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

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Abstract

Background. *Clostridioides difficile* is an anaerobic, spore-forming, Gram-positive pathogen that causes symptoms ranging from mild diarrhea and moderate pseudomembranous colitis to severe toxic megacolon and even death. Currently, the clinical treatment of *C. difficile* infection (CDI) still relies on Vancomycin and Metronidazole (MTZ). However, the high concentration of antibiotics used also contributes to increased gut microbiota dysbiosis, which results in a high probability of *C. difficile* recurrence. Therefore, the development of a novel therapeutic strategy is urgently required.

Methods. Mitomycin C (MMC), an FDA-approved chemotherapy drug, is frequently used to induce phage generation in lysogenic strains, thereby eliminating the host bacteria. Since 70% of *C. difficile* strains carry prophages, we aim to investigate whether MMC can enhance antibiotic treatment against *C. difficile* infection.

Results. The *in vitro* experimental results demonstrated that MMC synergistically helps MTZ inhibit the growth of *C. difficile* strain R20291. Additionally, this combined antibiotic strategy can decrease the resistance of vegetative cells within biofilms and lower the concentration of MTZ required to kill *C. difficile ex vivo* in the stool samples. Finally, *in vivo*, results in the CDI mouse relapse model further reveal that combining MMC with a low dose of MTZ can effectively improve the survival rate and reduce the number of spores in mice's feces after the antibiotic treatments.

Conclusions. Taken together**,** the low-dose combined antibiotic treatment of MMC and MTZ expresses a better therapeutic effect *in vitro* and *in vivo*, providing a novel treatment of CDI.

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

Introduction

Clostridioides difficile is an anaerobic, gram-positive, and spore-forming bacterium. The symptoms of *C. difficile* infection (CDI) progress from asymptomatic carriage, mild diarrhea to severe pseudomembranous colitis, and even death (1). Antibiotics, such as metronidazole (MTZ), vancomycin (VAN), and fidaxomicin, are clinically recommended against CDI (2-4). However, although antibiotics successfully treat CDI, the high dosage of antibiotic treatments has been linked to further host gut microbiota disruption, which would lead to the recurrence (5). Recurrent *C. difficile* infection (rCDI) is the greatest clinical challenge, as typically 20% to 35% of patients will experience a recurrence within 30 days after the initial CDI episode (6), and around 60% of patients will have a second recurrence (7). Furthermore, the mortality rate associated with CDI was 2.7% for patients who experienced primary infection alone and 25.4% for recurrent CDI, which is nearly ten times higher than that observed in individuals with primary CDI (8). Therefore, it is crucial to investigate effective ways to reduce CDI recurrence caused by antibiotic treatment while against CDI.

57 Mitomycin C (MMC), an FDA-approved anticancer drug, can cross-link DNA and inhibit
58 DNA synthesis and function (9). In addition to being used for treating gastric cancer, superficial
59 bladder cancer, and upper tract urothelial carcinoma (10-12), it has also been reported to have
60 antibacterial properties (13, 14). Furthermore, MMC exhibited potent activity against *C.*
61 *difficile* clinical strains (15). The treatment of combined two antibiotics used in infectious
62 diseases is common, such as tuberculosis and multidrug-resistant bacteria infections (16).
63 Since these effects on *C. difficile,* we hypothesize that MMC may assist the antibiotic
64 treatment against *C. difficile* infection. In this study, we aim to check the potential role of MMC
65 in CDI treatment. Our results demonstrated that MMC and MTZ could synergistically inhibit
66 *C. difficile* RT027 strain R20291 and RT078 clinical isolates, to reduce the minimal bactericidal
67 concentration (MBC) of MTZ to R20291 biofilm. Additionally, the trial in the CDI mouse
68 relapse model indicated that the combined treatment strategy of a low-dose MTZ and MMC
69 could effectively improve the survival rate of mice and reduce the spore number in the stool.
70

71 **Materials and Methods**

72 **Bacterial strains and culture conditions**

73 The bacterial strains used in this study are listed in Table S1. All *C. difficile* strains were
74 cultured anaerobically (10% H_2, 10% CO_2, and 80% N_2) at 37°C in the cultured media of BHI
75 (Thermo Fisher Scientific, Waltham, MA, USA) alone or BHI supplemented with 0.05% L-

76 cysteine (Sigma-Aldrich, St. Louis, USA) and 0.5% yeast extract (Thermo Fisher Scientific).
77 The anaerobic bacteria were maintained in an anaerobic workstation (DG250; Don Whitley
78 Scientific Ltd., West Yorkshire, UK).
79
80 **Checkerboard assay**
81 To determine the effects of MMC (Cyrusbioscience, New Taipei City, Taiwan) in
82 combination with MTZ (Cyrusbioscience) or VAN (Cyrusbioscience), the checkerboard assay
83 was conducted, following the steps described by Hu Y. et al. (17), with some modifications.
84 Briefly, MMC and antibiotic combinations were prepared in 96-well plates (SPL life sciences,
85 Pocheon, South Korea). The initial drug concentrations were set at 4-fold higher than their
86 respective MIC values, followed by a serial 2-fold dilution. Next, each well was inoculated with
87 100 μL of *C. difficile* cultures at a $1x10^6$ CFU/mL concentration in BHIS broth. After 18 to 20
88 hours of incubation at 37°C, the OD_{600} values were measured using the iMark™ Microplate
89 Absorbance Reader (Bio-Rad, Alfred Nobel Drive. Hercules, CA, USA). The combined effects
90 of the drugs were quantified by calculating the fractional inhibitory concentration (FIC) index
91 of the combination as follows:
92
93 $$\text{FIX index} = \frac{(\text{MIC of drug A in combination})}{(\text{MIC of drug A alone})} + \frac{(\text{MIC of drug B in combination})}{(\text{MIC of drug B alone})}$$
94

95 Synergy was defined as an FIC index of ≤0.5, additive or indifference was identified with
96 an FIC index of >0.5 but <4, and antagonism was defined as an FIC index of >4 (18). The
97 results represented three independent experiments with the average from triplicate
98 measurements.
99

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

101 The susceptibility of *C. difficile* to MTZ was determined using the E-test assay, following
102 the procedure described by Nachnani S. et al (19). In brief, the overnight cultures of strain
103 R20291 were 100-fold diluted using fresh BHIS broth and cultured until the value of OD_{600}
104 reached 1.0. These refreshed bacterial cultures were then spread on BHIS agar plates, with or
105 without MMC, using sterile glass plate spreaders. A single strip of MTZ (Biomérieux, Marcy-
106 l'Étoile, France) was positioned at the center of each plate and incubated at 37°C for 24 hr. The
107 inhibitory concentrations were directly determined at the point of intersection between the
108 inhibition zone and the test strip. This experiment was representative of three independent
109 experiments.
110

111 **Biofilm formation and MBC assay**

112 The biofilm formation assay was conducted as we previously described (16). In brief,
113 overnight cultures of strain R20291 were 100-fold diluted in fresh BHI broth containing 0.1 M

114 glucose (Avantor, Radnor Township, Pennsylvania, USA) and grown until the OD_{600} value
115 reached 0.1. Next, 200 μL of bacterial cultures were inoculated into each well of 96-well plates
116 and anaerobically incubated at 37°C for 72 hours. After 3 days of incubation, the supernatants
117 were carefully removed and replaced with 200 μL of fresh BHI-glucose broth containing
118 antibiotic treatments, followed by a further 48 hr incubation. Subsequently, the supernatants
119 were carefully removed, and each well was added with 200 μL of fresh BHIS broth to resuspend
120 the biofilm through pipetting. The MBC assay was then performed as described by Lambert
121 RJ., et al. (20) to determine the efficacy of biofilm disruption. The spot plate technique was
122 used, with three drops (2 μL) of the resuspended biofilm solution placed on BHIS agar. All
123 plates were incubated at 37°C for 24 hr. The MBC was the lowest biocide concentration at
124 which no bacterial growth was observed. This experiment was representative of three
125 independent experiments, each performed in triplicate.
126
127 ***Ex vivo* bacterial-fecal co-culture assay**
128 Fresh fecal samples were collected from C57BL/6 mice, and the fecal suspension was prepared
129 with sterile ddH_2O at a concentration of 0.01 g/mL. Overnight cultures of *C. difficile* strain
130 R20291 were refreshed 100-fold in BHIS broth until the OD_{600} reached 1.0. In each group,
131 $5x10^5$ CFU of R20291 vegetative cells in 800 μL BHIS broth were combined with or without
132 different antibiotic treatments (1 μg/mL of MMC, 0.375 μg/mL of MTZ, 3 μg/mL of MTZ, and

133 a combination of 1 μg/mL of MMC with 0.375 μg/mL of MTZ). These bacterial mixtures were
134 then gently mixed with 200 μL of fecal suspensions and anaerobically incubated for 24 hr. After
135 a day of co-incubation, the fecal-bacterial cultures were diluted 10^6-fold and spread on selective
136 cycloserine-cefoxitin fructose agar (CCFA) plates for bacterial CFU quantification. The
137 experiment was representative of four independent experiments with duplicate measurements.
138 Statistical analysis was performed using GraphPad Prism 10.0 (GraphPad, La Jolla, CA) and
139 significant differences between the groups were determined by One-Way ANOVA.
140

141 **Spore purification**

142 The spore purification process was referred to in our previous description (21). Briefly,
143 overnight cultures of *C. difficile* were 100-fold diluted in fresh BHIS broth and grown until the
144 OD_{600} value reached 0.8. The bacterial cultures were then inoculated on SMC agar (22) in 6-
145 well plates (SPL life sciences) and incubated anaerobically at 37°C for 7 days. Subsequently,
146 bacterial spores were harvested using ice-cold sterile ddH_2O and stored overnight at 4°C. The
147 spores were then centrifuged at 2,330 ×g for 16 min at 4°C and washed five times with ice-cold
148 sterile ddH_2O to separate spores from cell debris. *C. difficile* spores were then purified using
149 the universal density gradient medium Nycodenz (48%; Axis Shield, Oslo, Norway) and
150 centrifuged at 2,500 ×g for 10 min. The pellet containing spores was collected and washed five
151 times with ice-cold sterile ddH_2O, followed by centrifugation at 2,500 ×g for 7 min at 25°C.

152 The pellet was resuspended in 1x PBS and stored at 4°C for *in vivo* experiments. Purified spores
153 were 10-fold serially diluted and plated on BHIS agar supplemented with 0.1% taurocholic acid
154 (Sigma-Aldrich) to determine the counts of spore-forming CFU.
155
156 **Experimental rCDI animal model and sample collection**
157 7-weeks-old male C57BL/6 mice were obtained from the Laboratory Animal Center of
158 NCKU. All mice were managed in accordance with the guidelines of the Institutional Animal
159 Care and Use Committee (IACUC) of NCKU. Furthermore, all animal experiments followed
160 the protocol approved by the IACUC of NCKU (approval number: NCKU-IACUC-113-068)
161 and the NCKU Biosafety and Radiation Safety Management Division. To determine the effects
162 of combined antibiotic treatment, a mouse CDI relapse model was performed, following the
163 procedure previously described by Bublitz A. et al (23). Briefly, mice were weighed and
164 intraperitoneally injected with 10 mg/kg of clindamycin 24 hr before infection. On the
165 following day, mice were orally challenged with 100 uL containing $1x10^4$ CFU spores of *C.*
166 *difficile* strain R20291. On days 2 and 3 post-infection, mice were treated with different kinds
167 of antibiotic treatment through oral gavage. To determine whether MMC can synergically assist
168 low dose of MTZ against *C. difficile* infection and recurrence, mice were divided into six groups
169 and respectively treated with (1) sterile 1x PBS (Mock group, 3 mice and R20291 group, 8
170 mice), (2) 50 mg/kg of MTZ dissolved in 1x PBS (MTZ_{HC} group, 8 mice), (3) 7 mg/kg of MTZ

171 dissolved in 1x PBS (MTZ_{LC} group, 8 mice), (4) 5 mg/kg of MMC dissolved in 1x PBS (MMC
172 group, 8 mice), and (5) 7 mg/kg of MTZ combined with 5 mg/kg MMC dissolved in 1x PBS
173 (MTZ_{LC}+MMC group, 8 mice). In the animal experiment to compare the efficacy of combined
174 MTZ_{LC} and MMC treatment with VAN treatment in a mouse CDI relapse model, mice were
175 divided into three groups and respectively treated with (1) 50 mg/kg of MTZ dissolved in 1x
176 PBS (MTZ_{HC} group, 9 mice), (2) 20 mg/kg of VAN dissolved in 1x PBS (VAN group, 9 mice),
177 (3) 7 mg/kg of MTZ combined with 5 mg/kg MMC dissolved in 1x PBS (MTZ_{LC}+MMC group,
178 9 mice). During the infection, mice were monitored daily for CSS (24), and fecal samples were
179 collected to quantify the number of spores on days 3, 5, and 7 post-infection. Mice were
180 humanely euthanized with CO_2 asphyxiation followed by cervical dislocation on day 7 post-
181 infection. Subsequently, mice serial samples were used to measure the amount of glutamic
182 pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT), blood urea nitrogen
183 (BUN) and creatinine (CRE) using NX-600 dry biochemistry analyzer (Fujifilm, Minato,
184 Tokyo, Japan). After observation of colonic morphology, the resected colon tissue samples
185 were fixed in 10% formaldehyde (BS Chemical, Kretinga, Lithuania), and harvested organs
186 were stored at -80°C for further analysis. Statistical analysis was performed using GraphPad
187 Prism 10.0, and significance was determined by t-test and One-Way ANOVA.
188
189 **Quantification of *C. difficile* spore-forming CUFs**

190 Fresh fecal samples were collected on days 3, 5, and 7 post-infection, and fecal weights
191 were recorded. The fecal samples were then diluted by 1 mL of ddH_2O, and 100 mg of 0.1 mm
192 zirconia beads (BioSpec Products, Inc., Bartlesville, Oklahoma, USA) were added, followed
193 by a homogenization using the BeadBeater (Bertin Technologies, avenue Ampère, Montigny-
194 le-Bretonneux, France) for 50 sec (23). To quantify the number of spores, *C. difficile* vegetative
195 cells were first heat-killed at 65°C for 20 min and plated on BHIS agar containing 0.1% TCA.
196 The plates were then anaerobically incubated at 37°C for 24 hr. The CFUs of *C. difficile* were
197 calculated after normalization to the weight of mice feces. This experiment was representative
198 of three independent experiments, and each experiment was performed in duplicate. Statistical
199 analysis was performed using GraphPad Prism 10.0, and significance was determined by t-test
200 and One-Way ANOVA.
201
202 **Results**
203 **Mitomycin C showed a synergistic antibacterial activity in combination with**
204 **metronidazole against *C. difficile* RT027 strain R20291 and RT078 clinical strains**
205 In our previous study, MMC was used to induce phage production from *C. difficile* strain
206 NCKUH-21 and promote host bacteria entering the lytic cycle (21). Additionally, more than
207 70% of *C. difficile* strains naturally carry prophage (25). Therefore, we wondered if MMC could
208 be combined with VAN or MTZ as a novel therapeutic strategy against CDI. Therefore, we
209 performed the checkerboard assay. Antibiotics were serially diluted and co-cultured with fresh

210 RT027 *C. difficile* strain R20291 cultures in a 96-well plate (Figure 1A). As shown in Figure
211 S1A, the MIC of MMC and MTZ were determined to be 2 μg/mL and 3 μg/mL, respectively.
212 The result also demonstrated that MMC expresses a synergistic antibacterial activity in
213 combination with MTZ in inhibiting the growth of R20291, as a fractional inhibitory
214 concentration (FIC) index was less than 0.5. The combination of MMC with MTZ could
215 synergistically inhibit the growth of *C. difficile*; the required concentration of MTZ to inhibit
216 *C. difficile* growth could be reduced from 3 μg/mL to 0.375 μg/mL (an 8-fold reduced MIC
217 value), with the presence of 1 μg/mL MMC (Figure S1A). In addition, the combination of MMC
218 and VAN exhibited an additive activity only, with the FIC index between 0.5 and 1, inhibiting
219 R20291 (Figure 1B). Furthermore, the combination of MMC and MTZ could also
220 synergistically against both of RT027 and RT078 *C. difficile* clinical strains (Figure 1C); MMC
221 additively assisted VAN in inhibiting RT078 strains (Figure S1B). These results demonstrated
222 that MMC effectively enhances the effects of CDI-treating antibiotics.

223 To confirm the synergistic effects of MMC and MTZ, antibiotic susceptibility testing with
224 E-test strips was also performed to determine the feasibility of combining MMC with MTZ to
225 combat strain R20291. The minimum inhibitory concentration (MIC) of MTZ strip alone was
226 0.125 μg/mL (Figure 1D, left). However, 0.25 μg/mL of MMC can effectively reduce the MIC
227 of MTZ to 0.023 μg/mL (a 5.4-fold reduced MIC value; Figure 1D, middle), and the growth of
228 bacteria is not affected at a concentration of 0.25 μg/mL MMC only (Figure 1D, right).

229 Moreover, we also combined higher concentration of MMC with MTZ to determine the
230 inhibitory effects of *C. difficile*. As shown in Figure S1C, although 0.5 μg/mL of MMC could
231 reduce the MIC of MTZ reach 0.016 μg/mL, this concentration of MMC might slightly
232 influence the basic growth of *C. difficile*, and this phenomenon was strongly observed when
233 combining with 1 μg/mL of MMC. Taken together, these *in vitro* experimental results indicate
234 that the combination of MTZ and MMC can more effectively inhibit the growth of *C. difficile*
235 strain R20291 than MTZ alone.
236

237 **Mitomycin C could effectively enhance the metronidazole effect on the strain R20291**
238 **biofilms**

239 *C. difficile* strain R20291 strain has previously been observed to form aggregates and a
240 glycan-rich biofilm during the CDI mouse model (26). Moreover, the formation of biofilm by
241 *C. difficile* has previously been associated with increased antibiotic resistance and a higher
242 recurrence of CDI (27, 28). Therefore, we tried to clarify whether MMC can enhance MTZ
243 effectively to decrease the required MTZ concentration to kill biofilm. To assess the resistance
244 of *C. difficile* to antibiotics in the biofilm form, we conducted a minimum bactericidal
245 concentration (MBC) assay (Figure 2A). As shown in Figure 2B, the MBC of MTZ for the
246 vegetative cells of strain R20291 was 3 μg/mL. However, when biofilm formation, the MBC
247 of MTZ required to eliminate strain R20291 increased to 18 μg/mL (Figure 2C). Interestingly,

248 when MTZ was combined with 1μg/mL of MMC, which could synergistically inhibit the
249 growth of *C. difficile* in Figure 1, the MBC of MTZ markedly decreased to 9 μg/mL (Figure
250 2D). MMC did not exhibit any toxic effects on the R20291 vegetative cells until the
251 concentration reached 4 μg/mL (Figure 2E). To determine whether any *C. difficile* vegetative
252 cells were released outside the biofilm and would affect the experimental results, we also
253 dropped the biofilm supernatant on BHIS agar plates. As shown in Figure S2, after treatment
254 with antibiotics, no live bacteria could be detected outside the biofilm. Taken together, MMC
255 could enhance MTZ effectively to reduce the MBC of MTZ on strain R20291 biofilm.

256

257 **Combination of mitomycin C with metronidazole inhibits *C. difficile* colonization in fresh**
258 **mouse feces**

259 To accurately simulate the diverse microbial environment within the host gut, *C. difficile*
260 strain R20291 was mixed with fresh mouse fecal suspensions with several different antibiotic
261 treatments to assess the efficacy of a low concentration of MTZ combined with MMC against
262 *C. difficile ex vivo*. After co-culturing for 24 hours, 1 x 10^6 dilutions of bacterial-fecal cultures
263 were spread on BHIS agar to calculate the bacterial colony-forming units (CFUs) (Figure 3A).
264 As shown in Figure 3B, no *C. difficile* CFU was observed on BHIS agar in the group treated
265 with 3 μg/mL of MTZ to indicate that the number of *C. difficile* was less than 1x10^6 CFU/mL.
266 To demonstrate the efficacy of the combination therapy of MTZ and MMC, the concentration

267 of MTZ was reduced to 0.375 μg/mL with the presence of 1μg/mL MMC, and an average of
268 $4.5x10^6$ CFU/mL was observed. In contrast, the average *C. difficile* number under the treatment
269 of 0.375 μg/mL MTZ (MTZ_{LC}) was $7.8x10^7$ CFU/mL, and the one under the treatment of
270 1μg/mL MMC was $4.9x10^7$ CFU/mL. The *C. difficile* number in these two groups was higher
271 than in the group without any antibiotic treatment (R20291 group) significantly, which might
272 be because the treatment of low-concentration antibiotics impacted other bacteria that competed
273 with *C. difficile* in mouse feces. In summary, these results demonstrated that combining MTZ
274 with MMC is a potential strategy to inhibit the colonization of *C. difficile ex vivo*.

275

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

278 Given that the combination of low-dose MTZ and MMC effectively inhibited *C. difficile*
279 in various *in vitro* and *ex vivo* experiments, we next want to determine whether this therapeutic
280 strategy can against severe CDI and recurrence using a mouse model (Figure 4A). We
281 performed a CDI recurrence mouse model, as previously described by Arne Bublitz et al. (23),
282 and orally infected mice with purified spores from *C. difficile* strain R20291 (Figure S3A, B).
283 In mice survival rate, only the noninfected mock group and the infected group treated with a
284 combination of MTZ and MMC provided 100% survival, while the survival rates of other
285 groups were consistently under 80% (Figure 4B). Furthermore, we noticed that the groups

286 infected with R20291 spores alone (R20291 group) and those treated with MMC only (MMC
287 group) exhibited the lowest weight on the second day post-infection. However, the rate of body
288 weight change for all surviving mice in each group consistently recovered to over 95% of the
289 initial weight at day 7 post-infection (Figure 4C).

290 To determine the recurrence of *C. difficile* in mice, we collected feces on days 3, 5, and 7
291 post-infection and plated them on BHIS agar supplemented with 0.1% taurocholic acid (TCA).
292 After two days of antibiotic treatment, there was no significant difference in the number of
293 spore-forming CFUs in the feces between the MTZ_{HC} and MTZ_{LC}+MMC groups on day 3 post-
294 infection (Figure 4D). However, the CFUs formed by spores in other infected groups were
295 significantly higher than those in the MTZ_{LC}+MMC group (Figure 4D). On day 5 post-infection,
296 we observed that the amount of spore-forming CFUs in both the R20291 and the MTZ_{LC}+MMC
297 groups was significantly lower than that of other infected groups (Figure 4E). Notably, the
298 MTZ_{HC} group had a significantly higher amount of CFUs than the MTZ_{LC}+MMC group and
299 had the highest amount among all groups on day 5 post-infection (Figure 4E). On day 7 post-
300 infection, despite a decrease in the CFUs formed by spores from all infected groups, the CFUs
301 in the group treated with high-dose MTZ remained significantly higher than that in the
302 MTZ_{LC}+MMC group (Figure 4F). During the *C. difficile* infection, we also monitored the daily
303 clinical sickness score (CSS) of each surviving mouse (24). In the result, the CSS of mice in
304 the MTZ_{LC}+MMC group was lower than other infection groups during the CDI (Figure S3C).

305 The colonic morphology of infected mice was an important indicator for assessing intestinal
306 inflammation (29). As shown in Figure 4G, all surviving mice had recovered at day 7 post-
307 infection, and there was no significant difference in the length and weight of cecum between
308 each group. The colon length of the MTZ_{LC}+MMC group was significantly higher than the
309 MTZ_{LC} and MMC groups. Taken together, the CDI recurrence *in vivo* experiments
310 demonstrated that the combined treatment strategy of MTZ and MMC can not only express the
311 highest mouse survival rate but also prevent the relapse of *C. difficile*.
312

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

315 The previous study has suggested that vancomycin exhibits superior clinical cure rates,
316 particularly in severe cases of CDI (30). Moreover, mice treated with vancomycin alone for
317 CDI demonstrated better outcomes than those treated with metronidazole (31). However,
318 vancomycin treatment was associated with a significant disruption of the intestinal microbiota,
319 thereby increasing the risk of CDI recurrence (32, 33). Therefore, we wanted to assess whether
320 the combination of MTZ with MMC can have a better outcome than VAN in the mouse CDI
321 relapse model.

322 After being infected with *C. difficile* R20291 spores, mice were given VAN, MTZ alone,
323 or low-dose MTZ combined with MMC treatments, respectively (Figure 5A). As the result

324 shown in Figure 5B, compared to the groups treated with VAN (67%) or MTZ (67%) alone,
325 mice treated with low-dose MTZ combined with MMC (89%) provided a higher survival rate
326 after the infection. In bodyweight change rate, two groups treated with VAN (VAN group) and
327 MTZ (MTZ_{HC} group) alone exhibited an appreciable decrease on day 3 to 4 post-infection, a
328 phenomenon that was less obvious in MTZ_{LC}+MMC group (Figure 5C). However, on day 7
329 post-infection, all groups of mice had recovered to over 90% of their initial weight (Figure 5C).
330 To monitor the recurrence of *C. difficile* in the mouse intestine, we collected feces for spore
331 plating on days 3, 5 and 7 post-infection. As shown in Figure 5D, although the spore-forming
332 CFUs in mice feces of the MTZ_{LC}+MMC group were significantly higher than the other two
333 groups on day 3 post-infection. However, on days 5 and 7 post-infection, the fecal spore CFUs
334 of the MTZ_{LC}+MMC group were significantly lower than the other two groups (Figure 5E, F).
335 In addition, the VAN group had increased nearly ten times of spore number in their feces on
336 day 5 post-infection (Figure 5E). The MTZ_{HC} group maintained a high level of spore content in
337 their feces from day 3 to 7 post-infection (Figure 5F). Furthermore, mice intestinal
338 inflammation was also observed, and except for the cecal weight in MTZ_{LC}+MMC was
339 significantly higher than the MTZ_{HC} group, the morphology of mice cecum and colon showed
340 no significant difference between each group (Figure 5G). Finally, to ensure that the strategy
341 of combining low-dose MTZ with MMC for treating CDI would not lead to additional damage
342 to the liver and kidneys of mice. Serum was collected on day 7 post-infection and analyzed for

343 the indicator, including glutamic pyruvic transaminase (GPT), glutamic oxaloacetic
344 transaminase (GOT), blood urea nitrogen (BUN), and creatinine (CRE) using a dry biochemical
345 analyzer. As shown in Figure 5H, the values of each indicator exhibited no significant
346 difference between the groups. In summary, our results demonstrated that compared with the
347 usage of VAN or MTZ alone, the treatment strategy of combining low-dose MTZ with MMC
348 could not only provide the highest survival rate for CDI mice but also effectively reduce the
349 spore content in the intestine of infected mice.

350

351 **Discussion**

352 Patients infected with *C. difficile* still rely on antibiotic treatments, such as VAN and MTZ
353 (2-4). However, these treatments significantly disrupt the gut microbiota, leading to a high
354 probability of CDI recurrence (34). In this study, we found that MMC, an FDA-approved
355 chemotherapeutic agent frequently used to induce prophage from lysogenic bacteria (11, 21,
356 35-37), can synergistically cooperate with MTZ against *C. difficile* infection. The bacterial
357 biofilm protects *C. difficile* vegetative cells and spores from antibiotics, making it difficult to
358 treat and leading to recurrent infections (26-28, 38). Notably, our results revealed that MMC
359 can enhance the effect of MTZ in killing *C. difficile* within the biofilm. However, the
360 mechanism of MMC in assisting MTZ to eliminate *C. difficile* in biofilm remains unclear.
361 Fidaxomicin has previously been reported to effectively kill vegetative cells within *C. difficile*

362 biofilm by penetrating biofilms and disrupting their structure, thereby enhancing the
363 bactericidal activity (39, 40). In the future, the biofilm morphology should be observed to
364 determine whether MMC-MTZ treatment can directly disrupt biofilm structures to kill *C.*
365 *difficile* vegetative cells.

366 In our *in vivo* experiments, we referred to the recurrent mouse model described by A.
367 Bublitz et al (23). Mice infected with *C. difficile* will experience decreased body weight, severe
368 diarrhea, high mortality rates, and reduced activity levels (41-43). Remarkably, the combination
369 of MTZ with MMC provided mice with higher survival rates and less daily CSS after the spore
370 challenge. However, the recovery of body weight in this group was not as good as in other
371 infected groups. We consider that most mice of other groups with severe body weight loss have
372 died in CDI, leading to the lived mice having a higher average body weight than the MTZ-
373 MMC-treated group. We quantified the CFU number of spores from mice fecal samples to
374 evaluate CDI recurrence (23, 44) and there was no significant difference in CFU counts between
375 the R20291-infected and MTZ-MMC-treated groups after day 5 post-infection. Colonization
376 resistance has been suggested to play a critical role in recovery from CDI and preventing
377 recurrence through the healthy microbiota in inhibiting *C. difficile* colonization and overgrowth
378 (45-47). However, the antibiotic treatment will disrupt the diversity and richness of microbiota
379 (48). Accordingly, we consider that mice in both groups may suffer less damage in their
380 microbiota and could recover more rapidly against *C. difficile* colonization than other

381 antibiotic-treated groups. Nevertheless, further analysis of intestinal microbial composition
382 between each group should be performed to confirm this hypothesis. Finally, we found that
383 mice treated with VAN significantly increased *C. difficile* spores in their fecal contents
384 compared to other groups at day 5 post-infection. This is consistent with previous studies
385 suggesting that VAN treatment leads to more severe alterations in the composition of symbiotic
386 microorganisms, potentially complicating the recovery process and resulting in prolonged
387 susceptibility to *C. difficile* compared to treatment with MTZ (32).

388 MTZ targets bacteria by disrupting DNA synthesis (49), while the anticancer agent MMC
389 cross-links DNA to prevent bacterial replication (50). The results of our study indicate that the
390 combination of MTZ and MMC seems to provide better action in inhibiting bacterial DNA
391 replication against *C. difficile* infection.

392 In summary, our findings reveal a novel therapeutic strategy that the combination of MMC
393 significantly enhances the efficacy of MTZ against *C. difficile* infection. This combination not
394 only improves the survival rate of CDI mice but also reduces the spore count in their feces. The
395 reduced efficacy of MTZ in treating CDI has previously been suggested to *C. difficile* mutant
396 strains that overexpress the NimB enzyme, leading to the inactivation of MTZ and antibiotic
397 resistance (51). This issue did not occur in our MTZ-MMC combination therapy against *C.*
398 *difficile*. Furthermore, the low dosage of MTZ combined with MMC may effectively decrease
399 *C. difficile* recurrence by minimizing severe disruptions to the gut microbiota composition of

400 the infected host (45, 47). Nevertheless, further experiments are necessary to confirm the safety
401 profile, optimal dosage, and potential host microbial side effects of the combined antibiotic
402 therapy before its application in the clinical management of CDI.
403

404 **Notes**

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412 investigation, visualization, and original manuscript writing. I. H. H. contributed to
413 conceptualization and methodology. Y. P. H. contributed to conceptualization, methodology,
414 and resources. Y. W. C. contributed to conceptualization, methodology, and visualization. Y.
415 C. L. contributed to the methodology and investigation. J. W. C. contributed to the
416 conceptualization, methodology, supervision, project administration, funding acquisition, and
417 manuscript editing.

418 ***Potential Conflict of Interest.*** All authors declare that they have no conflicts of interest.

419

420 **Figure Legends**

421 **Figure 1. Evaluating the ability of MMC combined with MTZ or Van to combat *C. difficile***

422 **strain R20291 *in vitro*.** (A) Flowchart of checkerboard assay (Created with BioRender.com).

423 FIC index of MMC combined with MTZ or VAN co-culturing with *C. difficile* (B) RT027 strain

424 R20291, (C) RT027 and RT078 clinical strains. The MIC of (D) MTZ alone or (E) combination

425 of MMC with MTZ to against *C. difficile* was determined using E-test strips. MTZ,

426 metronidazole. VAN, vancomycin. MMC, mitomycin C. FIC index, fractional inhibitory

427 concentration index. MIC, minimum inhibitory concentration. The results were representative

428 of three independent experiments with the average from triplicate measurements.

429

430 **Fig 2. Combining MMC and MTZ therapy effectively reduced the antibiotic resistance of**

431 **R20291 biofilms.** (A) The flowchart of antibiotic treatments against *C. difficile* biofilm

432 formation (Created with BioRender.com). (B) MBC assay was used to determine the

433 concentration of MTZ that can kill R20291 vegetative cells. After 3 days of incubation to form

434 biofilms, the MBC concentration of (C) MTZ alone, (D) MTZ combined with 1 μg/mL MMC,

435 and (E) MMC alone to combat R20291 within the biofilm was determined. MBC, minimum

436 bactericidal concentration. This experiment was representative of three independent

437 experiments and each experiment was performed in triplicate.

438

439 **Fig 3. The combination of MMC and low-dose MTZ significantly inhibited the growth of**

440 **R20291 in mouse feces.**

441 (A) *Ex vivo* model of R20291 co-cultured with mouse feces and treated with different kinds of

442 antibiotic treatment (Created with BioRender.com). (B) Fecal-bacterial mixtures cultured for

443 24 hr were 10^6-fold diluted and the CFU of *C. difficile* were calculated by spreading on CCFA

444 plates. CFU, colony-forming units. MTZ_{HC}, high concentration of MTZ (3 μg/mL). MTZ_{LC},

445 low concentration of MTZ (0.375 μg/mL). MMC, 1 μg/mL MMC. MTZ_{LC}+MMC, 0.375

446 μg/mL MTZ combined with 1 μg/mL MMC. The experiment was representative of four

447 independent experiments with duplicate measurements. Statistical analysis was performed

448 using the one-way analysis of variance test (*$p \leq 0.05$; ****$p \leq 0.0001$; ns, not significant)

449

450 **Fig 4. Application of combining MMC with low-dose MTZ treatment in the mouse CDI**

451 **recurrence model.**

452 (A) Schematic diagram of the mouse CDI recurrence model (Created with BioRender.com).

453 C57BL/6 mice were given clindamycin (10 mg/kg) and orally challenged with 10^4 CFU spores.

454 After two days treating with (n=8/group) or without (Mock, n=3; R20291, n=8) antibiotics, the

455 severity of infection symptoms was observed, and feces were collected to assess the number of

456 spores. (B) The mice survival rate was monitored daily from the day of infection (pi-day 0)

457 until being sacrificed. (C) The body weight of each group was monitored daily from the day
458 before infection until the day 7 post-infection. (D-F) The CFU of *C. difficile* spore was
459 determined using mouse feces collected on the 3rd, 5th, and 7th days post-infection. (G) The
460 morphology and quantification of mice's cecal and colonic tissues. The colon length, cecum
461 length, and cecum weight were harvested when the mice were sacrificed on the day 7 post-
462 infection. Each point represents the data of one mouse. Statistical analysis was performed using
463 GraphPad Prism 10.0, and significance was determined by the one-way analysis of variance
464 (**≤ 0.01; ***≤ 0.001; ****$p \leq 0.0001$; ns, not significant)
465
466 **Fig 5. Comparing the effect of MMC-MTZ$_{LC}$ treatment and clinical antibiotics against *C.***
467 ***difficile* infection and recurrence *in vivo*.**
468 (A) Diagram of the mouse CDI recurrence model (Created with BioRender.com). Mice (n =
469 9/group) were orally challenged with *C. difficile* spores, followed by two days of antibiotic
470 treatment. (B) Mice survival rate and (C) the daily percentage of mouse bodyweight were
471 recorded after infected with R20291 spores. Mice feces were collected on (D) day 3, (E) day 5,
472 and (F) day 7 post infection. The homogenized feces were diluted and plated on CCFA plates
473 to measure the spore forming CFU. (G) The morphology of mice cecum and colon. The degree
474 of intestinal inflammation was evaluated by the quantification of the colonic length, cecum
475 length and weight. Each point represents the data of a mouse. (H) The values of GPT, GOT,

BUN, and CRE in mice serum. The dry biochemical analyzer was used to measure various indicators in the serum to evaluate the damage and impact of different treatment processes on the liver and kidney functions of mice. GPT, glutamic 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)

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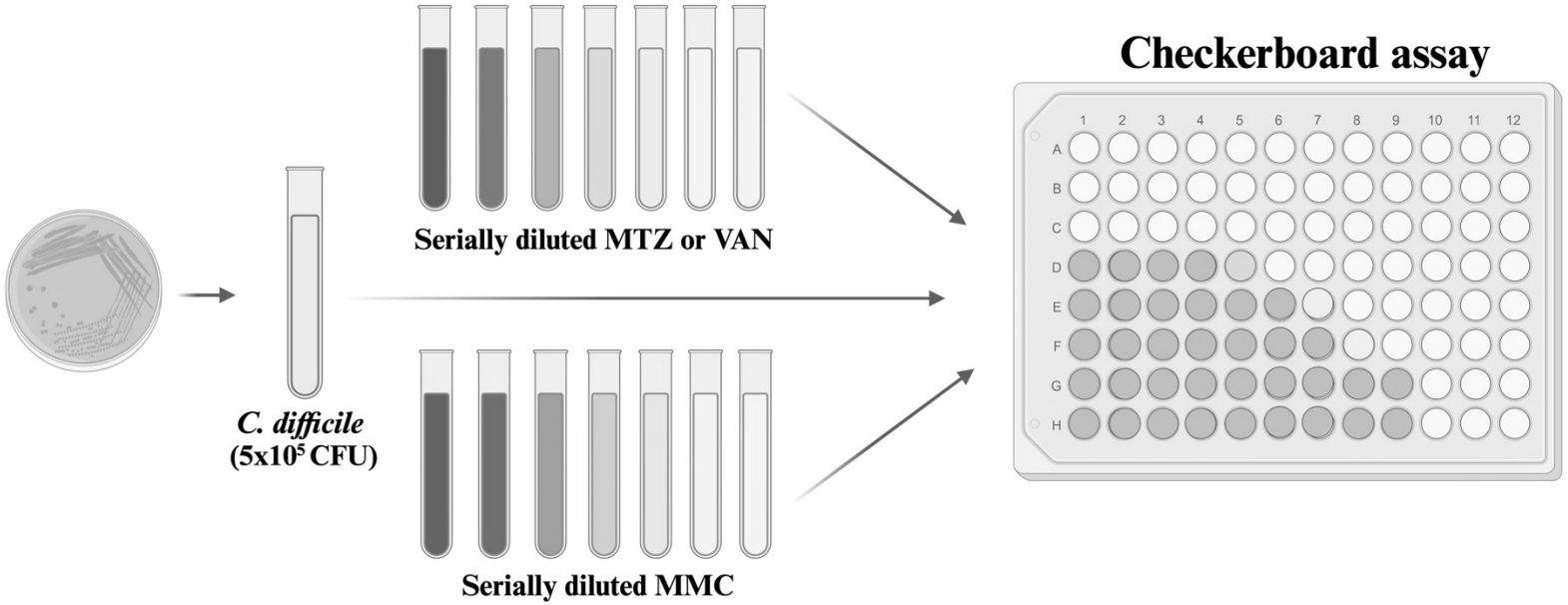
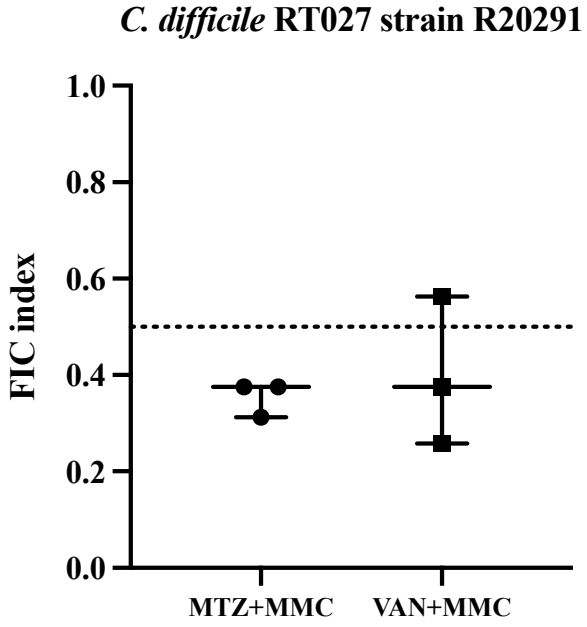
629

Fig. 1

A.


Checkerboard assay
Serially diluted MTZ or VAN
C. difficile
(5x10^5 CFU)
Serially diluted MMC

B.


C. difficile RT027 strain R20291
FIC index
1.0
0.8
0.6
0.4
0.2
0.0
MTZ+MMC
VAN+MMC

C.

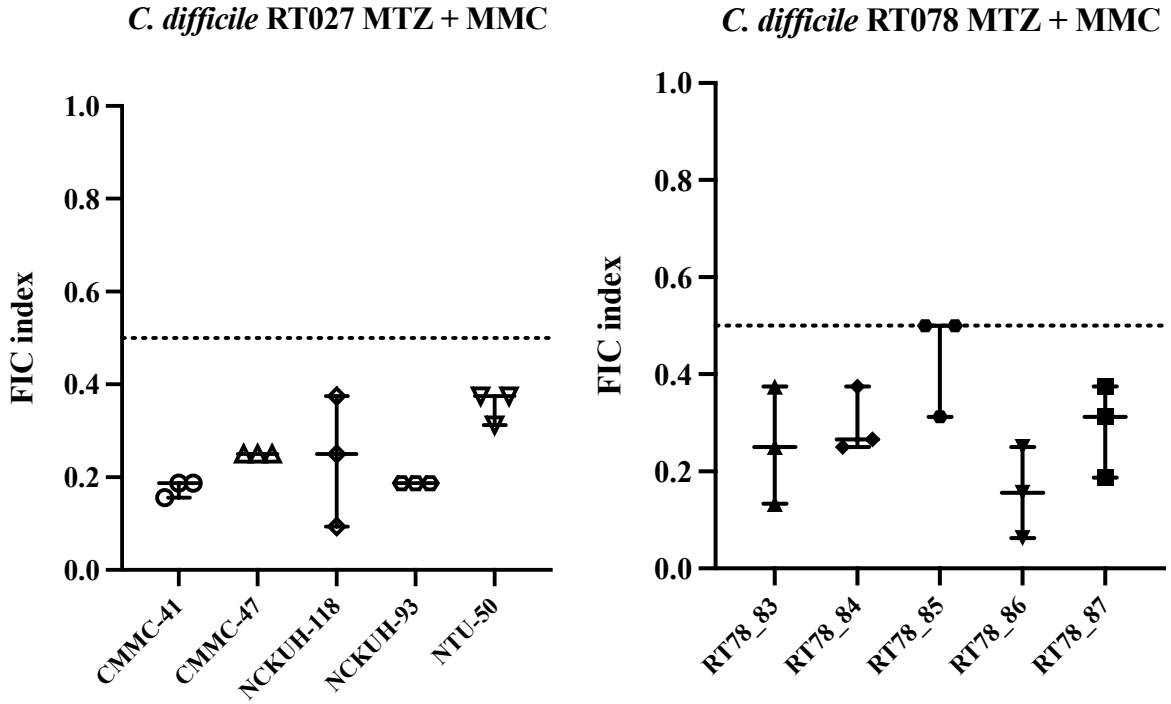
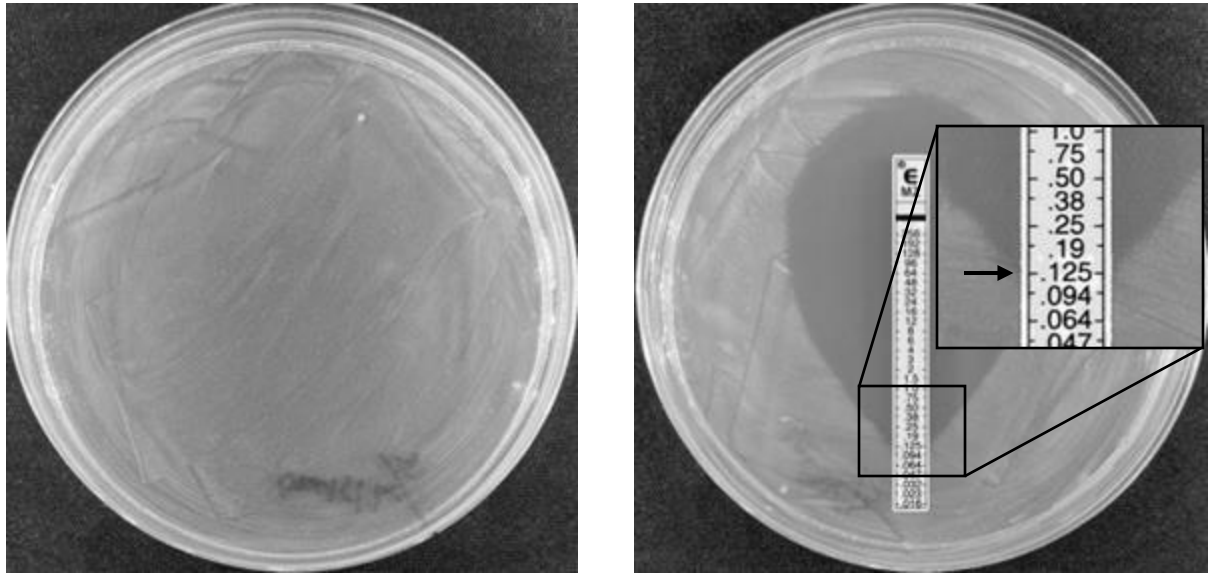

C. difficile RT027 MTZ + MMC
C. difficile RT078 MTZ + MMC
FIC index
1.0
0.8
0.6
0.4
0.2
0.0
CMMC-41
CMMC-47
NCKUH-118
NCKUH-93
NTU-50
RT78_83
RT78_84
RT78_85
RT78_86
RT78_87

Fig. 1

D.

BHIS agar only



E.

BHIS + 0.25 µg/mL MMC

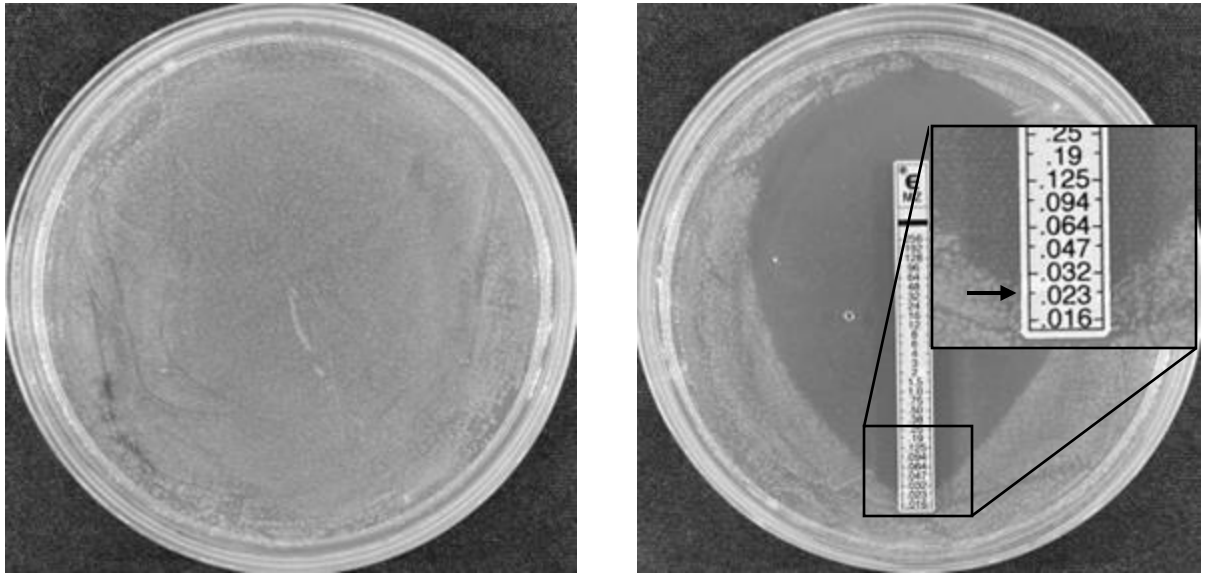
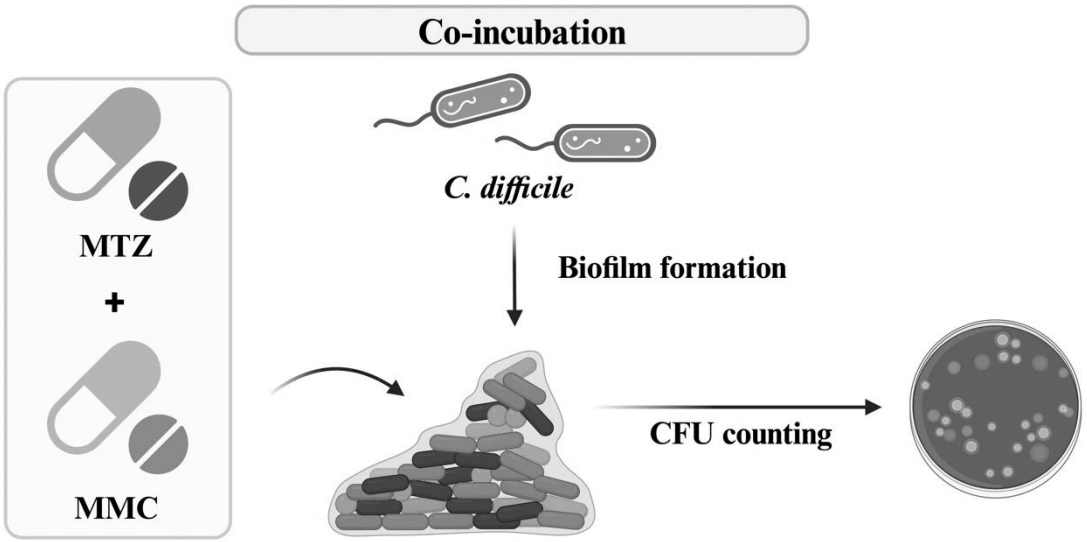
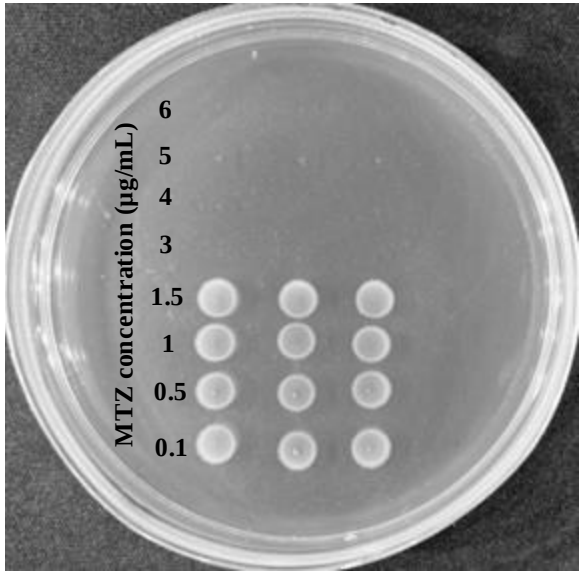


Fig. 2

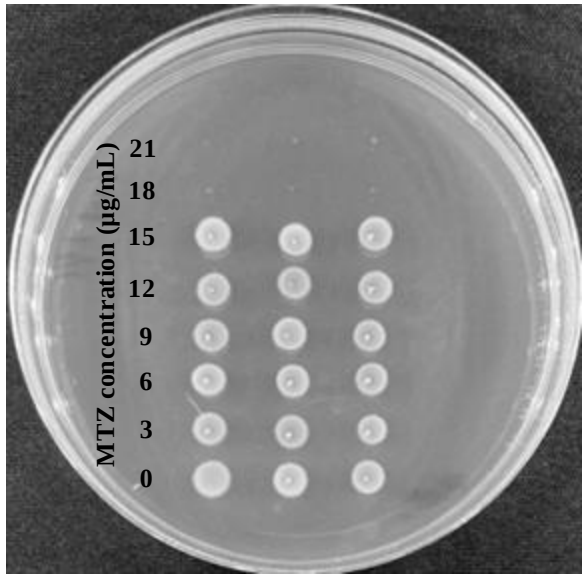
A.



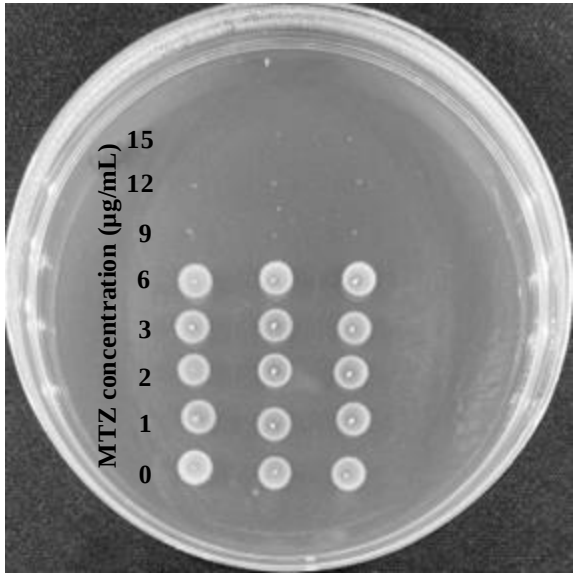
B. **MTZ only**



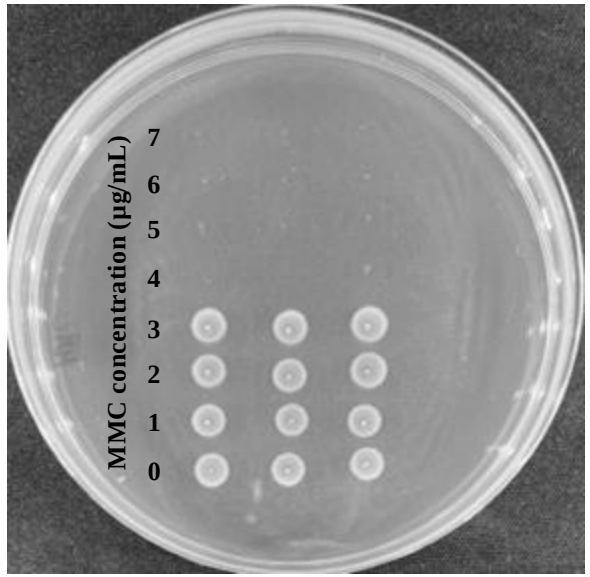
C. **MTZ only**



D. **MTZ + 1 μg/mL MMC**



E. **MMC only**

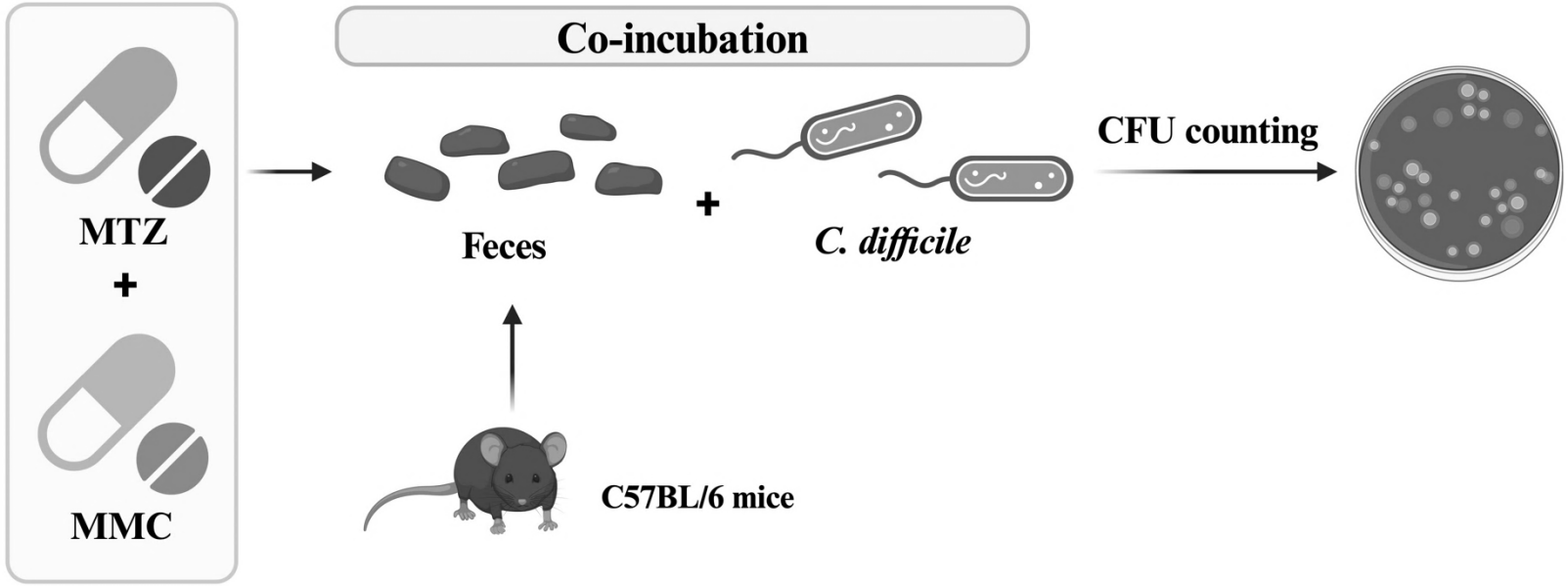


R20291 vegetative cell

R20291 Biofilm

Fig. 3

A.


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

B.

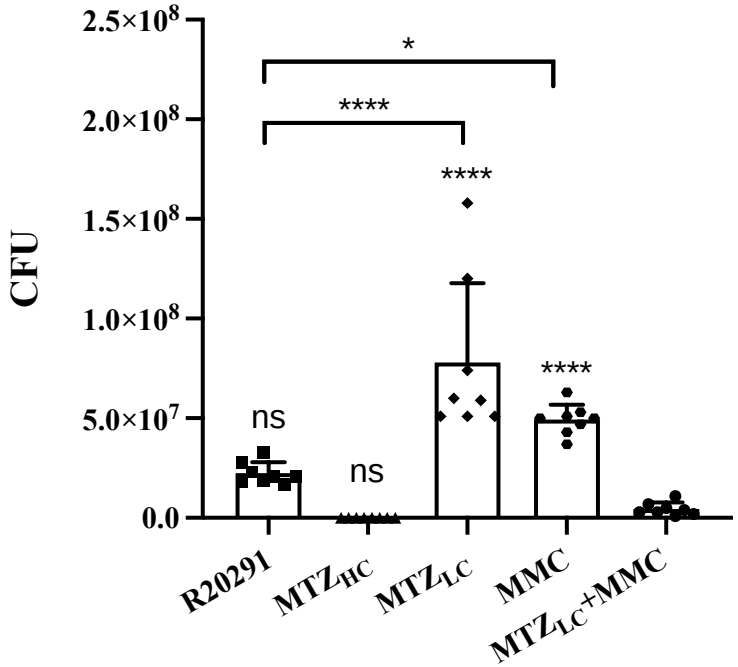
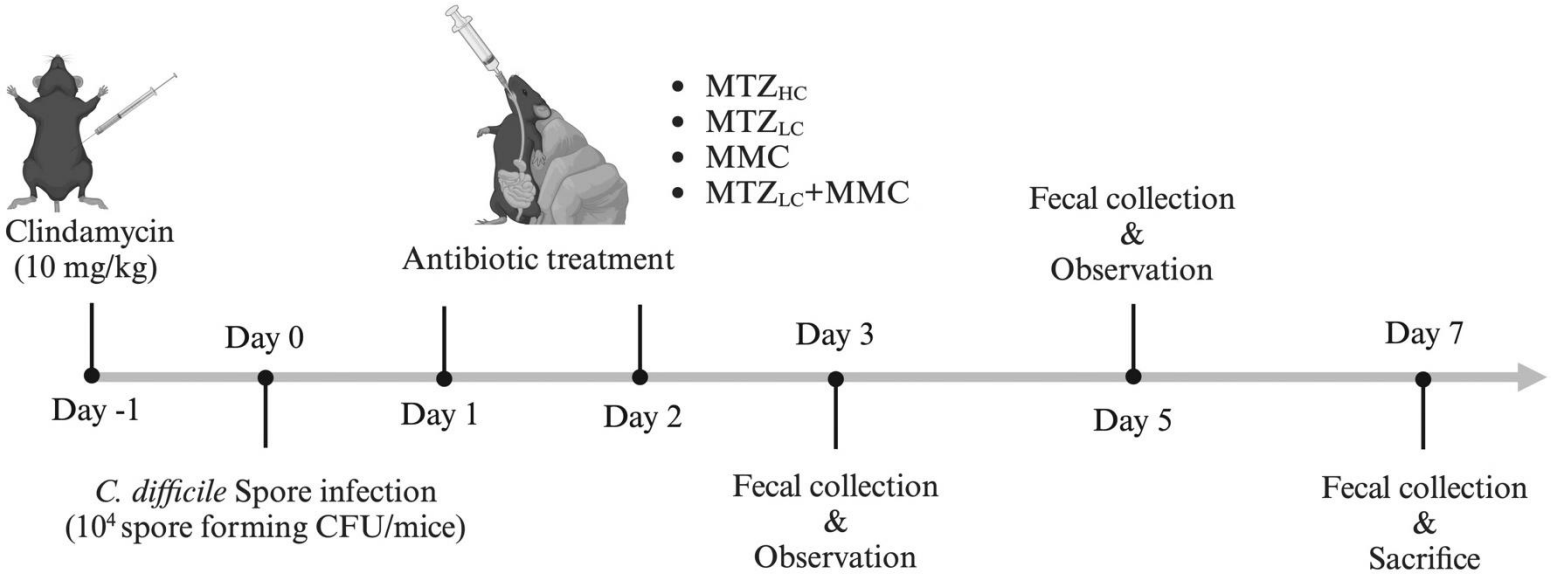
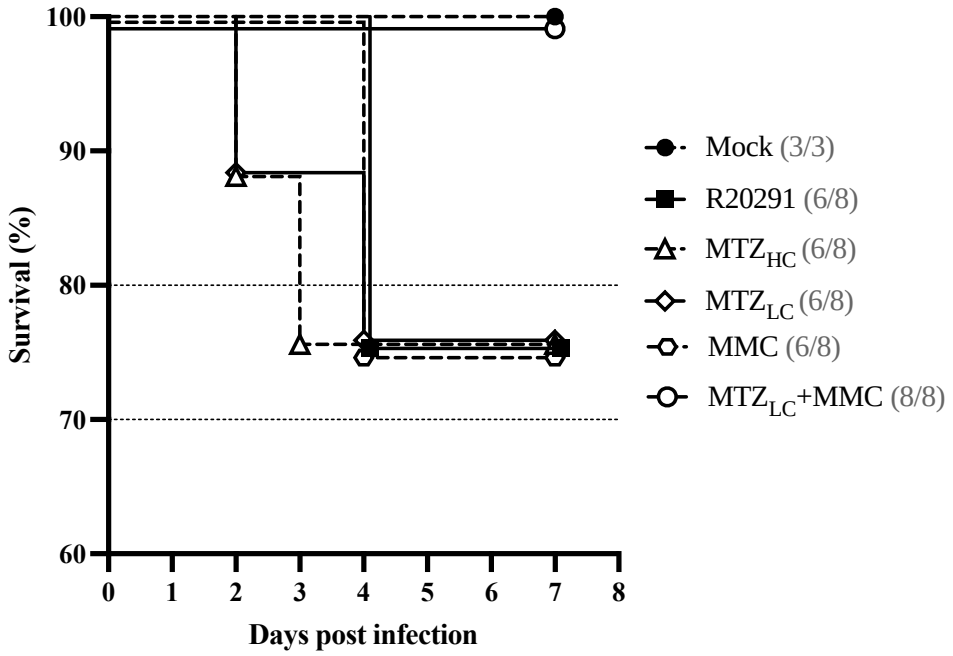

CFU
2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0.0
*
****
****
****
ns
ns
R20291
MTZ_HC
MTZ_LC
MMC
MTZ_LC+MMC

Fig. 4

A.


Clindamycin
(10 mg/kg)
Antibiotic treatment
MTZHC
MTZLC
MMC
MTZLC+MMC
Fecal collection
&
Observation
Day -1
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7
C. difficile Spore infection
(10⁴ spore forming CFU/mice)
Fecal collection
&
Observation
Fecal collection
&
Sacrifice

B.


Survival (%)
Days post infection
Mock (3/3)
R20291 (6/8)
MTZHC (6/8)
MTZLC (6/8)
MMC (6/8)
MTZLC+MMC (8/8)

C.

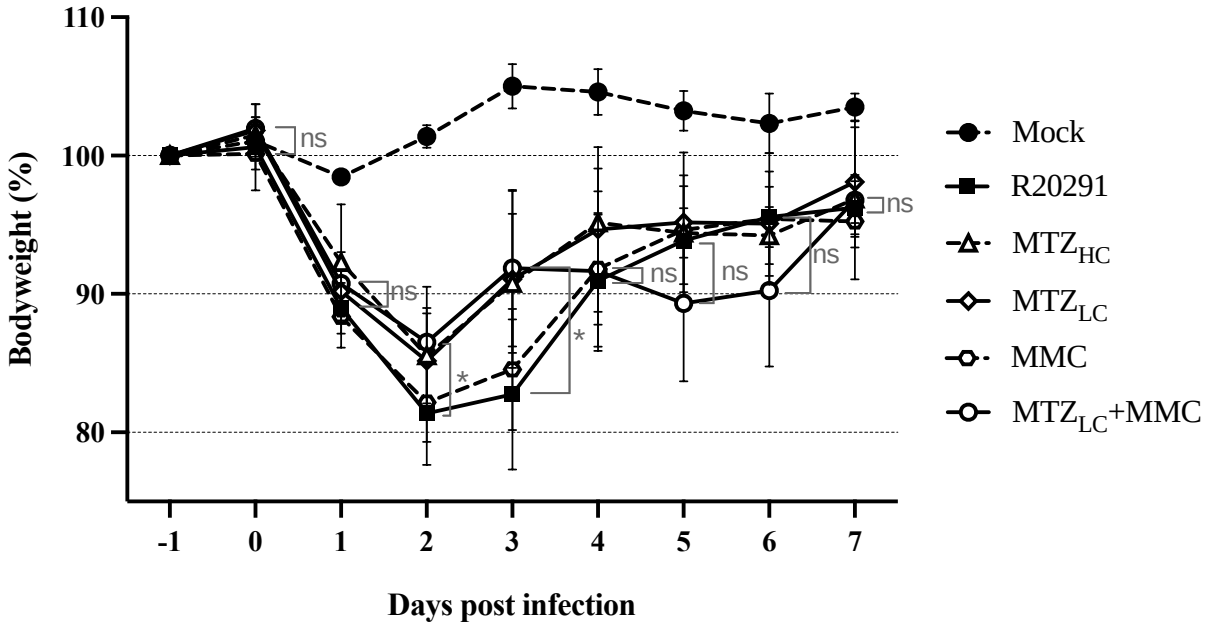

Bodyweight (%)
Days post infection
ns
*
Mock
R20291
MTZHC
MTZLC
MMC
MTZLC+MMC

Fig. 4

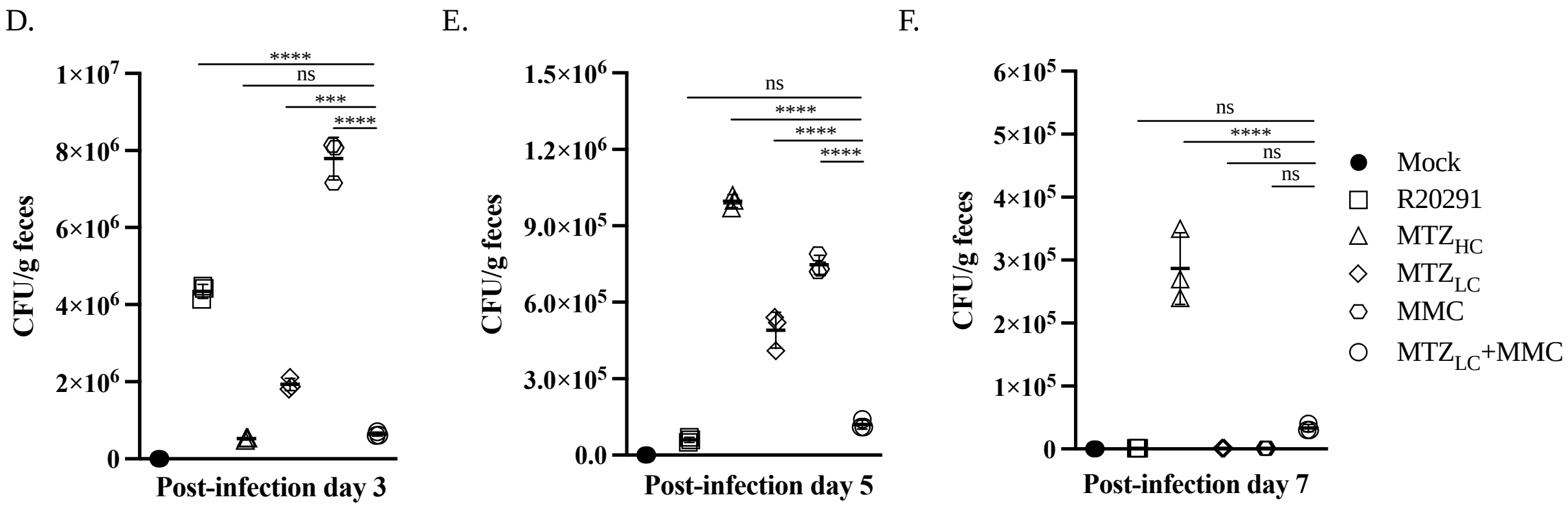
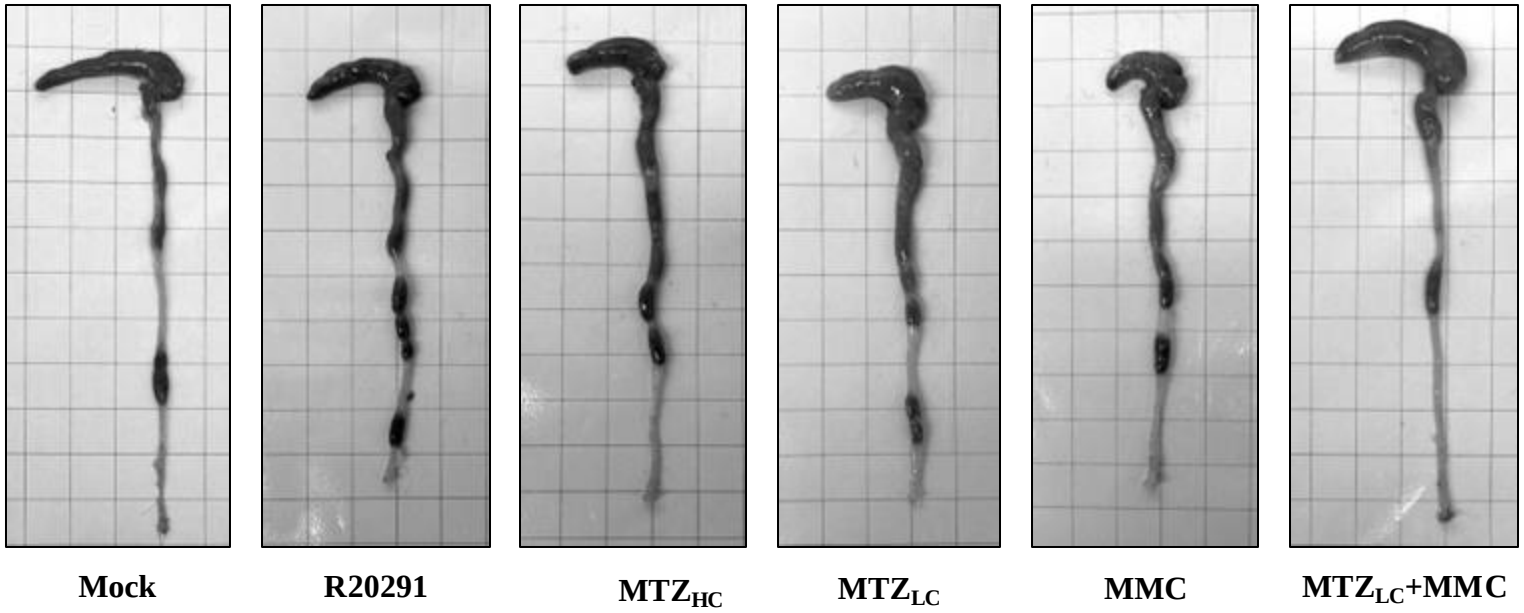

D.
E.
F.
CFU/g feces
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
****
ns
***
****
Post-infection day 3
1.5×10^6
1.2×10^6
9.0×10^5
6.0×10^5
3.0×10^5
0.0
ns
****
****
****
Post-infection day 5
6×10^5
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
ns
****
ns
ns
Post-infection day 7
Mock
R20291
MTZ_HC
MTZ_LC
MMC
MTZ_LC+MMC

Fig. 4

G.

Colonic morphology



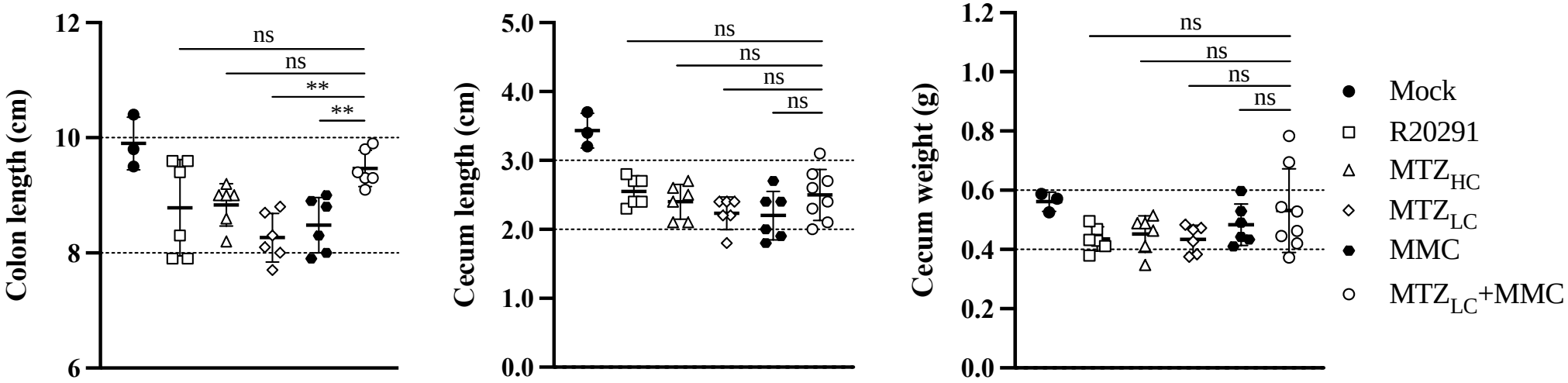
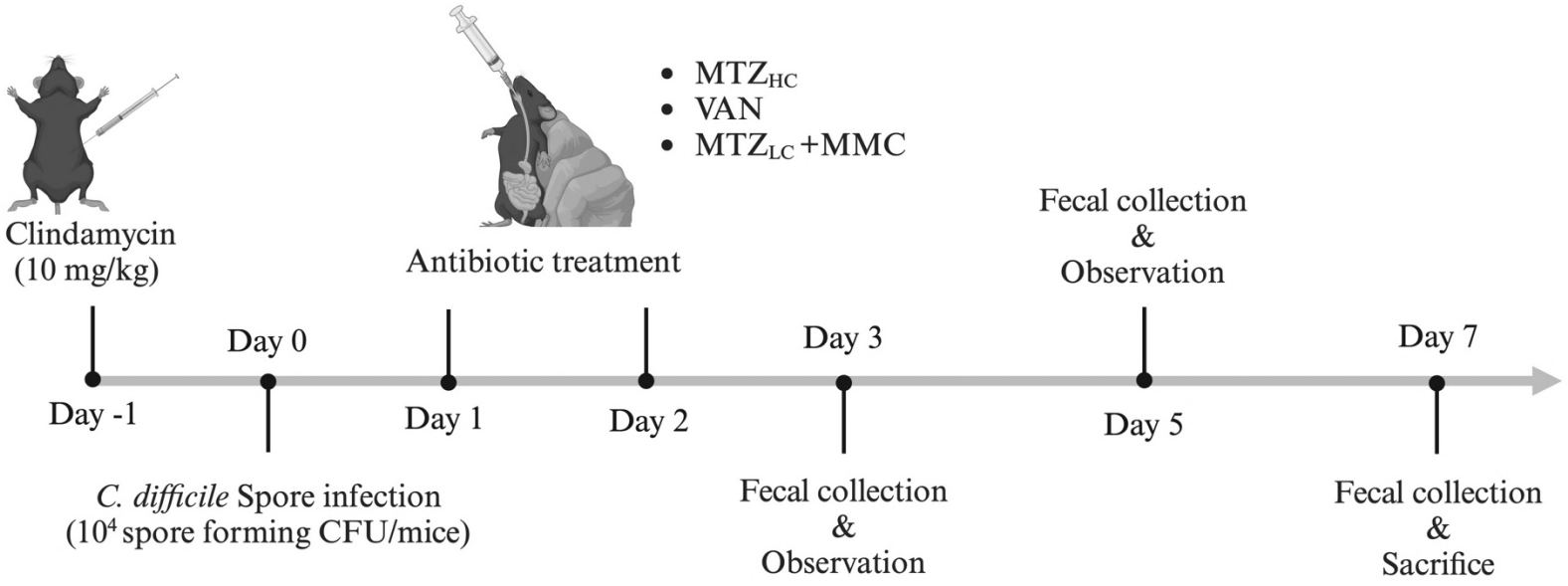
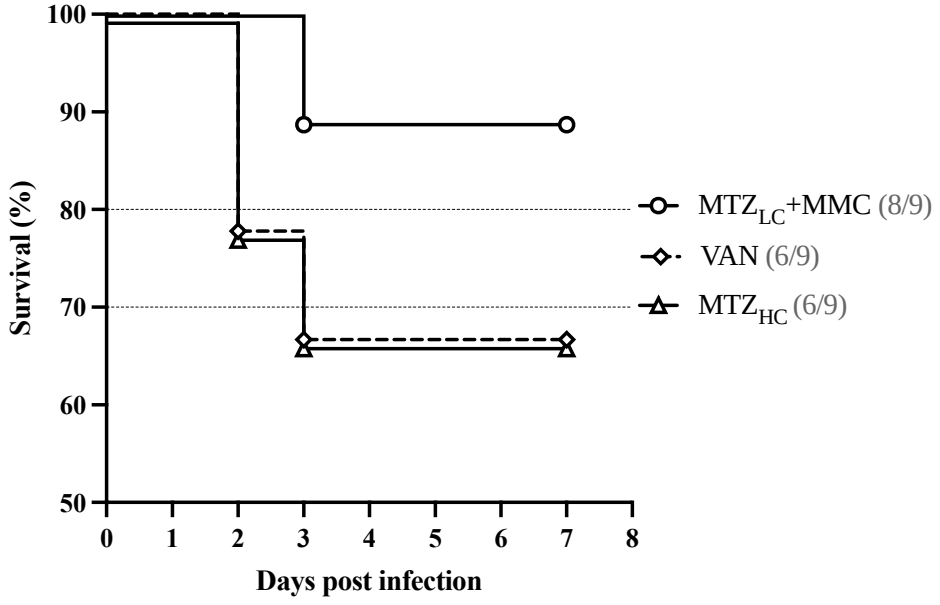


Fig. 5

A.


MTZ$_{HC}$
VAN
MTZ$_{LC}$+MMC
Clindamycin
(10 mg/kg)
Antibiotic treatment
Fecal collection
&
Observation
Day -1
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7
C. difficile Spore infection
(10^4 spore forming CFU/mice)
Fecal collection
&
Observation
Fecal collection
&
Sacrifice

B.


Survival (%)
100
90
80
70
60
50
0
1
2
3
4
5
6
7
8
Days post infection
MTZ$_{LC}$+MMC (8/9)
VAN (6/9)
MTZ$_{HC}$ (6/9)

C.

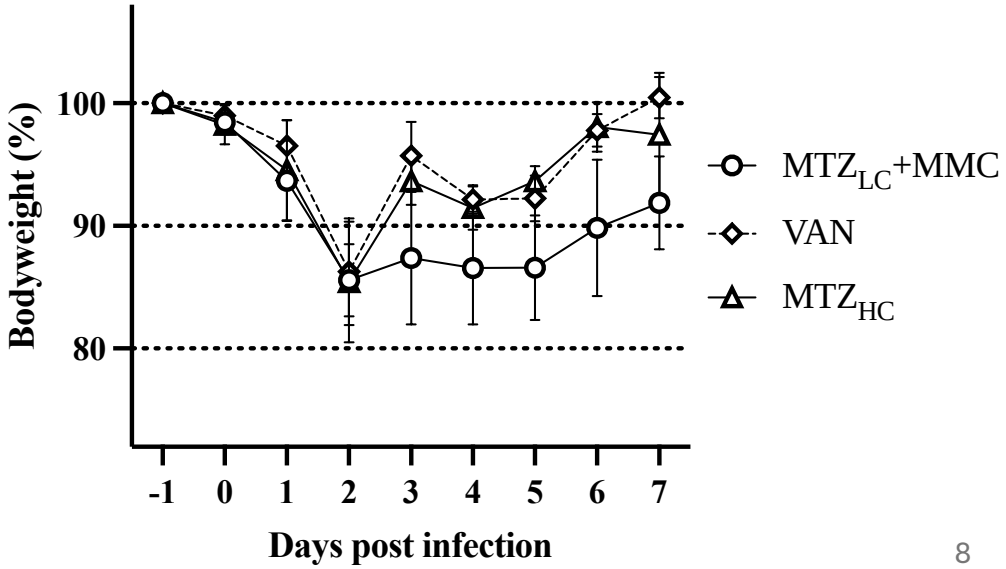

Bodyweight (%)
100
90
80
-1
0
1
2
3
4
5
6
7
Days post infection
MTZ$_{LC}$+MMC
VAN
MTZ$_{HC}$

Fig. 5

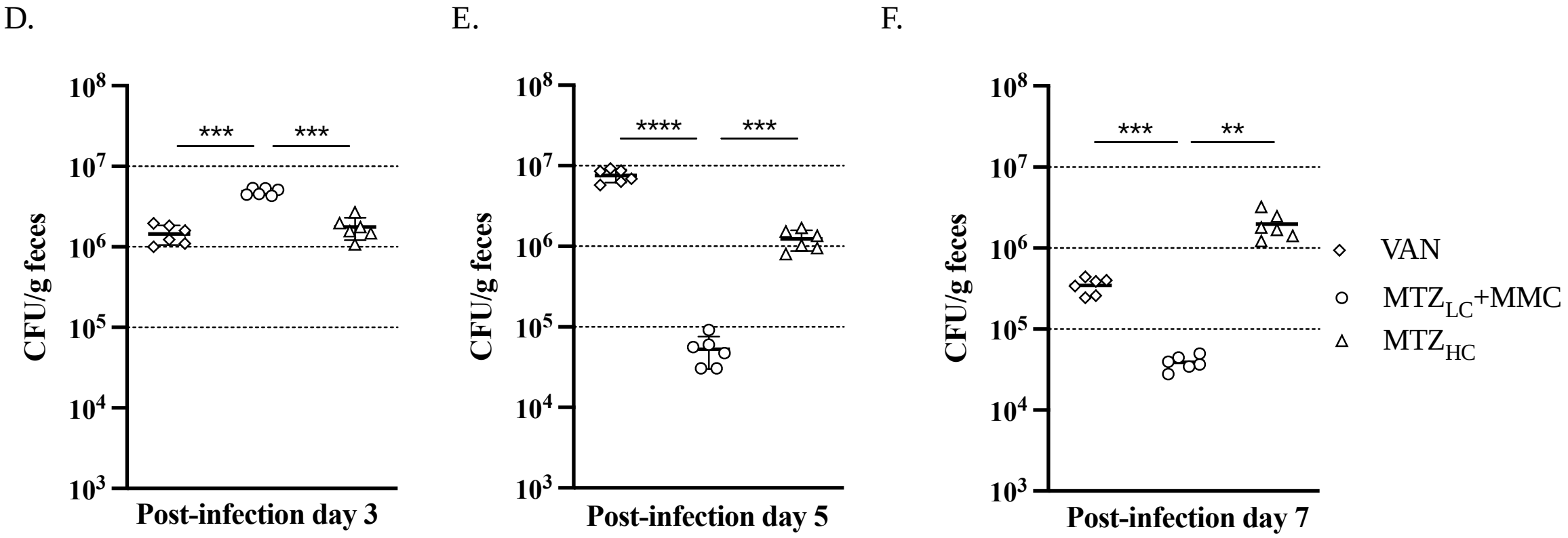
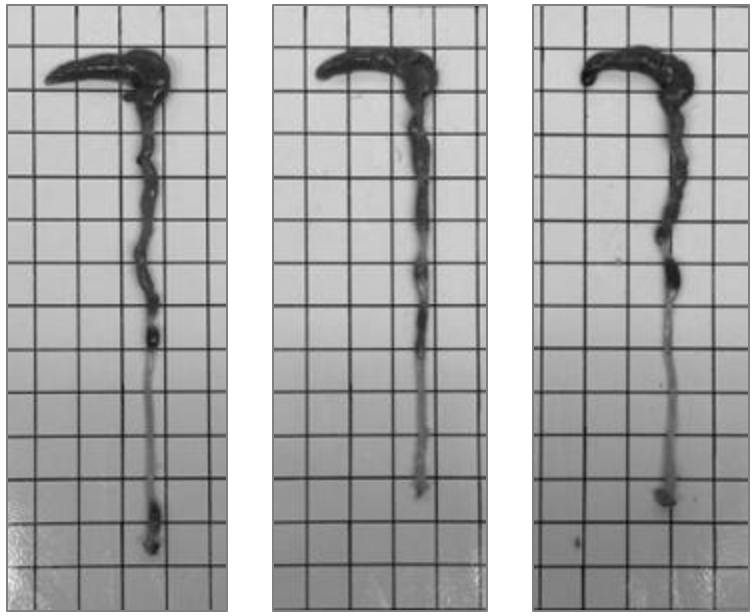

D.
E.
F.
CFU/g feces
10^8
10^7
10^6
10^5
10^4
10^3
***
***
****
***
***
**
Post-infection day 3
Post-infection day 5
Post-infection day 7
VAN
MTZ_{LC}+MMC
MTZ_{HC}

Fig. 5

G.

Cecal and colonic morphology



MTZ_{LC}+MMC VAN MTZ_{HC}

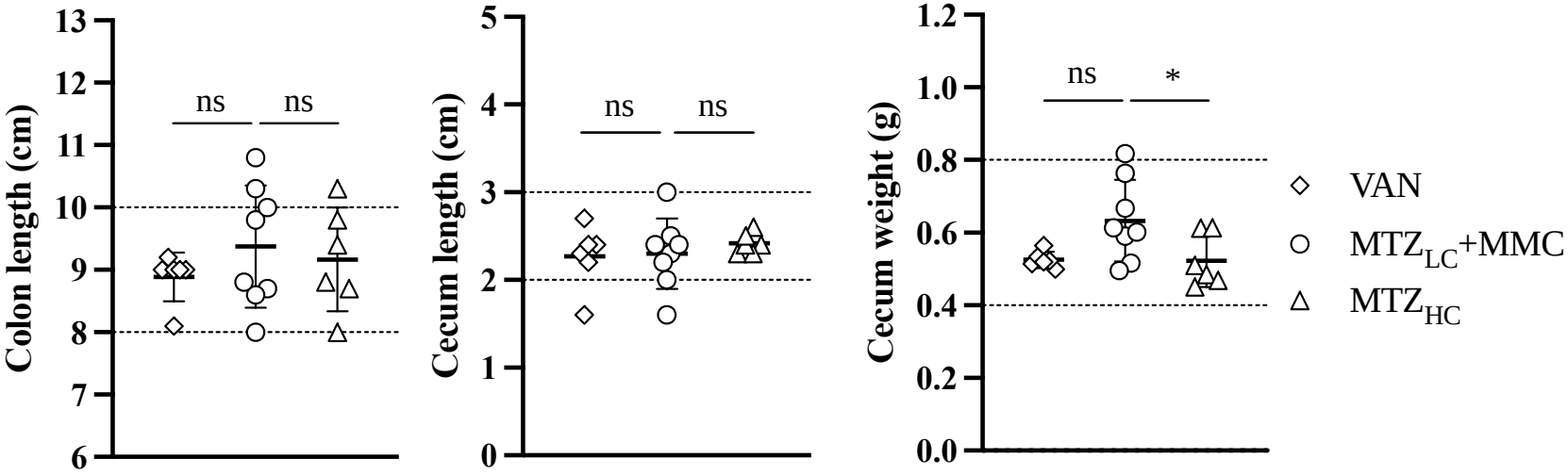
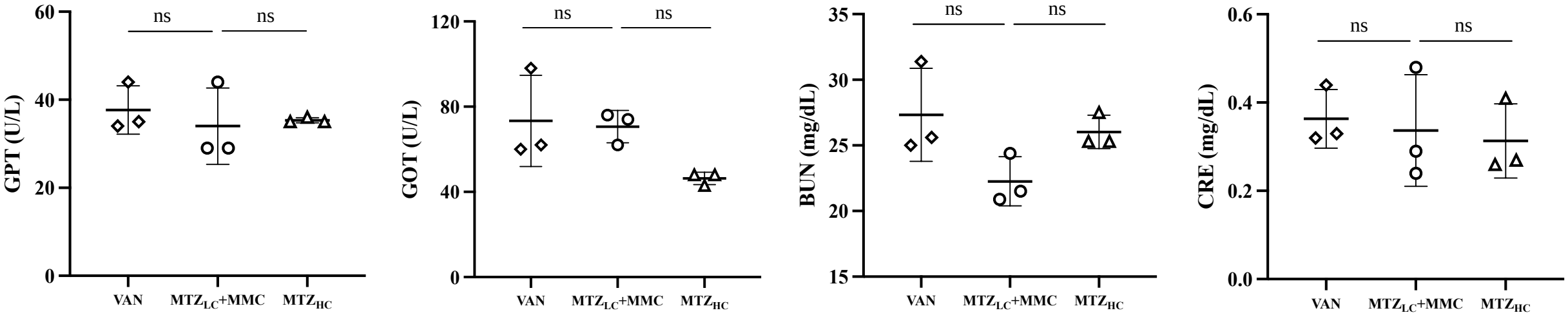


Fig. 5

H.


GPT (U/L)
60
40
20
0
ns
ns
VAN
MTZLC+MMC
MTZHC
GOT (U/L)
120
80
40
0
ns
ns
VAN
MTZLC+MMC
MTZHC
BUN (mg/dL)
35
30
25
20
15
ns
ns
VAN
MTZLC+MMC
MTZHC
CRE (mg/dL)
0.6
0.4
0.2
0.0
ns
ns
VAN
MTZLC+MMC
MTZHC