Combination of mitomycin C and low-dose metronidazole synergistically against *Clostridioides difficile* infection and recurrence prevention

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Abstract

Clostridioides difficile is an anaerobic, spore-forming, Gram-positive pathogen responsible for various conditions from mild diarrhea to severe toxic megacolon and potentially death. Current treatments for *C. difficile* infection (CDI) rely on antibiotics like vancomycin and metronidazole (MTZ); however, the high doses required often disrupt gut microbiota, leading to a high recurrence rate. Mitomycin C (MMC), a chemotherapy drug approved by the FDA, is known for inducing phage production in lysogenic bacterial strains, effectively targeting the host bacteria. Given that 70% of *C. difficile* strains harbor prophages, this study investigates MMC's potential to enhance antibiotic efficacy against CDI. Our in vitro experiments indicate that MMC acts synergistically with MTZ to inhibit the growth of *C. difficile* strain R20291. Furthermore, this combination decreases biofilm-resident vegetative cell resistance and reduces the MTZ concentration needed to kill *C. difficile* in stool samples ex vivo. In a CDI mouse relapse model, in vivo results show that MMC combined with a low dose of MTZ significantly improves survival rates and reduces fecal spore counts after antibiotic treatment. Overall, these findings suggest that a low-dose combination of MMC and MTZ offers enhanced therapeutic efficacy for CDI, both in vitro and in vivo, and may provide a promising new approach for treatment.

Keywords: *Clostridioides difficile*, mitomycin C, metronidazole, spore, recurrence

39 **Introduction**

40 *Clostridioides difficile* is an anaerobic, gram-positive, spore-forming bacterium. The
41 symptoms of *C. difficile* infection (CDI) progress from asymptomatic carriage, diarrhea to
42 severe pseudomembranous colitis, and even death (1). Antibiotics, such as metronidazole
43 (MTZ), vancomycin (VAN), and fidaxomicin, are clinically recommended against CDI (2-4).
44 However, a high dose of antibiotics disrupts the host gut microbiota, leading to recurrent *C.*
45 *difficile* infection (rCDI) (5). rCDI is a serious clinical challenge, with over 20% of patients
46 experiencing recurrence within 30 days (6), and 60% will relapse again (7). The mortality rate
47 for primary CDI is 2.7%, while for rCDI is 25.4%, which is nearly ten times higher (8).
48 Therefore, investigating effective ways to reduce rCDI caused by antibiotics is crucial.

49 Mitomycin C (MMC), an FDA-approved anticancer drug, inhibits DNA synthesis by
50 cross-linking DNA (9). In addition to treating several cancer diseases (10-12), it has
51 antibacterial properties (13, 14) and exhibits potent activity against *C. difficile* (15). The
52 combination of two antibiotics is common for treating infections such as tuberculosis and
53 multidrug-resistant bacteria (16).

54 Since these effects on *C. difficile,* we hypothesize that MMC may assist antibiotic
55 treatment against CDI, and we aim to check the potential role of MMC in CDI treatment. Our
56 study demonstrated that MMC and MTZ synergistically inhibited *C. difficile* RT027 and RT078
57 strains, reducing the minimal bactericidal concentration (MBC) of MTZ for R20291 biofilm.

58 Additionally, the rCDI animal model trial indicated that combining low-dose MTZ with MMC
59 effectively improved mice survival rates and reduced the spore counts in their stool.
60

61 **Materials and Methods**

62 **Bacterial strains and culture conditions**

63 The bacterial strains used in this study are listed in Table S1. All *C. difficile* strains were
64 cultured anaerobically (10% H_2, 10% CO_2, and 80% N_2) at 37°C in BHI media (Thermo Fisher
65 Scientific, Waltham, MA, USA), alone or with 0.05% L-cysteine (Sigma-Aldrich, St. Louis,
66 USA) and 0.5% yeast extract (Thermo Fisher Scientific). The anaerobic bacteria were
67 maintained in an anaerobic workstation (DG250; Don Whitley Scientific Ltd., West Yorkshire,
68 UK).
69

70 **Checkerboard assay**

71 To determine the effects of MMC (Cyrusbioscience, New Taipei City, Taiwan) with MTZ
72 or VAN (Cyrusbioscience), we conducted the checkerboard assay in 96-well plates (SPL life
73 sciences, Pocheon, South Korea) (17). The initial drug concentrations were prepared at 4-fold
74 minimum inhibitory concentration (MIC) values, followed by 2-fold serial dilutions. Next, each
75 well was inoculated with 100 μL of *C. difficile* at 10^6 colony-forming unit (CFU)/mL in BHIS
76 broth. After 18 hours of incubation at 37°C, the OD_{600} values were measured using the iMark^{TM}

77 Microplate Absorbance Reader (Bio-Rad, Alfred Nobel Drive. Hercules, CA, USA). The
78 combined drug effects were quantified by the fractional inhibitory concentration (FIC) index as
79 follows:
80

81 $$\text{FIX index} = \frac{(\text{MIC of drug A in combination})}{(\text{MIC of drug A alone})} + \frac{(\text{MIC of drug B in combination})}{(\text{MIC of drug B alone})}$$

82

83 Synergy, FIC index ≤0.5; additive or indifference, FIC index >0.5 but <4; antagonism,
84 FIC index >4 (18). The results represented three independent experiments with the average
85 from triplicate measurements.
86

87 **Epsilometer test (E-test) assay**

88 The susceptibility of *C. difficile* to MTZ was determined using the E-test assay (19). The
89 overnight cultures of R20291 were 100-fold diluted using BHIS broth and cultured until the
90 value of OD_{600} reached 1.0. The refreshed cultures were then spread on BHIS agar, with or
91 without MMC, using sterile glass plate spreaders. A single strip of MTZ (Biomérieux, Marcy-
92 l'Étoile, France) was positioned at the center of each plate and incubated at 37°C for 24 hours.
93 The inhibitory concentrations were directly determined at the point of intersection between the
94 inhibition zone and the test strip. This experiment was representative of three independent
95 experiments.

96

97 **Biofilm formation and MBC assay**

98 The biofilm formation assay was conducted as previously described (16). In brief,
99 overnight cultures of strain R20291 were 100-fold diluted in BHI broth containing 0.1 M
100 glucose (Avantor, Radnor Township, Pennsylvania, USA) and grown until the OD_{600} value
101 reached 0.1. 200 µL of cultures were inoculated into 96-well plates and anaerobically incubated
102 at 37°C for 72 hours. The supernatants were then replaced with 200 µL of BHI-glucose broth
103 containing antibiotics, followed by a 48-hour incubation. Subsequently, the supernatants were
104 removed, and 200 µL of BHIS broth was used to resuspend the biofilm. The Minimum
105 Bactericidal Concentration (MBC) Assay was performed to determine the efficacy of biofilm
106 disruption (20). Two µL of resuspended biofilm solution were dropped into BHIS agar and
107 incubated at 37°C for 24 hours. This experiment was representative of three independent
108 experiments, each performed in triplicate.

109

110 **Ex vivo bacterial-fecal co-culture assay**

111 Fresh feces were collected from C57BL/6 mice and dissociated in sterile ddH_2O at a ratio of
112 0.01 g stool per 1 mL ddH_2O. Overnight cultures of *C. difficile* strain R20291 were refreshed
113 100-fold in BHIS broth until the OD_{600} reached 1.0. In each group, $5x10^5$ CFU of R20291 in
114 800 µL BHIS broth were combined, with or without different antibiotics (1 µg/mL of MMC,

115 0.375 μg/mL of MTZ, 3 μg/mL of MTZ, or 1 μg/mL of MMC+0.375 μg/mL of MTZ). These
116 mixtures were then gently mixed with 200 μL of fecal suspensions and anaerobically incubated
117 for 24 hours. The fecal-bacterial cultures were then diluted 10^6-fold and spread on selective
118 cycloserine-cefoxitin fructose agar (CCFA) plates for CFU quantification. The experiment was
119 representative of four independent experiments with duplicate measurements. Statistical
120 analysis was performed using One-Way ANOVA with GraphPad Prism 10.0 (GraphPad, La
121 Jolla, CA).
122
123 **Spore purification**
124 The spore purification process was performed as previously described (21). Briefly,
125 overnight cultures of *C. difficile* were 100-fold diluted in BHIS broth and grown until the OD_{600}
126 value reached 0.8. The bacterial cultures were then inoculated on SMC agar (22) in 6-well plates
127 (SPL life sciences) and incubated anaerobically at 37°C for 7 days. *C. difficile* spores were
128 harvested using ice-cold sterile ddH_2O and stored overnight at 4°C. Spores were centrifuged at
129 2,330 ×g for 16 min at 4°C and washed five times with ice-cold sterile ddH_2O to separate spores
130 from cell debris. Spores were purified using Nycodenz (48%; Axis Shield, Oslo, Norway) and
131 centrifuged at 2,500 ×g for 10 min to collect the pellet. After five times wash with ice-cold
132 sterile ddH_2O, the pellet was resuspended in 1x PBS and stored at 4°C. Purified spores were
133 10-fold serially diluted and plated on BHIS agar with 0.1% taurocholic acid (Sigma-Aldrich)

134 to determine the CFUs formed by spores.

135

136 **Experimental rCDI animal model and sample collection**

137 7-weeks-old male C57BL/6 mice were obtained from the Laboratory Animal Center of
138 NCKU. All mice were managed following the Institutional Animal Care and Use Committee
139 (IACUC) guidelines of NCKU. All animal experiments followed the protocol approved by the
140 IACUC (approval number: NCKU-IACUC-113-068) and the NCKU Biosafety and Radiation
141 Safety Management Division. To determine the effects of combined antibiotic treatment, a
142 rCDI mouse model was performed as previously described (23). Briefly, mice were
143 intraperitoneally injected with 10 mg/kg of clindamycin 24 hours before infection, then orally
144 challenged with 100 μL containing 10^4 CFU R20291 spores. Different antibiotic treatments
145 were orally given on days 2 and 3 post-infection. To determine whether MMC can synergically
146 assist low-dose of MTZ against *C. difficile* infection and recurrence, mice were divided into six
147 groups and respectively treated with (1) sterile 1x PBS (Mock group, 3 mice and R20291 group,
148 8 mice), (2) 50 mg/kg of MTZ dissolved in 1x PBS (MTZ_{HC} group, 8 mice), (3) 7 mg/kg of
149 MTZ dissolved in 1x PBS (MTZ_{LC} group, 8 mice), (4) 5 mg/kg of MMC dissolved in 1x PBS
150 (MMC group, 8 mice), and (5) 7 mg/kg of MTZ combined with 5 mg/kg MMC dissolved in 1x
151 PBS (MTZ_{LC}+MMC group, 8 mice). In the animal experiment to compare the efficacy of
152 MTZ_{LC}+MMC treatment and VAN treatment, mice were divided into three groups and

153 respectively treated with (1) 50 mg/kg of MTZ dissolved in 1x PBS (MTZ_{HC} group, 9 mice),
154 (2) 20 mg/kg of VAN dissolved in 1x PBS (VAN group, 9 mice), (3) 7 mg/kg of MTZ combined
155 with 5 mg/kg MMC dissolved in 1x PBS (MTZ_{LC}+MMC group, 9 mice). During the infection,
156 mice were monitored daily for clinical sickness score (CSS) (24), and fecal samples were
157 collected to quantify the number of spores on days 3, 5, and 7 post-infection. Mice sera were
158 analyzed for glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT),
159 blood urea nitrogen (BUN), and creatinine (CRE) using NX-600 dry biochemistry analyzer
160 (Fujifilm, Minato, Tokyo, Japan). After colonic morphology was observed, harvested organs
161 were fixed in 10% formaldehyde (BS Chemical, Kretinga, Lithuania) or stored at -80°C for
162 further analysis. Statistical analysis was performed using GraphPad Prism 10.0, and
163 significance was determined by t-test and One-Way ANOVA.
164

165 **Quantification of *C. difficile* spore-forming CUFs**

166 Fresh fecal samples collected on days 3, 5, and 7 post-infection were weighted and diluted
167 by 1 mL of ddH_2O with 100 mg of 0.1 mm zirconia beads (BioSpec Products, Inc., Bartlesville,
168 Oklahoma, USA). After the homogenization using the BeadBeater (Bertin Technologies,
169 avenue Ampère, Montigny-le-Bretonneux, France) for 50 sec (23), fecal samples were heat-
170 killed at 65°C for 20 min and plated on BHIS agar containing 0.1% TCA to quantify the CFUs.
171 The plates were anaerobically incubated at 37°C for 24 hours and CFU numbers were calculated

172 after normalizing to the fecal weight. This experiment was representative of three independent
173 experiments and each in duplicate. Statistical analysis was performed using GraphPad Prism
174 10.0, and significance was determined by t-test and One-Way ANOVA.
175
176 **Results**

177 **Mitomycin C showed a synergistic antibacterial activity in combination with**
178 **metronidazole against *C. difficile* RT027 strain R20291 and RT078 clinical strains**

179 The previous study used MMC to induce phage production in *C. difficile* and promote the
180 lytic cycle (21). Interestingly, over 70% of *C. difficile* strains naturally carry prophages (25).
181 Therefore, we wanted to check whether MMC could be combined with VAN or MTZ as a novel
182 CDI therapeutic strategy. First, we performed the checkerboard assay (Figure 1A), and the MIC
183 of MMC and MTZ were determined to be 2 μg/mL and 3 μg/mL, respectively (Figure S1A).
184 The combination of MTZ with MMC showed synergistic antibacterial activity in inhibiting the
185 growth of R20291, as the FIC index was less than 0.5 (Figure 1B). MTZ combined with 1
186 μg/mL MMC could reduce the MIC of MTZ for inhibiting *C. difficile* growth from 3 μg/mL to
187 0.375 μg/mL (an 8-fold reduced MIC value) (Figure S1A). While the combination of VAN and
188 MMC exhibited an additive activity only (Figure 1B). Furthermore, the MTZ-MMC treatment
189 also synergistically against RT027 and RT078 *C. difficile* clinical strains (Figure 1C); MMC

190 additively assisted VAN in inhibiting RT078 strains (Figure S1B). These results demonstrated
191 that MMC enhances the effects of CDI-treating antibiotics.
192 E-test strips were used to confirm the synergistic effects of MTZ and MMC for combating
193 R20291. The MIC of MTZ strip alone was 0.125 μg/mL (Figure 1D, left). Notably, 0.25 μg/mL
194 of MMC would not influence the growth of bacteria (Figure 1D, middle), but effectively
195 reduced the MIC of MTZ to 0.023 μg/mL (a 5.4-fold reduced MIC value; Figure 1D, right).
196 These in vitro experimental results indicate that the combination of MTZ and MMC can more
197 effectively inhibit *C. difficile* strain R20291 than MTZ alone.
198

199 **Mitomycin C could effectively enhance the metronidazole effect on the strain R20291**
200 **biofilms**

201 In the CDI mouse model, *C. difficile* strain R20291 can aggregate and form a glycan-rich
202 biofilm, which is associated with increased antibiotic resistance and rCDI (26-28). Therefore,
203 we tried to clarify whether MMC can help MTZ reduce the required concentration against
204 biofilm by the MBC assay (Figure 2A). As shown in Figure 2B, the MBC of MTZ for R20291
205 vegetative cells was 3 μg/mL. However, the MBC of MTZ required to eliminate R20291 in
206 biofilm increased to 18 μg/mL (Figure 2C, left). Interestingly, combining MTZ with 1 μg/mL
207 of MMC markedly decreased the MBC of MTZ to 9 μg/mL (Figure 2C, middle). MMC showed
208 no toxicity on R20291 vegetative cells until the concentration reached 4 μg/mL (Figure 2C,

209 right). To determine whether any vegetative cells were released outside the biofilm, we dropped
210 the biofilm supernatant on BHIS agar plates. As shown in Figure S2, no lived bacterium was
211 detected outside the biofilm after treatment with antibiotics. Taken together, MMC could
212 enhance MTZ to effectively reduce its MBC against R20291 biofilm.

213

214 **Combination of mitomycin C with metronidazole inhibits *C. difficile* colonization in fresh**
215 **mouse feces**

216 To simulate the diverse microbial gut environment, *C. difficile* strain R20291 was mixed
217 with fresh mouse fecal suspensions to assess the efficacy of a low concentration of MTZ
218 combined with MMC against *C. difficile* ex vivo. After co-culturing for 24 hours, 10^6 dilutions
219 of bacterial-fecal cultures were spread on BHIS agar to assess the bacterial CFU count (Figure
220 3A). As shown in Figure 3B, no CFU was observed in the 3 μg/mL of MTZ-treated group,
221 indicating that the number of *C. difficile* was less than 10^6 CFU/mL. With the combination
222 treatment of 0.375 μg/mL MTZ and 1 μg/mL MMC, an average of 4.5×10^6 CFU/mL was
223 observed. By comparison, the average CFU counts with 0.375 μg/mL of MTZ (MTZ_{LC}) or
224 1μg/mL of MMC alone were 7.8×10^7 CFU/mL and 4.9×10^7 CFU/mL, respectively. The
225 difference may be due to the low concentration of antibiotic treatments, which affect other
226 bacteria competing with *C. difficile* while having no direct impact on *C. difficile* in the mouse

227 feces. In summary, these results demonstrated that combining MTZ with MMC is a potential
228 strategy to inhibit *C. difficile* colonization ex vivo.

229

230 **Treating metronidazole with mitomycin C successfully increased the survival rate and**
231 **decreased the spore number in a recurrence CDI mouse model**

232 Given that the combination of low-dose MTZ and MMC effectively inhibited *C. difficile*
233 in vitro and ex vivo, we want to determine whether this strategy can be applied in vivo (Figure
234 4A). We referred to the rCDI mouse model (23), and challenged mice with purified R20291
235 spores (Figure S3A, B). Only the non-infected and infected group treated with MTZ_{LC}+MMC
236 demonstrated a 100% survival rate, while other groups consistently fell below 80% (Figure 4B,
237 left). Furthermore, both R20291 group and MMC group exhibited the lowest weight on day 2
238 post-infection. However, by day 7 post-infection, all surviving mice had recovered to over 95%
239 of their initial weight (Figure 4B, right).

240 To determine the recurrence of *C. difficile* in mice, we collected feces on days 3, 5, and 7
241 post-infection and plated them on BHIS agar with 0.1% taurocholic acid (TCA). The spore-
242 formed CFU counts showed no significant difference between MTZ_{HC} and MTZ_{LC}+MMC
243 groups on day 3 post-infection (Figure 4C, left). However, the CFU counts in other infected
244 groups were significantly higher than those in the MTZ_{LC}+MMC group (Figure 4C). On day 5
245 post-infection, the spore-formed CFU counts in R20291 and MTZ_{LC}+MMC groups were

246 significantly lower than in other infected groups (Figure 4C, middle). Notably, the MTZ_{HC}
247 group had a significantly highest CFU number among all groups (Figure 4C, middle). On day
248 7 post-infection, despite a decrease in CFU number from all infected groups, the CFU number
249 in the MTZ_{HC} group remained significantly higher than that in the MTZ_{LC}+MMC group (Figure
250 4C, right). During the CDI, the daily clinical sickness score of mice in the MTZ_{LC}+MMC group
251 was lower than other infection groups (Figure S3C). The colonic morphology of infected mice
252 was an important indicator for assessing intestinal inflammation (29). As shown in Figure 4D,
253 all surviving mice recovered at day 7 post-infection with no significant difference in their cecum
254 length and weight. The colon length of the MTZ_{LC}+MMC group was significantly higher than
255 that of the MTZ_{LC} and MMC groups. Taken together, the rCDI in vivo experiments
256 demonstrated that the MTZ_{LC}+MMC treatment could express the highest mouse survival rate
257 and prevent the relapse of CDI.
258

259 **Synergistic effects of mitomycin C and metronidazole enhanced mice survival rate and**
260 **reduced stool spore number compared to vancomycin treatment**

261 Previous studies suggest that VAN has superior cure rates for severe CDI and better
262 outcomes for infected mice than those treated with MTZ (30, 31). However, VAN treatment
263 was associated with a significant disruption of the intestinal microbiota, thereby increasing the

264 risk of the rCDI (32, 33). Therefore, we wanted to assess whether combining MTZ with MMC
265 could express a better effect than VAN in the rCDI mouse model.

266 After being infected with R20291 spores, mice received VAN, MTZ_{HC}, or MTZ_{LC}+MMC
267 treatments, respectively (Figure 5A). The MTZ_{LC}+MMC treatment provided a higher survival
268 rate (89%) compared to the VAN (67%) or MTZ_{HC} (67%) groups (Figure 5B, left). In body
269 weight change rate, VAN and MTZ_{HC} groups exhibited an appreciable decrease on day 3 to 4
270 post-infection; this trend was less obvious in MTZ_{LC}+MMC group (Figure 5B, right), all groups
271 of mice recovered to over 90% of their initial weight on day 7 post-infection (Figure 5B, right).
272 Although the CFU counts of spore in feces from MTZ_{LC}+MMC group were significantly higher
273 than the other groups on day 3 post-infection (Figure 5C, left). However, on days 5 and 7 post-
274 infection, the CFU counts in MTZ_{LC}+MMC group were significantly lower than other two
275 groups (Figure 5C, middle and right). In addition, the VAN group had increased nearly ten
276 times spore number in their feces on day 5 post-infection (Figure 5C, middle), while the MTZ_{HC}
277 group maintained a high spore level in feces from day 3 to 7 post-infection (Figure 5C). In mice
278 intestinal inflammation, except for the cecal weight in MTZ_{LC}+MMC group was significantly
279 higher than in the MTZ_{HC} group; mice cecum and colon morphology showed no significant
280 difference between each group (Figure 5D). Finally, to ensure that combining low-dose MTZ
281 with MMC for CDI would not cause liver and kidney damage in mice. Serum was collected on
282 day 7 post-infection and analyzed for glutamic pyruvic transaminase (GPT), glutamic

283 oxaloacetic transaminase (GOT), blood urea nitrogen (BUN), and creatinine (CRE) using a dry
284 biochemical analyzer, and the values of each indicator exhibited no significant difference
285 between the groups (Figure 5E). In summary, our results demonstrated that combining low-
286 dose MTZ with MMC showed the highest survival rate and effectively reduced intestinal spore
287 count in CDI mice compared to VAN or MTZ-treated groups.

288

289 **Discussion**

290 The CDI patients still rely on VAN and MTZ treatments (2-4), however, these antibiotics
291 disrupt gut microbiota to cause the recurrence (34). In this study, we found that MMC can
292 synergistically cooperate with MTZ against *C. difficile*. Moreover, MMC can boost MTZ's
293 effectiveness in eliminating *C. difficile* within biofilms, despite biofilm formation providing
294 protection for *C. difficile* against antibiotic attacks, which can result in recurrent infections (26-
295 28, 35). Fidaxomicin can penetrate biofilms and disrupt their structure, thereby enhancing the
296 bactericidal activity (36, 37). Future work should observe the biofilm morphology to determine
297 whether the MTZ-MMC treatment directly disrupts biofilms to kill *C. difficile* vegetative cells.

298 Our in vivo experiments showed that combining MTZ_{LC} with MMC increased mice
299 survival rates after the spore challenge. However, the recovery of body weight in this group
300 was not as good as in other infected groups. Most mice of other groups with severe body weight
301 loss have died in CDI, leading to the lived mice having a higher average body weight than the

302 MTZ_{LC}+MMC group. The CFUs formed by spore in mice fecal samples to evaluate CDI
303 recurrence showed no significant difference between the R20291 and MTZ_{LC}+MMC groups
304 after day 5 post-infection. Colonization resistance is critical for recovery and preventing rCDI
305 through the healthy microbiota in inhibiting *C. difficile* colonization and overgrowth (38-40).
306 However, antibiotics disrupt microbiota diversity (41). Accordingly, we consider that mice in
307 both groups might suffer less damage in their microbiota and recover more rapidly to prevent
308 *C. difficile* colonization. Nevertheless, further analysis of intestinal microbial composition
309 between each group is needed to confirm our hypothesis.

310 MTZ disrupts DNA synthesis (42), while MMC cross-links DNA to prevent bacterial
311 replication (43). Our study indicated that combining MTZ with MMC provides better action in
312 inhibiting bacterial DNA replication against *C. difficile* infection.

313 In summary, our findings reveal a novel therapeutic strategy that MMC enhances the
314 efficacy of MTZ against CDI. The overexpression of NimB in some mutant strains reduces the
315 ability of MTZ to treat CDI, leading to antibiotic resistance (44). This issue did not occur in our
316 MTZ-MMC combination therapy for *C. difficile*. Furthermore, the low dose of MTZ combined
317 with MMC may effectively decrease rCDI by minimizing gut microbiota disruption (38, 40).
318 Nevertheless, further experiments are necessary to confirm the safety, optimal dosage, and
319 potential side effects of the combined antibiotic therapy before the clinical application.

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334 **Figure Legends**

335 **Figure 1. MMC and MTZ demonstrated synergistic inhibition against specific *C. difficile***
336 **strains, including RT027 (e.g., R20291) and RT078 in vitro.** (**A**) Flowchart of checkerboard
337 assay (Created with BioRender.com). FIC index of MMC combined with MTZ or VAN co-
338 culturing with (**B**) *C. difficile* strain R20291, (**C**) RT027 and RT078 clinical strains. The MIC
339 of MTZ alone or a combination of MMC with MTZ against *C. difficile* was determined using
340 E-test strips (**D**). MTZ, metronidazole. VAN, vancomycin. MMC, mitomycin C. FIC index,
341 fractional inhibitory concentration index. MIC, minimum inhibitory concentration. The results
342 represented three independent experiments with the average from triplicate measurements.

343

344 **Fig 2. Combining MMC and MTZ therapy effectively reduced the antibiotic resistance of**
345 **R20291 biofilms.** (**A**) The flowchart of antibiotic treatments against *C. difficile* biofilm
346 formation (Created with BioRender.com). (**B**) MBC assay was used to determine the
347 concentration of MTZ that can kill R20291 vegetative cells. After 3 days of incubation to form
348 biofilms, the MBC concentration of (**C**) MTZ alone, MTZ combined with 1 μg/mL MMC, and
349 MMC alone to combat R20291 within the biofilm was determined. MBC, minimum
350 bactericidal concentration. This experiment was representative of three independent
351 experiments, each performed in triplicate.

352

353 **Fig 3. The combination of MMC and low-dose MTZ significantly inhibited the growth of**
354 **R20291 in mouse feces.** (**A**) Ex vivo model of R20291 co-cultured with mouse feces and
355 treated with different kinds of antibiotic treatment (Created with BioRender.com). (**B**) Fecal-
356 bacterial mixtures cultured for 24 hours were 10^6-fold diluted, and the CFU counts of *C. difficile*
357 were determined by spreading on CCFA plates. CFU, colony-forming units. MTZ_{HC}, high
358 concentration of MTZ (3 μg/mL). MTZ_{LC}, low concentration of MTZ (0.375 μg/mL). MMC, 1
359 μg/mL MMC. MTZ_{LC}+MMC, 0.375 μg/mL MTZ combined with 1 μg/mL MMC. The
360 experiment was representative of four independent experiments with duplicate measurements.
361 Statistical analysis was performed using the one-way analysis of variance test (*$p \leq 0.05$; ****p
362 ≤ 0.0001; ns, not significant)
363
364 **Fig 4. Application of combining MMC with low-dose MTZ treatment in the mouse CDI**
365 **recurrence model.** (**A**) Schematic diagram of the mouse CDI recurrence model (Created with
366 BioRender.com). C57BL/6 mice were given clindamycin (10 mg/kg) and orally challenged
367 with 10^4 CFU spores. After two days of treatment with (n=8/group) or without (Mock, n=3;
368 R20291, n=8) antibiotics, the severity of infection symptoms was observed, and feces were
369 collected to assess the number of spores. (**B**) The survival and body weight change rate of mice
370 were monitored daily from the day of infection (pi-day 0) until being sacrificed. (**C**) The CFU
371 of *C. difficile* spore was determined using mouse feces collected on the days 3, 5, and 7 post-

372 infection. (**D**) The morphology and quantification of cecal and colonic tissues. The colon length,
373 cecum length, and cecum weight were harvested when the mice were sacrificed on day 7 post-
374 infection. Each point represents the data of one mouse. Statistical analysis was performed using
375 GraphPad Prism 10.0, and significance was determined by the one-way analysis of variance
376 (**≤ 0.01; ***≤ 0.001; ****$p \leq 0.0001$; ns, not significant)
377
378 **Fig 5. Comparing the effect of MMC-MTZ$_{LC}$ treatment and clinical antibiotics against *C.***
379 ***difficile* infection and recurrence in vivo.** (**A**) Diagram of the mouse CDI recurrence model
380 (Created with BioRender.com). Mice (n = 9/group) were orally challenged with *C. difficile*
381 spores, followed by two days of antibiotic treatment. (**B**) Mice survival rate and the daily
382 percentage of mouse body weight were recorded after being infected with R20291 spores. (**C**)
383 Mice feces were collected on day 3, 5, and 7 post-infection. The homogenized feces were
384 diluted and plated on CCFA plates to measure the spore-forming CFU counts. (**D**) The
385 morphology of mice cecum and colon. The degree of intestinal inflammation was evaluated by
386 quantifying the colonic length, cecum length, and weight. Each point represents the data of a
387 mouse. (**E**) The values of GPT, GOT, BUN, and CRE in mice serum. The dry biochemical
388 analyzer was used to measure various indicators in the serum to evaluate the damage and impact
389 of different treatment processes on the liver and kidney functions of mice. GPT, glutamic
390 pyruvic transaminase. GOT, glutamic oxaloacetic transaminase. BUN, blood urea nitrogen.

CRE, creatinine. Statistical analysis was performed using GraphPad Prism 10.0, and significance was determined by the t-test and one-way analysis of variance (*≤ 0.05; **≤ 0.01; ***≤ 0.001; ****$p \leq 0.0001$; ns, not significant)

394 **References**

395 1. Mounsey A, Lacy Smith K, Reddy VC, Nickolich S. 2020. Clostridioides difficile
396 Infection: Update on Management. Am Fam Physician 101:168-175.

397 2. Kelly CR, Fischer M, Allegretti JR, LaPlante K, Stewart DB, Limketkai BN, Stollman
398 NH. 2021. ACG Clinical Guidelines: Prevention, Diagnosis, and Treatment of
399 Clostridioides difficile Infections. Am J Gastroenterol 116:1124-1147.

400 3. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke
401 ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox
402 MH. 2018. Clinical Practice Guidelines for Clostridium difficile Infection in Adults and
403 Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and
404 Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis 66:e1-e48.

405 4. Surawicz CM, Brandt LJ, Binion DG, Ananthakrishnan AN, Curry SR, Gilligan PH,
406 McFarland LV, Mellow M, Zuckerbraun BS. 2013. Guidelines for diagnosis, treatment,
407 and prevention of Clostridium difficile infections. Am J Gastroenterol 108:478-98; quiz
408 499.

409 5. Chilton CH, Pickering DS, Freeman J. 2018. Microbiologic factors affecting
410 Clostridium difficile recurrence. Clin Microbiol Infect 24:476-482.

411 6. Feuerstadt P, Theriault N, Tillotson G. 2023. The burden of CDI in the United States: a
412 multifactorial challenge. BMC Infect Dis 23:132.

413 7. Nelson WW, Scott TA, Boules M, Teigland C, Parente A, Unni S, Feuerstadt P. 2021.
414 Health care resource utilization and costs of recurrent Clostridioides difficile infection
415 in the elderly: a real-world claims analysis. J Manag Care Spec Pharm 27:828-838.
416 8. Feuerstadt P, Nelson WW, Drozd EM, Dreyfus J, Dahdal DN, Wong AC, Mohammadi
417 I, Teigland C, Amin A. 2022. Mortality, Health Care Use, and Costs of Clostridioides
418 difficile Infections in Older Adults. J Am Med Dir Assoc 23:1721-1728.e19.
419 9. Verweij J, Pinedo HM. 1990. Mitomycin C: mechanism of action, usefulness and
420 limitations. Anticancer Drugs 1:5-13.
421 10. Metcalfe M, Wagenheim G, Xiao L, Papadopoulos J, Navai N, Davis JW, Karam JA,
422 Kamat AM, Wood CG, Dinney CP, Matin SF. 2017. Induction and Maintenance
423 Adjuvant Mitomycin C Topical Therapy for Upper Tract Urothelial Carcinoma:
424 Tolerability and Intermediate Term Outcomes. J Endourol 31:946-953.
425 11. Read MD, Drake J, Hashemipour G, Powers BD, Mehta R, Sinnamon A, Pimiento JM,
426 Dineen SP. 2024. Initial Experience Using Laparoscopic HIPEC for Gastric Cancer with
427 Peritoneal Metastasis: Safety and Outcomes. Ann Surg Oncol 31:3750-3757.
428 12. Al-Humairi RMA, Hashim Mohammad T, Thanoon Ahmed S, Ad'hiah AH. 2023.
429 Systemic Interleukin-6 Response after Intravesical Instillation of Bacillus Calmette-
430 Guérin and Mitomycin C in Superficial Bladder Cancer. Arch Razi Inst 78:353-360.
431 13. Svedholm E, Bruce B, Parcell BJ, Coote PJ. 2024. Repurposing Mitomycin C in

432 Combination with Pentamidine or Gentamicin to Treat Infections with Multi-Drug-
433 Resistant (MDR) Pseudomonas aeruginosa. Antibiotics (Basel) 13.
434 14. Pacios O, Fernández-García L, Bleriot I, Blasco L, González-Bardanca M, López M,
435 Fernández-Cuenca F, Oteo J, Pascual Á, Martínez-Martínez L, Domingo-Calap P, Bou
436 G, Tomás M. 2021. Enhanced Antibacterial Activity of Repurposed Mitomycin C and
437 Imipenem in Combination with the Lytic Phage vB_KpnM-VAC13 against Clinical
438 Isolates of Klebsiella pneumoniae. Antimicrob Agents Chemother 65:e0090021.
439 15. Pal R, Seleem MN. 2020. Screening of Natural Products and Approved Oncology Drug
440 Libraries for Activity against Clostridioides difficile. Sci Rep 10:5966.
441 16. Worthington RJ, Melander C. 2013. Combination approaches to combat multidrug-
442 resistant bacteria. Trends Biotechnol 31:177-84.
443 17. Hu Y, Liu Y, Coates A. 2019. Azidothymidine Produces Synergistic Activity in
444 Combination with Colistin against Antibiotic-Resistant Enterobacteriaceae. Antimicrob
445 Agents Chemother 63.
446 18. Odds FC. 2003. Synergy, antagonism, and what the chequerboard puts between them. J
447 Antimicrob Chemother 52:1.
448 19. Nachnani S, Scuteri A, Newman MG, Avanessian AB, Lomeli SL. 1992. E-test: a new
449 technique for antimicrobial susceptibility testing for periodontal microorganisms. J
450 Periodontol 63:576-83.

451 20. Lambert RJ, Pearson J. 2000. Susceptibility testing: accurate and reproducible minimum
452 inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. J Appl
453 Microbiol 88:784-90.
454 21. Gong JJ, Huang IH, Su MS, Xie SX, Liu WY, Huang CR, Hung YP, Wu SR, Tsai PJ,
455 Ko WC, Chen JW. 2024. Phage transcriptional regulator X (PtrX)-mediated
456 augmentation of toxin production and virulence in Clostridioides difficile strain R20291.
457 Microbiol Res 280:127576.
458 22. Permpoonpattana P, Tolls EH, Nadem R, Tan S, Brisson A, Cutting SM. 2011. Surface
459 layers of Clostridium difficile endospores. J Bacteriol 193:6461-70.
460 23. Bublitz A, Brauer M, Wagner S, Hofer W, Müsken M, Deschner F, Lesker TR,
461 Neumann-Schaal M, Paul LS, Nübel U, Bartel J, Kany AM, Zühlke D, Bernecker S,
462 Jansen R, Sievers S, Riedel K, Herrmann J, Müller R, Fuchs TM, Strowig T. 2023. The
463 natural product chlorotonil A preserves colonization resistance and prevents relapsing
464 Clostridioides difficile infection. Cell Host Microbe 31:734-750.e8.
465 24. Shelby RD, Tengberg N, Conces M, Olson JK, Navarro JB, Bailey MT, Goodman SD,
466 Besner GE. 2020. Development of a Standardized Scoring System to Assess a Murine
467 Model of Clostridium difficile Colitis. J Invest Surg 33:887-895.
468 25. Hargreaves KR, Colvin HV, Patel KV, Clokie JJ, Clokie MR. 2013. Genetically diverse
469 Clostridium difficile strains harboring abundant prophages in an estuarine environment.

470 Appl Environ Microbiol 79:6236-43.

471 26. Soavelomandroso AP, Gaudin F, Hoys S, Nicolas V, Vedantam G, Janoir C, Bouttier S.
472 2017. Biofilm Structures in a Mono-Associated Mouse Model of Clostridium difficile
473 Infection. Front Microbiol 8:2086.

474 27. Morais M, Santos MGC, Costa CL, Martins CS, Leitão RFC, de Melo Pacífico D,
475 Quesada-Gómez C, Castelo Branco D, Ferreira EO, Brito GAC. 2022. Comparative
476 biofilm-forming ability between Clostridioides difficile strains isolated in Latin
477 America and the epidemic NAP1/027 strain. Front Cell Infect Microbiol 12:1033698.

478 28. Đapa T, Leuzzi R, Ng YK, Baban ST, Adamo R, Kuehne SA, Scarselli M, Minton NP,
479 Serruto D, Unnikrishnan M. 2013. Multiple factors modulate biofilm formation by the
480 anaerobic pathogen Clostridium difficile. J Bacteriol 195:545-55.

481 29. Huang J, Kelly CP, Bakirtzi K, Villafuerte Gálvez JA, Lyras D, Mileto SJ, Larcombe S,
482 Xu H, Yang X, Shields KS, Zhu W, Zhang Y, Goldsmith JD, Patel IJ, Hansen J, Huang
483 M, Yla-Herttuala S, Moss AC, Paredes-Sabja D, Pothoulakis C, Shah YM, Wang J,
484 Chen X. 2019. Clostridium difficile toxins induce VEGF-A and vascular permeability
485 to promote disease pathogenesis. Nat Microbiol 4:269-279.

486 30. Di X, Bai N, Zhang X, Liu B, Ni W, Wang J, Wang K, Liang B, Liu Y, Wang R. 2015.
487 A meta-analysis of metronidazole and vancomycin for the treatment of Clostridium
488 difficile infection, stratified by disease severity. Braz J Infect Dis 19:339-49.

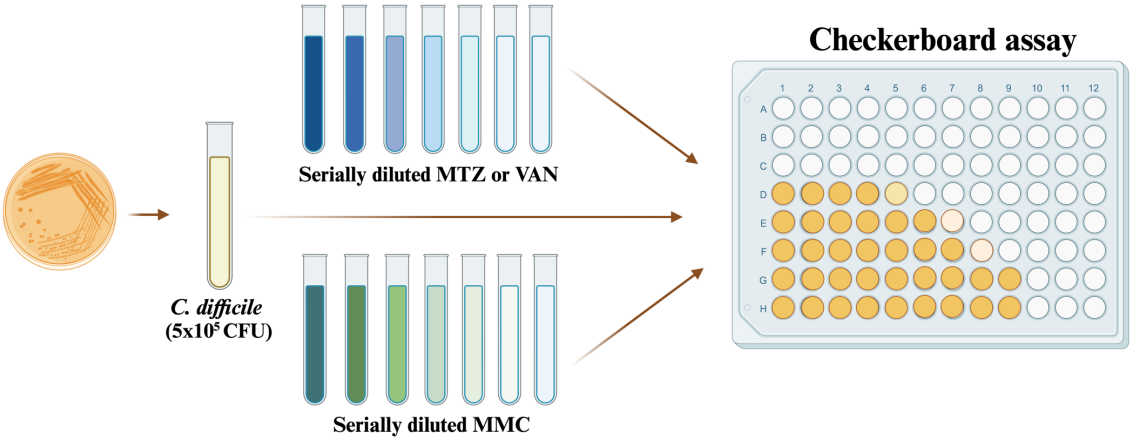
489 31. Erikstrup LT, Aarup M, Hagemann-Madsen R, Dagnaes-Hansen F, Kristensen B, Olsen
490 KE, Fuursted K. 2015. Treatment of Clostridium difficile infection in mice with
491 vancomycin alone is as effective as treatment with vancomycin and metronidazole in
492 combination. BMJ Open Gastroenterol 2:e000038.
493 32. Lewis BB, Buffie CG, Carter RA, Leiner I, Toussaint NC, Miller LC, Gobourne A, Ling
494 L, Pamer EG. 2015. Loss of Microbiota-Mediated Colonization Resistance to
495 Clostridium difficile Infection With Oral Vancomycin Compared With Metronidazole.
496 J Infect Dis 212:1656-65.
497 33. Warren CA, van Opstal EJ, Riggins MS, Li Y, Moore JH, Kolling GL, Guerrant RL,
498 Hoffman PS. 2013. Vancomycin treatment's association with delayed intestinal tissue
499 injury, clostridial overgrowth, and recurrence of Clostridium difficile infection in mice.
500 Antimicrob Agents Chemother 57:689-96.
501 34. Berkell M, Mysara M, Xavier BB, van Werkhoven CH, Monsieurs P, Lammens C,
502 Ducher A, Vehreschild M, Goossens H, de Gunzburg J, Bonten MJM, Malhotra-Kumar
503 S. 2021. Microbiota-based markers predictive of development of Clostridioides difficile
504 infection. Nat Commun 12:2241.
505 35. Dawson LF, Peltier J, Hall CL, Harrison MA, Derakhshan M, Shaw HA, Fairweather
506 NF, Wren BW. 2021. Extracellular DNA, cell surface proteins and c-di-GMP promote
507 biofilm formation in Clostridioides difficile. Sci Rep 11:3244.

508 36. James GA, Chesnel L, Boegli L, deLancey Pulcini E, Fisher S, Stewart PS. 2018.
509 Analysis of Clostridium difficile biofilms: imaging and antimicrobial treatment. J
510 Antimicrob Chemother 73:102-108.
511 37. Hamada M, Yamaguchi T, Ishii Y, Chono K, Tateda K. 2020. Inhibitory effect of
512 fidaxomicin on biofilm formation in Clostridioides difficile. J Infect Chemother 26:685-
513 692.
514 38. Theriot CM, Koenigsknecht MJ, Carlson PE, Jr., Hatton GE, Nelson AM, Li B,
515 Huffnagle GB, J ZL, Young VB. 2014. Antibiotic-induced shifts in the mouse gut
516 microbiome and metabolome increase susceptibility to Clostridium difficile infection.
517 Nat Commun 5:3114.
518 39. Britton RA, Young VB. 2014. Role of the intestinal microbiota in resistance to
519 colonization by Clostridium difficile. Gastroenterology 146:1547-53.
520 40. Theriot CM, Young VB. 2015. Interactions Between the Gastrointestinal Microbiome
521 and Clostridium difficile. Annu Rev Microbiol 69:445-61.
522 41. Binyamin D, Nitzan O, Azrad M, Hamo Z, Koren O, Peretz A. 2021. The microbial
523 diversity following antibiotic treatment of Clostridioides difficile infection. BMC
524 Gastroenterol 21:166.
525 42. Edwards DI. 1979. Mechanism of antimicrobial action of metronidazole. J Antimicrob
526 Chemother 5:499-502.

527 43. Tomasz M, Palom Y. 1997. The mitomycin bioreductive antitumor agents: cross-linking
528 and alkylation of DNA as the molecular basis of their activity. Pharmacol Ther 76:73-
529 87.
530 44. Olaitan AO, Dureja C, Youngblom MA, Topf MA, Shen WJ, Gonzales-Luna AJ,
531 Deshpande A, Hevener KE, Freeman J, Wilcox MH, Palmer KL, Garey KW, Pepperell
532 CS, Hurdle JG. 2023. Decoding a cryptic mechanism of metronidazole resistance
533 among globally disseminated fluoroquinolone-resistant Clostridioides difficile. Nat
534 Commun 14:4130.
535

Figure 1

A.



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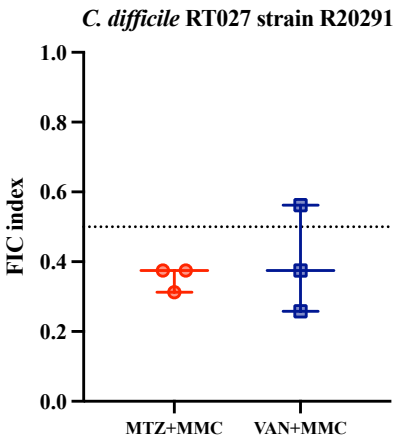

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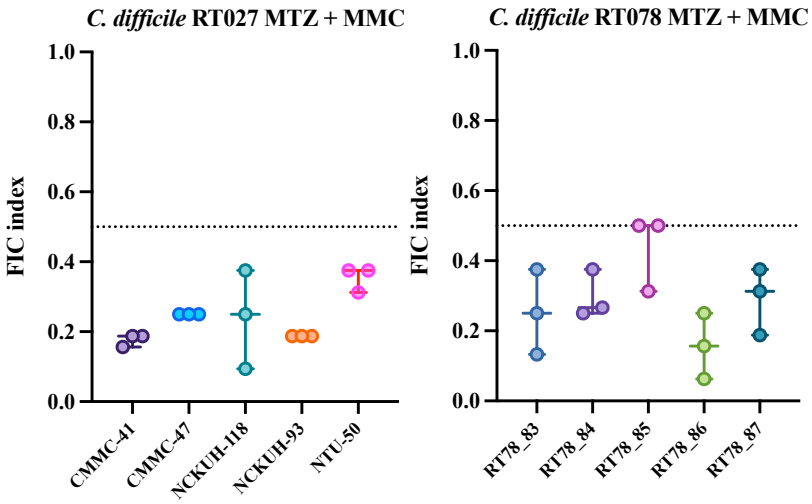

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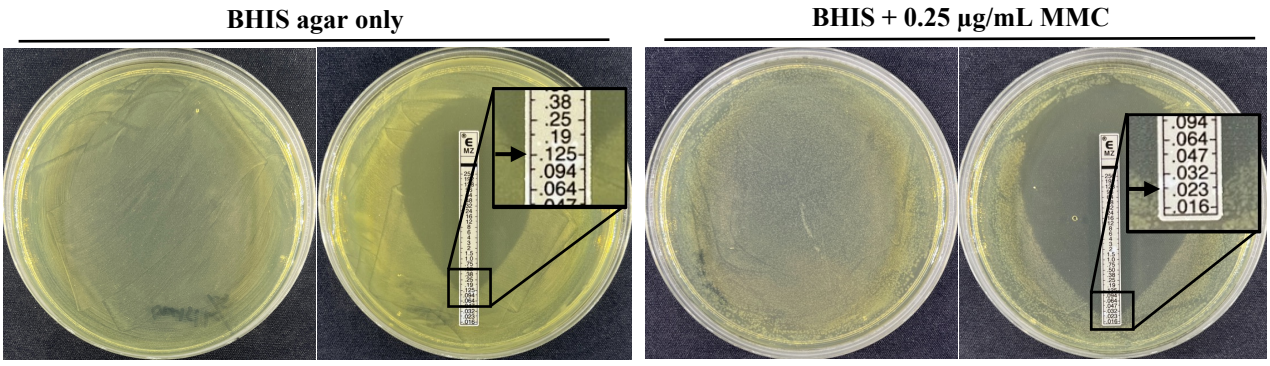


Figure 2

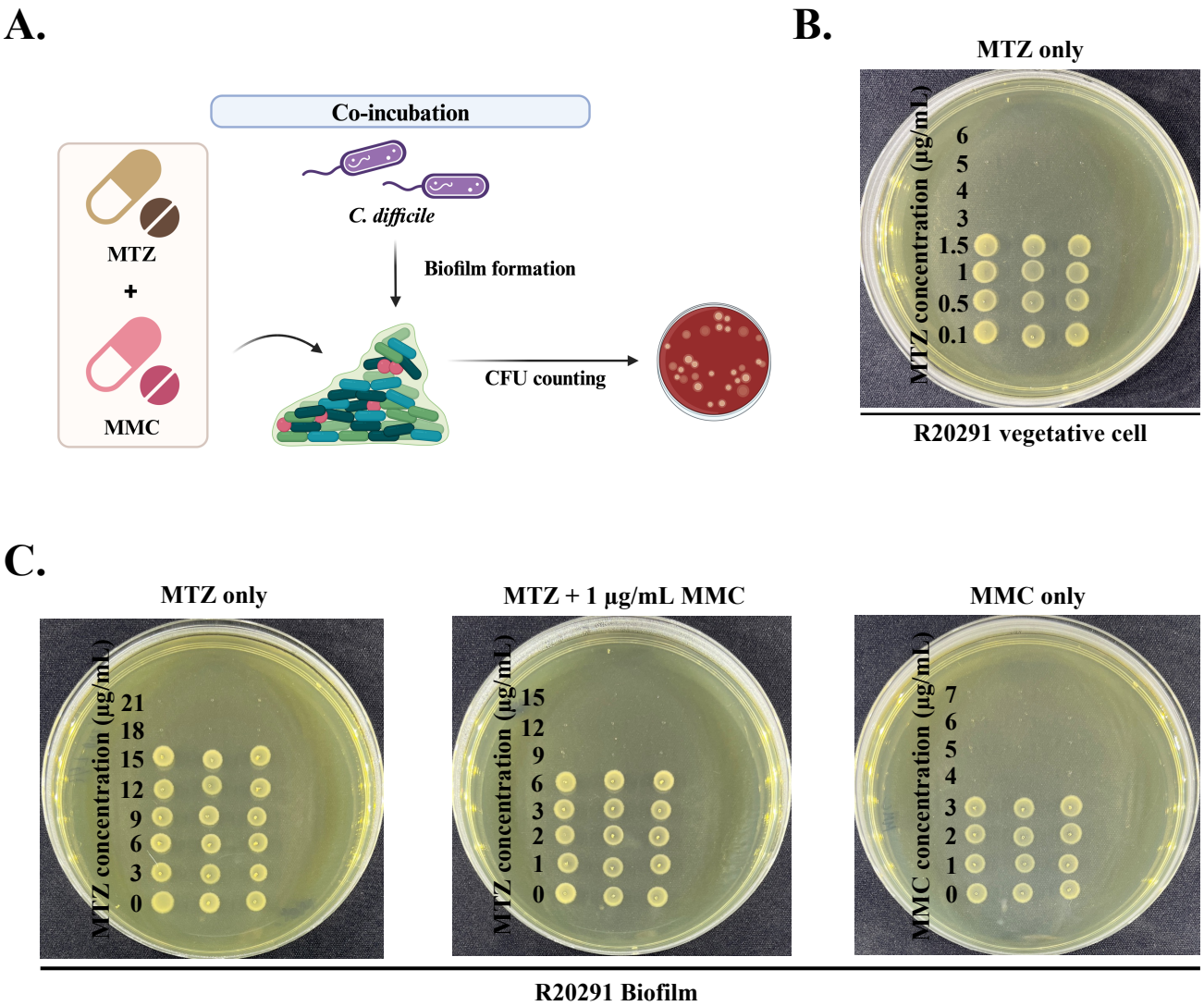
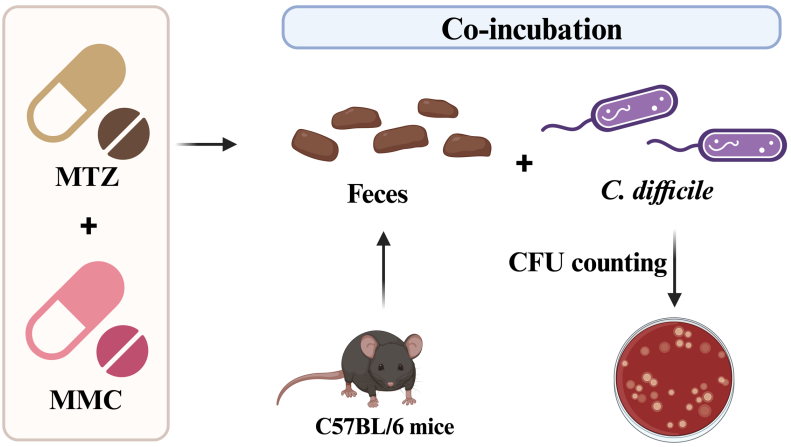

A.
Co-incubation
MTZ
+
MMC
C. difficile
Biofilm formation
CFU counting
B.
MTZ only
MTZ concentration (μg/mL)
6
5
4
3
1.5
1
0.5
0.1
R20291 vegetative cell
C.
MTZ only
MTZ concentration (μg/mL)
21
18
15
12
9
6
3
0
MTZ + 1 μg/mL MMC
MTZ concentration (μg/mL)
15
12
9
6
3
2
1
0
MMC only
MMC concentration (μg/mL)
7
6
5
4
3
2
1
0
R20291 Biofilm

Figure 3

A.


Co-incubation
MTZ
+
MMC
Feces
+
C. difficile
CFU counting
C57BL/6 mice

B.

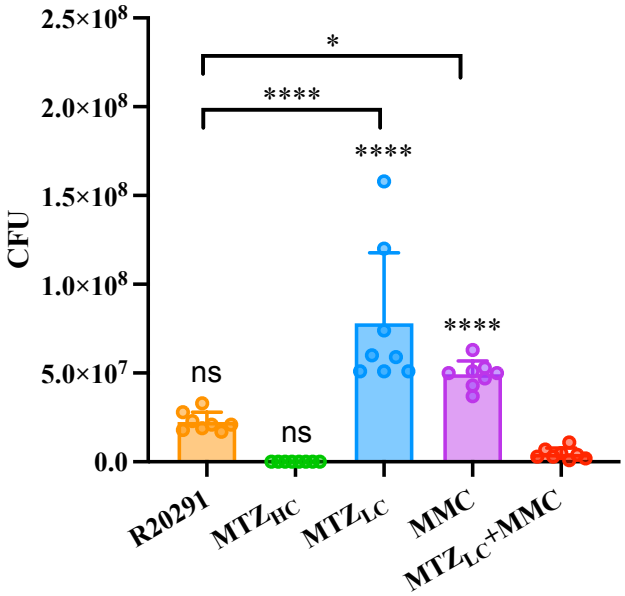
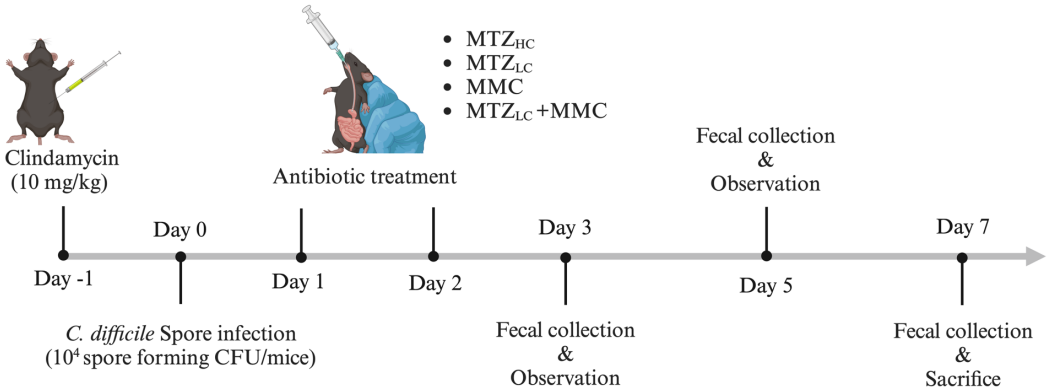

2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0.0
CFU
*
****
****
****
ns
ns
R20291
MTZ_HC
MTZ_LC
MMC
MTZ_LC+MMC

Figure 4

A.



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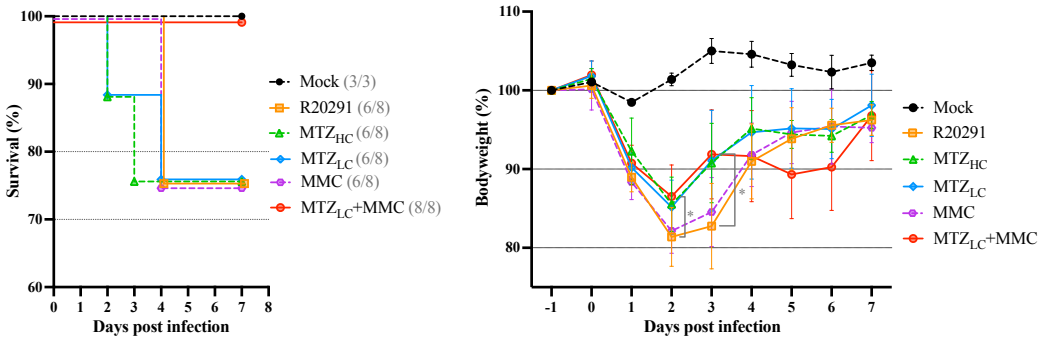

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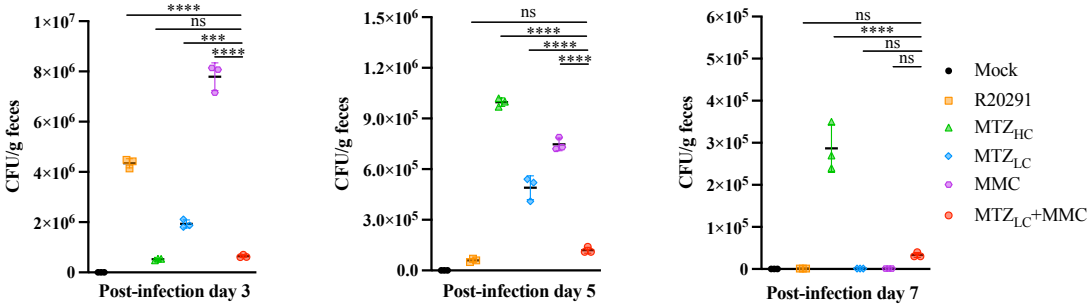

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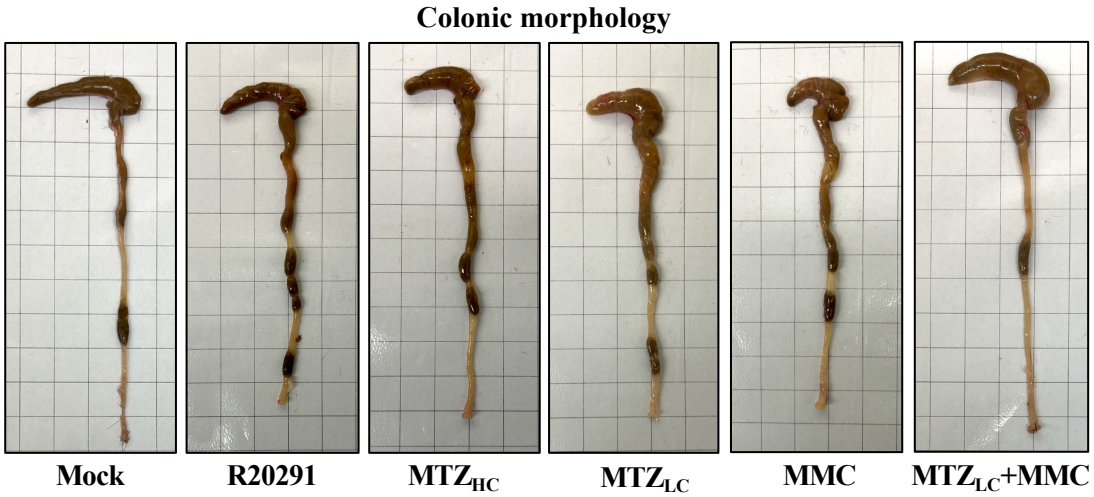


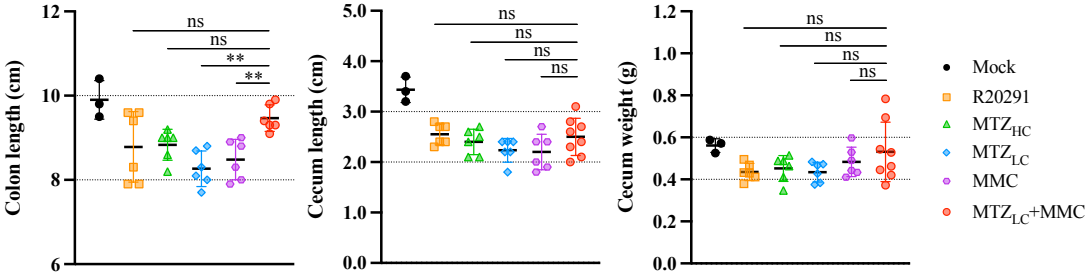
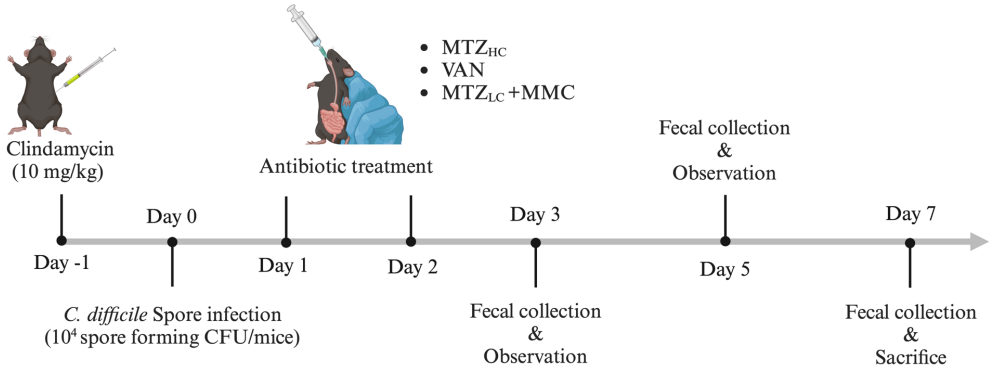


Figure 5

A.



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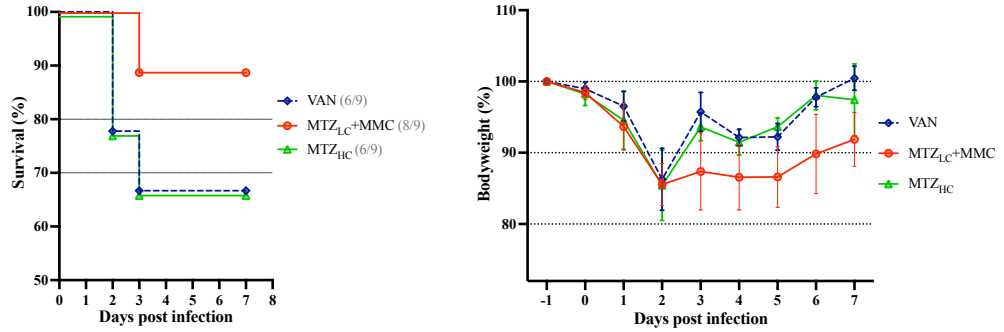

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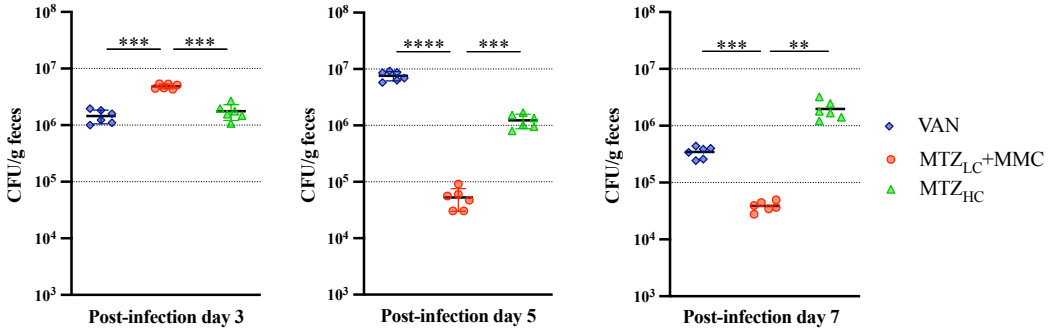

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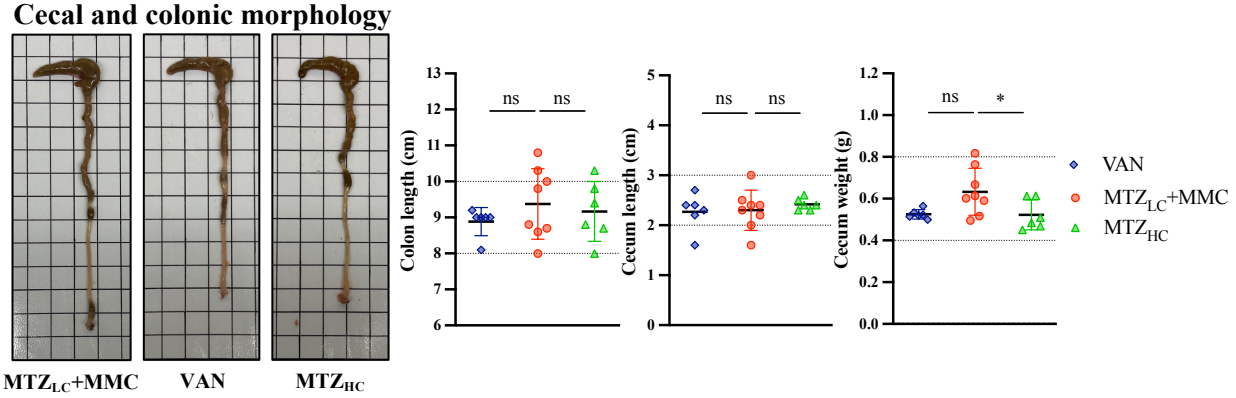
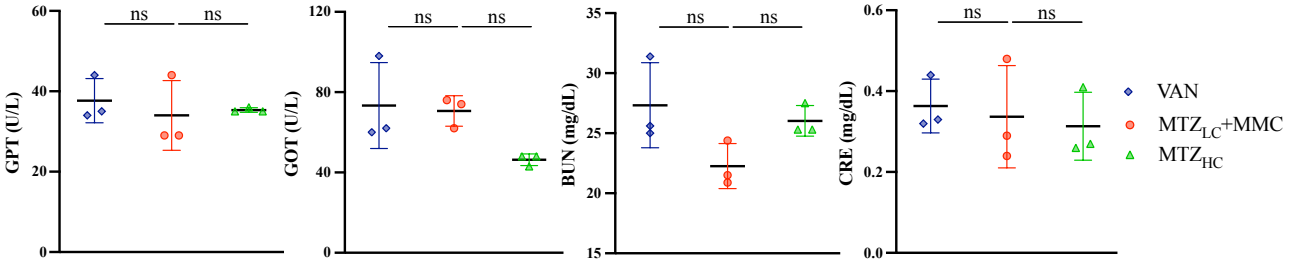
E.