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

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11 Running title: Eravacycline does not induce simulated CDI

12

13 **Abstract**

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

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

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

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

35

36 **Introduction**

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

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

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

67 Here, we used an *in vitro* human gut model to assess the impact of eravacycline on a healthy
68 microbiota and the propensity to induce CDI, alongside a comparator antibiotic,
69 moxifloxacin.¹⁹ This model consists of three chemostat vessels arranged in a weir cascade
70 fashion, where each vessel mimics the physiological conditions of the proximal to distal
71 colon.⁶ Our gut model has previously been used to study antibiotic predisposition to
72 simulated CDI and the results correlate well with higher^{5,20,21} and lower^{19,22} clinical CDI risk.
73

74 **Materials and methods**

75 *Gut model – gut model setup and ethics*

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

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

100 *Gut model – Experimental design*

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

106 against *C. difficile* germination. One week later, another dose of *C. difficile* spores was added
107 to the model and the microbiota were disrupted with either eravacycline or moxifloxacin.
108 Eravacycline was dosed at 15.4 mg/L per dose, twice daily for 7 days²⁵ and moxifloxacin was
109 dosed at 43 mg/L per dose, once daily for 7 days.¹⁹ These concentrations are reflective of the
110 concentration found in the human colon. The recovery of the microbiota, and any subsequent
111 induction of simulated CDI, were monitored post antibiotic exposure.

112 *Preparation of C. difficile RT027 strain 210 spores*

113 *C. difficile* spores for gut model inoculation were prepared as previously described.²⁶ Briefly,
114 *C. difficile* RT027 was grown in BHI broth anaerobically at 37 °C for 6 days and removed
115 from the incubator and incubated aerobically at room temperature overnight to further induce
116 sporulation. Growth was harvested by centrifugation and incubated with PBS supplemented
117 with 10 mg/mL lysozyme at 37 °C overnight. Samples were separated using a sucrose
118 gradient and spores were treated with PBS supplemented with 20 ng/mL protease K and 200
119 nm EDTA. Spores were separated using a sucrose gradient and washed with PBS twice
120 before a final resuspension in 30 mL. These were enumerated and diluted to approximately
121 1×10^7 spores/mL for use in the models.

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

123 Gut microbiota populations were monitored using viable enumeration on selective and non-
124 selective agars as described previously.¹⁹ Microbial colonies were enumerated and identified
125 based on colony morphology and MALDI-TOF identification. Each bacterial population was
126 measured in triplicate (three technical replicates of a single biological replicate) in vessels 2
127 and 3. *C. difficile* total viable counts and spore counts were measured from all vessels; spore
128 counts were obtained through plating serial dilutions of model fluid after alcohol shock. The
129 limit of detection for either total viable counts or spores were 1.2 or 1.5, respectively, \log_{10}
130 cfu/mL.

131 *C. difficile* cytotoxin was monitored using a semi-quantitative Vero cell cytotoxicity assay as
132 described.¹⁹ Cytotoxin titre was expressed as \log_{10} relative units at the highest dilution with
133 >70% cell rounding, i.e. 10^0 , 1 RU; 10^1 , 2 RU etc.

134 *Antibiotic bioassays*

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

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

140

141 **Results**

142 *Established microbiota populations conferred colonisation resistance against C. difficile* 143 *spore germination*

144 Each set of duplicate gut models were seeded with a pool (n=5 individual donors; *C. difficile*
145 negative) of human faecal slurry at the start of the experiment, and the microbial populations
146 stabilised for two weeks prior to further intervention (**Figure 1a**). Bacterial enumeration
147 within vessel 3 are presented in figures 1-3, as this vessel represents the distal colon, the most
148 physiologically relevant for CDI. At this time, the recoveries of the bacterial populations
149 present in each model were similar to those enumerated from the faecal slurry (**Table 1**);
150 however, the recovery of *Lactobacillus* spp. from each model was higher compared with the
151 faecal slurry, 7.16 and 5.28 log₁₀ cfu/mL, respectively. Once the microbial populations had
152 stabilised, enumeration of the individual bacterial populations were highly similar between
153 the models (**Table 1**), with *Bacteroides* spp. showing the most variation (± 0.6 log₁₀ cfu/mL).
154 To determine if the established microbiota within each model conferred colonisation
155 resistance against *C. difficile* spore germination, we exposed each model to 10⁷ spore/mL and
156 monitored for germination and outgrowth. Once added to the model, *C. difficile* cells
157 remained in spore form, where no evidence of germination was seen. Formation of
158 colonisation resistance against *C. difficile* spore germination in the models ensures that any
159 deleterious effect on this phenotype is due to the administration of the antibiotic and not the
160 stability of the microbiota.

161 *Effects of eravacycline on the microbiota*

162 Instillation of eravacycline had a pleotropic effect on the bacterial populations recovered in
163 each vessel. A bioassay was used to determine the bioactive concentrations of eravacycline
164 within each vessel over the course of antibiotic instillation. Peak concentration of
165 eravacycline was 10.6, 9.4 and 5.7 mg/L, in vessels 1, 2 and 3 respectively, during antibiotic
166 instillation (peaking between days 27 and 28). The levels of eravacycline were undetectable
167 4, 5 and 5 days after cessation of antibiotic instillation in vessels 1, 2 and 3, respectively.

168 These levels of eravacycline were associated with an overall decrease in the bacterial
169 populations, with decreases of 1.8, 2.9 and 2.2 log₁₀ cfu/mL observed in the obligate
170 anaerobes, *Enterococcus* spp. and *Lactobacillus* spp. respectively (**Figure 1b**). *Clostridium*
171 populations were depleted and only detectable as spores. More severe effects on *Bacteroides*
172 spp. and *Bifidobacterium* spp. were observed, where bacterial numbers decreased to

173 undetectable levels during eravacycline dosing (**Figure 2a**). Post-eravacycline, a bloom of
174 aerobic bacterial species occurred, including Enterobacteriaceae, compared with pre-
175 antibiotic, which remained high for the duration of the experiment (**Figure 1b**).

176 *Bifidobacterium* spp. and *Clostridium* spp. levels recovered once eravacycline bioactivity fell
177 to undetectable levels in the model. *Bacteroides* spp. populations never recovered to pre-
178 eravacycline levels and were only detected transiently until the end of the experiments
179 (**Figure 2a**).

180 *Effects of moxifloxacin on the microbiota*

181 Instillation of moxifloxacin reached peak concentrations of 13.1, 16.9 and 16.2 mg/L in
182 vessels 1, 2 and 3, respectively, during instillation (peaking after 3 days of antibiotic
183 instillation). After which, the levels decreased over time and were undetectable in vessel 1, 2
184 and 3 by 4, 4 and 5 days, respectively, post cessation of antibiotic. Exposure of the microbial
185 populations to moxifloxacin had a pleotropic effect on the microbial populations. Enumerated
186 levels of Enterobacteriaceae, *Enterococcus* spp. and *Lactobacillus* spp. reduced by 5.0, 1.9
187 and 3.0 log₁₀ cfu/mL, respectively during moxifloxacin instillation (**Figure 1c**). Several
188 obligate anaerobic bacterial species were affected by moxifloxacin; *Bacteroides* spp. were
189 reduced to undetectable levels, *Clostridium* spp. populations were only detectable as spores,
190 whilst *Bifidobacterium* spp. decreased by 4.3 log₁₀ cfu/mL (**Figure 2b**). Post-moxifloxacin,
191 most bacterial populations recovered to pre-antibiotic levels, although *Bifidobacterium* spp.
192 did not fully recover until 13 days after antibiotic exposure (**Figure 1c and 2b**).

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

194 Prior to antibiotic instillation, *C. difficile* spores reached peak recoveries in vessel 3 between
195 4.3-4.4 log₁₀ cfu/mL across both models, which decreased before increasing again after the
196 second *C. difficile* dose (**Figure 3**). Moxifloxacin-induced microbiota disruption caused *C.*
197 *difficile* spore germination two days after cessation of antibiotics, with proliferation and peak
198 growth reaching 6.6 log₁₀ cfu/mL 3 days later (day 32 of the model) in vessel 3 (**Figure 3**
199 **black solid line**). Simulated CDI in the model dosed with moxifloxacin was confirmed
200 following detection of toxin activity 3 days after cessation of antibiotics. Toxin levels peaked
201 at 5.5 log₁₀ reciprocal titre 14 days after antibiotics. Following instillation of eravacycline, *C.*
202 *difficile* spores remained quiescent for the duration of the experiment (16 days post-
203 antibiotic), where no vegetative cells were detected, thus no toxin activity was detected as
204 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

208 **Discussion**

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

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

237 The microbial disruption upon exposure to eravacycline observed here is similar to the effects
238 seen with other closely related antibiotics, such as tigecycline²² and omadacycline.¹⁹ These
239 tetracycline derivatives also did not induce CDI in the gut model. Conversely, in a separate
240 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 eravacycline, 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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254 model experiments to determine the propensity of eravacycline, and a comparator antibiotic,
255 to induce *Clostridium difficile* infection). The funders had no role in the design of the study
256 or the interpretation of the data.

257

258 **Transparency declarations**

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267

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- 367

368 **Figure legends**

369 *Figure 1.*

370 Schematic timeline of the *in vitro* triple stage chemostat gut model and experimental design
371 for each model (**A**). *C. difficile* (CD) spores were added to each model (black lines) before
372 addition of antibiotics (blue arrow). Facultative anaerobic microbial populations were
373 monitored after exposure to either eravacycline (**B**) or moxifloxacin (**C**). In each graph, black
374 lines are the total facultative anaerobic bacteria, red lines are the lactose-fermenting (LF)
375 Enterobacteriaceae, green lines are the *Enterococcus* spp. and purple lines are the
376 *Lactobacillus* spp. Results shown are mean log₁₀ cfu/mL from three technical replicates.
377 Limit of detection for this assay is 1.2 log₁₀ cfu/mL.

378 *Figure 2.*

379 Dynamics of obligate anaerobic bacterial populations upon exposure to either eravacycline
380 (**A**) or moxifloxacin (**B**). In each graph, black lines are the total bacteria, red lines are the
381 *Bacteroides* spp., green lines are the *Clostridium* spp., purple lines are the *Bifidobacterium*
382 spp., and orange lines are the total spore-formers. Results shown are mean log₁₀ cfu/mL from
383 three technical replicates. Limit of detection for this assay is 1.2 log₁₀ cfu/mL.

384 *Figure 3.*

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

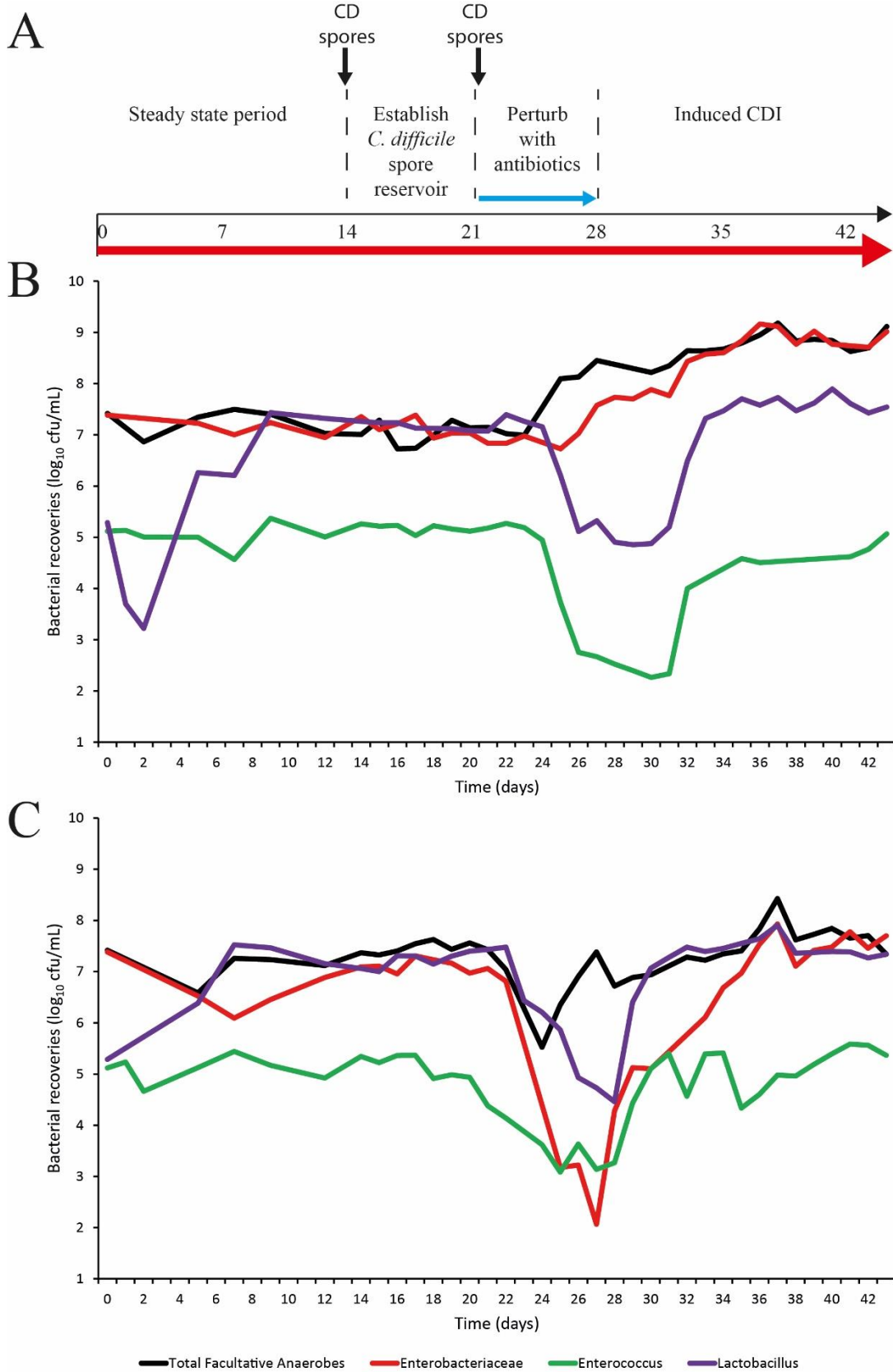
390

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

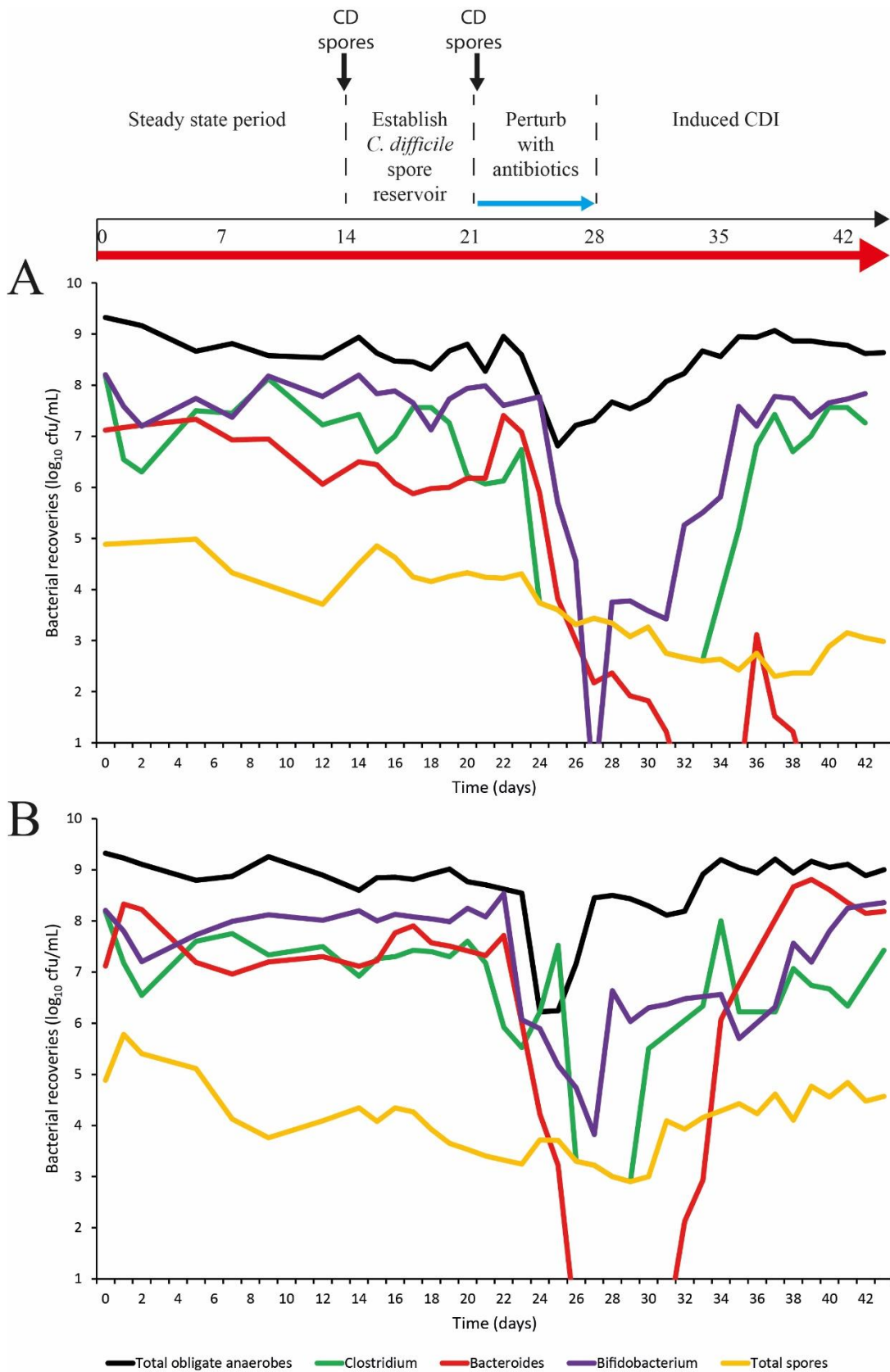
Bacterial population	Faecal slurry	Models ^b	
		Eravacycline	Moxifloxacin
Total facultative anaerobes	7.42	7.01	7.12
LF <i>Enterobacteraceae</i> ^a	7.38	6.95	6.89
<i>Enterococcus</i> spp.	5.12	5.01	4.92
Total bacteria	9.32	8.94	8.85
<i>Clostridium</i> spp.	8.18	7.43	7.5
<i>Bacteroides</i> spp.	7.12	6.5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

^a Lactose fermenting *Enterobacteriaceae*

^b log₁₀ cfu/mL value is from day 14 vessel 3 only



397 **Figure 2**



398

399

400 **Figure 3**

