

1 **Outbreak of murine infection with *Clostridium difficile* associated with the administration
2 of a pre- and peri-natal methyl-donor diet**

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20 Running Head: *C. difficile* outbreak in murine methyl-diet study

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24 **Abstract**

25 Between October 2016 and June 2017, a C57BL/6J mouse colony that was undergoing a pre- and
26 peri-natal methyl-donor supplementation diet intervention to study the impact of parental
27 nutrition on offspring susceptibility to disease was found to suffer from an epizootic of
28 unexpected deaths. Necropsy revealed the presence of severe colitis, and further investigation
29 linked these outbreak deaths to a *Clostridium difficile* strain of ribotype 027 we term 16N203. *C.*
30 *difficile* infection (CDI) is associated with antibiotic use in humans. Current murine models of
31 CDI rely on antibiotic pretreatment to establish clinical phenotypes. In this report, the *C. difficile*
32 outbreak occurs in F1 mice linked to alterations in the parental diet. The diagnosis of CDI in the
33 affected mice was confirmed by cecal/colonic histopathology, presence of *C. difficile* bacteria in
34 fecal/colonic culture, and detection of *C. difficile* toxins. F1 mice from parents fed the methyl-
35 supplementation diet also had significantly reduced survival ($p<0.0001$) than F1 mice from
36 parents fed the control diet. When we tested the 16N203 outbreak strain in an established mouse
37 model of antibiotic-induced CDI, we confirmed that this strain is pathogenic. Our serendipitous
38 observations from this spontaneous outbreak of *C. difficile* in association with a pre- and peri-
39 natal methyl-donor diet suggest the important role diet may play in host defense and CDI risk
40 factors.

41

42 **Importance**

43 *Clostridium difficile* infection (CDI) has become the leading cause of infectious diarrhea in
44 hospitals worldwide, owing its preeminence to the emergence of hyperendemic strains, such as
45 ribotype 027 (RT027). A major CDI risk factor is antibiotic exposure which alters gut
46 microbiota, resulting in the loss of colonization resistance. Current murine models of CDI also

47 depend on pretreatment of antibiotics of animals to establish disease. The outbreak we report
48 here is unique in that the CDI occurred in mice with no antibiotic exposure and is associated to a
49 pre- and peri-natal methyl-supplementation donor diet intervention study. Our investigation
50 subsequently reveals that the outbreak strain we term 16N203 is an RT027 strain, and this
51 isolated strain is also pathogenic in an established murine model of CDI (with antibiotics). Our
52 report of this spontaneous outbreak offers additional insight into the importance of
53 environmental factors, such as diet, and CDI susceptibility.

54

55 **Introduction**

56 *Clostridium difficile* is a spore-forming, gram positive obligate anaerobe that has become the
57 leading cause of infectious diarrhea in hospitals worldwide. On a yearly basis, nearly half a
58 million cases of *Clostridium difficile* infection (CDI) are reported in the United States (US), with
59 an approximated 29,000 CDI-related deaths (1). Exposure to *C. difficile* can have varied
60 outcomes ranging from asymptomatic intestinal colonization, to severe diarrhea, development of
61 pseudomembranous colitis, and death (2). CDI risk is associated with disruption of the gut
62 microbiota, for example following antibiotic administration (2–4), that leads to a loss of
63 resistance towards *C. difficile* colonization.

64

65 Following spore germination and establishment of the vegetative form of *C. difficile*, disease
66 results from the production of the large clostridial toxins TcdA and TcdB (5). These enterotoxins
67 inactivate small GTPases (6–9) (e.g. Rho, Rac, Cdc42) and lead to the rearrangement of the actin
68 cytoskeleton of intestinal epithelial cells. Consequently, mucosal epithelium damage ensues
69 from cellular apoptosis (10) and the induction of colitis via toxin-mediated inflammation. In

70 humans, the increasing clinical presence of *C. difficile* epidemic strains in the US and West
71 Europe (11) is attributed to antibiotic-use and the rise of endemic strains such as NAP1/027/BI.
72 Ribotype 027 (RT027) strains produce higher quantities of TcdA and TcdB (12) and express an
73 additional *C. difficile* transferase (CDT) binary toxin encoded by *cdtA/cdtB* (13). The binary
74 toxin also disrupts the cytoskeleton and increases bacterial adhesion to intestinal epithelial cells
75 (14). The exact role of CDT in *C. difficile* virulence remains undefined, but it has been
76 suggested that CDT plays a significant role in worsened clinical patient outcomes (15) and
77 increases of CDI recurrence (16).

78

79 Mouse models have been developed to study the pathogenesis of CDI. These models generally
80 require administration of antibiotics to disrupt the microbiota prior to *C. difficile* exposure. A
81 number of antibiotic regimens have been employed to render animals susceptible to CDI (17–
82 19). The importance of the indigenous microbiota in mediating colonization resistance against
83 CDI is highlighted by the fact that germ free animals are inherently sensitive to colonization and
84 disease when exposed to *C. difficile* (20).

85

86 Here, we report a spontaneous outbreak of *C. difficile* due to a strain of RT027 that occurred in a
87 mouse colony that was associated with the administration of a specific pre- and peri-natal diet (in
88 the original study, a methyl-supplementation donor diet (21–24) is administered to F0 mice and
89 we study diet-induced obesity in F1 mice). Given that CDI mouse models typically rely on prior
90 gut microbiota disruption via antibiotics to establish disease (25), this outbreak is unique in that
91 it occurred in the absence of antibiotic use and instead, in association with an altered diet.

92

93 **Materials and Methods**

94 **Animals**

95 The animal care and use program at the University of Michigan is AAALAC-accredited. All
96 procedures involving the animals and their care were approved by the University of Michigan
97 Institutional Animal Care and Use Committee. Mice were housed in autoclaved positive-pressure
98 individually-ventilated cages (P/NV IVC, Allentown, Allentown, NJ) with corncob bedding (The
99 Andersons, Frontier) and provided with reverse osmosis-deionized (RO/DI) water through
100 automated water systems (Edstrom; Waterford, WI). Animal housing rooms were maintained on
101 a 12:12-h light:dark cycle, relative humidity of 30-70%, and temperature of $72 \pm 2^{\circ}\text{F}$ ($22.2 \pm$
102 1.1°C). For specific-pathogen free (SPF) rooms, cage changing and experimental procedures
103 were performed under a laminar flow cage change station (AniGARD[®] VF, Baker Company,
104 Sanford, ME) or in laminar flow benches, using a cold sterilant (Spor-Klenz[®], Steris, St Louis,
105 MO) for disinfecting gloved hands or transfer forceps. For biocontainment rooms (animal
106 biosafety level 2), procedures were performed under a biosafety cabinet (SterilGARD[®], Baker
107 Company, Sanford, ME) using a sporicidal disinfectant cleaner (Perisept, Triple S 48027).
108 Health surveillance program for SPF colonies included quarterly testing of dedicated soiled-
109 bedding sentinel animals via fecal and perianal swab PCR or serology, and PCR of exhaust
110 plenum swabs for fur mites. Health surveillance results indicated that the mice were negative for
111 mouse rotavirus, mouse hepatitis virus, minute virus of mice, ectromelia virus, Theiler mouse
112 encephalomyelitis virus, lymphocytic choriomeningitis virus, mouse adenovirus, mouse
113 parvovirus, mouse polyoma virus, pneumonia virus of mice, reovirus, Sendai virus, *Mycoplasma*
114 *pulmonis*, pinworms (*Syphacia* spp. and *Aspicularis* spp.) and fur mites (*Myobia musculi*,
115 *Myocoptes musculinus*, and *Radfordia affinis*).

116

117 **Dietary Study**

118 In the initial dietary study, young adult (8 wk) C57BL/6J mice were purchased from Jackson
119 Laboratories. All custom diets “TD” are produced and distributed by Harlan-Teklad (Madison,
120 WI). Mice were acclimated for 2 weeks before feeding either control (TD.06689) or methyl-
121 donor supplementation (MS) diet (TD.110144). The MS diet contained 12g/kg methionine, 16.5
122 g/kg choline, 15 g/kg betaine, 16.5 mg/kg folic acid, 1.56 mg/kg vitamin B12, and 200 mg/kg
123 Zn. Two weeks after starting diet, female and male mice were paired for mating. Mated F0 mice
124 were fed the diet throughout pregnancy and lactation. F1 mice were weaned at 28 days. The F1
125 mice were then placed on a standard chow diet (PicoLab Laboratory Rodent Diet 5L0D,
126 LabDiet, St. Louis, MO) or a 42% high-fat diet (HFD) (TD.88137).

127

128 **Outbreak necropsy and assessment**

129 Dead or clinically affected animals were detected during routine daily health checks. Veterinary
130 staff assessed for any clinical sign of gastrointestinal or systemic disease, such as diarrhea,
131 lethargy or unkempt appearance, and euthanized animals via CO₂ inhalation followed by
132 induction of bilateral pneumothorax. Complete necropsy was performed with select tissues
133 processed for histologic examination.

134

135 Fecal PCR of selected mice in the colony was performed by a commercial laboratory (Charles
136 River Laboratories, Wilmington, MA) to confirm SPF status. Fecal and/or cecum and colonic
137 samples were submitted for ELISA for *C. perfringens* type A toxin and *C. difficile* toxins A and
138 B (Veterinary Diagnostic Laboratory, Michigan State University, Lansing, MI) or for real-time

139 fluorogenic PCR for *C. difficile* targeting the 23S rRNA gene (Charles River Laboratories,
140 Wilmington, MA). Cecum and colon tissues as well as tissues from other organs were fixed in
141 10% neutral buffered formalin for a minimum of 24 hours and then routinely processed to
142 paraffin, sectioned, and stained with hematoxylin and eosin by the University of Michigan In
143 Vivo Animal Core (IVAC) histology laboratory. A board-certified veterinary pathologist blinded
144 to the diet groups evaluated the tissues descriptively in the initial outbreak and subsequently
145 performed severity scoring of cecal and colonic tissues according to our previously published
146 scoring system for experimentally-induced *C. difficile*-associated typhlitis and colitis (25). In
147 brief, slides were evaluated on a 0-4 scale for the individual parameters of edema, inflammation,
148 and epithelial damage and an overall severity score was generated by summing these parameters
149 (scale 0-12).

150

151 To investigate the outbreak source and the extent of contamination, environmental and fecal
152 pellet PCR was performed as described above. Swabs were taken from various areas including
153 cardboard rolls collected for mouse enrichment and the interior of the respective collection bins
154 and supply and exhaust plenums of four autoclaved racks. Within the ABSL2 room, door knobs,
155 biosafety cabinet used for changing cages, clean lixits, and rack supply ducts connected to the
156 building ventilation system were sampled. Each PCR sample tested for the affected mouse
157 colony comprised of pooled fecal pellets (a fecal pellet collected from a representative mouse in
158 each cage in each rack row) combined with the associated rack row's exhaust plenum swab.

159

160 ***C. difficile* strain isolation, growth conditions, and colony identification**

161 *C. difficile* strain 16N203 was isolated from feces frozen at -80°C after collection from a
162 spontaneously affected animal in the dietary study mouse colony. 1.5 fecal pellets were thawed,
163 passed into an anaerobic chamber, and diluted in 200µL sterile anaerobic PBS. 20µL was placed
164 into 3mL of taurocholate cefoxitin cycloserine fructose broth (TCCFB) and incubated at 37°C
165 overnight. A 10µL loop of the TCCFB enrichment culture was streaked onto taurocholate
166 cefoxitin cycloserine fructose agar (TCCFA) to get an isolated colony of *C. difficile*. The
167 isolated colony was used for downstream applications.

168

169 For colony identification, a 16N203 *C. difficile* colony was diluted in 15µL UltraPure Water
170 (Invitrogen 10977-015), heated to 95°C for 20 min and then used for colony PCR to determine
171 identity and toxin type of the isolated organism (26, 27). PCR was performed using the
172 following primers for the *C. difficile* specific band F: 5'-
173 TTGAGCGATTACTTCGGTAAAGA-3' and R: 5'-CCATCCTGTACTGGCTCACCT-3'
174 along with the universal 16S primers 515F: 5'-GTGCCAGCMGCCGCGTAA-3' and E939R:
175 5'-CTTGTGCGGGCCCCCGTCAATT-3' (26). Toxin specific PCR followed previously
176 published primers omitting the 16s rDNA primers (27). The PCRs were run in a total volume of
177 25uL containing GoTaq Green Master Mix (Promega M712), primers, and nuclease free water.

178

179 ***C. difficile* cytotoxin assay**

180 Cytotoxicity assay was performed as previously described with the following modifications (25).
181 Briefly, ATCC CCL-81 Vero cells were grown to confluence in Dulbecco modified Eagle
182 medium (Gibco 11965) supplemented with 10% fetal bovine serum (16140) and 1% Penicillin
183 streptomycin (Gibco 15070). Cells were plated to a density of 10⁵ cells/well. Mouse cecal

184 content was diluted 1:10 in sterile PBS, passed through a 0.22 μ m filter, and serially diluted to 10⁻
185⁶. Filtered samples were tested in duplicate with a corresponding control which both antitoxin
186 (Techlab T5000) and sample were added. A positive control of *C. difficile* TcdA (List
187 Biologicals 152C) was used. Samples were incubated overnight at 37°C and the cytotoxic titer
188 was determined as the reciprocal of the highest dilution that produced 80% cell rounding.

189

190 **Genome sequencing, variant identification, and comparative genomics**

191 A colony of *C. difficile* 16N203 was cultured 18 hours anaerobically at 37°C in 13mL Brain
192 Heart Infusion Broth (BD 211059) + 0.01% L-cysteine (Sigma C6852). The culture was spun at
193 4500g for 12min, washed one time with sterile PBS, spun at 4500g for 12 min and the pellet was
194 re-suspended in 300uL of Dneasy UltraClean Microbial kit Microbead solution (Qiagen 12224-
195 50). The extracted DNA was then prepared for sequencing on an Illumina MiSeq instrument
196 using the NexteraXT kit and sample-specific barcoding. Library preparation and sequencing
197 were performed at the Center for Microbial Systems at the University of Michigan. Quality of
198 reads was assessed with Fastqc (28), and Trimmomatic (29) was used for trimming adapter
199 sequences and low quality bases. Genome assemblies were performed using Spades (30).
200 Variants were identified by: 1) mapping filtered reads to the assembled *C. difficile* strain 630
201 reference sequence (NC_009089.1) using the Burrows–Wheeler short-read aligner (BWA), 2)
202 discarding PCR duplicates with Picard, and 3) calling variants with SAMtools and bcftools.
203 Variants were filtered from raw results using GATK’s VariantFiltration (QUAL > 100, MQ >
204 50, > 10 reads supporting variant, FQ <0.025). In addition, a custom python script was used to
205 filter out single nucleotide variants that were: 1) <5 bp in proximity to indels 2) <10 bp in
206 proximity to another variant or 3) not present in the core genome. Maximum likelihood trees

207 were constructed using core genome variants among the outbreak strain and a representative set
208 of previously sequenced *C. difficile* genomes in FastTree (31). MLST predictions were made by
209 BLASTing genome assemblies against the PubMLST database for *C. difficile* (downloaded on
210 January 3rd, 2017).

211

212 **Experimental *C. difficile* infection**

213 8-wk old C57BL/6J wild-type (WT) mice (6 males and 3 females) were obtained from an in-
214 house breeding colony that was established originally with animals from Jackson Laboratories
215 (Bar Harbor, ME). Mice were treated 10 days with 0.5g/L cefoperazone (MP Biomedicals
216 219969505) in sterile distilled water (Gibco 15230-147) as previously described (25). Briefly,
217 animals were allowed to drink antibiotic amended water *ad libitum* and the antibiotic water was
218 changed every other day. After 10 days, antibiotic water was switched to sterile distilled water.
219 After 2 days on water without antibiotics, 3 male and 3 female mice (total treated n=6) were
220 challenged via oral gavage with 500 spores of *C. difficile* strain 16N203. Spores (preparation
221 described below) were suspended in 50μl Gibco sterile distilled water and 3 male mice (total
222 mock n=3) were treated with 50μl of Gibco water only. Mice were monitored 16 hours post
223 infection and feces were collected and plated to confirm spore inoculation. Mice were observed
224 every 3 hours for 36 hours for clinical signs of disease until clinical signs appeared. Mice were
225 euthanized by CO₂ inhalation when clinical signs appeared or at study endpoint. Mice were then
226 evaluated at 48 hours after *C. difficile* infection for weight loss, activity, posture, coat, diarrhea
227 and nose/eyes appearance (32). Animals were euthanized and cecal contents and tissues from
228 the animals were collected for culture and ELISA.

229

230 ***C. difficile* spore preparation**

231 16N203 was streaked onto TCCFA and an isolated colony was incubated overnight anaerobically
232 at 37°C in 2mL Columbia broth (BD 294420). The next day the culture was placed into 40mL of
233 Clospore (33) and incubated anaerobically at 37°C for 8 days. The culture was spun at 3200rpm
234 for 20min at 4°C. The pellet was washed two times with sterile water (Gibco 15230-147), once
235 with sterile 0.05% Tween 20 (Fisher BP337), and then once with sterile water. The final pellet
236 was resuspended in 1mL sterile water. The spore stock was stored at 4°C in sterile water. Prior
237 to gavage, spores were heated to 65°C for 20min. Spores were plated on TCCFA to determine
238 dose administered to animals.

239

240 **Results**

241 **Recognition of an outbreak of severe typhlocolitis in a cohort of mice undergoing pre- and
242 peri-natal diet manipulation**

243 Typhlocolitis was observed in a cohort of F1 mice from a study involving parental (F0) pre- and
244 peri-natal methyl-donor supplementation (MS) and post-weaning intake of normal diet (ND) or
245 42% high-fat diet (HFD) chow. The purpose of the original study was to evaluate MS diet
246 epigenetic effects on obesity development in later-life and at various life stages of the F1 mice.

247

248 Over the course of 254 days from October 4th, 2016—June 15th, 2017, 57 out of 207 mice in the
249 MS diet study were found dead with no premonitory signs (n=39) or were euthanized for
250 moribund condition (n=18), including hunched posture, lethargy, dyspnea, and mucoid stool with
251 perineal staining. Severe necrotizing typhlocolitis with pseudomembrane formation and severe
252 submucosal edema was observed at necropsy in early cases and was suggestive of *C. difficile*

253 colitis (25). This led to screening of affected animals for *C. difficile*, which was subsequently
254 identified in a number of these mice, suggesting a potential outbreak. To facilitate investigation
255 of this outbreak, we developed the following case definition: confirmed cases had 1) presentation
256 of a compatible clinical syndrome and either, 2) histopathologic cecal/colonic lesions consistent
257 with *C. difficile* infection or, 3) tested positive for *C. difficile* bacteria or *C. difficile* toxin. Of the
258 57 animals that died during the study period, we were able to complete analysis on 36 mice and
259 determined 25 of these mice fit our case definition. We found 12 mice have causes of death
260 unrelated to the outbreak and 20 mice had no tissues available for examination (**Table 1**).

261
262 We constructed an epidemic curve to follow the course of this outbreak. The initial 14 deaths in
263 the colony that led to the outbreak investigation were included in this epidemic curve (**Figure**
264 **1A**), but as most of these animals were found dead, or intestinal tissue was not examined, case
265 definitions could not be assigned to these mice. Over the course of the outbreak study, there
266 were 86 MS diet and 97 control diet F1 mice. We excluded all F0 mice and F1 mice that had no
267 tissue available or were examined but did not meet case definition and generated a Kaplan-Meier
268 survival curve analysis on *only the confirmed cases* of CDI shows lower survival in the MS diet
269 mice (n=66) compared to control diet mice (n=85) (**Figure 1B**). Curve analysis indicates that on
270 day 254, which is the day on which the last outbreak case was identified, 96.5% of the control
271 mice survived while only 66.7% of the MS mice remained ($p < 0.0001$). A curve comparison of
272 the confirmed CDI cases based on F1 diets—HFD (n=50) versus ND (n=101) group suggests
273 that HFD fed mice had higher survival percentages ($p < 0.02$), (**Figure 1B**); however, there were
274 65 F1 mice on HFD mice while 118 F1 mice were on ND in the initial colony (**Table 1**). For

275 comparison, we also included a Kaplan-Meier survival curve analysis on all F1 mice,
276 incorporating them as censored (lost) for analyses (**Figure 1C**).
277

278 While attempting to control this outbreak, breeders on the MS diet and control diet were
279 transferred to a separate room in January 2017 based on negative fecal ELISA results. The
280 remainder of the colony was ultimately transferred to containment housing. On 4/14/17, PCR of
281 pooled plenum and fecal pellets revealed 8/8 rows on one rack and 1/2 rows on a second rack
282 were positive for *C. difficile*. Environmental samples on 3 separate dates (3/9/17, 3/15/17, and
283 10/24/17) tested negative for *C. difficile*. The timeline of the plenum swabs, fecal sampling, and
284 environmental tests is also included on the epidemic curve (**Figure 1A**).
285

286 **Identification of *C. difficile* in animals with typhlocolitis**

287 Gastrointestinal (cecum and colon) tissues were available for histologic evaluation in 24 of the
288 total 57 animals found dead or euthanized during the outbreak (**Figure 2**). 17 of 24 animals were
289 found to have necrotizing and pseudomembranous typhlocolitis consistent with CDI as seen in
290 experimental murine models (25). Histological findings included striking submucosal edema of
291 the cecum and colon, accompanied by marked neutrophilic inflammation and necrosis and
292 sloughing of the superficial to mid-portions of the mucosa, often with pseudomembrane
293 formation (**Figure 2A, 2B**). Of the 17 animals with histologically evident typhlocolitis, we were
294 also able to collect and submit fecal and/or cecum and colonic samples from 14 animals for
295 testing. 12 of the 14 animals tested positive for *C. difficile* TcdA and TcdB via ELISA and 2
296 tested positive for *C. difficile* bacteria via PCR. 14 were F1 mice from the MS diet and 3 were
297 from the control diet groups (**Figure 2C**). The cecum summary scores (**Figure 2C**) in the MS

298 diet (n=13) was 6.1 ± 2.6 and 9.3 ± 2.1 in the control diet (n=3). The colon summary scores
299 (**Figure 2C**) shows that MS diet (n=14) was 4.0 ± 2.2 and control diet (n=2) was 4.5 ± 2.1 . One
300 MS-diet F1 mouse had missing colon scores and one control diet F1 mouse had missing cecum
301 scores due to tissue autolysis. Given that significantly fewer control diet F1 mice ($p<0.0001$)
302 were affected in the outbreak (**Figure 1B**), we collected fewer control samples and thus cannot
303 draw conclusions on the effect of the F0 diet on disease severity between the two groups of F1
304 mice.

305

306 Overall, we observed that the histology score was higher in cecum than colon in most animals,
307 consistent with experimental murine *C. difficile* models (25). Of the remaining 7 out of 24
308 histologically evaluated animals that did not have evidence of typhlocolitis, alternate causes of
309 death/morbidity were identified, including tumors (n=2), bacterial enteritis (n=2), or
310 undetermined by histology (n=3). Finally, there were 12 out of 36 analyzed animals that had no
311 gastrointestinal tissue available to be histologically evaluated, and 8 of these animals tested
312 positive for *C. difficile* bacteria (n=2) and *C. difficile* toxins A and B (n=6) which then allows us
313 to include these 8 as confirmed cases of *C. difficile*-related deaths. The 4 animals that tested
314 negative for *C. difficile* toxin were found to have an alternative cause of death (endometritis n=1)
315 or undetermined (n=3), and naturally, these were not included in the confirmed cases of *C.*
316 *difficile*.

317

318 Isolation of *C. difficile* was performed by the use of selective culture. A *C. difficile* strain
319 (designated 16N203 for the animal from which it was initially isolated) was obtained in pure
320 culture. A PCR (26) confirmed the *C. difficile* bacteria was present in fecal sample (**Supp.**

321 **Figure 1A)**, and this strain was also positive for the toxin genes *tcdA/tcdB* and *cdtA/cdtB* by
322 PCR (27) (**Supp. Figure 1B**). Whole genome sequencing of strain 16N203 was undertaken, and
323 a whole genome phylogeny was constructed (**Figure 3A**), including a representative set of
324 previously sequenced *C. difficile* isolates. These isolates contain publicly available clinical
325 genomes (34–36), clinical isolates collected at the University of Michigan, and two mouse
326 strains (16N203 and LEM1). LEM1 is an indigenous murine spore-forming *C. difficile* strain
327 identified and isolated from mice acquired from common mouse vendors, Jackson Laboratories
328 and Charles River Laboratories (37). LEM1 appears to not be highly virulent and can protect
329 against the closely related but more virulent strain VPI10463, at least in mice with C57BL/6J or
330 BALB/c background (37). In our data, the murine strain LEM1 clusters with Clade 1, which
331 contains reference strain CD630, while the outbreak strain fell within the diversity of Clade 2,
332 which contains RT027 isolates (**Figure 3A**). When a phylogenetic tree was constructed with only
333 RT027 isolates, the outbreak 16N203 strain clustered with isolates derived from human patients
334 with clinical CDI (38) (**Figure 3B**).

335
336 ***C. difficile* strain 16N203 is fully virulent in a mouse model of antibiotic-induced CDI**
337 We had previously demonstrated that *C. difficile* strains have variable virulence in an established
338 mouse model where CDI susceptibility is conveyed by treatment with the antibiotic cefoperazone
339 (25). To determine whether the pathogenicity of the outbreak strain 16N203 was unique to the
340 dietary model, we assessed whether the outbreak strain 16N203 was virulent in this established
341 model of CDI (**Figure 4A**). C57BL/6J mice on a standard diet and without previous dietary
342 manipulations were treated with cefoperazone in drinking water for 10 days and then switched
343 back to plain water. Two days after stopping antibiotics, mice were challenged with either 500

344 spores of the 16N203 outbreak strain or vehicle via oral gavage. Significant weight loss
345 ($p<0.05$) was observed in mice challenged with 16N203 spores during the 48-hour study time
346 course while mock-treated mice maintained weight (**Figure 4B**). Mice challenged with *C.*
347 *difficile* 16N203 had higher levels of cecal colonization ($p<0.03$) with detectable toxin in
348 intestinal contents ($p<0.05$) and developed clinical signs compatible with severe CDI (**Figure**
349 **4C**).

350

351 **Discussion**

352 In this report, we describe an outbreak of *C. difficile* infection (CDI) and colitis in laboratory
353 mice associated with diet manipulation. This outbreak is interesting because spontaneous,
354 symptomatic CDI is unusual in laboratory mice, and symptomatic colitis due to experimental
355 CDI generally requires pretreatment with antibiotics (37, 39). Subclinical colonization of mice
356 has been reported previously. In one study it was noted that mice could be colonized with *C.*
357 *difficile* with or without prior antibiotic treatment but this colonization was at a very low level
358 and not associated with any clinical disease (19). Administration of the antibiotic clindamycin
359 was associated with increases in the levels of colonization but again without development of
360 clinical colitis. It has been also reported that wild rodents can harbor toxigenic *C. difficile* strains,
361 again without any overt disease (40). One group reported that laboratory mice obtained from
362 multiple sources appear to harbor LEM1, an indigenous *C. difficile* strain (37). Colonization with
363 LEM1 in the same study did not result in disease and in fact was associated with protection from
364 disease following challenge with another strain of *C. difficile* that generally produces severe
365 colitis in experimental models of CDI. In contrast, while LEM1 clusters with Clade 1 (containing
366 reference strain CD630 (RT 12)), the outbreak strain in our study clustered in Clade 2 with

367 patient-derived RT 027 isolates and was associated with clinical disease in both spontaneously
368 infected and experimentally infected mice. The source of this strain remains unclear, and our
369 attempt at revealing a source with environmental sampling did not yield conclusive results.
370 Ribotyping and genetic analyses suggest a potential source was through fomites or human
371 carriers, but the source may have been gone by the time of environmental investigation.

372

373 The mechanism underlying the unexpected increased susceptibility of these mice is also not
374 entirely clear. Small animal models to recapitulate CDI pathogenesis generally rely on antibiotic
375 pretreatment prior to experimental challenge with *C. difficile*, even with virulent strains (39). As
376 noted, the current outbreak is unusual in that there were no antibiotics administered to the
377 animals and instead susceptibility to colonization and colitis was associated with dietary (peri-
378 and pre-natal) manipulation. Our current understanding of the pathogenesis of CDI attributes loss
379 of colonization resistance to altered structure and function of the indigenous intestinal microbiota
380 (41). However, given the unexpected nature of the outbreak, we were not positioned to determine
381 if there were changes in the microbiota associated to the increased susceptibility we observed in
382 MS F1 mice. Antibiotics, which can cause widespread changes in the indigenous microbiota, are
383 associated with the development of clinical CDI in humans, but other factors that alter microbial
384 populations may also convey susceptibility.

385

386 In our study, increased *C. difficile*-associated deaths were seen in the offspring of mice receiving
387 the methyl-supplementation (MS) diet. Diet may be a factor that can alter the community
388 structure and function of the intestinal microbiota, and diet-related susceptibility to CDI has been
389 evaluated in some studies (42, 43). Patients receiving enteral tube feeding had increased risk of

390 developing CDI (44). Tube feeds given in the form of an elemental diet (i.e. a diet that is entirely
391 absorbed within the small bowel) are thought to deprive the colonic bacteria of nutrition in the
392 form of fiber and resistant starch, increasing the risk of CDI (45). Microbiota accessible starches
393 may protect against CDI as in murine studies where feeding such carbohydrates can suppress
394 experimental CDI (46). This protection was associated with an increase in short chain fatty acids
395 which are the metabolic products of microbial fermentation of non-digestible carbohydrates.
396 Another study showed that a low protein diet (which had reciprocal increases in carbohydrate
397 composition) could be protective in a mouse model of CDI (47). In addition to macronutrients, it
398 was recently demonstrated that zinc deficiency could alter the microbiota and increase
399 susceptibility to experimental CDI (48).

400

401 It should be noted that, in contrast to our study, the diet-related susceptibility or amelioration of
402 experimental CDI discussed above occurred in models where the microbiota was still altered via
403 antibiotic administration. An earlier study in hamsters fed an atherogenic diet in the absence of
404 antibiotic administration, unexpected diarrhea and death due to colitis was observed in animals
405 starting 45 days after diet manipulation (49). Similar to our study, toxigenic *C. difficile* was
406 isolated from affected animals, although typing of the strain was not performed. It is interesting
407 to note that in these hamsters, the atherogenic diet increased susceptibility to CDI, but in our
408 study, F1 mice that were on a 42% high fat diet (HFD) exhibited increased survival—though it
409 should be noted that this observation was limited by the fact that there were significantly more
410 normal diet mice than HFD mice in the mouse colony at the time.

411

412 While hamsters are exquisitely susceptible to *C. difficile*, symptomatic murine *C. difficile* colitis
413 outbreaks in the absence of antibiotic administration are very rare. There is precedence for
414 disease outbreaks in mice associated with *C. difficile*, but the affected mice have had significant
415 immune alterations that increased their susceptibility (50, 51). One such outbreak occurred in a
416 murine experimental autoimmune encephalomyelitis model generated via the administration of
417 pertussis toxin and a myelin oligodendrocyte glycoprotein fragment (51). These authors
418 speculated that the stress of this experimental manipulation or the development of the
419 autoimmune disease altered the microbiota in a manner that leads to susceptibility to CDI, but
420 they did not profile the microbiota in these animals. Although we were unable to characterize the
421 microbiota in our study due to the unexpected nature of the outbreak, a future avenue to explore
422 would be diet-associated microbial community alterations that may convey susceptibility.

423

424 Specific mechanisms by which our methyl-donor diet alters susceptibility to CDI are unclear. A
425 similar methyl-supplementation maternal diet was shown to increase colitis risk in a chemically-
426 induced inflammatory bowel disease (IBD) using dextran sulfate sodium (DSS) (52, 53). F1
427 mice from methyl-fed dams had worsened colitis and significantly higher mortality than control
428 F1 mice (52). Colonic mucosal bacterial diversity analyses indicated striking composition
429 variation, including significantly higher prevalence of *Clostridia* in methyl diet F1 mice. In
430 addition, there was an increase in *Firmicutes* (*Lachnospira*, *Oscillospira*, *Ruminococcus* and
431 *Catonella*) and a decrease in *Parabacteroides* and *Eubacterium* in methyl F1 mice compared to
432 controls. This suggests that dysbiosis between colitogenic (*Firmicutes*) and anti-inflammatory
433 bacteria (*Parabacteroides*) following prenatal methyl supplementation may have led to the
434 colitis prone phenotype (52). A fecal microbiome transfer (FMT) via cage swapping (between

435 methyl F1 mice and germ free mice) was sufficient to worsen DSS-induced colitis in germ free
436 mice, indicating that the maternal methyl-donor diet alone may enhance a colitis-prone
437 microbiota profile. While this study cannot be directly compared to *C. difficile* colitis, it strongly
438 suggests that a prenatal methyl-donor diet is sufficient in generating a pro-colitic F1 microbiota.
439 In the context of CDI, it remains to be determined whether susceptibility is due strictly to altered
440 community composition, creating a niche for *C. difficile* colonization, or whether microbial
441 changes directly promote colitis-enhancing inflammatory responses. While unexpected, the
442 current spontaneous outbreak of CDI in the absence of antibiotics in mice with parents fed an
443 altered pre- and peri-natal diet may ultimately provide additional insight into the interplay of
444 diet, host responses, and the microbiota that mediates CDI and colitis susceptibility.

445

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451

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461

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- 626

627

628 **Figure Legends**

629

630 **FIG1 Methyl-donor diet mice had higher total mortality during outbreak. A)** Epidemic
631 curve depicting the first set of unconfirmed cases that triggered the outbreak investigation and
632 the subsequent cases determined to be consistent with *C. difficile* infection. ^ - denotes
633 *environmental swab tests on 3/9/17, 3/15/17, 10/24/17 (not shown)*. # - denotes plenum + fecal
634 pellet testing on 4/14/17. **B)** Kaplan-Meier survival curves of mouse colony comparing the
635 percent survival of the pre-/peri- natal control diet (n=85, excluding 8 for not meeting case
636 definition and 4 for unavailable tissues) versus pre-/peri- natal methyl-supplementation diet
637 (n=66, excluding 3 for not meeting case definition and 16 for unavailable tissues) F1 mice; F1
638 normal chow diet (n=101, excluding 5 for not meeting case definition and 11 for unavailable
639 tissues) versus 42% high fat diet (n=50, excluding 6 for not meeting case definition and 9 for
640 unavailable tissues) mice over the course of 8.5 months. 95% CI are graphed (light dashed
641 lines). **C)** Kaplan-Meier survival curves including all F1 mice (n=183) in analyses but entering
642 previously excluded mice as “0” censored. Survival curves compare Control diet (fed to F0) F1
643 mice (n=97) to MS diet (fed to F0) F1 mice (n=86). A second curve compares normal chow (fed
644 to F1) F1 mice (n=118) to 42% HFD chow (fed to F1) F1 mice (n=65).

645 * $p<0.05$, **** $p<0.0001$, Mantel-Cox log-rank test.

646

647 **FIG2 Necropsies and histopathology indicate typhlocolitis as primary diagnosis of affected
648 animals. A)** Representative histology of mouse cecum in a *C. difficile* culture-positive case
649 exhibiting severe submucosal edema and inflammation (arrow). H&E stain, Bar=200 μm . **B)**

650 Representative histology of an area of cecum in the same case displaying mucosal necrosis
651 (arrow) and extensive pseudomembrane formation (*), consisting of neutrophils and sloughed
652 enterocytes in a fibrinous matrix. H&E stain, Bar=100 µm. **C)** Histological severity scores for
653 the 17 mice showing typhlocolitis, out of 24 clinically affected mice for which tissues were
654 available. Severity was scored by a board-certified veterinary pathologist using an established
655 murine *C. difficile* scoring system. Each category—edema, inflammation, or epithelial damage
656 was individually scored 0-4 and a summary score was generated (range 0-12). Of the 17 mice, 1
657 mouse had no cecum score and 1 mouse had no colon score due to tissue autolysis of those
658 samples. As such, for cecum scores, control diet F1 mice n=3 and methyl-supplementation diet
659 (MS) F1 mice n= 13. For colon scores, control diet n=2 and MS diet n=14.

660

661 **FIG3 Isolation and genomic sequencing confirms NAP1/027/BI *Clostridium difficile* strain**
662 **A)** Phylogenetic relationship between the mouse outbreak strain and representative *C. difficile*
663 isolates. Isolates include publicly available clinical genomes, clinical isolates collected at the
664 University of Michigan, and two mouse strains (16N203 and LEM1). A maximum likelihood
665 tree was generated from the recombination filtered, polymorphic core genome positions in all
666 isolates (N = 438). Clade 1 (purple) includes reference strain CD630 and mouse strain LEM1
667 (gray); clade 2 (red) includes epidemic RT 027. Clades 1-5 are as previously defined (36, 54,
668 55). **B)** Tree in (A) was subset to include only 027 isolates. The mouse outbreak “16N203” strain
669 (blue) clusters with the RT 027 isolates (pink), a RT in Clade 2 (red).

670

671 **FIG4 *C. difficile* ‘outbreak’ strain induces CDI in a standard mouse model with antibiotics**
672 **A)** CDI mouse model with cefoperazone in drinking water used to assess virulence of the

673 outbreak 16N203 strain. Wild-type (WT) mice were administered cefoperazone in drinking
674 water for 10 days on day -12 (D -12) and then orally gavaged either 16N203 spores (n=6) or the
675 vehicle (distilled water) for mock (n=3) controls on day 0 (D0). Mice were monitored for
676 disease between D0 and D2. **B)** Body weight loss over the course of 48 hours; total percentage
677 of body weight loss shown in column graph. **C)** Clinical score, colonization, and toxin scores at
678 48 hours. For weight loss, clinical, colonization, and toxin scores, Mann-Whitney test was used
679 for statistical analyses, * p<0.05.

680

681

682 **Supplementary FIG 1. PCR identification of *Clostridium difficile* and its associated toxins**
683 (**TcdA, TcdB, and the binary cdtA/cdtB toxin**) **A)** PCR (26) confirming *C. difficile* specific
684 identification. Lane 1 = 100bp ladder, Lane 2 = 16N203-1, Lane 3 = 16N203-2, Lane 4 =
685 16N203-3, Lane 5 = 16N203-4, Lane 6 = *C. difficile* 630, Lane 7 = water. **B)** PCR (27)
686 confirming presence of CDI toxins: Toxin A (*TcdA*), Toxin B (*TcdB*), and binary toxin
687 (*cdtA/cdtB*). Lane 1 = 100bp ladder, Lane 2 = 16N203-1, Lane 3 = 16N203-1, Lane 4 =
688 16N203-2, Lane 5 = 16N203-3, Lane 6 = 16N203-4, Lane 7 = *C. difficile* R20291, Lane 8 = *C.*
689 *difficile* 630.

690

Table 1 – Enumeration of outbreak confirmed and excluded deaths

	Total	Dead	Examined,	Examined,	No tissue available
			Met case definition*	Did not meet case	
**All F0 mice	24	1	0	1	0
All F1 mice	183	56	25	11	20
Control diet (fed to F0) F1 mice	97	15	3	8	4
MS diet (fed to F0) F1 mice	86	41	22	3	16
Normal chow (fed to F1) F1 mice	118	38	22	5	11
HFD chow (fed to F1) F1 mice	65	18	3	6	9

1 *Met case definition either with histopathology or presence of *C. difficile* or *C. difficile* toxins.

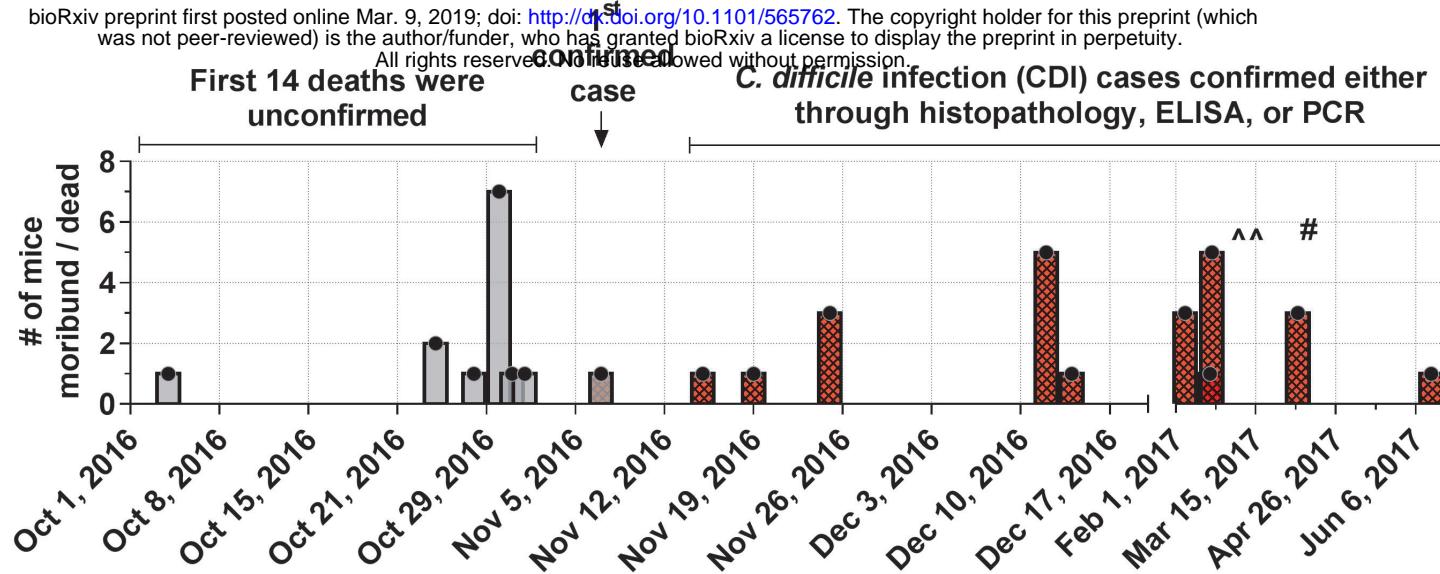
2 **F0 mice not included in survival curve analysis.

3

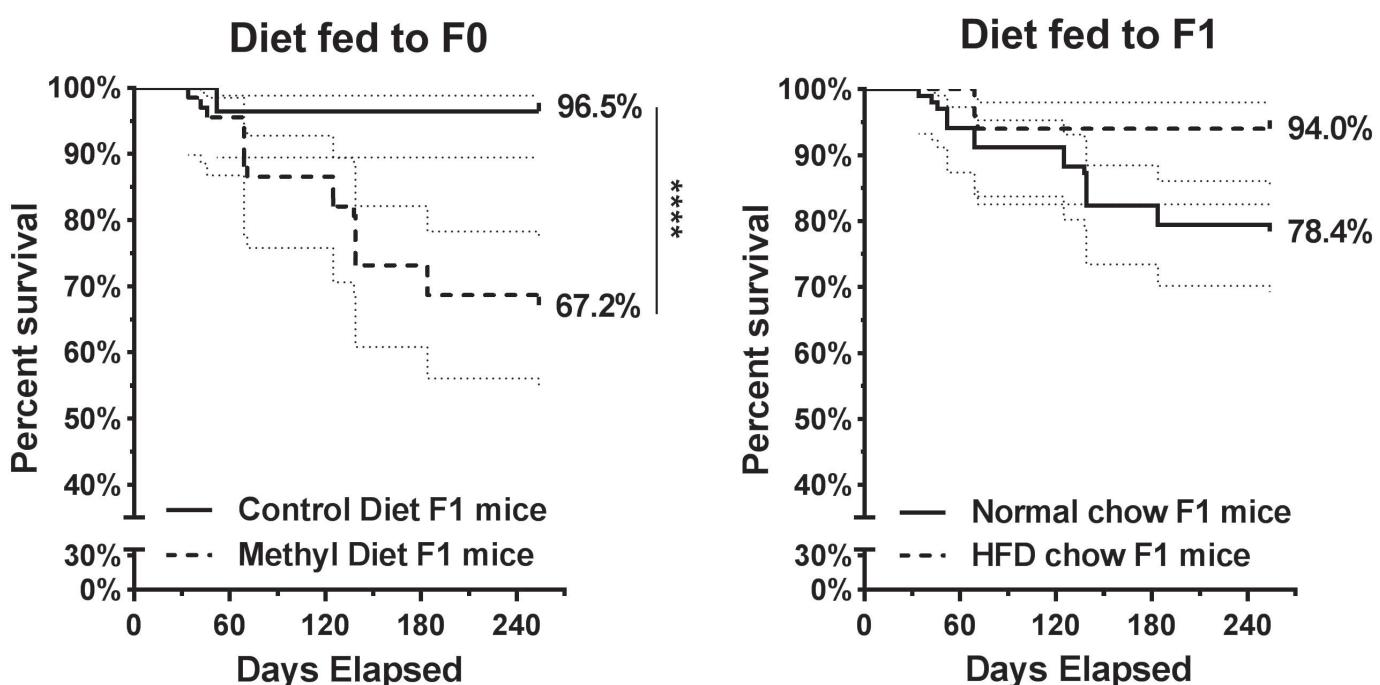
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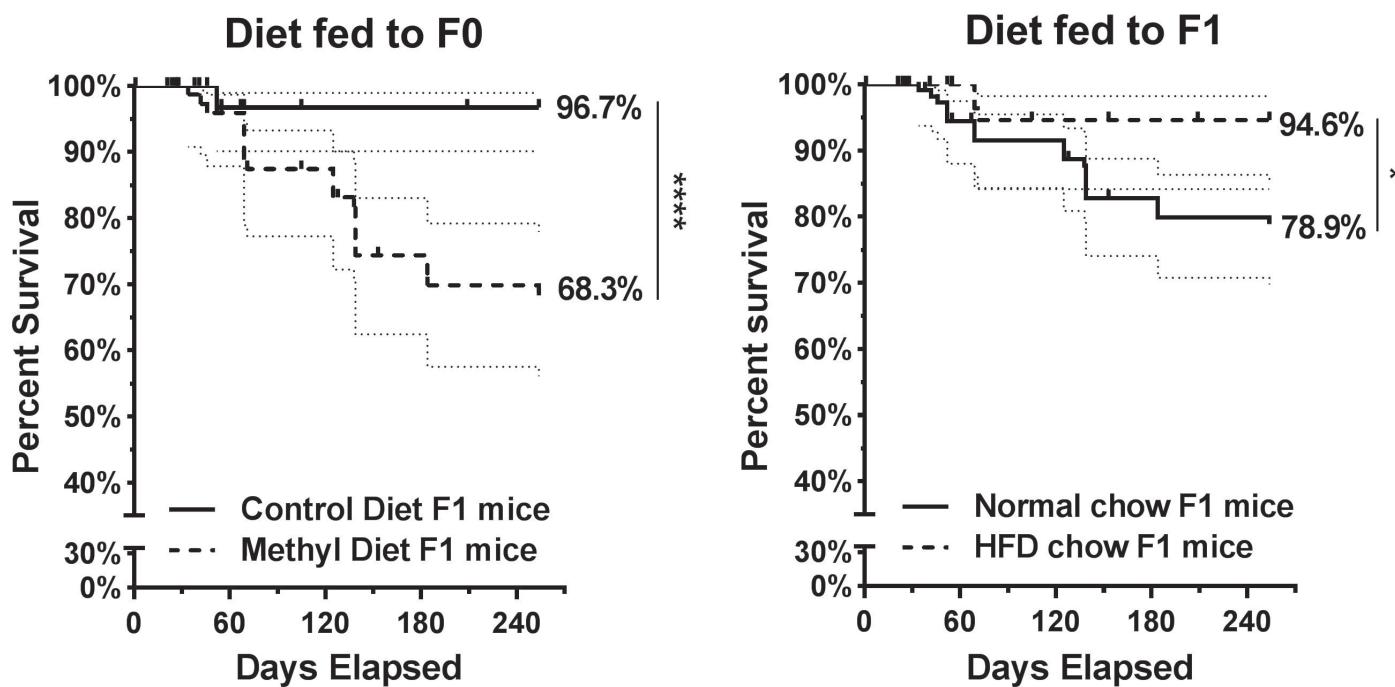
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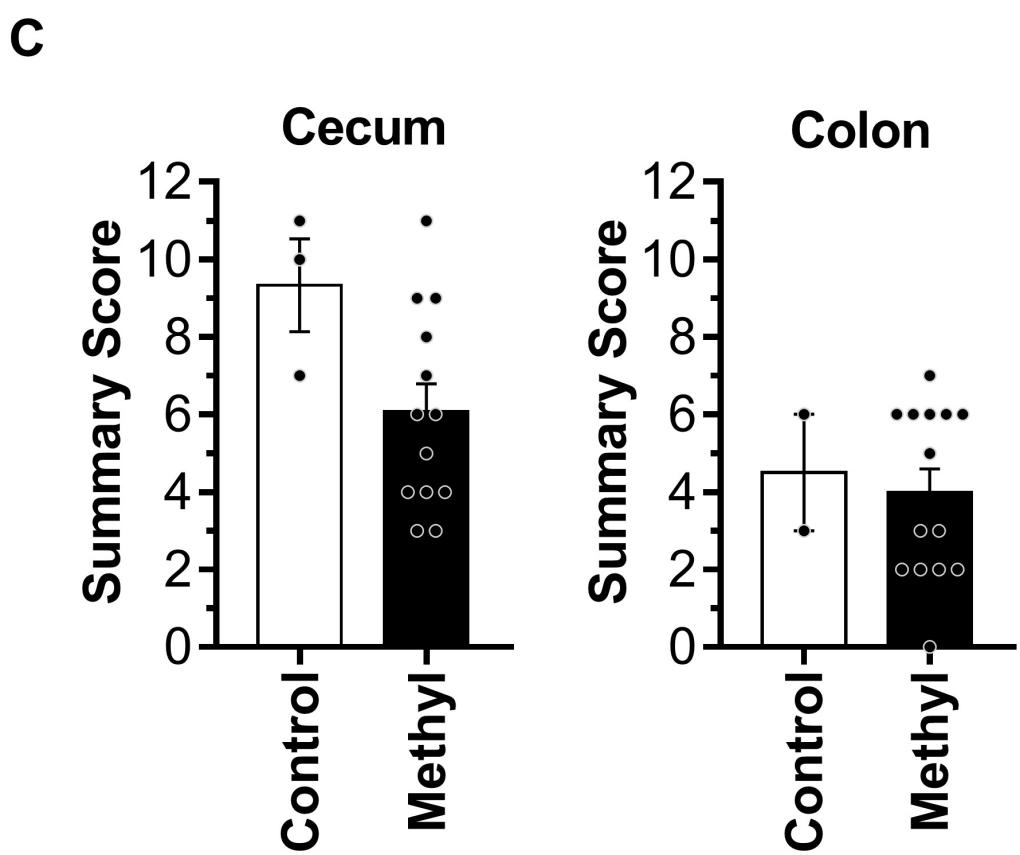
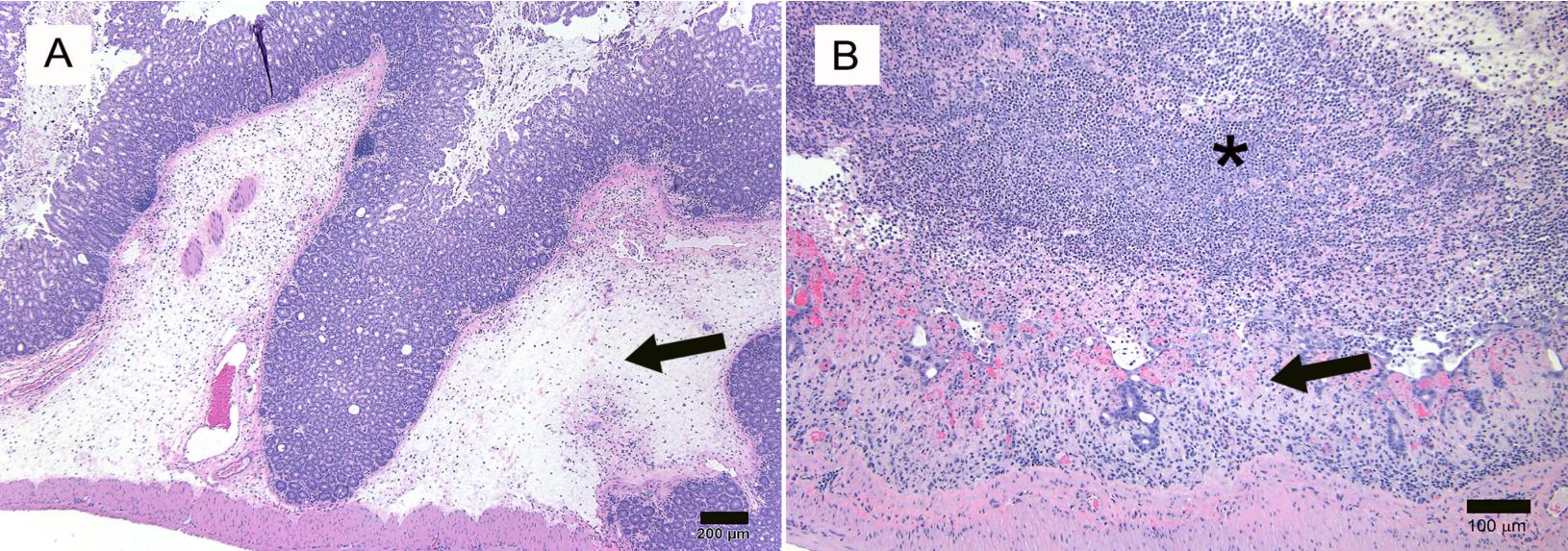


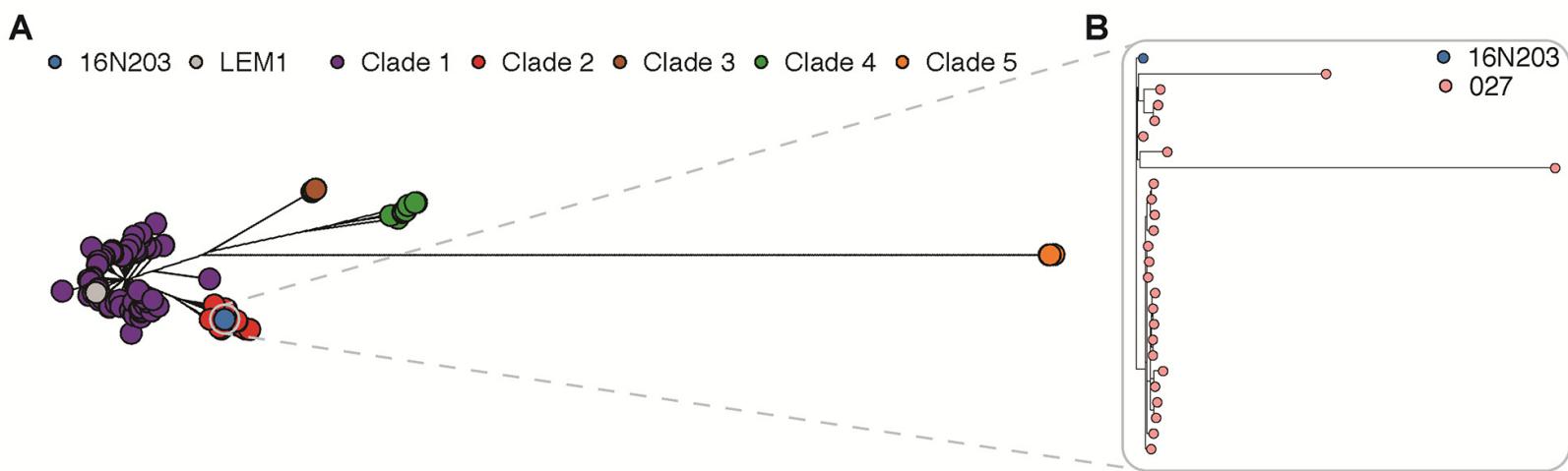
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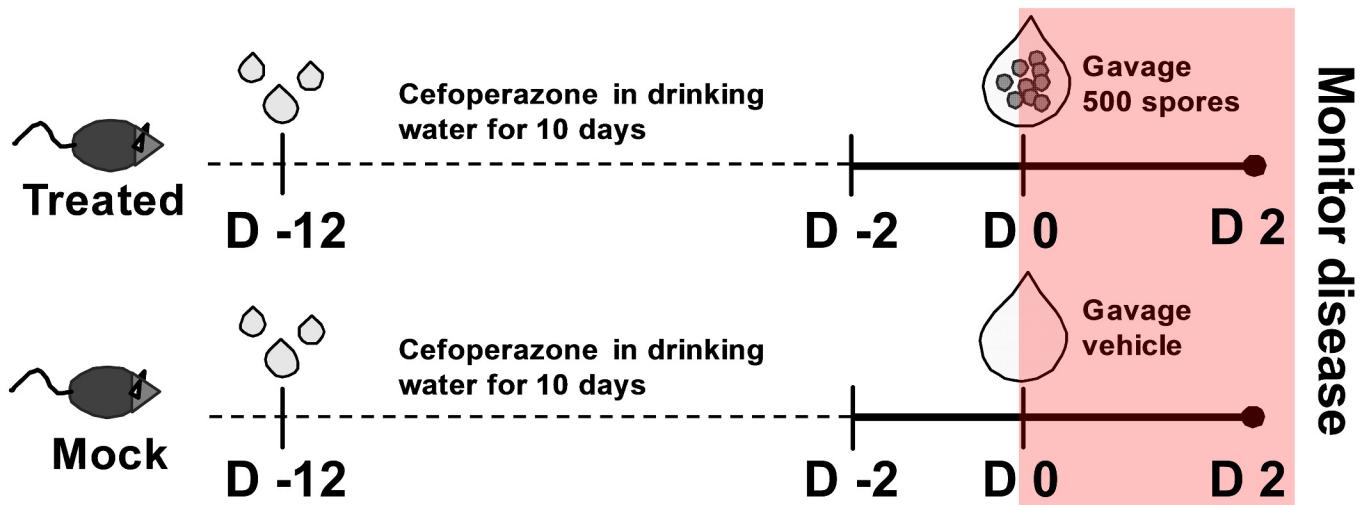
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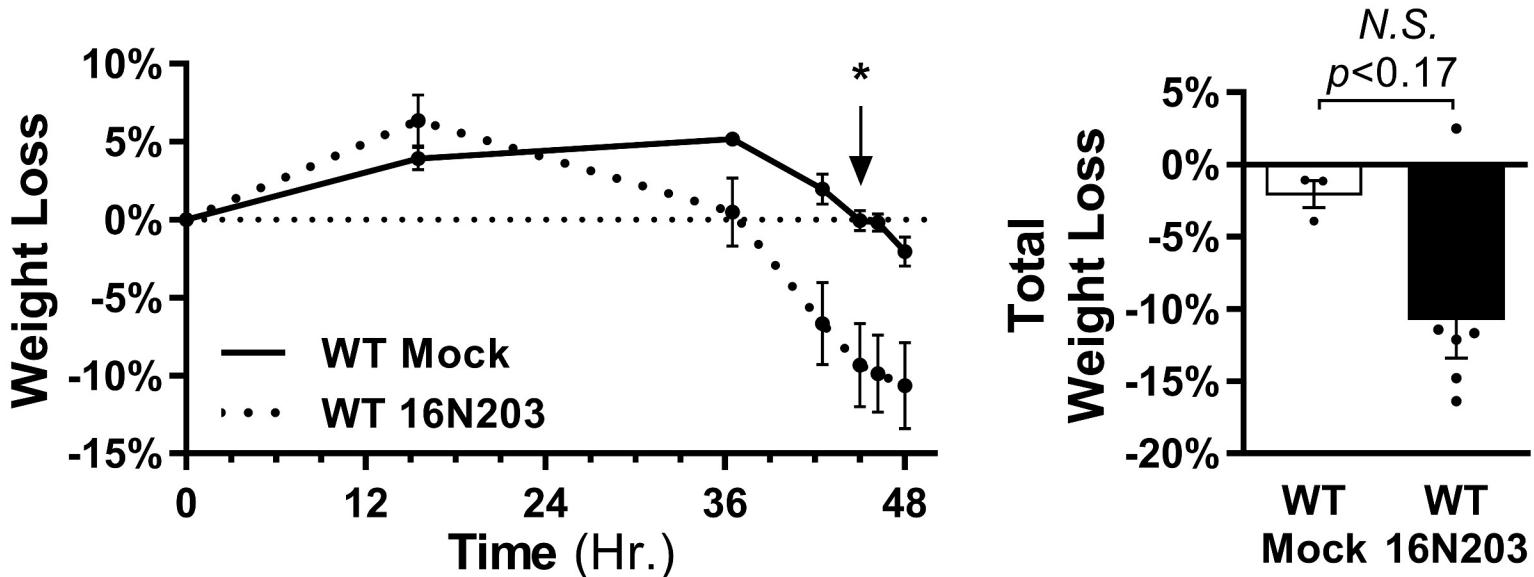




A



B



C

