Investigation of the effect of adsorbent DAV131A on the propensity of moxifloxacin to induce simulated *Clostridium difficile* infection (CDI) in an *in vitro* human gut model

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Running title: DAV131A to prevent moxifloxacin-induced *C. difficile* infection

Abstract

Background *C. difficile* infection (CDI) remains a high burden worldwide. DAV131A, a novel adsorbent product, reduces residual gut antimicrobial levels, reducing CDI risk in animal models.

Objectives We used a validated human gut model to investigate the efficacy of DAV131A in preventing moxifloxacin-induced CDI.

Methods *C. difficile* (CD) spores were inoculated into two models populated with pooled human faeces. Moxifloxacin was instilled (43 mg/L, once daily, 7 days) alongside DAV131A (5g in 18mL PBS, 3 times daily, 14 days, Model A), or PBS (18mL, 3 times daily, 14 days, Model B). Selected gut microbiota populations, CD total counts, spore counts, cytotoxin titre and antimicrobial concentrations (HPLC) were monitored daily. We monitored for reduced susceptibility of CD to moxifloxacin. Growth of CD in faecal filtrate and BHI in the presence/absence of DAV131A, or in medium pre-treated with DAV131A, was also investigated.

Results DAV131A instillation reduced active moxifloxacin levels to below the limit of detection (50 ng/mL), and prevented microbiota disruption, excepting *B. fragilis* group populations, which declined by ~3 $\log_{10}$ cfu/mL. DAV131A delayed onset of simulated CDI by ~2 weeks, but did not prevent CD germination and toxin production. DAV131A prevented emergence of reduced susceptibility of CD to moxifloxacin. In batch culture, DAV131A had minor effects on CD vegetative growth, but significantly reduced toxin/spores ($p<0.005$).

Conclusions DAV131A reduced moxifloxacin-induced microbiota disruption and emergence of antibiotic-resistant CD. Delayed onset of CD germination and toxin production indicates further investigations are warranted to understand the clinical benefits of DAV131A in CDI prevention.

Introduction

Clostridium difficile (*Clostridioides difficile*[1]) infection (CDI) is a leading cause of antibiotic-associated diarrhoea[2] and a major worldwide burden.[3, 4] Treatment options are limited and are associated with high recurrence rates (~20%).[5, 6] Many antimicrobial classes can induce CDI,[7-9] and notably fluoroquinolones.[10, 11] This is likely due to disruption of normal gut microbiota, so reducing colonisation resistance.[12] DAV132 is a non-specific adsorbent formulated to irreversibly capture antibiotics in the late ileum, caecum and colon of humans before they can significantly alter the microbiota. It is particularly efficacious in binding fluoroquinolones, e.g. levofloxacin or moxifloxacin, and protects the gut microbiota from antibiotic-mediated disruption, without affecting plasma antimicrobial levels.[13] Thus, co-administration of DAV132 alongside fluoroquinolones may reduce their propensity to induce CDI. Indeed, it has been demonstrated in animal models that DAV131A, the rodent-adapted version of DAV132, can prevent moxifoxacin-mediated microbiota disruption in hamsters and protection from lethal CDI.[14, 15]

The *in vitro* gut model has been used to investigate the propensity of multiple antibiotics to induce CDI,[16-20] and results correlate well with clinical data. For example, fluoroquinolones and cephalosporins induce simulated CDI in the model, whereas piperacillin-tazobactam does not.[16-20] Here we have used this *in vitro* gut model system to investigate the effects of DAV131A instillation on moxifloxacin-mediated gut microbiota disruption, *C. difficile* growth and toxin production, and whether emergence of reduced susceptibility to fluoroquinolone occurs in *C. difficile*. The test item used here was DAV131A, which is the non-formulated adsorbent of the DAV132 product, to be used for *in vitro* studies.

Methods

In vitro gut model

C. difficile strains

The PCR ribotype 027 strain used in this experiment (027 210) was isolated during an outbreak of CDI at the Maine Medical Centre (Portland, MA, USA - kindly supplied by Dr Rob Owens).

Gut model

The model consisted of three chemostat vessels, pH controlled (vessel 1 5.5±0.2; vessel 2 6.2±0.2; vessel 3 6.8±0.2) and arranged in a weir cascade system. Vessel 1 was top fed with a complex growth medium,[18] and all vessels were sparged with nitrogen to maintain an anaerobic atmosphere. The model was inoculated with a pooled faecal slurry (10% in pre-reduced PBS). Faeces was from elderly volunteers (>60 years) (n=3-5) with no history of antimicrobial therapy (last 3 months), and was screened for *C. difficile* (by culture on selective agar). Only faecal samples confirmed as *C. difficile* negative were used to create the faecal slurry.

Gut model experimental design

Two gut models were then run simultaneously as outlined in Figure 1.

The models were set up and primed with pooled faecal slurry, then left for 2 weeks to reach steady state, before addition of an aliquot of *C. difficile* spores (~10^7 cfu). One week later, moxifloxacin instillation commenced (43mg/L, once daily, 7 days). Another aliquot of *C. difficile* spores (~10^7 cfu) was added with the first moxifloxacin dose. Model B was instilled with moxifloxacin only, and Model A was instilled with moxifloxacin and DAV131A (5g in 18mL pre-reduced PBS, 3 times daily, 14 days). 18mL of pre-reduced PBS diluent (3 times daily, 14 days) was added to Model B to keep the flow rates of the two models comparable.

Bacterial enumeration

Gut microbiota populations and *C. difficile* total viable and spore counts were enumerated by culture onto solid media. Colonies were identified to genus level on the basis of colony morphology, Gram

reaction, microscopic appearance and/or MALDI-TOF identification on selective and non-selective agars as follows: fastidious anaerobe agar supplemented with 5% horse blood (total anaerobes); Beerens agar - 42.5g/L Columbia agar, 5g/L agar technical, 0.5g/L cysteine HCl, 5g/L glucose, 5mL propionic acid, adjusted to pH 5 (bifidobacteria); *Bacteroides* bile aesculin agar supplemented with 5mg/L haemin, 10µL/L vitamin K, 7.5mg/L vancomycin, 1mg/L penicillin G, 75mg/L kanamycin and 10mg/L colistin (*Bacteroidies fragilis* group); LAMVAB agar – 20g/L agar technical, 52.2g/L MRS broth, 0.5g/L cysteine HCl, 20mg/L vancomycin, adjusted to pH 5 (lactobacilli); Nutrient agar (total facultative anaerobes); MacConkey's agar No. 3 (lactose fermenting Enterobacteriaceae); kanamycin aesculin azide agar supplemented with 10mg/L nalidixic acid, 10mg/L aztreonam and 20mg/L kanamycin (enterococci); Alcohol shock and Brazier's CCEYL agar supplemented with 2% lysed horse blood, 5mg/L lysozyme, 250mg/L cycloserine and 8mg/L cefoxitin (*C. difficile* spores); Brazier's CCEYL agar as above and supplemented with 2mg/L moxifloxacin (*C. difficile* total viable counts).

Cytotoxin Testing

The presence of *C. difficile* cytotoxins was determined by Vero cell cytotoxicity assay (CA).[19] Gut model fluid (1mL) was centrifuged at 16,000*g* , 4°C for 15 minutes. Supernatants were then serially diluted 1:10 in sterile PBS to 10^{-6}. Twenty microlitres of each dilution was added to Vero cell monolayers and a further 20µL of *C. sordellii* antitoxin (diluted 1:10 in sterile distilled water) placed in to the corresponding antitoxin row. Monolayers were examined after 24 and 48 hours incubation at 37°C in 5% CO_2, with a positive result indicated by the presence of cell rounding with concurrent neutralisation of effect by *C. sordellii* antitoxin. Cytotoxin titres (relative units, RU) were an arbitrary $\log_{10}$ scale and the cytotoxin titre reported in the highest dilution with >70% cell rounding, *i.e.* 10^{0}=1RU, 10^{-1}=2RU, 10^{-2}=3RU.

Measurement of antimicrobial concentrations by HPLC

Samples (1mL) from all vessels of each gut model were centrifuged (16,000 g, 10 min) and the supernatants sterilized by filtration through 0.22µm syringe filters, resulting in elimination of charcoal and any particular material from the medium, before being stored at -20˚C for measurement of antimicrobial concentrations. This was achieved by HPLC coupled with fluorescence detection, and was performed by AmatsiAvogadro (Fontenilles, France). Samples were spiked with 2.5µg/mL enrofloxacin used as an internal standard, and extracted by solubilisation with 4% phosphoric acid followed by loading onto a solid phase cation exchange sorbent (Oasis MCX 60mg 3cc cartridges, Waters) that was successively washed with 2% formic acid and methanol, dried and finally eluted with 5% ammonia in methanol. Dried samples were reconstituted with 0.1% formic acid in 90:10 water:acetonitrile, and separated by HPLC onto a Kinetex PhenylHexyl 100 x 3 mm 0.26µm (Phenomenex) column that was eluted with a gradient from 90:10 to 30:70 of mobile phases respectively consisting in 20mM ammonium formate and 0.1 formic acid in acetonitrile. Fluorometric detection of the eluted products (excitation at 290nm, emission at 500nm) enabled to reach a lower limit if detection of 50ng/mL for moxifloxacin. Non-interference of the matrix with the assay was ensured by the fact that control as well as calibration samples with known amounts of moxifloxacin made in matrix or buffer gave similar results in the assay.

Emergence of reduced susceptibility

The emergence of *C. difficile* populations showing reduced susceptibility to moxifloxacin was monitored on antibiotic-containing agar plates as described previously.[16] Brazier's CCEYL agar containing 32 or 64mg/L moxifloxacin, as well as the usual supplements, was used in addition to normal agars to enumerate *C. difficile* TVC and spores. The MIC of moxifloxacin for the *C. difficile* strain used here was 32mg/L.

C. difficile growth and toxin production in batch culture

Three clinical isolates, submitted to the *C. difficile* ribotyping network (CDRN) in 2013 were selected for batch culture growth experiments. Isolates were chosen to represent the epidemic ribotypes 027, 001 and 078. The growth of each strain was investigated in both brain heart infusion (BHI) broth and faecal filtrate prepared from faeces provided by healthy volunteers aged >60 (at-risk population for CDI). A 10% (w/v) faecal slurry was prepared in pre-reduced PBS. Faecal slurry was centrifuged and filtered through 0.22µm filters to remove all viable organisms.

Each media was treated in 4 different ways; **A - Control** (sterile broth); **B - Spun control** (sterile broth centrifuged and filtered through 0.22µm filters before use); **C - DAV131A exposed** (sterile broth preincubated with 0.05g/ml of DAV131 for 2 hours before use); **D - Spun DAV131A exposed** (sterile broth preincubated with 0.05g/ml of DAV131A for 2 hours then centrifuged and filtered through 0.22µm filters before use to eliminate DAV131A from the resulting broth).

Broths were pre-reduced overnight and either incubated with 0.05g/ml of DAV131A (C- DAV131A exposed and D – Spun DAV131A exposed only) for 2 hours, or not (A – Control and B – Spun control); B-spun control and D-Spun DAV131A exposed were centrifuged (16000g, 15 minutes) and decanted into new tubes. All media were then filter sterilised and inoculated with *C. difficile* as follows: *C. difficile* was grown on CCEYL agar for 48 hours, and the growth was suspended in pre-reduced saline to ~0.5 McFarlane suspension; 200µl of the *C. difficile* suspension was added to all broths, and incubated anaerobically at 37°C. Samples were taken for *C. difficile* enumeration (total viable counts (TVCs), spore counts) and toxin quantification at 48h. The supernatant from the A broths after 48 h growth was used as a toxin positive control. This supernatant was then incubated anaerobically with 0.05g/mL DAV131A for 2 hours, filtered and assayed for toxin (**E – DAV131A exposed supernatant**)

C. difficile total viable and spore counts were enumerated (in triplicate) as described above. Toxin levels were assayed using a cell CA described above (in duplicate). Experiments were repeated in biological duplicate for each different ribotype. Statistical significance was determined using a paired t-test using Stata/IC 13.1 software.

Ethics statement

The collection/use of faecal donations from healthy adult volunteers following informed consent was approved by the Leeds Institute of Health Sciences and Leeds Institute of Genetics, Health and Therapeutics and Leeds Institute of Molecular Medicine, University of Leeds joint ethics Committee (reference HSLTLM/12/061).

Results

In vitro gut model

Antimicrobial concentrations

In Model A, the instillation of DAV131A prevented the detection of any moxifloxacin throughout the gut model experiment in vessels 1 and 3 (vessel 3 data shown in Figure 2a). Moxifloxacin was detected (0.3µg/mL) only on a single day (day 21) in vessel 2 of Model A (data not shown). Concentrations of moxifloxacin in Model B detected by HPLC peaked at ~120µg/mL in vessel 1, ~90 µg/mL in vessel 2 (data not shown) and ~100µg/mL in vessel 3 (vessel 3 data shown in Figure 2b).

Gut microbiota populations

Without the addition of DAV131A (Model B), moxifloxacin instillation caused substantial disruption to microbiota populations (Figure 3b, 3d). Decreases were observed in populations of lactose fermenters and *B. fragilis* group (~6 $\log_{10}$ cfu/mL), bifidobacteria (~4 $\log_{10}$ cfu/mL), and lactobacilli (~3 $\log_{10}$ cfu/mL). All populations recovered to steady state levels approximately 10 days after the end of moxifloxacin infusion. In Model A, the effects of moxifloxacin instillation on the gut microbiota were greatly reduced (Figure 3a, 3c), with only a small, temporary decline in *Bacteroides* spp. counts observed (~3 $\log_{10}$ cfu/mL).

*C. difficile total and spore counts and toxin titre*s

In the absence of DAV131A (Model B), moxifloxacin instillation caused rapid (1-2 days into instillation) germination and proliferation of *C. difficile*. Toxin production was also very rapid (1-2 days into instillation). In Model A, germination was delayed by approximately 7 days in comparison to Model B, occurring 1 day after the end of moxifloxacin instillation. Toxin production was not detected until 7 days after the end of moxifloxacin instillation, when DAV131A instillation also ceased.

Emergence of reduced susceptibility to moxifloxacin

In both models, *C. difficile* counts on agar containing 32mg/L moxifloxacin were comparable to those on agar containing 2 mg/L moxifloxacin (moxifloxacin MIC of strain 027 210 = 32mg/L) (Figure 4). In Model A, no *C. difficile* was isolated on 64mg/L agar (Figure 4a); however, in Model B, following moxifloxacin instillation there was an increase in *C. difficile* isolated on agar containing 64mg/L (Figure 4b) reaching ~3 $\log_{10}$ cfu/mL.

C. difficile growth and toxin production in batch culture

Although variation was observed between growth characteristics of different *C. difficile* strains (data not shown), pooled data are presented here to indicate overall effects of DAV131A on *C. difficile* growth and toxin production. As expected, no differences were observed in *C. difficile* growth or toxin production between control samples and spun control samples in either BHI or faecal filtrate (Fig 5).

Inclusion of DAV131A in the BHI media had no effect on total viable counts ($p=0.38$), but significantly reduced spore counts ($p<0.005$) and toxin levels ($p<0.005$). Inclusion of DAV131A in BHI followed by centrifugation and filtration before inoculation of *C. difficile* affected total viable counts to some extent ($p=0.03$), and significantly reduced spore counts ($p<0.005$) and toxin levels ($p<0.005$).

208 Incubation of BHI toxin positive supernatant with DAV131A followed by centrifugation decreased
209 toxin detection slightly (mean decrease 3.93 to 3.42 RU; not significant, p=0.16).

210 Pre-incubation of DAV131A in faecal filtrate, followed or not by centrifugation and filtration before
211 *C. difficile* inoculation, significantly reduced total viable and spore counts and toxin levels ($p<0.005$ in
212 all cases). Incubation of faecal filtrate toxin-positive supernatant with DAV131A followed by
213 centrifugation decreased mean toxin detection in the supernatant from 2.33 to 1 RU (p=0.007).

Discussion

This study investigated the effects of a novel non-specific absorbent, DAV131A, on moxifloxacin-induced simulated CDI in an *in vitro* gut model. We have previously demonstrated that moxifloxacin administration instilled at 43mg/L, once daily for 7 days, to reflect a standard clinical dosing regimen and achieve faecal antibiotic levels,[21] induces simulated CDI in our *in vitro* model system.[16] This observation is consistent with clinical data that fluoroquinolone administration is a risk factor for CDI.[10, 11]

We show here that DAV131A instillation prevented detection of active moxifloxacin for the duration of the gut model experiment, indicating that DAV131A successfully adsorbed and inactivated substantial quantities of moxifloxacin. This is reflected in the fact that the majority of changes in gut microbiota populations observed following moxifloxacin instillation were not seen in the presence of DAV131A. These data are consistent with the recently reported clinical trial where DAV132, the targeted-release product for humans containing the same adsorbent as DAV131A, reduced exposure of the intestinal microbiota to moxifloxacin by ~99%, and largely preserved the richness and composition of the microbiota seen in healthy volunteers.[13]

However, despite the presence of DAV131A, some microbiota disruption was observed, specifically a ~3 $\log_{10}$ decline in *B. fragilis* group populations. This was substantially less than the ~6 $\log_{10}$ cfu/mL decrease observed in the absence of DAV131A, but indicates that some active, but undetected moxifloxacin (i.e. below the limit of detection, LOD, of the HPLC method used here, 50ng/mL), may still be present despite DAV131A instillation. The detection of 0.3µg/mL of moxifloxacin in vessel 2 on day 21 supports the suggestion that some level of active moxifloxacin is persisting. Whilst the MIC of moxifloxacin for *B. fragilis* ATCC25285 is 0.25µg/mL, it is expected that the range of *B. fragilis* group species within the gut microbiota would have a range of MIC values, and so it is possible that moxifloxacin concentrations could be supra-MIC for some *Bacteroides* spp. populations in the model, but still below the HPLC assay LOD as discussed above. Alternatively, this minimal disruption could

be due to the presence of DAV131A. As a non-specific adsorbent, DAV131A will sequester other components of the microbiota milieu, which may affect the growth of certain populations. This work has demonstrated that DAV131A appears to have minimal effects on cultivable microbiota, supporting the findings of clinical studies.[13]

Instillation of DAV131A delayed the onset of *C difficile* spore germination by ~1 week and *C. difficile* toxin production by ~2 weeks. In the absence of DAV131A, toxin production occurred simultaneously to germination, very soon after moxifloxacin instillation commenced. This is similar to previously reported observations following moxifloxacin instillation in the gut model.[16] However, with the co-administration of DAV131A, germination was not observed until ~1 week after moxifloxacin instillation ended, and toxin production was delayed until ~5 days after germination was observed. DAV131A was instilled for a further 7 days after the end of moxifloxacin instillation. Germination occurred during DAV131A instillation, but interestingly toxin detection was delayed until after DAV131ADAV131A instillation ceased. This may represent delayed toxin production, or because DAV131A adsorbed toxin while it was being instilled, so preventing its subsequent detection in the CA.

In order to facilitate instillation of DAV131A into the model, an increased fluid volume (54mL/day) was required. This is a notably higher fluid instillation than used in previous gut model experiments and will have increased the flow rate of the system. It is possible that the increased flow rate may have some effects on the growth/behaviour of microbiota populations, including *C. difficile*. However, the instillation of pre-reduced PBS in the non-DAV131A exposed model ensured that the flow rate of the two model systems was identical, allowing the effects of DAV131A exposure to be examined.

Batch culture experiments were utilised to try to further elucidate the mechanisms by which DAV131A might affect toxin detection. Data indicate that although DAV131A appears to sequester some toxin (from a toxin-positive culture supernatant), this did not reduce detected toxin to the

same extent as when DAV131A was either included in the growth media, or simply used to treat the media before *C. difficile* inoculation, suggesting that DAV131A may be affecting toxin production and/or detection. Since DAV131A is a non-specific adsorbent, the fact that inclusion of DAV131A in the growth media, or simple pre-treatment of the media by DAV131A similarly reduced sporulation and toxin production/detection, suggests it is likely acting by adsorbing media components, thereby altering the environmental conditions in which *C. difficile* is growing. Many nutritional and environmental factors have been reported to affect toxin production, including temperature,[22] bicarbonate concentration,[23] sub-inhibitory antimicrobials concentrations,[24, 25], short chain fatty acids,[26] amino acid concentrations,[23] and glucose or other rapidly metabolised carbon sources.[27] Spo0A is the master regulator of sporulation in *Clostridium* (and *Bacillus*) species and has been reported to play a role in toxin mediation,[28-31] again linking sporulation and toxin production. In a complex, multispecies gut environment (such as the gut model or host gut), these nutritional and environmental factors are mediated by members of the microbiota communities. As these communities are altered by antibiotic exposure, this may affect resistance to colonisation, in particular to *C. difficile*, and predispose to CDI, as has been demonstrated for *Clostridium scindens* mediated bile acid metabolism.[32] This suggests a potential two-fold mechanism of action by which DAV132 administration in humans may help to prevent antibiotic-induced CDI: in addition to preventing antibiotic-induced changes to the microbiota leading to loss of colonisation resistance, DAV132 could sequester key nutrients and germinants in the colonic environment, thereby reducing *C. difficile* germination and toxin production by the few *C. difficile* that could develop, not withstanding these unfavourable conditions.

Notably, DAV131A instillation prevented the emergence of *C. difficile* with elevated resistance to moxifloxacin. In the absence of DAV131A, instillation of moxifloxacin caused a population (~3 $\log_{10}$ cfu/mL) of *C. difficile* with moxifloxacin MIC >64mg/L to emerge and persist. This was not observed following DAV131A co-administration, consistent with the considerable lowering of antibiotic

289 selective pressure by DAV131A. In humans, such a mechanism might reduce the emergence of highly
290 fluoroquinolone resistant strains of *C. difficile* consequential to moxifloxacin administration.[33]

291 In the gut model, DAV131A successfully reduced detectable active moxifloxacin levels, substantially
292 reduced moxifloxacin-induced deleterious effects on gut microbiota populations, prevented
293 moxifloxacin-induced emergence of *C. difficile* with reduced susceptibility to moxifloxacin, and
294 delayed, but did not totally prevent, the onset of simulated CDI. DAV131A successfully prevented
295 moxifloxacin-induced CDI in hamsters.[15] Both the hamster model and the human gut model have
296 been shown to correlate with clinical use of CDI therapeutics. The gut model includes a human
297 colonic microbiome, but does not simulate a humoral or cell-mediated immune response (beyond
298 that present in the faecal samples used to prime the system). It is therefore possible that the
299 observed delay in germination and toxin production caused by DAV131A instillation in the gut
300 model, in conjunction with an effective (anti-toxin antibody) immune response in
301 immunocompetent hosts, may prevent the actual development of CDI, and/or allow greater
302 recovery of the gut microbiota thereby improving colonisation resistance to CD.

303 The *C. difficile* 027 strain used in the experiments described here is a highly virulent epidemic strain,
304 whereas the strain used in the hamster model was non-epidemic. It is possible that the differences
305 in outcomes between the two studies may be strain/ribotype specific. It should also be noted that
306 the inoculum of *C. difficile* spores (10^7 cfu/mL) reflects levels of spores in the faeces of an infected
307 patient, and is likely to be significantly higher than the exposure level of an 'at-risk' patient in a
308 healthcare setting. Thus, the model may have provided a very stringent test of the capacity of
309 DAV131A to adsorb moxifloxacin and to prevent its deleterious effects. In this context, DAV131A
310 was at least partially protective.

311

312 **Conclusion**

These gut model results complement hamster data in indicating that DAV131A may provide some protection against moxifloxacin-induced CDI. Whilst instillation of DAV131A did not prevent the onset of simulated CDI in this experiment, it caused a ~2 week delay. It also substantially protected the gut microbiota examined in this study from the deleterious effects of moxifloxacin, and prevented emergence of *C. difficile* populations displaying reduced susceptibility to moxifloxacin. These results confirm clinical findings indicating that DAV132 has potential clinical benefit in humans, in reducing antibiotic-induced disruption of the gut microbiota.[13] Whether DAV132 may confer a clinical benefit in prevention of CDI remains to be shown, but these results indicate that further investigation is warranted.

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Transparency declaration

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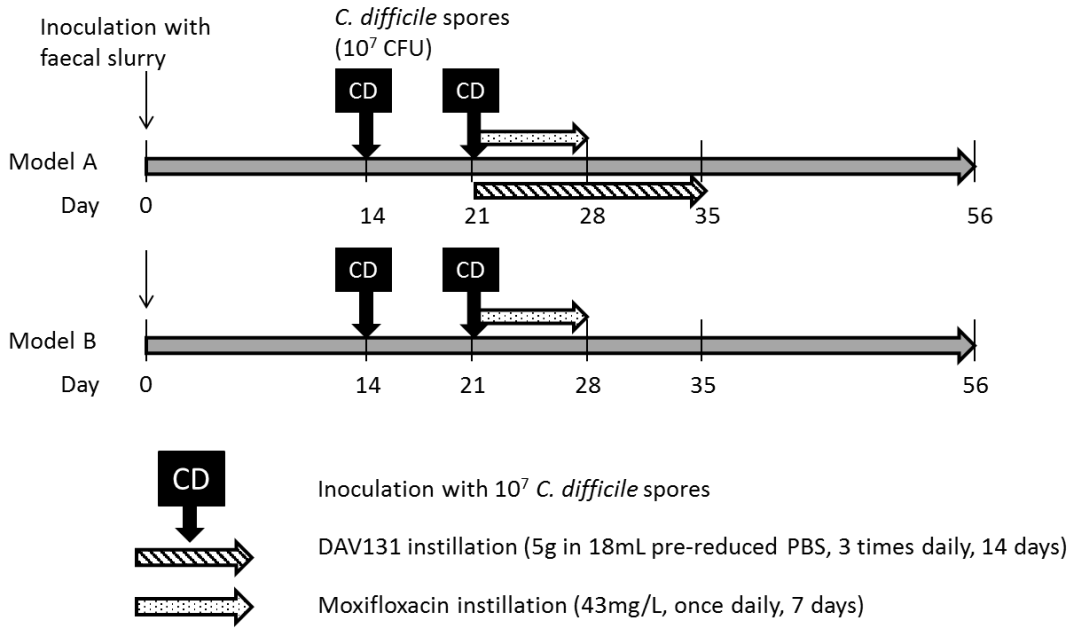
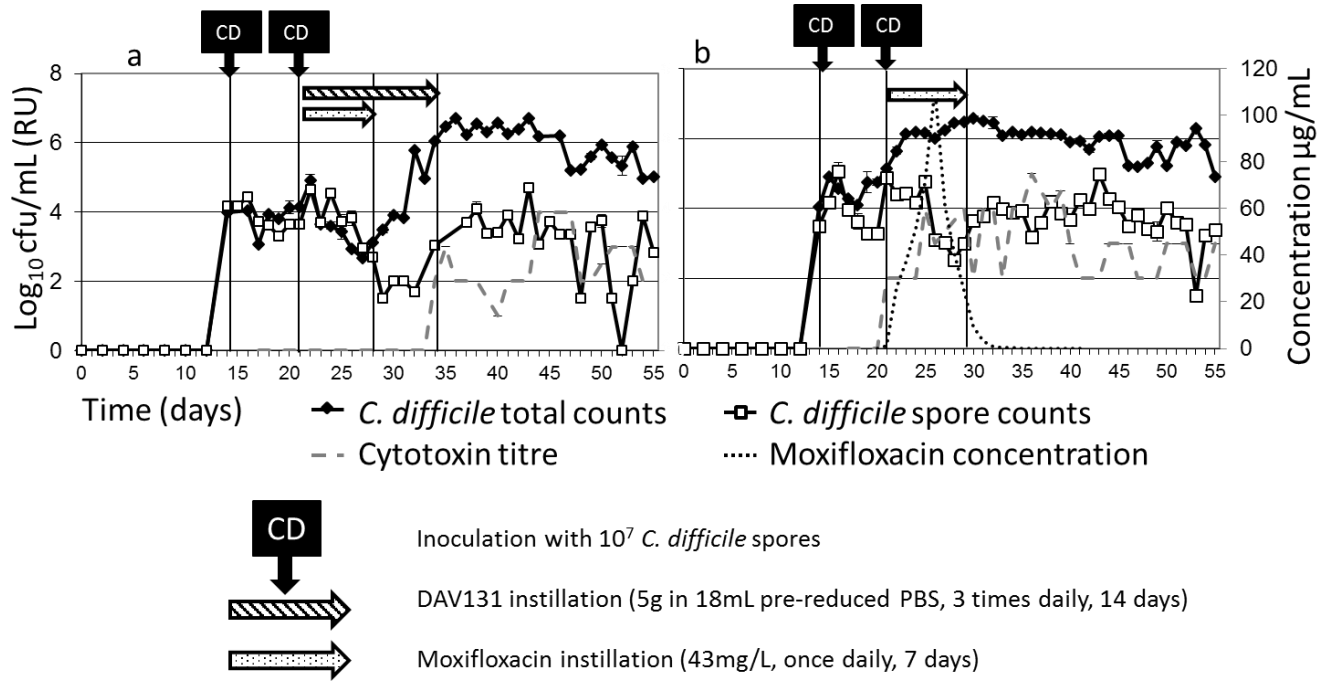


420

421 *Figure 1 – Gut model experimental design.*



422

423 *Figure 2 – Mean (±SE) C. difficile total viable counts (log_{10} cfu/mL), spore counts (log_{10} cfu/mL),*

424 *cytotoxin titre (RU) and moxifloxacin concentration (µg/mL) in vessel 3 of (a) Model A (instilled with*

425 *DAV131A and moxifloxacin) and (b) Model B (instilled with moxifloxacin alone).*

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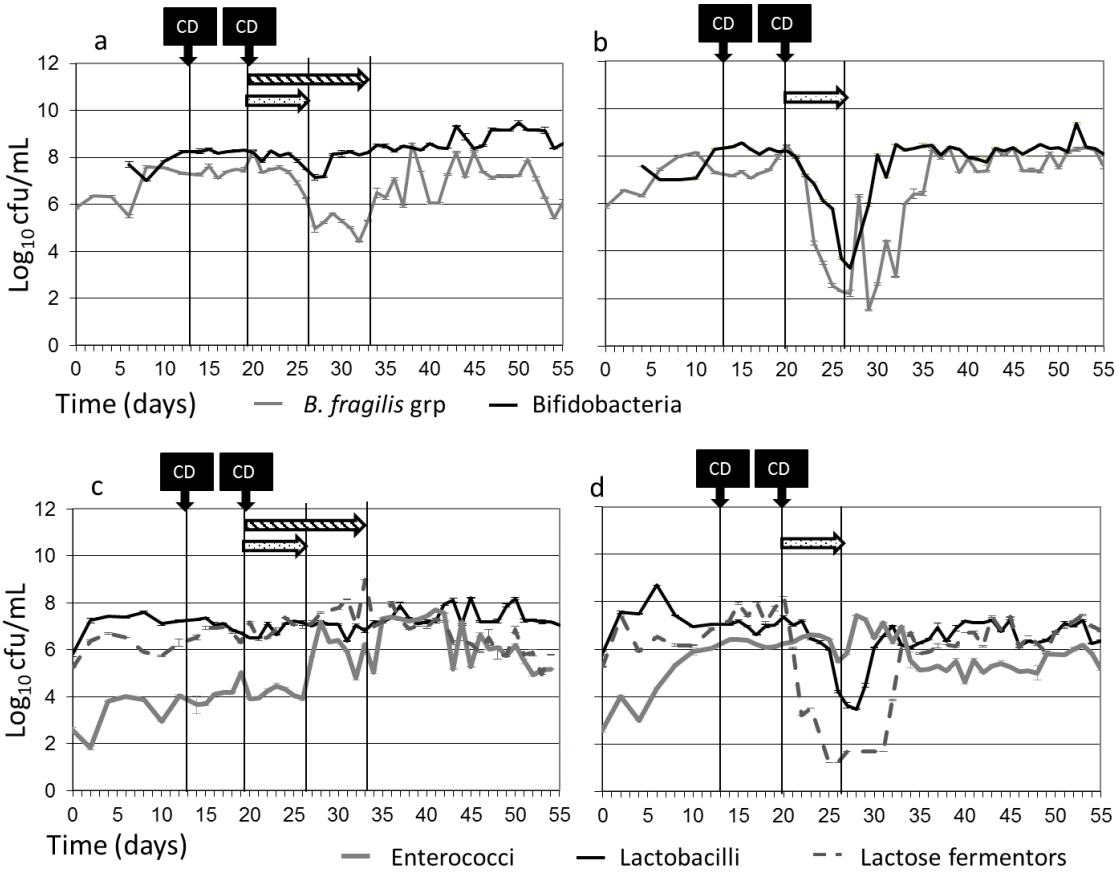

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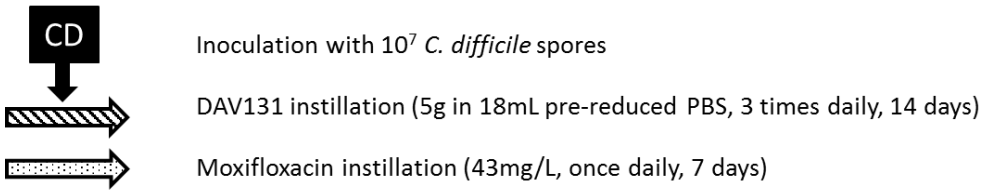


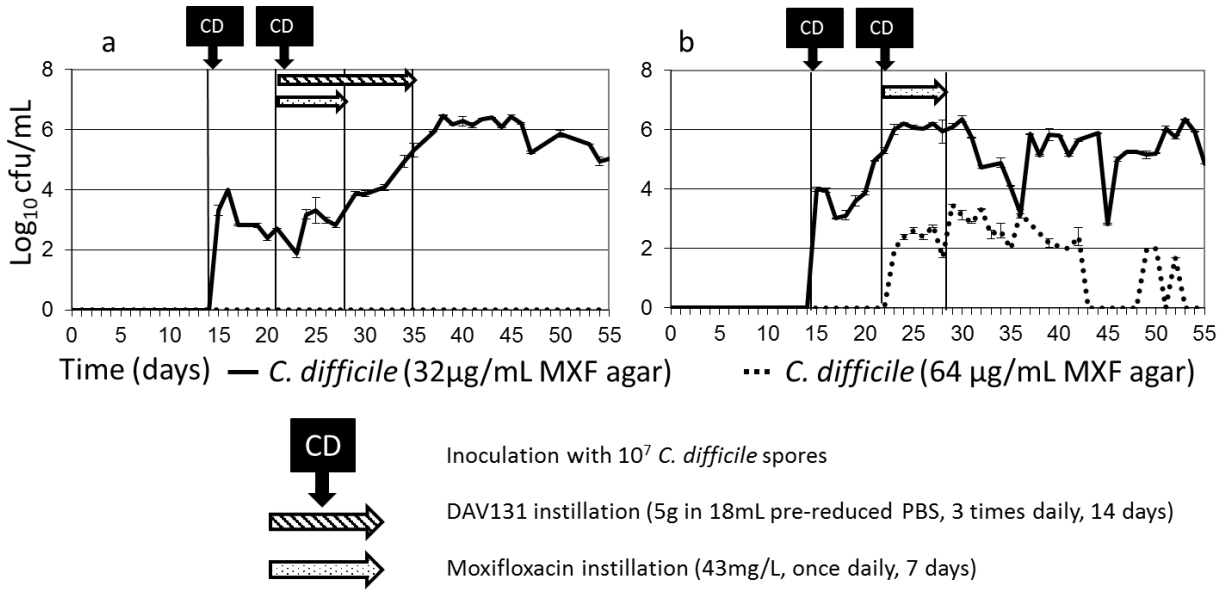
428

429 *Figure 3 – Mean (±SE) viable counts of selected microbiota populations from vessel 3 of the gut*

430 *models. (a) – obligate anaerobes in Model A (instilled with DAV131A and moxifloxacin); (b) obligate*

431 *anaerobes in Model B (instilled with moxifloxacin alone); (c) – facultative anaerobes in Model A; (d) -*

432 *facultative anaerobes in Model B.*

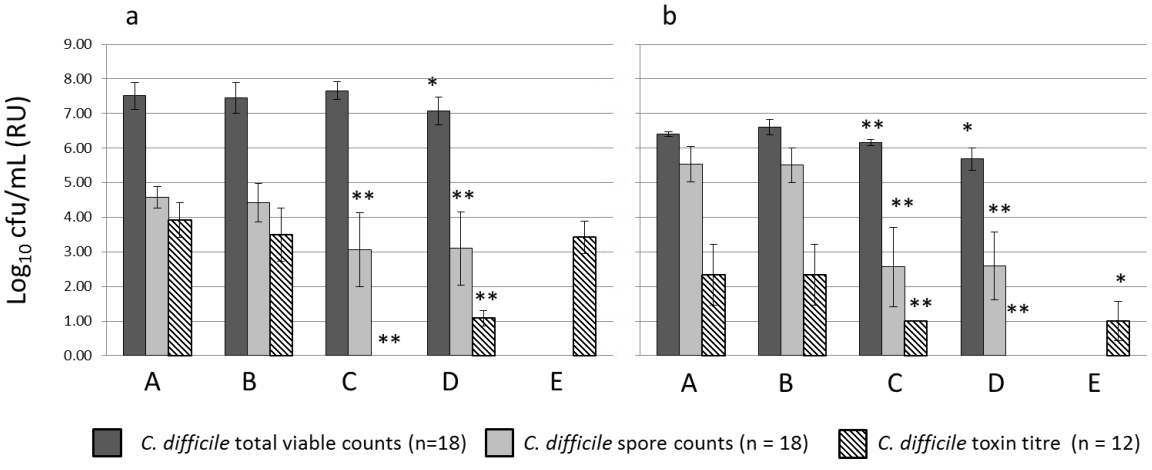


433

434 *Figure 4 – Mean (±SE) C. difficile total viable counts (log_{10} cfu/mL), isolated on breakpoint agar (32*

435 *mg/L and 64 mg/L) from vessel 3 of (a) Model A (instilled with DAV131A and moxifloxacin) and (b)*

436 *Model B (instilled with moxifloxacin alone).*



437

438 *Figure 5 – Mean (±SE) C. difficile total viable counts (log_{10} cfu/mL), spore counts (log_{10} cfu/mL) and*

439 *cytotoxin titre (relative units (RU)) of three different C. difficile strains (of PCR ribotype 027, 001, 078)*

440 *grown in (a) BHI and (b) Faecal filtrate. A = Control (sterile broth); B = Spun control (sterile broth*

441 *centrifuged and filtered through 0.22µm filters before use); C = DAV131A exposed (sterile broth*

442 *exposed to 0.05g/ml of DAV131ADAV131A for 2 hours before use); D = Spun DAV131A exposed*

443 *(sterile broth exposed to 0.05g/ml of DAV131A for 2 hours then centrifuged and filtered through*

*0.22µm filters before use); E = DAV131A exposed supernatant (toxin positive supernatant from control broths incubated anaerobically with 0.05g/mL DAV131A for 2 hours, centrifuged and filtered through 0.22µm filters before use). Significant differences from the control (A) samples are indicated by **(p<0.005) or * (p<0.05). Each strain was assayed in biological duplicate. TVCs were enumerated in technical triplicate (n=18), and toxin in technical duplicate (n=12).*