Infection and Drug Resistance

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ORIGINAL RESEARCH

Fosfomycin Enhances the Inhibition Ability of Linezolid Against Biofilms of Vancomycin-Resistant *Enterococcus faecium* in vitro

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Purpose: We explored the inhibition ability of linezolid/fosfomycin combination against biofilms of vancomycin-resistant *Enterococcus faecium* (VRE*fm*) and tried to provide a theoretical basis for the treatment of VRE*fm* biofilm-associated infections.

Methods: Four clinical isolates of VRE*fm* (No.2, No.4, No.5, and No.6) were used for this study, which were collected from the First Affiliated Hospital of Anhui Medical University. The checkerboard method was used to assess the synergistic effect of linezolid and fosfomycin. The inhibition ability of biofilm biomass was evaluated by crystal violet staining, and the metabolic activity was tested by an Alamar blue cell viability assay. Changes in biofilm formation-related genes of the strains after incubating with drugs were investigated via the quantitative real-time polymerase chain reaction (RT-qPCR).

Results: The fractional inhibitory concentration index (FICI) showed that linezolid combined with fosfomycin had a synergistic effect on all four VRE*fm* isolates. Compared with linezolid monotherapy, linezolid combined with fosfomycin led to a significant decrease in biofilm biomass and metabolic activity, especially in the mature biofilm. The results of RT-qPCR showed linezolid combined with fosfomycin inhibition biofilm formation through the inhibition of *cylA, ebpA*, and *gelE* transcription in VRE*fm* in the initial and mature stages. To the mature biofilm, the combination also reduced the expression of *asa1, atlA*, and *esp*.

Conclusion: The combination of linezolid and fosfomycin represented stronger inhibitory effect on the biofilm formation of VRE*fm* than linezolid alone.

Keywords: linezolid, fosfomycin, biofilm-formation genes, vancomycin-resistant *Enterococcus faecium*

Introduction

Enterococcus faecium is an important conditionally pathogenic bacteria of nosocomial infections in recent years which usually causes urinary tract, abdominal, and bloodstream infections.[1] Of particular concern is the increasing difficulty of treating *E. faecium*, due to its resistance to some antimicrobial agents, including vancomycin, the first-line treatment for *Enterococcus*. However, its inappropriate use has contributed to an increasing detection rate of vancomycin-resistant E. *faecium* (VRE*fm*) and has caused high mortality and clinical failure rates.[2]

Considering rising incidences of invasive VRE*fm* diseases, linezolid has become a vital compound to treat VRE*fm* infections. VRE*fm* strains are generally susceptible to linezolid. However, it had been reported that high variability of serum linezolid concentration was detected in critically ill patients who received standard dosing of 600 mg linezolid intravenously twice a day.[3] These might lead to the development of resistance and drug-related toxicity. Moreover, prior exposure to linezolid proved to be an independent risk factor for drug-induced tolerance.[4] A report by the German National Reference Center indicated an increasing prevalence of linezolid resistance among VRE, passing from a prevalence of <1% in 2008 to >9% in 2014.[5]

Received: 1 November 2023
Accepted: 14 December 2023
Published: 19 December 2023

E. faecium is also a bacterial species most commonly capable of producing biofilms (a pivotal virulence factor in the pathogenesis).[6] Biofilm is a microbial population of cells attached irreversibly to the surface of a wide variety of medical devices, these living tissues are encased in extracellular polymeric substances (EPSs) consisting of proteins, extracellular DNA, and polysaccharides.[7] Bacteria associated with a biofilm are up to 1000 times more resistant to antibiotics in comparison to their planktonic counterparts and are insensitive to the host immune response, allowing them to persist and promote continued infection despite aggressive antibiotic therapy.[8] In confronting VRE*fm* infections (especially biofilm-forming *E. faecium*), monotherapy is often ineffective and may cause adverse drug reactions due to the need for long-term medication.[9] Thus, it is urgent to find antibiotic combinations to treat VRE*fm* infections.

Treatment choices should consider adverse effects, antibiotic penetration, and drug interactions. It has been proved that an increasing dose and treatment time of linezolid may lead to hematologic toxicity and the development of resistance during linezolid therapy.[10] Considering the limitations of linezolid monotherapy, a combination of drugs may be a good approach. Fosfomycin is an old antibiotic, with activity against several bacteria, including multidrug-resistant Gram-positive and Gram-negative bacteria, by irreversibly inhibiting an early stage in cell wall synthesis.[11] However, fosfomycin monotherapy may raise the MIC (minimum inhibitory concentration) of the susceptible bacterium. Many studies have demonstrated synergistic effects on *Enterococcus* between fosfomycin and many antibiotics.[12] Our team also did some research in this area. We confirmed that linezolid combined with fosfomycin had a significant synergistic effect on vancomycin-susceptible and -resistant *Enterococci* treatment in vitro and the vivo via a time-kill curve study and the *Galleria mellonella* infection model, prevented E*nterococcus* resistance and weakened the virulence of fosfomycin-susceptible and -resistant *Enterococcus* strains.[13,14] We also got the conclusion that, for VRE strains, neither linezolid nor fosfomycin monotherapy inhibited amplification of the resistant sub-populations, and the development of fosfomycin resistance was at the expense of the virulence of VRE*fm*.[15] Fosfomycin plus linezolid or tigecycline showed synergism on VRE in time-kill studies of approximately 10% and 30%, respectively.[16] The combination of oritavancin and fosfomycin increased drug susceptibility and showed a synergistic effect in 80% of isolates and an additive effect in the remaining isolates. In addition, the combination manifested a synergistic or additive effect in a biofilm assay.[17]

Fosfomycin is active against the adherent *Enterococcus faecalis* isolates.[18] High-dose (16×MIC) fosfomycin combined with daptomycin demonstrated significantly more anti-biofilm activities than daptomycin or fosfomycin alone and effectively killed the adherent cells in the mature biofilms of linezolid-resistant isolates of *E. faecalis*.[19] Linezolid could also inhibit *E. faecalis* biofilm formation and the addition of gentamicin significantly increased activity for linezolid.[20,21] However, whether linezolid combined with fosfomycin has a synergistic effect against VRE*fm* biofilm is still unknown.

Biofilm formation goes through four steps.[22] (1) Planktonic bacteria adhere to the surface and start to form biofilm; (2) Attached bacteria secrete EPSs resulting in a conglomeration of bacteria and matrix production; (3) Biofilms grow in multiple layers by forming micro-colonies and water channel structures; (4) Biofilms are basically mature and start releasing bacterial micro-colonies from the primary community to new sites and spreading the infection. Recent studies[23] focused on the eradication ability of reagents in mature biofilms, and few articles tried to investigate the effect of drugs against immature biofilms. Due to the growth property of the biofilms, even if the mature biofilms are eradicated, bacteria released from the biofilms can migrate to new sites and form biofilms again. So, if we can inhibit the formation of biofilms in the initial stage, it will drastically improve the cure rate of biofilm-related infections.

Therefore, in this study, we assessed the combination of linezolid with fosfomycin against VRE*fm* immature and mature biofilms by biofilm biomass and the metabolic activity of bacteria in the biofilms. The difference between linezolid alone and linezolid-plus-fosfomycin against selected biofilm-formation-associated genes evaluated by RT-qPCR.

Materials and Methods

Bacterial Isolates

All eight clinical isolates of VRE*fm* collected from patients' urine at the First Affiliated Hospital of Anhui Medical University have been tested for the ability of biofilm formation, and four clinical isolates (No.2, No.4, No.5, and No.6) have been selected for the study. All strains were identified by the automated VITEK-2 system (BioMerieux, Marcy

I'Etoile, France). Vancomycin-resistant *Enterococcus* ATCC 51299 was used as the quality control strain. These strains were part of the routine hospital laboratory procedure, which was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University and was conducted in accordance with the principles outlined Declaration of Helsinki. According to the national guidelines in China, isolates were collected as part of the routine clinical management of patients. Therefore, informed consent was not required. The isolates used in the research were not specifically isolated for this research.

Antimicrobial and Media

Linezolid, fosfomycin, and vancomycin were purchased from the National Institute for Food and Drug Control of China (Beijing, China). Mueller–Hinton broth (MHB) (Oxoid, England) and MH agar (MHA) (Oxoid, England) were used for all experiments, and Brain Heart Infusion agar (BHIA, Oxoid, United Kingdom) was only used for the susceptibility testing of vancomycin. In addition, all media with fosfomycin was added in 25 mg/L glucose-6-phosphate (Sigma-Aldrich).

In vitro Susceptibility Test and Fractional Inhibitory Concentration Index (FICI) Assay

Minimum inhibitory concentrations (MICs) of linezolid, fosfomycin and vancomycin were determined using the agar dilution method according to Clinical and Laboratory Standards Institute guidelines.[24] Briefly, Mueller–Hinton agar and Brain Heart Infusion agar plates containing a series of two-fold concentration antibiotics of each agent were prepared. The agar plates containing fosfomycin needed to add in glucose-6-phosphate with the final concentration of 25 mg/L. Then, 5×10^5 colony forming units (CFU) of bacterial cells were inoculated with these plates and 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 2020 guidelines,[24] drug resistance (R) was defined as vancomycin MIC≥32 mg/L, linezolid MIC≥8 mg/L, and fosfomycin MIC≥256 mg/L. Vancomycin-resistant *Enterococcus* ATCC 51299 was used as the quality control strain for these experiments. MIC determinations were performed in triplicate for each strain.

A checkerboard assay was used to evaluate the synergistic effects of linezolid and fosfomycin at different concentrations. The final concentration of linezolid and fosfomycin ranged from 1/16×MIC to 2×MIC. Each well of a 96-well plate contained 10^5 CFU/mL bacterial suspension. The plates were incubated under aerobic conditions at 37°C for 24 h.

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 was explained as follows: FICI ≤ 0.5, synergistic effect; 1 < FICI ≤ 4, no difference; and FICI > 4, antagonistic effect.[25]

Minimum Biofilm Eradication Concentrations (MBECs) in vitro

The in vitro minimum biofilm eradication concentrations were determined according to the aforementioned reference.[26] Suspensions of all strains at initial inocula of 5×10^6 CFU/mL in MHB were added to the plates and incubated at 37◦C for 24 h to form the mature biofilms. The wells were then washed three times with PBS and MHB supplemented with serial dilutions of linezolid or fosfomycin, ranging from 2×MIC to 64×MIC, was added. After 24 h of exposure, the wells were washed three times with PBS again and a solution of 0.01% Alamar blue (Maokang Bio, China) in MHB was added. Alamar blue, also named resazurin, is a redox indicator that represents cell viability. After 3 h of incubation at 37◦C, the absorbance was recorded at 570_{nm} and 600_{nm} with a microplate reader (Nanodrop, USA). The% of metabolic activity (MA) when compared with positive (sample with bacteria suspended in pure MHB) and negative (pure MHB) controls, which were taken as 100% and 0%, respectively, was calculated according to the specification as following formula:

$$MA(\%) = ((117216 * A570 - 80586 * A600) / (117216 * P570 - 80586 * P600)) * 100$$

A570 was the 570_{nm} absorbance value of treated sample, and A600 was the 600_{nm} absorbance value of treated sample; P570 was the 570_{nm} absorbance value of untreated sample, and P600 was the 600_{nm} absorbance value of untreated sample.

The results presented as $MBEC_{90}$ and $MBEC_{50}$, which were regarded as the lowest concentrations of antibacterials that reduced the MA of bacteria by at least 90±5% and 50±5%, respectively. The MA taken for determining the MBEC was the mean of three results obtained on three different days.

Biofilm Biomass Assay

The effects of linezolid monotherapy or combined with fosfomycin against VRE*fm* biofilm were analyzed by crystal violet staining as previously described.[19] Briefly, VRE*fm* isolates were inoculated into 96-well polystyrene microtiter plates with MHB containing antimicrobial for 48 h to evaluate the inhibition capability of antibiotics against biofilms. In order to compare the effects of drugs on immature and mature biofilms, antibiotics were added to the plates after incubating for 2 h and 24 h, respectively. After 24 h or 48 h of static incubation, with the medium containing antibiotics replaced daily, the supernatant liquid was discarded and the wells were gently washed with PBS thrice, the remaining biofilm biomass was determined by crystal violet staining and was measured at 590_{nm} with a microplate reader. Each assay was performed in triplicate three times.

Biofilm Metabolic Activity Assay

The Alamar blue cell viability assay was used as a reliable, reproducible, and recommended method to evaluate the bacterial activity of biofilm cells.[27] After 24 h or 48 h incubation as mentioned above, the supernatant liquid was removed, and the wells were gently washed with PBS thrice. Then, fresh MHB media containing 10% (v/v) Alamar blue staining reagent was added and incubated at 37°C for 3 h, as recommended by the manufacturer (Maokang Bio, China). The metabolic activity (MA) was calculated as above. Each assay was performed in triplicate on different days.

RT-qPCR to Determine the RNA Levels of VRE*fm* Biofilm Formation-Related Genes

The RNA levels of six biofilm formation-related genes of No.5 and No.6 clinical isolates were determined by RT-qPCR based on published reports.[28] The VRE*fm* clinical isolates were inoculated into 100 mm×20 mm nonpyrogenic polystyrene cell culture dishes with MHB containing 1/8×MIC linezolid and 1/2×MIC fosfomycin for 24 h to investigate the inhibition to the biofilms in initial stage and containing 2×MIC linezolid and 1×MIC fosfomycin for 48 h to test the eradication to the mature biofilms. After static incubation, total RNA was extracted from planktonic and biofilm cells for RT-qPCR. RT-qPCR is 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 are normalized to that of the housekeeping gene (16srRNA) and determined via the $2^{-\Delta\Delta CT}$ calculation method, where CT is the threshold cycle. The primers used for RT-qPCR are listed in Table 1. Each assay was performed in triplicate.

Statistical Analysis

All data were analyzed in SPSS version 20.0 (SPSS, Inc., Chicago, IL, United States). All figures were performed using GraphPad Prism, version 8.0 (GraphPad Software, Inc., San Diego, CA, United States). The data were analyzed using One-way ANOVA, and the significance was cited as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Results

MIC and FICI Results

The MICs of linezolid and fosfomycin against four isolates are shown in Table 2. The MICs of linezolid against the four strains were 2mg/L and that of fosfomycin was 128mg/L except the No.4 strain which MIC of fosfomycin was 256mg/L. By the way, all strains were resistant to vancomycin.

The FICI results represented the interaction between linezolid and fosfomycin are listed in Table 2, too. The FICI results of reagents against four strains were all below 0.5 meaning that linezolid and fosfomycin showed a synergistic effect on the VRE*fm* strains.

Table 1 PCR Primers Used for Determining the Expression Levels of Selected Genes of Vancomycin-Resistant *E. faecium* Isolates by RT-qPCR

Target	Primer	Primer Sequence (5´-3´)	Reference
asa1	*asa1*-F	GCACGCTATTACGAACTATGA	[19]
	asa1-R	TAAGAAAGAACATCACCACGA	
atlA	*atlA*-F	AACAGCACCAACGGATTAC	[27]
	atlA-R	CATAGTCAGCATAGTTATTCATTG	
cylA	*cylA*-F	GGAGGATATGGTGACAAT	[19]
	cylA-R	TTACTTCTGGAGTTGCTAA	
esp	*esp*-F	AATTGATTCTTTAGCATCTGG	[28]
	esp-R	AGATTCATCTTTGATTCTTGG	
ebpA	*ebpA*-F	ATAATAACAACGCCATTCAA	[19]
	ebpA-R	ACATATCACCAGCATCTC	
gelE	*gelE*-F	TACACCATTATCCAGAACT	[19]
	gelE-R	CATCGCCATATTGAACTT	

Table 2 MICs and FICI of Antimicrobial Reagents Against Four Strains

Isolates	MIC (mg/L)			MIC in Combination	
	LIN	FOS	VAN	LIN+FOS	FICI
ATCC51299	2	128	128	NE	NE
No.2	2	128	128	0.125+8	0.125
No.4	2	256	512	0.125+16	0.125
No.5	2	128	512	0.125+16	0.1875
No.6	2	128	512	0.125+8	0.125

Abbreviations: LIN, linezolid; FOS, fosfomycin; VAN, vancomycin; LIN+FOS, linezolid-fosfomycin combination; NE, not estimable.

Minimum Biofilm Eradication Concentrations (MBECs) in vitro

According to the MBECs listed in Table 3, VRE*fm* biofilms showed a high level of resistance to the antibiotics compared with planktonic cells in Table 2. The biofilms of VRE*fm* exhibited stronger resistance to fosfomycin than to linezolid. The $MBEC_{50}$ of four strains against fosfomycin were 8×MIC and 64×MIC respectively, in contrast, that of linezolid ranged from 4×MIC to 32×MIC. The situation of $MBEC_{90}$ was similar to that in $MBEC_{50}$. The biofilms formed by No.4 and No.5 strains showed a high level of resistance on account of the $MBEC_{90}$ of the two isolates against linezolid and fosfomycin were nearly higher than 64×MIC. The biofilm of No.6 isolate was the most susceptible to the two antibiotics, while the $MBEC_{90}$ against linezolid and fosfomycin was 8×MIC and 32×MIC, respectively.

Table 3 $MBEC_{90}$ and $MBEC_{50}$ of Antimicrobial Agents Against Four Strains

Isolates	$MBEC_{90}$ (mg/L)		$MBEC_{50}$ (mg/L)	
	LIN	FOS	LIN	FOS
No.2	64	4096	16	1024
No.4	>128	>16,384	64	>16,384
No.5	128	>8192	32	8192
No.6	16	4096	8	1024

Abbreviations: LIN, linezolid; FOS, fosfomycin.

Biofilm Biomass Assay

First, we compared the inhibition potential of linezolid combined with fosfomycin and linezolid monotherapy against the four VRE*fm* isolates' biofilms in the initial stage. When we added the antibiotics, planktonic bacteria started to adhere to the surface and secrete EPSs to form a biofilm. Sub-minimum inhibitory concentrations of antibiotics inhibited the formation of biofilm in Figure 1. Linezolid (1/8×MIC or 1/4×MIC) combined with fosfomycin (1/4×MIC) efficiently inhibited VRE*fm*-biofilm formation and to a greater extent than linezolid alone. This trend was observed in all four isolates. In the No.5 isolate, linezolid (1/4×MIC) combined with fosfomycin (1/4×MIC) showed inhibition potential better than linezolid (1/2×MIC).

We also compared high concentrations (2, 4, or 8×MIC) of linezolid combined with fosfomycin (1×MIC) eradicated VRE*fm*-formed biofilm in the mature stage with linezolid alone. As shown in Figure 2, combined administration demonstrated excellent ability to clear mature biofilms. Even compared with the high concentration group at 8×MIC of linezolid, linezolid combined with fosfomycin eradicated formed biofilms effectively.

Biofilm Metabolic Activity Assay

In the initial stage, linezolid at medium and high concentration groups (1/4 and 1/2×MIC) inhibited bacterial metabolic activity in the biofilms above 50% in the four isolates. As shown in Figure 3, fosfomycin significantly enhanced the inhibitory effect of linezolid at low and medium concentration groups (1/8 and 1/4×MIC). In the No.6 isolate, the

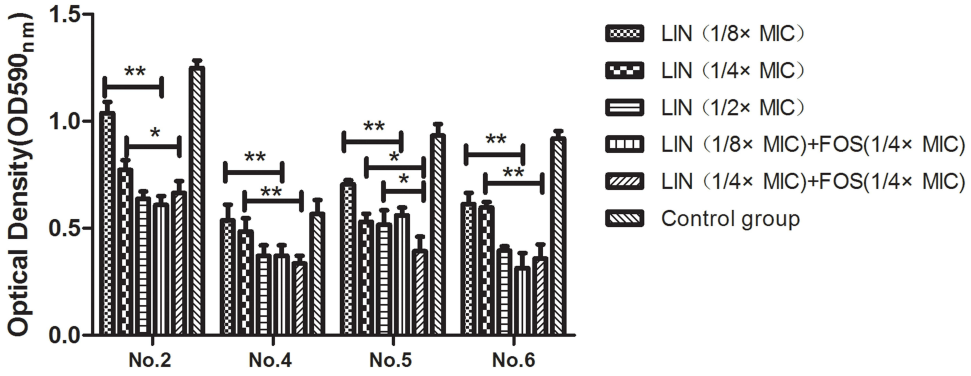

Figure 1 Sub-minimum inhibitory concentrations (1/8, 1/4, or 1/2×MIC) of linezolid combined with 1/4MIC of fosfomycin inhibited VRE*fm*-biofilm formation in the initial stage.
Notes: Data represent the average of three independent experiments (mean±SD). *P < 0.05, **P < 0.01.
Abbreviations: LIN, linezolid; FOS, fosfomycin; LIN+FOS, linezolid-fosfomycin combination.

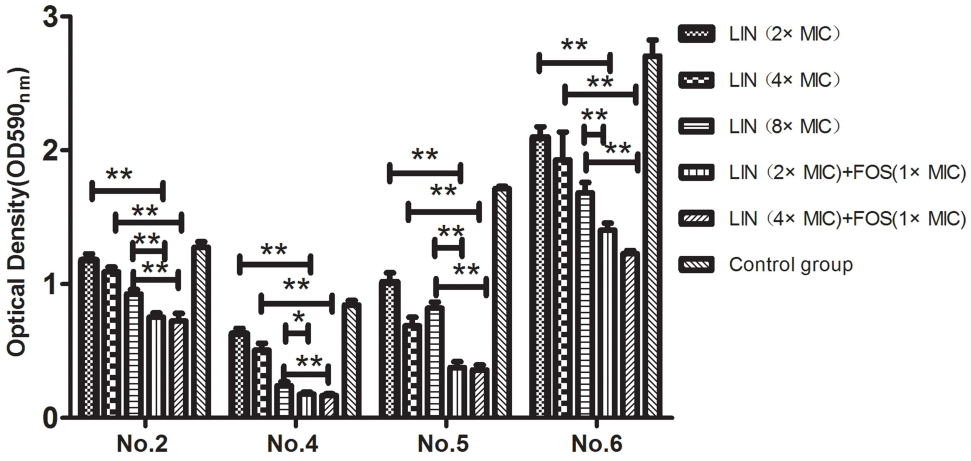

Figure 2 High concentrations (2, 4, or 8×MIC) of linezolid combined with 1×MIC of fosfomycin inhibited VRE*fm*-biofilm formation in the mature stage.
Notes: Data represent the average of three independent experiments (mean±SD). *P < 0.05, **P < 0.01.
Abbreviations: LIN, linezolid; FOS, fosfomycin; LIN+FOS, linezolid-fosfomycin combination.

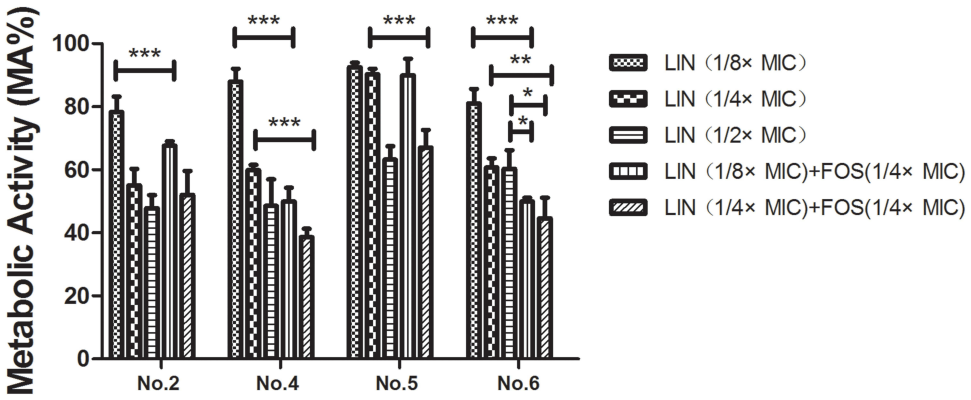

Figure 3 Sub-minimum inhibitory concentrations (1/8, 1/4, or 1/2×MIC) of linezolid combined with 1/4MIC of fosfomycin inhibited cells in VRE*fm*-biofilm in the initial stage.
Notes: Data represent the average of three independent experiments (mean±SD). *P < 0.05, **P < 0.01, ***P < 0.001.
Abbreviations: LIN, linezolid; FOS, fosfomycin; LIN+FOS, linezolid-fosfomycin combination.

inhibition of linezolid at low and medium concentration groups (1/8 and 1/4×MIC) combined with fosfomycin (1/4×MIC) was stronger than linezolid at high concentration group (1/2×MIC).

Antibiotics exhibited weak inhibitory effects on bacterial metabolism in mature biofilms in Figure 4. In No.2, No.4, and No.5 isolates, whether linezolid was used alone or combined with fosfomycin, the inhibition rate of metabolism of bacteria in the mature biofilm was lower than 50%. Fosfomycin (1×MIC) enhanced the effect of linezolid in the four isolates, especially in the No.4 and No.5 isolates. We observed medium concentration (4×MIC) of linezolid plus fosfomycin (1×MIC) showed a stronger inhibitory effect than the high concentration group (8×MIC) of linezolid alone.

Relative Quantification of Biofilm Formation Gene Expression

Last, No.5 and No.6 isolates were selected to clarify the relative expression levels of six biofilm formation genes by RT-qPCR. In Figure 5, fosfomycin had no effect on the expression of *asa1* and *esp* in two isolates, as well as *atlA* in the No.6 isolate. The gene expression levels of *cylA, ebpA*, and *gelE* showed a significant decrease when isolates were treated with 1/8×MIC linezolid and 1/4×MIC fosfomycin in the initial stage for 24 h.

In Figure 6, six gene expressions showed a significant decline except for *atlA* in the No.5 isolate. The *asa1* and *gelE* expression were markedly decreased both in two isolates when the isolates were treated with 2×MIC linezolid and 1×MIC fosfomycin for 48 h in the mature stage.

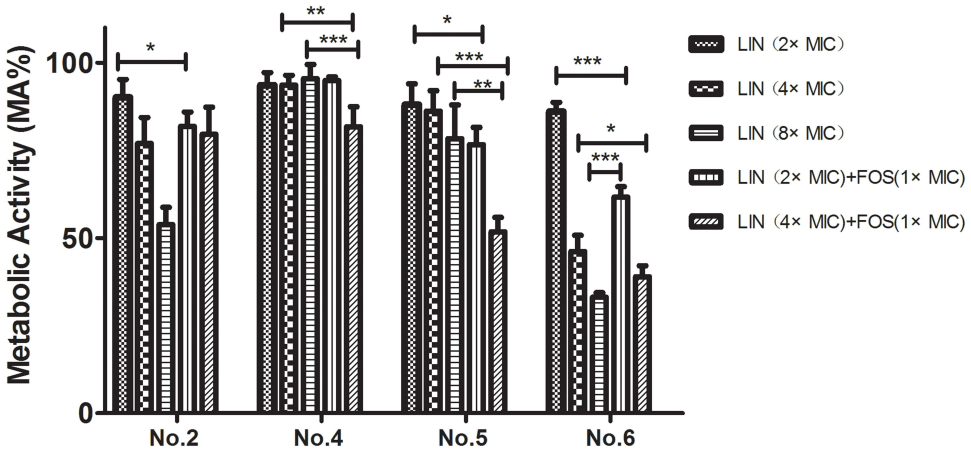

Figure 4 High concentrations (2, 4, or 8×MIC) of linezolid combined with 1×MIC of fosfomycin inhibited cells in VRE*fm*-biofilm in the mature stage.
Notes: Data represent the average of three independent experiments (mean±SD). *P < 0.05, **P < 0.01, ***P < 0.001.
Abbreviations: LIN, linezolid; FOS, fosfomycin; LIN+FOS, linezolid-fosfomycin combination.

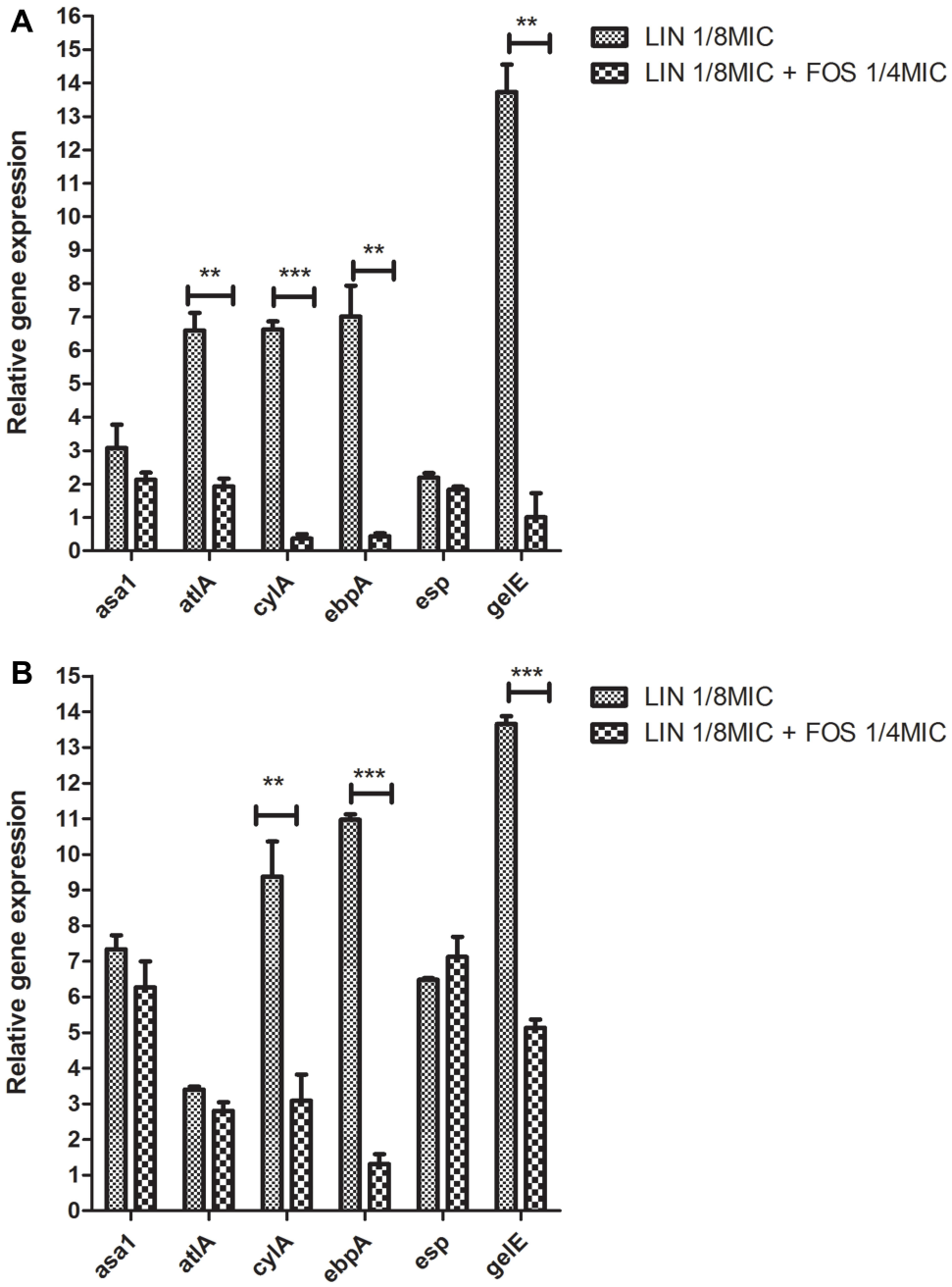

Figure 5 Relative gene expression of biofilm formation-related genes of isolates No.5 and No.6 with linezolid alone or linezolid combining with fosfomycin treatment in the initial stage.
Notes: Data represent the average of three independent experiments (mean±SD). **P < 0.01, ***P < 0.001; No.5 strain (**A**); No.6 strain (**B**).
Abbreviations: LIN, linezolid; FOS, fosfomycin; LIN+FOS, linezolid-fosfomycin combination.

Discussion

Biofilm-associated infections have caused increasing concerns, which include device-related infections, chronic infections in the absence of a foreign body, and even malfunction of medical devices. *Enterococci* prefer to survive as microbial colonies and cause 25% of all catheter-associated urinary tract infections. *Enterococci* are frequently isolated in wounds and increasingly found in infective endocarditis, and all of these infections are associated with biofilms.[29] Due to the properties of biofilms, biofilm-associated enterococcal infections are hard to eradicate and serve as a nidus for bacterial dissemination and as a reservoir for antibiotic resistance genes.[30]

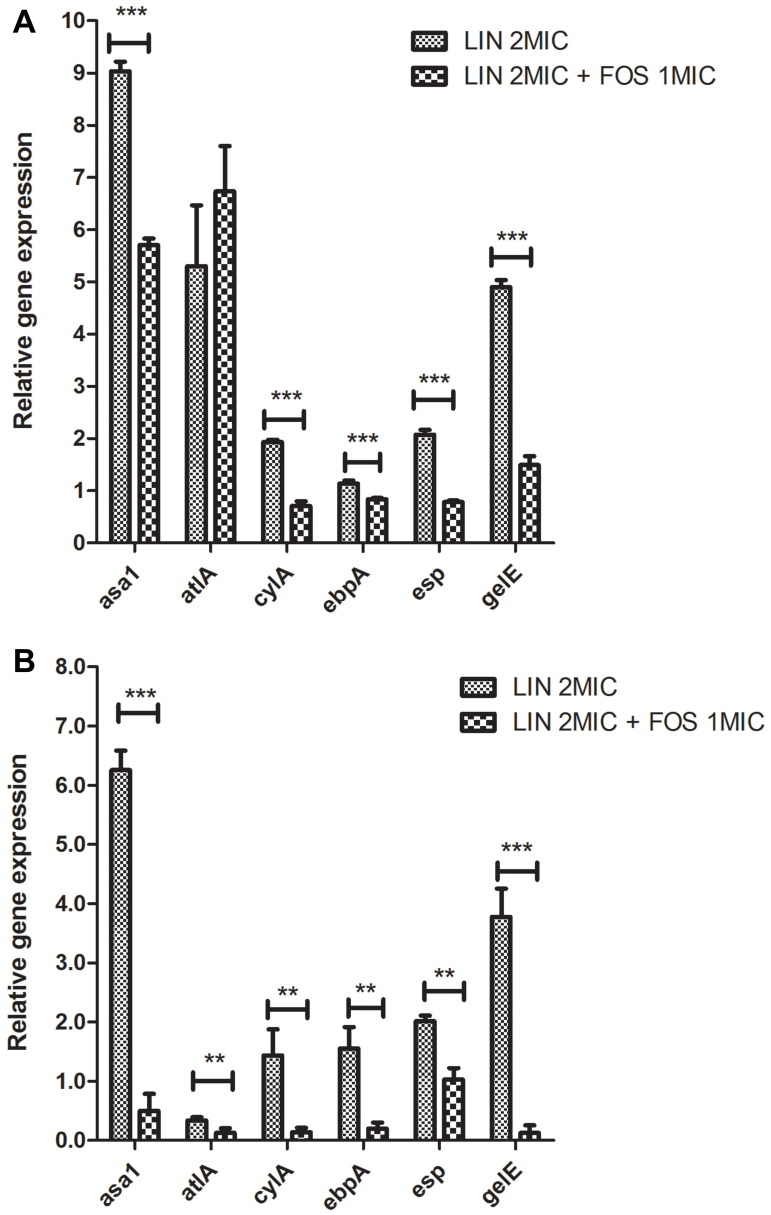

Figure 6 Relative gene expression of biofilm formation-related genes of isolates No.5 and No.6 with linezolid alone or linezolid combining with fosfomycin treatment in the mature stage.

Notes: Data represent the average of three independent experiments (mean±SD). **P < 0.01, ***P < 0.001; No.5 strain (**A**); No.6 strain (**B**).

Abbreviations: LIN, linezolid; FOS, fosfomycin; LIN+FOS, linezolid-fosfomycin combination.

Multi-resistant bacteria increase the difficulty of biofilm-associated infections treatment. As a first-line treatment for VRE infections, many studies have indicated that linezolid suppressed the formation of *enterococci* biofilms, whether when administered alone or in combination with rifampicin or gentamicin.[21,31] Fosfomycin also has inhibitory effects on biofilms of various bacteria, including *Enterococcus spp*.[32] In the study, we verified that linezolid and fosfomycin have synergistic antibacterial effects on VRE, which was consistent with the conclusion of the previous study of our research group.[14] Fosfomycin has also been proven to have either a synergistic or additive effect with linezolid against 32 VRE*fm* urinary isolates in a time-kill study.[33] It was worth noting that most of these strains collected from urine. Another investigation in 26 cases of VRE-infected patients from a hospital in Thailand found a synergistic effect of fosfomycin combined with linezolid.[34] These isolates in the research were collected from different infection sites, like the bloodstream, urine, and gastrointestinal system. In general, most articles evaluating the effectiveness of the combination of fosfomycin and linezolid on VRE were

conducted on clinical isolates collected from the urinary tract, and more research on *Enterococcus* from different infection sites is needed.

The results of the biofilm biomass assay showed that fosfomycin enhanced the inhibitory effect of linezolid on VRE biofilm, especially in the mature stage. In Figure 2, the inhibition against the biofilm grown 24 h was about fold increased in the co-administration group over the linezolid alone, especially in No.4 and No.5 isolates. What was important was that the $OD590_{nm}$ in the combination group was lower than the high-dose linezolid alone group (8×MIC), which was very meaningful for clinical treatment. That suggested that combining with fosfomycin may substitute for the high-dose linezolid alone in VRE infection treatment because high-dose linezolid was liable to cause adverse drug reactions such as lactic acidosis.[35] In the initial adhesion stage, the inhibitory effect of the drugs was not as strong as the effect on the mature biofilm, the combination treatment and the linezolid monotherapy group reduced the biofilm biomass by about 50% and 30%, respectively.

In the biofilm metabolic activity assay, linezolid showed bacterial killing ability against bacteria in the biofilm, fosfomycin enhanced the bactericidal capacity of linezolid. The bactericidal effect in different isolates was not the same, in the No.6 isolate, the combination of linezolid and fosfomycin resulted in a higher degree of bacterial kill than monotherapy no matter in the initial biofilm or in the mature biofilm. The situation in the No.2 isolate was that only low dose linezolid (1/8×MIC or 2×MIC) combined with fosfomycin killed more bacteria in the biofilm than linezolid alone, there was no significant difference between combination treatment at 1/4×MIC, 1/2×MIC, or 4×MIC, 8×MIC linezolid and combination treatment (Figures 3 and 4). This was inconsistent with the results of the biofilm inhibition experiment, suggesting that besides directly killing bacteria to reduce biofilm formation, drugs may also have other ways to reduce biofilm formation. This was confirmed by the results of the RT-qPCR experiments. Many genes are involved in the formation of enterococci biofilm. We chose six biofilm formation-related genes in the RT-qPCR experiments. The *gelE* gene, part of the *gelE-sprE* operon, encodes gelatinase which plays an important role in the initial stage of biofilm formation via the ability to degrade the collagen adhesion protein (Ace).[36] Gelatinase helps the bacteria adhere to surfaces and contributes to dissemination and colonization by degrading Ace. Gelatinase also functions as a stimulant for N-acetylglucosaminidase (AtlA) release, which is a vital autolysin that prompts lysis of a bacterial subpopulation (autolysis) and releases extracellular DNA (eDNA). eDNA acts as an adhesive and is significant for biofilm attachment and stability.[37] In Figure 5, linezolid and fosfomycin reduce gelatinase production in order to lessen bacterial adhesion at the surface by inhibiting *gelE* expression in the initial stage of biofilm formation. After 24 h stationary incubation, the biofilm has basically formed, and gelatinase secretion decreases which may reduce the release of eDNA due to the reduction of autolysis. The adhesion ability and stability of the biofilm decrease, which makes the biofilm easy to destroy (Figure 6). Linezolid combined with fosfomycin can inhibit the level of *cylA* expression to decrease biofilm formation. The *cylA*, a gene related to toxin structure and function, composes one of the cytolysin operon promoters, the P_L promoter, together with *cylLL, cylLS, cylM, cylB*, and *cylI*.[38] The cytolysin can compromise target cell membranes leading to lysis, which is necessary for eDNA formed.

When the biofilm matures, linezolid combined with fosfomycin also reduced aggregation substance (AS) production, a hair-like glycoprotein on the bacterial surface from the *asa1* gene (Figure 6). Linezolid-combined-fosfomycin can reduce the expression level of the *asa1* gene compared with linezolid alone. The expression of AS led to bacterial clumping and higher antibiotic resistance, helping in biofilm formation.[39] It is consistent with our observation of a decline of the biofilm biomass and MIC in the co-administration group.

The *esp* gene that encodes the surface-associated enterococcal surface protein (Esp) is located on a 153-kb pathogenicity island, and its expression significantly increases the bacterial cell surface hydrophobicity and attachment on a substratum.[40] It has been reported the *esp* gene of *E. faecalis* was critical for biofilm formation, but not indispensable.[41] However, there is no doubt that Esp can enhance the formation of biofilm.[42] Esp has also been demonstrated that it can highly enhance primary binding to polystyrene and polyvinyl chloride plastic from urine collection bags,[43] and *esp* expression enhanced in vitro binding to bladder and kidney epithelial cells in mice.[44] Therefore, the reduced level of *esp* expression in combination treatment may be beneficial to reduce the occurrence of VRE-associated urinary tract infections in clinical. Endocarditis- and biofilm-associated pili (Ebp), cotranscribed at the *ebpABC* locus, are composed of 3 subunits, EbpA, EbpB, and EbpC, and play a role in biofilm formation as well as in endocarditis. The tip adhesin EbpA mediates attachment to host fibrinogen and collagen and contributes to urinary tract infections, catheter-associated urinary tract infections, and endocarditis.[45] We

observed a substantial reduction of *ebpA* expression in combination treatment, especially in the initial stage of biofilm formation (Figure 5). These may be because the genes *ebpA* to *srtC* were transcribed as a polycistronic operon, and the *ebpA* disruption mutant showed highly reduced transcription of downstream *ebpB* and *ebpC* genes. Furthermore, *ebpA, ebpB, ebpC*, and *srtC* mutant strains showed significant differences with the wild type in the initial attachment step of biofilm formation using phase-contrast microscopy, indicating that these genes played a role in the early cell-surface interactions of the multistep biofilm formation process.[46]

However, there were some limitations in the study. First, only four clinical isolates were studied in the assays and were all collected from patients' urine. These isolates might not be representative of all VRE*fm*. To be more universal and representative, more isolates from different infection sites with dissimilar MIC values should be studied. Second, all assays were performed in vitro and failed to take account of the host immune response. Therefore, more animal studies may be needed to assess the role of the immune system further. Furthermore, we quantitatively examined six biofilm formation-related genes expressions in RT-qPCR but ignored the remaining genes and other potential influencing factors.

Conclusion

In conclusion, linezolid combined with fosfomycin had a synergistic effect on VRE*fm*. The combination inhibited the biofilm biomass of four isolates of VRE*fm* and reduced the metabolic activity of live bacteria in the biofilm. The results of RT-qPCR informed linezolid combined with fosfomycin had a significant decrease in the expression of *cylA, ebpA*, and *gelE*, which played an important role in promoting biofilm formation. For the mature biofilm, the transcription of *asa1, atlA*, and *esp* was also inhibited by the combination. This suggested that linezolid combined with fosfomycin may be a viable therapeutic option for the treatment of VRE*fm* biofilm-associated infections.

Acknowledgments

This study was supported by Health Commission of Anhui Province (AHWJ2022c042).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Gao W, Howden BP, Stinear TP. Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. *Curr Opin Microbiol*. 2018;41:76–82. doi:10.1016/j.mib.2017.11.030
2. Joshi S, Shallal A, Zervos M. Vancomycin-resistant Enterococci: epidemiology, infection prevention, and control. *Infect Dis Clin North Am*. 2021;35(4):953–968. doi:10.1016/j.idc.2021.07.002
3. Zoller M, Maier B, Hornuss C, et al. Variability of linezolid concentrations after standard dosing in critically ill patients: a prospective observational study. *Crit Care*. 2014;18(4):R148. doi:10.1186/cc13984
4. Smith TT, Tamma PD, Do TB, et al. Prolonged linezolid use is associated with the development of linezolid-resistant *Enterococcus faecium*. *Diagn Microbiol Infect Dis*. 2018;91(2):161–163. doi:10.1016/j.diagmicrobio.2018.01.027
5. Klare I, Fleige C, Geringer U, et al. Increased frequency of linezolid resistance among clinical *Enterococcus faecium* isolates from German hospital patients. *J Glob Antimicrob Resist*. 2015;3(2):128–131. doi:10.1016/j.jgar.2015.02.007
6. Azeredo J, Sutherland IW. The use of phages for the removal of infectious biofilms. *Curr Pharm Biotechnol*. 2008;9(4):261–266. doi:10.2174/138920108785161604
7. Blackledge MS, Worthington RJ, Melander C. Biologically inspired strategies for combating bacterial biofilms. *Curr Opin Pharmacol*. 2013;13(5):699–706. doi:10.1016/j.coph.2013.07.004
8. Rogers SA, Huigens RW, Cavanagh J, Melander C. Synergistic effects between conventional antibiotics and 2-aminoimidazole-derived antibiofilm agents. *Antimicrob Agents Chemother*. 2010;54(5):2112–2118. doi:10.1128/AAC.01418-09
9. Goi´c-Bariši´c I, Radi´c M, Novak A, et al. Vancomycin-resistant *Enterococcus faecium* COLONIZATION and *Clostridium difficile* infection in a HEMATOLOGIC patient. *Acta Clin Croat*. 2020;59(3):523–528. doi:10.20471/acc.2020.59.03.17
10. Ye JJ, Shie SS, Cheng CW, et al. Clinical characteristics and treatment outcomes of vancomycin-resistant *Enterococcus faecium* bacteremia. *J Microbiol Immunol Infect*. 2018;51(6):705–716. doi:10.1016/j.jmii.2017.08.025
11. Dijkmans AC, Zacarías NVO, Burggraaf J, et al. Fosfomycin: pharmacological, clinical and future perspectives. *Antibiotics*. 2017;6(4):24. doi:10.3390/antibiotics6040024
12. Tong J, Jiang Y, Xu H, et al. In vitro antimicrobial activity of fosfomycin, rifampin, vancomycin, daptomycin alone and in combination against vancomycin-resistant *Enterococci*. *Drug Des Devel Ther*. 2021;15:3049–3055. doi:10.2147/DDDT.S315061
13. Qi C, Xu S, Wu M, et al. Pharmacodynamics of linezolid-plus-fosfomycin against vancomycin-susceptible and -resistant *Enterococci* in vitro and in vivo of a galleria mellonella larval infection model. *Infect Drug Resist*. 2019;12:3497–3505. doi:10.2147/IDR.S219117

14. Li Y, Peng Y, Zhang N, et al. Assessing the emergence of resistance in vitro and invivo: linezolid combined with fosfomycin against fosfomycin-sensitive and resistant *Enterococcus*. *Infect Drug Resist*. 2022;15:4995–5010. doi:10.2147/IDR.S377848
15. Wang S, Liu H, Mao J, et al. Pharmacodynamics of linezolid plus fosfomycin against vancomycin-resistant *Enterococcus faecium* in a hollow fiber infection model. *Front Microbiol*. 2021;12:779885. doi:10.3389/fmicb.2021.779885
16. Tang HJ, Chen CC, Zhang CC, et al. In vitro efficacy of fosfomycin-based combinations against clinical vancomycin-resistant *Enterococcus* isolates. *Diagn Microbiol Infect Dis*. 2013;77(3):254–257. doi:10.1016/j.diagmicrobio.2013.07.012
17. Lagatolla C, Mehat JW, La Ragione RM, Luzzati R, Di Bella S. In vitro and in vivo studies of oritavancin and fosfomycin synergism against vancomycin-resistant *Enterococcus faecium*. *Antibiotics*. 2022;11(10):1334. doi:10.3390/antibiotics11101334
18. Oliva A, Furustrand Tafin U, Maiolo EM, Jeddari S, Bétrisey B, Trampuz A. Activities of fosfomycin and rifampin on planktonic and adherent *Enterococcus faecalis* strains in an experimental foreign-body infection model. *Antimicrob Agents Chemother*. 2014;58(3):1284–1293. doi:10.1128/AAC.02583-12
19. Zheng JX, Sun X, Lin ZW, et al. In vitro activities of daptomycin combined with fosfomycin or rifampin on planktonic and adherent linezolid-resistant isolates of *Enterococcus faecalis*. *J Med Microbiol*. 2019;68(3):493–502. doi:10.1099/jmm.0.000945
20. Zheng J, Chen Z, Lin Z, et al. Radezolid is more effective than linezolid against planktonic cells and inhibits *Enterococcus faecalis* biofilm formation. *Front Microbiol*. 2020;11:196.
21. Luther MK, Arvanitis M, Mylonakis E, LaPlante KL. Activity of daptomycin or linezolid in combination with rifampin or gentamicin against biofilm-forming *Enterococcus faecalis* or E. faecium in an in vitro pharmacodynamic model using simulated endocardial vegetations and an in vivo survival assay using Galleria mellonella larvae. *Antimicrob Agents Chemother*. 2014;58(8):4612–4620. doi:10.1128/AAC.02790-13
22. Davies D. Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov*. 2003;2(2):114–122. doi:10.1038/nrd1008
23. Del Pozo JL. Biofilm-related disease. *Expert Rev Anti Infect Ther*. 2018;16(1):51–65. doi:10.1080/14787210.2018.1417036
24. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; 30th Informational Supplement. CLSI M100-S30*. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.
25. Davis H, Brown R, Ashcraft D, Pankey G. In vitro synergy with fosfomycin plus doxycyclin against linezolid and vancomycin-resistant *Enterococcus faecium*. *J Glob Antimicrob Resist*. 2020;22:78–83. doi:10.1016/j.jgar.2020.01.014
26. Paduszynska MA, Greber KE, Paduszynski W, Sawicki W, Kamysz W. Activity of temporin a and short lipopeptides combined with gentamicin against biofilm formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Antibiotics*. 2020;9(9):566. doi:10.3390/antibiotics9090566
27. Pettit RK, Weber CA, Pettit GR. Application of a high throughput Alamar blue biofilm susceptibility assay to *Staphylococcus aureus* biofilms. *Ann Clinic Microbiol Antimicrob*. 2009;8(1):28. doi:10.1186/1476-0711-8-28
28. Dubrac S, Boneca IG, Poupel O, Msadek T. New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *J Bacteriol*. 2007;189(22):8257–8269. doi:10.1128/JB.00645-07
29. Ch'ng JH, Chong KKL, Lam LN, Wong JJ, Kline KA. Biofilm-associated infection by *enterococci*. *Nat Rev Microbiol*. 2019;17(2):82–94. doi:10.1038/s41579-018-0107-z
30. Zheng JX, Wu Y, Lin ZW, et al. Characteristics of and virulence factors associated with biofilm formation in clinical *Enterococcus faecalis* isolates in China. *Front Microbiol*. 2017;8:2388. doi:10.3389/fmicb.2017.02388
31. Holmberg A, Mörgelin M, Rasmussen M. Effectiveness of ciprofloxacin or linezolid in combination with rifampicin against *Enterococcus faecalis* in biofilms. *J Antimicrob Chemother*. 2012;67(2):433–439. doi:10.1093/jac/dkr477
32. Bari AK, Belalekar TS, Poojary A, Rohra S. Combination drug strategies for biofilm eradication using synthetic and natural agents in KAPE pathogens. *Front Cell Infect Microbiol*. 2023;13:1155699. doi:10.3389/fcimb.2023.1155699
33. Descourouez JL, Jorgenson MR, Wergin JE, et al. Fosfomycin synergy in vitro with amoxicillin, daptomycin, and linezolid against vancomycin-resistant *Enterococcus faecium* from renal transplant patients with infected urinary stents. *Antimicrob Agents Chemother*. 2013;57(3):1518–1520. doi:10.1128/AAC.02099-12
34. Hemapanpairoa J, Changpradub D, Thunyaharn S, Santimaleeworagun W. Vancomycin-resistant *enterococcal* infection in a Thai university hospital: clinical characteristics, treatment outcomes, and synergistic effect. *Infect Drug Resist*. 2019;12:2049–2057. doi:10.2147/IDR.S208298
35. Mao Y, Dai D, Jin H, Wang Y. The risk factors of linezolid-induced lactic acidosis: a case report and review. *Medicine*. 2018;97(36):e12114. doi:10.1097/MD.0000000000012114
36. Dundar H, Brede DA, La RSL, El-Gendy AO, Diep DB, Nes IF. The *fsr* quorum-sensing system and cognate gelatinase orchestrate the expression and processing of proprotein EF_1097 into the mature antimicrobial peptide enterocin O16. *J Bacteriol*. 2015;197(13):2112–2121. doi:10.1128/JB.02513-14
37. Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. *Mol Microbiol*. 2009;72(4):1022–1036. doi:10.1111/j.1365-2958.2009.06703.x
38. Van TD, Martin MJ, Gilmore MS. Structure, function, and biology of the *Enterococcus faecalis* cytolysin. *Toxins*. 2013;5(5):895–911. doi:10.3390/toxins5050895
39. Süssmuth SD, Muscholl-Silberhorn A, Wirth R, Susa M, Marre R, Rozdzinski E. Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. *Infect Immune*. 2000;68(9):4900–4906. doi:10.1128/IAI.68.9.4900-4906.2000
40. Tendolkar PM, Baghdayan AS, Shankar N. Putative surface proteins encoded within a novel transferable locus confer a high-biofilm phenotype to *Enterococcus faecalis*. *J Bacteriol*. 2006;188(6):2063–2072. doi:10.1128/JB.188.6.2063-2072.2006
41. Kristich CJ, Li YH, Cvitkovitch DG, Dunny GM. Esp-independent biofilm formation by *Enterococcus faecalis*. *J Bacteriol*. 2004;186(1):154–163. doi:10.1128/JB.186.1.154-163.2004
42. Tendolkar PM, Baghdayan AS, Gilmore MS, Shankar N. Enterococcal surface protein, Esp, enhances biofilm formation by *Enterococcus faecalis*. *Infect Immune*. 2004;72(10):6032–6039. doi:10.1128/IAI.72.10.6032-6039.2004
43. Toledo-Arana A, Valle J, Solano C, et al. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Appl Environ Microbiol*. 2001;67(10):4538–4545. doi:10.1128/AEM.67.10.4538-4545.2001
44. Leendertse M, Heikens E, Wijnands LM, et al. Enterococcal surface protein transiently aggravates *Enterococcus faecium*-induced urinary tract infection in mice. *J Infect Dis*. 2009;200(7):1162–1165. doi:10.1086/605609

45. Nielsen HV, Flores-Mireles AL, Kau AL, et al. Pilin and sortase residues critical for endocarditis- and biofilm-associated pilus biogenesis in *Enterococcus faecalis*. *J Bacteriol*. 2013;195(19):4484–4495. doi:10.1128/JB.00451-13
46. Nallapareddy SR, Singh KV, Sillanpää J, et al. Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. *J Clin Invest*. 2006;116 (10):2799–2807. doi:10.1172/JCI29021