Acidification-dependent suppression of *C. difficile* by enterococci in vitro

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Abstract: *Clostridioides difficile* and Vancomycin-resistant *Enterococcus faecium* (VRE) are commonly co-isolated from hospitalized patients. We sought to develop *in vitro* co-culture systems to identify interactions and study biofilm formation by these two opportunistic pathogens. We found that VRE quickly acidifies media containing glucose, fructose and trehalose to a pH that is not compatible with growth of *C. difficile*. Acidification was necessary and sufficient for VRE-mediated suppression of *C. difficile* and this mechanism is conserved among a panel of enterococci and *Clostridia*. Robust co-culture conditions and biofilm formation were achieved by managing the available carbon source.

***Importance*:** The steady state pH of the colon in humans and mice is below the growth limiting pH for *C. difficile* found in this study and others. Following treatment with antibiotics, pH of the colon rises to neutral or slightly alkaline, which correlates with the loss of colonization resistance to *C. difficile*. These data suggest that manipulation of pH by managing the available carbon source may affect colonization by pathogenic and commensal *Clostridioides*.

Introduction

High levels of population diversity in the gut microbiota is generally correlated with health across gastro-intestinal diseases. Treatment with antibiotics can disrupt the gut microbiota to the point that a single species can expand and dominate the gut, making up a high percentage of the reads in a 16S sequencing data set. Treatment with broad-spectrum antibiotics can lead to expansion of VRE (Donskey et al. 2000) and domination of the gut microbiota by the genus *Enterococcus*. This was observed in humans (Taur et al. 2012) and mice (Ubeda et al. 2010). This domination occurs commonly in human antibiotics-treated patients, such as hematopoietic stem cell transplant patients where a low diversity microbiota is correlated with mortality (Liao et al. 2021; Taur et al. 2012). Vancomycin-resistant *Enterococcus faecium* (VRE) is a rising clinical concern that causes difficult-to-treat systemic infections. But it is often forgotten that *E. faecium* is also a common member of the human gut microbiota, a lactic acid bacterium, and a commonly used probiotic in humans and agricultural animal production (Dubin and Pamer 2014; Krawczyk et al. 2021).

Patients with a low-diversity gut microbiota following antibiotic treatment are also at elevated risk for *Clostridioides difficile* infection (CDI). Colonization of the gut with VRE and the genus *Enterococcus* has been correlated with increased risk of CDI in hospitalized human patients (Fujitani et al. 2011; Lee et al. 2017; Smith et al. 2022). *Enterococcus* species in the gut microbiota were predicted to enhance CDI risk in mathematical modeling that combined data from human and mouse infections (Buffie et al. 2015). In mouse models, genus *Enterococcus* was also correlated with increased persistence of *C. difficile* in the gut (Tomkovich et al. 2020) and increased toxin production and virulence under high dietary zinc conditions (Zackular et al. 2016). One other study described correlation of genus *Enterococcus* with attenuation of *C. difficile* virulence (De Wolfe et al. 2019). Two direct tests of the effects of *Enterococcus* colonization on the CDI mouse model reported higher toxin titre and pathology (Keith et al. 2020; Smith et al. 2022). The mechanism of enhancement of CDI severity in mice was linked to metabolic cross-talk between *E. faecalis* OG1RF and *C. difficile* in which arginine supplementation was sufficient to reduce toxin production and pathology, without significantly affecting colonization by either organism (Smith et al. 2022).

Both *C. difficile* and enterococci form biofilms (Ch'ng et al. 2019; Tremblay and Dupuy 2022). In both species biofilms contribute to antibiotic tolerance (Dapa and Unnikrishnan 2013; Holmberg and Rasmussen 2016) and may be a reservoir for infection recurrence in CDI (Frost, Cheng, and Unnikrishnan 2021; Normington et al. 2021). This study began as an attempt to establish an in vitro co-culture biofilm model of VRE and *C. difficile*. We quickly noticed that the byproducts of primary metabolism produced by VRE negatively affected *C. difficile* growth. Here we have established that VRE and commensal enterococci are capable of acidifying unbuffered bacterial growth media below a pH that is growth inhibitory to *C. difficile* and other commensal and opportunistic *Clostridia* species. Under these conditions, we show that acidification of a carbon source is necessary and sufficient for inhibition of *C. difficile* and *Clostridia* growth. Finally, we demonstrate that alteration of the available carbon source creates a permissive environment for co-culture and biofilm formation by *C. difficile* and VRE. These data suggest that in conditions where lactic acid bacteria such as VRE dominate the local environment, alteration of acid production and pH levels in a carbon source-dependent manner may help to control expansion of *C. difficile*.

Results

74 We began this work as an attempt to establish a dual-species in vitro biofilm model for VRE and
75 *C. difficile*. Typically for Gram-positive bacteria, glucose is added to the culture media to a final
76 concentration of 0.2-1%, which promotes attachment and production of exopolysaccharide matrix
77 (Kristich et al. 2004; Pillai et al. 2004; Toledo-Arana et al. 2001; Donelli et al. 2012; Dapa and
78 Unnikrishnan 2013). When we performed a pilot experiment of co-culture biofilm growth in
79 Supplemented Brain Heart Infusion (BHI) broth with 0.4% added glucose (total glucose = 0.6%)
80 we found a significant decrease in *C. difficile* growth at 8 and 24 hours post-inoculation when in
81 co-culture with VRE (Figure 1A). We observed a similar elimination of *C. difficile* when co-cultured
82 with VRE in Sporulation Media (SM) which lacks an added carbon source, when it was
83 supplemented with 0.6% glucose in co-culture (Figure 1C).
84
85 Next to determine if the inhibitory factor produced by VRE is soluble and filterable, we generated
86 conditioned media by growing VRE to exhaustion (OD600 ~ 1.8) and filtered it through a 0.22 μm
87 filter. To determine if the inhibition was dose-dependent, we generated VRE conditioned media
88 with escalating levels of added glucose. The conditioned media was then inoculated with *C.*
89 *difficile* and grown for 48-hours. We found that increasing the concentration of glucose results in
90 inhibition of *C. difficile* growth and that the inhibitory factor is soluble and filterable in both BHI and
91 SM (Figure 1B,1D). Enterococci are closely related to lactic acid bacteria and are known to
92 produce large amounts of acid when cultured in glucose (Ramsey, Hartke, and Huycke 2014).
93 We tested the pH of the VRE conditioned media and found that pH is inversely correlated with
94 glucose concentration. In BHI conditioned media the starting pH was < 5.3 for all tested glucose
95 concentrations, which did not support *C. difficile* growth (Figure 1B). In conditioned SM with 0.4%
96 added glucose and an average starting pH of 6.3, *C. difficile* growth was not supported (Figure
97 1D). These data suggest a inhibitory pH threshold of around 6 for *C. difficile* in VRE conditioned
98 media.
99
100 The inhibition of *C. difficile* growth described above could be the result of an inhibitory factor
101 produced by VRE or it could be due to nutrient limitation. To differentiate between these two
102 mechanisms, we first generated VRE conditioned SM in a range of glucose concentrations. Then
103 we created a dilution series of those conditioned media in sterile PBS. VRE conditioned media
104 inhibits *C. difficile* growth with 0.4% and 0.6% total glucose concentration (Figure 2). When VRE
105 conditioned media containing 0.4% glucose is diluted 1:2 in PBS, it no longer inhibits *C. difficile*
106 growth. These data confirm that VRE produces an inhibitor of *C. difficile* in a glucose

107 concentration-dependent manner, which can be titrated below its inhibitory concentration through
108 dilution.
109
110 To determine if VRE-mediated acidification is necessary for inhibition of *C. difficile* we neutralized
111 VRE conditioned BHI + 0.4% glucose (pH 5) with sodium hydroxide to pH 7. In the neutralized
112 conditioned media *C. difficile* grew to similar levels as in naive BHI + 0.4% glucose (Figure 3A).
113 To determine if acidification of media is sufficient to inhibit *C. difficile* growth we acidified naive
114 BHI and SM (pH 7.0) with hydrochloric acid and found that *C. difficile* growth was inhibited at a
115 pH between 5.0-4.5 in acidified BHI and 6.0-5.5 in acidified SM (Figure 3B). Finally, to determine
116 if acidification has the potential to synergize with other secreted effectors in VRE conditioned
117 media, we used HCl to acidify VRE-conditioned SM, which had a starting pH of 7.6, to a range of
118 pH from 7.0 to 4.5 Here we observed an inhibitory pH between 6.0-5.5, similar to the threshold
119 observed in HCl acidification of naive SM (Figure 3C). These data suggest that acidification is the
120 primary inhibitor of *C. difficile* under these in vitro conditions.
121
122 Next we tested the hypothesis that the acidification of any sugar is sufficient to cause VRE to
123 inhibit the growth of *C. difficile*. We used a panel of simple sugars that differed in their reported
124 ability to be metabolized to acid by enterococci. Glucose and fructose were reported to be
125 acidified, while fucose and xylose were not acidified (Schleifer and Kilpper-Balz 1984). Tagatose
126 and arabinose acidification was reported to differentiate between *E. faecalis* and *E. faecium*
127 (Schleifer and Kilpper-Balz 1984). We also included the disaccharide trehalose and the
128 polysaccharide inulin which have been implicated experimentally in mouse models of *C. difficile*
129 infection (Hryckowian et al. 2018; Collins et al. 2018). We generated VRE conditioned SM with
130 0.6% of each sugar and grew *C. difficile* for 48 hours before plating to simulate biofilm culture
131 conditions. We found that only VRE conditioned SM containing glucose, fructose and trehalose
132 inhibited growth of *C. difficile* when compared to growth in the same naive media (Figure 4A).
133 Glucose, fructose and trehalose lowered pH of the conditioned media significantly with mean pH
134 of 6.1, 6.0 and 5.7, respectively. These pH levels are consistent with the inhibitory pH levels
135 described above.
136
137 Toxin production is regulated in part by glucose-mediated catabolite repression via the
138 transcription factor CcpA (Dupuy and Sonenshein 1998; Antunes, Martin-Verstraete, and Dupuy
139 2011; Hofmann et al. 2021). We performed cytotoxicity assays using filtered supernatants from
140 the carbon source screen described above. We did not observe a significant change in toxin titre

141 in excess glucose as previously reported, however, our samples were collected at 48 hours post-
142 inoculation, which is 12-36 hours later than previously reported results on catabolite repression
143 (Dupuy and Sonenshein 1998; Antunes, Martin-Verstraete, and Dupuy 2011). Thus, our assay
144 represents total accumulated toxin following growth of *C. difficile* to exhaustion. Another study
145 examined TcdA protein levels by ELISA at 48 hours and found a decrease in TcdA in 0.5%
146 glucose at 48 hours, whereas we found no difference in toxin titre by cytotoxicity at 48 hours
147 (Hofmann et al. 2021). Our most significant reductions in toxin production came in VRE
148 conditioned SM containing the acidified carbon sources, glucose, fructose or trehalose, which
149 reflects the killing of *C. difficile* by VRE (Figure 4B). Additionally, we observed a trend toward
150 reduction in toxin titre when *C. difficile* was grown in VRE conditioned SM containing arabinose
151 ($p = 0.062$), which is slightly acidified by VRE, without a significant reduction in *C. difficile* CFUs
152 (Figure 4A).
153
154 To test the effects of carbon source availability in a more physiologically relevant in vitro system,
155 we utilized extracts from cecal content of germ free mice and compared growth of *C. difficile* in
156 naive and VRE conditioned filter-sterilized cecal extracts. In cecal content conditioned by VRE,
157 we observed a slight, but statistically significant increase in *C. difficile* growth after 48 hours
158 (Figure 5A). However, when we added 0.6% glucose as a carbon source to the cecal extract
159 before VRE conditioning, subsequent growth of *C. difficile* was not supported and pH was reduced
160 to 4.0. Addition of 0.6% fucose as a carbon source did not result in a significant difference in *C.*
161 *difficile* growth between naive and VRE conditioned media. These data suggest that the
162 byproducts of carbon source metabolism by VRE can suppress *C. difficile* growth in cecal
163 extracts. In these conditions we did not observe a significant reduction in toxin titre of VRE
164 conditioned cecal content extract containing glucose, despite suppression of *C. difficile* growth
165 below the level of detection (Figure 5B). We did, however, observe a significant decrease in toxin
166 titre in cecal content without an added carbon source.
167
168 We then tested if acidification-mediated suppression of *C. difficile* is conserved among
169 enterococci. We generated conditioned SM + 0.6% glucose from a panel of enterococci including
170 *E. hirae*, and vancomycin sensitive isolates of *E. faecuim* and *E. faecalis*. All of the tested strains
171 completely suppressed *C. difficile* growth and produced conditioned media with a pH below 6
172 (Figure 5A). We then tested a small panel of *Clostridioides* for growth in VRE conditioned media
173 containing glucose (Figure 5B). Growth of *C. scindens, C. inoccuum, C. citrone, and C. difficile*
174 ribotype 027 strain R20291 were completely inhibited, while *C. bifermentans* showed significantly

175 reduced growth. Acidification was necessary for growth inhibition of all strains as growth was
176 restored when VRE conditioned media containing glucose was neutralized with NaOH. These
177 data suggest that acidification is conserved among enterococci and is necessary and sufficient to
178 inhibit growth of diverse *Clostridioides* under these conditions.
179
180 This study began with an attempt to establish an *in vitro* co-culture biofilm model of *C. difficile* and
181 VRE. We again attempted to establish a co-culture polystyrene plate biofilm model using SM
182 liquid media containing fucose or xylose as a carbon source to avoid media acidification (Figure
183 7). We also added deoxycholic acid which stimulated formation of *C. difficile* and VRE
184 monoculture biofilms in previous studies (McKenney et al. 2019; Dubois et al. 2019) and pre-
185 coated the plates with human fibrinogen (Flores-Mireles et al. 2014). After 48 hours of growth,
186 mono-cultures of *C. difficile* grew in all sugar conditions (Figure 7A). VRE grew in all mono- and
187 dual-species biofilm carbon sources. *C. difficile* grew in dual-species culture with VRE only in non-
188 acidifying sugars, fucose and xylose. As expected, *C. difficile* was inhibited by VRE in the
189 presence of glucose. We used confocal microscopy to image biofilms after 48 hours of growth in
190 SM with and without 0.6% glucose or 0.6% fructose (Figure 7B). Single species biofilms of VRE
191 resembled a monolayer of cells with distinct microcolony clumps of cells under all three media
192 conditions. Single species biofilms of *C. difficile* also formed a monolayer of cells in all three media
193 conditions. However, SYTO-9 staining resulted in discrete foci of fluorescence in SM and SM +
194 fucose, which we hypothesize to be condensed DNA in *C. difficile* sporangia. These foci were
195 absent from biofilms grown in SM + Glucose which appeared as a mat of cell strands, similar to
196 a previous report (Dubois et al. 2019). In co-culture biofilms in SM and SM + 0.6% fucose, we
197 observed a mixed monolayer containing C. difficile and VRE with comparatively smaller biofilm
198 microcolonies when compared to VRE alone. In co-culture biofilms in 0.6% glucose, we observed
199 a monolayer of cells primarily made up of C. difficile with microcolonies of VRE. Thus, by
200 controlling the available carbon source we were able to produce robust *C. difficile* - VRE co-culture
201 biofilms.

202 **Discussion**

203 We initially set out to develop an in vitro co-culture biofilm model of VRE and *C. difficile* using
204 excess glucose to promote adherence of the bacteria to plastic plates. Growth in excess glucose
205 was not supported in co-culture (Figure 1). We found that acidification of glucose is necessary,
206 sufficient and appears to be the primary mechanism by which VRE affects *C. difficile* growth in

207 the presence of excess glucose in vitro (Figure 3). To test if VRE conditioned media inhibits *C.*
208 *difficile* under all growth conditions we altered the available carbon source and found that growth
209 inhibition is limited to contexts in which the carbon source is acidified by VRE. We observed a
210 pH-mediated inhibition of growth in the presence of glucose, fructose and trehalose (Figure 4A).
211 Our results were similar when the growth medium was germ-free mouse cecal content extract
212 (Figure 5A). These data suggest that control of the available carbon source is critical to
213 developing in vitro co-culture assays with lactic acid bacteria. Substitution of glucose for fucose
214 or xylose, neither of which were acidified by VRE, allowed for the formation of a stable co-
215 culture biofilm (Figure 7A).

216 We also measured the toxin titre of *C. difficile* in the carbon source screen and did not find
217 significant changes in toxin production without an underlying inhibition of growth (Figure 4B).
218 This is likely due to the 48 hour time point chosen for analysis. In the case of added l-arabinose
219 there was a slight trend towards reduced toxin expression ($p = 0.062$). Arabinose was acidified
220 by VRE (*E. faecium* ATCC 700221), which contains a predicted arabinose operon (McKenney et
221 al. 2016; van Schaik et al. 2010). VRE conditioned media containing arabinose was acidified to
222 an average pH of 6.9 ($p = 0.053$ compared to naive SM) but did not inhibit *C. difficile* growth, so
223 the nature of this reduction of cytotoxicity is unknown. *E. faecium* and *E. faecalis* type strains
224 generally grow when l-arabinose is the carbon source on phenotype culture arrays (Lebreton et
225 al. 2017). A recent characterization of vancomycin-resistant *E. faecium* isolates from pediatric
226 cancer patients found a high level of variation in the ability of isolates to utilize l-arabinose as a
227 sole carbon source (Chilambi et al. 2020). Data from clinical carbohydrate acidification assays,
228 phenotype culture arrays (Collins et al. 2018; Scaria et al. 2014; Norsigian et al. 2020) as well as
229 continuous flow culture (Wilson and Perini 1988) suggest that *C. difficile* does not utilize l-
230 arabinose as a sole carbon source. Together these data suggest that metabolites produced by VRE
231 grown in the presence of l-arabinose may suppress *C. difficile* toxin production or stability in a
232 48-hour culture. We also note high levels of variability in *C. difficile* growth in SM containing
233 added tagatose. We did not find a significant decrease in cytotoxicity in VRE conditioned cecal
234 content containing added glucose ($p = 0.136$), despite our inability to recover *C. difficile* CFUs at
235 48 hours post inoculation. We hypothesize that toxin was produced by the inoculated *C. difficile*
236 early during the 48 hour culture period and that toxin was stable under these conditions.

237 In mouse models of infection, *C. difficile* first begins to accumulate in the caecum 6-12 hours
238 post infection (Koenigsknecht et al. 2015). The colon, particularly the cecum, is densely
239 colonized and is a site of high degrees of population diversity and countless metabolic products.
240 A general feature of the mouse (Shimizu et al. 2021; Sorbara et al. 2019) and human cecum
241 (Koziolek et al. 2015; Nugent et al. 2001) is an acidic pH of 5.0-6.5 at steady state. During *in*
242 *vitro* culture *C. difficile* growth and sporulation is limited at pH of less than 6 (Woo et al. 2011;
243 Wetzel and McBride 2020). Treatment with antibiotics raises colon pH from acidic to slightly
244 alkaline conditions, suggesting that the gut microbiota are necessary for maintaining acidic pH
245 (Shimizu et al. 2021; Sorbara et al. 2019). These data suggest that pH of the gut lumen, unlike
246 the pH of blood, is not tightly buffered and may be among the many factors that contribute to *C.*
247 *difficile* colonization resistance mediated by the gut microbiota. Most bacteria are capable of
248 living in a pH range of 3-4 units and pH may act as a constraint on growth of commensals and
249 opportunistic pathogens (Jin and Kirk 2018). Furthermore, significant variations in fecal pH,
250 both alkaline and acidic were associated with a significantly increased risk of subsequent
251 bacteremia in critically ill patients (Osuka et al. 2012).

252 The pH of the gut lumen is modulated and constrained by inputs from the host diet, and the
253 metabolism of the gut microbiota and the colonic epithelium. The colonic epithelium produces
254 and secretes bicarbonate, which increases the alkalinity of the gut lumen (Alka and Casey 2014).
255 The colonic epithelium also produces lactic acid as a byproduct of glycolysis which can be
256 converted into short chain fatty acids by the gut microbiota (Louis et al. 2022). Primary
257 metabolism by the gut microbiota produces weak organic acids such as lactic acid and short
258 chain fatty acids when grown in a fermentable carbon source (Gregory, Pensinger, and
259 Hryckowian 2021). Multiple groups have reported that changes in pH affect growth and survival
260 of core taxa of the gut microbiota. For example, during in vitro culture at pH 5.5 multiple human
261 gut isolates from genus *Bacteroides* failed to grow (Duncan et al. 2009). This pH sensitivity of
262 *Bacteroides* has been replicated in several batch culture fermenter studies seeded with donor
263 human feces (Firrman et al. 2022; Ilhan et al. 2017; Wang et al. 2020). These studies also
264 consistently reported reductions in levels of genus *Clostridioides* (or *Clostridium*) under low pH
265 conditions.

266 Sporadic clinical trials have tested the efficacy of probiotic lactic acid bacteria on *C. difficile*
267 infections with mixed results (Goldstein et al. 2017; Mills, Rao, and Young 2018). However it is
268 clear that strains from genus *Bifidobacterium* and *Lactobacillus* can inhibit *C. difficile* in vitro by
269 decreasing pH (Fredua-Agyeman et al. 2017) and in some cases similar probiotics have reduced
270 *C. difficile* virulence in animal models of infection (Wei et al. 2018). For example, lactic acid
271 production by *Streptococcus thermophilus* lowered the pH of conditioned media and was
272 inversely correlated with *C. difficile* growth and toxin production *in vitro* and in a mouse model
273 of infection (Kolling et al. 2012).

274 In certain cases, such as in critically ill patients treated with antibiotics who become dominated
275 by enterococci (Liao et al. 2021), it may seem desirable to enhance the acidity of the gut lumen
276 through administration of a diet that results in acid production and suppression of *C. difficile*. In
277 keeping with the batch fermentation studies mentioned above, we also found that commensal
278 *Clostridioides* are subject to a similar pH-mediated sensitivity to VRE-conditioned media as *C.*
279 *difficile* (Figure 6). Therefore, efforts to remediate dysbiosis in recurrent *C. difficile* infection by
280 reintroducing commensal spore-forming anaerobes (Feuerstadt et al. 2022, Louie et al. 2023)
281 may benefit from a different strategy. Here, temporarily limiting acid production by enterococci
282 and other lactic acid bacteria through dietary intervention may favor engraftment of the
283 bacteriotherapy. These types of individually tailored prebiotic & probiotic combination therapies
284 are a long way from clinical reality, however, gut lumen pH levels may be a useful biomarker for
285 states of dysbiosis and permissiveness to colonization by commensals.
286

Figure1

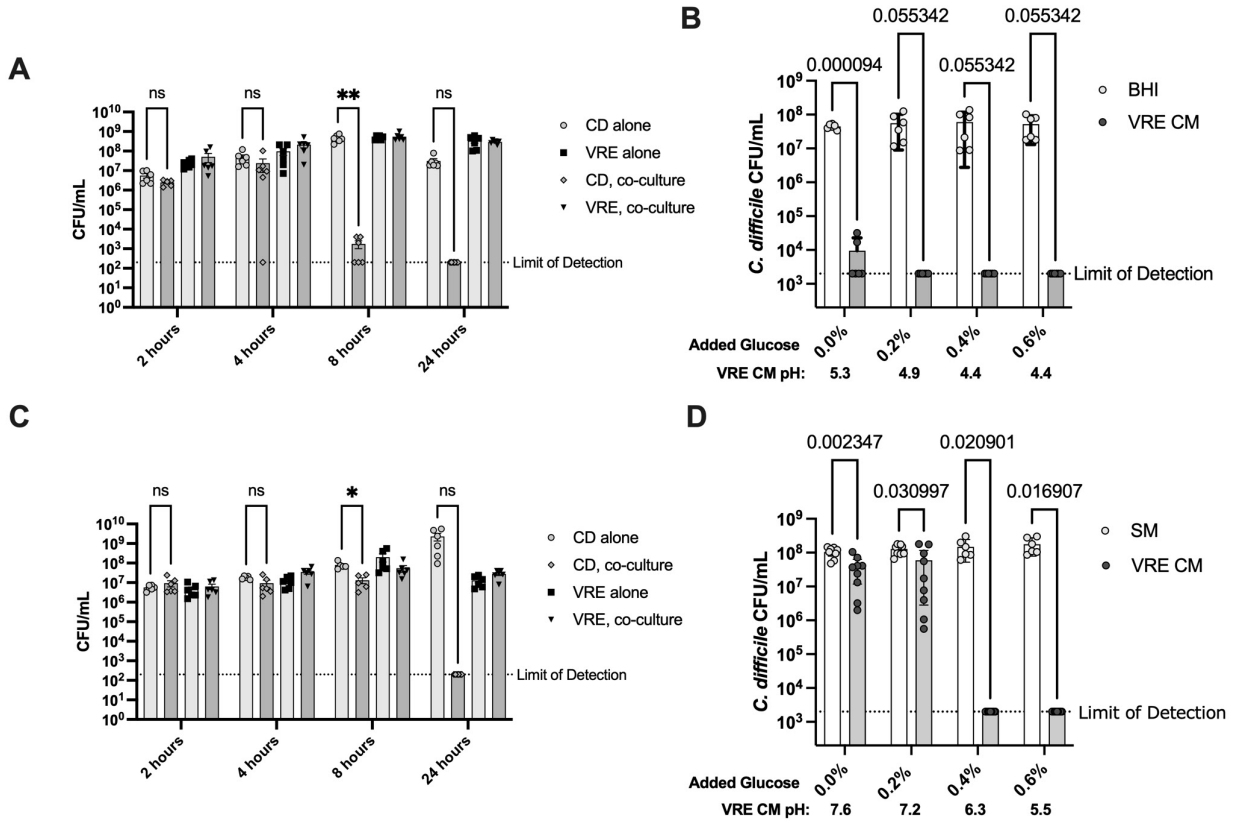

Figure1 *C. difficile* – VRE co-culture A) Time course CFUs of C. difficile and VRE single co-culture in BHI + 0.4% glucose (0.6% total glucose). Data are combined from 2 independent experiments (n=5-6 per time point). B) VRE was grown for 48 hours in BHI + 0, 0.2, 0.4 or 0.6% additional glucose, then filter sterilized to create conditioned media (VRE CM) which was then inoculated with *C. difficile*. The average pH of the VRE CM is shown below the graph (n=2). CFU data are combined from 2 independent experiments (n=6). C) Time course CFUs of C. difficile and VRE single and co-culture in SM + 0.6% glucose. Data are combined from 2 independent experiments (n=5-6 per time point). D) VRE was grown for 48 hours in SM + 0, 0.2, 0.4 or 0.6% additional glucose, then filter sterilized to create VRE CM which was then inoculated with *C. difficile* and grown for 48 hours. The average pH of the VRE CM (n=3) is shown below the graph. CFU data are combined from 3 independent experiments (n=6-9 per condition). * p = 0.04, ** p =0.008, All comparisons: Unpaired t-test with Welch correction

305 **Figure 2**
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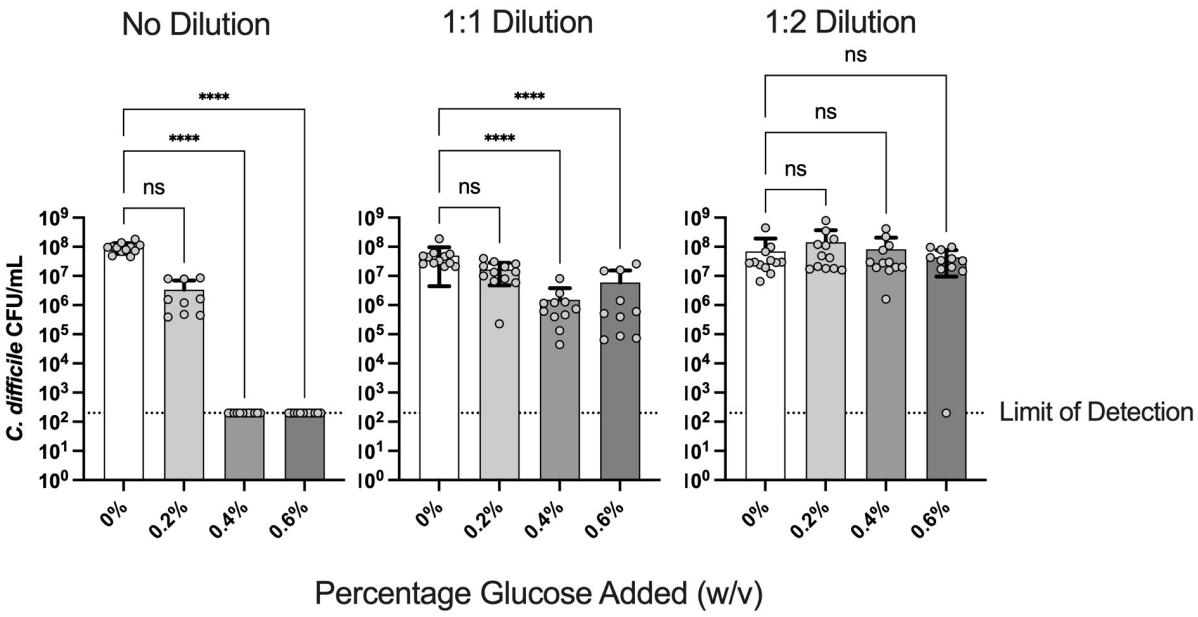

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309 **Figure 2: Dilutions of Inhibition by VRE-conditioned media.** VRE conditioned media were generated
310 with increasing concentrations of added glucose, filter sterilized, then diluted 1:1 or 1:2 with reduced
311 PBS before inoculation with *C. difficile* and plating at 48 hours of growth. Data are combined from 3
312 independent experiments (n=9-12 per condition). Statistics: Kruskal-Wallace 1-way ANOVA with Dunn's
313 correction vs. 0% glucose. **** $p < 0.0001$, ns $p > 0.05$
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333 **Figure 3**
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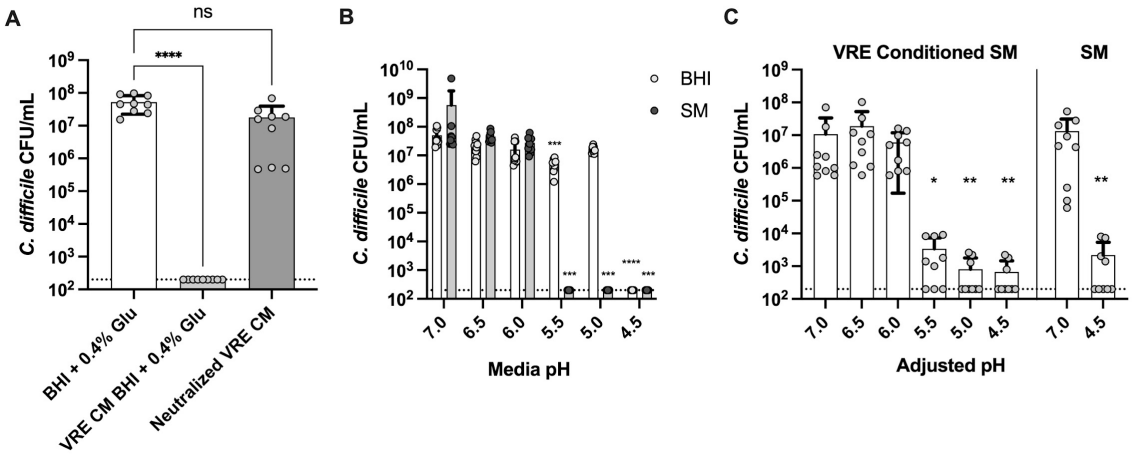

335
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337 **Figure 3: Acidification is necessary and sufficient for inhibition of *C. difficile*** a) VRE-conditioned BHI +
338 0.4% glucose was neutralized with NaOH to a pH of 7 and inoculated with *C. difficile* for 48 hours before
339 plating. Data are combined from 3 independent experiments (n=9). **** $p < 0.0001$ Kruskal-Wallis One-
340 way ANOVA with Dunn's correction. B) BHI and SM were acidified with reduced HCl in 0.5 unit pH
341 increments before inoculation with *C. difficile* and growth for 48 hours. Data are combined from 3
342 independent experiments (n=9). *** $p < 0.0003$, **** $p < .0001$ Kruskal-Wallis One-way ANOVA with
343 Dunn's correction versus pH 7.0 CFUs for each medium. C) VRE conditioned SM was filter-sterilized and
344 acidified with reduced HCl before inoculation with *C. difficile* for 48 hours. Data are combined from 3
345 independent experiments (n=9). ** $p < 0.006$, * $p = 0.026$ Kruskal-Wallis One-way ANOVA with Dunn's
346 correction versus pH 7.0 VRE Conditioned SM CFUs for each condition.

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349 **Figure 4**
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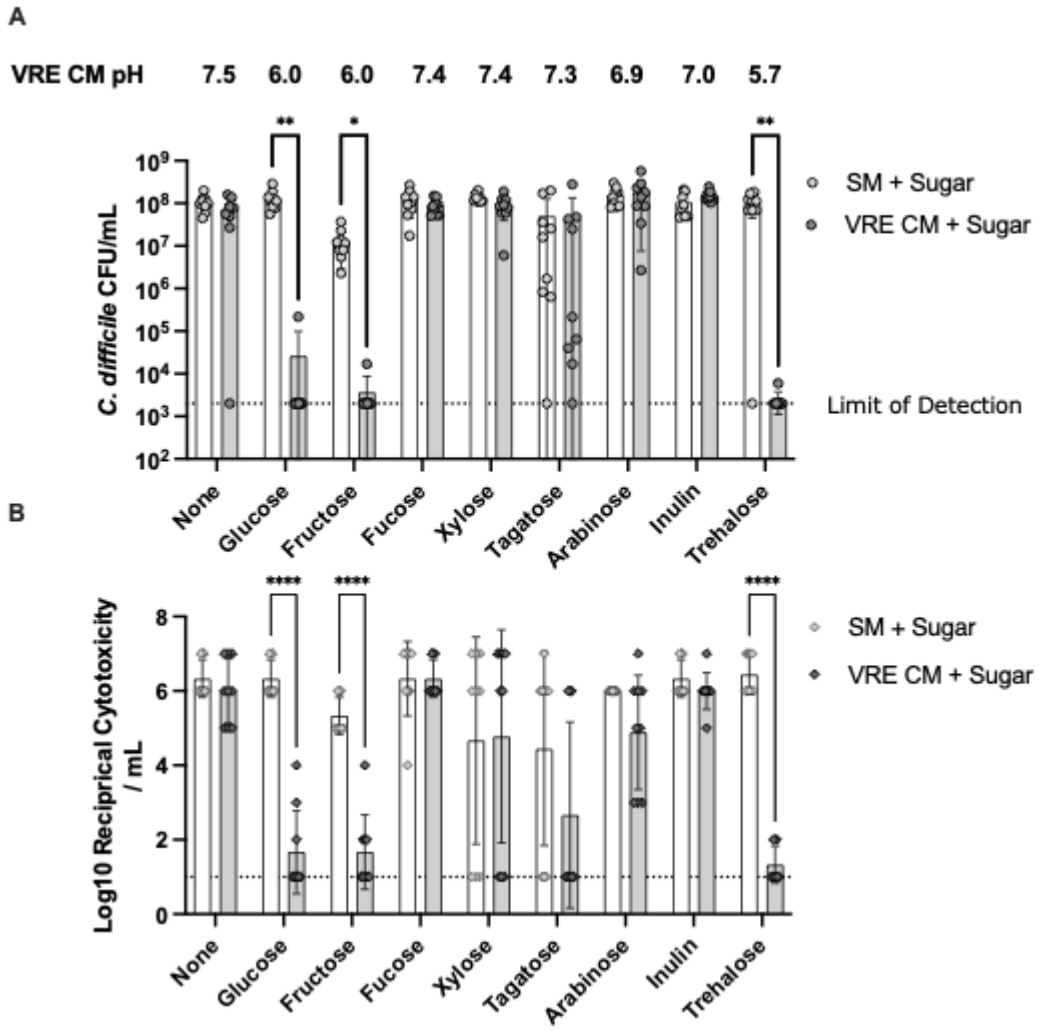

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353 **Figure 4: Effects of VRE Carbon Source Metabolism on C. difficile Growth**. A) C. difficile
354 was grown in fresh SM + 0.6% of the carbon source (SM + Sugar) indicated on the x-axis or
355 VRE conditioned SM + 0.6% carbon source (VRE CM + Sugar). Starting pH of VRE CM +
356 Sugar for each condition is reported above each pair of bars and is the mean of 3
357 measurements. Data are combined from 3 independent experiments (n=9). Unpaired Welch's t-
358 tests between SM + Sugar and VRE CM + Sugar ** $p < 0.006$, * $p = 0.035$ B) Cytotoxicity on
359 Vero cells of filtered supernatants from A. Data are combined from 3 independent experiments.
360 Unpaired Welch's t-tests naive SM + sugar vs VRE CM + sugar. **** $p < 0.00001$
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367 **Figure 5**
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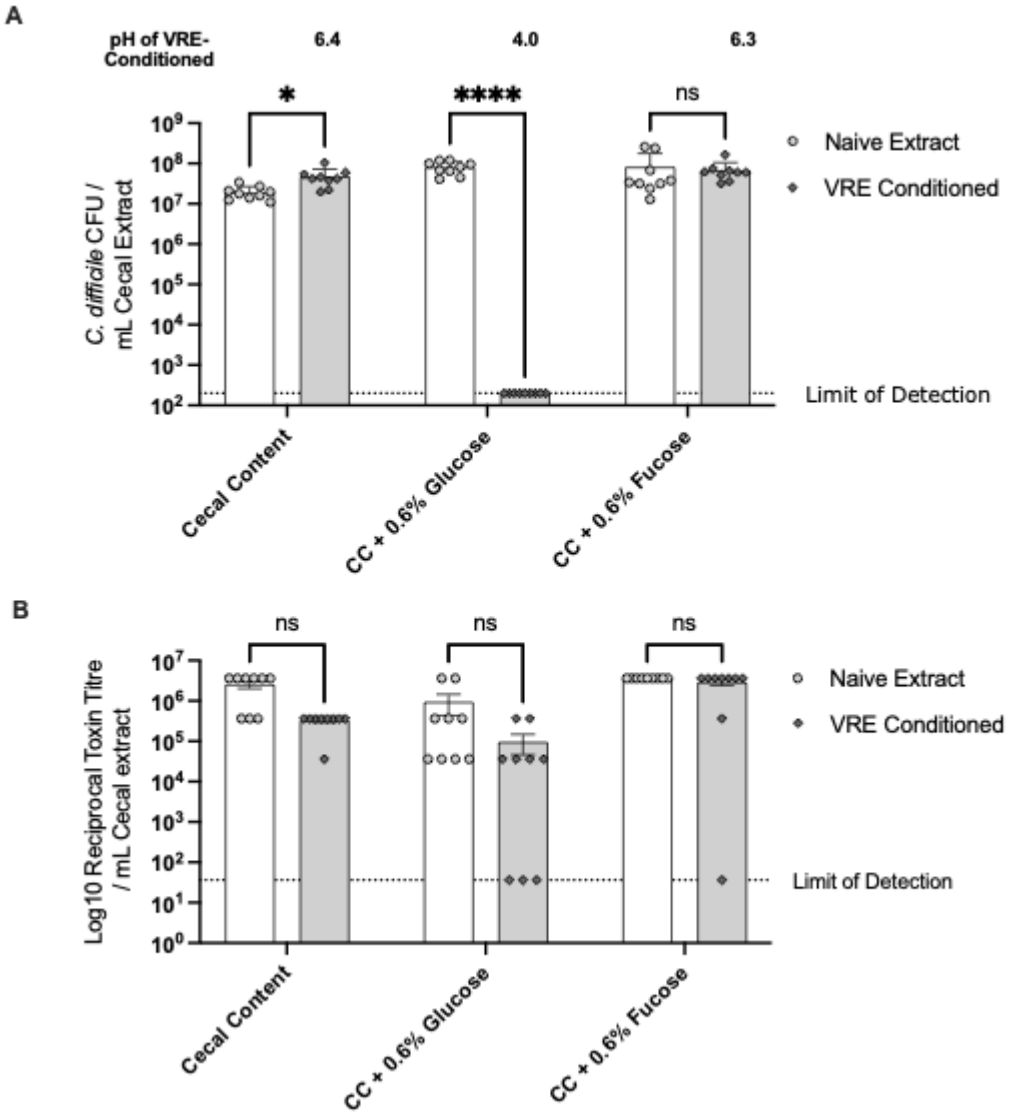

369
370 **Figure 5 Effects of VRE Carbon Source Metabolism on *C. difficile* Growth in Cecal**
371 **Content Extracts.** A) Sterile filtered extracts of cecal content from germ-free mice were
372 prepared with reduced PBS and the indicated added carbon source or equivalent volume PBS
373 and inoculated with VRE and grown for 24 hours. Conditioned extracts were sterile filtered then
374 inoculated with *C. difficile* and grown for 48 hours followed by plating on selective media. Data
375 are combined from 3 independent experiments (n = 9) B) Vero cell cytotoxicity assay on filtered
376 supernatants following *C. difficile* culture. Assay was performed twice on extracts from A, Data
377 are representative of one assay (n = 9). * $p < 0.02$, **** $p = 0.00009$, Unpaired Welch's t-Test.
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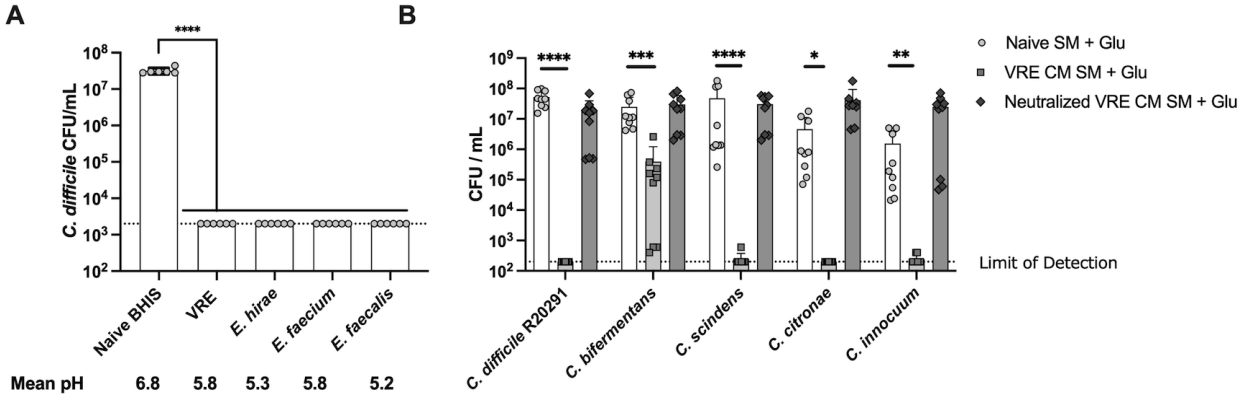
384 **Figure 6**
385



386
387

388 **Figure 6: Conservation of Mechanism.** A) SM + 0.6 % glucose was conditioned by incubation with the
389 indicated strains on the x-axis followed by inoculation with *C. difficile* and growth for 48 hours. Mean pH
390 of the conditioned media are displayed below the x-axis (n=2). CFU data are combined from 2
391 independent experiments (n=6) **** $p < 0.0001$ Kruskal-Wallis One-way ANOVA with Dunn's correction
392 vs. Naive BHI B) VRE conditioned SM + 0.6% glucose was created by 48 hours of VRE growth followed by
393 filter sterilization or was neutralized with NaOH to pH7 before filter sterilization inoculation with *C.*
394 *difficile* for 48 hours. Data are combined from 3 independent experiments (n=9). Kruskal-Wallis One-way
395 ANOVA with Dunn's correction vs. Naive SM + Glu **** $p < 0.0001$, *** $p = 0.0002$, ** $p = 0.006$, * $p =$
396 0.017
397

Figure 7

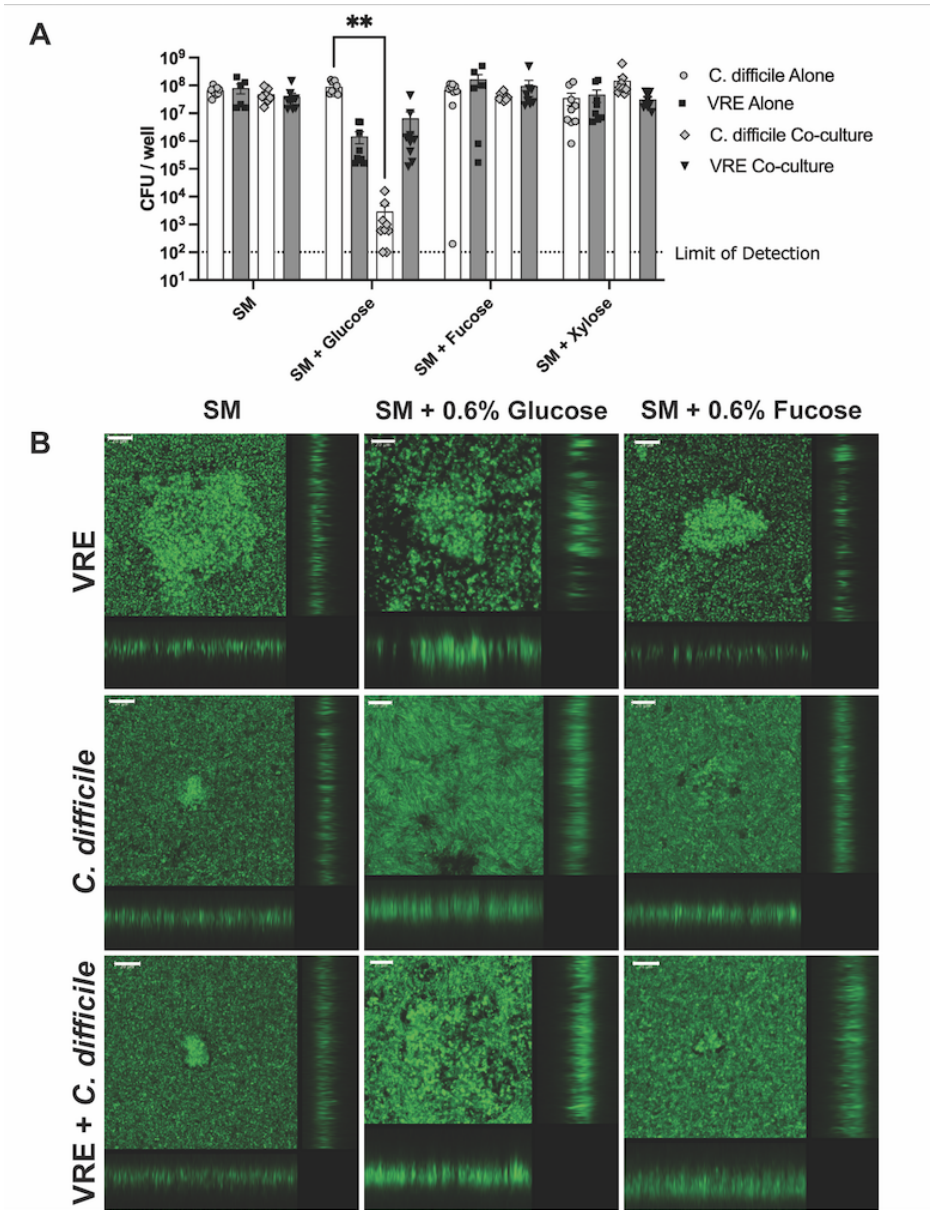


Figure 7 - Co-culture biofilm growth under different carbon sources. A) Biofilms were generated in polystyrene plates with 5mg/mL fibrinogen coating 24 hours before inoculation in SM + 125 μM DCA + 0.6% of the indicated sugar for 48 hours followed by selective plating. Data are combined from 3 independent experiments (n=9). ** p = 0.001 Unpaired Welch's t-test of Alone vs. Co-culture B) Confocal microscopy of 48 hour biofilms stained with SYTO-9. Images show one representative field of equal size, with projected z-intensity vertically and horizontally. Scale bar = 20 μm, Images are representative of 2 experiments (n = 6 per condition).

414 **Figure Legends**
415
416 **Figure1 *C. difficile* – VRE co-culture** A) Time course CFUs of C. difficile and VRE single co-
417 culture in BHI + 0.4% glucose (0.6% total glucose). Data are combined from 2 independent
418 experiments (n=5-6 per time point). B) VRE was grown for 48 hours in BHI + 0, 0.2, 0.4 or 0.6%
419 additional glucose, then filter sterilized to create conditioned media (VRE CM) which was then
420 inoculated with *C. difficile*. The average pH of the VRE CM is shown below the graph (n=2).
421 CFU data are combined from 2 independent experiments (n=6). C) Time course CFUs of C.
422 difficile and VRE single and co-culture in SM + 0.6% glucose. Data are combined from 2
423 independent experiments (n=5-6 per time point). D) VRE was grown for 48 hours in SM + 0, 0.2,
424 0.4 or 0.6% additional glucose, then filter sterilized to create VRE CM which was then
425 inoculated with *C. difficile* and grown for 48 hours. The average pH of the VRE CM (n=3) is
426 shown below the graph. CFU data are combined from 3 independent experiments (n=6-9 per
427 condition). * p = 0.04, ** p =0.008, All comparisons: Unpaired t-test with Welch correction
428
429 **Figure 2: Dilutions of Inhibition by VRE-conditioned media.** VRE conditioned media were generated
430 with increasing concentrations of added glucose, filter sterilized, then diluted 1:1 or 1:2 with reduced
431 PBS before inoculation with *C. difficile* and plating at 48 hours of growth. Data are combined from 3
432 independent experiments (n=9-12 per condition). Statistics: Kruskal-Wallace 1-way ANOVA with Dunn's
433 correction vs. 0% glucose. **** p < 0.0001, ns p > 0.05
434
435 **Figure 3: Acidification is necessary and sufficient for inhibition of *C. difficile*** a) VRE-conditioned BHI +
436 0.4% glucose was neutralized with NaOH to a pH of 7 and inoculated with *C. difficile* for 48 hours before
437 plating. Data are combined from 3 independent experiments (n=9). **** p < 0.0001 Kruskal-Wallis One-
438 way ANOVA with Dunn's correction. B) BHI and SM were acidified with reduced HCl in 0.5 unit pH
439 increments before inoculation with *C. difficile* and growth for 48 hours. Data are combined from 3
440 independent experiments (n=9). *** p < 0.0003, **** p < .0001 Kruskal-Wallis One-way ANOVA with
441 Dunn's correction versus pH 7.0 CFUs for each medium. C) VRE conditioned SM was filter-sterilized and
442 acidified with reduced HCl before inoculation with *C. difficile* for 48 hours. Data are combined from 3
443 independent experiments (n=9). ** p < 0.006, * p = 0.026 Kruskal-Wallis One-way ANOVA with Dunn's
444 correction versus pH 7.0 VRE Conditioned SM CFUs for each condition.
445
446 **Figure 4: Effects of VRE Carbon Source Metabolism on C. difficile Growth**. A) C. difficile
447 was grown in fresh SM + 0.6% of the carbon source (SM + Sugar) indicated on the x-axis or
448 VRE conditioned SM + 0.6% carbon source (VRE CM + Sugar). Starting pH of VRE CM +
449 Sugar for each condition is reported above each pair of bars and is the mean of 3
450 measurements. Data are combined from 3 independent experiments (n=9). Unpaired Welch's t-
451 tests between SM + Sugar and VRE CM + Sugar ** p < 0.006, * p = 0.035 B) Cytotoxicity on
452 Vero cells of filtered supernatants from A. Data are combined from 3 independent experiments.
453 Unpaired Welch's t-tests naive SM + sugar vs VRE CM + sugar. **** p < 0.00001
454
455 **Figure 5 Effects of VRE Carbon Source Metabolism on *C. difficile* Growth in Cecal**
456 **Content Extracts.** A) Sterile filtered extracts of cecal content from germ-free mice were

457 prepared with reduced PBS and the indicated added carbon source or equivalent volume PBS
458 and inoculated with VRE and grown for 24 hours. Conditioned extracts were sterile filtered then
459 inoculated with *C. difficile* and grown for 48 hours followed by plating on selective media. Data
460 are combined from 3 independent experiments (n = 9) B) Vero cell cytotoxicity assay on filtered
461 supernatants following *C. difficile* culture. Assay was performed twice on extracts from A, Data
462 are representative of one assay (n = 9). * $p < 0.02$, **** $p = 0.00009$, Unpaired Welch's t-Test.
463
464 **Figure 6: Conservation of Mechanism.** A) SM + 0.6 % glucose was conditioned by incubation with the
465 indicated strains on the x-axis followed by inoculation with *C. difficile* and growth for 48 hours. Mean pH
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468 vs. Naive BHI B) VRE conditioned SM + 0.6% glucose was created by 48 hours of VRE growth followed by
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470 *difficile* for 48 hours. Data are combined from 3 independent experiments (n=9). Kruskal-Wallis One-way
471 ANOVA with Dunn's correction vs. Naive SM + Glu **** $p < 0.0001$, *** $p = 0.0002$, ** $p = 0.006$, * $p =$
472 0.017
473
474 **Figure 7 - Co-culture biofilm growth under different carbon sources.** A) Biofilms were
475 generated in polystyrene plates with 5mg/mL fibrinogen coating 24 hours before inoculation in
476 SM + 125 μM DCA + 0.6% of the indicated sugar for 48 hours followed by selective plating.
477 Data are combined from 3 independent experiments (n=9). ** $p = 0.001$ Unpaired Welch's t-test
478 of Alone vs. Co-culture B) Confocal microscopy of 48 hour biofilms stained with SYTO-9.
479 Images show one representative field of equal size, with projected z-intensity vertically and
480 horizontally. Scale bar = 20 μm, Images are representative of 2 experiments (n = 6 per
481 condition).
482

483
484 **Table 1: Table of Strains**
485
486

Species	Strain	Source
Clostridioides difficile	VPI 10463	ATCC
Enterococcus faecium (VRE)	700223	ATCC
Enterococcus hirae	9790	ATCC
Enterococcus faecium	19434	ATCC
Enterococcus faecalis	19433	ATCC
Clostridioides difficile	R20291	DSMZ
Paraclostridium bifermentans	638	ATCC
Clostridium scindens	35704	ATCC
Clostridium citronae	BAA-1317	ATCC
Clostridium innocuum	14501	ATCC

487

488 **Methods**

489 # Methods

490 ## Strains and Growth Conditions

491 Strains are listed in table 1. All experiments were conducted in an anaerobic chamber (Coy Laboratory Products),
492 with an atmosphere of 90% N_2, 5% CO_2 and 5% H_2. *C. difficile* and other *Clostridia* strains were routinely cultured
493 on BHI plates and liquid media (Brain Heart Infusion + 0.5% yeast extract) supplemented with 0.1% taurocholate.
494 VRE and other enterococci were routinely cultured in BHI liquid media and Enterococcosel (BD Biosciences)
495 plates. For differential selection during co-culture VRE and enterococci were plated on Enterococcosel while *C.*
496 *difficile* was plated on BHI plates supplemented with 0.1% taurocholate, 250 ug/mL D-cycloserine, 16 ug/mL
497 cefoxitin. Commensal *Clostridia* were enumerated by plating Columbia Blood Agar and counting by distinguishing
498 between colony morphology of VRE.

499 ## Media

500 Brain heart infusion (BHI) and Sporulation Media (SM) were used in the described experiments. BHI was made
501 with 36g/L Bacto BHI powder with 5g/L yeast extract, bacterial, autoclaved, then 3 mL filter sterilized 10% L-
502 cysteine was added to the media before aliquoting and reducing in the anaerobic chamber. Sporulation media was
503 made with 90g Bacto peptone, 5 g Bacto protease peptone, 1 g ammonium sulfate and 1.5 g tris base, then 3 mL
504 10% L-cystine was added after autoclaving. All liquid and plate media were pH adjusted to 7.0 with HCl unless
505 otherwise specified before autoclaving. Glucose and other sugars used were dissolved into water at a 6% weight per
506 volume ratio before being filter sterilized. Sugars were then added into the media before being placed in the
507 anaerobic chamber or allowed to reduce in the chamber before being added to the media. Inulin was added as
508 described with heating to allow full solubility before being filtered. All plates were poured in 18mL volumes and
509 kept at 4°C until needed. 8-24 hours before using, plates were placed in the anaerobic chamber to reduce fully.

510 ## *C. difficile* Growth in VRE and enterococci conditioned media

511 Mid-log VRE overnight culture was OD-normalized to 0.5 before being inoculated (1:20 dilution) into SM + 0.6%
512 sugar. The culture was grown for at least 24 hours anaerobically at 37°C until the OD_{600} was > 2 to ensure growth to
513 saturation. Cultures were spun at 1500g x 15 minutes before being filter sterilized with a 0.22 µm filter syringe into
514 3 mL aliquots into glass tubes. 1.5 mL aliquots were also frozen at -80C for further use in cytotoxicity assays. *C.*
515 *difficile* was inoculated 1:60 from mid-log into the VRE-conditioned media and incubated at 37°C for 48 hours
516 before being serially diluted and drop plated for CFU/mL enumeration.

517 ## Glucose Titration in BHI and SM

518 BHI and SM were used to create media containing the following glucose concentrations: 0.0%, 0.2%, 0.4% and
519 0.6%. Media was placed in the anaerobic chamber to reduce before using. VRE was inoculated 1:20 into each
520 glucose concentration of both BHI and SM. Cultures were incubated for 24 hours before being filtered sterilized as
521 described above. *C. difficile* was inoculated 1:60 into VRE-conditioned media and fresh BHI and SM conditions and
522 incubated for 24 hours before serial dilutions and drop plating for enumeration.

523 ## Dilution of inhibition by VRE Conditioned Media

524 VRE conditioned media was prepared by inoculating VRE 1:20 into BHI with 0.0%, 0.2%, 0.4% and 0.6% added
525 glucose. Cultures were grown for 24-36 hours to an OD_{600} >2 before being spun down and filter sterilized. A subset
526 of the supernatant was taken out of the chamber for pH recording. The VRE-conditioned media was then diluted 0,
527 1:1, 1:2, 1:4 and 1:8 with sterile PBS. After dilution, 3 mL aliquots were divided into test tubes per each biological
528 replicate. C. difficile mid-log cultures were diluted 1:10 in sporulation media, then added to the aliquots in a 1:60
529 dilution and grown between 24 hour before drop plating for enumeration.

530 ## Acidification and Neutralization of SM and BHI

531 Acidification of SM and BHI was created by titrating HCl into the media to a pH of 7.0, 6.5, 6.0, 5.5, 5.0 and 4.5
532 before being autoclaved and aliquoted. *C. difficile* was inoculated into the reduced media 1:60 and incubated at 37°C
533 for 48 hours before being serial diluted and drop plated for enumeration.

534 Neutralization was performed by growing VRE in SM + 0.6% glucose or BHI + 0.4% glucose to exhaustion before
535 being spun down and filter sterilized. The filtered media pH was recorded to ensure full acidification at or below
536 5.5, then neutralized to a pH of 7.0 with NaOH. Neutralized media was filter sterilized again and aliquoted into 3
537 mL test tubes then placed in an anaerobic incubator for 24 hours to ensure full media reduction. 3 mL aliquots of the
538 acidified media were collected to be used as a negative control. After being reduced, *C. difficile* was inoculated 1:60
539 into the media conditions. *C. difficile* was also inoculated 1:60 into fresh media at a pH of 7.0 as a positive control.
540 All media conditions were incubated at 37°C for 48 hours before drop plating and enumeration.

541 ## Cytotoxicity Assays

542 Vero cells were grown at 10,000 cells per well in a 96-well plate in Eagle's minimum essential media (EMEM) +
543 10% Fetal Bovine Serum and 1x Penicillin & Streptomycin overnight. Culture supernatants were frozen at -80C
544 until assay. All samples were spun down for 15 minutes at 15,000g before being filter sterilized into new tubes.
545 Each sample was serial diluted down to 10^{-6} in a fresh 96-well plate with PBS. *C. difficile* purified toxin (TechLab)
546 was diluted into 1 mL of sterile water as a positive toxin control. 96 µL of each sample was placed in the top row of
547 a 96-well plate and 4 µL of the anti-toxin (TechLab) was added and incubated for at least 20 minutes to ensure full
548 toxin neutralization from the anti-toxin. Samples with antitoxin were serially diluted in the remaining rows down to
549 10^{-6} in PBS. Once all toxin, aliquoted samples and antitoxin samples were completed, 100uL of each sample and the
550 dilutions were placed onto their respective Vero-cell wells. Vero cells were incubated at 37°C with 5.0% CO_2
551 overnight. Cytotoxicity was then analyzed with microscopy. Cells that showed rounding were positive for
552 cytotoxicity. To calculate toxin cytotoxicity, dilutions with less than 80% cell rounding were considered cytotoxic-
553 negative, and the previous dilution was considered cytotoxic positive. This cytotoxic positive dilution number was
554 then used to calculate the Log10 reciprocal cytotoxicity value.

555 ## Conservation of Mechanism

556 We first grew each enterococcal in BHI+0.6% glucose overnight, filter sterilized and inoculated *C. difficile* VPI to
557 grow for 48 hours at 37°C before serial dilution and drop plating on *C. difficile* plates for enumeration. We then used
558 VRE to condition SM + 0.6% glucose, spun down and filter sterilized as previously described. We then inoculated
559 mid-log cultures of the remaining Clostridia panel into the VRE-conditioned media in a 1:60 dilution and grew C.
560 difficile for 48 hours at 37°C before drop plating. We also performed neutralization and acidification as described
561 above with this clostridia panel.

562 ## In-vitro Biofilms

563 24-well polystyrene cell culture plates were treated with 100 µL of 5mg/mL human fibrinogen overnight or until dry
564 before being placed in the anaerobic chamber. All SM used for these experiments also contained 125 µM
565 deoxycholic acid (DCA). 2 mL aliquots of each condition, SM, SM + 0.6% glucose, SM + 0.6% fucose, SM + 0.6%
566 xylose was placed into the middle wells of the 24-well fibrinogen treated plate with 3 technical replicates as well as
567 one replicate for a contamination blank. *C. difficile* and VRE overnight mid-log cultures were normalized to 0.5
568 OD_{600}. Monoculture inoculation of *C. difficile* and VRE in a 1:60 dilution was used as positive controls in each
569 media condition. Dual cultures were created by combining the normalized culture 1 to 1 in a new tube before being
570 inoculated 1:60 into the media conditions. The remaining empty wells around each 24-well treated plate were 2/3
571 filled with sterile water to mitigate media evaporation. The plates were incubated at 37°C for 48 hours without
572 media changes. After 48 hours, the plates were tilted at a 45° angle and the media was slowly discarded in 200 µL
573 aliquots, taking care to only take off the media from the edge and just below the air-liquid interface to ensure the
574 biofilms stayed intact. 500 µL sterile PBS was then slowly added into the biofilm wells, maintaining the 45° angle,

575 and adding the water to the bottom edge of the well to not disturb the biofilm. The plate was then tilted the opposite
576 direction twice, before discarding the liquid in sets of 200 µL slowly to wash the biofilm. 500 µL of sterile PBS was
577 then added to each biofilm and the plate was gently vortexed for 10 minutes to resuspend the biofilm in liquid. After
578 resuspension, the liquid was used for serial dilutions and drop plating for enumeration. ***Microscopy Details***

579 Live/Dead Staining of Biofilms

580 Biofilms were grown as described above in SM, SM + 0.6% glucose or SM + 0.6% fucose, with 125 uM DCA
581 added to the media. C. difficile was grown alone and in a 1:1 ratio with VRE, diluted 1:100 into the biofilm wells.
582 Biofilms were grown undisturbed for 48 hours. Biofilm supernatant was discarded as described above and washed
583 once with 200uL PBS. 1 mL of PBS was gently placed on top of the biofilm samples. For fluorescent confocal
584 microscopy, biofilms were stained with Invitrogen™ LIVE/DEAD™ BacLight™ Bacterial Viability Kit, for
585 microscopy (Catalog number: L7007). CYTO-9 and Propidium Iodide solutions were mixed 1:1 before adding 3 uL
586 to each biofilm sample for 15 minutes in the dark in the anaerobic chamber. Wells were swirled gently to ensure the
587 stain was homogeneously mixed on top of the biofilms. After staining, the supernatant was discarded, wells were
588 washed with 200 uL PBS and 300uL of fresh 4% paraformaldehyde (PFA) and incubated for 20 minutes.

589 Confocal Imaging of Biofilms

590 Biofilm samples were kept in their original 24 well plate for microscopy. The Leica-Sp5 confocal scanning
591 microscope was used for fluorescent imaging of the Live/Dead stain. The stains SYTO-9 and Propidium Iodide used
592 in the Live/Dead staining procedure have emission wavelengths of 490 and 480 and excitation wavelengths of 635
593 and 500 nm, respectively. These emission and excitation wavelengths were programmed into the Leica LAS-AF set
594 up, with the Argon laser being set to a total power of 30%. Channel 1 was set to the SYTO-9 (green) wavelength of
595 500nm and Channel 2 was set to the propidium iodide (red) wavelength of 635 nm. A 10x objective lens was used
596 for all confocal imaging with a resolution of 2046 x 2046. A 10x zoom was used to visualize individual biofilm
597 formations and 2-D images were taken using z-stacking of 3.00 micron thickness to image the bottom to the top of
598 an individual microcolony. Imaging was also taken of unstained biofilms, SM stained and unstained with and
599 without DCA and empty wells to verify a lack of background fluorescence. LAS X application version 3.7.4.23463
600 was used to visualize the z-stacks and compile snapshots as seen in figure 7.

601

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