Clostridioides difficile colonization is not mediated by bile salts and requires Stickland fermentation of proline in an *in vitro* model of infection

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ABSTRACT

Treatment with antibiotics is a major risk factor for *Clostridioides difficile* infection, likely due to depletion of the gastrointestinal microbiota. Two microbiota-mediated mechanisms thought to limit *C. difficile* colonization include conversion of conjugated primary bile salts into secondary bile salts toxic to *C. difficile* growth, and competition between the microbiota and *C. difficile* for limiting nutrients. Using a continuous flow model of the distal colon, we investigated how treatment with six clinically-used antibiotics influenced susceptibility to *C. difficile* infection in 12 different microbial communities cultivated from healthy individuals. Antibiotic treatment reduced microbial richness; disruption varied by antibiotic class and microbiota composition, but did not correlate with *C. difficile* susceptibility. Antibiotic treatment also disrupted microbial bile salt metabolism, increasing levels of the primary bile salt, cholate, and decreasing levels of the secondary bile salt, deoxycholate. However, decreased levels of deoxycholate did not correlate with increased *C. difficile* susceptibility. Further, bile salts were not required to inhibit *C. difficile* colonization. We tested whether amino acid fermentation contributed to persistence of *C. difficile* in antibiotic-treated communities. *C. difficile* mutants unable to use proline as an electron acceptor in Stickland fermentation due to disruption of proline reductase (Δ*prdB*) had significantly lower levels of colonization than wild-type strains in four of six antibiotic-treated communities tested. This data provides further support for the importance of bile salt-independent mechanisms in regulating colonization of *C. difficile.*

IMPORTANCE

C. difficile is one of the leading causes of hospital-acquired infections and antibiotic-associated diarrhea. Several potential mechanisms through which the microbiota can limit *C. difficile* infection have been identified and are potential targets for new therapeutics. However, it is unclear which mechanisms of *C. difficile* inhibition represent the best targets for development of new therapeutics. These studies demonstrate that in a complex *in vitro* model of *C. difficile* infection, colonization resistance is independent of microbial bile salt metabolism. Instead, the ability of *C. difficile* to colonize is dependent upon its ability to metabolize proline, although proline-dependent colonization is context-dependent and is not observed in all disrupted communities. Altogether, these studies support the need for further work to understand how bile-independent mechanisms regulate *C. difficile* colonization.

INTRODUCTION

Clostridioides difficile is one of the leading causes of nosocomial infections due to its transmissibility as environmentally-resistant spores and its ability to infect patients who have been treated with antibiotics (1, 2). Many classes of antibiotics can disrupt the colonic microbiota (3–5); disruption can decrease production of inhibitory metabolites (6–9) and reduce competition for limiting nutrients (10–13), providing favorable conditions for *C. difficile* infection. Although multiple mechanisms for colonization resistance have been identified, an understanding of the hierarchical importance of these mechanisms in *C. difficile* colonization and disease is just beginning to emerge.

Microbial bile salt metabolism has been extensively studied for its role in limiting *C. difficile* colonization. The primary bile salts cholate and chenodeoxycholate are synthesized in the liver

67 and are conjugated to glycine or taurine to improve solubility (14). Once secreted into the
68 intestine, microbial enzymes begin modifying these bile salts, removing conjugated amino acids
69 and dehydroxylating primary bile salts into secondary bile salts (14). *C. difficile* spore germination
70 is stimulated by cholate-family bile salts (cholate, taurocholate, glycocholate, deoxycholate) and
71 inhibited by chenodeoxycholate-family bile salts (15, 16), although there is variation in these
72 responses between strains (17). Germination is enhanced by amino acid (15, 18) and calcium co-
73 germinants, which act synergistically with bile salts to enhance germination (19). Secondary bile
74 salts (deoxycholate, lithocholate) inhibit growth of vegetative *C. difficile* in vitro (7, 15, 20), and
75 low levels of secondary bile salts correlate with *C. difficile* infection in humans (21, 22) and in
76 mouse models (7, 23, 24).

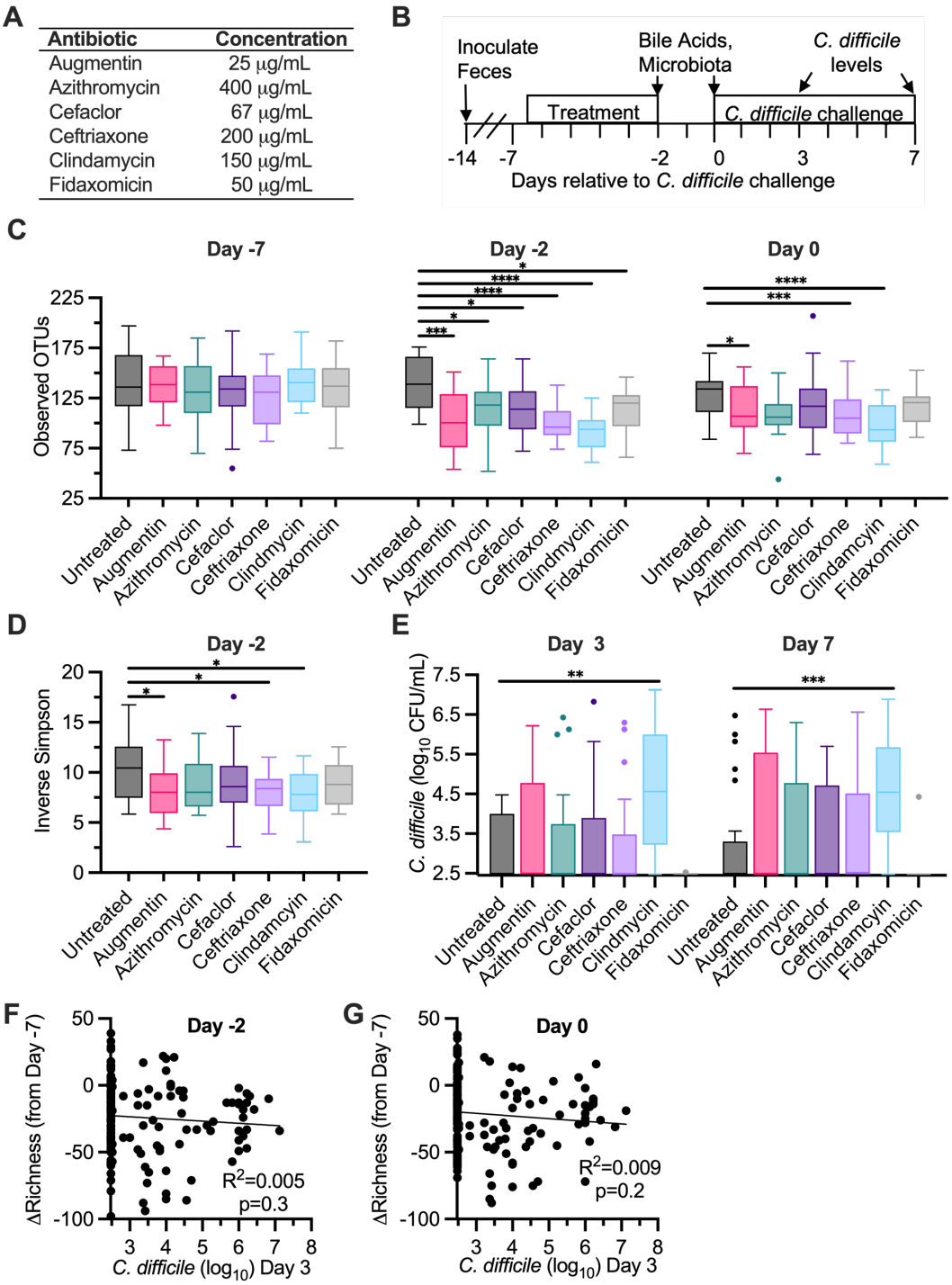
77 However, recent studies have demonstrated that our understanding of the role of bile salts
78 in *C. difficile* colonization resistance may be incomplete. *C. difficile* spore colonization and
79 fulminant disease was observed in *Cypb8b1*$^{-/-}$ mice unable to make cholate-family bile salts (25),
80 and resistance to *C. difficile* colonization was observed even though these mice do not produce
81 the secondary bile salts deoxycholate or lithocholate. *Clostridium scindens*, a microbe inferred to
82 inhibit *C. difficile* through dehydroxylation of primary bile salts to secondary bile salts (24), was
83 also shown to inhibit *C. difficile in vitro* through production of tryptophan-derived antibiotics (9)
84 and other uncharacterized metabolites (26) and to potentially compete with *C. difficile* for
85 nutrients required for growth in *Cypb8b1*$^{-/-}$ monocolonized mice (25). In addition, both cholate
86 and deoxycholate were shown to induce similar *C. difficile* stress responses, although 10X higher
87 concentrations of cholate were used to observe these effects (27).

88 Competition for nutrients between commensal microbes and *C. difficile* has long been
89 postulated as a mechanism for colonization resistance, with Wilson and colleagues utilizing
90 continuous-flow culture systems to demonstrate competition between the microbiota and *C.*
91 *difficile in vitro* (28, 29). *C. difficile* can metabolize mucin monosaccharides *in vitro* (30), and
92 preferentially expresses pathways for degradation of mucin monosaccharides in mouse models
93 (31, 32), indicating that metabolism of mucin monosaccharides may be a niche open to *C. difficile*
94 during infection. Similarly, *C. difficile* metabolism of carbohydrates and sugar alcohols from the
95 host and its diet may be another niche open to *C. difficile* during infection, as expression of genes
96 in these metabolic pathways increase during infection in germ-free and antibiotic-treated mice
97 (11, 31, 32). In addition to carbohydrate metabolism, *C. difficile* also efficiently utilizes amino
98 acids through Stickland fermentation (33), which is a metabolic pathway limited primarily to
99 proteolytic Clostridial species (34), providing a unique metabolic niche within the GI tract.
100 Increasing evidence from human (10, 35) and mouse (11, 13, 25, 31, 32, 36) studies points to
101 Stickland fermentation with proline as an electron acceptor as a preferred nutritional niche for
102 *C. difficile* in the GI tract.
103 Previously, we described an *in vitro* minibioreactor array (MBRA) model for characterization
104 of *C. difficile* colonization resistance in the presence of human fecal microbial communities under
105 nutritional conditions that simulate the distal colon (37), that has subsequently been used to
106 characterize microbes that inhibit *C. difficile* colonization and/or toxin production (38–40). In this
107 study, we used the MBRA model to investigate how treatment with six clinically-used antibiotics
108 differentially impacted susceptibility to *C. difficile* infection in microbial communities cultivated
109 from 12 healthy individuals. As expected, we observed that antibiotic treatment reduced

110 microbial diversity, although the extent of antibiotic-mediated disruption varied across individual
111 communities and was not well correlated with susceptibility to *C. difficile* infection. Antibiotic
112 treatment also reduced microbial metabolism of cholate to deoxycholate, although there was no
113 correlation between deoxycholate levels and susceptibility to *C. difficile* infection. To further test
114 whether bile salts contributed to *C. difficile* colonization resistance, we cultivated fecal
115 communities in the presence and absence of bovine bile. Similar to what was observed by Aguirre
116 et al. (25) in *Cypb8b1*$^{-/-}$ mice, we observed bile salts were not required for *C. difficile* colonization
117 resistance and that the low level of *C. difficile* spore germination that occurred in the absence of
118 bile was sufficient to allow colonization of antibiotic-treated communities by *C. difficile* spores.
119 Using a *C. difficile* mutant unable to utilize proline as an electron acceptor during Stickland
120 fermentation due to a mutation in proline reductase (Δ*prdB* (41)), we demonstrated that proline
121 reduction was required for *C. difficile* to colonize a subset of antibiotic-disrupted microbial
122 communities. These results can serve as a foundation for further mechanistic characterization of
123 the hierarchical importance of different nutritional environments in limiting *C. difficile*
124 colonization.
125
126 **RESULTS**
127 **Loss of microbial diversity is not sufficient to promote *C. difficile* colonization.** Previously, we
128 demonstrated that microbial communities cultured from the fecal samples of healthy individuals
129 in MBRAs can resist colonization by *C. difficile*; treatment with clindamycin could induce
130 susceptibility to *C. difficile* colonization in these complex fecal communities (37). To better
131 understand how this model can be used to measure effects of antibiotics on microbiota

132 disruption and *C. difficile* colonization, we compared treatment with six individual antibiotics (Fig.

133 1A) on microbial community composition and *C. difficile* susceptibility of communities cultured



134

135 **Figure 1**: **Alterations in microbiota diversity and levels of *C. difficile* varied by type of antibiotic**
136 **administered.** (A) Antibiotics administered during experiment. (B) Experimental timeline
137 indicating points where samples were collected; time is indicated relative to the point of *C.*
138 *difficile* challenge (Day 0). In (C)-(G), samples were collected from duplicate reactors inoculated
139 with one of twelve healthy human fecal samples and treated as indicated. (C) Microbiota richness
140 (Observed OTUs with $\geq$ 97% identity) and (D) diversity (Inverse Simpson measure) were
141 determined from 16S rRNA gene sequences. Microbiota richness from Day -7 and Day 0 are
142 reported in Supplementary Figure S1. (E) *C. difficile* levels on Day 3 and 7 of infection. Boxes
143 represent the interquartile range (IQR), cross lines represent the median values, whiskers
144 indicate 1.5X IQR, and data outside this range were indicated by discrete points. Statistical
145 significance of differences from untreated samples was determined by Kruskal-Wallis testing with
146 Dunn's correction for multiple comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.
147 In (F)-(G), the change in richness from baseline (Day -7) on (F) Day -2 and (G) Day 0 were plotted
148 as a function of $\log_{10}$ *C. difficile* levels on Day 3 of infection. Simple linear regression was used to
149 evaluate the relationship between the variables.

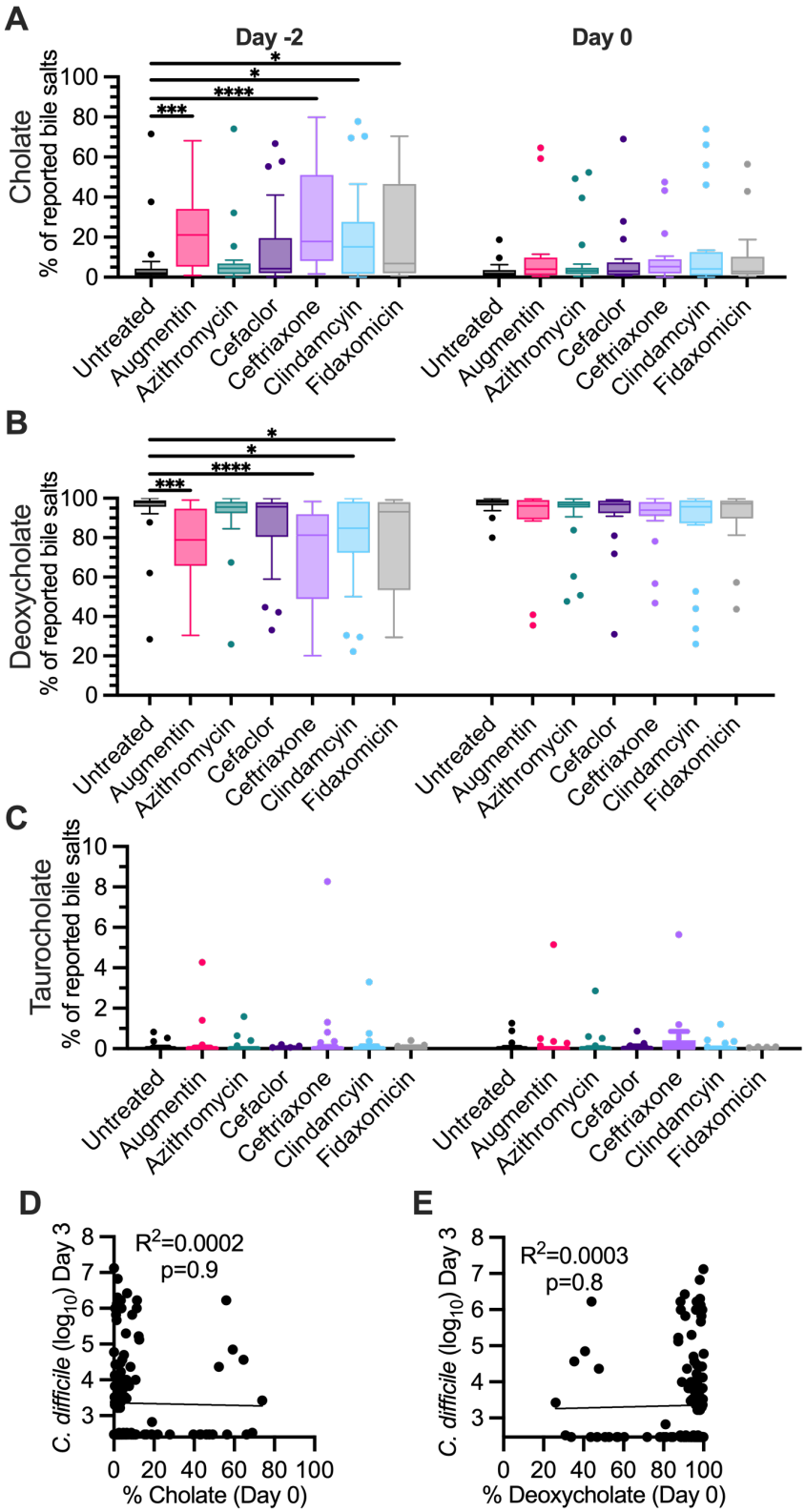
150 from twelve different healthy individuals. These antibiotics were selected because they are all
151 used clinically, but vary in antimicrobial spectrum. Fourteen independent bioreactors were
152 inoculated with each fecal sample and allowed to stabilize in continuous culture for seven days.
153 Reactors were treated in duplicate with one of six antibiotics twice daily for five days (Figure 1B);
154 two reactors served as untreated controls. Two days after the end of antibiotic treatment,
155 communities were challenged with vegetative cells of *C. difficile* strain 2015 (CD2015), a ribotype
156 027 clinical isolate (37). Samples were collected prior to administration of antibiotics, at the end
157 of antibiotic treatment, and two days later, just prior to *C. difficile* challenge, for analysis of
158 microbial community composition by 16S rRNA gene sequencing. Samples were additionally
159 collected for analysis of *C. difficile* levels on days 3 and 7 post-infection.

160 Prior to antibiotic treatment (Day -7), there were no differences in richness (Figure 1C) or
161 microbial diversity between treatment groups (Supplementary Figure 1A). Treatment with
162 antibiotics significantly reduced microbiota richness for all antibiotics tested at the end of
163 antibiotic treatment (Day -2; Figure 1C), with median richness declining from 1.2-fold (cefaclor)
164 to 1.5-fold (clindamycin). Microbial diversity declined significantly following treatment with just
165 three antibiotics – clindamycin, ceftriaxone, and augmentin (Figure 1D), which was consistent
166 with the largest declines in microbial richness. Richness and diversity had partially recovered two
167 days following antibiotic treatment (Figure 1C; Supplementary Figure S1), although communities
168 treated with clindamycin, ceftriaxone, and augmentin still exhibited significantly lower microbial
169 richness compared to untreated controls (Figure 1C, Day 0).

170 *C. difficile* susceptibility, measured as levels of *C. difficile* detected on day 3 or day 7 following
171 challenge with *C. difficile* cells, varied across different antibiotic treatments (Figure 1E) and fecal
172 donors (Supplementary Figure S2). Of the twelve fecal samples tested, untreated communities
173 from seven fecal samples were resistant to colonization ($<10^4$ *C. difficile* colony forming units
174 (CFU)/mL at both time points in both replicates), untreated communities from four communities
175 exhibited variable susceptibility to colonization ($>10^4$ CFU/mL in one or more replicates/time
176 points), and untreated communities from one fecal samples was susceptible to colonization ($>10^4$
177 CFU/mL in both replicates/time points). Clindamycin was the only antibiotic that significantly
178 increased levels of *C. difficile* colonization above levels observed in untreated communities
179 (Figure 1E), with communities from nine of twelve fecal samples exhibiting median levels of
180 colonization $>10^4$ CFU/mL following clindamycin-treatment (Supplementary Figure S2).
181 Susceptibility to infection varied across communities from different fecal samples that were

182 treated with other antibiotics (Supplementary Figure S2). We tested whether changes in richness
183 from baseline following antibiotic treatment (Day -2) or on the day of *C. difficile* challenge (Day
184 0) were correlated with levels of *C. difficile* on day 3 (Figure 1F-1G) or day 7 of infection
185 (Supplementary Figure S3); we found that there were no significant correlations between
186 changes in richness and susceptibility to *C. difficile* colonization. When we tested for taxa whose
187 differences in abundance at Day 0 correlated with susceptibility to *C. difficile* colonization
188 (Supplementary Figure S4), we observed that five taxa were significantly higher in resistant
189 communities and three taxa were significantly lower in resistant communities.
190
191 **Antibiotic treatment alters bile salt metabolism.** Bioreactor medium (BRM3) contains bovine
192 bile as a complex source of bile salts. Analysis of bioreactor medium indicated that cholate family
193 bile salts predominated, with approximately 36% taurocholate (198 μM), 34% glycocholate (190
194 μM), 10% cholate (56 μM), and 2% deoxycholate (13 μM) of total bile salts measured
195 (Supplementary Figure S5A-B). To understand how microbiota cultivation and antibiotic
196 treatment altered bile salt pools in bioreactor cultures, we focused on quantification of the amino
197 acid conjugated primary bile salt, taurocholate, the primary bile salt, cholate, and the secondary
198 bile salt, deoxycholate across the communities described in Figure 1. We observed that
199 microbiota cultivation led to a >500-fold decrease in taurocholate, a 3-fold decrease in cholate,
200 and a 20-fold increase in deoxycholate in spent culture supernatant compared to fresh medium
201 (Supplementary Figure S5C). Treatment with four antibiotics – augmentin, ceftriaxone,

202 clindamycin, and fidaxomicin - significantly shifted the composition of bile salts compared to



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204 **Figure 2: Cholate, deoxycholate, and taurocholate levels in untreated and antibiotic-treated**
205 **bioreactors.** Levels of (A) cholate, (B) deoxycholate, and (C) taurocholate were measured from
206 the communities described in Figure 1 at the end of antibiotic treatment (Day -2) and just prior
207 to *C. difficile* challenge (Day 0). Boxes represent the IQR, cross lines represent median values, and
208 whiskers indicate 1.5 X IQR; data outside this range were indicated by discrete points. Significance
209 of differences from untreated samples was determined by Kruskal-Wallis testing with Dunn's
210 correction for multiple comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.
211 Relative proportions of bile salts were reported because the coefficient of variation in untreated
212 samples was lower compared to absolute concentrations (Supplementary Figure S5D); absolute
213 concentrations are reported in Supplementary Figure S6A-C. Simple linear regression of (D)
214 percent cholate and (E) percent deoxycholate on Day 0 relative to levels of *C. difficile* on Day 3 of
215 infection.

216 untreated communities, with significant increases in cholate and decreases in deoxycholate two
217 days prior to *C. difficile* challenge (Figure 2A-2B). No significant differences in taurocholate were
218 observed across communities treated with antibiotics (Figure 2C). As with microbial diversity
219 measures, bile salt levels had recovered two days following antibiotic treatment and there was
220 no significant correlation between levels of cholate on Day 0 of infection and *C. difficile*
221 colonization on Day 3 (Figure 2D) or Day 7 (Supplementary Figure S6D) of infection.

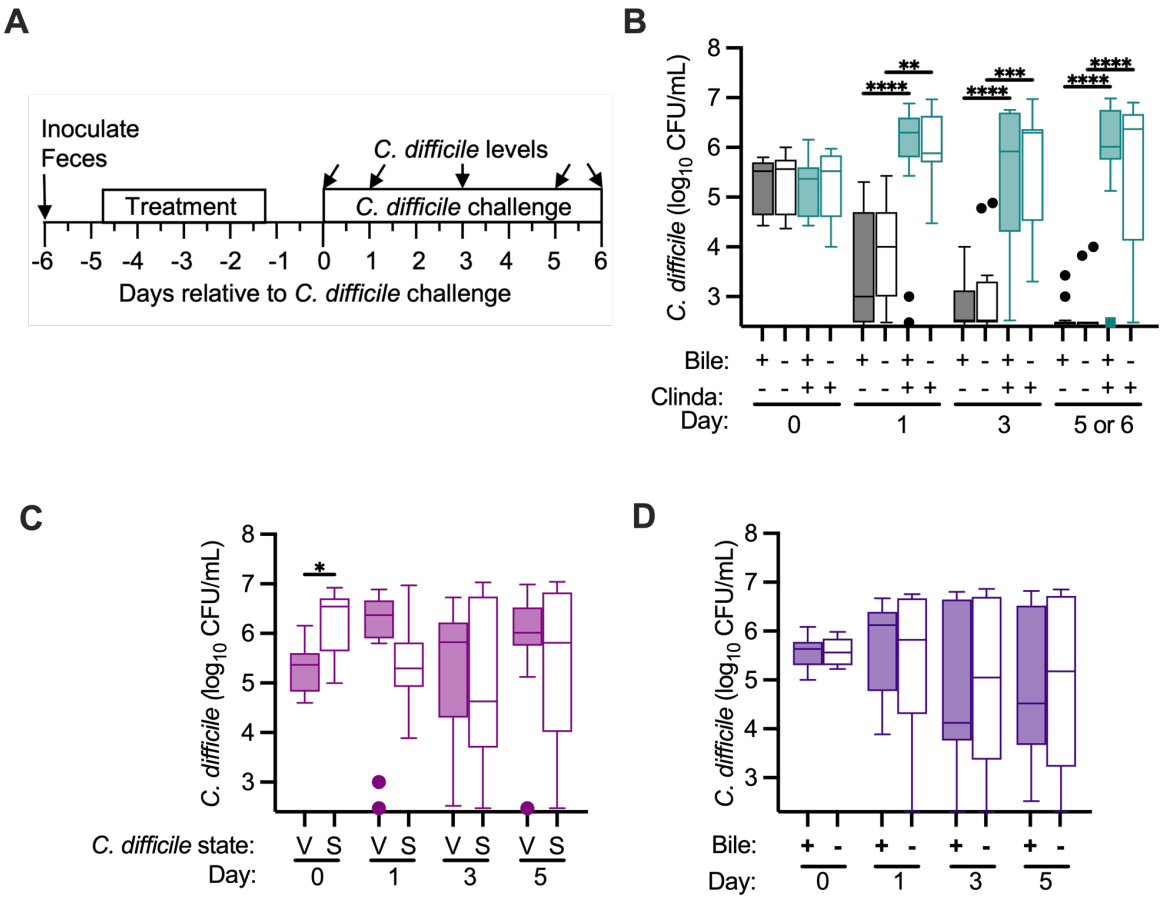
222 Previous studies have found that deoxycholate inhibits the growth of vegetative *C. difficile*
223 cells (7, 15, 20), with inhibitory concentrations ranging from 0.01 – 0.1% (250 - 2500 μM). The
224 median concentration of deoxycholate measured in untreated communities on the day of *C.*

225 *difficile* challenge was 602 μM (Supplementary Figure S6B); median concentrations in antibiotic
226 treated communities ranged from 431 μM (augmentin) to 608 μM (clindamycin), but were not
227 significantly different than those observed in untreated communities on Day 0. Because both *C.*
228 *difficile* susceptibility and deoxycholate levels varied by fecal donor and antibiotic treatment, we
229 tested whether *C. difficile* levels observed on Day 3 or Day 7 of infection correlated with
230 deoxycholate levels on Day 0. We found no correlation between deoxycholate levels on the day
231 of *C. difficile* challenge and the levels of *C. difficile* on Day 3 (Figure 2E) or Day 7 (Supplementary
232 Figure S6E-F) and that 18% of communities with high *C. difficile* levels (>10^5 CFU/mL) on Day 7 of
233 infection had deoxycholate levels ≥1000 μM on Day 0 of infection.
234
235 **Bile was not required for *C. difficile* colonization resistance *in vitro*.** While the previous results
236 were consistent with the hypothesis that secondary bile salts do not mediate colonization
237 resistance in communities cultured in bioreactors, we could not rule out effects of other bile salts
238 that were not measured. To test whether bile salts were required, we monitored colonization
239 resistance of microbial communities established from five fecal samples cultured in parallel in
240 media with and without added bile. Specifically, replicate bioreactors were established for each
241 fecal sample in media either containing or lacking bovine bile. Triplicate communities were
242 treated with clindamycin as previously described (42), while the remaining three replicates for
243 each type of media were left untreated (Figure 3A). Communities were challenged with
244 vegetative cells of CD2015 and *C. difficile* levels were monitored over time. We found *C. difficile*
245 colonization was similarly suppressed in communities cultured in the presence or absence of bile
246 and that treatment with clindamycin led to similar levels of *C. difficile* colonization (Figure 3B).

247

248 **Bile was not required for *C. difficile* spores to colonize *in vitro*.** Cholate family bile salts are

249 known to greatly enhance *C. difficile* spore germination during *in vitro* culture (15, 43, 44),



250

251 **Figure 3. Bile acids were not required for colonization resistance and did not enhance**

252 **colonization in communities of human fecal microbes cultured in bioreactors.** (A) Experimental

253 timeline indicating points where samples were collected; time is indicated relative to the point

254 of *C. difficile* challenge. (B) *C. difficile* levels in untreated and clindamycin-treated communities

255 cultured in the presence and absence of bile. (C) *C. difficile* levels in clindamycin-treated

256 communities challenged with *C. difficile* vegetative (V) cells or spores (S). (D) *C. difficile* levels in

257 clindamycin-treated communities cultured in the presence or absence of bile challenged with *C.*

258 *difficile* spores. Boxes represent the IQR, cross lines represent the median values, whiskers

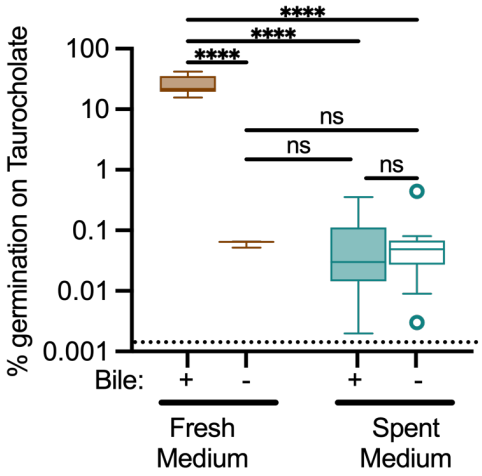
259 indicate 1.5 X IQR; data outside this range were indicated by discrete points. Significance of

260 differences from untreated samples was determined by Kruskal-Wallis testing with Dunn's
261 correction for multiple comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

262 although they are not required for spore germination in a mouse model of infection (25).
263 Previously, we reported that we were unable to colonize a clindamycin-treated microbial
264 community cultured in an MBRA model with *C. difficile* spores (37). Because these studies were
265 performed using a microbial community formed from pooling twelve fecal donors, we tested the
266 ability of the spores to colonize clindamycin-treated communities established from five individual
267 fecal samples. We found that the majority of clindamycin-treated communities could be
268 colonized following challenge with *C. difficile* spores (Figure 3C), although communities from one
269 fecal sample were colonized at a lower frequency by spores than by vegetative cells
270 (Supplementary Figure S7). We then tested whether bile salts were required for *C. difficile* spores
271 to colonize clindamycin-treated fecal microbial communities cultured in MBRAs. We observed
272 that colonization by spores was not significantly different between communities cultured in
273 media with and without bile (Figure 3D).

274 Because cholate family bile salts were previously reported to enhance germination, we
275 compared the ability of media with and without bile to enhance colony formation of spores in
276 pure culture. Consistent with previous observations (15, 43), incubation of CD2015 spores in
277 bioreactor medium containing bile for one hour enhanced colony formation by germinated *C.*
278 *difficile* spores by >400-fold compared to incubation in the presence of bioreactor medium
279 without bile (Figure 4). We next tested how spent culture medium collected from clindamycin-
280 treated communities cultured in the presence or absence of bile would impact spore
281 germination. Low levels of spore germination were observed in spent culture medium from fecal

282 communities cultured in the presence and absence of bile (Figure 4). Altogether, this data
283 indicates that the levels of bile salts present in spent culture medium from communities cultured
284 in the presence of bile do not have significant impacts on germination. Further, while the levels
285 of spore germination and colony formation in spent culture medium are low, these low levels of
286 germination would result in ~10^3 vegetative cells, a level previously shown to infect antibiotic
287 disrupted communities (37).



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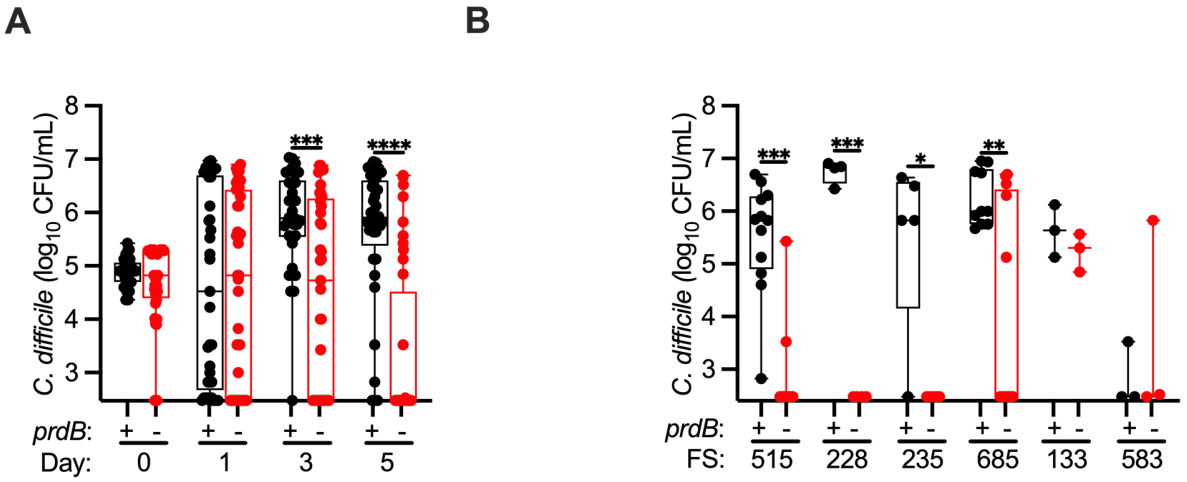
289 **Figure 4. Low levels of spore germination in spent culture medium from communities cultured**
290 **in the presence of bile**. Germination of CD2015 spores was determined in filter-sterilized spent
291 culture medium from clindamycin-treated fecal communities cultured in the presence or absence
292 of bile. Spores were incubated in medium for one hour followed by enumeration on agar medium
293 with or without added taurocholate to determine the percent germination on taurocholate.
294 Fresh bioreactor medium made with or without bovine bile were included as controls. Boxes
295 represent the IQR, cross lines represent the median values, and whiskers indicate 1.5 X IQR; data
296 outside this range were indicated by discrete points. Significance of differences between samples
297 were determined by one-way ANOVA with Brown-Forsythe correction for unequal variances and
298 Dunnett's correction for multiple comparisons. ns, $p>0.05$; ****, $p<0.0001$.

299 **Proline metabolism was required for *C. difficile* to colonize a subset of fecal samples *in vitro*.**
300 Proteolytic metabolism through Stickland fermentation has previously been reported as an
301 important nutritional niche for *C. difficile* in human (10, 35) and mouse models of infection (11,
302 13, 25, 31, 32, 36). As bioreactor medium contains low levels of fermentable carbohydrates and
303 does not contain mucin-associated monosaccharides, we hypothesized that Stickland
304 Fermentation with proline as an electron acceptor could be important for colonization of
305 antibiotic-disrupted fecal communities. To test this hypothesis, we compared the colonization of
306 mutants defective in the ability to metabolize proline (Δ*prdB*::CT (41)) to a wild type (wt) strain.
307 Fecal samples from six healthy individuals were cultured in replicate bioreactors, treated with
308 clindamycin, and challenged with wt or Δ*prdB* strains using the approach described in Fig. 3A.



309

310 **Figure 5. *C. difficile* Δ*prdB* mutants fail to persist in most clindamycin-treated communities.** (A)
311 Levels of wt (*prdB*$^+$) and Δ*prdB* (*prdB*$^-$) strains were measured over time following introduction
312 into fecal communities treated with clindamycin as outlined in Figure 3A. Data reported are
313 pooled from all six fecal samples tested. (B) Levels of wt and Δ*prdB* strains measured on day 5 of
314 infection from each of the six fecal samples tested (FS515, FS228, FS235, FS685, FS133, and
315 FS583).

316 Overall, we observed that fecal communities challenged with Δ*prdB* mutants showed decreasing
317 levels of colonization over time, with significantly lower levels observed on day 3 and 5 of
318 infection compared to wild type (Figure 5A). However, the ability of wt and Δ*prdB* strains to
319 colonize was dependent upon the fecal community tested (Figure 5B). Wild-type strains
320 colonized five of the six clindamycin-disrupted communities (FS515, FS228, FS235, FS685, FS133)
321 whereas Δ*prdB* strains failed to colonize two of these susceptible communities (FS228, FS235).
322 In the other three susceptible communities, there was either a lack of Δ*prdB* colonization in the
323 majority of replicates tested (FS515: 10/12 replicates; FS685; 8/13 replicates), or there was no
324 difference between levels of wt and Δ*prdB* colonization levels (FS133). Based on these results,
325 we conclude that Stickland fermentation with proline as an electron acceptor can play an
326 important role in the ability of *C. difficile* to persist in complex communities *in vitro*, but this is
327 dependent upon the composition of the fecal community and its ability to limit *C. difficile*
328 colonization through other mechanisms.
329
330 **DISCUSSION**
331 The factors that govern whether or not an individual exposed to *C. difficile* will go on to develop
332 symptomatic disease are complex. While it is clear that disruption of the GI microbiota is a key
333 risk factor for infection and disease, the extent to which different microbes interact with each
334 other and the host to limit *C. difficile* infection and disease progression are not completely
335 understood. Developing tools that allow microbe and metabolite interactions to be investigated
336 in the absence of a host can help to provide insights into whether mechanisms that inhibit or
337 promote *C. difficile* colonization are more likely to be causative or correlative. This level of

338 understanding is necessary as new therapeutic approaches that more narrowly target *C. difficile*,
339 such as defined microbial consortia are developed.

340 To gain greater insights into factors that govern *C. difficile* colonization resistance in a fecal
341 MBRA model, we investigated how six different clinically-used antibiotics impacted the ability of
342 the microbiota to resist *C. difficile* colonization. As expected, we observed that antibiotic
343 treatment led to loss of microbial richness, although the extent of microbiota disruption varied
344 both by the class of antibiotic tested and by the composition of the microbial community.
345 Consistent with previous meta-analyses of human antibiotic exposure and risk for *C. difficile*
346 infection in non-hospitalized patients (45, 46), clindamycin was most strongly associated with
347 susceptibility to infection in antibiotic-treated communities, with ten of twelve communities
348 tested showing susceptibility following treatment. However, the magnitude of microbiota
349 disruption across antibiotic treatments did not correlate with susceptibility to *C. difficile*
350 infection, with some communities with significant losses in richness resisting *C. difficile* infection
351 and other communities with modest richness losses exhibiting susceptibility to infection. This
352 data provides further support for the hypothesis that *C. difficile* colonization resistance is likely
353 due to the presence of specific microbes and their functions that limit *C. difficile* colonization
354 rather than overall microbiota diversity.

355 Due to the compositional diversity between fecal communities tested, there were relatively
356 few conserved taxa whose presence or absence correlated with susceptibility to *C. difficile*
357 infection, which is consistent with the model that differences in microbiota function rather than
358 composition are important for understanding *C. difficile* susceptibility. Similar to previous studies,

359 however, levels of *Clostridium scindens* and *Bacteroides thetaiotaomicron* were higher in
360 resistant communities.

361 To better understand which microbiota functions may be important for colonization
362 resistance in this model, we investigated two mechanisms previously proposed to regulate *C.*
363 *difficile* colonization: microbial metabolism of bile acids and Stickland fermentation with proline
364 as an electron acceptor. We observed that microbial communities cultured in bioreactors were
365 able to convert the taurocholate in bovine bile into deoxycholate. Treatment with antibiotics had
366 no significant impacts on levels of taurocholate. Microbes encoding bile salt hydrolases, which
367 remove taurine and glycine from primary bile salts (14), are broadly distributed amongst
368 members of the GI microbiota (47), which may have contributed to the ability of this function to
369 persist during antibiotic treatment.

370 In contrast, enzymes necessary to perform 7α-dehydroxylation of cholate to deoxycholate
371 are restricted to a smaller subset of the GI microbiota, primarily members of the Clostridiales
372 family and the *Clostridium* genus (48). Treatment with augmentin, ceftriaxone, clindamycin, and
373 fidaxomicin significantly increased levels of cholate and decreased levels of deoxycholate
374 compared to untreated controls, although levels were not significantly different from untreated
375 communities two days following the cessation of antibiotics. There was also no correlation
376 between levels of cholate or deoxycholate and susceptibility to *C. difficile* infection, indicating
377 that secondary bile salt mediated inhibition was unlikely to be a mechanism preventing
378 colonization in this model. This was further supported by experiments investigating colonization
379 resistance in the presence and absence of bile, which demonstrated bile was not required for *C.*
380 *difficile* colonization resistance.

381 Lack of inhibition by secondary bile salts was surprising, as levels of deoxycholate measured
382 in spent culture medium (431 - 608 μM) were within the range shown to inhibit *C. difficile* growth
383 in pure culture *in vitro* (250 - 2500 μM; (7, 15, 20)). Nevertheless, 18% of communities with high
384 *C. difficile* levels (>10^5 CFU/mL) on Day 7 of infection had deoxycholate levels ≥ 1000 μM on Day
385 0 of infection, suggesting inhibitory effects observed in pure culture may not be the same in
386 complex culture in the presence of other microbes. Further studies are needed to understand
387 whether the presence of other microbes could mitigate some of the toxic effects of deoxycholate
388 on *C. difficile* growth.

389 We also observed that spores were capable of infecting communities treated with
390 clindamycin and this was independent of the presence of bile. Consistent with what has been
391 previously reported, low levels of germination were observed in the absence of bile and in spent
392 culture medium cultured in the presence or absence of bile. However, these low levels of
393 germination would result in ~10^3 vegetative cells, a level previously shown to infect antibiotic
394 disrupted communities cultured in bioreactors (37). This data provides further support to prior
395 studies that have proposed that control of *C. difficile* germination and growth (12, 25) through
396 restoration of microbial bile salt metabolism may not be sufficient to limit *C. difficile* colonization.

397 While levels of bile salt did not correlate with susceptibility to infection, the ability of *C.*
398 *difficile* to metabolize proline as an electron acceptor for Stickland fermentation was required for
399 persistence in the majority of disrupted communities tested. These results are consistent with
400 evidence from human (10, 35) and mouse (11, 13, 25, 31, 32, 36) studies that point to Stickland
401 fermentation with proline as an electron acceptor as a preferred nutritional niche for *C. difficile*
402 in the GI tract. Stickland fermentation is a metabolic pathway limited primarily to proteolytic

403 Clostridial species (34). This includes microbes such as *C. scindens*, which can compete with *C.*
404 *difficile* for proline (25), dehydroxylate primary bile salts to secondary bile salts (24), and can
405 produce a bacteriocin that limits *C. difficile* growth (9). Identifying microbes that effectively
406 compete with *C. difficile* for proline and other amino acids following antibiotic treatment may be
407 one approach to limit *C. difficile* colonization.

408 It is unclear which metabolites were supporting *C. difficile* growth in antibiotic-treated
409 communities where Δ*prdB* mutants persisted at higher levels over time. Stickland fermentation
410 with glycine as an electron acceptor is one potential niche that could have been utilized by Δ*prdB*
411 mutants. Alternatively, *C. difficile* may have utilized carbohydrate fermentation pathways.
412 Further studies are needed to more fully understand how *C. difficile* colonizes disrupted
413 communities.

414 Altogether, these studies highlight the importance of improving our understanding of bile-
415 independent mechanisms regulating *C. difficile* colonization. While it is clear that Stickland
416 fermentation of amino acids with proline as an electron acceptor is important for *C. difficile*
417 persistence in many disrupted communities, other nutritional niches should also be explored.
418 Studies similar to those described here, examining variations in fecal microbial communities and
419 environmental conditions, will further clarify the hierarchical importance of different nutritional
420 environments in limiting *C. difficile* colonization.

421

422

423

424

425 **MATERIALS AND METHODS**

426 **Fecal sample collection and preparation**. Fecal samples were collected from 18 healthy
427 individuals who had not been treated with oral antibiotics within the previous six months.
428 Samples were collected from children aged 4-17 (n=3), adults aged 18-65 (n=12), and older adults
429 aged >65 (n=2). Similar numbers of male and female participants agreed to provide samples.
430 However, only donor age categorization (child, adult, older adult) remained linked to specific
431 fecal samples following de-identification. Studies were designed to collect samples across the age
432 span rather than specifically powered to evaluate differences in community composition by age.
433 All adult participants provided consent to participate in the study. Children provided assent to
434 participate along with parental consent to participate. Protocols for collection and use of fecal
435 samples were reviewed and approved by Institutional Review Boards at Baylor College of
436 Medicine (protocol number H-38014) and University of Nebraska-Lincoln (protocol numbers
437 18585 and 20186).

438 Samples were self-collected by participants in commode specimen collection containers,
439 sealed in a plastic bag containing a gas pack (BBL GasPak Anaerobe sachet), packed in ice packs,
440 placed in a sealed container, and returned to the lab within 24 hours as previously described (37).
441 Fecal samples were manually homogenized and subdivided under anaerobic conditions
442 (Anaerobic Chamber with 5% H_2, 5%CO_2, 90% N_2 atmosphere) and frozen at -80°C until later
443 resuspended at 25% w/v in reduced phosphate buffered saline (PBS). Fecal suspensions were
444 vortex-mixed for 5 min at $\geq$ 2500 rpm, centrifuged at 200 X *g* for 5 min to settle large particulates,
445 and the supernatants were either used immediately or amended with 7.5% dimethylsulfoxide

446 and preserved at -80°C until use. Previous work had shown that freezing samples at -80°C did not
447 significantly change the composition of communities cultured in bioreactors (49).
448
449 **Bioreactor experiments.** MBRAs were assembled and operated under anoxic conditions (5% H_2,
450 5% CO_2, 90% N_2) at 37°C as described previously (37). MBRAs are strips of six independent
451 continuous flow bioreactors that operate at a 15 mL volume and are continuously stirred with
452 small magnetic stir bars positioned over a stir plate. Each independent reactor in the array
453 contains three ports, one for delivery of fresh medium at a slow continuous rate, one for removal
454 of waste, and a sample port for sampling of medium and bacteria in suspension. Bioreactor
455 medium version 3 (BRM3;(39)), which simulates nutritional conditions of the distal colon was
456 used for all studies, with the exception of studies testing the effect of bovine bile, when this
457 component was excluded from BRM3. Fecal suspensions from individual donors were inoculated
458 into sterile, anoxic BRM3 medium at 1% w/v final concentration and allowed to grow for 16-24
459 hr in batch, prior to initiation of continuous flow of medium at 1.875 mL/hr (16 hr retention time
460 for 15 mL bioreactors). Communities were allowed to equilibrate under continuous flow for 1 or
461 6 days as indicated in figures. Antibiotics were either administered twice daily to individual
462 bioreactors or added directly to source medium as indicated in figures. Research grade antibiotics
463 were obtained from the following sources: augmentin (5:1 amoxicillin sodium salt to potassium
464 clavulanate; Research Products International); azithromycin dihydrate (Thermo Scientific
465 Chemicals); cefaclor (Thermo Scientific Chemicals); ceftriaxone sodium salt hemiheptahydrate
466 (Thermo Scientific Chemicals); clindamycin phosphate (TCI America); and fidaxomicin (Apexbio
467 Technology, LLC). Antibiotic concentrations used for twice daily dosing were estimated based

468 upon previously published measurements in human feces and/or bile (50–56), although
469 fidaxomicin dosing was limited by its maximal solubility. Clindamycin concentrations used in
470 media were previously described (40). One day following cessation of antibiotics, *C. difficile*
471 spores or vegetative cells was administered to reactors at ~ 1 X 10^5 CFU/mL (vegetative cells) or
472 1 X 10^6 CFU/mL (spores) and levels were enumerated over time. Samples were collected from
473 bioreactors at the time points indicated using a sterile needle and syringe to collect bacteria in
474 suspension. When appropriate, an aliquot of this sample was used to enumerate *C. difficile* by
475 serial dilution and plating on taurocholate cefoxitin cycloserine fructose agar (TCCFA (37)) or
476 TCCFA supplemented with 50 μg/mL rifampicin and 20 μg/mL erythromycin when CD2015 was
477 enumerated. Sample were centrifuged at ~3000 X *g* for 5 min to pellet cells. Supernatants were
478 removed, and pellets and supernatants were stored at ≤ -20°C.
479
480 **Microbial community analysis by 16S rRNA gene sequencing.** DNA was extracted from cell
481 pellets using the BioSprint 96 One-For-All Vet processing kit (Qiagen) according to instructions
482 with the following modifications. Prior to extraction, cells were resuspended in Buffer ASL
483 (Qiagen) and added to sterile deep well plates (Axygen) containing 0.1 mm Zirconia beads
484 (BioSpec Products). Cells were disrupted by bead beating for 2 minutes at 1800 rpm on a FastPrep
485 96 homogenizer (MP Biomedicals). DNA was amplified in duplicate with Phusion polymerase
486 using barcoded primers 515F and 806R that target the 16S rRNA gene as previously described
487 (39, 49), then sequenced on an Illumina MiSeq using 2 x 250 kits according to manufacturer's
488 protocol. Fastqs were processed by mothur 1.41.3, removing chimeras identified by uchime,
489 mapping sequences against Silva release 132, and clustering OTUs at 99% identity using the

490 OptiClust algorithm (57–59). To remove sample bleedover, the median + quartile value for a given
491 OTU in 47 negative controls were removed from all samples prior to rarefaction. The level of
492 rarefaction was chosen to maximize the number of samples and the reads/sample. Following
493 removal of bleedover, bioreactor samples were rarefied to 6332 reads/sample. When necessary,
494 the identity of OTUs were manually confirmed by BLAST (60).
495
496 **Bile salt measurements by liquid chromatography-tandem mass spectrometry (LC-MS/MS).**
497 Quantitative analysis of the bile salt levels contained in conditioned-BRM3 culture media sample
498 supernatants was performed by the Texas Children's Microbiome Center Metabolomics and
499 Proteomics Mass Spectrometry Laboratory (TCMC-MPMSL) using previously published
500 methods(61, 62). Briefly, conditioned-BRM3 growth media samples were removed from the fecal
501 communities cultured in the individual bioreactors using sterile needles and syringes. Samples
502 were added into sterile tubes, and the bacterial cells were pelleted by centrifugation at 3,000 X
503 *g* for 5 min. Clarified supernatants were then sterile filtered using 96-well 0.2 μM
504 polyvinyldifluoride (PVDF) filter plates by centrifugation at 200 X *g* for 5 min. The 96-well, deep
505 well plates used to capture sample filtrates were covered with silicone cap mats and the capped
506 samples were wrapped in parafilm to ensure the fixture of the capmats during shipment. The
507 cell-free conditioned media samples were stored frozen at -80°C pending shipment to the TCMC-
508 MPMSL. The cell-free conditioned media samples were shipped frozen on dry ice, and upon
509 receipt by the TCMC-MPMSL, were stored at -80°C until analysis.
510 All cell-free conditioned media samples were thawed at ambient temperature on a
511 laboratory benchtop. Once thawed, the entire sample volume was transferred into individual 0.6

512 or 1.5 mL Eppendorf tubes, and all samples were vortex-mixed using a multi-tube vortexer.
513 Preliminary work indicated that 10-fold and 1,000-fold dilutions were suitable to measure the
514 TCA content, and the CA and DCA content, respectively, in each of the cell-free conditioned media
515 samples using a common linear dynamic range of 0.977-1,000 ng/mL for CA, DCA, and TCA – the
516 two different dilution procedures for each metabolite were described previously (62). These
517 sample dilution steps were performed using a working internal standard (WIS) solution prepared
518 in 1:1 methanol: water that contained 250 ng/mL each of D4-CA and D4-DCA as described (62).
519 A 5 μL volume of sample was injected onto the SCIEX QTRAP 6500-based LC-MS/MS system, and
520 bile acid concentrations in the conditioned media samples were back calculated using the
521 regression parameters of the standard curve as described previously (62).

522 A broad screen of the bile acids/salt content of a fresh preparation of BRM3-based
523 bioreactor medium (with bovine bile) was performed using another previously reported targeted
524 metabolomics method that is capable of quantitatively measuring 16 bile acids/salts (63). This
525 expanded method can quantify the bile acid/salt content of microbial media samples: CA,
526 glycocholic acid (GCA), TCA, beta-muricholic acid (β-MCA), DCA, glycodeoxycholic acid (GDCA),
527 taurodeoxycholic acid (TDCA), chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid
528 (GCDCA), taurochenodeoxycholic acid (TCDCA), ursodeoxycholic acid (UDCA),
529 glycoursodeoxycholic acid (GUDCA), tauroursodeoxycholic acid (TUDCA), lithocholic acid (LCA),
530 glycolithocholic acid (GLCA), and taurolithocholic acid (TLCA). Briefly, samples were prepared by
531 diluting a 10 μL volume of a thawed cell-free, conditioned BRM3 media sample in a 90 μL volume
532 of a WIS solution that contains deuterated analogs of each of the analytes listed above at
533 concentrations of 250 nM for each prepared in 1:1 methanol: water (63). The samples were

534 prepared directly in glass autosampler vials and the samples were vortex-mixed briefly prior to
535 injecting a 10 µL volume onto the SCIEX QTRAP 6500-based LC-MS/MS system. Bile acid/salt
536 concentrations in the fresh BRM3 medium samples were back calculated using the linear
537 regression parameters of the standard curve as described previously (63).
538
539 **Data visualization and statistical analysis.** The statistical tests used to evaluate significance of
540 differences are listed for each figure legend. Except where indicated, significance of differences
541 between groups was evaluated with Kruskal-Wallis testing with Dunn's correction for multiple
542 comparisons. One-way ANOVA with Brown and Forsythe correction for unequal variances and
543 Dunnet's correction for multiple comparisons was also used as indicated. These analyses were
544 performed in Graphpad Prism version 10.2.2, which was also used to generate graphs. LEfSe (64)
545 was used to identify OTUs whose abundance differed significantly between susceptible and
546 resistant communities. LEfSe analysis was performed using Mothur version 1.44.0.
547
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555 was performed by X.H., A.E.J., T.A.A, S.J.H., K.M.H., T.D.H., A.M.H. and J.M.A. J.M.A. and A.M.H

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DATA AVAILABILITY

16S rRNA gene data has been deposited in NCBI's Sequence Read Archive under BioProject ID PRJNA729569.

ETHICS APPROVAL

Protocols for collection and use of fecal samples were reviewed and approved by Institutional Review Boards at Baylor College of Medicine (protocol number H-38014) and University of Nebraska-Lincoln (protocol numbers 18585 and 20186).

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583

584 **CONFLICTS OF INTEREST**

585 J.M.A. and T.A.A. have a significant financial interest in Synbiotic Health. T.D.H. is a member of
586 the Editorial Advisory board and is contracted as an Associate Editor for a Cell Press Journal called
587 STAR Protocols. There are no other conflicts of interest to declare.

588

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