9th International *C. difficile* Symposium

SEPTEMBER 8-10, 2026, BLED, SLOVENIA

NACIONALNI LABORATORIJ ZA ZDRAVJE, OKOLJE IN HRANO

ABSTRACT BOOK

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9th International *C. difficile* Symposium Abstract book
September 8-10, 2026 Bled, Slovenia

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CONTENTS

WELCOME

Dear friends and colleagues,

It is a great pleasure to welcome you to the 9th International *C. difficile* Symposium (ICDS 2026) in Bled.

For more than 20 years ICDS brings together researchers, clinicians, public health professionals, veterinarians, and industry representatives from around the world to share new findings, exchange experience, and discuss current issues related to *C. difficile*. The programme reflects the broad scope of the field, covering epidemiology, diagnostics, treatment, infection prevention, genomic research, microbiota, antimicrobial resistance, and One Health.

We hope that the symposium will provide an open and productive setting for scientific discussion, the exchange of ideas, and the development of new collaborations. We also hope that the symposium will provide an opportunity to reconnect with colleagues, strengthen existing relationships, and welcome new members into the close and collaborative *C. difficile* community.

We sincerely thank all speakers, abstract presenters, participants, members of the Organizing committee, and sponsors, whose contributions and continued support are essential to the organization and success of ICDS.

We wish you an interesting and rewarding meeting and a pleasant stay in Bled.

Welcome to ICDS 2026.

On behalf of the Organizing Committee

Maja Rupnik and Sandra Janezic

GENERAL INFORMATION

CONGRESS VENUE

Rikli Balance Hotel, Cankarjeva cesta 4, 4260 Bled, Slovenia

REGISTRATION DESK OPENING HOURS

Tuesday, 8 September: 10:00–16:00 and during coffee breaks
Wednesday, 9 September: 08:00–09:00 and during coffee breaks
Thursday, 10 September: 08:00–09:00 and during coffee breaks

MEALS AND SOCIAL EVENTS

Lunch is included in the registration fee and will be provided on Wednesday, 9 September, and Thursday, 10 September. It will be served in the restaurant of the Rikli Balance Hotel.

Coffee breaks will be held in the foyer in front of the lecture hall and in the poster area.

The **Welcome reception**, held on Tuesday, 8 September, is included in the registration fee and will take place at the Rikli Balance Hotel.

The **Congress dinner**, held on Thursday, 10 September, is also included in the registration fee and will take place at the Grand Hotel Toplice.

NAME BADGES

Participants are required to wear their name badges during all scientific sessions and social events.

CERTIFICATE OF ATTENDANCE

Certificates of attendance will be available on site upon request at the Registration desk.

INFORMATION FOR SPEAKERS

Speakers are kindly requested to upload their presentations to the computer in the lecture hall before their scheduled session. Technical assistance will be available. The use of personal laptops for presentations is not recommended.

INFORMATION FOR POSTER PRESENTERS

Posters will remain on display throughout the Congress. Posters may be set up from Tuesday, 8 September, and should be removed after the final poster session on Thursday, 10 September. Materials for mounting posters will be provided.

Your assigned poster number is listed in the **poster overview** table. A list of abstracts with the corresponding poster numbers will also be available in the poster area.

9TH ICDS - INVITED SPEAKERS

Michael Abt *(United States)*

Qiwen Dong *(United States)*

Paul Feuerstadt *(United States)*

Dazhi Jin *(China)*

Caroline Le Maréchal *(France)*

Diana López-Ureña *(Costa Rica)*

Ines Moura *(United Kingdom)*

Johann Peltier *(France)*

Christian Seyboldt *(Germany)*

Wiep Klaas Smits *(The Netherlands)*

Maria Vehreschild *(Germany)*

ICDS AND ESGCD ATTENDANCE GRANTS

Frederico Alves
(National Institute of Health Dr. Ricardo Jorge [INSA], Portugal)

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(São Paulo State University, Brazil)

Daniela Javorská
(Pavol Jozef Šafárik University in Košice and Louis Pasteur University Hospital, Slovakia)

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ICDS PROGRAMME

TUESDAY, SEPTEMBER 8, 2026

14:00–14:10			**Opening**
14:10–15:40	**SESSION 1**		**Innovations in the *C. difficile* field**
14:10–14:40	INV1	Wiep Klaas Smits	Experimental colonization and infection with *C. difficile* in healthy volunteers
14:40–15:10	INV2	Diana López-Ureña	Characterisation of a novel *C. difficile* virulence factor that alters host activity
15:10–15:40	INV3	Ines Moura	Assessing individual risk of CDI using a Miniature Gut (MiGut) model
15:40–16:20			**COFFEE BREAK**
16:20–18:00	**SESSION 2**		**Epidemiology and OneHealth**
16:20–16:50	INV-4	Caroline Le Maréchal	*C. difficile*: a foodborne pathogen?
16:50–17:20	INV-5	Christian Seyboldt	*C. difficile* in small animals and its role in community CDI
17:20–17:40	OP-1	Sarah Willis	*C. difficile* infection among adults with immunocompromising and chronic health conditions in the United States, 2018–2024: an administrative claims-based study
17:40–18:00	OP-2	Elisabeth Dietz	Risk factors for *C. difficile* infection recurrence across three decades
18:00		**Welcome reception, Rikli Balance Hotel (Hall Arnold and Restaurant)**	

WEDNESDAY, SEPTEMBER 9, 2026

08:30–10:00	**SESSION 3**		***C. difficile* and public health**
08:30–09:00	INV-6	Dazhi Jin	*C. difficile* epidemiology in China
09:00–10:00			Open forum discussion
10:00 - 10:30			**COFFEE BREAK**
10:30–12:10	**SESSION 4**		**CDI Treatment**
10:30–11:00	INV-7	Maria Vehreschild	New microbiota based therapies – pipeline and legislation
11:00–11:30	INV-8	Paul Feuerstadt	Is there still a place for FMT in a world with LBP?
11:30–11:50	OP-3	Carolina Altaf Figueiredo	*C. difficile* biofilm formation in the gut context
11:50–12:10	OP-4	Bastien Castagner	Small-molecule allosteric triggers of *C. difficile* toxins auto-proteolysis as a therapeutic strategy
12:10 - 13:00			**POSTER SESSION**
13:00 - 14:00			**LUNCH (Hotel Rikli Balance)**
14:00 - 15:00			**POSTER SESSION + COFFEE**
15:00–16:30	**SESSION 5**		**Molecular biology and physiology**
15:00–15:30	INV-9	Johann Peltier	Beyond classical resistance mechanisms: antibiotic resistance in *C. difficile*
15:30–15:50	OP-5	Claire Morvan	Cyclic fatty acids in *C. difficile* cyclic life
15:50–16:10	OP-6	Isabel Roseiro	A single amino acid substitution in CspA increases germination sensitivity and broadens bile salt germinant specificity in *C. difficile* spores
16:10–16:30	OP-7	Janet Wackenreuter	A stress-responsive sRNA modulates sporulation in *C. difficile* via Spo0A
16:30 - 17:00			**COFFEE BREAK**
17:00–18:30	**PARALLEL SESSIONS**		
17:00–18:30	Session 6A		*C. difficile* on the bench — Open forum discussion
17:00–18:30	Session 6B		*C. difficile* in preclinical and clinical settings — Open forum discussion
EVENING			**Free evening**

THURSDAY, SEPTEMBER 10, 2026

08:30–09:50	**SESSION 7**		**Clinical trials and developmental research**
08:30–08:50	OP-8	Mark Riddle	A phase 2 randomized study to evaluate the safety, tolerability and immunogenicity of a *C. difficile* adjuvanted vaccine in healthy adults
08:50–09:10	OP-9	Jason Norman	Biological predictors of response to VE303, a defined bacterial consortium, in recurrent *C. difficile* infection: phase 2 consortium study
09:10–09:30	OP-10	Mayur Ramesh	Evaluation of the safety, tolerability and pharmacokinetics of AZD5148, a long-acting monoclonal antibody against *C. difficile* toxin B
09:30–09:50	OP-11	Erik Dubberke	Development of a novel colonic release metronidazole formulation for treatment of *C. difficile* infection
9:50 - 10:30			**COFFEE BREAK**
10:30–12:10	**SESSION 8**		***C. difficile* virulence**
10:30–11:00	INV-10	Qiwen Dong	Phenotypic heterogeneity in *C. difficile* virulence
11:00–11:30	INV-11	Michael Abt	Impact of *C. difficile* toxins on developing immunity
11:30–11:50	OP-12	Ashley Weiss	*C. difficile* remodels the intestinal epithelium through persistent epigenetic reprogramming spanning acute and recurrent infection
11:50–12:10	OP-13	Tor Savidge	Microbial GABA and pharmacologic GABA_A receptor activation increase host susceptibility to *C. difficile* infection
12:10 - 13:00			**POSTER SESSION**
13:00 - 14:00			**LUNCH (Hotel Rikli Balance)**
14:00 - 15:00			**POSTER SESSION + COFFEE**
15:00–16:30	**SESSION 9**		**Emerging therapeutics and biological insights**
15:00–15:20	OP-14	Andrew Skinner	From antimicrobial resistance to metabolic adaptation: quantifying the shift toward endemicity in *C. difficile*
15:20–15:50	OP-15	Liam Keogh	Baseline microbiota determines *C. difficile* infection severity and prebiotic response
15:50–16:10	OP-16	Alyssa Ehni	Multi-specific nanobody-Fc fusions against toxin B protect in vivo from diverse *C. difficile* strains
16:10–16:30	OP-17	Nebojsa Janjic	Narrow spectrum of CRS3123 is rooted in target phylogeny and translates to low recurrence rates in CDI patients with preservation of healthy gut microbiota
16:30 - 18:30			**NETWORKING**
19:00			**CONGRESS DINNER (Grand Hotel Toplice)**

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ABSTRACTS OF INVITED PRESENTATIONS

INV1

EXPERIMENTAL COLONIZATION AND INFECTION WITH *C. difficile* IN HEALTHY VOLUNTEERS

Wiep Klaas Smits[1], Annefleur Hensen[1], Devika Kalika[1], Céline Harmanus[1], Christine Voorvelt[1], Meta Roestenberg[1].

[1]Leiden University Center for Infectious Diseases (LUCID), Leiden University Medical Center (LUMC), Leiden, The Netherlands.

Experimental colonization and/or infection studies with bacterial pathogens are gaining traction for investigating difficult to study aspects of disease, and to assess the efficacy of novel treatments. At present, no controlled human infection model (CHIM) has been established for *C. difficile*. We previously has established a framework for such a model (1) and the production of *C. difficile* challenge material (2).

We have set up an experimental colonization model for *C. difficile* in which healthy volunteers are colonized after oral administration of capsulized spores of a non-toxigenic strain, L-NTCD03 (clade 4, PCR ribotype 416, ST39), in an out-patient setting (3). The CloDiCo study (NCT05693077) was a adaptive dose design, placebo-controlled randomized clinical trial to assess safety, tolerability and colonization as primary endpoints in groups without and with antimicrobial pretreatment. We found a large impact of pretreatment on colonization, no clear dose-effect and colonization with non-challenge strains in a subset of subjects. Despite relatively small study groups, meaningful differences in microbiome composition were detected.

Informed by the results from the CloDiCo study, we are currently performing experimental infection with a toxigenic *C. difficile* strain, L-TCD-01 (clade 1, PCR ribotype 020, ST2), in healthy volunteers. This study is part of the IHI2-funded Inno4vac project that aims to establish novel CHIM models (4). The CloDiCHI study (NCT06702345) is an open-label, adaptive design, escalating trial to assess safety, tolerability, microbiological and clinical endpoints. Preliminary results from sentinel groups will be presented.

References

1. Hensen et al (2025) Clin Microbiol Infect. doi: 10.1016/j.cmi.2024.08.025.
2. Hensen et al (2026) J Inf Dis. doi: 10.1093/infdis/jiaf612
3. Hensen et al (2026) Nat Commun. doi: 10.1038/s41467-026-74327-y
4. https://www.inno4vac.eu/

INV2

CHARACTERISATION OF A NOVEL *C. difficile* VIRULENCE FACTOR THAT ALTERS HOST ACTIVITY

Diana López-Ureña[1,2], Kaitlyn Gallagher[3], Lena Le[1], Kim Bourke[1], Daniel Foxwell[1], Melanie Hutton[1], Korakrit Imwattana[4], Daniel Knight[4,5], Ashleigh Rogers[1], Alexandra Donlan[6], Yogitha Srikhanta[1], William A. Petri[6], César Rodríguez-Sánchez[2], Tom Riley[4], Borden Lacy[7], Steven Mileto[1], Dena Lyras[1]

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[2]Centro de Investigación en Enfermedades Tropicales, Facultad de Microbiología, Universidad de Costa Rica, San Pedro de Montes de Oca, San José, Costa Rica;
[3]Microbe-Host Interactions PhD Program, Vanderbilt University Medical Center, Nashville, TN, USA;
[4]School of Biomedical Science, University of Western Australia, Perth, WA, Australia;
[5]Department of Microbiology, PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, WA, Australia;
[6]University of Virginia, Division of Infectious Diseases and International Health, Charlottesville, VA, USA; [7]Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA

Background and Aims: Clostridioides difficile causes gastrointestinal disease primarily through toxin-mediated epithelial damage. To date, three toxins, TcdA, TcdB, and CDT, have been implicated in pathogenesis. Here, we aimed to describe a previously unrecognized virulence factor produced by *C. difficile*, named CdV (*C. difficile* virulence factor), and investigate its role during infection.

Methods: Genomic analyses, murine infection models, transcriptomics, untargeted metabolomics, in vitro and cellular imaging approaches were combined to characterize CdV function and distribution in *C. difficile* strains. Continuous monitoring of infected mice using digital ventilated cages enabled non-invasive assessment of locomotor activity throughout infection.

Results: CdV is encoded within a conserved chromosomal locus containing genes encoding proteins related to those found in a growing family of multiprotein complexes capable of delivering effector proteins into host cell. By screening over 30,000 *C. difficile* genomes, we identified the locus in five phylogenetic clades including emerging cryptic clades, mainly deriving from production animals and environmental samples. This locus is also present in strains isolated from human samples, suggesting a possible zoonotic origin and in non-toxigenic *C. difficile* strains. Using an isogenic CdV mutant, we found this factor alters host physiology independently of bacterial burden or feeding behaviors. Mice infected with a CdV-producing strain exhibited increased weight loss and reduced locomotor activity, particularly during dark cycles when rodents are normally most active. These effects occurred in the absence of diarrhea, substantial inflammation, or histological evidence of intestinal damage. Colonic transcriptomic analyses revealed alterations in pathways associated with circadian regulation, cellular stress responses, and mitochondrial biology. In parallel, serum metabolomics identified changes in amino acid and purine metabolism. By expressing the effector region of CdV and using bacterial-free supernatants, we found that CdV localizes to mitochondria and alters mitochondria morphology.

Conclusions: CdV represents a previously unrecognized virulence factor that alters host activity without causing the tissue destruction classically associated with *C. difficile* infection. These findings reveal a stealth strategy by which an enteric pathogen manipulates host responses likely through mitochondrial network disruption.

INV3

ASSESSING INDIVIDUAL RISK OF CDI USING A MINIATURE GUT (MIGUT) MODEL

Ines B. Moura[1]

[1]*School of Medicine, University of Leeds, Leeds, UK*

Antibiotics such as clindamycin or moxifloxacin are known for their high risk of inducing *Clostridioides difficile* infection (CDI), particularly in patients colonised with the hypervirulent strain PCR ribotype 027 (RT027). However, the correlation between colonisation resistance, antibiotic-mediated dysbiosis, antimicrobial resistance and the individual gut microbiome composition is less clearly established for newly epidemic strains.

One such example is the virulent strain PCR ribotype 955 (RT955), one of the few strains associated with hospital outbreaks in the UK over the past two decades. While RT027 and RT955 strains share genetic markers of virulence, the latter have shown antibiotic resistance profiles (e.g. metronidazole) which can impact disease epidemiology and patient treatment.

In vitro human gut models have long provided an effective way to study CDI pathogenesis, as they offer strict process control, and some are able to use human ex vivo prokaryotic and/or eukaryotic cells. However, simulating the colonic environment for long periods can be challenging, with many existing systems showing throughput and parameter control limitations that restrict their use for modelling CDI physiology and disease prognosis.

In Leeds, we developed a scalable miniaturised gut model (MiGut), that maintains the parameter control and clinical relevance of existing triple-stage chemostat models, while allowing the study of individual microbiotas and antibiotic-microbe interactions with higher throughput. We used this system to investigate CDI risk to three individual donors when exposed to RT955 spores and to moxifloxacin, ceftriaxone or clindamycin.

Using MiGut, we observed simulated CDI all models exposed to clindamycin. However, not all microbiotas developed CDI when exposed to ceftriaxone or moxifloxacin. Interestingly, despite the moxifloxacin-resistant profile of RT955, one microbiota did not show *C. difficile* proliferation following moxifloxacin exposure, even as all models were similarly affected by the antibiotic (namely, declines in Lachnospiraceae, Bifidobacteriaceae and Lactobacillaceae and increases in Enterobacteriaceae were observed).

In summary, using a scalable chemostat colonic model, we were able to simulate donor- and antibiotic-specific microbiota responses to RT955 strain colonisation. Different classes of antibiotics induced different microbiota responses; however, these were not always associated with CDI even in cases of inherent antimicrobial resistance.

As *in vitro* models evolve to become more physiologically relevant, there is potential in using single-donor *in vitro* models to identify patient-specific microbiota markers that can support a stratified antibiotic treatment approach to reduce CDI risk.

INV4

Clostridioides difficile: A FOODBORNE PATHOGEN?

Caroline Le Maréchal[1], Olivier Firmesse[2], Frédéric Barbut[3]

[1]Hygiene and Quality of Poultry and Pig Products (HQPAP) Unit, Ploufragan-Plouzané-Niort Laboratory, French Agency for Food, Environmental and Occupational Health & Safety (ANSES), 22440 Ploufragan, France
[2]Staphylococcus, Bacillus & Clostridium (SBCL) Unit, Laboratory for Food Safety, French Agency for Food, Environmental and Occupational Health & Safety (ANSES), 94701 Maisons-Alfort, France
[3]National Reference Laboratory (NRL) for Clostridioides difficile, AP-HP, Assistance Publique-Hôpitaux de Paris, Hôpital Saint Antoine, 75012 Paris, France.

Clostridioides difficile is an opportunistic pathogen causing gastrointestinal infections in humans, often associated with antimicrobial therapy. Once considered primarily a healthcare-associated pathogen, the incidence of community-acquired *C. difficile* infections (CA-CDI) has dramatically increased worldwide, raising major public health concerns. The reasons underlying this epidemiological shift and the factors driving the rise in CA-CDI remain poorly understood.

Food has been proposed as a potential route of *C. difficile* transmission to humans and has been extensively investigated over the past decade. Numerous studies have reported the presence of *C. difficile* in a wide range of food products, including vegetables, meat and meat products, seafood, dairy products, and ready-to-eat foods. Molecular typing of food isolates has identified ribotypes commonly associated with human infections, as well as evidence of genomic relatedness between food and human isolates. In addition, the spore-forming ability of *C. difficile* enables it to withstand and survive under adverse conditions, including heat treatment, refrigeration, and freezing. Together, these findings provide strong evidence that humans are frequently exposed to *C. difficile* through food.

Despite its detection in a variety of food products, no foodborne outbreaks or confirmed human cases directly attributable to contaminated food have been conclusively documented so far. One explanation may be that *C. difficile* is not currently included in routine food surveillance programs, limiting opportunities to systematically compare food isolates with clinical strains. Furthermore, epidemiological investigations are rarely conducted following cases of CA-CDI. and foods consumed by patients are generally not tested for *C. difficile*. As a result, there is currently no definitive evidence that consumption of food contaminated with *C. difficile* directly causes CDI in humans.

It should also be emphasized that CA-CDI is a multifactorial disease. Its development depends not only on exposure to *C. difficile* but also on host-related factors, such as gut microbiota dysbiosis and immune status. Therefore, the mere presence of *C. difficile* is not sufficient to consistently trigger disease.

In conclusion, while available data support the hypothesis that *C. difficile* is a plausible foodborne pathogen, direct foodborne transmission to humans remains unproven and warrants further integrated surveillance and research efforts.

INV5

Clostridioides difficile IN SMALL ANIMALS AND ROLE IN COMMUNITY CDI

Christian Seyboldt1[1]

[1]*Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Bacterial Infections and Zoonoses, Jena, Germany*

In recent years, community-associated (CA) Clostridioides (C.) difficile Infection (CDI) has received increasing attention, as the importance of CA-CDI in the epidemiology of CDI in humans has also grown. There are many ways to be exposed to *C. difficile*, as it can be found in environmental, food, animal, and human samples. Certain animal species, as well as humans, can become infected with *C. difficile* and develop enteritis of varying severity. However, asymptomatic carriage is also possible, particularly in animals but also humans. Infection or carriage in humans is possible through many transmission routes. Since individuals can have particularly close contact with pets such as dogs and cats, it is necessary to collect information on potential infection risks and transmission routes in this context.

Although there are still relatively few studies on companion animals, dogs and cats are the most studied pets in terms of *C. difficile* prevalence, occurring (ribo)types and potential infection risk for humans. Remarkably, the occurrence of interspecies transmission of toxigenic *C. difficile* was reported for dogs and humans. Factors most likely associated with *C. difficile* colonization in dogs and cats are age, enteric disease, antibiotic treatment and hospitalization. Despite some case reports of *C. difficile* infection in dogs and cats, an association with diarrhea was not obvious in a number of studies. Dogs and cats can carry *C. difficile* strains with virulence factors but the duration of *C. difficile* shedding is barely addressed. It is not clear weather a *C. difficile* carriage can be a result of a longer lasting colonization or is just connected with a short transient passage. However, it is not only fecal samples that can test positive for *C. difficile*; the pathogen was also found on dog paws and nasal discharge from dogs, pointing to its environmental presence. Not least, dogs and cats are an important reservoir for *C. difficile*, and an increasing number of studies show that there is direct or indirect interspecies transmission; however, many questions remain to be investigated.

INV6

Clostridioides difficile EPIDEMIOLOGY IN CHINA

Dazhi Jin[1,2]

[1]*School of Laboratory Medicine and Bioengineering, Hangzhou Medical College, Hangzhou, Zhejiang, 310059, China;*
[2]*Laboratory Medicine Center, Department of Clinical Laboratory, Zhejiang Provincial People's Hospital, Hangzhou Medical College, Hangzhou, Zhejiang, 310014, China*

Epidemiology of Clostridioides difficile infection (CDI) in China has been studied in healthcare- and community- acquired patients and animals in last two decades. The molecular characteristics of *C. difficile* are relatively clear, including antimicrobial resistance profile, genotypes, and genome evolution. Here, I aim to summarize *C. difficile* epidemiology in China from patients and animals, respectively.

Most studies on molecular epidemiology of CDI in China published in recent years were carefully reviewed. The main genotypes and antimicrobial resistance profiles of *C. difficile* and risk factors on CDI in China were comprehensively presented. Genomic evolution of two main Chinese *C. difficile* genotypes from human and animals were also investigated, including ST37 and ST35.

Among most of studies on CDI conducted in China, the summarized data showed that CDI had a prevalance of approximately 10%-16% in China, where ST2, ST3, ST35, ST37, and ST54 were predominate genotypes. *C. difficile* isolates from China exhibited region-dependent characteristics on antimicrobial resistance profile, with no resistance to metronidazole, fidaxomicin and vancomycin, and higher resistance rates to erythromycin and clindamycin. Except well-recognized risk factors, the advanced age threshold of patients with CDI was 55 years, which was much younger for Chinese patients than that reported for patients in developed countries. In addition, ST37 genomes from China were divided into four distinct sublineages, showing five importation times and international sources. Isolates associated with severe infections exhibited significantly higher toxin productions, tcdB mRNA levels, and sporulation capacities ($P < 0.001$). Phylogenetic analysis of ST35 genomes reveals two distinctive lineages without time-, region-, or source-dependent distribution, but with different distribution of antimicrobial resistance genes. Nosocomial and communal transmission occurred in humans with the isolates differed by ≤ two core-genome single nucleotide polymorphism (cgSNPs) and clonal circulation was found in pigs with the isolates differed by ≤ four cgSNPs. Notably, interspecies clonal transmission was identified among three patients with community acquired CDI and pigs with epidemiological links, differed by ≤ nine cgSNPs.

This study provided a systematic characterization of CDI epidemiology from human and animals in China, characterising antimicrobial resistance profiles, genotypes, and risk factors. The genome evolution of ST37 and ST35, the 2 main *C. difficile* genotypes from China was also described with intraspecies and interspecies clonal transmission.

INV7

NEW MICROBIOTA BASED THERAPIES FOR *C. difficile* – PIPELINE AND LEGISLATION

Vehreschild Maria[1,2,3]

[1]*University Medicine Frankfurt, Department of Internal Medicine, Infectious Diseases, Goethe University Frankfurt, Frankfurt am Main, Germany;*
[2]*University of Cologne, Faculty of Medicine and University Hospital of Cologne, Department I of Internal Medicine, Cologne, Germany;*
[3]*German Center for Infection Research (DZIF), Partner Site Bonn-Cologne, Germany*

Live biotherapeutic products (LBPs) have emerged as a promising therapeutic modality, harnessing defined microbial organisms for the prevention and treatment of disease. As the field matures beyond early proof-of-concept, several challenges must be addressed to bring these products reliably from bench to bedside. This talk provides an overview of three key dimensions shaping the development of LBPs. First, the evolving regulatory landscape is discussed, including the frameworks established by the FDA and EMA for the classification, characterization, and approval of LBPs, as well as remaining uncertainties around nomenclature and pharmacovigilance requirements. Second, manufacturing and quality control considerations are addressed. Third, the clinical development of LBPs for *Clostridioides difficile* infection (CDI) is reviewed in detail, examining the rationale for microbiome-based approaches in recurrent CDI, key efficacy and safety findings from recent pivotal trials, and the lessons these first approved LBPs offer for the broader field. The talk aims to provide a comprehensive picture of where the field stands and what hurdles remain on the path toward broader clinical adoption of LBPs.

INV8

IS THERE STILL A PLACE FOR FMT IN A WORLD WITH LBP?

Paul Feuerstadt

Associate Clinical Professor of Medicine, Yale School of Medicine; Attending Gastroenterologist, PACT-Gastroenterology Center

Background and Aims: Recurrence of *C. difficile* infection (CDI) after standard of care antimicrobial (SOC) is a major problem with approximately 25% experiencing a recurrence after initial treatment and even higher frequency thereafter. Fecal microbiota transplantation (FMT) as an "add on therapy" replenishes deficient microorganisms in the colonic microbiota, altering the metabolome and decreasing recurrence of CDI. FMT, by definition, involves administration of a broad consortium of microorganisms from whole stool. With the approval of live biotherapeutic products (LBPs) by the Food and Drug Administration in the United States in November of 2022 and April of 2023, more standardized formulations that underwent rigorous clinical trial assessment became more widely available. Parallel to the revolution of microbiota restoration for prevention of recurrent *C. difficile* (rCDI) is the potential of microbiota alteration impacting other diseases, such irritable bowel syndrome, ulcerative colitis, Crohn's disease, and mild-moderate immunocompromise; diseases that are risk factors for CDI and rCDI. The question of impact of FMT or LBP usage in these populations with rCDI on the underlying disease and prevention of rCDI is important and there is emerging data showing that FMT and LBP seem safe and effective for those with these medical co-morbidities. LBP are available mostly in the United States, with some small availability in Canada. As such, the question of whether there is a role for FMT in a world with LBP is an evolving one. Where LBP is not available, FMT remains the gold-standard choice for with rCDI to prevent recurrence. Where LBPs are available, this is a matter of debate, but governmental interventions might limit access to FMT where regulatory approval has been achieved, thereby answering the question for clinicians without them being able to choose.

INV9

BEYOND CLASSICAL RESISTANCE MECHANISMS: ANTIBIOTIC RESISTANCE IN *Clostridioides difficile*

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Antibiotic resistance in Clostridioides difficile has mainly been explained by horizontally acquired resistance genes or mutations in target genes. Recent studies have highlighted clinically relevant changes in susceptibility, including reduced susceptibility to fidaxomicin driven by mutations in RNA polymerase, as well as emerging pathways of vancomycin resistance.

Recent work highlights the bacterial cell envelope as an important site of adaptation under antibiotic stress, where regulation of peptidoglycan metabolism, rather than specific transpeptidation pathways, contributes to changes in antibiotic susceptibility. In particular, the plasticity of the *C. difficile* peptidoglycan, driven by coordinated enzymatic activities involved in its synthesis and maturation, is associated with survival under antibiotic pressure. Findings from our laboratory and others suggest that remodeling of cell envelope architecture can modulate antibiotic susceptibility and contribute to adaptive responses during exposure.

Together, these results support a broader view of antibiotic resistance in *C. difficile* in which genetic determinants and physiological adaptation processes both contribute to survival under antimicrobial pressure. This perspective provides a more integrated understanding of resistance mechanisms and may help identify new strategies for therapeutic intervention.

INV10

PHENOTYPIC HETEROGENEITY IN *C. difficile* VIRULENCE

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Bacteria exhibit phenotypic heterogeneity that generates subpopulations of varied traits to promote adaptation to a given environmental condition. Such phenotypic heterogeneity may influence bacterial motility, antibiotic resistance, and virulence. *Clostridioides difficile* exhibits phenotypic heterogeneity in bacterial colony morphology, flagellar gene expression, and toxin production, impacting the outcomes of disease pathogenicity. For example, the site-specific DNA recombinase RecV regulates multiple phase-variable loci, including the cell wall protein CwpV, which promotes bacterial aggregation and may facilitate intestinal colonization. RecV also controls the CmrRST signaling system, which induces a cell-chaining phenotype that enhances surface motility and contributes to disease severity in hamsters.

Our laboratory recently performed genomic and phenotypic analyses of a panel of RT027 clinical isolates to define factors contributing to *C. difficile* virulence. While we observed a weak correlation between increased flagellar gene expression and virulence, we determined that the heterogeneity of flagellar gene expression is a key contributor to *in vivo* virulence. Flagellar gene expression is regulated by RecV-mediated inversion of the DNA switch upstream of the *flgB* operon, generating flagella-ON and flagella-OFF subpopulations. We discovered that naturally occurring sequence variation within the inverted repeats flanking the flagellar switch alters the efficiency of RecV-mediated recombination, thereby controlling the proportion of flagella-ON and flagella-OFF cells during infection. Notably, strains capable of maintaining both subpopulations exhibited greater virulence than strains biased toward either the flagella-ON or flagella-OFF state, demonstrating that population heterogeneity, rather than a single expression state, maximizes virulence.

Looking forward, single-cell approaches, including fluorescent-labeled reporters and single-cell RNA-seq, will further delineate the functional significance of individual bacterial subpopulations during infection. Taken together, our findings propose a mechanism that may explain the variable disease outcomes observed in *C. difficile* patients, while highlighting the importance of potential cooperative interactions within bacterial subpopulations during disease.

INV11

IMPACT OF *C. difficile* TOXIN ON DEVELOPING IMMUNITY

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Multiple recurrent episodes of *C. difficile* transforms this infection into a chronic, debilitating intestinal disease. It is estimated that up to one quarter of patients that recover from primary *C. difficile* infection will experience a recurrence, often within a month of initial infection and with the same strain. These clinical patterns indicate a breakdown in the generation of protective immune memory against *C. difficile*. Clinical data finds that the ability to generate antibody responses to toxin A (TcdA) and toxin B (TcdB), two of the primary virulence factors that drive *C. difficile* disease, negatively correlates with likelihood of disease recurrence. However, the anti-toxin antibody responses are highly variable in patients and the factors that determine the quality of the antibody response is unknown. Mouse studies that have investigated adaptive immunity report a weak, non-neutralizing, predominantly IgM anti-TcdB antibody response following infection. In contrast, vaccination mouse studies demonstrate that the host has the capacity to generate antibodies that protect against *C. difficile* infection. These studies suggest natural infection actively impairs induction of the adaptive immune response. We have conducted a systemic characterization of the mucosal TcdA and TcdB specific CD4+ T cell and antibody response following infection in mice. Specifically, we find that the toxins impair formation of both the TcdA and TcdB specific effector CD4+ T cell as well as circulating and mucosal antibody response. Inactivation of the glycosyltransferase domain of either toxin restores the toxin-specific effector CD4+ T cell and antibody response and protects the host against *C. difficile* rechallenge. Combined, these data suggest that the toxins are actively disrupting the immunologic chain of events that lead to toxin-specific protective adaptive immunity and may provide a mechanistic explanation for poor immune memory formation in patients that experience high recurrence rates following primary *C. difficile* infection.

9th International *C. difficile* Symposium

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ABSTRACTS OF SHORT ORAL PRESENTATIONS

OP1

Clostridioides difficile INFECTION AMONG ADULTS WITH IMMUNOCOMPROMISING AND CHRONIC HEALTH CONDITIONS IN THE UNITED STATES, 2018-2024: AN ADMINSTRATIVE CLAIMS-BASED

Sarah J. Willis[1], Natalie McCarthy[1], Margaret A. Olsen[2], Pingping Zhang[3], Frederick J. Angulo[1], Constantina Boikos[4], Stephanie Duench[5], Holly Yu[6], Jamie Findlow[4], Erik R. Dubberke[2]

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Background and Aims: Few, current data exist on *Clostridioides difficile* infection (CDI) incidence among adults with immunocompromising and chronic health conditions in the United States (US). We used an administrative claims database to 1) identify CDI cases and describe their demographic and clinical characteristics and 2) estimate CDI incidence among adults, overall and by health conditions.

Methods: We conducted a retrospective cohort study using Optum Clinformatics® between 2018–2024. Adults ≥18 years of age enrolled on January 1 of at least one calendar year were included. CDI was defined as ≥1 inpatient CDI diagnosis code, ≥1 outpatient CDI diagnosis code and metronidazole, oral vancomycin, or fidaxomicin claim ±14 days, or ≥1 CDI-toxin test procedure code and outpatient claim for listed antibiotics ±14 days. We examined demographics and clinical characteristics of adults with and without CDI. We calculated annual incidence rates by following adults from January 1st of the calendar year until the earliest of: CDI, death, health plan disenrollment, or December 31st.

Results: Between 2018–2024, an annual average of 14,703,980 adults were enrolled in the health plan and 30,725 (0.2%) experienced CDI. Adults with CDI were more likely to be ≥65 years of age (69% vs. 32%, p <0.001) and White race (73% vs. 50%, p<0.001) compared to adults without CDI. More than half of individuals with CDI had ≥1 immunocompromising condition (56% vs. 17% without CDI, p<0.001); the most common immunocompromising conditions were chronic kidney disease (34%), solid tumor cancer (19%), and immunodeficiencies (19%) among cases. Three-quarters of adults with CDI had ≥1 chronic condition (75% vs. 35% without CDI, p<0.001); the most common chronic conditions were heart disease (61%), diabetes (37%), and chronic lung disease (33%) among cases. CDI incidence decreased from 286 per 100,000 person-years (PY) in 2018 to 225 per 100,000 PY in 2024. CDI incidence was higher among adults with ≥1 immunocompromising condition (average annual incidence = 808 per 100,000 PY) and adults with ≥1 chronic condition (average annual incidence = 493 per 100,000 PY).

Discussion: Individuals with immunocompromising and chronic health conditions have higher CDI incidence than the general population. Identifying populations at risk for CDI is essential to develop targeted educational and prevention strategies.

OP2

RISK FACTORS FOR *C. difficile* INFECTION RECURRENCE ACROSS THREE DECADES

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Background and aims: *C. difficile* infection (CDI) remains a concern for the NHS, particularly with the increases in incidence seen in England since around 2020. Recurrence is also a persistent challenge in CDI management, given the elevated complication and mortality risks for patients and the strain placed on healthcare resources. Identifying risk factors for CDI recurrence could help identify vulnerable patients for targeted treatment, limit further increases to incidence, and improve understanding of evolving CDI epidemiology. The aim of our study was therefore to identify risk factors for CDI recurrence and evaluate associations over time.

Methods: We analysed electronic health records from Oxfordshire hospitals between 2000 and 2025, to investigate whether CDI recurrence risk factors had changed over time. A number of previously identified risk factors from the literature were evaluated, including hospital exposures, demographics, chronic and acute conditions, procedure history, prior testing, and baseline severity. Using Fine-Gray regression models accounting for competing risks, we compared associations with recurrence and death before recurrence across different epidemiological periods, including before and after the COVID-19 pandemic.

Results: Our results suggest a shift towards increased recurrence risk among community-associated cases, especially compared to the "high virulence" era of the early 2000s. Apart from surveillance classification, we did not detect other significant changes in associations by time period. We identified overall associations with recurrence for several risk factors including age, emergency bed-days, outpatient appointments, immunosuppression, chronic kidney disease, diverticular disease, feeding tubes, and recent sepsis, but not for baseline severity as measured by blood test results.

Conclusions: Recurrence risks increased for community-onset community-associated CDI cases over time, as compared to hospital-onset cases. However, this shift appears to have begun prior to the start of the 2020 pandemic. Despite increased risks for community-associated cases, hospital exposures such as emergency bed-days also remained significantly associated with recurrence. Further research is needed to better understand the complex interplay between infection setting, healthcare exposure, and recurrence risk for CDI patients.

OP3

Clostridioides difficile BIOFILM FORMATION IN THE GUT CONTEXT

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Clostridioides difficile proliferates in the colon upon gut dysbiosis, causing mild-to-severe diarrhea. *C. difficile* infection (CDI) has a high relapse rate that increases with every episode, which has been linked to sporulation and, more recently, to biofilm formation. We already showed that some metabolites, such as deoxycholate, whose levels are altered in dysbiosis, induce formation of strong biofilm *in vitro*. However, the triggers and the genetics and physiology of *C. difficile* induced biofilms are still poorly characterized. Here, we use simple and complex *in vitro* models, as well as microscopy and transcriptomic studies to characterize this process. We have found that volatile short-chain fatty acids, salts with electrolyte function and a heterocyclic aromatic compound are also relevant gut-inducers of biofilm formation at physiological levels. We demonstrate that the induction of biofilms by these metabolites depends on different dietary sugars used by *C. difficile*, which have varying effects on growth kinetics and biomass levels. These inducers may act synergistically, having similar preferred carbon sources, strain specificity and majority of transcriptomic profile. However, a smaller portion of the transcriptome analysis, matrix organization and the gene-knockout profiles suggest that different pathways are involved in biofilm induction in response to each metabolite. In addition, we observed how different inducers shape *C. difficile*'s biofilm matrix structure, its behavior on a gut cellular model and interspecies co-biofilms. Thus, *C. difficile* biofilm formation appears to be a fine-tuned phenotype resulting from the integration of various gut signals during infection. The implication of SCFA in biofilm formation should be considered in ongoing clinical research on pre- and probiotics treatments for CDI. Overall, these signals, provided by the host or beneficial microbial interactions, may promote a protective/adherent biofilm lifestyle, enabling *C. difficile* to defend itself against antimicrobial elements and spatially adapt near the epithelial border, which supports a link between biofilms and carriage between relapses.

OP4

SMALL-MOLECULE ALLOSTERIC TRIGGERS OF *Clostridioides difficile* TOXINS AUTO-PROTEOLYSIS AS A THERAPEUTIC STRATEGY

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Background and Aims: Antibiotics are an important risk factor for *Clostridioides difficile* infection (CDI) because they deplete the gut microbiota that normally protects against *C. difficile* colonization. As a result, current antibiotic treatments plague patients with disease recurrence. The pathogenesis of *C. difficile* is driven by two large protein toxins: TcdA and TcdB, the latter considered to be the primary virulence determinant in human infections. Targeting *C. difficile* toxins is an attractive therapeutic approach since it avoids the use of antibiotics. We hypothesized that analogues of inositol hexakisphosphate (IP6), a natural co-factor responsible for toxin auto-processing inside human intestinal cells, would be able to pre-emptively induce auto-processing in the gut lumen, prior to toxin cell uptake.

Methods: We synthesized and characterized inositol phosphate analogues, where phosphate groups on the inositol core are replaced with sulfates and thiophosphates. The analogs' ability to induce toxin cleavage in the presence of intestinal concentrations of calcium was determined by gel electrophoresis and Western blot. Molecular dynamic simulation was used to understand the allosteric activation of TcdB in response to IP6.

Results: A structure-activity relationship (SAR) study revealed that an analog with 3 sulfates and 3 thiophosphates (IT3S3) displayed the optimal binding affinity to the toxin cysteine protease domain (CPD) in the presence of calcium.[1] Interestingly, the analogs bind to and stabilize the CPD to a greater extent than the natural co-factor IP6 and could displace IP6. Importantly, IT3S3 was more effective in inducing TcdB cleavage in the presence of calcium than our previous candidate that showed activity in a CDI mouse model.[2] Furthermore, molecular dynamic simulation revealed the allosteric network and highlighted the importance of key residues in the activation that is consistent with our SAR.[3]

Conclusion: We have identified a superior second-generation analog that induce TcdA and TcdB cleavage *in vitro*. Further evaluation in vivo is currently underway. These inositol analogs could be interesting tools to studies other bacterial toxins and effectors that use IP6 as a co-factor.[4]

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OP5

CYCLIC FATTY ACIDS IN *C. difficile* CYCLIC LIFE

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Clostridioides difficile is an enteric pathogen responsible for diarrhea and colitis. Initially linked to microbiota disruption following antibiotic treatments, nearly half of current *C. difficile* infections (CDI) are community-associated. CDI represent a major global healthcare and economic burden, notably due to high relapse rates.

C. difficile exhibits two life forms: vegetative cells, which colonize the gut and produce toxins causing symptoms, and spores, which are dormant, highly resistant cells, and responsible for the dissemination and persistence in the environment, contributing to relapses. Survival in the gastrointestinal tract requires adaptation to multiple stresses such as different pH, oxidative and osmotic conditions.

Bacterial membrane plays a key role in sensing environmental stresses and initiating adaptive responses. Cyclopropane fatty acids (CFAs) are modified fatty acids, cyclized by cyclopropane fatty acid synthase (Cfa) directly in membrane phospholipids. Such changes modulate membrane properties by maintaining the fluidity while decreasing the permeability. While CFAs are known to contribute to stress resistance in other enteric pathogens, their roles in anaerobic and sporulating bacteria remain poorly understood. This study thus investigates the role of CFAs in *C. difficile* physiology.

Using a *cfa::erm* mutant in the 630Δ*erm* strain, we examined the role of CFAs in vegetative cells and spores. In vegetative cells, *cfa* disruption led to a reduced survival under acidic, oxidative, osmotic, and detergent stresses. However, Cfa absence did not affect spore resistance to hypochlorite or acidic conditions, nor sporulation efficiency. In contrast, a significant germination defect was observed in the *cfa* mutant spores, suggesting a role for membrane cyclopropanation in the exit from dormancy.

RT-qPCR analyses showed strong induction of *cfa* expression during stationary phase and under sporulation conditions. Fluorescent reporter assays indicated enrichment of *cfa* expression and Cfa protein in forespores.

Overall, membrane cyclopropanation has a context-dependent role, contributing to stress adaptation in vegetative cells and playing a key role in spore germination, highlighting CFAs as important players in *C. difficile* physiology and potentially in environment sensing and infection persistence.

OP6

A SINGLE AMINO ACID SUBSTITUTION IN CspA INCREASES GERMINATION SENSITIVITY AND BROADENS BILE SALT GERMINANT SPECIFICITY IN *Clostridioides difficile* SPORES

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Once regarded primarily as a healthcare-associated pathogen, *Clostridioides difficile* is increasingly associated with community-acquired infection, raising questions about how animal and environmental reservoirs contribute to persistence and transmission. Ribotype 033 (RT033) strains are predominantly linked to these reservoirs, but the traits underlying their ecology remain poorly defined. Here, we show that RT033 spores exhibit a naturally occurring rewiring of germinant sensing, characterized by enhanced germination sensitivity and expanded bile-salt specificity. This enables germination at low germinant concentrations and in response to bile salts typically considered inhibitory, including bile salts prominent in animal hosts. Genetic and functional analyses identify a single amino acid substitution, R1036I, in the pseudoprotease CspA, a component of the CspBAC germination complex, as the determinant of this phenotype. Transfer of the RT033 *cspBAC* operon into a laboratory strain is sufficient to confer increased sensitivity and expanded bile-salt responsiveness. Structural modelling suggests that disruption of a conserved salt bridge at the CspA:CspC interface, caused by the R1036I substitution, alters signalling thresholds without gross destabilization of the complex in the absence or presence of germinants and co-germinants. Together, these findings reveal spore germinant sensing as an evolvable trait in *C. difficile* and suggest that adaptation to animal-associated bile-acid environments may influence persistence in agricultural settings, transmission, and the zoonotic potential of RT033.

OP7

A STRESS-RESPONSIVE sRNA MODULATES SPORULATION IN *Clostridioides difficile* VIA Spo0A

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Clostridioides difficile produces highly resistant endospores by activating a gene expression program controlled by the transcription factor *Spo0A*. Initiation of this sporulation cascade requires tight regulation, as it inevitably leads to the demise of the mother cell. Using RIL-seq (Fuchs et al., 2023), we previously identified 10 small RNAs (sRNAs) that bind *spo0A* and affect its expression. Five were predicted to bind to the ribosome binding site (RBS) of *spo0A* mRNA and therefore likely inhibit translation. Individual deletion of three of these sRNAs significantly increased sporulation frequency. To understand why spo0A is redundantly targeted by multiple sRNAs to downregulate translation, we characterised one of these sRNAs, nc105.

In-line probing confirmed that nc105 binds the RBS of *spo0A* mRNA and *in vivo* reporter assays demonstrated repression of *spo*0A translation via the first C-stretch of the sRNA. nc105 also targets a transcriptional activator of Spo0A, the sigma factor of the transition phase, SigH. In-line probing confirmed binding to the RBS of *sig*H mRNA, and a reporter assay showed that both C-stretches of nc105 enable binding to *sigH*, which reduced translation. Deletion of nc105 increased SigH-driven *spo0A* mRNA levels (northern blot), Spo0A protein levels (western blot), and sporulation frequency, identifying nc105 as a negative regulator of sporulation.

We identified the general stress response sigma factor SigB as the main driver of nc105 expression. SigB induction led to nc105 expression as shown by northern blotting. A fluorescent reporter driven by the promoter of nc105 revealed heterogeneous nc105 expression. Notably, SigB deletion activated an alternative regulatory pathway inducing nc105 expression in a subset of cells during later growth stages. As SigB is activated in response to environmental stresses, we tested relevant conditions and found that nc105 is induced by oxygen exposure, nitric stress and osmotic stress.

Our findings provide mechanistic insights into sporulation control in *C. difficile*, and may explain previous observations (Kint et al., 2017) that SigB deletion increases sporulation frequency. We propose a stress-responsive mechanism in which a sRNA acts as a molecular dimmer of *spo0A* expression, favouring a reversible stress response under conditions where irreversible differentiation into spores is disadvantageous.

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OP8

A PHASE 2 RANDOMIZED STUDY TO EVALUATE THE SAFETY, TOLERABILITY, AND IMMUNOGENICITY OF A *C. difficile* ADJUVANTED VACCINE IN HEALTHY ADULTS

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Background and aims. *Clostridioides difficile* infection (CDI) presents a substantial health burden with limited preventive options. Pfizer's original investigational vaccine formulation, containing genetically modified and chemically detoxified toxins (toxoids), designated TxdA DS and TxdB DS with $Al(OH)_3$ as an adsorbent, was safe and well tolerated and demonstrated encouraging protective efficacy against primary CDI that required medical attention. In this study, we assessed the safety, tolerability and immunogenicity of a new investigational *C. difficile* vaccine, containing the same toxoids, with the addition of CpG adjuvant to elicit an improved immune response.

Methods: In this Phase 2, randomized, multicenter study, 615 adults ≥65 years were assigned to five CpG-adjuvanted vaccine regimens, including two-dose (0,6 and 0,12 months) and three-dose schedules (0,1,6; 0,2,6; and 0,4,12 months). Safety and tolerability were assessed alongside neutralizing antibody responses to toxins A (TcdA) and B (TcdB), and compared with the original $Al(OH)_3$-adsorbed *C. difficile* vaccine.

Results: Reactogenicity was comparable across all *C. difficile* adjuvanted vaccine groups. Most local reactions were mild to moderate, with injection site pain being reported most. Fatigue and headache were the most frequent systemic events. Severe reactogenicity events were uncommon. The adverse event profile across vaccination groups from the time of each vaccination up to 2 months after vaccination reflected events to be expected in individuals ≥65 years of age, consisting primarily of infections, falls, and fractures. Notably, the CpG-adjuvanted vaccine induced **substantially higher neutralizing antibody responses**: for the 2-dose (Months 0,6) regimen, geometric mean ratios (GMRs) were 4.87 (95% CI: 3.59–6.61) for TcdA and 6.67 (95% CI: 4.24–10.50) for TcdB, relative to the original *C. difficile* vaccine in a 3-dose regiment (Months 0,1,6) at Month 7.

Conclusions: The CpG-adjuvanted *C. difficile* vaccine demonstrated a favorable safety profile, with enhanced immunogenicity when compared to the original Al(OH)3-adsorbed formulation. These findings informed selection of a simplified 2-dose schedule (0- and 6-month regimen) for ongoing Phase 3 evaluation and highlight the potential for improved preventive strategies against CDI.

OP9

BIOLOGICAL PREDICTORS OF RESPONSE TO VE303, A DEFINED BACTERIAL CONSORTIUM, IN RECURRENT *C. difficile* INFECTION: PHASE 2 CONSORTIUM STUDY

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Background and Aims: Donor-derived therapies such as fecal microbiota transplantation (FMT) promote a gut environment resistant to *C. difficile* but carry inherent limitations including variable composition, complex donor and stool screening, inconvenient administration, and pathogen transmission risk. VE303 is a first-in-class live biotherapeutic product consisting of eight commensal bacterial strains from Clostridium clusters IV, XIVa, and XVII, manufactured from pure clonal cell banks. In the Phase 2 CONSORTIUM Study, high-dose VE303 was generally well tolerated, reduced risk of CDI recurrence at Week 8 to 13.8% (vs. 45.5% with placebo; absolute risk reduction 31.7%), and achieved robust strain colonization, which was associated with clinical efficacy.

Methods: CONSORTIUM was a Phase 2, randomized, double-blind, placebo-controlled trial. Following standard-of-care antibiotics for a lab-confirmed CDI episode, 79 subjects were randomized to low-dose VE303, high-dose VE303, or placebo, dosed orally once daily for 14 days. Subjects were followed for 24 weeks to assess safety and CDI recurrence; samples collected during dosing and at Weeks 4 and 8 underwent metagenomic, metabolomic, and immune analysis. Machine learning and statistical models identified variables predictive of strain colonization and response to VE303.

Results: Multi-omic modeling identified antibiotic history, baseline stool metabolites, and serum cytokines as predictors of both on-study CDI recurrence and VE303 colonization. VE303 accelerated post-antibiotic recovery of the host microbiota and microbiota-derived metabolites, reducing Gram-negative pathobionts and increasing short-chain fatty acids, secondary bile acids, and bile salt hydrolase genes.

Conclusions: VE303 may protect against recurrence of CDI through multiple mechanisms, including durable strain colonization and restoration of a gut environment inhospitable to *C. difficile*. A registrational Phase 3 study (RESTORATiVE303) is ongoing.

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OP10

EVALUATION OF THE SAFETY, TOLERABILITY AND PHARMACOKINETICS OF AZD5148, A LONG-ACTING MONOCLONAL ANTIBODY AGAINST *C. difficile* TOXIN B

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Background and aim: AZD5148, a half-life extended, humanised, IgG1κ monoclonal antibody (mAb) targeting *C. difficile* Toxin B, is being developed for the prevention of *C. difficile* infection recurrence.

Methods: This was a Phase I, first-time-in-human, double-blind, placebo-controlled, dose-escalation, 12-month study evaluating the safety, tolerability, and pharmacokinetics (PK) of AZD5148 in healthy adults ≥18–≤65 years of age. Participants were randomised 10:2 (AZD5148:placebo) across 5 dosage cohorts (Intramuscular [IM]: Dosages 1–2; intravenous [IV]: Dosages 3–5). PK samples were taken on Days 1 (pre- and post-dose), 2, 5, 8, 15, 29, 91, 181, and 361.

Results: Of 84 participants (mean age: 37.5 years [standard deviation: 10.4 years]; 69% male), 70 received AZD5148 and 14 received placebo. Twenty-two (31.4%) AZD5148 and 8 (57.1%) placebo recipients had ≥1 treatment-emergent adverse event (AE; 8 [11.4%] vs 2 [14.3%] treatment-related, respectively). Related AEs reported in >1 participant were nausea (2 [2.9%]) and injection-site bruising (2 [2.9%]). Three participants (1 each in Dosages 1, 2 and 4) experienced AEs within 1 hour of dosing. No deaths, serious AEs, AEs of special interest, Grade ≥3 AEs, or AE-related discontinuations occurred. The PK profile was consistent across all routes of administration. ADA were detected in a subset of AZD5148 recipients but had no meaningful impact on AZD5148 exposure, and no ADA-related safety concerns were observed.

Conclusions: AZD5148 was found to have a favourable safety profile with a consistent PK across all routes of administration and no clinically meaningful ADA-related concerns. Taken together, these data support advancing AZD5148 to Phase 2b study.

OP11

DEVELOPMENT OF A NOVEL COLONIC RELEASE (CR) METRONIDAZOLE (MTZ) FORMULATION FOR TREATMENT OF *Clostridioides difficile* INFECTION (CDI)

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Background and Aims: MTZ is no longer recommended to treat CDI because of a prolonged time to diarrhea resolution versus vancomycin (VAN). The likely mechanism for this is decreased MTZ excretion into the colon as CDI resolves. MTZ is also poorly tolerated due to systemic side effects. The recommended first line treatment for CDI is fidaxomicin (FDX) followed by VAN. There are reports of increasing resistance and treatment failures to FDX and VAN. Additional options to treat CDI are urgently needed. CR-MTZ may overcome the downsides of immediate release (IR) MTZ. CR-MTZ may achieve higher and more consistent fecal MTZ concentrations and fewer side effects with less systemic MTZ absorption. In this abstract we describe the development of a CR-MTZ formulation.

Methods: Seven CR coating formulations were evaluated in a canine model by X-Ray with barium sulfate. Based on these results 2 formulations were selected to formulate with MTZ for single-dose studies, and one formulation was selected for multidose studies. Systemic bioavailability and fecal MTZ concentrations of these two formulations were compared to IR-MTZ. The formulation with the best CR from the canine studies was further optimized in humans and used in single dose CR-AC phase 1 human study.

Results: Based on the time-to and site-of-dissolution results in the canine barium sulfate experiments, formulas 09E and 10B were used for the canine CR-MTZ studies. In the single dose studies, the systemic MTZ Tmax was at 5 hours for 09E and 10B compared to 1-1.5 hours for IR-MTZ, consistent with delayed absorption. The Cmax was 70.7% (09E) and 46.9%(10B) compared to IR-MTZ . Based on this, 10B was selected for the multi-dose study. The Cmax of 10B was confirmed at 46.8% compared to IR-MTZ, and the AUC at steady state was 30.6% compared to IR-MTZ. Steady state fecal MTZ concentrations were 24.6 µg/g and 1.2 µg/g for 10B and IR-MTZ, respectively. Similar findings were observed in phase 1 human study with the optimized formula with Cmax 42.8% and AUC 60.1% compared to IR-MTZ and mean fecal concentrations of 32.3 µg/g and 1.62 µg/g for CR-MTZ and IR-MTZ, respectively.

Conclusions: We have developed a CR-MTZ formulation that results in less systemic absorption and higher fecal concentrations of MTZ compared to IR-MTZ in healthy humans. This may result in a MTZ formulation with greater efficacy for treating CDI.

OP12

Clostridioides difficile REMODELS THE INTESTINAL EPITHELIUM THROUGH PERSISTENT EPIGENETIC REPROGRAMMING SPANNING ACUTE AND RECURRENT INFECTION

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Background and Aims: *Clostridioides difficile* is the most common cause of nosocomial diarrheal disease, where production of two exotoxins damages the intestinal epithelium and causes dramatic inflammatory responses. 20-30% of *C. difficile* infection (CDI) patients experience recurrent CDI (rCDI), where the risk of recurrence increases after each episode of disease. While it is known that repeated antibiotic use diminishes colonization resistance and opens a niche for *C. difficile*, it is not well understood how *C. difficile* impacts the host intestinal epithelium to contribute to persistent disease. This study investigates the impact of acute CDI on susceptibility to and the severity of rCDI through modifications to the host intestinal epithelial transcriptional memory. Furthermore, a bivalent mRNA vaccine strategy is utilized to assess if vaccination against *C. difficile* toxins can prevent the long-term effects of *C. difficile* on host epithelial remodeling.

Methods: Intestinal epithelial cells (IECs) from mice with acute and rCDI were collected for ATAC- and RNA-sequencing. Uninfected and *C. difficile* infected mice were compared in the context of acute and rCDI along with mice treated with an anti-*C. difficile* toxin mRNA vaccine to determine the effect of toxin-mediated damage on IECs. Immunofluorescence staining was performed to further detect and localize differential histone marks within the colon.

Results: A large subset of genes displayed altered chromatin accessibility and expression in acute CDI that persisted or amplified in rCDI, suggesting *C. difficile* imprints on colonic IECs during a primary episode, predisposing the host to the development and heightened severity of rCDI. Furthermore, mice that experience rCDI have increased expression of genes regulating TNF-α signaling via NF-κB, inflammatory responses, and IL-6/JAK/STAT3 signaling compared to uninfected or acute infected mice. Moreover, vaccination against *C. difficile* toxins reduces the impact of toxin-dependent epigenetic imprints and inflammation-induced transcriptional reprogramming.

Conclusions: This work reveals novel, enduring consequences of *C. difficile* infection on host epigenetic regulation and transcriptional reprogramming and highlights vaccination as an effective strategy for reducing the effect of toxin-dependent epigenetic changes to the intestinal epithelium.

OP13

MICROBIAL GABA AND PHARMACOLOGIC GABA_A RECEPTOR ACTIVATION INCREASE HOST SUSCEPTIBILITY TO *Clostridioides difficile* INFECTION

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Background and aims: *C. difficile* infection (CDI) arises following antibiotic-induced disruption of the gut microbiome, yet how dysbiosis is translated into host susceptibility to toxin-mediated disease remains unclear. We aimed to determine whether microbiota-derived metabolites contribute to CDI susceptibility and define the microbial and host mechanisms involved.

Methods: Integrated microbiome, metabolomic, metatranscriptomic, and fecal microRNA analyses were performed across 6,044 subjects from 38 independent cohorts including CDI, antibiotic-associated diarrhea (AAD), IBD, IBS, cancer and case-controls. Functional studies were conducted in human fecal microbiota bioreactors and murine CDI models. Microbial glutamate decarboxylase pathways were characterized using metagenomics, metatranscriptomics, isotope tracing, and heterologous expression studies. Pharmacologic modulation and epithelial-specific GABA_A receptor α1 knockout mice were used to define receptor-specific mechanisms. Human epidemiologic analyses examined associations between GABAergic medications and CDI risk.

Results: Multi-omics integration identified GABAergic signaling as the most significantly dysregulated host-associated pathway linked to CDI susceptibility. Targeted metabolomics demonstrated elevated fecal GABA concentrations in CDI and AAD that normalized following successful FMT. Antibiotic exposure induced microbial GABA accumulation through enrichment of pH-independent glutamate decarboxylase pathways and depletion of GABA-consuming commensals. Microbiota-derived GABA did not alter *C. difficile* colonization but significantly exacerbated toxin-mediated inflammation and mortality in CDI models. Pharmacologic activation of GABA_A receptors worsened disease severity, whereas antagonism or epithelial-specific deletion of the GABA_A receptor α1 subunit conferred protection. Epidemiologic propensity analyses further demonstrated that GABA_A receptor agonist exposure in patients was independently associated with increased CDI risk.

Conclusions: These findings identify a microbiome-derived GABAergic signaling axis linking antibiotic-induced dysbiosis to epithelial susceptibility during CDI. Luminal GABA promotes pathogenic epithelial GABA_A receptor signaling independently of pathogen burden, revealing a mechanism by which microbial neurotransmitters regulate host vulnerability to infection and identifying clinically actionable host–microbiome interactions relevant to CDI risk and therapy.

OP14

FROM ANTIMICROBIAL RESISTANCE TO METABOLIC ADAPTATION: QUANTIFYING THE SHIFT TOWARD ENDEMICITY IN *Clostridioides difficile*

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Background and Aims: The U.S molecular epidemiology of *Clostridioides difficile* has shifted from the epidemic, fluoroquinolone-resistant RT027 strain to a diverse group of endemic lineages such as RT014/020. The genomic mechanisms underlying this transition remain unknown. This study aims to identify the genomic mechanisms of this transition by comparing the Core Carbohydrate Active enzymes (CAZyme) repertoires, prophage auxiliary metabolic genes (AMGs), and the evolution of fluoroquinolone and cephalosporin resistance between the lineages.

Methods:We conducted a phylogenomic analysis of 486 *C. difficile* ST1 (RT027) isolates and 1034 isolates comprised of the common STs associated with RT014/020 collected from 1985 - 2023. In silico antimicrobial resistance to fluoroquinolones and cephalosporins was determined and defined as mutations in *gyr*A/B or PBP2 (V497L), respectively.

Results: The core CAZomes were stable within both lineages, but RT014/020 enriched plant-glycan foraging (Glycoside Hydrolase Family 1 [GH1]) while RT027 enriched host-mucin degradation (GH129) (PERMANOVA, $p<0.01$). Cysteine/methionine AMGs mapped to the RT014/020 root (MPP=0.97) and persisted in 93.6% of contemporary genomes, versus 3.3% in RT027 ($p<0.01$). RT014/020 expanded purine AMGs (Odds ratio: 1.05/year, $p<0.01$) despite root absence (MPP=0.09). Conversely, RT027 lacked these metabolic AMGs (MPPs <0.05). In RT027, PBP2 V497L co-emerged with gyrA mutations across four independent events between 1998-2001. PBP2 V497L and *gyrB* mutations were transient and co-occurred in 1.3% of RT027 genomes indicating incompatibility. While *gyrA/B* mutations were transient in RT014/020, PBP V497L were found in 11.3% of genomes but were only found within a single sub-clade (MPP = 0.98).

Conclusions: RT027's historic dominance likely resulted from resistance to and exposure to fluoroquinolones. These data reveal that PBP2 mutations co-occurred with *gyr*A mutations in RT027, but not in RT014/020, suggesting PBP2 may be required to compensate for decreased fitness associated with *gyr*A mutations. Unlike RT027, RT014/020 relies on plant-glycan foraging systems and metabolic AMGs proving a mechanism for persistent colonization further explaining the lineages long-term persistence. The lineage's ongoing acquisition of purine AMGs further highlights continuous, non-antimicrobial evolutionary adaptation.

OP15

BASELINE MICROBIOTA DETERMINES *C. difficile* INFECTION SEVERITY AND PREBIOTIC RESPONSE

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Background and Aims: Antibiotic therapies for *C. difficile* infection (CDI) reduce acute disease but prolong dysbiosis, contributing to recurrence and highlighting the need for microbiome-targeted therapies. Prebiotics such as inulin may benefit CDI, however baseline microbiota composition likely influences both CDI susceptibility and therapeutic response. We hypothesized that microbiota structure determines CDI outcome and response to inulin, and identification of taxa involved in inulin-mediated protection will allow the development of a precision synbiotic (combination prebiotic and probiotic). The aims of this project were to (1) demonstrate microbiota-dependent differences in CDI susceptibility; and (2) assess microbiota-dependent efficacy of inulin as a treatment for CDI.

Methods: (1) Conventional C57BL/6 mice from two vendors were housed separately or cohoused for two weeks prior to antibiotic administration and challenge with *C. difficile* spores. Microbiota composition was analyzed by full-length 16S rDNA sequencing. (2) Germ-free C57BL/6 mice underwent FMT using human donor stool prior to antibiotic administration and *C. difficile* challenge. Mice received a low-fibre diet ± inulin supplementation. Metabolomics, immunophenotyping, and 16S rDNA sequencing were performed to characterize inulin response.

Results: (1) Conventional mice from different vendors demonstrated marked differences in CDI severity and survival. Resistance to infection was transferable by cohousing, indicating that microbiota composition was the primary determinant of disease outcome. (2) Humanized mice colonized with different donor microbiomes also exhibited variable CDI susceptibility. Importantly, not all microbiomes responded to inulin supplementation. In one donor microbiome, inulin administration beginning at antibiotic treatment completely protected mice from CDI-associated mortality. Protection was associated with expansion of three inulin-consuming bacterial taxa, representing a candidate precision synbiotic.

Conclusions: These findings demonstrate that CDI susceptibility and prebiotic efficacy are microbiota dependent. Integration of conventional and humanized mouse models identifies baseline community composition as a key determinant of disease outcome and response to inulin. Determining the mechanism of protection by the synbiotic candidate is currently underway.

OP16

MULTI-SPECIFIC NANOBODY-FC FUSIONS AGAINST TOXIN B PROTECT IN VIVO FROM DIVERSE *Clostridioides difficile* STRAINS

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Background and Aim: The sole FDA-approved monoclonal antibody (mAb) for the treatment of recurrent *C. difficile* infection (CDI), ZINPLAVA™ (bezlotoxumab), was discontinued in January 2025. This underscores a need for new mAb based therapeutics for CDI. The target of bezlotoxumab is the dominant virulence factor of *C. difficile*, Toxin B (TcdB). However, TcdB sequences vary across strains and encompass twelve subtypes, with TcdB1/2/3 being of the highest clinical relevance. Sequence variation between TcdB1/2/3 presents a challenge for the identification of pan-neutralizing mAb therapeutics. The objective of this study was to assess the preclinical efficacy of multi-specific nanobody human Fc fusions (Nb-hFc) to overcome sequence divergence and provide greater efficacy than bezlotoxumab for the treatment of CDI across diverse strains.

Methods: We selected three anti-TcdB nanobodies (Nbs) previously characterized by our lab that had therapeutic potential. These Nbs were fused to a human IgG1 Fc to form Nb-hFc homodimers to increase half-life and valency. We utilized a "knobs-into-holes" approach to generate three bi-specific and one tri-specific Nb-hFc fusions. Neutralization potency against TcdB1/2/3 was determined by Vero cell viability assays. Binding affinities were determined by SPR. In vivo efficacy was established by prophylactic studies in a murine model of CDI against representative strains for TcdB1/2/3, as well as a therapeutic study with a TcdB2 representative strain.

Results: All multi-specific fusions neutralized TcdB1/2/3 *in vitro* with greater potency than homodimer Nb-hFcs and bezlotoxumab. Further, the multi-specific fusions improved apparent binding affinities for TcdB1/2/3. When administered prophylactically in a mouse model of CDI, three out of four multi-specific fusions improved infection outcomes over bezlotoxumab against three diverse strains. Additionally, one fusion improved survival in a therapeutic mouse model of CDI.

Conclusions: These results highlight that our multi-specific fusions outperform the standards established by bezlotoxumab both *in vitro* and *in vivo* across TcdB subtypes. These data substantiate multi-specific Nb-hFc fusions as a strategy to improve neutralization potency and overcome sequence variation within TcdB, ultimately providing a foundation for the translation of multi-specific broad-spectrum anti-TcdB mAbs.

OP17

NARROW SPECTRUM OF CRS3123 IS ROOTED IN TARGET PHYLOGENY AND TRANSLATES TO LOW RECURRENCE RATES IN CDI PATIENTS WITH PRESERVATION OF HEALTHY GUT MICROBIOTA

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Background and Aims: Selective attenuation of *C. difficile* while preserving recovering healthy intestinal microbiota is the hallmark of optimal treatments for CDI. Our drug candidate, CRS3123, exhibits narrow spectrum due to confined distribution of the susceptible variant of its target, methionyl-tRNA synthetase type 1 (MetRS1), among primarily pathogenic bacteria, including *C. difficile*. Low impact on healthy intestinal microbiota is due to the presence of the type 2 enzyme, MetRS2, in non-susceptible species (almost all Gram-negative bacteria and Actinobacteria) and in Gram-positive species that have both type 1 and type 2 MetRS. Safety and efficacy of CRS3123 in CDI patients was established in a multicenter, randomized, double-blind, vancomycin-controlled, Phase 2 study conducted in USA and Canada. Both doses of CRS3123, 200 mg and 400 mg BID, as well as vancomycin 125 mg QID exhibited high cure rates after a 10-day treatment, but CRS3123 showed much lower recurrence rates at day 40 (4% with the two doses combined) compared with 23% with vancomycin.

Methods: To explore the impact of treatment in our Phase 2 study on the microbiota of CDI patients, we characterized the fecal microbiome, metabolome, and soluble fecal immune factors of collected fecal samples from this trial.

Results: Both a 200 mg and 400 mg dose of CRS3123 caused less disruption of the commensal microbiome compared to vancomycin, with a less pronounced drop in alpha diversity and a smaller compositional deviation from healthy controls with treatment. CRS3123 better preserved *Bifidobacterium* and *Bacteroides* while effectively clearing both *C. difficile* and vancomycin-resistant enterococci (VRE) and showing a decrease in fecal inflammatory markers. In contrast, vancomycin shifted communities toward *Escherichia-Shigella* and other *Enterobacteriaceae*.

Shotgun metagenomics coupled with targeted metabolomics showed that the microbiome with CRS3123 treatment retained the capacity to produce lithocholic acid, a secondary bile

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acid known to suppress *C. difficile*, whereas the bile acid pool with the vancomycin treatment accumulated conjugated primary bile acids that are known to promote *C. difficile* germination.

Conclusions: Narrow spectrum of CRS3123 coupled with high potency against *C. difficile* translates to high clinical cure rates but much lower recurrence due to limited disruption of healthy intestinal microbiota

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P3	Barbut, Frédéric	Intra-hospital transmission of *Clostridioides difficile* using whole-genome sequencing
P4	Barbut, Frédéric	Epidemiological trends in *Clostridioides difficile* infections in France, 2019–2024: TRACE national population-based study
P5	Savidge, Tor	Microbiome-informed diagnostics distinguish *Clostridioides difficile* infection from asymptomatic carriage
P6	Wang, Xianjun	Accelerated detection of *Clostridioides difficile* sequence type 37 by integrating MALDI-TOF mass spectrometry with artificial neural network
P7	Corver, Jeroen	Cwp20 as potential biomarker to detect *Clostridioides difficile* in feces of CDI patients
P8	Kimani, Andrew Mwaura	*Clostridioides difficile* in the dairy farm environment of southwest Australia
P9	Anantharajah, Ahalieyah	Exploring the circulation of *Clostridioides difficile* in Brussels wastewater
P10	Anantharajah, Ahalieyah	Distinct clinical and antimicrobial resistance profiles of binary toxin genes–positive *Clostridioides difficile* isolates circulating in Belgium
P11	Mrozinski, Alexandre	Treated wastewater irrigation and pathogen dissemination across the food chain and environment: a One Health study of Clostridioides difficile
P12	Kalogeropoulou, Eleni	Increasing burden of *Clostridioides difficile* infection: a twelve-year surveillance study, 2014–2025
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P14	Angulo, Frederick	Systematic literature review of the *Clostridioides difficile* infection (CDI) disease burden in mainland China
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P20	Callies, Milena	Integrating antimicrobial consumption and *Clostridioides difficile* infection surveillance in Belgian hospitals: a decade-long retrospective study
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P23	Henigman, Urška	Detection of *Clostridioides difficile* in pasteurized poultry products
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P25	Lonkeu Njouboussi, Yelena Fadela	Assessment of contamination of point-of-sale foodstuffs with *Clostridioides difficile* in the United Kingdom
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ABSTRACTS OF POSTER PRESENTATIONS

P1

A RETROSPECTIVE STUDY OF *Clostridioides difficile* INFECTION IN HORSES

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Background and Aims: *Clostridioides difficile* causes enterocolitis, with diarrhea and high mortality in horses. The disease is frequently linked to hospitalization and antimicrobial use, although cases without these risk factors also occur. This study describes the clinical, pathologic, and diagnostic findings of *C. difficile*–associated disease (CDAD) in horses in California.

Material and Methods: A retrospective review of 59 equine CDAD cases (2014–2024) submitted for post-mortem examination was performed. The cohort included 34 adults, 16 juveniles, and 9 neonatal foals. Inclusion criteria were detection of TcdA and/or TcdB and/or positive culture for *C. difficile*. Complete necropsies were performed, HE and Gram-stained tissue sections were examined, toxin (A/B) detection was performed by ELISA, and selective culture for *C. difficile* was performed.

Results: The most common clinical presentation was acute diarrhea and/or colic (71%). Other clinical signs included sudden death (12%), respiratory disease (8%), hemorrhagic syndromes (5%), catastrophic orthopedic trauma (5%), neurologic/metabolic disease (5%). 11,9 % of cases had received antimicrobial treatment and 14 % had a history of hospitalization. Gross and histopathological examination revealed necrohemorrhagic colitis (25.4%), enterocolitis (18.6%), enteritis (18.6%), typhlocolitis (16.9%), multiorgan hemorrhages (16.9%), fibrinous peritonitis (8.5%), hemoabdomen (3%), gastrointestinal displacements (6,5%), suppurative bronchopneumonia (8.5%), hepatic necrosis (3.4%) and lipidosis (3.4%), adrenal gland infarction (3.4%). myocardial necrosis (5.1%), interstitial nephritis (5.1%), and bone fractures (3.4. Four cases (6.8%) were positive for *C. difficile* by culture alone, 14 (23,7%) by toxin de tection alone, and 41 (69,5%) by both methods. Clinical findings and gastrointestinal lesions were similar across groups: colic and gastrointestinal lesions were observed in 29.3% of culture-positive/toxin-negative cases, in 31.7% of toxin-positive/culture-negative cases, and in 39.0% of cases positive by both methods, indicating similar clinical and pathological profiles regardless of the diagnostic assay used.

Conclusion: Equine CDAD shows heterogeneous clinical and pathological presentations and limited agreement between culture and toxin detection. These findings support the complementary use of both diagnostic approaches for accurate diagnosis and improved epidemiological interpretation.

P2

EVALUATION OF VIVALYTIC *C. difficile* ASSAY AND VIVALTIC NORO-, ROTAVIRUS, *C. difficile* ASSAY FOR THE DETECTION OF TOXIGENIC *Clostridioides difficile* STRAINS IN STOOLS

Yasmine Roukoz-Dia[1], Muriel Ehmig M[1], Solweig Luce[1], Catherine Eckert[2], Imane Mostaghat[1,2,3], Jeanne Couturier[1,2], Frédéric Barbut [1,2]

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Background and Aims: *Clostridioides difficile* is the major cause of healthcare-associated diarrhea. European guidelines for the diagnosis of *C. difficile* infections recommend the use of a two-step algorithm starting with a sensitive screening method such as GDH detection of nucleic acid amplification technique (NAAT). The objective of this study was to compare the performances (sensitivity and specificity) of the Vivalytic *C. difficile* simplex assay (Bosch), Vivalytic Noro-, Rotavirus *C. difficile* triplex assay and the Xpert *C. difficile* (Cepheid) assays for the detection of toxigenic *C. difficile* strains.

Methods: 300 consecutive fresh diarrheal stool samples were included. Following routine testing by the C. diff. complete assay (TechLab), the 2 Vivalytic assays and Xpert assay were tested in parallel alongside with toxigenic culture (reference standard) on selective medium (ChromId, Biomérieux).

Results: 59/300 stool specimens (19.7 %) were positive for toxigenic culture, including 22 stools positive for free toxins (7.2%). Both the Vivalytic assays and Xpert demonstrated high sensitivity and specificity as shown in the table below. The cdtB cycle threshold (Ct) values obtained with both Vivalytic assays (simplex and triplex) and Xpert assays were highly correlated with a Pearson correlation coefficient of 0.83 and 0.86, respectively ($p<0.001$). The median tcdB Ct values for toxin EIA-positive/NAAT-positive specimens were significantly lower than those for toxin EIA-negative/NAAT-positive specimens with Vivalytic simplex (26.28 vs 33.01), Vivalytic triplex (26.28 vs 32.96) and Xpert assays (23.07 vs 29.05) ($p< 0.005$). Receiver operating characteristic curve analysis, assessing the predictive ability of Ct values for the detection of free toxins exhibited an area under the curve of 0.767 for the Vivalytic simplex, 0.823 for the Vivalytic triplex and 0.875 for the for Xpert.

	Sensitivity (CI95%)	Specificity (CI95%)	NPV (CI95%)	PPV (CI95%)	Accuracy (CI95%)
Simplex	89.8 (79.2-96.2)	98.59 (97.7-99.9)	97.56 (94.9-98.8)	98.15 (88.2-99.7)	97.67 (95.2-99.1)
Triplex	89.8 (79.2-96.2)	98.59 (97.7-99.9)	97.56 (94.9-98.8)	98.15 (88.2-99.7)	97.67 (95.2-99.1)
Xpert	100 (93.9-100)	98.3 (95.8-99.6)	100 (98.5-100)	93.6 (88.8-97.5)	98.67 (96.6-99.6)

Conclusions: Both Vivalytic assays have good analytical performances and can be used as screening tests in a two-step algorithm diagnostic strategy.

P3

INTRA-HOSPITAL TRANSMISSION OF *Clostridioides difficile* USING WHOLE-GENOME SEQUENCING

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Background and aims: *Clostridioides difficile* is the leading cause of healthcare-associated diarrhea in adults. Transmission occurs via the oral route through spores, which can survive in the environment for months. PCR-ribotyping, a reference method routinely used to characterize *C. difficile* strains, lacks discriminatory power to accurately study transmission. The objective of this study was to determine the frequency of transmission of *C. difficile* strains outside of an epidemic context using cgMLST (core-genome Multi-Locus Sequence Typing), a highly discriminatory typing method based on the comparison of allelic variations among strains.

Materials and Methods: All *C. difficile* strains isolated between January 1, 2024, and October 10, 2024, in our 650-beds university-affiliated hospital were included. The strains were characterized by PCR ribotyping and cgMLST. The cgMLST analysis was performed under Unix on the cluster of the French Institute of Bioinformatics. The sequences were cleaned using fastp v0.12.1 and then assembled using shovill v1.0.9. cgMLST analysis was performed using the ChewBBACA v3.3.4 tool, and the results were visualized as a minimum spanning tree in the GrapeTree software. Two strains were considered clonal when they differed by no more than 3 alleles. The different types of transmission were defined as follows: patient-to-patient (overlapping hospitalization in the same ward), environmental (same ward at different times), intra-hospital (overlapping hospitalization in different wards), or cryptic (no epidemiological link but identical strains).

Results: A total of 181 strains isolated from 125 patients were analyzed. Three strains were excluded from the analysis due to poor sequencing quality. The strains primarily belonged to PCR ribotypes 106 (n=14), 020 (n=13), and 002 (n=13). cgMLST analysis of 178 strains showed that 25 pairs of strains were clonal, including 22 pairs of strains of the same PCR ribotype. Twelve pairs of strains had been isolated from the same patient and corresponded to relapses. The 13 events of potential transmission (10.4%) between two patients included 4 patient-to-patient transmissions, 1 environmental transmission, 3 nosocomial transmissions, and 5 cryptic transmissions.

Conclusion: Among clusters identified by cgMLST, 38% corresponded to transmission with unidentified epidemiological link between patients.

P4

EPIDEMIOLOGICAL TRENDS IN *Clostridioides difficile* INFECTIONS IN FRANCE, 2019-2024: TRACE NATIONAL POPULATION-BASED STUDY

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Background and aims: *Clostridioides difficile* infection (CDI) is a major cause of healthcare-associated diarrhea with 124,000 cases per year in Europe and 3% of attributable mortality. In France, most recent data (2019) indicated an incidence of 34.0/100,000 of community-treated CDI episodes and 29.1/100,000 of hospitalized CDI episodes. However, since 2019 no national estimates have been available. The TRACE study aimed to assess national CDI trends using the French National Health Data System (SNDS) from 2019 to 2024.

Methods: Patients ≥2 years old with CDI in hospital and community settings were included. SNDS definition was based on adapted ECDC-based algorithm combining (1) ICD-10 code A04.7 for hospitalized patients (2) a *C. difficile* tests AND a targeted CDI antibiotics within a 7-day window for patients from the community. ICD-10 diagnoses, surgical procedure codes for digestive CDI complications, antibiotic dispensing and microbiology exams allowed estimation of annual incidence, new episodes and recurrent cases through a one-year back and one-year follow-up.

Results: Between 2019 and 2024, 206,640 distinct patients had at least one episode of CDI in France. In-hospital incidence increased from 2.99/10,000 hospitalization-days (HD) (n=14,532) in 2019 to 3.76/10,000 HD (n=16,633) in 2024 (+26%). Outpatient CDI showed a marked increase from approximately 8,300 cases in 2019 (incidence 31/100,000 inhabitant) to 25,800 in 2024 (incidence 41/100,000 inhabitants). Over one third (38%) of the new cases occurred in hospital, mainly in older patients with comorbidities, and the majority (85%) were healthcare associated CDI. In addition, 62% of CDI cases occurred in the community, in younger adults, and 57% of the new cases were community CDI

Conclusions: This study suggests a rising trend in both hospital and community settings. A validated SNDS-based CDI detection tool could complement existing surveillance systems and strengthen national monitoring capacities.

P5

MICROBIOME-INFORMED DIAGNOSTICS DISTINGUISH *Clostridioides difficile* INFECTION FROM ASYMPTOMATIC CARRIAGE

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Background and Aims: *C. difficile* infection (CDI) is a leading cause of healthcare-associated disease driven by disruption of the gut microbiome. Current molecular diagnostics detect toxigenic *C. difficile* but cannot reliably distinguish active infection from asymptomatic carriage in patients with alternative diarrheal etiologies, resulting in overdiagnosis and unnecessary antimicrobial treatment. We aimed to develop a rapid microbiome-informed molecular diagnostic capable of differentiating active CDI from asymptomatic carriage and non-CDI diarrhea.

Methods: Microbiome signatures associated with CDI were identified by metagenomic sequencing from 8,044 individuals across 36 independent cohorts and incorporated into supervised machine-learning classifiers. Top discriminatory taxa were translated into a nested multiplex PCR panel implemented on the Research-Use-Only BIOFIRE® SPOTFIRE® FILMARRAY® platform. Independent validation was performed in 2,358 fecal specimens spanning CDI and multiple non-CDI diarrheal cohorts. Functional correlations with metabolomic profiles and treatment outcomes were additionally evaluated.

Results: CDI-associated microbiomes were characterized by depletion of health-associated commensals and pathobiome enrichment. Machine-learning classifiers demonstrated high discrimination between CDI and non-CDI diarrhea (AUC = 0.997). The multiplex PCR assay reproduced sequencing-based microbial profiles with high fidelity in an approximately 20-minute sample-to-answer time. Combined classifiers distinguished IDSA-defined CDI from non-CDI diarrhea with 95.4% sensitivity and 100% specificity, and differentiated ROME III-defined asymptomatic carriage from CDI with 99.1% sensitivity and 93.6% specificity. Strong classifier performance was retained even when toxigenic *C. difficile* abundance was excluded, indicating that broader ecological dysbiosis signatures underlie disease classification and favorable treatment outcomes. Functional screening of top-ranked taxa identified potent anti-*C. difficile* activity associated with *Anaerostipes hadrus*, leading to discovery of hadromycin, a new ether-class glycolipid with selective antimicrobial activity.

Conclusions: We developed a rapid microbiome-informed multiplex PCR diagnostic that accurately differentiates active CDI from asymptomatic carriage and alternative diarrheal etiologies. Integration of ecological microbiome context with pathogen detection substantially improves diagnostic precision beyond conventional toxin-targeted assays and identifies protective commensal pathways linked to endogenous antimicrobial resistance against *C. difficile*.

P6

ACCELERATED DETECTION OF *Clostridioides difficile* SEQUENCE TYPE 37 BY INTEGRATING MALDI-TOF MASS SPECTROMETRY WITH ARTIFICIAL NEURAL NETWORK

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Background and Aims: Rapid identification of *Clostridioides difficile* (*C. difficile*) Sequence Type 37 (ST37) is crucial due to its association with severe infections and antibiotic resistance. Existing methodologies are labor-intensive and costly. The modeling approach combining matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) with machine learning offers a promising alternative for fast and cost-effective subtyping.

Methods: This work gathered 1155 mass spectra representing 385 distinct clinical *C. difficile* isolates from multiple regions, including 118 ST37 isolates (30.65%) and 267 non-ST37 isolates (69.35%). An artificial neural network (ANN) model was created using MALDI-TOF MS data, trained on 80% of the dataset, and validated on the remaining 20%.

Results: The constructed ANN model demonstrated exceptional diagnostic precision and reliable generalizability, achieving an area under the receiver operating characteristic curve (AUROC) of 0.96 and an area under the precision-recall curve (AUPRC) of 0.94 for detecting *C. difficile* ST37 in the validation set. Furthermore, we identified the top 15 potential biomarkers of *C. difficile* ST37 strains with mass-to-charge (m/z) ratios of 6729, 12013, 12012, 6731, 7296, 12085, 14716, 7292, 3104, 7293, 16966, 15360, 7259, 18488, and 14660 Da.

Conclusions: Our model provides a rapid, reliable, and economical option for identifying *C. difficile* ST37. The integration of our model into the clinical laboratory workflow enables the swift identification of the ST37 strain in about 10 seconds. This facilitates accurate clinical diagnosis, mitigating the risk of severe *C. difficile* infections.

P7

CWP20 AS POTENTIAL BIOMARKER TO DETECT *Clostridioides difficile* IN FECES OF CDI PATIENTS

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Clostridioides difficile is a bacterium present in relatively low numbers in the feces of asymptomatic individuals. Antibiotic treatment, however, is a major cause of *C. difficile* overgrowth, infection (CDI) and diarrhea, due to disruption of the intestinal microbiota (1). Despite the clinical burden of CDI, current diagnostic tools often lack sensitivity and/or specificity.

Here, we characterized the enzymatic activity and substrate specificity of the *C. difficile* serine protease Cwp20, using proteomics and activity-based protein profiling. Cwp20 selectively cleaved D-alanine-containing peptides and biochemical analysis revealed structural similarities to penicillin-binding proteins. Fecal screening showed detectable activity of Cwp20 in fecal samples from twelve CDI patients, but not in samples from twelve individuals with non-CDI associated diarrhea. Further, Cwp20 activity correlated strongly with *C. difficile* bacterial load, showing its diagnostic potential. The enzyme's high specificity, stability, and direct detectability in feces highlight its suitability for non-invasive CDI diagnostics.

In conclusion, Cwp20 activity represents a promising and specific biomarker for active *C. difficile* infection. Its discovery opens new possibilities for accurate, rapid, and non-culture-based diagnostic approaches, which are critically needed for the effective clinical management and control of CDI.

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P8

Clostridioides difficile IN THE DAIRY FARM ENVIRONMENT OF SOUTHWEST AUSTRALIA

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Background and Aims: Antimicrobial exposure is a well-established driver of *Clostridioides difficile* infection (CDI) in hospitalised patients. However, over the last three decades, CDI outside healthcare settings has increased significantly, making CDI a growing global public health concern. *C. difficile* has been detected in a range of environmental settings, highlighting its ability to persist outside healthcare and raising concerns about the role of animal and environmental reservoirs/sources in its persistence and transmission. Given the use of antimicrobials in dairy production, this study investigated the prevalence, strain types and toxin gene profiles of *C. difficile* recovered from the dairy farm environments in the Southwest region of Western Australia and assessed their susceptibility to a panel of clinically important antimicrobial agents including metronidazole, vancomycin, clindamycin, ceftriaxone and moxifloxacin.

Methods: Using a cross-sectional study design, a total of 171 environmental samples, including soil, water and effluent, was collected from three dairy farms in the Southwest of Western Australia (WA) in January 2026. Samples were transported to the laboratory under ambient conditions, cultured on/in selective and enrichment media and incubated in a Don WhitleyA35 anaerobic chamber to isolate *C. difficile*. Genomic DNA was extracted from pure cultures and screened for *C. difficile* toxin genes by PCR. Ribotyping was performed and ribotypes (RTs) identified by comparing banding patterns with those of a database of reference strains using BioNumerics version 7.6. Antimicrobial susceptibility of the identified strains was assessed using disc diffusion and E-test methods.

Results: *C. difficile* was present in all three farms and overall, 32.2% of samples contained *C. difficile*. Soil had the highest prevalence (45.7%), followed by effluent (26.7%) and water (5.9%), highlighting soil as the principal environmental source in this study. Sixty isolates of *C. difficile* representing 10 distinct RTs were recovered; half the RTs had been reported in humans previously. *C. difficile* RTs 033 (A–B–CDT+) and 127 (A+B+CDT+) predominated, accounting for 33.3% and 28.3% of isolates, respectively. For antimicrobial susceptibility testing, 42 isolates were selected. Of these, all remained susceptible to vancomycin, moxifloxacin and clindamycin, however, five (12%) exhibited low-level clindamycin resistance with elevated MICs.

Conclusions: Australian dairy farms may serve as reservoirs or sources of toxigenic *C. difficile* strains of epidemiological and possible public health relevance. The elevated clindamycin MICs in a few isolates suggest that antimicrobial resistance can emerge in such environments and susceptibility patterns of *C. difficile* isolated from dairy farms should be monitored over time.

P9

EXPLORING THE CIRCULATION OF *Clostridioides difficile* IN BRUSSELS WASTEWATER

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Background and Aims: *Clostridioides difficile* is a spore-forming pathogen capable of environmental persistence. Spores excreted in faeces may enter wastewater systems and contribute to environmental dissemination. However, little is known about the circulation of *C. difficile* ribotypes in wastewater in Belgium. We aimed to investigate the presence and diversity of *C. difficile* in Brussels wastewater samples and compare the identified ribotypes with Belgian clinical isolates.

Methods: Eighteen wastewater samples collected on Mondays by auto-samplers (24-hour composite) at the inlet of two wastewater treatment plants covering the Brussels-Capital region were analysed using an enrichment culture protocol adapted from previous wastewater studies (Chisholm et al.,2022). Following filtration and anaerobic enrichment in supplemented Brain Heart Infusion broth, alcohol shock and culture on ChromID agar were performed. Up to 10–12 colonies per sample were subcultured and characterised by PCR ribotyping. Identified ribotypes were compared with Belgian National Reference Centre surveillance data from 2024–2025. In Brussels, clinical surveillance included isolates received from five hospitals.

Results: Clostridioides difficile was isolated from wastewater samples, revealing ribotype diversity, with 16 ribotypes identified. Clinically relevant ribotypes included RTs 014, 020, 001, 012 and 078. Most identified strains carried both major toxins (*tcdA* and *tcdB*), while RT078 and RT695 additionally carried binary toxin genes. Three non-toxigenic ribotypes were also identified (RTs 010, 031 and 073). Several ribotypes infrequently detected in Brussels clinical surveillance were nevertheless identified in wastewater, including RT081 (0.7% Brussels), RT047 (0.5%), RT207 (0.7%), RT073 (0.5%), RT695 (0.2%), RT031 (0%) and RT296 (0%). Conversely, several common clinical ribotypes, including RTs 002, 106, 015, 005 and 023 were not detected in wastewater samples.

Conclusions: This pilot study highlights the potential utility of wastewater analysis for *C. difficile* in Belgium. Wastewater samples showed a diverse range of ribotypes, including clinically relevant strains and uncommon ribotypes rarely identified in national surveillance. Further longitudinal and One Health investigations are needed to better understand relationships between human, animal and environmental reservoirs of *C. difficile*.

P10

DISTINCT CLINICAL AND ANTIMICROBIAL RESISTANCE PROFILES OF BINARY TOXIN GENES–POSITIVE *Clostridioides difficile* ISOLATES CIRCULATING IN BELGIUM

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Binary toxin (CDT)–positive *Clostridioides difficile* strains have been associated with increased virulence, yet their epidemiology in Belgium remains poorly characterised. We investigated the genomic diversity, clinical characteristics, and antimicrobial resistance of CDT-positive strains collected through the National Reference Centre between 2016 and 2025.

All isolates underwent PCR ribotyping and toxin gene detection. A subset of 211 CDT-genes positive isolates underwent whole-genome sequencing and antimicrobial susceptibility testing. Clinical and epidemiological data were compared between clades.

CDT-positive isolates represented 14.0% (1117/7975) of isolates between 2016 and 2025. RTs 078 (47.2%), 023 (16.1%), 126 (13.8%), and 027 (13.4%) predominated. Clade 5/ST11 showed the highest diversity (13 ribotypes; n=125), mainly RT078 and RT126, with clindamycin (91.7%) and erythromycin (59.0%) resistance; tetracycline resistance (31.3%) was restricted to this clade. Clade 3 (6 ribotypes; n=37), dominated by RT023/ST5, remained susceptible except for clindamycin resistance (58.5%). Clade 2 (mainly ST1), comprised 10 ribotypes (n=46), including RTs 027, 181 and 176. RT027 declined after 2020, whereas RT955 and RT244 were first detected in Belgium in 2025. This clade showed resistance to moxifloxacin (69.8%), rifampicin (18.6%) erythromycin (67.4%) and clindamycin (93.0%). Among clade 2, 36/46 isolates carried *Pnim*BG and *nim*B L155I mutations suggestive of haem-dependent metronidazole resistance despite preserved phenotypic susceptibility in all but one strain. One RT955 isolate showed a metronidazole MIC at the susceptibility breakpoint (MIC 2-4 mg/L), and one RT027 isolate carried the *vanR-Cd_T115A* mutation associated with reduced vancomycin susceptibility (MIC 4 mg/L). Clade 3 was significantly associated ($p<0.05$) with younger patients, non-healthcare-associated CDI, and complicated infection, whereas clades 2 and 5 were associated with elderly patients and HA-CDI ; recurrent CDI was more common in clade 2. CDT-positive *C. difficile* strains circulating in Belgium show a distinct clade-associated clinical and antimicrobial resistance profile. Ongoing circulation and emergence of epidemic ribotypes, together with detection of mutations associated with reduced metronidazole and vancomycin susceptibility highlight the need for continued genomic surveillance.

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TREATED WASTEWATER IRRIGATION AND PATHOGEN DISSEMINATION ACROSS THE FOOD CHAIN AND ENVIRONMENT: A ONE HEALTH STUDY OF *Clostridioides difficile*

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Water scarcity is placing increasing pressure on agriculture and driving the search for alternative irrigation sources. Treated wastewater (TWW) reuse represents a promising solution within a circular economy framework; however, its microbiological safety remains a concern. TWW may contain pathogens such as *Clostridioides difficile*, a spore-forming bacterium causing severe intestinal infections and persisting in the environment. Its presence in wastewater treatment plants (WWTP) has been reported in the literature, but it has not been studied in France yet, and the impact of TWW reuse on the contamination of irrigated crops by *C. difficile* remains unclear. This study aimed to evaluate *C. difficile* presence in TWW according to treatment processes and to assess contamination of vegetables following irrigation with TWW.

Five WWTPs using different treatment processes were sampled over one year (180 samples) to monitor *C. difficile* during wastewater treatment over time. Isolates were recovered using culture-based methods and characterized for virulence genes, antimicrobial resistance and PCR ribotyping. Greenhouse experiment was conducted using radish and rocket grown in soils irrigated either with conventional TWW or clean water (10 replicates per condition). The presence of *C. difficile* was investigated in irrigation water and harvested vegetables. Vegetable-derived isolates were sequenced using Oxford Nanopore long-read technology.

C. difficile was consistently detected in TWW from WWTPs using conventional treatments, with toxigenic strains present throughout the year. In contrast, no *C. difficile* was detected in TWW from a WWTP equipped with final membrane filtration. Greenhouse experiments demonstrated partial contamination of crops following irrigation with conventional TWW: *C. difficile* was recovered from radishes (3/10) and rockets (1/10). Most isolates recovered from TWW and crops were toxigenic (6/10, and none showed phenotypic resistance to clinically relevant antibiotics. Genomic analysis revealed the presence of numerous mobile genetic elements carrying other antibiotic resistance genes. From a One Health perspective, irrigation with conventionally TWW may contribute to the dissemination and persistence of toxigenic *C. difficile* in agricultural environments. Advanced treatments such as membrane filtration could reduce the risk of transmission to irrigated crops.

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INCREASING BURDEN OF *Clostridioides difficile* INFECTION: A TWELVE-YEAR SURVEILLANCE STUDY, 2014-2025

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Background and Aim: *Clostridioides difficile* infection (CDI) remains an important cause of healthcare-associated morbidity and mortality. Long-term surveillance is essential for evaluating temporal trends in CDI burden, diagnostic activity, acquisition origin, recurrence, and outcomes. We evaluated CDI trends over 12 years in a tertiary-care hospital.

Methods: We conducted a twelve-year CDI surveillance study at Attikon University Hospital, Athens, Greece. CDI data were prospectively collected from 2014 to 2025 and analyzed according to the ECDC CDI surveillance protocol, version 2.4. CDI episodes were classified as new or recurrent, and new episodes as healthcare-associated, community-associated, or unknown. Incidence and testing activity were calculated per 10,000 patient-days. Patient characteristics, length of stay among survivors, and crude all-cause in-hospital mortality were assessed.

Results: Overall, 1,753 CDI episodes were identified, including 1,599 new and 154 recurrent episodes. Annual CDI episodes increased from 62 in 2014 to 270 in 2023, followed by 259 in 2024 and 244 in 2025. New CDI incidence increased from 3.2 per 10,000 patient-days in 2014 to 10.7 in 2023 and remained elevated in 2024 and 2025, at 10.8 and 9.8, respectively. Healthcare-associated CDI accounted for most new episodes, representing 1,394/1,599 cases; community-associated and unknown-origin CDI accounted for 199 and 6 cases, respectively. Testing activity increased from 48.2 tests per 10,000 patient-days in 2014 to 125.7 in 2025. Recurrent CDI increased from 11 episodes in 2022 to 46 in 2023 and remained elevated in 2024–2025, with 30 episodes annually. Crude all-cause in-hospital mortality was substantial, with 406 deaths overall, increasing from 19 in 2014 to 69 in 2023, before declining to 54 in 2025. Median age ranged from 74.5 to 79 years, and median stay among survivors ranged from 12 to 17 days.

Conclusions: Over twelve years, CDI burden increased substantially, particularly after 2021, alongside intensified testing and a predominance of healthcare-associated episodes. Although incidence stabilized or modestly declined after the 2023–2024 peak, rates remained above early-period levels. These findings support sustained surveillance, interpretation of incidence trends in relation to testing activity, and strengthened infection prevention and antimicrobial stewardship.

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RECENT INCREASE IN THE INCIDENCE OF *Clostridioides difficile* INFECTION (CDI) IN MOST EUROPEAN COUNTRIES WHICH CONDUCT POPULATION-BASED CDI SURVEILLANCE, 2017-2024

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Background and Aims: *Clostridioides difficile* infection (CDI) is an important cause of morbidity and mortality worldwide. Unlike hospital-based CDI surveillance, population-based CDI surveillance provides a more complete estimate of the population-level CDI disease burden (i.e., CDI cases, hospitalizations, deaths) because it captures CDI cases occurring in both the hospital and outpatient settings. Although many European countries conduct hospital-based CDI surveillance, fewer conduct population-based surveillance. In European countries that conducted population-based CDI surveillance, we assessed temporal trends in population-based CDI incidence in the pre-COVID19 pandemic period from 2017-2020 and in the post-pandemic period from 2022-2024.

Methods: We searched PubMed and publicly available reports from national governmental agencies responsible for infectious disease surveillance to identify population-based CDI surveillance data. We extracted surveillance-reported laboratory-confirmed CDI case counts and population-based incidence estimates (cases per 100,000 population per year [PPY]) from 2017-2024. For surveillance programs reporting only CDI case counts, population-based CDI incidence was calculated using EuroStat mid-year population estimates.

Results: Eight European countries (England, Finland, Ireland, Northern Ireland, Norway, Scotland, Sweden, and Wales) conducted population-based surveillance for laboratory-confirmed CDI cases between 2017-2024. CDI incidence in 2024 ranged from 33/100,000 PPY in England to 64/100,000 PPY in Norway. In the post-pandemic period from 2022-2024, the CDI incidence increased by 22% in England, 21% in Ireland, 5% in Norway, 37% in Scotland, and 35% in Wales. In each of these five countries, CDI incidence in 2024 exceeded the CDI incidence in 2017: England (33/100,000 PPY v. 24/100,000 PPY), Ireland (35/100,000 PPY v. 32/100,000 PPY), Norway (64/100,000 PPY v. 58/100,000 PPY), Scotland (26/100,000 PPY v. 25/100,000 PPY), and Wales (50/100,000 PPY v. 34/100,000 PPY).

Conclusion: Population-based CDI incidence increased in several European countries during the post-pandemic period. These surveillance data describe ongoing CDI disease burden in Europe and highlight the need for enhanced prevention strategies to reduce the CDI disease burden in the community and healthcare settings.

P14

SYSTEMATIC LITERATURE REVIEW OF THE *Clostridioides difficile* INFECTION (CDI) DISEASE BURDEN IN MAINLAND CHINA

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Background and Aims: *Clostridioides difficile* infection (CDI) is an important cause of morbidity and mortality worldwide. In many countries, CDI is a leading cause of healthcare-associated infections and an increasing cause of community-associated infections. There is limited information, however, on CDI disease burden in China. Hospital-based studies in Hong Kong reported a population-based hospitalized CDI incidence comparable to that observed in the United States, but public health surveillance for CDI is not conducted in China and the CDI disease burden is not well defined in mainland China. We conducted a systematic literature review to better understand the available data on CDI disease burden in mainland China.

Methods: Three global (MEDLINE including using PubMed, Embase, and Cochrane) and three Chinese (National Knowledge Infrastructure, Science Citation, Wanfang) databases were searched in August 2025 using CDI-related and epidemiological search terms with no date or language limits. Outcomes of interest for CDI disease burden in mainland China, abstracted or calculated from the available studies, included hospital-based CDI incidence (cases per 10,000 patient-days), population-based CDI incidence (cases per 100,000 persons per year), and CDI admission rates (cases per 1,000 admissions).

Results: Searches yielded 563 unique articles. Following the exclusion of 547 (97.2%) in accordance with the exclusion criteria, 16 articles were assessed at the full text review level, of which 11 articles reported outcomes of interest. The 11 articles were published between 2014-2023 and were each a single-hospital study; the studies were conducted in six cities in mainland China (map). While none of the studies reported population-based CDI incidence, 10 (90.9%) studies reported hospital-based CDI incidence, and 4 (36.4%) reported CDI admission rates. The reported hospital-based CDI incidence ranged from 0.2-58.0/10,000 patient-days and the CDI admission rate ranged from 0.2-10.0/1,000 admissions.

Conclusion: A limited number of studies demonstrate the presence of CDI in hospitals in mainland China but there are no published estimates of population-based CDI incidence for mainland China. Future population-based studies are needed to quantify the CDI disease burden in mainland China and to guide prevention strategies aimed at reducing the CDI disease burden in China.

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PROTOCOL FOR A PROSPECTIVE MULTI-HOSPITAL STUDY OF THE *Clostridioides difficile* INFECTION (CDI) DISEASE BURDEN IN FIVE CITIES IN CHINA

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Background and Aims: *Clostridioides difficile* infection (CDI) is an important cause of morbidity and mortality worldwide. Data on CDI disease burden are important for understanding the unmet medical need for the prevention of CDI. However, there is a paucity of data on CDI disease burden in China. We describe the study protocol for conducting a prospective multihospital study to estimate the population-based and hospital-based CDI incidence in mainland China.

Methods: Active surveillance will be conducted in all wards of tertiary hospitals in five cities in mainland China (Yinchuan, Changzhou, Chengdu, Shanghai, and Xiamen). The median (range) number of hospital beds in the participating hospitals is 3,800 (720 - 4,900) beds. Surveillance will be conducted for 12 months, beginning in March 2026, to identify hospitalized laboratory-confirmed CDI cases among residents ≥ 50 years-of-age. Laboratory testing to identify toxigenic *C. difficile* will use a standardized two-step testing method with polymerase chain reaction (PCR) nucleic acid amplification test (NAAT) testing and Quik Chek toxin testing. Cases will be defined as diarrhea (≥ 3 stools within a 24-hour period with a consistency of ≥5 on the Bristol Scale) lasting for ≥ 2 days with laboratory-confirmed toxigenic *C. difficile* infection. Population-based CDI incidence will be reported as laboratory-confirmed CDI cases per 100,000 population per year and the hospital-based CDI incidence will be reported as laboratory-confirmed CDI cases per 10,000 patient-days.

Results: This study has been approved by the local ethics committees of all participating hospitals. Upon completion of the study, results will be disseminated through conference presentations and publication in peer-reviewed journals. Study limitations include potential

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incomplete enrollment and stool collections, and possible preferential capture of more severe cases. Systematic case ascertainment and standardized stool testing, however, is expected to reduce under-ascertainment of CDI cases and support a more accurate estimate of CDI incidence in China.

Conclusion: We are conducting a large prospective multihospital study to estimate the population-based and hospital-based CDI incidence in mainland China. Data from this study will be important for understanding the CDI disease burden in China and the unmet medical need for the prevention of CDI.

P16

INVESTIGATION ON THE CURRENT STATUS OF *Clostridioes difficile* INFECTION PREVENTION AND CONTROL IN CHINESE HEALTHCARE FACILITIES: A CROSS-SECTIONAL SURVEY OF 2,306 HEALTHCARE FACILITIES

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Background and Aims: To investigate the current status of laboratory testing, surveillance, infection prevention and control practices, and perceived disease burden of *Clostridioides difficile* infection (CDI) in Chinese healthcare facilities.

Methods: A nationwide cross-sectional questionnaire survey was conducted among infection prevention and control professionals. The questionnaire was developed after two rounds of expert consultation involving 16 experts and covered five domains: facility characteristics, perceived CDI burden, laboratory testing and case surveillance, epidemiology and cluster response, and emerging technologies. Facilities were regrouped into seven geographical regions for regional analyses. Categorical data were expressed as frequency and percentage, and the comparison between groups was conducted using the χ^2 test.

Results: A total of 2,306 valid facility-level questionnaires were included. Among 2,306 healthcare facilities, 1,750 (75.9%) were general hospitals, 2,089 (90.6%) were public hospitals, 789 (34.2%) were tertiary hospitals, and 1065 (46.2%) were secondary hospitals. According to the seven major geographical regions of China, the numbers of surveyed institutions in East China, Southwest China, North China, Northwest China, Central China, South China, and Northeast China were 587, 445, 374, 337, 329, 140, and 94, respectively. Among the respondents, the awareness rate of CDI prevention and control was the highest (91.5%), while the awareness rates of treatment principles and regimens, as well as diagnostic methods and criteria, were 59.5% and 61.8%, respectively. Overall, 229 facilities reported conducting CDI laboratory testing, corresponding to a testing implementation rate of 9.9% (95%CI: 8.8%-11.2%). The implementation rate differed significantly across geographical regions (χ^2=13.16, P=0.041). Among 229 facilities conducting CDI laboratory testing, 111 (48.5%) conducted CDI case surveillance and 138 (60.3%) considered that CDI cases were not fully detected in their own institutions. Self-reported CDI testing samples and detected cases increased from 2023 to 2025.

Conclusion: Although perceived CDI disease burden is high among infection prevention and control professionals, laboratory testing and case surveillance remain insufficient and heterogeneous across regions. Standardized diagnostic algorithms, active surveillance, staff training, contact precautions, environmental disinfection and antimicrobial stewardship should be strengthened to support national CDI burden assessment and future prevention strategies.

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COMPARATIVE IN VITRO KILLING KINETICS OF IBEZAPOLSTAT AND COMPARATOR ANTIBIOTICS IN *Clostridioides difficile* AND VANCOMYCIN-RESISTANT *Enterococcus* CO-CULTURES

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Background: The targeted-spectrum antibiotic ibezapolstat (IBZ) is under clinical development to treat *Clostridioides difficile* infection (CDI) while preserving the healthy gut microbiome. The microbiome of patients experiencing recurrent CDI is frequently dominated by overgrowths of *Enterococcus* species. This study evaluated the *in vitro* bactericidal and molecular efficacy of IBZ, vancomycin (VAN), and fidaxomicin (FDX) against *C. difficile* and *Enterococcus* in standalone and complex co-culture environments.

Methods: Ten clinical vancomycin-resistant *Enterococcus* (VRE) strains and *C. difficile* (R20291 and CD630) were exposed to therapeutic concentrations (50 µg/mL) of IBZ, VAN, or FDX in monoculture and co-culture configurations for 24 hours. Eradication kinetics were quantified using colony-forming unit (CFU) counts and a multiplex qPCR assay quantifying *tcdB* and *vanA* gene abundance.

Results: All VRE strains demonstrated high-level resistance to VAN (MIC >128 µg/mL), whereas IBZ and FDX exhibited equivalent lower susceptibility thresholds (MIC range: 2–16 µg/mL). In monoculture, IBZ displayed potent bactericidal activity against *C. difficile* non-inferior to FDX ($p \geq 0.2381$). *Enterococcus* co-culture with *C. difficile* created severe microbial interference that impaired *C. difficile* killing across all antibiotic arms ($p = 0.0054$), decreasing killing by up to 2.90 logs; however, IBZ maintained the highest residual anti-*C. difficile* performance (5.51-log reduction), significantly outperforming VAN ($p = 0.0112$). Conversely, *C. difficile* presence exerted negligible interference on VRE killing ($p = 0.3805$), where IBZ and FDX maintained robust anti-VRE killing while VAN remained inactive. At the transcriptional level, all treatments significantly suppressed tcdB abundance compared to control ($p < .0001$). Notably, co-culture with Enterococcus improved IBZ effect on TcdB with a 0.33-log additional reduction ($p=0.0082$). IBZ decreased VanA by 3.87-logs compared to VAN ($p<.0001$) and 1.74 logs improvement compared to FDX ($p<0.0001$).

Conclusion: These findings demonstrate that *Enterococcus* creates a protective microenvironment that actively impairs *C. difficile* killing using standard antibiotic regimens. IBZ maintained superior bactericidal activity under co-culture conditions, including further decreasing TcdB expression while simultaneously eradicating vancomycin-resistant reservoirs.

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DIVERSITY OF *Clostridioides difficile* IN MARINE SEDIMENTS OF EASTERN ADRIATIC SEA

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C. difficile can persist in the environment as resistant spores, yet its occurrence in marine sediments remains poorly understood. This study aimed to determine the prevalence and PCR ribotype diversity of *C. difficile* in marine sediments from the eastern Adriatic Sea.

In April 2021, 12 surface sediment samples were collected at water depths of 2–40 m from three anthropogenically impacted marine areas: Pula, Šibenik, and Vranjic. Each sediment sample was heat-shocked and selectively enriched under anaerobic conditions. Following ethanol shock, the samples were cultured on CHROMID™ *C. difficile* agar. Isolate identity was confirmed by MALDI-TOF. Isolates were characterized by PCR ribotyping and whole-genome sequencing (WGS) followed by resistance gene detection and analysis of genomic relatedness using core-genome MLST (cgMLST).

C. difficile was recovered from all 12 sediment samples. In total, 107 isolates were obtained, with 4–11 isolates per sample. PCR ribotyping identified 30 distinct PCR ribotypes, indicating high strain diversity. PCR ribotype 001/072 was the most widely distributed and was detected in five samples across all three sampling areas.

WGS was performed on 28 isolates from ten PCR ribotypes. Most isolates belonged to clade 1 (22/28), while three RT078 isolates belonged to clade 5. Three isolates of PCR ribotypes SLO 322, SLO 028 and SLO 324 were assigned to cryptic clades. Nineteen isolates had an A+B+ CDT- toxin-gene profile, three RT078 isolates were A+B+ CDT+, and six isolates lacked the major toxin genes. Overall, the detected resistance gene profiles included determinants also reported in human isolates. Using a cgMLST cluster threshold of ≤3 allelic differences, three clusters comprising six isolates were identified. Two clusters consisted of pairs of RT001/072 isolates with identical cgMLST profiles, one pair from Vranjic and one from Šibenik. The third cluster comprised RT078 isolates from Šibenik and Vranjic with three allelic differences. High similarity was also detected with deposited sequences from clinically relevant strains from geographically distinct sites in Croatia.

These findings show that marine sediments can harbour diverse populations of toxigenic and non-toxigenic *C. difficile*, including clinically relevant strains, and may serve as an additional environmental reservoir contributing to the spread of *C. difficile*.

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THE RISE AND FALL OF RT027 – THE CHANGING RIBOTYPE LANDSCAPE OF *Clostridioides difficile* IN AUSTRIA, 2008-2024

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Background and Aims: *Clostridioides difficile* (formerly *Clostridium difficile*) is an anaerobic, gram-positive bacterium capable of producing toxins that cause diarrhoea and severe gastrointestinal diseases. In Europe, approximately 190.000 cases are reported annually, accounting for about 3,6% of all healthcare-associated infections and more than half (54,6%) of hospital-acquired gastrointestinal infections. Molecular classification of *C. difficile* isolates for epidemiological surveillance is based on PCR ribotyping. This study provides a comprehensive overview of the temporal distribution of *C. difficile* ribotypes in Austria over a 17-year period.

Methods: Isolates collected between 2008 and 2024 within the Austrian national *C. difficile* surveillance system were subjected to PCR ribotyping. Ribotype assignment and comparative analysis were performed using the web-based application Webribo.

Results: A total of 5.643 isolates collected between 2008 and 2024 were included in the analysis. In total, 475 distinct ribotypes were identified. The numbers of ribotypes detected per year ranged between 37 and 215, reflecting substantial temporal variability in ribotype diversity. Overall, RT027 was the most frequently detected ribotype, accounting 787 isolates (14% of all samples). However, the majority of RT027 isolates were detected between 2009 and 2019. Beginning around 2018, the relative abundance of ribotypes RT078, RT106, and RT126 increased. In the most recent years of the study, RT181 emerged as the predominant ribotype, accounting for the largest proportion of isolates in both 2023 and 2024.

Conclusions: The data demonstrate a marked shift in the epidemiology of *C. difficile* in Austria over the past decade. While RT027 dominated between 2008 and 2019, its frequency declined sharply after 2020. This decline was accompanied by the emergence and increasing relative abundance of several other hypervirulent ribotypes, including binary-toxin producing RTs 078, 126 and the closely 027-related ribotype 181, as well as the non-binary-toxin-producing RT106. The limited overlap of dominant ribotypes within individual years suggests successive waves of epidemic lineages rather than stable long-term co-circulation. These findings indicate a transition from a surveillance landscape previously driven by a single dominant epidemic ribotype to a more heterogeneous population structure composed of multiple hypervirulent lineages. Continuous national surveillance is therefore essential to detect emerging strains early and to understand the evolving epidemiology of *C. difficile* infections in Austria.

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INTEGRATING ANTIMICROBIAL CONSUMPTION AND *Clostridioides difficile* INFECTION SURVEILLANCE IN BELGIAN HOSPITALS: A DECADE-LONG RETROSPECTIVE STUDY

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Antimicrobial consumption (AMC) is a well-established risk factor for *Clostridioides difficile* infection (CDI). As CDIs occur predominantly in healthcare settings, are highly recurrent, and are associated with antimicrobial resistance, reducing their burden remains a public health priority. This study examined the association between consumption of high-risk antimicrobials, including beta-lactam/beta-lactamase inhibitor combinations, cephalosporins, fluoroquinolones, carbapenems, glycopeptides and clindamycin, and hospital-associated (HA)-CDI in Belgian hospitals between 2014 and 2024.

We conducted a retrospective longitudinal study using AMC and CDI data from national surveillance. Hospital-level AMC surveillance relies on reimbursement data. Antimicrobial agents were categorised according to the World Health Organisation Anatomical Therapeutic Chemical classification, and consumption was expressed as Defined Daily Dose (DDD). Patient-level CDI surveillance is organised per semester through voluntary hospital participation. HA-CDI was defined as CDI occurring ≥ 2 days after admission. Patient-days (PD) were collected separately within each surveillance and used as denominators. Population-averaged negative binomial models with generalised estimating equations were fitted, adjusting for hospital type and region. Annual HA-CDI trends were modelled with piecewise linear splines.

Average annual consumption of high-risk antimicrobials reached 341 DDD/1,000PD. A hospital's yearly high-risk AMC, as well as cephalosporins, carbapenems and glycopeptides consumption, was significantly associated with HA-CDI incidence. Consuming 100 DDD/1,000PD more high-risk antimicrobials yearly than the average hospital was associated with a 19% increase in HA-CDI rate ($p<.01$). Carbapenem consumption showed the strongest association with HA-CDI rate (IRR 1 DDD/1,000PD=1.01, 95%CI 1.00-1.02, $p<.01$). Secondary (IRR=1.40, 95%CI 1.17-1.65, $p<.001$) and tertiary hospitals (IRR=1.59, 95%CI 1.11-2.28, $p<.05$) exhibited higher HA-CDI rates than primary hospitals, even after adjusting for region and high-risk AMC.

Our findings suggest that hospital-level AMC plays an important role in HA-CDI incidence. Strengthening antimicrobial stewardship programmes and limiting high-risk antimicrobial use, such as carbapenems, cephalosporins, and glycopeptides, may help prevent HA-CDI.

P21

REBOUND IN ESTIMATED NUMBER OF *Clostridioides difficile* INFECTION CASES, HOSPITALIZATIONS, AND DEATHS IN THE UNITED STATES FOLLOWING COVID-19 PANDEMIC, 2017-2023

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Background and Aims: *Clostridioides difficile* infection (CDI) is a leading cause of healthcare-associated diarrheal infection worldwide. Although the Centers for Disease Control and Prevention (CDC) considers CDI an "urgent public health threat", the most recent published estimate of the US CDI disease burden (defined by cases, hospitalizations, and deaths) is for 2017. We therefore estimated the national CDI disease burden from 2017-2023 and evaluated temporal trends before and after the COVID-19 pandemic.

Methods: Age-stratified, population-based incidence of laboratory-confirmed CDI cases reported in the CDC Emerging Infections Program (EIP) surveillance annual reports were multiplied by corresponding US Census population estimates to derive national estimates of community- and healthcare-associated CDI cases for 2017-2023. The percentage of CDI cases hospitalized on the day of or within 6 days of specimen collection, and the in-hospital mortality rates reported in EIP annual reports, were multiplied by the estimated number of CDI cases to calculate CDI hospitalizations and CDI deaths in the US for corresponding years. A similar approach was used to estimate burden among individuals ≥65 years. Estimates derived for 2017 were compared with published data, and trends in incidence were evaluated for 2017-2023.

Results: We estimate that there were 414,613 CDI cases, 178,284 CDI hospitalizations and 12,438 CDI deaths in the US in 2023 with most of the CDI disease burden among individuals ≥65 years. Among all ages, in the time prior to the COVID-19 pandemic (2017-2020), the estimated number of CDI cases, hospitalizations, and deaths declined by 22%, 33%, and 58%, respectively. In contrast, from 2020 to 2023 the estimated number of cases, hospitalizations, and deaths increased by 16%, 13%, and 35%, respectively. From 2017-2020, healthcare-associated CDI disease burden was higher than community-associated burden, while from 2020-2023 the community-associated CDI disease burden was more common than healthcare-associated burden. Our estimates of the US CDI disease burden in 2017 were similar to published estimates.

Conclusions: The US CDI disease burden is high and increasing in community and healthcare settings, particularly since the pandemic. Interventions are needed to reduce the US CDI disease burden, particularly individuals ≥65 years.

P22

COMMUNITY, COMPANIONS AND *Clostridioides difficile* - A CASE OF *C. difficile* INFECTION IN THE DOMESTIC ENVIRONMENT

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Background and Aims: In Australia, ~10,500 cases of *Clostridioides difficile* infection (CDI) and 750 deaths are reported annually with an economic burden of ~$200 million. Although traditionally considered a healthcare-related disease, 80% of Australian CDI cases in 2022-2023 were community derived. Factors driving this change remain unclear, warranting investigation into the emerging role of non-traditional sources/reservoirs of transmission.

Methods: This case report describes a 42-year-old Australian woman with laboratory-confirmed CDI that developed after antimicrobial therapy for an apparent post-influenza respiratory infection, in the absence of recent hospital exposure. Household environment samples were collected at both the time of diagnosis and 2-weeks post diagnosis, including household surface swabs (n=12), pet faeces (n=4), and garden (n=2) and pet enclosure samples (n=4). Patient and household samples were enriched for 7 days in supplemented brain heart infusion broth, followed by ethanol shock and 48h anaerobic culture on ChromID agar. Isolates were characterised by PCR ribotyping and toxin gene profiling.

Results: *C. difficile* was isolated from initial cat and dog faeces, as well as pet enclosure samples. The two cats (3 years) and two dogs (15 years) were asymptomatic, and no veterinary intervention was sought. Follow-up household sampling 2 weeks post patient diagnosis, again detected *C. difficile* in dog faeces, pet enclosure and the house floor adjacent to the enclosure, suggesting ongoing environmental presence. Confirmatory PCR analysis determined isolates from the patient, pets, household floor and pet enclosure were all toxigenic ribotype (RT) 014/020 (tcdA+ tcdB+ cdtA/B-). A second toxigenic strain, RT 095 (tcdA+ tcdB+ cdtA/B-), was also detected in enclosure samples. The patient reported initially being motivated to seek medical advice on her symptoms after taking part in a recent *C. difficile* community education initiative.

Conclusions: This case highlights the potential role of household pets and environments as sources/reservoirs for community-associated CDI. It underscores the need to broaden diagnostic and surveillance strategies to address epidemiological changes and emerging sources of transmission and emphasises a growing need for enhanced public health education at both community and clinical levels to reflect the changing disease landscape.

P23 DETECTION OF *Clostridioides difficile* IN PASTEURIZED POULTRY PRODUCTS

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Mechanically separated meat (MSM) from poultry is widely used in pasteurized ready-to-eat products, yet its potential role as a vehicle for *Clostridioides difficile* remains insufficiently explored. To address this gap, we examined a range of pasteurized poultry sausages (n = 33) made either from a single production batch of chicken MSM or from diverse retail formulations containing MSM or non-MSM meat. They originated from various EU manufacturers, brands, recipes and batches. Sampling was performed in the first half of 2025. We employed both qualitative (cultivation after sample enrichment) and quantitative (direct plating, most probable number [MPN] method) approaches to assess *C. difficile* contamination rates, while whole-genome sequencing (WGS) was used for isolate characterization and assessment of genetic relatedness.

C. difficile was identified in 20/33 (60.6%) of the samples, indicating a notable level of contamination. In positive samples, enumeration using the MPN method revealed *C. difficile* levels ranging from < 0.090 to 1.600 MPN/g. Despite relatively low pathogen counts, genetic profiling of the obtained isolates revealed substantial diversity and virulence potential. Namely, 10 different sequence types (STs) were identified, with ST1255 as a novel ST. Two cgMLST clusters were identified: one comprising 19 isolates from products made from the same MSM by a single food business operator (FBO), and another comprising four isolates from three different FBOs. Most isolates (28/32; 87.5 %) were $A^+B^+CDT^+$, harbouring *C. difficile* toxin genes *tcdA, tcdB, cdtA* and *cdtB*, respectively. Although antimicrobial resistance (AMR) genes or AMR-associated mutations were detected at relatively low frequencies, they were associated with phenotypic resistance to several antimicrobial classes.

While the contamination levels found were low, the frequent detection of viable *C. difficile* spores in pasteurized, ready-to-eat products raises important questions for public health. Although the infectious dose for humans remains poorly defined, such low-level exposures may be relevant for susceptible individuals.

P24

EPIDEMIOLOGY AND RISK FACTORS OF HOSPITAL-ONSET *Clostridioides difficile* INFECTION IN ADULT INPATIENTS: A RETROSPECTIVE CASE–CONTROL STUDY

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Background and aims: *Clostridioides difficile* infection (CDI) is a major healthcare-associated infection worldwide, yet epidemiological data from China remain limited. We aimed to describe the epidemiology of hospital-onset CDI (HO-CDI) and identify modifiable risk factors among adult inpatients.

Methods: We conducted a retrospective matched case–control study at Zhongshan Hospital, Fudan University, Shanghai, China. Adult inpatients with diarrhea and a positive *C. difficile* PCR result obtained ≥48 hours after admission were classified as HO-CDI cases. Controls were diarrheal inpatients who tested negative for CDI, matched 1:1 by ward and age (±5 years). To improve temporal attribution and reduce reverse causation, exposures were restricted to predefined pre-test windows, including medications within 30 days and laboratory parameters within 7 days before CDI testing. Conditional logistic regression was used to identify independent risk factors.

Results: During 2024, the overall incidence density of CDI was 4.1 cases per 10,000 patient-days among adult inpatients. The highest incidence densities were observed in the gastroenterology ward, intensive care unit, and colorectal surgery ward. Recurrent CDI occurred in 8.9% of PCR-positive patients. A total of 411 matched pairs were included in the risk factor analysis. In multivariable analysis, higher neutrophil percentage (aOR 1.03, 95% CI 1.01–1.05), lower serum albumin (aOR 0.92, 95% CI 0.88–0.97), fluoroquinolone exposure (aOR 11.09, 95% CI 1.64–75.03), ertapenem exposure (aOR 3.43, 95% CI 1.38–8.52), and H2-receptor antagonist use (aOR 2.56, 95% CI 1.51–4.36) were independently associated with CDI.

Conclusions: HO-CDI was associated with both host clinical status and specific antimicrobial exposures, particularly fluoroquinolones and ertapenem. The use of predefined pre-test exposure windows strengthened temporal inference in this retrospective analysis. These findings support targeted antimicrobial stewardship and early identification of high-risk hospitalized patients.

P25

ASSESSMENT OF CONTAMINATION OF POINT-OF-SALE FOODSTUFFS WITH *Clostridioides difficile* IN THE UNITED KINGDOM

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Background and aims: *Clostridioides difficile* is a major cause of healthcare associated infection but increasing reports of community associated disease suggest transmission routes beyond clinical settings. Although international studies have reported *C. difficile* contamination of food, UK surveillance is predominantly in the clinical setting, resulting in limited understanding of retail foodstuffs as potential *C. difficile* reservoirs.This PhD project aims to determine the prevalence of point of sale food contamination with *C. difficile* in the UK and to characterise isolates to inform public health and industry stakeholders. As part of this work, the present study evaluates detection sensitivity and preservative susceptibility of *C. difficile* isolates.

Methods: A culture based detection protocol incorporating alcohol shock, direct culture ± selective enrichment was applied to meat matrices to validate recovery of *C. difficile* spores, including quantitative recovery from frozen and unfrozen samples. Additionally, MICs (0.0078–1024mg/L) of common food preservatives, sodium benzoate, potassium benzoate, potassium nitrite, sodium metabisulphite, sodium nitrite and sodium nitrate were determined against vegetative and spore forms of *C. difficile*, using an agar incorporation method. *C. difficile* from ribotypes -005, 014, 027, 078, 015, 002, and 001, (N=70) were evaluated.

Results: Using direct plating, the limit of detection was approximately 100 spores/mL, based on recovery at the 10^{-1} dilution. Selective enrichment improved sensitivity, enabling recovery from approximately 2–400 spores/mL, demonstrating that enrichment reduced the number of spores required for detection from ~100 to as few as ~2 spores, representing an approximate one log order increase in detection sensitivity.

MIC testing showed generally high MIC values across preservatives. Most isolates were not inhibited at the highest concentration tested (1024 mg/L), although limited inhibition at 256–1024 mg/L was observed in some isolates, including ribotypes RT078, RT027 and RT014. MIC_{50} and MIC_{90} exceeded 1024 mg/L, indicating low overall susceptibility.

Conclusions: These findings demonstrate reliable recovery of *C. difficile* from retail meat matrices and no detrimental effects of freezing on *C. difficile* spores. High MIC values indicates that food associated *C. difficile* isolates exhibit low susceptibility to commonly used preservatives, with inhibitory concentrations exceeding levels typically applied to preserve food. This suggests that these preservatives are unlikely to inhibit spore viability, germination, or outgrowth, and are similarly ineffective against vegetative *C. difficile*. Consequently, *C. difficile* spores may persist in food products, supporting the potential for transmission through the food chain.

P26

UNDERSTANDING *C. difficile* TCDB CONSERVATION THROUGH SURVEILLANCE OF DIVERSE AND GLOBAL CONTEMPORARY ISOLATES

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Background and Aims: *C. difficile* infection (CDI) is the leading cause of antibiotic and healthcare-associated infective diarrhoea. AZD5148 is a humanised IgGκ monoclonal antibody (mAb) in development for the prevention of CDI recurrence. AZD5148 neutralises *C. difficile* Toxin B (TcdB) by binding an epitope on the glucosyltransferase domain (GTD). The AZD5148 binding epitope was highly conserved among a collection of 9,134 global *C. difficile* genomes obtained from a public repository. Herein, we expanded this to evaluate AZD5148 binding epitope conservation using a collection of over 21,800 global *C. difficile* genomes obtained from Enterobase, the US National Center for Biotechnology Information (NCBI) and regional microbial surveillance, to evaluate the conservation of residues crucial for neutralisation.

Methods: A total of 11,286 assembled *C. difficile* genomes collected between 01 Jan 2015 – 31 Dec 2025 were obtained from Enterobase, 10,331 from NCBI and 241 isolates from South Korea and Taiwan. All sequences were annotated to identify the TcdB gene coding sequence and aligned to the R20291 reference. From this alignment, variants were identified and conservation or variance were calculated at each TcdB residue, both regionally and temporally. Sequences were characterised using multilocus sequence type (MLST) and clade.

Results: The majority of the *C. difficile* genomes obtained from both datasets were from the US. Among the genomes, MLST Clade 1 was the most prevalent, followed by Clade 2 and Clade 5. The GTD (96.03% Enterobase; 95.90% NCBI; 95.04% South Korea/Taiwan) and AZD5148 binding epitope (97.27%; 97.19%; 96.39%, respectively) were both found to be highly conserved. Among the AZD5148 binding residues, one substitution was identified (Y323H) at low variance (2.89% Enterobase; 3.50% NCBI). In both datasets, this substitution only occurred in Clade 4 sequences predominantly found in Asia. In the South Korea and Taiwan dataset, Y323H occurred in only 16 of the 241 isolates (6.64%).

Conclusions: Our updated analysis of contemporary global sequences from 3 unique datasets found that the AZD5148 epitope continues to be highly conserved.

P27

AZD5148 PREVENTS *C. difficile* TOXIN B-INDUCED CYTOTOXICITY IN A HUMAN COLON TRANSWELL SYSTEM

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Background and Aims: AZD5148 is an anti–Toxin B (TcdB) neutralising monoclonal antibody (mAb) in clinical development for the prevention of *C. difficile* infection (CDI) recurrence. AZD5148 previously exhibited potent neutralising activity *in vitro* in cytotoxic assays with Vero and Caco 2 cell lines and *in vivo* efficacy in CDI mouse models. To bridge *in vitro* and *in vivo* findings, we used a human colon-derived transwell system that reflects in situ patient conditions to evaluate the protective effects of AZD5148 following *C. difficile* TcdB exposure. A preliminary quantitative systems pharmacology (QSP) model was also developed to mechanistically capture the dynamics between mAb and TcdB-driven epithelial damage.

Methods: Colon epithelial monolayers from patient-derived organoids were cultured on 96-well transwell plates. Monolayers were pre-treated basally with AZD5148, bezlotoxumab or R347 (negative control) at 0.2, 1, and 40 µg/mL. After 24 hours, TcdB was added apically at 1, 20, and 50 ng/mL. Barrier integrity was assessed via transepithelial electrical resistance and cytotoxicity was quantified by high-content imaging of ethidium homodimer-1 (EthD-1) staining at 0, 8, 18, and 24 hours post-exposure.

Results: In R347 pre-treated cells, TcdB caused concentration-dependent barrier disruption and cytotoxicity. Up to 1 µg/mL, AZD5148 or bezlotoxumab preserved barrier integrity after exposure to 1 and 20 ng/mL TcdB at all time points. At 50 ng/mL TcdB exposure, barrier integrity was maintained with 40 µg/mL AZD5148 or bezlotoxumab. EthD-1 staining showed that 40 µg/mL AZD5148 maintained low cytotoxicity that was similar to healthy controls across all TcdB concentrations. AZD5148-treated transwells exhibited consistent protection against cytotoxicity, even at high Toxin B concentrations with prolonged exposure, indicating robust viability preservation versus bezlotoxumab.

Conclusions: AZD5148 is a potent neutralising anti-TcdB mAb, exhibiting epithelial barrier protection and prevention of toxin-induced cytotoxicity in a human colon transwell model. A preliminary mechanistic QSP model developed from the transwell data captured the observed temporal dynamics of TcdB-induced epithelial damage and demonstrated the protective effect of AZD5148. The model will continue to be developed to inform AZD5148 clinical development.

P28

COMPARISON OF *Clostridioides difficile* RIBOTYPES FROM WASTEWATER ISOLATES AND CLINICAL ISOLATES: PRELIMINARY RESULTS OF IZS VE 02/24 RC RESEARCH PROJECT

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The incidence and severity of hospital-acquired *Clostridioides difficile* (CD) infections is currently gaining an increased recognition due to the diffusion of virulent and multi-drug resistant ribotypes (RTs). Moreover, the recent recognition of cases in non-hospitalized subjects suggests other potential sources of infection occurring in community. Wastewater-based surveillance (WWBS) has become a complementary tool for routine surveillance of human infectious disease, capable to provide aggregated data from an entire community.

The IZS VE 02/24 RC is a proof of concept study aiming to explore whether WWBS may support the clinical surveillance of CD RTs and act as a rapid and early warning system for the introduction of new and public health relevant RTs, with no aim to estimate ribotypes' prevalence.

A 6-month-long fortnight sampling protocol has been performed on three WW basins of a high-bed capacity hospital (H) and on the incoming WW in an urban treatment plant. CD isolation was performed by applying the method of Chisolm et al. 23 CD strains from hospitalized clinical cases occurring at H were enrolled in this study. Five CD colonies per each WW sample and all human isolates have been submitted to ribotyping (172 CD strains). Overall, 16 different RTs were identified from the WW urban treatment plant, and 14 RTs from human isolates.

In agreement with the literature, our results confirm the detection of several RTs in the wastewater and some of these resulted relevant to public health. RT014/20, RT010, and RT002, defined as highly virulent RTs associated with severe clinical cases and high mortality, were detected on both environmental samples and human strains. Of note, the hypervirulent RT 027 was not among the WW or human RTs. Being aware that CD RTs early detection based on WWBS requires sensitivity estimation, these preliminary results are promising for WWBS of hospital and urban treatment plants to complement CD clinical cases surveillance.

P29

CLINICAL AND WASTEWATER *Clostridioides difficile* PROFILES OBTAINED FROM CAPE TOWN, SOUTH AFRICA

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Background and Aims: *C. difficile* remains a global public health issue. The detection of toxigenic *C. difficile* from clinical, community and environmental settings underscore the need for a One Health approach to combat this pathogen. Effluent, the product of wastewater obtained from communities, hospitals and industry, is a known resource containing residual microbes of human origin (including pathogens). It is a useful resource to trace and characterise pathogens of concern. This study characterised *C. difficile* isolates from patients with confirmed diarrhoea as well as isolates obtained from wastewater.

Methods: The pathogen was cultured from 56 patients with laboratory confirmed *C. difficile*, as well as 10 bottles of effluent. BHI agar containing *C. difficile* selective supplement was used for isolation of the organism. Presumptive colonies were screened using PCR targeting the *C. difficile* tpi and toxin encoding genes (tcdA, tcdB, cdtA and cdtB). Genomic DNA was subjected to PCR-ribotyping, as well as whole genome sequencing (via the Oxford Nanopore sequencing platform).

Results: A set of 84 clinical and 36 effluent colonies were selected. Toxin gene profiling of clinical isolates identified 15/84 (17.86%) A^+B^+, 1/84 (1.19%) A^+, 54/84 (64.29%) $A^+B^-CDT^+$ and 14/84 (16.67%) non-toxigenic isolates. Effluent isolates comprised 19/36 (52.78%) A^+B^+, 1/36 (2.78%) $A^+B^+CDT^+$, 13/36 (36.11%) $A^+B^-CDT^+$, 1/36 (2.78%) *cdtA* only, and 2/36 (5.56%) non-toxigenic isolates. PCR-ribotyping and toxin profile revealed diversity across both clinical and effluent isolates. Preliminary WGS identified MLST ST1, ST54, and ST58 sequence types, including one hypervirulent ST1 isolate carrying binary toxin genes and a *tcdC* deletion. Genomic analysis of sequenced isolates identified antimicrobial resistance genes and mutations associated with resistance to tetracyclines, MLSB-class antibiotics, fluoroquinolones, and rifamycins.

Conclusions: This study demonstrates the presence of both toxigenic and non toxigenic *C. difficile* in clinical and effluent samples. Effluent thus could be a valuable resource to monitor this organism in local settings, especially to trace disease-causing strains.

P30

MOLECULAR EPIDEMIOLOGY OF *Clostridioides difficile* COLONIZATION IN PATIENTS WITH CHRONIC KIDNEY DISEASE IN EASTERN CHINA

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Background and Aims: *Clostridioides difficile* colonization (CDC) is considered an important potential reservoir for subsequent infection, yet its role in patients with chronic kidney disease (CKD) remains unclear.

Methods: A over two-year cross-sectional study was conducted at one hospital in Zhejiang, China. Fecal specimens collected from outpatients in the nephrology department were subjected to *C. difficile* isolation. Isolates were further characterized through toxin gene detection, multilocus sequence typing, whole-genome sequencing, and antimicrobial susceptibility testing. Demographic data were also collected for statistical analysis.

Results: Among the 323 patients with CKD enrolled in this study, approximately 5.0% (16/323) were *C. difficile* colonization. The 16 *C. difficile* isolates comprised 9 toxin A-negative, toxin B-positive, and binary toxins-negative ($A^-B^+CDT^-$), 1 $A^-B^+CDT^+$, 2 $A^+B^+CDT^-$, and 4 $A^-B^-CDT^-$, yielding 8 sequence types. All the isolates were susceptible to vancomycin and metronidazole, and significant associations were found between resistance to erythromycin and tetracycline and toxin genotypes (P = 0.009 and 0.002). Multivariable regression analysis revealed that hyperphosphatemia (OR = 1.18; 95% CI: 1.048-1.065) and hemodialysis frequency (OR = 1.10; 95% CI: 1.023-1.467) were independent risk factors for CDC.

Conclusions: This study provides the first systematic characterization of the epidemiological profile of CDC in patients with CKD in China.

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SODIUM METABISULFITE ENHANCES *Clostridioides difficile* VIRULENCE THROUGH ACTIVATION OF THE LYTR REGULATOR

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Background and Aims: *Clostridioides difficile* infection (CDI) poses a significant healthcare burden. However, the impact of dietary components, particularly food preservatives, on *C. difficile* pathogenesis remains poorly understood. This study aimed to investigate the effect of sodium metabisulfite ($Na_2S_2O_5$), a ubiquitous food preservative, on the virulence of hypervirulent *C. difficile* strains.

Methods: We assessed virulence phenotypes including adhesion, sporulation, biofilm formation, and toxin production in RT027 strains exposed to subinhibitory concentrations of $Na_2S_2O_5$. The impact on disease severity and colonization was evaluated using a gerbil infection model. RNA sequencing was performed to identify transcriptional alterations, and the functional role of key regulators was validated via CRISPRi-mediated knockdown.

Results: $Na_2S_2O_5$ significantly enhanced all tested virulence traits in vitro. In vivo, bacteria pre-exposed to $Na_2S_2O_5$ induced more severe disease, characterized by exacerbated weight loss, histopathology, and intestinal colonization. Transcriptomic analysis revealed the upregulation of the LytR family regulator. Crucially, CRISPRi-mediated knockdown of lytR abolished the $Na_2S_2O_5$ induced virulence enhancement, confirming LytR as the critical mediator.

Conclusions: Sodium metabisulfite exacerbates *C. difficile* pathogenicity by activating the LytR regulator. This study uncovers a novel mechanism linking a common food preservative to enhanced bacterial virulence, highlighting a potential dietary risk factor for CDI.

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NITROREDUCTASE NFP-MEDIATED METRONIDAZOLE RESISTANCE IN *Clostridioides difficile*

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Background and Aims: Nitroreductases that catalyze the reduction of nitroaromatic compounds play a crucial role in microbial metabolism and the regulation of metronidazole resistance. However, the biological function of the putative nitroreductase encoded by the CDR20291_3199 gene in *Clostridioides difficile* strain R20291 temporarily designated as the Nfp protein (NCBI ID: CBE07100.1) remains unknown.

Methods: The expressed Nfp protein exhitited enzymatic reductive activity. UV spectroscopy and LC-MS/MS analyses revealed that the Nfp catalytically reduces the nitro group of metronidazole (MTZ) to non-cytotoxic amine derivatives, thereby attenuating the bactericidal activity of MTZ.

Results: The presence of Nfp significantly increased *C. difficile* tolerance to MTZ, raising the minimum inhibitory concentration (MIC) from 0.25-0.5 to 4-8 μg/mL ($P < 0.001$) in vitro. Moreover, MTZ resistance correlated with the nfp gene in vivo, with the MIC increasing from 0.5 μg/mL to 4 μg/mL ($P < 0.001$).

Conclusions: These findings provide novel evidence of MTZ resistance mechanisms in *C. difficile*, potentially informing improved treatment strategies for *C. difficile* infections.

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Clostridioides difficile IN DAIRY CATTLE

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Clostridioides difficile is a spore-forming anaerobe of significant concern as a zoonotic pathogen, with widespread environmental persistence and rising community-associated infection rates. Historically, *C. difficile* is thought of as a healthcare-related infection, however, emerging evidence suggests that many production animals may be key amplifiers of *C. difficile*, shedding large quantities of toxigenic and antimicrobial-resistant spores into shared farm environments. Amongst these animals, dairy calves may be an under-appreciated and under-studied group. Their immature gastrointestinal physiology, poorly developed immunity and limited microbial diversity create permissive conditions for colonisation and asymptomatic shedding. This review explores the interplay between gut development and antimicrobial exposure in shaping colonisation risk. It further assesses the strain-level diversity and genomic adaptability enabling *C. difficile* to persist across hosts and in environments. Although molecular surveillance has improved, major gaps remain in understanding asymptomatic carriage, environmental transmission and compartment-specific colonisation. Within a One Health framework, this review identifies critical research priorities and control strategies to reduce zoonotic and environmental transmission from dairy systems that may be applicable to other areas of agriculture.

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SEALING THE ANAEROBE GAP IN AFRICA

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Backgrounds and aims: Routine diagnosis of anaerobic infections remains limited in many African laboratories, contributing to the under-recognition of *Clostridioides difficile* and its role in diarrhoeal disease. Limited molecular epidemiological data further hinder understanding of circulating strains and their implications for vaccine development. We aim to establish *C. difficile* diagnostic and surveillance capacity across laboratories in Malawi, South Africa, Nigeria, and Kenya by strengthening diagnostic infrastructure and implementing standardised workflows through collaboration.

Methods: Prospective studies will be conducted at the Malawi Liverpool Wellcome Research Programme (n ≥ 800, ~25% prevalence) and University College Hospital, Nigeria (n ≥ 544, ~15% prevalence), while archived stool specimens will be analysed at the University of Cape Town in South Africa (n ≥ 683, ~20% prevalence) and the Kenya Medical Research Institute/ Walter Reed Army Institute of Research in Kenya (n ≥ 800, ~25% prevalence). Sample sizes were calculated using a 95% confidence level and a 3% margin of error. Specimens will be tested for *C. difficile* using glutamate dehydrogenase detection and toxigenic culture, and screened with the Seegene Allplex™ Gastrointestinal Panel to identify alternative enteric pathogens. *C. difficile* isolates will undergo agar dilution testing and PCR ribotyping (RT), with selected RT characterised by whole-genome sequencing. In collaboration with the *C. difficile* Reference Laboratory in Western Australia and a UK-based deep technology partner, a prototype RT software platform will be developed to enable local RT analysis.

Results: Don Whitley anaerobic workstations have been installed in Malawi and South Africa and BACTRON anaerobic chambers established in Kenya and Nigeria. Standard operating procedures and diagnostic workflows have been developed, and ethics applications submitted to support implementation of the multicountry programme. Laboratory personnel training is underway in preparation for the study commencement in November 2026.

Conclusion: This research addresses a critical gap in *C. difficile* diagnostics in Africa by establishing standardised diagnostic workflows and locally developed analytical software to generate vaccine-relevant data, strengthen AMR surveillance, improve patient care, and inform public health policy and vaccine development.

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C. difficile INFECTION SENTINEL SURVEILLANCE IN THE NETHERLANDS: EPIDEMIOLOGY, WGS- AND RIBOTYPING.

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Background and Aims: The Dutch sentinel surveillance started in 2009 and is conducted from 2022 onward in 5 centers geographically spread over the Netherlands. We here describe (1) epidemiologic and (2) molecular trends of the 2025 surveillance, and (3) resistance to anti-CDI antibiotics in patients with recurrent CDI (rCDI) identified in the 2023-2024 surveillance.

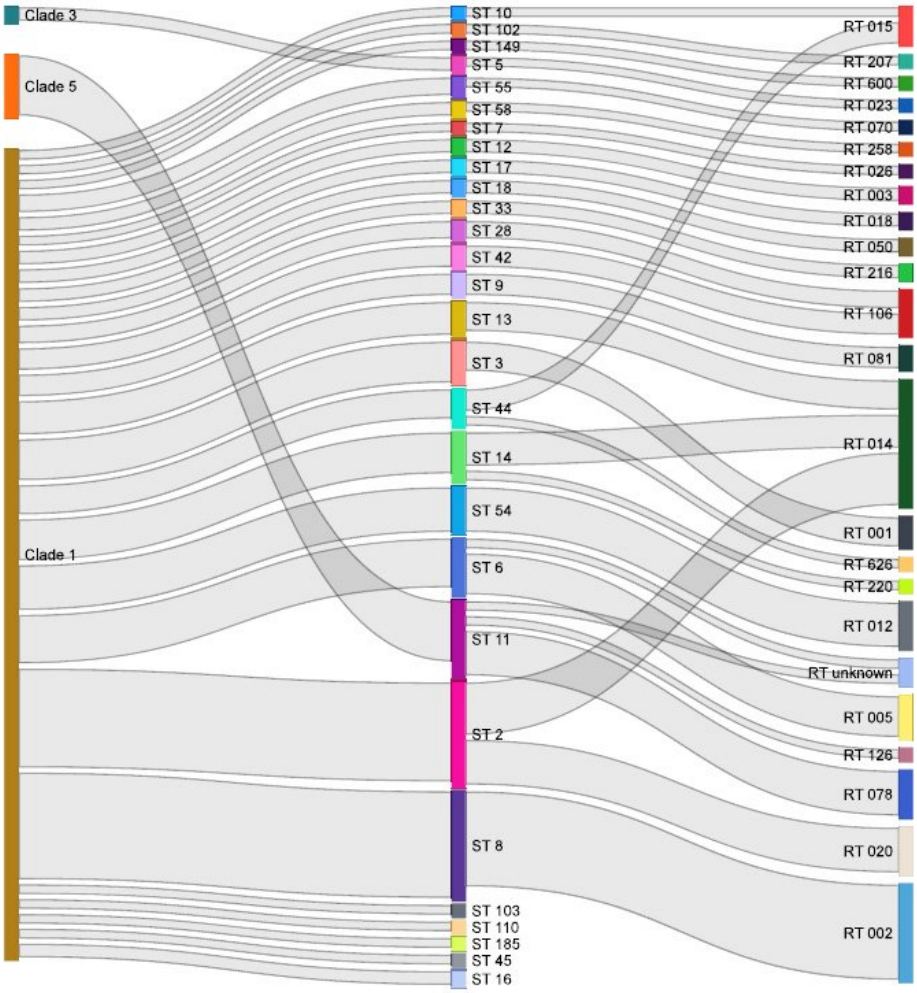
Methods: Surveillance inclusion criteria are: hospitalization with clinical CDI and a positive CDI diagnostic test (toxin enzyme-immunoassay, or toxigenic *C. difficile* PCR), or presence of pseudomembranous colitis. A web-based system (OSIRIS) is used to capture epidemiological data, e.g. location of onset, CDI severity, disease course (ICU admission, and/or surgery), and CDI-attributable mortality. Surveillance cohort isolates were subjected to WGS for cgMLST typing, and PCR ribotyping (still included for historical comparisons). *Ad hoc* typing is available for all medical centers for samples/isolates of severe CDI cases or from a suspected outbreak. The 2023-2024 surveillance included 539 cases, 64 being rCDI: 60 isolates were available for susceptibility testing with agar dilution.

Results: 280 cases (median age 69 years, IQR 58-77) met surveillance inclusion criteria; 198 isolates could be cultured and typed. CDI incidence was 3.4 cases/10,000 patient days, which is stable compared to pre-COVID19 pandemic years. The percentage of patients with severe disease was 21% (15.9-25.5). Of 244 cases, CDI treatment data was available: 44% of patients were treated with vancomycin for 10-14 days, 28% with fidaxomicin 10-14 days, 4.5% metronidazole, 2.9% extended fidaxomicin, 1.2% vancomycin taper and pulse, while 18% did not receive treatment. The distribution of circulating types was stable compared to previous years (Figure 1). Sequence Type (ST) 2 (n=25, 12.6%, includes RT014/020), ST8 (n=27, 13.6%, includes RT002), and ST11 (n=15, 7.6% includes RT078/126) were dominant. One isolate belonged to ST1,RT18, not previously been detected in the Netherlands. We received 25 samples for *ad hoc* typing; one transmission event was confirmed. MIC90 for the rCDI cohort was 0.25 mg/L (range 0.0625-0.25) for metronidazole, 2 mg/L (1-2) for vancomycin and 0.25 mg/L (0.0625-0.5) for fidaxomicin. No resistant isolates were identified.

Discussion: Epidemiological characteristics were stable. The ST1,RT181 isolate encountered belongs to the hypervirulent ST1, clade 2 lineage which includes RT016/027/036/176/244/955. Reports from Eastern Europe indicate that RT181 is an emerging, multidrug-resistant lineage;

a severe CDI outbreak was reported in Spain (ECDC EpiPulse). No resistance to anti-CDI antibiotics was identified in patients with rCDI, though MIC90 for vancomycin was one step below the EUCAST breakpoint.

Figure Sankey diagram of Clade, Sequence Type (ST) and Ribotype (RT) of *C. difficile* isolates of the 2025 sentinel surveillance. Isolates with available typing data and count >1 are shown



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COMPARISON OF FOUR CULTURE MEDIA FOR ISOLATION OF *Clostridioides difficile* FROM NEONATAL FOAL FECES

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Background and Aims: Isolation of *Clostridioides difficile* from neonatal foal feces depends on culture media with variable performance. Although several selective media are available, there is limited information regarding their comparative performance for isolating *C. difficile* from foal feces. This study aimed to compare the performance of four culture media for the isolation of *C. difficile* from fecal samples of neonatal foals.

Methods: Seventy fecal samples from neonatal foals (≤1 month of age) were inoculated into non-selective fructose broth supplemented with 0.1% sodium taurocholate and incubated anaerobically at 37°C for 5 days. Following enrichment, 2 mL of broth was mixed with an equal volume of absolute ethanol for 30 min (alcohol shock) and centrifuged at 5,000 × g for 10 min. The pellet was inoculated onto four culture media plates: Phenylethyl Alcohol Agar (PEA), Columbia Blood Agar (CAB), *Clostridioides difficile* Moxalactam Norfloxacin Agar (CDMN), and chromID *C. difficile* agar (CHRO). PEA, CAB, and CDMN were supplemented with 5% horse blood. Plates were incubated anaerobically (10% CO_2, 5% H_2, 85% N_2) at 37°C for 5 days. Colonies exhibiting characteristic morphology were subcultured and identified by MALDI-TOF mass spectrometry. Isolation frequencies were compared using Cochran's Q test and pairwise McNemar's tests with Bonferroni correction. Each individual medium and the CHRO+CDMN combination were compared with the combined results of all four culture media using McNemar's exact test ($P < 0.05$).

Results: *C. difficile* was isolated from 17/70 (24.3%) fecal samples. Isolation rates were 2.9% (CAB), 5.7% (PEA), 11.4% (CDMN), and 20.0% (CHRO), with significant differences among media (Cochran's Q, $P < 0.001$). CHRO recovered significantly more isolates than CAB ($P = 0.0005$) and PEA ($P = 0.006$), but not CDMN ($P = 0.109$). Compared with the combined results of all four culture media, isolation using CHRO alone did not differ significantly (McNemar's exact test, $P = 0.25$), whereas CAB, PEA, and CDMN recovered significantly fewer isolates (all $P < 0.05$). The combination of CHRO and CDMN recovered 94.1% (16/17) of all isolates detected by the combined use of the four media.

Conclusions: The combination CHRO+CDMN provided recovery equivalent to the combined results of all four culture media for the isolation of *C. difficile* from neonatal foal feces.

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NOVEL TOXIN AND NON-TOXIN BASED ADJUVANTED PROTEIN VACCINES FOR *C. difficile* PROTECT AGAINST TOXIN B AND SPORE CHALLENGE IN MICE AND HAMSTERS

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Background and Aims: *C. difficile* infection (CDI) causes toxin-mediated colitis, with significant morbidity and mortality. Prior vaccine development efforts using inactivated toxoid approaches have been unsuccessful in Phase 3 studies. Currently there are no FDA-approved vaccines for prevention of CDI.

Methods: Matrix-M® adjuvanted novel vaccine candidates, using a recombinant protein vaccine platform technology were evaluated in mouse and hamster models: "Q-Tox", a quadrivalent chimeric construct containing receptor binding domains (RBDs) from *C. difficile* toxin A (TcdA), toxin B (two RBDs), and binary toxin (CDTb); "P-Tox", a pentavalent construct containing the domains in Q-Tox plus an inactivated *C. difficile* protease domain; and "C-Tox", a pentavalent construct containing the domains in Q-Tox plus a *C. difficile* cell wall protein.

Results: *C. difficile* adjuvanted vaccine candidates induced both cellular and humoral immune responses in mice and hamsters. Robust humoral immune responses (anti-toxin IgG and toxin neutralizing antibodies), and strong Th1-dominant polyfunctional CD4+ T cell responses were observed. In lethal challenge models of mice and hamsters with toxin B and *C. difficile* spores, immunization with the lead *C. difficile* vaccine candidate resulted in 75-100% survival at the end of the study (compared to 0% survival in the placebo group) and markedly reduced the severity of cecal histopathological findings. Vaccination also elicited antigen-specific IgA antibodies in ceca, a measure of mucosal immune response. These findings indicate the potential for our novel *C. difficile* vaccines to confer protection against *C. difficile*-induced intestinal damage.

Conclusions: Our studies demonstrated that Matrix-M adjuvanted *C. difficile* vaccines generated robust and protective immune responses, including neutralizing antibodies against toxins, and conferred protection in two lethal challenge models (mice and hamsters using toxin B and spore challenge). These data support further nonclinical and clinical evaluation of the vaccines.

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OUTPATIENT *C. difficile* INFECTIONS: EVIDENCE FROM A GERMAN PHYSICIAN SURVEY ON CLINICAL MANAGEMENT AND MEDICAL CODING PRACTICES

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Background and Aims: *Clostridioides difficile* infection (CDI) is a leading healthcare-associated infection with increasing relevance in outpatient care, yet data on diagnosis, treatment, and ICD coding practices in primary care remain limited.

Objectives: To assess real-world clinical management and coding practices for CDI in primary care and identify potential sources of misclassification in administrative data.

Methods: A weighted cross-sectional survey with general practitioners in Germany (GPs; n=50; April– June 2026) descriptively assessed diarrhea cases, suspected CDI, and CDI-related diagnosis, treatment, and ICD coding practices.

Results: Diarrheal diseases were common in primary care with ~50% of physicians reporting 11–20 cases/month and ~20% of physicians reporting >50 cases/month. Before diagnostic confirmation, CDI was often suspected, i.e. 31% of physicians regarded *C. difficile* in 11-20% of diarrheal patients as the causative agent. Testing and treatment practices of CDI varied between GPs: 38.0% tested for *C. difficile* in 21–50% of suspected cases and 17.4% tested >80%; antibiotic therapy was initiated in ~44% depending on clinical severity, while 45% awaited laboratory confirmation. Most CDI courses were mild/moderate, with only 7% being predominantly severe. Over half of suspected cases remained in care of GPs, while 34% were referred to specialists. Substantial deviations were observed in coding practices: 27% of physicians assigned CDI- specific ICD codes (e.g., A04.7) without laboratory confirmation, while approximately 45% did not correct these codes after negative test results. Only about one-third reported switching to a non-specific ICD code, such as "diarrhea" or "other infection," when test results were negative.

Conclusions: The observed variability in clinical management and coding practices suggests potential misclassification of outpatient CDI in administrative datasets. If these datasets are used for epidemiological studies this may lead to both over- and underestimation of the CDI disease burden. Researchers using claims or administrative data should consider potential coding- related misclassification when estimating CDI incidence and burden.

Disclosures
This study was sponsored by Pfizer

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CHARACTERIZATION OF THE REGULATORY RNA RCd2 IN THE PHYSIOLOGY AND THE PATHOGENESIS OF *Clostridoides difficile*

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Clostridioides difficile is a Gram-positive, strictly anaerobic, spore-forming bacterium and the leading cause of nosocomial diarrhea associated with antibiotic therapy. In recent years, the incidence of community-acquired cases has significantly increased, establishing this bacterium as a major public health concern.

Although significant progress has been made in understanding the pathophysiology of *C. difficile*, the molecular mechanisms governing its infection cycle are still not fully understood. Among these, **regulatory RNAs** have emerged as key players in **controlling gene expression**. Multi-omics approaches, including high-throughput sequencing and transcription start site (TSS) mapping, have led to the identification of more than 250 regulatory RNAs encoded in the *C. difficile* genome [1].

In this work, we focused on **RCd2**, a regulatory RNA expressed during the exponential phase of growth. We identified both RNA and protein in partners of RCd2 using a co-immunoprecipitation based approach, and showed that the majority of its targets are involved in cell wall biogenesis and degradation. We further showed that RCd2 expression regulation is required for optimal bacterial growth and sporulation.

Altogether, our data suggest that RCd2 acts as a key regulator of cell wall dynamic thereby controlling bacterial growth and sporulation. Ongoing studies aim to better understand how RCd2 modulates these critical physiological processes.

A deeper understanding of these regulatory mechanisms could lead for novel strategies for controlling and treating *C. difficile* infections.

[1] Soutourina OA, Monot M, Boudry P, Saujet L, Pichon C, Sismeiro O, Semenova E, Severinov K, Le Bouguenec C, Coppée JY, Dupuy B, Martin-Verstraete I. Genome-wide identification of regulatory RNAs in the human pathogen Clostridium difficile. PLoS Genet. 2013 May;9(5):e1003493. doi: 10.1371/journal.pgen.1003493.

P40

EXPLORING THE SECONDARY METABOLISM OF *Clostridioides difficile*

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Natural products, also called secondary metabolites (SMs), are compounds produced by microorganisms such as bacteria and fungi. Although not essential for short-term survival, an organism's secondary metabolism plays an important role in a variety of processes including intra- and interspecies communication and long-term adaptation. Among bacteria, the Actinobacteria, and particularly the *streptomycetes* are prolific producers of SMs providing pharmaceutically and industrially important products. The secondary metabolism of obligate anaerobic bacteria such as the gram-positive *Clostridioides difficile*, however, has thus far been understudied due to the belief that limitations in energy supply render these anaerobes incapable of producing complex small molecules. Despite the notoriety of *C. difficile* as a major threat to public health, little is known about crucial aspects of its virulence. Considering the importance of the host microbiota in infection as well as insights gained from genomic data, it is evident that *C. difficile* possesses yet undiscovered metabolic potential, producing SMs that may play crucial roles in virulence and infection, driving host-pathogen and microbial interactions and influencing disease outcomes. In collaboration with the group of Prof. Christian Hertweck (Leibniz Institute for Natural Product Research, Jena) we have identified several biosynthetic gene clusters (BGCs) in phylogenetically diverse strains of *C. difficile* using bioinformatic BGC-discovery platforms. The identified BGCs are suspected to lead to the production of SMs with quorum sensing, antimicrobial, and virulence-related properties. Among them, several were found to be conserved across the phylogeny of *C. difficile* while others are likely highly specialized products restricted to a select clade or only a handful of isolates. Through targeted genomic engineering, elucidation of inducing conditions, and sophisticated transcriptome and metabolome profiling, this work aims to investigate BGC expression and SM production in *C. difficile*. The introduction of a tetracycline inducible promoter in a previously unmodified strain has enabled the induction of a thus far uncharacterized BGC which can subsequently be investigated. Through the analysis of this underexplored area of *C. difficile* biology new insights into virulence, pathogenesis, and adaptation will be uncovered.

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EXPANDING THE ROLE OF CRISPR-CAS SYSTEMS IN *Clostridioides difficile*

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Clostridioides difficile is subject to continuous environmental pressures, including invasion by mobile genetic elements such as bacteriophages and plasmids. To counter these threats, *C. difficile* has evolved diverse defense mechanisms, ranging from innate immune strategies (e.g., abortive infection and restriction-modification systems) to the adaptive immune response of CRISPR-Cas systems. Notably, most *C. difficile* strains harbor multiple cas operons of the same subtype, type I-B. These typically include one "full" operon encoding both adaptation and interference modules, and one or more "partial" operons lacking adaptation genes. In addition, *C. difficile* genomes contain an unusually large number of CRISPR arrays, many of which are located within prophage regions, while prophages themselves can encode anti-CRISPR proteins such as AcrIB2. Together, these observations suggest a complex interplay between host immunity and mobile genetic elements and raise the possibility that type I-B CRISPR-Cas systems in *C. difficile* may perform non-canonical functions beyond classical defense. To investigate this possibility, we performed growth analyses and transcriptomic profiling of cas operon deletion mutants. Deletion of the "partial" operon resulted in a significant growth delay, whereas deletion of the "full" operon had no detectable effect on growth. Transcriptomic analyses further revealed widespread alterations in the expression of coding and non-coding RNAs compared with the wild-type strain, particularly following deletion of the "partial" operon. Complementary AlphaFold3 and Protenix structural predictions further suggest that the "full" operon may form a weaker Cas3 interaction interface, potentially impairing interference activity and supporting differential functional activity between the two systems. Together, our results indicate that the type I-B CRISPR-Cas systems may play a previously unrecognized role in cellular physiology, potentially through gene regulatory functions rather than canonical immunity. Ongoing work aims to validate the direct targets of these systems and elucidate the underlying mechanisms, which may reveal a broader role for CRISPR-Cas systems in bacterial gene regulation and host-phage interactions.

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SWARMING-ASSOCIATED MORPHOLOGICAL ADAPTATION OF *Clostridioides difficile* CELLS

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Background and aims: Motility contributes to colonization and persistence of *Clostridioides difficile*, while morphological adaptations associated with different motility states are not well characterized. In many motile bacteria, swarming is associated with cellular differentiation, but whether comparable adaptations occur in *C. difficile* is unclear. This study aimed to assess morphological changes during swimming and swarming, focusing on cell elongation and structural features analyzed using microscopy. To our knowledge, this is the first study describing morphological differences between swimming and swarming cells in *C. difficile*.

Methods: Swimming and swarming were assessed in one clinical *C. difficile* strain: JH/20/B belonging to new PCR-ribotype RT955. Non-motile strain M120 (RT078) was used as a control. Assays were performed on BHIs medium (BHI supplemented with 1,5% yeast extract and 1% glucose) solidified with 0,3% agar (Sigma-Aldrich, no. 05040) for swimming and 0,4% agar for swarming. Cultures were incubated for 48 h (swimming) and 120 h (swarming) under anaerobic conditions. Cell morphology was analyzed by fluorescence microscopy after DAPI and FM4-64 staining. Nanoscale cell structure was examined by atomic force microscopy (AFM). Image and data analysis were performed using Fiji and STATISTICA 13.1 software.

Results: The motile strain JH/20/B showed a swimming diameter of 24,0 ± 3,61 mm and clear surface expansion during swarming, whereas the control strain M120 exhibited neither swimming nor swarming motility and remained localized at the inoculation site. Cell length significantly increased during swarming in JH/20/B (11,92 ± 5,02 µm vs 4,05 ± 0,51 µm; $p < 0,01$), while no significant changes were observed in M120. Under swimming conditions, M120 cells were significantly longer than those of JH/20/B ($p = 0,01$). Fluorescence microscopy revealed multinucleoid, elongated, non-septated cells during swarming in both strains, suggesting altered cell division.

Conclusions: Swarming is associated with cell elongation in JH/20/B, while multinucleoid cells occur in both strains, suggesting altered cell division during swarming independent of motility. These findings indicate that morphological adaptation in *C. difficile* involves elongation and changes in cell cycle regulation.

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ELUCIDATING THE PLEIOTROPIC EFFECTS OF CLPC DELETION IN *Clostridioides difficile*: PROTEOMIC AND PHENOTYPIC INSIGHTS

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Background and Aims: In gram-positive bacteria, ClpP protease is typically associated with the ATPases ClpX, ClpE, or ClpC. Clp proteases play a crucial role in bacterial stress responses and protein homeostasis. Recent studies have proposed the involvement of ClpC in sporulation, motility, and toxin production in the ribotype 027 R20291 strain. However, the molecular mechanisms underlying substrate selection by the ClpC ATPase in *C. difficile* are not understood.

Methods: In this study, we investigated the role of the Clp ATPase, ClpC, in *C. difficile* 630 Δ*erm* physiology using genetic, phenotypic, and proteomic analyses.

Results: Deletion of *clpC* reduced heat shock survival but did not affect growth or stationary phase survival under non-stress conditions. Comparative proteomics revealed that ClpC influences the abundance of proteins involved in sporulation, motility, metabolism, and cell wall biosynthesis. The Δ*clpC* mutant exhibited faster sporulation and increased motility compared to the parental strain and in contrast to the R20291 strain. Peptidoglycan quantification showed a significant increase in the Δ*clpC* mutant, suggesting ClpC's involvement in cell wall homeostasis. The mutant also displayed altered sensitivity to cell wall-targeting antibiotics. Unlike in other bacteria, ClpC did not control the level of MurA, a key enzyme in peptidoglycan precursor synthesis. Instead, the SEDS protein RodA, a transglycosylase involved in peptidoglycan polymerization, accumulated in the Δ*clpC* mutant.

Conclusions: Our findings highlight the pleiotropic role of ClpC in *C. difficile*, particularly in sporulation, motility, and cell wall metabolism, likely through the degradation of key proteins. Understanding the molecular mechanisms of ClpC-mediated proteolysis in *C. difficile* stress responses and virulence may provide insights for the development of novel strategies to combat this important pathogen.

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STRUCTURAL SIMILARITY AND IMMUNOLOGICAL CROSS-REACTIVITY OF LIPOTEICHOIC ACIDS FROM *Clostridioides difficile* AND *Peptostreptococcus anaerobius*

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Background and Aims: Over the past decade, immunization utilizing polysaccharide II (PSII) or lipoteichoic acid (LTA), also referred to as PSIII, has been conducted in animal models to confer protection against *Clostridioides difficile* infection. Both polysaccharides are highly conserved among *C. difficile*, and similar immunogenic responses have been observed when synthetic PSII or LTA were employed as antigens. Vaccination using LTA appears promising; however, LTAs from *C. difficile* and *Peptostreptococcus anaerobius* (analyzed by Stortz et al. (1990)) exhibit structural similarity. The objective of our study was to determine whether the LTA from *C. difficile* is indeed similar to that of *P. anaerobius* and to ascertain whether antibodies specifically directed against the *C. difficile* LTA could cross-react or not with the LTA from *P. anaerobius*.

Methods: The LTAs from *C. difficile* and *P. anaerobius* were extracted and subjected to nuclear magnetic resonance (NMR) analysis. Antibodies generated against LTAs from *C. difficile* were employed to assess cross-reactivity with LTAs from both bacterial species using dot blot techniques and immunofluorescence microscopy.

Results: NMR analysis indicates that LTA from *C. difficile* exhibits a degree of N-acetylation that is tenfold lower than that of *P. anaerobius* LTA. This finding suggests that while the LTA of P. anaerobius is structurally analogous to that of *C. difficile*, it is fully N-acetylated. Furthermore, the dot blot technique demonstrated a reduced recognition of *P. anaerobius* LTA compared to *C. difficile* LTA. Additionally, immunofluorescence assays revealed that the labeling intensity for *P. anaerobius* was also lower than that observed for *C. difficile*.

Conclusions: In this study, we have verified that the LTA of *P. anaerobius* and *C. difficile* exhibit structural similarities. Nonetheless, we have identified a previously unreported variation in the LTA of P. anaerobius. Furthermore, antibodies generated against *C. difficile* LTA demonstrated diminished recognition of *P. anaerobius* LTA, despite the pronounced structural similarities between the LTA structures.

***Reference**: C.A. Stortz, R. Cherniak, R.G. Jones, T.D. Treber, D.J. Reinhardt, Polysaccharides from Peptostreptococcus anaerobius and structure of the species-specific antigen, Carbohydrate Research 207 (1990) 101–120.*

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SH3-MEDIATED SUBSTRATE RECOGNITION DIRECTS YABG LOCALIZATION AND PROTEOLYSIS DURING *Clostridioides difficile* SPORULATION

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Clostridioides difficile is a Gram-positive, strict anaerobic, spore-forming bacterium and a leading cause of antibiotic-associated intestinal disease. Infection is initiated by ingestion of spores, which mediate transmission and contribute to relapse through persistence in host and environment. YabG is a conserved cysteine protease essential for spore morphogenesis and germination. Structurally, YabG comprises an N-terminal SH3 domain fused to a C-terminal CheY-like catalytic domain. While the wild-type YabG ($YabG^{WT}$) undergoes auto-proteolysis, the inactive mutant $YabG^{C207A}$ is cleaved by $YabG^{WT}$ in trans into SH3 and catalytic domains, suggesting release of an inhibitory prodomain. However, the isolated catalytic domain is inactive, suggesting that the SH3 domain is required for activity. This study aimed to define the role of the SH3 domain in YabG activity during *C. difficile* spore development.

We used a combination of activity assays with $YabG^{WT}$ and its variants, designed on the basis of AlphaFold 3 structural models, together with studies of YabG subcellular localization during sporulation and activity at the spore surface, to investigate the role of the SH3 domain.

We found that the SH3 domain contains key determinants for YabG localization to the developing spore surface. This localization was impaired in mutants lacking YabG substrates, indicating dependence on interactions with pre-assembled substrates. YabG-substrate interactions involve the β4-strand of the SH3 domain, where single amino acid substitutions impaired localization and processing. Notably, YabG and its substrates contain xxx[PV]xxP motifs recognized by SH3 domains, and alanine substitutions in these motifs stabilize YabG, consistent with impaired proteolytic activation. Furthermore, mutation of the first motif within CspBA significantly reduced YabG-dependent processing in both *E. coli* and during *C. difficile* sporulation.

Therefore, the SH3 domain is essential for YabG to engage its substrates and for the subcellular localization of the protease, both processes mediated through interactions between SH3-β4 and xxx[PV]xxP motifs in YabG and its other substrates.

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SPATIALLY RESOLVED ANALYSIS OF SPORULATION DECISION-MAKING IN *Clostridioides difficile*

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Unlike model sporulating bacteria, sporulation in *C. difficile* is highly asynchronous, resulting in heterogeneous populations in which only a subset of cells commit to spore formation at any given time. While the transcriptional regulation of sporulation has been extensively characterised, the role that post-transcriptional mechanisms contribute to this heterogeneity at the single-cell level remain largely unexplored. Recent work has demonstrated that small regulatory RNAs (sRNAs) modulate the availability of Spo0A, the master regulator of sporulation, thereby influencing the probability that individual cells initiate sporulation. However, these regulatory interactions have so far been studied exclusively at the population level, obscuring potential spatial and temporal differences in sRNA activity during spore development. Here, we aim to resolve the heterogeneity of sporulation in *C. difficile* by visualising the spatial and temporal expression of Spo0A-regulating sRNAs at single-cell resolution. We are establishing **H**ybridisation **C**hain **R**eaction **F**luorescence ***I**n **S**itu* **H**ybridisation (HCR-FISH) to sensitively detect sRNAs within individual cells and across distinct cellular compartments formed during asymmetric division. This approach enables multiplexed detection of low-abundance transcripts and provides spatial information that is inaccessible to bulk transcriptomic methods and obscured by protein-based reporters. By applying HCR-FISH to sporulating populations, this work will define when and where Spo0A-regulating sRNAs are active during spore development and will provide mechanistic insight into mechanisms that contributes to phenotypic heterogeneity in this pathogen.

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Phi027 PROPHAGE SHAPES *Clostridioides difficile* VIRULENCE THROUGH THE SinR/SinR' AXIS AND THE FLAGELLAR SWITCH NETWORK

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Background and Aims: *Clostridioides difficile* is a leading nosocomial pathogen whose virulence depends on its glucosylating toxins TcdA/TcdB, sporulation and intestinal colonization. The temperate myovirus phi027, highly conserved among hypervirulent RT027/RT176 lineages, was shown to enhance sporulation, adhesion to intestinal epithelial cells, TcdB production and cytopathic effects in strain 500/12, but the underlying mechanisms remained unclear. We investigated how phi027 engages the SinR/SinR'–SigD–flagellar switch network and whether this translates into altered virulence in vivo.

Methods: RNA seq was performed on an isogenic strain pair: phi027 lysogenic 500/12 and its prophage free CRISPR Cpf1 derivative CKH08 (four biological replicates each). Differential expression was analyzed with DESeq2 (adjusted $p < 0.05$, $|log2FC| \geq 1$). Flagellar switch orientation was quantified by allele specific qPCR normalized to rpoA. Flagellar architecture was visualized by atomic force microscopy, and swimming motility by soft agar assay (0.3% BHIS, anaerobic). Virulence was assessed in the *Galleria mellonella* model (10^6 CFU/larva; 75 larvae/group; three replicates), recording survival and melanization over 72 h (log rank test, Bonferroni correction).

Results: We identified 166 differentially expressed genes, including 114 chromosomal host genes. CKH08 showed reduced *sinR/sinR'* expression, downregulation of 26 flagellar biosynthesis genes spanning the flgB locus and decreased *tcdA* transcripts. The flg ON/flg OFF ratio was significantly higher in 500/12 than in CKH08 ($p < 0.01$). Flagella were detected in 93% of 500/12 cells versus 11% of CKH08, yet swimming motility did not differ significantly ($p = 0.69$). Larvae infected with 500/12 showed reduced survival ($p < 0.001$) and more pronounced melanization than CKH08 infected larvae.

Conclusions: Phi027 induces broad transcriptional reprogramming in *C. difficile* via the SinR/SinR'–SigD–flagellar switch axis, coordinating flagellar gene expression, sporulation and toxin associated gene regulation. In vivo, phi027 confers a measurable virulence advantage, supporting prophage mediated regulation as a key driver of the pathogenic potential of epidemic *C. difficile* RT027/RT176 lineages.

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DIMERIC V_HH BINDING PROTEINS MITIGATE *CLOSTRIDIOIDES DIFFICILE* TOXIN-MEDIATED EPITHELIAL DAMAGE

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Background and Aims: *Clostridioides difficile* proliferation in the dysbiotic gut is driven by exotoxins TcdA and TcdB that disrupt tight junctions and impair colonic barrier function. We developed two gastric-stable, dimeric V_HH binding proteins: BL5-6.2 (anti-TcdB) and BL8-9.2 (anti-TcdA). We aimed to evaluate their capacity to support gut barrier integrity across in vitro models of increasing complexity – from monolayer and transwell intestinal models to a dynamic gut flow-cell system – and assessed synergy with butyrate and effects of combining binding proteins.

Methods: VHH binding proteins were evaluated in differentiated Caco-2 and T84 monolayers (WST-1) and T84 transwell barrier models. Butyrate synergy was tested in Caco-2 transwells ± 5 mM butyrate (all challenged with purified toxins). Binding protein interplay was evaluated in a mucus-producing T84:HT29-MTX transwell challenged with toxigenic *C. difficile* (VPI 10463). Barrier model endpoints were barrier strength (TEER), tight-junction integrity (occludin, ZO-1/DAPI imaging), permeability (FD4), toxin levels (ELISA) and *C. difficile* abundance (qPCR). Performance was assessed in a novel anaerobic dynamic gut flow-cell *C. difficile* challenge model by confocal microscopy (ZO-1, actin, MUC2).

Results: Both BL5-6.2 and BL8-9.2 neutralized target toxins and maintained >95% cell viability. In T84 transwells BL5-6.2 preserved barrier function more effectively than BL8-9.2 (≈98% vs ≈65%). In Caco-2 transwells butyrate alone was ineffective; BL5-6.2 partially preserved TEER at 24 h and the combination significantly improved TEER and prevented FD4 flux at 48 h. In T84:HT29-MTX transwells challenged with toxigenic *C. difficile* both binders attenuated TEER loss and preserved occludin; combining binders showed protection but no clear synergistic gain. Flow-cell confocal imaging showed maintained occludin continuity, preserved actin organization and retained MUC2 with V_HH binding proteins versus challenge control.

Conclusions: BL5-6.2 and BL8-9.2 efficiently mitigate toxins and preserve epithelial barrier structure across in vitro models. V_HH binding proteins robustly mitigate toxin-induced gut barrier disruption, with synergistic enhancement by butyrate. These results support further development of V_HH binding proteins as functional food ingredients for proactive gut health support.

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C. difficile TOXINS AND THEIR INTERPLAY WITH THE HEALTHY GUT MICROBIOTA: SUSTAINING A HEALTHY GUT WITH FUNCTIONAL IGG-DERIVED BINDING PROTEINS

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Background and Aims: Maintaining a healthy gut microbiota is key to mitigating *C. difficile* overgrowth and gut disruption. Gut microbes ferment dietary carbohydrates to SCFAs, which support gut health by reducing inflammation and modulating luminal pH. Binding Protein CBB concentrate (BP-CBB) is a precision-fermented, IgG-derived (V_HH) functional food ingredient, discovered and developed for its capacity to support the healthy gut against *C. difficile* toxin B. Here, we aimed to investigate the gut microbiota dynamics of Binding Protein CBB supplementation, with and without *C. difficile* challenge, using the ex vivo SIFR® technology.

Methods: A two phase SIFR study was performed using colonic microbiota from healthy adults (n = 6) with three experimental groups; Control, BP-CBB (0.9 g/L), and BP-CBB (0.9 g/L) + *S. boulardii* (CNCM I-745, 5×10^7 CFU/mL). **Phase 1 (healthy state, 0–24 h)**: healthy microbiota SIFR simulation. **Phase 2 (challenge state, 24–48 h)**: re inoculation of the pre grown gut microbiota and addition of *C. difficile* (ATCC 9689, 1×10^7 CFU/mL). Metrics evaluated; fermentative parameters (GC), pH and gas production, microbial composition (quantitative shallow shotgun sequencing), and untargeted semi polar metabolomics (LC MS/MS). Host–microbiota interactions were assessed in a T84 colonic epithelial transwell model. Toxin B levels were quantified by ELISA (tgcBiomics).

Results: In Phase 1, BP-CBB stimulated SCFA production by **23% (±2%)**, while metabolomics showed moderate increases in health associated metabolites derived from proline, arginine, and tryptophan. Compositional shifts were mainly associated with increase in Bacteroidota (<2 fold). In Phase 2, BP-CBB reduced *C. difficile* proliferation by **~62%**, lowered toxin B levels by **~85%**, and preserved epithelial barrier integrity **(~90%)** relative to challenge control. Combination with probiotic *S. boulardii*, routinely used for *C. difficile* mitigation, reduced dysbiosis (decreased Pseudomonadota) but did not expand on *C. difficile*-specific effects.

Conclusions: BP-CBB enhanced SCFA production and reduced *C. difficile* proliferation and toxin expression in a simulated gut model. This research points to a role of toxin B beyond the direct interaction with gut epithelial receptors, contributing to microbial crosstalk during *C. difficile* proliferation driving pathobiont expansion in situ.

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CHARACTERIZATION OF GENOTYPIC DIVERSITY IN CONTEMPORARY *Clostridioides difficile* ISOLATES FROM ATLAS 2020-2024

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Background and Aims: *Clostridioides difficile* infection (CDI) is a major global public health burden, affecting nearly 500,000 individuals and causing ~30,000 deaths annually in the United States alone. Antimicrobials, including those used in CDI treatment, can increase the risk of both primary and recurrent CDI; therefore, a preventive vaccine is needed. The genotypic diversity in a contemporary collection of *C. difficile* isolates was evaluated with a goal of identifying virulence biomarkers and establishing a platform to assess the breadth of coverage of Pfizer's investigational vaccine.

Methods: 2,461 contemporary (2020–2024) clinical *C. difficile* isolates were collected through the Antimicrobial Testing Leadership and Surveillance (ATLAS) program. Approximately 40% of isolates were from the United States, followed by Japan (18%), Germany (9%), and five additional European countries. These isolates were analyzed by WGS followed by multilocus sequence typing, clade assignment, determination of large toxin and binary toxin gene status, identification of Toxin A (TcdA) and Toxin B (TcdB) variants and evaluation of related regulatory elements.

Results: ST2, ST3, ST8, and ST1 (collectively 37%) were the most frequently identified sequence types. Clade 1 predominated (76%), followed by clades 2 (12%) and 5 (4%). TcdA010 (34%) and TcdB012 (39%) were the most prevalent toxin variants, and 83 novel TcdA and 70 novel TcdB variants were identified. Overall, 17% of isolates carried tcdA, tcdB and binary toxin genes ($A^+B^+CDT^+$) and 67% were $A^+B^+CDT^-$ isolates. Most STs (93%), TcdA (96%) and TcdB (90.3%) variants in $A^+B^+CDT^+$ isolates were not found in $A^+B^+CDT^-$ strains. Moreover, 92% of $A^+B^+CDT^+$ isolates versus 5% of $A^+B^+CDT^-$ isolates harbored a disrupted tcdC.

Conclusions: In our contemporary isolate collection, we identified a large genomic diversity and numerous novel TcdA and TcdB variants. A correlation of specific STs, large toxin variants, and tcdC disruption with CDT^+ status was also observed. While the CDT^+ lineage alone has been an inconsistent surrogate marker of hypervirulence, the TcdA and TcdB genotypes provide putative markers for further evaluation. These results will inform further investigation of the correlation of large toxin variants with virulence and assessment of the breadth of coverage of Pfizer's investigational *C. difficile* vaccine.

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EXOGENOUS BUTYRATE INDUCES *C. difficile* TOXIN RELEASE AND SPORULATION

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Infection with the gastrointestinal pathogen *Clostridioides difficile* represents a major concern to global health. Current treatments for *C. difficile* infections (CDIs) are antibiotics and fecal microbiota transplants (FMTs) for recurrent cases. However, antibiotics contribute to recurrence and antibiotic resistance and the long-term sustainability and accessibility of FMTs remain to be determined. CDI is often engendered by dysbiosis of the gut microbiota, allowing access to vacant nutrient niches. These limitations highlight the need for more precise strategies for coping with CDI, which requires a deeper understanding of microbiome dynamics to develop. One of the major metabolic products of the gut microbiome is the short chain fatty acid, butyrate. Butyrate has pleiotropic beneficial effects on human health and GI butyrate is associated with protection against CDI in humans and animal models. Despite these protective effects, butyrate exposure leads to elevated toxin release and increased sporulation by *C. difficile*, suggesting that *C. difficile* senses and responds to butyrate in order to outcompete inflammation-sensitive microbiome members and transmit to new hosts. Our ongoing work is revealing previously uncharacterized microbiome-responsive post-transcriptional regulatory mechanisms of toxin release and sporulation in *C. difficile*. Insights gained from this work will expand our understanding of how the microbiome impacts *C. difficile* fitness and virulence and will hone future microbiome-sparing strategies against CDI.

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CHARACTERIZATION OF THE NATIVE AUTOINDUCING PEPTIDE(S) IN *C. difficile*

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Background and Aims: Quorum sensing (QS) is a density-dependent regulatory mechanism that enables bacteria to coordinate gene expression via secreted signaling molecules. In Gram-positive organisms, QS is typically mediated by autoinducing peptides (AIPs) that activate two-component regulatory systems, leading to transcriptional changes associated with virulence. In *Clostridioides difficile*, toxin production is regulated in part by the accessory gene regulator (*agr*) system, which also influences additional virulence traits. Although targeting of QS has been explored as a thera-peutic avenue in other Gram-positive pathogens, its potential in *C. difficile* remains largely un-explored. This study aims to characterize the native AIP(s) produced by *C. difficile* and to determine their role in regulating toxin production. Furthermore, we investigate whether disruption of QS signaling, including inhibition of the sensor kinase, can modulate the toxin-producing phe-notype. These findings may inform the development of quorum quenching as an alternative therapeutic strategy against *C. difficile* infection.

Methods: Supernatants from stationary phase cultures of *C. difficile* CD630 and R20291 were fraction-ated by solid phase extraction (SPE) on a C18 functionalized silica gel column. The AIP(s) were eluted with 80% acetonitrile in water. Fractions were analyzed by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) analysis. Synthetic AIP analogs were synthesized by solid-phase peptide synthesis (SPPS) on an MeDbz-functionalized resin. Toxin production was assessed by qPCR and "Cdifftox" assay. Agr activity was assessed by qPCR.

Results: Using FTICR-MS we were able to show the mass 612.31 Da present in CD630 and R20291 supernatant, which corresponds to the mass of the cyclic pentamer with the sequence CPWII. Following treatment with the synthetic peptide (CPWII) we were able to induce transcription of *agrBD1*, as well as *tcdA* and *tcdB* visualized by qPCR. Additionally, levels of TcdA and TcdB were elevated in cultures treated with synthetic AIP.

Conclusion: In *C. difficile*, the level of TcdA and TcdB increases upon treatment with the homodetic pen-tamer, CPWII, indicating that toxin production is at least partially regulated by the Agr sys-tem. Moreover, we suggest that the cyclic peptide with the sequence CPWII is an AIP pro-duced from the *agrBD1* loci in CD630 and R20291.

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FUNCTIONAL CHARACTERIZATION OF THE AUTOINDUCING PEPTIDE(S) IN *C. difficile* – A CHEMICAL APPROACH

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Background and Aims: Quorum sensing (QS) is a density-dependent regulatory mechanism that enables bacteria to coordinate gene expression via secreted signaling molecules. In Gram-positive organisms, QS is typically mediated by autoinducing peptides (AIPs) that activate two-component regulato-ry systems, leading to transcriptional changes. In *Clostridioides difficile*, toxin production is regulated in part by the accessory gene regulator (agr) system, which also influences addi-tional virulence traits. Although AIPs of other Clostridia, such as *Clostridium acetobutylicum* and *Clostridium perfringens*, have been characterized by extraction or bioinformatic predic-tions followed by functional assays, only a handful of studies address the AIP produced by *C. difficile*. At present, the AIP produced by *C. difficile* is described as a peptide containing a thiolactone with a mass >1000 Da, yet its full structure remains elusive. This study aims to characterize the native AIP produced by *C. difficile* based on bioinformatic predictions. Furthermore, we investigate the stability of the thiolactone motif, as studies in-vestigating QS in other Gram-positive pathogens suggest a rapid shift of the thiolactone motif in tailless AIPs resulting in a homodetic peptide at physiological pH.

Methods: Based on reported *agrD1* sequences across *C. difficile* strains, potential AIPs were synthe-sized by solid-phase peptide synthesis (SPPS) to obtain both the homodetic peptide and the cyclic thiolactone variants. The predicted S-to-N acyl shift of the cyclic thiolactone was as-sessed at pH 4–7, by monitoring the change in retention time in ultra high-performance liquid chromatography.

Results: Cyclic thiolactone and homodetic peptides were successfully synthesized (purity >95%) using SPPS. The cyclic thiolactone undergoes a rapid S-to-N acyl shift at pH 7, resulting in homo-detic peptide formation. At pH 7, the half-life of the thiolactone is 3.3 minutes, whereas it increases to 128 min at pH 4.

Conclusion: The thiolactone motif of the predicted AIP produced in *C. difficile* readily undergoes an S-to-N acyl shift at pH 7, resulting in a homodetic peptide. This raises the question whether homo-detic or thiolactone-containing peptide is the predominant AIP at physiological pH. The ef-fects of the homodetic peptide as well as the thiolactone-containing peptide on toxin produc-tion, are currently being examined by qPCR.

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DISSECTING THE BIOSYNTHETIC PATHWAY OF THE FLAGELLIN TYPE A GLYCAN IN *Clostridioides difficile* STRAIN 630Δ*erm*

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Glycosylation of bacterial surface proteins, such as flagellin (FliC), is important for their function and is often involved in virulence of pathogens. Glycans can be further modified by so-called post-glycosylation modifications (PGMs) often resulting in exclusive molecular structures. In *Clostridioides difficile* a unique glycan structure (Type A) decorates FliC (which forms the flagellar filament) that consists of an *O*-linked N-acetyl-β-D-glucosamine (GlcNAc) modified with an N-methyl-L-threonine via a phosphodiester linkage (1). This PGM is synthesized by a set of four enzymes encoded in one operon (*cd0241-cd0244*, we will refer to the enzymes as Fta (Flagellin Type A) ABCD), but the exact biosynthesis pathway and biosynthetic intermediates remain unknown. Based on mass spectrometry-based proteomics experiments we recently provided a revised model for the Type A biosynthesis (2). Here, we aim to provide experimental evidence for the proposed pathway.

Fundamental to our model is the prediction of CDP-threonine (CDP-Thr) and CDP-*N*-methyl-threonine (CDP-Me-Thr) as novel biosynthetic intermediates. In this study, we chemically synthesized both predicted biosynthetic intermediates. We showed that they are involved in the Type A PGM biosynthesis, as evidenced by mass spectrometric analyses of extracts of a set of *C. difficile* mutant strains. Furthermore, we recombinantly expressed and purified FtaC and FtaD. Using vitro assays, we characterized FtaC to be a SAM-dependent CDP-threonine N-methyltransferase, that installs the methyl group on CDP-threonine. Next, we revealed FtaD as the CDP-N-methylthreonine:GlcNAc N-methylthreoninephosphotransferase, transferring the full PGM to GlcNAc-peptide. Finally, using both FtaC and FtaD in combination with synthetic CDP-threonine, we reconstituted the biosynthesis pathway of the Type A PGM in vitro.

Overall, our results open avenues to explore these unique biosynthesis enzymes in molecular detail to provide new points of entry for the development of biosynthesis inhibitors and tools to study the role of this PGM in virulence and flagellar function.

1. A. Faulds-Pain et al, Mol Microbiol 94, 272-289 (2014)
2. B. Claushuis et al., ACS Infect Dis 9, 2665-2674 (2023)

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SMART COLON-ON-A-CHIP: A HIGH-FIDELITY *IN VITRO* MODEL FOR STUDYING *C. difficile* PATHOGENESIS

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Clostridioides difficile is a leading cause of antibiotic-associated diarrhoea and healthcare-related infections, yet our understanding of its interaction with the host epithelium remains minimal. Physiologically relevant in vitro models, such as 2D cultures and vertical diffusion chambers, lack controlled fluid flow, which is essential for mimicking the gut's dynamic environment. These models fail to recapitulate the complex interactions between the gut epithelium, microbiota, and immune cells. Advanced microfluidic Gut-on-a-Chip systems, which address this limitation by incorporating flow and mechanical forces are often expensive, requiring specialized engineering facilities. Additionally, culturing cells within these devices are challenging and they are often restrictive in the retrieval of host and bacterial cells, complicating downstream analyses.

To overcome these challenges, we have developed a Smart Colon-on-a-Chip, a millifluidic platform that replicates key aspects of the human gut microenvironment using widely available and cost-effective components. This bioengineered system integrates gut epithelial cells, a mucus layer, and fibroblasts under controlled fluid flow, and simulates gut peristalsis and transepithelial barrier functions. Furthermore, this platform enables real-time monitoring, providing high-resolution insights into *C. difficile* colonization. We have optimised the design of the Smart Colon-on-a-Chip device to enable culture of polarised epithelial cells up to 5 days. We have demonstrated successful *C. difficile* infection over time, recapitulating the transepithelial barrier and its disruption during *C. difficile* infection. We are currently analysing the physical parameters that control initial stages of *C. difficile* colonization.

Thus, our Colon-on-a Chip represents a cost-effective, scalable, and user-friendly system for host-anaerobe interaction studies, which has broader applications in studying the gut microbiota and other anaerobic pathogens. Importantly, it offers an excellent system for screening drug and vaccine targets in a physiologically relevant setting.

P56

STRUCTURAL BASIS OF SECONDARY POLYSACCHARIDE BIOSYNTHESIS IN *C. difficile*

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PSII is a major secondary polysaccharide extending from the peptidoglycan that mediates non-covalent anchoring of the 2D proteinaceous S-layer to the cell surface and is essential for *C. difficile* survival. Disruption of PSII biosynthetic enzymes causes severe growth defects and impairs peptidoglycan, S-layer and biofilm formation, ultimately reducing virulence. This study aimed to validate these enzymes as targets for specific treatment of *C. difficile* infection. Structures of recombinant PSII biosynthetic enzymes, including mannose-converting enzymes, glycosyltransferases and enzymes anchoring PSII to peptidoglycan, were determined by X-ray crystallography. Ligand analogues of predicted PSII building blocks were synthesised chemically, and protein-ligand complexes were characterised after co-crystallisation and crystal soaking. Molecular simulations were used to elucidate catalytic mechanisms and guide inhibitor design.

Four representative crystal structures revealed similarities to homologous proteins and conserved as well as unique ligand-binding regions associated with PSII assembly. The Pgm2 α-D-phosphohexomutase structure showed an open four-domain conformation, with each domain contributing a conserved functional loop to the active site containing R50 and K421 as the general acid and base, respectively. The identity of the bound metal cation remains unresolved, and molecular docking identified glucose-1-phosphate as the most favorable substrate. The structure of spontaneously cleaved CD630_27760 glycosyltransferase revealed an N-terminal glycosyltransferase domain bound with unidentified metal cation and a disordered substrate access loop, whereas the C-terminal putative acceptor (saccharide-UndPP)-binding domain was absent. Soaking with putative donor UDP-GlcNAc yielded a UDP complex. LcpA and LcpB, redundant pyrophosphatases/phosphotransferases that anchor PSII to peptidoglycan, exhibited a single-domain structure with a putative acceptor (peptidoglycan)-binding groove and a hydrophobic pocket occupied by an unidentified ligand, likely an analogue of the donor (saccharide-UndPP).

Structural analysis of PSII biosynthetic enzymes elucidates the PSII biosynthesis pathway and paves the way for its complete reconstitution and novel targeted treatment strategies against *C. difficile* and other Gram-positive bacteria possessing homologous proteins.

P57

S-LAYER DELETION IN *C. difficile* CLINICALLY RELEVANT STRAINS

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Background and Aims: Many bacteria are coated with a proteinaceous surface layer (S-layer) formed by the self-assembly of monomeric proteins into a two-dimensional array. S layers are implicated in growth and survival, cell integrity, enzyme display and interaction with the host and its immune system. In *C. difficile*, the principal S-layer protein, SlpA, displays considerable sequence diversity between strains with 11 distinguishable SlpA isoforms[1], associated with 13 S-layer cassette types[2]. We determined the structure and assembly of SlpA, revealing a tightly packed array[3]. Spontaneous SlpA point mutants strains failed to cause disease in hamsters4 and secondary point mutants with a restored *slpA* gene quickly outcompete an SlpA-deficient strain in a mouse model[5]. A CRISPR-Cas9 deletion of slpA in the lab strain (CD630) recently reported pleiotropic effects on fitness and virulence-related traits[6].

Methods: We report *slpA* deletion mutants obtained using CRISPR-Cas9 in clinically relevant strains and characterise their fitness, sporulation, toxin production and biofilm capabilities. Resistance to antibiotics and host antimicrobials was assessed to determine the role of SlpA in antimicrobial resistance (AMR).

Results: Deletion of *slpA* in the clinically relevant R20291 strain (027 ribotype, SlpA 4 isoform) has limited effect on growth, but cell morphology is altered with shorter, wider cells observed. A delay in toxin production and sporulation and increase in biofilm formation accompanies the morphology changes. Absence of SlpA leads to sensitivity to host antimicrobials lysozyme and LL-37, an antimicrobial peptide. Importantly, absence of SlpA increases sensitivity to antibiotics targeting the cell envelope, such as vancomycin and daptomycin, with observed increase of cell lysis onset.

Conclusions: SlpA is involved in several key pathogenicity aspects, from toxin production to biofilm formation and sporulation. AMR is impacted by the presence of the S-layer and the mechanisms involved are exciting avenues for new investigation and as potential therapeutic targets.

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ADDING FUNCTIONALITY TO *C. difficile's* ARMOUR – INVESTIGATION OF CWPS

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Background and Aims: The surface layer (S-layer) is a 2D paracrystalline coat that covers the entire cell surface. A mature S-layer in *C. difficile* is mainly composed of SlpA, decorated with minor components called cell wall proteins (CWPs) that provide additional functionality1. The S-layer has been implicated in adherence to gut epithelial cells and evasion of host immune system2. Although research into some CWPs has elucidated their role as proteases and adhesins, the function and exact composition of these minor components is still unknown. We are interested in investigating the functional modules of selected CWPs including Cwp6, 16 and 17, which are predicted to possess a C-terminal N-acetylmuramyl-L-alanine amidase (amidase 3) domain. We hypothesise that these CWPs may possess autolysin activity and therefore play important roles in cell growth and survival.

Methods: The CWPs of interest will be expressed and purified in E. coli systems to be structurally and functionally characterised. Here, we will assess the role of these CWPs in *C. difficile* by investigating fitness and phenotypes of deletion of Δ*cwp6/16/17* in WT and in a Δ*slpA* mutant recently obtained in the lab using CRISPR-Cas9.

Results: Preliminary proteomics analysis of the surface protein extracts from the R20291 wildtype (WT) and R20291 Δ*slpA* revealed that different CWPs are overexpressed in the absence of SlpA. Fitness and antimicrobial resistance will elucidate the role of these autolysins in *C. difficile* biology. Determining the structure will provide a thorough understanding of whether Cwp16 and Cwp17 share the same domain organization to previously determined Cwp6[3].

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P59

D,D-CARBOXYPEPTIDASES CONTROL THE MODE OF PEPTIDOGLYCAN TRANSPEPTIDATION IN *C. difficile*

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Clostridioides difficile (CD), the leading cause of healthcare-associated diarrhea, displays intrinsic resistance to β-lactam antibiotics, particularly cephalosporins, through mechanisms that remain largely unresolved. A distinctive feature of CD is its unusual peptidoglycan (PG) architecture, characterized by a predominance of 3→3 peptide cross-links generated by L,D-transpeptidases (LDTs). LDT-mediated cross-linking depends on tetrapeptide donor substrates generated from pentapeptide precursors by D,D-carboxypeptidases (DD-CPases), suggesting that these enzymes regulate PG assembly in CD and control the balance between classical (4→3) and non-classical (3→3) cross-linking. Although LDTs are poorly inhibited by most β-lactams, we recently showed that β-lactams inhibit the LDT-dependent pathway and alter PG composition in CD, likely through DD-CPase inactivation.

This study aims to investigate the role of DD-CPases in PG homeostasis in CD. Biochemical assays using purified recombinant proteins and disaccharide penta- and tetrapeptide substrates identified two class C penicillin-binding proteins, DacA and DacB, as the principal vegetative D,D-carboxypeptidases. We also identified two VanY metallopeptidases displaying D,D- and L,D-carboxypeptidase activity, respectively. A *dacA dacB* double mutant showed marked alterations in PG composition, including accumulation of pentapeptide-containing muropeptides and a strong reduction in 3→3 cross-links, demonstrating that D,D-CPase activity is required for LDT-dependent PG assembly. However, residual 3→3 cross-links suggested compensatory mechanisms sustaining LDT-mediated cell wall synthesis. Complementation by *dacA*, but not *dacB*, restored a wild-type PG profile, indicating that DacA plays a predominant role during vegetative growth. Deletion of the VanY-family D,D-carboxypeptidase gene had no detectable impact on PG structure. However, overexpression of this enzyme in the *dacA dacB* mutant eliminated pentapeptide-containing muropeptides while generating a PG profile distinct from the wild-type strain, indicating partial functional compensation.

Our results reveal that distinct DD-CPases differentially shape PG composition, with specific enzymes strongly promoting LDT-dependent cross-linking. These findings identify DD-CPases as central regulators of cell wall architecture in CD.

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PREVALENCE OF MULTIDRUG-RESISTANT *Clostridioides difficile* RIBOTYPES ISOLATED FROM FOALS IN BRAZIL

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Background and Aims: *Clostridioides difficile* is an important cause of diarrhea in foals, often requiring intensive care and antimicrobial therapy. Antimicrobial resistance among *C. difficile* isolates from horses has increased in recent years. This study aimed to determine the phenotypic and genotypic antimicrobial resistance profiles of *C. difficile* recovered from diarrheic foals.

Methods: Twenty-five *C. difficile* isolates from foals (≤ 1 year old), obtained from the FMVZ/Unesp biobank, were investigated. Toxin genes were detected using multiplex PCR, followed by PCR ribotyping. Whole-genome sequencing (WGS) was performed using an Illumina MiSeq platform. Sequence assembly and analysis were conducted to determine multilocus sequence typing (MLST), antimicrobial resistance genes, and mobile genetic elements. Antimicrobial susceptibility testing was performed by inoculating strains onto Brucella blood agar supplemented with vitamin K and hemin. Initial screening was conducted using disk diffusion, with confirmation by epsilometer testing for erythromycin, rifampicin, tetracycline, metronidazole, moxifloxacin, and vancomycin. Statistical associations were analyzed using chi-square or Fisher's exact test, with $p < 0.05$ considered statistically significant.

Results: Isolates were classified as follows: $A^+B^+CDT^+$ RT126/ST11/Clade 5 (64%); $A^+B^+CDT^+$ RT AI-60/ST11/Clade 5 (4%); $A^+B^+CDT^-$ RT046/ST35/Clade 1 (20%); $A^-B^-CDT^-$ RT009/ST1/Clade 1 (4%); $A^-B^-CDT^-$ RT012/ST3/Clade 1 (4%); and $A^+B^+CDT^+$ novel RT/ST149/Clade 1 (4%). Antimicrobial resistance determinants were identified for aminoglycosides (72%) *(aph(2'')-If, ant(6)-Ia, aph(3')-III, and nmpA)*, tetracyclines (80%) *(tet(40) and tet(M))*, macrolides (16%) *(erm(B)* and *mef(A))*, streptogramins (16%) *(msr(D))*, and lincosamides (12%) *(erm(G))*. The plasmid *repUS43* (60%) and the transposon *Tn6009* (44%) were also detected. Phenotypic antimicrobial susceptibility testing revealed resistance to erythromycin (88%), rifampicin (88%), moxifloxacin (60%), and tetracycline (4%). All isolates were susceptible to metronidazole and vancomycin. Based on these results, 17 isolates were classified as multidrug-resistant (MDR), 14 of which belonged to RT126/ST11/Clade 5. A significant association was identified between RT126/ST11 and MDR ($p = 0.009$).

Conclusions: The high prevalence of RT126/ST11/Clade 5 strains in foals with diarrhea, along with their association with multidrug resistance and antimicrobial resistance genes, is concerning. These strains are considered hypervirulent and may pose a significant threat to equine health, while also highlighting the potential role of animals as reservoirs of clinically relevant *C. difficile* strains.

P61

DEFINING TETRACYCLINE ECOFF AND RESISTANCE DETERMINANTS IN *Clostridioides difficile* ACROSS ONE HEALTH RESERVOIRS

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Background and Aims: Antimicrobial resistance has shaped *Clostridioides difficile* epidemiology. Interpretation of tetracycline resistance is limited by the lack of species-specific breakpoints, with studies relying on generic anaerobe breakpoints or *tet(M)* detection. This study characterizes resistance phenotypes and genotypes and proposes an epidemiological cut-off value (ECOFF) for tetracycline.

Methods: Isolates from animal (n = 119) and environmental (n = 51) sources and a subset of human isolates (n = 385) were screened for *tet(M)* gene by PCR; minimum inhibitory concentrations (MIC) were determined by Etest® for *tet(M)*-positive isolates. A control group of 23 *tet(M)*-negative was also included. From January 2025, tetracycline MIC testing was added to the antimicrobial surveillance panel, yielding additional 258 human isolates. In this group, *tet(M)* PCR was performed on isolates with MIC >0.094 mg/L (n=37) and selected controls with MIC ≤ 0.094 mg/L (n=6). A representative subset (n=161) underwent WGS.

Results: *tet(M)*-positive isolates (n = 61), showed MICs of 1–32 mg/L, while *tet(M)*-negative controls had MICs of 0.023–0.094 mg/L, except two isolates at 4 mg/L. An ECOFF of 0.094 mg/L was proposed. Among prospective human isolates, 37/258 (14.3%) were resistant (MIC range: 1–12 mg/L), including 26 *tet(M)*-positive. Susceptible isolates (n=221) had MICs of 0.016–0.094 mg/L. WGS confirmed *tet(M)* in 60 isolates and identified 13 allelic variants (A1–A13), with A2 being the most prevalent (n=15). Additional tet genes, tet(40) (n=11), tet(44) (n=5) and tet(O) (n=3) were ribotype specific (RT126, RT078, and RT039, respectively). *tet(M)*-negative resistant isolates (n=9, WGS) harboured *tetA(P)/tetB(P)*, supporting an independent role in tetracycline resistance. Several tet-associated transposons were identified.

Conclusions: An 0.094 mg/L ECOFF reliably distinguishes wild-type (susceptible) from non-wild-type (resistant) isolates, supporting standardized susceptibility interpretation in *C. difficile*. This species-specific breakpoint appears more appropriate than relying exclusively on *tet(M)* detection, as additional determinants contribute to resistance. Validation across human, animal, and environmental strains underscores reservoir interconnectedness and reinforces the importance of a One Health approach for surveillance of community-circulating pathogens.

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A NEW ANTIBIOTIC RESISTANCE ENZYME FOR A CLASSIC TARGET: MRMA REDIRECTS RADICAL SAM METHYLATION TO 23S RRNA A2058 IN *C. difficile*

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The ongoing evolution and spread of antimicrobial resistance necessitate a deeper understanding of how resistance mechanisms emerge and diversify. The radical SAM methyltransferase family, exemplified by RlmN and Cfr, modifies the bacterial ribosome but with divergent outcomes: RlmN methylates C2 of 23S rRNA nucleotide A2503 as part of cellular physiology, while Cfr methylates C8 at the same position, conferring broad resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (PhLOPSA).

Here, through comparative genomics of *C. difficile*, we identify a novel radical SAM enzyme, MrmA, which represents a dramatic evolutionary shift in substrate specificity. Using genetic, biochemical and structural approaches we demonstrated that MrmA methylates the nucleotide A2058—a canonical antibiotic-binding site whose dimethylation by Erm-family enzymes confers MLSB resistance. Remarkably, MrmA is the first radical SAM enzyme shown to target this position. By installing a novel C2 methylation at A2058, MrmA specifically confers resistance to macrolides and streptogramin B but not to lincosamides.

The comparative analysis of MrmA, Cfr, and RlmN reveals how conserved enzyme scaffolds evolve novel functions. Together, these three enzymes provide a powerful model to elucidate the molecular determinants that dictate which nucleotide position is selected for methylation, offering crucial insights into the general mechanism of substrate selection by ribosomal methyltransferases and their role in the evolution of antibiotic resistance.

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LONG-TERM TRENDS IN ANTIMICROBIAL RESISTANCE AND RIBOTYPE DISTRIBUTION OF *C. difficile* ISOLATES FROM TWO KOREAN HOSPITALS, 2018–2025

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Background and Aims: Since 2018, monitoring of the genotypes and antimicrobial resistance of *C. difficile* isolated from two hospitals in Seoul and Gyeonggi Province has been conducted as part of the national antimicrobial resistance surveillance program, KorGLASS. We aimed to report the antimicrobial resistance patterns and genotypic changes of *C. difficile* isolates collected from two hospitals between 2018 and 2025, and to analyze the isolates according to their epidemiologic origin (community vs hospital).

Methods: All *C. difficile* strains isolated from diarrheal samples of patients suspected of CDI at the two hospitals were analyzed. Identification of *C. difficile* was performed using MALDI-TOF, followed by PCR ribotyping for strain typing. The minimum inhibitory concentration (MIC) for ampicillin, cefotetan, clindamycin, imipenem, chloramphenicol, tetracycline, moxifloxacin, vancomycin, metronidazole, and rifaximin were determined using agar dilution method. Electronic medical records of patients with isolated *C. difficile* were reviewed.

Results: Between 2018 and 2025, a total of 2,184 non-duplicate toxigenic *C. difficile* strains were isolated, of which 667 (30.5%; range, 23.4%–37.7% annually) were of community origin. In 2018, ribotype 018 (R018) was predominant among hospital-origin cases at Hospital A and among both community-onset and hospital-onset cases at Hospital B. By 2025, the predominant ribotypes had shifted to R014/020 in both hospital-origin and community-origin cases at Hospital A, and to R002 in hospital-origin cases and AB24 in community-origin cases at Hospital B. Antimicrobial resistance showed a gradual decrease over the 8-year period, particularly in moxifloxacin non-susceptibility, which decreased from 48.4% to 23.1%, and rifaximin non-susceptibility, which decreased from 22.0% to 6.3%. None of the isolates showed MICs > 2 µg/mL for metronidazole or vancomycin.

Conclusions: The observed changes in antimicrobial resistance were associated with shifts in genotype distribution rather than year-to-year variation within the same genotype.

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THE PREDICTION OF ANTIMICROBIAL RESISTANCE IN *Clostridioides difficile* BASED ON GENOMIC DATA

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Background and Aims: Whole-genome sequencing (WGS) has increasingly been implemented in the surveillance of *Clostridioides difficile* infection (CDI), although antimicrobial susceptibility testing (AST) is not routinely performed. In this study, we aimed to compare phenotypic and genotypic data to determine whether antimicrobial resistance (AMR) can be reliably predicted from genomic AMR determinants detected using publicly available tools.

Methods: AST (E-test) and Illumina genomic data from 535 *C. difficile* isolates were analysed. Two assemblers (SPAdes and Unicycler) and three AMR prediction tools (AMRFinderPlus, CARD, and ResFinder) were compared.

Results: Clindamycin and erythromycin resistance prediction based on the erm and cfr genes showed high PPV (99–100%), with lower NPV for erythromycin (73–79%) than clindamycin (86–93%), likely due to the absence of the mrmA gene in databases. Linezolid prediction varied across tools. ResFinder and AMRFinderPlus achieved high NPV (99–100%) but moderate PPV (46%). CARD showed lower NPV (80%) and PPV (24–25%). For fluoroquinolones, *gyrA* (T82I) showed perfect PPV (100%) but lower NPV for ciprofloxacin (47%), while maintaining high NPV for moxifloxacin (98–99%). For ciprofloxacin, adding *gyrB* mutations in AMRFinderPlus increased NPV to 56% while keeping high PPV (89%). For rifamycin (*S. aureus* breakpoint MIC ≥ 0.06 mg/L), PPV and NPV were near-complete (98–99%). For tetracyclines, PPV was very low (13%) despite perfect NPV (100%), suggesting the need for a reassessment of the breakpoint. AMRFinderPlus and CARD detect genes not correlated with phenotypic resistance: *van* (vancomycin), *dfr* (cotrimoxazole), *nimB* (metronidazole), *cplR* (clindamycin). CARD also detects C656T in 23S rRNA (erythromycin, clindamycin), and AMRFinderPlus the PnimB^{G} promoter mutation (metronidazole), both of which are poorly predictive.

Conclusions: Current tools do not achieve sufficiently reliable prediction of *C. difficile* resistance from WGS data, primarily due to the inclusion of resistance determinants with limited phenotypic support and the omission of newly described or established determinants.

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BEYOND THE BINDING SITE: HIDDEN DRIVERS OF FIDAXOMICIN RESISTANCE

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Antibiotics are currently the primary therapeutic option available for the treatment of *Clostridioides difficile* infection (CDI). Treatment with Fidaxomicin (FDX), an antibiotic targeting RNA polymerase (RNAP), is the recommended first-line therapy. FDX acts similar to rifampicin (RIF), another widely used RNAP inhibitor but differs in the mechanism of action. FDX-resistant clinical isolates of *C. difficile* strains are emerging but the present knowledge on FDX resistance mechanisms is limited only to mutations in RNA polymerase (RNAP) subunits. Besides RNAP mutations, different mechanisms of resistance have been reported for RIF (e.g. RIF modification, efflux, target protection etc.). We envisage that mechanisms of resistance analogous to RIF exist in *C. difficile*. To identify such mechanisms, we performed a proteomic study exposing *C. difficile* to sub-inhibitory concentration of FDX. From the proteomic analysis, we identified 60 proteins that are significantly upregulated after FDX treatment. We performed CRISPRi to knock down expression from selected top upregulated proteins. Agar-dilution susceptibility assays using FDX showed that knockdown of two genes, CD630_08470 and CD630_08480, impaired bacterial growth in the presence of sub-inhibitory concentrations of FDX. CD630_08470 and CD630_08480 were not previously known to be associated with FDX resistance. We hypothesize possible mechanism(s) of FDX resistance involving these two genes.

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DIFFERENTIAL IMPACT OF VANCOMYCIN AND FIDAXOMICIN ON DIGESTIVE CLEARANCE OF *Clostridioides difficile*

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Background and Aims: Recurrent *Clostridioides difficile* infection (CDI) can occur in up to 20% of patients. Recurrency is multifactorial and commonly driven by suboptimal immune response, persistent dysbiosis, and/or persistent *C. difficile* carriage. This study aimed to compare the impact of vancomycin (VAN) and fidaxomicin (FDX) on the dynamics of digestive clearance of *C. difficile* during and after CDI treatment.

Methods: In this prospective cohort (September 2023-December 2025), adult patients with clinically and microbiologically confirmed CDI were enrolled. The diagnostic stool sample (D0) was stored at -80°C. Follow-up samples were collected at D5, D10, D17, D31, and D56 (rectal swab if no stool available). Qualitative cultures (vegetative forms/spores), free toxin detection, and detection of tcdB gene by PCR were performed on all samples.

Results: Sixty-eight patients were included: 9 treated with VAN, 59 with FDX. Recurrence rates were comparable: 11% (VAN) vs 12% (FDX). During treatment, vegetative forms persisted at D5 (25% under VAN vs 11% under FDX) and cleared by D10 in both groups. Spore persistence was more pronounced under FDX (37% at D5, 45% at D10) vs under VAN (0% at D5 and D10). After treatment discontinuation, secondary recolonization by vegetative forms was significantly more frequent under VAN than FDX at D17 (75% vs 9%) and D31 (50% vs 29%), and by spores at D17 (50% vs 22%). All patients tested positive for free toxins at D0 were negative by D10. PCR remained positive in 65% of patients at D5 and 52% at D17.

Conclusion: This study confirms persistent *C. difficile* carriage after treatment, with a significant rebound effect under vancomycin at D17, suggesting a differential impact of both molecules on recolonization dynamics. The rapid negativation of free toxins supports their potential value for early recurrence diagnosis, complementing current strategies.

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ABSENCE OF RESISTANCE TO ANTI-CDI ANTIBIOTICS IN PATIENTS WITH RECURRENT *Clostridioides difficile* INFECTION (CDI) IN THE NETHERLANDS

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Background and Aims: Decreased susceptibility and resistance of *C. difficile* to anti-CDI antibiotics has been reported in North America. We aimed to determine resistance to anti-CDI antibiotics in (1) patients with recurrent CDI (rCDI) identified through the Dutch sentinel surveillance (2023-2024) and (2) patients with multiple rCDI treated with fecal microbiota transplantation (FMT) using donor feces from the Netherlands Donor Feces Bank (2016-2021).

Methods: For both cohorts previous CDI episodes were documented. Data regarding prior antibiotic use was available for the FMT cohort only. Available isolates were tested with EUCAST recommended agar dilution for antibiotic susceptibility to metronidazole, vancomycin and fidaxomicin in the surveillance cohort, and vancomycin and fidaxomicin in the FMT cohort. Surveillance cohort isolates were sequenced (Illumina short read 150bp pair-end) and after *de novo* assembly (Velvet) CARD/resfinder/MEGARES/AMRfinder+ databased were used to identify resistance determinants, while *rpoB* and *rpoC* loci were manually inspected (aligned to CD630).

Results: The 2023-2024 surveillance included 539 CDI cases (5 medical centers), of which 81 represented rCDI episodes. Sixty isolates were available for susceptibility testing and 62 for whole genome sequencing (WGS). Between 2016-2021, 217 patients were treated with FMT. Isolates were available for 42 patients (mean 4,1 CDI episodes). Among FMT recipients, prior exposure to oral metronidazole (79%), vancomycin (88%) and fidaxomicin (38%) was common. MIC90 for the surveillance cohort was 0,25 mg/L (range 0,0625-0,25) for metronidazole, 2 mg/L (1-2) for vancomycin and 0,25 mg/L (0,0625-0,5) for fidaxomicin. For the FMT cohort MIC90 was 1,0 mg/L (1,0-2,0) for vancomycin and 0,5 mg/L (0,06-0,5) for fidaxomicin. No resistant isolates were identified (EUCAST breakpoints). WGS identified a *PnimB*^{G} mutation in one isolate and seven distinct *rpoB/rpoC* non-synonymous mutations in 29 isolates. None of these showed reduced susceptibility to fidaxomicin.

Conclusions: No resistance to metronidazole, vancomycin and fidaxomicin was identified in isolates of Dutch patients with rCDI, despite frequent prior exposure in the FMT cohort. While *rpoB* or *rpoC* variants were observed in nearly half of surveillance isolates, their relevance remains unclear, given all isolates were susceptible to fidaxomicin.

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Clostridioides difficile IMMUNITY DURING PREGNANCY AND PASSIVE ANTIBODY TRANSFER TO NEONATES FROM CORD BLOOD AND BREAST MILK

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Background and Aims: Infants are frequently exposed to *Clostridioides difficile* (Cd) and colonized in early life, but they rarely develop symptomatic disease. Although mechanisms underlying this infancy paradox remain poorly understood, maternal immunity transferred during pregnancy and breastfeeding may contribute to neonatal protection. This study aimed to characterize maternal humoral immunity to Cd toxins and some well-known colonization-associated surface proteins, and to investigate the transplacental and breast milk transfer of antigen-specific antibodies to their neonates.

Methods: We conducted a prospective observational study enrolling 58 healthy mother-neonate dyads. Cd carriage was systematically investigated in maternal stools collected during the peripartum period and in stools of their newborns. Antibody responses (IgM, IgG, IgA) against Cd toxins TcdA and TcdB, and the surface proteins FliD and Cwp84, were quantified using a standardized quantitative ELISA. Samples included maternal serum, cord blood collected at delivery, and breast milk collected during early lactation. Maternal antibody profiles were also compared with those of age-matched healthy non-pregnant women to assess the impact of pregnancy on Cd-specific humoral immunity.

Results: We reported a high seroprevalence of IgG specific to the four antigens in healthy pregnant women, regardless of Cd carriage status. Compared with non-pregnant women, pregnant women showed significantly lower concentrations of TcdA-specific IgM and IgG, while responses to TcdB and surface proteins were comparable. Cord blood samples contained high levels of IgG for all four antigens, with strong correlations between maternal and cord blood IgG concentrations, suggesting a transplacental transfer of Cd-specific IgG antibodies to neonates. In breast milk, a high seroprevalence of IgA specific to the two toxins was detected, and positive correlations between maternal serum and breast milk antibody levels highlight a preferential transfer of TcdB- and Cwp84-specific IgG to breast milk.

Conclusions: Pregnant women exhibit highly prevalent humoral immunity to Cd, with antigen-specific antibodies efficiently transferred to neonates via both the placenta and breast milk. Maternal antibody transfer, particularly against TcdB and surface proteins, may contribute to passive antibody-mediated protection of infants from Cd-associated disease despite frequent early-life colonization. Nevertheless, larger studies exploring immune factors involved in protection from CDI during pregnancy and infancy are needed.

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HIGH SEROPREVALENCE OF ANTI-TOXIN ANTIBODIES TO *Clostridioides difficile*: A MULTICENTER PROSPECTIVE STUDY ACROSS THREE FRENCH REGIONS

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Background and Aims: Population-level data on seroprevalence of antibodies directed against *Clostridioides difficile* (Cd) toxins remain scarce and largely outdated. Early work (Viscidi et al., 1983) demonstrated frequent seropositivity in adults and children, but relied on qualitative assays with limited sensitivity and no quantitative resolution. Modern weight-based ELISA now enables precise measurement of antibody concentrations across populations. This study aimed to quantify anti-toxin IgG responses across three French regions, comparing age groups, sex, and community versus hospitalized individuals, and to identify profiles potentially associated with lower antibody levels.

Methods:PREVADIFF is a prospective multicenter study conducted across nine centers in three French regions (Île-de-France, Hauts-de-France, PACA), including acute care medicine units, rehabilitation and continuing care wards, and French Blood Service collection sites. Clinical and demographic data were collected for all participants. Serum concentrations of anti-TcdA and anti-TcdB IgG were measured by quantitative ELISA (positivity threshold: 0.1 mg/L) in 550 subjects (330 community-based, 220 hospitalized).

Results: Seroprevalence of anti-Cd toxin IgG was near-universal across all populations. Significant interregional differences were observed for both anti-TcdA and anti-TcdB levels ($p < 0.01$): mean anti-TcdA concentrations were 117.0 µg/mL (95% CI: 84.8 - 149.3) in Hauts-de-France, 103.5 µg/mL (83.5 - 123.5) in PACA, and 53.9 µg/mL (29.6 - 78.3) in Île-de-France; anti-TcdB concentrations followed a similar gradient. Wide interindividual variability was noted throughout. Among hospitalized individuals, IgG levels increased with age. Hospitalized subjects exhibited higher antibody concentrations than community-based individuals, particularly for anti-TcdB. Anti-TcdA and anti-TcdB levels were significantly correlated.

Conclusions: This study demonstrates near-universal seroprevalence of anti-Cd toxin antibodies in the French population, with marked interindividual variability and significant differences across regions, age groups, and population categories. Future analyses will investigate additional determinants — including length of hospital stay — to refine exposure patterns and identify groups with comparatively lower antibody profiles.

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FUNCTIONAL PROFILING OF MICROBIOME RECOVERY REVEALS PATIENT-SPECIFIC RESTORATION OF COLONIZATION RESISTANCE TO *Clostridioides difficile*

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**Share Equal Responsibility in this work*

Background and aims: Fecal microbiota transplantation (FMT) is an effective treatment for recurrent *Clostridioides difficile* infection (CDI). However, the key microbial interactions underlying microbiota-mediated colonization resistance remain poorly understood, limiting the rational development of defined, scalable live biotherapeutic products as alternatives to FMT. Most patients with primary CDI achieve clinical cure following first-line antibiotic therapy, indicating that spontaneous restoration of colonization resistance is generally robust. We hypothesized that identifying and experimentally recapitulating the microbial taxa and functions associated with spontaneous recovery of colonization resistance could inform the rational design of functionally representative and potentially therapeutic bacterial communities.

Methods: We longitudinally profiled CDI patients treated with fidaxomicin or vancomycin and functionally interrogated post-therapy microbiomes using humanized gnotobiotic mice, in which cryopreserved patient-derived microbial communities were reproducibly engrafted into germ-free recipients. Engrafted communities clustered primarily by donor and exhibited low inter-mouse variability, enabling replicated functional phenotyping of *C. difficile* colonization resistance. Microbiota composition and functional potential were analyzed in relation to colonization resistance phenotypes.

Results: Post-therapy microbiomes conferred a broad spectrum of colonization resistance, ranging from minimal to complete suppression of *C. difficile* colonization, with post-fidaxomicin communities tending toward greater resilience. Resistance phenotypes were highly donor-specific and reproducible across replicate mice, and correlated with distinct taxonomic configurations.

Conclusions: Our study establishes a reproducible experimental platform to functionally dissect spontaneous microbiome recovery following CDI therapy and reveals patient-specific recovery trajectories underlying restoration of colonization resistance. These findings provide a framework for the mechanistically informed design of simplified microbial communities with defined colonization resistance functions.

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UNCOVERING *Clostridioides difficile* INFECTION RISK IN COVID-19 PATIENTS THROUGH PREDICTIVE MODELING

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Background and Aims: The COVID-19 pandemic was associated with significant antibiotic exposure and prolonged hospital stays, which possibly contributed to the increased incidence of *Clostridioides difficile* infection (CDI). Early identification of patients in increased risk of CDI remains challenging, especially in heterogeneous populations and changing epidemiology of this infection. The aim of this study was to evaluate predictive modelling methods for the onset of CDI in patients hospitalized with COVID-19 and to find clinically relevant risk factors for developing an infection.

Methods: This retrospective, single-center study was performed among 3848 adult patients hospitalized with COVID-19 at the University Hospital L. Pasteur in Košice, Slovakia, between 2020 and 2024. Several machine learning approaches for CDI risk prediction were developed and evaluated, including XGBoost, logistic regression, Random Forest and multilayer perceptron neural network models. The prevalence of CDI in the analyzed cohort was 2.68%. The developed system was then tested on an independent cohort of patients that were not included in the model training before.

Results: Patients who developed CDI were generally older and had a higher burden of comorbidities compared with CDI-negative patients. Validation of the developed application on an independent cohort of previously unseen patients comprising 15 CDI-positive and 15 CDI-negative cases, demonstrated correct classification in 27 out of 30 patients (90.0%). Three CDI-positive cases were misclassified as low-risk cases, predominantly due to lower age. Individual risk estimation and explainability analysis were done for each patient to identify parameters with the highest impact on the final prediction. In general, elevated inflammatory markers and exposure to antibiotics were identified as the strongest predictors of CDI development.

Conclusions: Machine learning models may be a useful tool for identifying hospitalized patients at increased risk of Clostridioides difficile infection. The study was conducted in patients hospitalized with COVID-19, and the proposed approach could potentially be applicable to other patient populations and could provide guidance for future clinical decision support systems and antimicrobial stewardship strategies. The main limitations of the study were the low prevalence of CDI in the cohort and the difficulty of obtaining follow-up data on patients treated for CDI outside the study hospital.

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PROGNOSTIC SIGNIFICANCE OF LEUKOCYTE DIFFERENTIAL COUNTS IN *Clostridioides difficile* INFECTION

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Background and Aims: *Clostridioides difficile* infection (CDI) continues to be a significant cause of healthcare-associated morbidity and mortality, particularly in elderly and hospitalized patients. The early identification of patients in increased risk of severe disease and poor outcome remains a significant clinical challenge. Regularly performed laboratory parameters may serve as a simple and easily available prognostic tool. The aim of this study was to evaluate the association between leukocyte differential count parameters and 90-day mortality in CDI patients.

Methods: We retrospectively evaluated 165 patients diagnosed with CDI during the hospitalization at the Department of Infectology and Travel Medicine in Košice, Slovakia. The laboratory results were collected in the shortest possible time following CDI diagnosis, within 48 hours before or after a positive *C. difficile* test. The parameters analyzed subsequently were total count of leucocytes (WBC), absolute count of neutrophils, lymphocytes, eosinophils and neutrophil to lymphocyte ratio (NLR). Patients at risk were stratified according to 90-day mortality.

Results: Most of the patients were aged ≥ 65 years with a median age of 70 years. The 90-day mortality was 40.0%. Patients with 90-day mortality were significantly older (median age 79 vs. 64 years; $p < 0.001$). We observed significantly higher total leukocyte counts among patients who died within 90 days, including elevated WBC (median 16.94 versus 11.37 $\times 10^9$/l; $p < 0.001$), absolute neutrophil counts (13.97 versus 7.85 $\times 10^9$/l; $p < 0.001$), and neutrophil-to-lymphocyte ratio (NLR) (16.52 versus 5.79; $p < 0.001$). On the other hand, non-survivors had lower absolute lymphocyte (1.03 vs 1.21 $\times 10^9$/l; $p = 0.008$) and eosinophil counts (0.02 vs 0.07 $\times 10^9$/l; $p < 0.001$). The optimal cut off value of NLR for predicting mortality was 9.13.

Conclusions: The parameters of the leukocyte differential count, especially NLR, neutrophilia and eosinopenia, are strongly associated with mortality in patients with CDI. These routinely available laboratory markers may have a role in early risk stratification and identification of patients requiring intensified monitoring and management.

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SUBLINGUAL IMMUNISATION AGAINST *C. difficile* ENHANCES INTESTINAL IGA WHILE SUBCUTANEOUS IMMUNISATION BOOSTS SERUM IGG

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Background and Aims: There are no licensed vaccines against *C. difficile*. Vaccine clinical trials have faced setbacks. In all the past trials, the vaccines were injected and targeted *C. difficile* toxins. New approaches are needed. We hypothesised that vaccines which induce mucosal immunity in the large intestine (site of *C. difficile* colonisation) could prevent the infection taking place in the first instance. Given that mucosal immunity is induced to a much greater extent when antigens are administered at mucosal surfaces (rather than when they are injected), we investigated sublingual immunisation. We selected the pathogen's surface-associated proteins, FliC and Cwp84, as antigens. These two proteins have previously shown promise when given orally in a hamster challenge model. In contrast to the oral route, the sublingual route avoids the pitfalls of the stomach acid and gastrointestinal enzymes which can denature and digest the antigens respectively. Our aims were to determine: (i) whether sublingual immunisation elicits antigen-specific systemic and mucosal antibody responses to Cwp84 and FliC, and (ii) whether a thermoresponsive gel elicits higher antigen-specific systemic and mucosal antibody responses compared to a liquid vaccine formulation. Theoretically, the gel could result in enhanced immune responses by prolonging the vaccine's residence under the tongue which could lead to increased antigen uptake by local immune cells.

Methods: Antigens and adjuvants were formulated in sublingual and control subcutaneous preparations and immunogenicity was assessed in a mouse model, by measuring the antigen-specific antibody levels in serum, small and large intestines, and nasal wash. Sublingual preparations included liquids which gelled when placed under the tongue, and those which did not.

Results: Sublingual immunisation, especially with the gel formulation, elicited higher mucosal antigen-specific IgA while subcutaneous immunisation elicited higher antigen-specific serum IgG levels. For example, for Cwp84, the geometric mean IgA titres in the large intestine were 45,264 for sublingual gel, 9,667 for the sublingual liquid and 7,133 for the subcutaneous formulation.

Conclusions: Sublingual immunisation, using a suitable vaccine formulation and the pathogen's surface antigens, is promising and deserves further investigation.

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BIOVIGILANCE IN FAECAL MICROBIOTA TRANSPLANTATION: 7-YEAR COHORT STUDY AND FRAMEWORK FOR MICROBIOLOGICAL ASSESSMENTS OF INFECTIOUS ADVERSE EVENTS

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Background and Aims: Faecal microbiota transplantation (FMT) is increasingly used. However, no systematic approach exists to assess infectious risks after FMT, leading to underreporting. We evaluated infectious complications at the Netherlands Donor Feces Bank (NDFB) and proposed a structured biovigilance approach aligned with the EU Regulation on Substances of Human Origin (SoHO).

Methods: We conducted a prospective observational cohort study of all patients receiving frozen donor faecal suspensions from the NDFB (May 2016–December 2023) for recurrent *Clostridioides difficile* infection (rCDI) or via an extended access programme (EAP) for non-CDI indications. (Serious) adverse events ((S)AEs) were reported by physicians and recorded at 3 weeks, 3 months, and 6 months. Suspected infectious events underwent predefined microbiological investigation, including donor–recipient traceability and whole-genome sequencing when indicated. Serious adverse reactions (SARs) were defined as SAEs probably or definitely related to FMT.

Results: We included 290 rCDI patients (322 FMTs) and 35 EAP patients (75 FMTs). FMT efficacy was favourable overall: 92% of rCDI patients remained free of rCDI within 8 weeks, and 49% of EAP patients achieved at least a partial response. Sixty-one per cent of rCDI patients and 34% of EAP patients had mild gastrointestinal AEs. AE incidences were comparable across groups (rCDI: 5.1 vs. EAP: 4.4 per 100 patient–weeks). SAEs were more frequent in EAP patients (0.66 vs. 0.28 per patient), reflecting higher immunosuppression and comorbidity. FMT-attributable SARs occurred after 6 of 322 FMTs (1.9%) in the rCDI group and after 3 of 75 FMTs (4.0%) in the EAP group. Two donor-derived Escherichia coli infections in EAP patients with

predisposing conditions were confirmed, consistent with early donor–strain colonisation rather than a direct FMT effect. Of 15 suspected infectious events investigated, only these two were confirmed as donor-derived. Most SAEs were unrelated.

Conclusions: In this 7-year cohort, donor-derived infections were rare but present, particularly in non-CDI patients with substantial comorbidity, whereas overall safety remained favourable. A SoHO-compliant biovigilance protocol incorporating microbiological investigation and donor/sample traceability is essential for the safe clinical use of FMT and faecal microbiota products.

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DO CDI OUTCOMES IMPROVE AFTER THE INTRODUCTION OF A FMT STOOL BANK: A SINGLE CENTER RETROSPECTIVE COHORT STUDY

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Background and Aims: Fecal microbiota transplantation (FMT) is a safe and effective treatment for *Clostridioides difficile* infection (CDI) and is mostly used to cure recurrent disease. Still, use of FMT is hampered by low general availability and practical difficulties. At our clinic, a stool bank with donated, tested and prepared feces was established in February 2016, replacing patient recruited donors. Here, we evaluate whether outcomes for patients with CDI have improved after the introduction of a local stool bank.

Methods: This is a retrospective cohort study, including all patients treated for CDI at the Department of Infectious diseases, Umeå University Hospital, Sweden, from 2008 to 2024. The study period was divided into the time before (Period 1) and after (Period 2) introduction of the stool bank. CDI cases were defined in accordance with ESCMID 2021 guidelines for CDI. Clinical data was retrieved from the electronical medical records. The primary outcome was the number of treatment episodes before cure. Key secondary outcomes included the number of CDI-attributable nights in hospital, and outpatient visits related to CDI.

Results: 488 CDI cases from 452 individual patients with 751 treatment episodes were included in the study. The median age was 75,5 years. 53,5% were women. 52,7% of the cases occurred during Period 2. Charlson comorbidity index, age and sex distribution was similar between periods, while an immunosuppressed state and use of proton pump inhibitors were significantly higher in Period 2. Although fulminant CDI was rare overall, it was more common in Period 2 (20 vs 11 cases). During Period 2, FMT was used more frequently as well as earlier in the course of the disease for each patient. Significantly fewer treatment episodes were required to reach cure and the number of hospital nights per CDI case decreased in Period 2, compared to Period 1. The number of CDI-related outpatient visits increased in Period 2, likely because of outpatient FMT treatments.

Conclusions: Our study indicates that having access to a stool bank can improve outcomes for patients with CDI. Further results and analyses will be presented.

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INGESTION OF DEODORIZED DOUBLE-WASHED ALCOHOL SHOCKED GUT MICROBES WITHOUT ENCAPSULATION OR BOWEL PREPARATION AS FMT FOR YOUNG PEDIATRIC PATIENTS WITH MULTIPLE RECURRENT *C. difficile* INFECTION

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Background and Aims: Young pediatric patients who have multiple recurrent *C. difficile* infection present challenges providing FMT. Patients who are aged 2-5 years in particular are not able to swallow capsules containing gut microbes. Bowel preps, retention enemas and colonoscopic delivery of fecal slurries are logistical and patient tolerance challenges. An alternative approach to provide FMT in this needed. An oral liquid microbial preparation that provides Bacillota microbes, is culture negative for pathobionts, deodorized to sidestep objectionable smell and taste was formulated.

Methods: Feces from a healthy donor is processed to separate gut microbes from fecal matter via serial dilution, centrifugation and sedimentation. The microbial pellet is resuspended and incubated 1:1 vol ratio in 3% phenylethyl alcohol: 97% ethyl alcohol x 1 h, followed by two serial washings with dH2O, retrieving the final pellet which has a faint rose petal odor. BAP and MacConkey agar quantitative cultures verified absence of Enterobacteriaceae, streptococci and staphylococci. Anaerobe cultures retrieved counts of Bacillota organisms ~10^{6-7} /ml, dispensed as 10 or 15 ml dH2O 10% glycerol, 15 ml conical test tubes, stored at -80° C. FMT protocol: 24 hr after completion of a 10-14 day course of vancomycin, patients ingested 10-15 ml per day in drinks or food for 9 consecutive days, and were followed for recurrence for 3 months. 16s and bile salt profiles during follow up will be compared with results from fecal samples obtained from adult rCDI patients undergoing full spectrum and ethyl alcohol shock capsular FMT.

Results: ***Case 1*** is 23 month old M with AML diagnosed at 10 months age, remission 16 mo., acquired CDI at age 13 mo. with 5 rCDI episodes over 10 months, maintained on metronidazole due to vancomycin allergy ***Case 2*** is 3 yr and 10 months Trisomy 21, Hirschsprung's disease resected, anorectal malformation. rCDI of 1 year duration with 6 episodes. Case 3 is aged 5 years with medulloblastoma, shunted hydrocephalus and 1 year duration of rCDI x 6. ***Outcomes***: All patients recovered uneventfully. Parents found the preparation was well tolerated and easily disguised.

Conclusion: For young pediatric patients with recurrent CDI, oral ingestion of deodorized washed gut microbes without encapsulation or bowel preparation is an effective FMT method.

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Faecalibacterium prausnitzii EXL-01 MODULATES *Clostridioides difficile* GROWTH *IN VITRO*

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Clostridioides difficile is responsible for 15-25% of cases of antibiotic-associated bacterial diarrhea and is considered the leading cause of healthcare-associated diarrhea in adults. The risk of recurrence is very high and problematic (about 20%). Treatment of *C. difficile* infection (CDI) is mainly based on antibiotics, but other promising approaches aimed at preventing CDI are being evaluated, such as the use of probiotics. In this study, we tested the *Faecalibacterium prausnitzii* EXL-01 strain as a probiotic. *F. prausnitzii* is the most abundant bacterium in the human intestinal microbiota of healthy adults, representing more than 5% of the total bacterial population. A decrease in *F. prausnitzii* abundance has been linked to dysbiosis in several human disorders. This abundant and ubiquitous bacterium could play a major role in new preventive and curative strategies for CDI management. Although there is increasing evidence that *F. prausnitzii* is an important regulator of intestinal homeostasis, data regarding its *in vitro* effects on *C. difficile* remain limited. Our aim was to test the impact of *F. prausnitzii* EXL-01 strain on *C. difficile*. To assess *F. prausnitzii* EXL-01 effects on *C. difficile in vitro*, we monitored the growth of *C. difficile* R20291 strain with or without *F. prausnitzii* EXL-01 supernatant and pellet for 24 h in planktonic culture and for 48 h for biofilm production. Biofilm biomass was quantified after staining with crystal violet. We also evaluated whether *F. prausnitzii* EXL-01 could impact *C. difficile* R20291 sporulation after 72 h of incubation or germination after 1 h.

Compared to controls, *F. prausnitzii* EXL-01 supernatant significantly disrupted the *C. difficile* R20291 growth kinetics, and both supernatant and pellet significantly reduced biofilm formation. *F. prausnitzii* EXL-01 supernatant had a significant impact on sporulation and no significant effect on germination. Together, these results suggest that *F. prausnitzii* EXL-01 has an impact on *C. difficile* growth, biofilm formation, and sporulation. Further investigations into the composition of the supernatant and the bacterial surface could shed light on these results and enable us to determine whether *F. prausnitzii* EXL-01 could be a good candidate as a next-generation probiotic to reduce the impact of CDI.

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INDIVIDUAL VS POOLED GUT MICROBIOME RESPONSES TO SIMULATED *C. difficile* PCR RIBOTYPE 955 INFECTION

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Clostridioides difficile infection (CDI) is associated with antibiotic-mediated dysbiosis and is a high-risk disease in healthcare settings. From 2021-2025, a virulent strain genetically close to *C. difficile* ribotype 027 caused an outbreak in the UK affecting 65 patients. Classified as PCR ribotype 955 (RT955), the strain shows multi-drug resistance to commonly prescribed antibiotics (e.g. moxifloxacin/clindamycin). Research is required to elucidate RT955-induced CDI disease dynamics. *In vitro* human gut models have been widely used to study CDI in pooled faecal samples however, their large size and low throughput hinders the study of individual microbiomes, making it difficult to stratify intervention responses. Using a novel triple-stage miniaturised gut model (MiGut) we investigated microbial dysbiosis and RT955 CDI risk in both individual and pooled microbiotas treated with different classes of antibiotics.

Twelve MiGut models were inoculated in triplicate with faecal slurries from 3 individual donors (>55 years) and a pooled slurry comprising all 3 faecal samples. Bacterial populations were allowed to stabilise before challenge with RT955 spores. Simulated CDI was induced by exposing each microbiota to either moxifloxacin, ceftriaxone or clindamycin. Microbiota and *C. difficile* dynamics were monitored thrice weekly for up to 44 days using bacterial culture, toxin testing, qPCR, 16S rRNA sequencing and bile acid metabolomic analysis.

CDI was observed in every pooled model investigated however; toxin production was delayed by ~3 days with lower titres when compared with individual donors. Declines in *Bifidobacterium spp.* and *Bacteroides spp.* following antibiotics were seen in both pooled and individual microbiomes, whilst Enterobacteriaceae levels increased in all models treated with ceftriaxone or clindamycin. Simulated CDI was successfully induced in all except two individual microbiotas after treatment with either ceftriaxone or moxifloxacin. Bile acid production will further our understanding of these individual responses.

Induction of simulated CDI was donor- and antibiotic-specific, highlighting the importance of stratified antimicrobial chemotherapy and the potential of single-donor *in vitro* models in revealing individual microbiota responses. This study showcases the potential of high-throughput chemostat models to investigate CDI treatment options.

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PRECLINICAL SAFETY PROFILE OF AJ-024, A NOVEL ORAL ANTIBIOTIC FOR *C. difficile* INFECTION

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The U.S. CDC identifies *Clostridioides difficile* as an urgent public health threat; however, available therapies remain limited. First-line standard-of-care antibiotics show a high recurrence rate (20-30%) with reduced activity against emerging hypervirulent ribotypes, including RT027 and RT078. These challenges underscore the need for next-generation antibiotics with potent antibacterial activity while minimizing disruption of the gut microbiome.

We have identified AJ-024, a novel thiopeptide antibiotic with potent activity against *C. difficile*. AJ-024 demonstrates superior in vitro antibacterial activity compared with vancomycin and fidaxomicin against a comprehensive collection of clinically isolated *C. difficile* strains. In addition, AJ-024 exhibits rapid bactericidal activity against the hypervirulent RT027 strain in a time-kill assay and a longer post-antibiotic effect than fidaxomicin up to 24hr.

The general toxicity of oral AJ-024 was assessed in acute and repeated dose, up to 28 days, in rats and dogs. For a single oral dose in rats and dogs, the maximum tolerated dose was determined to be 2000 mg/kg. In the repeated oral dose (once daily) for 28 days with 14-day recovery follow-up, the no-observed-adverse-effect level (NOAEL) was determined to be 1000 mg/kg/day in male/female rats and 500 mg/kg/day in male/female beagle dogs. The maximum serum concentrations (C_{max}) at NOAEL doses were 12.4 ng/ml at 6hr post-dose in rats and 54.4 ng/ml at 24hr post-dose in dogs. In a quantitative whole-body tissue distribution study of oral [^{14}C]AJ-024 (30mg/kg) in rats, radioactivity was largely confined to the gastrointestinal tract for up to 48hr, with minimal distribution to other tissues. Therefore, AJ-024 is well-tolerated in rats and dogs with limited systemic exposure following oral administration.

Currently, pharmaceutical techniques have been studied to develop the AJ-024 drug product for evaluation in the clinic. Collectively, these findings support continued development of AJ-024 as a promising therapeutic candidate for *C. difficile* infection and justify progression to a first-in-human Phase I trial to evaluate safety and tolerability.

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EVALUATING ASOS AS ANTIBACTERIAL AGENTS FOR TREATMENT OF *C. difficile* INFECTION

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Although antibiotic treatment is still the main therapeutic option for *C. difficile* infections (CDI), it frequently results in disease recurrence and further exacerbates dysbiosis of the gut microbiome. Therefore, strategies aimed at interfering specifically with *C. difficile* survival and pathogenicity are earnestly needed. Synthetic nucleic acid analogues have recently emerged as a promising new strategy to target bacterial pathogens in a sequence-specific manner. These antisense oligonucleotides (ASOs) enable selective targeting of a bacterial species of interest within a complex community through ASO-mediated mRNA repression. Our study aimed to systematically evaluate peptide nucleic acid (PNA) based ASOs targeted against essential and virulence-associated genes in *C. difficile*.

We screened a library of more than 100 peptides for their capacity to deliver cargo across the *C. difficile* cell envelope using flow cytometry and super-resolution microscopy. The three lead peptides were combined with PNA ASOs targeting the translation initiation region (TIR) of essential and virulence-related genes. Using growth curves and CFU counting, one promising peptide-PNA was selected for optimization and RNA-seq.

A 10-mer PNA targeting the start codon region of RNA polymerase subunit α (*rpoA*) coupled to the TAT peptide shows bactericidal activity against a variety of *C. difficile* isolates from all 5 genomic clades. Transcriptomic response of the model strain 630 exposed to peptide-PNA suggests a mechanism of action at the posttranscriptional level, consistent with current understanding of ASO based gene silencing in other bacteria.

In this study, we designed and characterized a PNA-based antisense oligonucleotide capable of effectively killing *C. difficile*. Considering the urgent need for alternatives to classic broad-spectrum antibiotics, novel ideas and approaches to antimicrobials are highly relevant. The ASO presented here represents a novel class of targeted antimicrobial therapeutics that could fill this need. This study marks a first step in systematically evaluating the future use of asobiotics in CDI treatment.

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AMPHIPHILIC CATIONIC COPOLYPEPTOIDS: NOVEL ANTIBACTERIAL AGENTS TARGETING *Clostridioides difficile*

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Only three antibiotics are currently marketed against CDI: vancomycin, fidaxomicin, and metronidazole, although the latter is no longer recommended as a first-line treatment for ICD. Moreover, these treatments do not prevent recurrent infections, (up to 25 % of patients after a primoinfection), which is a major issue of CDI. The limited antibiotic arsenal against *C. difficile* and the emergence of highly resistant strains highlight the critical need for the development of new antimicrobial agents. Antimicrobial peptides (AMPs), which are naturally occurring macromolecules made up of amino acids, have emerged as promising novel therapeutic candidates due to their broad-spectrum antibiotic activity. These cationic amphiphilic peptides present antimicrobial activity by membrane destabilization and are biodegradable, minimizing environmental persistence and reducing the risk of promoting antibiotic resistance. However, their clinical application is hindered by limitations such as degradation by enzymes when taken orally and potential systemic toxicity when administered intravenously. To expand our antibiotic arsenal, we generated polypeptoid polymers, a class of synthetic peptides based on N-substituted glycine backbones, produced via ring-opening polymerization (ROP) of N-alkyl-N-carboxyanhydride (NNCA) monomers, which offer an alternative approach to develop simplified orally active analogs of AMPs, overcoming their limitations. Synthesized polymer candidates have undergone screening, with one hit compound "TRO4" showing bactericidal activity with a narrow spectrum, elucidated mechanism of action and promising *in vivo* results after oral administration in infected mice. TRO4 is composed of 20 N-alkylated glycine units bearing benzylamine ("phenylalanine"-like) as hydrophobic side chains, butylamine ("lysine"-like) as cationic side chains, and a hexylamine moiety at the C_{ter} extremity. TRO4 presents effectiveness against vegetative form, spore and biofilm of *C. difficile*, significantly reducing intestinal persistence and environmental dissemination, which in turn lowers the recurrence rate of CDI.

P82

MICROBIOLOGICAL AND PHYSIOLOGICAL ANALYSIS OF FITNESS COSTS OF FIDAXOMICIN RESISTANCE IN *C. difficile*

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Background and Aims: Fidaxomicin is recommended for *C. difficile* infection, but resistance has emerged in clinical isolates and is linked to mutations in the RNA polymerase fidaxomicin-binding pocket, including RpoB Val1143. We examined how two clinical substitutions, Val1143Leu (MIC = 16 mg/L) and Val1143Asp (MIC = 128 mg/L) affect *C. difficile* physiology and virulence.

Methods: Paired fidaxomicin-susceptible and -resistant *C. difficile* isolates collected longitudinally from treated patients were analyzed. Whole-genome comparisons confirmed resistance-associated mutations. Resistant isolates were compared with paired susceptible counterparts using competitive fitness assays, sporulation and toxin measurements, mouse and hamster CDI models, and RNA-seq.

Results: Each resistant isolate differed from its susceptible counterpart by a single nonsynonymous RpoB Val1143 mutation. Both showed modest in vitro fitness reductions, but phenotypes differed by allele and strain background. Val1143Asp reduced sporulation, toxin production *in vitro* and *in vivo*, and virulence in mouse and hamster models. Val1143Leu retained sporulation and colonization but reduced toxin-associated disease severity in hamsters. RNA-seq showed broad remodeling in Val1143Asp, including RNAP-associated genes, Stickland fermentation, redox and energy metabolism, sporulation, and virulence pathways; Val1143Leu showed fewer transcriptional changes.

Conclusions: Clinically occurring RpoB Val1143 substitutions may mitigate fitness costs through physiological remodeling. Although Val1143Asp is among the more frequently reported clinical mutations, it markedly reduced virulence in two clinically relevant animal models. These findings suggest that Val1143 substitutions impose allele- and strain-dependent fitness costs that may influence which fidaxomicin-resistance mutations persist or become dominant in clinical settings.

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STRUCTURAL INSIGHTS INTO THE POTENCY, SELECTIVITY AND FITNESS COST OF RESISTANCE TO CRS3123, A CLINICAL CANDIDATE FOR TREATMENT OF *C. difficile* INFECTIONS

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Background and Aims: CRS3123 is a clinical candidate that exhibits 10^4- to 10^6-fold selectivity for *C. difficile* methionyl-tRNA synthetase1 (CdMetRS1) compared to MetRS2 orthologs that are prevalent in commensal gut flora. Phylogenetic distribution of the two orthologs provides a clear explanation for its narrow spectrum. CRS3123 demonstrated excellent efficacy and low recurrence in phase 2 trials. This study examines the structural basis for ortholog selectivity and the fitness cost of resistance.

Methods: CRS3123 inhibition was measured using purified CdMetRS1 and 3D structures were solved by Xray crystallography.

Results: CRS3123 exhibited strong synergy with ATP in inhibition studies of CdMetRS1 in marked contrast to published studies showing no effect of ATP on CRS3123 binding to MetRS2. To better understand the selectivity, potency, and mechanism of resistance to CRS3123, we solved 3D structures of CdMetRS1 bound to CRS3123 with and without ATP and also in its apo and Met-bound forms. Structural insights into ATP cooperativity include differentially conserved MetRS1 residues that stabilize the CSR3123-ATP-MetRS1 complex. The exceptional CRS3123 potency (K_i = 20pM) arises from hydrophobic interactions supported by key hydrogen bonds; the inhibitor occludes both the L-Met and tRNA acceptor arm binding sites and exhibits extensive shape complementarity with ATP to fully occupy the enzyme active site. Comparison of CdMetRS1 structures with published MetRS1 and MetRS2 structures supports a conformational selection model for CRS3123 binding and further illuminates the mechanisms driving MetRS1 selectivity. Mutant residues that confer elevated MICs result in substantial fitness costs. These cluster along a critical interface between the active site and the conformationally labile CP domain which plays a pivotal role in both substrate binding and catalysis, providing structural insights into the enzymatic impairment and growth fitness burden associated with resistance. Interestingly, spontaneous resistance isolates generally do not mutate toward MetRS2 sequences, emphasizing the wide evolutionary gap between the two orthologs.

Conclusions: These studies provide a strong structural rationale for the excellent potency and selectivity of CRS3123 and for better understanding the fitness constraints that limit emergence and persistence of resistance to CRS3123.

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GUT MATURATION STATE DIFFERENTIALLY INFLUENCES *Clostridioides difficile* TCS REPERTOIRE

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Clostridioides difficile is one of the leading causes of infectious diarrheal disease worldwide. The primary risk factor for *C. difficile* infection is antibiotic exposure, which perturbs the resident gut microbiota and reduces colonization resistance to this pathogen. Although *C. difficile* is the leading cause of hospital-acquired infections, recent data suggest that asymptomatic carriage of *C. difficile* is common in high-risk patients and asymptomatic carriers have a 6-fold increased risk of severe disease. Thus, suggesting that asymptomatic carriers are an underappreciated reservoir for *C. difficile*. Moreover, asymptomatic colonization may enable the pathogen to adapt and favor severe disease. Surprisingly, we know very little about the factors that shape pathogen fitness and virulence in the asymptomatic niche, and the mechanisms by which *C. difficile* responds to dynamic changes in the gut ecosystem have not been extensively explored.

To respond to environmental changes, bacteria utilize an elegant sensory mechanism known as two-component systems (TCSs), which consist of a membrane-embedded histidine kinase and a cytosolic response regulator. *C. difficile* encodes as many as 52 highly conserved TCSs, yet the role of these TCSs in controlling *C. difficile* fitness and virulence across pathogenic and carrier niches has not been explored. To address this, we have leveraged a novel murine model of neonatal asymptomatic carriage of *C. difficile* in comparison to acute disease in adult mice to investigate TCS activation repertoire across disease states. *We hypothesize that* C. difficile *alters its TCS repertoire in response to gut environmental factors that contrastingly impact pathogen fitness during carriage and acute disease*. We observe shifts in the expression of eight uncharacterized TCSs between neonates and adults, suggesting that TCSs may play a critical role in sensing these two divergent environments. Interactions between *C. difficile* and the resident gut microbiota are a major driver of *C. difficile* fitness in the gut ecosystem. We have previously shown that expansion of opportunistic pathogens, including enterococci, is associated with severe *C. difficile* infection in adults. We thus postulated that TCSs may be responsive to polymicrobial interactions in the gut ecosystem.

To begin investigating this, we interrogated the expression of these previously unidentified TCSs *in vitro* in the presence of *Enterococcus faecalis*. Indeed, co-culture with *E. faecalis* stimulates these novel TCSs, dependent on the growth phase. While no novel TCS conferred growth defects in rich media when knocked down in monoculture, knockdown of an adult-associated TCS using a CRISPRi system demonstrates significant susceptibility to detergent-induced autolysis. Thus, suggesting these TCSs are not essential for growth, but may be critical for dynamic responses to environmental changes and pathogen lifestyle shifts. Future directions include understanding the clinical relevance of *C. difficile* TCS repertoire, leveraging banked patient stool samples, as well as humanized mice to interrogate the role of TCSs on *C. difficile*-microbiota interactions. Further investigation into the role of these uncharacterized TCSs has the potential to broaden our understanding of *C. difficile* physiology and pathogenesis, with the potential to identify

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PREVALENCE OF *Clostridioides difficile* IN HOUSEHOLDS IN PERTH, WESTERN AUSTRALIA

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Background and Aims: *Clostridioides difficile* infection (CDI) has traditionally been associated with healthcare settings; however, community-associated CDI accounts for ~80% of CDI cases in Australia, including in individuals without conventional risk factors. The domestic environment may represent an under-investigated source/reservoir of *C. difficile* spores contributing to community transmission. This study aimed to determine the prevalence, distribution, toxin profiles, ribotype (RT) and antimicrobial susceptibility of *C. difficile* recovered from households in Perth, Western Australia.

Methods: A total of 183 household samples were collected from 15 homes across 14 suburbs of Perth between April and August 2021, including kitchen surfaces, toilets, refrigerators, entrances, vacuum dust, work shoe soles, chopping boards, root vegetables, companion animal-related sites and other surfaces. Sterile Polywipe sponges (Medical Wire & Equipment, UK) were used for sample collection. *C. difficile* was isolated following enrichment culture using a Don Whitley A35 anaerobic chamber. Presumptive isolates were confirmed using standard microbiological methods, followed by toxin gene profiling, PCR ribotyping and antimicrobial susceptibility testing against clinically relevant agents.

Results: *C. difficile* was recovered from 38/183 samples, an overall prevalence of 21%, with positives obtained from multiple sources, indicating widespread contamination. Seventeen RTs were identified, demonstrating substantial strain diversity. Toxigenic strains represented 32% of isolates. The most common RTs were 014/020 and 002, both associated with human CDI in Australia; 7 esculin hydrolysis-negative strains producing atypical white colonies on chromogenic agar were detected, highlighting diagnostic challenges. All isolates were susceptible to fidaxomicin, metronidazole, moxifloxacin and rifaximin; however, clindamycin resistance was common (66% of isolates).

Conclusions: This study demonstrates presence of *C. difficile* in household environments in Western Australia, with significant RTs. The recovery of antimicrobial-resistant strains and atypical colony phenotypes suggests that domestic environments may act as a source of *C. difficile* and contribute to the epidemiology of community-associated CDI. These findings support the need for broader One Health surveillance.

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PREVALENCE OF *Clostridioides difficile* IN MEAT AND MEAT PRODUCTS IN PERTH, WESTERN AUSTRALIA

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Background and Aims: *Clostridioides difficile* is a spore-forming enteric pathogen of increasing One Health importance. While *C. difficile* infection (CDI) is classically associated with healthcare settings, community-associated cases have focused attention on non-healthcare reservoirs/sources, including food animals, retail meat and domestic environments. Because *C. difficile* spores persist, meat and meat products may represent plausible but under-investigated exposure sources. This study investigated the prevalence, ribotype (RT) diversity, toxin profiles and antimicrobial susceptibility of *C. difficile* isolated from meat in Perth, Western Australia.

Methods: Between September 2020 and May 2022, 213 raw meat and meat products were collected from stores and butchers in nine Perth suburbs, including pork mince, piglet intestine, piglet meat, pig stomach, pig uterus, veal meat, veal intestine, beef mince, sausages, salami and other meat products. Samples were processed with enrichment and selective media using a Don Whitley A35 anaerobic chamber. Presumptive isolates were confirmed using toxin gene profiling, PCR ribotyping and antimicrobial susceptibility testing against selected agents.

Results: *C. difficile* was recovered from 10/213 meat samples, an overall prevalence of 5%. Samples containing *C. difficile* were identified from three of nine suburbs. Recovery was restricted to pork mince (4/27, 15%) and piglet intestine (6/64, 9%). Six PCR RTs were identified, demonstrating strain diversity despite low prevalence. Toxigenic strains accounted for 30% of isolates and RT014/020, commonly found in Australian pigs, represented 30% of meat isolates, highlighting a RT of clinical and One Health relevance. All isolates were susceptible to fidaxomicin, metronidazole, moxifloxacin and rifaximin. However, clindamycin resistance was frequent (80%) and multidrug resistance (MDR) was observed in one non-toxigenic piglet intestine isolate.

Conclusions: Retail meat and meat products in Perth, Western Australia can be contaminated with *C. difficile*, including toxigenic, RT-diverse and AMR strains. Although prevalence was low, detection suggests possible food-chain exposure potentially linked to contamination during slaughter or processing. Further surveillance and genomic comparison of food, animal, environmental and human CDI isolates is required to clarify transmission pathways.

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RILEY, Tom	Australia	INV2, P8, P22, P33, P34, P85, P86
ROSEIRO, Isabel	Portugal	OP6
ROSSI PACCANI, Silvia	Italy	
RUPNIK, Maja	Slovenia	P18
SAIKATENDU, Kumar	United States	P37
SALGADO, Paula	United Kingdom	P57, P58
SAVIDGE, Tor	United States	OP13, P5
SCHLEIFE, Laura	Austria	P19
SEGHROUCHNI BENT HOUSSINE, Imane	France	P39
SEPHIRI, Jwalane	South Africa	P29
SERGEANT, Madeline	France	
SEYBOLDT, Christian	Germany	INV5
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SHEN, Aimee	United States	
SHEN, Steven	Canada	P13, P14, P15, P21
SHEVCHUK, Veronika	Switzerland	
SHINDE, Vivek	United States	P37
SKINNER, Andrew	United States	OP14
SMIT, Floor	Netherlands	P35, P67
SMITS, Wiep Klaas	Netherlands	INV1, P35, P54, P62, P65, P67
SOUTOURINA, Olga	France	P39, P41, P59
STANLEY, Ann Marie	United States	OP10, P26, P27
TAUCHER, Christian	Austria	
TERVEER, Liz	Netherlands	P67, P74
TOMINC, Kaja	Slovenia	
TONGIANI, Serena	Italy	
UNNIKRISHNAN, Meera	United Kingdom	P55
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UZAL, Francisco A.	United States	P1
VAN IMSCHOOT, Leila	Belgium	P20
VAN PREHN, Joffrey	Netherlands	P35, P67, P74
VEHRESCHILD, Maria	Germany	INV7
VON EICHEL-STREIBER, Christoph	Germany	
WACKENREUTER, Janet	Germany	OP7
WAN, Wenbo	China	P24
WANG, Xianjun	China	P6
WEIDENFELLER, Christian	Switzerland	
WEISS, Ashley	United States	OP12
WEISS, Kayla	United States	P50
WILCOX, Mark	United Kingdom	OP2, OP17
WILLIS, Sarah	United States	OP1
WINGAARD THRANE, Sandra	Denmark	P48, P49
YU, Holly	United States	OP1
ZAMPARO, Joann	United States	
ZHABKO, Anastasiia	Ukraine	
ZHANG, YouYi	China	
ZHAO, Genming	China	P14, P15

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