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In vitro synergy of 5-nitrofurans, vancomycin and sodium deoxycholate against Gram-negative pathogens

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Abstract

Introduction. There is an urgent need for effective therapies against bacterial infections, especially those caused by antibiotic-resistant Gram-negative pathogens.

Hypothesis. Synergistic combinations of existing antimicrobials show promise due to their enhanced efficacies and reduced dosages which can mitigate adverse effects, and therefore can be used as potential antibacterial therapy.

Aim. In this study, we sought to characterize the *in vitro* interaction of 5-nitrofurans, vancomycin and sodium deoxycholate (NVD) against pathogenic bacteria.

Methodology. The synergy of the NVD combination was investigated in terms of growth inhibition and bacterial killing using checkerboard and time-kill assays, respectively.

Results. Using a three-dimensional checkerboard assay, we showed that 5-nitrofurans, sodium deoxycholate and vancomycin interact synergistically in the growth inhibition of 15 out of 20 Gram-negative strains tested, including clinically significant pathogens such as carbapenemase-producing *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, and interact indifferently against the Gram-positive strains tested. The time-kill assay further confirmed that the triple combination was bactericidal in a synergistic manner.

Conclusion. This study demonstrates the synergistic effect of 5-nitrofurans, sodium deoxycholate and vancomycin against Gram-negative pathogens and highlights the potential of the combination as a treatment for Gram-negative and Gram-positive infections.

INTRODUCTION

Antimicrobial resistance that renders existing antimicrobial therapies ineffective poses a significant threat to public health with an enormous social and economic burden globally. The issue is particularly more severe with regard to Gram-negative bacterial pathogens than their Gram-positive counterparts because the former group is inherently resistant to many antimicrobial agents due to a highly impermeable outer membrane and a range of powerful efflux pumps [1]. This is reflected in the World Health Organization (WHO)'s list of priority pathogens, against which research and development of new antibiotics is urgently needed [2]. The majority of these pathogens are Gram-negative bacteria, including three that are deemed critical: carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*. As multidrug resistance continues to emerge and spread globally, and with the current clinical development pipeline insufficient to keep pace [3], this problem needs to be rectified through alternative strategies. In this study, we propose the revival of 'old' drugs by employing them in novel synergistic antibacterial combinations.

One such example of 'old' drugs are 5-nitrofurans, a broad-spectrum class of antibacterials. Although they have had a controversial past due to their mutagenicity and carcinogenicity [4, 5], they remain effective, have low prevalence of resistance [6–10] and are still clinically used today. The

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Abbreviations: CLSI, Clinical and Laboratory Standards Institute; 3D, three-dimensional; DOC, sodium deoxycholate; FICI, fractional inhibitory concentration index; FZ, furazolidone; NFZ, nitrofurazone; NIT, nitrofurantoin; NVD, 5-nitrofurans, vancomycin and sodium deoxycholate; Van, vancomycin; WHO, World Health Organization.

5-nitrofuran class of antibacterials includes commercially available furazolidone (FZ), nitrofurantoin (NIT) and nitrofurazone (NFZ). FZ is used to treat diarrhoea and as a component of combination therapy against *Helicobacter pylori*, NIT to treat urinary tract infections, and NFZ as a topical treatment for burns and wounds [11].

The glycopeptide vancomycin (Van) is another 'old' antibiotic that is clinically used to treat Gram-positive infections. However, due to its toxicity [12–14] and complications with its intravenous administration [15], it is not a first-choice antibiotic. Over the past few years, new glycopeptides with lipophilic substituents, termed lipoglycopeptides, have been developed and introduced to the market. They include telavancin, dalbavancin and oritavancin [16]. Having lipophilic side-chains and other modifications give these drugs better antimicrobial properties and safety profiles compared to Van [17]. The glycopeptide antibiotics exert their antibacterial activity by inhibiting peptidoglycan synthesis [18]. Large molecule size (≥1449 Da) prevents the passage of glycopeptides across the Gram-negative outer membrane, blocking their access to the target and rendering them ineffective against this group of organisms at sub-toxic concentrations.

Sodium deoxycholate (DOC) is a secondary bile salt, playing important roles in lipid digestion, intestinal homeostasis and antimicrobial protection [19, 20]. Gram-negative bacteria have evolved resistance to DOC through mechanisms such as preventing the accumulation of DOC inside the cells by expulsion mediated by multidrug efflux pumps [21–23], and activation of stress responses including DNA repair mechanisms [24, 25].

Having identified pairwise synergies between nitrofurans and DOC [26], and between nitrofurans and Van in a high-througput screen of 180000 small molecules that included known antibiotics (J. Spagnuolo, F. Glickman and J. Rakonjac, 2012, unpublished data) and in light of a published nitrofurantoin–Van synergy against *Escherichia coli* [27] we explored whether combining these three antibacterials will further decrease the inhibitory concentration of individual components. We now demonstrate the *in vitro* three-way synergy of 5-nitrofurans, Van and DOC (NVD) in growth inhibition and killing of a range of clinical Gram-negative pathogenic isolates, including multidrug-resistant and carbepenemase-producing enterobacteria for which new therapies are urgently needed [2]. To explore the possibility of also using the combination in treating WHO priority Gram-positive pathogens, the interaction was also evaluated in Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus*.

METHODS

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1. The bacterial cell cultures were grown in trypticase soy agar at 37 °C. For *Staphylococcus, Streptococcus* and *Pasteurella* strains, the growth medium was further supplemented with 5% sheep blood. Cation-adjusted Mueller Hinton II broth (Becton-Dickinson) was used in checkerboard and time-kill assays. Because 5-nitrofuran stocks were made in DMSO, final DMSO concentrations for all treatments and controls in the following assays were maintained at 1%.

Susceptibility testing

The broth microdilution assay for antimicrobial susceptibility was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [28] in a 384-well plate format. Each well contained 5×10^5 c.f.u. ml^{-1} at exponential growth phase, 1% DMSO, and the antimicrobial at specific concentrations prepared by two-fold serial dilutions, in a total volume of 50 µl. Each treatment was performed in triplicate. The plate was incubated at 37 °C for 18 h for enterobacteria and *Staphylococcus* and 24 h for *Acinetobacter* and *Streptococcus* as recommended by the CLSI guidelines [28]. The optical density at 600 nm was measured to assess growth inhibition [29]. The MIC is defined as the concentration that inhibits at least 90% of growth at the endpoint.

Checkerboard assay

The synergy between the test antimicrobials was examined using a growth inhibition three-dimensional (3D) checkerboard microdilution assay in 384-well plates according to the CLSI guidelines [28]. Two-fold dilutions with a concentration range of 2× MIC to 0.06× MIC of the antimicrobials and exponential-phase bacteria were prepared in BBL cation-adjusted Mueller Hinton II broth (Becton-Dickinson). Each well contained 5×10^5 c.f.u. ml^{-1}, 1% DMSO and the antimicrobials in a total volume of 50 µl. Each plate was incubated at 37 °C for the required time according to CLSI guidelines, and the optical density at 600 nm was measured to quantify growth [29]. Each combination of concentrations was performed in triplicate and the mean growth inhibition was used to determine MIC values which correspond to concentrations of combinations that result in at least 90% of growth inhibition [29]. For most of the strains tested, FZ was used as a representative of the 5-nitrofurans in the checkerboard assays.

The fractional inhibitory concentration index (FICI) was calculated using the equation [30]:

$$\mathrm{FICI}=\frac{\mathrm{MIC}_{5\text{-nitrofuran(com)}}}{\mathrm{MIC}_{5\text{-nitrofuran(alone)}}}+\frac{\mathrm{MIC}_{\mathrm{DOC(com)}}}{\mathrm{MIC}_{\mathrm{DOC(alone)}}}+\frac{\mathrm{MIC}_{\mathrm{Van(com)}}}{\mathrm{MIC}_{\mathrm{Van(alone)}}}$$

where $\mathrm{MIC}_{5\text{-nitrofuran(com)}}$, $\mathrm{MIC}_{\mathrm{DOC(com)}}$ and $\mathrm{MIC}_{\mathrm{Van(com)}}$ is the MIC of 5-nitrofuran, DOC or oxgall bile salts, and Van or lipoglycopeptides (dalbavancin or oritavancin) when used in combination, and $\mathrm{MIC}_{5\text{-nitrofuran(alone)}}$, $\mathrm{MIC}_{\mathrm{DOC(alone)}}$ and $\mathrm{MIC}_{\mathrm{Van(alone)}}$ is the MIC when used alone. Using the lowest FICI, the interactions were interpreted as synergistic if FICI≤0.5, indifferent if 0.5<FICI≤4.0 and antagonistic if FICI>4.0 [31].

Time-kill assay

Exponentially growing bacterial cultures were prepared at 5×10^5 c.f.u. ml^{-1} and treated with individual antimicrobial agents (FZ, DOC, Van) or combinations thereof at specified concentrations in a final volume of 10 ml. The cultures treated with 1% DMSO were included as vehicle controls. Each treatment was performed in triplicate. The bacterial cultures were incubated at

Table 1. Bacterial strains used in this study

Strain	Genotype/description	Source
E. coli ATCC 25922	Antibiotic susceptibility reference/quality control strain	ATCC 25922; NZRM 916*
E. coli K12 strain K1508	MC4100 [*F*⁻ *araD*⁻ Δ*lac* U169 *relA*⁻ *thiA rpsL* (Str^R)] Δ*lamB106*	[41]
E. coli ERL034336	O157, human isolate	Dr Ann Midwinter, School of Veterinary Sciences, Massey University, Palmerston North, New Zealand (NZ)
E. coli UPEC P191	Isolate from a feline urinary tract infection	New Zealand Veterinary Pathology (NZVP) diagnostic labs, Palmerston North, NZ
E. coli NZRM 4402	Plasmid-mediated AmpC *β*-lactamase-producing (*CMY-2*)	NZRM 4402
E. coli NZRM 4364	Carbapenemase (*IMP-4*) producing. Human isolate from hospital outbreak (Melbourne, Australia)	NZRM 4364
E. coli NZRM 4457	NDM-1 metallo-*β*-lactamase-producing	ARL09/232 [42]; NZRM 4457
E. coli NZRM 4524	Verotoxin-producing *E. coli* (VTEC) Serotype 045: H Rough	NZRM 4524
Citrobacter gillenii PMR001	Isolate from a municipal sewage processing (water purification) plant, Palmerston North, NZ	[26]
Klebsiella pneumoniae PMR001	Isolate from a municipal sewage processing (water purification) plant, Palmerston North, NZ	[26]
Klebsiella pneumoniae NZRM 4387	Beta-lactamase-producing. *SHV-11, TEM-1, CTX-M-15*	NZRM 4387
Klebsiella pneumoniae NZRM 4412	*Klebsiella pneumoniae* carbapenemase-producing	ATCC BAA-170; NZRM 4412
Klebsiella pneumoniae NZRM 4498	OXA-181 carbapenemase-producing	NZRM 4498
Salmonella enterica LT2	Type strain, *S. enterica* subsp. *enterica*, serovar Typhimurium	ATCC 43971
Salmonella enterica NZRM 4533	Human isolate (NZ)	NZRM 4533
Shigella dysenteriae 1015	Type strain	ATCC 13313; NZRM 1015
Acinetobacter lwoffi NZRM 1218	Knee wound isolate (NZ)	NZRM 1218
Acinetobacter baumannii NZRM 3697	Human isolate from outbreak (Christchurch, NZ)	NZRM 3697
Acinetobacter baumannii NZRM 4408	OXA-27 carbapenemase-producing	NCTC 13304; NZRM 4408
Pasteurella dagmatis NZRM 959	Isolate from a feline oral cavity	NZRM 959
Staphylococcus aureus NZRM 3478	Methicillin-resistant. human wound isolate (Auckland, NZ)	NZRM 3478
Staphylococcus aureus NZRM 4315	Human wound isolate. Erythromycin (*ermA*) resistant	ATCC BAA-977; NZRM 4315
Staphylococcus aureus NZRM 4548	WR/AK1 strain. Panton-Valentine-leukocidin (PVL)-positive methicillin-resistant. Human isolate (NZ)	NZRM 4548
Staphylococcus aureus NZRM 4549	AK3 methicillin-resistant. Human isolate (NZ)	NZRM 4549
Streptococcus pneumoniae NZRM 2764	Susceptibility testing control	CDC 78-008107; NZRM 2764
Streptococcus pyogenes NZRM 4366	Exotoxin positive control	NZRM 4366

*NZRM, The New Zealand Reference Culture Collection: Medical Section.

37 °C with shaking at 200 r.p.m. At time points 0, 2, 4, 6, 8, and 24 h, 500 µl was sampled from each culture and centrifuged at 10000 ***g*** for 1 min. The pellet was then resuspended in 100 µl of the maximum recovery diluent (0.1% peptone, 0.85% NaCl), and 10-fold serial dilutions were drop-plated (10 µl) on 2×YT agar. Plates were incubated at 37 °C overnight before counting colonies. The limit of detection is 60 c.f.u. ml^{-1} or 1.78 $\log_{10}$ c.f.u. ml^{-1}. The synergy of the combination was defined as a ≥2$\log_{10}$ decrease in the cell count (c.f.u. ml^{-1}) compared to the most potent single drug at 24 h.

Table 2. MICs against Gram-negative pathogens

	MIC (μg ml^{-1})				
Strain	**FZ**	**DOC**	**Van**	**NIT**	**NFZ**
E. coli ATCC 25922	1.25	80000	500	16	8
E. coli K1508	1.25	>80000	250	16	8
E. coli ERL034336	1.25	80000	250	16	8
E. coli UPEC P191	1.25	80000	250	16	8
E. coli NZRM 4364	0.25	>80000	125	NT	NT
E. coli NZRM 4402	0.625	40000	500	NT	NT
E. coli NZRM 4457	>128	80000	250	128	64
E. coli NZRM 4524	1.25	80000	500	NT	NT
C. gillenii PMR001	5	80000	500	16	16
K. pneumoniae PMR001	1.25	80000	1000	64	32
K. pneumoniae NZRM 4387	2.5	80000	2000	NT	NT
K. pneumoniae NZRM 4412	32	80000	2000	>128	128
K. pneumoniae NZRM 4498	16	>80000	2000	>128	64
Salmonella enterica sv. Typhimurium LT2	2.5	40000	500	16	8
Salmonella enterica NZRM 4533	2	80000	1000	32	8
Shigella dysenteriae NZRM 1015	4	>80000	250	8	8
A. lwoffi NZRM 1218	16	40000	62.5	NT	NT
A. baumannii NZRM 3697	32	80000	125	NT	NT
A. baumannii NZRM 4408	>128	80000	250	>128	32
P. dagmatis NZRM 959	2	1250	31.25	4	4

FZ, furazolidone; DOC, sodium deoxycholate, Van, vancomycin; NIT, nitrofurantoin; NFZ, nitrofurazone; NT, not tested.

RESULTS

Synergy of 5-nitrofurans, Van and DOC against Gram-negative bacteria

Growth-inhibition checkerboard assays were performed to evaluate the synergy between 5-nitrofurans, Van and DOC against a range of Gram-negative bacteria. The MICs for these antibacterials were determined according to the CLSI guidelines and are summarized in Table 2. As a proof of concept that the 5-nitrofuran drug, as an antibacterial class, is synergistic with Van and DOC, we evaluated the antibacterial effect of combinations of these two agents with either of the three 5-nitrofurans (FZ, NIT and NFZ) in a checkerboard assay against the reference strain *E. coli* ATCC 25922. We found that all the three 5-nitrofurans were synergistic with DOC and Van, with FICIs ranging from 0.11 to 0.15 (Fig. S1).

Among the 5-nitrofurans included in this study, FZ was the most potent against the strains tested (Table 2). Therefore, we chose FZ as a representative of the 5-nitrofurans in the expanded strain profiling by checkerboard and time-kill assays. The FICIs for the two-drug and three-drug combinations of FZ, Van and DOC are listed in Table 3. For some of the strains, where the MIC could not be determined, the FICI was calculated using the highest tested concentration. In this case, the actual FICI would be lower than the calculated value, and for some strains, it may not be possible to classify an interaction when the calculated FICI is bordering between synergistic and indifferent, such as in *E. coli* NZRM 4457. With the 20 Gram-negative bacterial strains tested, the FZ, Van and DOC combination was synergistic against 15 strains, indifferent against four strains (*S.* Typhimurium LT2, *K. pneumoniae* PMR001, *K. pneumoniae* NZRM 4412 and *P. dagmatis* NZRM 959), and unable to be classified as synergistic or indifferent against one strain (*E. coli* NZRM 4457). Of importance is the synergy of the combination against some of the WHO critical priority pathogens, namely carbapenemase-producing *E. coli* NZRM 4364 and *Acinetobacter baumannii* NZRM 4408, and extended-spectrum β-lactamase-producing *K. pneumoniae* NZRM 4387 (Table 3).

To further investigate the interaction between FZ, Van and DOC in terms of bacterial killing, we performed time-kill

Table 3. FICI values for the two-drug and three-drug combinations of furazolidone, vancomycin and sodium deoxycholate

	FICI			
Strain	**FZ+DOC**	**FZ+Van**	**DOC+Van**	**FZ+Van+DOC**
E. coli ATCC 25922	**0.16**	0.75	0.52	**0.11**
E. coli K1508	**<0.50**	0.75	<1.02	**<0.13**
E. coli ERL034336	**0.27**	0.50	1.01	**0.30**
E. coli UPEC P191	**0.19**	0.75	**0.28**	**0.17**
E. coli NZRM 4364	**<0.31***	0.9	<0.52*	**<0.35***
E. coli NZRM 4402	**0.19**	0.74	**0.23**	**0.19**
E. coli NZRM 4457	<0.75*	<1*	1	<0.54*
E. coli NZRM 4524	**0.19**	**0.35**	0.53	**0.17**
C. gillenii PMR001	**0.17**	0.5	1	**0.27**
K. pneumoniae PMR001	1	0.53	1	0.51
K. pneumoniae NZRM 4387	**0.31**	0.63	1	**0.28**
K.pneumoniae NZRM 4412	1	1	0.53	0.54
K. pneumoniae NZRM 4498	<0.52*	**0.38**	<1*	**<0.26***
Salmonella enterica sv. Typhimurium LT2	**0.31**	0.56	1	0.53
Salmonella enterica NZRM4533	**0.38**	**0.5**	1	**0.27**
Shigella dysenteriae NZRM 1015	**<0.25***	**0.5**	<0.52*	**<0.28***
A. lwoffi NZRM 1218	**0.25**	0.82	**0.46**	**0.29**
A. baumannii NZRM 3697	**0.28**	0.75	1	**0.32**
A. baumannii NZRM 4408	**<0.09***	0.56	1	**<0.13***
P. dagmatis NZRM 959	0.75	0.75	0.63	0.66

FZ, furazolidone; DOC, sodium deoxycholate; Van, vancomycin.
*MIC is higher than the highest tested concentration which was used to calculate the FICI, and therefore the actual FICI is lower than the calculated value; values in bold indicate synergy (FICI≤0.5).

assays on some representative pathogens for which the triple combination showed growth inhibition synergy in the checkerboard assay. The strains were exposed to subinhibitory concentrations of FZ, Van and DOC or two-drug and three-drug combinations of these concentrations over a time course of 24 h. The time-kill analysis for *E. coli* ATCC 25922, *Shigella dysenteriae* NZRM 1015, *Citrobacter gillenii* PMR001 and *Acinetobacter baumannii* NZRM 3697 is shown in Fig. 1. The combination of subinhibitory concentrations of FZ, Van and DOC resulted in >2log$_{10}$ reduction in viable cell count after 24 h of exposure in comparison with the most active single drug, demonstrating the synergy in the bacterial killing of the three-drug combination in these strains. In these four examples, the triple combination led to the extinction of the challenged bacterial population at the end of the assay (i.e. below the limit of detection), which was not achieved by single drugs or double combinations at those same concentrations.

We also performed the time-kill assay for three multidrug-resistant pathogens, *E. coli* NZRM 4364, *A. baumannii* NZRM 4408 and *K. pneumoniae* NZRM 4387. Interestingly, bactericidal synergy was still retained against these strains, although the effect was less profound than against the drug-sensitive strains (Fig. 2).

Indifferent interaction of FZ, DOC and Van against Gram-positive bacteria

Van is not the first choice for the treatment of Gram-positive infections due to its adverse effects [32]. We questioned whether the triple combination would be synergistic against Gram-positive bacteria, achieving eradication with a lower dose of Van, and thus mitigating its adverse effects. To do so, we examined the interaction between FZ, Van and DOC against some important Gram-positive pathogens such as *Staphylococcus aureus, Streptococcus pneumoniae* and *Streptococcus pyogenes* using a checkerboard assay. In the six Gram-positive strains tested, all two-drug and three-drug combinations of FZ, Van and DOC were classified as indifferent, with FICIs ranging from 0.53 to 1.05 (Table 4). The

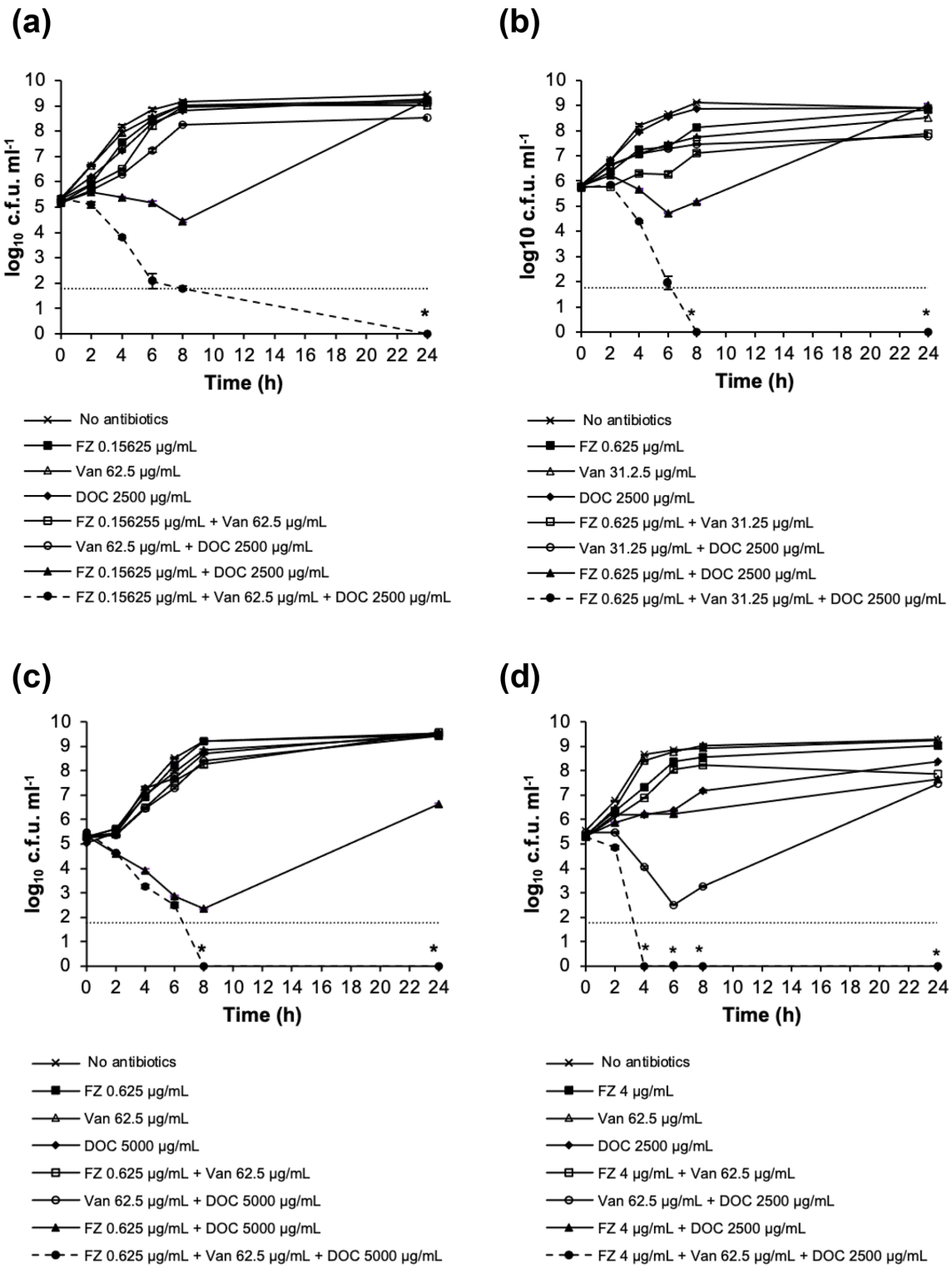

Fig. 1. Time-kill analysis of furazolidone (FZ), vancomycin (Van) and sodium deoxycholate (DOC) combinations in killing (a) *E. coli* ATCC 25922, (b) *Shigella dysenteriae* NZRM 1015, (c) *C. gillenii* PMR001 and (d) *A. baumannii* NZRM 3697. The data are presented as mean±SEM of three independent measurements. The lower limit of detection was 60 c.f.u. ml^{-1}; an asterisk (*) indicates a data point that is below the limit of detection.

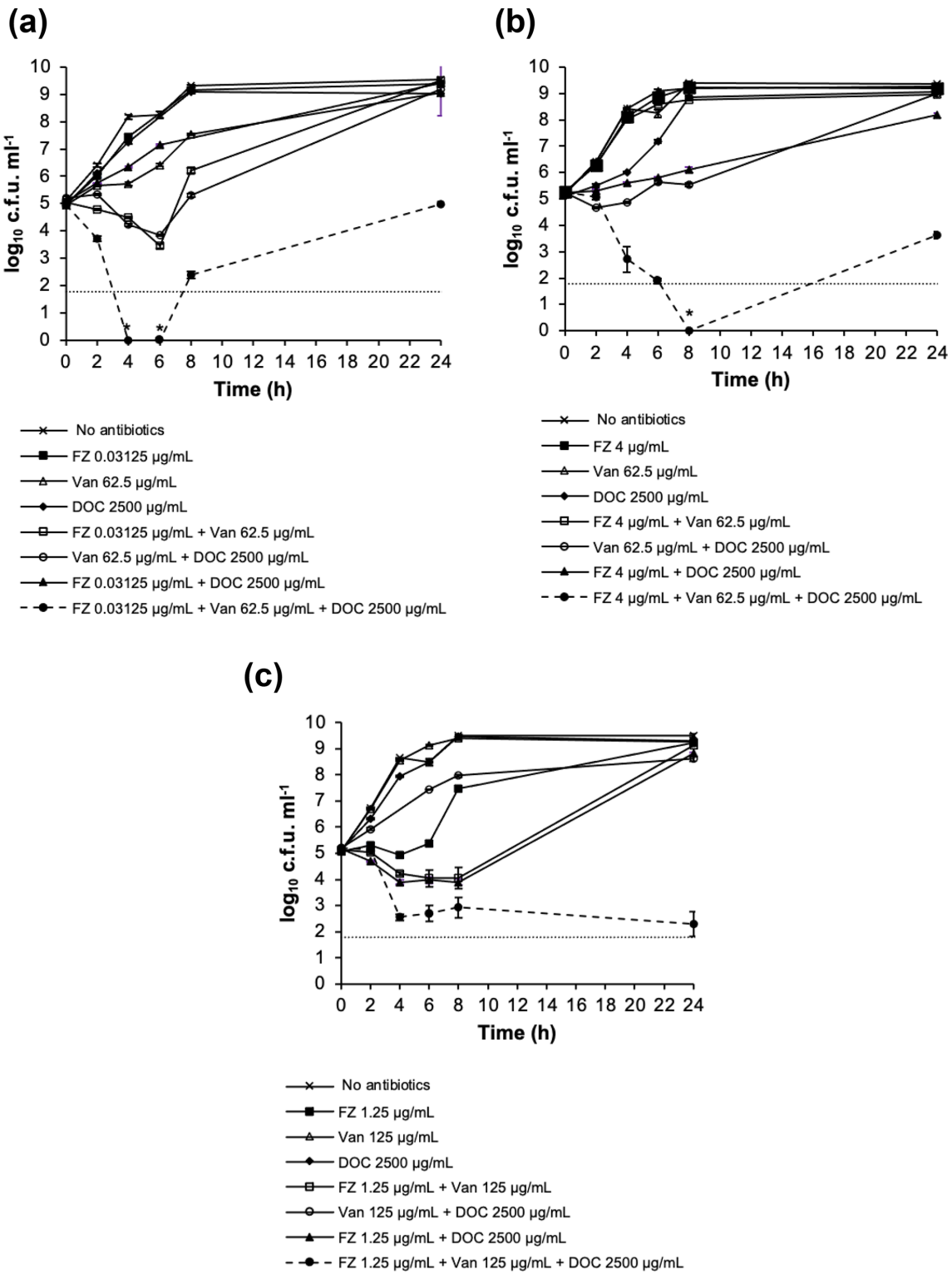

Fig. 2. Time-kill analysis of furazolidone (FZ), vancomycin (Van) and sodium deoxycholate (DOC) combinations in killing carbapenemase-producing (a) *E. coli* NZRM 4364 and (b) *A. baumannii* NZRM 4408, and (c) multidrug-resistant extended-spectrum β-lactamase-producing *K. pneumoniae* NZRM 4387. The data are presented as mean±SEM of three independent measurements. The lower limit of detection was 60 c.f.u. ml^{-1}; an asterisk (*) indicates a data point that is below the limit of detection.

Table 4. MICs and FICIs for the combinations of FZ, Van and DOC against Gram-positive pathogens

	MIC* (μg ml^{-1})		
Strain	**FZ**	**DOC**	**Van**
Staphylococcus aureus NZRM 3478	4	312.50	2
Staphylococcus aureus NZRM 4315	2	625	1
Staphylococcus aureus NZRM 4548	2	625	1
Staphylococcus aureus NZRM 4549	2	625	1
Streptococcus pyogenes NZRM 4366	16	156.25	0.78
Streptococcus pneumoniae NZRM 2764	2	625	0.78

	FICI			
	FZ+DOC	**FZ+Van**	**DOC+Van**	**FZ+Van+DOC**
Staphylococcus aureus NZRM 3478	1	1	1	0.81
Staphylococcus aureus NZRM 4315	1	1.03	1	0.88
Staphylococcus aureus NZRM 4548	1	1.03	1.02	1.05
Staphylococcus aureus NZRM 4549	1	1.03	1	1.03
Streptococcus pyogenes NZRM 4366	0.63	0.63	0.53	0.56
Streptococcus pneumonia NZRM 2764	0.53	0.75	1	0.58

*MIC of antibacterials when used as monotherapy.

inhibitory concentrations of at least two out of these three antibacterials were lower against Gram-positive bacteria than concentrations within the Gram-negative-inhibitory triple combination, and hence the combination would be effective against these pathogens despite an indifferent interaction.

Interactions of bile salts mixture with FZ and Van against *E. coli*

We further investigated whether a natural bile combination present in the human gut is able to aid treatment of enterobacteria-caused gastrointestinal illness in combination with FZ and Van. Ox gall powder from Sigma-Aldrich (Cat. No. B3883) was reported to have bile salt ratios closest to human bile [33] and was therefore used in this study. The content of DOC in the ox gall powder, according to Hu *et al.* [33], is 0.09% (w/w) or 2.09 mmol kg^{-1}.

The MIC of the ox gall powder for the *E. coli* strain ATCC 25922 was 200 mg ml^{-1}, a much higher value than that of DOC (80 mg ml^{-1}). A checkerboard assay was performed to investigate interactions between FZ, Van and ox gall bile salts. In the two-way combinations of the ox gall bile salts with FZ or Van, the FICIs were both 1.02, indicating an indifferent interaction in the double combinations. The three-way combination of ox-gall bile salts, Van and FZ led to a modest decrease in the FICI (0.63), yet this value corresponds to an indifferent interaction.

Interactions of lipoglycopeptides with FZ and DOC against *E. coli*

Dalbavancin and oritavancin are two modified versions of Van that have been reported to possess higher efficacy against Gram-positive bacteria and a better safety profile than Van [17]. Given that they share the same mechanism of action with Van, we hypothesized that dalbavancin and oritavancin were also synergistic with FZ and DOC in inhibiting Gram-negative pathogens. The highest achievable concentration of dalbavancin and oritavancin, due to limited solubility, is 200 μg ml^{-1}. This concentration was too low to inhibit the

growth of *E. coli* ATCC 25922. The combination of 200 µg ml^{-1} dalbavancin or oritavancin with FZ (0.04 µg ml^{-1}) and DOC (625 µg ml^{-1}) resulted in an FICI of <1.04, indicating indifferent interactions.

DISCUSSION

Novel effective therapies are urgently needed against Gram-negative bacteria, in particular carbapenemase-producing *Acinetobacter baumannii* and enterobacteria. The latter two groups top the list of WHO priority pathogens for which development of novel therapies is urgent. With the conventional approach of small-molecule antibiotic development unable to keep pace with the continuing emergence and spread of multidrug resistance, alternative strategies are necessary to expand the therapeutic space. Synergistic combinations of currently available antibiotics show promise due to their enhanced activity with the advantage of improved clearance of pathogens, slowed resistance development and decreased toxicity [34].

Van and DOC, on their own, are not effective against Gram-negative bacteria, as evidenced by their high MICs when applied individually. Due to the relative impermeability of the outer membrane, large antibiotics such as Van are not able to reach their targets in Gram-negative bacteria [35, 36]. These bacteria have also evolved to be highly resistant to bile salts, including DOC [37–39]. As shown in this study, a Gram-negative-active agent, 5-nitrofuran, undermines tolerance of these bacteria to Van and DOC, thereby allowing the use of these antimicrobials to be expanded beyond Gram-positive bacteria.

Because of the synergistic interaction, effective doses of the individual drugs in the combinations have been significantly reduced. This reduction is especially significant for drugs that have adverse effects, such as Van and 5-nitrofurans. Decline in the use of these drugs after their introduction to the market is due to their toxicity. Adverse reactions of Van, including nephrotoxicity and ototoxicity [12, 14], are partly the reasons why this antibiotic is considered a last resort treatment for Gram-positive infections. Similarly, 5-nitrofuran use has been controversial due to its mutagenicity [11]. Taking advantage of the NVD synergy and using these antibiotics in a combination at lowered concentrations will allow the revival of these 'old' drugs due to the reduction or elimination of adverse effects.

In addition to testing the combination in Gram-negative pathogens, we also investigated the drug interaction in Gram-positive bacteria that cause skin and soft tissue infections such as *Staphylococcus aureus* and *Streptococcus pyogenes*. Although the three molecules are not synergistic against Gram-positive pathogens, the individual MICs of DOC and Van are very low in comparison with those for Gram-negative bacteria. The combination therapy effective against Gram-negative bacteria will therefore contain at least two molecules at concentrations above the Gram-positive MIC and will therefore inhibit these latter organisms. The NVD combination therapy could therefore be considered an alternative therapeutic option for the Gram-positive WHO priority pathogens such as methicillin-resistant *Staphylococcus aureus*. Besides these Gram-positive bacteria, some Gram-negative pathogens, such as enterobacteria, can also cause skin and soft tissue infections, albeit at lower rates compared to Gram-positive pathogens [40]. This study therefore provides evidence for the potential of the NVD combination as a broad-spectrum treatment for Gram-negative and Gram-positive skin infections.

Bile salts are present along the gastrointestinal tract. To assess the potential contribution of endogenous bile salts to NVD therapy of gastrointestinal infections, we tested the combination of FZ, Van and a human-gut-like *in vitro* bile mixture (ox gall) against *E. coli* and have found that this combination interacts indifferently. These findings suggest that components other than DOC (e.g. other bile salts) of ox gall powder have antibacterial properties, which act only additively with FZ and/or Van, and that the DOC content of ox gall powder is not sufficient to result in synergy. An important consideration, however, is that bile salts undergo transformations such as deconjugation and dehydroxylation along the gastrointestinal tract after they are released from the gall bladder [20]. Therefore, ox gall powder may not be a suitable human bile model due to under-representation of DOC in comparison with the human intestine, where bile salt transformations lead to a higher DOC concentration. Notwithstanding this caveat, it can be concluded from the ox gall experiment that for treating gastrointestinal infections, DOC would need to be supplemented together with FZ and Van to achieve the synergistic effect of the triple therapy.

Because the lipoglycopeptide antibiotics dalbavancin and oritavancin have better safety profiles than Van, we sought to expand their use beyond Gram-positive bacteria by employing them in the triple combination instead of Van. Therefore, we tested the combination of these lipoglycopeptides with FZ and DOC, and found that at the highest soluble concentration in liquid media, dalbavancin and oritavancin are not inhibitory to *E. coli*. The FICI values of these lipoglycopeptides could not be calculated because insolubility precluded determination of their MIC for *E. coli*. Using maximal soluble concentrations as proxies, checkerboard analyses showed at least indifferent interaction with FZ and DOC; a synergistic interaction, however, cannot be excluded.

The variation among strains in MIC and type of interaction between 5-nitrofurans, Van and DOC was observed in this study. No obvious pattern in terms of individual drug MICs can be seen that would dictate whether synergy would be observed or not. In addition, the mechanism of the NVD combination synergy is unknown. These reasons make it difficult to predict whether the NVD combination will be synergistic in a specific bacterial strain. Elucidation of the triple synergy mechanism will help understand why the combination is not synergistic in some of the Gram-negative strains.

A limitation of our study is that time-kill assays were only performed to confirm synergistic interaction identified by the checkerboard assay. It is known that FICIs from checkerboard

tests can differ depending on the various interpretation criteria. This could mean that in some strains where the combination was interpreted as indifferent, it could actually be synergistic, or vice versa. In this regard, for strains showing borderline interactions between synergistic and indifferent interactions by checkerboard growth inhibition assays, further time-kill analyses are expected to define more precisely the NVD interaction with respect to their bactericidal effect. Finally, this was an *in vitro* study, and the ability of *in vitro* combination assays to predict clinical synergy is unknown. *In vivo* studies are therefore needed to confirm the clinical relevance of our findings.

In summary, we present here a synergistic combination of 5-nitrofurans, Van and DOC (NVD) against Gram-negative pathogens. This synergistic interaction allows the use of antimicrobials, such as Van and DOC, that would otherwise be ineffective against Gram-negative bacteria.

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Author contributions
Conceptualization, C.O., V.L. and J.R. Methodology, C.O. Investigation, C.O. and C.D. Original draft preparation, C.O. Review and editing, V.L. and J.R.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Breijyeh Z, Jubeh B, Karaman R. Resistance of Gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules* 2020;25:1340.
2. World Health Organization. *Prioritization of Pathogens to Guide Discovery, Research and Development of New Antibiotics for Drug-Resistant Bacterial Infections, Including Tuberculosis*. Geneva, Switzerland: World Health Organization; 2017.
3. World Health Organization. *Antibacterial Agents in Clinical Development: an Analysis of the Antibacterial Clinical Development Pipeline*, 2019. Geneva, Switzerland: World Health Organization; 2019.
4. McCalla DR, Voutsinos D. On the mutagenicity of nitrofurans. *Mutat Res-Fund Mol M* 1974;26:3–16.
5. Obaseiki-Ebor EE, Akerele JO. Nitrofuran mutagenicity: induction of frameshift mutations. *Mutat Res Lett* 1986;175:149–152.
6. Ny S, Edquist P, Dumpis U, Gröndahl-Yli-Hannuksela K, Hermes J *et al*. Antimicrobial resistance of *Escherichia coli* isolates from outpatient urinary tract infections in women in six European countries including Russia. *J Glob Antimicrob Re* 2019;17:25–34.
7. Raja NS. Oral treatment options for patients with urinary tract infections caused by extended spectrum *β*eta-lactamase (ESBL) producing Enterobacteriaceae. *J Infect Public Heal* 2019;12:843–846.
8. Kwon DH, Lee M, Kim JJ, Kim JG, El-Zaatari FAK *et al*. Furazolidone- and nitrofurantoin-resistant *Helicobacter pylori*: Prevalence and role of genes involved in metronidazole resistance. *Antimicrob Agents Chemother* 2001;45:306–308.
9. Choi YI, Jeong S-H, Chung J-W, Park DK, Kim KO *et al*. Rifabutin and furazolidone could be the candidates of the rescue regimen for antibiotic-resistant *H. pylori* in Korea. *Can J Infect Dis Med* 2019;2019:1–7.
10. Zamani M, Rahbar A, Shokri-Shirvani J. Resistance of *Helicobacter pylori* to furazolidone and levofloxacin: A viewpoint. *WJG* 2017;23:6920–6922.
11. Vass M, Hruska K, Fránek M. Nitrofuran antibiotics: a review on the application, prohibition and residual analysis. *Vet Med (Praha)* 2008;53:469–500.
12. Filippone EJ, Kraft WK, Farber JL. The nephrotoxicity of vancomycin. *Clin Pharmacol Ther* 2017;102:459–469.
13. Marinho DS, Huf G, Ferreira BLA, Castro H, Rodrigues CR *et al*. The study of vancomycin use and its adverse reactions associated to patients of a Brazilian university hospital. *BMC Res Notes* 2011;4:1.
14. Forouzesh A, Moise PA, Sakoulas G. Vancomycin ototoxicity: a reevaluation in an era of increasing doses. *Antimicrob Agents Chemother* 2009;53:483–486.
15. Roszell S, Jones C. Intravenous administration issues: a comparison of intravenous insertions and complications in vancomycin versus other antibiotics. *J Infus Nurs* 2010;33:112–118.
16. Van Bambeke F. Lipoglycopeptide antibacterial agents in gram-positive infections: a comparative review. *Drugs* 2015;75:2073–2095.
17. Crotty MP, Krekel T, Burnham C-AD, Ritchie DJ. New gram-positive agents: the next generation of oxazolidinones and lipoglycopeptides. *J Clin Microbiol* 2016;54:2225–2232.
18. Zeng D, Debabov D, Hartsell TL, Cano RJ, Adams S *et al*. Approved glycopeptide antibacterial drugs: mechanism of action and resistance. *Cold Spring Harb Perspect Med* 2016;6:a026989.
19. Begley M, Gahan CGM, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev* 2005;29:625–651.
20. Urdaneta V, Casadesús J. Interactions between bacteria and bile salts in the gastrointestinal and hepatobiliary tracts. *Front Med* 2017;4.
21. Lin J, Sahin O, Michel LO, Zhang Q. Critical role of multidrug efflux pump CmeABC in bile resistance and *in vivo* colonization of *Campylobacter jejuni*. *Infect Immun* 2003;71:4250–4259.
22. Thanassi DG, Cheng LW, Nikaido H. Active efflux of bile salts by *Escherichia coli*. *J Bacteriol* 1997;179:2512–2518.
23. Venter H, Mowla R, Ohene-Agyei T, Ma S. RND-type drug efflux pumps from gram-negative bacteria: molecular mechanism and inhibition. *Front Microbiol* 2015;06.
24. Prieto AI, Ramos-Morales F, Casadesús J. Repair of DNA damage induced by bile salts in *Salmonella enterica*. *Genetics* 2006;174:575–584.
25. Hernández SB, Cota I, Ducret A, Aussel L, Casadesús J. Adaptation and preadaptation of *Salmonella enterica* to bile. *PLoS Genet* 2012;8:e1002459.
26. Le VVH, Olivera C, Spagnuolo J, Davies IG, Rakonjac J. In vitro synergy between sodium deoxycholate and furazolidone against enterobacteria. *BMC Microbiol* 2020;20.
27. Zhou A, Kang TM, Yuan J, Beppler C, Nguyen C *et al*. Synergistic interactions of vancomycin with different antibiotics against *Escherichia coli*: trimethoprim and nitrofurantoin display strong synergies with vancomycin against wild-type *E. coli*. *Antimicrob Agents Chemother* 2015;59:276–281.
28. Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 10th ed. M07-A10 Wayne, PA: Clinical and Laboratory Standards Institute; 2015.
29. Campbell J. High-throughput assessment of bacterial growth inhibition by optical density measurements. *Curr Protoc Chem Biol*2011;3.

30. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Dieases (ESCMID). Eucast definitive document E.Def 1.2, may 2000: terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. *Clin Microbiol Infect* 2000;6:503–508.
31. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. *J Antimicrob Chemother* 2003;52:1.
32. Bruniera F, Ferreira F, Saviolli L, Bacci M, Feder D. The use of vancomycin with its therapeutic and adverse effects: a review. *Eur Rev Med Pharmacol Sci* 2015;19:694–700.
33. PL H, Yuan YH, Yue TL, Guo CF. Bile acid patterns in commercially available oxgall powders used for the evaluation of the bile tolerance ability of potential probiotics. *PLoS One* 2018;13:e0192964-e.
34. Bollenbach T. Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution. *Curr Opin Microbiol* 2015;27:1–9.
35. Hammes WP, Neuhaus FC. On the mechanism of action of vancomycin: inhibition of peptidoglycan synthesis in *Gaffkya homari*. *Antimicrob Agents Chemother* 1974;6:722–728.
36. Nieto M, Perkins HR. Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. *Biochem J* 1971;123:789–803.
37. Gunn JS. Mechanisms of bacterial resistance and response to bile. *Microbes Infect* 2000;2:907–913.
38. Raphael BH, Pereira S, Flom GA, Zhang Q, Ketley JM *et al*. The *Campylobacter jejuni* response regulator, CbrR, modulates sodium deoxycholate resistance and chicken colonization. *J Bacteriol* 2005;187:3662–3670.
39. Paul S, Alegre KO, Holdsworth SR, Rice M, Brown JA *et al*. A single-component multidrug transporter of the major facilitator superfamily is part of a network that protects *Escherichia coli* from bile salt stress. *Mol Microbiol* 2014;92:872–884.
40. Moet GJ, Jones RN, Biedenbach DJ, Stilwell MG, Fritsche TR. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: report from the SENTRY antimicrobial surveillance program (1998–2004). *Diagn Microbiol Infect Dis* 2007;57:7–13.
41. Spagnuolo J, Opalka N, Wen WX, Gagic D, Chabaud E *et al*. Identification of the gate regions in the primary structure of the secretin pIV. *Mol Microbiol* 2010;76:133–150.
42. Williamson DA, Sidjabat HE, Freeman JT, Roberts SA, Silvey A *et al*. Identification and molecular characterisation of New Delhi metallo-*β*-lactamase-1 (NDM-1)- and NDM-6-producing Enterobacteriaceae from New Zealand hospitals. *Int J Antimicrob Agents* 2012;39:529–533.