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Activities of Fosfomycin and Rifampin on Planktonic and Adherent *Enterococcus faecalis* Strains in an Experimental Foreign-Body Infection Model

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Enterococcal implant-associated infections are difficult to treat because antibiotics generally lack activity against enterococcal biofilms. We investigated fosfomycin, rifampin, and their combinations against planktonic and adherent *Enterococcus faecalis* (ATCC 19433) *in vitro* and in a foreign-body infection model. The MIC/MBC$_{log}$ values were 32/>512 μg/ml for fosfomycin, 4/>64 μg/ml for rifampin, 1/2 μg/ml for ampicillin, 2/>256 μg/ml for linezolid, 16/32 μg/ml for gentamicin, 1/>64 μg/ml for vancomycin, and 1/5 μg/ml for daptomycin. In time-kill studies, fosfomycin was bactericidal at 8× and 16× MIC, but regrowth of resistant strains occurred after 24 h. With the exception of gentamicin, no complete inhibition of growth-related heat production was observed with other antimicrobials on early (3 h) or mature (24 h) biofilms. In the animal model, fosfomycin alone or in combination with daptomycin reduced planktonic counts by ≈4 log$_{10}$ CFU/ml below the levels before treatment. Fosfomycin cleared planktonic bacteria from 74% of cage fluids (i.e., no growth in aspirated fluid) and eradicated biofilm bacteria from 43% of cages (i.e., no growth from removed cages). In combination with gentamicin, fosfomycin cleared 77% and cured 58% of cages; in combination with vancomycin, fosfomycin cleared 33% and cured 18% of cages; in combination with daptomycin, fosfomycin cleared 75% and cured 17% of cages. Rifampin showed no activity on planktonic or adherent *E. faecalis*, whereas in combination with daptomycin it cured 17% and with fosfomycin it cured 25% of cages. Emergence of fosfomycin resistance was not observed *in vivo*. In conclusion, fosfomycin showed activity against planktonic and adherent *E. faecalis*. Its role against enterococcal biofilms should be further investigated, especially in combination with rifampin and/or daptomycin treatment.

Treatment of implant-associated infections is challenging because bacteria form biofilms on implant surfaces (1). Microorganisms in a biofilm are up to 1,000-fold more resistant to antibiotics than their planktonic counterparts (2). Thus, successful treatment requires antimicrobials that retain activity against adherent and metabolically less active bacteria and thus persist during the stationary growth phase (3).

Enterococci are an important global cause of health care-associated infections, as they are increasingly associated with endocarditis, urinary tract, intra-abdominal, catheter-related, surgical site, and central nervous system infections (4). In addition, enterococci have emerged as multidrug-resistant pathogens, causing infections in patients with intravascular or extravascular implants (5, 6). Several virulence and genetic factors involved in the immunity and pathogenesis of enterococcal infections have been identified (7).

Enterococci cause 3 to 10% of prosthetic joint infections, and the treatment of these infections is associated with high failure rates, mainly due to antimicrobial tolerance and the slow bactericidal activities of β-lactam antibiotics and glycopeptides (8). While rifampin combination treatment has been established for staphylococcal implant-associated infections (9), an optimal treatment for enterococcal infections has not been determined. Despite gentamicin-containing regimens showing activity against *Enterococcus faecalis* biofilms in an experimental foreign-body infection model, the failure rates remain high (10).

Fosfomycin is a bactericidal agent with a broad spectrum of activity against Gram-positive and Gram-negative microorganisms, including *E. faecalis* (11). Although for fosfomycin the main drawback is the high rate of *in vitro* emergence of resistance, which limits its use in the clinic, the rate of *in vivo* resistance remains low (12, 13). Despite fosfomycin's main use for the treatment of uncomplicated urinary tract infections, activities against biofilms and bone infections have also been demonstrated, as fosfomycin penetrates well into soft tissue and bone tissue (14, 15).

In this study, we investigated the activities of fosfomycin, rifampin, gentamicin, ampicillin, vancomycin, daptomycin, linezolid, and their combinations against planktonic and adherent *E. faecalis in vitro* and in a guinea pig foreign-body infection model. This animal model has been previously used for the evaluation of antimicrobial agents against biofilms and has been predictive for clinical outcomes in implant-associated infections (10, 16–20).

MATERIALS AND METHODS

Study organism. The biofilm-forming *E. faecalis* ATCC 19433 strain was used for all *in vitro* and *in vivo* experiments (21). Bacteria were stored in a cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) at −80°C. An inoculum was prepared by spreading one cryovial bead on a blood agar plate and incubating the plate overnight at 37°C. One colony was resuspended in 5 ml tryptic soy broth (TSB) and incubated at 37°C without shaking. Overnight cultures

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were then adjusted to a turbidity of 0.5 McFarland, which corresponded to $\approx 5 \times 10^7$ CFU/ml.

Antimicrobial agents. Fosfomycin was provided as purified powder by the manufacturer (InfectoPharm, Heppenheim, Germany). A 50-mg/ml stock solution was prepared in sterile and pyrogen-free 0.9% saline. Daptomycin for injection was supplied by the manufacturer (Novartis Pharma Schweiz, Bern, Switzerland). A stock solution of 50 mg/ml was prepared in sterile and pyrogen-free 0.9% saline. Vancomycin was supplied as 10-mg powder ampoules (Teva Pharma, Aesch, Switzerland). A stock solution of 50 mg/ml was prepared in sterile and pyrogen-free 0.9% saline. Rifampin (Sandoz, Steinhausen, Switzerland) was prepared as a 60-mg/ml stock solution in sterile water. Ampicillin was provided as a purified powder by the manufacturer (Roche Diagnostics, Mannheim, Germany), and a 5-mg/ml stock solution was prepared in sterile water.

Antimicrobial susceptibility testing. The MICs and the logarithmic MBC (MBC_{log}) values for fosfomycin, ampicillin, rifampin, gentamicin, linezolid, daptomycin, and vancomycin were determined by the broth macrodilution method in cation-adjusted Mueller-Hinton broth (CAMHB), according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (22). An inoculum of $\sim 3 \times 10^5$ CFU/ml was used. Two-fold serial dilutions of each antimicrobial agent were prepared in 2 ml Mueller-Hinton broth (MHB) in plastic tubes and incubated for 18 h at 37°C. The MIC was defined as the lowest concentration of antibiotic that completely inhibited visible growth. After the incubation, all tubes without visible growth were vigorously vortexed and incubated for an additional 4 h at 37°C without shaking. Aliquots of 100 μl were plated on blood agar plates, and the numbers of bacteria were determined. The MBC_{log} was defined as the lowest antimicrobial concentration that killed ≥99.9% of the initial bacterial count (i.e., $\geq 3 \log_{10}$ CFU/ml) in 24 h. For bacteria in the stationary phase, MBC_{stat} was determined in phosphate-buffered saline (PBS) with the addition of 0.1% TSB. Media were supplemented with 25 mg/liter glucose-6-phosphate for testing of fosfomycin and with 50 mg/liter Ca^{2+} for testing of daptomycin. All experiments were performed in triplicate.

Time-kill studies. The activities of fosfomycin, rifampin, ampicillin, and linezolid were investigated in time-kill studies performed with cells in the logarithmic growth phase. The tests were performed in plastic tubes in a final volume of 10 ml CAMHB and were further incubated at 37°C. At time points of 2, 4, 6, 8, and 24 h, 1-ml aliquots were sampled and washed with 0.9% saline solution in order to prevent the antibiotic carryover effect. Ten-fold dilutions were then plated on Muller-Hinton agar, and the numbers of CFU were determined. Medium without antibiotics was used as the growth control. Bactericidal activity was defined as a ≥99.9% (i.e., ≥ 3-$\log_{10}$ CFU/ml) reduction of the initial bacterial count after 24 h. The initial inoculum was $\sim 5 \times 10^5$ CFU/ml, and the medium used was CAMHB supplemented with glucose-6-phosphate at 25 mg/liter for the fosfomycin studies.

Microcalorimetry testing of antimicrobial activity against planktonic and adherent *E. faecalis*. A 48-channel isothermal microcalorimeter (thermal activity monitor, model 3102 TAM III; TA Instruments, New Castle, DE) was used as described for previous studies (23–26). Microcalorimetry is a highly sensitive method for assessment of bacterial growth throughout the measurements of bacterial heat production over time (the lower limit of heat flow detection is 0.25 μW). The antimicrobial activity against planktonic and adherent *E. faecalis* can be quantitatively assessed in real time by the impact on the heat flow curve, i.e., the delay of heat production, reduction of peak heat flow, and total heat. All experiments were performed in triplicate.

For planktonic *E. faecalis*, microcalorimetry ampoules containing CAMHB with different concentrations of each tested antibiotic were inoculated with a standard inoculum size (6.7×10^5 CFU/ml). Media were supplemented with 25 mg/liter glucose-6-phosphate for fosfomycin and 50 mg/liter Ca^{2+} for daptomycin testing. The heat flow was recorded during 24 h, and the results were plotted as the heat flow (in microwatts) versus time. The minimal heat inhibition concentration (MHIC) was defined as the lowest antimicrobial concentration that inhibited heat production during 24 h.

For *E. faecalis* biofilms, porous sintered glass beads (diameter, 4 mm; pore size, 60 μm; surface area, approximately 60 cm^2) were incubated in CAMHB, which was inoculated with 2 to 3 colonies of *E. faecalis* and incubated at 37°C for 3 h (early biofilm) and 24 h (mature biofilm). Then, beads were washed three times with sterile 0.9% saline to remove planktonic bacteria and exposed to serial dilutions of fosfomycin, rifampin, ampicillin, gentamicin, linezolid, daptomycin, or vancomycin in 1 ml of CAMHB and incubated for a further 24 h at 37°C. CAMHB was supplemented with 25 mg/liter glucose-6-phosphate for fosfomycin testing and with 50 mg/liter Ca^{2+} for daptomycin testing. After antimicrobial exposure, beads were rinsed three times with 0.9% saline and placed in microcalorimetry ampoules containing 3 ml of CAMHB. Sterile beads served as a negative control. Heat production was recorded for 24 h to detect recovering bacteria. The minimal biofilm eradication concentration (MBEC) was defined as the lowest antimicrobial concentration that killed biofilm bacteria on beads and led to an absence of regrowth after 24 h of incubation in the microcalorimeter.

Animal model. A previously described foreign-body infection model in guinea pigs was used (27). Experiments were performed according to the Swiss veterinary law regulations. Male albino guinea pigs (Charles River, Sulzfeld, Germany) were used and their well-being was checked daily, and the experiments were started when the animals weighed at least 450 g. Briefly, four sterile polytetrafluoroethylene (Teflon) cages (32 mm by 10 mm) with 130 regularly spaced perforations 1 mm in diameter (Angst-Pfister, Zurich, Switzerland) were subcutaneously implanted under aseptic conditions in the flanks of the guinea pigs. After the complete healing of the surgical wounds, which took approximately 2 weeks, the sterility of the cages was verified by culturing aspirated cage fluid on blood agar plates. Contaminated cages were excluded from the experiments.

Antimicrobial treatment regimens. Cages were infected by percutaneous injection of 200 μl containing 5.9×10^4 CFU of *E. faecalis*. Before the start of treatment, the infection was determined by quantitative culture of aspirated cage fluid. Three hours after infection, antimicrobial treatment was initiated (day 1). Three animals were randomized into each of the following treatment groups (with 4 cages per animal): untreated (control) group, fosfomycin (250 mg/kg), rifampin (12.5 mg/kg), gentamicin (10 mg/kg) plus fosfomycin (250 mg/kg), daptomycin (40 mg/kg) plus fosfomycin (250 mg/kg), daptomycin (40 mg/kg) plus rifampin (12.5 mg/kg), vancomycin (15 mg/kg) plus fosfomycin (250 mg/kg), and vancomycin (15 mg/kg) plus rifampin (12.5 mg/kg). All antimicrobials were administered intraperitoneally every 12 h, with the exception of daptomycin, which was given once daily. The treatment was administered for 4 days. The dosing regimens for all tested antimicrobials were chosen according to results of pharmacokinetic experimental studies that mimicked drug concentrations in humans (14, 28–30).

Antimicrobial activities on planktonic *E. faecalis* in the animal model. To determine the activities of antimicrobials on planktonic *E. faecalis*, each cage fluid was aspirated during treatment (preceding the last dose, i.e., day 4) and 5 days after completion of treatment (i.e., day 9). The treatment efficacy against planktonic bacteria was assessed based on the reduction of bacterial counts in the cage fluid (expressed as the $\log_{10}$ CFU/ml) and the clearance rate (expressed as a percentage), which was defined as the number of cage fluid cultures without growth of *E. faecalis* divided by the total number of cages in the individual treatment group.

Antimicrobial activities on adherent *E. faecalis* in the animal model. Five days after completion of treatment, animals were sacrificed and cages were removed under aseptic conditions. Each cage was then incubated in 5 ml TSB at 37°C for 48 h. Aliquots of 100 μl of these mixtures were spread on blood agar plates and incubated for an additional 48 h. The treatment efficacies against biofilm bacteria were assessed through the "cure" rate, defined as the number of cage cultures without *E. faecalis* growth divided by the total number of cages in the treatment group (expressed as a percentage).

TABLE 1 Antimicrobial susceptibility of planktonic and adherent *E. faecalis* (ATCC 19433) determined by conventional broth macrodilution and microcalorimetry

Antimicrobial agent	Susceptibility[a] (μg/ml) based on:					
	Broth macrodilution			Microcalorimetry		
	MIC	MBC_{log}	MBC_{stat}	MHIC	$MBEC_{3\ h}$	$MBEC_{24\ h}$
Fosfomycin	32	>512	>512	64	>512	>512
Rifampin	4	>64	>64	4	>512	>512
Ampicillin	1	2[b]	2[b]	2	>512	>512
Linezolid	2	>256	>256	4	>512	>512
Gentamicin[c]	16	32	4	16	128	512
Vancomycin[c]	1	>64	>64	1	>512	>512
Daptomycin[c]	1	5	>20	1	>512	>512

[a] MBC_{log}, the MBC during the logarithmic growth phase; MBC_{stat}, the MBC during the stationary growth phase; MHIC, minimal heat inhibition concentration; $MBEC_{3\ h}$, the minimal biofilm eradication concentration in a 3-h biofilm; $MBEC_{24\ h}$, the minimal biofilm eradication concentration in a 24-h biofilm. Values represent medians of triplicate measurements.

[b] At ampicillin concentrations above 2 μg/ml, the number of *E. faecalis* organisms recovered after 24 h of incubation increased with higher ampicillin concentrations (a paradoxical effect).

[c] Broth macrodilution data for this agent were extracted from reference 10.

Emergence of antimicrobial resistance *in vivo*. Susceptibilities against fosfomycin, rifampin, and daptomycin were determined in *E. faecalis* cells growing in TSB from explanted cages (i.e., in treatment failures) to screen for emergence of antimicrobial resistance. A gradient strip diffusion test (Etest) was used, following the manufacturer's instructions (AB Biodisk, Solna, Sweden).

Statistical analyses. Comparisons for continuous variables were performed by using the Mann-Whitney U test for continuous variables and by using Fisher's exact test for categorical variables. For all tests, differences were considered significant when *P* values were <0.05. The graphs in the figures were plotted using Prism software (version 6.01; GraphPad Software, La Jolla, CA).

RESULTS

Antimicrobial susceptibility. Table 1 summarizes the *in vitro* susceptibilities of planktonic and adherent *E. faecalis* cells. Fosfomycin, rifampin, and linezolid exhibited bacteriostatic activities in the logarithmic and stationary growth phases, even at the highest tested concentrations (64 to 512 μg/ml). The MIC, MBC_{log}, and MBC_{stat} for ampicillin were 1, 2, and 2 μg/ml, respectively; however, at ampicillin concentrations above 2 μg/ml, the number of bacteria recovered after 24 h of incubation increased with higher ampicillin concentrations (paradoxical effect).

Time-kill studies. Fosfomycin inhibited bacterial growth at 1× MIC and was bactericidal at ≥2× MIC (Fig. 1). However, at 2× and 4× MIC, regrowth occurred after 24 h, and in these strains fosfomycin resistance emerged (MIC, >1,024 μg/ml). Rifampin and linezolid inhibited growth at any concentration above 1× MIC. Ampicillin was bactericidal at 2×, 4×, and 8× MIC but showed better killing activity at 2× MIC than at 4× and 8× MIC (paradoxical antimicrobial effect).

Antimicrobial activities on planktonic *E. faecalis* cells based on microcalorimetry. The MHICs correlated well with MICs obtained with the standard broth macrodilution method (Table 1). The antimicrobial activities were evaluated by the delay and reduction of

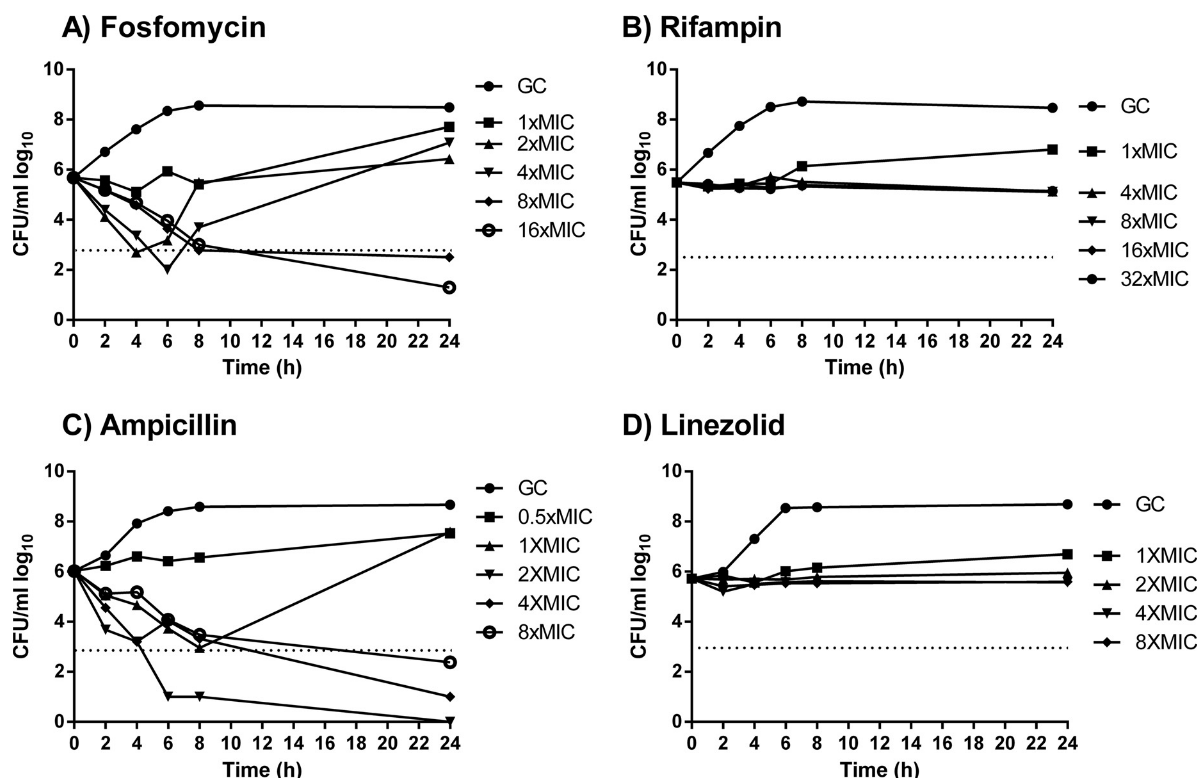

FIG 1 Time-kill studies for fosfomycin (A), rifampin (B), ampicillin (C), and linezolid (D) during logarithmic growth. The horizontal dashed line represents the reduction of 3 log_{10} CFU/ml compared to the initial bacterial count. GC, growth control.

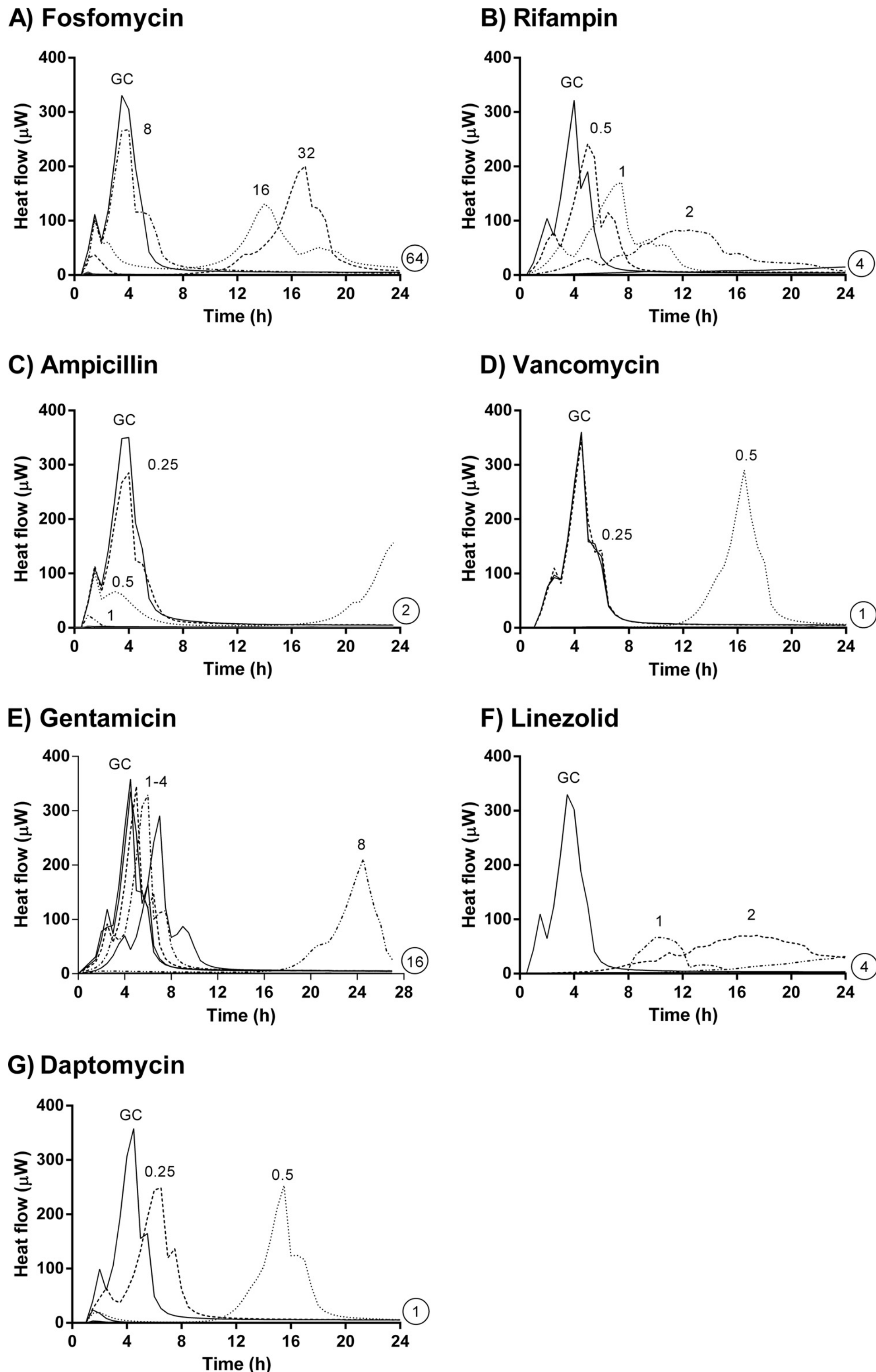

FIG 2 Microcalorimetry of planktonic *E. faecalis*. Numbers represent concentrations (in µg/ml) of fosfomycin (A), rifampin (B), ampicillin (C), vancomycin (D), gentamicin (E), linezolid (F), and daptomycin (G). Circled values represent the MHIC, defined as the lowest antimicrobial concentration that inhibited heat production during 24 h. GC, growth control.

the heat flow peak compared to the growth control in the absence of antibiotic (Fig. 2). Fosfomycin showed a reduction of the heat flow peak at 0.5× MIC. However, after 12 h, regrowth was observed at 0.5× and 1× MIC, corresponding to the findings in time-kill studies. Rifampin and linezolid caused delays in growth-related heat production and a reduction of the heat flow peak at 0.125×, 0.25×, and 0.5× MIC. Ampicillin showed activity at 0.125× MIC, mainly in the reduction of the heat flow peak.

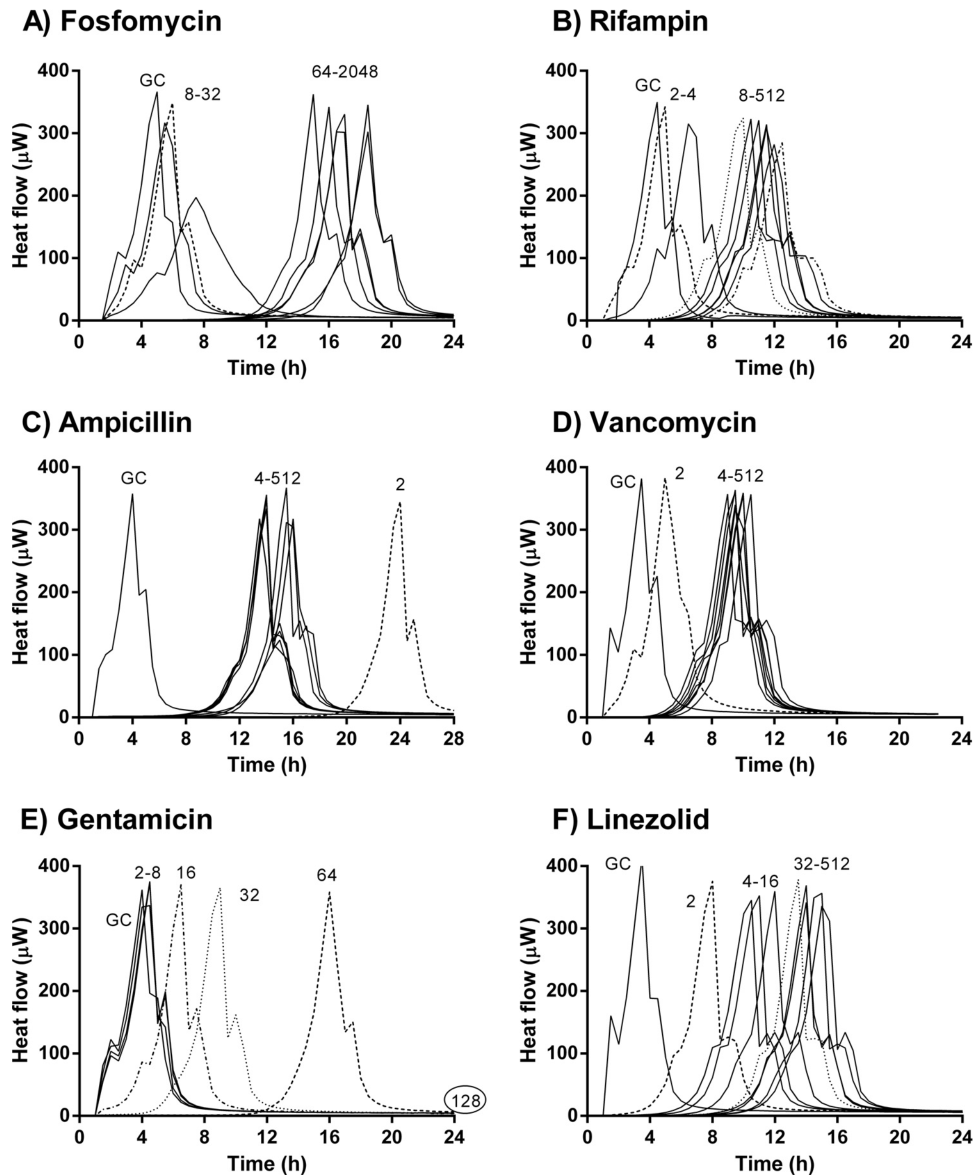

FIG 3 Microcalorimetry results for adherent *E. faecalis* (3-h biofilm). Numbers represent concentrations (in μg/ml) of fosfomycin (A), rifampin (B), ampicillin (C), vancomycin (D), gentamicin (E), and linezolid (F). Circled values represent the MBEC. GC, growth control.

Antimicrobial activity on adherent *E. faecalis* based on microcalorimetry evaluation. The antimicrobial activities on early (3 h) and mature (24 h) biofilms are shown in Fig. 3 and 4, respectively. With the exception of gentamicin, which suppressed heat production at 128 μg/ml at 3 h (Fig. 3E) and at 512 μg/ml at 24 h (Fig. 4E), no complete inhibition of heat production was observed with other antibiotics, even at concentrations up to 512 μg/ml. However, the activity of each antibiotic against early biofilm was stronger than against a mature biofilm. At concentrations below the MIC, fosfomycin had no effect on *E. faecalis* biofilm, whereas at higher concentrations (64 to 2,048 μg/ml), the heat production was delayed. This effect was more evident in early than in mature biofilm (Fig. 3A and 4A, respectively). With fosfomycin, no concentration-dependent activity was observed. In contrast, rifampin showed concentration-dependent activity (Fig. 3B and 4B). In the early biofilms, there was a difference in the drug activity between low (2 to 4 μg/ml) and high (8 to 512 μg/ml) concentrations. With ampicillin, the longest delay in heat production was found at 2 μg/ml, especially in early biofilms. No concentration-dependent differences in the antibiofilm activity were observed between 4 μg/ml and 512 μg/ml, in either early or mature biofilms (Fig. 3C and 4C). For vancomycin, no differences were found between early and mature biofilms, and its activity did not improve at higher concentrations (Fig. 3D and 4D). Linezolid showed a concentration-dependent activity from 2 μg/ml to 32 μg/ml on early biofilms (Fig. 3F) and from 2 μg/ml to 64 μg/ml on mature biofilms (Fig. 4F); at higher concentrations, no improved activity of linezolid was observed.

The combination of fosfomycin and gentamicin showed a complete inhibition of heat production, in both early and mature biofilms at concentrations of fosfomycin of 1,024 μg/ml plus gen-

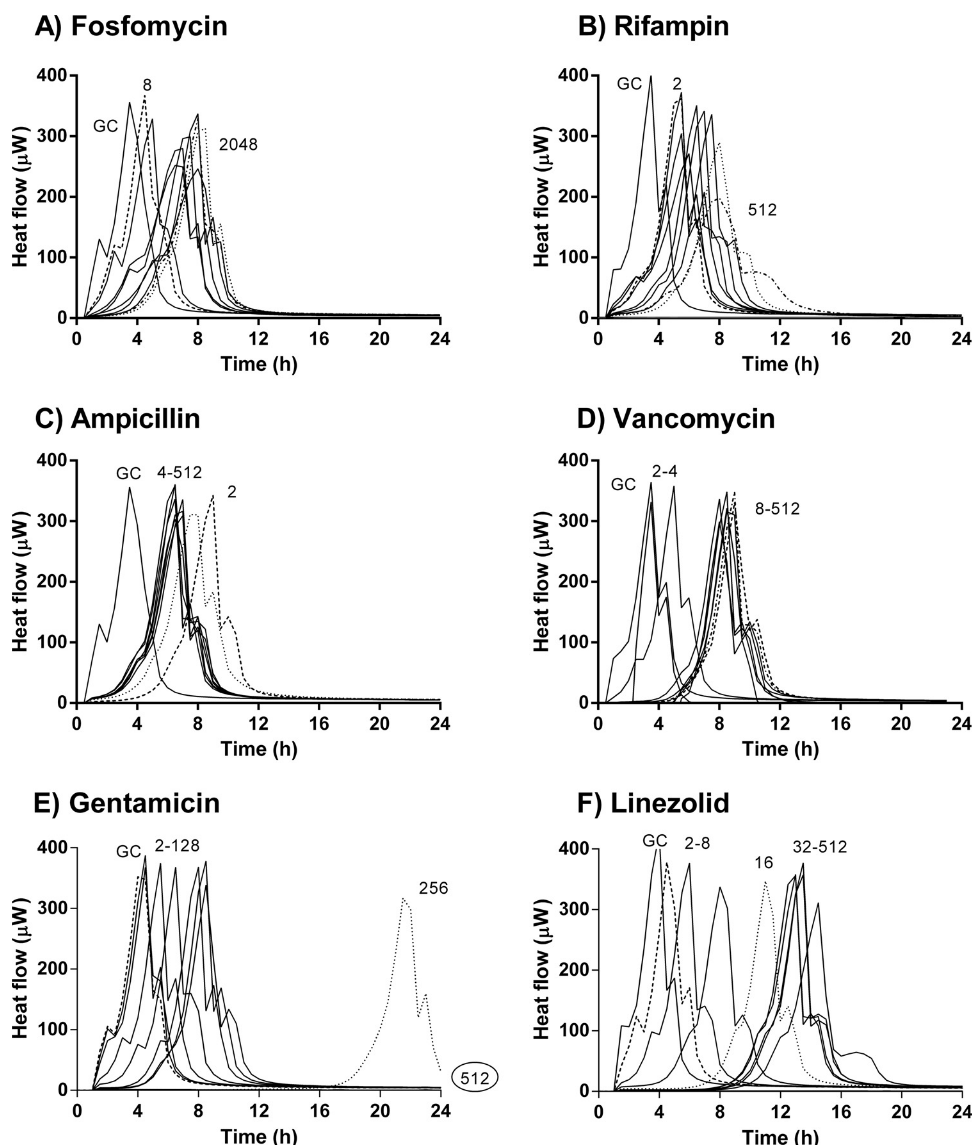

FIG 4 Microcalorimetry results for adherent *E. faecalis* (24-h biofilm). Numbers represent concentrations (in μg/ml) of fosfomycin (A), rifampin (B), ampicillin (C), vancomycin (D), gentamicin (E), and linezolid (F). Circled values represent the MBEC. GC, growth control.

tamicin at 16 to 32 μg/ml and fosfomycin at 1,024 μg/ml plus gentamicin at 64 μg/ml, respectively.

Antimicrobial activities on planktonic *E. faecalis* in the animal model. Before start of treatment (i.e., day 1), cage fluid contained 1.6×10^5 CFU/ml (i.e., 5.20 $\log_{10}$ CFU/ml). Figure 5A shows the counts of planktonic bacteria in cage fluid during treatment (i.e., day 4) and 5 days after the end of treatment (i.e., day 9). In the untreated (control) animals, the bacterial load was 4.08 and 4.16 $\log_{10}$ CFU/ml on day 4 and day 9, respectively. During treatment, the bacterial count decreased to 1.29 $\log_{10}$ CFU/ml with fosfomycin alone ($P < 0.001$), 0.75 $\log_{10}$ CFU/ml with fosfomycin plus gentamicin ($P < 0.001$), 1.32 $\log_{10}$ CFU/ml with fosfomycin plus rifampin ($P < 0.001$), 0.78 $\log_{10}$ CFU/ml with fosfomycin plus daptomycin ($P < 0.001$), 1.86 $\log_{10}$ CFU/ml with fosfomycin plus vancomycin, 2.01 $\log_{10}$ CFU/ml with rifampin plus daptomycin, and 2.4 $\log_{10}$ CFU/ml with rifampin plus vancomycin. With rifampin alone, the bacterial count was 4.65 $\log_{10}$ CFU/ml (during treatment) and 4.75 $\log_{10}$ CFU/ml (after treatment). Compared to the bacterial count during treatment, the count increased after treatment to 0.97 $\log_{10}$ CFU/ml with fosfomycin plus gentamicin, to 2.17 $\log_{10}$ CFU/ml with fosfomycin plus rifampin, to 2.91 $\log_{10}$ CFU/ml with fosfomycin plus vancomycin, and to 3.72 $\log_{10}$ CFU/ml with rifampin plus vancomycin, whereas the count remained stable and low at 0.73 $\log_{10}$ CFU/ml with fosfomycin plus daptomycin ($P < 0.001$) and decreased to 0.92 $\log_{10}$ CFU/ml with fosfomycin alone ($P < 0.001$) and to 1.12 $\log_{10}$ CFU/ml with rifampin plus daptomycin.

Figure 5B shows the clearance rate of planktonic bacteria from the cage fluid. No spontaneous clearance was observed in the untreated (control) animals. Fosfomycin alone showed a clearance rate of 74%, in combination with gentamicin the rate was 77%, with daptomycin it was 75%, with vancomycin it was 33%, and with rifampin the rate was 58%. Rifampin alone showed a clear-

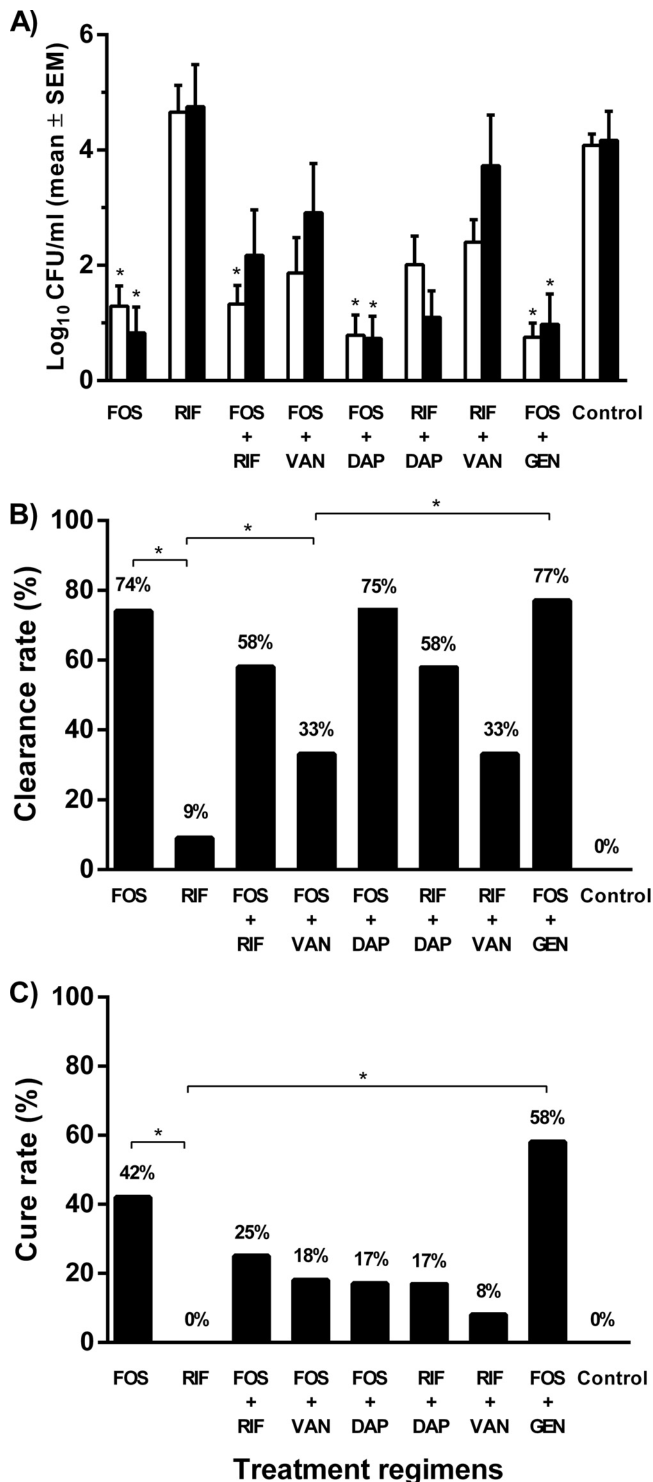

FIG 5 Antimicrobial activity on planktonic and adherent *E. faecalis* cells in the animal model. (A) Planktonic bacterial counts in cage fluid during treatment (white bars) and 5 days after treatment (black bars). *, $P < 0.001$ (compared to control animals). SEM, standard error of the mean. (B) Clearance of planktonic bacteria in cage fluid. *, $P < 0.05$. (C) Cure rate of adherent *E. faecalis* from explanted cages. *, $P < 0.05$. FOS, fosfomycin; RIF, rifampin; DAP, daptomycin; VAN, vancomycin; GEN, gentamicin; control, untreated animals.

ance rate of 9%, which increased to 33% in combination with vancomycin and to 58% with daptomycin.

Antimicrobial activity on adherent *E. faecalis* in the animal model. In the control (untreated) animals, no spontaneous cure occurred. Fosfomycin alone eradicated adherent *E. faecalis* in 43% of cages. Fosfomycin in combination with gentamicin increased the cure rate to 58%. Despite the high activities of fosfomycin and daptomycin against planktonic bacteria, this combination eradicated adherent bacteria in only 17% of cages. Rifampin alone had no effect on adherent *E. faecalis*, whereas the addition of fosfomycin and daptomycin enhanced the cure rate to 25% and 17%, respectively.

Emergence of antimicrobial resistance *in vivo*. Among the explanted cages that showed bacterial growth (treatment failures), no resistant strains to fosfomycin, rifampin, or daptomycin were detected.

DISCUSSION

Spread of resistance to penicillin derivatives, glycopeptides, and aminoglycosides has reduced the available treatment options for enterococcal infections (31, 32). In the presence of a foreign body, enterococcal infections present an additional challenge, since most antibiotics lack antibiofilm activity against enterococci (3, 8). In this study, the activities of fosfomycin and rifampin, alone and in combination with antibiotics active against enterococci, such as vancomycin, daptomycin, ampicillin, and linezolid, were tested against *E. faecalis* biofilms *in vitro* and in an established animal model.

In vitro, the macrodilution method and a microcalorimetry assay showed comparable results, with the advantage of microcalorimetry providing real-time data on bacterial growth (or inhibition thereof in the presence of antibiotic combinations) by continuous recording of bacterial heat production. Importantly, microcalorimetry showed that regrowth of *E. faecalis* occurred in the presence of fosfomycin at 16 and 32 μg/ml already after 12 h of antimicrobial exposure, which was also observed in the time-kill studies. The rapid emergence of fosfomycin resistance during treatment is the main limiting factor for the use of fosfomycin in clinical practice (33). The development of chromosomal resistance to fosfomycin implies a biological cost in virulence and fitness of Gram-negative pathogens (12, 34–36). Whether this applies also to enterococci is possible, but has not yet been demonstrated.

Both microcalorimetry and time-kill studies showed the presence of a paradoxical effect for *E. faecalis* and ampicillin. The paradoxical effect (Eagle phenomenon), which was first described by Eagle in 1948, is defined as a bactericidal activity that decreases when the concentration of the antibiotic increases. The mechanism of this phenomenon has not been clearly established; defective autolytic activity when exposed to high concentrations of antibiotics has been implicated for *E. faecalis* (37). However, the clinical relevance of this phenomenon is still unclear and needs further investigation.

The activity against *E. faecalis* in biofilms was evaluated *in vitro* by microcalorimetry using glass beads, which simulated the Calgary biofilm device method (38) and determined the biofilm recovery after 24 h of antibiotic challenge. With the exception of gentamicin, no other antibiotic inhibited bacterial heat production in early or mature *E. faecalis* biofilms, even at concentrations exceeding the achievable values in clinical practice. Antibiotics

were more active against the early (3 h) rather than mature (24 h) biofilm, highlighting the importance of a rapid start of treatment for implant-associated infections caused by enterococci.

In the present study, the experimental conditions were modified from those used previously in the animal model (16–19). First, the infection inoculum was reduced from 10^5 to 10^4 CFU/cage, and second, the duration of infection was shortened to 3 h, based on a previous study involving *E. faecalis* (10). If treatment was started after an infection duration of 24 h, no cure of cage infections was achieved with any antibiotic or combination regimen (data not shown). However, even with the short, 3-h duration of infection, the highest cure rate did not exceed 42%, highlighting that *E. faecalis* is a difficult-to-treat microorganism that tends to adhere and persist on foreign bodies, explaining the high rate of treatment failure.

Fosfomycin showed activity against both Gram-positive and Gram-negative biofilms, *Pseudomonas aeruginosa*, extended-spectrum β-lactamase-producing *E. coli*, and methicillin-resistant *S. aureus* (MRSA) (13, 39–42; R. Mihailescu, U. Furustrand Tafin, S. Corvec, A. Oliva, B. Betrisey, O. Borens, A. Trampuz, submitted for publication). However, the role of fosfomycin in enterococcal biofilm infections has not been widely investigated. In the present study, fosfomycin alone eradicated adherent *E. faecalis* from 42% of infected cages, which could be explained by the immunomodulatory effect of fosfomycin (34, 43). However, due to the risk of emergence of fosfomycin resistance, fosfomycin is not recommended for monotherapy in clinical practice (13).

In contrast to staphylococci, for which the combination of an antistaphylococcal agent with rifampin has been shown to improve the cure rate in implant-associated infections (9, 16, 17, 44, 45), the role of rifampin in enterococcal infection remains controversial. Rifampin was investigated against enterococcal biofilms in combination with ciprofloxacin and linezolid *in vitro* (46) and in combination with tigecycline *in vivo* (47). In our study, rifampin showed no activity against enterococcal biofilms, either *in vitro* or *in vivo*. While alone a cure rate of 0% was observed, rifampin activity against biofilms was improved to 8% in combination with vancomycin, to 17% with daptomycin, and to 25% with fosfomycin.

Despite the combination of fosfomycin and daptomycin showing the highest clearance rate of planktonic bacteria (75%), the cure rate (eradication of adherent bacteria from cages) was only 17%. Similar results were found in a recent study using the same guinea pig model (Trampuz et al., submitted), where the combination fosfomycin plus daptomycin was active only on planktonic and not biofilm MRSA, despite use of a higher daptomycin dose (50 mg/kg), which was equivalent to ≈10 mg/kg in humans. The daptomycin dose used in our animal experiments (40 mg/kg) corresponds to ≈8 mg/kg in humans (17, 48–50). This dose is higher than the currently recommended dose for staphylococcal infections (4 to 6 mg/kg) (51). However, daptomycin MICs for enterococci are in general 1- to 2-fold higher than those for *S. aureus*, and higher daptomycin doses are probably needed for treatment of enterococcal infections, especially in immunocompromised patients, device-related infections, and infective endocarditis (52–56). Thus, higher daptomycin doses (equivalent to 10 to 12 mg/kg in humans) may be needed in order to penetrate into biofilms and kill adherent enterococci.

In the present study, the most efficient regimen for killing planktonic and adherent *E. faecalis* was the combination of fosfomycin and gentamicin. Previous studies had demonstrated that gentamicin improves the activities of daptomycin and vancomycin against *E. faecalis* (10), and the combination of fosfomycin and gentamicin has been studied for multidrug-resistant Gram-negative bacteria (57). However, with regard to enterococcal infections, clinical data are lacking.

In conclusion, fosfomycin showed activity against planktonic and adherent *E. faecalis*. Its role in the treatment of enterococcal biofilms should be further investigated, especially in combination with gentamicin, rifampin, and/or daptomycin.

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