Eravacycline, a novel tetracycline derivative, does not induce *Clostridioides difficile* infection in an *in vitro* human gut model

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Running title: Eravacycline does not induce simulated CDI

Abstract

Objectives. The approval of new antibiotics is essential to combat infections caused by antimicrobial resistant pathogens; however, such agents should be tested to determine their effect on the resident microbiota and propensity to select for opportunistic pathogens, such as *C. difficile*. Eravacycline is a new antibiotic for the treatment of complicated intra-abdominal infections. Here, we determined the effects of eravacycline compared with moxifloxacin on the microbiota and if these were conducive for induction of *C. difficile* infection.

Methods. We seeded *in vitro* chemostat models, which simulate the physiological conditions of the human colon, with a human faecal slurry and instilled gut reflective concentrations of either eravacycline or moxifloxacin.

Results. Eravacycline instillation was associated with decreased *Bifidobacterium*, *Lactobacillus* and *Clostridium* species which recovered 1 week after exposure. However, *Bacteroides* spp. levels decreased to below the limit of detection and did not recover prior to the end of the experiment. Post-eravacycline, a bloom of aerobic bacterial species occurred, including Enterobacteriaceae, compared with pre-antibiotic, which remained high for the duration of the experiment. These changes in microbiota were not associated with induction of CDI, as we observed a lack of *C. difficile* spore germination, thus no toxin was detected. Moxifloxacin exposure sufficiently disrupted the microbiota to induce simulated CDI, where *C. difficile* spore germination, outgrowth and toxin production was seen.

Conclusions. These model data suggest that, despite initial impact of eravacycline on the intestinal microbiota, similar to clinical trial data, this novel tetracycline has a low propensity to induce CDI.

Introduction

Our intestinal microbiota plays an essential role in homeostasis of the immune system and in preventing the expansion and colonisation of enteric pathogens, such as *Clostridioides difficile*, a term called colonisation resistance. If this colonisation resistance phenotype is disrupted, i.e. through the use of antibiotics, this provides nutrients and space for the expansion of pathogens and the progression of disease phenotypes. The impact of different antibiotics and prescription practices on our microbiota has contributed to a rise in *C. difficile* infection (CDI) cases and recurrent infections.[1,2] The development of new antimicrobials is essential; however, it is imperative to investigate the impact of new antimicrobials on the microbiota, specifically the propensity to induce CDI, which has the capacity to perpetuate the number of CDI cases. The spectrum of activity of an antibiotic does not correlate with the propensity of an antibiotic to induce CDI. For example, piperacillin/tazobactam is active against Gram-positive, Gram-negative and anaerobic bacteria but is considered low risk for CDI induction.[3,4] Conversely, 3rd generation cephalosporins have a similar spectrum of activity and some are considered high risk for CDI induction.[5–7]

C. difficile is the leading cause of infective antibiotic-associated diarrhoea and a significant cause of patient morbidity and mortality. The financial burden CDI cases place on healthcare systems are estimated to be €3 billion in Europe and $4.8 billion in USA.[8–10] Depletion of the microbiota allows the germination of ingested *C. difficile* spores to proliferate and produce toxins, TcdA and TcdB, that are responsible for the clinical manifestations of CDI.[11,12]

Eravacycline (previously known as TP-434) is a tetracycline-based, fully synthetic fluorocycline antibiotic that, like all tetracyclines, inhibits the protein elongation phase of protein synthesis by binding to the 16S ribosomal subunit to block attachment of aminoacyl tRNAs.[13] This antibiotic has recently gained Food and Drug Administration approval, and is under review by the European Medicines Agency, for treatment of complicated intra-abdominal infections.[14–16] Eravacycline has potent *in vitro* activity against aerobic and anaerobic Gram positive and Gram negative pathogens, including carbapenemase-producing Enterobacteriaceae, *Acinetobacter baumannii*, vancomycin-resistant Enterococci, *Staphylococcus aureus* (including methicillin resistant isolates), *Stenotrophomonas maltophilia* and *Bacteroides* spp.;[17,18] however, is not efficacious against *Pseudomonas aeruginosa.*[13]

Here, we used an *in vitro* human gut model to assess the impact of eravacycline on a healthy microbiota and the propensity to induce CDI, alongside a comparator antibiotic, moxifloxacin.[19] This model consists of three chemostat vessels arranged in a weir cascade fashion, where each vessel mimics the physiological conditions of the proximal to distal colon.[6] Our gut model has previously been used to study antibiotic predisposition to simulated CDI and the results correlate well with higher[5,20,21] and lower[19,22] clinical CDI risk.

Materials and methods

Gut model – gut model setup and ethics

Two triple-staged gut models were run in parallel and assembled to simulate CDI induction as previously described;[6,19] models were done in duplicate. Briefly, each model was composed of three chemostat vessels and maintained at physiological conditions; vessel 1 (pH 5.5 ± 0.1, 280 mL; proximal colon), vessel 2 (pH 6.2 ± 0.1, 300 mL; medial colon), and vessel 3 (pH 6.7 ± 0.1, 300 mL; distal colon). An anaerobic environment was maintained by sparging each vessel with nitrogen, and a complex growth medium connected to vessel 1 at a preestablished rate of 0.015 h^{-1}.[6]

Faecal samples from 5 healthy donors (aged ≥ 60 years old with no history of antimicrobial usage in previous 6 months) were individually screened for the presence of glutamine dehydrogenase (GDH), a *C. difficile* specific protein constitutively expressed, as determined by EIA C. DIFF CHEK™ 60 test (Techlab, U.S.). The age of participants who provided faecal donations were chosen to represent a common risk factor for CDI.[23] Each donor faecal sample was screened negative for *C. difficile* by EIA C. DIFF CHEK™. Following this, samples were pooled and diluted 1:10 with pre-reduced PBS. This slurry was filtered to remove large particulate matter. Each vessel, of each model, was seeded with ~160 ml of this slurry to start the experiment. A small aliquot of faecal slurry was kept anaerobically at 37 °C and the bacterial populations were enumerated (as described below). The collection and use of human faeces in our gut model has been approved by the School of Medicine Research Ethics Committee, University of Leeds (MREC 15-070 – Investigation of the Interplay between Commensal Intestinal Organisms and Pathogenic Bacteria). Participants were provided with a 'Participant Information Sheet' (PIS) detailing a lay summary of the *in vitro* gut model and the scientific work they are contributing to by providing a faecal donation. Within this PIS, it is explained that by providing the sample, the participant is giving informed consent for that sample to be used in the gut model.

Gut model – Experimental design

The experimental timeline for these models is depicted in **Figure 1a**. After addition of the faecal slurry, microbial populations were monitored for 14 days without further intervention to ensure the populations reach steady state. A 1 mL aliquot of *C. difficile* spores (10^7 spores/mL) strain 210 (BI/NAP1/PCR ribotype 027/toxinotype III)[24] was added to vessel 1 of each model. This was done to establish that the microbiota had formed colonisation resistance

against *C. difficile* germination. One week later, another dose of *C. difficile* spores was added to the model and the microbiota were disrupted with either eravacycline or moxifloxacin. Eravacycline was dosed at 15.4 mg/L per dose, twice daily for 7 days[25] and moxifloxacin was dosed at 43 mg/L per dose, once daily for 7 days.[19] These concentrations are reflective of the concentration found in the human colon. The recovery of the microbiota, and any subsequent induction of simulated CDI, were monitored post antibiotic exposure.

Preparation of C. difficile RT027 strain 210 spores

C. difficile spores for gut model inoculation were prepared as previously described.[26] Briefly, *C. difficile* RT027 was grown in BHI broth anaerobically at 37 °C for 6 days and removed from the incubator and incubated aerobically at room temperature overnight to further induce sporulation. Growth was harvested by centrifugation and incubated with PBS supplemented with 10 mg/mL lysozyme at 37 °C overnight. Samples were separated using a sucrose gradient and spores were treated with PBS supplemented with 20 ng/mL protease K and 200 nm EDTA. Spores were separated using a sucrose gradient and washed with PBS twice before a final resuspension in 30 mL. These were enumerated and diluted to approximately $1x10^7$ spores/mL for use in the models.

Enumeration of endogenous bacteria and quantification of C. difficile *toxin*

Gut microbiota populations were monitored using viable enumeration on selective and non-selective agars as described previously.[19] Microbial colonies were enumerated and identified based on colony morphology and MALDI-TOF identification. Each bacterial population was measured in triplicate (three technical replicates of a single biological replicate) in vessels 2 and 3. *C. difficile* total viable counts and spore counts were measured from all vessels; spore counts were obtained through plating serial dilutions of model fluid after alcohol shock. The limit of detection for either total viable counts or spores were 1.2 or l.5, respectively, $\log_{10}$ cfu/mL.

C. difficile cytotoxin was monitored using a semi-quantitative Vero cell cytotoxicity assay as described.[19] Cytotoxin titre was expressed as $\log_{10}$ relative units at the highest dilution with >70% cell rounding, i.e. 10^0, 1 RU; 10^1, 2 RU etc.

Antibiotic bioassays

The concentration of moxifloxacin in each vessel was determined by antibiotic bioassays as previously described.[27] To measure the concentration of eravacycline, a range of bacterial indicator organisms and different agars were tested to determine the optimum combination to

use. Using *Staphylococcus aureus* (ATCC 29213) and Mueller-Hinton agar to determine eravacycline concentrations in the vessels gave the lowest limit of detection of 0.9 mg/L.

Results

Established microbiota populations conferred colonisation resistance against C. difficile *spore germination*

Each set of duplicate gut models were seeded with a pool (n=5 individual donors; *C. difficile* negative) of human faecal slurry at the start of the experiment, and the microbial populations stabilised for two weeks prior to further intervention (**Figure 1a**). Bacterial enumeration within vessel 3 are presented in figures 1-3, as this vessel represents the distal colon, the most physiologically relevant for CDI. At this time, the recoveries of the bacterial populations present in each model were similar to those enumerated from the faecal slurry (**Table 1**); however, the recovery of *Lactobacillus* spp. from each model was higher compared with the faecal slurry, 7.16 and 5.28 $\log_{10}$ cfu/mL, respectively. Once the microbial populations had stabilised, enumeration of the individual bacterial populations were highly similar between the models (**Table 1**), with *Bacteroides* spp. showing the most variation (± 0.6 $\log_{10}$ cfu/mL). To determine if the established microbiota within each model conferred colonisation resistance against *C. difficile* spore germination, we exposed each model to 10^7 spore/mL and monitored for germination and outgrowth. Once added to the model, *C. difficile* cells remained in spore form, where no evidence of germination was seen. Formation of colonisation resistance against *C. difficile* spore germination in the models ensures that any deleterious effect on this phenotype is due to the administration of the antibiotic and not the stability of the microbiota.

Effects of eravacycline on the microbiota

Instillation of eravacycline had a pleotropic effect on the bacterial populations recovered in each vessel. A bioassay was used to determine the bioactive concentrations of eravacycline within each vessel over the course of antibiotic instillation. Peak concentration of eravacycline was 10.6, 9.4 and 5.7 mg/L, in vessels 1, 2 and 3 respectively, during antibiotic instillation (peaking between days 27 and 28). The levels of eravacycline were undetectable 4, 5 and 5 days after cessation of antibiotic instillation in vessels 1, 2 and 3, respectively.

These levels of eravacycline were associated with an overall decrease in the bacterial populations, with decreases of 1.8, 2.9 and 2.2 $\log_{10}$ cfu/mL observed in the obligate anaerobes, *Enterococcus* spp. and *Lactobacillus* spp. respectively (**Figure 1b**). *Clostridium* populations were depleted and only detectable as spores. More severe effects on *Bacteroides* spp. and *Bifidobacterium* spp. were observed, where bacterial numbers decreased to

undetectable levels during eravacycline dosing (**Figure 2a**). Post-eravacycline, a bloom of aerobic bacterial species occurred, including Enterobacteriaceae, compared with pre-antibiotic, which remained high for the duration of the experiment (**Figure 1b**). *Bifidobacterium* spp. and *Clostridium* spp. levels recovered once eravcycline bioactivity fell to undetectable levels in the model. *Bacteroides* spp. populations never recovered to pre-eravacycline levels and were only detected transiently until the end of the experiments (**Figure 2a**).

Effects of moxifloxacin on the microbiota

Instillation of moxifloxacin reached peak concentrations of 13.1, 16.9 and 16.2 mg/L in vessels 1, 2 and 3, respectively, during instillation (peaking after 3 days of antibiotic instillation). After which, the levels decreased over time and were undetectable in vessel 1, 2 and 3 by 4, 4 and 5 days, respectively, post cessation of antibiotic. Exposure of the microbial populations to moxifloxacin had a pleotropic effect on the microbial populations. Enumerated levels of Enterobacteriaceae, *Enterococcus* spp. and *Lactobacillus* spp. reduced by 5.0, 1.9 and 3.0 $\log_{10}$ cfu/mL, respectively during moxifloxacin instillation (**Figure 1c**). Several obligate anaerobic bacterial species were affected by moxifloxacin; *Bacteroides* spp. were reduced to undetectable levels, *Clostridium* spp. populations were only detectable as spores, whilst *Bifidobacterium* spp. decreased by 4.3 $\log_{10}$ cfu/mL (**Figure 2b**). Post-moxifloxacin, most bacterial populations recovered to pre-antibiotic levels, although *Bifidobacterium* spp. did not fully recover until 13 days after antibiotic exposure (**Figure 1c and 2b**).

Dynamics of C. difficile *populations and induction of CDI*

Prior to antibiotic instillation, *C. difficile* spores reached peak recoveries in vessel 3 between 4.3-4.4 $\log_{10}$ cfu/mL across both models, which decreased before increasing again after the second *C. difficile* dose (**Figure 3**). Moxifloxacin-induced microbiota disruption caused *C. difficile* spore germination two days after cessation of antibiotics, with proliferation and peak growth reaching 6.6 $\log_{10}$ cfu/mL 3 days later (day 32 of the model) in vessel 3 (**Figure 3 black solid line**). Simulated CDI in the model dosed with moxifloxacin was confirmed following detection of toxin activity 3 days after cessation of antibiotics. Toxin levels peaked at 5.5 $\log_{10}$ reciprocal titre 14 days after antibiotics. Following instillation of eravacycline, *C. difficile* spores remained quiescent for the duration of the experiment (16 days post-antibiotic), where no vegetative cells were detected, thus no toxin activity was detected as well (**Figure 3 – grey dotted line**). *C. difficile* spores gradually declined over time; however,

205 we still detected spores in the luminal phase of our model at levels approximately 2.6 $\log_{10}$
206 cfu/mL by the end of the model, 16 days post antibiotic instillation.
207

Discussion

Given the very limited development of new antibiotics and the challenge of multidrug resistant pathogens, eravacycline is a welcome additional agent, albeit at present limited to the indication of complicated intra-abdominal infections. However, the effects of this antibiotic on the intestinal microbiota, and potential risk for opportunistic hospital acquired infections, has not been established. Here we used an *in vitro* human gut model to assay the effects of clinically relevant concentrations of eravacycline on the microbiota and the propensity to induce simulated CDI. We have previously used our gut model to assess the impact of antimicrobials on the human microbiota and determined the risk of treatment to induce CDI. The microbiome present in each individual is unique, thus different faecal slurries can result in subtle responses to each antibiotic. We sought to overcome this limitation by using a pooled faecal slurry from 5 *C. difficile*-negative individuals, providing a broader range of microbiota present in the gut model, and two experimental replicates were performed.

In this study, eravacycline exposure caused reductions in many different bacterial populations, such as *Bacteroides* spp. and *Clostridium* spp., which is not surprising given its broad-spectrum activity.[17,18] Notably, however, despite the observed marked changes in bacterial populations, we did not observe *C. difficile* spore germination, pathogen outgrow or toxin production. Most of the bacterial populations sampled in this study had recovered by day 36, except for *Bacteroides* spp. *C. difficile* spore germination utilises environmental metabolic cues, such as the presence of glycine and abundance of primary bile acids. *Clostridium scindens* has been shown to metabolise primary bile acids into secondary bile acids, which act as an inhibitor of spore germination.[28] The recovery of different *Clostridium* species by day 36 could have depleted the primary bile acid pool, thus preventing *C. difficile* spore germination and CDI; however, other metabolic/environmental signals may contribute to CDI.[29] The metabolic activity of some *Bacteroides* spp. can enhance CDI. Succinate production by *B. thetaiotaomicron* can be utilised by *C. difficile* and enhances CDI progression.[30] Reduced recovery of *Bacteroides* after eravacycline exposure could eliminate this metabolite source, preventing succinate from being utilised by *C. difficile*.

The microbial disruption upon exposure to eravacycline observed here is similar to the effects seen with other closely related antibiotics, such as tigecycline[22] and omadacycline.[19] These tetracycline derivatives also did not induce CDI in the gut model. Conversely, in a separate control model, moxifloxacin instillation was followed by induction of CDI. Similar to

241 previous studies,[19,21] moxifloxacin created a niche in the microbiota whereby favourable
242 conditions for *C. difficile* germination and growth were observed two days post antibiotic
243 dosing. Enumerated *C. difficile* levels remained high and toxin activity was detected despite a
244 recovery in the enumerated microbial populations.

245 The lack of CDI induction seen in this gut model after eravacycline exposure suggests there
246 is a low risk of CDI induction *in vivo*. A very low risk of CDI after eravacycline treatment
247 was observed during clinical trials,[16,31,32] with no reported cases of CDI. In summary,
248 eravcycline, like other tetracycline-based antibiotics, appears to be low risk therapeutic for
249 CDI,[33] and so could be considered as part of an antimicrobial stewardship programme in
250 patients at increased risk of this iatrogenic complication.

251

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Transparency declarations

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Figure legends

Figure 1.

Schematic timeline of the *in vitro* triple stage chemostat gut model and experimental design for each model (**A**). *C. difficile* (CD) spores were added to each model (black lines) before addition of antibiotics (blue arrow). Facultative anaerobic microbial populations were monitored after exposure to either eravacycline (**B**) or moxifloxacin (**C**). In each graph, black lines are the total facultative anaerobic bacteria, red lines are the lactose-fermenting (LF) Enterobacteriaceae, green lines are the *Enterococcus* spp. and purple lines are the *Lactobacillus* spp. Results shown are mean $\log_{10}$ cfu/mL from three technical replicates. Limit of detection for this assay is 1.2 $\log_{10}$ cfu/mL.

Figure 2.

Dynamics of obligate anaerobic bacterial populations upon exposure to either eravacycline (**A**) or moxifloxacin (**B**). In each graph, black lines are the total bacteria, red lines are the *Bacteroides* spp., green lines are the *Clostridium* spp., purple lines are the *Bifidobacterium* spp., and orange lines are the total spore-formers. Results shown are mean $\log_{10}$ cfu/mL from three technical replicates. Limit of detection for this assay is 1.2 $\log_{10}$ cfu/mL.

Figure 3.

C. difficile induction and toxin production for vessel 3 of eravacycline (**grey**) and moxifloxacin (**black**). *C. difficile* spore populations are shown by a dotted line, but spore germination and vegetative cell growth are indicated by a solid line. Toxin production (arrows) was measured by cell toxicity assay – no toxin was detected from eravacycline model; thus, no arrow is visible.

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Table 1. Enumerated bacterial populations from the faecal slurry and within each model after reaching steady state.

Bacterial population	Faecal slurry	Models[b]	
		Eravacycline	Moxifloxacin
Total facultative anaerobes	7.42	7.01	7.12
LF *Enterobacteraceae*[a]	7.38	6.95	6.89
Enterococcus spp.	5.12	5.01	4.92
Total bacteria	9.32	8.94	8.85
Clostridium spp.	8.18	7.43	7.5
Bacteroides spp.	7.12	6.5	7.11
Bifidobacterium spp.	8.2	8.2	8.2
Lactobacillus spp.	5.28	7.26	7.06
Total spore-formers	4.89	4.5	4.34

[a] Lactose fermenting *Enterobacteriaceae*

[b] $\log_{10}$ cfu/mL value is from day 14 vessel 3 only

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393

Figure 1

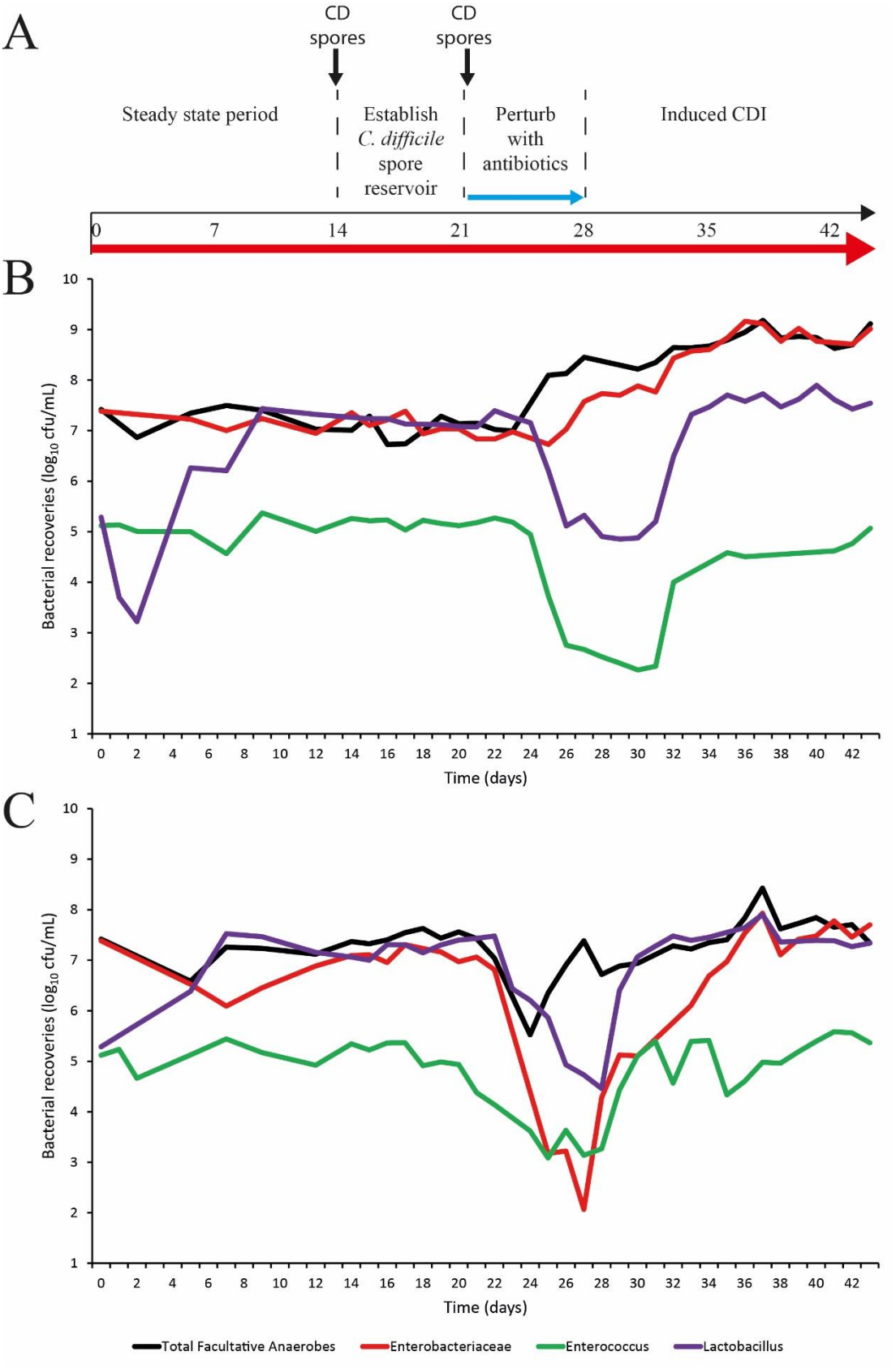
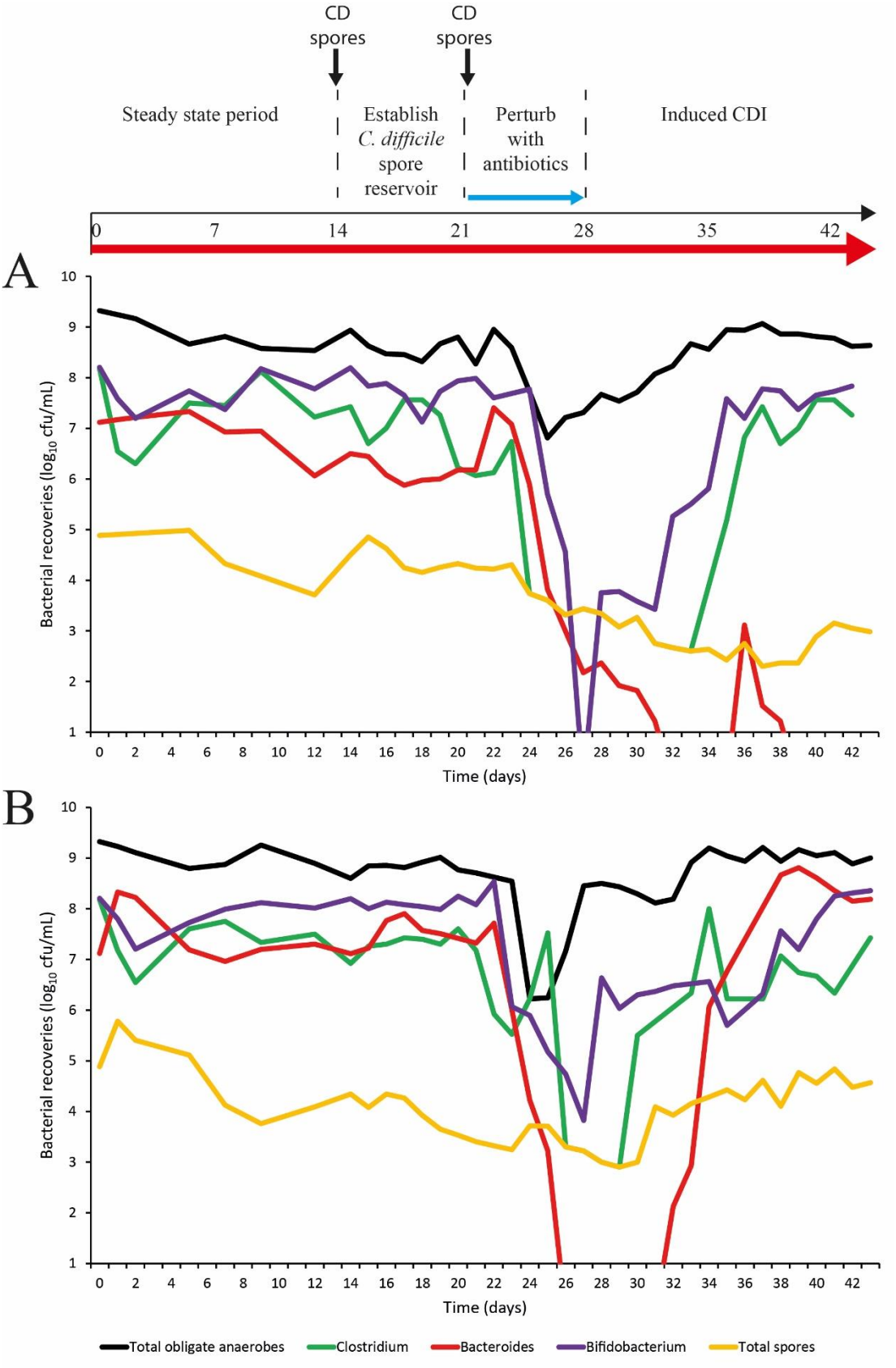
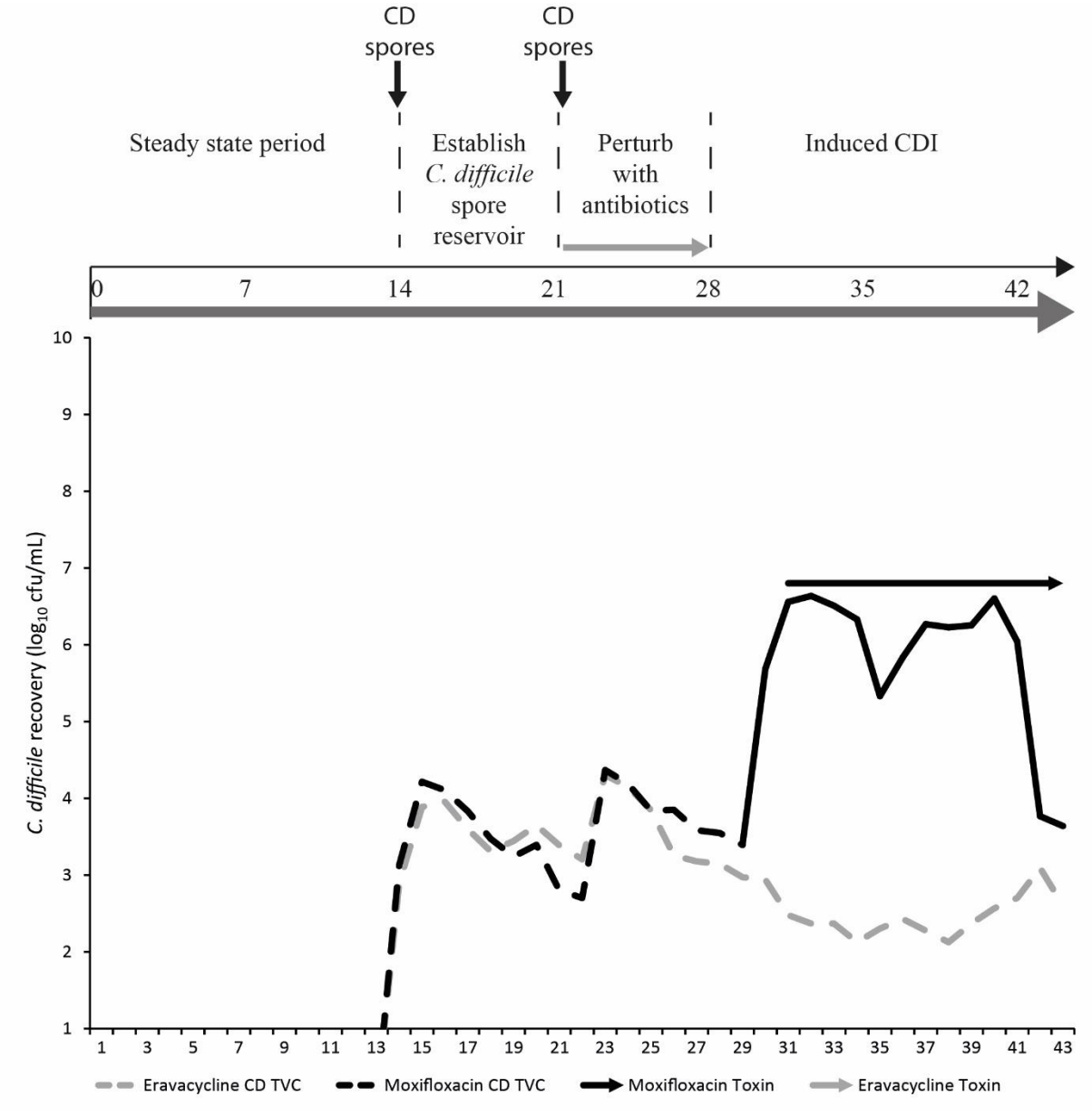

A
CD spores
CD spores
Steady state period
Establish C. difficile spore reservoir
Perturb with antibiotics
Induced CDI
0
7
14
21
28
35
42
B
Bacterial recoveries (log10 cfu/mL)
Time (days)
C
Bacterial recoveries (log10 cfu/mL)
Time (days)
Total Facultative Anaerobes
Enterobacteriaceae
Enterococcus
Lactobacillus

Figure 2


CD spores
CD spores
Steady state period
Establish C. difficile spore reservoir
Perturb with antibiotics
Induced CDI
0
7
14
21
28
35
42
A
B
Bacterial recoveries (log10 cfu/mL)
Time (days)
Total obligate anaerobes
Clostridium
Bacteroides
Bifidobacterium
Total spores

400 **Figure 3**



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