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Molecular Characterization of Clinical *Clostridioides difficile* Isolates Circulating in the German Healthcare System

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Declaration

The work presented in this thesis was carried out in the period from October 2019 till July 2022 at the Institute of Medical Microbiology and Hygiene (IMMH) at Saarland University. The results section contains data from the corresponding peer-reviewed publications after having the permission from the contributing authors. Unless specified otherwise, all images depicted in this thesis are owned by the author. The publications and conferences section involves a complete list of the author's publications.

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Abbreviations

Abbreviation	Full name
4C Antibiotics	[Clindamycin, Cephalosporins, fluoroQuinolones and aminopenicillin/Clavulanate]
ABC	ATP binding cassette
ABS	Antibiotic Stewardship
a.u.	Arbitrary Unit
AFLP	Amplified Fragment-Length Polymorphism
AMR	Antimicrobial Resistance
Arg	Argyrin
AST	Antimicrobial Susceptibility Testing
AUC	Area Under the Curve
B. bifidum	*Bifidobacterium bifidum*
B. fragilis	*Bacteroides fragilis*
BHQ	Black-Hole Quencher
BMN-5	Cyanine 5 analogue
Bp	Base pair
BTS	Bacterial Test Standard
C. butyricum	*Clostridium butyricum*
C. perfringens	*Clostridium perfringens*
C. scindens	*Clostridium scindens*
C. sporogenes	*Clostridium sporogenes*
Cy5	Cyanin-5 dye
CA-CDI	Community Associated-CDI
CCV	Compact Colony Variant
CDAD	*C. difficile*-Associated Diseases
CDC	Centers of Disease Control and Prevention
CDI	*Clostridioides difficile* Infection
CDKI	Cyclin-Dependent Kinase Inhibitor
CDT	Binary toxin protein
CdtAB	Binary toxin protein subunits
cdtA, cdtB	Binary Toxin subunits A and B genes
CdtLoc	Cdt Locus

CFU	Colony Forming Unit
CGE	Capillary Gel Electrophoresis
ClosER	*Clostridium difficile* European Resistance
CLR	Clarithromycin
CLSI	Clinical And Laboratory Standards Institute
COVID-19	Coronavirus Disease 2019
CPE	Cytopathic Effect
d-Ala-d-Ala	D-Alanine-D-Alanine amino acids
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DSM	Deutsche Sammlung von Mikroorganismen German Collection Of Microorganisms
ECDIS-Net	European *Clostridium difficile* Infection Study-Network
ECF	Energy-Coupling Factor
EF-G	Elongation Factor G
EIA	Enzyme Immunoassay
ESCMID	European Society Of Clinical Microbiology And Infectious Diseases
E-test	Epsilometer
EUCAST	European Committee On Antimicrobial Susceptibility Testing
EUCLID	European, Multicenter, Prospective, Biannual, Point-Prevalence Study Of *C. difficile* Infection In Hospitalized Patients With Diarrhea
FAM	6-Carboxyfluorescein Dye
FDA	Food and Drug Administration
FN	False Negative
FP	False Positive
Fw	Forward Primer
GDH	Glutamate Dehydrogenase Enzyme
gluD	Glutamate Dehydrogenase Gene
h	Hours
HA-CDI	Hospital Acquired-CDI
HAIs	Hospital Acquired Infections
HCCA	α-Cyano-4-Hydroxycinnamic Acid

HIPS	Helmholtz Institute For Pharmaceutical Research Saarland
HIV	Human Immunodeficiency Virus
HVR RTs	Hypervirulent Ribotypes
HX	HEX Dye
IDSA	Infectious Diseases Society Of America
IL	Interleukins
ISR	intergenic spacer region
KNN	K Nearest Neighbors Algorithm
kDa	kilo Dalton
LC-MS	Liquid Chromatography Mass Spectrometry
LEV	low elution volume
LPA gel	linear polyacrylamide gel
LSR	Lipolysis Stimulated Lipoprotein Receptor
m/z	Mass To Charge number Ratio
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-Of-Flight
McF	McFarland
MCV	Motile Colony Variant
mDAP	Meso-Diaminopimelic Acid
MDR	Multi-Drug Resistance
mEF-G1	Mitochondrial Elongation Factor G1
MET	Metronidazole
MFX	Moxifloxacin
MGEs	Mobile Genetic Elements
MIC	Minimal Inhibitory Concentrations
MIC	Minimal Inhibitory Concentrations
min	Minutes
ML	Machine Learning
MLST	Multilocus Sequence Typing
MLVA	Multi-Locus Variable Number Tandem-Repeats Analysis
MS	Mass Spectrometry
MSP	Main Spectra Profiles
NAAT	Nucleic Acid Amplification Tests
NAG	N-Acetyl Glucosamine
NAM	N-Acetyl Muramic Acid

Non-HVR	Non-Hypervirulent
NPV	Negative Predictive Value
NRZ	National Reference Center
NTC RTs	Non-Toxigenic Ribotypes
P	Probe Labeled At The 5′-End With The Reporter Dye
P. aeruginosa	*Pseudomonas aeruginosa*
PaLoc	Pathogenicity Locus
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PFGE	Pulsed Field Gel Electrophoresis
PLS-DA algorithm	Partial Least Squares–Discriminant Analysis Algorithm
PMC	Pseudomembranous Colitis
PPV	Positive Predictive Value
PRC	Precision Recall Curve
REA	Restriction Endonuclease Analysis
RF	Rifampicin
RF algorithm	Random Forest Algorithm
ROC curve	Receiver Operating Characteristic Curve
RT	Ribotype
Rv	Reverse Primer
slpAST	Surface-Layer Protein A Sequence Typing
SLS	Sample Loading Solution
ST	Sequence Type
SVM algorithm	Support Vector Machine Algorithm
TB	Tuberculosis
TBE buffer	Tris-Borate-EDTA Buffer
TC	Toxigenic Culture
tpi	Triose Phosphate Isomerase Gene
TCCA	Tissue Culture Cytotoxicity Assays
tcdA	Toxin A Gene
TcdA	Toxin A Protein
tcdB	Toxin B Gene

TcdB	Toxin B Protein
TIC	Total Ion Current
TN	True Negative
TP	True Positive
TR	Texas Red
TSBA	Tryptic Soy Blood Agar
UK	United Kingdom
UKS	Saarland University Hospital
USA	United States Of America
VA	Vancomycin
VIR RTs	Virulent Ribotypes
WGS	Whole Genome Sequencing
WHO	World Health Organization

Abstract

Clostridioides difficile Infection (CDI) is classified in the USA as an urgent threat by the Centers for Disease Control and Prevention (CDC). CDI is the leading cause of nosocomial diarrhea and accounts for 4% of all hospital acquired infections (HAIs) with ~ 15% case fatality rates. The CDI prevalence is increasing and associated with the rise of the hypervirulent ribotypes (HVR RTs) like RT023, RT027, and RT078. HVR RTs are nowadays responsible for numerous outbreaks in the hospital setting. Usually driven by antibiotic treatment of a hospitalized patient for other reasons, CDI is often difficult to treat by antimicrobials commonly used in the hospital setting, as HVR RT isolates are frequently multidrug-resistant (MDR) (*i.e*. resistant to three and more antibiotic classes), leaving only a few antibiotics such as vancomycin or fidaxomicin currently as treatments of choice against CDI.

Thus, the evaluation of new drug candidates against *C. difficile* is essential, particularly, in times of emerging antimicrobial resistance (AMR). Ideally, these drug candidates display a high activity against *C. difficile* but are less effective against other members of the gut microbiota thought to suppress the growth of this pathogen in the colon. My studies show that the natural compound argyrin B and some other argyrin derivatives display a promising *in vitro* antimicrobial activity against a wide variety of epidemiologically important RTs. Notably, these argyrin derivatives were clearly less effective against some members of the healthy gut flora such as *Clostridium scindens*, *Bacteroides fragilis*, and *Bifidobacterium bifidum*, suggesting that the latter bacterial species might be spared by these drug candidates when used for treatment of CDI and thus may help also to prevent the recurrence, which is a common feature of this disease. First preliminary drug susceptibility tests were also carried out for new nucleoside analogues and *C. difficile*, which suggest that some of these compounds might be also effective against this bacterial species in the low µg/mL range. Further *in vitro* drug testing studies revealed a promising activity for the Energy coupling factor transporters inhibitors K4104497 and HHPS77 against *Clostridium perfringens* in the low µg/mL range, while growth of other *Clostridium* spp. and *C. difficile* was less affected.

Knowledge about the *C. difficile* strain composition circulating in Germany and their AMR profiles is important for epidemiological and therapeutic reasons. The implementation of a standardized German-wide surveillance for *C. difficile* in the German hospital setting was thus an important aim of this work and was successfully attained to detect the circulating RTs and their AMR profiles. This study allows for the first time to get an idea about the real situation in the clinical setting by avoiding the bias by the disease severity or the random acquisition of isolates. This study revealed that isolates of the HVR RT027 are less frequently seen nowadays in the German healthcare system than before, and basically the same holds truth for the in Europe epidemiologically relevant RT001, for which the incidence also declined in recent years. MDR was encountered on a low level, but was particularly evident for RT027, supporting earlier findings suggesting that this HVR RT is a major driver for MDR.

The identification of *C. difficile* HVR RTs by ribotyping or whole genome sequencing is costly and time consuming. In order to find a cost-effective and swift way to reliably discriminate between HVR RTs and non-HVR RTs, MALDI-TOF MS was tested here in combination with bioinformatics as third part of my thesis for its suitability to distinguish between both aforementioned groups. Here, my studies revealed that epidemiologically relevant HVR RTs circulating in Europe, such as RTs 023, 027, 045, 078, 126 and 176, could be indeed distinguished by this method from non-HVR-RTs with an accuracy >95% when MALDI-TOF mass spectra were compared to a TICp-based peak matrix in combination with the RF (random forest) or PLS-DA (partial least squares-discriminant analysis) prediction algorithms. A closer look at the HVR RTs revealed that some of the *C. difficile* HVR RTs such as RTs 023 and 027/176 could be even further subcategorized with an accuracy >94% from other HVR RTs circulating in Germany such as RTs 045, 078, 126 and 127. These findings suggest that MALDI-TOF MS is a fast and powerful tool to inform the clinician if a CDI is caused by a HVR *C. difficile* isolate that may require special attention.

Zusammenfassung

Die *Clostridioides-difficile*-Infektion (CDI) wird in den USA von den Centers for Disease Control and Prevention (CDC) als dringende Gefahr für das Gesundheitssystem eingestuft. CDI ist die Hauptursache für nosokomiale Durchfallerkrankungen und macht 4% aller im Krankenhaus erworbenen Infektionen aus, wobei die Sterblichkeitsrate bei etwa 15% liegt. Die Prävalenz von CDI nimmt aktuell zu und dies steht vermutlich im Zusammenhang mit dem Auftreten und der Verbreitung hypervirulenter Ribotypen (RTs) wie den RTs 023, 027 und 078. Hypervirulente *C. difficile* RTs (HVR-RTs) sind heutzutage für zahlreiche Ausbrüche im Krankenhausumfeld verantwortlich. Da HVR-RT-Isolate häufig multiresistent (MDR) sind (d. h. resistent gegen drei und mehr Antibiotikaklassen), bleiben nur wenige Antibiotika wie Vancomycin oder Fidaxomicin als Mittel der Wahl zur Behandlung von CDI übrig.

Daher ist die Evaluierung neuer Wirkstoffkandidaten gegen *C. difficile*, insbesondere in Zeiten, in denen immer häufiger antimikrobielle Resistenzen (AMR) beobachtet werden, von entscheidender Bedeutung. Im Idealfall zeigen diese Wirkstoffkandidaten eine hohe Aktivität gegen *C. difficile*, sind aber gegen andere Mitglieder der Darmmikrobiota weniger wirksam, von denen angenommen wird, dass sie das Wachstum dieses Erregers im Dickdarm unterdrücken. Meine Studien zeigen, dass die natürliche Verbindung Argyrin B und einige andere Argyrin-Derivate eine vielversprechende In-vitro-Aktivität gegen eine Vielzahl von epidemiologisch wichtigen *C. difficile* RTs aufweisen. Bemerkenswert ist zudem, dass diese Argyrinderivate gegen einige Mitglieder der gesunden Darmflora wie *Clostridium scindens, Bacteroides fragilis* und *Bifidobacterium bifidum* deutlich weniger wirksam waren, was darauf hindeutet, dass die letztgenannten Bakterienarten bei der Behandlung von CDI durch diese Arzneimittelkandidaten verschont werden und somit auch zur Verhinderung von Rückfällen, die ein häufiges Merkmal dieser Krankheit sind, beitragen könnten. Für die neuen Nukleosidanaloga wurden auch erste vorläufige Empfindlichkeitstests gegenüber *C. difficile* durchgeführt, die darauf hindeuten, dass einige dieser Verbindungen auch gegen diese Bakterienart im niedrigen µg/mL-Bereich wirksam sein könnten. Weitere In-vitro-Wirkstofftests ergaben eine vielversprechende Aktivität der Energy Coupling Factor Transporter-Inhibitoren K4104497 und HHPS77 gegen *Clostridium perfringens* im niedrigen µg/mL-Bereich, während das Wachstum anderer *Clostridium* spp. und *C. difficile* weniger beeinträchtigt wurde.

Die Kenntnis der in Deutschland zirkulierenden *C. difficile*-Stämme und ihrer AMR-Profile ist aus epidemiologischen und therapeutischen Gründen wichtig. Die Implementierung einer standardisierten, deutschlandweiten Surveillance für *C. difficile* im deutschen Krankenhausumfeld war daher ein wichtiges Ziel dieser Arbeit, um die zirkulierenden RTs und deren AMR-Profile zu erfassen. Diese Studie ermöglicht es zum ersten Mal, sich ein Bild von der realen *C. difficile* Situation im klinischen Umfeld zu machen, indem die Verzerrung durch die Krankheitsschwere oder die zufällige Akquisition von Isolaten vermieden wurde. Diese Studie ergab, dass Isolate des HVR RT027 heute im deutschen Gesundheitswesen seltener

vorkommen als früher, und dasselbe gilt für den in Deutschland epidemiologisch relevanten RT001, dessen Inzidenz in den letzten Jahren ebenfalls zurückging. MDR wurde auf niedrigem Niveau angetroffen, war aber bei RT027 besonders deutlich, was frühere Befunde stützt, die darauf hindeuten, dass dieses HVR-RT ein wichtiger Treiber für MDR ist.

Die Identifizierung der HVR-RTs durch Ribotypisierung oder Ganzgenomsequenzierung ist kostspielig und zeitaufwendig. Um eine kosteneffiziente und schnelle Methode zur zuverlässigen Unterscheidung zwischen HVR-RTs und Nicht-HVR-RTs zu finden, wurde als dritter Teil meiner Dissertation die MALDI-TOF MS in Kombination mit Bioinformatik auf ihre Eignung zur Unterscheidung zwischen den beiden vorgenannten Gruppen getestet. Dabei zeigte sich, dass epidemiologisch relevante, in Europa zirkulierende HVR-RTs, wie die RTs 023, 027, 045, 078, 126 und 176, mit dieser Methode tatsächlich mit einer Genauigkeit von >95% von Nicht-HVR-RTs unterschieden werden konnten, wenn MALDI-TOF-Massenspektren mit einer TICp-basierten Peak-Matrix in Kombination mit den Vorhersagealgorithmen RF (Random Forest) oder PLS-DA (Partial Least Squares-Discriminant Analysis) analysiert wurden. Eine genauere Betrachtung der HVR-RTs ergab, dass einige der HVR-RTs von *C. difficile*, wie die RTs 023 und 027/176, mit einer Genauigkeit von >94% noch weiter von anderen in Deutschland zirkulierenden HVR-RTs wie den RTs 045, 078, 126 und 127 unterteilt werden konnten. Diese Ergebnisse deuten darauf hin, dass die MALDI-TOF MS ein schnelles und leistungsfähiges Werkzeug sein könnte, um den Arzt darüber zu informieren, ob eine CDI durch ein HVR *C. difficile*-Isolat verursacht wird, das möglicherweise besondere Aufmerksamkeit erfordert.

1. Introduction

1.1. *Clostridioides difficile (C. difficile)*

1.1.1. Historical perspective of *C. difficile*

C. difficile is an anaerobic, spore-forming, motile, Gram-positive bacilli (**Figure 1-1**) representing the major cause of nosocomial diarrhea in developed countries. The clinical picture may range from self-limiting diarrhea to fatal life threatening diseases such as pseudomembranous colitis (PMC) and toxic megacolon (Barbut and Petit, 2001; Paredes-Sabja *et al.*, 2014; Lawson *et al.*, 2016). In 1935, Hall and O´Toole isolated *C. difficile* from the stool of a neonate and described it as *Bacillus difficilis*, being not linked to any known disease at that time (Hall and O'Toole, 1935). Forty years later, Bartlett and his colleagues delineated the first association between *C. difficile* and PMC and established the connection between *C. difficile* and antibiotic induced diarrhea (Bartlett *et al.*, 1978). In the same year, another study found that *C. difficile* could be isolated from 80% of all PMC patients tested (Larson *et al.*, 1978), suggesting that this bacterium is a major driver for this disease.

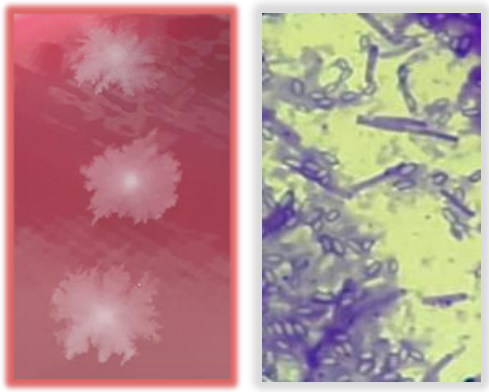

Figure 1-1: Left panel: Image of colonies formed by a clinical *C. difficile* strain on tryptic soy blood agar (TSBA) after 48 h of growth at 37°C (white centers indicate areas of sporulation). Right panel: Micrograph of a Gram-stained *C. difficile* cell population containing spores (transparent oval areas), vegetative cells (completely violet colored) and spore-forming cells (violet-colored rods displaying a transparent oval area).

The facultative pathogenic species *C. difficile* was for a long time also regarded as a member of the genus *Clostridium* and formally known as *Clostridium difficile*. Like other Gram-positive bacteria, the cell wall of *C. difficile* is made up of a thick peptidoglycan layer consisting of alternating monomers of N-acetyl glucosamine (NAG) together with N-acetyl muramic acid (NAM), which form a string-like meshwork that is cross-linked via pentapeptide chains composed of the amino acids; L-alanine, D-glutamate, meso-diaminopimeilic acid/L-lysine and D-alanine-D-alanine motif that are attached to the NAM (Vollmer *et al.*, 2008).

However, some features of the peptidoglycan sacculus formed by *C. difficile* are quite characteristic for this species:

- Around 90% of the NAG is N- deacetylated by peptidoglycan deacetylase (Ho *et al.*, 2014).
- The 3rd amino acid is a meso-diaminopimelic acid (mDAP), the epsilon-carboxy derivative of lysine (Turner *et al.*, 2014).
- The inter-chain cross linking occurs primarily between mDAP residues of adjacent chains and only to a smaller part between the 4th and the 3rd amino acids (Turner *et al.*, 2014).

The presence of mDAP in the cell wall peptidoglycan of *C. difficile* led to a reclassification of this species in 2016, which belongs now phylogenetically to the family *Peptostreptococcaceae* and the new formed genus *Clostridioides* that includes beneath *C. difficile* another species called *Clostridioides mangenotii* (Lawson *et al.*, 2016; Oren and Rupnik, 2018).

1.1.2. Pathogenesis of *C. difficile* infection (CDI)

C. difficile has been nowadays recognized worldwide as the main causative agent of infectious nosocomial diarrhea. Probably the main reason for this is antibiotic treatment of hospitalized patients to combat an underlying infection, which paves the way for *C. difficile*-associated diseases (CDAD) by damaging the gut flora of the patient (Rupnik *et al.*, 2009; Greathouse *et al.*, 2015). *C. difficile* is ubiquitous in nature and occupying a mixture of environmental niches as soil and sewage (Nikaeen *et al.*, 2015), and can be also found in the gut of a wide variety of mammals including many farm animals (Hensgens *et al.*, 2012). In humans, *C. difficile* asymptomatically colonizes between 0-15% of the healthy adult population, and the prevalence rate may reach up to 50% in neonates without any clinical relevance (Furuya-Kanamori *et al.*, 2015).

Horizontal transmission (*e.g.* in the hospital environment) is facilitated by the spores, which can survive in an aerobic milieu (Barra-Carrasco and Paredes-Sabja, 2014). To be infectious for humans, *C. difficile* needs to produce specific toxins (Lyras *et al.*, 2009), which are not formed by all *C. difficile* isolates. In fact, carriage of a non-toxigenic *C. difficile* isolate in the indigenous gut flora may serve as a colonization barrier against toxigenic *C. difficile* isolates and thus protect the carrier from CDI under antibiotic free conditions (Vincent *et al.*, 2016; Schäffler and Breitrück, 2018).

Although, the toxigenic *C. difficile* carriers usually do not develop a CDI under non-antibiotic treatment conditions, they may serve as reservoirs for these pathogenic strain types in the community (Eyre *et al.*, 2013). These asymptomatic toxigenic *C. difficile* carriers are at a higher risk than others for CDI development (Zacharioudakis *et al.*, 2015). However, colonization with toxigenic *C. difficile* variants may

also elicit a protective effect for the carrier by triggering the production of neutralizing antibodies directed against the toxins, which in turn may protect the host against CDI (Kyne *et al.*, 2000; Kyne *et al.*, 2001).

Toxigenic *C. difficile* strains may secrete two or more exotoxins that can be divided into different toxin families, and are considered to be the major virulence determinants of this bacterium. The key virulence traits of the pathogenic *C. difficile* strains are the large clostridial cytotoxins termed toxin A and toxin B, respectively. They are single protein chain toxins comprising three subdomains responsible for receptor-binding, translocation, and a catalytic domain. The latter subdomain renders the Rho and Ras-GTPases functionally inactive by glucosylation of a conserved threonine residue in the target GTPase, leading to actin cytoskeleton disruption and finally cell death (Orrell and Melnyk, 2021; Rupnik, 2008; Just and Gerhard, 2004).

In general, two distinct toxin genes are present (*tcdA* and *tcdB*), which encode toxin A and B (Hammond and Johnson, 1995). These two genes comprise with three further open reading frames (ORFs) (*tcdR, tcdC* and *tcdE*, respectively) a 19.6 kb toxigenic genetic element designated as the pathogenicity locus (PaLoc) (Hammond and Johnson, 1995). The *tcdR* gene however, encodes RNA polymerase sigma factor that induces the expression of toxin A and B (Mani and Dupuy, 2001). The *tcdE* gene encodes a holin-like protein that might be required for exotoxin release (Govind and Dupuy, 2012), while *tcdC* encodes for a negative regulatory protein that counteracts the action of TcdR (Matamouros *et al.*, 2007).

Whole genome sequencing (WGS) analyses of multiple clinical *C. difficile* isolates identified in rare occasions unusual variants of *C. difficile* with defective PaLoc, where only one toxin gene is functional. Isolates of the ribotype (RT) 017 (RT017) were repeatedly found to harbor a truncated *tcdA* and to secrete only toxin B (Collins *et al.*, 2013; Du *et al.*, 2014). Other clinical isolates have been described producing only toxin A (A^+B^- variants) (Monot *et al.*, 2015). Variation can be also found for the amino acid compositions of toxins A and B, respectively, with TcdB displaying a higher diversity than TcdA (14% vs. 2%) (Li *et al.*, 2020) that is primarily found in a specifically variable part of the TcdB polypeptide (Hernandez *et al.*, 2015; Roth *et al.*, 2022).

Some toxigenic *C. difficile* strains might express a third toxin termed binary toxin (CDT). CDT is encoded chromosomally by the Cdt locus (CdtLoc), which is a distinct region apart from the PaLoc. The CdtLoc contains the *cdtA* and *cdtB* toxin genes as well as the *cdtR* regulatory gene (Carter *et al.*, 2007). CDT is an ADP-ribosylating toxin formed by two subunits: the binding subunit CDTb, which translocates the enzymatic subunit CDTa into the host cytosol after binding to the lipolysis stimulated lipoprotein receptor (LSR) (Gerding *et al.*, 2013).

CDTa is capable of capping the monomeric G-actin by its ADP-ribose. This capping induces a depolymerization of F-actin and prevents further polymerization, leading to the destruction of the cellular cytoskeleton and the formation of microtubular protrusions that increase the bacterial adherence to the epithelial cells (Gerding *et al.*, 2013). CDT is commonly secreted by so-called "hypervirulent" isolates that may predispose to more severe courses of the disease (Merrigan *et al.*, 2010).

1.1.3. Microbiological diagnostics of *C. difficile*

C. difficile diagnosis may be tricky, as evidenced by the European, multicenter, prospective, biannual, point-prevalence study of *C. difficile* infection in hospitalized patients with diarrhea (EUCLID) conducted between 2012 and 2013 (Davies *et al.*, 2016). Owing to the defective testing approaches (*e.g.* single toxin testing), this study revealed that in Europe alone, ~ 60.000 CDI cases per year passed unnoticed (Davies *et al.*, 2014). The clinical symptoms together with the laboratory detection of toxigenic *C. difficile* isolates are essential for the CDI diagnosis (Kelly *et al.*, 2020).

A wide array of tests can be simply implemented in the routine diagnostics such as enzyme immunoassay (EIA) testing for glutamate dehydrogenase (GDH) of *C. difficile*, the toxin detection on the protein level via EIA or on the gene level through the nucleic acid amplification tests (NAAT), toxigenic culture (TC) and tissue culture cytotoxicity assays (TCCA) (Planche and Wilcox, 2011; Crobach *et al.*, 2016; Kelly *et al.*, 2020). TC and TCCA are considered the two gold standards for CDI diagnosis (Planche and Wilcox, 2011). TC comprises, firstly, culture of the patient stool sample and only if a *C. difficile* strain could be retrieved then toxin detection is mandatory (Thonnard *et al.*, 1996). TCCA applies the patient stool filtrate (*i.e.* supposed to contain the *C. difficile* toxin] to the tissue culture cells for the detection of the toxin cytopathic effect (CPE), which is then neutralized by the specific antibodies directed against the toxin (Delmée *et al.*, 2005).

Still both approaches are cumbersome, as they are time and resource consuming, thus, unfortunately, hamper their simple implementation in routine microbiological diagnostics (Planche and Wilcox, 2011; Berger *et al.*, 2020b). Since 2016, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommended to use a highly sensitive test for *C. difficile* detection, such as GDH detection followed by a toxin testing to identify toxigenic strains (Crobach *et al.*, 2016). To optimize the CDI testing, ESCMID encouraged a two-step algorithm, where NAAT testing is recommended if free toxin testing was found to be negative (Crobach *et al.*, 2016).

1.1.4. Molecular *C. difficile* typing methods

C. difficile strains circulating in the worldwide human population display a considerable genetic heterogeneity and can be distinguished by a number of molecular methods (**Table 1-1**) (van Dorp *et al.*, 2016).

Table 1-1: Molecular techniques implemented for *C. difficile* typing.

Typing technique	References
Restriction endonuclease analysis (REA)	(Sambol *et al.*, 2016)
DNA microarray	(Gawlik *et al.*, 2015)
Pulsed field gel electrophoresis (PFGE)	(Loo *et al.*, 2005)
Capillary gel electrophoresis (CGE) based PCR-Ribotyping	(Indra *et al.*, 2008; ECDIS-Net, 2012)
Multilocus sequence typing (MLST)	(Griffiths *et al.*, 2010)
Whole genome sequencing (WGS)	(Bletz *et al.*, 2018)
Real time PCRs against specific mutations of RT027 and RT078, respectively	(Wolff *et al.*, 2009; Persson *et al.*, 2011)
Surface-layer protein A sequence typing (slpAST)	(Kato *et al.*, 2005)
Matrix-Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF) mass spectrometry	(Reil *et al.*, 2011; Emele *et al.*, 2019)

For the determination of the *C. difficile* strain composition in outbreak and/or surveillance studies, genotyping methods such as capillary ribotyping (ribotyping), multilocus sequence typing (MLST), surface-layer protein A sequence typing (slpAST), and WGS are most commonly used within Europe, which all can detect a broader spectrum of genotypes (Berger *et al.*, 2020b). In ribotyping, non-coding inter spacer regions of ribosomal 16S–23S RNA are investigated, and resulting banding patterns are compared to in-house or public data bases such as webribo (Indra *et al.*, 2008; ECDIS-Net, 2012).

In MLST, nucleotide sequences of several housekeeping gene fragments are determined and compared with searchable Internet-accessible MLST databases such as at http://pubmlst.org/cdifficile/ (Griffiths *et al.*, 2010). In slpAST, the variable region of the *slpA* gene is determined by sequencing and compared to the database entries deposited in the public databases such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Kato *et al.*, 2005; Müller *et al.*, 2015). In WGS, the whole genome information of a strain is identified, and genetic information derived by this method can be used with all other aforementioned applications (Berger *et al.*, 2020a; Cho *et al.*, 2021) .

1.2. Antibiotics and CDAD/CDI

CDAD, including hospital acquired CDI (HA-CDI) and community associated CDI (CA-CDI), (Kuntz *et al.*, 2011; Chitnis *et al.*, 2013; Khanna and Gupta, 2014) typically follows the antibiotic treatment, particularly the **"4C"** provoking antibiotics [**C**lindamycin, **C**ephalosporins, fluoro**Q**uinolones and aminopenicillin/**C**lavulanate] (Lawes *et al.*, 2017; Davies *et al.*, 2020). Other major risk factors for CDI development include medication with proton pump inhibitors, an immunosuppression, a prolonged hospital stay, and a higher age of the patient (Eze *et al.*, 2017). HA-CDI is the principal cause of nosocomial contagious diarrhea (Rupnik *et al.*, 2009) accounting for 4% of all hospital acquired infections (HAIs) in the United States of America (USA) (Magill *et al.*, 2014; Haque *et al.*, 2018). CDI mortality rates range between 6% to 30% (Kelly and LaMont, 2008; Kuijper *et al.*, 2006). HA-CDI exerts an immense burden not only in terms of costs but also on the host morbidity and mortality (Choi *et al.*, 2019).

Increasing CDI prevalence is associated with the rise of hypervirulent lineages such as RT027 (Loo *et al.*, 2005). RT027 isolates are responsible for numerous outbreaks in the USA and the United Kingdom (UK), and have been associated with high case fatality rates (Loo *et al.*, 2005). CDI might be clinically represented by a limited course of watery diarrhea or the fatal complications are the first clinical representations (*e.g.* toxic megacolon, PMC, perforation of the intestinal wall and sepsis) (Leppkes *et al.*, 2015; Larson *et al.*, 1978; McDonald *et al.*, 2018; Bartlett *et al.*, 1978).

For the initial therapy of CDI, fidaxomicin or vancomycin are the treatment of choice according to the Infectious Diseases Society of America (IDSA) guidelines (McDonald *et al.*, 2018; Johnson *et al.*, 2021). Food and Drug Administration (FDA) approved the local application (oral or rectal) of vancomycin for its tolerability, high curing rates, the high colonic concentrations (1000 µg/mL) relative to the MIC (2µg/mL) required to suppress the growth of the vancomycin susceptible *C. difficile* isolates, and the comparably smaller side-effects on the gut microbiota (Carlson and Gonzales-Luna, 2020). In 2011, FDA approved fidaxomicin, another new antibiotic for the CDI treatment, which has an even more selective spectrum of activity than vancomycin (Louie *et al.*, 2011; Cornely *et al.*, 2012), and allows for lower rates of recurrence (Carlson and Gonzales-Luna, 2020; Okumura *et al.*, 2020).

Although the risk factors for CDI development and recurrence are similar, clinicians are more concerned with the recurrence (McDonald *et al.*, 2018; Garey *et al.*, 2008; D'Agostino *et al.*, 2014). Relapsing episodes are yet more challenging than the initial disease, either due to the dysbiosis persistence, lack of the protective immune response, dormant spores, and/or due to biofilm formation (Meehan *et al.*, 2016). Besides vancomycin, fidaxomicin and rifaximin are regularly used to combat recurrent episodes of CDI (Johnson *et al.*, 2021).

For recurrence prophylaxis, an anti-toxin B antibody (Bezlotoxumab) has been introduced recently and the recurrence rate decreased to ~ 20% (Mikamo *et al.*, 2018). Moreover, ESCMID recommended a bezlotoxumab therapy for the initial CDI episode in patients with a higher risk of recurrence (van Prehn *et al.*, 2021).

Multi-drug resistance (MDR) -being resistant towards three or more different classes of antibiotics- might develop as a consequence of chromosomal mutations or mobile genetic elements (MGEs) transfer to the *C. difficile* genome or both, further reducing the therapeutic options for handling the underlying infection (Peng *et al.*, 2017). In Spain, MDR isolates towards ertapenem, erythromycin, moxifloxacin and clindamycin were detected (Peláez *et al.*, 2013). In Poland, resistant strains to ertapenem, erythromycin, moxifloxacin and rifamycin were also reported (Lachowicz *et al.*, 2015). Of note, RT027 isolates are a major contributor to MDR compared to non-RT027 isolates (Tenover *et al.*, 2012; Wieczorkiewicz *et al.*, 2016). MDR in *C. difficile* necessitates the discovery of new antimicrobials against this bacterium. Promising novel candidates for CDI therapy are in the pipeline such as omadacycline (Zhanel *et al.*, 2020; Begum *et al.*, 2020) and eravacycline (both targeting the 30S small ribosomal subunit) (Bassères *et al.*, 2020; Zhao *et al.*, 2017; Buckley *et al.*, 2021). However, bringing new drugs to the market is an ongoing task for drug development to combat isolates that may have developed resistance against all antimicrobials currently in use.

Unfortunately, resistance testing of anaerobic, spore-forming bacteria is challenging and requires specific equipment such as an anaerobic chamber. The Clinical and Laboratory Standards Institute (CLSI) recommends agar and broth dilution based antimicrobial susceptibility testing (AST) as the reference control tools in AST of *C. difficile*, to which other tests should be correlated (CLSI, 2012). However, both techniques are laborious and not appropriate for routine testing, while antibiotic disk diffusion tests on agar plates and concentration gradient testing strips (E-tests) are more easily implemented, and thus used more frequently for AST of *C. difficile* in microbiological diagnostics (Balouiri *et al.*, 2016; Berger *et al.*, 2020b). For AST of *C. difficile*, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has determined the epidemiological cut off values for metronidazole, vancomycin and moxifloxacin to be 2, 2, and 4 µg/mL, respectively (EUCAST, 2021).

1.2.1. Metronidazole

Metronidazole is a nitroimidazole antibiotic which is effective against strictly anaerobic bacterial species and protozoa, and was for a long time considered as one of the first line choices to treat CDI. However, due to the low colonic concentration and increasing rates of reduced susceptibility (Baines *et al.*, 2008), IDSA does not recommend its use for non-severe CDI cases, unless other options are unavailable (Baines *et al.*, 2008; McDonald *et al.*, 2018; Okumura *et al.*, 2020).

Metronidazole attacks the DNA double helix and introduces strand breaks when present in a reduced state, which is one of the reasons why this antibiotic is particularly effective against anaerobic bacteria because this reduction usually happens only in absence of oxygen (Chong *et al.*, 2014; Müller, 1983).

Resistance of *C. difficile* to this antibiotic was reported and might be explained in part by the horizontal transfer of a 7-kb plasmid called pCD-METRO, which is carried only by resistant isolates, regardless of their toxigenic profiles (Boekhoud *et al.*, 2020). However, the molecular mechanism leading to the non-susceptibility phenotype found in pCD-METRO carrying *C. difficile* isolates has not been elucidated yet. Also, a putative multi-drug efflux pump (CD2068) was reported (Ngernsombat *et al.*, 2017).

1.2.2. Vancomycin

Vancomycin, an antibiotic member of the glycopeptide family, is active against numerous Gram-positive pathogens (Choo and Chambers, 2016; Sohn *et al.*, 2021). This reserve antibiotic is a substrate binder that targets the d-Ala-d-Ala motif of the peptidoglycan precursor (UDP-NAM pentapeptide), thereby preventing the cross linking between cell wall murein strands (Reynolds, 1989). It is nowadays one of the first line antimicrobials for the treatment of severe CDI cases (McDonald *et al.*, 2018; Johnson *et al.*, 2021).

However, *C. difficile* isolates that are not susceptible to this antibiotic are increasingly reported (Peng *et al.*, 2017; Eubank *et al.*, 2022). Reduced susceptibility towards vancomycin may be either due to mutations in genes involved in peptidoglycan biosynthesis such as *murG/cd2725* (Leeds *et al.*, 2014) or by acquiring resistance genes such as the *van* genes cluster (Saldanha *et al.*, 2020) via horizontal transfer of MGEs (O'Grady *et al.*, 2021), the acquisition of plasmid such as pX18-498 (Pu *et al.*, 2021), or transposon (*Tn*1549-like element) (Knight *et al.*, 2016a). Constitutive expression of the $vanG_{Cd}$ operon alters the target to d-Ala-d-Ser motif (Ramírez-Vargas *et al.*, 2017; Shen *et al.*, 2020). Nevertheless, harboring a functional *van* cluster does not always render the carrier resistant towards vancomycin, as in some isolates, *van* is phenotypically silent and these isolates remain sensitive to this antibiotic (Ammam *et al.*, 2013; Saldanha *et al.*, 2020; Depardieu *et al.*, 2015). Notably, a so-called Eagle effect (*i.e.* more drug→ kills less) was also described for this antibiotic at concentrations above and beyond the bactericidal level (Jarrad *et al.*, 2018; Dureja *et al.*, 2022).

1.2.3. Fidaxomicin and rifaximin

Fidaxomicin is a narrow spectrum macrocyclic antibiotic of the tiacumicin macrolide group (Gallagher *et al.*, 2015; Hattori *et al.*, 2018). Rifaximin is a semisynthetic derivative of the broad-spectrum antibiotic rifamycin, which accumulates almost exclusively within the lumen of the gut after oral application (O'Connor *et al.*, 2008). Both antibiotics target the bacterial RNA polymerase and display a good activity against *C. difficile*. While fidaxomicin is suggested as stand-alone first line therapy against CDI, usage of

rifaximin is only recommended in combination with vancomycin for the management of recurring CDI (Mattila *et al.*, 2013). *C. difficile* may become resistant to both antibiotics due to mutations in *rpoB*, encoding the RNA polymerase β-subunit (O'Connor *et al.*, 2008; Leeds *et al.*, 2014).

1.2.4. Tigecycline

Tigecycline is an antimicrobial belonging to the glycylcycline family that binds to the 30S ribosomal subunit. Utilization of this reserve antibiotic is suggested for the control of complicated intra-abdominal cases of severe CDI or MDR isolates (Lu *et al.*, 2010; Di Bella *et al.*, 2015; Gergely Szabo *et al.*, 2016; Freeman *et al.*, 2018; Petrosillo *et al.*, 2018). The expression of the ribosomal protection genes as *tetX*, *tetM* and *tetW* might increase the tigecycline non-susceptibility of *C. difficile* and is achieved by acquisition of MGEs that harbor these genes (Linkevicius *et al.*, 2016; Dingle *et al.*, 2019; He *et al.*, 2019; Sholeh *et al.*, 2020).

1.3. Global Epidemiology

1.3.1. *C. difficile* strains involved in human CDI and their antibiotic resistance profiles

As already mentioned above, isolates of the species *C. difficile* may display a large genetic heterogeneity, which can be observed on the global and regional level, although detailed information for some regions of the world is still missing. However, numerous studies demonstrated that the *C. difficile* strain composition of isolates being capable of causing CDI may differ substantially between regions/countries/continents (Stabler *et al.*, 2012; Davies *et al.*, 2016; Freeman *et al.*, 2020; Martínez-Meléndez *et al.*, 2021). Similarly, larger differences in antimicrobial resistance (AMR) profiles of *C. difficile* have been observed between geographic areas (Freeman *et al.*, 2018; Freeman *et al.*, 2020; Imwattana *et al.*, 2020; Berger *et al.*, 2020a).

1.3.1.1. Europe

For Europe, numerous studies have been conducted to explore the circulating RTs and to determine their AMR profiles. One Europe-wide approach was the EUCLID study, which was conducted between 2012/2013 in 20 European countries and identified among others a high prevalence of RT027 in Germany, the Czech Republic, Romania, and Poland (Davies *et al.*, 2016). The first introduction of this RT in Germany was most probably in 2005 (Steglich *et al.*, 2015), and isolates of this RT caused first outbreaks in South Germany in 2007 (Kleinkauf *et al.*, 2007). Since then, RT027 disseminated throughout Germany (Marujo and Arvand, 2020), and the local prevalence *e.g.* Hesse in Central Germany can exceed 30% (Arvand and Bettge-Weller, 2016). The EUCLID study demonstrated furthermore that the RT diversity across Europe is regional specific (Davies *et al.*, 2016).

In Poland and the Czech Republic, isolates of RT176, which are phylogenetically related to RT027, initiate severe CDI and display higher AMR rates as well (Krutova *et al.*, 2015). In Italy, RT018 account for more than 40% of all investigated isolates (Spigaglia *et al.*, 2010; Baldan *et al.*, 2015). This epidemic RT however, led to outbreaks not only in Italy but also in France (Gateau *et al.*, 2019) and Germany (Berger *et al.*, 2019). Interestingly, RT018 is also highly prevalent in Japan (Senoh *et al.*, 2015), suggesting a transfer between Asia and Europe. More recently, isolates of RT017, the most prevalent RT in East Asia, were detected in Portugal (Isidro *et al.*, 2018). Furthermore, RTs 001/014/020 are also important across Europe (Davies *et al.*, 2016).

In the ClosER study (*Clostridium difficile* European Resistance), which focused on AMR, high antibiotic resistance rates were observed in isolates belonging to RT001, RT018, and RT027/RT176. However, in the ClosER study, the sample recruitment mode was random and not consistent. Thus, this study cannot be considered suitable to gain deeper insights into the (representative) strain composition in the regions of origin analyzed (Freeman *et al.*, 2018; Freeman *et al.*, 2020) but gives a good overview on the AMR of *C. difficile* at the time point when the isolates were sampled. This and other investigations revealed that the proportion of MDR *C. difficile* isolates in Europe is alarming. Higher rates of resistance have been observed for instance towards carbapenems (Isidro *et al.*, 2018), and rifampicin, particularly within RT027 isolates (Färber *et al.*, 2017; Freeman *et al.*, 2018).

Fortunately, resistance to vancomycin – the first line treatment option for CDI - is yet sparse (Freeman *et al.*, 2018). A continuous, standardized surveillance system for *C. difficile* in Germany that includes AMR data is still lacking. However, the prompt discovery of RTs with major epidemiological significance like RT023 (Shaw *et al.*, 2020) and RT017 (Imwattana *et al.*, 2019), is of importance to take counter measures. Furthermore, early information on the AMR profiles of new emerging RTs is needed to allow for an evidence based empirical therapy of CDI.

1.3.1.2. Australia and Oceania

Studies conducted in New Zealand and Australia suggest that RTs 002, 014, and 020 are the dominating RTs in both countries (Roberts *et al.*, 2011; Johnston *et al.*, 2021; Huber *et al.*, 2014; Foster *et al.*, 2014). However, when looking for hypervirulent strains, RT023 seems to be an emerging RT in New Zealand (Shaw *et al.*, 2020) while RT126 is the dominant hypervirulent RT found in Australia (Hong *et al.*, 2020). In a recent study, resistance to vancomycin and moxifloxacin was reported in 5.5% of isolates sampled in Australia (Putsathit *et al.*, 2021), however, MDR isolates are still encountered with a low frequency of 1.7% in this region (Putsathit *et al.*, 2021).

1.3.1.3. North and South America

In 2002, the first RT027 driven outbreaks and severe CDI cases were observed in Quebec (Canada) (Pepin, 2004). Since then, isolates of this RT spread rapidly to other regions of America such as the USA and Mexico (Tenover *et al.*, 2011; Camacho-Ortiz *et al.*, 2015), where they exerted a higher burden on the healthcare system than other strains (Valiente *et al.*, 2014). Intriguingly, the RT027 prevalence seems to decline in the USA since recently, being replaced by other RTs such as RT106 (Tickler *et al.*, 2019; Roxas *et al.*, 2020). One reason for this might be that epidemic strains (*e.g.* RT027 and RT001) might be pushed back when antibiotic stewardship (ABS) measures are applied (Lawes *et al.*, 2017). In an antibiotic-rich environment, RT027 has a selective advantage, as many RT027 isolates were found to be resistant towards fluoroquinolones (*e.g.* moxifloxacin), macrolides (*e.g.* clarithromycin), and/or rifampicin (Tenover *et al.*, 2012). In the USA, about 27.5% of the analyzed RT027 isolates displayed a MDR phenotype, while it was rather infrequent in other RTs (Tenover *et al.*, 2012). In Mexico, some of the RT027 isolates exhibited even reduced susceptibility or resistance to vancomycin (~ 5%) and metronidazole (25%), respectively (Tamez-Torres *et al.*, 2017; Martínez-Meléndez *et al.*, 2021).

In Latin America, the RT profile is mosaic and contains some of the RTs found also in other regions of the world such as RT002, RT009, RT014, RT020, RT027 and RT106 together with a number of unique RTs such as RT133 and RT233, respectively (Balassiano *et al.*, 2011; Silva *et al.*, 2015). Isolates of RT591, RT002 and RT106 are dominating in Colombia (Salazar *et al.*, 2018), while RT027 was the main RT found in Chile in 2012-2015 (Hernández-Rocha *et al.*, 2012; Aguayo *et al.*, 2015), presumably emerging from Costa Rica (Wong-McClure *et al.*, 2013). Within the latter country, RT027 and RT012 were the most prominent RTs found (Ramírez-Vargas *et al.*, 2017). In Buenos Aires (Argentina), sequence type (ST) ST1 (corresponding to RT027) was also on the top of the list (Cejas *et al.*, 2018), however in Brazil, RT106 seems to be of epidemiological importance (Balassiano *et al.*, 2009; Diniz *et al.*, 2019), while RT027 appears to be of minor importance in this region (Pires *et al.*, 2018).

1.3.1.4. Africa

Most of the data for Africa were obtained from studies conducted in South Africa, emphasizing the high prevalence of RT017 along with a tremendously high resistance rate for rifampicin (99%), a selective advantage for *C. difficile* in tuberculosis (TB) patients (as rifampicin is used as anti-TB therapy) (Kullin *et al.*, 2017; Legenza *et al.*, 2018). The CDI risk increases 5 folds in TB patients (Jager *et al.*, 2021). Data obtained from this country suggest furthermore that CDI might impose a threat to children or adults with an impaired immune system *e.g.* HIV or cancer patients (Seugendo *et al.*, 2015; Plants-Paris *et al.*, 2019). Non-toxigenic strains seem to dominate the *C. difficile* strain composition in Zimbabwe (Berger *et al.*, 2020a), Ghana (Janssen *et al.*, 2016), Côte d'Ivoire (Becker *et al.*, 2015) and Algeria (Djebbar *et al.*, 2018).

In Kenya, >80% of the isolates were MDR isolates resistant to erythromycin, ciprofloxacin and rifampicin (Mutai *et al.*, 2021). Notably, in this study, only toxinotyping was carried out and >50% of the strains were found to be toxin A negative, which might be indicative for RT017 (Mutai *et al.*, 2021).

1.3.1.5. Asia

For the largest continent, a number of studies is available from different countries, especially for East and South Asia. Although RT027 was detected in Taiwan (Liao *et al.*, 2015) and China (Zhou *et al.*, 2019; Wang *et al.*, 2014), RT017 seems to be the main cause for CDI in this geographical area (Collins *et al.*, 2020; Wongkuna *et al.*, 2021; Hung *et al.*, 2016). This RT is associated with fluoroquinolone resistance (Imwattana *et al.*, 2020). As with other continents, regional variations in the genetic makeup are present. In Hong Kong, RT002 is prominent followed by RT017 (Chow *et al.*, 2017). With respect to the AMR profiles, isolates displaying MDR to clarithromycin, moxifloxacin and rifampicin along with a surprisingly higher rate of resistance to metronidazole (>20% of the isolates) were observed (Lin *et al.*, 2011). In China, a wide RT diversity is evident, however RT017 is the dominant and epidemiologically relevant genotype (Yan *et al.*, 2013; Luo *et al.*, 2018).

In Japan, isolates of RT018 are dominating (around 30%) followed by RT014 (Kato *et al.*, 2019). Interestingly, in South Korea, isolates of RT017 and RT018 are both epidemiologically important (Kim *et al.*, 2010; Kim *et al.*, 2013) with higher MIC values and MDR noted in the isolates of the former ribotypes (Byun *et al.*, 2019). In India, the strain composition was dominated by "hypervirulent" RTs 045 and 126, which were accompanied by other unclassified RTs (Hussain *et al.*, 2016). Still, only few data are available for many countries in Asia including Western Asia (*i.e.* the Middle East). RT027 was identified as a major RT in one study conducted in Saudi Arabia (Alzahrani and Johani, 2013), while RT001 and RT126 play a prominent role in Kuwait (Jamal and Rotimi, 2016) and Tehran (Iran) (Azimirad *et al.*, 2020).

1.3.2. Zoonotic potential of *Clostridioides difficile*

C. difficile may colonize the gut of different animal reservoirs (**Table 1-2**), which are thought to serve as a continuous source of CA-CDI spread (Lee *et al.*, 2018). In Southwestern Europe (Iberia), RT078 is frequently found in pigs (Álvarez-Pérez *et al.*, 2013) and the same RT was recently reported in China (Zhang *et al.*, 2019). Interestingly, the intercontinental spread of RT078 was demonstrated through multi-locus variable number tandem-repeats analysis (MLVA) genotyping. MLVA demonstrated that the *C. difficile* strains colonizing the farm piglets in Czech were clonally related. Notably, these strains also clustered with isolates sampled from other farms in Germany, Japan and Taiwan (Krutova *et al.*, 2018), indicating that the transfer of reservoirs such as animal livestock and food might have a role in the spread between countries (Knight and Riley, 2019).

Besides RT078, other RTs being frequently encountered in human disease such as RT014, RT027 and RT045, can be also detected (**Table 1-2)**.

Table 1-2: Examples of potential zoonotic sources for *C. difficile* infection.

Animal	Ribotype	References
Chicken	RT027	(Bingol *et al.*, 2020)
Calves	RT012/078/126	(Magistrali *et al.*, 2015)
Horses	RT001/078	(Schoster *et al.*, 2012)
Dogs	RT009/010/014	(Wetterwik *et al.*, 2013)
Cats	RT014/020/045	(Schneeberg *et al.*, 2012)
Rodents/insectivores	RT005/010/014/015/078/087	(Krijger *et al.*, 2019)

1.4. MALDI-TOF MS based subtyping of *C. difficile* ribotypes

Over the last decade, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has proved to be a unique and easy-to-use standard tool for the identification of bacteria in microbiological diagnostics (Biswas and Rolain, 2013; Candela *et al.*, 2022), which might be also used for subtyping purposes (among others for the identification of certain *C. difficile* RTs) (Rödel *et al.*, 2019; Calderaro *et al.*, 2021). In recent studies, several important RTs could be distinguished by MALDI-TOF, in particular the often with HA-CDI associated strains RT001 (Reil *et al.*, 2011; Carneiro *et al.*, 2021), RT017 (Li *et al.*, 2018), and the hypervirulent (HVR) RT027/RT176 (Reil *et al.*, 2011; Carneiro *et al.*, 2021; Emele *et al.*, 2019) and RT078/126 (Reil *et al.*, 2011). However, on a global scale, a broader variety of other HVR RTs exists besides RT027, *e.g.* RT023, which is an emerging HVR RT in Europe (Shaw *et al.*, 2020). Isolates of RT023 are nowadays identified in Europe with prevalence rates of 2.4% (Shaw *et al.*, 2020). It is currently unknown, whether HVR RTs can be distinguished reliably from each other by MALDI-TOF MS in a region including many different HVR RTs, and if HVR RTs can be distinguished reliably from non-HVR RTs by this method. Further research is therefore necessary to proof that MALDI-TOF is a tool of *C. difficile* subtyping.

Aims of the work

This work elaborated three aspects regarding *C. difficile* involving phenotypic and genotypic assays:

In an era of emerging drug resistance, the discovery of new antimicrobials poses a crucial task to ensure treatment options and this is particularly truth for *C. difficile*. Ideally, substances used for CDI therapy should be active against *C. difficile* but spare most of the gut microbiota to avoid recurrent episodes. In recent years only few new substances could be implemented into CDI therapy, underlining the need for new substances dedicated for the treatment of this disease. In this context, substances retrieved from different research laboratories of the Helmholtz Institute for Pharmaceutical Research Saarland (HIPS) and Saarland University were evaluated for their potential to combat *C. difficile* as the first aim of this work.

Knowledge about the *C. difficile* strain composition in Germany and their AMR profiles is of importance to identify RTs with major relevance for CDI in this region, and to suggest successful antibiotic treatment regimes. Up to this date, most studies concerning molecular epidemiology and AMR have been largely biased for Germany. Some studies only focused on *C. difficile* strains being isolated from outbreaks and severe cases, thus obscuring the real epidemiology, or were single-center based. In this context, the second aim of this thesis was the introduction of a robust surveillance scheme allowing to identify the *C. difficile* strain composition in our healthcare system and to identify their AMR rates.

For genotypic characterization of *C. difficile*, MALDI-TOF MS was reported to be able to differentiate between several RTs of higher epidemiological importance (*e.g.* RT027). Furthermore, this method is available in most modern laboratories. However, the majority of available studies reporting on the suitability of this method for RT detection were conducted only with a minor amount of isolates (~ 20) or a higher amount of isolates (~ 100) but under-representing many RTs. Utilizing the NRZ strain collection, MALDI-TOF MS was carried out for the first time in a far more diverse RT setting (23 RTs) with a relevant number of isolates (240) in order to elaborate this method. In particular, the discriminatory potential of this method (*e.g.* separation of “hypervirulent” from virulent and non-toxigenic RTs) was a major focus. If successful, this method might be especially helpful in the field of infection control and hospital hygiene to rapidly identify outbreak strains and those of higher epidemiological importance with an easy-to-use method.

2. Materials and Methods

2.1. *In vitro* activity testing of new substances against *C. difficile* strains

This part was directed towards testing the *in vitro* activity of new substances against *C. difficile*. They were provided by three collaborating groups, AG Ducho (Nucleoside analogues), AG Hirsch [inhibitors of Energy coupling factor (ECF) transporters], and AG Müller (Argyrins). Different assays were carried out to delineate the minimal inhibitory concentration of each compound.

2.1.1. Minimal inhibitory concentrations (MIC) detection using broth microdilution

Following the CLSI protocol (CLSI, 2012), the new substances were tested against a bacterial set that included four *C. difficile* strains (DSM 28645, DSM 27147, DSM 1296, and a clinical CDI isolate belonging to RT027), other predominately anaerobic spore-forming bacteria with probiotic potential such as *Clostridium butyricum* (DSM 10702) and *Clostridium sporogenes* (DSM 795), important members of the intestinal microbiota such as *Clostridium scindens* (DSM 5676), *Bacteroides fragilis* (DSM 2151) and *Bifidobacterium bifidum* (DSM 20456), and the spore-forming gut pathogen *Clostridium perfringens* (DSM 756). Strains were cultured in brain heart infusion (BHI) broth (COPAN Diagnostics Inc., California, USA) and the MIC testing was performed in a Whitley A35 Anaerobic workstation (Don Whitley Scientific Limited, West Yorkshire, UK) at 37°C and a gas mixture of N_2 (90.9%), CO_2 (9.0%) and O_2 (0.1%). The test compounds were dissolved in dimethyl sulfoxide (DMSO).

On the experiment day, 2x the desired final concentration was prepared in BHI in a way that DMSO final concentrations never exceeded 1%. The procedure was carried out in 96-well CellStar microtiter plates (Greiner Bio-One, Frickenhausen, Germany). The plate layout is depicted in **Table 2-1**. The wells from column 3 till column 11 were filled with 50 µL of BHI broth then the compound (2x the final concentration) was added to column 2. Drugs were diluted by serial two-fold dilution, starting from column 2 till column 11. The last 50 µL of the serial dilution from column 11 were pipetted into column 12 for checking the sterility of the compounds.

Standardized inocula were prepared by growing the bacterial test strains overnight in BHI broth and diluting the culture in BHI broth to a McFarland (McF) of 2.0 using a densitometer (BioMérieux, Marcyl'Étoile, France). Within 15 min after the preparation, the adjusted McF 2.0 solutions were diluted 1:100 in BHI broth [*i.e.* 4×10^5 Colony Forming Unit (CFU)/mL], and 50 µL aliquots of these bacterial suspensions were added to each well containing 50 µL of the drug/broth to achieve a final inoculum of ~ 2×10^4 CFU/well. The final volume after adding the compounds should be 100 µL. In the MIC_{50} measurements, a positive activity control containing DSM 28645 was added to ensure its action (row 1).

Positive viability controls contained the bacteria only in BHI + 1% DMSO (column 1). Negative controls (for confirming specificity) contained BHI + 1% DMSO (H11 and H12 wells). The outermost empty wells (in grey) were filled with 150 µL water to decrease the evaporation or a paper tissue was soaked with water and placed underneath the microplate. The CFU content of the adjusted McF solutions were determined (also used for a purity check) by plating out ten-fold serial dilutions onto tryptic soy blood agar (TSBA) plates and the plates were cultured anaerobically at 37°C for 48 h. (H3 till H7 wells were filled with 180 µL BHI). The tray was incubated in the anaerobic chamber for 48 h at 37°C.

Table 2-1: Microtiter plate layout of the broth microdilution assay for minimal inhibitory concentration (MIC) detection.

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td></td><td></td><td colspan="6">Compound activity control</td><td></td><td></td><td></td><td></td></tr>
<tr><td>B</td><td rowspan="6">Bacterial growth control</td><td rowspan="6">Compound (2X)</td><td colspan="8" rowspan="6">Compound (2-fold) serial dilution</td><td rowspan="6">Compound sterility control</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td></tr>
<tr><td>E</td></tr>
<tr><td>F</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td><td></td><td></td><td colspan="6">Bacterial (10-fold) serial dilution → CFU count</td><td></td><td></td><td colspan="2">BHI + DMSO</td></tr>
</table>

2.1.2. Minimal inhibitory concentrations (MIC) detection using agar dilution

In accordance with the CLSI protocol, MIC testing by the agar dilution was carried out (CLSI, 2012). First**,** 43 g of Brucella Agar powder (BD) was dissolved in 1 L of distilled water and supplemented with 1 mL of a 5 mg/mL hemin solution (Sigma-Aldrich, St. Louis, USA) and 1 mL of a 1 mg/mL vitamin K1 solution (Becton Dickinson, Franklin Lakes, USA). Then, the mixture was autoclaved at 121°C for 15 min and subsequently cooled down to 50° C in a water bath. 105 mL aliquots of the cooled agar broth were filled into sterile empty glass bottles equipped with a stir fly magnet to homogenize the medium, supplemented with 5 mL defibrinated, laked sheep blood, (BD), and kept at 50°C.

The respective antibiotic working solutions of metronidazole (5µg/µL; Fresenius, Bad Homburg, Germany) and argyrin B (1µg/µL; HIPS, Saarbrücken, Germany) were added. Each desired amount of the drug was dissolved in 1mL distilled water (for metronidazole) or DMSO (for argyrin B), and then added to the agar while stirring on the heater (**Table 2-2**). The antibiotic supplemented agar was then poured into sterile petri dishes (25 mL/petri dish) and allowed to solidify. The agar plate was divided into ten sections. Two µL of the bacterial suspension (McF 4.0) were inoculated onto the respective section (**Figure 2-1**). One agar plate, that did not contain any antibiotic, was used as a positive growth control.

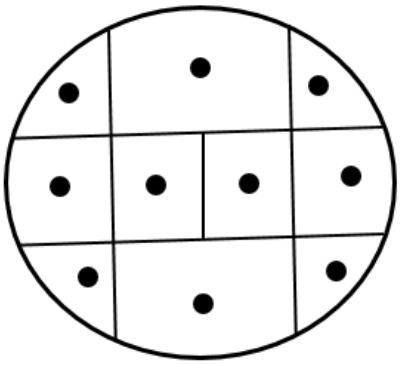

Figure 2-1: Layout of an agar dilution test plate containing the respective antibiotic. Each black circle represents a different *C. difficile* strain**.**

As this assay requires higher amounts of the test substance, this assay was used only if sufficient amounts of the compound could be provided by the cooperation partners (*e.g.* argyrin B). The respective compound concentrations can be found in **Table 2-2**.

Table 2-2: Metronidazole and argyrin B concentrations for the agar dilution assays. Each small bottle contained 105 mL of the molten Brucella agar supplemented with the antibiotic concentrations indicated.

Metronidazole (5 µg/µL)		**Argyrin B in DMSO (1 µg/µL)**	
Amount added (µg)	**End concentration (µg/mL)**	**Amount added (µg)**	**End concentration (ng/mL)**
840	**8**	**21**	**200**
420	**4**	**10.5**	**100**
210	**2**	**5.25**	**50**
105	**1**	**2.62**	**25**
52.5	**0.5**	**1.31**	**12.5**
26.25	**0.25**	**0.66**	**6.25**

2.1.3. Determination of the mutant frequency induced by argyrin B in *C. difficile*

The selection of argyrin B resistant mutants was done with the *C. difficile* reference strain DSM 28645 (CD630Δ*erm*) on Brucella agar plates supplemented with 4x MIC value of argyrin B (*i.e.* 100 ng/mL). A ten mL BHI broth culture of *C. difficile* DSM 28645 was grown (starting with a McF of 0.25) till its turbidity reached a McF of 4.0 and the bacterial cells were harvested by centrifugation at 5000 rpm/min for 10 min. After that, the cell pellet was washed once with phosphate-buffered saline (PBS), and resuspended in 0.5 mL PBS to reach a cell density of ~ 10^9-10^{10} CFU/mL. 100 µL aliquots of the bacterial solution (~ 10^8-10^9 cells/plate) were plated on argyrin B plates then anaerobically incubated at 37 °C for 48h.

Another 100 µL aliquot of the cell suspension was serially diluted (ten-fold) in PBS and plated out on Brucella agar plates without argyrin B for CFU enumeration. Mutant frequency of argyrin B resistance was calculated as the CFU number of the resistant mutants (being detected on the argyrin B containing plates) divided by the respective number of CFUs that were determined on the growth control plates (Nyfeler *et al.*, 2012).

For isolation of argyrin B resistant mutants, colonies grown on the argyrin B test plates were transferred to a fresh argyrin B plate (100 ng/mL) to confirm the ability of the mutant to grow in presence of an elevated argyrin B concentration (4x MIC) (Bielecki *et al.*, 2012). A colony of the wild strain served as an argyrin B activity control.

After confirmation of the argyrin B resistance, single colonies of the argyrin B resistant mutants were transferred to a fresh argyrin B plate and, after the purity check, they were used to inoculate two BHI broth cultures containing argyrin B (50 ng/mL). One of them was used for the DNA extraction as described in **section 2.2.3.1** to perform the WGS and the other was utilized for the MIC detection by the broth microdilution method as described in **section 2.1.1**.

2.2. Phenotypic and genotypic characterization of *C. difficile* strains

2.2.1. Sample collection

The ethical approval for conducting the investigations of the following studies was obtained by the Ethics Committee of the Medical Association of the German state of Saarland.

(Ärztekammer des Saarlandes, ethics vote 207/19)

- Non-standardized NRZ strain-set evaluation: Samples of preferentially severe CDI cases and outbreaks stemming from laboratories from all over Germany that were sent to the NRZ within 2014-2019 and a control strain-set sampled at Saarland University Hospital (UKS) within the same timeframe (2014-2019) were evaluated for MDR and a potential MIC creep for metronidazole and vancomycin, respectively.
- Standardized sentinel surveillance study: All university hospitals in Germany were requested to send the first ten stool samples from admitted patients who were tested positive for *C. difficile* by the implemented laboratory algorithm, starting at April first and October first, respectively. The earliest samples were received in October 2019, and participating laboratories were reminded per mail and email one month before the starting point of the next sampling period.

2.2.2. Phenotypic characterization of *C. difficile* strains

All samples were cultured on selective blood agar media (CLO-Agar, bioMérieux; Marcy L'Étoile, France) for 48 h in an anaerobic milieu at 37°C (Berger *et al.*, 2018). The laboratory work was accomplished in an anaerobic workbench (Don Whitley H35 Hypoxystation, West Yorkshire, UK). Bacterial cells of each strain were frozen at -80°C in cryovials containing chemically treated beads (Mast CRYOBANK® CRYO80/R tubes - Mast Diagnostica GmbH, Reinfeld, Deutschland).

2.2.2.1. Identification of *C. difficile*

All isolates received from outside the UKS were first confirmed using MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). One or two colonies of the isolates to be tested were spotted in a circular manner on the respective well of a target plate, covered with one µL of 70% formalin, and allowed to dry at room temperature. Afterwards, formalin-fixed bacteria were covered with one µL of the α-Cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker Daltonics, Bremen, Germany), then left to dry, and the dried target plate was placed into the MALDI-TOF biotyper for analysis (Candela *et al.*, 2022). Acquired spectra were compared with the Bruker database containing the 10184 species-specific main spectra profiles (MSP) using the MALDI Biotyper compass explorer software version 3.0 (Bruker Daltonics) for species identification. Only isolates with a MALDI score ≥2.0 were included into the study.

2.2.2.2. Antibiotic susceptibility testing (AST)

Susceptibility testing was carried out, whenever possible, on freshly cultured, yet unfrozen isolates as described previously (Berger *et al.*, 2018). Interpretation was done in reference to the epidemiologic breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), if available (EUCAST, 2021). Of note, the breakpoints for all antibiotics tested here did not differ between 2014 and 2021 (https://www.eucast.org/clinical breakpoints/).

Agar disk diffusion test (Kirby Bauer method)

Few bacterial colonies were dissolved in 0.9% NaCl to attain a McF of 4.0 using a densitometer (bioMérieux; Marcy L'Étoile, France), then a sterile cotton swab (Heinz Herenz, Hamburg, Germany) was immersed in the prepared inoculum, then taken out without pressing against the tube wall. The swab was streaked on Columbia agar plates (Becton Dickinson, Heidelberg, Germany) in three perpendicular directions. Clarithromycin (15µg) and rifampicin (5µg) loaded antibiotic disks (Oxoid, Wesel, Germany) were placed onto the inoculated plates, which were then incubated for 48 h at 37°C in an anaerobic environment. Lack of an inhibition zone was considered as resistant in accordance with previous studies (Müller *et al.*, 2012; Färber *et al.*, 2017; Berger *et al.*, 2018; Berger *et al.*, 2019).

Minimal inhibitory concentrations (MIC) detection using Epsilometry (E-test)

An E-test strip (Liofilchem; Roseto degli Abruzzi, Italy) containing an exponential antibiotic gradient was used for the testing of the moxifloxacin, metronidazole, and vancomycin MICs on Columbia agar plates (Oxoid, Wesel, Germany). The same cell suspension (McF 4.0) was used (as described above in the agar disk diffusion section) to inoculate the test plate (Berger *et al.*, 2020b).

The inoculated plates were allowed to dry for 10 min, and test strips were placed onto the agar surface of the plates. Plates were incubated for 48 h at 37°C in an anaerobic environment. The MIC was deduced as the first concentration above the intersection between the bacterial growth and the test strip (**Figure 2-2**).

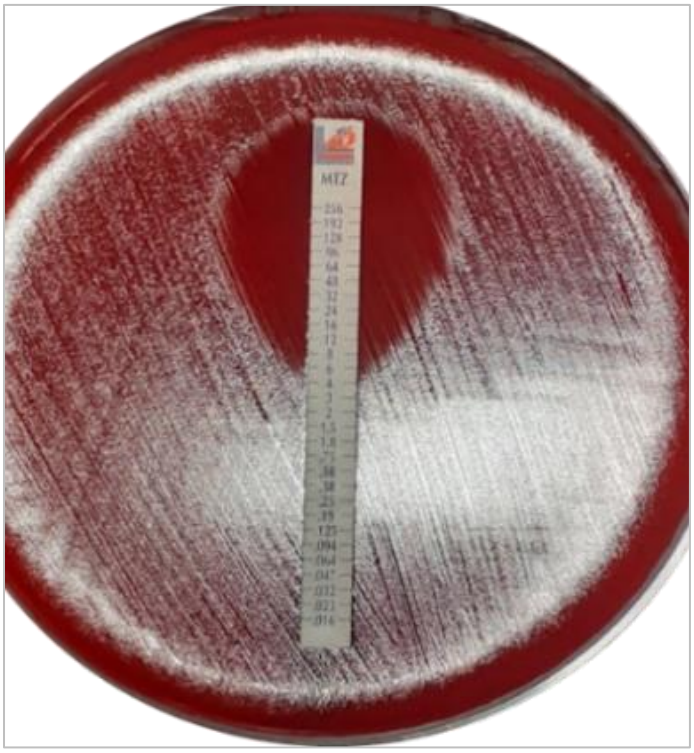

Figure 2-2: Antibiotic sensitivity testing of a *C. difficile* strain by epsilometry using a metronidazole E-test strip.

2.2.3. Genotypic characterization of *C. difficile* strains

Toxin genes detection and ribotyping were carried out as described previously (O'Neill, 1996; Bidet *et al.*, 1999; Bidet *et al.*, 2000; Berger *et al.*, 2018) using a standardized protocol (ECDIS-Net, 2012). *C. difficile* R20291 strain (RT027) was always used as a positive control and water as a negative control.

2.2.3.1. DNA extraction

One *C. difficile* colony grown on a Columbia agar plate was picked with a sterile plastic loop, dissolved in 300 µL molecular grade water, pipetted into the respective cartridge (Maxwell® 16 cell low elution volume (LEV) DNA purification kit) (Promega, Madison, Wisconsin, USA), and processed by the Promega Maxwell® 16 MDx system (Promega, Madison, Wisconsin, USA).

2.2.3.2. Toxin genes detection

A multiplex PCR was used for the detection of the respective five genes *tcdA, tcdB, cdtA, cdtB,* and *gluD*. The former four genes encode the three known *C. difficile* toxins (TcdA, TcdB and CdtAB) while the latter gene encodes for a specific glutamate dehydrogenase enzyme of *C. difficile*. Under cleanroom conditions (*i.e.* unidirectional workflow), the master mix was prepared using the primers listed in **Table 2-4** and the hotstart mix Y (Peqlab, Erlangen, Germany). Then in another room, the extracted DNA templates and controls were added to the corresponding sample. In a third room, the DNA fragments of interest were amplified using the Thermocycler peqSTAR (Peqlab, Erlangen, Germany). Materials, used volumes and PCR program are shown in **Tables 2-3, 2-4 and 2-5,** respectively. All reagents were left to equilibrate at room temperature, then they were mixed by vortexing and spinned down by centrifugation.

PCR amplicons were separated by slab-gel electrophoresis to detect different bands of the targeted genes, if present. During each run, a 100 bp ladder (Peqlab, Erlangen, Germany) was used for the size determination, and PCR fragments were separated for 90 min at 100 V in presence of the DNA dye SYBRGold (Invitrogen, Carlsbad, USA). SYBRGold-stained DNA fragments were visualized under UV-light (300 nm) and documented with a Gel Doc™ XR+ System, image lab version 4.0.1 (Bio-Rad Laboratories, Hercules, USA).

Table 2-3: Manufacturers of primers and reagents for PCR toxin genes detection.

Materials	Manufacturer
Primers of *tcdA, tcdB, cdtA, cdtB* and *gluD* genes	Biomers (Ulm, Germany)
Hot Start Mix Y	Peqlab (Erlangen, Germany)
ddH_2O (molecular grade water)	
peqGOLD 100bp DNA Ladder Plus (500 ng/µL)	
DNA Loading dye (6x), SYBRGold (10^4x in DMSO)	Invitrogen (Carlsbad, USA)

Materials	Manufacturer
TBE buffer (10x)	AppliChem (Darmstadt, Germany)
QA agarose	MP Bio (Derby, U.K)

Table 2-4: Sequences of utilized primers and the corresponding volumes of primers and reagents.

Primers	Target Gene (Amplicon size bp)	Oligonucleotide sequence 5'→ 3'	Volume (µL)
tcdA-F3345	*tcdA* (629)	GCATGATAAGGCAACTTCAGTGGTA	0.15
tcdA-R3969		AGTTCCTCCTGCTCCATCAAATG	0.15
tcdB-F5670	*tcdB* (410)	CCAAARTGGAGTGTTACAAACAGGTG	0.1
tcdB-R6079A		GCATTTCTCCATTCTCAGCAAAGTA	0.05
tcdB-R6079B		GCATTTCTCCGTTTTCAGCAAAGTA	0.05
cdtA-F739A	*cdtA* (221)	GGGAAGCACTATATTAAAGCAGAAGC	0.05
cdtA-F739B		GGGAAACATTATATTAAAGCAGAAGC	0.05
cdtA-R958		CTGGGTTAGGATTATTTACTGGACCA	0.1
cdtB-F617	*cdtB* (262)	TTGACCCAAAGTTGATGTCTGATTG	0.1
cdtB-R878		CGGATCTCTTGCTTCAGTCTTTATAG	0.1
908CLD_gluDs	*gluD* (158)	GTCTTGGATGGTTGATGAGTAC	0.05
909CLD_gluDas		TTCCTAATTTAGCAGCAGCTTC	0.05
Reagents			
Hotstart PCR Mix Y			12.5
ddH_2O (molecular grade water)			9
DNA template			2.5
Total volume			**25**

Table 2-5: PCR cycles for the toxin genes detection.

Amount of cycles	Reaction	Temperature	Duration
1	Initial denaturation	95° C	15 min
35	Denaturation	94° C	45 s
	Annealing	50° C	45 s
	Extension	72° C	1 min
1	Final extension	72° C	10 min
1	Cooling	4° C	-

Starting in April, 2021, the above described multiplex PCR protocol for toxin genes identification was replaced by a real-time PCR protocol (Boer *et al.*, 2011; Kilic *et al.*, 2015). Fluorescent labeled primers and probes were mixed with RotiPol (2x) HotTaqS-Mix (Carl Roth GmbH, Karlsruhe, Germany) and the target DNA samples as outlined in **Table 2-6**, and the CFX96 Deep Well™ Real-Time System-C1000™ Thermal Cycler (Bio-Rad, Hercules, USA) was used under the conditions described in **Table 2-7**.

Table 2-6: Utilized primers, TaqMan probes and reagents for multiplex real-time PCR. Primers were diluted 1:10 to achieve 10µM (working concentration).

Target genes (Amplicon size bp)	**Primers and Probes sequences**	**Volume (µL)**
tpi [#] (114 bp)	**Fw:** 5'-TGAATGTCCTATTACAACATAGTCC-3'	0.8
	Rv: 5'-ATAAAGATAGGTGCTCAAAATATGC-3'	0.8
	P: 5'FAM-AGAGGTGAAACTTCTCCTGTAAATGCTCCT-3'BHQ-1	0.5
tcdA (79 bp)	**Fw:** 5'-AAATAGCACCATACTTACAAGTAGG-3'	0.8
	Rv: 5'-GCATAAGCTCCTGGACCAC-3'	0.8
	P: 5'HX-ATGCCAGAAGCTCGCTCCACAATAAGTT-3'BHQ-1	0.5
tcdB (181 bp)	**Fw:** 5'-GCACCATCAATAACATATAGAGAGC-3'	0.8
	Rv: 5'-GTTTTGTGCCATCATTTTCTAAGC-3'	0.8
	P: 5'-TR-TGTCCATCCTGTTTCCCAAGCAAATACTCT-3'BHQ-2	0.5
cdtA (193 bp)	**Fw:** 5'-TATATTAAAGCAGAAGCATCTGT-3'	0.8
	Rv: 5'-CTGGACCATTTGATATTAAATAATT-3'	0.8
	P: 5'-Cy5-TCCTCCACGCATATAATCATTTACATCAGC-3'BHQ-3	0.5
Reagents		**Volume (µL)**
RotiPol (2x) HotTaqS-Mix		10.0
ddH_2O (molecular grade water)		0.6
DNA (diluted or not)		1.0
Total		**20**

[#]*tpi*, *C. difficile* specific triose phosphate isomerase gene

Fw = forward primer; Rv = reverse primer; P = probe labeled at the 5′-end with the reporter dye.

FAM = 6-carboxyfluorescein; HX = HEX; TR = Texas Red; Cy5 = Cyanin-5; BHQ = Black-Hole Quencher.

Table 2-7: Real Time-PCR cycles for toxin genes detection.

Amount of cycles	Reaction	Temperature	Duration
1	Initial denaturation	95° C	5 min
40	Denaturation	95° C	15 s
	Annealing/Extension	60° C	60 s

2.2.3.3. Ribotyping

Ribotyping was carried out according to the standardized protocol (ECDIS-Net, 2012; Fawley *et al.*, 2015). DNA extraction was done as described in **section 2.2.3.1**. Materials used are listed in **Table 2-8.** Under cleanroom conditions as described in **section 2.2.3.2**, PCR reactions in **Table 2-9** targeting the intergenic spacer region (ISR) (flanked by the 16S-23S rDNA) were prepared and run under the conditions described in **Table 2-10.**

Table 2-8: Manufacturers of the used materials in ribotyping.

Materials	Manufacturer
Roche Mix (2x) Hot Start-Mix	Roche (Mannheim, Germany)
Intergenic spacer region (ISR) primers	Biomers (Ulm, Germany)
Separation gel cartridge [linear polyacrylamide (LPA)], sample loading solution (SLS), DNA separation buffer, DNA size standard 600 bp (fluorescent internal marker)	AB Sciex (Darmstadt, Germany)
Thermowell PCR sample plate	Corning (Salt Lake City, Utah, USA)
GenomeLab GeXP^{TM}, separation buffer plate, mineral oil	Beckman Coulter (Brea, California, USA)
Thermocycler peqSTAR	peqlab (Erlangen, Germany)

Table 2-9: PCR-ribotyping primers & reagents with their respective volumes.

Reagent	Sequence (5'→ 3')	Volume (µL)
16S (Forward primer) 100µM	BMN5*-CTGGGGTGAAGTCGTAACAAGG	0.25
23S (Reverse primer) 100µM	GCGCCCTTTGTAGCTTGACC	0.25
Roche Mix (2x) Hot Start-Mix		12.5
ddH_2O (molecular grade water)		9.0
DNA template (diluted 1:10)		3.0
Total		**25.0**

*BMN-5 is Cyanine 5 analogue.

Table 2-10: Thermocycler program for PCR-ribotyping.

Cycles number	Reaction phase	Temperature	Duration
1	Initial denaturation	95° C	5 min
26	Denaturation	92° C	1 min
	Annealing	55° C	1 min
	Extension	72° C	90 s
1	Denaturation	95° C	1 min
1	Annealing	55° C	45 s
1	Final extension	72° C	5 min
1	Cooling	4° C	-

After the PCR reaction, the fluorescent labeled fragments (size range 200-600 bp) were separated via the capillary array system of the GenomeLab GeXP™ sequencer (capillary gel electrophoresis). Two 96 well-plates were prepared:

- Sample plate [Thermowell PCR plate (Corning, Salt lake city, Utah, USA)], where each reaction well contained:
  - 30 µL from a mixture of [250 µL of the Sample loading solution (SLS) + 1 µL of the DNA size standard 600].
  - 3µL of the diluted PCR products (10 µL of the amplicons + 90 µL molecular grade water).
  - One drop of mineral oil (~ 50 µL) per reaction well covered the reaction mixture.
- Separation buffer plate (Beckman Coulter, Brea, California, USA) contained only the separation buffer (~ 250 µL).

The plates were incubated for 30 min at room temperature, then placed in the sequencer. The sample injection time was 23 seconds at 1.2 kV. The electrophoresis run time was set at 1300 seconds and a separation voltage of 15 kV was applied.

The acquired spectra were imported from the GenomeLab GeXP Genetic Analysis System (Fragment Analysis software module) into the BioNumerics software, version 7.6.3 (Applied Maths N.V., Sint-Martens-Latem, Belgium). In each isolate profile, the highest intensity peak was used as a fragment calling control, *i.e.* any fragment displaying a peak intensity less than 10% of the peak intensity of the control fragment was not included. Overlaying peaks (split peak) were considered as two peaks only if they were separated by at least 1.5 bp, otherwise the peak with the lower intensity was excluded (Indra *et al.*, 2008; Fawley *et al.*, 2015).

To assign the strain RT, the isolate's banding pattern was compared to the reference RT profiles stored in the PCR-ribotyping library (database containing profiles of ~ 200 ribotypes). If there was no match in the NRZ database (*i.e.* the matching score <93%), the strain RT was categorized as unclassified RT (ECDIS-Net, 2012). Banding patterns of some RTs are shown in **Figure 2-3**. On a regular basis, the NRZ's strain collection is updated with new strains from the Reference Centers in Leeds (UK) and Leiden (Netherlands) (ECDIS-Net, 2012).

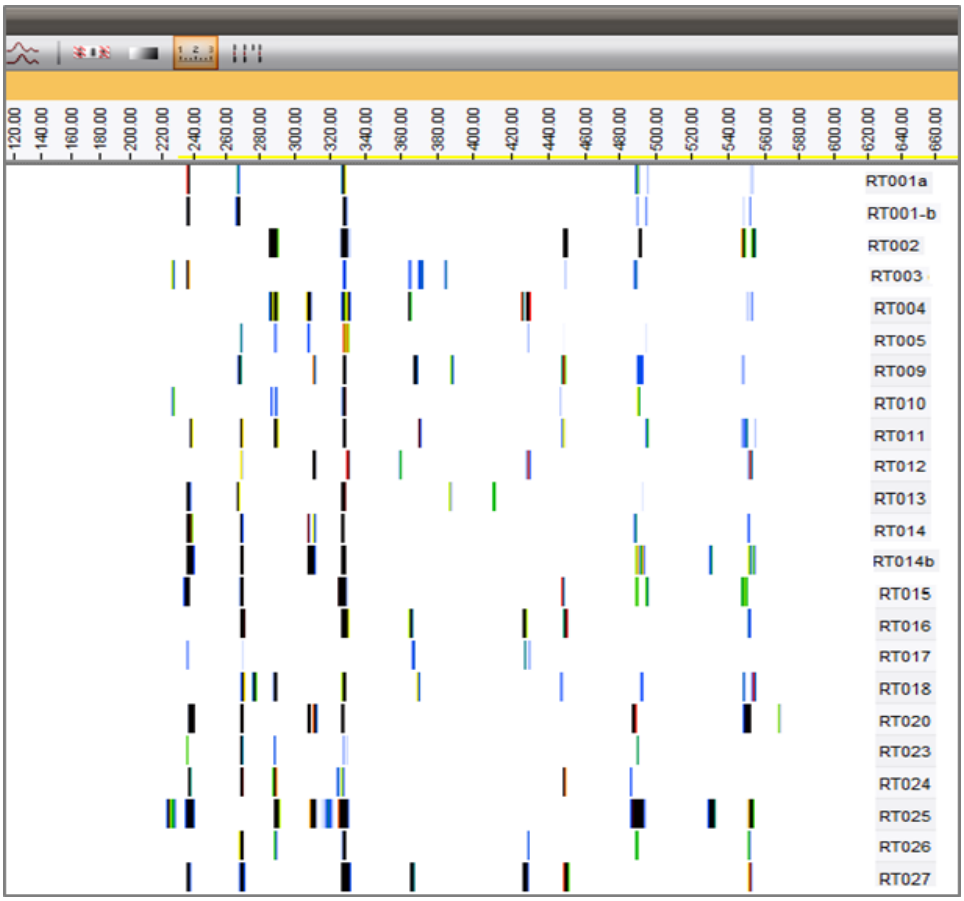

Figure 2-3: Some reference ribotypes and their corresponding banding patterns using BioNumerics version 7.6.3.

2.3. MALDI-TOF MS for *C. difficile* RTs subtyping

2.3.1. Strain collection

A panel of 240 *C. difficile* isolates of different RTs being of high epidemiological importance worldwide were selected from the strain collection of the NRZ. Internal strain names and strain characteristics are listed in **Tables 2-11 and 2-12.** All strains were characterized by PCR ribotyping (Berger *et al.*, 2018; Abdrabou *et al.*, 2021) and stored at -80°C.

Table 2-11: *C. difficile* 240 strains of epidemiologically important RTs that were included into the study: Hypervirulent ribotypes (HVR RTs), Virulent RTs (VIR RTs), Non-toxigenic ribotypes (NTC RTs). RT, ribotype.

RT	Total number (Discovery phase)	Group	Internal Code*
RT001	13 (8)	**VIR**	[P3, H2, H13, K42, P26, P42, R34, K1] (C32, E33, F54, G28, L18)
RT002	13 (6)	**VIR**	[W41, W65, C59, H16, P2, W28] (E30, M55, P19, P25, X56, XX171, XX647)
RT009	7 (6)	**NTC**	[A69, E73, F68, G41, K26, U45] (XX225)
RT010	8 (6)	**NTC**	[O59, O62, O73, O9, P55, P12] (P8, XX267)
RT012	6 (6)	**VIR**	[D16, X-1-54, X-1-75, X-9-57, X-9-60, CD630Δ*erm*]
RT014	10 (5)	**VIR**	[E20, J59, N70, V11, L60] (C33, E31, F52, H15, H44)
RT017	13 (7)	**VIR**	[B46, M38, R30, X-6-77, X-7-25, L56, S31] (J51, J52, J53, J54, J55, J56)
RT018	12 (9)	**VIR**	[G41, H30, U30, X-1-55, X-2-14, X-2-47, X-4-16, X-4-56, Q57] (N32, XX381, XX513)
RT020	13 (4)	**VIR**	[C23, J58, P1, O10] (F74, G50, H43, H61, J57, L29, M2, M56, XX224)
RT023	19 (10)	**HVR**	[N22, N23, P17, R18, T32, U60, U67, V80, W36, W8] (H75, J17, X47, XX59, XX216, XX234, XX235, XX478, XX536)
RT027	21 (14)	**HVR**	[X-5-51, P47, K27, P72, P75, CD2Q17, Q41, Q43, Q50, (R20291), S16, W18, Q69, X-5-41] (B12 / B40 / B64 / C6 / C12 / D4 / X51)
RT35	3 (3)	**NTC**	[B63, H66, M37]

RT	Total number (Discovery phase)	Group	Internal Code*
RT045	14 (10)	**HVR**	[B25, B65, H56, M9, N63, O52, O56, P63, P71, P73] (C3, J27, Q5, XX418)
RT073	4 (4)	**NTC**	[A10, A33, D21, F28]
RT078	21 (5)	**HVR**	[N71, H35, P79, W62, S79] (B74, F10, F78, I7, I37, J38, J59, P9, Q15, Q66, Q73, XX35, XX210, XX223, XX340, XX445)
RT084	2 (2)	**NTC**	[H46, M47]
RT085	2 (2)	**NTC**	[Q22, T23]
RT106	9 (6)	**VIR**	[X-5-29, X-5-55, L73, P45, S35, T69] (E38, G64, N39)
RT126	17 (14)	**HVR**	[F32, M19, M50, M53, N51, N53, N8, O64, Q63, R4, V62, V66, X-5-30, X-6-74] (C13, I6, XX168)
RT127	2 (2)	**HVR**	[G49, U01]
RT140	14 (11)	**NTC**	[D48, E27, P21, P55, R66, S25, S57, T15, T71, B47, V75] (XX15, XX153, C27)
RT176	10 (10)	**HVR**	[A1, A3, A5, A7, A9, E6, F31, G62, K44, W74]
RT207	7 (7)	**VIR**	[C48, V31, C74, T79, V10, V27, V30]

***Square Brackets: 157** isolates used for the discovery phase and the cross validation.

***Round brackets: 83** blind isolates used for the external validation.

Table 2-12: Number of isolates included in each phase. Hypervirulent ribotypes (HVR RTs), Virulent RTs (VIR RTs), Non-toxigenic ribotypes (NTC RTs). RT, ribotype.

Group	Discovery phase & Cross validation	External validation	Total
HVR RTs	65	39	104
VIR RTs	58	38	96
NTC RTs	34	6	40
Total	**157**	**83**	**240**

2.3.2. Culture

Selected isolates were thawed, sub-cultured twice on trypticase soy agar plates with 5% sheep blood (BD Biosciences, Franklin Lakes, USA), and incubated at 37° C for 48 h under anaerobic conditions using an anaerobic chamber (Whitley H35 Hypoxystation). Purity of the colonies was checked by MALDI-TOF MS analysis (Bruker Daltonics) as outlined in **section 2.2.2.1**.

2.3.3. MALDI-TOF MS

2.3.3.1. Protein extraction, spectra acquisition and species confirmation

Protein extracts of *C. difficile* colonies were obtained according to the protocol provided by Bruker Daltonics (Protein extraction for MSP creation protocol V1.1; Bruker Daltonics). Briefly, two to three colonies of an isolate were resuspended in 300 µL liquid chromatography–mass spectrometry (LC-MS) grade water (Merck, Darmstadt, Germany) and mixed with 900 µL of absolute ethanol (Merck, Darmstadt, Germany) by vortexing then centrifugation at 18,000 × g for 2 min.

Then, the supernatant was discarded. Afterwards, the acquired pellet was resuspended in 10 µL of 70% (v/v) formic acid, supplemented with 10 µL acetonitrile, and the mixture was carefully mixed, then centrifuged at 18,000×g for 2 min. One µL each of the (clear) supernatant was spotted in four technical replicates onto the MALDI target plate. After being air-dried, one µL of the saturated HCCA matrix solution (Bruker Daltonics) was added to each spot.

All measurements were carried out with the Microflex LT smart mass spectrometer utilizing the AutoXecute algorithm implemented in the Flexcontrol software version 3.4 (Bruker Daltonics). This procedure was reiterated with a new biological replicate to ensure the reproducibility. For the instrument calibration, an *Escherichia coli* protein extract [Bacterial Test Standard (BTS, Bruker Daltonics)] was used. Species confirmation was done as described in **section 2.2.2.1**.

2.3.3.2. MALDI-TOF MS parameters

The mass range for acquisition was 2 kDa to 20 kDa. To generate the spectra, forty laser shots at six random positions were applied in the positive ion mode with 200 Hz frequency, at 20 kV in 520 nanoseconds of pulsed ion extraction.

2.3.3.3. Spectra analysis and preprocessing

Raw spectra of *C. difficile* strains were first visualized using the FlexAnalysis software (Bruker Daltonics), then exported to the Clover MS Data Analysis platform for further analysis. Pre-processing step was performed to reduce the noise using the Savitzky-Golay filter (window length 11; polynomial order 3), then the baseline was removed using the top-hat filter method (factor 0.02) (Candela *et al.*, 2022). For the generation of the peak matrix, the processed spectra (smoothed and baseline removed) from each technical replicate were aligned (shift medium; constant mass tolerance 0.2 Da and linear mass tolerance 2000 ppm) to obtain one average spectrum per biological replicate, then this step was reiterated to acquire one average spectrum for each strain by combining the average spectra of the two biological replicates (Candela *et al.*, 2022). Group-specific peaks were detected (with or without threshold, see below) then merged by applying a constant mass tolerance of 0.5 Da and linear mass tolerance of 500 ppm.

Finally, a principal component analysis (PCA), based on the detected peaks, was performed to visualize the different clusters.

Three different approaches were assessed (Candela *et al.*, 2022):

- Full-spectrum method: all peaks separated by a constant mass tolerance of 0.5 Da, irrespective of the intensity (*i.e*. without threshold), were included, and a total ion current (TIC) normalization was used to standardize their intensities.
- (Threshold method TICp) → TIC normalization was applied first then a threshold factor of 0.01 applied (*i.e.* only peaks with intensities >1.0% of the maximum peak intensity were selected and finally merged).
- (Threshold method pTIC) → All peaks with intensities above 1% were selected and merged prior to the TIC normalization.

2.3.3.4. Machine learning validation phase

Peak matrices (Full spectrum, TICp and pTIC) were utilized as input data for four distinct supervised machine learning (ML) prediction algorithms. These algorithms include partial least squares–discriminant analysis (PLS-DA), kernilized-support vector machine (SVM), K nearest neighbors (KNN), and random forest (RF) (Candela *et al.*, 2022). Internal validation was tested using 10-fold cross validation (Zvezdanova *et al.*, 2020). Briefly, data of the discovery phase (spectra of 157 isolates) were randomly split into 10 equal-sized groups. Nine groups were used to train the algorithms and the remaining last group (the 10^{th} group) was used as a test set for the internally-validated algorithm. This procedure was repeated once for each of the 10 subgroups and the classification's confusion matrix was documented (Weis *et al.*, 2020). Next, spectra obtained from 83 new *C. difficile* isolates were used for the external validation of the most promising models.

2.4. Statistics and software programs

Qualitative data were described as numbers and percentages. The Fisher's exact test or the Chi-square test were used for the comparison between groups using Microsoft Excel program (MS Office 2016, Microsoft, Seattle, USA), as appropriate. Quantitative data were described as means or medians. After testing for normality, parametric tests (ANOVA test or Student's t-test) were used for the normally distributed data and non-parametric tests (Kruskal-Wallis or Mann-Whitney U) were utilized for the non-normally distributed data. *P*-values <0.05 were considered statistically significant. Software programs used in the studies are shown in **Table 2-13**.

Table 2-13: Software programs used and their developers.

Program	Developer
HighFlexX™	Intellitec (Kassel, Germany)
M/LAB Software; Version 32	Dorner Health IT Solutions (Müllheim, Germany)
BioNumerics Software; Version 7.6.3	Applied Maths N.V. (Sint-Martens-Latem, Belgium)
Clover MS data analysis software	Clover Bioanalytical Software (Granada, Spain)
MS Office 2016 (Excel)	Microsoft (Seattle, USA)

3. Results

3.1. *In vitro* activity testing of new compounds against *C. difficile*

This work was conducted to test the efficacy of new substances with proposed antimicrobial activity against *C. difficile*. They were received from three collaborating groups AG Ducho (Nucleoside analogues), AG Hirsch [inhibitors of energy coupling factor (ECF) transporters] and AG Müller (Argyrins). Most of the compounds were available only at a very low scale, which was a limiting factor to exclude certain assays (especially the agar dilution).

3.1.1. New argyrin derivatives provided by HIPS (AG Müller)

3.1.1.1. Minimal inhibitory concentrations (MIC) detection using broth microdilution

A small selection of *C. difficile* strains (DSM 1296, DSM 27147 and DSM 28645) and the anaerobic spore-forming bacterium with probiotic potential *C. butyricum* (DSM 10702) were first tested against a set of 22 argyrin derivatives. The MICs of the different *C. difficile* strains are depicted in **Table 3-1**. MIC values for *C. butyricum* (DSM 10702) were >400 ng/mL for all argyrin derivatives, which was the highest concentration of the antimicrobial compound that could be used in this assay.

Table 3-1: MIC values of four *C. difficile* strains for argyrin (Arg) derivatives. MICs of the compounds were determined in three independent experiments. PEG: Polyethylene glycol, DMSO: Dimethyl sulfoxide.

Species	***C. difficile***					
Strain	**DSM 28645 (CD 630Δ*erm*)**		**DSM 27147 (R20291) & clinical strain (0110)$^{+}$**		**DSM 1296**	
Ribotype	**RT012**		**RT027**		**RT001**	
MIC (ng/mL)						
Substance*	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**
Arg B	18.75	12.5-25	37.5	25-50	18.75	12,5-25
Arg B in PEG400/PBS	75	50-100	Nd	Nd	Nd	Nd
Arg C	25	25	25	25	Nd	Nd
Arg D	75	50-100	75	50-100	Nd	Nd
Arg 2	4.69	3.125 -6.25	4.68	3.125 – 6.25	4.68	3,125 – 6,25
Arg 4	100	100	100	100	150	100 – 200
Arg 5	150	100-200	200	200	300	200- 400
Arg 12	9.38	6.25 -12.5	6.25	6.25	6.25	6,25
Arg 13	150	100-200	75	50-100	75	50-100
Arg 14	75	50-100	100	100	100	100

Species	***C. difficile***					
Strain	**DSM 28645 (CD 630Δ*erm*)**		**DSM 27147 (R20291) & clinical strain (0110)$^{+}$**		**DSM 1296**	
Ribotype	**RT012**		**RT027**		**RT001**	
MIC (ng/mL)						
Substance*	**Mean**	**Range**	**Mean**	**Range**	**Mean**	**Range**
Arg 845$^{\#}$	400	400	400	400	Nd	Nd
Arg 875$^{\#}$	25	25	37.5	25-50	Nd	Nd
Arg 878$^{\#}$	150	100-200	200	200	Nd	Nd
Arg 1 / Arg 6 / Arg 7 Arg 8 / Arg 9 / Arg 10 / Arg 11 Arg 834$^{\#}$ / Arg 859$^{\#}$ / Arg 894$^{\#}$			**>400**			

*****All argyrin derivatives were dissolved in DMSO unless specified otherwise. MIC values for DMSO and PEG alone and DSM 28645 were 12.5% for each of them.

$^{\#}$ *C. difficile* DSM 1296 was not tested.

Nd: Not detected.

$^{+}$The RT027 clinical strain (0110) has the same MIC values as DSM 27147 (R20291) except for Arg B which was 18.75 ng/mL and for Arg 13 was 50 ng/mL.

The most promising argyrin derivatives; Arg 2, Arg B and Arg 12 were next tested against a set of 51 *C. difficile* strains of various RTs to delineate the MIC_{50} and MIC_{90}, respectively (**Table 3-2**).

Table 3-2: MIC values of 51 *C. difficile* strains of various ribotypes for Arg B, Arg 2 and Arg 12 (dissolved in DMSO). A selection of 13 compounds (indicated in bold) was also tested with Arg B dissolved in PEG400/PBS. PEG: Polyethylene glycol, DMSO: Dimethyl sulfoxide.

Strain ID	**Ribotype**	**Mean MIC ng/mL (Duplicates)**		
		Arg 2	**Arg B**	**Arg 12**
A	RT027	6.25	25	6.25
B	RT027	6.25	25	6.25
C	RT027	6.25	12.5	6.25
D	RT027	6.25	25	6.25
E	RT027	12.5	25	9.38
F	RT027	6.25	12.5	3.125
G	RT027	6.25	25	9.38
H	RT027	6.25	12.5	6.25
I	RT027	6.25	12.5	6.25

Strain ID	Ribotype	Mean MIC ng/mL (Duplicates)		
		Arg 2	Arg B	Arg 12
J	RT027	4.69	12.5	6.25
K	RT078	6.25	25	6.25
L	RT078	6.25	12.5	6.25
M	RT078	4.69	12.5	4.69
N	RT078	4.69	12.5	6.25
O	RT078	6.25	18.75	6.25
P	RT014	4.69	12.5	6.25
Q*	RT014	3.125	12.5	3.125
R*	RT014	6.25	12.5	4.69
S	RT014	6.25	25	12.5
T*	RT014	6.25	12.5	3.125
V	RT001	3.125	12.5	6.25
W	RT001	9.38	18.75	9.38
X	RT001	3.125	12.5	6.25
Y	RT001	3.125	12.5	3.125
Z*	RT001	4.69	25	3.125
AA*	RT001	6.25	12.5	6.25
AB	RT001	6.25	25	6.25
AC*	RT001	6.25	12.5	6.25
AD	RT018	4.69	12.5	3.125
AE	RT018	4.69	12.5	6.25
AF	RT176	6.25	50	12.5
AG*	RT176	6.25	18.75	6.25
AH*	RT176	6.25	25	4.69
AI*	RT176	6.25	25	3.125
AJ*	RT176	6.25	25	4.69
AK*	RT106	6.25	12.5	3.125
AL	RT106	4.69	18.75	4.69
AM	RT106	6.25	12.5	6.25
AN	RT106	4.69	12.5	6.25
AO	RT017	6.25	37.5	6.25
AP	RT017	4.69	12.5	3.125
AQ	RT010	6.25	12.5	6.25
AR	RT140	9.38	18.75	6.25
AS*	RT002	6.25	12.5	4.69

Strain ID	Ribotype	Mean MIC ng/mL (Duplicates)		
		Arg 2	**Arg B**	**Arg 12**
AT	RT002	4.69	12.5	9.38
AV	RT002	6.25	25	6.25
AW	RT002	6.25	12.5	6.25
AX	RT020	4.69	18.75	6.25
AY	RT020	6.25	25	6.25
AZ*	RT020	6.25	12.5	3.125
AAA	RT020	6.25	12.5	6.25
MIC_{50}		**6.25**	**12.5**	**6.25**
MIC_{90}		**6.25**	**25**	**9.38**

***Strains tested for Arg B in PEG400/PBS and their MIC values were between 100-200 ng/mL.

Anaerobic spore-forming bacteria with probiotic potential as *C. butyricum* (DSM 10702) and *C. sporogenes* (DSM 795) or important members of gut flora like [*C. scindens* (DSM 5676), *Bacteroides fragilis* (DSM 2151) and *Bifidobacterium bifidum* (DSM 20456)] or another spore-forming gut pathogen *e.g. C. perfringens* (DSM 756) were also tested against a subset of argyrin derivatives as illustrated in **Table 3-3**.

Table 3-3: MIC values of selected argyrin derivatives for some *Clostridium* spp. isolates and important gut microbiota members.

Species	MIC values (µg/mL) Argyrin derivatives													
	1	**2**	**B**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
C. butyricum DSM 10702	**>1**													
C. perfringens DSM 756	**>1**													
C. sporogenes DSM 795	**>1**													
C. scindens DSM 5676	**>1**													
Bacteroides fragilis DSM 2151	**>1**													
Bifidobacterium bifidum DSM 20456	**>1**													

For all bacterial species tested, growth was not suppressed even with the highest concentration of the drug that could be used in this assay (*i.e.* 1 µg/mL).

3.1.1.2. Minimal inhibitory concentrations (MIC) detection using agar dilution

In order to confirm my MIC findings made for the argyrin derivatives with the broth microdilution method, MICs for Arg B were retested with the agar dilution method, which is recommended by CLSI for MIC determinations with anaerobic bacteria (CLSI, 2012). All MIC values for *C. difficile* strains ranged between 25-50 ng/mL, and were thus about two-fold higher as those observed for Arg B with the broth microdilution method. For *C. butyricum*, *C. perfringens*, and *C. sporogenes*, MIC values were again much higher and >200 ng/mL, which was the highest concentration of the antimicrobial compound that could be tested in this compound-consumptive assay format.

3.1.1.3. Determination of the mutant frequency induced by argyrin B in *C. difficile*

Given the promising *in vitro* activity of Arg B against *C. difficile*, the mutant frequency of the *C. difficile* reference strain DSM 28645 was next tested by spotting $5x10^8$ to $1x10^9$ cells on Brucella blood agar plates supplemented with 100 ng/mL Arg B (~ 4x the MIC of ArgB for this strain) and counting the colonies that appeared on the plates after 48 h of incubation at 37°C in the anaerobic chamber. *C. difficile* DSM 28645 strain produced under these conditions about one to eight colonies in four biological replicates, yielding a mutant frequency of 4.62E-09 (**Table 3-4)** and (**Figure 3-1**).

Table 3-4: Mutant frequency of *C. difficile* (CD630Δ*erm*) strain for argyrin B.

Trial	Number of argyrin B resistant mutants (CD630Δ*erm* strain)	*C. difficile* cells inoculated/plate	Mutant Frequency
a	1	5E+08	2.00E-09
b	2	6E+08	3.33E-09
c	1	5E+08	2.00E-09
d	8	1E+09	8.00E-09
Total	**12**	**2.6E+09**	**4.62E-09**

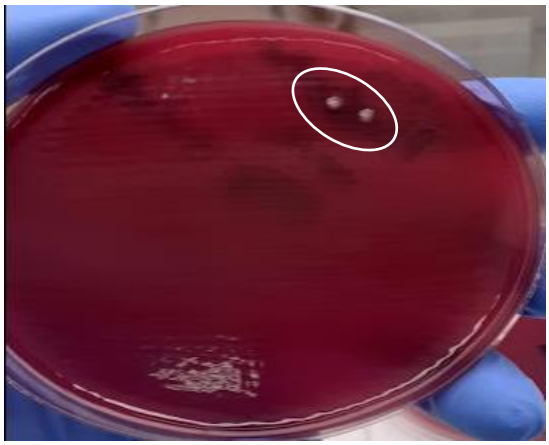

Figure 3-1: Two suspected argyrin B resistant mutants on Brucella blood agar supplemented with 100 ng/mL argyrin B after 48 h of growth at 37°C in the anaerobic chamber.

To confirm the increased resistance level of the suspected mutants, cells of these mutants were plated out on a fresh Brucella blood agar supplemented with 100 ng/mL argyrin B and checked for growth. Most of the colonies that appeared on the first 4x MIC argyrin B-selection plates also managed to grow on the second 4x MIC argyrin B selection plates, while this was not the case with the parental strain that served as a control (**Figure 3-2**).

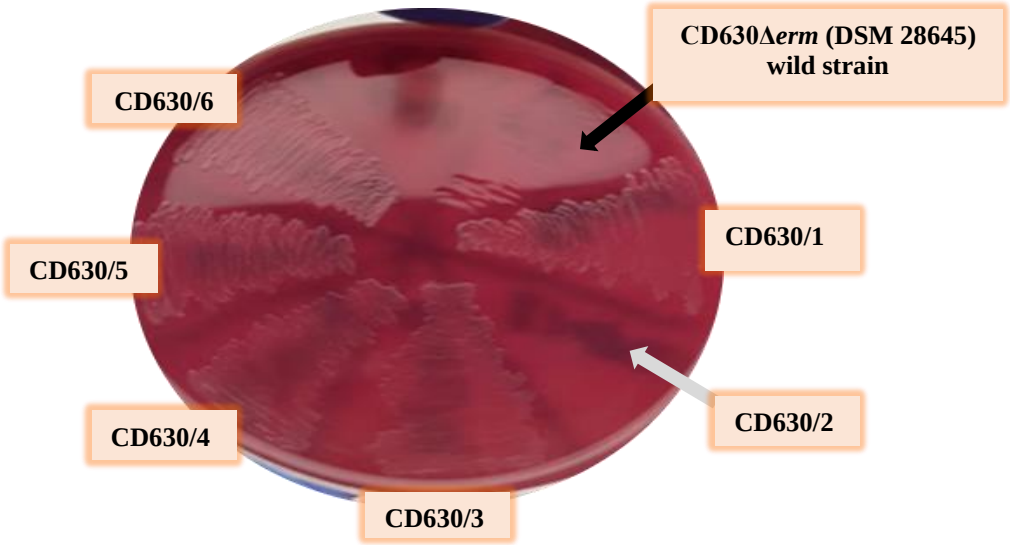

Figure 3-2: Resistance confirmation of argyrin B suspected mutants on Brucella blood agar supplemented with 100 ng/mL argyrin B. The black arrow is the DSM28645 (wild type) strain. The white arrow indicates the plating region of a colony that grew on the first selection plate but not on the second one used for confirmation.

All mutants that grew on both 4x MIC argyrin B plates were subsequently tested for their argyrin B MIC values by the broth microdilution method. MICs of all resistant mutants ranged between 250 ng/mL and >8 µg/mL (the highest concentration of argyrin B tested here), respectively.

3.1.2. Nucleoside analogues antibiotics provided by the Pharmaceutical and Medicinal Chemistry (AG Ducho)

Minimal inhibitory concentrations (MIC) detection using broth microdilution

Two strains of *C. difficile* (DSM 28645 and one RT027 clinical strain named 0110) were tested against a panel of 16 new nucleoside compounds. The MIC values of the *C. difficile* strains are depicted in **Table 3-5**. Due to the limited amounts of compounds provided, only broth microdilution testing was conducted.

Table 3-5: MIC values of two *C. difficile* strains for new nucleoside compounds. MICs ≤20 µg/mL are highlighted in bold. MICs of the compounds were determined in two independent experiments.

Species	***C. difficile***	
Strain	**Clinical strain (0110)**	**DSM 28645**
Substance	**MIC (µg/mL)**	
MWP 398	>20	>20
MWP 421	>20	>20
MWP 447	>20	>20
MWP 451	**2.5 - 5**	**2.5**
GN 142	**20**	**20**
GN 180	>20	>20
GN 184	>20	>20
GN 232	>20	>20
GN 243	**20**	**20**
GN 244	**20**	**20**
GN 245	**20**	**20**
GN 246	**5-10**	**2.5**
GN 249	>20	>20
CapA	>20	>20
CapB	>20	>20
CapF	>20	>20

The nucleoside-based compounds MWP 451 and GN246 showed the highest activity against the two *C. difficile* strains (2.5 – 10 µg/mL). Some compounds such as, GN 142, GN 243, GN 244, and GN 245 suppressed the growth in this assay at a concentration of 20 µg/mL (*i.e.* the highest concentration that could be used here), while other compounds failed to suppress the growth with the latter concentration.

3.1.3. ECF-Transporter inhibitors provided by HIPS (AG Hirsch)

Minimal inhibitory concentrations (MIC) detection using broth microdilution

A set of three *C. difficile* strains [DSM 1296, DSM 28645 and one RT027 clinical strain (0110)] and different *Clostridium* spp. strains [*C. butyricum* DSM 10702, *C. perfringens* DSM 756 and one *C. sporogenes* clinical strain (G33)] were tested against 6 substances thought to interfere with bacterial energy coupling factor (ECF) transporters inhibitors (Diamanti *et al.*, 2021). The MICs of the different strains are depicted in **Table 3-6**. Due to the limited amount of compounds provided, this assay was carried out only twice.

Table 3-6: MIC values of three *C. difficile* strains and three *Clostridium* spp. strains for new energy coupling factor (ECF)-transporters inhibitors. Promising results are highlighted in bold. MICs of the compounds were determined in two independent experiments.

Species	***C. difficile***			***C. butyricum***	***C. perfringens***	***C. sporogenes***
Strain	**DSM 1296**	**DSM 28645**	**clinical strain 0110**	**DSM 10702**	**DSM 756**	**clinical strain G33**
Ribotype	**RT001**	**RT012**	**RT0027**	**---**	**---**	**---**
Substance	**MIC (µM)**					
K4104497	**12.5**	**12.5**	**12.5**	**25-50**	**1.5-3**	**50**
HHPS77	**12.5 -25**	**12.5 -25**	**12.5 -25**	**12.5 -25**	**1.5-3**	**25-50**
E79	**25**	**25**	**25**	**100**	**12.5-25**	**100**
HIT1	>100					
SBO	>100	>100	>100	>100	12.5	>100
AKO	>100	>100	>100	25	12.5 -25	>100

Two of the compounds, HHPS77 and K4104497, showed an activity towards the *C. difficile* strains at concentrations of 12.5-25 µM, respectively, while two-fold higher concentrations (25-50 µM) were needed to suppress the growth of *C. butyricum* and *C. sporogenes*, respectively. Interestingly, both compounds worked best on the *C. perfringens* isolate tested (1.5-3 µM). Compound E79 was active on *C. difficile* and *C. perfringens* at MIC values of about 25 µM, while a 4-fold higher concentration was needed for *C. butyricum* and *C. sporogenes*, respectively.

3.2. Phenotypic and genotypic characterization of *C. difficile* strains

3.2.1. Evaluation of the *C. difficile* multi-drug resistance and $MIC_{50/90}$ towards metronidazole and vancomycin (toxigenic isolates from 2014-2019) with the confirmation of metronidazole resistance by agar dilution

In order to get an idea about the development of reduced susceptibility towards metronidazole and vancomycin in *C. difficile* isolates that were sent from German Hospitals to the NRZ in the years 2014 to 2019 for diagnostic purposes (NRZ strain-set; 1456 isolates) or collected during the same time period at Saarland University Hospital (UKS strain-set; 1131 isolates), the respective MIC_{50} and MIC_{90} were evaluated. These data were published in part in (Abdrabou *et al.*, 2021). Neither for metronidazole nor for vancomycin was a MIC creep noted in any of the strain-sets (**Table 3-7**). MICs epidemiologic EUCAST breakpoints for vancomycin, metronidazole, and moxifloxacin were 2, 2, and 4 µg/mL, respectively (EUCAST, 2021).

Table 3-7: MIC_{50} and MIC_{90} values of metronidazole and vancomycin per year in the national reference center (NRZ) and Saarland University Hospital (UKS) strain-sets. The table is modified after permission of Elsevier for non-commercial purposes (Abdrabou *et al.*, 2021).

Year	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
NRZ isolates						
Metronidazole (µg/mL)						
MIC range	0.125 - >256	0.125 - 32	0.094 - 4	0.094 - >256	0.094 - 4	0.094 - 2
MIC_{50}	0.75	0.75	0.75	0.75	0.75	0.75
MIC_{90}	1.5	2	1.5	1.5	1.5	1.5
Vancomycin (µg/mL)						
MIC range	0.19 - 2	0.094 - 2	0.125 - 2	0.094 - 2	0.094 - 2	0.125 - 2
MIC_{50}	0.5	0.38	0.5	0.38	0.5	0.5
MIC_{90}	1.5	1.0	1.0	0.75	1.0	1.0
UKS isolates						
Metronidazole (µg/mL)						
MIC range	0.047 - >32	0.094 - >32	0.094 – 1.5	0.125 – 1.5	0.094 – 2	0.094 – 2
MIC_{50}	0.5	0.5	0.38	0.5	0.5	0.5
MIC_{90}	1.5	1.0	1.0	1.0	1.0	1.0
Vancomycin (µg/mL)						
MIC range	0.125 - 2	0.125 - 2	0.125 - 2	0.19 – 1.5	0.125 – 1.5	0.19 – 1.5
MIC_{50}	0.5	0.5	0.5	0.38	0.38	0.5
MIC_{90}	1.0	1.0	0.75	0.75	0.75	0.75

When looking for MDR in the NRZ strain-set, 249 isolates were detected that featured resistances against the antibiotics rifampicin, moxifloxacin, and clarithromycin [96% of them belonging to RT027 (240/249 isolates) and four isolates belong to the genetically related RT176]. 14 isolates (all RT027) were resistant to all tested antibiotics except vancomycin. MDR was encountered more often in RT027 isolates (240/547) if compared to non-RT027 isolates (9/909; *p*-value <0.00001). In the UKS strain-set, only 18 MDR isolates were detected, with twelve of them belonging to RT027. MDR isolates were again significantly more often found in the RT027 subset (12/47, 26%) than in non-RT027 ribotypes (6/1084, 0.6%; *p*-value <0.00001).

From the 39 isolates displaying metronidazole MIC values above the EUCAST breakpoint by the E-test method, 32 isolates were available for retesting by agar dilution. This method confirmed a metronidazole resistance in 22/32 isolates. However, in 10 cases, discrepant results between the initial E-test and the agar dilution results were observed (**Table 3-8**). In order to address this discrepancy, E-tests were repeated for the latter isolates, which yielded in MIC values that were consistent for (8/10 isolates) with the agar dilution results.

Table 3-8: Comparing the minimal inhibitory concentration (MIC) values of the 32 metronidazole resistant isolates by E-test and agar dilution. Discrepant results are highlighted in bold and the E-test assay was repeated for them. *Strains displaying a metronidazole resistance (3-4 µg/mL) on repeated E-tests.

Strain Code	MIC (µg/mL)		Strain Code	MIC (µg/mL)	
	E-test	Agar		E-test	Agar
M1	>3	4	M17	>256	8
M2	8	>8	M18	3	4
M3	6	8	M19	4	4
M4	4	8	M20	4	4
M5	3	4	**M21**	**3**	**2**
M6	>30	>8	**M22**	**3**	**2**
M7	4	4	**M23**	**3**	**2**
M8	3	8	**M24**	**3**	**2**
M9	3	4	**M25***	**4**	**2**
M10	4	8	M26	4	4
M11	3	8	M27	4	4
M12	**3**	**1**	**M28**	**3**	**2**
M13	6	8	M29	3	4
M14*	**3**	**1**	M30	4	4
M15	3	4	**M31**	**4**	**1**
M16	12	4	**M32**	**3**	**1**

3.2.2. Establishment and evaluation of a standardized sentinel surveillance study (2019-2021)

In this part of my thesis, a standardized sentinel surveillance study for *C. difficile* among German University Hospitals should be established, in which the first 10 *C. difficile* isolates identified in April and October at the participating University Hospitals were sent to the NRZ for toxin-gene detection, PCR-ribotyping and AMR testing. 29 University medical Centers supplied samples, and the first strains were received during April 2019 and the last strains included into this study were received during October 2021. These data were published in the Journal Anaerobe (Abdrabou *et al.*, 2022).

A total of 1026 *C. difficile* strains were obtained during this time frame, from which 876 samples were identified as toxigenic *C. difficile* strains, in accordance with their respective toxin genes. PCR-ribotyping of this isolate set revealed that RTs 001, 002, 005, 014, 020, 027, and 078 were the most commonly encountered RTs (**Table 3-9**).

Table 3-9: Ribotypes diversity and the corresponding antimicrobial resistance (AMR) profiles. RT, ribotype; A, B, toxin A, B; C, CDT binary toxin; VA, vancomycin; MET, metronidazole; MFX, moxifloxacin; CLR, clarithromycin; RF, rifampicin. The table is modified after the permission of Elsevier for non-commercial purposes (Abdrabou *et al.*, 2022).

Genotyping results		**Antimicrobial susceptibility testing**				
RT (isolates' number)	**Toxins**	**VA**	**MET**	**MFX**	**CLR**	**RF**
RT001 (62)	A, B	0/62 (0%)	0/62 (0%)	23/62 (37%)	24/62 (39%)	1/62 (2%)
RT002 (48)	A, B	0/48 (0%)	0/48 (0%)	2/48 (4%)	1/48 (2%)	0/48 (0%)
RT003 (9)	A, B	0/9 (0%)	0/9 (0%)	0/9 (0%)	0/9 (0%)	0/9 (0%)
RT005 (44)	A, B	0/44 (0%)	0/44 (0%)	1/44 (2%)	1/44 (2%)	0/44 (0%)
RT011 (23)	A, B	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)	0/23 (0%)
RT012 (13)	A, B	0/13 (0%)	0/13 (0%)	1/13 (8%)	9/13 (69%)	1/13 (8%)
RT013 (4)	A, B	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
RT014 (153)	A, B	0/153 (0%)	0/153 (0%)	13/153 (8.5%)	10/153 (6.5%)	1/153 (0.7%)
RT015[&] (27)	A, B	0/26 (0%)	0/26 (0%)	1/26 (4%)	2/26 (8%)	0/26 (0%)
RT016 (2)	A, B, C	0/2 (0%)	0/2 (0%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
RT017 (3)	A*, B	0/3 (0%)	0/3 (0%)	2/3 (67%)	3/3 (100%)	0/3 (0%)
RT018 (7)	A, B	0/7 (0%)	0/7 (0%)	2/7 (29%)	3/7 (43%)	2/7(29%)
RT020 (38)	A, B	0/38 (0%)	0/38 (0%)	3/38 (8%)	2/38 (5%)	1/38 (3%)
RT023[&] (24)	A, B, C	0/23 (0%)	0/23 (0%)	3/23 (13%)	0/23 (0%)	1/23 (4%)
RT025 (1)	A, B	0/1 (0%)	0/1 (0%)	0/1(0%)	0/1(0%)	0/1 (0%)
RT026 (5)	A, B	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
RT027[&] (31)	A, B, C	0/30 (0%)	1/30 (3%)	26/30 (87%)	25/30 (83%)	19/30 (63%)

Genotyping results		Antimicrobial susceptibility testing				
RT (isolates' number)	**Toxins**	**VA**	**MET**	**MFX**	**CLR**	**RF**
RT029 (15)	A, B	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (0%)	0/15 (0%)
RT036 (1)	A, B, C	0/1 (0%)	0/1 (0%)	1/1 (100%)	1/1 (100%)	1/1 (100%)
RT043 (4)	A, B	0/4 (0%)	0/4 (0%)	0/4 (0%)	1/4 (25%)	0/4 (0%)
RT045 (8)	A, B, C	0/8 (0%)	0/8 (0%)	4/8 (50%)	6/8 (75%)	0/8 (0%)
RT046 (5)	A, B	0/5 (0%)	0/5 (0%)	1/5 (20%)	2/5 (40%)	1/5 (20%)
RT050 (7)	A, B	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	1/7 (14%)
RT053 (1)	A, B	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)
RT054 (2)	A, B	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
RT056 (7)	A, B	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)
RT057 (3)	A, B	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
RT062 (3)	A, B	0/3 (0%)	0/3(0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
RT070 (22)	A, B	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)
RT076 (5)	A, B	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
RT077 (8)	A, B	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)
RT078 (52)	A, B, C	0/52 (0%)	0/52 (0%)	17/52 (33%)	29/52 (56%)	1/52 (2%)
RT081 (14)	A, B	0/14 (0%)	0/14 (0%)	0/14 (0%)	0/14 (0%)	0/14 (0%)
RT087 (6)	A, B	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
RT097 (3)	A, B	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
RT103 (5)	A, B	0/5 (0%)	0/5 (0%)	1/5 (20%)	0/5 (0%)	0/5 (0%)
RT106 (16)	A, B	0/16 (0%)	0/16 (0%)	3/16 (19%)	2/16 (13%)	1/16 (6%)
RT120 (3)	A, B	0/3 (0%)	0/3 (0%)	2/3 (67%)	0/3 (0%)	0/3 (0%)
RT126 (19)	A, B, C	0/19 (0%)	0/19 (0%)	10/19 (53%)	17/19 (89%)	1/19 (5%)
RT127 (2)	A, B, C	0/2 (0%)	0/2 (0%)	1/2 (50%)	1/2 (50%)	0/2 (0%)
RT153 (2)	A, B	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
RT159 (21)	A, B	0/21 (0%)	0/21 (0%)	4/21 (19%)	0/21 (0%)	0/21 (0%)
RT176 (1)	A, B, C	0/1 (0%)	0/1 (0%)	1/1 (100%)	1/1 (100%)	0/1 (0%)
RT181 (2)	A, B, C	0/2 (0%)	0/2 (0%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
RT207 (7)	A, B	0/7 (0%)	0/7 (0%)	0/7 (0%)	1/7 (14%)	0/7 (0%)
RT216 (5)	A, B	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
RT220 (9)	A, B	0/9 (0%)	0/9 (0%)	0/9 (0%)	0/9 (0%)	0/9 (0%)
RT228 (2)	A, B	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
RT258 (7)	A, B	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)
RT276 (1)	A, B	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)
RT328 (6)	A, B	0/6 (0%)	0/6 (0%)	1/6 (17%)	2/6 (33%)	0/6 (0%)

Genotyping results		Antimicrobial susceptibility testing				
RT (isolates' number)	**Toxins**	**VA**	**MET**	**MFX**	**CLR**	**RF**
RT819 (1)	A, B	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)	0/1 (0%)
Other RTs[,$] (107)**	see footnote	0/107 (0%)	0/107 (0%)	4/107 (3.7%)	13/107 (12.1%)	1/106[$] (0.9%)
Total (876)	see for each RT	0/873 (0%)	1/873 (0.1%)	129/873 (14.8%)	159/873 (18.2%)	35/872 (4.0%)

[*] Isolates of this RT (RT017) usually harbor a truncating mutation in the *tcdA* gene, so that only toxin B is expressed.

[**] 95 isolates were positive for both toxins A and B. In twelve isolates, CDT was additionally found.

[&]AST for these RTs could not be done for one isolate per RT due to insufficient growth.

[$] For one isolate, rifampicin could not be tested due to insufficient growth.

The most frequently found RT was RT014 (17.5%), followed by RT001 and the hypervirulent RT078 (~ 7% and 6%, respectively). RT027 was detected only in 3.5% of the whole strain-set.

In a next step, the resistance profiles of the strain-set for clarithromycin, metronidazole, moxifloxacin, rifampicin, and vancomycin were determined. Within the whole toxigenic strain-set, AMR was the highest for clarithromycin (18%), followed by 15% and 4% for moxifloxacin and rifampicin, respectively. Among the RT027 isolates, 87% of the isolates were resistant towards moxifloxacin, 83% towards clarithromycin, and 63% towards rifampicin, respectively. Metronidazole resistance was scarce in this strain-set and encountered in one RT027 isolate only. Vancomycin resistance was not detected in the whole strain-set (**Table 3-9**).

MDR isolates that were non-susceptible towards rifampicin, moxifloxacin, and clarithromycin, were detected in 2.6% (23/873 isolates) over the whole time period monitored. Notably, 74% of the MDR isolates found in this strain-set belonged to RT027 (17/23 isolates). Similarly, RT027 isolates displayed significantly more often a MDR phenotype than non-RT027 isolates (57% vs. 0.7%, *p*-value <0.00001). One RT027 isolate was found in this strain-set that was resistant to clarithromycin, metronidazole, moxifloxacin, and rifampicin.

In order to assess whether there might be changes in MICs for metronidazole or vancomycin over time within the *C. difficile* strain population circulating in German University Hospitals, the respective MIC_{50} and MIC_{90} for the individual sampling phases were determined (**Table 3-10**).

Table 3-10: MIC_{50} and MIC_{90} values of metronidazole and vancomycin in the individual sampling phases of the sentinel surveillance study strain-set.

Sentinel surveillance phase	02/2019	01/2020	02/2020	01/2021
Metronidazole (µg/mL)				
MIC range	0.019-3	0.023-2	0.023-1.5	0.032-1.0
MIC_{50}	0.5	0.5	0.25	0.25
MIC_{90}	1.5	1	0.5	0.5
Vancomycin (µg/mL)				
MIC range	0.19-2	0.094-2	0.094-2	0.19-2
MIC_{50}	0.5	0.5	0.5	0.5
MIC_{90}	1.0	1.0	0.75	0.75

Unlike observed in a German point prevalence study, in which a metronidazole creep was noticed over the last years (Piepenbrock *et al.*, 2019), MIC_{50} and MIC_{90} values for metronidazole and vancomycin remained rather constant (vancomycin) or even declined over time (metronidazole) in this strain-set.

All RTs found within the sentinel surveillance study set (isolates from 2019-2021) were analyzed with respect to their geographic appearance. The regional distributions of these strains according to the source postal codes and the numbers of isolates provided by a certain area are shown in **Figure 3-3A**.

In all postal regions, non-epidemiologically important toxigenic RTs dominated the strain composition, followed by RT014 in most of the geographic areas. Notably, except for postal area 9, only a small proportion of RT027 isolates was observed within the sentinel surveillance study set, with no RT027 isolate being detected in the postal regions 0, 2, and 7, respectively (**Figure 3-3A**). However, it should not be left out that a low amount of isolates (<40) was received for some postal regions such as 1, 7 and 9, which might be one explanation for the overrepresentation of RT027 isolates noticed in postal region 9 if compared to other regions. In order to test whether changes in the RT composition occurred over time, the RT compositions were depicted for each time point analyzed (**Figure 3-3B**).

To exclude outbreaks as a confounding factor that might have compromised this data set, the Simpson's diversity index was calculated in the four time points analyzed in this study. It remained almost stable through all the phases (0.93, 0.93, 0.96, and 0.95, chronologically corresponding to each phase). This analysis revealed that the number of RT001 isolates decreased constantly from 26 isolates in autumn 2019 to 9 isolates in spring 2021 (12% to 3%, $P = 0.0002$). Of note, the RT027 prevalence rates remained rather constant and varied between 2.4 to 4.7% for individual collection phases, and a similar trend was also observed for the epidemiologically relevant RTs 002, 020, and 078.

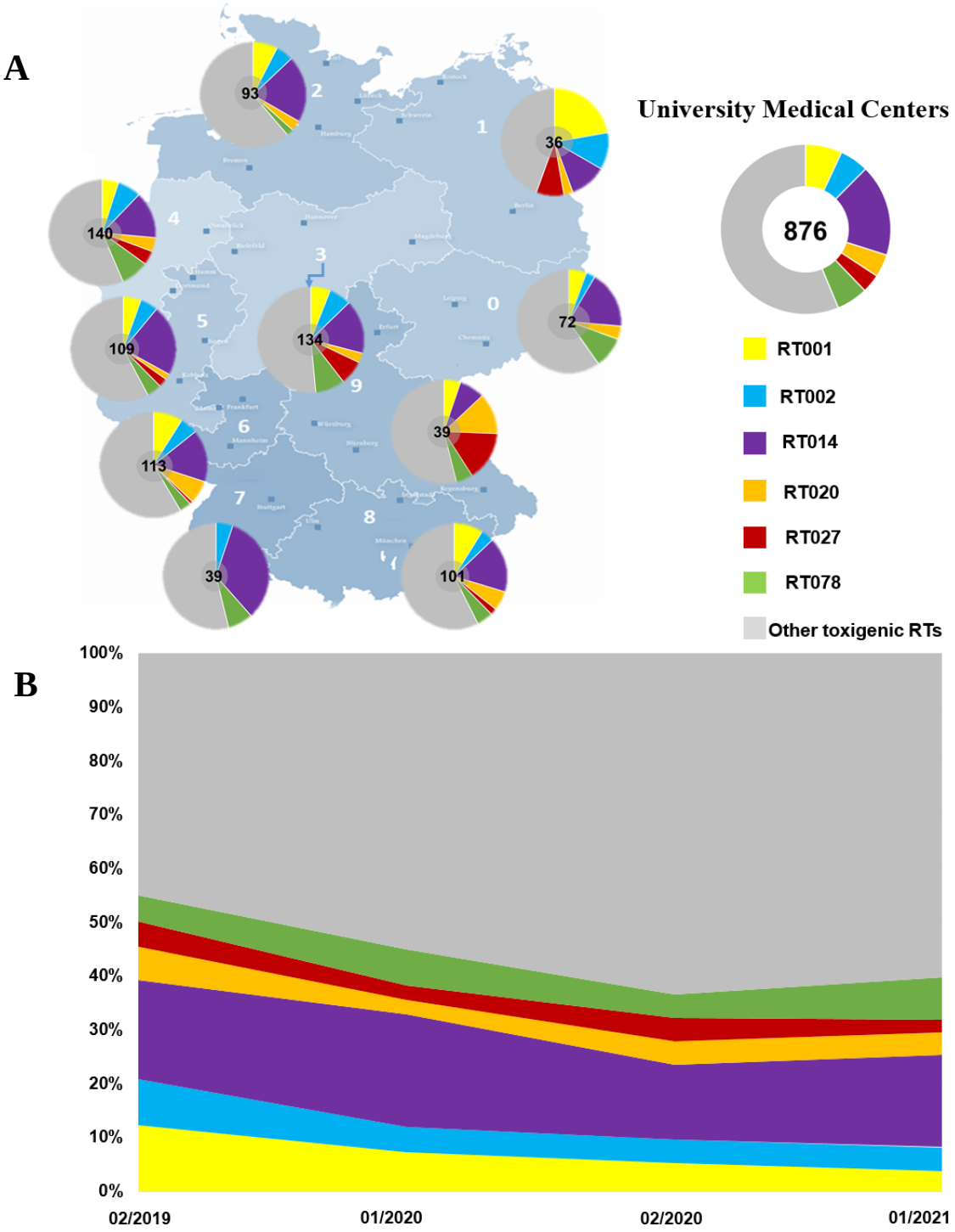

Figure 3-3: Spatial and temporal ribotype (RT) distribution of the *C. difficile* strains belonging to the sentinel surveillance study sampled between 2019 and 2021. RT abundances are displayed in accordance with the (German) postal code of the respective microbiological laboratories (A) and over time (B). Major RTs are indicated by different colors. Numbers in the centers of the map circles indicate the numbers of isolates that were provided by this region. Data are published in (Abdrabou *et al.*, 2022). Adapted with permission from Elsevier for non-commercial purposes.

3.3. MALDI-TOF MS analysis for *C. difficile* RTs subtyping

This part of my thesis was conducted to discover by MALDI-TOF MS potential biomarker peaks that allow for a separation of major RTs of hypervirulent *C. difficile* isolates (HVR) known to cause outbreaks and severe forms of CDI in Europe (*i.e.* RTs 023, 027, 045, 078, 126, 127, and 176) from other major RTs of epidemiological importance such as RTs 001, 014, and 020. To achieve this goal, two testing phases were conducted, a discovery phase in which 157 isolates were tested, and a validation phase, in which 83 additional isolates were used. All isolates were obtained from the stock collection at NRZ as described in **section 2.3.1**. For each isolate in the discovery phase, protein samples were isolated twice and tested by MALD-TOF-MS in four technical replicates. One protein sample, from the new isolates in the validation phase, was extracted and spotted twice on the MALDI-TOF target plate. After acquiring the mass spectra from the MALDI-TOF MS biotyper (**Figure 3-4**), they were uploaded and processed via the CloverBioSoft platform to generate an average spectrum per sample.

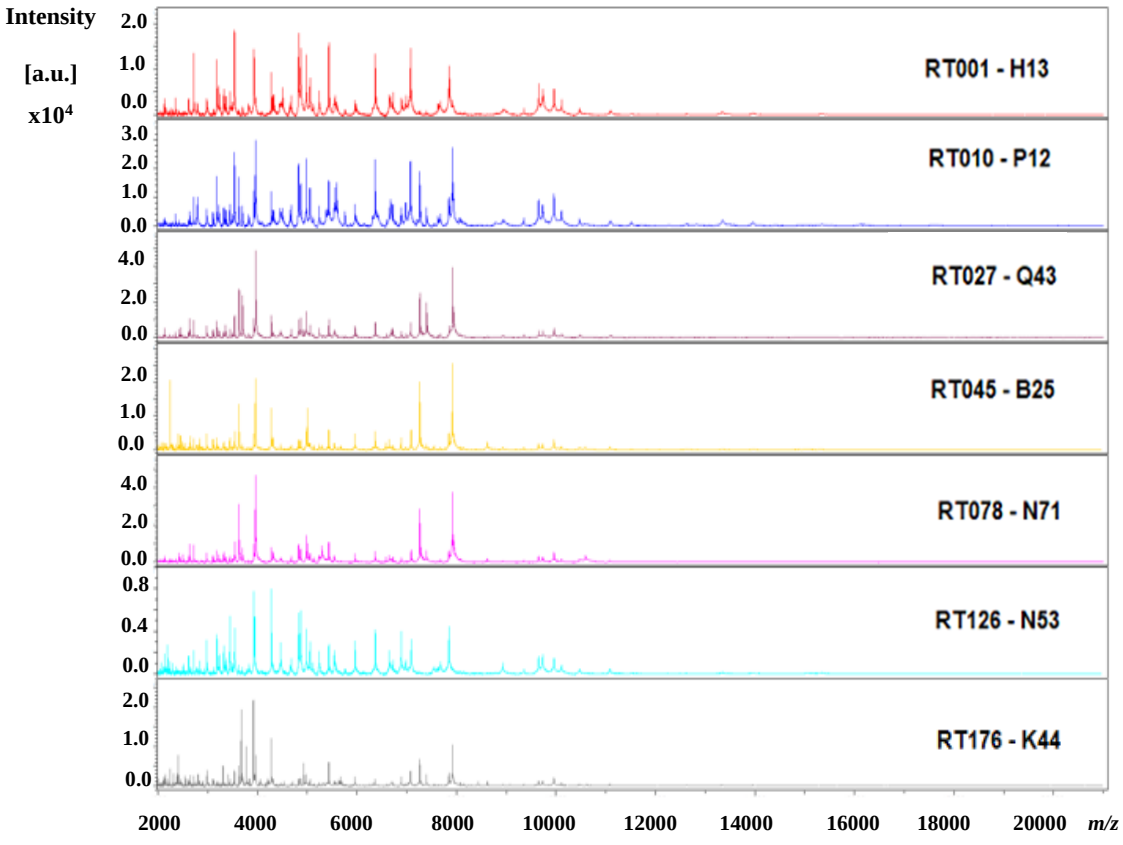

Figure 3-4: Representative spectral profiles of seven *C. difficile* isolates of different ribotypes (RTs) obtained with MALDI-TOF MS biotyper and the FlexAnalysis software. X-axis represents the mass to charge number ratio (*m/z*), and Y-axis represents intensity values in arbitrary unit (a.u.).

3.3.1. Discovery phase of *C. difficile* RTs subtyping

The processed average spectra of all 157 isolates used in this phase were annotated, aligned, and peaks were identified in three different modes (full-spectrum, TICp and pTIC) to generate the respective peak matrices (Candela *et al.*, 2022), which were subsequently used with the average spectra of all isolates as input for a principal component analysis (PCA) to identify potential isolate clusters (**Figure 3-5**).

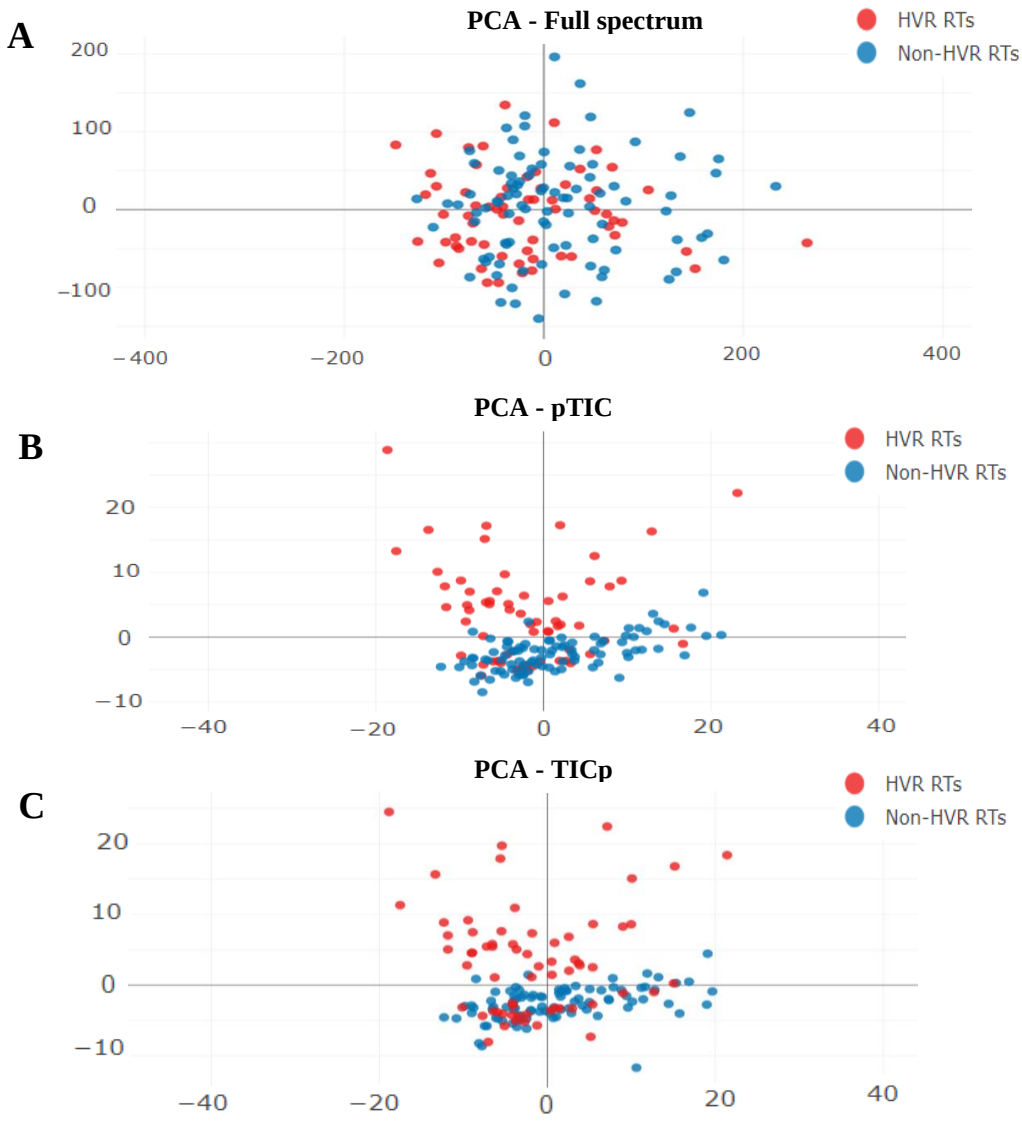

Figure 3-5: Separation of *C. difficile* isolates by principal component analysis (PCA) after applying three independent modes for peaks acquisition: (A) PCA based on the peak matrix obtained with the full spectrum mode (peaks were detected without threshold and then the spectra were normalized). (B) PCA based on the peak matrix obtained with the pTIC mode (peak finding with >1% threshold, followed by a total ion current (TIC) normalization). (C) PCA based on the peak matrix obtained with the TICp mode (TIC normalization followed by peak finding with >1% threshold). Each circle represented one isolate. HVR RTs, hypervirulent *C. difficile* ribotypes; Non-HVR RTs, non-hypervirulent RTs.

The PCA performed with peak matrix obtained with the full spectrum method failed to separate HVR-RTs from non-HVR RTs (**Figure 3-5A**), while the PCAs performed with peak matrices obtained with the TICp or pTIC methods yielded a better separation of both groups (**Figure 3-5B and C**). Interestingly, when looking at the PCA of the TICp mode (**Figure 3-6**), two clusters could be recognized, if isolates of RT027/176 were treated as an individual group; one cluster comprised almost exclusively of HVR RTs isolates belonging to RTs 023, 045, 078, 126, and 127, while the other cluster contained all RT027/176 isolates merging with the isolates of the non-HVR RTs (*i.e.* virulent and non-toxigenic *C. difficile* RTs).

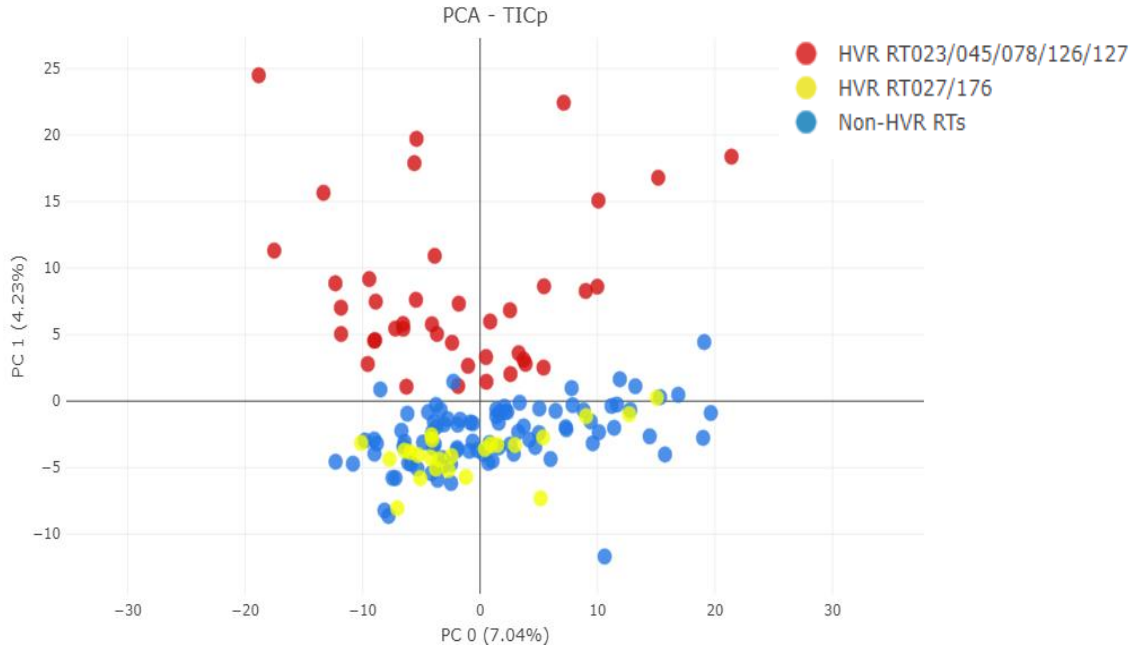

Figure 3-6: Separation of *C. difficile* isolates by PCA after applying the TICp mode. Each circle represented one isolate. Isolates belonging to HVR RTs (RT023, RT045, RT078, RT126, and RT127) are indicated in red, RT027/RT176 isolates in yellow, and isolates belonging to the non-HVR RTs are indicated in blue.

3.3.2. Supervised Machine learning (ML) validation phase of *C. difficile* RTs subtyping

Given the promising result obtained with the peak matrix generated by the TICp method, the TICp peak matrix was used as an input for all downstream analysis. For ML, the following four prediction algorithms were used: K nearest neighbors (KNN), support vector machine (SVM), random forest (RF), and partial least squares–discriminant analysis (PLS-DA). In a first step, the average spectra of the 157 isolates utilized in the discovery phase were re-used as a training set to try to segregate the HVR RTs from the non-HVR RTs. Similar to the PCA outcome presented in (**Figure 3-6**), the classification result of the SVM model failed to discriminate reliably between the HVR RTs and non-HVR RTs (**Figure 3-7A**), and this was again due to RT027/176. However, the other three models (RF, PLS-DA and, to a lesser extent, KNN) were much better in separating the HVR RTs from non-HVR RTs (**Figure 3-7B-D**).

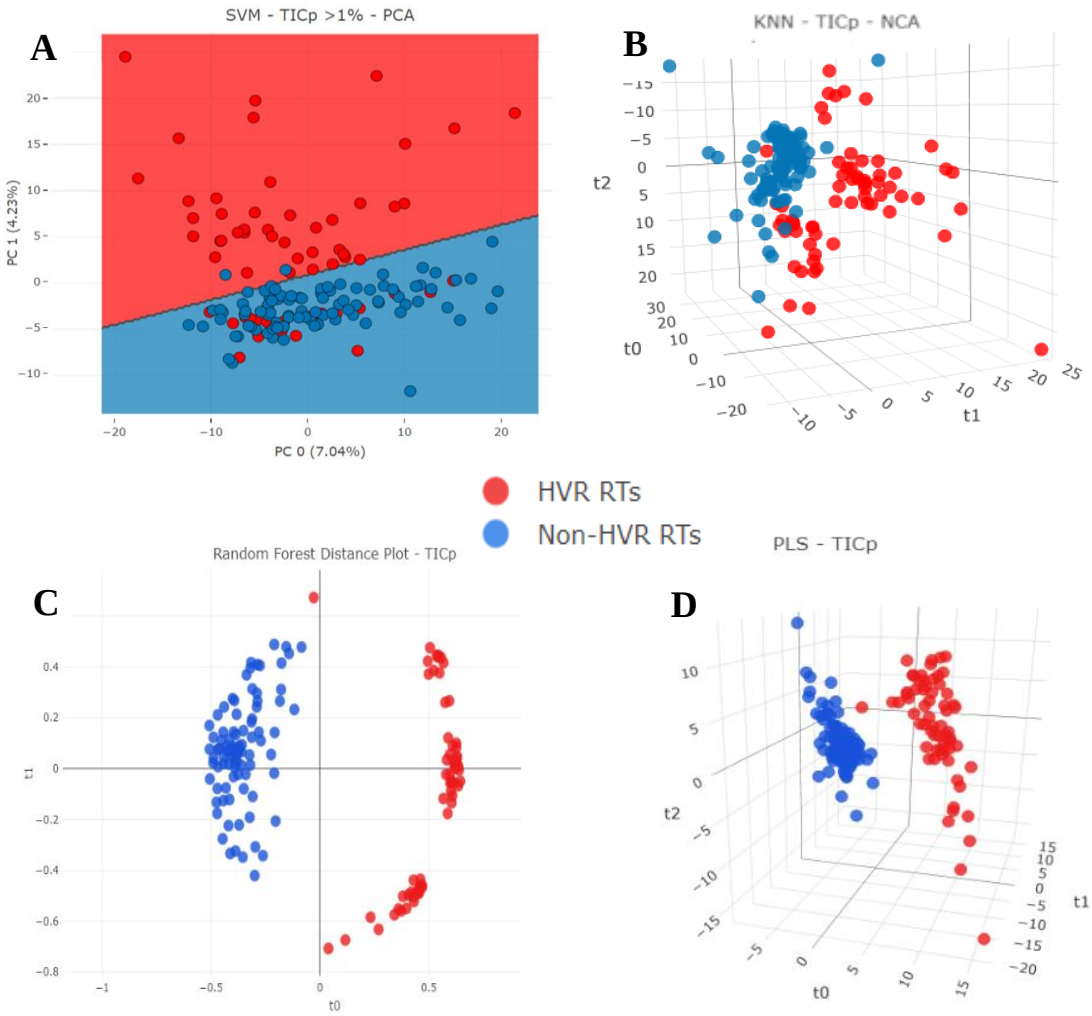

Figure 3-7: Clustering of *C. difficile* isolates based on the TICp peak matrix by machine learning. The TICp peak matrix and the average spectra of the 157 isolates were used as input for the following supervised ML models; (A) **s**upport vector machine (SVM) model, (B) K nearest neighbors (KNN) model, (C) random forest (RF) model, and (D) partial least squares–discriminant analysis (PLS-DA) model. Each circle represented one isolate. Isolates belonging to HVR RTs (RT023, RT027/176, RT045, RT078, RT126, and RT127) are indicated in red, and isolates belonging to the non-HVR RTs are indicated in blue.

When the 157 spectra obtained form the discovery phase strain-set were used in the internal validation of each model (using a 10-fold cross validation), the RF and PLS-DA algorithms achieved the best performance in terms of separation between HVR RT isolates and non-HVR RT isolates, followed by the KNN model with accuracies of 99%, 98% and 93%, respectively, while the SVM algorithm separated both groups with an accuracy of ~ 78% only (**Table 3-11**).

Table 3-11: *C. difficile* RTs subtyping. Cross validation (10-fold) of the prediction bases created by the **s**upport vector machine (SVM), K nearest neighbors (KNN), random forest (RF) and partial least squares–discriminant analysis (PLS-DA) models with 157 isolates. ROC curve: receiver operating characteristic curve, PRC: precision recall curve, AUC: area under the curve, PPV: positive predictive value, NPV: negative predictive value, TP: true positive, TN: true negative, FP: false positive, FN: false negative, HVR: hypervirulent strains, Non-HVR: non-hypervirulent *C. difficile* strains, RTs: ribotypes.

10-fold cross validation [(157 isolates) and HVR RTs is the selected category]			
SVM algorithm			
Actual / Predicted	**HVR RTs**	**Non-HVR RTs**	**% Correct**
HVR RTs	39 (TP)	26 (FN)	60% (Sensitivity)
Non-HVR RTs	8 (FP)	84 (TN)	91.3% (Specificity)
	82.98% (PPV)	76.36% (NPV)	78.34% (Accuracy)
mean ROC (AUC) → 0.74	mean PRC (AUC) → 0.79	F1-score → 69.64%	
KNN algorithm			
Actual / Predicted	**HVR RTs**	**Non-HVR RTs**	**% Correct**
HVR RTs	58 (TP)	7 (FN)	89.23% (Sensitivity)
Non-HVR RTs	4 (FP)	88 (TN)	95.65% (Specificity)
	93.55% (PPV)	92.63% (NPV)	92.99% (Accuracy)
mean ROC (AUC) → 0.94	mean PRC (AUC) → 0.96	F1-score → 91.34%	
RF algorithm			
Actual / Predicted	**HVR RTs**	**Non-HVR RTs**	**% Correct**
HVR RTs	64 (TP)	1 (FN)	98.46% (Sensitivity)
Non-HVR RTs	0 (FP)	92 (TN)	100% (Specificity)
	100% (PPV)	98.92% (NPV)	99.36% (Accuracy)
mean ROC (AUC) → 0.98	mean PRC (AUC) → 0.99	F1-score → 99.22%	
PLS- DA algorithm			
Actual / Predicted	**HVR RTs**	**Non-HVR RTs**	**% Correct**
HVR RTs	64 (TP)	1 (FN)	98.46% (Sensitivity)
Non-HVR RTs	1 (FP)	91 (TN)	98.91% (Specificity)
	98.46% (PPV)	98.91% (NPV)	98.73% (Accuracy)
mean ROC (AUC) → 0.99	mean PRC (AUC) → 1.00	F1-score → 98.46%	

The SVM algorithm struggled again mainly with the RT027/176 isolates, which were falsely identified as non-HVR RTs in 22/26 cases. Once more, all the seven cases misidentified as non-HVR RTs in the KNN model belonged to RT027/176 isolates. The same apply for the other two models regarding the misdiagnosed HVR isolate, which was RT176.

To externally validate the prediction bases generated by the aforementioned models in the discovery phase, average spectra of 83 additional blinded isolates were generated, and each spectrum was tested with the four prediction bases to assign them to the HVR RT or non-HVR RT groups (**Table 3-12**).

Table 3-12: *C. difficile* RTs subtyping. External validation of the prediction bases created by the **s**upport vector machine (SVM), K nearest neighbors (KNN), random forest (RF) and partial least squares–discriminant analysis (PLS-DA) models with 83 blinded isolates. ROC curve: receiver operating characteristic curve, PRC: precision recall curve, AUC: area under the curve, PPV: positive predictive value, NPV: negative predictive value, TP: true positive, TN: true negative, FP: false positive, FN: false negative, HVR: hypervirulent strains, Non-HVR: non-hypervirulent *C. difficile* strains, RTs: ribotypes.

External validation [(83 new isolates) and HVR RTs is the selected category]			
SVM algorithm			
Actual / Predicted	**HVR RTs**	**Non-HVR RTs**	**% Correct**
HVR RTs	31 (TP)	8 (FN)	79.49% (Sensitivity)
Non-HVR RTs	1 (FP)	43 (TN)	97.73% (Specificity)
	96.88% (PPV)	84.31% (NPV)	89.16% (Accuracy)
mean ROC (AUC) → 0.94	mean PRC (AUC) → 0.95	F1-score → 87.33%	
KNN algorithm			
Actual / Predicted	**HVR RTs**	**Non-HVR RTs**	**% Correct**
HVR RTs	33 (TP)	6 (FN)	84.62% (Sensitivity)
Non-HVR RTs	6 (FP)	38 (TN)	86.36% (Specificity)
	84.62% (PPV)	86.36% (NPV)	85.54% (Accuracy)
mean ROC (AUC) → 0.91	mean PRC (AUC) → 0.92	F1-score → 84.62%	
RF algorithm			
Actual / Predicted	**HVR RTs**	**Non-HVR RTs**	**% Correct**
HVR RTs	39 (TP)	0 (FN)	100% (Sensitivity)
Non-HVR RTs	1 (FP)	43 (TN)	97.73% (Specificity)
	97.5% (PPV)	100% (NPV)	98.8% (Accuracy)
ROC (AUC) → 0.98	PRC (AUC) → 0.98	F1-score → 98.73%	
PLS- DA algorithm			
Actual / Predicted	**HVR RTs**	**Non-HVR RTs**	**% Correct**
HVR RTs	38 (TP)	1 (FN)	97.44% (Sensitivity)
Non-HVR RTs	1 (FP)	43 (TN)	97.73% (Specificity)
	97.44% (PPV)	97.73% (NPV)	97.59% (Accuracy)
ROC (AUC) → 0.96	PRC (AUC) → 0.97	F1-score → 97.44%	

In this validation phase, the RF and PLS-DA models showed the best performance by assigning the blinded isolates to their groups with an accuracy >97%, while the KNN and the SVM models, in comparison, allowed for a correct assignment in only 85% and 89% of the cases, respectively. RT027 isolates, wrongly assigned to the non-HVR RTs group, were 75% and 50% of the cases in the SVM and the KNN models, respectively. The misidentified isolate in the PLS-DA model belonged to the RT078.

In the CloverBioSoft platform used, the RF model also allows to display the partial contribution of each peak of a given peak matrix to discriminate between the groups as illustrated in **Figure 3-8**.

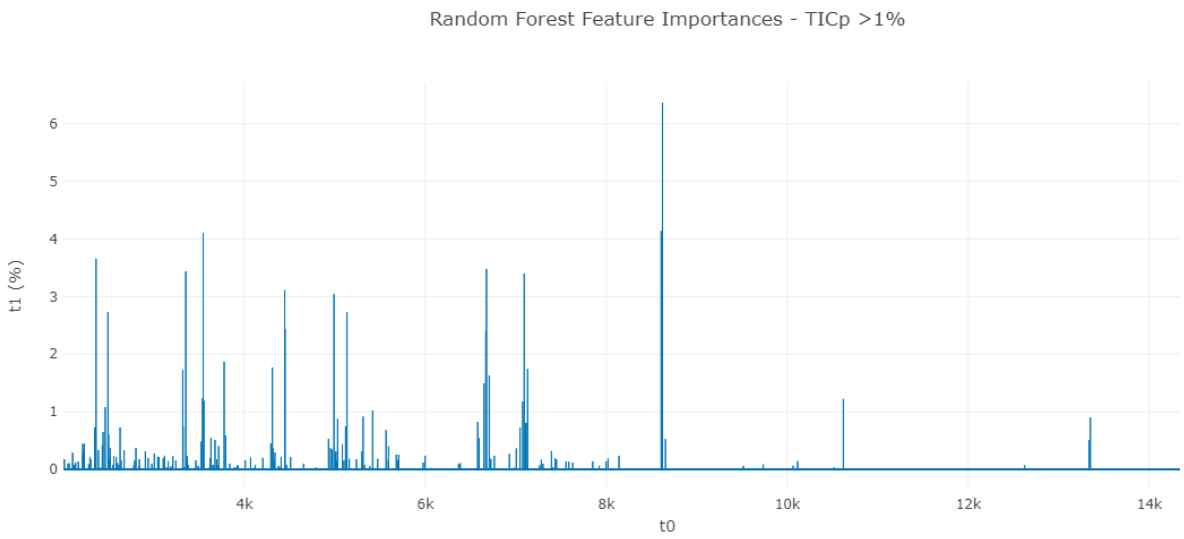

Figure 3-8: Marginal contribution of potential biomarker peaks identified by the random forest model based on TICp mode, which could discriminate between the isolates of the hypervirulent ribotypes (HVR RTs) and the non-HVR RTs.

The RF model showed that the following peaks 2361, 2493, 3353, 3545, 4445, 4990, 6675, 7092, 8605 and 8620 *m/z* are the top ten key features that contribute to the segregation of HVR RTs with the latter peak holding >5% of the total RF discriminatory power.

3.3.3. Discovery phase of HVR RTs subtyping

From the epidemiological point of view, it is crucial to delineate the circulating RTs in the hospital setting. In order to check for the MALDI-TOF MS capability to subtype the HVR *C. difficile* isolates, the processed average spectra of 65 HVR isolates used in this investigation were first annotated, aligned, and peaks were detected in TICp mode. The TICp peak matrix was used then to run a PCA to visualize the assorted clusters (**Figure 3-9**).

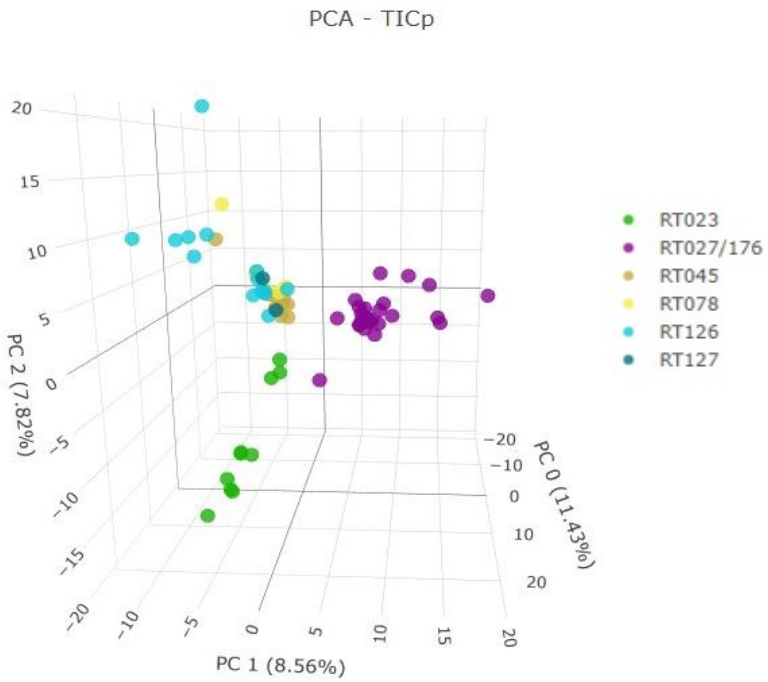

Figure 3-9: Separation of 65 hypervirulent *C. difficile* isolates by principal component analysis (PCA) after applying the TICp mode. Each circle represented one isolate. RT: ribotype.

Interestingly, when looking at the PCA, one can distinguish three clusters; one cluster comprising the RTs 045/078/126/127 isolates, another one for the RT023 isolates (except for three isolates that were slightly shifted towards the previous cluster) and the last one for the RT027/176 isolates.

3.3.4. Supervised Machine learning (ML) validation phase of HVR RTs subtyping

Given the promising PCA results with the HVR strain-set, the TICp peak matrix was re-utilized with the four ML prediction algorithms KNN, PLS-DA, and RF, and SVM. The average spectra of the 65 HVR isolates were used again here as a training set to subtype the HVR RTs isolates. All classification results obtained with the aforementioned algorithms supported the PCA outcome presented in **Figure 3-9** and were able to separate the three subgroups as illustrated in **Figure 3-10**.

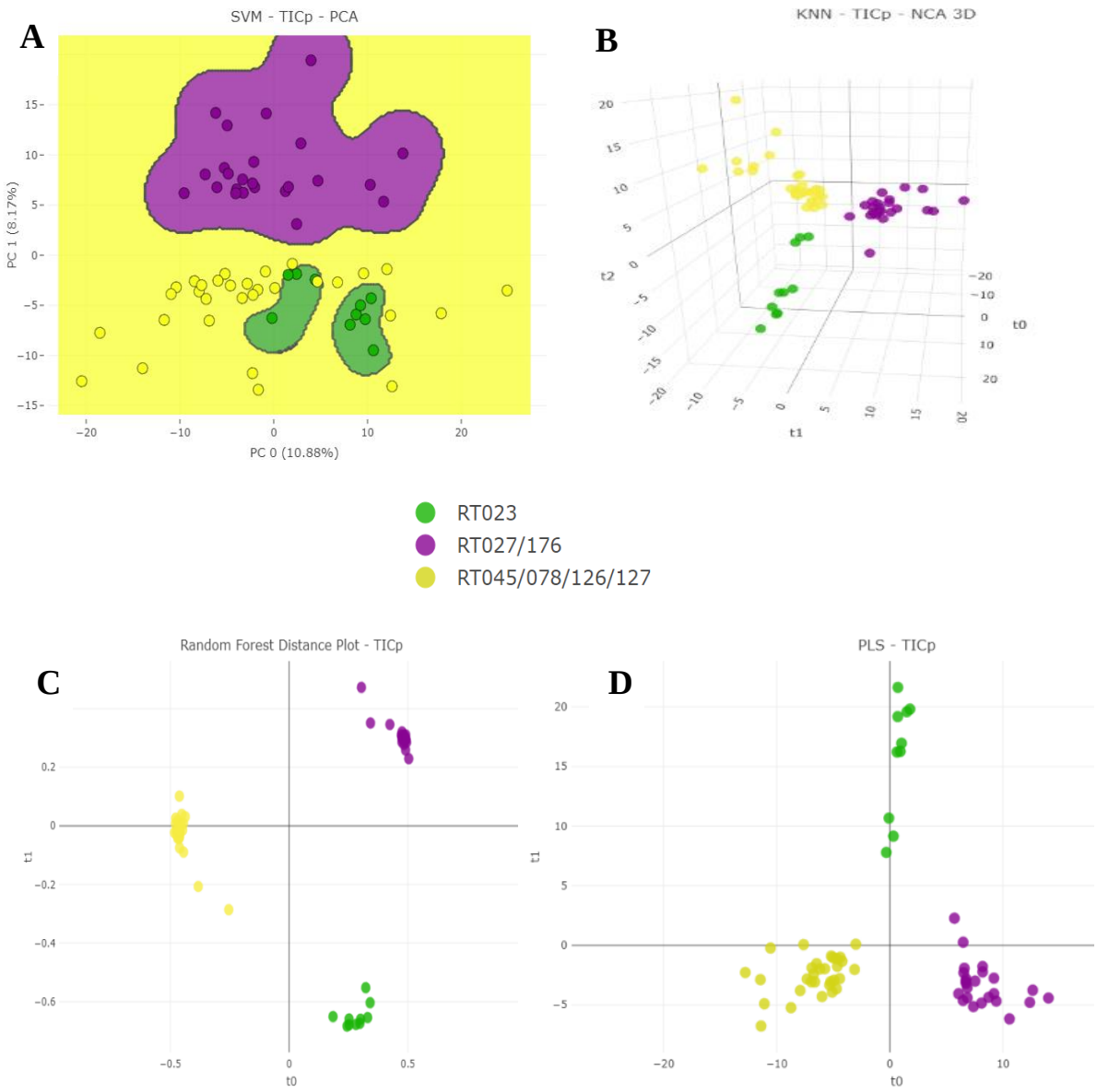

Figure 3-10: Clustering of hypervirulent (HVR) *C. difficile* isolates based on the TICp peak matrix by machine learning. The TICp peak matrix and the average spectra of the 65 isolates were used as input for the following supervised ML models; (A) **s**upport vector machine (SVM) model, (B) K nearest neighbors (KNN) model, (C) random forest (RF) model, and (D) partial least squares–discriminant analysis (PLS-DA) model. Each circle represented one isolate. Isolates belonging to RT023 are indicated in green, while isolates of RT027/176 are designated in violet. Other HVR RTs isolates are shown in yellow. RTs, ribotypes.

Once more as illustrated in the PCA result, except for the RF model, three isolates of the RT023 moved to some extent near the RTs 045/078/126/127 cluster in the other three models (SVM, KNN and, to a lesser extent, PLS-DA). The avarage spectra of the 65 HVR strains were used in the internal validation of each model using a 10-fold cross validation, and results of these analyses are presented in **Table 3-13**.

Table 3-13: Hypervirulent RTs subtyping. Cross validation (10-fold) of the prediction bases created by the **s**upport vector machine (SVM), K nearest neighbors (KNN), random forest (RF) and partial least squares–discriminant analysis (PLS-DA) models with 65 isolates. ROC curve: receiver operating characteristic curve, PRC: precision recall curve, AUC: area under the curve, RTs: ribotypes.

10-fold cross validation (65 isolates)				
SVM algorithm				
Actual/Predicted	**RT023**	**RTs 027/176**	**RTs 045/078/126/127**	**% Correct**
RT023	7	0	3	70%
RTs 027/176	0	24	0	100%
RTs 045/078/126/127	5	0	26	83.87%
AUC (ROC, PRC)	(0.94, 0.64)	(0.99, 1.0)	(0.90, 0.92)	87.69%
KNN algorithm				
Actual/Predicted	**RT023**	**RTs 027/176**	**RTs 045/078/126/127**	**% Correct**
RT023	10	0	0	100%
RTs 027/176	1	23	0	95.8%
RTs 045/078/126/127	0	0	31	100%
AUC (ROC, PRC)	(0.98, 0.99)	(0.99, 1.0)	(0.99, 1.0)	98.5%
RF algorithm				
Actual/Predicted	**RT023**	**RTs 027/176**	**RTs 045/078/126/127**	**% Correct**
RT023	10	0	0	100%
RTs 027/176	0	24	0	100%
RTs 045/078/126/127	0	0	31	100%
AUC (ROC, PRC)	(0.99, 1.0)	(0.99, 1.0)	(0.99, 1.0)	100%
PLS- DA algorithm				
Actual/Predicted	**RT023**	**RTs 027/176**	**RTs 045/078/126/127**	**% Correct**
RT023	10	0	0	100%
RTs 027/176	0	24	0	100%
RTs 045/078/126/127	0	0	31	100%
AUC (ROC, PRC)	(0.99, 1.0)	(0.99, 1.0)	(0.94, 0.96)	100%

The 10-fold cross validation results for the RF and PLS-DA algorithms both showed an accuracy of 100%, while the cross validations with the other two algorithms (KNN and SVM) felt slightly behind and showed accuracies of 98.5% and 88%, respectively. To externally validate the ML models, average spectra of 39 new blind isolates were included and aligned with the prediction model to assign them to the corresponding HVR RT subgroup (**Table 3-14**).

Table 3-14: Hypervirulent RTs subtyping. External validation of the prediction bases created by the support vector machine (SVM), K nearest neighbors (KNN), random forest (RF) and partial least squares–discriminant analysis (PLS-DA) models with 39 blinded isolates. ROC curve: receiver operating characteristic curve, PRC: precision recall curve, AUC: area under the curve, RT: ribotype.

External validation (39 new isolates)				
SVM algorithm				
Actual/Predicted	**RT023**	**RTs 027/176**	**RTs 045/078/126/127**	**% Correct**
RT023	0	0	9	0%
RTs 027/176	0	6	1	85.7%
RTs 045/078/126/127	5	0	18	87.3%
AUC (ROC, PRC)	(0.43, 0.24)	(0.99, 0.98)	(0.75, 0.82)	61.5%
KNN algorithm				
Actual/Predicted	**RT023**	**RTs 027/176**	**RTs 045/078/126/127**	**% Correct**
RT023	1	1	7	11.11%
RTs 027/176	0	7	0	100%
RTs 045/078/126/127	0	1	22	95.65%
AUC (ROC, PRC)	(0.6, 0.7)	(0.98, 0.94)	(0.84, 0.91)	76.92%
RF algorithm				
Actual/Predicted	**RT023**	**RTs 027/176**	**RTs 045/078/126/127**	**% Correct**
RT023	8	0	1	88.9%
RTs 027/176	0	7	0	100%
RTs 045/078/126/127	0	0	23	100%
AUC (ROC, PRC)	(1, 1)	(1, 1)	(1, 1)	97.44%
PLS- DA algorithm				
Actual/Predicted	**RT023**	**RTs 027/176**	**RTs 045/078/126/127**	**% Correct**
RT023	8	0	1	88.89%
RTs 027/176	0	7	0	100%
RTs 045/078/126/127	1	0	22	95.7%
AUC (ROC, PRC)	(0.96, 0.74)	(1.0, 1.0)	(0.96, 0.98)	94.87%

Utilization of the RF model allowed to correctly assign the blinded isolates to one of the three groups with an accuracy of 97%, followed by the PLS-DA model with an accuracy of 95%, while the KNN and SVM models correctly assigned a blind isolate to its correct group with accuracies of 77% and 62%, respectively, as they could not differentiate RT023 isolates from the RT045/078/126/127 subgroup, however they still can subtype the HVR RTs in two subclusters.

In addition, the RF model also allows to display the partial contribution of each peak of a given peak matrix to discriminate between the HVR RTs subgroups as illustrated in **Figure 3-11**.

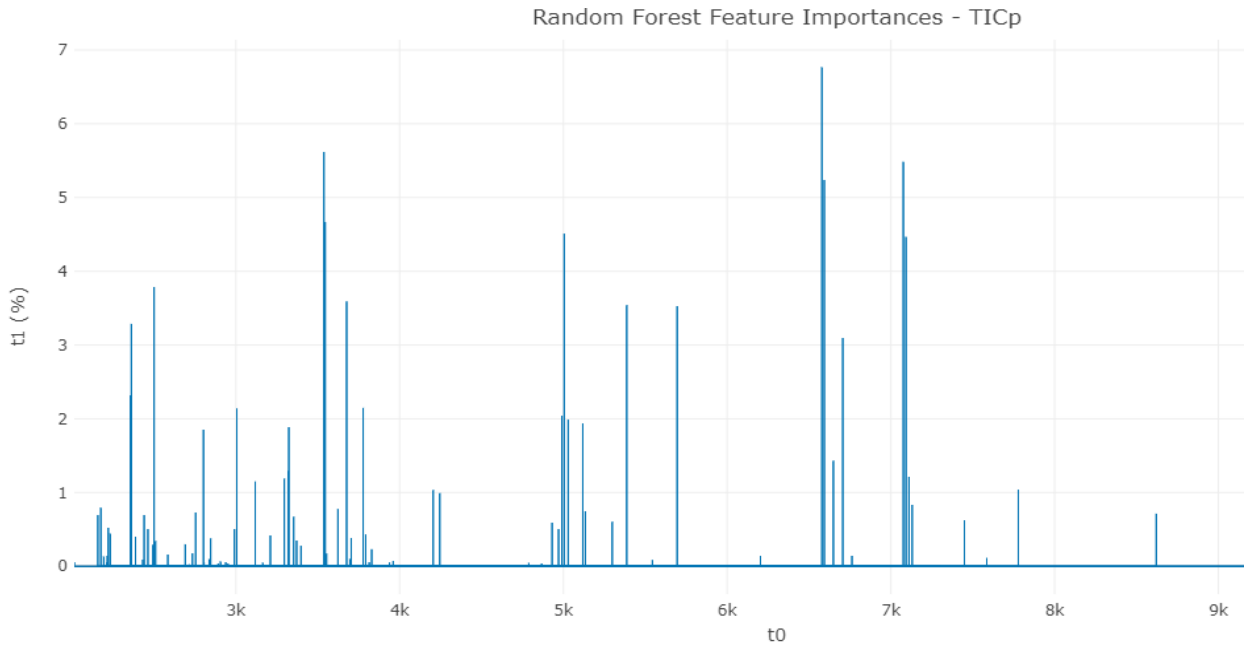

Figure 3-11: Marginal contribution of potential biomarker peaks identified by the random forest model based on TICp mode, which could discriminate between the isolates of the hypervirulent ribotypes (HVR RTs) subgroups.

The RF model identified the following peaks 2500, 3537, 3545, 3676, 5005, 5694, 6578, 6593, 7076 and 7092 *m/z* as the top ten key players that contribute to the subtyping of the HVR RTs, holding >45% of the total RF discriminatory power.

3.4. Disparate colony morphotype in certain hypervirulent RTs

On subculturing the *C. difficile* strains of various RTs on TSBA, two distinct colony morphotypes could be observed over time, a compact colony variant (CCV) phenotype displaying small, whitish, and rough colonies, which could be observed with isolates of the following RTs (RT045, 078, 126 and 127) as depicted in **Figure 3-12A**, while the majority of isolates of other RTs exhibited a motile colony variant (MCV) phenotype characterized by larger sized colonies that spread much faster over time, presumably due to swarming as in **Figure 3-12B.**

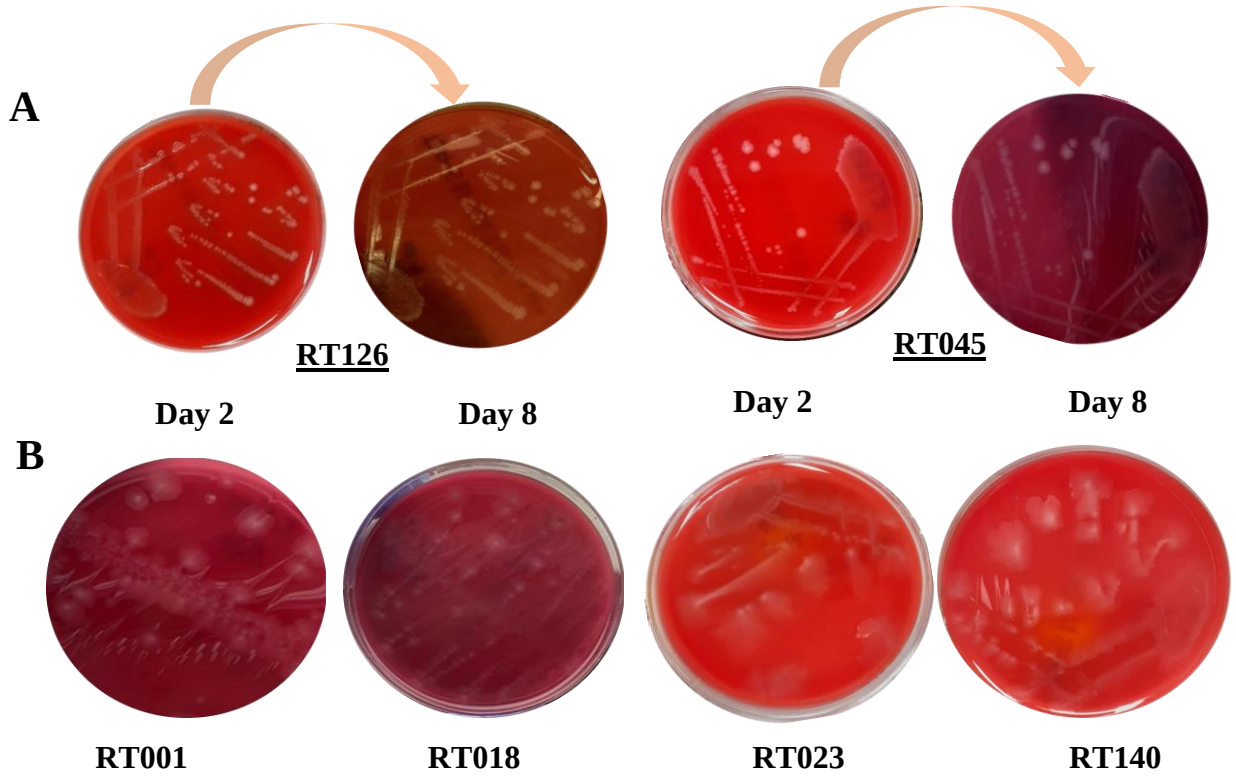

Figure 3-12: Distinct *C. difficile* morphotypes: Compact Colony Variant (CCV); non-motile, small, whitish and rough versus Motile Colony Variant (MCV); motile, large, grey, swarming and smooth. (A) CCV on culture day two and culture day eight for RT126 (left side) and RT045 (right side) strains. (B) Motile and frayed appearance of MCV on culture day two for representative RT001, RT018, RT023 and RT140 strains.

4. Discussion

4.1. New drug candidates against *C. difficile*

In times of emerging antibiotic resistance, the need for new substances is evident (WHO, 2021). One of the bacterial species where MDR is of particular concern is *C. difficile*, which is listed by the US Centers of Disease Control and Prevention (CDC) in 2019 as one of the major threats, for which new antibiotics are urgently needed (CDC, 2019). Several drug candidates for CDI therapy are in the pipeline of development. Most substances that have been tested in clinical trials so far, such as omadacycline and eravacycline, were found to be non-inferior to vancomycin or fidaxomicin (Begum *et al.*, 2020; Bassères *et al.*, 2020). However, cadazolid failed to show a good activity and will not be of use in CDI therapy (Gerding *et al.*, 2019).

Natural compounds can be used for treatment of a broad variety of diseases. Several different microbial species such as *Bacillus amyloliquefaciens* and some *Streptomyces* spp. excrete for instance products capable of modulating the immune response after the transplantation to prevent graft rejections or suppress the malignant tumors' growth (Chen *et al.*, 2009; Kong *et al.*, 2019; Medeot *et al.*, 2019). Prominent examples for natural substances used as an immunosuppressant medication are rapamycin and cyclosporine, respectively, which are also reported to exhibit antibacterial and/or antifungal activities (Mann, 2001; Newman and Cragg, 2016; Katz and Baltz, 2016; Medeot *et al.*, 2019).

Important contributors for natural compounds acting against bacteria are the soil-dwelling Gram-negative *Myxobacteria* or the Gram-positive *Actinomycetes* species, which produce a multitude of bioactive substances or secondary metabolites with biocide action (Wenzel and Müller, 2009; Schäberle *et al.*, 2014; Sucipto *et al.*, 2017). Terrestrial or marine *Myxobacteria* produce for instance hundreds of natural agents (Schäberle *et al.*, 2014). Some of them possess antifungal and antibacterial activity such as cyclopropyl-polyene-pyran acid ambruticin (Ringel *et al.*, 1977), while other compounds like epothilones A-F and their analogues exhibit promising anti-cancer activities (Li *et al.*, 2017).

An ideal therapeutic agent against CDI should attain the following features. Firstly, such a substance displays a high selectivity for *C. difficile*, at best without causing further dysbiosis of the already altered gut flora. Secondly, resistant mutants should appear (if at all) at a low frequency. Thirdly, the antibiotic should achieve a high bioactive level in the colonic lumen. Fourthly, it should not provoke *C. difficile* to express its virulence traits (*e.g.* the exotoxins). Lastly, the likelihood of a CDI relapse after a tapering regimen should be low (Baines and Wilcox, 2015). In the search for new antimicrobial compounds with an activity against *C. difficile*, the argyrin family could be identified.

Argyrin derivatives (A to H) are cyclic peptides (Vollbrecht *et al.*, 2002) produced by the genera *Archangium, Cystobacter,* and *Actinoplanes,* respectively (Sasse *et al.*, 2002; Selva *et al.*, 1996). Primarily, argyrins A and B were termed A21459 antibiotics (Selva *et al.*, 1996). Similar to vancomycin and penicillin, argyrins are peptides assembled by the megasynthetase machinery line of non-ribosomal peptide synthetases (NRPSs) encoded by five genes (*arg12345*) forming one operon called (argyrin gene cluster) (Süssmuth and Mainz, 2017; Pogorevc *et al.*, 2019).

Argyrin A was shown to exhibit an antitumor effect by stimulating the apoptosis and blocking the angiogenesis mediated by the cyclin-dependent kinase inhibitor (CDKI) $p27^{KIP1}$. Higher levels of $p27^{KIP1}$ can be maintained in the mammalian cell via inhibition of its proteasomal degradation by argyrin A (Nickeleit *et al.*, 2008; Stauch *et al.*, 2010). Argyrin B has been described to act as a strong suppressor of antibody production by murine B cells (Sasse *et al.*, 2002), and argyrin C was shown to improve the autoimmune response by binding to the mitochondrial elongation factor G1 (mEF-G1), thereby leading to downregulation of the T helper 17 (Th17) activation pathway, especially of interleukins (IL)17 and 23 (Almeida *et al.*, 2021). Argyrin A-D were also found to exert good antibiotic activity against *Pseudomonas* spp. with IC_{50} values around 50-140 ng/mL, and some of them were also active against *Staphylococcus aureus* (argyrin A and C, respectively), however, on a smaller level (Sasse *et al.*, 2002).

Subsequent work indicated that argyrin A and B interact with elongation factor G (EF-G) of *Pseudomonas aeruginosa* as the well-known steroid antibiotic fusidic acid, however, at a different allosteric pocket (Nyfeler *et al.*, 2012; Bielecki *et al.*, 2012; Wieland *et al.*, 2022). Argyrin B was also found to be active against other pathogens such as *Stenotrophomonas maltophila* and *Burkholderia multivorans* with MICs in the low µg/mL range, but ineffective against *Acinetobacter baumannii* and *E. coli* (MIC >64 µg/mL), probably due to efflux pumps in the latter pathogen that appear to extrude argyrin (Jones *et al.*, 2017). However, the impact of argyrins on *Clostridium* spp. and *C. difficile* has not been studied yet.

In order to fill this gap, work was carried out to verify the potential activity of 22 argyrin derivatives, against different *Clostridium* spp. type strains and a small selection of *C. difficile* isolates. My studies revealed that 12 out of the 22 argyrin derivatives tested showed a promising activity against *C. difficile* in the low to mid ng/mL range, if determined by broth microdilution testing. Of note, the lowest MIC was detected for *C. difficile* and argyrin 2 with 4.7 ng/mL, which is about 200 to 1000-fold below the MIC values reported for argyrin B and *P. aeruginosa, S. maltophila,* and *B. multivorans,* respectively, which all are in the µg/mL level (Jones *et al.*, 2017).

Given the promising initial screening results with small set of *C. difficile* strains, antimicrobial resistance testing was carried out for the three most promising argyrin candidates with a more representative strain-set. Specifically, MIC_{50} and MIC_{90} for argyrin 2, argyrin B, and argyrin 12 were determined with

51 representative *C. difficile* strains stemming from the Saarland University Hospitals (UKS) routine laboratory, which yielded MIC_{50} of 6.25, 12.5, 6.25 ng/mL and MIC_{90} of 6.25, 25, 9.38 ng/mL, respectively.

These findings suggest that argyrin derivatives such as **argyrin 2, argyrin B, and argyrin 12** are very promising drug candidates for the treatment of CDI, which fulfilled at least two of the five above mentioned criteria. However, it should not left unnoticed that these argyrin derivatives suppressed the growth of *C. difficile* strains with a comparable efficacy, irrespective of their toxin profile, thus eliminating a protective effect of non-toxigenic *C. difficile* strains when this agent is used for therapy. However, all three derivatives did not affect the growth of any of the probiotic *Clostridium* spp. type strains [*i.e. C. butyricum* (Stoeva *et al.*, 2021) and *C. sporogenes* (Guo *et al.*, 2020)] or *C. perfringens* [another gut pathogen causing food poisoning (Uzal *et al.*, 2014)] tested here at concentrations up to 1µg/mL (the highest concentration tested due to limitations in the compound availability), and it can be safely assumed that the MIC values for these species are in the µg/mL range as well. Up to this date, only very few drugs exhibit such low MIC values against *C. difficile*. One example that proved a persuasive *in vitro* activity against *C. difficile* is the tetracycline derivative omadacycline (sold under the brand name Nuzyra) with MIC_{50} and MIC_{90} values at 31 ng/mL each (Begum *et al.*, 2020).

One major factor for the success of antimicrobial CDI therapy is the orchestrated action by the gut microbiota colonization. This barrier should not be disturbed any further by the therapeutic agent, allowing for restoration of the healthy gut flora. This is one of the main advantage of fidaxomicin, which is far more selective than the other recommended CDI agents and does not kill bacterial members of the healthy gut flora such as *Bacteroides fragilis* (Chilton *et al.*, 2014; Baines and Wilcox, 2015), while vancomycin exhibits a rather broad activity against Gram-positive bacteria, negatively affects the host bile acid metabolism and the nephrotoxicity limits its usage especially in intensive care unit patients (Vrieze *et al.*, 2014; Modi *et al.*, 2014; Reijnders *et al.*, 2016; Park *et al.*, 2018).

To get an idea about the influence of argyrins on the intestinal microflora, three important bacterial members of the healthy gut flora (*C. scindens*, *B. bifidum* and *B. fragilis*) were tested against argyrin B, for which sufficient substance was available. All three strains tested displayed elevated MICs against argyrin B (>1 µg/mL), suggesting that they are, if at all, not much affected by this substance. The low activity of argyrins B, 2 and 12 against *C. scindens* is of particular interest, as this species is a key player in the synthesis of secondary bile acids, which by turns inhibit the germination of the *C. difficile* spores (Greathouse *et al.*, 2015).

One major problem associated with the usage of argyrin B might be the development of resistance (Martinez and Baquero, 2000). Earlier work demonstrated that exposition of *B. multivorans* and *P. aeruginosa* to higher concentration of argyrin B (4x MIC) led to point mutations in *fusA*, the gene encoding the argyrin A and B target EF-G, and yielded in mutants that displayed increased MIC values for

argyrin B (Nyfeler *et al.*, 2012; Jones *et al.*, 2017). Exposition of *S. maltophilia* to higher concentrations of argyrin B, on the other hand, induced mutations that inactivated FusA1 expression, probably because this species encodes a second *fusA* homolog (*fusA2*) that seems not to be affected by the argyrins and thus allows for the inactivation of *fusA1* rather than creating point mutations in *fusA1* (Jones *et al.*, 2017). Notably, when challenged with argyrin concentrations 4x the MIC, *P. aeruginosa* and *B. multivorans* formed mutants resistant to this substance with frequencies of 5×10^{-8} and 1×10^{-8} to 1×10^{-9}, respectively, while *S. maltophilia* produced under these conditions mutants able to grow on 4x the MIC with a frequency of 1×10^{-7} (Nyfeler *et al.*, 2012; Jones *et al.*, 2017).

In order to estimate the mutant frequency of argyrin B in *C. difficile*, type strain DSM28645 was challenged with 4x the MIC of this compound, which resulted in a mutant frequency of about 5×10^{-9}, suggesting that the resistance formation risk in this species is considerably lower than in *B. multivorans*, *P. aeruginosa* and *S. maltophilia*, but might still happen. With respect to this, it is worth mentioning that the same fear was expressed for fidaxomicin after its introduction into clinical use. Up to this date, no such development was reported yet (Freeman *et al.*, 2018; Putsathit *et al.*, 2021). However, most studies reporting on antimicrobial resistance testing of *C. difficile* did not regularly determine the resistance towards fidaxomicin, due to the expensive nature of the substance and the lack of a commercial testing scheme.

The *C. difficile* mutants that grew in presence of 100 ng/mL of argyrin B are currently under molecular investigation by using WGS (conducted by the NRZ Münster branch, University of Münster, Germany) to identify genetic changes that might be responsible for the increased argyrin B MICs of these mutants. In addition, binding studies are planned by HIPS for confirmation of the cellular target (EF-G), as encountered in *P. aeruginosa* (Nyfeler *et al.*, 2012).

Although this work clearly suggests that argyrin B exhibits an excellent antimicrobial activity against *C. difficile in vitro* while being rather ineffective against other members of the gut microbiota, this is only the first step for implementation of this substance as an option for antibiotic therapy. Animal models investigating the *in vivo* susceptibility, the pharmacokinetics as well as the clinical outcome are needed to confirm its usability and evaluate the CDI recurrence possibility. Furthermore, clinical trials with humans are mandatory as well to show non-inferiority towards the established therapy schemes, *i.e.* including, particularly the usage of vancomycin and fidaxomicin, respectively. In order to show that argyrin B does not affect the indigenous microbiota in a more negative way than other therapeutic agents do, further studies including state-of-the-art microbiome analysis are needed since only few important members of the normal gut flora of humans could be tested here.

In addition to the argyrins, a couple of further compounds were tested in this work for the first time against *C. difficile*. These candidates bear the advantage of selective toxicity, *i.e.* their target resides only in the prokaryotic cell and not the eukaryotic one. Nucleoside analogues target the viral polymerase or the species-specific bacterial deoxyribonucleoside kinases (Thomson and Lamont, 2019), and ECF transporters inhibitors attack the ECF one way transporters that are absent in humans (Rempel *et al.*, 2019; Bousis *et al.*, 2022). Nucleoside analogues are chemically modified pyrimidines or purines that are misincorporated in the nascent DNA chain by the non-human polymerase leading to a premature cessation of the cellular replication. Compounds of this class might be used in anticancer chemotherapy, and to treat viral, mycotic, and to a lesser extent, bacterial infections (Thomson and Lamont, 2019).

Halogenated pyrimidines like gemcitabine (fluorinated deoxycytidine analogue) or 5-fluorocytosine are prodrugs that need under *in vivo* conditions a phosphorylation by the deoxyribonucleoside kinases (Sun and Wang, 2013) to yield the active metabolites gemcitabine triphosphate and floxuridine triphosphate, respectively (Mini *et al.*, 2006; Bennet, 1977; Álvarez *et al.*, 2012). Both compounds exert the desired effect either solely (Sandrini *et al.*, 2007a; Zander *et al.*, 2010) or in combination with another nucleoside analogue (*e.g.* zidovudine) (Wambaugh *et al.*, 2017). Individual nucleoside analogues were reported to exert an antibacterial activity on a wide spectrum of bacteria; the liponucleoside caprazamycin was found to work on different members of the genus *Mycobacterium* (Kaysser *et al.*, 2009), zidovudine was shown to be active against the Gram-negative species *E. coli* and *K. pneumoniae* (Hu *et al.*, 2019), and the Gram-positive species *Streptococcus pyogenes*, *S. aureus* and *Bacillus cereus*, respectively (Sandrini *et al.*, 2007a; Sandrini *et al.*, 2007b; Zander *et al.*, 2010). Thiolated purines can be used as antibacterial agent against *Mycoplasma pneumonia* (Sun and Wang, 2013), *Bacillus anthracis* (Alvarez *et al.*, 2010), or *Campylobacter concisus* in the inflammatory bowel disease (Liu *et al.*, 2017), or as anticancer therapy (Hanauer *et al.*, 2019).

In this project, only two promising nucleoside candidates, **MWP 451** and **GN246**, were identified to be efficient against *C. difficile* in the low µg/mL range (≤10 µg/mL). However, to demonstrate their applicability in treating CDI, further characteristics of these compounds need to be determined: As resistance against nucleoside analogues might occur due to mutation in the deoxyribonucleoside kinase gene (Jordheim *et al.*, 2012), their mutant frequencies should be determined. Additionally, as myelosuppression (*e.g.* neutropenia) and pulmonary fibrosis are in some cases dose-dependent side effects observed with some nucleoside analogues (Galmarini *et al.*, 2002; Chi *et al.*, 2012), new nucleoside analogue-based compounds should be tested for these side effects.

The last group of novel candidates tested here were inhibitors of the ECF-part of the ATP binding cassette (ABC) superfamily-transporters, which transport essential micronutrients (e.g. vitamins and metal cations) from the extracellular milieu into the bacterial cytosol (Bousis *et al.*, 2019; Bousis *et al.*, 2022; Kiefer *et al.*, 2022). ECF transporters are considered as a promising antibacterial drug target, as these transporters are present in about 50% of prokaryotes but are absent in humans (Diamanti *et al.*, 2021). A new class of compounds effective against ECF transporters was recently shown to suppress growth of a *Streptococcus pneumoniae* test strain at concentrations of 2 µg/mL, while growth of *E. coli* strain K12 remained unaffected even at concentrations of 100 µg/mL (Diamanti *et al.*, 2021).

In the thesis project described here, two ECF transporter inhibitor candidates provided by HIPS could be identified that showed promising activities against *C. perfringens* and *C. difficile* in the low µM range, namely **HHPS77** and **K4104497.** Both compounds were particularly effective in suppressing growth of the tested *C. perfringens* strain (MICs of 1.5-3 µM), and allowed for a suppression of growth of *C. difficile* at MICs of 12.5-25 µM and 12.5 µM, respectively. Growth inhibition of the *C. sporogenes* test strain, on the other hand, required already concentrations of 25-50 µM, suggesting that this commensal gut inhabitant might be less strongly affected when these compounds are used for treatment of *C. perfringens*-induced infections. However, it is notable to consider, that only one *C. perfringens* isolate was tested yet, asking for additional *C. perfringens* isolates with independent repetitions of the experiment in order to draw valid conclusions.

It is currently unknown, why growth of *C. perfringens* is more effectively affected by HHPS77 and K4104497 than growth of other *Clostridium* spp. and *C. difficile*, respectively. One reason for this observation might be found in their targets, the ECF transporters, which might differ between the tested species in terms of the numbers of variants that are expressed in a given species, their amino acid composition and 3D structure, and their expression levels. Another reason for the observed differences in drug susceptibility might be differences in the dependence on vitamin uptake for growth of a certain species. Species such *Lactobacillus casei* or *Enterococcus faecium* are for instance known to depend on uptake of folate from external sources for growth, as they do not express enzymes for the *de novo* vitamin B9 biosynthesis (Bousis *et al.*, 2019). It is very likely that such species are more susceptible to ECF transporter inhibitors than species that can synthesize all vitamins needed for their growth through de novo biosynthesis. For the *Clostridium* spp. test strains tested here, one may speculate that the *C. perfringens* isolate might lack one or some of the factors needed for the de novo biosynthesis of the vitamins needed for its growth, and is thus more susceptible to ECF transporter inhibition that other *Clostridium* species such as the *C. sporogenes* test strain, which might be capable of producing all factors required for the de novo biosynthesis of the vitamins needed for its growth.

4.2. Establishment of a sentinel surveillance scheme for *C. difficile* in Germany

Epidemiologic data on *C. difficile* and their antibiotic resistance profiles are very crucial for the early detection of an import of new hypervirulent variants into a region of interest, and to treat CDI appropriately. Unfortunately, most epidemiologic data on *C. difficile* and their antibiotic resistance pattern in Germany were biased, as most studies were either monocentric (Piepenbrock *et al.*, 2019) or based on isolates almost exclusively originating from severe cases of disease and/or outbreaks (Müller *et al.*, 2015). When this thesis project started, only one study was available [termed "European, multicenter, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalized patients with diarrhea" (EUCLID)], which, however, was rather outdated by reporting on the RT composition of *C. difficile* isolates collected in 2012/2013 (Davies *et al.*, 2016). In the latter investigation, a comparably high RT027 prevalence was noticed, which accounted for approximately 20% of all isolates tested for Germany (Davies *et al.*, 2016). More recent data, making use of a standardized approach, were until now not available for Germany.

In order to fill this gap, a scheme was established by the NRZ, in which the strain sampling was biased neither by a monocentric approach nor by the patient case severity. Specifically, Tertiary Care German University Hospital Centers were asked to supply their first ten *C. difficile* positive samples or *C. difficile* isolates identified in their clinical routine in a biannual manner (April and October for each year) to the NRZ. This approach was quite similar to surveillance schemes that have been put into action e.g. in Great Britain (Wilcox *et al.*, 2012) and Australia (Hong *et al.*, 2020; Collins *et al.*, 2022).

Driven by the findings of the EUCLID study for Germany (Davies *et al.*, 2016) and other reports, it is assumed that isolates of RT027 play a major role as causal agent for CDI in Germany since its introduction in 2005 and its subsequent spread (Steglich *et al.*, 2015; Kleinkauf *et al.*, 2007; Marujo and Arvand, 2020). This assumption is strengthened by earlier findings made in Hesse, showing that RT027 might represent >30% of all *C. difficile* isolates on a regional level (Arvand and Bettge-Weller, 2016). Further support for this hypothesis is given by another regional investigation conducted in Cologne (West Germany), which detected RT027 in 21% of isolates in the year 2017 (Piepenbrock *et al.*, 2019).

This high ratio of RT027 was also observed in the direct precursor study, when isolates originating from severe CDI and outbreak isolates that were sent to the NRZ in 2014-2019 were analyzed (Abdrabou *et al.*, 2021). In the latter strain-set, a mean RT027 prevalence of 36% was observed over the whole study period, suggesting that RT027 isolates are still a major cause for severe CDI in Germany (Abdrabou *et al.*, 2021). It can also be noted since 2016, that RT027 prevalence decreased steadily, suggesting that this HVR RT is nowadays replaced by other, yet underestimated, RTs in Germany as major cause for severe CDI (Abdrabou *et al.*, 2021). A similar trend was also observed in other countries, including the USA, in which the RT027 prevalence was found to decline over the last years (Tickler *et al.*, 2019).

However, the German-wide strain collection used in the precursor study was largely influenced by the high prevalence of hypervirulent RTs in particular RT027 as pointed out above, leaving the question open, how prevalent RT027 isolates are in the German healthcare setting nowadays. A first idea on the real prevalence of RT027 in the German healthcare setting in recent past could be obtained from a *C. difficile* strain-set collected between 2014-2019 at Saarland University Hospital (termed UKS strain-set) that served as a control-set for the NRZ strain-set in the above-mentioned precursor study, albeit on a local level only. In this UKS strain-set, the RT027 prevalence rate of 4.2% was observed, indicating that the real RT027 prevalence is much smaller in Germany, if all CDI cases are included (Abdrabou *et al.*, 2021). This finding was essentially confirmed in the epidemiological study presented here as part of this thesis, in which the strain-set obtained by the standardized surveillance scheme was evaluated.

In this largely unbiased strain-set, the RT027 prevalence rate of only 3.5% was observed on the German-wide level for 2019-2021, and RT027 isolates were even completely absent in some postal regions (0, 2 and 7, respectively) (Abdrabou *et al.*, 2022). However, RT027 might have been undetected in the latter regions, since the amount of the received isolates from these regions was low (<100 isolates). Notably, the fact that the prevalence of RT027 isolates is generally low in the German healthcare setting nowadays but still very high among severe cases of CDI underlines once more the high pathogenicity of RT027 strains, as this lineage, despite being of minor incidence in patients developing CDI in German University Hospitals, accounts for more than 1/3 of all severe CDI cases occurring in this setting.

The low percentage of RT027 isolates in the German healthcare setting and the decrease in RT027 prevalence in severe CDI cases and outbreaks noticed in my studies since 2016 might have several explanations: It has been suggested that the overall impact of RT027 declined in recent years as in the USA due to a replacement by RTs such as RT106 (Tickler *et al.*, 2019). Furthermore, in a study by Lawes and colleagues (Lawes *et al.*, 2017), it was shown that the RT027 prevalence declined when ABS procedures (particularly, the reduction of "4C" antibiotics) were implemented. Such programs are nowadays established throughout Germany. Another important factor for the decrease of RT027 in German hospitals might be the reduced usage of the fluoroquinolone class of antibiotics, which is associated with RT027 selection (Wieczorkiewicz *et al.*, 2016). Fluoroquinolones are nowadays prescribed in much smaller quantities in Germany than the years before (Gradl *et al.*, 2021), presumably due to the reported severe side effects such as central nervous system disorders, tendinopathy and tendon rupture especially when prescribed with corticosteroids (Ellis *et al.*, 2021; Kim, 2010; Persson and Jick, 2019). Of note, the decline of fluoroquinolone use is congruent with lower RT027 rates being encountered in Germany since the year 2016 from outbreaks and severe cases (Abdrabou *et al.*, 2021). The implementation of ABS procedures in German hospitals might also account for the decline of RT001 seen in the standardized sentinel surveillance study, as RT001 isolates might be driven back by ABS as well (Lawes *et al.*, 2017).

From the epidemiological perspective, RT014 was the most prevalent RT found in the German healthcare setting in recent years. Notably, in the standardized sentinel surveillance study and the control group of the German precursor study, in which *C. difficile* isolates from Saarland University Hospitals were included, nearly identical prevalence rates were observed (17.5% vs. 18%) (Abdrabou *et al.*, 2022; Abdrabou *et al.*, 2021). This finding is not unexpected, since RT014 is an epidemiologically very successful RT that is widely distributed on the global scale (Davies *et al.*, 2016; Tickler *et al.*, 2019; Knight *et al.*, 2016b).

Notably, isolates of RT017, being the most abundantly isolated strain in East Asia, which are also frequently detected in Portugal (Isidro *et al.*, 2018), were not found in significant numbers (3 isolates) (Abdrabou *et al.*, 2022). However, since isolates of this RT seem to spread on the intercontinental level in recent past (Imwattana *et al.*, 2019; Imwattana *et al.*, 2022); awareness is recommended for the appearance of this RT in Germany. Isolates of RT018, which caused outbreaks in France (Gateau *et al*., 2019) and Germany (Berger *et al.*, 2019) in recent years, were encountered in <1% of all isolates, suggesting that the impact of this RT is currently limited in Germany, despite exhibiting high prevalence rates (>40%) in Northern Italy (Spigaglia *et al.*, 2010; Spigaglia *et al.*, 2015; Spigaglia, 2016). However, given the comparably close proximity between Germany and Northern Italy, a risk for transmission should be taken into account.

Isolates of RT176 are highly prevalent in the Czech Republic and Poland, respectively (Nyč *et al.*, 2011), however, this RT was detected in only one single case in the standardized sentinel surveillance study (Abdrabou *et al.*, 2022). Of note, this RT is related to RT027, representing another hypervirulent *C. difficile* lineage (van den Berg *et al.*, 2007; Stabler *et al.*, 2012; Valiente *et al.*, 2012; Karpiński *et al.*, 2022). As with RT018, it is currently unknown, why RT176 is only rarely detected in the German healthcare setting, given their prevalence in neighboring countries and the cultural interchange seen between these regions.

Isolates of RT023, which are associated with bloody diarrhea (Shaw *et al.*, 2020), were detected in 2.7% of all isolates in German University Hospitals. Interestingly, a similar prevalence has been detected for other European countries such as Great Britain and the Netherlands (Wilcox *et al.*, 2012; Shaw *et al.*, 2020). RT023 is also commonly found in countries outside Europe such as New Zealand and Australia (Johnston *et al.*, 2021; Shivaperumal *et al.*, 2022), suggesting that this RT might represent an upcoming challenge for our healthcare system in near future. From the clinical point of view, the AMR data concerning the circulating *C. difficile* RTs are crucial for an evidence-based antimicrobial therapy. Concerning antimicrobial resistance, several conclusions can be drawn from the two studies reported here. In general, there were lower resistance rates encountered in both studies than in the ClosER study, which was conducted in several European countries in 2011-2014 (Freeman *et al.*, 2018).

This was in particular evident for moxifloxacin (15% in the standardized sentinel surveillance study vs. 36% in the ClosER study), as well as for rifampicin (4% versus 13%) (Abdrabou *et al.*, 2022; Freeman *et al.*, 2018). Resistance rates towards antibiotics that are used for CDI therapy, however, were almost equal and only very rarely encountered in both studies (0.1% vs. 0.2% for metronidazole and 0% vs. 0.1% for vancomycin, respectively) (Abdrabou *et al.*, 2022; Freeman *et al.*, 2018). From these findings, it can be concluded that resistance towards metronidazole and vancomycin are not of major importance in Germany yet, and the same applies probably for whole Europe.

However, in the precursor study that investigated isolates from severe cases and outbreaks, metronidazole resistance (MIC ≥2 µg/mL) was significantly higher and encountered in 39 isolates (2.7%). Most interestingly, metronidazole non-susceptibility was almost entirely detected in the HVR RT027 strains (32/39 isolates) (Abdrabou *et al.*, 2021). This finding is in line with the ClosER study (Freeman *et al.*, 2018), suggesting that RT027 is a major driver for *C. difficile* resistance towards metronidazole, presumably by taking up and/or maintaining the plasmid pCD-METRO more easily than other RTs (Boekhoud *et al.*, 2020). As CLSI guidelines recommends to confirm the metronidazole resistance determined by other assays via the agar dilution method (CLSI, 2012), isolates displaying a metronidazole resistances by the E-test were retested by the agar dilution assay. However, with the latter assay, a metronidazole resistance was confirmed only in about 2/3 of the cases (22/32 isolates). This discrepancy is probably due to a known phenomenon encountered with frozen and thawed isolates, which often lose their resistance properties (Peláez *et al.*, 2008). Biological variance could be another possible explanation for a part of the isolates displaying MIC values situated around the metronidazole breakpoint (2µg/mL) (EUCAST, 2021). For the two isolates in which the metronidazole resistance remained above the breakpoint by the repeated E-test while being not detected by the agar dilution, no clear reason could be found, except for a potential non-homogenous distribution of the antibiotic in the agar dilution test plate.

Since a MIC creep for metronidazole was encountered recently in one single center study over a ten-year study where RT001 was replaced by RT027 (Piepenbrock *et al.*, 2019), MIC_{50} and MIC_{90} values obtained with strain-sets analyzed in this thesis were also monitored for metronidazole on a yearly basis. Conversely to findings made by Piepenbrock and colleagues (Piepenbrock *et al.*, 2019), such an effect was absent in both of my study sets, indicating that we don't have to expect an increase in the clinical breakpoint for this antibiotic in near future.

For vancomycin there were no resistant strains identified in the *C. difficile* isolate sets analyzed here (Abdrabou *et al.*, 2022; Abdrabou *et al.*, 2021), indicating that it is safe to use vancomycin as empirical treatment for CDI in Germany. Moreover, when looking at the MIC_{50} and MIC_{90} values for vancomycin per year, even a slight decline in the values was observed, indicating that the *C. difficile* population circulating

in the German healthcare system does not become more resistant towards this clinically important antibiotic over time.

When looking at the proportions of rifampicin resistant RT027 isolates, rates were 63% vs. 48%. They were even higher in the standardized sentinel surveillance study than in the precursor study (being composed of severe cases and outbreak strains from Germany) (Abdrabou *et al.*, 2022; Abdrabou *et al.*, 2021). However, this unexpected finding might be due to the comparably low number of RT027 isolates (*n*=30) in the standardized sentinel surveillance study.

In general, rifampicin usage might be a factor for the selection of rifampicin-resistant *C. difficile* isolates in certain patient groups such as orthopedic patients, where foreign body infections are regularly encountered which are often treated with rifampicin (Färber *et al.*, 2017). Among the RT027 isolates of the standardized sentinel surveillance study, very high resistance rates towards the macrolide antibiotic clarithromycin (87%) and the fluoroquinolone moxifloxacin (83%) were encountered, respectively (Abdrabou *et al.*, 2022), which is consistent with earlier findings made in USA (Wieczorkiewicz *et al.*, 2016) and with the findings of the precursor study where the resistance profiles of RT027 isolates exceeded 88% for both antibiotics (Abdrabou *et al.*, 2021).

MDR in *C. difficile* (*i.e.* being resistant against more than two classes of antibiotics) is a critical problem that limits the therapeutic options (Sebaihia *et al.*, 2006; Peng *et al.*, 2017). A higher proportion of MDR *C. difficile* was found in the NRZ strain-set (17%) when compared to the UKS and sentinel surveillance sets (1.6% and 2.6%, respectively). MDR was significantly driven by the RT027, which accounted for 96%, 67% and 74% of the MDR isolates, respectively (Abdrabou *et al.*, 2022; Abdrabou *et al.*, 2021). These observations are in agreement with other studies emphasizing the role of RT027 as a major driver of severe CDI courses and MDR in different geographical areas (Hidalgo-Villeda *et al.*, 2018; Tenover *et al.*, 2012; Spigaglia *et al.*, 2011; Freeman *et al.*, 2020; Freeman *et al.*, 2018; Freeman *et al.*, 2010; López-Ureña *et al.*, 2014; Lopardo *et al.*, 2015).

Hence, the NRZ strain-set is dominated by severe cases and RT027 accounts for 36% of all cases, this can explain the significant rise of MDR in this set compared to the RT027 prevalence in the other two sets (4.2% and 3.4%, respectively) (Abdrabou *et al.*, 2021; Abdrabou *et al.*, 2022). MDR was significantly more often found among RT027 isolates (26-57%) when compared to the non-RT027 isolates (0.6-1%), and this difference was evidenced in all strain-sets tested here (Abdrabou *et al.*, 2022; Abdrabou *et al.*, 2021). MDR was also evident for other RTs in other regions of the world *e.g.* RT017 in Kenya (Mutai *et al.*, 2021), RT001 and RT126 in Iran (Baghani *et al.*, 2020) and RT017, RT001 and RT012 in Eastern China (Jin *et al.*, 2017).

Also representing a major step towards a reliable picture of the *C. difficile* RT composition and AMR in the German healthcare setting, the surveillance study presented here - although being conducted in a standardized fashion - still featured a couple of limitations. The first limitation is the fact that exclusively University Medical Centers participated in this investigation. The set-up of these centers might have affected the RT distribution, and in particular, isolates stemming from more severe cases are probably overrepresented. Differences might also be evident on a regional level. With only few isolates being included for some regions while other contributed much larger numbers, the generalizability is probably biased by regions contributing with larger numbers of isolates. However, this issue is difficult to address, as in some areas only a low number of study sites is present.

Another bias might have been introduced is the current situation regarding the coronavirus disease 2019 (COVID-19) crisis. In the standardized sentinel surveillance study, strains only from one phase (late 2019), just before the pandemic, were available. In fact, changes in the strain composition induced by COVID-19 cannot be evaluated properly within the study period monitored (2019-2021). However, it can be assumed that changes due to the COVID-19 pandemic might have occurred. This could be attributed for instance to more rigid hygiene measures in the hospitals during the ongoing COVID-19 pandemic. Of note, the overall strain make-up seemed not to alter markedly during the conduct of the study, with a stable diversity score being found for all sampling periods.

A third limitation is due to the fact that fidaxomicin, the currently favored therapy regimen for CDI (McDonald *et al.*, 2018; Johnson *et al.*, 2021), could not be evaluated due to the absence of a commercial and easy-to-use testing method. The latter limitation was solved only very recently, since the NRZ has implemented an adjusted testing scheme for this substance by the end of 2021. In order to overcome these limitations, further centers are going to be recruited also from non-University sites. This should at best comprise of primary care centers and, if feasible, also include isolates from the out-patient setting that has been a rather neglected field in recent years. In summary, a robust surveillance network for *C. difficile* isolates has been put into action that will be further advanced in the future to facilitate the detection of new emerging strains and antimicrobial resistance, which should be of major help for clinicians and Hospital Hygiene.

4.3. MALDI-TOF MS for *C. difficile* RTs subtyping

MALDI-TOF MS is an easy-to-use method and has changed the microbial diagnostic workflows due to its handling, short turnaround time, accuracy, and low costs (Biswas and Rolain, 2013). In clinical diagnostics, MALDI-TOF MS allows for a differentiation of a broad variety of bacterial species (Angeletti and Ciccozzi, 2019; Rodríguez-Sánchez *et al.*, 2019). Moreover, several reports emphasized its capability in distinguishing clinically relevant subtypes within a certain species, *i.e.* virulent, toxigenic or antibiotic resistant subgroups (Wolters *et al.*, 2011; Christner *et al.*, 2014).

Subtyping of *C. difficile* can be for instance crucial when confirmation or exclusion of an outbreak scenario is needed. The primary methods currently in use to subtype *C. difficile* isolates are ribotyping (Indra *et al.*, 2008) and WGS (Bletz *et al.*, 2018), which both are laborious, time-consuming and costly. However, to some extent, subtyping by MALDI-TOF MS is also possible for *C. difficile*. In particular, RT027 is one of the best-known examples for an outbreak strain, which can be differentiated by MALDI-TOF MS (Emele *et al.*, 2019). Of note, isolates of RT027 cannot be distinguished from RT176, so that these two RTs are usually reported as one group (Emele *et al.*, 2019; Krutova *et al.*, 2014; Krutova *et al.*, 2015). We could confirm this finding within our study by observing a clustering of these two RTs away from the non-HVR RTs (**Supplementary Figure S1)**. Besides RT027/176, other strains of high epidemiologic importance might be differentiated by MALDI-TOF MS as well, including the pandemic lineage RT001 (Reil *et al.*, 2011; Carneiro *et al.*, 2021), RT017 (Li *et al.*, 2018) and the hypervirulent lineage RT078/126 (Reil *et al.*, 2011).

From the standpoint of hospital hygiene, it is essential to discriminate between hypervirulent RTs (HVR RTs) and non-hypervirulent RTs (non-HVR RTs), but it is unknown yet, whether this is possible by MALDI-TOF MS in an environment where several unrelated HVR RTs must be considered. Thus, as third part of this thesis, a strain-set of HVR- and non-HVR isolates that are commonly found in the German healthcare system was selected from the stock collection of the NRZ, and protein extracts obtained from these isolates were subjected to MALDI-TOF MS. When a peak matrix based on the nine biomarker peaks previously identified by Emele *et al.* for the discrimination of RT027/176 from other RTs was used with our strain-set to discriminate between RT027/176 isolates and other RTs (**Supplementary Figure S2**), a reliable separation of RT027/176 isolates from isolates of other RTs was not possible. Hence, this peak matrix alone is not suitable for an identification of RT027/176 isolates in an environment containing other HVR lineages beside the non-HVR RTs. Thus, a machine learning analysis of the MALDI-TOF mass spectra using the CloverBioSoft MS-based approach was conducted to test whether the HVR RTs (RT023/027/045/078/126/127/176) can be distinguished from the non-HVR RTs based on their MALDI-TOF mass spectra.

When employing the peak matrices based on average spectra normalized by the full-spectrum, pTIC and TICp mode, respectively, PCA did again not allow to discriminate reliably between RT027/176 and other RTs, unlike previously reported (Emele *et al.*, 2019). It is important to notice here that Emele and colleagues (Emele *et al.*, 2019) included only nine non-RT027/176 HVR isolates (two strains from RT016, one RT023, four RT078, one RT126, and one RT127 isolate) into their study, a number probably too low to interfere with clustering. However, when the average spectra of the RT027/176 isolates were indicated as an individual group, the PCA done with the peak matrix obtained with the TICp mode allowed to discriminate between two clusters, one composed of non-HVR RTs merging with the RT027/176, and another composed of the remaining HVR RTs (*i.e.* RTs 023, 045, 078, 126 and 127).

Based on this promising observation, machine learning was applied next to test whether a reliable discrimination between HVR RTs and non-HVR-RTs could be achieved when the average spectra of the isolates were analyzed with the peak matrix obtained with the TICp mode in combination with the prediction algorithms KNN, SVM, RF, and PLS-DA, respectively. In fact, when the peak matrix obtained by the TICp mode was used in combination with the prediction algorithms RF and PLS-DA, an almost complete separation of HVR RTs from non-HVR RTs could be achieved.

The 10-fold cross validation performed with the spectra of the 157 discovery phase isolates allowed allocating the isolates to the HVR or non-HVR groups with a total accuracy of 99% and 98%, respectively. When the predictive power of both models was tested with 83 new blinded isolates, these isolates were correctly assigned to their respective groups with accuracies >97% and ROC curve AUCs >0.96. Furthermore, the positive and negative predictive values (PPV and NPV, correspondingly) of both validation levels (cross and external) exceeded 97%, indicating that a reliable discrimination between major HVR RTs and non-HVR-RTs circulating in the German healthcare system is possible by MALDI-TOF MS, when applying the peak matrix obtained in the TICp mode in combination with one of the two prediction models.

However, it should not be left out here that for some HVR RTs such as RT127, only a small number of isolates was tested due to availability reasons, and the same holds truth for isolates of the non-toxigenic RTs (NTC isolates), which were underrepresented in the external validation phase. Thus, it cannot be excluded at this stage that PPV and NPV might change if further isolates of RT127 and the NTC group would be included.

From the epidemiological prospective, further classification is highly crucial for tracing back the source of infection. RT045/078/126 carry a continuous zoonotic potential for CA-CDI (Schneeberg *et al.*, 2012; Schneeberg *et al.*, 2013b; Schneeberg *et al.*, 2013a). RT023 is an emerging clade 3 strain (Shaw *et al.*, 2020) being preferentially detected in Europe with prevalence rates up to 2.5% (Shaw *et al.*, 2020), and it can be assumed that the true prevalence rate is even higher, since some selective media do not identify

this RT properly (Connor *et al.*, 2016; Reigadas *et al.*, 2017). RT023 is also in favor of a more severe course of disease (bloody diarrhea) (Shaw *et al.*, 2020).

Interestingly, isolates of the HVR RTs group could be further separated into smaller subgroups when a peak matrix based on the TICp mode was utilized in combination with the PLS-DA and RF prediction algorithm, respectively. Here, RT027/176 formed one cluster, RT023 formed a second cluster, and isolates of RT045/078/126/127 clustered together with a predictability of >94% after external validation. This clustering is in principle in line with the MLST clades formed by these HVR RTs (Zhao *et al.*, 2021), where RT023 belongs to clade 3 (Shaw *et al.*, 2020), and RT027/176 is found in clade 2 (Badilla-Lobo and Rodríguez, 2021; Emele *et al.*, 2019) and the last cluster RT045/078/126/127 to clade 5 (Knight and Riley, 2016; Stabler *et al.*, 2012; Knight *et al.*, 2015).

The identification of HVR strains by MALDI-TOF MS represents an interesting option for a swift, preliminary *C. difficile* outbreak investigation and for surveillance purposes, which, however, should be confirmed by other subtyping methods (*e.g.* ribotyping or WGS) to allow for a clear discrimination between different RTs. In this work, a search algorithm was established via trained reference sets using a machine learning approach that allowed to distinguish between HVR RTs from non-HVR RTs on the basis of MALDI-TOF mass spectra generated with a standard MALDI-TOF MS utilized by many medical microbiology diagnostic laboratories for genus/species identification. Moreover, within the HVR group, a further subtyping of RTs RT023, RT027/176 and (RT045, RT078, RT126, and RT127) might be possible.

4.4. Disparate colony variant

Besides separation by MALDI-TOF, another phenotypic feature of some *C. difficile* strains was detected. The MALDI-TOF MS results presented here show that RT078 isolates cluster together with RT045, RT126, and RT127 isolates, mirroring other genetic analysis studies conducted previously (Knetsch *et al.*, 2011; Elliott *et al.*, 2014; Schneeberg *et al.*, 2013b; Schneeberg *et al.*, 2012). These studies also reported that the former RTs, together with RT033 and RT066, form a phylogenetic coherent group based on their amplified fragment-length polymorphism (AFLP) profiles, carriage of *cdtAB*, and a specific deletion in *tcdC* (Knetsch *et al.*, 2011; Schneeberg *et al.*, 2013b).

Interestingly, when comparing the colony morphologies of isolates belonging to HVR RTs, an easily visible difference in colony morphology could be observed for certain RTs. While isolates of the RTs 045, 078, 126, and 127 formed whitish, compact and rough colonies on blood agar, a different phenotype was observed for RT023 and RT027/176 isolates, which produced large, greyish colonies, presumably due to swarming (Karpiński *et al.*, 2022). Although still of preliminary nature, this observation might be of particular relevance for areas lacking advanced diagnostics to rapidly discriminate between certain HVR RTs.

5. Future perspectives

This work consisted of three aspects. The first aspect was the evaluation of several natural antimicrobial compounds against *C. difficile* and other bacteria, which identified argyrin B to suppress growth of this bacterium under in vitro conditions at low ng/mL concentrations. Importantly, this compound was not only highly active against selected reference strains but also against a broad variety of epidemiologically important RTs. However, to underline its potential as therapeutic agent to treat CDI, more studies are needed including human cell culture-based toxicity studies and *in vivo* infection models in animals. A major drawback of this compound might be that the extraction process of argyrin B is highly complex and expensive. Thus, pharmaceutical industry will be challenged to find ways for an effective production process of this compound once an implementation in human therapy is envisioned. Furthermore, *in vitro* testing of argyrin B in combination with vancomycin and fidaxomicin should be done to identify potential synergistic effects between this test substance and other compounds used to treat CDI.

The second aspect was dedicated to the implementation of a standardized surveillance scheme for *C. difficile* which is not heavily biased by disease severity and/or outbreak events. As a start point for this scheme, Medical Microbiology units of all University Hospitals located within Germany were asked to supply their first ten isolates seen in the laboratories at a given date. It is worth mentioning that this was the first time that a *C. difficile* sentinel surveillance scheme could be implemented in Germany covering the whole country. However, as only University Hospitals were asked to send in strains, this scheme might not reflect the complete strain composition seen in the German healthcare setting, as smaller hospitals and the outpatient settings were not yet included. To account for this, the sentinel scheme should be extended in the next phases to include other hospitals and healthcare institutions than University centers. With this profound data base and with the transition from ribotyping towards WGS, a German-wide real-time surveillance will be possible that should allow for a rapid detection of emerging new RTs and fluctuations in the AMR profiles of *C. difficile* circulating in the German healthcare system. These aspects are now being addressed by the NRZ in order to allocate these important data. Furthermore an orienting resistance scheme concerning the antimicrobial susceptibility testing of fidaxomicin has been implemented and will be used in the future sampling periods of this important surveillance approach. With fidaxomicin being now the new antimicrobial of choice against CDI according to current ESCMID guidelines, it is likely that resistance will rise as well.

The third aspect of this thesis was the evaluation of MALDI-TOF MS as a suitable tool to differentiate between hypervirulent *C. difficile* RTs (HVR RTs) and non-hypervirulent RTs (non-HVR RTs) commonly found within Germany. My studies demonstrate that protein-based *C. difficile* MALDI-TOF mass spectra can be indeed used in combination with bioinformatics to reliably discriminate between the HVR RTs and non-HVR RTs used in the discovery and validation phases. However, as the *C. difficile* RT

composition is changing over time on the global and regional levels, it is important to constantly include new RTs seen in Germany into this test scheme to assure that emerging HVR RTs will not be misinterpreted by this technique. From the clinical perspective, it would be also very interesting to test whether this method might be also applied to discriminate between the toxigenic RTs and non-toxigenic RTs, which was unfortunately not possible in this project due to the inadequate representation of the non-toxigenic isolates, particularly in the external validation strain-set. However, this might become possible when a larger and more diverse isolate set will be used in combination with the machine learning algorithms.

6. References

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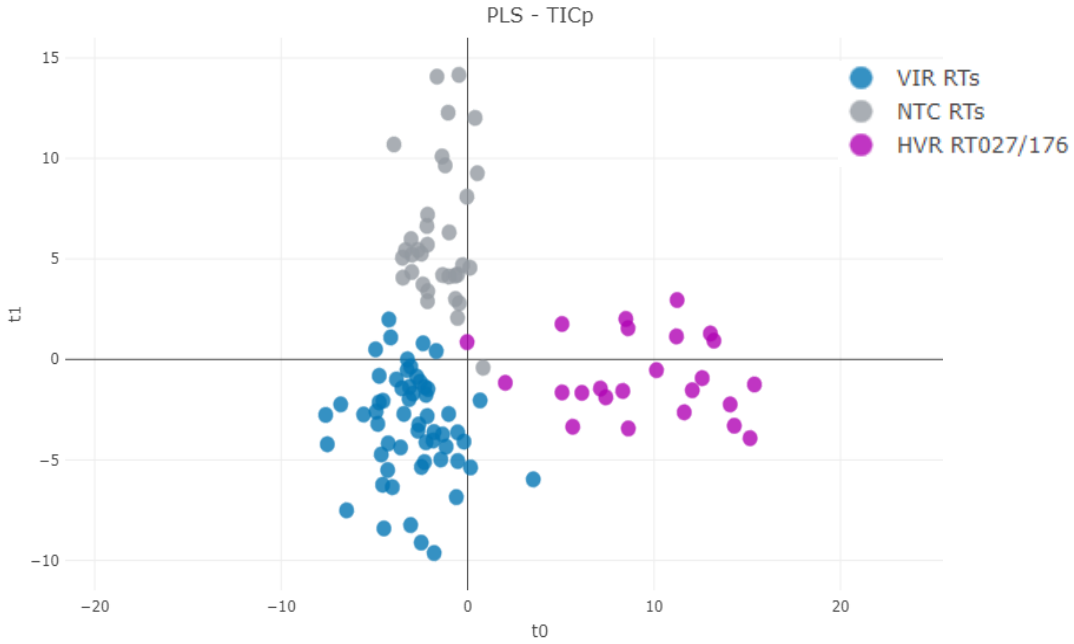
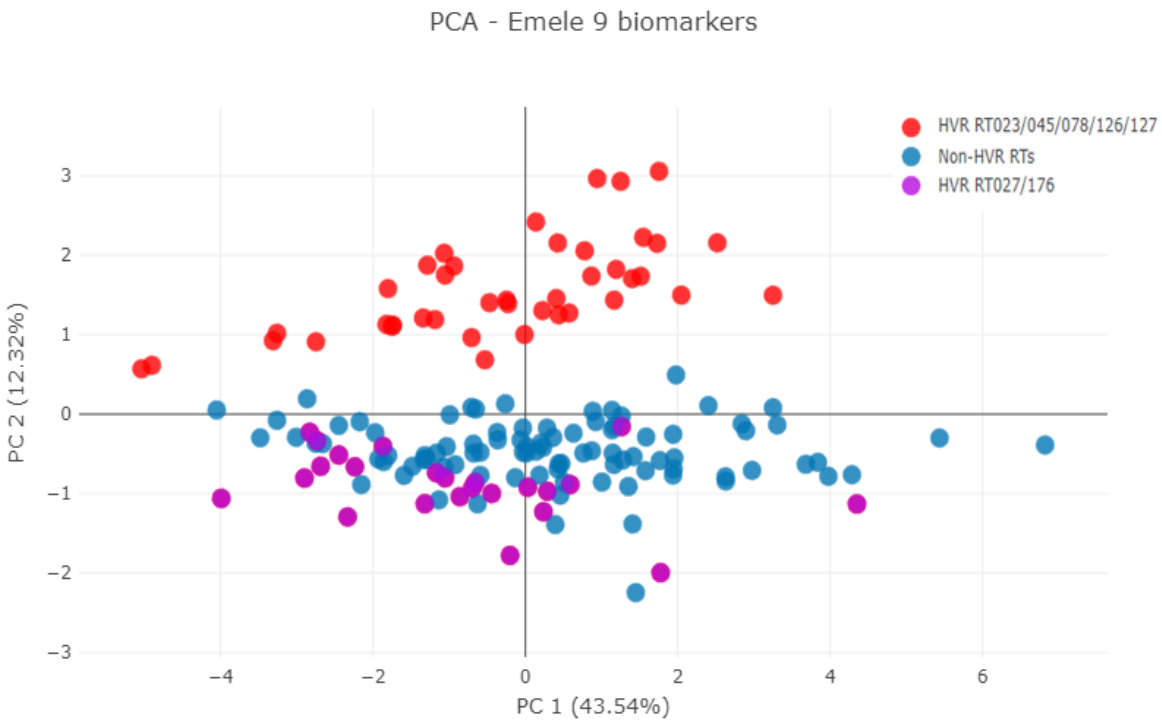
7. Supplementary data



Supplementary Figure S1: Supervised machine learning model represents partial least squares–discriminant analysis (PLS-DA) after applying the TICp mode. It was able to separate the HVR RT027/176 isolates (violet) from the non-hypervirulent group that includes the VIR (blue) and NTC (grey) groups. HVR, hypervirulent strains; VIR, virulent strains; NTC, non-toxigenic *C. difficile* strains; RT, ribotype.



Supplementary Figure S2: Separation of *C. difficile* isolates principal component analysis (PCA) after applying the nine peaks (4277, 5566, 5959, 6366, 6648, 6722, 6888, 7092, 9651 *m/z*) previously reported by Emele *et al*. (Emele *et al.*, 2019). Each circle represented one isolate. Isolates belonging to HVR RTs (RT023, RT045, RT078, RT126, and RT127) are indicated in red, RT027/RT176 isolates in violet, and isolates belonging to the non-HVR RTs are indicated in blue. RT027/176 isolates cannot be separated from th non-HVR RTs. HVR RTs, hypervirulent ribotypes; Non-HVR RTs, non-hypervirulent ribotypes; RT, ribotype.

Publications and conferences

Publications (in peer-reviewed journals)

- **Abdrabou, Ahmed Mohamed Mostafa;** Bischoff, Markus; Mellmann, Alexander; von Müller, Lutz; Margardt, Lena; Gärtner, Barbara C.; Berger, Fabian K.; Haase, Gerhard; Häfner, Helga; Hoffmann, Reinhard; Simon, Valeska; Stappmanns, Hannes; Hischebeth, Gunnar T.R.; Büchler, Christian; Rößler, Susann; Hochauf-Stange, Kristina; Pfeffer, Klaus; MacKenzie, Colin; Kunz, Caroline; Alsalameh, Rayya; Dziobaka, Jan; Le Chapot, Valérie Saout; Sanabria, Erwin; Hogardt, Michael; Komp, Johanna; Imirzalioglu, Can; Schmiedel, Judith; Pararas, Michael; Sommer, Frank; Groß, Uwe; Bohne, Wolfgang; Kekulé, Alexander S.; Dagwadordsch, Urantschimeg; Löffler, Bettina; Rödel, Jürgen; Walker, Sarah Victoria; Tobys, David; Weikert-Asbeck, Simone; Hauswaldt, Susanne; Kaasch, Achim J.; Zautner, Andreas E.; Joß, Nadja; Siegel, Ekkehard; Kehr, Katharina; Schaumburg, Frieder; Schoeler, Sarah; Hamprecht, Axel; Hellkamp, Josef; Hagemann, Jürgen Benjamin; Kubis, Jan; Hering, Silvio; Warnke, Philipp **(2022)**: Implementation of a *Clostridioides difficile* sentinel surveillance system in Germany: First insights for 2019–2021. In *Anaerobe*, p. 102548. DOI: 10.1016/j.anaerobe.2022.102548.

- **Abdrabou, Ahmed Mohamed Mostafa;** Ul Habib Bajwa, Zia; Halfmann, Alexander; Mellmann, Alexander; Nimmesgern, Anna; Margardt, Lena; Bischoff, Markus; von Müller, Lutz; Gärtner, Barbara; Berger, Fabian K. **(2021)**: Molecular epidemiology and antimicrobial resistance of *Clostridioides difficile* in Germany, 2014–2019. In *International Journal of Medical Microbiology* 311 (4), p. 151507. DOI: 10.1016/j.ijmm.2021.151507.

- Elhawy, Mohamed Ibrahem; Huc-Brandt, Sylvaine; Pätzold, Linda; Gannoun-Zaki, Laila; **Abdrabou, Ahmed Mohamed Mostafa;** Bischoff, Markus; Molle, Virginie **(2021)**: The Phosphoarginine Phosphatase PtpB from *Staphylococcus aureus* Is Involved in Bacterial Stress Adaptation during Infection. In *Cells* 10 (3). DOI: 10.3390/cells10030645.

- El-Mahdy, Rasha; **Mostafa, Ahmed;** El-Kannishy, Ghada **(2018)**: High level aminoglycoside resistant *enterococci* in hospital-acquired urinary tract infections in Mansoura, Egypt. In *Germs* 8 (4), pp. 186–190. DOI: 10.18683/germs.2018.1145.

Publications (in preparation)

- **Abdrabou, Ahmed Mohamed Mostafa[*];** Sy, Issa[*]; Bischoff, Markus; Becker, Sören L.; Mellmann, Alexander; von Müller, Lutz; Gärtner, Barbara; Berger Fabian K. **(2022)**: MALDI-TOF mass spectrometry-based subtyping of *Clostridioides difficile* ribotypes. (Submittted, under review)
- Pogorevc, Domen; Popoff, Alexander; Panter, Sophia; Rasheed, Sari; Abou Fayad, Antoine; **Abdrabou, Ahmed Mohamed Mostafa;** Berger, Fabian K.; Bischoff, Markus; Herrmann, Jennifer; Müller Rolf **(2022)**: Production of novel argyrin derivatives against *Clostridioides difficile*. (Manuscript writing).
- **Abdrabou, Ahmed Mohamed Mostafa;** Bischoff, Markus; Berger Fabian K. **(2022)**: Disparate colony morphotype by hypervirulent *Clostridioides difficile* RTs. (Manuscript writing).

Conferences (Poster-presentations)

- **Mostafa, Ahmed;** Eldars, Waleed M.; Elbadrawy, Mohamed K.; Abou Elela, Mohamed A. Plasmid control of carbapenem resistance in pandrug resistant *klebsiella pneumoniae* strains in intensive care units of Mansoura University Hospitals. The 4th International Conference on Prevention & Infection Control (ICPIC). 20-23 June **2017**, Geneva, Switzerland.
- El-Mahdy, Rasha; **Mostafa, Ahmed;** El-Kannishy, Ghada. High level aminoglycoside resistant *enterococci* in hospital-acquired urinary tract infections in Mansoura, Egypt. The 4th International Conference on Prevention & Infection Control (ICPIC). 20-23 June **2017**, Geneva, Switzerland.

Curriculum Vitae

The curriculum vitae was removed from the electronic version of the doctoral thesis for reasons of data protection.

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