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Recurrence of dual strain *Clostridium difficile* infection in an *in vitro* human gut model

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Abstract

Objectives: *Clostridium difficile* infection (CDI) is still a major challenge to healthcare facilities. The detection of multiple *C. difficile* strains has been reported in some patient samples during initial and recurrent CDI episodes. However, the behaviour of individual strains and their contribution to symptomatic disease is unclear.

Methods: An *in vitro* human gut model was used to investigate the germination and proliferation of two distinct *C. difficile* strains during initial and recurrent simulated CDI, as well as their response to vancomycin treatment. The gut model was inoculated with a pooled human faecal emulsion and indigenous gut microbiota, *C. difficile* populations (vegetative and spore forms), cytotoxin and antimicrobial activity was monitored throughout the experiment.

Results: Both *C. difficile* strains germinated and proliferated in response to ceftriaxone instillation, with cytotoxin detection during peak vegetative growth. Vancomycin instillation resulted in a rapid decline in vegetative forms of both strains, with only spores remaining 2 days after dosing commencement. Recrudescence of both strains occurred following cessation of vancomycin, although this was observed in one strain sooner, and to a greater extent, than the other strain.

Conclusions: Within a human gut model multiple *C. difficile* strains are able to germinate and proliferate concurrently in response to antibiotic challenge (onset of simulated CDI). Similarly, more than one strain can proliferate during simulated recurrent CDI, although with differences in germination and growth rate and timing. It appears probable that multiple strains can contribute to CDI within an individual patient with possible implications for management and bacterial transmission.

Introduction

Clostridium difficile infection (CDI) continues to pose a major burden on healthcare facilities worldwide.[1] Standard therapy for CDI includes the administration of vancomycin, metronidazole, or fidaxomicin, although the former two agents in particular are associated with recurrent infection.[2,3] Recurrent CDI can be due to reinfection with another strain,[3] or relapse of infection due to the existing infecting strain.[4] Accurate epidemiological data pertaining to the rates of re-infection versus relapsing recurrent infection, frequency of strain types, and possible routes of transmission are needed. *C. difficile* typing and fingerprinting methods are used to monitor strain prevalence and transmission, especially in outbreak settings. However, it is rare that multiple colonies from the same sample are analysed. PCR ribotyping and multilocus variable number tandem repeat analysis (MLVA) have been used to demonstrate the existence of multiple strains of *C. difficile* within a patient (mixed infection) at initial[5] and recurrent[6] symptomatic episodes, indicating that in some patients more than one strain may be contributing to disease.

The pathogenesis and interaction of individual strains within a multi-strain CDI is not well understood. We recently demonstrated the germination and proliferation of two distinct *C. difficile* isolates within a human gut model,[7] indicating that multiple strains may contribute to disease in an *in vitro* model system. However, response to treatment and possible infection recrudescence was not examined. We have now re-studied the *C. difficile* isolates used previously,[7] to examine response to therapy with vancomycin and the nature of recurrent infection.

Methods

68 ***Clostridium difficile* strains**

69 We examined two *C. difficile* PCR ribotype 001 strains (11/11 and P62). Both strains were clinical
70 isolates from cytotoxin-positive faeces of patients with CDI at Leeds General Infirmary, UK. Isolate
71 11/11 (CD-RM) demonstrates reduced susceptibility to metronidazole (MIC 8 mg/L), and is
72 clindamycin susceptible (MIC 2 mg/L); strain P62 (CD-CR) is clindamycin resistant (MIC ≥ 256 mg/L),
73 and fully metronidazole susceptible (MIC ≤ 0.25 mg/L). The ceftriaxone MIC for CD-RM is 64 mg/L
74 and CD-CR is > 256 mg/L. The vancomcyin MIC for CD-RM is 2 mg/L and CD-CDR is 1 mg/L.

75

76 ***In vitro* human gut model**

77 The use of a triple stage model of the human to gut to study CDI has previously been described.[8-10]
78 This model has been validated against physicochemical and microbiological measurements of the
79 intestinal contents of sudden death victims,[11] and comprises three chemostat glass vessels (Soham
80 Scientific, Cambridge, UK) (V1 – 280 mL; V2 – 300 mL; V3 – 300 mL), connected in a weir cascade
81 formation, top-fed with a complex growth medium[7] (flow rate = 13.2ml/h), maintained in an
82 anaerobic atmosphere and at a controlled pH (V1 – pH 5.5 ± 0.2; V2 – pH 6.2 ± 0.2; V3 – pH 6.8 ± 0.2).
83 Each vessel is inoculated with a *C. difficile*-negative, pooled human faecal emulsion, donated from
84 healthy adult volunteers (n = 5, aged ≥60 years).[12]

85

86 **Experimental design**

87 The experimental design is outlined in Figure 1. After inoculation of the faecal emulsion into the gut
88 model (day 0 – period A), the media pump was started and no interventions were made until *C.*
89 *difficile* CD-RM (~ 10^7 cfu) and CD-CR (~ 10^7 cfu) spores were inoculated into vessel 1 on day 14
90 (period B). After seven days (day 21) a further inoculum of *C. difficile* CD-RM and CD-CR spores were
91 added to vessel 1, along with 150 mg/L ceftriaxone, once daily, for seven days (period C) to induce
92 simulated CDI. Ceftriaxone instillation was designed to reflect concentrations observed in faeces of
93 volunteers.[13] Once high level cytotoxin was detected within the model (indicating simulated CDI),

instillation of vancomycin commenced (125 mg/L, four times daily, seven days – period E). No interventions were made for a further 21 days (period F). Indigenous gut microbiota (periods A-F, vessels 2 and 3), *C. difficile* total viable counts, spores and cytotoxin (periods B-F, vessels 1-3), and antimicrobial activity (periods C-F, vessels 1-3) were monitored daily (every 2-days period A only).

Enumeration of gut microbiota and *C. difficile* populations and cytotoxin

Gut microbiota populations were identified and enumerated, using selective and non-selective agars as described previously.[14] Cultures were plated onto solid media in triplicate and average populations (±standard error) obtained. *C. difficile* isolates were distinguished by culture onto antimicrobial-containing CCEYL. CD-CR was isolated on CCEYL containing 8 mg/L clindamycin (growth of CD-RM was inhibited) and CD-RM was isolated on CCEYL containing 1 mg/L metronidazole (growth of CD-CR was inhibited). *C. difficile* spores were enumerated after alcohol shock.[9] Cytotoxin was quantified using Vero cell cytotoxin assay.[9]

Quantification of antimicrobial activity

Concentrations of active ceftriaxone and vancomycin were determined by large plate in house bioassay. Briefly, 1 mL aliquots of gut model culture fluid were centrifuged (16,000g, 15 min), filter-sterilised by filtration through 0.22 µm syringe filters and stored at -20˚C. For the ceftriaxone and vancomycin bioassays, the indicator organisms were *E. coli* (ATCC 25922) and *K. rhizophilia* (ATCC 9341)(~10^8 cfu/mL), seeded into Mueller-Hinton and Antibiotic medium number 1 agar, respectively. The agars (100 mL volumes) were autoclaved and cooled to 50˚C and poured into bioassay plates (245 mm x 245 mm). Twenty microliters of gut model aliquots or antimicrobial calibration series (ceftriaxone 1-256 mg/L; vancomycin 4-256 mg/L) were assigned randomly in triplicate into wells (9 mm). Bioassay plates remained at ambient temperature for four hours prior to aerobic incubation at 37˚C overnight. The limit of detection of the ceftriaxone and vancomycin bioassay was 1 and 8 mg/L,

respectively. Zone diameters were measured with callipers accurate to 0.1 mm. Calibration lines were plotted from squared zone diameters and unknown concentrations from culture supernatants determined.

Results

Gut microbiota populations

Vessel 3 is of most physiological relevance for CDI, and therefore data from this vessel are discussed and shown graphically in this report (Figures 2 and 3). All monitored gut microbiota populations reached steady state by the end of period A (Figure 2), with *B. fragilis* group and *Clostridium* spp. predominating (~ 8.0-8.5 $\log_{10}$cfu/mL). *C. difficile* inoculation (period B – Figure 2) did not affect indigenous microbiota populations. Ceftriaxone instillation resulted in a decrease in *Bifidobacterium* spp. (~2.5 $\log_{10}$cfu/mL), whilst other microbiota remained unaffected. Vancomycin instillation (period E – Figure 2) elicited a rapid and profound reduction in *Bifidobacterium* spp. (~6 $\log_{10}$cfu/mL) and *B. fragilis* group (~6 $\log_{10}$cfu/mL) populations, with minor declines in *Clostridium* spp. (~2 $\log_{10}$cfu/mL). *B. fragilis* group and *Clostridium* spp. populations recovered to pre-vancomycin levels three days after dosing cessation, whilst *Bifidobacterium* spp. remained below the limit of detection (~1.5 $\log_{10}$cfu/mL) for the remainder of the experiment. Lactose-fermenting Enterobactericeae and *Lactobacillus* spp. populations temporarily increased (~4 and 2 $\log_{10}$cfu/mL, respectively) during vancomycin instillation.

C. difficile populations

Both CD-CR and CD-RM *C. difficile* spores remained quiescent before antimicrobial agent instillation (period B - Figure 3). CD-RM populations were ~1 $\log_{10}$cfu/mL greater than CD-

CR populations by the end of period B, although populations were equal (~6.5 $\log_{10}$cfu/mL) mid-way through period C. *C. difficile* total counts began to increase relative to spore counts on day six of ceftriaxone instillation for both CD-CR and CD-RM isolates. Maximum *C. difficile* total counts of ~5.5 $\log_{10}$cfu/mL for both *C. difficile* isolates were obtained on day seven of ceftriaxone instillation, with a concurrent maximum toxin titre of 3 RU (period D). Vancomycin instillation resulted in a rapid decrease in vegetative cells, with only spores of both CD-CR and CD-RM remaining two days after commencement of antibiotic; cytotoxin was undetectable four days after vancomycin dosing started. Both *C. difficile* strain populations remained as spores at a level of ~3.5 $\log_{10}$cfu/mL until CD-RM and CD-CR began to germinate nine and 11 days after vancomycin cessation, respectively. CR-RM total counts peaked at ~6.5 $\log_{10}$cfu/mL before declining to ~5.5 $\log_{10}$cfu/mL. CD-RM spore populations remained at ~3.5 $\log_{10}$cfu/mL for the remainder of the experiment. CD-CR total cells peaked at a population of ~5 $\log_{10}$cfu/mL before declining to ~3.5 $\log_{10}$cfu/mL, with only spores remaining. A peak cytotoxin titre of 4 RU was detected 12 days after vancomycin cessation and remained at ≥ 2RU for the remainder of the experiment.

Antimicrobial concentrations

Ceftriaxone was detected in vessels 1 and 2 only (data not shown). Concentrations peaked at 102.3 mg/L in vessel 1 on day five of ceftriaxone instillation and were no longer detectable two days after dosing cessation. Vancomycin was detected in all three vessels of the gut model, with peak concentrations of 225.7 mg/L in vessel 3, six days after vancomycin instillation commenced (Figure 3). Vancomycin activity was no longer detectable in any vessel six days after instillation ceased.

Discussion

As has been observed in previous gut model experiments, indigenous gut microbiota populations present within the faecal emulsion reached steady state by the end of period A, and were not affected by instillation of *C. difficile* spores. However a deleterious effect was observed on *Bifidobacterium* spp. populations during ceftriaxone instillation, as described previously.[10] A more profound response to vancomycin instillation occurred, with reductions in *Bifidobacterium* spp., *B. fragilis* group and *Clostridium* spp. populations, again as previously described.[10,15,16]

Both *C. difficile* strains (CD-RM and CD-CR) remained as spores following inoculation into the *in vitro* human gut model in the absence of antimicrobial intervention. Although both CD-RM and CD-CR strains initially comprised spore populations (~5.5 $\log_{10}$cfu/mL), strain CD-RM established a spore population of ~6.5 compared with ~5.5 $\log_{10}$cfu/mL for strain CD-CR by the end of period B (six days after spore inoculation). Differences in initial spore populations of these two strains were not evident in a previous gut model experiment,[7] and reasons for this observation in this study are unclear. However, following the addition of second aliquots of both strains, spore populations of both strains were again equal.

We have previously used ceftriaxone instillation within an *in vitro* gut model to induce simulated CDI.[7,10,17] The dosage regimen aims to reflect *in vivo* antibiotic concentrations in the faeces of patients (152-258 mg/kg),[13] as reflected by similar concentrations observed within vessel 1 of the gut model (data not shown). The sporadic detection of active ceftriaxone within vessel 2 and the absence of active agent in vessel 3 (limit of detection 1

191 mg/L ceftriaxone) has been previously observed within the gut model,[7,10,17] likely due to β-
192 lactamase-mediated deactivation or because of adsorption.[18]
193 Initial germination of both strains within the gut model was concurrent, as observed
194 previously.[7] Peak total counts of both *C. difficile* strains were equal, indicating the potential
195 for an equal contribution to simulated CDI. Toxin production was detected concurrent with
196 maximal *C. difficile* populations. Maximal PCR ribotype 001 *C. difficile* total counts of
197 individual strains following CDI-inducing antimicrobial instillation during previous gut model
198 experiments typically reached ~6.5 $\log_{10}$cfu/mL,[7,12,19] similar to those measured in the
199 present study. This suggests that growth of the two strains was not limited by competition
200 for resources. Total toxin levels produced by two strains within this experiment and in a
201 previous dual strain gut model[7] were no greater than those measured in single strain
202 investigations,[12,19] despite 2-fold greater combined total cell counts in the dual strain
203 studies. *C. difficile* toxin production is a complex, multifactorial process that is dependent
204 on various external stimuli as well as cell growth phase. As toxin production of individual
205 strains can not be monitored by the present experimental design, it remains unclear
206 whether both strains were producing toxin during this experiment. It is possible that only
207 one of the two strains within the model was actively producing toxin due to preferential
208 ability to respond to external stimuli, thus allow transcription of toxin genes and/or
209 regulator genes. Alternatively, competition between the strains may lead to reduced toxin
210 generation or for reduced lengths of time by both strains. It should be noted, however, that
211 the measured toxin titre units in these experiments follow a 10-fold dilution series; thus, a
212 2-fold increase in toxin production (corresponding to a 2-fold increase in vegetative *C.*
213 *difficile* populations) may well go undetected.
214

Instillation of vancomycin rapidly reduced vegetative populations of both *C. difficile* strains, leaving total counts equal to spore counts, and comparable spore population levels for the two strains. *C. difficile* remained as spores until total counts began to increase relative to spore counts, 9 and 11 days after vancomycin cessation for strains CD-RM and CD-CR, respectively. This was followed by cytotoxin detection. Recurrence of vegetative growth occurred in strain CD-RM two days before that of strain CD-CR. In addition, maximum total counts of strain CD-RM were ~1.5 $\log_{10}$cfu/mL greater than those of strain CD-CR, with both strains exhibiting similar spore populations (~3.5 $\log_{10}$cfu/mL). This indicates that CD-RM vegetative cell populations were greater, potentially providing a greater contribution to simulated recurrent CDI, than for strain CD-CR. Modest differences in strain growth within the gut model were seen during recurrent infection. Growth curves of these strains within batch culture experiments indicated similar growth and germination rates (data not shown), suggesting that the growth cycles of the strains are not the cause of the differences observed. The post-ceftriaxone and vancomycin environment of the gut model is likely to differ considerably from that of the relatively unstressed period during initial simulated CDI, with marked differences in bacterial composition evident. Other differences such as concentrations of fermentation products and substrate composition are unknown; these are likely to vary during different periods of the gut model experiment and could be expected to affect germination and proliferation processes. Minor differences in susceptibility of the strains to vancomycin were noted (CD-RM 2 mg/L; CD-CR 1 mg/L). The bioactive vancomycin concentration declined to 2.37 mg/L 5 days after dosing cessation. From hereon the levels of vancomycin were below the limit of detection for the bioassay protocol (≤ ~2mg/L). It is likely that further decline in active vancomycin concentration would occur after dosing cessation due to dilution in the model system (akin to excretion *in vivo*). The

bioactive vancomycin concentration would therefore presumably decrease to sub-inhibitory levels for CD-RM (2 mg/L) sooner than for CD-CR (1 mg/L), possibly allowing preferential germination and proliferation of CD-RM. It is notable that despite similar populations of these strains prior to antimicrobial intervention (period B), post-vancomycin (period F) CD-RM populations remained greater than those of CD-CR for the remainder of the experiment. This suggests that the ability of CD-RM to germinate and proliferate sooner than CD-CR in the stressed environment of a post infection and treatment gut model, may provide it with a sustained advantage over CD-CR and is potentially explained by the differential susceptibility of these isolates to vancomycin.

The existence of mixed infections within primary and recurrent CDIs has been reported in ~8-15% of cases.[5,6,20] The relative contribution to symptomatic disease of individual strains within a mixed culture infection is not well understood. *C. difficile* may possess strain-specific virulence factors or stimulators of the immune response, thereby giving some strains a competitive advantage. Whole genome sequencing has been utilised to identify differences in the proportions of multiple strains within mixed infections comprised of single- and mixed- MLST sequence types.[6] Whilst these data described the relative abundance of individual strains,[6] the contribution of strains to disease was not investigated. In the present study we have provided evidence of the proliferation of multiple strains within both the initial and recurrent episodes of simulated CDI, and have identified differences in abundance of vegetative cells of different strains during the recurrent, but not initial disease episode. However, the relative contribution to disease (i.e. toxin production) remains unclear.

This study describes a mixed CDI infection of two distinct *C. difficile* PCR ribotype 001 isolates. The use of two isolates of the same ribotype within this study possibly limits the conclusions that can be drawn in regards to multiple ribotype infections. However, despite the high level of relatedness of these two strains based on their ribotype profiles, within the gut model they displayed differential behaviour in their growth profiles post-vancomycin instillation. Thus, less related strains could reasonably be expected also to behave differently in the gut when under antimicrobial stress..

This study demonstrates the existence of two distinct vegetative *C. difficile* strains within a simulated disease state. This data, along with reports of mixed infections within CDI patients [5,6,20], highlight the possibility of transmission of one or multiple strains from a symptomatic patient. In order to fully understand *C. difficile* transmission routes the possibility of multiple strains being present and indeed contributing to CDI should not be discounted. Lastly, the significance of dual strain CDI to patient management requires further study.

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Transparency declaration

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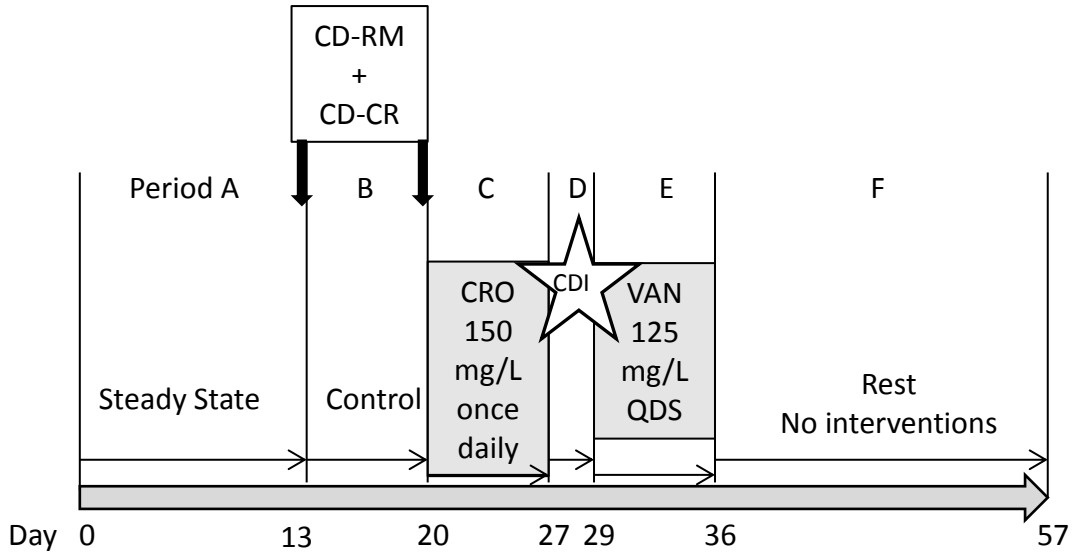
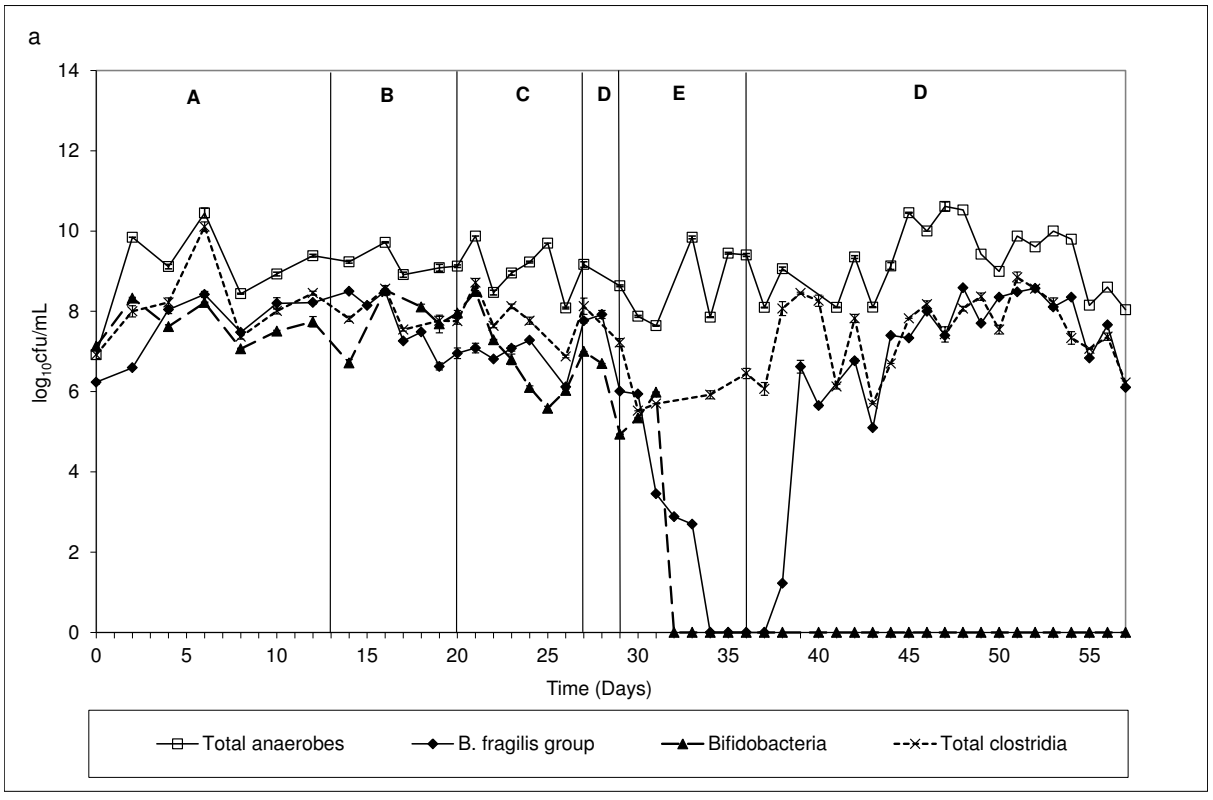


355

356 **Figure 1:** Schematic diagram outlining the experimental design. CRO – ceftriaxone, VANC –

357 vancomycin, QDS – four times daily.

358



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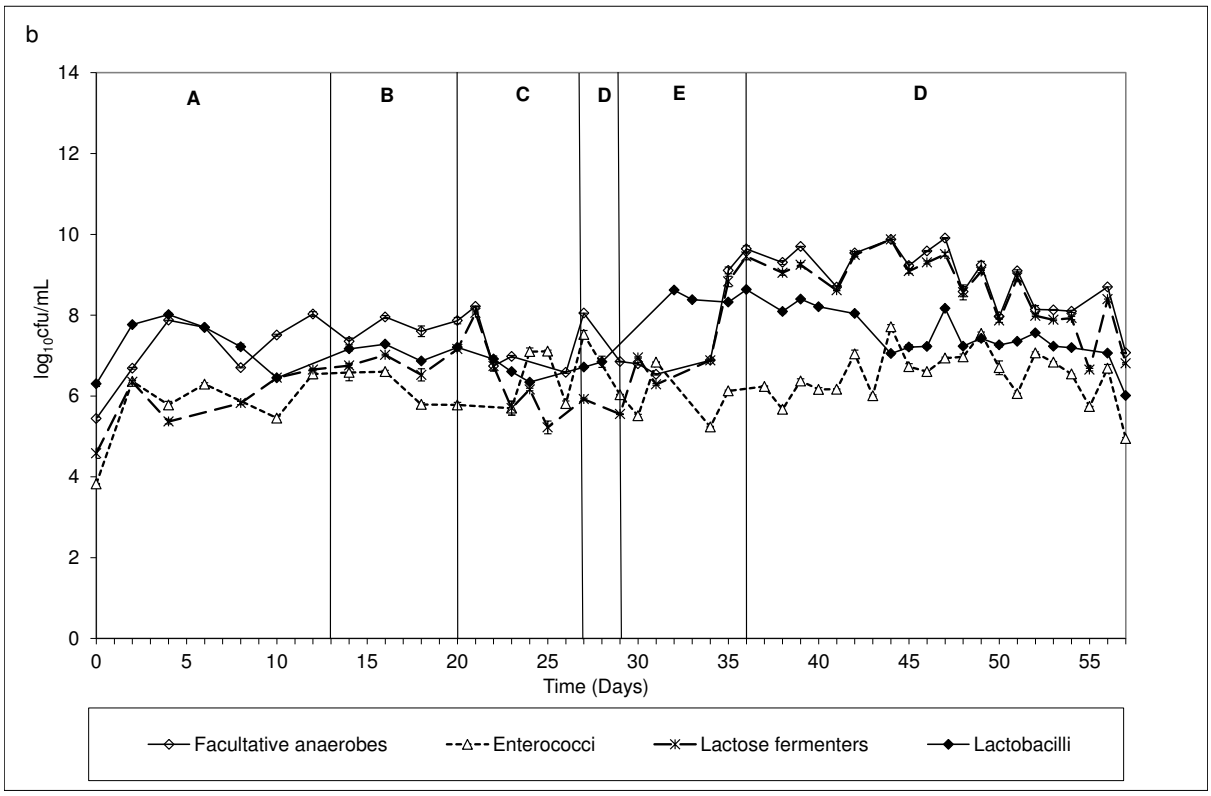

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362 **Figure 2a and b:** Mean (±SE) populations (log_{10}cfu/mL) of obligate (a) and facultative (b) anaerobes

363 within vessel 3 of the gut model. Vertical line represents the last day of each time period.

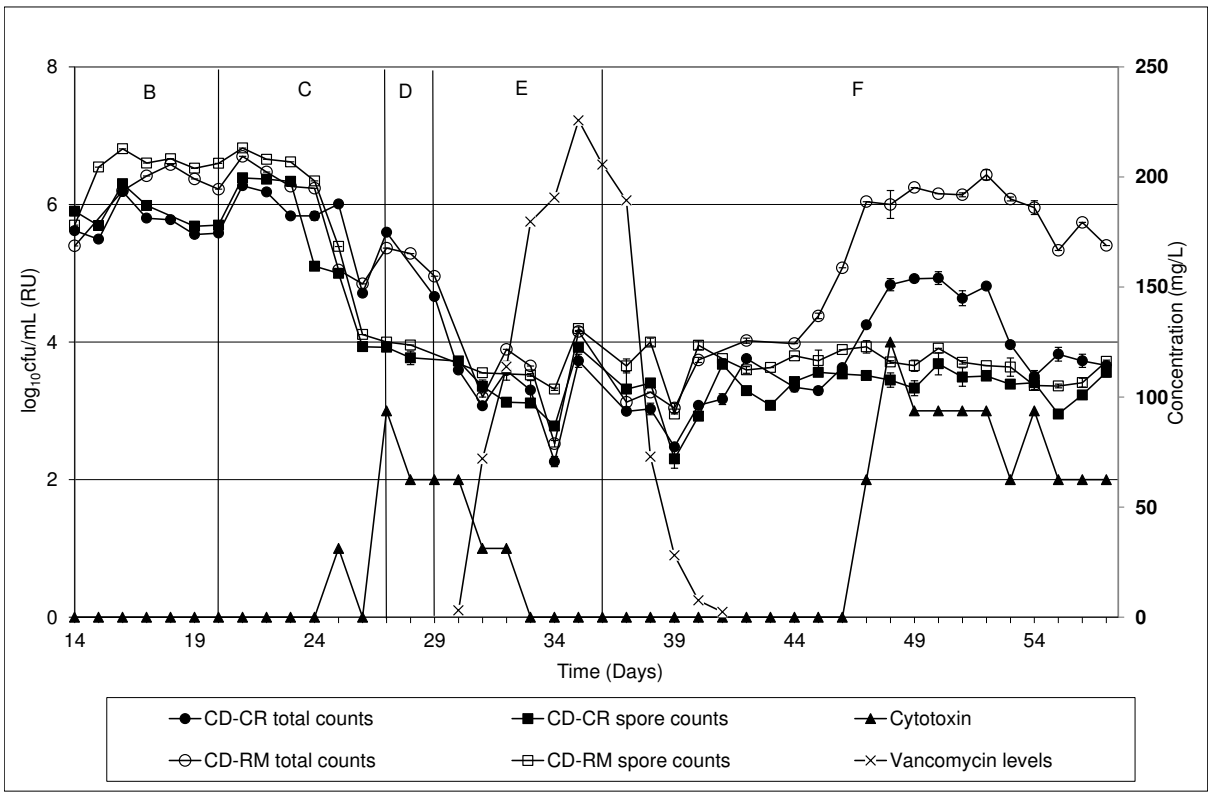

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Figure 3: Mean (±SE) populations of *C. difficile* total viable counts and spores (log_{10}cfu/mL), cytotoxin (RU) and vancomycin concentration (mg/L) within vessel 3 of the gut model. Vertical line represents the last day of each time period. CD-CR – *C. difficile* clindamycin resistant, fully metronidazole susceptible. CD-RM – *C. difficile* reduced susceptibility to metronidazole, clindamycin susceptible.