Comparison of planktonic and biofilm-associated communities of Clostridium difficile and indigenous gut microbiota in a triple-stage chemostat gut model

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Background: Biofilms are characteristic of some chronic or recurrent infections and this mode of growth tends to reduce treatment efficacy. Clostridium difficile infection (CDI) is associated with a high rate of recurrent symptomatic disease. The presence and behaviour of C. difficile within intestinal biofilms remains largely unexplored, but may factor in recurrent infection.

Methods: A triple-stage chemostat gut model designed to facilitate the formation of intestinal biofilm was inoculated with a pooled human faecal emulsion. Bacterial populations were allowed to equilibrate before simulated CDI was induced by clindamycin (33.9 mg/L, four times daily, 7 days) and subsequently treated with vancomycin (125 mg/L, four times daily, 7 days). Indigenous gut microbiota, C. difficile total viable counts, spores, cytotoxin and antimicrobial activity in planktonic and biofilm communities were monitored during the 10 week experimental period.

Results: Vancomycin successfully treated the initial episode of simulated CDI, but ~18 days after therapy cessation, recurrent infection occurred. Germination, proliferation and toxin production were evident within planktonic communities in both initial and recurrent CDI. In contrast, sessile C. difficile remained in dormant spore form for the duration of the experiment. The effects of and recovery from clindamycin and vancomycin exposure for sessile populations was delayed compared with responses for planktonic bacteria.

Conclusions: Intestinal biofilms provide a potential reservoir for C. difficile spore persistence, possibly facilitating their dispersal into the gut lumen after therapeutic intervention, leading to recurrent infection. Therapeutic options for CDI could have increased efficacy if they are more effective against sessile C. difficile.

Keywords: sessile, recurrence, spores

Introduction

Clostridium difficile infection (CDI) is associated with significant morbidity, mortality and financial burden worldwide.1 For many years, metronidazole and vancomycin have been the only first-line treatment options for CDI; notably, both are associated with recurrence in ~20% of patients.2 Fidaxomicin is a new therapeutic option for CDI that is associated with significantly lower recurrence rates for most but not all C. difficile types.3 It is known that antibiotic treatment of CDI does not achieve microbiological cure in some patients even when symptoms do cease.4-5 Persistence of C. difficile spores is believed to be a key explanation for the high rates of recurrent CDI.

The human gastrointestinal tract harbours a complex microbial community, existing both as planktonic, free-floating bacteria in the gut lumen and mucosal, sessile microorganisms present in biofilm structures.6-8 The biofilm mode of growth is ubiquitous in nature and often considered the natural state for bacterial growth. Sessile bacteria often possess distinct characteristics from their planktonic counterparts, such as displaying a reduced susceptibility to antimicrobial agents.9 Intestinal biofilm structures remain largely unexplored, due in part to the physical
inaccessibility of healthy colonic mucosa. However, studies of colonic biopsies have identified distinct differences between mucosal- and faeces-associated bacterial populations.\(^6\)

*C. difficile* is known to adhere to mucus and intestinal cell lines, with the apparent formation of cellular aggregates on the epithelia of diseased mice.\(^{10–12}\) The adherence of pure *C. difficile* cultures to abiotic surfaces has recently been characterized.\(^{13–15}\) However, these simple model systems cannot accurately simulate the complex bacterial communities and environmental conditions of the human colon. Here, we report the use of an *in vitro* model of the human gastrointestinal tract, which has been validated to facilitate the formation and analysis of complex, mixed-species biofilms.\(^{16}\) This model system has been utilized to investigate both planktonic and biofilm-associated communities of *C. difficile* and the indigenous gut microbiota, allowing the evaluation of the possible roles of these respective microbial communities in primary and recurrent CDI. Furthermore, the activity of clindamycin and vancomycin against these two distinct modes of growth has been investigated.

**Methods**

*C. difficile* strains

The *C. difficile* strain evaluated in this study was PCR ribotype 027 (NAP1/BI), isolated during an outbreak of CDI at Maine Medical Centre (Portland, MA, USA) in 2005, and was supplied courtesy of Dr Robert Owens (Maine Medical Centre).

**Triple-stage chemostat biofilm human gut model**

A validated chemostat gut model of simulated CDI has been utilized by our group to investigate the interaction between antimicrobial agents, planktonic indigenous gut microbiota and *C. difficile* populations.\(^{17–20}\) More recently, we have described the adaptation and validation of this original model system to facilitate the independent formation and analysis of mature, mixed-species biofilm formation.\(^{16}\) Briefly, the gut model comprises three pH-maintained (pH 5.5 ± 0.2, vessel 1; pH 6.2 ± 0.2, vessel 2; and pH 6.8 ± 0.2, vessel 3) fermentation vessels inoculated with a faecal emulsion [\(\sim 10\%\) (w/v) in pre-reduced PBS] prepared from *C. difficile*-negative faeces of three healthy elderly (\(\geq 60\) years) volunteers with no history of antimicrobial therapy for \(\geq 3\) months prior to the study. Vessels were connected in a weir cascade formation, maintained within an anaerobic atmosphere and top-fed by a complex growth medium at a controlled rate (\(D = 0.015\ h^{-1}\)). Constituents and preparation of growth medium for the gut model was as described previously.\(^{17}\) Vessel 3 comprised a design that incorporated 18 glass rods to facilitate biofilm formation. Each rod could be removed and analysed independently whilst maintaining the integrity of the system.

**Experimental design**

Following inoculation of faecal emulsion into all three vessels of the model, the media pump was started and the system was left without interventions for 20 days to allow bacterial populations to equilibrate (Figure 1, period A). A single inoculum of *C. difficile* PCR ribotype 027 spores (\(\sim 10^7\) cfu) was inoculated into vessel 1 of the model on day 21 (period B). The system was left without further intervention until rods 1–3 were sampled on day 27 (timepoint Z). On day 28, a further inoculum of *C. difficile* spores (\(\sim 10^7\) cfu) was instilled into vessel 1 to re-establish *C. difficile* populations, along with a dosing regimen of clindamycin (33.9 mg/L, four times daily, 7 days—period C). Rods 4–6 and 7–9 were sampled on days 35 (timepoint Y) and 41 (timepoint X), respectively. Once high-level cytotoxin titre (\(\geq 4\) relative units [RU]) was observed within the planktonic fluid (period D), instillation of vancomycin commenced on day 43 (125 mg/L, four times daily, 7 days—period E). The model was left without intervention for a further 24 days (period F). A further three rods were sampled at each timepoint; W, V and U on days 52, 59 and 73, respectively. Planktonic 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). Sessile indigenous gut microbiota (timepoints Z–U), *C. difficile* total viable counts, spores and cytotoxin (timepoints Z–U) and antimicrobial activity (timepoints Y–U) were monitored during the experiment.

**Enumeration of gut microbiota populations, *C. difficile* total viable counts, spores and cytotoxin titre**

The planktonic and sessile bacterial groups and culture media used in this study are detailed in Table 1. Briefly, planktonic culture fluid was serially diluted in pre-reduced peptone water to \(10^{-7}\) and 20 \(\mu\)L of appropriate dilution inoculated onto quarter plates of each solid culture medium in

![Figure 1](image-url)

**Figure 1.** Experimental design of the triple-stage biofilm gut model of simulated CDI. Vertical lines represent the last day of each period. CD, *C. difficile*; CLI, clindamycin; VAN, vancomycin; QD, four times daily.
**Results**

**Planktonic indigenous gut microbiota populations**

Vessel 3 of the gut model represents the distal colon and is therefore of most physiological relevance to the study of CDI; hence, results from this vessel only will be detailed here. The majority of indigenous gut microbiota bacterial groups reached steady-state midway through period A, with the exception of Enterococcus spp., which continued a gradual increase until the end of period B (Figure 2a).

**Detection and quantification of antimicrobial activity**

One millilitre planktonic (all vessels) and 500 μL sessile (all rods) samples were centrifuged (15 min, 16000 g) and the supernatants sterilized by filtration through 0.22 μm syringe filters and stored at −20°C. The concentration of active clindamycin and vancomycin was determined by separate in-house, large-plate bioassays. Briefly, 1 mL of indicator organism (≏10^8 cfu/mL) was added to 100 mL of molten (50°C) agar (Table 2). Inoculated agars were mixed by inversion, poured into 245 mm agar plates and allowed to solidify for 30 min. Inoculated agars were dried (37°C for 8 h) and a 10 mm diameter stab was made in the agar. Twenty microlitres of serially diluted sample from the gut model was randomly assigned to bioassay wells in triplicate. Bioassay plates remained at ambient temperature for 24 h prior to overnight aerobic incubation at 37°C. Zone diameters were measured using callipers accurate to 0.1 mm. Calibration lines were plotted for squared zone diameters and unknown concentrations from culture supernatants determined. Coefficient-of-variation values were typically 10% and R^2^ values for calibration lines were all ≥0.97. Quantification of biofilm-associated antimicrobial activity was determined using the assumption that 1 g of biofilm was equal to 1 mL of planktonic culture fluid; therefore, the results obtained utilizing this method should be interpreted with caution.

### Table 1. Culture media used for the isolation and enumeration of indigenous gut microbiota and *C. difficile*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Target species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fastidious anaerobe agar (FAA) supplemented with 5% horse blood</td>
<td>total anaerobes and total clostridia</td>
</tr>
<tr>
<td>Bacteroides bile aesculin agar supplemented with 5 mg/L haemin, 10 μg/L vitamin K, 7.5 mg/L vancomycin, 1 mg/L penicillin G, 75 mg/L kanamycin and 10 mg/L colistin</td>
<td>B. fragilis group</td>
</tr>
<tr>
<td>LAMMAB agar: 52.5 mg/L MRS broth and 20 mg/L agar technical supplemented with 0.5 g/L cysteine HCl and 20 mg/L vancomycin, adjusted to pH 5</td>
<td><em>Lactobacillus</em> spp.</td>
</tr>
<tr>
<td>Beerens agar: 42.5 mg/L Columbia agar and 5 mg/L agar technical supplemented with 5 mg/L glucose, 0.5 g/L cysteine HCl and 5 mL propionic acid, adjusted to pH 5</td>
<td><em>Bifidobacterium</em> spp.</td>
</tr>
<tr>
<td>Nutrient agar</td>
<td>total facultative anaerobes</td>
</tr>
<tr>
<td>MacConkey agar</td>
<td>lactose-fermenting <em>Enterobacteriaceae</em></td>
</tr>
<tr>
<td>Kanamycin aesculin azide agar supplemented with 10 mg/L nalidixic acid, 10 mg/L aztreonam and 20 mg/L kanamycin</td>
<td><em>Enterococcus</em> spp.</td>
</tr>
<tr>
<td>Alcohol shock followed by Brazzer’s CCEY agar supplemented with 2% lysed horse blood, 5 mg/L lysozyme, 250 mg/L cycloserine and 8 mg/L cefoxitin</td>
<td><em>C. difficile</em> spores</td>
</tr>
<tr>
<td>Brazzer’s CCEY agar as described above supplemented with 2 mg/L moxifloxacin</td>
<td><em>C. difficile</em> total viable counts</td>
</tr>
</tbody>
</table>

All agar bases are supplied by Oxoid, with the exception of CCEY and FAA supplied by LabM, and made according to the manufacturer’s instructions.

### Table 2. Indicator organisms and agars used in large plate bioassay

<table>
<thead>
<tr>
<th>Target antimicrobial agent</th>
<th>Indicator organism</th>
<th>Agar</th>
<th>Range of concentrations evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clindamycin</td>
<td><em>Kocuria rhizophila</em> ATCC 9341</td>
<td>Wilkins-Chalgren</td>
<td>2–128 mg/L</td>
</tr>
<tr>
<td>Vancomycin</td>
<td><em>S. aureus</em> ATCC 29213</td>
<td>Mueller-Hinton</td>
<td>8–512 mg/L</td>
</tr>
</tbody>
</table>

triplicate. Inoculated plates were incubated anaerobically (obligate anaerobes) or aerobically (facultative anaerobes) at 37°C for 48 h. Bacterial colonies were identified, based on colony morphology, Gram stain reaction and microscopic morphology, and enumerated. Indigenous gut microbiota populations from vessel 1 of the gut models were not determined because the proximal colon is of less physiological relevance in CDI than the distal colon.

Biofilm-associated bacterial populations were enumerated following removal of rods from vessel 3 of the model. Rods were transferred into 5 mL of pre-reduced saline, thoroughly vortexed for ~2 min and the rod discarded. Total resuspended biofilm was then centrifuged at 4000 g for 10 min and the pellet resuspended in 2 mL of pre-reduced saline.

Planktonic and biofilm aliquots were centrifuged and stored at 4°C for cytotoxin assay as described previously and −20°C for quantification of antimicrobial activity. Planktonic and biofilm aliquots (1 mL) were centrifuged (16 000 g, 10 min) in a pre-weighed Eppendorf and the pellet weight determined. Gut microbiota and *C. difficile* populations were enumerated as described for planktonic populations and units were reported as log_{10} cfu/g of culture pellet weight.
and b). The instillation of C. difficile spores on day 21 (period B) did not appear to affect gut microbiota populations, with levels remaining steady. Clindamycin instillation (Figure 2a and b, period C) elicited a precipitous decline in Bifidobacterium spp. (≏8 log_{10} cfu/mL decline), with populations declining to below the limit of detection (LOD) 4 days after instillation commenced. A less marked decline was evident in Bacteroides fragilis group (≏1 log_{10} cfu/mL), Clostridium spp. (≏2 log_{10} cfu/mL) and Lactobacillus spp. (≏1.5 log_{10} cfu/mL). Vancomycin instillation (Figure 2a and b, period E) commenced 8 days after clindamycin cessation and resulted in profound decreases in viable counts of B. fragilis group (≏6–6.5 log_{10} cfu/mL) and Bifidobacterium spp. (≏6 log_{10} cfu/mL). Smaller declines in Enterococcus spp., lactose-fermenting Enterobacteriaceae and Clostridium spp. were evident, although Lactobacillus spp. populations increased (≏1–1.5 log_{10} cfu/mL). Most bacterial groups recovered to or exceeded pre-vancomycin levels ~9 days after dosing cessation, except Bifidobacterium spp., which recovered after 18–21 days (period F).

Figure 2. Mean planktonic populations (log_{10} cfu/mL) of (a) facultative and (b) obligate anaerobic indigenous gut microbiota in vessel 3 of the triple-stage biofilm gut model. Z–U biofilm sampling points.
Sessile indigenous gut microbiota populations

Rod-associated bacterial populations were monitored at six different timepoints (Z–U, Figure 3a and b). Bacterial group populations on each rod at timepoint Z displayed little variation (Figure 3a and b). Lactose-fermenting Enterobacteriaceae (mean $9.2 \log_{10}$ cfu/g) dominated sessile bacterial populations, with $B. fragilis$ group and $Lactobacillus$ spp. (mean $8.1$ and $8.2 \log_{10}$ cfu/g, respectively) the least populous groups.

Clindamycin instillation commenced 8 days prior to timepoint Y. Sessile $Bifidobacterium$ spp. populations were diminished (≏$4.5 \log_{10}$ cfu/g), but maintained an average population of $3.9 \log_{10}$ cfu/g. More modest decreases in sessile $Lactobacillus$ spp. (≏$1.5 \log_{10}$ cfu/g), $Clostridium$ spp. (≏$1 \log_{10}$ cfu/g), $Enterococcus$ spp. (≏$1 \log_{10}$ cfu/g) and $B. fragilis$ group (≏$1 \log_{10}$ cfu/g) were also seen. Sessile populations at timepoint X remained at similar levels to those at timepoint Y, although $Bifidobacterium$ spp. increased by $\sim 1.5 \log_{10}$ cfu/g. Vancomycin instillation commenced 10 days before biofilm sampling point W, resulting in a marked decline in sessile $B. fragilis$ group and $Bifidobacterium$ spp. to below the LOD and lactose-fermenting Enterobacteriaceae by $\sim 2 \log_{10}$ cfu/g, whilst $Lactobacillus$ spp. increased by $\sim 2.5 \log_{10}$ cfu/g. Sessile $Bifidobacterium$ spp. populations remained below the LOD.

Figure 3. Mean (±SE) facultative obligate (a) and obligate (b) anaerobic microbiota populations ($\log_{10}$ cfu/g) from rod/biofilm sampling points Z–U within vessel 3 of the triple-stage biofilm human gut model. Planktonic populations of each bacterial group ($\log_{10}$ cfu/g) present at each timepoint are represented by a single line. The horizontal broken line represents the approximate LOD.

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at timepoint V. *B. fragilis* group began to recover, with sessile populations increasing to $5.1 \log_{10} \text{cfu/g}$. All bacterial groups re-established sessile populations by the completion of the experiment, although facultative anaerobic groups and *B. fragilis* group and *Bifidobacterium* spp. populations remained marginally inferior to initial (timepoint Z) levels.

**Planktonic C. difficile populations**

*C. difficile* populations comprised primarily of spores during the internal control period (period B, Figure 4), declining from $\sim 5.2$ to $\sim 4 \log_{10} \text{cfu/mL}$ as the vessel contents were diluted. Cytotoxin was not detected (period B). A second inoculum of *C. difficile* spores concurrent with the start of clindamycin dosing (period C) was instilled on day 28. *C. difficile* populations initially existed primarily as spores until a marked increase in total viable counts relative to spore counts was observed 5 days after clindamycin instillation cessation. Total viable counts peaked at $\sim 6.5 \log_{10} \text{cfu/mL}$ 1 day before the cytotoxin titre peaked at 4 RU. Vancomycin instillation (period E) elicited a rapid decline in total viable counts ($\sim 2 \log_{10} \text{cfu/mL}$), with only spores remaining after 2 days. Cytotoxin decreased to below the LOD 5 days after the start of vancomycin instillation. Total viable counts increased relative to spores $\sim 18$ days after vancomycin cessation. A subsequent increase in cytotoxin was observed, reaching peak titres of 4 RU by the end of the experiment.

**Sessile C. difficile populations**

Rod-associated *C. difficile* populations remained primarily as spores for the duration of the experiment (Figure 5). At timepoint Z, *C. difficile* rod-associated population levels were consistent across the three rods and existed primarily as spores ($\sim 5.8 \log_{10} \text{cfu/g}$). Instillation of a second inoculum of *C. difficile* spores did not greatly affect sessile *C. difficile* populations, although variation in populations (as measured by standard error (SE)) between different rods increased modestly (SE timepoint Z, $\pm 0.05$–$0.09 \log_{10} \text{cfu/g}$; SE timepoint Y, $\pm 0.36$–$0.37 \log_{10} \text{cfu/g}$). Cytotoxin was not detected within biofilm structures at timepoints Z and Y. During simulated planktonic CDI, sessile *C. difficile* (timepoint X) continued to maintain a stable population, comprised primarily of spores ($\sim 5.5 \log_{10} \text{cfu/g}$). Cytotoxin was detected at low levels (1 RU) within one of the three rods. During timepoints W and V, *C. difficile* populations remained as spores at $\sim 5.5 \log_{10} \text{cfu/g}$ with no toxin detected. During recurrent planktonic infection (timepoint U), sessile *C. difficile* populations remained in spore form with only a modest decline ($\sim 0.5 \log_{10} \text{cfu/g}$). Cytotoxin was detected in one of the three rods at low levels (2 RU).

**Antimicrobial activity**

Clindamycin was detected within the planktonic phase of all three vessels of the gut model, peaking at a concentration of 64.9, 50.7 and 50.8 mg/L in vessels 1, 2 (data not shown) and 3 (Figure 4), respectively. Clindamycin activity in the planktonic phase continued to be detected 1, 2 and 3 days after dosing cessation in vessels 1, 2 and 3, respectively. Rod-associated biofilm at timepoint Y (day 35) was sampled and analysed 1 day after dosing cessation. Clindamycin activity was not detected within rod-associated biofilm structures. Vancomycin activity was detected within the planktonic fluid throughout period E and continued to be detected for...
3 days after dosing cessation in all three vessels (Figure 4). Concentrations peaked on the final day of instillation (day 49) at concentrations of 330.2, 317.8 and 270.3 mg/L in vessels 1, 2 (data not shown) and 3 (Figure 4), respectively. Vancomycin activity within biofilm structures per rod (timepoint W, day 52) ranged from 38.7 to 43.4 mg/L (Figure 6; mean 40.4 mg/L). Vancomycin activity on day 52 in the planktonic phase of vessel 3 was 6.05 mg/L.

Discussion

Recurrence of symptoms is a disruptive and costly feature of CDI. Biofilm communities are known to play a role within chronic infection, such as chronic otitis media and periodontitis, and we therefore hypothesized that biofilm structures within the gut could play a role in the recurrence of CDI. We have recently validated the chemostat gut model to facilitate the formation and analysis of mature, mixed-species intestinal biofilms. Using our original CDI gut model, we have previously simulated recurrent infection after initial successful vancomycin treatment, but did so without specific measurement and study of the planktonic versus sessile modes of growth. The modified biofilm gut model has now been utilized to investigate the behaviour of C. difficile and the indigenous gut microbiota in these distinct modes of growth during CDI.

In the absence of antimicrobial intervention (period B), sessile and planktonic gut microbiota and C. difficile behaved in a similar manner. Planktonic populations of obligate anaerobe species were marginally greater than sessile populations, with the converse true for facultative anaerobe populations. A similar trend was previously observed during biofilm model validation studies. Increased sessile compared with planktonic facultative anaerobe populations have been previously observed both in vivo and in vitro. Whilst there are consistencies here, we cannot rule out the possibility that the observation may be (in part) mediated by experimental methods, i.e. increased aerobic handling time required to process biofilm samples. In addition, it should be noted that in order to compare planktonic and biofilm populations, the weight of the pellet from a 1 mL suspension of planktonic culture fluid or resuspended biofilm was used to determine the cfu per gram of biomass. Planktonic cultures are likely to contain a high proportion of bacteria within this biomass whilst biofilm comprises extracellular material in addition to the bacterial mass.
The effects of clindamycin instillation on planktonic gut microbiota populations within the model were similar to those observed previously within the original gut model,28 with a notable deleterious effect on *Bifidobacterium* spp. and more modest declines in *B. fragilis* group and *Clostridium* spp. Similar declines in these groups were also evident in sessile communities. However, although planktonic *Bifidobacterium* spp. were below the LOD at timepoint Y, sessile communities, whilst reduced from ~8.6 log cfu/g, were still detected at ~3.9 log_{10} cfu/g. At biofilm sampling point Y (period D), planktonic *B. fragilis* group and *Clostridium* spp. had recovered to pre-clindamycin levels. However, sessile populations of these groups remained at decreased levels relative to pre-dosing populations. The reduced susceptibility of biofilms to antimicrobial agents is a well-defined characteristic of sessile communities.29 To date, little work exists investigating the exposure of mixed-species intestinal biofilms to antimicrobial agents, with the majority of groups focusing on single species biofilms. Here, we present evidence of the differences in behaviour of sessile and planktonic gut microbiota populations in response to antimicrobial exposure within mixed-species biofilms. We postulate that clindamycin elicits an antimicrobial effect on both communities, although the effects of and recovery from antimicrobial exposure for sessile populations appears to be delayed compared with responses for planktonic bacteria. Clindamycin was used to induce *C. difficile* germination, proliferation and toxin production. Simulated planktonic CDI was evident 5 days after cessation of clindamycin dosing, whilst gut microbiota remained perturbed. Clindamycin demonstrates an in vitro activity against vegetative *C. difficile* (PCR ribotype 027 planktonic MIC of 1 mg/L), potentially preventing germination until only sub-MIC levels remained within the system. This was achieved 4 days after dosage cessation within vessel 3.

The behaviour of *C. difficile* within planktonic communities was as previously observed.28,30 However, distinct differences between the planktonic and biofilm modes of growth were evident, with biofilm-associated *C. difficile* communities remaining in spore form for the duration of the experiment, despite germination and proliferation of planktonic *C. difficile* populations. Mechanisms and conditions necessary to induce CDI remain to be fully elucidated. CDI is frequently associated with the use of antimicrobial agents, leading to the assumption that the disruption of the gut microbiota may play a role in the pathogenesis of *C. difficile*. Clindamycin-induced perturbation of planktonic gut microbiota is evident within the gut model. Instillation of spores concurrent with administration of clindamycin consistently leads to CDI within the gut model, whilst this is not the case in the absence of antimicrobial administration. This gut microbiota disruption was also evident within sessile communities but did not elicit germination and proliferation of sessile *C. difficile*. On day 35 (sample point Y), clindamycin was detected within planktonic fluid at a level of 50.8 mg/L, but no antibiotic activity was detected within biofilm structures. Despite the absence of detectable clindamycin within biofilm, antimicrobial disruption of sessile communities was observed. However, it should be noted that the protocol used to resuspend rod-associated biofilm in this study included multiple centrifugation and vortexing steps. As a result, only toxin and antimicrobial agents securely bound to biofilm structures will be detected using the cytotoxin assay and antimicrobial bioassay.

Studies on exposure of *Staphylococcus aureus*31 and *Propionibacterium acnes*32 biofilms to clindamycin highlight the differences in planktonic MIC/sessile MBC values (0.25/>128 and 0.125/512 mg/L, respectively), indicating the reduced susceptibility of sessile versus planktonic bacteria to antimicrobials. Another study reported that 20%–99% (mean 62%) of *S. aureus* biofilm-associated cells survived after clindamycin exposure.33 This highlights the complex and variable nature of the biofilm mode of growth. Heterogeneity in metabolic and reproductive activity exists amongst cells located in different parts of biofilm. We clearly observed a differential response of sessile bacteria to antimicrobial administration, with *C. difficile* spores being largely unresponsive either to clindamycin instillation or perturbation of gut microbiota.

Dawson et al.13 demonstrated that 6-day-old biofilms contained greater numbers of spores compared with 3-day-old biofilms, thus providing evidence of heterogenous *C. difficile* composition in distinct biofilm lifecycle stages. In addition, Ethapa et al.15 demonstrated that *C. difficile* SpOA mutants were defective for biofilm formation, demonstrating a possible link between biofilm formation and sporulation in *C. difficile*. Similar observations in other spore-forming sessile bacteria have been noted.14–16 However, Ethapa et al.15 demonstrated extremely low numbers of spores in biofilm and planktonic phases. It is possible that germinants required for efficient germination may be prevented from accessing the spores within biofilms. In addition to the putative role of SpoOA in biofilm formation, Deakin et al.37 have demonstrated that SpoOA is required for recurrence of CDI within a murine model. Whilst the majority of spores within a planktonic population initiate rapid germination in response to germinants, a subpopulation of spores is refractory to these stimuli and the germination process is slow. This response is termed ‘superdormancy’ and has been most extensively characterized in *Bacillus* spp.38–40 The persistence of a superdormant fraction of *C. difficile* spores after exposure to germinants has been described,41 although understanding in this area remains basic. Sessile spores may display analogies to superdormant spores and display increased recalcitrance to germination, resulting in high spore populations.

Following planktonic *C. difficile* germination, proliferation and toxin production, planktonic *Bifidobacterium* spp. populations began to recover to pre-clindamycin levels, although this was at a greater rate than sessile recovery, providing further evidence for the delayed reaction of sessile versus planktonic communities. The increase in biofilm populations is likely mediated predominantly by recruitment of planktonic cells rather than the active proliferation of sessile populations, due to the inherent dormant, slow-growing nature of the latter communities. Recruitment of planktonic cells requires the planktonic population to increase in number to facilitate transfer to the biofilm mode of growth, possibly explaining the delayed recovery of sessile compared with planktonic communities.

Planktonic *C. difficile* viable counts declined rapidly and converged with spore counts 2 days after exposure to vancomycin. Cytotoxin was reduced to undetectable levels 5 days after vancomycin commencement. Sessile *C. difficile*, which remained as spores, were not affected by vancomycin instillation, potentially due to the absence of antispor activity of vancomycin. Vancomycin instillation elicited a marked deleterious effect on planktonic *B. fragilis* group and *Bifidobacterium* spp. and increase in *Lactobacillus* spp., as previously observed within the gut model.18,30 Similar declines and increases in sessile communities were also observed during period W (and period V), demonstrating
the proficient effect of vancomycin on sessile populations. Delay in the response of sessile communities to vancomycin instillation was not evident at timepoint W, despite a post-clindamycin delay observed at timepoint Y. This may be due to different antimicrobial agents eliciting different responses in sessile communities or due to differential timing of sampling. Timepoint W was 3 days after cessation of vancomycin, whereas timepoint Y was only 1 day after clindamycin cessation. It is possible that a delay in the response of sessile communities to vancomycin may still occur pre-timepoint W, but may no longer be apparent by the time rods were sampled. Additionally, it has been reported that vancomycin displays a more profound action against sessile communities compared with clindamycin.\(^{33}\) Vancomycin activity within the rod-associated biofilm structures at timepoint W averaged \(\sim 40\) mg/L, demonstrating the successful diffusion of this agent into biofilm structures. This level is similar to the in vitro MIC (16–32 mg/L) of vancomycin for \textit{B. fragilis}. Increased vancomycin concentration within biofilms versus the planktonic phase, as observed within this study, has previously been reported.\(^4\) One study reported that exposure of sessile \textit{Staphylococcus epidermidis} to increasing concentrations of vancomycin elicited greater antibiotic levels within biofilms.\(^{43}\)

Vancomycin treatment of simulated CDI was initially successful, but planktonic \textit{C. difficile} populations remained as spores after cessation of antibiotic instillation. Recurrence of CDI was evident \(\sim 18\) days after vancomycin cessation, with an increase in \textit{C. difficile} viable counts and toxin. Sessile \textit{C. difficile} populations remained in spore form throughout. Population levels were maintained at \(\sim 5 \log_{10}\) cfu/g from timepoint Z through to U, despite fluctuations in planktonic populations, demonstrating the highly stable nature of sessile \textit{C. difficile} populations. Early signs of CDI recurrence were evident on day 67 (18 days after vancomycin instillation ceased). However, at this point most gut microbiota populations had recovered to pre-dosing levels. \textit{Bifidobacterium} spp. levels began to recover, reaching populations of \(\sim 6.5 \log_{10}\) cfu/g on day 67. Despite recovery of gut microbiota, subsequent CDI recurrence was evident, suggesting recurrence is a complex process not only reliant on gut microbiota-associated colonization resistance.

Biofilm dispersal mechanisms are important factors for microbial survival, allowing single organisms or biofilm aggregates to colonize new niches. Many factors are thought to mediate this dispersal, including regulatory systems (e.g. quorum sensing),\(^{44}\) active dispersal mechanisms (e.g. matrix-degrading enzymes)\(^{45}\) and changes in nutrient levels.\(^{46}\) Various dispersal mechanisms are in continued operation within the biofilm lifecycle. The persistence of a stable population of \textit{C. difficile} spores within biofilms was observed in this study. Within \textit{Bacillus subtilis}, gene expression is described as a dynamic process during biofilm development. Cells expressing certain genes localized at distinct regions of a biofilm, with spores located on the outer edge of biofilm structures. This provides an ideal nidus for \textit{B. subtilis} spores and their seeding into the surrounding environment. \textit{B. subtilis} and \textit{C. difficile} sporation (and potentially biofilm formation) are regulated by Spo0A, for which they share 56% amino acid homology. \textit{C. difficile} may potentially display similar cellular expression pathways and spatial biofilm architecture to \textit{B. subtilis}, thereby providing a mechanism for reseeding of \textit{C. difficile} spores into the planktonic form.

The maintenance of a dormant \textit{C. difficile} spore state within the modified biofilm model, but presence of low levels of biofilm-associated toxin and high levels of planktonic toxin, indicates the diffusion of planktonic-associated toxin into the biofilm. Mucosal biofilms potentially cover toxin receptors; therefore, the presence of toxin within these structures may be required to localize toxin in close proximity to receptors. However, the persistence of biofilm-associated toxin was not prolonged and detected only at low levels, suggesting a minimal impact on contribution to disease. The presence of pseudomembranous lesions on the mucosal surface in vivo is evident that the action of toxin occurs at this site. It is possible that disruption of the mucosal biofilm, possibly by antimicrobial therapy, allows access of toxins to the mucosal epithelia and their receptors. Alternatively, extensive mucosal biofilms in vivo may facilitate the sequestration of toxin at concentrations greater than those observed in this study.

We have demonstrated clear differences between planktonic versus biofilm-associated \textit{C. difficile} populations. The factors responsible for these differences remain to be fully elucidated. This project has identified the potential role of biofilms in the recurrence of CDI, which is a major clinical challenge.\(^2\) A better understanding of the role of biofilm structures in CDI and recurrent infection could facilitate novel treatment regimes or agents that have improved efficacy against sessile and planktonic communities.

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