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Title: Efficacy of rifampicin combination therapy for the treatment of enterococcal infections assessed *in vivo* using a *Galleria mellonella* infection model

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Abstract

Enterococci are a leading cause of healthcare-associated infections world-wide and display increasing levels of resistance to many of the commonly used antimicrobials, making treatment of their infections challenging. Combinations of antibiotics are occasionally employed to treat serious infections allowing for the possibility of synergistic killing.

The aim of this study was to evaluate the effects of different antibacterial combinations against enterococcal isolates using an *in vitro* approach and *in vivo Galleria mellonella* infection model.

Five *Enterococcus faecalis* and three *E. faecium* strains were screened by paired combinations of rifampicin, tigecycline, linezolid, or vancomycin, using a chequerboard dilution method. Antibacterial combinations that displayed synergy were selected for *in vivo* testing using a *G. mellonella* larvae infection model.

Rifampicin was an effective antibacterial enhancer when used in combination with tigecycline or vancomycin, with minimum inhibitory concentrations (MICs) of each individual antibiotic being reduced by between 2- and 4-doubling dilutions, generating fractional inhibitory concentration index (FICI) values between 0.31 and 0.5. Synergy observed with the chequerboard screening assays was subsequently observed *in vivo* using the *G. mellonella* model, with combination treatment demonstrating superior protection of larvae post-infection in comparison to antibiotic monotherapy. In particular, rifampicin in combination with tigecycline or vancomycin significantly enhanced larvae survival.

The addition of rifampicin to anti-enterococcal treatment regimens warrants further investigation and may prove useful in the treatment of enterococcal infections, whilst prolonging the clinically useful life of currently active antibiotics.

Keywords: rifampicin, *Galleria mellonella*, enterococci, antibiotic combinations, antibiotic resistance

Abbreviations:

BSAC, British Society for Antimicrobial Chemotherapy

CFU, colony forming unit

FIC, fractional inhibitory concentration

FICI, fractional inhibitory concentration index

LZD, linezolid

MIC, minimum inhibitory concentration

RIF, rifampicin

TGC, tigecycline

VAN, vancomycin

1. Introduction

Enterococci cause a spectrum of infections from uncomplicated urinary tract infection to life-threatening endocardial and device-related infections. These pathogens are intrinsically resistant to a number of commonly used antimicrobials and have a remarkable ability to acquire new resistance mechanisms. This situation has fuelled global concern over future treatment options for serious enterococcal infections caused by multi-drug resistant strains.

Antibacterial agents commonly utilised or recently developed for the treatment of enterococcal infections include vancomycin, linezolid, daptomycin, telavancin and dalbavancin. Tigecycline has been suggested as an alternative therapy, but with the exception of intra-abdominal infections, a current lack of clinical data has impeded greater use [1]. As resistance to many of these antibacterials increases, including tigecycline [2], therapeutic options become progressively more limited and the need for strategies to protect against further loss of activity becomes paramount.

Prescribing antibacterial combinations is established clinical practice for treatment of serious infections. There are several potential advantages to combined therapy: enhanced killing effect and the possibility of synergy, a reduction in the concentration of individual agents required (reduced toxicity, selection pressure), and of vital importance, the ability to protect against development of resistance. Daptomycin is, for example, recommended as part of a combined therapy for serious infections [3, 4]. Rifampicin is rapidly bactericidal against many Gram-positive bacteria and displays good tissue penetration, but the rapid development of resistance precludes its use as monotherapy [4] leading to this old agent often being considered specifically for use in combination therapy [5, 6].

The aim of this study was to describe the killing effect of different antibacterial combinations against clinical enterococcal isolates using standard methods and the *in vivo G. mellonella* infection model.

2. Material and methods

2.1 Bacterial isolates and growth conditions

Eight enterococcal isolates were studied: including three vancomycin sensitive strains; *E. faecalis* ATCC 29212, *E. faecalis* clinical isolates E019 and E045; as well as four vancomycin resistant strains; *E. faecalis* ATCC 51299, *E. faecium* ATCC 51559, *E. faecium* clinical isolates E022, E039; and one tigecycline resistant *E. faecalis* UW6940 (supplied by Dr Werner) (Table 1).

2.2 Preparation of antibiotics

Vancomycin and rifampicin were purchased from Sigma-Aldrich (Sigma-Aldrich, Dorset, UK). Linezolid and tigecycline were gifted from Pfizer (Pfizer Ltd, Surrey, UK). Antibiotic stocks of 10,000 mg/L (except linezolid 1,000 mg/L) were freshly prepared using distilled water each day (linezolid and tigecycline) or stored at -20°C for a maximum of one week (vancomycin and rifampicin).

2.3Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) of antibiotics was determined by the broth microdilution method described by the British Society for Antimicrobial Chemotherapy (BSAC) for each of the eight isolates [7]. *E. faecalis* ATCC 29212 was included as a control strain in each experiment; all results were within guideline limits. MICs were performed in duplicate and repeated on two further occasions.

2.4 Antibacterial combination assays

Standard chequerboard assays were performed in 96-well microtitre plates with doubling dilutions of each antibiotic prepared in Muller Hinton broth. The final sub-MIC ranges used for vancomycin, rifampicin, tigecycline and linezolid were 0.12-1024 mg/L, 0.12-32 mg/L, 0.007-4 mg/L and 0.12-8 mg/L, respectively. An equal volume of standardised bacterial suspension

$5x10^5$ CFU/ml was added before plates were incubated at 37°C in air for 24 h. Fractional inhibitory concentrations (FICs) were calculated as the MIC of drug A or B in combination divided by the MIC of drug A or B alone, respectively, and the FIC index (FICI) was obtained by adding the two FIC values. The drug combination that consistently generated the lowest FICI after repeating the experiment in duplicate on two further occasions was used to categorise results as follows: FICIs of $\leq$0.5 were interpreted as synergistic, those >0.5 but <4 were considered as no interaction, and those above $\geq$4 were interpreted as antagonistic [8]. Combinations demonstrated to be synergistic were assessed using the *G. mellonella* infection model.

2.5 *G. mellonella* infection model

G. mellonella wax moth larvae (Livefood UK Ltd) in their final instar stage of development were stored at room temperature in the dark and used within one week of delivery. Healthy larvae, without grey markings and of a similar weight (200-300 mg), were selected and split into experimental groups of 15 [9]. Bacterial suspensions of each isolate were prepared based on a pre-optimised dose ($1x10^6$–$5x10^6$ CFU/larva) which caused >80% larvae deaths at 72 h post-infection (Supplemental Data Fig. 1).

After sterilisation of the inoculation site with 70% (v/v) ethanol, the last right proleg was used to deliver 10 µl of bacterial suspension (recorded in Fig. 1) into the hemocoel (primary body cavity) using a 1/2 inch, 30 gauge needle (BD precisionglide® syringe needle, Sigma) attached to a 50 µl Hamilton syringe (1705 TLL, Jaytee Biosciences, UK). Antimicrobial drugs (used at 1xMIC as monotherapy or between 1/4xMIC and 1/16xMIC for antibacterial combinations) were delivered into the hemocoel via a 10 µl injection into the last left proleg (n=15). The larvae were incubated in vented plastic petri dishes at 37°C in air and deaths were scored through observation of melanisation and failure of larvae to move in response to touch at

time points 12, 24, 48, 72 and 96 h. Appropriate uninfected and vehicle controls were included for each experiment.

Pooled data from three independent experiments, using *G. mellonella* larvae obtained from different batches, was assessed using the Kaplan-Meier method and treatment groups were compared using the logrank (Mantel-Cox) test (GraphPad Prism® 6). P values <0.05 were considered statistically significant.

3. Results

3.1 Assessing the *in vitro* antimicrobial sensitivities of eight enterococcal isolates using the chequerboard dilution method

Results of susceptibility testing are shown in Table 1. No antimicrobial combination showed an antagonistic effect against any of the strains evaluated. Out of the six antimicrobial combinations, tested against eight strains, synergy was seen in six cases, but in each case it was a rifampicin-containing combination. Synergy was demonstrated against all vancomycin resistant enterococci by at least one rifampicin containing antibacterial combination; all *E. faecium* isolates and one *E. faecalis* isolate (Tables 1 and 2).

3.2 Antimicrobial treatment of infected *G. mellonella* larvae

Dose-dependent killing of *G. mellonella* was achieved when larvae were infected with $1x10^6$, $3x10^6$ or $5x10^6$ CFU/larva of each isolate. *E. faecalis* ATCC 51299 was highly virulent at all three doses tested, whilst *E. faecium* ATCC 51559, *E. faecium* E022 and *E. faecium* E039 were less virulent in the *G. mellonella* model (Supplemental Data Fig. 1).

Treatment of infected larvae with an antibacterial combination of sub-MIC agents consistently led to an increased level of survival (20–73% larvae survival at 96 h with a median value of 57%) compared to those treated with a higher concentration of a single agent (7–53% survival with a median value of 13%) (Fig. 1). A statistically significant greater level of survival

was observed with four of the six combinations tested compared to the untreated control group (Fig. 1a, c, e, f), whilst only two vancomycin monotherapies administered at 1xMIC significantly improved survival of the *G. mellonella* compared to the control group (Fig. 1c p=0.003, 1e p=0.0159). Furthermore, the combination of rifampicin with tigecycline was statistically superior to tigecycline alone (13% larval survival when treated with tigecycline for infection with strain E039 versus 60% larval survival when treated with the antimicrobial combination; p=0.0237) (Fig. 1f).

4. Discussion

Antibacterial combination are often utilised during treatment of serious infections but the superiority of one antibiotic combination over another for the treatment of enterococcal infections remains unproven. In this study, it was demonstrated that antimicrobial combinations including rifampicin can be synergistic against enterococci but this is not a consistent finding among enterococci.

Though traditionally used in the investigation of antibacterial combinations for synergistic activity, chequerboard assays have reproducibility issues and may not adequately reflect activity *in vivo*. The *G. mellonella in vivo* infection model however, shares some basic immunity characteristics with mammals, including the deployment of proteolytic cascades (clotting and melanisation) following pathogen recognition [10]. An additional advantage and in contrast to mammalian models, *G. mellonella* can be inexpensively sourced and is not subject to animal research legislature [11].

In this study the larval model corroborated the *in vitro* data; treatment with antibacterial combinations that were synergistic *in vitro* improved the survival rate of larvae against those treated with a single agent.

All combinations where synergy was detected were performed with concentrations of antibacterial below the MIC (1/4-1/16th x MIC) and contained rifampicin; rifampicin with either vancomycin or tigecycline being the most effective combinations. Of the five vancomycin or tigecycline combinations trialled, a statistically greater proportion of the larvae treated by combination therapy survived in four of the assays compared to the wax moths which were either untreated or received only one antibiotic.

Rifampicin is effective against a range of Gram positive pathogens, but the rapid development of resistance necessitates that it be used in combination with another agent. Several studies have highlighted the synergistic activity of rifampicin with others agents [6, 12], indeed rifampicin based combinations, including vancomycin, are recommended for the treatment of staphylococcal endocarditis [3]. Combinations incorporating rifampicin are not, however, in routine use for treatment of enterococcal infections.

The classic combination of a cell wall active agent (such as vancomycin or a beta-lactam) plus an aminoglycoside results in a synergistic effect against enterococci [13]. High-level aminoglycoside resistance has however, led to a reconsideration of this standard treatment (gentamicin and ampicillin) resulting, in some instances, to the recommendation of an unusual double beta-lactam combination (ampicillin plus ceftriaxone) [14, 15]. In addition, the continual rise of vancomycin resistance has limited the value of this agent in the classic treatment and, as a consequence, excluded it in many instances from combination therapy studies. Yet a vancomycin-based combination has shown efficacy against resistant enterococci [16]. In this study, a vancomycin plus rifampicin combination improved the survival rate of *G. mellonella* larvae infected with selected rifampicin and/or vancomycin resistant enterococcal strains.

Typically, the interaction of tigecycline with other agents results in indifference or occasionally antagonism, an exception being with rifampicin [17]. High rates of *in vitro* synergism have been described for tigecycline plus rifampicin against *E. faecalis* and *E. faecium*

isolates using the chequerboard dilution method [5, 18]. More recently, Silvestri *et al*. (2012) tested tigecycline plus rifampicin combinations in an animal model of surgical wound infections and reported good activity against enterococcal isolates [19] supporting the observations reported here with the wax moth infection model.

The failure to assess any reduction in susceptibility to agents during treatment was a limitation of the current study since the rapid development of resistance against rifampicin in particular will always remain a concern and the observations of Holmberg and Rasmussen (2014) indicate that combined therapy might not be sufficient to prevent this from developing [20].

In conclusion, the study has revealed the efficacy of rifampicin-based combination therapies against some highly-resistant enterococci and further investigation *in vivo* with additional clinically relevant strains is warranted.

Antibiotic combinations offer the potential to treat problematic antimicrobial resistant bacteria with lower concentration of antibiotic without compromising efficacy and with a lower risk of adverse side effects. Moreover, combination therapy has the potential to reduce selective pressures and help protect the clinical life of agents, particularly newer agents for which little if any resistance exists. In addition, the chequerboard assay remains a useful screening tool for detection of potentially synergistic antimicrobial combinations, with the wax moth model being a practical and superior technique for providing quantitative *in vivo* data.

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Competing Interests

None declared.

Ethical approval

Not required.

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Table 1. Standard antibiotic susceptibility and antibacterial combination results for eight enterococcal isolates.

Enterococcal isolate		**Antibiotic susceptibility MIC (mg/L)**				**Lowest FICI generated by each drug combinations**					
		VAN	**RIF**	**LZD**	**TGC**	**VAN + TGC**	**VAN + RIF**	**TGC + RIF**	**LZD + VAN**	**LZD + TGC**	**LZD + RIF**
E. faecalis	ATCC 29212	2	2	2	0.12	0.62	0.62	0.62	1	1	0.62
	E019	1	4	2	0.25	1	1	0.75	1	1	0.62
	E045	2	1	2	0.12	0.75	0.75	0.75	1.5	1	0.75
	ATCC 51299	64	1	1	0.12	0.62	**0.37**	0.75	0.75	0.75	0.62
	UW6940	1	2	1	2	0.75	1	0.75	1	0.75	0.75
E. faecium	ATCC 51559	256	8	2	0.12	1	0.75	0.62	0.56	0.75	**0.5**
	E022	512	4	1	0.12	0.62	**0.37**	**0.5**	0.56	0.56	0.62
	E039	512	16	2	0.12	0.75	**0.31**	**0.37**	0.62	0.75	0.62

Antibiotics; VAN – vancomycin, RIF – rifampicin, LZD – linezolid, TGC – tigecycline. Antibiotic susceptibilities: VAN, RIF and LZD – sensitive ≤4 mg/L, resistant >4 mg/L; TGC – sensitive ≤0.25 mg/L, intermediate 0.5 mg/L, resistant >0.5 mg/L. Impact of antibacterial combinations; synergy = FICI ≤0.5 (shown in bold), no interaction = FICI >0.5 - <4, antagonism = >4.

Table 2. Enterococcal strains which displayed synergy with antibiotic combinations.

Enterococcal isolate	**Antibiotic synergistic combination with RIF - Drug A**	**MIC of Drug A and RIF alone (mg/l)**		**Concentration of the drug in combination (mg/l)**		**Fractional inhibitory concentration (FIC)**		**Fractional inhibitory concentration index (FICI)**
		Drug A	**RIF**	**Drug A**	**RIF**	**Drug A**	**RIF**	
E. faecalis ATCC 51299	VAN	64	1	16	0.12	0.25	0.12	0.37
E. faecium ATCC 51559	LZD	2	8	0.5	2	0.25	0.25	0.5
E. faecium E022	VAN	512	4	64	1	0.125	0.25	0.37
	TGC	0.12	4	0.03	1	0.25	0.25	0.5
E. faecium E039	VAN	512	16	128	1	0.25	0.06	0.31
	TGC	0.12	16	0.03	2	0.25	0.12	0.37

Antibiotics; VAN – vancomycin, RIF – rifampicin, LZD – linezolid, TGC – tigecycline.

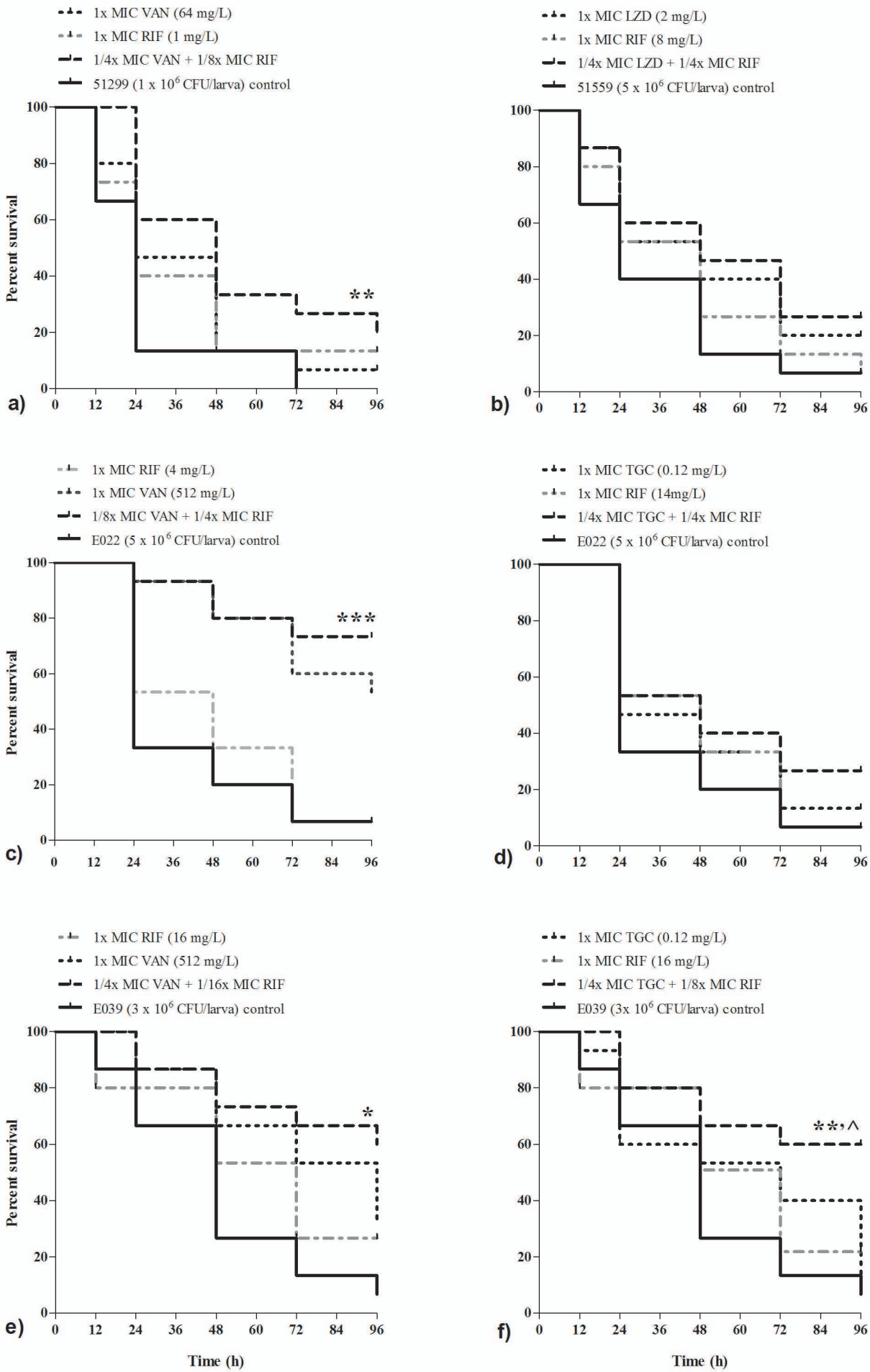
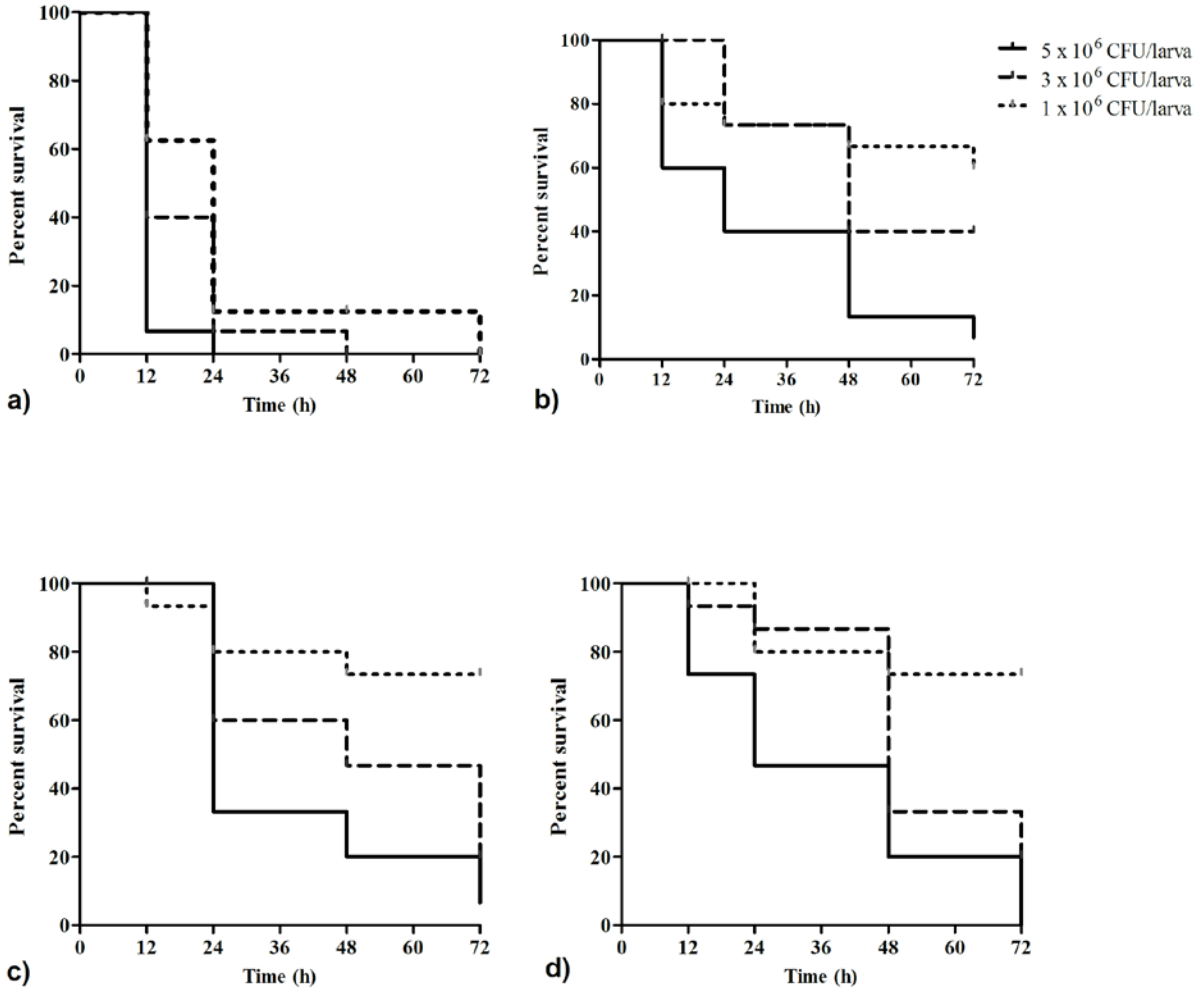

1x MIC VAN (64 mg/L)
1x MIC RIF (1 mg/L)
1/4x MIC VAN + 1/8x MIC RIF
51299 (1 x 10^6 CFU/larva) control
Percent survival
**
a)
1x MIC LZD (2 mg/L)
1x MIC RIF (8 mg/L)
1/4x MIC LZD + 1/4x MIC RIF
51559 (5 x 10^6 CFU/larva) control
b)
1x MIC RIF (4 mg/L)
1x MIC VAN (512 mg/L)
1/8x MIC VAN + 1/4x MIC RIF
E022 (5 x 10^6 CFU/larva) control
***
c)
1x MIC TGC (0.12 mg/L)
1x MIC RIF (14mg/L)
1/4x MIC TGC + 1/4x MIC RIF
E022 (5 x 10^6 CFU/larva) control
d)
1x MIC RIF (16 mg/L)
1x MIC VAN (512 mg/L)
1/4x MIC VAN + 1/16x MIC RIF
E039 (3 x 10^6 CFU/larva) control
*
e)
1x MIC TGC (0.12 mg/L)
1x MIC RIF (16 mg/L)
1/4x MIC TGC + 1/8x MIC RIF
E039 (3x 10^6 CFU/larva) control
**,^
f)
0 12 24 36 48 60 72 84 96
0 20 40 60 80 100
Time (h)

Fig. 1. Effect of antibiotic treatment on survival of *G. mellonella* larvae infected with *E. faecalis* ATCC 51299 (a), *E. faecium* ATCC 51559 (b), *E. faecium* E022 (c and d), and *E. faecium* E039 (e and f) (n = 45). VAN, vancomycin; RIF, rifampicin; LZD, linezolid; TGC, tigecycline. *** $p<0.001$, ** $p<0.01$, *$p<0.05$ for antibiotic combinations tested compared to the untreated control; ^$p<0.05$ for antibiotic combinations tested compared to tigecycline alone.



Supplemental Data Fig. 1. Effect of inoculum dose on *G. mellonella* larva survival. Larvae were inoculated with three bacterial concentrations (1x10^6 CFU/larva, 3x10^6 CFU/larva and 5x10^6 CFU/larva in MH broth) of (a) *E. faecalis* ATCC 51299, (b) *E. faecium* ATCC 51559, (c) *E. faecium* E022 and (d) *E. faecium* E039 and deaths recorded at 12, 24, 48 and 72 h to assess lethal doses. Experiments were performed three times using different batches of fifteen *G. mellonella* and the average larvae death at each time point were determined (n=45).