

Assessing the Emergence of Resistance in vitro and In vivo: Linezolid Combined with Fosfomycin Against Fosfomycin-Sensitive and Resistant *Enterococcus*

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Purpose: We aimed to evaluate the synergistic effect of linezolid and fosfomycin on fosfomycin-sensitive and -resistant *Enterococcus* clinical isolates in vitro and in vivo and whether the emergence of fosfomycin resistance in *Enterococcus* is associated with changes in strain virulence, from the perspective of fitness cost.

Methods: The synergistic effect of linezolid and fosfomycin was studied via in vitro checkerboard and static time-kill assays, as well as based on the in vivo survival rate and hemolymph load of a *Galleria mellonella* infection model. Fosfomycin resistance was induced via a stepwise increase in concentration. Changes in the virulence of the strains after drug resistance were investigated using the *G. mellonella* infection model and reverse transcription quantitative polymerase chain reaction (RT-qPCR). In vitro and in vivo growth curves and competitive experiments were used to study the fitness cost of the strain. Finally, a static time-kill assay was performed to explore the effect of the combined medication.

Results: In vitro and in vivo data showed that linezolid combined with fosfomycin had a good synergistic effect on *Enterococcus* treatment. The *G. mellonella* infection model and RT-qPCR data showed that the virulence of the resistant strains was weakened to varying degrees. A survival curve and competition experimental data showed that this was related to the fitness cost of strains while acquiring resistance and negatively impacted linezolid treatment; however, the combination still showed a good synergistic effect in drug-resistant strains.

Conclusion: Linezolid combined with fosfomycin had a synergistic effect on both fosfomycin-sensitive and -resistant *Enterococcus* strains. Strains incur fitness costs as they develop drug resistance, which leads to a decrease in virulence. There is an interaction between fitness cost, virulence, and drug resistance, which indirectly affects drug treatment.

Keywords: linezolid, fosfomycin, resistance, fitness cost, virulence

Introduction

Enterococcus is a gram-positive bacterium that can cause bacteremia, endocarditis, urinary tract infection, etc.^{1–3} Because of its rapid adaptation to human hosts and the environment, it is the main cause of community and hospital infections.^{4,5} Moreover, owing to its resistance to almost all main first-line clinical antibiotics, such as cephalosporins, aminoglycosides, clindamycin, and trimethoprim-sulfamethoxazole, antimicrobial selection is currently difficult.^{6–8} Linezolid is an oxazolidinone antibiotic that significantly affects the treatment of vancomycin-resistant *Enterococcus* (VRE) infection.⁹ However, with the excessive use of antibiotics in hospitals in recent years, linezolid-resistant

Enterococcus strains are gradually appearing in various places.¹⁰ Drug combinations have been widely used to reduce the development of drug resistance. In vitro studies have shown that linezolid combined with fosfomycin has a good synergistic effect on clinical isolates of vancomycin-susceptible *Enterococcus* and VRE strains.^{11,12} However, the efficacy of this combination in the treatment of infection with fosfomycin-resistant *Enterococcus* has not been reported.

Galleria mellonella has been widely used to evaluate the pathogenicity of bacterial and fungal pathogens and determine the in vivo efficacy of established and novel antimicrobial agents.^{13,14} Because of its structural and functional similarity with the mammalian immune system, the infection process can be carried out in a certain temperature range (from 15 °C to over 37 °C).¹² The infection model of *G. mellonella* was studied using larval survival, bacterial load, and blood cell density.¹⁵ In a previous study, Qi and Li et al observed a synergistic effect of drugs, mainly by observing the survival rate of larvae,^{12,16} but hemolymph load was used as an important supplement for single- and combined-drug therapy, which can more intuitively embody medicaments to the curative effect of organisms.^{17,18}

When judging the synergistic effect, it is necessary to not only select susceptible strains but also study resistant strains and whether drug resistance will affect treatment. Meanwhile, a study on the treatment of enterococcal infection with fosfomycin by Mao et al using a static time-kill assay and an in vitro dynamic PK/PD model showed excellent bactericidal activity of fosfomycin in the first 4–8 h; however, in the dynamic process of treatment, a duration of 6–8 h leads to the regeneration of resistant mutants, which affect the linezolid treatment.¹⁹ Few reports have discussed fosfomycin resistance. The characteristics of easy resistance to fosfomycin were demonstrated by a static time-kill assay in vitro in previous studies, but the interaction between fosfomycin-resistant *Enterococcus* strains and the body and its related mechanisms have rarely been reported. The development of antibiotic resistance is often accompanied by fitness costs, defined as changes in competitiveness in antibiotic-free environments.^{20,21} This phenomenon often leads to the selection of winners, which may lead to the replacement of susceptible with resistant strains, thus leading to the proliferation of resistant strains. Competitive experiments provide the “gold standard” for studying fitness costs.²² The fitness cost of *Escherichia coli* carrying resistant genes may be associated with the virulence of the strain, increasing the potential risk of infection, while indirectly impairing the therapeutic effect of colistin.²³ Thus, the difference in treatment may be related to this hypothesis, as shown by Wang’s study; VRE virulence gene expression (especially *acm*) and mortality of *G. mellonella* decreased with an increase in fosfomycin resistance, and virulence of the strain presumably decreased with an increase in fosfomycin resistance.²⁴ In addition, competition of *Enterococcus* strains to acquire fosfomycin resistance has not been reported yet. Studies on *Enterococcus* have mostly focused on in vitro rather than in vivo competition.

Therefore, in this study, we explored the synergistic effect of linezolid combined with fosfomycin on fosfomycin-susceptible strains via checkerboard and static time-kill assays. The synergistic effect of the drug combination was further verified based on the survival rate and hemolymph load of the *G. mellonella* infection model. Through artificial induction of fosfomycin-resistant strains, the virulence of susceptible bacteria and corresponding resistant bacteria was studied using the *G. mellonella* infection model and quantitative real-time PCR. Growth curves and competition experiments were used to measure the fitness costs of the strain. Finally, NO.22 was selected for the static time-kill assay to study the influence of the fitness cost of strains on drug therapy.

Materials and Methods

Strains, Medium, and Antibiotics

The strains were derived from 19 clinical isolates of *Enterococcus*, mainly collected from patients’ urine and blood samples at the First Affiliated Hospital of Anhui Medical University in 2020. They were part of the routine hospital laboratory procedure. This study was approved by the institutional review board of the First Affiliated Hospital of Anhui Medical University. All strains were identified using the automated Vitek-2 system (Marcy l’Etoile BioMérieux, France). ATCC 51299 was used as the quality control strain.

Linezolid and fosfomycin were purchased from the National Institute for Food and Drug Control of China (Beijing). Glucose-6-phosphate (G-6-P) was purchased from Sigma-Aldrich. Mueller–Hinton broth (MHB) (Oxoid, England) and MH agar (MHA) (Oxoid, England) were used for the susceptibility, checkerboard, and time-kill assays.

In vitro Sensitivity Test and Checkerboard Assay

The minimal inhibitory concentrations (MICs) of linezolid and fosfomycin were determined via the agar dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) Standard Methods guidelines.²⁵ Briefly, 0.1 mL of antibiotics and 0.1 mL of a final bacterial inoculum of 5×10^5 colony-forming units (CFU)/mL made from fresh broth were placed in each well. The antibiotic concentration was diluted using the doubling dilution method, and the fosfomycin agar plate also included G-6-P at a final concentration of 25 mg/L. The plates were incubated at 37 °C for 18–24 h. The MIC was defined as the lowest drug concentration without visible colony growth. According to the CLSI 2021 guidelines,²⁵ drug resistance (R) was defined as MIC \geq 8 mg/L linezolid and MIC \geq 32 mg/L fosfomycin. MIC assays were performed in triplicate for each strain. ATCC 29212 was used as the quality control strain.

The synergistic effects of linezolid and fosfomycin at different concentrations were evaluated via a checkerboard assay. The concentration of linezolid ranged from 0.03125 mg/L to 8 mg/L, and that of fosfomycin ranged from 0.5 mg/L to 256 mg/L. Each strain was inoculated into a 96-well plate to obtain suitable suspension (10^5 CFU/mL) with a final volume of 200 μ L (25 mg/L G-6-P) and incubated at 37 °C for 18–22 h. All experiments were repeated in triplicate. The fractional inhibitory concentration index (FICI) was defined as follows: FICI = (drug A combined MIC/Drug A alone MIC)/(drug B combined MIC/Drug B alone MIC). The effect of FICI on *Enterococcus* was explained as follows: FICI \leq 0.5, synergistic effect; $1 < \text{FICI} \leq 4$, no difference; and FICI > 4 , antagonistic effect.²⁶

Static Time-Kill Assays

The static time-kill assays followed the method described above.²⁷ The initial vaccination of bacteria in the 10-mL MHB system was $\sim 1 \times 10^6$ CFU/mL, the designed concentrations of linezolid were 2 mg/L and 4 mg/L, and those of fosfomycin were 128 mg/L and 256 mg/L. The bacterial suspension was incubated at 37 °C with moderate shaking, and CFU were counted at 0, 2, 4, 6, 8, 10, and 24 h. Three replicates were performed for each strain. The synergistic effect was defined as a reduction of more than 2 log₁₀ CFU/mL at 24 h compared to that of the most active single drug.

Galleria Mellonella Infection Model

G. mellonella (Tianjin, China) was used to study the effect of linezolid combined with fosfomycin on strain NO.22. As previously described,²⁸ *Enterococcus* was centrifugally precipitated in overnight cultures using phosphate buffer saline (PBS) to adjust the concentration, using a 20- μ L Hamilton syringe (Hamilton, Shanghai, China) to enter the last left front leg of larvae, followed by inoculation of 10 μ L of different bacterial suspensions (10^6 , 10^7 , and 10^8 CFU/larva), and the concentration that caused 80% mortality in larvae at 24 h was determined; the bacterial colony count was used to confirm the consistency of the inoculum. After 2 h of infection, we applied antibiotics (linezolid 5 and 10 mg/kg, fosfomycin 100 and 200 mg/kg) alone or in combination (linezolid 5 mg/kg + fosfomycin 100 mg/kg, linezolid 10 mg/kg + fosfomycin 200 mg/kg); 10 mg/kg of linezolid and 200 mg/kg of fosfomycin were based on human doses, and larvae injected with PBS alone were used as controls.²⁹ We selected 16 larvae from each group weighing 250–350 mg without gray spots. The number of dead caterpillars was recorded every 24 h for 120 h. When the body blackens or shrivels and there is no movement and no response to touch, they are considered dead. At 120 h, the hemolymph of surviving larvae was collected, the bacterial load was counted, and the treatment effect was completed in triplicate. The primary outcome of the insect model was to assess the rate and extent of death of *G. mellonella* using Kaplan–Meier analysis and Log rank test.

In vitro Induction of Fosfomycin-Resistant Strains

In vitro artificial induction of drug-resistant strains was carried out in accordance with the above method.³⁰ Briefly, the concentrations were increased stepwise to induce drug-resistant strains in vitro. First, the strain was incubated overnight in $1/2 \times \text{MIC}$ (64 mg/L) drug-containing MHB, and the centrifuged strain was added to $1 \times \text{MIC}$ (128 mg/L) drug-containing medicine broth for 24 h to gradually increase the concentration of fosfomycin up to 1024 mg/L. After incubation for 24 h, the bacterial liquid was uniformly spread on an MHA plate containing 1024 mg/L fosfomycin, and the bacterial liquid was placed in a 37 °C incubator overnight. Then, the selected drug-resistant mutants were confirmed using an antibacterial agent-free medium, and serial passages were passaged 20 times. Finally, the MIC was determined via strain analysis using linezolid and fosfomycin.

Survival of *G. mellonella*

To study whether strain virulence changes correspondingly after acquired drug resistance, the above methods were used to inject 10^8 CFU/mL strains into *G. mellonella* and observe the survival rate.

Relative Quantification of Virulence Gene Expression

RNA from the parent and drug-resistant strains was extracted using *RNAex Pro* Reagent (Agbio Co., Hunan, China) in accordance with the manufacturer's instructions. cDNA was synthesized using *Evo M-MLV* RT Master Mix (Agbio Co., Hunan, China). Real-time PCR was performed using SYBR[®] Green *Pro Taq HS* Premix II (Rox Plus) (Agbio.co, Hunan, China) on a fluorescence quantitative PCR instrument (Roche LightCycler 96, Switzerland). The target gene expression levels were normalized to that of the housekeeping gene (*gyrb*) mRNA and determined via the $2^{-\Delta\Delta CT}$ calculation method, where *CT* is the threshold cycle. The primers used in this study are listed in [Supplementary Table 1](#).

Bacterial Growth Curve in vitro and in vivo

The parental strain NO.22 and the drug-resistant strain NO.22R were selected, and fresh overnight cultured strains were diluted to 1×10^6 CFU/mL in 10 mL MHB and placed in a 37 °C incubator for shaking. Ten microliters of the bacterial solution were extracted from the culture system at 0, 4, 8, 12, 24, and 48 h and coated on an agar plate with a sterile L-shaped plastic coated rod. The samples were incubated at 37 °C for 24 h and counted. In the in vivo experiment, the hemolymph of infected larvae was collected at the same time points as above, diluted, and spotted.

In vitro and in vivo Competition Experiments

As mentioned previously,³¹ a competition experiment of the strains was carried out. Briefly, the mixed bacterial liquid of NO.22 and NO.22R (1:1) was cultured under the above conditions, and cultured bacteria were sucked at 0, 4, 8, 12, 24, and 48 h. The CFU of the culture solution was counted on an agar plate and an agar plate containing $1 \times$ MIC fosfomycin for 24 h. Fitness was calculated using a competition experiment, and the relative fitness (*W*) of the mutant to wild type was calculated as follows:

$$W = \frac{\log_{10}(RF/RI)}{\log_{10}(SF/SI)}$$

Where RF is the number of resistant bacteria at the end of culture, RI is the number of resistant bacteria before culture, SF is the number of susceptible bacteria at the end of culture, and SI is the number of susceptible bacteria before culture. $W < 1$ indicates that the competitive fitness of S is better than that of R, $W = 1$ indicates that the competitive fitness of S and R is similar, and $W > 1$ indicates that the competitive fitness of R is better than that of S.³²

To further study the competition between the mutant and parent body, the same volume of NO.22 and NO.22R suspensions was adjusted with PBS to a bacterial concentration with lethality of 80% and injected into the larvae. The hemolymph was collected at the same time point as in vitro, and the hemolymph was uniformly spread on a common agar plate and an agar plate containing $1 \times$ MIC fosfomycin and incubated at 37 °C for 24 h. A colony count was performed to calculate the load of hemolymph fluid in each node.

Static Time-Kill Assay of Fosfomycin-Resistant Strain

NO.22R was selected for static time-kill assay. The concentrations of linezolid were 2 mg/L and 4 mg/L and those of fosfomycin were 2048 mg/L and 4096 mg/L.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism, version 8.0 (GraphPad Software, Inc., San Diego, CA, United States). One-way ANOVA was used to evaluate significant differences in mRNA expression. Survival analyses were performed using Kaplan–Meier survival curves, and significant differences between groups were tested using the Log rank test. Statistical significance was set at $P < 0.05$.

Results

MIC results and Checkerboard Assay

The susceptibility and FICI data of the 19 clinical isolates of linezolid and fosfomycin are shown in Table 1. The MIC assay for linezolid showed that 89.5% (17/19) of *Enterococcus* isolates were susceptible, 5.26% (1/19) were intermediate, and 5.26% (1/19) were resistant. In the determination of the fosfomycin MIC, 57.9% (11/19) of *Enterococcus* were susceptible, 36.8% (7/19) were intermediate, and 5.26% (1/19) were resistant. The FICI value of the experimental strains revealed that the combination of linezolid and fosfomycin had a synergistic or additive effect on 68.4% (13/19) of the tested strains in vitro, and there was no antagonism between the two drugs. Among them, NO.22, NO.23, and NO.24 showed a better synergistic effect on linezolid and fosfomycin than the other strains; therefore, three strains were selected as the main experimental objects.

Table 1 MICs of Antimicrobial Agents Against Nineteen Strains of *Enterococcus*

Isolates	MIC (mg/L)		MIC in Combination	
	LZD	FOS	LZD+FOS	FICI
22	2	64	1+8	0.625
23	2	64	1+8	0.625
24	2	64	1+8	0.625
116	4	64	1+32	0.75
118	2	64	1+32	1.0
226	2	64	1+16	0.75
231	2	128	1+128	1.5
300	2	64	1+8	0.625
593	8	64	4+8	0.625
605	2	128	2+16	1.125
633	2	128	1+16	0.625
643	1	64	0.25+32	0.75
662	2	256	0.25+128	0.625
673	2	128	1+32	0.75
674	2	64	2+16	1.25
694	2	64	1+32	1.0
680	2	128	2+16	1.125
681	2	128	1+32	0.75
29,212	2	128	1+64	1.0

Abbreviations: FOS, fosfomycin; LZD, linezolid; FICI, fractional inhibitory concentration index; MIC, minimal inhibitory concentration of antibiotics.

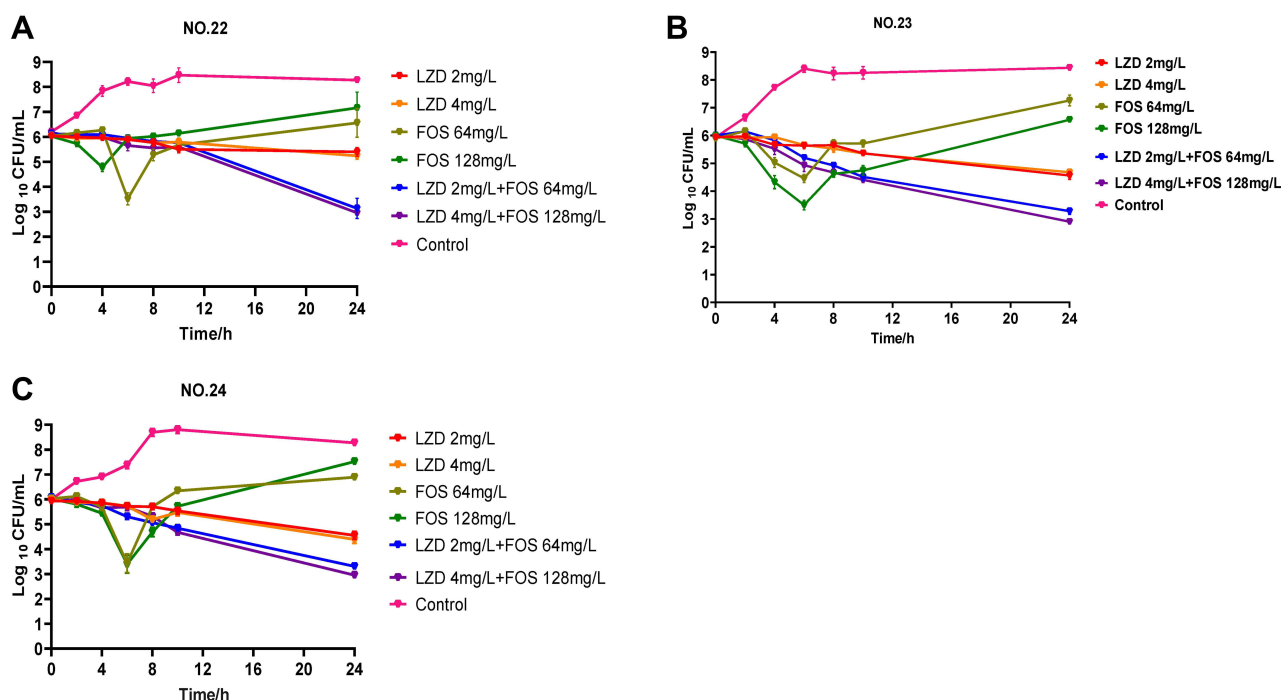


Figure 1 Static time-kill assays displaying the activity of linezolid, fosfomycin and their combination against *Enterococcus*. NO.22 (A), NO.23 (B), and NO.24 (C); Control: no drug.

Abbreviations: FOS, fosfomycin; LZD, linezolid.

In vitro Static Time-Kill Assays

The results of the static time-kill assay are shown in Figure 1 and Table 2. Linezolid at 2 mg/L and 4 mg/L had similar therapeutic effects and could cause ~ 1 \log_{10} value of bacterial killing in 24 h. For all strains, all concentrations of fosfomycin (64 mg/L and 128 mg/L) resulted in 1–2 \log_{10} bacterial kills in 4–8 h. However, at 8–12 h, all strains regrew after fosfomycin treatment, although the final population density was still slightly lower (~ 1 \log_{10} CFU/mL) than that of the untreated control group. In contrast, all combination groups resulted in sustained bacterial killing at 24 h of treatment, and the total bacterial count did not exceed 4 \log_{10} values at 24 h. Among them, the low-dose combination group (2 mg/L LZD + 64 mg/L FOS) showed an excellent bactericidal effect, and there was no significant difference in the therapeutic effect compared with the high-dose combination group (4 mg/L LZD + 128 mg/L FOS). Compared with the initial colony count ($\Delta \log_{10} \text{CFU}_{0-24\text{h}}$), changes in bacterial counts after 24 h of treatment in the low-dose combination groups were -2.99 ± 0.3 , -2.73 ± 0.07 , and -2.77 ± 0.06 \log_{10} values, respectively. The final results showed that the low-dose combination groups had additive effects on all three strains, with -2.35 , -1.35 , and -1.38 \log_{10} values, of which NO.22 was the most significant, showing a synergistic effect.

Table 2 $\Delta \log \text{CFU}_{0-24\text{h}}$ Values of Linezolid and Fosfomycin as Monotherapy and in Combination

Strains ($\text{MIC}_{\text{LZD}} / \text{MIC}_{\text{FOS}}$)	$\Delta \log \text{CFU}_{0-24\text{h}}$ by Antibiotic Therapy (Mean \pm SD) (n = 3)						
	No drug	Linezolid		Fosfomycin		Combination	
Concentration (mg/L)	–	2	4	64	128	2+64	4+128
NO.22 (2/64)	2.07 \pm 0.10	-0.64 \pm 0.14	-0.81 \pm 0.11	0.53 \pm 0.48	1.14 \pm 0.51	-2.99 \pm 0.3	-3.22 \pm 0.07
NO.23 (2/64)	2.5 \pm 0.09	-1.38 \pm 0.10	-1.27 \pm 0.06	1.37 \pm 0.17	0.57 \pm 0.11	-2.73 \pm 0.07	-3.11 \pm 0.07
NO.24 (2/64)	2.27 \pm 0.07	-1.39 \pm 0.13	-1.61 \pm 0.14	0.86 \pm 0.07	1.49 \pm 0.04	-2.77 \pm 0.06	-3.02 \pm 0.02

Galleria mellonella Infection Model

To verify the therapeutic effect of the combination of linezolid and fosfomycin against *Enterococcus* in vivo, we used a *G. mellonella* infection model. As shown in Figure 2A, exploration of the preliminary concentration showed that 1×10^8 CFU/mL was 80% of the lethal dose. Almost the entire control group with injected strains died within 120 h, while the survival rate was significantly improved by drug treatment ($P < 0.05$). As shown in Figure 2B, the survival rate in the monotherapy group was significantly lower than that in the combination therapy group ($P < 0.05$), and there were slight differences between the monotherapy groups. The mortality in the high-dose linezolid group was similar to that in the low-dose group. The fosfomycin monotherapy group showed excellent antibacterial efficacy in vivo, with similar survival rates in the fosfomycin high-dose monotherapy (200 mg/kg) and low-dose combination therapy groups (5 mg/kg + 100 mg/kg), although not as good as high-dose linezolid combined with fosfomycin (10 mg/kg + 200 mg/kg). Although the combination of high-dose linezolid and fosfomycin was superior to the low-dose combination, no significant differences were observed.

Figure 2C shows the hemolymph load of larvae treated with either monotherapy or combined therapy; the bacterial burden of the latter was significantly lower than that of the former ($P < 0.05$), and the efficacy of linezolid was similar. Fosfomycin, as shown in previous survival data, significantly reduced the bacterial load compared with linezolid ($P < 0.05$). The effect of high-dose fosfomycin monotherapy was similar to that of low-dose combined therapy and caused a decrease of -3.85 and -4.98 \log_{10} values, respectively, compared to the control group. Both combination groups could achieve significant bactericidal effects, but high-dose combination therapy improved survival rates; however, the bactericidal effect was slightly inferior to that of the low-dose combination group. Overall, this suggests that linezolid combined with fosfomycin has a synergistic effect in the treatment of *Enterococcus* infection in vivo.

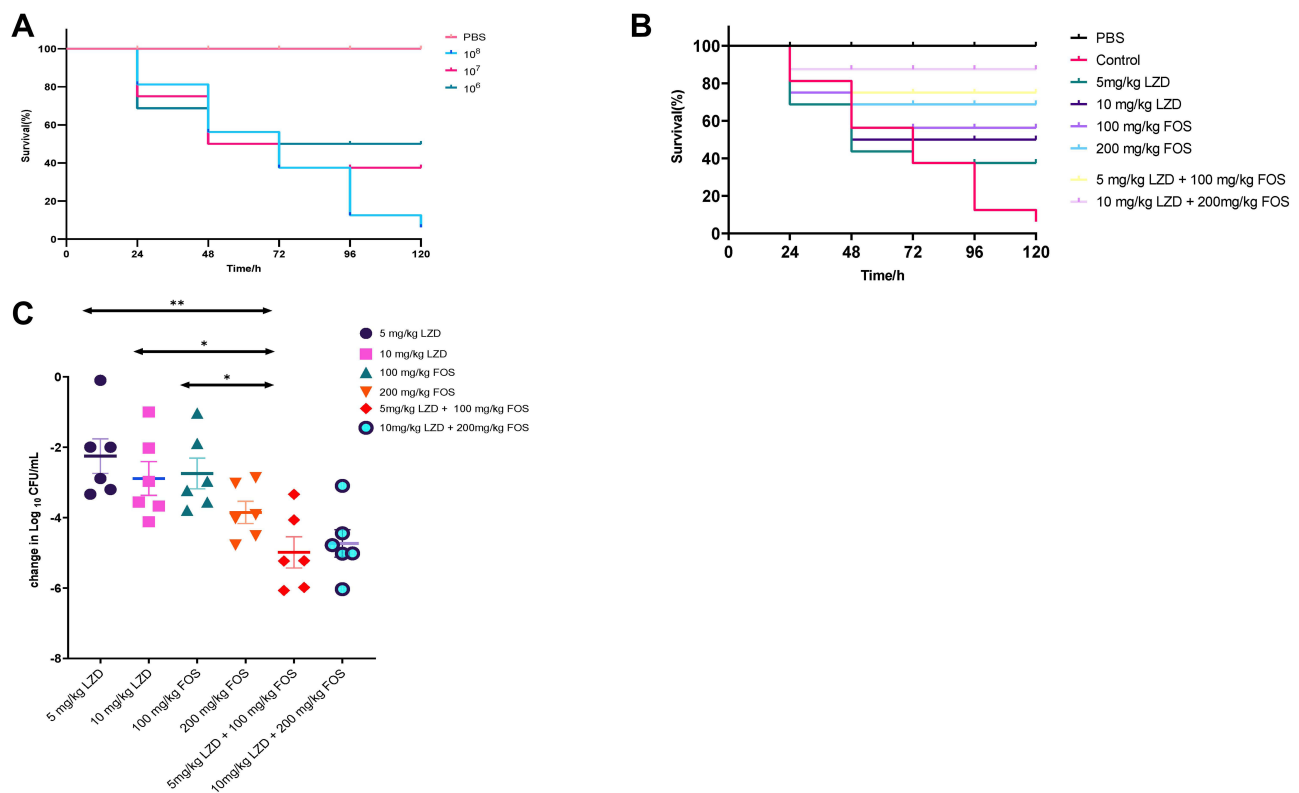


Figure 2 In vivo assays using *G. mellonella* model. (A) exploration of the concentration of 50% lethal bacteria (B) survival curves of infected *G. mellonella* larvae treated with with linezolid and fosfomycin at different concentrations alone or in combination. (C) Haemolymph CFU burden of *G. mellonella* after infection with NO.22 followed by antibiotic treatment. CFU/mL values are shown for the drug treatment groups in relation to the mean CFU/mL value for the control group, indicating the reduction in \log_{10} CFU/mL due to treatment. Data expressed as mean \pm standard deviation of three independent experiments (* $P < 0.05$, ** $P < 0.01$).

Table 3 MICs of Antimicrobial Agents Against Four Strains

Stains	MIC (LZD) mg/L	MIC (FOS) mg/L
NO.22R	2	2048
NO.23R	2	1024
NO.24R	2	1024
29,212	2	128

In vitro Induction of Resistance of Fosfomicin

The susceptibility of induced strains was measured by the method described above. The sensitivity of the resistant strain did not change when it was passed 20 times on the blank plate, indicating good stability of the bacteria, and the MICs are shown in Table 3.

Survival of *Galleria mellonella* Infection Model

To determine the effect of fosfomicin resistance on the virulence of *Enterococcus faecalis*, we used the *G. mellonella* infection model to test virulence. The induced drug-resistant strains were inoculated with *G. mellonella* at a density of 10 μ L and 1×10^8 CFU/mL. A comparison of the survival rate curves showed that the virulence of the three strains before and after drug resistance was significantly downregulated ($P < 0.05$). As shown in Figure 3A, which NO.22R was the most representative. The survival rate of this strain was significantly increased by 75%. Figure 3B shows that NO.23R, the survival rate increased by only 31.25%, and Figure 3C showed that NO.24R increased by 56.25%.

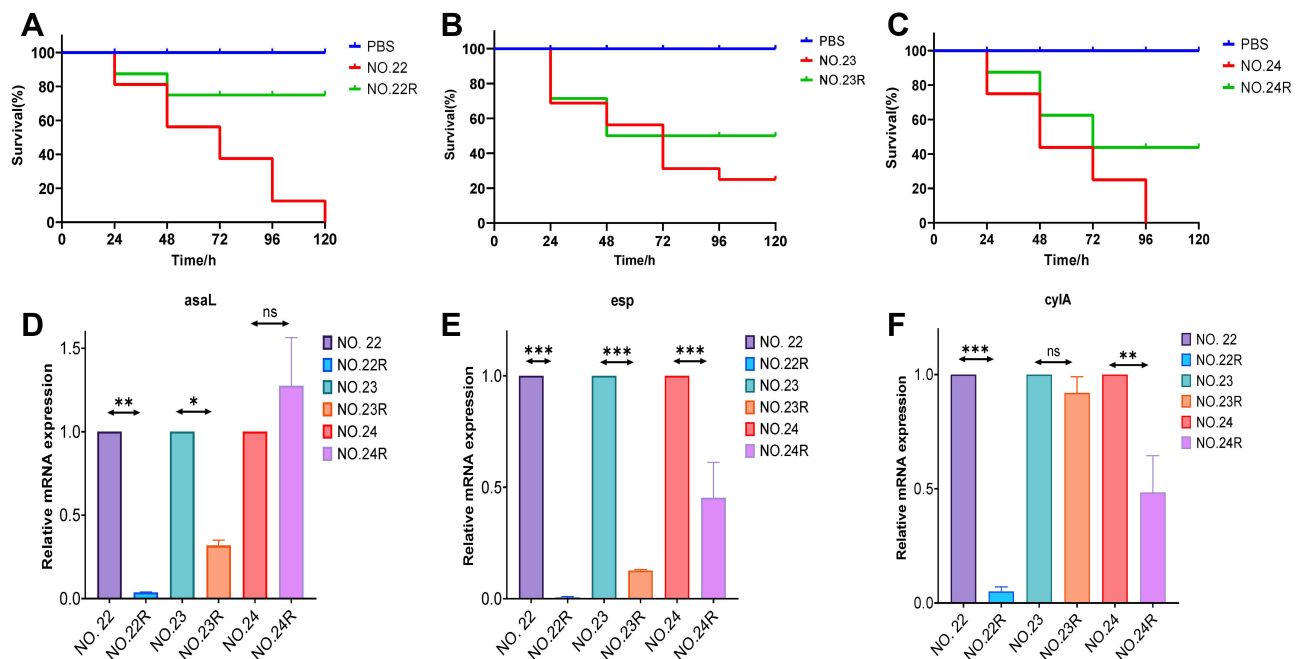


Figure 3 Virulence analysis of fosfomicin-resistant mutants (A–C). The *Galleria mellonella* infection model was used to explore the difference in pathogenicity between baseline isolates (NO.22, NO.23, NO.24) and fosfomicin-resistant mutants (NO.22, NO.23, NO.24). (D–F) The relative mRNA expression levels of virulence genes (D) *asaL*, (E) *esp*, (F) *cyaA* were compared between baseline isolates and fosfomicin-resistant isolates mutants. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). NO.22R, isolate resistant to NO.22; NO.23R, isolate resistant to NO.23; NO.24R, isolate resistant to NO.24.

Relative Quantification of Virulence Gene Expression

Parental and drug-resistant strains also differ in the expression of virulence genes. Virulence genes of the strains were downregulated after drug resistance. As illustrated in Figure 3D, compared with that of the parental strains, the expression of *asaL* in NO.22R and NO.23R decreased by 0.03 and 0.31 times respectively, while that of NO.24R increased by 1.27 times. Figure 3E depicted the expression of *esp* decreased by 0.005, 0.12, and 0.29 times, respectively, and Figure 3F showed that of *cylA* decreased by 0.07, 0.86, and 0.48 times, respectively. Compared to the parental strain, there were significant differences ($P < 0.05$). This corresponded to the survival data of the *G. mellonella* infection model, indicating a correlation between resistance to fosfomycin and the release of virulence factors. Increasing exposure to fosfomycin causes a decrease in virulence factors.

Growth Curves of Fosfomycin-Resistant Strain

As shown in Figure 4A, in vitro data showed that the growth rate of drug-resistant strain NO.22 was slower than that of the parental strain, but it was flat with no significant change. Figure 4B shows that the growth rate of drug-resistant strains in vivo was slower than that in the in vitro culture, but there was no significant difference in the growth rate of the parental strains ($P > 0.05$).

Competitiveness of Fosfomycin-Resistant Strain

To further investigate whether the emergence of drug-resistant strains during treatment affects the growth of normal strains, we adopted a co-cultivation method. Both in vitro and in vivo adaptive data showed that NO.22 had a fitness cost after drug resistance. As depicted in Figure 4C, In vitro studies confirmed that the competition of drug-resistant strains showed the best result at 4 h, with a value of 0.62; then, the adaptability increased and competition declined and tended to level off. The in vivo data indicated that adaptivity was higher (over 0.7), the competition was lower, and the peak value was 0.74 at 8 h in Figure 4D. These data suggest that susceptible strains gradually replace resistant strains during co-culture; therefore, susceptible strains are still the main target of treatment. The in vitro static time-kill assay showed that the single-agent treatment with fosfomycin would be resistant within 4 h, which might be related to the low adaptation in vitro.

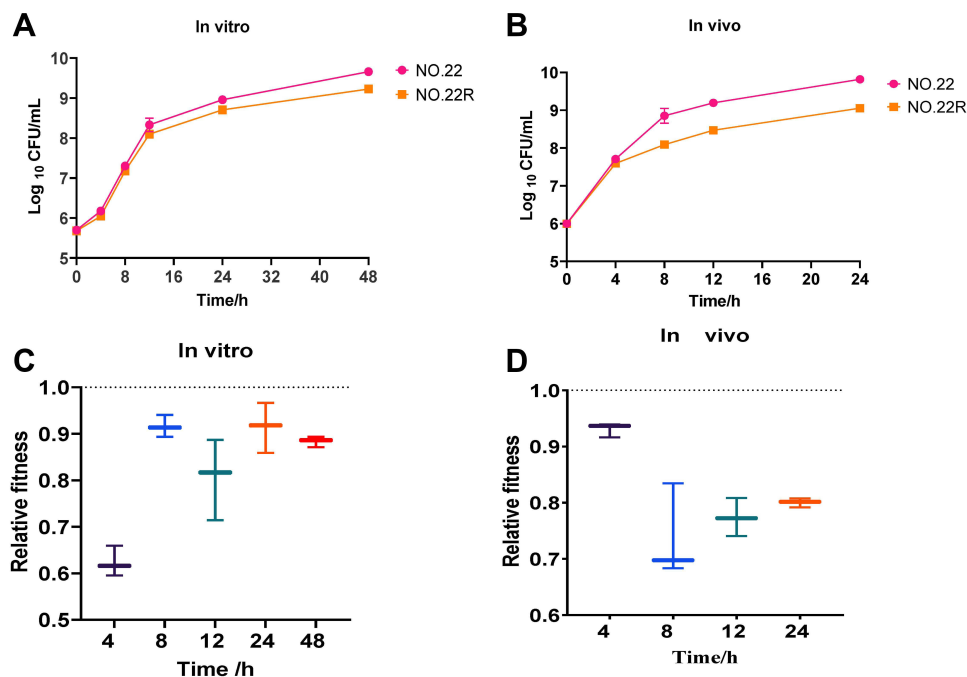


Figure 4 Fitness cost analysis of fosfomycin-resistant mutant NO.22. (A and B) Growth curve of drug-resistant mutant NO.22 and parental strain NO.22 cultured separately in vitro and in vivo. (C and D) Relative fitness of fosfomycin-resistant mutant NO.22R and parental strain NO.22 in co-culture in vitro and in vivo. Relative fitness value less than 1 indicates fitness defect, which incurs fitness cost and a value greater than 1 indicates fitness benefit.

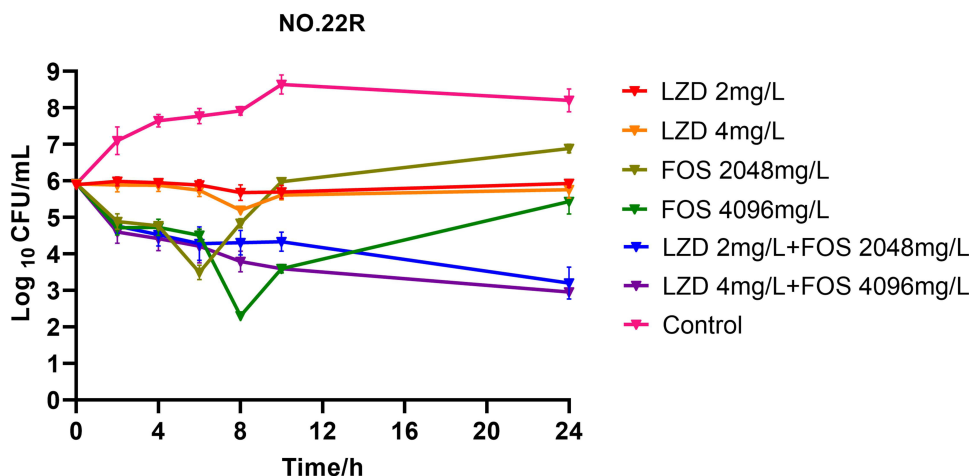


Figure 5 Static time-kill assays displaying the activity of linezolid, fosfomycin and their combination against *Enterococcus* NO.22R; Control: no drug. **Abbreviations:** FOS, fosfomycin; LZD, linezolid.

Static Time-Kill Assay of the Fosfomycin-Resistant Strain

We selected strain NO.22 in this assay, which induced the least virulent and lethal strains of the drug-resistant strains. To observe the bactericidal effect of linezolid combined with fosfomycin on drug-resistant strains, we performed a static time-kill assay in vitro. As shown in Figure 5, the treatment effect of the linezolid monotherapy group changed, showing a slight or no bacterial killing effect, which showed an increase of 0.03 \log_{10} value compared to the previous value ($-0.64 \log_{10}$). However, the resistance of fosfomycin was controlled and the 4096 mg/L fosfomycin group showed a bactericidal effect, which decreased by 0.49 \log_{10} value at 24 h compared with the previous value ($+1.14 \log_{10}$). The sterilization effect of the combined treatment medication group was still significant, and all of them could decrease by $2 \times \log_{10}$ value, which was $-2.72 \log_{10}$ and $-2.98 \log_{10}$, respectively. The drug combination still showed an excellent synergistic effect in the resistant strains, and the combined medication could effectively inhibit fosfomycin resistance.

Discussion

We confirmed that linezolid combined with fosfomycin has a good synergistic effect on susceptible *Enterococcus* based on an in vitro checkerboard assay, static time-kill assay, and an in vivo infection model of *G. mellonella*. The virulence of fosfomycin-resistant strains was studied using the survival curve of *G. mellonella* and RT-qPCR, and the therapeutic effect of linezolid combined with fosfomycin on drug-resistant mutants was investigated using an in vitro static time-kill assay. Finally, the fitness cost of the strains acquiring drug resistance was analyzed using in vitro and in vivo survival curves and competitive experiments. These studies help assess the resistance risk of resistant strains and thus elucidate the resistance mechanism of *Enterococcus*, which will provide important guidance for the development of future therapeutic regimens.

First, in vitro assay data showed that linezolid combined with fosfomycin had a highly synergistic effect, of which the linezolid group showed similar bacteriostatic effects, causing a $\sim 1 \log_{10}$ decrease in the three clinical strains after 24 h compared to the initial concentration at 0 h. However, Yan et al showed a trend of gradual drug resistance when a sub-inhibitory concentration of 1 mg/L linezolid was used in the treatment of VRE.¹¹ According to a survey of 1442 clinically collected *Enterococcus* isolates in the Czech Republic from 2009 to 2019, linezolid-resistant *Enterococcus faecalis* (LREF) increased from 0 in 2009 to 36 in 2019.³³ Subsequently, a global statistical analysis showed that of 69,291 *Enterococcus* isolates, 1646 were LREF isolates, indicating a prevalence rate of 2.2%.³⁴ Linezolid-resistant strains emerged gradually in VRE, suggesting a possible association with long-term linezolid exposure.^{35,36} Although LREF did not appear in this study, the data suggest that linezolid-resistant *Enterococcus* also began to proliferate.^{37,38} Therefore, the public should strictly control the dose and time of linezolid in the course of treatment. The single fosfomycin group showed a good sterilization effect in the early stages and regrowth in the later stages. Qi et al

further showed that the phenomenon of fosfomycin also appears in VRE,¹² indicating that fosfomycin may not be directly applied to the treatment of *Enterococcus*. Surprisingly, linezolid combined with fosfomycin exhibited a good synergistic effect and exerted a sustained bactericidal effect. Yan et al further suggested that linezolid combined with fosfomycin could prolong the post-antibiotic effect,¹¹ which was beneficial for prolonging the treatment time. Jiang et al speculated that the combination of drugs might be a beneficial result of the synergistic closure of their respective mutation selection windows (MSW).³⁹ Xie et al found that the combination of drugs significantly reduced the thickness of bacterial cell walls,²⁹ while Chai et al found that the combination of drugs reduced biofilm formation,⁴⁰ which may be the reason for the improved efficacy of the treatment. Combination therapy with fosfomycin drugs has shown effective benefits in clinical treatment. Clinical data have shown that fosfomycin combination therapy can improve the clinical success rate of MRSA lung infections, VRE urinary tract infections, etc.^{41,42} These data support further investigation of the effects of fosfomycin on infections caused by gram-positive cocci with advanced antimicrobial resistance. Physicians have successfully used linezolid and fosfomycin to rescue patients with severe VRE bacteremia.⁴³ This suggests the clinical utility of linezolid combined with fosfomycin.

In contrast to the in vitro studies, the in vivo data showed a significant improvement in the efficacy of fosfomycin alone, with fosfomycin being more effective than linezolid in improving the survival rate. A similar situation emerged in Li's study on fosfomycin in the MRSA *G. mellonella* infection model,¹⁶ suggesting that this may be related to the interaction of fosfomycin with the host. Studies have shown that fosfomycin has an immunomodulatory effect,⁴⁴ which can enhance the killing effect on host cells and reduce the emergence of drug-resistant mutants, thereby significantly shrinking MSW and reducing the emergence of drug resistance.⁴⁵ For synergism, the survival rate was often used in the previous infection model of *G. mellonella*; however, this single performance metric may not be sufficient to account for the synergistic treatment effect of the drug. Bacterial load, as the ultimate drug therapy target, is particularly important for the evaluation of efficacy. In a previous study on the synergistic effect of novel β -lactams against *Enterococcus*, Thieme et al assessed the synergistic effect of the drug by increasing the hemolymph load of the larvae,⁴⁶ so that the in vivo additive and synergistic effect can be better displayed. Therefore, in this study, the hemolymph load of the larvae was extracted and the combined treatment effect was verified by observing the bacterial load between the treatment groups. The combination group also showed the best effect in terms of bacterial killing, as indicated by the previous survival rate, in which the low-dose combination group (5 mg/kg + 100 mg/kg) could cause a killing effect of $-4.98 \log_{10}$ value compared with the growth group. However, the in vivo bactericidal effect of fosfomycin was surprising, as the bactericidal effect in the high-dose group (200 mg/kg) was similar to that in the low-combination group. When studying the basic efficacy and synergy of the drug, the infection model of *G. mellonella* became a suitable choice because of its easy operation and breeding. However, owing to the lack of studies on immune metabolism, there may be insufficient studies on host immunity compared with mouse infection models.^{47,48} More experiments are needed in the future to explore the applicability of *G. mellonella*.

The broad-spectrum activity of fosfomycin, the efficacy of intravenous and oral preparations, and the safety of tolerability make it a clinically superior option for the treatment of multidrug-resistant bacteria.^{49,50} However, in vitro and clinical studies have frequently observed resistance to fosfomycin, which is worthy of our attention. Recent studies have shown that when fosfomycin is used in the clinical treatment of *Enterococcus* infection, resistance at 1024 mg/L or even higher doses will occur,⁵¹ which will seriously hinder treatment and increase the pressure of antimicrobial drug selection. This suggests that continuous monitoring and in-depth research on fosfomycin resistance are required. In this study, we found that the in vitro static time-kill assay for all single-dose fosfomycin groups in the treatment process will have a good therapeutic effect in the early stage, but gradually resist over time, which will affect the outcome of treatment. Karsten et al monitored the growth curve of *Enterococcus* exposed to fosfomycin every 15 min and revealed that low doses of fosfomycin may even promote the growth of the strain itself at 48 h; in contrast, high doses completely and irreversibly inhibit the growth of *Enterococcus*.⁵² However, we determined from the hemolymph load counts in the *G. mellonella* infection model that the in vivo resistance disappears for all fosfomycin dose groups.

Although the cause of this occurrence of *Enterococcus* is unclear, exposure of the strain to fosfomycin can be induced to expand the enrichment, survival, and promotion of fosfomycin mutants through known selection mechanisms. Scotti et al found that the *fosX* enzyme can inactivate fosfomycin entering *Listeria monocytogenes* cells through a non-characteristic transport

mechanism, resulting in high levels of bacterial resistance.⁵³ However, through genome research, the combined effect of the virulence genes *prfA* and *hpt* on fosfomycin transport in vivo inhibits the effect of *fosX* on fosfomycin resistance, which causes the strain to be susceptible to fosfomycin in vivo.⁵⁴ This suggests that fosfomycin-resistant *Enterococcus* may also regulate the susceptibility in vitro and in vivo in this way. If there is an interaction between virulence and resistance gene regulatory networks in strains, a detailed understanding of these interactions is critical for predicting the development of strain resistance and the impact of antimicrobial formulation on resistant strains.⁵⁵ Therefore, we need in-depth information on the relationship between fosfomycin resistance and virulence in *Enterococcus*. Thus, we induced high levels of fosfomycin resistance in these strains. Based on the survival curve of *G. mellonella*, we found that the virulence of *Enterococcus* decreased after acquiring resistance, which is similar to that of fosfomycin in *E. coli*, where the virulence of acquired resistant mutants tended to decrease.⁵⁶ Wang et al further found that the downregulation of the virulence of fosfomycin was concentration-dependent on the fosfomycin-resistant mutants obtained from the hollow fiber model.²⁴ Subsequently, RT-qPCR revealed that the overall virulence factor of the mutant decreased, with *cylA* and *esp* showing a decreasing trend on average, while *asaL* showed a slightly increasing trend at NO.24. A similar situation was found in previous studies of daptomycin-resistant methicillin-resistant *Staphylococcus aureus* and artificially induced *Streptococcus* that acquired daptomycin resistance.^{57,58} The resistance gene *VraSR* encoding daptomycin also affects daptomycin adhesion of daptomycin-resistant strains to epithelial cells, which affects the colonization and survival of daptomycin-resistant strains in the host. Therefore, the impact of decreased virulence factors on the host is complicated, and *asaL* has been associated with mortality in clinical case studies;⁵⁹ thus, the acquisition of resistance may lead to a decrease in the killing rate of the strain. However, if the mutant can spread and infect more easily and adversely affect the choice of therapeutic agents, it could pose a deeper challenge to treatment.

There is an urgent need to understand the underlying causes of drug resistance because of its frequent emergence. Among the different factors contributing to the rise in antibiotic resistance, the fitness cost is considered to be the most prominent, which is a close indicator of the relationship between the frequency of antimicrobial use and the prevalence of drug-resistant bacteria in the future.^{60,61} The fitness cost theory and studies have shown that in an environment absent of or low in antibiotics, drug-resistant strains will be disadvantaged in a competitive environment with susceptible strains, thereby maintaining or even prolonging the effective time of the corresponding antibiotics.⁶² Therefore, it is particularly critical to evaluate the fosfomycin-resistant *Enterococcus*; the adaptability of strains will change with the change in environment, which leads to the differences between in vitro and in vivo competitive experiments.⁶³ Thus, we studied the adaptability of drug-resistant strains based on in vitro and in vivo growth curves and competition experiments. The data show that although the growth rate of the strain has a slight tendency to slow down after acquiring resistance, there will be an fitness cost in the process of acquiring fosfomycin resistance. The adaptability of the strain in vitro for 4 h was extremely low, and the body maintained a high degree of adaptability and weak competitiveness in vivo. This may explain why drug-resistant strains develop resistance at an early stage of in vitro therapy. However, in recent years, there have been no or high-adaptive-cost mutations in strains and compensatory evolution to promote the survival of drug-resistant strains in the environment. In a previous study of the rifampicin resistance mutation site *rpoB* H526Y, this binding site was found to increase the transcriptional gene, thereby increasing the adaptive cost of resistance. This also suggests that the use of drugs that can inhibit *rpoB* H526Y in combination with rifampicin reduces drug resistance.⁶⁴ In this study, we speculated that linezolid may inhibit drug resistance by inhibiting the fosfomycin mutation site. In the future, it will reveal the potential molecular mechanism of fitness cost through genomic research to better understand the resistance of the strain and speed up the discovery of new combined therapies. In general, fitness cost, drug resistance, and virulence may have complex links or even overlaps in genetics, which has significant research potential.

Whether the emergence of drug-resistant mutants will affect the efficacy of previous antimicrobial agents is also of concern and is related to clinical drug selection. Therefore, we studied the efficacy of linezolid, fosfomycin, and their combination against fosfomycin-resistant *Enterococcus* through in vitro static time-kill assays. These data suggested that linezolid does not have a bactericidal effect on resistant strains as it does on susceptible strains. Xie also found similar results,²⁹ suggesting that linezolid may no longer be the dominant option in the clinical treatment of fosfomycin-resistant mutants. Therefore, increasing the drug dose may be an appropriate choice. Continuous infusion of linezolid is one of the choices for clinical response, as it can avoid the toxic side effects caused by an increased dosage. It may also help maintain the serum level required for treatment and limit plasma concentration fluctuations.⁶⁵ However, the advantages

of continuous infusion may only occur in special clinical environments and special populations, and side effects are frequent.^{66,67} Continuous infusion greatly increases the time of patients in the hospital and the possibility of cross infection. Also, there may be an increased risk of drug-resistant strains because the continuous infusion of linezolid will continue to maintain drug concentrations around the MIC.⁶⁸

Fosfomycin also induces drug resistance. The advantages of linezolid combined with fosfomycin are fully demonstrated, which can directly inhibit the occurrence of fosfomycin resistance. Previously, fosfomycin combined with chloramphenicol showed a good synergistic bactericidal effect in vitro and in the *G.mellonella* infection model,⁶⁹ and fosfomycin combined with daptomycin for the treatment of linezolid-resistant VRE also showed an excellent bacteriostatic effect;⁷⁰ therefore, fosfomycin has great potential as a combination therapy. The high resistance to fosfomycin suggests that the dose of the drug needs to be strictly controlled clinically.³² Recent studies on vancomycin in the treatment of enterococcal infection revealed that parameters such as AUC/MIC and valley concentration can guide the concentration control of vancomycin, thereby improving treatment results and reducing side effects such as nephrotoxicity.^{71,72} This suggests that we need to conduct in-depth studies of relevant PK/PD models to identify drug-related therapies and resistance targets to optimize the clinical choice of individualized doses.

However, there were some limitations to our study. Although artificially induced strains are the appropriate means to study the fitness cost of strains, there are still differences between them and drug-resistant strains in clinical practice. In the future, further exploration can be carried out using highly drug-resistant and sensitive strains screened in clinical practice. Simultaneously, this experiment only studied drug resistance from the perspective of fitness cost, which belongs to the classification of heteroresistance. In reality, there are very complex classifications for the resistance mechanism of fosfomycin, including mutations in *murA* genes conferring intrinsic resistance to fosfomycin, resistance by changes in fosfomycin transport caused by mutations in structural genes encoding *GlpT* and *UhpT* membrane transporters, and acquisition of plasmid-encoded genes *fosA*, *fosX*, etc. that inactivate antibiotics, also resulting in fosfomycin resistance.⁷³ This suggests a need for future studies of relevant resistance mechanisms from a genetic perspective. Some genes encoding drug resistance have the same recognition site as the virulence gene encoding *Enterococcus*, which indicates that there is an association between drug-resistance genes and the upstream control of virulence genes.⁵⁴ At present, the association between *Enterococcus* fosfomycin-resistance genes and virulence genes is unclear, and further research is needed. Further animal and clinical trials are needed to verify the utility of this drug combination.

Conclusion

The combination of linezolid and fosfomycin has a significant synergistic effect in the treatment of fosfomycin-susceptible and -resistant *Enterococcus*, and linezolid alone may negatively affect fosfomycin-resistant *Enterococcus*. The treatment of *Enterococcus* infections with fosfomycin showed that increasing the dose of the drug does not address bacterial resistance. When the drug concentration changes, bacteria can develop resistance in various ways, which has a complex impact on virulence. Therefore, clinicians require targeted drug selection in the face of drug-resistant strains. In addition, although the fosfomycin-resistant *Enterococcus* mutants were not as competitive as the susceptible strains in this study, the generation of adaptive costs was an important factor to consider in treatment planning, requiring continuous monitoring and research. Therefore, further in vivo and in vitro studies will be conducted to investigate the relationship between the existence of adaptive costs and the resistance of different fosfomycin genes. Moreover, there is a complex relationship between *Enterococcus* drug resistance and virulence gene networks; thus, it is necessary to further study the control of fosfomycin-resistance genes through genomics and the potential association between adaptive cost and *Enterococcus* virulence and other genes.

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Disclosure

The authors report no conflicts of interest in this work.

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