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Efficacy of vancomycin extended dosing regimens for treatment of simulated *Clostridium difficile* infection (CDI) within an *in vitro* human gut model

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Running title: Extended dosing of vancomycin for treatment of *Clostridium difficile* infection

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Abstract

Objectives: Effects of two vancomycin extended-dosing regimens on microbiota populations within an *in-vitro* gut model of simulated *C. difficile* infection (CDI) were evaluated.

Methods: Two chemostat gut models were inoculated with faecal emulsion, and clindamycin instilled to induce CDI. Simulated CDI was treated with vancomycin (125mg/L four-times daily, 7 days) followed by different vancomycin dosing extensions totalling 7g (lower dose) or 9.5g (higher dose) over 6 weeks in models A and B respectively. Microbiota populations, CD vegetative cells (VC) and spores (SP), cytotoxin (CYT), antimicrobial concentrations, and vancomycin-tolerant enterococci (VTE) were measured every 1-2 days.

Results: In both models, vancomycin instillation caused a rapid decline in VC and CYT, and declines in *Bacteroides fragilis* group, Bifidobacteria, and Clostridia populations to the lower limit of detection. Bifidobacteria failed to recover for the remainder of the experiment. *B .fragilis* group populations recovered to pre-dosing levels during the dosing extension in model A and after dosing ceased in model B. Recurrent CDI was observed on the penultimate day of model B, but not model A. VTE were observed throughout the experiment in both models, but populations increased during- and post-vancomycin instillation.

Conclusion:

The two vancomycin extended-dosing regimens were efficacious in initial treatment of simulated CDI. Both had a prolonged deleterious effect on the indigenous gut microbiota, a factor which may contribute to recurrence; recurrence was observed only in model B, although the potential for vegetative regrowth within model A cannot be excluded. Vancomycin exposure appeared to select for VTE populations.

Introduction

Clostridium difficile is the leading infective cause of antibiotic-associated diarrhoea and a major public health concern. Treatment options for *C. difficile* infection (CDI) are limited. Guidelines published by PHE[1] list extended vancomycin as an option for patients with multiple recurrences, however, no specific regimen is recommended. Tedesco *et al* [2] evaluated a different (lower dose) vancomycin extended-dosing regimen to that outlined by PHE guidelines, and reported a high response rate in 22 patients with CDI. Further studies are required to provide definitive evidence of the efficacy of such regimens.

Colonisation resistance, provided by the gut microbiota, plays an important role in suppression of *C. difficile* proliferation.[3] Recovery of the microbiota following antimicrobial treatment may be of key importance in recurrence prevention, and is likely to differ significantly between individuals depending on host factors, microbiota composition and previous antimicrobial exposure / recurrent episodes. Vancomycin instillation within the gut model elicits profound deleterious effects on the gut microbiota, notably *B. fragilis* group and *Clostridium* spp.,[4] which may contribute to recurrence of vegetative growth;[5-7] vancomycin extended-dosing regimens may further exacerbate this effect. Furthermore, prolonged exposure of gut microbiota to vancomycin may select for vancomycin resistant enterococci (VRE).

Two vancomycin extended-dosing regimens outlined by Tedasco *et al*[2] and PHE guidelines[1] (comprising totals of 7 g[2] (lower-dose) and 9.5 g[1] (higher-dose) vancomycin respectively), were investigated using an *in vitro* gut model of CDI to determine the effects on the gut microbiota, *C. difficile,* and VRE populations.

Methodology

Triple stage i*n vitro* human gut model

Two gut models were set up and run in parallel, as previously described.[8] Models were inoculated with a 10% faecal emulsion prepared from *C. difficile*-negative faeces of three healthy volunteers (≥60 years) with no history of antimicrobial therapy for 3 months.

Experimental Design (Figure 1)

Following inoculation with faecal emulsion models were left without intervention for 2 weeks to reach a steady state (period A). A single aliquot of *C. difficile* PCR ribotype 027 spores[5] (~10^7 cfu) were inoculated into vessel 1. Seven days later a second inoculum of spores (~10^7 cfu), were added to vessel 1 alongside clindamycin (33.9 mg/L, four-times daily, 7 days – period C). After *C. difficile* germination, proliferation and toxin detection (3 relative units), vancomycin instillation commenced (Period E). Initially the vancomycin dosing regimens of the two models were the same (125 mg/L, four-times daily, 7 days). Subsequent dosing is outlined in Figure 1. Following treatment, models were observed for a further 21 days with no interventions.

Enumeration of gut microbiota and *C. difficile* and quantification of toxin and antimicrobial

Indigenous gut microbiota (periods A-F, vessels 2&3), *C. difficile* total viable counts (TVC) and spores (periods A-F, all vessels), cytotoxin (periods B-F, all vessels), and antimicrobial concentrations (periods C-F, all vessels) were monitored daily as previously described in detail.[9]

Vancomycin tolerant enterococci surveillance

Vancomycin tolerant enterococci (VTE) were monitored by enumeration on kanamycin azide agar[9] supplemented with 4 mg/L vancomycin, periodically identified by MALDI-TOF and stored at -80˚C. Enterococci with vancomycin MIC of >4mg/L are described as resistant (EUCAST guidelines[10]). Enterococci isolated on the breakpoint agar utilised here may have an MIC value of ≥4 mg/L, and are described as vancomycin tolerant.

Ethics statement

The collection/use of faecal donations from healthy adult volunteers 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

Vessel 3 represents the distal colon (of most physiological relevance in CDI); therefore results from vessel 3 are reported here.

Antimicrobial concentrations

In both models clindamycin concentrations peaked at 90-100 mg/L and rapidly washed out of the model (figure 2a, 2b). Vancomycin activity peaked at ~190 mg/L within the first week of antibiotic instillation. In the lower-dosage model, vancomycin activity declined during weeks 2-3 of instillation to ~30 mg/L and persisted at this level for weeks 4-6 of treatment, coinciding with instillation of vancomycin every 3 days. In the higher-dosage model vancomycin activity gradually declined for the remainder of the treatment period, as the frequency of dosing decreased.

Effect on simulated *Clostridium difficile* infection

In both models *C.difficile* spores remained quiescent during period B, and were gradually diluted out of the model. Following clindamycin instillation *C. difficile* total viable counts (TVC) increased relative to spore counts, peaking at ~6 $\log_{10}$cfu/mL , and cytotoxin (3 RU) was detected (Figure 2a, 2b). In both models, vancomycin instillation (period E) resulted in a rapid decline in *C. difficile*

vegetative populations and toxin levels. In the lower-dosage model *C.difficile* spores were only sporadically detected at around the limit of detection for the remainder of the experiment. However in the higher-dosage model an increase in *C. difficile* TVCs relative to spores (recurrence of simulated CDI) was observed on the final 2 days of the experiment (days 119-120), peaking at 5.0 log_{10}cfu/mL in vessel 3, although toxin remained undetectable (figure 2a).

Effect of treatment regimens on indigenous gut microbiota and vancomycin tolerant *Enterococci*

In both models, gut microbiota populations reached steady state by the end of period A and remained relatively stable throughout period B. Clindamycin instillation (period C) elicited disruptions similar to those previously reported.[9, 11] Vancomycin instillation resulted in a precipitous decline in *B. fragilis* group populations in both models (figure 2c, 2d). These recovered to pre-vancomycin levels ~ 1 week before the end of the vancomycin dosing in the lower-dosage model, but not until 5 days post treatment (period F) in the higher-dosage model. *Bifidobacterium* spp. declined to below the limit of detection (LOD) (~1.2 log_{10}cfu/mL) in both models and were not detected for the remainder of the experiment.

Prior to vancomycin instillation, VTE were sporadically detected at ~2-3 log_{10}cfu/mL in both models (at least ~2 log_{10}cfu/mL lower than total enterococci populations). However, the proportion of VTEs increased during vancomycin exposure. In both models, total enterococci populations were largely equal to VTE populations (~4-6 log_{10}cfu/mL) following the end of vancomycin dosing (Figure 2e, 2f).

Discussion

Standard vancomycin dosing regimens to treat CDI are associated with failure and recurrent infection.[12-14] Such regimens have been extensively evaluated in the gut model, and whilst they lead to rapid initial clinical cure, recurrent vegetative cell proliferation and toxin production is frequently observed.[4-6, 15, 16] The vancomycin extended-dosing regimens evaluated here also led to a rapid decrease in vegetative *C. difficile* populations and toxin initially, and inhibited growth during instillation. However, in the higher-dosage model, recurrence of *C. difficile* germination and proliferation was observed on the penultimate day of the experiment.

Extended vancomycin instillation had a profound deleterious effect on *B. fragilis* species and *Bifidobacterium* spp. within both models, as previously observed following standard vancomycin

157 instillation.[4, 5, 16,] *Bifidobacterium* spp. failed to recover after vancomycin exposure in both models,
158 which is sometimes[5, 16,] but not always[4, 6,] observed following standard vancomycin instillation.
159 Recovery of *B. fragilis* group populations occurred during vancomycin instillation, but not until the
160 dosing frequency was considerably reduced, and active vancomycin concentrations were ~30-20
161 mg/L (higher-dosage model) and ~66-34 mg/L (lower-dosage model). During standard vancomycin
162 therapy typical active levels are 300-400 mg/L during instillation, and *B. fragilis* species do not
163 recover until approximately 1 week post-standard vancomycin instillation.[4-6, 16]
164
165 The proportion of enterococci showing vancomycin tolerance increased during extended exposure.
166 Tolerant isolates were periodically identified by MALDI-TOF as *Enterococcus casseliflavus*, with a
167 vancomycin MIC of 4-8 mg/L (agar dilution method[17]). Intrinsic low-level glycopeptide resistance is a
168 characteristic of *Enterococcus casseliflavus* and is associated with the *vanC* gene.[18] It is likely that the
169 presence of this VTE strain was due to natural carriage within the donor stool, but its subsequent
170 proliferation was due to the selective pressure of vancomycin.
171
172 In conclusion, like standard vancomycin therapy, the two vancomycin extended-dosing regimens
173 investigated here were efficacious in initial treatment and suppression of *C. difficile* vegetative
174 growth immediately following therapy. The higher-dosage model was exposed to 36% more
175 vancomycin than the lower-dosage model; *B. fragilis* group populations took longer to recover in
176 this model, and recurrent CDI was observed. Although recurrent CDI was not observed following the
177 lower-dosage regimen, the possibility of similar vegetative regrowth with a longer post-treatment
178 observation period cannot be ruled out. Vancomycin exposure appeared to select for VTE
179 populations.
180
181 Data on appropriate strategies to manage patients with recurrent CDI are restricted, with patients
182 often suffering from multiple comorbidities and often on concomitant antibiotics, making recurrent
183 CDI management complex. The data we present here suggest that vancomycin extended therapies
184 (like standard vancomycin therapy) may not be conducive to sustained clinical cure. Compared to
185 standard vancomycin therapy, vancomycin extended-dosing regimens may further exacerbate
186 intestinal microbiota dysbiosis in a dose-dependant manner, and may select for vancomycin
187 resistance. Therefore, other treatment strategies to treat recurrent CDI should be considered;
188 therapeutic options that are less prone to cause microbiota dysbiosis may be preferable considering
189 that CDI usually develops in the setting of antibiotic-mediated microflora disturbance.
190

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Transparency declarations

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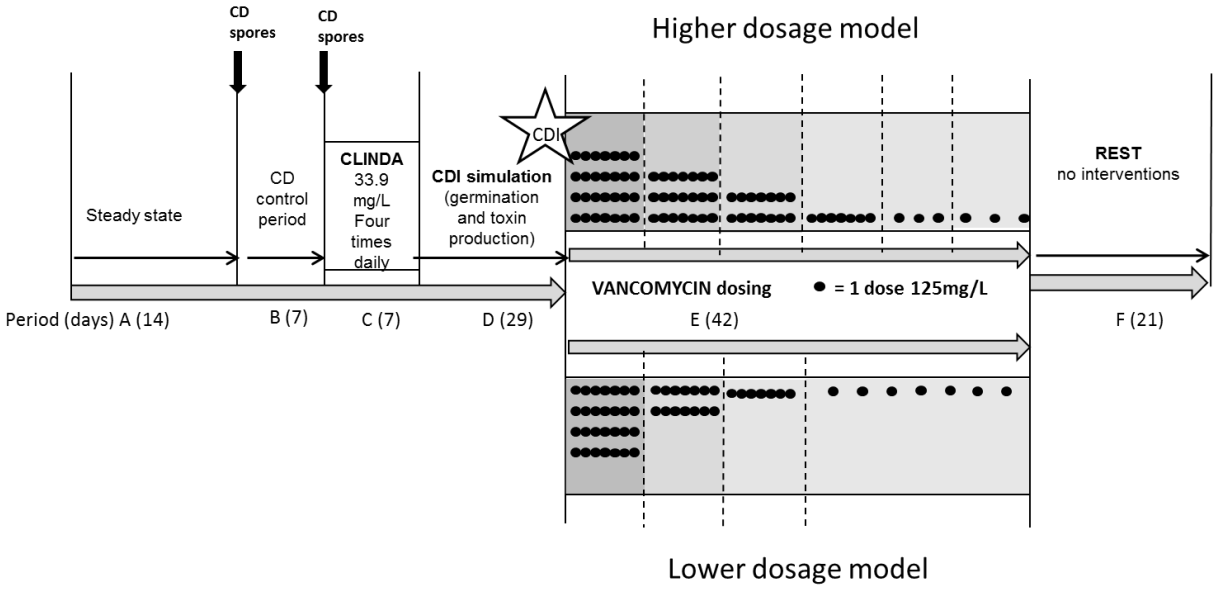
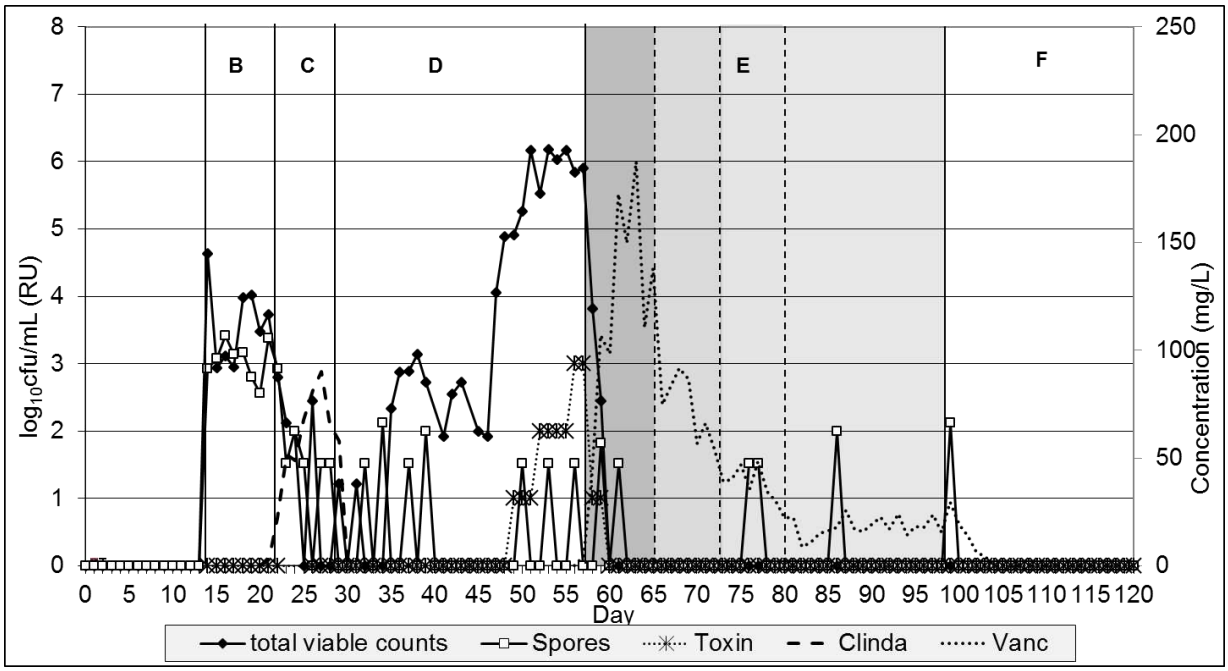
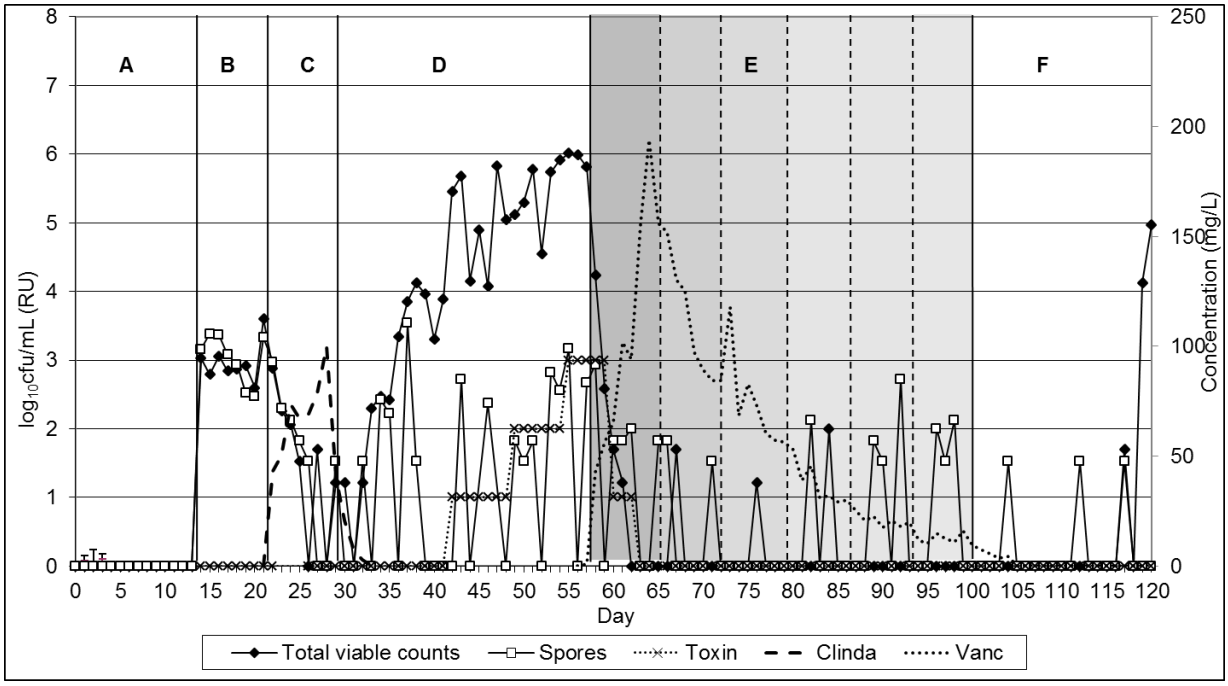


Figure 1: Experimental timeline for the higher and lower dosage gut models.

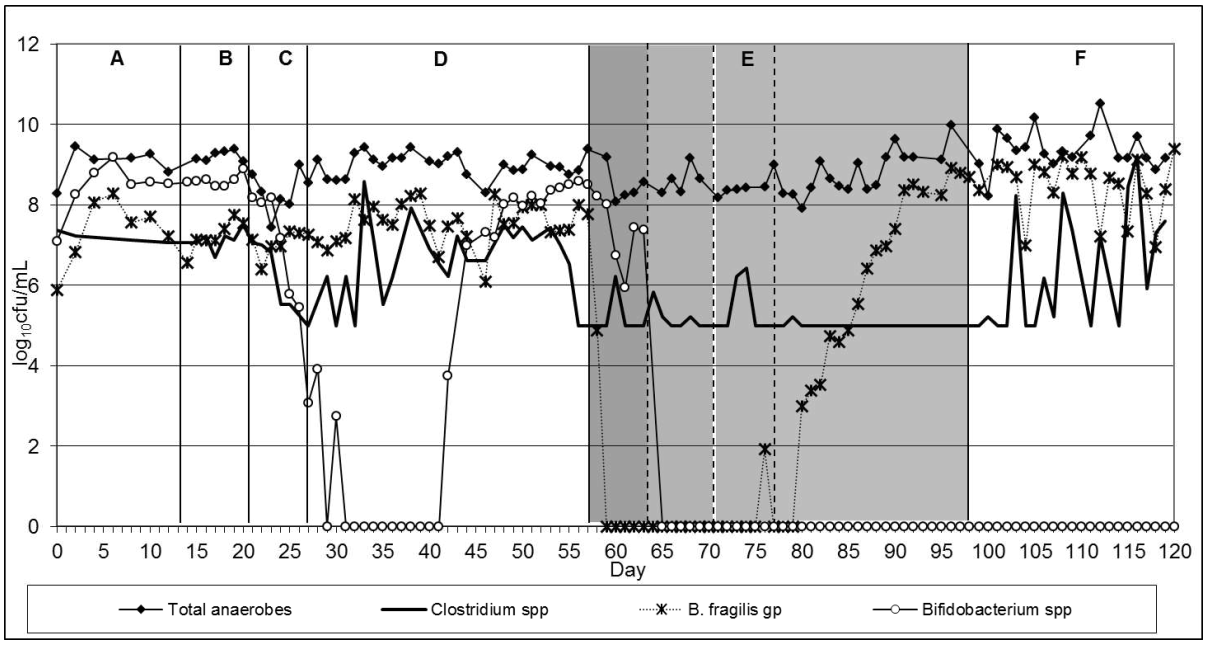
a)



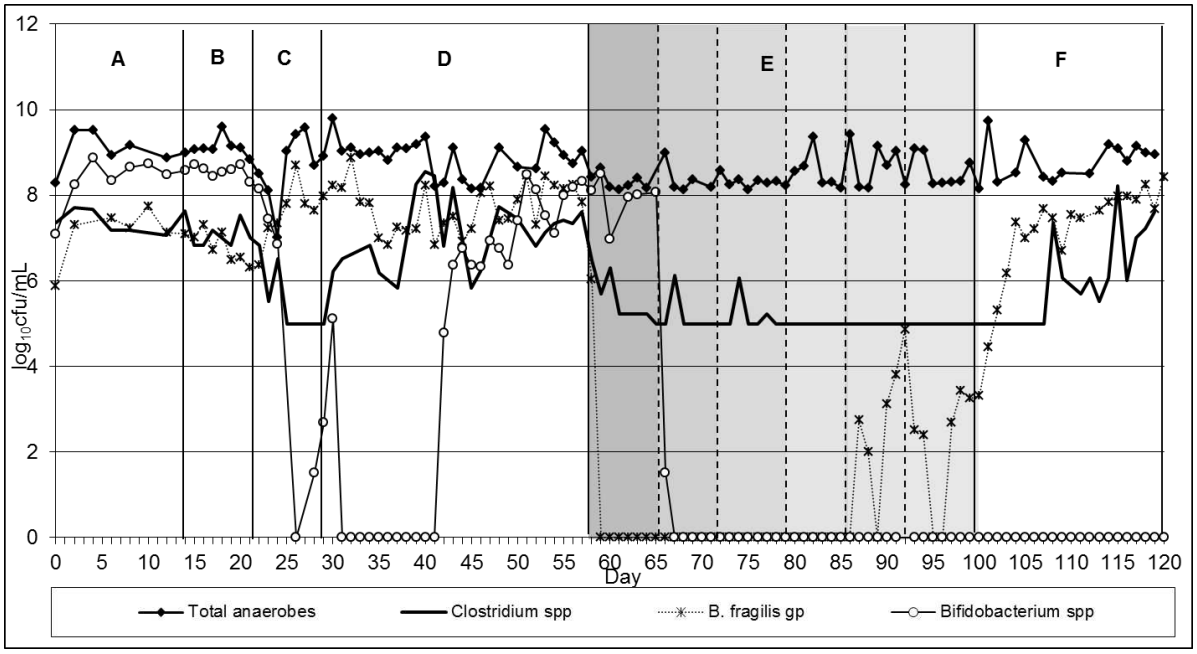
b)


A
B
C
D
E
F
log10cfu/mL (RU)
Concentration (mg/L)
Day
Total viable counts
Spores
Toxin
Clinda
Vanc

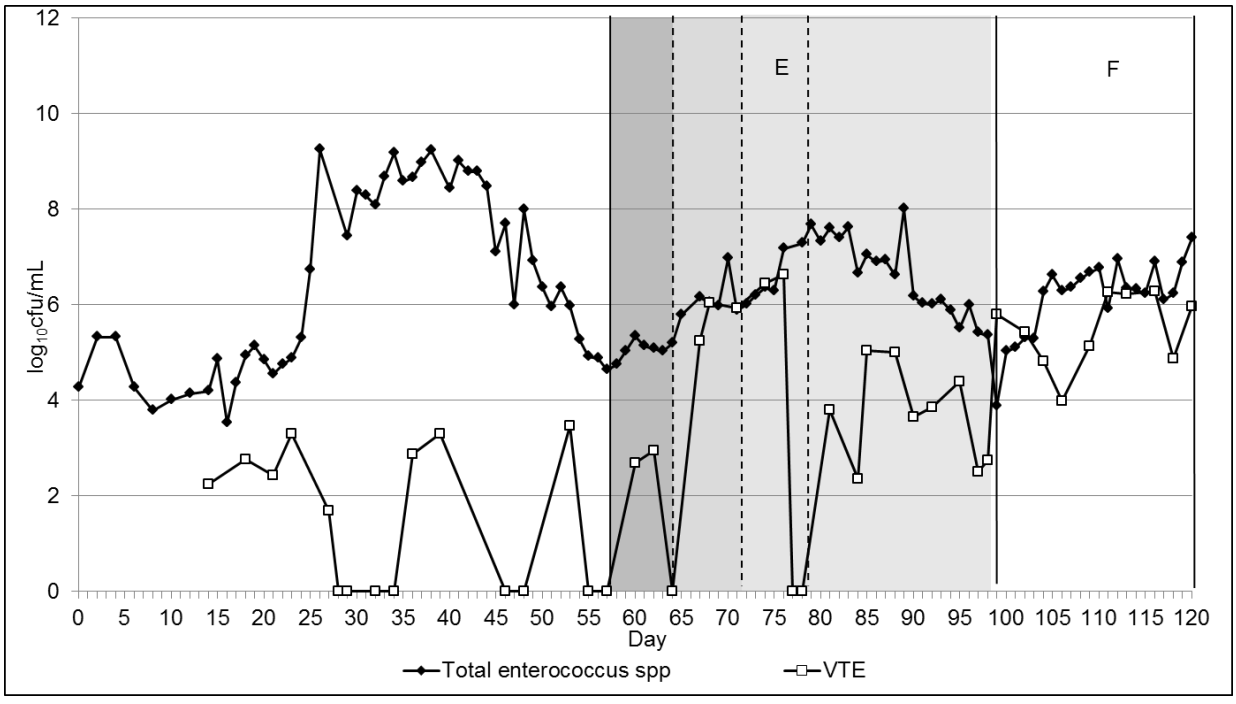
c)


A
B
C
D
E
F
log10cfu/mL
Day
Total anaerobes
Clostridium spp
B. fragilis gp
Bifidobacterium spp

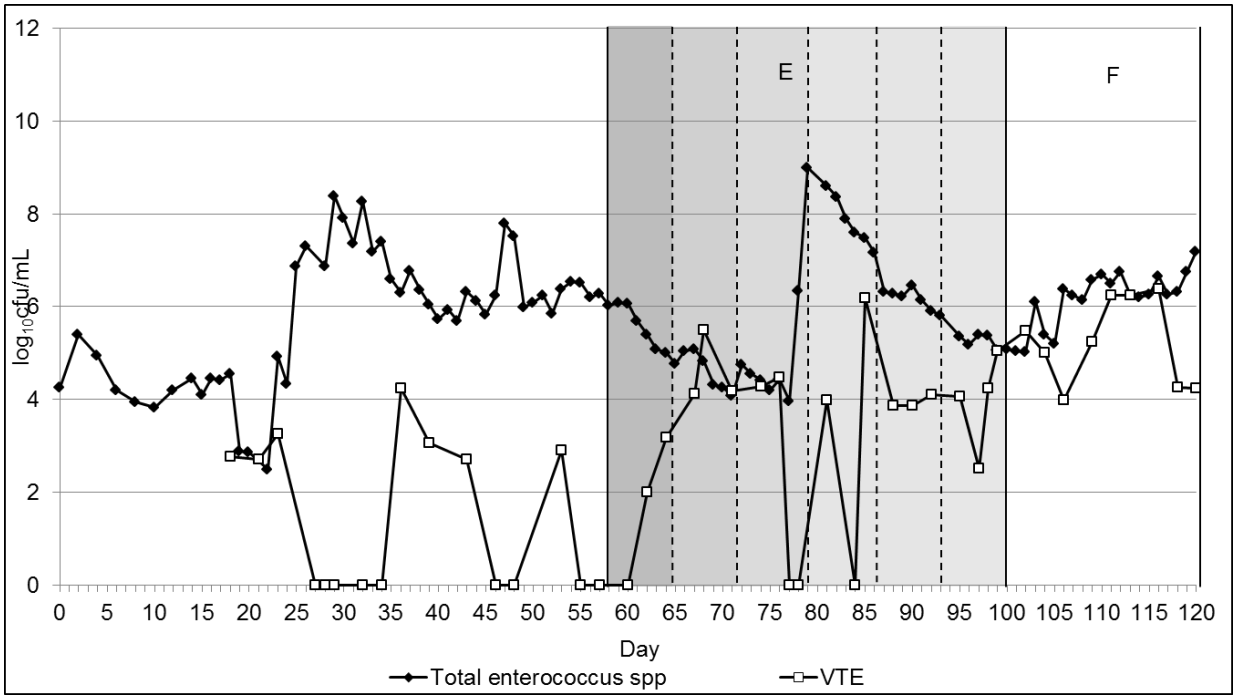
d)


A
B
C
D
E
F
log10cfu/mL
Day
Total anaerobes
Clostridium spp
B. fragilis gp
Bifidobacterium spp

e)


E
F
log10cfu/mL
Day
Total enterococcus spp
VTE

f)



274

275 Figure 2: (a, b) *C. difficile* total viable counts, spores (log_{10}cfu/mL), cytotoxin levels (relative units - RU)
276 and clindamycin (clinda) and vancomycin (vanc) concentrations (mg/L) in vessel 3 of (a) the lower
277 dosage and (b) the higher dosage model. (c, d) Obligate anaerobic indigenous gut microbiota
278 populations (log_{10}cfu/mL) in vessel 3 of (c) the lower dosage and (d) the higher dosage model
279 (*Clostridium* spp. limit of detection = 5.22 log_{10}cfu/mL, limit of detection other microbiota = 1.2
280 log_{10}cfu/mL). (e, f) Total *Enterococcus* spp. and vancomycin tolerant enterococci (VTE) populations in
281 vessel 3 of (e) the lower dosage and (f) the higher dosage models.

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