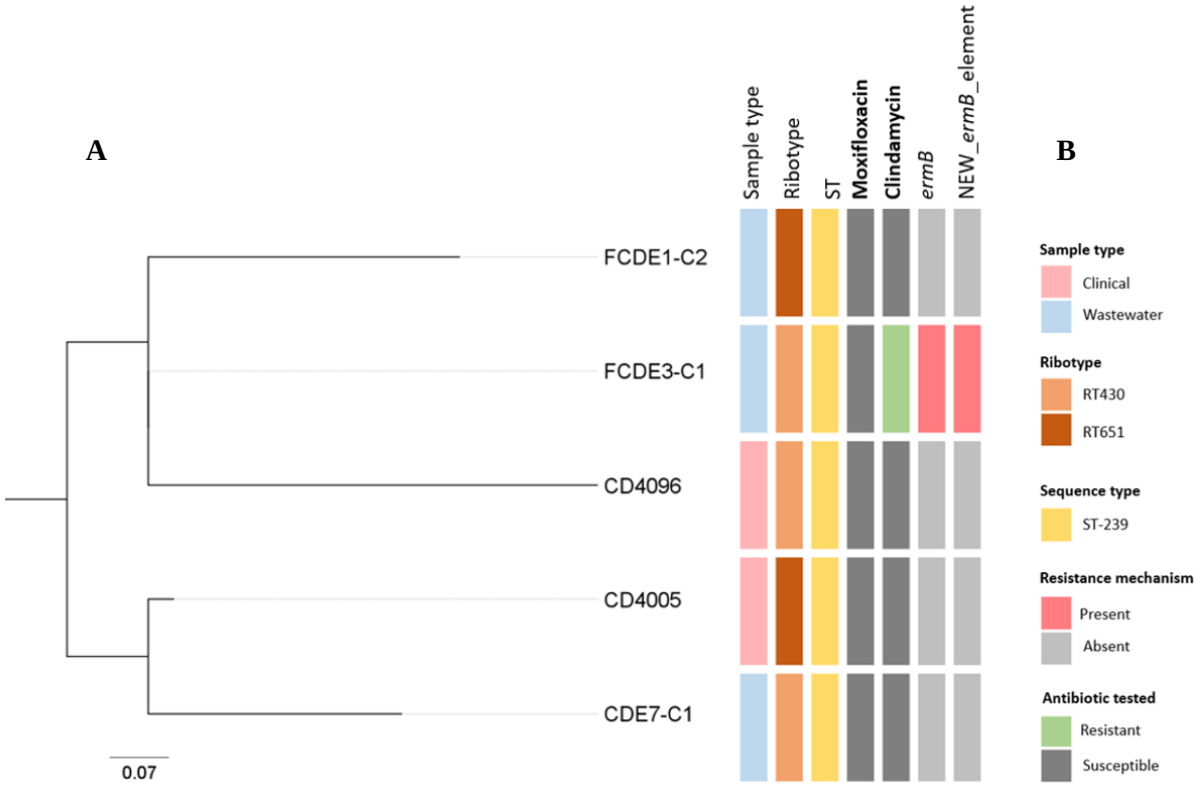

Figure 4.15. Phylogeny of *Clostridioides difficile* isolates of ribotypes RT430 and RT651, from both clinical and wastewater samples, and genetic determinants of antibiotic resistance based on WGS sequences. **(A)** Maximum likelihood tree based on 27 single-nucleotide variant positions of three RT430 and two RT651 *Clostridioides difficile* isolates, two from clinical samples and three from wastewater samples, generated by mapping reads against the genome of clinical isolate CD4096, used as reference. **(B)** Heatmap exhibiting the type of sample, sequence type (ST) and distribution of antimicrobial resistance determinants, as well as the associated phenotypes, for each isolate.

Despite the small number of isolates analyzed by WGS, it was observed across all four ribotypes that no clones were found among the included wastewater isolates. The closest pair of isolates of the same ribotype was separated by only one SNV, while the furthest was distanced by 297 SNVs, demonstrating significant diversity amongst isolates, despite their shared ribotype profile.

5. DISCUSSION

Given that CDI continues to represent a significant burden on healthcare systems worldwide, studying the epidemiology of this pathogen is crucial for countries to develop effective strategies to manage infection. Additionally, establishing a national surveillance network for CDI is essential for collecting data on the issue, as demonstrated by its implementation in Portugal, which facilitated this study. By analyzing *C. difficile* strains circulating in various Portuguese hospitals, detailing prevalent ribotypes, antimicrobial resistance rates and resistance mechanisms, this study provides an overview of the current CDI paradigm in Portugal. Nevertheless, it is important to note that the findings are based on a convenience sample of public hospitals that voluntarily participate in the national CDI surveillance network. Therefore, these results may not fully represent the broader national context of healthcare facilities.

In this study, out of the samples received from the 17 hospitals integrating the national CDI surveillance programme, 384 isolates were recovered as *C. difficile* through the golden standard method, culture (Crobach et al., 2016). All *C. difficile* isolates from clinical samples were characterized by ribotype, toxin profile and antimicrobial susceptibility.

Regarding ribotype distribution, and consistent with recent studies, RT106 was the most prevalent amongst isolates. Although RT106 was not previously considered one of the most common ribotypes in Europe in general (Davies et al., 2016), or specifically in Portugal (Santos et al., 2016), a more recent study in Portugal (Alves et al., 2023) reports a significant increase in its prevalence over the last eight years, being one of the top three most common ribotypes since 2017. Notably, RT106 was firstly reported in the United Kingdom (UK), in Stubbs et al. (1999), comprising one of the area's most prevalent ribotypes in the early 2000's (Brazier et al., 2008). However, more recent data indicates a decrease in the incidence of this RT in the UK in the last decade, as reported in Davies et al. (2016), with only three RT106 isolates accounted for in the latest ECDC's annual CDI surveillance report (ECDC, 2024). Interestingly, in the USA, the rise of RT106 occurred after its peak in the UK, between 2011 and 2017 (Tickler et al., 2019). Furthermore, this ribotype is typically associated with CA-CDI (Lessa et al., 2015; Tickler et al., 2019) and animal infection, with Alves et al. (2023) reporting RT106 as the most commonly found profile in pet samples in Portugal, and its rise reflects the shift in the epidemiology of *C. difficile*. This underscores the importance to keep investigating the presence of this pathogen in environments beyond healthcare facilities.

The most frequent ribotypes in this study also include RT014/020, a ribotype that has consistently been ranked among the most common over the years, as shown by studies from Europe (Viprey et al., 2021), Portugal (Nazareth et al., 2022), Scotland (Banks et al., 2016), Australia (Collins & Riley, 2016) and the USA (Tickler et al., 2019). Once again, this ribotype is more frequently associated with CA-CDI and is increasingly overtaking epidemic strains, such as RT027, while being common amongst pets in Portugal (Alves et al., 2023), as well.

Another commonly found ribotype, RT002, has been reported in both hospital-associated CDI cases and environmental samples. Across Europe, between 2011 and 2013, both Davies et al. (2014) and Freeman et al. (2015) identified RT002 as one of the most prevalent ribotypes. In the USA, Tickler et al. (2019) reported that RT002 showed the most significant increase in prevalence among ribotypes, rising from 3.0% to 10.0%, between 2011 and 2017. Additionally, it was also one of the most frequently reported ribotypes in CDI cases in Australia (Collins & Riley, 2016). By 2017, RT002 was already

among the five most commonly detected ribotypes in Portugal (Nazareth et al., 2022), and this study showed that its prevalence has been steady. Aside from being detected in lakes and rivers (Cizek et al., 2022; Zidaric et al., 2010), an RT002 isolate was found in effluent from a Wastewater Treatment Plant in Italy (Romano et al., 2018). Notably, in a recent study from Europe (Dost et al., 2024), a new European RT002 data collection was created. Identical genomes were revealed between human samples from Germany and France and non-human samples from Germany, while isolates obtained from UK wastewater clustered with isolates from human samples both in the UK and other countries. This underscores not only the zoonotic potential of this ribotype, as well as its significance within the One Health approach. Furthermore, Dost et al. (2024) described that RT002 sporulates more frequently, and germinates more easily without certain bile salts, compared to other ribotypes, being able to cause severe CDI, though not as severe as hypervirulent ribotypes. Interestingly, based on the genetic clones found amongst isolates, the study also suggests the possible existence of a single common source.

In addition to the ones previously mentioned, RT012 and RT013 are also among the most commonly found ribotypes in clinical samples from this study. According to the ECDC's annual epidemiological reports for *Clostridioides difficile* infections for 2016-2017 (ECDC, 2022) and for 2018-2020 (ECDC, 2024), both ribotypes ranked among the top 20 most frequently RTs associated with CDI in Europe. These results align with the findings of this study. Moreover, RT012 is notably linked to hospital-acquired infections outside Europe as well. Tickler et al. (2019) reported that RT012 consistently ranked among the top 15 most common ribotypes in the USA between 2011 and 2017. Similarly, Collins et al. (2013) identified RT012 as one of the most frequently detected ribotypes in China. This RT is also one of the leading causes of CDI in children in Vietnam (Khun et al., 2023). Additionally, RT012 has been identified in samples collected from WWTPs in the Czech Republic (Cizek et al., 2022) and Australia (Chisholm et al., 2022).

Despite all the most found ribotypes in this study being toxigenic, only RT078/126 is positive for the *cdtA*/*cdtB* genes, encoding the binary toxin. This hypervirulent ribotype is one of the most common profiles in Europe (Viprey et al., 2021), typically associated with CA-CDI and farm animals (Blau et al., 2023). Over the years, RT078/126 has been associated with the infection in swine, having been reported in Italy (Spigaglia et al., 2015), Ireland (Stein et al., 2017), and more recently in the Czech Republic (Krutova, Zouharova, et al., 2018) and China (Zhang et al., 2019). A recent European study (Freeman et al., 2024) has also reported this RT as the most predominant amongst 126 isolates from samples of pigs and rodents representing over 32.0%. This establishes it as one of the ribotypes with the highest zoonotic potential. However, its association with CDI in hospitalized patients has been increasing (Alves et al., 2022), being responsible for more severe cases in younger individuals (Chisholm et al., 2022). Moreover, RT078/126 was first reported in wastewaters in Switzerland (Romano et al., 2012).

The presence of RT027 in this study is attributed to an active outbreak, confirmed by WGS analysis, in one of the hospitals within the national CDI surveillance network. Hence, although it statistically represents the third most prevalent RT (at 5.7%) among clinical isolates, it cannot be included in the RT analysis to avoid biased results. The emergence of RT027, driven by the widespread use of fluoroquinolones, led to increased concern over CDI monitoring and kept this toxigenic and *cdtA*/*cdtB* positive ribotype consistently among the most common RTs for several years, as observed in Europe (Davies et al., 2016) in general, Portugal (Santos et al., 2016), and the USA (Lessa et al., 2015). Aside from the outbreak, no occurrences of RT027 were found between September 2023 and May 2024 in any other context. This aligns with more recent data from Portugal (Alves et al., 2023) and the USA (Tickler et al., 2019), showing a decline in the prevalence of RT027, making way for ribotypes with greater zoonotic potential over more epidemic ones in recent years. However, this outbreak is of concern

as it suggests a reemergence of this hypervirulent ribotype in Portugal, since the last reported outbreak that occurred in 2012 (Oleastro et al., 2014).

The first major study on the epidemiology of CDI in Portugal analyzed 498 *C. difficile* isolates from samples collected between the years 2010 and 2015 (Santos et al., 2016). Similarly to the current study, samples were received within the scope of the national CDI surveillance programme, from 20 Portuguese hospitals, covering the North, Center, Lisbon Metropolitan Area and Algarve regions (Santos et al., 2016). At the time, amongst the 96 RT profiles identified in Santos et al. (2016), RT014 was the most common in the North, while RT027 was the most prevalent in the Center, Lisbon Metropolitan Area, and Algarve. In comparison, the current study confirmed that RT014/020 remained the dominant strain in the North, and the Center and Lisbon Metropolitan Area continue to share the same most prevalent ribotype, now being RT106. These findings further support the shift in *C. difficile* epidemiology. Although RT027 was previously the most prevalent ribotype in Portugal, its absence in more recent samples from Portuguese hospitals reflects the spread of ribotypes that were once only found in environmental settings and are now responsible for human CDI cases. Nevertheless, the ongoing outbreak of RT027 at a hospital located in the North region indicates the reemergence of this hypervirulent ribotype in the country. Moreover, in Alentejo, the most prevalent ribotype was RT643, previously found in animal and wastewater samples from a pig farm in Santarém, Portugal (Alves et al., 2022). Meanwhile, RT050 and RT056 were the most commonly identified ribotypes in the Azores region, both usually linked to hospital-associated CDI cases. RT050 has been documented in European CDI surveillance studies (Abdrabou et al., 2022; Freeman et al., 2015). In contrast, RT056 was one of the most prevalent ribotypes in Australian hospitals (Collins & Riley, 2016), with its incidence rising between 2011 and 2017 in the USA (Tickler et al., 2019). Nevertheless, RT056 has also been reported in cattle samples (Alves et al., 2022) and wastewater samples, once again in Australia (Chisholm et al., 2022).

Lastly, it is worth mentioning that the latest annual report for CDI surveillance by the European Centre for Disease Prevention and Control (ECDC, 2024), which covered seven countries, identified the following top 10 most common ribotypes among 2838 *C. difficile* isolates from CDI: RT014/020 (17.7%), RT002 (14.5%), RT078/126 (9.1%), RT005 (5.1%), RT015 (5.0%), RT001 (3.9%), RT023 (3.8%), RT106 (2.4%), RT012 (1.9%) and RT017 (1.7%). Notably, five of the six most prevalent RTs (RT106, RT014/020, RT002, RT012, RT013 and RT078/126) from clinical isolates in this study are within this list, while RT013 represents only 1.0% of isolates in the European study. Moreover, RT027 was also reported by the ECDC's report, accounting for 1.6% of strains, whereas between 2016 and 2017, it was the third most common ribotype (8.1%) (ECDC, 2022). Once again, the ongoing shift in this pathogen's epidemiology is highlighted, with the top six RTs found in Portugal also having been detected among *C. difficile* isolates from various sources outside healthcare facilities. These include swine (RT002, RT013, RT014/020 and RT078/126), mollusks and vegetables (RT002, RT012 and RT014/020), and beef and chicken (RT014/020), as reported by Bolton & Marcos (2023). Moreover, these RTs have been found in samples from WWTPs, both RT012 (Cizek et al., 2022) and RT078/126 (Romano et al., 2012), pets (RT012, RT014/020, RT078/126 and RT106) and wild animals (RT002, RT013, RT014/020 and RT078/126) and wastewater effluent (RT002 and RT014/020), as described in Rodriguez-Diaz et al. (2024). While the most epidemic RTs associated with CDI in humans, as RT027, are currently in decline. Nevertheless, they may reemerge, as observed in the present study.

Regarding antimicrobial susceptibility, it is important to note that the presence of the 22 isolates of outbreak-associated RT027 can influence results. For instance, a recent study (Freeman et al., 2024) within the scope of the Combatting Bacterial Resistance in Europe - *Clostridioides difficile* infections (COMBACTE-CDI) project, which aims to contribute to the understanding of the epidemiology of CDI

across Europe, illustrates this point. Freeman et al. (2024) reports an increase in overall resistance levels to the antibiotics tested (including clindamycin, moxifloxacin, rifampicin, vancomycin and metronidazole) due to the presence of RT027 isolates from Eastern Europe.

In this study, 21.4% of the total clinical isolates demonstrated resistance to clindamycin, making it the antibiotic with the highest resistant rate. This pattern was also observed globally. In a study conducted in the USA (Tickler et al., 2019), which analyzed 940 *C. difficile* isolates from 26 different hospitals, collected between 2011 and 2017, the proportion of clindamycin-resistant isolates was 41.5% between 2013 and 2014, and 56.8% between 2015 and 2017. Additionally, a European study found resistance to clindamycin in all 22 participating countries, representing 49.6% of the 953 *C. difficile* isolates examined (Freeman et al., 2015). Moreover, a study in China (Collins et al., 2013) revealed a higher percentage of clindamycin-resistant isolates compared to other tested antibiotics. Furthermore, the high rate of resistance to clindamycin in *C. difficile* isolates can also be attributed to the mechanism underlying this resistance. In other words, the presence of the *ermB* gene within MGEs in the *C. difficile* genome, such as transposons Tn5398, Tn9164, and Tn6215 (Isidro et al., 2017), facilitates the dissemination of clindamycin resistance through horizontal gene transfer (HGT). Overall, these studies show that clindamycin resistance is prevalent among common ribotypes, such as RT014/020 and RT106, which aligns with the findings of the current study, given the diversity of ribotypes exhibiting this resistance.

Resistance to fluoroquinolones in *C. difficile* continues to be reported worldwide. As previously mentioned, and detailed in Oleastro et al. (2014), fluoroquinolones emerged in the USA as the most frequently prescribed class of antibiotics in the early 2000s. The selective pressure imposed by their widespread use contributed to an increase in the prevalence of hypervirulent and resistant strains of RT027, which are linked to more severe cases of CDI. Consequently, a major outbreak occurred in North America and Europe, raising global concerns regarding the monitoring of CDI, and establishing RT027 as one of the primary causes of infection in the subsequent years. However, this resistance is no longer exclusively due to the prevalence of RT027 strains. This shift may be related to the reduced use of fluoroquinolones, as seen in Germany, where its administration decreased by approximately 50.0% between 2014 and 2019 (Abdrabou et al., 2022). Such reduction reflects a significant decrease in the number of RT027 strains identified in subsequent years. In Portugal, already between 2014 and 2015, the high percentage of resistance to moxifloxacin was associated with a high diversity of ribotypes (23 distinct ribotypes), including ones not typically linked to this resistance, such as RT014/020, RT078/126 and RT203 (Santos et al., 2016). In the current study, moxifloxacin ranks second (with 15.6%) in terms of resistant isolates, similar to recent data from Germany (Abdrabou et al., 2022) and China (Collins et al., 2013), being associated with 15 distinct ribotypes, including the commonly found RT106 (if RT027 is excluded).

Lower percentages of resistance in *C. difficile* isolates are typically observed for rifampicin. This is evident from the study on the implementation of a CDI surveillance system in Germany (Abdrabou et al., 2022), where rifampicin-resistant isolates account for 4.0% of the total. Similarly, in the European study of Freeman et al. (2020), rifampicin resistance was found in 13.5% of the isolates in 2011. In the following years included in the study (2015 and 2016) this percentage slightly decreased, ranging from 10.2% to 11.8%, with this value varying among the 28 countries included. Furthermore, this finding is corroborated by the present study, which reports a 6.5% resistance rate to this antibiotic. However, when excluding the 22 outbreak-associated RT027 isolates, only three isolates with this resistance remain, corresponding to ribotypes RT085, RT181, and RT284.

Regarding vancomycin and metronidazole, all isolates from this study were found to be susceptible, consistent with previous research (Abdrabou et al., 2022; Collins et al., 2013; Nazareth et al., 2022). Once again, this finding aligns with earlier studies conducted in Europe (Freeman et al., 2020 and 2024) and Portugal (Santos et al., 2016).

The RT027 isolates linked to the described outbreak exhibit a resistance profile that slightly differs from what is typically reported for strains of this RT in literature, including in Portugal. The antimicrobial susceptibility tests and further WGS analysis revealed that moxifloxacin resistance was associated with known point mutation in *gyrA*, the GyrA T82I mutation, previously reported in Gargis et al. (2023), while resistance to rifampicin was attributed to the R505K amino acid substitution in *rpoB*, which according to Isidro et al. (2017), is the most commonly found. Additionally, this strain showed MLS_B resistance, which was caused by the presence of transposon Tn6189, harboring the *ermB* gene, reported by Kartalidis et al. (2021). Regarding gentamicin, despite the elevated MIC value indicating a resistant phenotype, no associated antimicrobial resistance mechanisms were identified through WGS. Furthermore, the RT027 outbreak-associated clone exhibited the recently described VanRCd Thr115Ala mutation in the cytoplasmic response regulator, *vanR*, which is responsible for the reduced susceptibility to vancomycin observed in the strain analyzed in the present study. This is the first report of such an RT027 strain circulating in Portugal. This mutation has only been described in the USA, in two RT027 *C. difficile* clinical isolates obtained from symptomatic CDI-diagnosed patients from Florida, in 2016 (Wickramage et al., 2023). The VanRCd Thr115Ala mutation was also independently reported in seven *C. difficile* clinical isolates in Texas, USA, as well as in two RT027 *C. difficile* clinical isolates from Israel (Shen et al., 2020). According to the authors, RT027 isolates developed vancomycin resistance through the constitutive expression of the typically phenotypically silent *vanG* gene, following a mutation in the two-component regulatory system, VanSR. They further note that, while the discovery of constitutive vancomycin resistance mechanisms is recent for *C. difficile*, it is not new for enterococci, where this phenomenon has been commonly reported. Thus, based on the mechanisms observed in bacteria of the *Enterococcus* genus, Shen et al. (2020) described that the Thr115Ala mutation in *vanR* of *C. difficile* enhances the stability of the effector domain when interacting with *C. difficile* DNA, thereby activating the resistance gene transcription.

The spread of *C. difficile* strains non-susceptible to vancomycin, a first-line antibiotic for CDI, poses a serious therapeutic challenge, corroborating the need for routine susceptibility testing in the context of CDI surveillance.

Resistance rates associated with each antibiotic tested varied across the regions of Portugal studied, showing differences with the data of the previous study regarding CDI epidemiology. In Santos et al. (2016), the Center region was identified as the region with the highest percentage (53.4%) of moxifloxacin-resistant isolates, while the Northern region recorded the lowest resistance rate (11.9%) to this antibiotic amongst the four regions studied. Interestingly, in the current study, the Center region not only exhibited the highest resistance rate (20.3%) to moxifloxacin as well, however, alongside the Northern region, but also housed the highest percentage (29.7%) of clindamycin-resistant isolates. This suggests that the Central region continues to display concerning resistance rates compared to other areas of the country. Additionally, there has been an increase in the prevalence of strains resistant to fluoroquinolones in the Northern region over the years (even after excluding the outbreak-associated RT027).

Furthermore, it is important to note that isolates from CDI cases were predominantly toxigenic, as expected. Nevertheless, non-toxigenic strains were also detected in clinical samples. Although these non-toxigenic strains do not express the virulence factors associated with disease, they can still play a

significant role in CDI. These strains often harbor important antibiotic-resistance genes acquired by HGT from other *C. difficile* strains, or even other species of bacteria present in the same environment, such as the human gut, being a reservoir of MGE associated with antibiotic resistance, that can be transferred to toxigenic strains, as demonstrated by Brouwer et al. (2013). Moreover, non-toxigenic and toxigenic strains can coexist and simultaneously colonize the patient's gut, thus explaining the presence of non-toxigenic isolates in stool samples from CDI cases, supporting the findings of the present study. It is, therefore, important to highlight the non-toxigenic clinical strains resistant to clindamycin found in this study. As previously mentioned, resistance to this antibiotic is acquired by the presence of the *ermB* gene, located in MGEs such as the transposon Tn6189, reported by Kartalidis et al. (2021) in both toxigenic and non-toxigenic strains. Consequently, co-colonization of the intestine by these resistant isolates alongside a toxigenic *C. difficile* strain may facilitate the transfer of transposons between them, thereby contributing to the spread of this resistance profile to disease-causing strains. This emphasizes the importance of also monitoring the *C. difficile* non-toxigenic strains.

Community-associated CDI cases arise from exposure to *C. difficile* reservoirs or sources within the community, rather than the typical hospital settings. While the impact of the environment on *C. difficile* epidemiology has been recognized globally, the information available remains limited, particularly in Portugal. Therefore, searching for new potential reservoirs is crucial. This study aimed to shed light on *C. difficile* surveillance in municipal wastewater treatment plants (WWTPs) in Portugal for the first time, by assessing the presence of *C. difficile* in untreated and treated wastewater, characterizing the circulating strains, and understanding the overlap between environmental and human strains. Alongside the national CDI surveillance programme carried out at INSA, this approach is intended to enhance our understanding on the epidemiology of CA-CDI and the potential risk to Public Health that *C. difficile* strains found in wastewater samples may comprise.

In this study, out of the samples collected from a WWTP in the Lisbon Metropolitan Area, mainly treating domestic and industrial wastewater, 140 isolates were recovered as *C. difficile*, 99 from influent and 41 from treated effluent, while no *C. difficile* was uncovered in ApR (water for reuse) samples. All *C. difficile* isolates from wastewater samples were characterized by ribotype, toxin profile and antimicrobial susceptibility.

C. difficile prevalence was of 100.0% in influent samples, but the isolation rate in effluent samples was lower, at 87.5%. *C. difficile* was not detected in ApR samples, likely due to samples undergoing tertiary treatment, which included microfiltration or ultrafiltration, followed by disinfection with sodium hypochlorite or UV light, in addition to conventional treatments. Thus, treatment proved effective in eliminating *C. difficile* spores, suggesting that water reused for irrigation or public space cleaning does not pose a risk to Public Health. However, it is important to note the small number of samples analyzed in this study (n = 3), indicating that further investigation is necessary. Overall, these results are consistent with those found in Chisholm et al. (2022).

Discrepancies in isolation success rates between studies may result not only from variations in the concentration of *C. difficile* in samples, but also from the use of different techniques for isolating this pathogen from environmental matrices. This highlights the need for a standardized universal method. For instance, in Iran (Baghani et al., 2020), only one *C. difficile* isolate was identified from samples collected at three different WWTPs between 2016 and 2017. Oppositely, in Australia, a study involving 12 different WWTPs found an average of 10 *C. difficile* isolates per WWTP in influent and four isolates per WWTP in effluent samples (Chisholm et al., 2022). In the present study, while direct inoculation of the filter onto selective media was employed, performing a prior enrichment step would have enhanced the detection of *C. difficile* strains present in lower concentrations, given the

environmental nature of the matrix. Contrary to what was theoretically expected, the direct plating method proved more effective in terms of positivity rates for both influent and effluent samples. This finding aligns with Cizek et al. (2022), where *C. difficile* was obtained in five samples when using direct plating and only in one sample when performing prior enrichment. Nevertheless, the enrichment broth method provided a greater diversity of ribotypes in both influent and effluent samples. Ultimately, these results emphasize the importance of optimizing *C. difficile* isolation protocols for environmental samples. However, using both methods concurrently proved beneficial, as it was observed that when one method failed to isolate *C. difficile*, the other method succeeded, thereby maximizing its recovery.

Regarding ribotype diversity in wastewater samples, analysis showed that six ribotypes, three non-toxigenic (RT009, RT010 and RT084) and three *toxA*/t*oxB* positive (RT103, RT081, and RT104) were identified in both influent and effluent samples. They were all identified in the clinical samples studied. Amongst toxigenic, previous studies have reported RT103 in both wastewater samples (Chisholm et al., 2022) and human stool samples (Abdrabou et al., 2022; Collins & Riley, 2016), while RT081 has only been found in hospital-associated CDI cases (Abdrabou et al., 2022; Romano et al., 2018). Additionally, an isolate of RT081 with a multi-resistance profile to clindamycin, moxifloxacin, rifampicin, and tetracycline was reported in the USA (Tickler et al., 2019), however, this was not observed in this study. No information was found on RT104, however, this ribotype was also identified amongst the clinical isolates of this study. Regarding non-toxigenic ribotypes, both RT010 and RT009 are commonly associated with *C. difficile* colonization in companion animals (Alves et al., 2023) and are frequently found in bodies of water (Cizek et al., 2022). Although these ribotypes do not express the *tcdA*, *tcdB* or *cdtA*/*cdtB* genes, reducing its risk to Public Health, they still play a significant role in the epidemiology of CDI, as previously mentioned. For instance, when analyzing wastewater samples, Cizek et al. (2022) reported an RT010 isolate with metronidazole resistance acquired through plasmid pCD-METRO, which could potentially be transferred to other strains, whether non-toxigenic or toxigenic, and resistant or susceptible. Once introduced into the environment, this strain could have a significant long-term impact on community-associated *C. difficile* infections. Additionally, RT084 was described in 2020 as one of the most prevalent RTs circulating in children in both the UK and Tanzania (Riley & Perumalsamy, 2021).

Surprisingly, five PCR-ribotype profiles were found exclusively in effluent samples: RT154, RT430, RT651 and two distinct unknown RTs. This observation may be due to the procedure for identifying suspected colonies, which was not exhaustive. Furthermore, all three known RTs were found in the clinical isolates as well. Notably, all five RTs are toxigenic, expressing the *tcdA* and *tcdB* genes. Both RT430 and RT651 are associated with cases of community-acquired CDI (Santos et al., 2016), while RT651 has also been previously identified in wastewaters (Cizek et al., 2022). No information was found regarding RT154, regardless, this ribotype was also identified amongst the clinical isolates of this study.

Regarding antimicrobial susceptibility, resistance to clindamycin was the most common phenotype amongst the *C. difficile* isolates from wastewater, consistent with findings in clinical isolates. Of the four antibiotics tested, clindamycin was the only one with resistant isolates in both influent (14.1% of influent isolates) and effluent (12.2% of effluent isolates) samples. In addition, the resistant isolates found in the effluent samples showed resistance exclusively to clindamycin. It is noteworthy that, while in influent samples seven different RTs showed resistance to clindamycin, a single non-toxigenic ribotype, RT009, presented this phenotype in effluent. A study conducted in Australia between January and June 2020, including 284 *C. difficile* isolates, also found that 56.7% were resistant to clindamycin (Chisholm et al., 2022), although not distinguishing influent from effluent isolates. Moxifloxacin-resistant isolates were identified as well at the entrance of the WWTP, representing 9.1%

of influent isolates, and including ribotypes RT1078/126, RT106 and RT014/020. Similar to clinical isolates, the number of rifampicin-resistant isolates in influent samples was lower (1.0%) compared to other antibiotics. This phenotype was found in only one influent sample, in an RT009 isolate. Furthermore, this isolate also showed resistance to clindamycin, making it the only isolate (and the only RT) resistant to more than one antibiotic, further emphasizing the risk non-toxigenic strains may pose for Public Health. All isolates from both influent and effluent samples were susceptible to vancomycin and metronidazole, not only consistent with other similar studies (Baghani et al., 2020; Chisholm et al., 2022; Rivas et al., 2020), but also with the findings in clinical isolates.

The overlap between *C. difficile* ribotypes found in wastewater samples and those isolated from human samples in Portugal is substantial. Ribotypes frequently known to be prevalent in humans were notably present in wastewater samples, with only two ribotypes (RT202 and RT434, both from influent samples) out of 25 known RTs not detected in the human CDI cases included in this study. Specifically, the vast majority of RTs found in the inlet (20/22; 90.9%) and all RTs from outlet (9/9) of the WWTP were consistent with the PCR-ribotype profiles identified in clinical isolates. In agreement with the first study of *C. difficile* in wastewater samples (and subsequent comparison with clinical isolates) conducted in Switzerland (Romano et al., 2012), the current study also found an overlap between the PCR-ribotypes identified in wastewater samples and those in clinical isolates from the same area where the WWTP is located (Lisbon Metropolitan Area). A considerable proportion of ribotypes from the influent (14/22; 63.6%) and the effluent (7/9; 77.8%) corresponded to the RTs identified in isolates associated with CDI in this region. More specifically, the seven ribotypes common to both effluent and CDI in the Lisbon Metropolitan Area were the non-toxigenic RT009 and RT010, along with the toxigenic RT103, RT104, RT154, RT430, and RT651. This suggests a strong correspondence between these environments, which is expected given their shared geographic location. Furthermore, in one of the most recent European studies (Viprey et al., 2021), which included 12 countries, the most common ribotypes among 82 *C. difficile* isolates from CA-CDI were RT078/126 (9.0%), RT039 (9.0%), RT001 (6.0%), RT014/020 (6.0%), RT009 (5.0%) and RT010 (5.0%). Notably, all aforementioned ribotypes were isolated from the clinical samples in this study, and in wastewater samples as well, except for RT001, the only ribotype not detected.

Overall, although hypervirulent ribotypes, as well as antibiotic resistance, were found in influent samples, the analysis of *C. difficile* in samples from the exit of the WWTP shows a significant decrease in RT diversity and in resistance rates. This suggests that wastewater treatment processes are effective in reducing the presence of this pathogen, though not completely. In the current study, both non-toxigenic and toxigenic strains of *C. difficile* were identified in effluent samples. These strains can therefore be released into the environment, posing a risk to Public Health. Most of the ribotypes found in effluent are toxigenic and are also linked to CDI cases in humans, which is concerning. As previously mentioned, although not expressing the toxin encoding genes, non-toxigenic strains may acquire MGEs via HGT from other bacteria from the gut, or from the consortium of pathogenic or commensal microorganisms within the WWTP. Moreover, the presence of various selective pressures, including antibiotics, in WWTPs can create ideal conditions for these processes to occur (Chisholm et al., 2022). Thus, findings demonstrate and reinforce that effluent discharge could potentially lead to contamination by pathogenic agents, including *C. difficile*, potentially contributing to CA-CDI.

Although a significant overlap between clinical and wastewater RTs was demonstrated, the classical differentiation of *C. difficile* isolates through ribotyping is not discriminatory enough to compare isolates from different environments (Seth-Smith et al., 2021). To address this limitation, a WGS analysis was conducted, providing further insights into the genomic diversity and relatedness of isolates from clinical samples and urban wastewater samples.

In 2018, a study in the East of England sequenced the whole genomes of 70 clinical isolates and 186 wastewater isolates, followed by phylogenetic analysis (Moradigaravand et al., 2018). This study demonstrated a high degree of genetic similarity between clinical and wastewater isolates, with some pairs showing particularly close relationships. When consisting of a clinical isolate and a wastewater isolate from a WWTP handling hospital effluent, the pair differed by only one SNP. However, when the wastewater isolate came from a WWTP with no hospital waste treatment, this genetic difference increased to four SNPs. In 2019, Numberger et al. (2019) was the first study in Germany to compare clinical isolates and isolates from urban water sources using WGS analysis. However, only three environmental isolates were sequenced: one toxigenic isolate from a lake and two non-toxigenic isolates from urban wastewaters. All three environmental isolates were closely related to distinct human isolates. More recently, in Australia, Su-Chen et al. (2022) conducted a genomic analysis comparing isolates from water sources with clinical isolates. The analysis revealed a close relationship (≤10 SNPs) between them, further supporting the significant role of environmental contamination by *C. difficile* in the transmission of CDI. However, this study only included lakes, rivers, estuaries, and seawater, and did not analyze wastewaters.

In the present study, the WGS-based analysis offered insights not only on the genetic proximity between clinical and urban wastewater isolates, but also on the genetic similarity of same ribotype profiles within the studied group of isolates. It is also important to highlight the geographic locations of the analyzed isolates. All clinical isolates of RT106, as well as those of RT430 and RT651, were obtained from hospitals in the Lisbon Metropolitan Area, sharing this geographic location with the wastewater isolates. In the RT078/126 group, only one isolate was obtained from this region of Portugal. The common geographic location of isolates may increase the probability of identifying potential links between environmental contamination by *C. difficile* and CDI.

Clustering analysis suggested that the transmission of *C. difficile* strains between the two distinct environments is possible, with several clusters containing isolates from both hospitals and wastewater in all three phylogenetic trees. Notably, the RT078/126 SNV-based analysis revealed that two isolates — one from human samples and another from wastewater samples — differed by only one SNV. Additionally, one wastewater isolate showed no SNV differences compared to two clinical isolates, both of which also shared the same resistance profile. However, it is not possible to assert that these three are in fact the same isolate, as the total number of samples analyzed is very small. In addition, the limited sampling period for wastewater also hampers the ability to draw definitive conclusions. Therefore, it is clear that a much broader analysis would be necessary. Overall, a high genetic similarity was observed among clinical and wastewater isolates, consistent with the previously presented literature (Moradigaravand et al., 2018; Numberger et al., 2019).

As expected, *in silico* antimicrobial resistance (AMR) analysis revealed the presence of the respective AMR determinants, confirming all the resistance phenotypes previously described. Specifically, the presence of *ermB* in all clindamycin-resistant isolates, and a single mutation in *gyrA* was identified in moxifloxacin-resistant isolates.

Regarding the RT106 group, all strains harbored the genetic island GI1, which is specific to this ribotype profile and was recently described in Roxas et al. (2020). The American study details that GI1 contains genes that may confer a competitive advantage to *C. difficile* isolates of this ribotype, as they are associated with both antimicrobial multi-resistance and biofilm formation. Thus, these factors have allowed RT106 to quickly establish itself as one of the most common in CDI cases in Europe (Davies et al., 2016), the USA (Tickler et al., 2019), and specifically in Portugal (Alves et al., 2023), as previously mentioned and now observed. Additionally, the presence of an additional genetic island, GI3,

was found in seven of the isolates (including two from wastewater), which, according to Roxas et al. (2020), also contains genes associated with both AMR and cell adhesion. In contrast to the findings of the previous study, none of the isolates in the present research harbored GI2. It is also noteworthy that these genetic islands were identified in RT106 isolates of different sequence types, ST-42 and ST-28, consistent, once again, with Roxas et al. (2020).

Furthermore, the genome sequencing of RT106 isolates enabled the identification of the plasmid pCD-ECE6 in isolates from both sources. This plasmid had been previously described in *C. difficile* clinical isolates (Adamczyk et al., 2024), but to the best of our knowledge, not yet in environmental isolates. Plasmid pZ1323HCD0047-3 was also identified, though only in a single isolate, from wastewater samples. The aforementioned has been detected in the genome of a clinical strain (Z1323HCD0047) isolated in 2023, in South Korea (Bio Project: PRJNA1087341), exhibiting a 100% identity match and query coverage upon using Nucleotide BLAST. However, this plasmid has not yet been documented in European strains of *C. difficile* and is not included in the Clostyper database used for the analysis in the present study. Thus, it represents a novel finding not only at a European level, but also in environmental isolates of *C. difficile*. This plasmid constitutes an additional MGE that could potentially be transferred to other strains, both in aquatic environments and within the host gut, thereby possibly conferring some competitive advantage to the strain that harbors it.

An extremely rare mobile genetic element of *ermB* was also uncovered in a clinical isolate of RT106, as well as in an effluent isolate of RT430. The presence of this MGE in isolates from distinct ribotypes further supports the notion of active genetic exchange through HGT, leading to the dissemination of MGEs. These resistance determinants confer antibiotic resistance and, in turn, facilitate the spread of resistant strains. Moreover, the presence of this element in both human and environmental strains suggests that such dissemination can occur across different environments.

The novel *ermB* MGE was uniquely described in the aforementioned project from South Korea, however, it was found in a different strain (Z1322HCD0006) than the one that harbored the plasmid pZ1323HCD0047-3. Nonetheless, it was also derived from a human sample, demonstrating a 100% identity match and query coverage upon resorting to Nucleotide BLAST. Similarly, this MGE has not yet been documented in European strains of *C. difficile* and is not included in the database utilized in this study, representing another novel MGE in environmental isolates of *C. difficile*. Given that it harbors the *ermB* gene, its presence in wastewater strains poses a significant risk for the dissemination of resistance to the macrolide-lincosamide-streptogramin B (MLS_B) antibiotic group in the environment.

Additionally, it was determined that this *ermB* MGE is present in other recently described bacterial species, such as *Blautia coccoides*, *Coprococcus phoceensis*, and *Faecalimonas umbilicata*, with a 99% identity match and query coverage, uncovered when using Nucleotide BLAST. Interestingly, all these species are part of the human intestinal microbiota, suggesting that genetic information exchange via HGT may occur not only in the external environment, but also within the human gut. Furthermore, this applies to resistant isolates obtained at both the inlet and outlet of the WWTP, insofar as both the influent and effluent (as the strain harboring the novel *ermB* MGE found in this study) isolates may acquire resistance either in the intestine of a previous host or later within the WWTP itself. Ultimately, the emergence of this MGE in an environmental isolate raises concern, as it further contributes to the dissemination of MLS_B-resistant strains.

Regarding the RT078/126 group, *in silico* AMR analysis revealed that six isolates (including two from wastewater) harbored the *tetM* gene, and all isolates carried the Tn916 transposon, both of which are associated with tetracycline resistance (Spigaglia et al., 2006). Tetracycline is not used in the

treatment of CDI, so it was not included in the AST performed in this study. Nevertheless, this antibiotic is widely used in Europe, further supporting the hypothesis that the acquisition of MGEs from other microorganisms in shared environments can lead to the emergence of resistant *C. difficile* isolates (Kartalidis et al., 2021). Moreover, the presence of Tn916 in all RT078/126 isolates, and its absence in isolates from the other three ribotypes studied, may suggest a greater tendency for strains of this PCR-ribotype profile to acquire this specific MGE, and thus develop tetracycline resistance. It is also worth noting that RT078/126 is one of the ribotypes with the greatest zoonotic potential and highly associated with CA-CDI. Dingle et al. (2019) suggests that tetracycline resistance was acquired by common ancestors of *C. difficile* RT078 strains, showing a high proportion (76.5%) of their analyzed RT078 genomes testing positive for the *tetM* gene. The study also highlights the close genetic relatedness between human and swine RT078/126 genomes, regardless of geographic origin, indicating a widespread, international dissemination of tetracycline-resistant strains. Although tetracycline use in human medicine has decreased due to emerging resistance, its extensive application in agriculture, particularly in livestock, likely created selective pressure, facilitating the spread of resistant RT078 strains through the food chain (Dingle et al., 2019). Thus, contributing to the emergence of this ribotype as a human pathogen. The study further suggests that indirect transmission of *C. difficile* strains from agricultural environments to humans could occur via contaminated water, supporting the findings in the present study and, once again, emphasizing that strains may have acquired AMR determinants either in the intestine of a host or in the environment.

Overall, *C. difficile* surveillance in urban wastewaters has proven to be highly relevant for enhancing the understanding of CA-CDI epidemiology and has uncovered new insights that could significantly impact research on this pathogen as a whole.

6. CONCLUSION

In conclusion, the analysis of *C. difficile* clinical isolates not only highlighted the change in CDI epidemiology described across the literature, with the increasing prevalence of ribotypes such as RT106, typically found in the environment, but also supports a new paradigm shift, with the re-emergence of the epidemic, multidrug-resistant, and hypervirulent RT027 in Portugal. This study also demonstrated that implementing *C. difficile* monitoring upon urban wastewater treatment, in association with the national CDI surveillance programme, could significantly contribute to our understanding of the epidemiology of CA-CDI. Some of the strains found in wastewater exhibited resistance to antimicrobials that may or may not be used in CDI treatment, with some genetic determinants being found in MGEs, thus, being easily transferred to other bacteria by HGT. This further expands our understanding of this pathogen, as well as its genetic repertoire, both in Portugal and across Europe. Overall, the results obtained in this study strongly support the need for a continuous surveillance of *C. difficile*, and to assess the risk of treated effluent discharge into the environment, it is strongly recommended that more studies be conducted in Portugal. Moreover, the present study further highlights that *C. difficile* should be considered a One Health Public Health issue and provides a foundation for more extensive future research. Indeed, considering the changing epidemiology of CDI, and the very preliminary results of this study regarding wastewaters, it becomes necessary to carry out a more in-depth investigation of environmental reservoirs of *C. difficile* in the One Health context.

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