Infection and Drug Resistance

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ORIGINAL RESEARCH

In vitro and in vivo activity of ciprofloxacin/fosfomycin combination therapy against ciprofloxacin-resistant *Shigella flexneri* isolates

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Objective: Ciprofloxacin resistance (CIP^R) for *Shigella* isolates is becoming more prevalent. This study systematically investigated the antibacterial activity of ciprofloxacin (CIP)/fosfomycin (FOS) combination in vitro and in vivo against CIP^R *S. flexneri* isolates.

Method: Eighty CIP^R *S. flexneri* isolates were selected for synergy studies by the microtiter plate checkerboard assay. Two *S. flexneri* isolates (GN120471, CIP^RFOS^R; GN120454, CIP^RFOS^S) were used to investigate the efficacy of the CIP/FOS combination by the time-kill methodology. Clinically relevant concentrations (CIP, 0.5, 1, or 2.5 μg/mL; FOS, 30, 150, or 300 μg/mL) were combined, and the colony counts were conducted at 3, 5, 8, and 24 hours. The in vivo activity of the CIP/FOS combination was assessed using a *Galleria mellonella* larvae model.

Results: In checkerboard assays, 31 strains (38.75%) showed synergy for the CIP/FOS combination. For the isolate GN120471, monotherapy with CIP or FOS at all concentrations produced little or no bacterial killing, while the CIP/FOS combination produced enhanced bacterial killing with FOS concentrations of 150 and 300 μg/mL, especially when combined with CIP at 2.5 μg/mL. For the isolate GN120454, the CIP/FOS combination at all concentrations produced more rapid and extensive killing (up to $5\log_{10}$ colony forming units (CFU)/mL with many combinations) than with either antibiotic alone. Mortality at 96 hours was around 80% at approximately 10^4 CFU/larva for GN120471 and GN120454. When CIP at 2.5 μg/mL was combined with FOS at 150 μg/mL for the bactericidal activity in vivo, the survival rates for CIP/FOS combination against GN120471-infected and GN120454-infected larvae were significantly higher than that of CIP (68.75% vs 25%, P=0.013; 81.25% vs 37.5%, P=0.012, respectively).

Conclusion: Against CIP^R *S. flexneri* isolates, the CIP/FOS combination induced synergy, and increased bacterial killing in vitro and in a simple invertebrate model of infection.

Keywords: ciprofloxacin, fosfomycin, antimicrobial synergy, *Shigella*

Introduction

Shigellosis is an acute invasive enteric infection caused by bacteria belonging to the genus *Shigella*: *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei. Shigella flexneri* is the chief cause of endemic shigellosis in developing countries, causing nearly two-thirds of infections.[1] While shigellosis is typically self-limiting, treatment with appropriate antimicrobial therapy can shorten the duration of illness and prevent transmission. In the guidelines for the control of shigellosis by the WHO in 2005, ciprofloxacin (CIP) was considered the first-line treatment for shigellosis. However, the widespread use of CIP has led to the development of resistance to CIP and other

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fluoroquinolones for *Shigella* isolates, especially in Asia.[2–4] Thus, combination therapy has been employed to treat shigellosis caused by CIP-resistant (CIP^R) *Shigella* isolates.

Some studies have demonstrated that fosfomycin (FOS) is a promising agent, especially as part of combination therapy.[5–7] FOS was discovered in 1969 and is a phosphonic acid derivative with a molecular mass of 138 Da.[8] The mechanism of action is to disrupt the formation of the peptidoglycan precursor uridine diphosphate *N*-acetylmuramic acid, which is unaffected by other antimicrobials, meaning that there is no cross-resistance with other classes of antimicrobials.[9,10] It has a broad spectrum of activity against many bacteria, including multidrug-resistant (MDR) strains, and is generally well tolerated at therapeutic doses.[11–13] However, FOS resistance (FOS^R) emerges quickly when used as monotherapy.[14,15] For this reason, FOS is usually administered in combination with other classes of antimicrobials for the treatment of systemic infections.[16] More recently, some studies have demonstrated that the CIP/FOS combination exhibits enhanced in vitro activity against CIP^R *Pseudomonas aeruginosa* strains.[17,18] However, no study has examined the in vitro and in vivo activity of the CIP/FOS combination against CIP^R *Shigella* isolates.

Animal studies for this combination are necessary to predict its suitability for clinical use in humans. However, mammalian models of infection are associated with high cost, ethical constraints, and specialized training requirements. Therefore, invertebrate models, such as *Galleria mellonella* larvae, have been proposed as an alternative to investigate the in vivo activity of antimicrobial agents.[19–21] In this study, we demonstrate the in vitro and in vivo efficacy of the CIP/FOS combination against CIP^R *Shigella* isolates in an attempt to gain insights into whether it should be explored further for the treatment of CIP^R *Shigella* infections.

Materials and methods

Bacteria and antimicrobial agents

Eighty CIP^R *S. flexneri* isolates were obtained from the stool samples of patients at 34 hospitals in Anhui, People's Republic of China. Two clinical isolates of *S. flexneri* (GN120471, CIP^RFOS^R; GN120454, CIP^RFOS^S) were employed, which had synergistic effects for the CIP/FOS combination. According to the Clinical and Laboratory Standards Institute (CLSI) guidelines in 2017, susceptibility (S) and resistance (R) were defined as minimal inhibitory concentrations (MICs) of ≤1 and ≥4 μg/mL CIP and ≤64 and ≥256 μg/mL FOS, respectively. In addition, 25 μg/mL glucose-6-phosphate was supplemented when the sensitivity of FOS was detected. All antibiotics were obtained from Sigma-Aldrich China (Shanghai, China).

The study was conducted in accordance with the guidelines of the Declaration of Helsinki, the principles of Good Clinical Practice, and Chinese regulatory requirements, and was approved by the local Ethics Committees of the First Affiliated Hospital of Anhui Medical University (Hefei, People's Republic of China). All patients gave written informed consent.

Synergy testing by the checkerboard assay

MIC values of CIP and FOS were determined with the agar dilution method according to CLSI guidelines. *Escherichia coli* ATCC 25922 was used as a control. Synergy between CIP and FOS was assessed by the microtiter plate checkerboard assay. In brief, 96-well microtiter plates were set up with increasing concentrations of CIP (1/64MIC-4MIC) in the horizontal wells and FOS (1/32MIC-4MIC) in the vertical wells, and were inoculated with 5×10^5 CFU/mL of *S. flexneri* isolates prepared in cation-adjusted Mueller–Hinton broth (CAMHB; with 23.0 μg/mL Ca^{2+} and 12.2 μg/mL Mg^{2+}; Oxoid [Basingstoke, UK]). Plates were incubated at 37°C overnight and were visually inspected for turbidity to determine growth. Synergy was assessed by calculation of the fractional inhibitory concentration index (FICI). An FICI ≤0.5 was defined as showing a synergistic effect, 0.5< FICI <4.0 as showing no interaction, and FICI ≥4.0 as showing an antagonistic effect.[22]

Time-kill assays and pharmacodynamic analysis

Bacterial killing was examined using time-kill studies with each antibiotic alone or in combination at an initial inoculum of approximately 10^6 CFU/mL (GN120471 and GN120454) over 24 hours, according to a previously described methodology.[17] The employed concentrations were 0.5, 1, or 2.5 μg/mL CIP and 30, 150, or 300 μg/mL FOS. Nine experiments (three concentrations of CIP × three concentrations of FOS) for combination were performed. Each tube was incubated at 37°C. Serial samples were collected for viable counting at 0, 3, 5, 8, and 24 hours.

Microbiological responses were quantified by the log-change method, which compared the change in $\log_{10}$

CFU/mL from 0 hours (CFU_0) to time t (3, 5, 8, or 24 hours; CFU_t): log change = $\log_{10}$ (CFU_t) – $\log_{10}$ (CFU_0). Synergy was defined as a reduction of ≥2 $\log_{10}$ CFU/mL for the combination compared with the most active monotherapy at the specified time, and additivity as a reduction of 1 to <2 $\log_{10}$ CFU/mL for the combination.[17]

Galleria mellonella treatment assays

The *G. mellonella* infection model for *S. flexneri* isolates was performed as previously described.[20] Batches of *G. mellonella* (Kaide Ruixin Co., Tianjin, People's Republic of China) in their final instar stage were stored in the dark at 4°C and were used within 7 days. Larvae masses (250–300 mg) were used to calculate treatment doses.

To establish the lethal inoculum required to cause 80% of the larvae to die over 96 hours, 40 larvae were inoculated with 10 µL of bacterial suspensions (10 larvae for 10^5, 10^6, 10^7, and 10^8 CFU/mL, respectively) and 10 larvae were inoculated with PBS as controls (Figuire S1). Bacteria were injected into the hemocoels through the last left proleg using a 25 µL Hamilton syringe (Hamilton, Shanghai, People's Republic of China). Larvae were incubated at 37°C and were observed daily for 4 days.

Sixteen larvae were infected with a lethal dose of *S. flexneri* GN120471 or GN120454 as described above. CIP (the steady-state plasma concentration 2.5 µg/mL) and FOS (the steady-state plasma concentration 150 µg/mL) were administered via 10 µL injections for 4 days into the last right proleg within 2 hours of inoculation of bacterial suspensions. The following doses were used: 100 µg/mL every 12 hours for CIP and 1,000 µg/mL every 6 hours for FOS. Sixteen mock-inoculated (sterile PBS) larvae were used as controls. The larvae were observed for survival every 24 hours for 4 days.

Statistical analysis

Data were analyzed using SPSS, version 16.0 (SPSS, Chicago, IL, USA). Univariate analysis was performed by the chi-squared test or Fisher's exact test when appropriate. *P*-values were based on two-tailed test results, and *P*-values <0.05 were considered statistically significant.

Results

FICI of CIP/FOS combination treatment

The MICs of CIP and/or FOS against 80 CIP^R *S. flexneri* are shown in Table 1. The CIP/FOS combination showed a synergistic effect in 31 (38.75%) strains and no interaction in 49 (61.25%) strains, while no antagonism was observed in any strains.

Time-kill assays

The complete time-kill data for GN120471 and GN120454 and concentrations (monotherapy and combination therapy) are presented in Figure 1. Log changes in viable cell counts with monotherapy and combination therapy are shown in Table 2.

For the isolate GN120471 (CIP^RFOS^R), monotherapy with CIP or FOS at all concentrations produced little or no bacterial killing (Figure 1A, C and E). The CIP/FOS combination produced enhanced bacterial killing with FOS concentrations of 150 and 300 µg/mL, especially when combined with CIP at 2.5 µg/mL (Figure 1E). With these two combinations (CIP at 2.5 µg/mL plus FOS at 150 or 300 µg/mL), >5 $\log_{10}$ CFU/mL additional killing was achieved over equivalent monotherapy at 24 hours (Table 2). For this isolate, the combination was additive or synergistic in four (11.1%) and 10 (27.8%) of 36 cases (nine combinations at four time-points: 3, 5, 8, and 24 hours), respectively. Furthermore, all combinations containing FOS at 150 or 300 µg/mL at some time-points were additive or synergistic, with enhanced killing primarily across the initial 8 hours of therapy (Table 2 and Figure 1A, C and E). At 24 hours, regrowth was observed with all combinations.

For the isolate GN120454 (CIP^RFOS^S), FOS at the different concentrations produced varying degrees of initial killing, and CIP at all concentrations produced little or no bacterial killing (Figure 1B, D and F). The CIP/FOS combination at all concentrations produced more rapid and extensive killing (up to 5 $\log_{10}$ CFU/mL with many combinations) than with either antibiotic alone, especially when combined with FOS at 150 or 300 µg/mL (Table 2 and Figure 1B, D and F). For this isolate, the combination was additive or synergistic in six (16.7%) and eight (22.2%) of 36 cases, respectively (Table 2). Furthermore, all combinations at 24 hours were synergistic, except for CIP at 0.5 µg/mL plus FOS at 30 µg/mL.

Activities of CIP and FOS in the *G. mellonella* infection model

The concentration of bacteria was approximately 10^4 CFU in the body that could cause approximately 80% of the larvae to die at 96 hours for GN120471 (Figure 2A) and GN120454 (Figure 2B) (bacterial suspension

Table 1 MICs of ciprofloxacin and/or fosfomycin against CIP^R *Shigella flexneri* isolates

No.	Strain	MIC (μg/mL)				FICI
		CIP		FOS		
		Alone	Combined with FOS	Alone	Combined with CIP	
1	GN110052	16	4	2,048	512	0.5
2	GN110066	32	4	512	128	0.375
3	GN110123	16	8	8	2	0.75
4	GN110124	16	1	32	16	0.563
5	GN110130	16	8	256	16	0.536
6	GN110132	16	4	128	16	0.375
7	GN110157	8	2	16	8	0.75
8	GN110161	8	2	256	64	0.5
9	GN110184	8	4	8	4	1
10	GN110194	8	2	4	2	0.75
11	GN110201	8	4	2,048	256	0.625
12	GN110205	8	2	64	32	0.75
13	GN110208	32	8	64	16	0.5
14	GN110210	8	4	32	16	1
15	GN110216	16	2	16	8	0.625
16	GN110226	8	4	128	8	0.563
17	GN110227	16	8	4	2	1
18	GN110248	16	4	512	128	0.5
19	GN110250	16	8	128	16	0.625
20	GN110925	16	8	64	1	0.516
21	GN120002	8	4	128	64	1
22	GN120030	8	2	512	128	0.5
23	GN120158	8	4	32	8	0.75
24	GN120202	32	16	4	2	0.75
25	GN120207	32	4	256	128	0.625
26	GN120242	8	4	4	2	1
27	GN120252	8	4	256	32	0.625
28	GN120255	8	4	16	8	1
29	GN120267	8	2	64	16	0.5
30	GN120269	16	4	2,048	256	0.375
31	GN120278	16	8	64	2	0.531
32	GN120281	32	8	128	32	0.5
33	GN120290	16	4	8	4	0.75
34	GN120291	16	4	16	8	0.75
35	GN120301	16	4	256	32	0.375
36	GN120313	16	4	128	32	0.5
37	GN120454	32	8	64	16	0.5
38	GN120471	32	8	512	128	0.5
39	GN121842	16	8	256	32	0.625
40	GN122107	16	4	32	8	0.5
41	GN130003	8	4	16	4	0.75
42	GN130005	8	4	256	64	0.75
43	GN130014	8	1	4	2	0.625
44	GN130027	8	4	128	32	0.75
45	GN130032	16	4	64	4	0.313
46	GN130044	8	2	512	128	0.5
47	GN130047	32	4	512	128	0.375
48	GN130048	16	8	1,024	128	0.625

(*Continued*)

Table 1 (Continued).

No.	Strain	MIC (μg/mL)				FICI
		CIP		FOS		
		Alone	Combined with FOS	Alone	Combined with CIP	
49	GN130050	16	2	2,048	512	0.375
50	GN130052	16	4	256	32	0.375
51	GN130057	8	4	2,048	128	0.563
52	GN130146	16	4	256	64	0.5
53	GN130151	16	8	128	16	0.625
54	GN130176	32	4	64	16	0.375
55	GN130179	32	8	128	32	0.5
56	GN130199	8	4	32	16	1
57	GN130234	8	2	64	16	0.5
58	GN130238	8	2	1,024	128	0.375
59	GN130255	8	2	8	4	0.75
60	GN131887	16	8	4	2	1
61	GN140017	8	4	16	2	0.625
62	GN140026	32	16	32	8	0.75
63	GN140028	16	4	32	16	0.75
64	GN140029	32	8	32	8	0.5
65	GN140045	32	8	32	8	0.5
66	GN140064	8	2	128	64	0.75
67	GN140066	8	1	128	64	0.625
68	GN140069	8	4	32	8	0.75
69	GN140070	32	4	64	32	0.625
70	GN140071	8	2	16	4	0.5
71	GN140082	8	2	64	32	0.75
72	GN140083	8	4	32	16	0.75
73	GN140096	16	2	256	64	0.375
74	GN140102	8	4	16	1	0.563
75	GN140110	16	4	2,048	512	0.5
76	GN140169	8	2	64	32	0.75
77	GN140197	8	2	4	2	0.75
78	GN140201	8	4	64	8	0.625
79	GN140211	8	4	128	32	0.75
80	GN140224	8	2	128	32	0.5

Abbreviations: MIC, minimal inhibitory concentration; CIP, ciprofloxacin; FOS, fosfomycin; FICI, fractional inhibitory concentration index.

concentration for ~10^6 CFU/mL). Based on these data, 1×10^4 CFU/larva was selected as the inoculum for the subsequent treatment experiments. According to the above research results, the following antimicrobial concentrations were used, simulating human doses: CIP (the steady-state plasma concentration 2.5 μg/mL) and FOS (the steady-state plasma concentration 150 μg/mL). The survival rate for the CIP/FOS combination against GN120471-infected larvae was 68.75% compared with 25% for CIP (χ^2=6.149, P=0.013) and 43.75% for FOS (χ^2=2.032, P=0.154) (Figure 3A). The survival rate for the CIP/FOS combination against GN120454-infected larvae was 81.25% compared with 37.5% for CIP (χ^2= 6.348, P=0.012) and 62.5% for FOS (P>0.05) (Figure 3B).

Discussion

With the increased resistance to CIP, unorthodox combination therapies are increasingly being considered against CIP^R *Shigella* infections. FOS, an old antibiotic, is increasingly used for treatment of infections due to MDR organisms.[23,24] Some studies have shown that the CIP/FOS combination had potent synergy effect in vitro against CIP^R *P. aeruginosa*.[17,18] Although this combination appears to be a promising treatment option based on

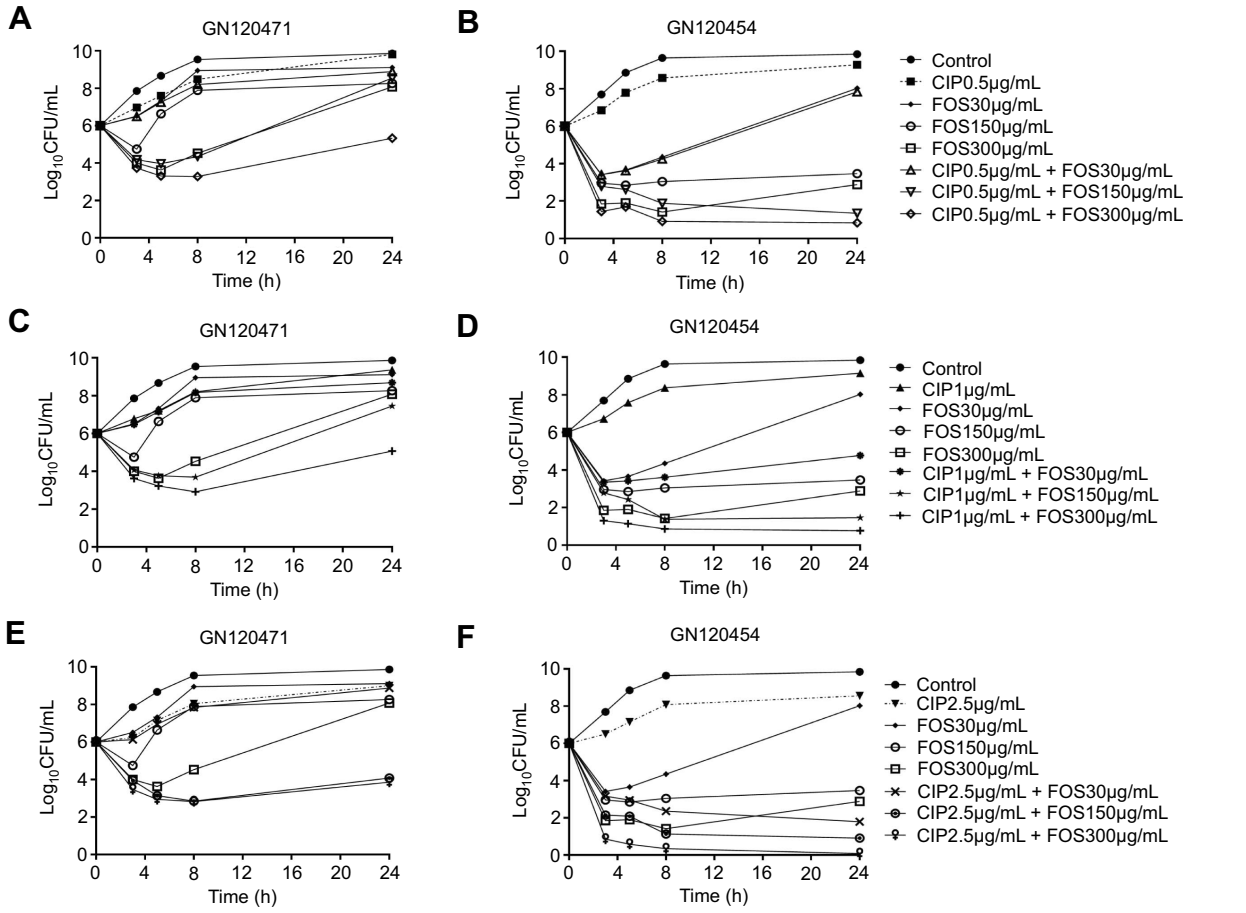

Figure 1 Representative time-kill curves with low, medium, and high concentrations of ciprofloxacin and fosfomycin alone and in combination against CIP^R *Shigella flexneri* isolates. (**A**) CIP (0.5 μg/mL) and FOS (30, 150, 300 μg/mL) alone and in combination against GN120471 (CIP^{R}FOS^R); (**B**) CIP (0.5 μg/mL) and FOS (30, 150, 300 μg/mL) alone and in combination against GN120454 (CIP^{R}FOS^S); (**C**) CIP (1 μg/mL) and FOS (30, 150, 300 μg/mL) alone and in combination against GN120471 (CIP^{R}FOS^R); (**D**) CIP (1 μg/mL) and FOS (30, 150, 300 μg/mL) alone and in combination against GN120454 (CIP^{R}FOS^S); (**E**) CIP (2.5 μg/mL) and FOS (30, 150, 300 μg/mL) alone and in combination against GN120471 (CIP^{R}FOS^R); (**F**) CIP (2.5 μg/mL) and FOS (30, 150, 300 μg/mL) alone and in combination against GN120454 (CIP^{R}FOS^S).
Abbreviations: CIP, ciprofloxacin; FOS, fosfomycin; R, resistance; S, susceptibility.

in vitro data, further preclinical work is clearly needed before it can be considered for clinical use. Therefore, we systematically investigated the effectiveness of the CIP/FOS combination in vitro and in vivo against CIP^R *S. flexneri* isolates.

To the best of our knowledge, this study is the first to confirm that the CIP/FOS combination enhanced in vitro and in vivo activity against CIP^R *S. flexneri* isolates. This may be due to enhancement of FOS cell-wall permeability, which could enhance the activity of CIP. In checkerboard assays, our results provided evidence that CIP possessed a considerable synergy with FOS against CIP^R *S. flexneri* isolates. In time-kill assays, the concentrations of CIP and FOS represented low, medium, and high average steady-state plasma concentrations typically achieved in patients receiving standard doses.[25,26] With the majority of combinations, the addition of FOS to CIP generally resulted in substantial improvements in bacterial killing (up to 5 $\log_{10}$ CFU/mL) over 24 hours for CIP^R *S. flexneri* isolates, especially when CIP at 2.5 μg/mL was combined with FOS at 150 or 300 μg/mL. Moreover, all combinations of CIP/FOS at some time-points were synergistic in vitro for CIP^R *S. flexneri* isolates, with the exception of combinations containing CIP at all concentrations plus FOS at 30 μg/mL for CIP^R *S. flexneri* isolate. This indicated that substantial improvements in bacterial killing were possible with the higher concentrations of the CIP/FOS combination against CIP^R *S. flexneri* isolates.

Larvae of *G. mellonella* have been used as an alternative to study a number of important human pathogens.[27–30] Barnoy et al[31] initially developed a *G. mellonella* model to investigate the pathogenicity of *Shigella* infection and to show that *S. flexneri* reside within a vacuole of the insect hemocytes that ultrastructurally

Table 2 Log changes at 3, 5, 8, and 24 hours with various clinically relevant concentrations of ciprofloxacin and/or fosfomycin against CIP^R *Shigella flexneri* isolates

Isolate	Inoculum (CFU/mL)	Time (hours)	Log change[=$\log_{10}(CFU_t) - \log_{10}(CFU_0)$]														
			CIP 0.5 µg/mL	CIP 1 µg/mL	CIP 2.5 µg/mL	FOS 30 µg/mL	FOS 150 µg/mL	FOS 300 µg/mL	CIP 0.5 µg/mL + FOS 30 µg/mL	CIP 0.5 µg/mL + FOS 150 µg/mL	CIP 0.5 µg/mL + FOS 300 µg/mL	CIP 1 µg/mL + FOS 30 µg/mL	CIP 1 µg/mL + FOS 150 µg/mL	CIP 1 µg/mL + FOS 300 µg/mL	CIP 2.5 µg/mL +FOS 30 µg/mL	CIP 2.5 µg/mL + FOS 150 µg/mL	CIP 2.5 µg/mL + FOS 300 µg/mL
GN120471	~10^6	3	0.97	0.78	0.24	0.51	0.25	−0.64	0.49	−1.82	−2.27	0.47	−1.93	−2.38	0.13	−2.05	−2.53
		5	1.60	1.20	1.14	1.32	0.63	−1.35	1.25	−2.03	−2.69	1.15	−2.24	−2.78	0.95	−2.86	−3.06
		8	2.48	2.20	2.04	2.95	1.89	−1.47	2.20	−1.65	**−2.72**	2.17	−2.31	**−3.08**	**1.84**	−3.15	**−3.18**
		24	3.81	3.36	3.00	3.11	2.26	2.07	2.89	2.55	−0.67	2.68	1.45	−0.93	2.87	−1.92	−2.14
GN120454	~10^6	3	0.85	0.73	0.50	−2.58	−3.04	−4.15	−2.59	−3.21	−4.56	−2.65	−3.22	−4.70	−2.84	−3.85	−5.14
		5	1.79	1.58	1.15	−2.35	−3.15	−4.10	−2.37	−3.38	−4.31	−2.58	−3.57	−4.86	−3.05	−3.91	**−5.42**
		8	2.57	2.37	2.08	−1.65	−2.96	−4.58	−1.75	**−4.12**	−5.08	−2.39	**−4.62**	−5.14	**−3.64**	**−4.87**	**−5.66**
		24	3.28	3.15	2.56	2.02	−2.53	−3.12	1.84	−4.65	−5.16	−1.23	−4.54	−5.23	−4.21	−5.09	−5.92

Note: Underlining indicates synergy (≥2 $\log_{10}$ decrease in the CFU/mL with the combination compared with its most active monotherapy) and bold formatting indicates additivity (1 to <2 $\log_{10}$ decrease in the CFU/mL with the combination compared with its most active monotherapy).

Abbreviations: CIP, ciprofloxacin; FOS, fosfomycin.

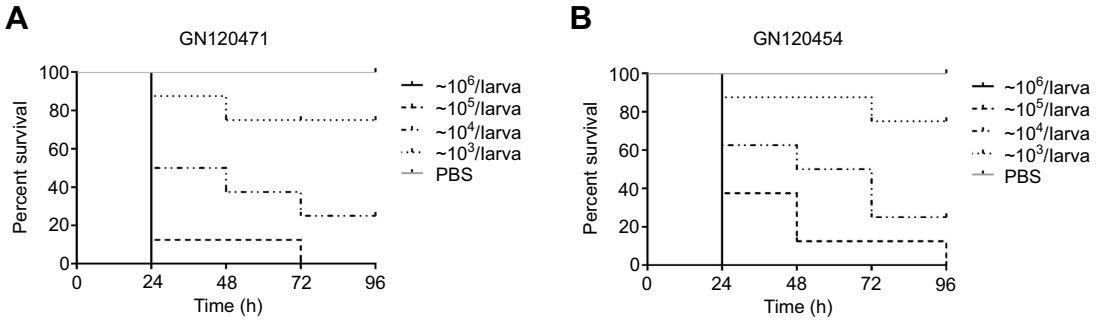

Figure 2 Survival curves of various inoculum doses of *Shigella flexneri* GN120471 (**A**) or GN120454 (**B**) in *Galleria mellonella* larvae during incubation at 37°C for 96 hours. Curves were plotted using 10 larvae for every experiment. Larvae were inoculated with 10 μL of bacterial suspensions. Mortality at 24 hours was 100% at 10^6 CFU/larva (bacterial suspension concentration for ~10^8 CFU/mL).

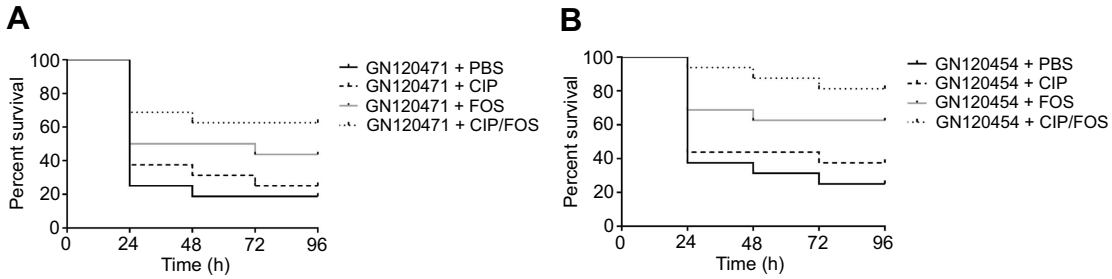

Figure 3 Survival curves for *Galleria mellonella* larvae by CIP (steady-state plasma concentration 2.5 μg/mL) and FOS (steady-state plasma concentration 150 μg/mL) alone and in combination against *Shigella flexneri*isolates GN120471 (**A**) or GN120454 (**B**). Curves were plotted using 16 larvae for every experiment during incubation at 37°C for 96 hours. Bacterial suspension concentration for 1×10^6 CFU/mL (1×10^4 CFU/larva) was selected as the inoculum for the treatment experiments. Univariate analysis was performed by the chi-squared test or Fisher's exact test when appropriate. The survival rate for CIP/FOS combination against GN120471-infected larvae was 68.75% compared with 25% for CIP (χ^2=6.149, *P*=0.013) and 43.75% for FOS (χ^2=2.032, *P*=0.154). The survival rate for CIP/FOS combination against GN120454-infected larvae was 81.25% compared with 37.5% for CIP (χ^2=6.348, *P*=0.012) and 62.5% for FOS (*P*>0.05).
Abbreviations: CIP, ciprofloxacin; FOS, fosfomycin.

resemble vacuoles, with mouse and human macrophage cell lines. We applied this model to study the in vivo activities of the CIP/FOS combination against CIP^R *S. flexneri* isolates. In this study, mortality at 96 hours was around 80% at approximately 10^4 CFU/larva for GN120471 and GN120454 (bacterial suspension concentration for ~10^6 CFU/mL). The CIP/FOS combination (CIP 2.5 μg/mL and FOS 150 μg/mL) was significantly more effective than CIP monotherapy (P<0.05) in protecting larvae from lethal infections with CIP^R *S. flexneri* isolates, which was consistent with the in vitro results of the CIP/FOS combination against CIP^R *S. flexneri* isolates. An interesting finding was that the CIP/FOS combination in the larvae model showed no significant difference compared to FOS monotherapy (P>0.05), despite its performance in vitro being better than any drug individually. It was speculated that this phenomenon might result from immunomodulatory activities of FOS in *G. mellonella* larvae because FOS exerted immunomodulatory effects by altering lymphocyte, monocyte, and neutrophil function.[32] It is possible that certain immunological effects augment the antibacterial activity of FOS, leading to better efficacy than observed in vitro. Furthermore, although preliminary evidence of in vivo efficacy has been obtained through the use of invertebrate infection models, additional studies using mammalian infection models are required.

Conclusion

Our in vitro and in vivo results provide clues to understanding the synergistic effect of CIP combined with FOS and to developing more effective therapy against CIP^R *S. flexneri* isolates. One possible mechanism for the enhanced bacterial killing is through the increased uptake of one antibiotic by the other. Further studies are required to clarify the underlying mechanism of their synergistic effect.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

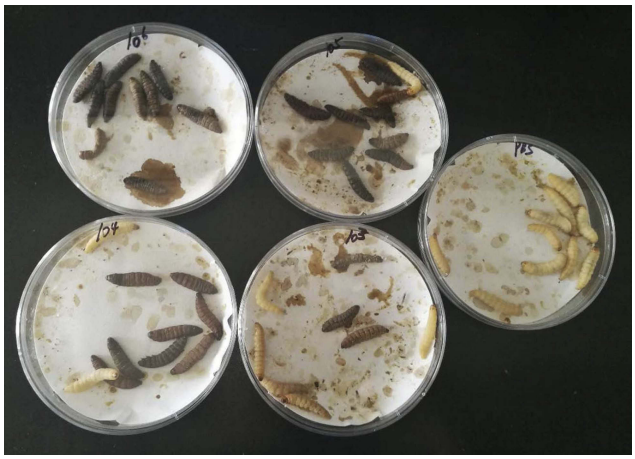

Figure S1 Larvae infected with *Shigella flexneri* (10^3, 10^5, 10^6, and 10^7 CFU/larva) at 96 hours.