Evaluation of treatment options for *Enterococcus faecalis* endocarditis by assessing synergistic and anti-biofilm effects

Dissertation

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Abbreviations

Ace	adhesin to collagen
AS	aggregation substance
CDC	Centre for Disease Control
CEO	Chief Executive Officer
CF	cystic fibrosis
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CLSM	confocal laser scanning microscope
ebp	endocarditis and biofilm-associated pili
eDNA	extracellular DNA
EFIE	*Enterococcus faecalis* infective endocarditis
EPS	extracellular polymeric substance
ESC	European Society of Cardiology
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FBEC	fractional biofilm eradication concentration
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
gelE	gelatinase E
Gm	*Galleria mellonella*
HACEK	*Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, Kingella*
HLAR	high level aminoglycoside resistance
HTP	high-throughput
IE	infective endocarditis
iv	intravenous

log	logarithmic
MBEC	minimal biofilm eradication concentration
MIC	minimum inhibitory concentration
MRSA	methicillin resistant *Staphylococcus aureus*
OPAT	outpatient parenteral antibiotic therapy
PBP	penicillin binding protein
PCR	polymerase chain reaction
POET	Partial Oral Treatment of Endocarditis
PSI	pathology score index
SCV	small colony variant
SGT	Start-Growth-Time
sprE	serine protease E
TKA	time-kill curve assay
UTI	urinary tract infection
UV	ultraviolet
VRE	vancomycin resistant enterococci

Summary

Enterococcus faecalis is increasingly found as the causative organism of infective endocarditis, an infection of the heart valves and endocardium with mortality rates up to 30 %. Traditionally, antibiotic combination therapy has been used for treatment of *E. faecalis* endocarditis, but it is still not clear if and which combination therapy is truly superior to monotherapy. Further, the chronic, treatment-resistant nature of infective endocarditis strongly suggests the involvement of biofilms, an aspect so far neglected in the treatment recommendation based on antibiotic susceptibility testing of planktonic bacterial cultures. The main aim of this thesis was therefore to compare the recommended but nephrotoxic standard gentamicin/ampicillin combination with the recently recommended alternative ceftriaxone/ampicillin and the novel possibly effective ceftaroline/ampicillin combination by evaluating their synergistic interactions against planktonic and biofilm-embedded enterococci.

To compare the antibiotic combinations, both the biofilm and the synergy methodology needed to be further developed. Assessment of anti-biofilm activities of antibiotics is currently hampered by the lack of standardized biofilm susceptibility methods. Biofilm eradicative and preventive activities of the antibiotic combinations were measured by colony forming unit quantification, confocal laser scanning microscopy and an image-based biofilm analysis algorithm. A novel method for biofilm analysis, the Start-Growth-Time method, including standardized biofilm susceptibility endpoint parameters, was developed to allow for future high-throughput anti-biofilm measurements. To analyse synergistic effects between the antibiotics on planktonic level in a simple *in vivo* model, the *Galleria mellonella* larval infection model was established in the course of this thesis. A novel method for standardized synergism testing in larvae based on a partial transfer of an *in vitro* checkerboard assay into the larvae was suggested. Synergy results of *in vivo* larval analysis were compared to those of *in vitro* checkerboard analysis.

On biofilm level, no synergistic effects between neither a cephalosporin- nor aminoglycoside-based combination could be observed, since none of the antibiotic combinations succeeded in eradicating mature *E. faecalis* biofilms. However, in planktonic *E. faecalis* cells synergism could be detected for ceftriaxone/ampicillin both *in vitro* and in the larvae, while for ceftaroline/ampicillin synergism was only detected *in vitro*. Gentamicin/ampicillin showed no synergistic effects for both *in vitro* and *in vivo*

synergy analysis.

In conclusion, the choice of antibiotic combination or monotherapy for treatment of *E. faecalis* endocarditis should ideally be based on the maturity and progression of biofilm development. While patients with acute disease and a proven bacteraemia (i.e. planktonic cells) may benefit from a synergistic antibiotic combination therapy, combination therapy does not seem to be superior to monotherapy for treatment of mature biofilms. The results of this thesis favour the replacement of gentamicin/ampicillin by a cephalosporin-based combination due to the lack of synergistic effects. However, novel cephalosporins, i.e. ceftaroline instead of ceftriaxone, do not seem to provide additional benefit. Future antibiotic treatment options of *E. faecalis* endocarditis should be directed towards biofilm eradication to possibly avoid surgical valve replacement. Therefore, the further development of clinically meaningful biofilm susceptibility assays is mandatory.

Zusammenfassung

Enterococcus faecalis wird zunehmend als Erreger der infektiösen Endokarditis diagnostiziert. Bei der infektiösen Endokarditis handelt es sich um eine Infektion der Herzklappen und des Endokards mit einer hohen Sterblichkeitsrate von bis zu 30 %. Traditionell wird eine Antibiotika-Kombinationstherapie zur Behandlung der *E. faecalis*-Endokarditis eingesetzt, wobei noch nicht geklärt ist, ob und welche Kombinationstherapie der Monotherapie überlegen ist. Darüber hinaus deutet die chronische, behandlungsresistente Natur der infektiösen Endokarditis stark auf die Beteiligung von Biofilmen hin. Der Biofilm-Aspekt wurde bisher in den Behandlungsrichtlinien vernachlässigt, die auf Sensitivitätstestungen bei planktonischen Zellen beruhen. Das Hauptziel dieser Arbeit war es daher, die empfohlene, aber nephrotoxische Standardkombination Gentamicin/Ampicillin mit der kürzlich empfohlenen alternativen Kombination Ceftriaxon/Ampicillin und der neuartigen Kombination Ceftarolin/Ampicillin zu vergleichen, indem ihre synergistischen Wechselwirkungen gegen planktonische und in Biofilmen eingebettete Enterokokken bewertet wurden.

Um die Antibiotika-Kombinationen zu vergleichen, mussten sowohl die Biofilm- als auch die Synergiemethodik weiterentwickelt werden. Die Beurteilung von Anti-Biofilm-Aktivitäten von Antibiotika wird derzeit durch das Fehlen standardisierter Methoden zur Sensitivitätstestung von Biofilmen erschwert. Die Biofilm-eradizierenden und -präventiven Aktivitäten der Antibiotika-Kombinationen wurden mittels der Quantifizierung von koloniebildenden Einheiten, der konfokalen Laser-Scanning-Mikroskopie und einem bildbasierten Biofilm-Analysealgorithmus bestimmt. Die Start-Wachstumszeit-Methode einschließlich standardisierter Endpunktparameter für die Sensitivitätstestung von Biofilmen wurde als neuartige Methode für zukünftige Hochdurchsatz-Anti-Biofilm-Messungen entwickelt. Um synergistische Effekte auf planktonischer Ebene zwischen den Antibiotika in einem einfachen *In-vivo*-Modell zu analysieren, wurde im Rahmen dieser Arbeit das *Galleria mellonella*-Larveninfektionsmodell etabliert. Ferner wurde eine neuartige Methode für standardisierte Synergismus-Testungen in Larven eingeführt, welche auf einer Überführung des *In-vitro*-Checkerboard-Aufbaus in die Larve basiert. Die Synergieergebnisse der *In-vivo*-Larvenanalyse wurden mit denen der *In-vitro*-Checkerboardanalyse verglichen.

Auf Biofilmebene konnten keine synergistischen Effekte zwischen einer Kombination auf Cephalosporin- oder Aminoglykosidbasis beobachtet werden, da es keiner der Antibiotika-Kombinationen gelang, ausgereifte *E. faecalis*-Biofilme anzugreifen. In planktonischen *E. faecalis*-Zellen konnte jedoch sowohl *in vitro* als auch in den Larven ein Synergismus für Ceftriaxon/Ampicillin nachgewiesen werden, während für Ceftarolin/Ampicillin ein Synergismus nur *in vitro* nachgewiesen wurde. Gentamicin/Ampicillin zeigten sowohl in der *In-vitro*- als auch in der *In-vivo*-Synergieanalyse keine synergistischen Effekte.

Zusammenfassend sollte die Wahl, ob eine Antibiotika-Kombinations- oder Monotherapie zur Behandlung der *E. faecalis*-Endokarditis eingesetzt wird, idealerweise auf der Reife und dem Grad der Biofilmentwicklung beruhen. Während Patienten mit einer akuten Endokarditis mit nachgewiesener Bakteriämie (d.h. planktonische Zellen) von einer synergistischen Antibiotika-Kombinationstherapie profitieren könnten, scheint die Kombinationstherapie der Monotherapie zur Behandlung ausgereifter Biofilme nicht überlegen zu sein. Die Ergebnisse dieser Arbeit sprechen für einen Wechsel von Aminoglykosid- zu Cephalosporin-Kombinationen, da keine synergistischen Effekte zwischen Gentamicin/Ampicillin beobachtet werden konnten. Jedoch scheint der Wechsel zu neuen Cephalosporinen, d.h. von Ceftriaxon zu Ceftarolin, keine weiteren Vorteile zu bieten. Zukünftige Behandlungsoptionen der *E. faecalis*-Endokarditis sollten auf Strategien zur Eradikation von Biofilmen zielen, um einen chirurgischen Klappenersatz zu vermeiden. Dafür ist die Weiterentwicklung klinisch anwendbarer Methoden für die Sensitivitätstestung von Biofilmen zwingend erforderlich.

1 Introduction

1.1 *Enterococcus faecalis* infective endocarditis

1.1.1 Clinical aspects of infective endocarditis

Infective endocarditis (IE) is defined as a potentially deadly infection and inflammation of the heart valves and endocardium. The pathophysiology is characterized by the formation of infective endocardial vegetations, consisting of platelets, fibrin and microorganisms (Marrie et al. 1987, Pierce et al. 2012). These vegetations form when bacteria enter the bloodstream and attach to abnormal, damaged or replaced (i.e. prosthetic) valves. Infection of healthy valves occurs either due to high bacterial inoculum, e.g., in septic patients, or aggressive bacterial strains (Cahill et al. 2017). Risk factors for IE include immunosuppression, increased age, prosthetic valve replacement, insertion of cardiac implantable electronic devices (e.g., pacemakers, defibrillator), haemodialysis, venous catheters and intravenous (iv) drug use (Slipczuk et al. 2013). There are two forms of IE: acute IE develops suddenly with life-threating symptoms of heart failure within days, while subacute or chronic IE develops gradually over weeks or months with initially mild clinical symptoms (McDonald 2009). The principles of diagnosis are largely identical between acute and subacute IE both relying on the modified Duke criteria (Habib et al. 2015). These criteria involve clinical information, microbiological findings (blood culture, in some cases PCR) and imaging results (transthoracic and/or transesophageal echocardiography). Although the incidence of IE with 3 to 10 cases per 100.000 per year is rather low, mortality rates still approach up to 30 % (Bin Abdulhak et al. 2014, Holland et al. 2016, Pant et al. 2015). Despite earlier diagnosis, surgical intervention, antibiotic prophylaxis and optimized antibiotic treatment, the 1-year mortality has not improved in two decades (Cahill et al. 2017, Habib et al. 2019). The European Society of Cardiology (ESC) has therefore proposed a collaborative approach for management of IE by an “Endocarditis Team”, composed of cardiologists, cardiac surgeons, infectious disease physicians, microbiologists, radiologists and other experts depending on the patient’s symptoms (Habib et al. 2015). Since IE is frequently acquired as a nosocomial infection, health care-associated organisms have been increasingly found as causative microbes (Cahill et al. 2017). Staphylococci and streptococci comprise the majority of IE cases

with roughly 70 %, while enterococci account for a further 10 % (Habib et al. 2015, Murdoch et al. 2009). HACEK (*Haemophilus, Aggregatibacter, Cardiobacterium, Eikenella, Kingella*) organisms, zoonoses and fungi can be identified as causative agents of IE in approximately 5 % of cases, while 10-20 % of patients have negative blood culture findings (Cahill et al. 2017).

1.1.2 Treatment options of enterococcal endocarditis

Enterococci are Gram-positive, facultative anaerobe bacteria found as commensal opportunists in the human gastrointestinal flora (Arias and Murray 2012). While enterococci are usually not highly virulent or aggressive, they have an exceptional ability to adapt to harsh environmental conditions, such as high temperatures (up to 60°C), high salt concentrations and wide pH ranges (Lebreton et al. 2014). Their extraordinary intrinsic resistance to many antibiotics and tolerance to common disinfectants, UV radiation, starvation and desiccation allow enterococci to persist for extended periods in the hospital environment (Lebreton et al. 2017). If enterococci escape the microbiome, they can cause a variety of infections, e.g., urinary tract infections (UTIs), bacteraemia, intra-abdominal infections, and endocarditis. Enterococci are one of the leading hospital pathogens and the third most common cause of IE (9-17 % of all cases) (Dahl and Bruun 2013). However, recent studies suggest that enterococcal IE numbers are rising and approaching staphylococcal IE due to changing demographics, more gastrointestinal and urinary tract instrumentations and increased numbers of health-care associated infections (Fernandez-Hidalgo et al. 2020, Hill et al. 2007, Pericás et al. 2015, Scudeller et al. 2009). Enterococcal IE patients are older than patients with streptococcal or staphylococcal IE (Dahl and Bruun 2013, Pericás et al. 2015). In addition, enterococci are responsible for 13 % of prosthetic valve endocarditis cases (Dahl and Bruun 2013, Nagpal et al. 2012). *Enterococcus faecium* and *Enterococcus faecalis* are the most clinically relevant species among enterococcal infections in general, with *E. faecalis* being the predominant species responsible for approximately 90 % of all enterococcal IE cases (Habib et al. 2015). Although *E. faecium* endocarditis cases remain the minority, treatment is strongly limited by intrinsic and acquired antibiotic resistances, i.e. β-lactam or vancomycin resistant enterococci (VRE). Treatment guidelines recommend vancomycin combined with gentamicin for non-VRE and daptomycin-

based combinations for VRE cases (Habib et al. 2015).

In contrast to *E. faecium*, most *E. faecalis* isolates remain susceptible to cell wall-active β-lactam antibiotics such as ampicillin (Arias and Murray 2008). Effective treatment of deep-seated, high-inoculum *E. faecalis* infective endocarditis (EFIE) requires, however, bactericidal antibiotic combination therapy (Habib et al. 2015). Consequently, ampicillin has been supplemented with high-bactericidal aminoglycosides such as gentamicin or streptomycin for treatment of EFIE, based on *in vitro* synergy results and observational clinical data (Beganovic et al. 2018). However, acquisition of high-level aminoglycoside resistance (HLAR) and severe side effects, e.g., nephrotoxicity and ototoxicity, has prompted the need for alternative treatment options of EFIE. Dual β-lactam therapy, i.e. ampicillin combined with a cephalosporin, has emerged as a novel treatment alternative with clinically similar efficiency but a better safety profile. Although enterococci are intrinsically resistant to cephalosporins, two observational clinical studies published 2007 and 2013 have shown non-inferiority of ceftriaxone/ampicillin compared to gentamicin/ampicillin while reducing the risk of early treatment withdrawal due to less toxic side effects (Fernandez-Hidalgo et al. 2013, Gavaldà et al. 2007). No differences in mortality or relapse rates were found between both treatment groups. In 2015, the ESC has therefore adapted their guidelines for treatment of EFIE by recommending ceftriaxone/ampicillin for both HLAR and non-HLAR *E. faecalis* infections (Habib et al. 2015). However, due to the lack of randomized controlled trials, the limited number of observational studies and further limitations in the conducted studies, the replacement of gentamicin by ceftriaxone remains controversial among many clinicians and researchers (Koehler et al. 2019, Lebeaux et al. 2019, Peterson et al. 2017, Beganovic et al. 2018). It remains questionable whether the similar efficiency of ceftriaxone/ampicillin and gentamicin/ampicillin as proposed by retrospective studies is due to true synergistic effects or significantly reduced toxic side effects. Further unsolved questions of antibiotic treatment of EFIE include i) the duration of treatment, i.e. for which subgroups of patients may treatment be shortened from 6 weeks to 4 or even 2 weeks (Fernandez-Hidalgo et al. 2020), ii) combination versus monotherapy, i.e. is prolonged combination therapy truly superior to monotherapy (Koehler et al. 2019), iii) the potential safety risks associated with ceftriaxone, i.e. is the prolonged use of ceftriaxone worth the risk of side effects such as VRE colonization (Beganovic et al. 2018).

Most *E. faecalis* isolates are intrinsically resistant against 3rd generation cephalosporins such as ceftriaxone due to a decrease in binding affinity to essential enterococcal penicillin binding proteins (PBP) (Miller et al. 2014). The mechanism of action of the ceftriaxone/ampicillin combination exploits the differential affinities of the individual antibiotics to different PBP homologues (Gavalda et al. 1999). Novel 5th generation cephalosporins such as ceftaroline show enhanced *in vitro* activity against Gram-positive bacteria, overcoming the intrinsic cephalosporin resistance of enterococci by providing increased affinity to former low-affinity PBP homologues (Henry et al. 2013). Accordingly, ceftaroline might be a superior combination partner for treatment of EFIE. Further, unlike ceftriaxone, ceftaroline does not seem to promote the same negative side effects, e.g., VRE and *Clostridium difficile* infection, increasing its' tolerability during long-term use (Beganovic et al. 2018). The Food and Drug Administration (FDA) as well as the European Commission have approved ceftaroline for treatment of acute bacterial skin and skin structure infections and community-acquired bacterial pneumonia caused by *Staphylococcus aureus*, including methicillin-resistant strains (MRSA) (Laudano 2011). While some *in vitro* studies have shown synergistic effects between ampicillin and ceftaroline against selected planktonic *E. faecalis* isolates (Luther et al. 2016, Werth and Abbott 2015, Werth and Shireman 2017), the clinical potential for treatment of EFIE with ceftaroline alone and in combination with ampicillin remains unknown.

1.1.3 Infective endocarditis is a biofilm-associated infection

Clinical behaviour and *in vitro* studies strongly suggest that IE is a biofilm-associated infection, explaining the poor treatment outcomes and the frequent need of surgical intervention. Biofilms are microbial communities consisting of aggregated bacteria surrounded by a polymeric matrix (Hall-Stoodley et al. 2012). Reasons why bacteria switch from the free-floating, planktonic to the biofilm mode of growth are believed to be shelter from the host immune system or antibiotics, colonization in a nutrient-dense niche and benefits of a cooperative community (Jefferson 2004). Clinically, they occur on both natural (e.g., teeth, skin, endothelium) and inanimate surfaces (e.g., catheters, implants, foreign devices such as pacemakers). Following hallmarks are characteristic for biofilm-associated infections: i) chronic infection with persistent inflammation and tissue damage, ii) presence of implants or biomaterial, iii) localized infection with the

capability to spread, iv) negative cultures despite signs of infection, v) general tolerance to appropriate antibiotics and immune responses, often vi) interval response to antibiotics with relapse of infection with same organism when stopped and vii) resolution of infection with a combination of surgery and antimicrobial treatment (Elgharably et al. 2018, Høiby et al. 2015). Consequently, typical biofilm infections include both device-related and tissue infections, e.g., prosthetic joint infections, wound infections, UTIs, chronic lung infections in cystic fibrosis (CF) patients, and both prosthetic and native valve endocarditis (Høiby et al. 2011). Despite adequate high-dose and long-term antibiotic therapy according to standard antimicrobial susceptibility testing, biofilm-embedded bacteria remain viable leading to treatment failure and increased mortality (Bjarnsholt et al. 2013). The problem of biofilm-associated infections in human medicine has increased with the rising life expectancy of the population, the frequent use of implanted medical devices and the progress in intensive care medicine (intravascular catheters, ventilation tubes, urinary tract catheters) in recent decades. While acute infections are associated with free-living, planktonic bacteria, 60-80 % of all human infections are believed to be biofilm-associated (Macia et al. 2014).

Although high-resolution images of IE-derived heart valves have suggested decades ago that IE is a biofilm-associated disease, it has only recently been acknowledged in clinical practice that biofilm formation plays a major role in IE pathogenicity (Elgharably et al. 2016, Schäfers 2016). While some biofilm diagnostic markers have been developed, e.g., in biofilm-related UTIs (Antypas et al. 2018), there is currently no standardized approach to establish an endocarditis biofilm infection diagnosis without direct evaluation of the infected cardiac structures. No biofilm-specific treatment guidelines are recommended in the ESC guidelines (Habib et al. 2015). In contrast to *E. faecium*, *E. faecalis* shows increased rates of biofilm formation (Di Rosa et al. 2006, Beganovic et al. 2018). *E. faecalis* biofilms were shown *in vitro* to be highly tolerant to almost all clinically applied antibiotics, including ampicillin and gentamicin, whereas the potential of an anti-biofilm effect of ceftaroline is currently unknown (Sandoe et al. 2006, Di Domenico et al. 2019, Holmberg and Rasmussen 2016).

1.2 Medical microbiology of biofilm-associated infections

1.2.1 Current state-of-the-art of medical biofilms

The concept of biofilm-associated infections was introduced into medicine in 1985 by observations of aggregated bacteria in chronically infected CF patients and odontology (Høiby 2014). Biofilms have been classically defined as 'aggregates of microbial cells adherent to a living or non-living surface, embedded within a matrix of extracellular polymeric substance (EPS) of microbial origin' (Hall-Stoodley et al. 2012). However, many features of this definition have nowadays been revised since many different types of biofilms depending on the niche and infection have been observed. Biofilms can occur as sheets, where one layer is in direct contact with the substratum (e.g., implant-associated infections), as flocs or small aggregates, which are non-surface attached, 'mobile' biofilms (e.g., wound infections), or can develop mushroom-like structures with open voids, as seen for *in vitro Pseudomonas aeruginosa* biofilms (Bjarnsholt et al. 2013, Flemming et al. 2016). Differences in biofilm formation have been especially noted between *in vitro* and *in vivo* biofilms. *In vivo* biofilms occur as small aggregates without mushroom-like structures and their constitution and shape is a result of the interaction with the host (Bjarnsholt et al. 2013). Their matrix contains 'extramicrobial' host-derived components, e.g., fibrin and collagen in IE, in addition to bacterial-derived, extracellular macromolecules such as polysaccharides, proteins or extracellular DNA (eDNA) (Hall-Stoodley et al. 2012). Inflammatory cells of the host such as neutrophils produce oxygen gradients around the single bacterial aggregates, creating a distinct environment around the biofilm called secondary matrix (Sønderholm et al. 2017). In contrast, *in vitro* biofilms are usually surface-attached and follow a distinct developmental cycle of attachment, maturation and dispersion without the influence of host factors (Bjarnsholt et al. 2013). Nevertheless, the recalcitrance of biofilms towards antibiotic treatment is easily reproduced *in vitro*, indicating that host factors are not primarily involved in the characteristic antibiotic tolerance of biofilms. Biofilm-mediated tolerance is not caused by genetic alterations but by adaptive mechanisms involving several physical and chemical traits. The phenotypic resistance of biofilms is attributable to i) a downregulated metabolism shutting down the targets of many antibiotics, ii) formation of persister cells, iii) decrease in antibiotic penetration due to the primary and selective barrier of the EPS matrix, iv) accumulation of

antibiotic-modifying enzymes like β-lactamases and v) enhanced horizontal gene transfer (Macia et al. 2014, Stewart 2002).

1.2.2 Enterococcal biofilm formation

Enterococcal biofilms constitute an increasing clinical problem since they are frequently involved in a variety of biofilm-associated opportunistic infections such as catheter-associated UTIs, wound infections or endocarditis. However, little is known about their spatiotemporal formation (Ch'ng et al. 2019). Many factors contributing to the single steps of biofilm formation have been identified for *E. faecalis*, while biofilm formation in *E. faecium* is less well described.

Biofilm formation in general is divided into four stages (I-IV), whereby it is not yet understood whether this fixed developmental cycle also occurs *in vivo* (Coenye et al. 2020). Once enterococci gained access to the bloodstream by e.g., surgical procedures, ascending UTIs or intestinal bacterial translocation, biofilm formation in EFIE starts with the initial attachment (I) of single bacterial cells to the injured endothelial cell layer of the heart valve (Figure 1). While several surface adhesins, proteases and glycolipids of *E. faecalis* were shown to be involved in the adherence process, their individual contribution depends on the niche of infection (Ch'ng et al. 2019). For EFIE, the surface adhesins *aggregation substance* (AS) and *adhesin to collagen* (Ace) as well as the *endocarditis and biofilm-associated pilus* (Ebp) were shown to be involved in binding to fibrinogen, collagen and platelets (Madsen et al. 2017). Once attached, biofilm formation continues by production of microcolonies (II), consisting of aggregates of single bacteria surrounded by small quantities of biofilm matrix. The resulting structure of the matrix protects the biofilm from phagocytosis and removal by mechanical shear forces. It is not yet clear which enterococcal factors drive microcolony formation (Ch'ng et al. 2019). *In vitro*, *E. faecalis* usually forms 2D biofilm sheets, while observation of IE vegetations on heart valves suggest that the microcolonies further develop into mature, small aggregate biofilms as observed in wound infections or pulmonary infection in CF patients. *E. faecalis* biofilm maturation (III) involves the production of extracellular matrix, from which the best characterized enterococcal component is eDNA. The release of eDNA is done by metabolically active cells and tightly controlled by the two enterococcal proteases *gelatinase E* (gelE) and *serine protease E* (sprE) (Paganelli et al. 2012). Both are regulated by quorum sensing,

the population density-dependent signalling within a biofilm. Cell-to-cell communication upregulates the virulence profile of the *E. faecalis* biofilm, leading to invasion and destruction of the endothelial tissue, followed by the clinically observed hyperinflammatory state of the host immune response (Elgharably et al. 2018). Gelatinase-mediated degradation of the fibrin layer of the biofilm vegetation leads to dissemination and embolization (IV) of planktonic cells in the bloodstream (Madsen et al. 2017). Factors leading to the switch from mature biofilms to the dispersal state are still elusive in enterococci.

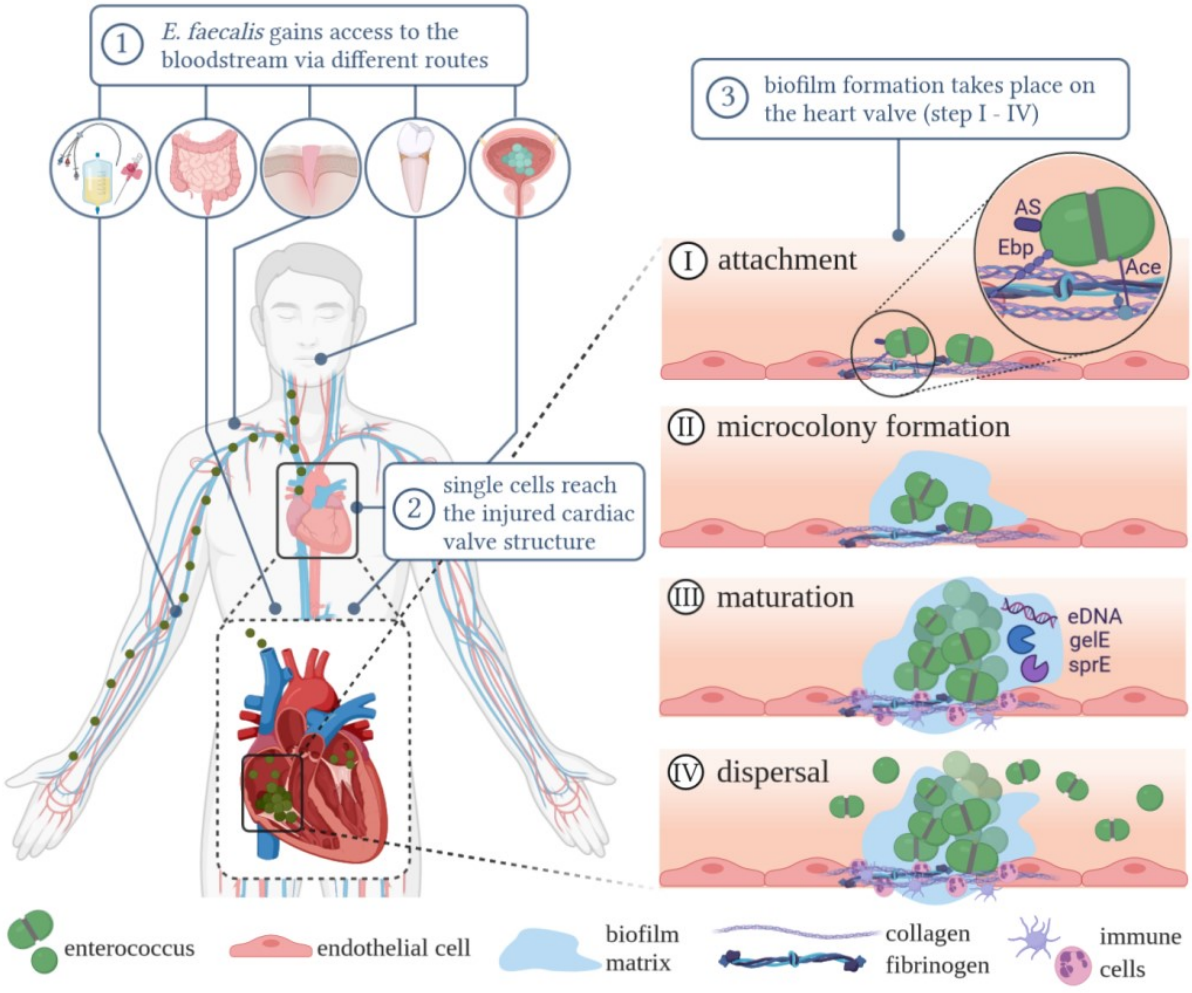

Figure 1: Enterococcal biofilm formation in infective endocarditis.
(1) *E. faecalis* uses different ports of entry into the bloodstream, e.g., intravenous catheters, dental sources, wound infections or the gastrointestinal and urinary tract. (2) *E. faecalis* adheres to an abnormal cardiac valve surface, starting the biofilm formation process (3). Initial attachment (I) is mediated by bacterial adhesins such as endocarditis and biofilm-associated pilus (Ebp), aggregation substance (AS) and adhesin to collagen (Ace). The attached bacteria start to aggregate and produce small quantities of biofilm matrix, leading to the formation of microcolonies, which develop into infective vegetations (II). Mature enterococcal biofilms are characterized by the accumulation of extracellular DNA (eDNA) and the proteases gelatinase E (gelE) and serine protease E (sprE) in the matrix (III). Vegetation particles and planktonic

cells can detach and disseminate into the bloodstream, may leading to strokes and infarcts, as well as to renewed biofilm cycles at distant niches. Own figure created with Biorender (www.biorender.com).

1.2.3 Biofilm susceptibility and synergy testing

The biofilm lifestyle differs significantly from that of free-living, planktonic cells, following that the study of free-living bacterial cells does not necessarily predict biofilm susceptibilities. An array of *in vitro* and *in vivo* models to study enterococcal biofilms exist, each with its' own benefits and limitations (Azeredo et al. 2017, Magana et al. 2018). Biofilm models can be generally classified into closed static and open dynamic systems depending on nutrient delivery (Macia et al. 2014). While flow cells or Centre for Disease Control (CDC) biofilm reactors resemble more closely the *in vivo* situation, they are highly resource-consuming and thus inappropriate for high-throughput (HTP) biofilm antimicrobial susceptibility testing. Major drawbacks of more simple methods like the microtitre plate model or the Calgary biofilm device is their poor mimicking of various host niches. Besides different methods of biofilm growth, different methods for assay readout and quantification of anti-biofilm effects exist (Peeters et al. 2008). The gold standard for determination of viable cells is still colony forming unit (CFU) counting, while other methods, such as biofilm mass staining by crystal violet, measurement of the metabolic activity by resazurin or fluorescein diacetate, or biofilm imaging followed by image software analysis, are used as well for quantification of microbial biofilms. However, despite the diversity of methods to assess anti-biofilm effects, no standardized biofilm susceptibility test has been introduced into routine diagnostics until today. The development of clinically meaningful biofilm susceptibility assays (i.e. a positive correlation between test results and treatment outcome) - similar to minimal inhibitory concentration (MIC) testing for planktonic cells - remains one of the major challenges in the management of biofilm-associated infections. The pendant of the MIC in biofilm testing is the minimal biofilm eradication concentration (MBEC), albeit official agencies such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have not yet determined standardized definitions of biofilm endpoint parameters and according breakpoints. Consequently, there is currently also no standard method available for synergy testing of antimicrobials in biofilms, while researchers have suggested to use the fractional biofilm eradication concentration

(FBEC) index similar to the fractional inhibitory concentration (FIC) index for planktonic cells (Dall et al. 2018) as elucidated in the following.

1.3 *In vitro* synergy testing of antimicrobial combinations

The goal of *in vitro* synergy testing is to assess the interaction of two (or more) antimicrobial substances, which can be classified into synergistic, additive or antagonistic interactions. An antimicrobial combination is said to be synergistic if the observed total effect is greater than the sum of the individual effects of each combination partner (Tallarida 2011, Tallarida 2016). If the combination produces an effect that is equal to what has been expected by the individual drug potencies, the combination is classified as additive between the single agents. Antagonistic interactions are observed when the combination effect is less than additive. While it is clinically essential to avoid antagonistic combination therapies, the risk-benefit ratio of combination over monotherapy can be further estimated by analysing whether an antimicrobial combination produces additive or synergistic effects.

Three different methods are commonly used *in vitro* to assess the interaction between antimicrobials, namely the checkerboard method, E-tests and time-kill curve assays (TKA). While both checkerboard and E-testing assess microbial eradication at a fixed time point, TKAs provide dynamic information about pathogen killing over time. Similar as for biofilm susceptibility testing, no gold standard for synergism testing on planktonic level has been set by official agencies such as the EUCAST or CLSI (Clinical and Laboratory Standards Institute). All three *in vitro* synergy testing methods are complex, labour-intensive and not easy to interpret. Clinical studies correlating these methods with treatment outcome are limited.

For the study presented in this thesis, checkerboard analysis was used to analyse synergistic effects. The checkerboard assay is based on broth microdilution testing and can be regarded as 2-dimensional MIC testing (Figure 2). Serial dilutions of each antimicrobial compound as well as combined concentrations of both agents are tested in a microtitre plate set-up for their efficiency in inhibiting microbial growth overnight. The interaction of both compounds is evaluated by comparison of the combination-derived MICs versus the single agent MICs. Mathematically, this is depicted by the fractional inhibitory concentration index (FICI), e.g.:

$$\text{FICI}_{\text{ ampicillin/ceftaroline}} = \frac{MIC\ ampicillin\ (combination)}{MIC\ ampicillin\ (alone)} + \frac{MIC\ ceftaroline\ (combination)}{MIC\ ceftaroline\ (alone)}$$

FICI values are calculated along the growth/no-growth (turbid/non-turbid) interface of the checkerboard assay. If an antimicrobial combination results in an at least 4-fold reduction in the MIC compared with the MICs of the compounds alone, FICI values equal or below the synergy threshold of ≤ 0.5 are reached (Odds 2003). FICIs in the range of 0.5 to 4 are considered non-synergistic, indifferent or additive, while FICIs over 4 indicate antagonistic effects (Odds 2003). Integrated in the checkerboard assay set-up and FICI threshold analysis is the concept of dose equivalence (Lederer et al. 2018). This concept is essential for demonstrating that the combination effect is greater than the individual drug potencies, i.e. to prove that the antibiotic combination shows truly synergistic and not additive effects. The term “dose equivalent” equates all doses of different compounds with the same effect, in case of the checkerboard the MIC. This means if one antibiotic is replaced by its’ dose equivalent of the other antibiotic, no change in the observed effect will be seen. Following this concept, the checkerboard set-up analyses combinations of antibiotics equalling 1x MIC of each antibiotic, e.g., 0.5x MIC_A + 0.5x MIC_B, 0.75x MIC_A + 0.25x MIC_B, 0.25x MIC_A + 0.75x MIC_B etc., to check for combined expected (= additive) effects. If the wells of these combinations turn out to form the growth/non-growth interface of the checkerboard assay, FICI values around 1 will be reached indicating solely additive and not synergistic effects (Figure 2a). If the growth/non-growth interface forms at lower combined concentrations than the expected ones, the interaction of the tested antibiotics is regarded synergistic (Figure 2b).

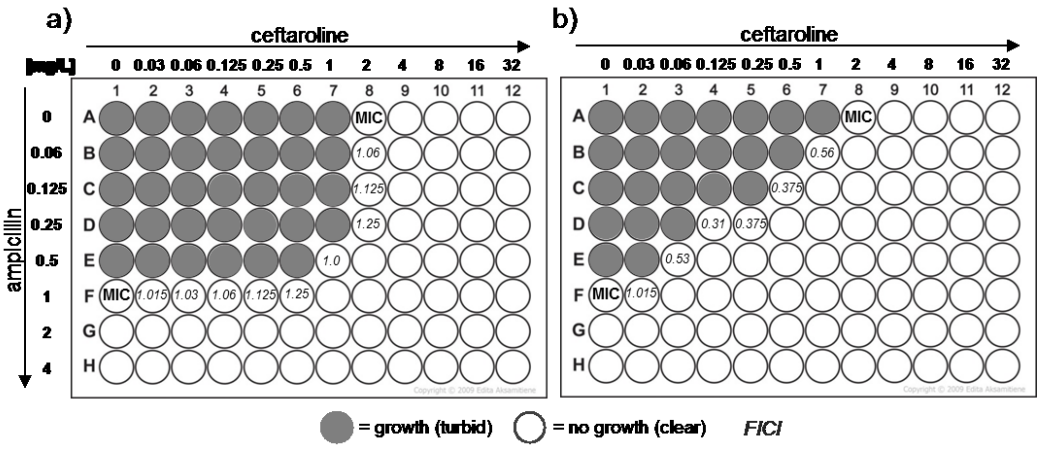

Figure 2: Additive versus synergistic checkerboard assay.

Shown are examples of checkerboard assays of ampicillin and ceftaroline against *E. faecalis* showing additive (a) or synergistic (b) interaction. Fractional inhibitory concentration indices (FICI) were calculated along the turbid/non-turbid interface. Own figure.

1.4 The *Galleria mellonella* larvae infection model

While originally known as parasites of honeybees' honeycombs, hence the name greater wax moth or honeycomb moth, several advantages make *G. mellonella* (Gm) larvae well-suited for different applications in research. Larval research models in general are associated with low costs and are easy to handle as in contrast to mammalian experiments, no specialized training is required. Gm larvae can be efficiently inoculated through their proleg structures on their abdominal site without leakage of haemolymph (Ramarao et al. 2012) (Figure 3a). In contrast to vertebrates, insects possess an open circulatory system with the organs being distributed throughout the haemocoel (body cavity) and surrounded by haemolymph (functional analogue to mammalian blood). Injection in the proleg leads to direct inoculation in the haemolymph. Insect larva have the advantage over mammalian testing that no animal testing application, ethical approval or inspection of housing conditions is needed (Tsai et al. 2016). A special benefit of Gm larvae is their ability to perform infection assays at human body temperature or even higher up to 42° C (Tsai et al. 2016). In contrast to other invertebrate models, this allows the study of temperature-dependent expression profiles of virulence factors (Konkel and Tilly 2000). Further unique to Gm larvae is that they share many core principles of their innate immune response with mammals, while they - as all invertebrates - lack an adaptive immune response (Pereira et al. 2018). At innate cellular level, Gm larvae possess a subset of six types of hemocytes, which are involved in phagocytosis, encapsulation and clotting and are the functional equivalents of mammalian neutrophils. Once recognized and bound to larval opsonins that act like mammalian pathogen recognition receptors, pathogens are phagocytosed and eliminated by several mechanisms including reactive oxygen species or lysosomal enzymes. As part of their humoral innate immune response, the larvae secrete a variety of broad-spectrum antimicrobial peptides (e.g., Galleria defensin, gallerimycin, lysozyme) into their haemolymph. A second feature of the humoral immune system is the stepwise melanisation response via a phenoloxidase cascade upon contact with a pathogen, increasing with the pathogenic load (Schmit et al. 1977, Tsai et al. 2016) (Figure 3b-d). The synthesis and deposition of melanin

around invading pathogens and wounds helps to encapsulate the pathogen analogous to abscess formation in mammalian infections. The visible Gm larval melanisation response allows for a varied endpoint analysis as part of a pathology score index (PSI) system in bacterial and fungal virulence studies and antimicrobial drug screenings. So far, no universal Gm PSI system has been established, but most scores assess the health status of the larvae by evaluating larval survival, mobility, cocoon formation and the melanisation response (Tsai et al. 2016). Further accessible endpoints include the determination of pathogenic load in the haemolymph, alterations in hemocyte composition, larval genomic/proteomic changes, histopathological screening or X-ray micro-chromatography. An imaging platform to record larval health in real time is currently under development (personal communication Dr. Olivia Champion, CEO BioSystems Technology, 11th October 2019).

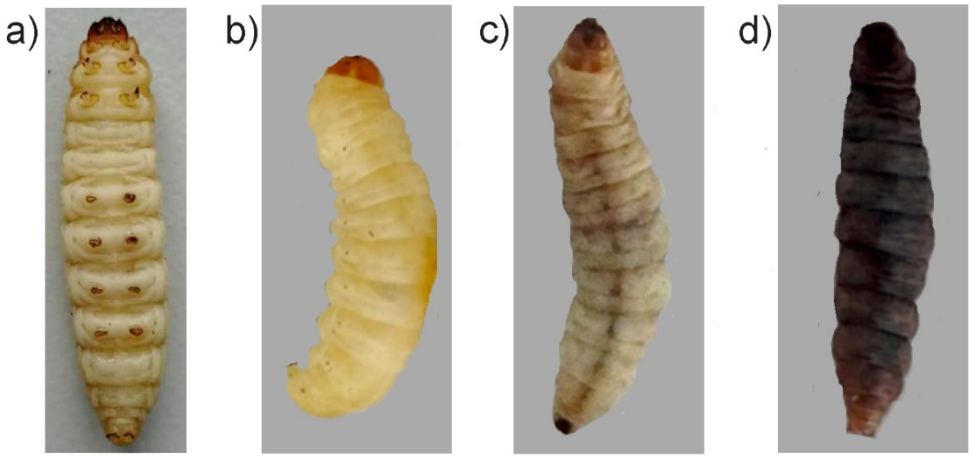

Figure 3: Melanisation response in the *G. mellonella* infection model.
After inoculation of the bacterial suspension via one of the prolegs on the abdominal site (a), beige coloured larvae (b) start to develop black spots (c), which can turn into completely melanised, black larvae (d). Own images.

The introduction of a standardized, quality controlled inbred Gm larval line (TruLarv™) has further contributed to the larva's adoption as a model organism. TruLarv larvae are age and weight defined, grown without the addition of antibiotics or hormones and genome sequenced, including the mitochondrial genome (Lange et al. 2018, Park et al. 2017).They overcome the variability associated with bait-shop larvae, reducing the sample size and number of total experiments (Champion et al. 2018). Gm larvae have been used in innate immune system studies, for chemical toxicity screening and in various microbiological applications, including bacterial pathogenicity, antimicrobial efficiency and microbiomes studies (Cutuli et al. 2019). Gm larvae have been widely

used as an infection model with various bacterial and fungal clinical strains, including enterococcal isolates (Cutuli et al. 2019, Yuen and Ausubel 2014). A Gm infection model with *E. faecalis* has first been described in 2007, while *E. faecium* causes none to weak lethality in the larvae (Lebreton et al. 2012, Park et al. 2007). Not only for enterococci, but also for other species the Gm model has been used to assess synergistic interactions between antimicrobials (Luther et al. 2014, Skinner et al. 2017, Tsai et al. 2016). However, these studies lack the differentiation between synergistic and additive interactions. Until now, no standardized protocol for synergy testing in larvae has been established.

2 Aims of this thesis

The overall aim of this thesis was the comparison of the novel antibiotic combination ceftaroline/ampicillin with the recommended standard gentamicin/ampicillin and the recently recommended alternative ceftriaxone/ampicillin for treatment of *E. faecalis* endocarditis. Two retrospective studies have shown non-inferiority of ceftriaxone/ampicillin compared to gentamicin/ampicillin treatment, but to date it remains controversial which subgroups of EFIE patients can be treated successfully with which antibiotic combination. Novel double β-lactam therapies such as ceftaroline/ampicillin have been discussed as potentially more effective and better-tolerated treatment options for EFIE. Comparison of the three antibiotic combinations was done by evaluating their anti-biofilm activity and their synergistic interaction on planktonic and biofilm-embedded enterococci. Following sub-goals for evaluation of the treatment options for EFIE were addressed:

- Testing of biofilm eradicative and preventive activities of the single antibiotics and their combinations (Publication I, Thieme et al. 2018)
- Translational development of the biofilm antibiotic susceptibility testing methods towards clinical routine diagnostics, i.e.
    - Practical application of an in-house biofilm algorithm (qBA) and comparison with CFU analysis for measurement of anti-biofilm activities (Publication I, Thieme et al. 2018)
    - Development of a novel high-throughput method –the Start Growth Time method- for biofilm analysis (manuscript in preparation)
    - Contribution to standardization of biofilm susceptibility endpoint parameters to improve the clinical validity of anti-biofilm assays (Publication II, Thieme et al. 2019)
- Development of *in vivo* synergy methodology
    - Establishment of the *Galleria mellonella* (Gm) larvae infection model at the Jena University Hospital followed by establishment of synergy testing in Gm larvae (Publication III, Thieme et al. 2020)
- Synergy testing of the antibiotic combinations on planktonic bacteria i) *in vitro* by checkerboard analysis (Publication I, Thieme et al. 2018), ii) *in vivo* in the Gm larvae model (Publication III, Thieme et al. 2020)

3 Publications

3.1 Publication I:

In vitro synergism and anti-biofilm activity of ampicillin, gentamicin, ceftaroline and ceftriaxone against *Enterococcus faecalis*

Authors: **Lara Thieme**, Mareike Klinger-Strobel, Anita Hartung, Claudia Stein, Oliwia Makarewicz, Mathias W. Pletz

Published in: Journal of Antimicrobial Chemotherapy (2018), 73(6), 1553–1561.

5-year Impact Factor (July 2020): 5.191

Own contribution to publication: 80 % (design and performance of experiments; analysis and interpretation of data; writing of the manuscript)

The aim of this publication was to compare the *in vitro* effectiveness of the currently applied gentamicin/ampicillin and ceftriaxone/ampicillin combination therapies for treatment of EFIE with a novel, potentially more effective ceftaroline/ampicillin combination. Synergism between ceftaroline and ampicillin had only been analysed in a limited number of strains and only at the planktonic level so far, but infective endocarditis is a biofilm-associated infection, explaining its resistance to antibiotic treatment. Therefore, biofilm-eradicating and biofilm-preventing activities of the single and combined antibiotics were analysed and compared to those at planktonic level. Synergism analysis at the planktonic level was done by checkerboard analysis, while anti-biofilm activities were measured by CFU plating and image acquisition at the confocal laser scanning microscope followed by analysis with the in-house qBA biofilm algorithm (Klinger-Strobel et al. 2016). Both ceftaroline/ampicillin and ceftriaxone/ampicillin showed synergistic effects against most *E. faecalis* isolates, while gentamicin/ampicillin exhibited additive effects. No biofilm-eradicating effects were observed for any antibiotic combination.

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Journal of Antimicrobial Chemotherapy

In vitro synergism and anti-biofilm activity of ampicillin, gentamicin, ceftaroline and ceftriaxone against *Enterococcus faecalis*

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Background: Enterococci frequently cause severe biofilm-associated infections such as endocarditis. The combination of ampicillin/ceftriaxone has recently been clinically evaluated as non-inferior compared with the standard therapy of ampicillin/gentamicin for treatment of *Enterococcus faecalis* endocarditis. Ceftaroline is a novel cephalosporin with enhanced activity against Gram-positive bacteria.

Objectives: To compare the *in vitro* effectiveness of the ceftaroline/ampicillin combination with those of gentamicin/ampicillin and ceftriaxone/ampicillin in planktonic and biofilm cultures of clinical *E. faecalis* isolates.

Methods: Synergistic effects at the planktonic level were analysed by chequerboard assays in 20 *E. faecalis* isolates. Biofilm-eradicating and biofilm-preventing activities of the antibiotics and their combinations were determined by confocal laser scanning microscopy with quantification by quantitative biofilm analysis (qBA) algorithm and cfu/mL determination.

Results: Comparable synergistic effects were observed for both β-lactam combinations in most isolates, in contrast to gentamicin/ampicillin. However, none of the antibiotic combinations succeeded in eradicating mature biofilms. Gentamicin showed promising biofilm-preventing activity, but at concentrations above those clinically tolerable. The β-lactams showed a U-shape dose–response relationship in biofilm prevention. Only exposure to cephalosporins caused alterations in cell morphology, which resulted in cell elongation and reclustering in a concentration-dependent manner. Reclustering was associated with high occurrences of small colony variants (SCVs), especially at high ceftriaxone concentrations.

Conclusions: This study suggests that combinations of cephalosporins or gentamicin with ampicillin may be advantageous only while bacteraemia persists, whereas combinations have no advantage over monotherapy regarding the treatment of mature biofilms. The selection of SCVs at high ceftriaxone concentrations is worth further study.

Introduction

Enterococci frequently cause biofilm-associated infections such as catheter-related bloodstream infections, urinary tract infections and infective endocarditis (IE).[1] Biofilms are matrix-embedded communities that aggregate on artificial or natural surfaces and exhibit increased resistance to attacks from the host's immune system and antibiotic therapy, often resulting in treatment failure, relapsing infections and increased lethality.[2,3]

Because the majority of clinical *Enterococcus faecalis* isolates remain susceptible to β-lactams, the most recommended antibiotic treatment of IE caused by *E. faecalis* involves ampicillin combined with gentamicin for 4–6 weeks.[4] However, this therapy is limited due to the severe side effects of aminoglycosides (nephrotoxicity and ototoxicity) and the emergence of high-level aminoglycoside resistance (HLAR) among *E. faecalis* isolates.[5] A combination of two β-lactam antibiotics, ampicillin and ceftriaxone, was recently proven in a retrospective cohort study as non-inferior and well-tolerated compared with the standard therapy and has therefore been recommended for treatment of infections caused by high-level aminoglycoside-resistant *E. faecalis*.[6]

Ceftriaxone is a third-generation cephalosporin and, as most cephalosporins, is ineffective individually in enterococci, but synergistically supports ampicillin by differential and stepwise saturation of PBPs.[7] Ceftaroline is a novel, broad-spectrum, fifth-generation cephalosporin that, in contrast to ceftriaxone, shows

enhanced *in vitro* activity against Gram-positive bacteria, including enterococci.[8] It is approved for the treatment of acute *Staphylococcus aureus*-related skin infections and community-acquired pneumonia. *In vitro* data have demonstrated that ceftaroline has anti-biofilm activity against nascent and mature staphylococcal biofilms.[9,10] We thus hypothesized that in line with current antibiotic combination therapies for enterococcal IE, ceftaroline plus ampicillin might be an effective treatment option for *E. faecalis* biofilm-associated infections. Recently, synergistic effects between ceftaroline and ampicillin against *E. faecalis* isolates were demonstrated in time–kill experiments[11] as well as in pharmacokinetic/pharmacodynamic (PK/PD) models, including simulated endocardial vegetations.[12,13] However, the effectiveness of this combination in enterococcal biofilms is still unknown.

The aim of this study was to elucidate the *in vitro* effectiveness of the ceftaroline/ampicillin combination in comparison with the current standard gentamicin/ampicillin treatment and the recommended alternative ceftriaxone/ampicillin treatment in biofilms of clinical *E. faecalis* isolates. We therefore analysed the biofilm-eradicating and biofilm-preventing activity of these antibiotics and their combinations, the latter in terms of their biofilm prevention concentrations (BPCs), and conducted chequerboard assays to analyse synergistic effects in planktonic cultures and biofilms.

Materials and methods

Enterococcal strains, liquid cultures and antimicrobials

Clinical *E. faecalis* ($n = 20$) isolates were acquired from blood cultures or from swabs of mitral valves by the Institute of Medical Microbiology at Jena University Hospital, Germany (Table 1). *E. faecalis* ATCC 29212 served as a reference strain for the chequerboard assays. Bacterial liquid cultures were prepared in Todd Hewitt (TH) broth (Karl Roth, Karlsruhe, Germany) and incubated at 37°C at constant rotation speed (approximately 13 **g**) for 2–3 h. Test solutions of ampicillin (Karl Roth, Karlsruhe, Germany), ceftriaxone (TCI Europe, Zwijndrecht, Belgium), gentamicin (TCI Europe, Zwijndrecht, Belgium) and ceftaroline (Forest Laboratories, New York City, USA) were prepared immediately before usage.

MLST

MLST was performed according to Ruiz-Garbajosa *et al.*[14] For DNA isolation, 2–3 colonies of each isolate were resuspended in RNase-free H_2O and denatured at 95°C for 10 min. The DNA was separated from cell debris by centrifugation at 12 000 **g** for 5 min. PCR was performed in a 25 μL reaction mixture containing 2 mM $MgCl_2$, 1×KCl buffer, 1.5 U/μL Taq standard polymerase (all Thermo Fisher Scientific, Massachusetts, USA), 0.2 mM dNTPs (Karl Roth, Karlsruhe, Germany), 0.4 μM of each forward and reverse primer and 150 ng of chromosomal DNA. PCR products were purified using a NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany) and quantified in the Infinite® 200 plate reader (Tecan, Männedorf, Switzerland) using a NanoQuant plate, both following the manufacturers' protocols. Samples were sequenced with forward and reverse primers at the EZ-Seq service (Macrogen, Amsterdam, The Netherlands). Allelic profiles and STs were assigned in accordance with the *E. faecalis* MLST database (http://efaecalis.mlst.net/) and analysed by the Draw Tree module applying the neighbour joining algorithm.

Antimicrobial synergism testing by chequerboard assays

The chequerboard assays were performed with 11 double-dilution steps of either ceftaroline, ceftriaxone or gentamicin and 7 double-dilution steps of ampicillin, as described previously.[15] Antibiotic concentration ranges were selected based on MIC values determined by the broth microdilution method according to EUCAST guidelines (ISO 20776-1:2006), except that TH broth was used instead of Mueller–Hinton broth. Each chequerboard assay was performed in duplicate.

The effects of the combined antibiotics were evaluated by calculating the fractional inhibitory concentration indices (FICI) along the turbidity/non-turbidity interface as described previously.[15] The lowest FICI value was used for interpretation and we assumed synergism occurred at FICI ≤ 0.5, antagonism at FICI ≥ 4 and no interaction at $0.5 < \text{FICI} < 4$.

Anti-biofilm testing

Biofilms were grown in optical microtitre plates (0.54 cm^2/well) with glass bottoms (Greiner Bio-one, Kremsmünster, Austria) in triplicate by applying 100 μL of a bacterial suspension (0.5 McFarland) to each well. Plates were placed in a humidified chamber and incubated at 37°C, 5% CO_2 without shaking. To assess biofilm-eradicating effects, we grew the biofilms for 24 h, carefully removed the supernatant and added 100 μL (2×50 μL each for the combinations) of the antibiotic solution prepared in TH medium. Medium was changed in the untreated growth controls. Plates were incubated for a further 24 h before analysis.

For assessment of the BPC, the antibiotics in sub-MICs were directly added to the bacterial suspension (0.5 McFarland/well). The plates were grown for 48 h without the medium being changed. For quantification of antibiotic effects, the viable cell number was determined by counting the number of viable bacteria (corresponding to cfu/cm^2). The supernatant of the biofilms was therefore carefully removed; the biofilms were washed twice with 100 μL of sterile NaCl, scraped off and resuspended in TH medium. Selected 10-fold dilutions were plated on TH-agar plates and incubated overnight at 37°C, followed by counting of cfu/mL. The BPC was defined as the first concentration at which the viable cell count decreased significantly by 1 log magnitude compared with the untreated control, referring to Macia *et al.*[16]

Biofilm imaging and computed analysis

Biofilms were stained using the LIVE/DEAD BacLight Bacterial Viability Kit for microscopy (Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer's protocol. Stained biofilms were analysed under vital conditions using an inverse confocal laser scanning microscope (CLSM), LSM510 (Carl Zeiss AG, Jena, Germany), as described previously.[17] The biofilm images were visualized by ZEN 9.0 software (Carl Zeiss AG, Jena, Germany). The biofilm experiments [eradication and prevention (BPC)] were independently performed in duplicate for each strain and in triplicate for each assay. Quantitative analysis of biofilm images was performed by an algorithm termed qBA (quantitative biofilm analysis) that determined the number of bacterial counts/cm^2.[17]

Statistical analysis

The correlation of the MIC was analysed by non-parametric Spearman's rank correlation coefficient (r_s) with a two-tailed CI of 95%. The non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison post-test with a CI of 95% was used for statistical analysis of the cfu/mL or cfu/cm^2 quantification. Differences were considered significant at P values <0.05.

Results

Characterization of isolates

Most of the clinical isolates (14/20) were obtained from patients with biofilm-associated infections, including three isolates from patients with endocarditis (Table 1). Among the 20 isolates,

Table 1. Results of susceptibility testing (MIC), synergy testing (FICI) and clinical data of the patient cohort

Isolate[a]	ST	Clinical background	Sex	Age (years)	MIC of ampicillin (mg/L)	MIC of ceftaroline (mg/L)	MIC of ceftriaxone (mg/L)	MIC of gentamicin (mg/L)	FICI of ampicillin/ceftaroline[b]	FICI of ampicillin/ceftriaxone	FICI of ampicillin/gentamicin
va67230	579	endocarditis	male	39	1	1	16	16	**0.50**	**0.25**	1.02
va245	280	endocarditis	male	76	0.5	0.125	2	16	0.73	**0.50**	1.02
bk5597	40	opportunistic infection without clinical signs	female	55	1	0.5	8[c]	16	**0.50**	**0.31**[c]	1.00
bk848	19	opportunistic infection after liver transplant rejection	male	60	1	0.25	8	16	**0.49**	**0.31**	1.02
bk905	41	endocarditis	male	75	1	1	256	8	**0.38**	**0.27**	1.03
bk2164	74	opportunistic infection due to acute renal failure	female	78	2	0.5	32[c]	4	**0.37**	**0.25**[c]	1.06
ATCC 29212	30	/	/	/	1	0.5	4	16	**0.50**	**0.50**	1.00
bk8653	6	opportunistic infection after liver transplantation	male	56	1	8	>512	>512	**0.25**	ND	ND
bk3043	6	opportunistic infection due to respiratory insufficiency, liver cirrhosis	female	74	0.5	1	>512	>512	**0.37**	ND	ND
bk3062	6	urosepsis due to permanent urinary catheter	male	77	1	4	>512	>512	**0.31**	ND	ND
bk7183	6	urosepsis (with prostate carcinoma)	male	76	1	8	512	>512	**0.37**	**0.38**	ND
bk6037	6	urosepsis due to permanent urinary catheter	male	86	1	2	512[c]	>512	**0.37**	**0.10**[c]	ND
bk9190	6	wound infection	female	59	1	2	>512	>512	**0.50**	ND	ND
bk281	498	sepsis due to biliary tract infection	female	79	1	1	32[c]	8	**0.50**	**0.25**[c]	1.03
bk9367	64	recurrent bacteraemia	female	85	2	0.5	16[c]	32	**0.37**	**0.38**[c]	0.63
bk6747	64	biliary tract infection (with Klatskin tumour)	male	81	1	0.5	256[c]	32	0.62	**0.10**[c]	1.01
bk1653	179	urosepsis due to urinary tract infection	female	87	1	2	16[c]	64	**0.31**	**0.25**[c]	**0.50**
bk4497	16	urosepsis due to permanent urinary catheter	female	67	1	0.5	64[c]	>512	**0.50**	**0.10**[c]	ND
bk5187	16	urosepsis post-operative	male	79	1	0.25	16	256	**0.49**	**0.19**	0.63
bk6886	16	wound infection	female	68	2	1	16[c]	32	**0.50**	**0.19**[c]	1.00
bk8669	23	opportunistic infection due to metastasizing rectal carcinoma	male	80	1	4	32[c]	32	**0.50**	**0.25**[c]	0.56

[a]Isolates with various backgrounds of infection were obtained from the Institute of Medical Microbiology in Jena, Germany. bk = blood culture. va = mitral valve. / = not applicable to the laboratory standard strain.
[b]FICI values are given as the lowest observed FICI. Synergistic FICI values (lowest FICI ≤0.5) are indicated in bold. ND = not determined due to MICs exceeding 512 mg/L.
[c]Labelled isolates started to regrow as visible aggregates at concentrations above the determined turbid/non-turbid interface of ceftriaxone (alone and in combination with ampicillin). FICIs were calculated using the concentrations in the first non-turbid well found in each row and column, neglecting the regrowth.

9 isolates exhibited unique STs and variable resistance profiles. Six isolates, all highly resistant to gentamicin and ceftriaxone (≥512 mg/L), belonged to ST6, whereas ST64 ($n = 2$) and ST16 ($n = 3$) also showed resistance to those antibiotics, but had different MIC values.

Synergy testing on planktonic bacteria

To analyse synergistic effects between ceftaroline/ampicillin, ceftriaxone/ampicillin and gentamicin/ampicillin, we performed chequerboard assays for the 20 clinical *E. faecalis* isolates and the laboratory standard strain ATCC 29212 (Table 1). All isolates

showed ampicillin MIC values of 1 mg/L ± 1× MIC. The gentamicin MICs ranked between 4 and >512 mg/L, with eight isolates exhibiting an HLAR profile (MIC of gentamicin >128 mg/L).

One isolate exhibiting low-level resistance against gentamicin (MIC of gentamicin <128 mg/L) experienced synergistic effects from gentamicin plus ampicillin (FICI = 0.5), while all other isolates (non-HLAR and HLAR) showed FICI values from >0.5 to 1, indicating no interaction between these two antibiotics [Table 1 and Figure S2 (available as Supplementary data at *JAC* Online)].

The MIC values of ceftriaxone ranked between 2 and >512 mg/L, while the MIC values of ceftaroline were generally lower, with values between 0.125 mg/L and 8 mg/L (Table 1). Both the MIC of ceftriaxone and the MIC of ceftaroline showed no correlation with the MIC of ampicillin, but the MIC of ceftaroline positively correlated with the MIC of ceftriaxone ($r_s = 0.705$, $P < 0.001$).

Synergistic effects were observed for ceftaroline and ampicillin in 19 of 21 tested isolates, with FICI values of ampicillin/ceftaroline between 0.25 and 0.50 (Table 1). Ceftriaxone and ampicillin synergistically inhibited the growth of 17 isolates, with FICI values of ampicillin/ceftriaxone between 0.1 and 0.5; 15 of these isolates also showed synergistic effects for ceftaroline and ampicillin. At higher concentrations of ceftriaxone (alone and in combination with ampicillin), visible turbid clusters were observed beyond the turbid/non-turbid interface (greater than the MIC of ceftriaxone) for individual isolates (marked in Table 1), suggesting regrowth. This phenomenon was not observed for ceftaroline.

Low concentrations of ceftaroline or ceftriaxone were sufficient to strongly reduce the effective concentrations of ampicillin (Figure S2 and Table 1). This effect was most apparent in isolates with high MICs of ceftriaxone, as shown by a weak correlation between the FICI of ampicillin/ceftriaxone and the MIC of ceftriaxone ($r_s = -0.54$, $P = 0.025$).

No correlations between the other MICs and the FICI values or between FICI values and clonality based on MLST (Figure S1) were observed.

Biofilm eradication by ceftaroline, ceftriaxone, ampicillin and gentamicin

Biofilm-eradicating effects were assessed by CLSM imaging of five biofilm-associated isolates from patients with endocarditis (va245, va67230, bk905) or urosepsis (bk1653, bk6037); these isolates all exhibited synergistic FICI values for the ceftaroline/ampicillin combination (Table 1). Except for isolate bk6037, the isolates produced strong biofilms after 24 h of growth (data not shown). None of the antibiotics in concentrations up to 1000× MIC showed visible biofilm-eradicating effects in any of the isolates, neither alone nor in combination, compared with the untreated control after 24 h of exposure. The biofilms' thicknesses did not decrease, and the number of dead bacteria did not increase after antibiotic exposure (Figure S3).

Biofilm prevention by ceftaroline, ceftriaxone, ampicillin and gentamicin

The BPC was tested at sub-MICs of ceftaroline, ceftriaxone, ampicillin and gentamicin for selected isolates (va245, va67230, bk1653 and bk6037). Subinhibitory concentrations of both cephalosporins (ceftaroline and ceftriaxone) induced morphological alterations at the single-cell level in a concentration-dependent manner (Figure 1). As shown in isolate bk1653, the enterococci elongated with increasing ceftaroline and ceftriaxone concentrations in all Z-layers, adapting a long rod shape at ≤1/8× MIC that resulted in filamentous structures. This effect, albeit not as strongly, was also observed for isolates va245 and va67230 (data not shown). The elongated cells were viable, but their density decreased with increasing cephalosporin concentrations as confirmed by cfu/mL count and qBA (Figure 2). As per definition (see the Materials and methods section), the BPCs of both cephalosporins were reached at the elongated stage at 1/8× MIC (0.125 mg/L ceftaroline and 2 mg/L ceftriaxone). At cephalosporin concentrations higher than the BPC, the elongated enterococci reverted to the coccal shape and appeared as clusters in the CLSM images (Figure 1). Ceftaroline-induced cell clusters disappeared at 4× MIC of ceftaroline (4 mg/L), while ceftriaxone-induced clusters were still present at the highest concentration tested (16× MIC of ceftriaxone, 256 mg/L) (data not shown).

In contrast to the response to the cephalosporins, cell elongation was not observed with ampicillin exposure (Figure 1). The number of viable bacteria remained stable at increasing concentrations until a sudden drop at the BPC at 1/2× MIC of ampicillin (0.5 mg/L ampicillin) (Figure 2). However, ampicillin concentrations above the BPC led to increased bacterial counts, which correlated with cluster formation observed in the CLSM images as under cephalosporin treatment (Figure 1). The clusters disappeared at 2× MIC of ampicillin (2 mg/L) (data not shown).

In contrast to the β-lactams, gentamicin killed bacterial cells in the nascent biofilm in a concentration-dependent manner without the clustering effect. Gentamicin did not prevent biofilm formation below the MIC (64 mg/L), but at the MIC of gentamicin (= BPC) no cfu/mL were detected.

Synergism in biofilm prevention by ampicillin combined with ceftaroline or gentamicin

Only a minor synergism between ampicillin and ceftaroline in biofilm prevention was detected at ≤1/8× MIC of ampicillin because the viable cell numbers per cm^2 from the untreated control value were not reduced by 1 log magnitude (Figure 3a). Combining sub-MICs of ceftaroline with increasing ampicillin concentrations ≤1/8× MIC of ampicillin led to the same cell shape change from elongation to coccal clusters as was observed with only sub-MIC ceftaroline exposure (Figure S4). However, the clustering effect was achieved at lower ceftaroline concentrations in combination than with treatment with ceftaroline alone. The BPC for the ceftaroline/ampicillin combination was first reached at 1/4× MIC of ampicillin and 1/32× MIC of ceftaroline, with a reduction in viable cells/cm^2 of at least 1.5 log magnitudes (Figure 3a). No elongated cells or clusters were observed under these conditions (Figure S4). The minimum number of viable cells/cm^2 was reached at 1/2× MIC of ampicillin plus 1/32× MIC of ceftaroline. Synergy inversion was surprisingly observed with increasing ceftaroline concentrations, i.e. cell numbers were higher than under 1/2× MIC of ampicillin alone (Figure 3a).

The ampicillin/gentamicin combination indifferently affected biofilm prevention (Figures 3b and S5). The combination of gentamicin at concentrations below the MIC of gentamicin and

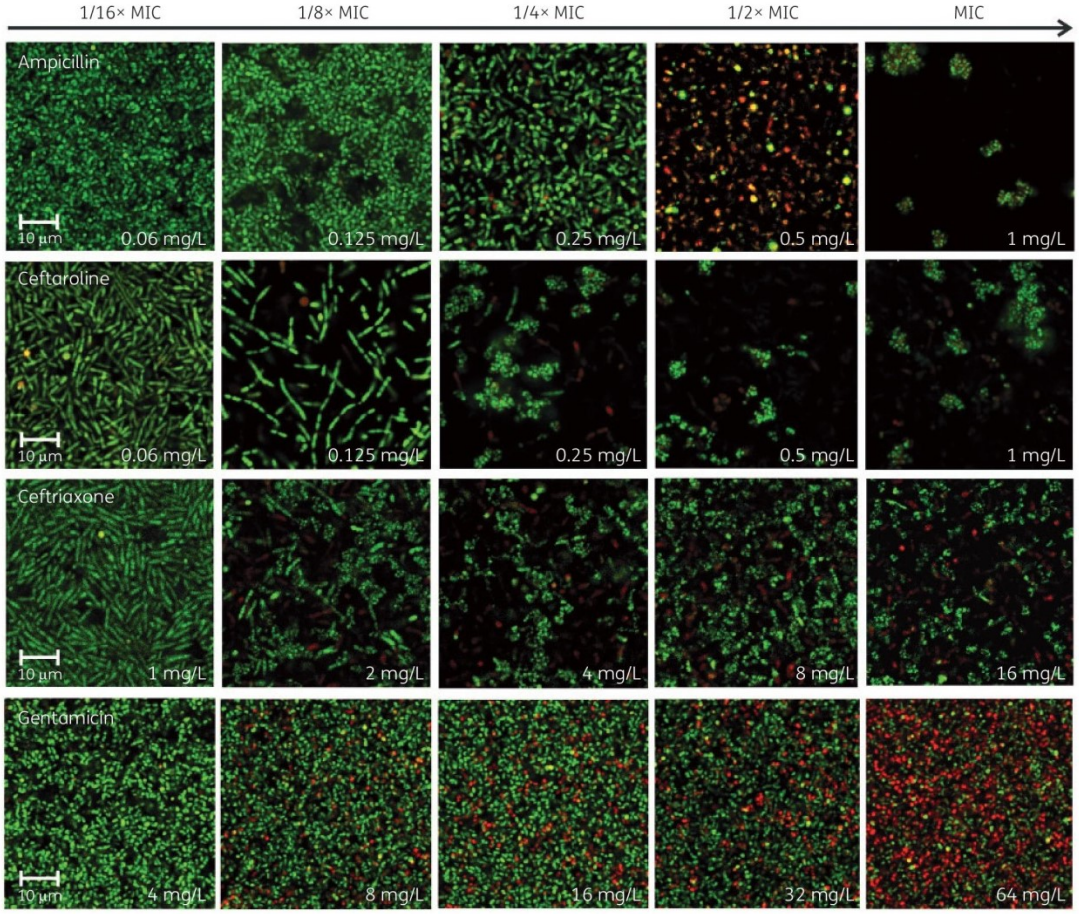

Figure 1. Effects of antibiotics on biofilm formation. *E. faecalis* isolate bk1653 was grown for 48 h under static conditions in a 96-well glass-bottom plate in the presence of sub-MICs of ampicillin, ceftaroline, ceftriaxone and gentamicin. CLSM images show viable bacteria in green (SYTO 9) and dead bacteria in red (propidium iodide). The concentration corresponding to the fold MIC is noted in each image. Scales in the images of 1/16× MIC apply for all corresponding images to the right.

ampicillin at 1/2× MIC of ampicillin increased the bacterial count by 1 log magnitude, indicating an antagonistic effect (Figure 3b).

Formation of small colony variants (SCVs) under antimicrobial treatment

The formation of clusters above the BPC of ceftaroline, ceftriaxone and ampicillin led us to quantify the proportion of SCVs[18] in the viable bacteria (isolates bk1653 and va67230) (cfu/mL) after biofilm growth in the presence of 0.5×, 1× and 2× MIC of each antibiotic. After 48 h, biofilms were resolved and plated on agar to allow for cfu/mL and SCV determination. SCVs were distinguished from normal bacteria as pinpoint colonies[18] at higher dilutions of the bacterial suspension. Isolate bk1653 showed a high proportion of SCVs (between 40% and 70%; Figure 4) in the presence of 0.5× and 1× MIC of each β-lactam. At 2× MIC, only the cephalosporins selected SCVs, while under ampicillin exposure, the SCV proportion was strongly reduced to 1.5%. In contrast to isolate bk1653, isolate va67230 produced SCVs only at 0.5× MIC of ampicillin and at all tested ceftaroline concentrations, but not under ceftriaxone exposure (data not shown). Gentamicin led to low or undetectable levels of SCV formation at all concentrations tested in both isolates. In the untreated control and at concentrations lower than 0.5× MIC, the SCV phenotype was not detected for either isolate.

Discussion

In the present study, 20 clinical *E. faecalis* isolates and one laboratory standard strain were analysed *in vitro* for synergism between

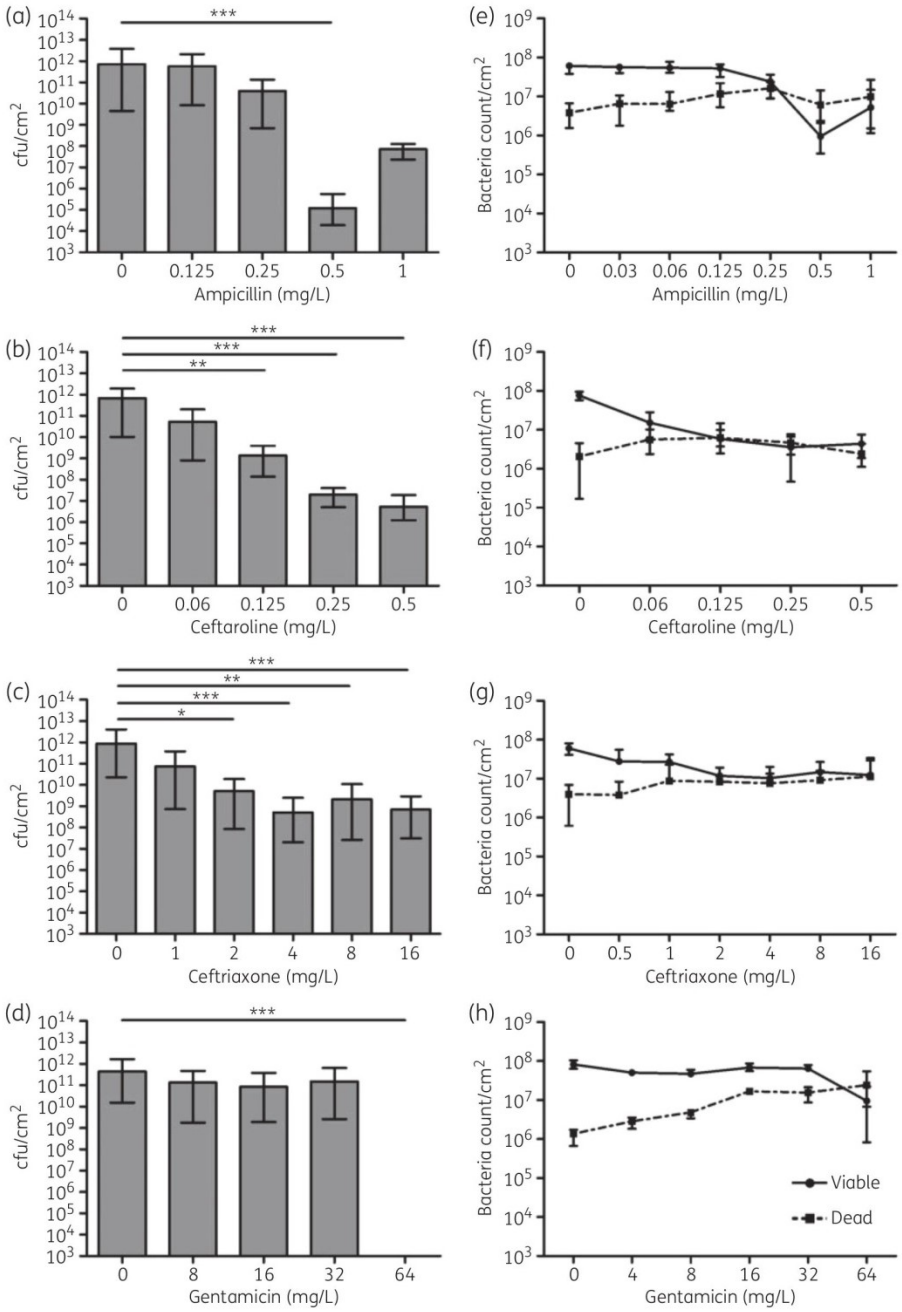

Figure 2. Biofilm prevention activity of the antibiotics. *E. faecalis* isolate bk1653 was grown for 48 h under static conditions in a 96-well glass-bottom plate in the presence of sub-MICs of ampicillin, ceftaroline, ceftriaxone and gentamicin. (a) to (d) Quantification of the cfu/cm^2. Statistical analysis was performed by one-way ANOVA (Kruskal–Wallis test) followed by Dunn's multiple comparison test. (e) to (h) Quantification of the bacteria counts/cm^2 in the microscope images by qBA. The quantifications were determined independently twice and then in triplicate for each antibiotic. The mean values and the ranges are shown in the diagrams.

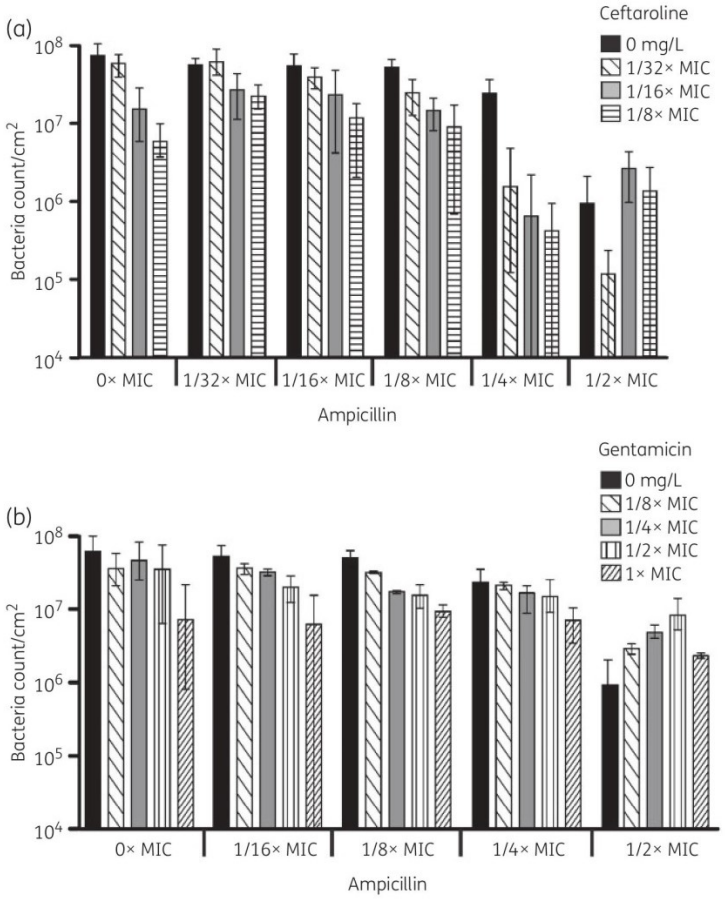

Figure 3. Quantification of the effects on biofilm formation of combining ampicillin with ceftaroline (a) or gentamicin (b). The viable cell counts were determined by the qBA algorithm based on CLSM images (approximately 100 μm×100 μm) and scaled up to an area of 1 cm^2 (10^4 cells/cm^2 represents the limit of detection of this method). The experiments were performed in triplicate and the means and standard deviations are shown.

ampicillin and ceftaroline, ceftriaxone or gentamicin in the planktonic and biofilm-embedded status, respectively. Nine isolates were not related to each other and most likely not of nosocomial origin. In contrast, we cannot clearly exclude a nosocomial source for the three clonal clusters, with ST6 being a prevalent clade often found in wild animals[19] and livestock,[20] but also spreading endemically in hospitals.[19,21]

Compared with the current standard therapy of gentamicin/ampicillin for *E. faecalis* IE, the ceftaroline/ampicillin combination showed a superior synergistic effect *in vitro* against planktonic cultures of clinical *E. faecalis* isolates, including all HLAR isolates, but FICI values were similar compared with those of the recently recommended ceftriaxone/ampicillin combination.[4] These results are in accordance with those of two recently published smaller studies that reported synergism of ceftaroline/ampicillin combinations in different PK/PD models and concluded that only in a limited number of strains ceftaroline might have better activity than ceftriaxone in combination with ampicillin.[12,13]

However, *E. faecalis* infections associated with biofilm formation are less susceptible to antibiotics due to biofilm-specific antimicrobial tolerance mechanisms, such as reduced antibiotic penetration and metabolic dormancy.[22] The routinely assessed MICs of antibiotics that are sufficient to kill planktonic bacteria are thus usually insufficient to eradicate their biofilm-embedded counterparts.[23] Biofilm susceptibility testing is not currently part of diagnostic routines because neither standards for biofilm analysis nor specific breakpoints have been established by official agencies such as EUCAST. We therefore used a microscopy approach to evaluate the efficacy of ampicillin combined with ceftaroline, ceftriaxone or gentamicin in biofilm eradication and the inhibition of biofilm formation and compared the results with the resource-consuming method of cfu/mL determination. The reduction in viable bacterial counts/cm^2 in the CLSM images obtained by qBA corresponded to the cfu/cm^2 values, but the qBA values were generally lower because the analysed areas of the images were at the centres of the wells, which bear fewer cells than the edges of

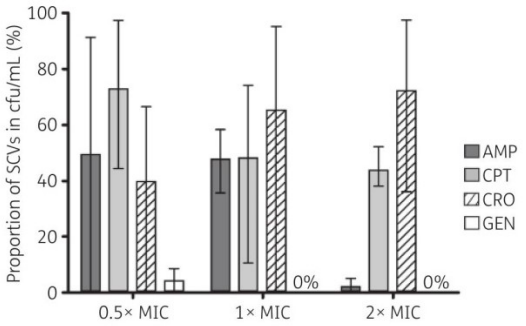

Figure 4. Proportion of SCVs in viable bacteria (cfu/mL) in dependence on the antibiotics present during biofilm formation. Biofilms of *E. faecalis* isolate bk1653 were grown in the presence of 0.5×, 1× and 2× MIC of ampicillin (AMP), ceftaroline (CPT), ceftriaxone (CRO) or gentamicin (GEN). After 48 h, biofilms were resolved and plated on agar to allow for cfu/mL and SCV determination. Experiments were performed in triplicate and the means and ranges are shown. The untreated control biofilms never exhibited SCVs.

the wells; in contrast, the edges were included in the cfu/mL determination.

Neither the individual antibiotics nor their combinations were sufficient to eradicate established *E. faecalis* biofilms *in vitro*. It is still unclear whether β-lactams and aminoglycosides efficiently penetrate the biofilm matrix of *E. faecalis* because we are not aware of any studies of *E. faecalis* biofilm penetration. Both antibiotic classes exhibit limited anti-biofilm activity in many species.[24] Because β-lactams are inhibitors of cell wall synthesis, the effectiveness of the β-lactam combinations in biofilm eradication is likely hampered by the strongly slowed cell division in metabolically dormant biofilms.[24]

Gentamicin alone showed promising biofilm-preventing activity but was only effective outside clinically tolerable concentrations. No reduction in BPC of gentamicin was achieved when it was used in combination with ampicillin. All tested β-lactams reduced biofilm formation at sub-MIC concentrations. The combination of ampicillin with ceftaroline, compared with each β-lactam alone, improved the inhibition of biofilm formation in a concentration-dependent manner, suggesting a synergistic interaction between these β-lactams. PBPs 2 and 3 have been identified as primary targets of cephalosporins, while aminopenicillins target PBPs 4 and 5.[7] Ampicillin and ceftaroline, similar to the ampicillin/ceftriaxone combination, are thus likely to synergize by inhibiting complementary PBPs, thereby disrupting the cooperation of the PBPs necessary for cell wall biosynthesis.[25] Ceftaroline has a higher affinity than ceftriaxone to PBP 5 while maintaining a high affinity for PBPs 2 and 3,[8] which explains the lower MIC values of ceftaroline compared with ceftriaxone. The most effective dosing of ceftaroline/ampicillin was achieved at 1/32× MIC of ceftaroline and 1/4× to 1/2× MIC of ampicillin in the BPC experiments, but synergy inversion was observed with increasing ceftaroline concentrations. The effectiveness of ampicillin was thus increased by very low ceftaroline concentrations but was hampered by higher sub-MIC concentrations of ceftaroline. Lower concentrations of ceftaroline likely saturate PBPs 2 and 3, complementing PBP 4 and 5 saturation by ampicillin, while at higher concentrations, ampicillin and ceftaroline may compete for binding of PBP 5, leading to an antagonistic effect. However, this behaviour was not observed at the planktonic level in the chequerboard assays, which displayed synergistic concave isoboles (Figure S2). This result might be caused by the different inocula that were used, i.e. 5×10^5 cfu/mL for MIC and synergism assays versus 1×10^8 cfu/mL in BPC assays.

The cephalosporins exhibited a U-shape dose–response relationship in biofilm prevention, which resulted in cell elongation and reclustering in a concentration-dependent manner. Both ceftaroline and ceftriaxone at sub-MICs led to filamentation, suggesting a similar mode of action for both cephalosporins. This hypothesis is further supported by the significant correlation of the MIC of ceftriaxone with the MIC of ceftaroline and the lack of correlation of both with the MIC of ampicillin. The observed filamentation may be explained by an inhibition of cell separation. Specific PBPs of *Streptococcus pneumoniae* have been involved in septal ring closure in previous studies.[26] PBPs 2 and 3 of *E. faecalis*, which become saturated at very low concentrations of cephalosporins,[7] might also be involved in this process. Elongation of bacterial cells exposed to cephalosporins has been described for different Gram-negative bacterial species,[27,28] but to our knowledge this report is the first regarding this behaviour in enterococci. Both cephalosporins strongly induced the formation of SCVs at concentrations close to their respective MICs, but with different intensities and not in all isolates. The observed clusters of viable cells escaping the cephalosporins most likely represented the SCVs, and the clustering might be the result of the reduced SCV growth rate.[18] Further, the disappearance of the cell clusters at 2× MIC of ampicillin correlated with the decrease in the SCV rate of some isolates (e.g. bk1653) at 2× MIC of ampicillin. Clusters were still observed at 16× MIC of ceftriaxone, indicating that ceftriaxone is unable to kill SCVs.

This study suggests that combinations of cephalosporins or gentamicin with ampicillin may be only advantageous for the treatment of persistent bacteraemia (i.e. planktonic cells) but seem to be non-superior compared with monotherapy against mature biofilms. In this regard, the recommendation to treat patients that have enterococcal endocarditis over the entire 4–6 weeks with an antibacterial combination may be questioned, particularly in the face of increased risks of prolonged cephalosporin (e.g. *Clostridium difficile* colitis) and gentamicin treatment (e.g. nephrotoxicity).[4] Furthermore, high cephalosporin concentrations seem to favour selection of SCVs, suggesting that higher doses of combined ceftriaxone (e.g. 2×2 g daily) might even be detrimental, at least for some strains. Clinical studies are therefore mandatory to elucidate whether the selection of SCVs composes a risk of using combined therapies against enterococcal biofilm-associated infections.

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Transparency declarations

None to declare.

Forest Laboratories, Inc. supplied the ceftaroline powder, but had no involvement in: the design of the study; the collection, analysis and interpretation of the data; or the decision to present these results.

Supplementary data

Figures S1 to S5 are available as Supplementary data at *JAC* Online.

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Supplementary data

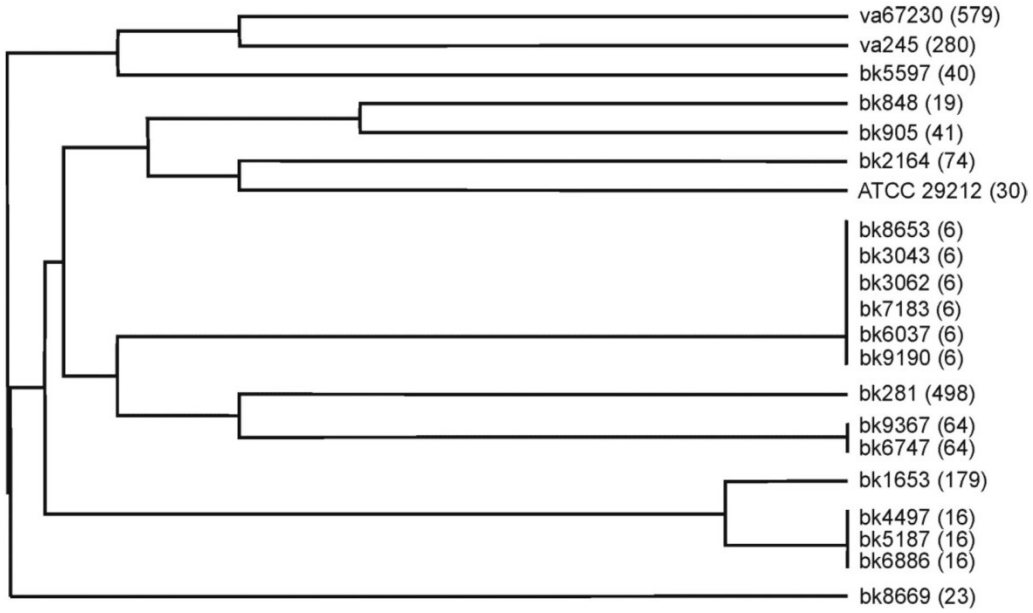

Figure S1. MLST based phylogram of the *E. faecalis* strains used in this study. Multilocus sequence type is indicated in brackets. The relationship of the different STs was analysed using the MLST database Draw Tree module applying the neighbour joining algorithm (http://efaecalis.mlst.net/sql/uniquetree.asp?).

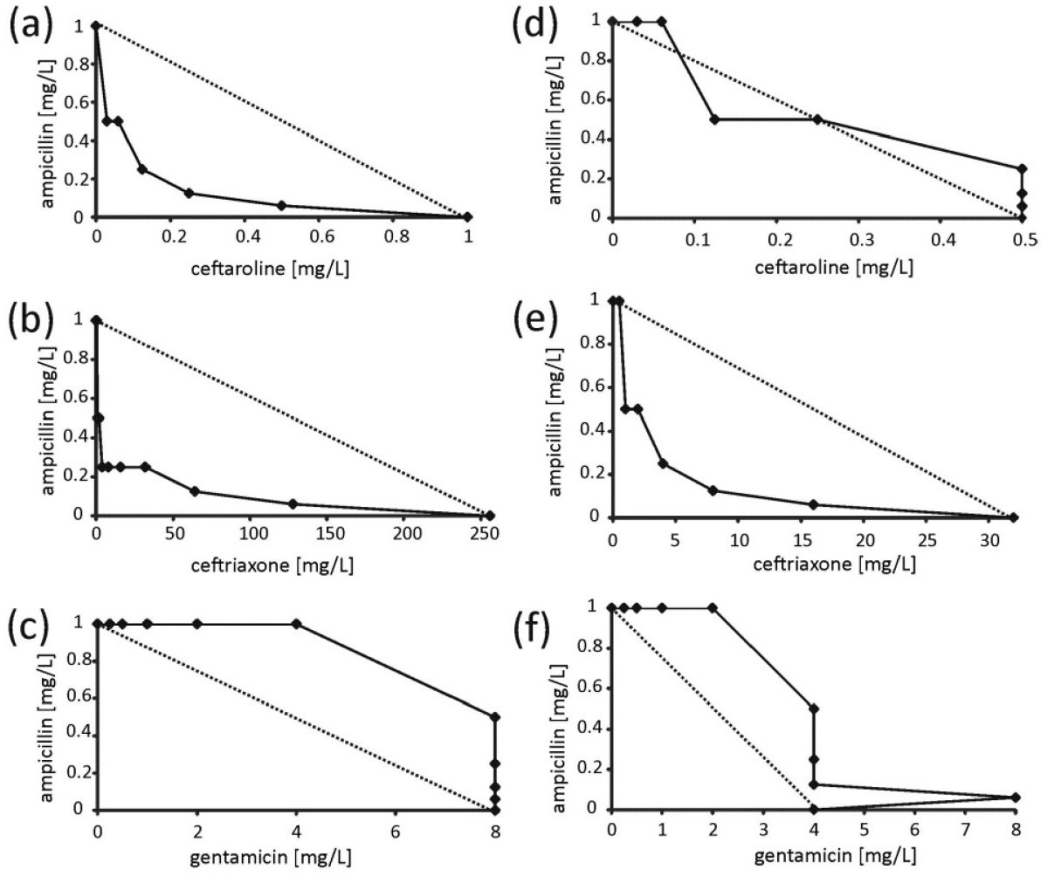

Figure S2. Exemplary isoboles of the MICs for antibiotic combinations. (a) - (c): Isoboles of isolate bk905 exhibiting synergistic effects for ceftaroline/ampicillin and ceftriaxone/ampicillin (gentamicin/ampicillin indifferent effect). (d) - (f): Isoboles of isolate bk2164 exhibiting synergistic effects only for ceftriaxone/ampicillin. Straight dotted lines indicate the theoretical concentrations of additive interactions (endpoints are the MIC values of the single antibiotics).

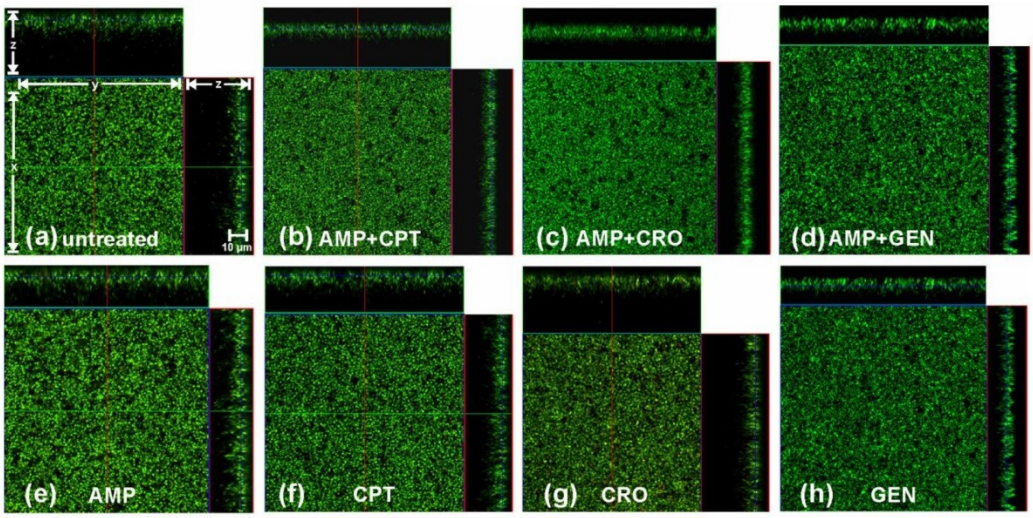

Figure S3. Effects of antibiotics on mature biofilms. CLSM images after SYTO9/propidium iodide staining (viable = green, dead = red) for the different antibiotic treatments (24 h) of 24 h-old biofilms of *E. faecalis* isolate va67230. Images represent intersections of the biofilms (x/y) and the corresponding cross sections (z). An area of approximately 100 µm (X) x 100 µm (Y) was recorded in 1 µm Z-intervals (Z-stacks) in the green (excitation 488 nm/emission filter 501–545 nm) and red (excitation 488 nm/emission filter 570–670 nm) channels, respectively. (a): Untreated 48 h-old biofilm. (b) - (d): Treatment combining ampicillin with ceftaroline (AMP+CPT), ceftriaxone (AMP+CRO) or gentamicin (AMP+GEN), with 256 mg/L of each antibiotic. (e) - (h): Single antibiotic treatment with 512 mg/L of ampicillin (AMP), ceftaroline (CPT), ceftriaxone (CRO) or gentamicin (GEN). Scale applies to all images. Experiments were performed twice in triplicate.

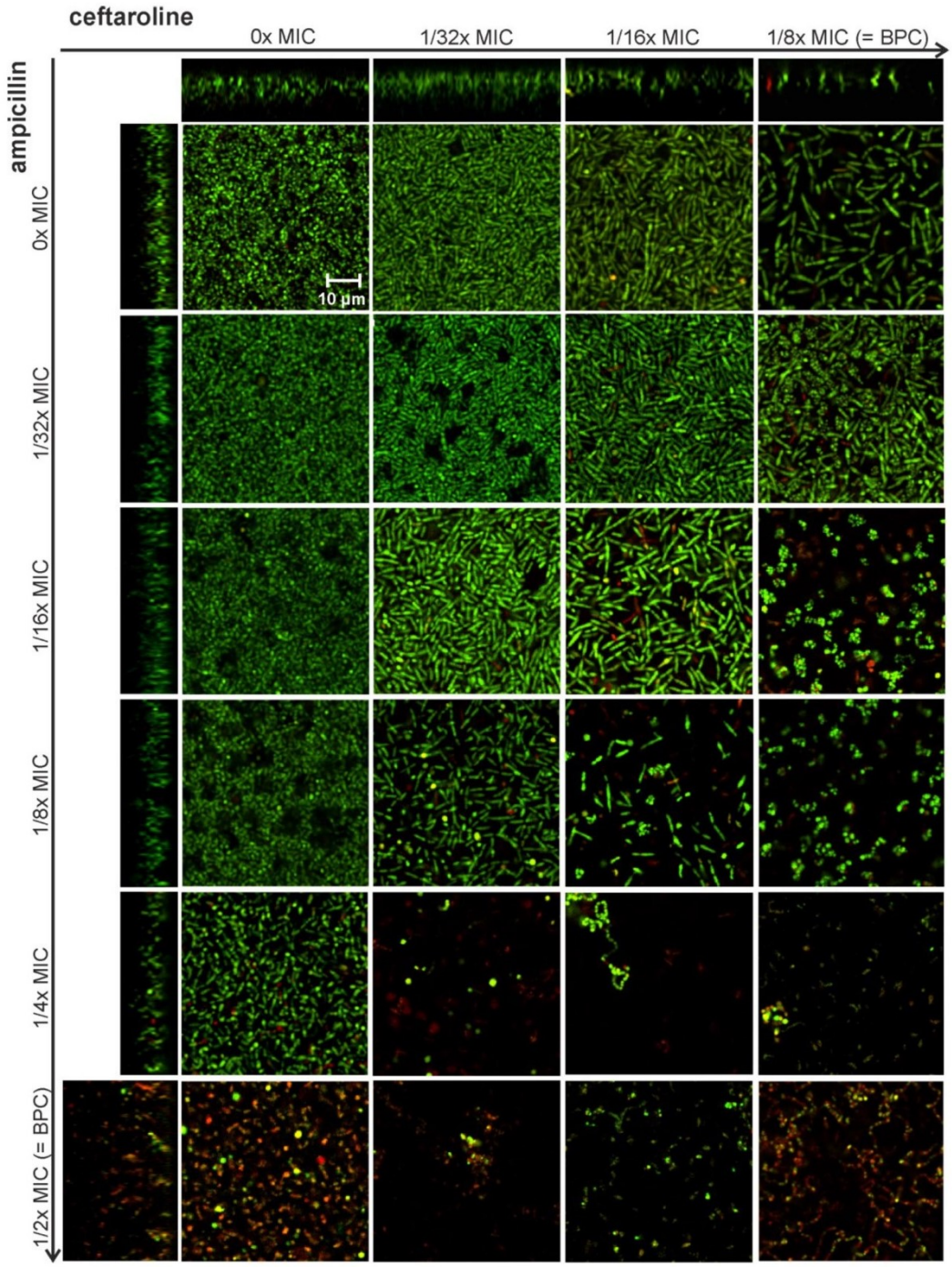

Figure S4. Effects of ceftaroline and ampicillin in combination on biofilm formation. Biofilm formation by *E. faecalis* isolate bk1653 in the presence of sub-MICs of ceftaroline and/or ampicillin (both MIC = 1 mg/L), in 96 well glass bottom plates after 48 h, assessed by confocal laser scanning microscope analysis. Viable bacteria are visible in green (SYTO9), and dead bacteria in red (propidium iodide). Scale applies to all images. BPC = biofilm prevention concentration.

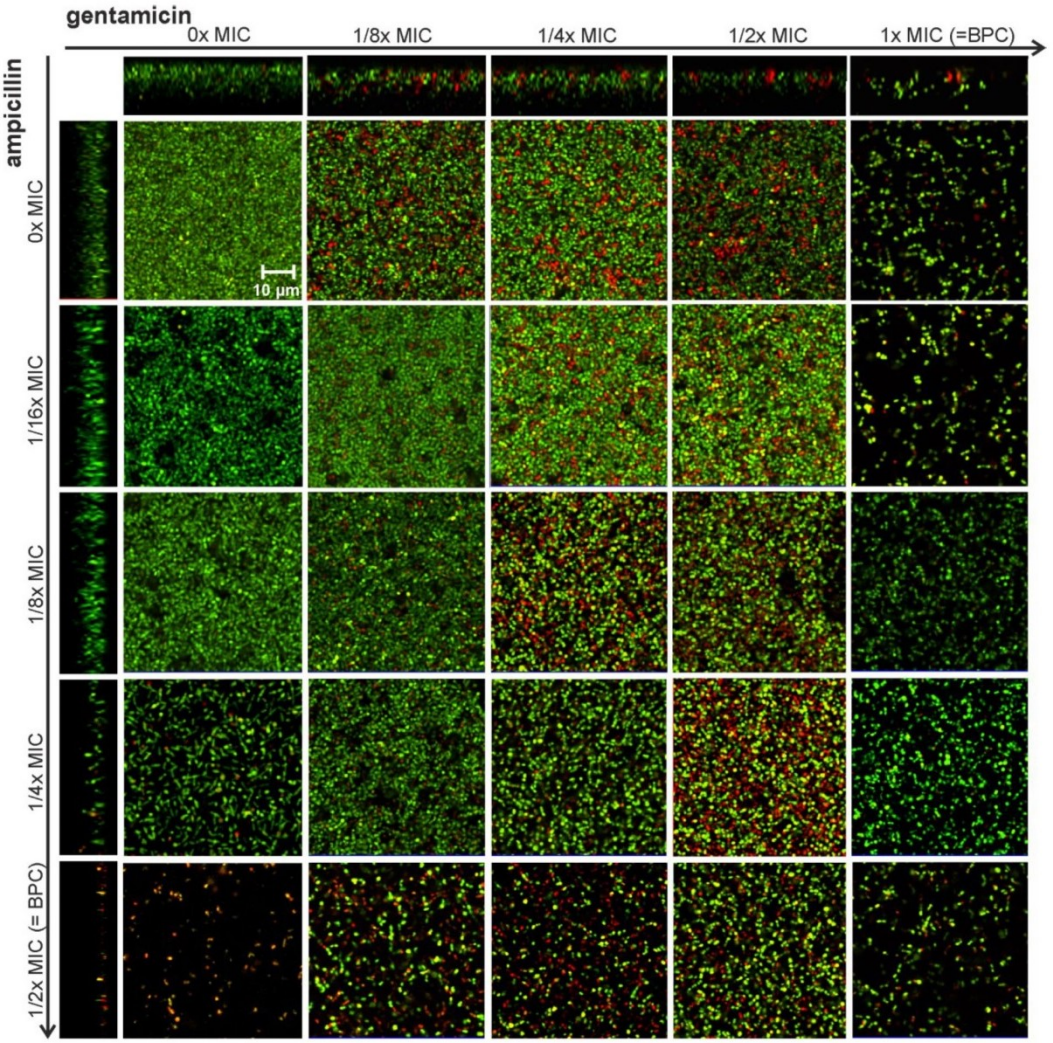

Figure S5. Effects of gentamicin and ampicillin in combination on biofilm formation. Biofilm formation by *E. faecalis* isolate bk1653 in the presence of sub-MICs of gentamicin and/or ampicillin (MIC_{GEN} = 64 mg/L, MIC_{AMP} = 1 mg/L), in 96 well glass bottom plates after 48 h, assessed by confocal laser scanning microscope analysis. Viable bacteria are visible in green (SYTO9), and dead bacteria in red (propidium iodide). Scale applies to all images. BPC = biofilm prevention concentration.

3.2 Publication II:

MBEC versus MBIC: The Lack of Differentiation between Biofilm Reducing and Inhibitory Effects as a Current Problem in Biofilm Methodology

Authors: **Lara Thieme**, Anita Hartung, Kristina Tramm, Mareike Klinger-Strobel, Klaus D. Jandt, Oliwia Makarewicz, Mathias W. Pletz

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Own contribution to publication: 75 % (partly performance of experiments; analysis and interpretation of data; conceptualization, writing and revision of the manuscript)

This commentary aims to contribute to a standardized use of biofilm susceptibility endpoint parameters. A major challenge in current biofilm research is the development of adequate biofilm susceptibility testing assays that are clinically meaningful, i.e. that their results correlate with treatment outcome. The biofilm susceptibility endpoint parameters determined in such tests, e.g., the minimal biofilm eradication concentration (MBEC) or the minimal biofilm inhibitory concentration (MBIC), are inconsistently perceived, used and interpreted among biofilm researchers. This hinders the comparability of results and progress in the development of clinically meaningful anti-biofilm assays. In this commentary we point out the importance of individual quantification of mature, established biofilms before antimicrobial treatment – and not only after the antibiotic incubation period - for each biofilm model in order to draw conclusions on the measured biofilm effect size, i.e. biofilm reducing (“MBEC”) or biofilm inhibitory (“MBIC”) effects. Bacterial quantification results of a theoretical anti-biofilm study illustrate that also a quantification of the untreated biofilm before antimicrobial exposure is required, before reliable conclusions on biofilm eradicating or inhibiting effects can be made. The assessment of pre-treatment biofilms will contribute to a standardized use of biofilm susceptibility endpoint parameters, which is urgently needed to improve the clinical validity of anti-biofilm assays.

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Biological Procedures Online

COMMENTARY Open Access

MBEC Versus MBIC: the Lack of Differentiation between Biofilm Reducing and Inhibitory Effects as a Current Problem in Biofilm Methodology

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Abstract

Background: Biofilms are communities of aggregated, matrix-embedded microbial cells showing a high tolerance to an in principle adequate antibiotic therapy, often resulting in treatment failure. A major challenge in the management of biofilm-associated infections is the development of adequate, standardized biofilm susceptibility testing assays that are clinically meaningful, i.e. that their results correlate with treatment outcome. Different biofilm susceptibility endpoint parameters like the minimal biofilm eradication concentration (MBEC) or the minimal biofilm inhibitory concentration (MBIC) have been suggested as a guide for treatment of biofilm-associated infections, however with inconsistent perception and use among biofilm researchers, leading to confusion and contradictions among different anti-biofilm component studies and clinical trials.

Findings: Evaluation of anti-biofilm effects is mostly based on the untreated reference growth control biofilm measured at the same endpoint as the treated biofilm, neglecting the possible change of the untreated reference biofilm from the time point of pre-antimicrobial exposure to the measured endpoint. In this commentary, we point out the importance of individual quantification of mature, established biofilms before antimicrobial treatment for each biofilm model in order to draw conclusions on the measured biofilm effect size, i.e. biofilm reducing (MBEC) or biofilm inhibitory (MBIC) effects.

Conclusion: The assessment of pre-treatment biofilms contributes to a standardized use of biofilm susceptibility endpoint parameters, which is urgently needed to improve the clinical validity of future anti-biofilm assays.

Keywords: Biofilm-associated infections, Biofilm susceptibility testing, Biofilm susceptibility endpoint parameters, MBEC, MBIC

Background

Biofilms are matrix-embedded communities of microbial cells that are attached to each other and/or on a surface [1]. Biofilms protect enclosed bacterial cells against the immune system and an in principle adequate antibiotic therapy, often resulting in treatment failure, relapsing infections and increased mortality [1]. The minimal inhibitory concentrations (MIC) of antibiotics are routinely determined using planktonic bacteria and do not match the concentrations that are required to prevent, inhibit, diminish or eradicate biofilms [2].

A major challenge in the management of biofilm-associated infections (BAI) is the development of adequate, standardized biofilm susceptibility testing assays that are clinically meaningful, i.e. that their results correlate with treatment outcome [3, 4]. Over the last years, a multitude of diverse laboratory methods to assess anti-biofilm treatments has been developed. Each method has its own benefits and drawbacks as critically discussed elsewhere, with the overall consensus that

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there is currently no optimal biofilm method available mimicking the in vivo biofilm setting of human BAI [3, 5, 6]. Different biofilm susceptibility endpoint parameters have been suggested as a guide for treatment of BAI, like the minimal biofilm eradication concentration (MBEC), the minimal biofilm inhibitory concentration (MBIC), the biofilm bactericidal concentration (BBC) or the biofilm prevention concentration (BPC) [2]. However, the definition and interpretation of these parameters differ greatly among publications and none of the official agencies, e.g. EUCAST or CLSI, have yet set up standardized definitions of biofilm endpoint parameters likewise the MIC. While some researchers define the MBEC as the lowest concentration of an antimicrobial substance that eradicates 99.9% of biofilm-embedded bacteria (3 $\log_{10}$ reduction in CFU/mL) compared to growth controls [7], others define the former as the BBC in line with the minimal bactericidal concentration (MBC) on planktonic level and refer to the MBEC in the context of complete eradication of the biofilm [2, 8]. Inhibitory effects on biofilm formation are commonly assessed by the MBIC, which is the lowest concentration of an antimicrobial substance at which there is no time-dependent increase in the mean number of biofilm viable cells [2]. In contrast to the MBIC, the BPC determines at which antimicrobial substance concentration the cell density of a planktonic culture is sufficiently reduced in order to prevent biofilm formation [2].

In this commentary, we point out the importance of individual quantification of mature, established biofilms before antimicrobial treatment for each biofilm model in order to draw conclusions on the measured biofilm effect size, i.e. biofilm reducing or biofilm inhibitory effects.

Findings

Importantly, all of the above parameters, except for the BPC, analyse the activity of antimicrobial substances on mature, established biofilms, so the experimental set-up to assess either biofilm reducing or inhibitory effects is in principle the same, regardless of the method of choice of biofilm growth and assay readout (Fig. 1). After establishment - while even the time of biofilm maturation varies strongly between different research groups -, biofilms are treated with the respective antimicrobial substance for a variable period of time (hours to days), followed by assessment of the treated and untreated biofilms by e.g. CFU/mL determination, image acquisition or staining and photometric measurement (e.g. resazurin or crystal violet). Evaluation of anti-biofilm effects is thereby mostly based on the untreated reference growth control biofilm measured at the same endpoint as the treated biofilm [7, 9–11], assuming that the constitution (e.g. viable cell numbers, total biomass etc.) of the untreated reference growth control biofilm is stable from the time point of pre-antimicrobial exposure to the measured endpoint. Four theoretical scenarios showing the consequences of stable and unstable quantities of untreated reference biofilm viable cells (CFU/mL) over the course of the experiment for the interpretation of anti-biofilm effects are listed in Table 1. Provided the established biofilm had a starting quantity of 10^5 CFU/mL before antimicrobial treatment (Table 1, scenario A), the quantification of 10^8 CFU/mL of the untreated biofilm at the measured endpoint reveals that the biofilm without the addition of antibiotics increased by 3 $\log_{10}$ in CFU/mL. The treated biofilm with 10^5 CFU/mL at the measured endpoint, however, implies no increase in the mean number of biofilm viable cells, making this scenario a classic example for the determination of inhibitory effects. The further growth of the biofilm was inhibited with addition of the antimicrobial substance by 3 $\log_{10}$ in CFU/mL in scenario A, but the biofilm was not reduced by 3 $\log_{10}$ in CFU/mL, which would be the conclusion if the untreated reference growth control biofilm is regarded only at the measured endpoint, but not before antimicrobial exposure. Scenario B illustrates an unstable quantity of the untreated reference biofilm over the course of the experiment as well, but in a smaller magnitude. If the untreated reference biofilm at the time point of pre-antimicrobial exposure and at the measured

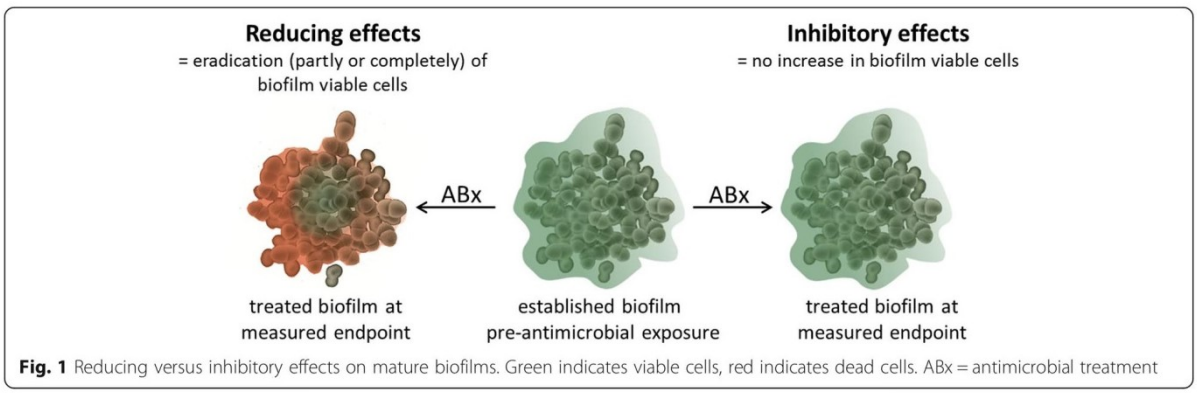

Fig. 1 Reducing versus inhibitory effects on mature biofilms. Green indicates viable cells, red indicates dead cells. ABx = antimicrobial treatment

Table 1 Interpretation of the anti-biofilm effect size based on different scenarios of starting viable cell numbers before treatment

	At measured endpoint	Scenario A	Scenario B	Scenario C	Scenario D
		Pre-antimicrobial exposure	Pre- antimicrobial exposure	Pre- antimicrobial exposure	Pre- antimicrobial exposure
Untreated	10^8 CFU/mL	10^5 CFU/mL	10^7 CFU/mL	10^8 CFU/mL	10^9 CFU/mL
Treated	10^5 CFU/mL	10^5 CFU/mL	10^7 CFU/mL	10^8 CFU/mL	10^9 CFU/mL
Interpretation	3 $\log_{10}$ biofilm reduction	3 $\log_{10}$ inhibition of biofilm growth (MBIC)	2 $\log_{10}$ biofilm reduction	3 $\log_{10}$ biofilm reduction (MBEC or BBC)	3 $\log_{10}$ biofilm reduction (MBEC or BBC)

MBIC Minimal biofilm inhibitory concentration, *MBEC* Minimal biofilm eradication concentration, *BBC* Biofilm bactericidal concentration

endpoint is composed of 10^7 CFU/mL and 10^8 CFU/mL, respectively, the untreated biofilm increased by 1 $\log_{10}$ CFU/mL in this time span. With the same readout of the treated biofilm at the measured endpoint of 10^5 CFU/mL, this scenario indicates that the biofilm was reduced by 2 $\log_{10}$ in CFU/mL (from 10^7 to 10^5 CFU/mL) with the addition of the antimicrobial substance. Notably, only the starting quantity of the established, mature biofilm changed, but not the final results at the measured endpoint of this theoretical anti-biofilm assay. Only if the untreated reference biofilm is stable in CFU/mL numbers in the time span of pre-antimicrobial exposure and assay readout (scenario C), the interpretation of the anti-biofilm effect size (3 $\log_{10}$ reduction with a decrease of 10^8 to 10^5 CFU/mL) is the same when evaluating the effect based on the reference biofilm at the measured endpoint or the time point before antimicrobial exposure. If the untreated reference biofilm shows a higher viable cell quantity before antimicrobial exposure than at the time point of readout (scenario D), the decrease of viable cell numbers independent on antimicrobial treatment needs to be considered for the interpretation of the anti-biofilm effect size. In scenario D, this means the biofilm quantity decreased treatment-independent from 10^9 CFU/mL to 10^8 CFU/mL, leading to a 3 $\log_{10}$ reduction from 10^8 to 10^5 CFU/mL due to antimicrobial treatment. Above scenarios illustrate that only *after* the anti-biofilm experiment has been performed and, essentially, had included an assessment of the established biofilm before antimicrobial exposure, one can clearly say i) whether a biofilm reducing or inhibitory effect has taken place, ii) how high the magnitude of the analysed effect is. Researchers should therefore match the according biofilm susceptibility parameter to the observed effect based on the quantification of the reference growth control biofilm before and after treatment.

To assess how the starting number of biofilm viable cells may influence the interpretation of the anti-biofilm effects measured in our biofilm model, we determined the CFU/mL of untreated reference biofilms of five different bacterial species before and after potential antimicrobial exposure (Fig. 2). In our model, biofilms are grown for 48 h followed by incubation of antimicrobial substance for 24 h, resulting in 72 h of growth of untreated reference biofilms at the measured endpoint. Bacterial suspensions (0.5 McFarland) of three clinical isolates of each *Enterococcus faecium, Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* prepared in Müller Hinton broth or Todd Hewitt broth (both Karl Roth, Karlsruhe, Germany) for enterococci, respectively, were inoculated

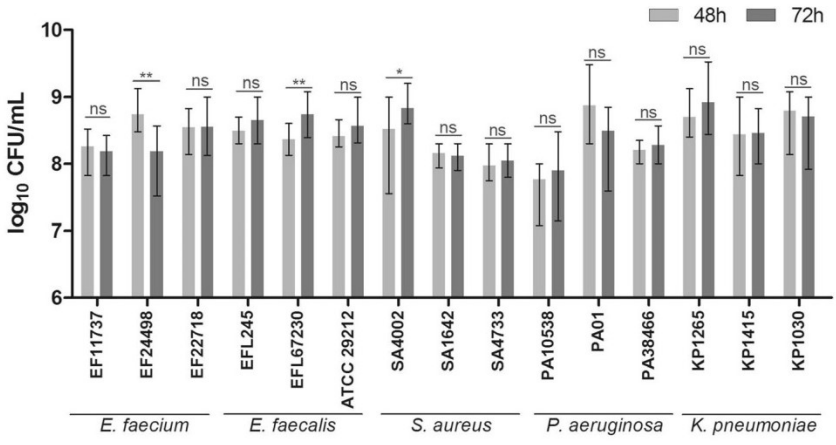

Fig. 2 Biofilm viable cell numbers (CFU/mL) after 48 h and 72 h of growth. Shown are the mean values with ranges of triplicates. An unpaired t-test was performed to analyse significant differences (*P*-value < 0.05) between 48 h and 72 h of growth. ns = no significance

in triplicates in plastic microtiter plates (Greiner Bio-one, Frickenhausen, Germany). Biofilms were grown at 37 °C, 5% CO_2 without shaking for 48 h and 72 h with change of medium after 48 h to mock antimicrobial treatment. After 48 h and 72 h, respectively, biofilms were washed, resuspended and selected 10-fold dilutions were plated for determination of CFU/mL. 80% of the tested isolates showed no significant increase in CFU between both time points (Fig. 2). For two strains (EFL67230 and SA4002), CFU_{72h} was significantly increased compared to CFU_{48h}, however below 1 $\log_{10}$ and therefore not influencing the interpretation of the results in terms of reducing or inhibiting effects. One *E. faecium* isolate (EF24498) showed a significant decrease in CFU/mL, but again below 1 $\log_{10}$. The constancy of biofilm cell numbers from the time point of pre-antimicrobial exposure to the measured endpoint implies the determination of biofilm reducing instead of inhibiting effects in our model (Table 1, scenario C). A decrease in viable cell numbers of the treated biofilm compared to the untreated reference biofilm at the measured endpoint (which has the equal quantity as the untreated reference biofilm pre-antimicrobial exposure) can clearly be related to a reduction of the biofilm due to antimicrobial treatment. If viable biofilm cell numbers were increasing between 48 h and 72 h, either the effect magnitude of the reducing effect would change (Table 1, scenario B) or inhibiting instead of reducing effects would be analysed (Table 1, scenario A), making it necessary to determine the anti-biofilm effect size based on the quantification of the established, mature biofilm before antimicrobial treatment. Importantly, the constancy of the reference biofilm may not be the case for other methods of biofilm growth, e.g. dynamic biofilm reactors where biofilms are grown under constant nutrient flow [3], highlighting the importance of individual quantification of mature, pre-treatment biofilms for each biofilm model.

Conclusions

Above scenarios elucidate another point of many current difficulties in biofilm methodology. Presently, biofilm susceptibility endpoint parameters are inconsistently perceived, used and interpreted among biofilm researchers. For example, Sandoe et al. quantified their peg biofilms before and after exposure to ampicillin, showing a significant reduction in CFU/mL numbers, yet using the MBIC as biofilm susceptibility endpoint parameter to describe their results [11]. To overcome this lack of consistency, standardized methods with accurate and precise definitions of biofilm susceptibility endpoint parameters are urgently needed, reducing confusion and contradictions among different anti-biofilm component studies. For the clinical evaluation of anti-biofilm compounds intended for therapy of BAI, it is crucial to determine whether a drug is able to penetrate and eradicate, in part or completely, the biofilm structure or is only able to inhibit its further growth.

The current insufficient evidence to recommend antibiotics on the basis of biofilm susceptibility testing is mainly attributed to the deficit of proper methodology representing in vivo biofilms [5]. The fact that the very few clinical trials addressing BAI have not measured biofilm eradicative but inhibitory effects might contribute to the poor observed correlation between biofilm susceptibility testing and clinical outcome [10–12]. Commercially available anti-biofilm test kits like the MBEC Assay®, formerly the Calgary Biofilm Device (Innovotech, Edmonton, Canada), show increasing rates of use in biofilm research [6], however neglect the potential problem of not measuring reducing, but inhibitory effects. Although the datasheet of the MBEC Assay® recommends a biofilm growth check before antimicrobial treatment [13], most publications do not take those values into account for the interpretation of anti-biofilm effects [10–12, 14]. We therefore highly encourage biofilm researchers to assess established biofilms before antimicrobial exposure, independent on the method of choice for biofilm growth and assay readout, to bring more clarity to their measured biofilm effect size and biofilm susceptibility parameters. The assessment of pre-treatment biofilms will contribute to a standardized use of biofilm susceptibility endpoint parameters, which is urgently needed to improve the comparability of anti-biofilm studies and to make progress in the development of clinically meaningful anti-biofilm assays.

Abbreviations

BAI: Biofilm-associated infection; BBC: Biofilm bactericidal concentration; BPC: Biofilm prevention concentration; CFU: Colony forming unit; CLSI: Clinical & Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing; MBEC: Minimal biofilm eradication concentration; MBIC: Minimal biofilm inhibitory concentration; MIC: Minimal inhibitory concentration

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Authors' Contributions

LT conceptualized the article; designed and performed the experiments; analysed and interpreted the data; wrote and revised the manuscript. AH and KT revised and critically discussed the article; performed the experiments; analysed the data. MKS, KDJ, OM and MWP revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Authors' Information

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Not applicable.

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Publisher's Note

3.3 Publication III:

In vivo synergism of ampicillin, gentamicin, ceftaroline and ceftriaxone against *Enterococcus faecalis* assessed in the *Galleria mellonella* infection model

Authors: **Lara Thieme**, Anita Hartung, Oliwia Makarewicz, Mathias W. Pletz

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This work continued the *in vitro* studies and analyzed for the first time the antibiotic combinations discussed for the treatment of EFIE in a larval infection model. Methodically, the study served as a proof-of-concept for the general establishment of the *Galleria mellonella* larvae infection model at the Jena University Hospital. To assess synergistic effects between gentamicin/ampicillin, ceftriaxone/ampicillin and ceftaroline/ampicillin against *E. faecalis in vivo*, the *in vitro* checkerboard assay set-up was partially transferred into the larvae. Three output parameters for analysis were used: larval survival, bacterial quantity in the haemolymph and visual markers of the larvae. Globally, no standardization of synergism testing in larvae had been established so far. To implement a multiple antibiotic dosing regimen based on the half-lives of the antibiotics, the pharmacokinetics of ampicillin, gentamicin, ceftriaxone and ceftaroline were assessed for the first time in the larvae. In contrast to the *in vitro* results, only ceftriaxone/ampicillin showed synergistic effects in a strain-dependent manner, while ceftaroline/ampicillin and gentamicin/ampicillin exhibited additive effects against *E. faecalis* in the larvae.

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In vivo synergism of ampicillin, gentamicin, ceftaroline and ceftriaxone against *Enterococcus faecalis* assessed in the *Galleria mellonella* infection model

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Background: The unfavourable safety profile of aminoglycosides and the synergistic effects observed *in vitro* have prompted the development of novel dual β-lactam therapies, e.g. ampicillin/ceftriaxone or ampicillin/ceftaroline, for the treatment of *Enterococcus faecalis* endocarditis.

Objectives: For comparison with *in vitro* chequerboard assay results, a partial chequerboard setup of ampicillin/gentamicin, ampicillin/ceftriaxone and ampicillin/ceftaroline against *E. faecalis* was established in the *Galleria mellonella* larval infection model.

Methods: Discrimination of synergistic and additive interactions was based on the evaluation of larval survival, bacterial quantity in the haemolymph and a pathology score index (internal to the workgroup). Single and multiple dosing schemes based on the half-life of ampicillin were applied. Pharmacokinetic data of the antibiotics in the larvae were determined via agar plate diffusion assays.

Results: Ampicillin and ceftriaxone exhibited strain-specific synergistic interactions in the larvae under both dosing regimens, while the other two combinations showed additive effects. Ampicillin/ceftaroline was inferior to ampicillin/ ceftriaxone. Not all synergistic effects observed *in vitro* could be replicated in the larvae.

Conclusions: Our results suggest superior efficacy of ampicillin/ceftriaxone for the treatment of high-inoculum enterococcal infections, for at least some strains, but question the benefit of the current standard of adding the nephrotoxic gentamicin compared with the safer ceftriaxone. This is the first study to develop a scheme for differentiation between additive and synergistic effects in larvae and apply a multiple-antibiotic dosing scheme based on the pharmacokinetics of ampicillin. The model allows the analysis of synergistic effects of antimicrobials in an *in vivo* setting, but the clinical correlation warrants further study.

Introduction

Enterococcus faecalis is the third most common pathogen causing infective endocarditis (IE), a deep-seated, high-inoculum infection that has had unchanging mortality rates of up to 30% for several decades.[1] In addition to cardiac surgery, guidelines recommend antibiotic combination therapies, but further research is urgently needed to identify the optimal combination regimens.[2,3] Currently, ampicillin combined with gentamicin or ceftriaxone is used for the treatment of *E. faecalis* IE but each have significant drawbacks, as elucidated elsewhere.[2–4] We recently compared these combinations with that of ampicillin and ceftaroline, which is a novel cephalosporin with intrinsic activity against *E. faecalis*, by *in vitro* chequerboard assays,[5] that we now report as partially transferred into the *in vivo Galleria mellonella* larval infection model. The *G. mellonella* infection model has gained popularity among non-mammalian infection models in recent years, with the main advantages of relatively few ethical constraints, low usage of resources and the ability to perform infection assays at human body temperature.[6] A new research-grade *G. mellonella* breeding line (TruLarv™, BioSystems Technology, Exeter, UK) overcomes the variability associated with larvae from bait shops, allowing the use of smaller sample sizes.[7–9] Although this model has been used to assess the interaction of several antimicrobials against different bacteria, including *E. faecalis*,[6,10,11] standardized protocols for synergy testing in larvae have not been established. Many studies define synergistic interaction by showing that lower doses of the agents in combination result in the same or higher survival rates compared with monotreatment with each agent at higher concentrations,[10–13] but a clear distinction between synergistic and additive effects is frequently not made. An additive effect is defined by no significantly greater antimicrobial efficacy of the

combination than that of each antibiotic individually.[14] Essentially, the term additivity is not based on the simple addition of the effect magnitudes of the single agents but is derived from the concept of dose equivalence.[14,15] This concept assumes that two doses of different compounds having the same effect are equivalent and it follows that the replacement of one component by the other or vice versa does not influence the effect.[16] Synergistic and antagonistic effects are seen as departures from additivity, presenting as superadditive or subadditive effects, respectively.[14] Clinically, synergistic interactions of antibiotic combinations are desired to enhance the bactericidal effects of individual antibiotics, allowing the use of lower doses of each agent, which reduces the risk of side effects and selection pressure. The risk–benefit ratio of purely additive effects is less convincing than that of synergistic combinations, particularly if the combination partner has an unfavourable safety profile, as is the case for aminoglycosides.

The aim of this study was to establish synergism testing in *G. mellonella* larvae with an emphasis on the differentiation from additive effects and to determine the synergistic effects of ampicillin combined with gentamicin, ceftriaxone or ceftaroline against *E. faecalis in vivo*. The interactions of the antibiotics were examined based on three parameters: survival curves, viable bacteria (cfu/mL) in the haemolymph and a modified pathology score index (PSI). Single and triple dosing schemes were applied, wherein the latter were based on predetermined pharmacokinetic data of the antibiotics in the larvae.

Materials and methods

Bacterial strains and antibiotics

The clinical *E. faecalis* isolates (bk1653, bk9367 and va245) were obtained from the Institute of Medical Microbiology at Jena University Hospital, Germany. The clinical background, *in vitro* antibiotic susceptibility and synergism of the isolates were assessed in a previous study (Table 1).[5] Test solutions of ampicillin (Karl Roth, Karlsruhe, Germany), ceftriaxone (TCI Europe, Zwijndrecht, Belgium), gentamicin (TCI Europe), ceftaroline (Pfizer, New York City, USA) and ceftaroline fosamil (Zinforo™, Pfizer) were prepared immediately before usage.

G. mellonella model of E. faecalis infection

Synergism experiments were performed with *G. mellonella* wax moth larvae (TruLarv™, BioSystems Technology) of similar weight. Bacterial suspensions were grown for 1 h in Todd Hewitt (TH) broth (Karl Roth), washed in 1× PBS and diluted with PBS to a bacterial cell density that caused ≥80% larval deaths at 24 h post-infection (p.i.) (for details, see Figure S1, available as Supplementary data at *JAC* Online): 10^6 cfu/10 μL (isolates bk1653 and bk9367) or 5×10^6 cfu/10 μL (isolate va245). Inoculation of the larvae was performed via injection of 10 μL of the respective bacterial suspension into a proleg of the larva using a Hamilton syringe (701N; Merck, Darmstadt, Germany). Antibiotics were delivered in the same way 1 h p.i. (single dosing) or 1, 3 and 5 h p.i. (multiple dosing). Different prolegs of the larvae were used to inject bacterial or antibiotic suspensions to minimize leakage of the haemolymph (Figure S2). Antibiotic concentrations were based on *in vitro* MICs considering an average haemolymph volume of 50 μL and the increase in volume caused by bacterial and antibiotic injection (each 10 μL), e.g. for a final concentration of 2× MIC in the larvae, 10 μL of an antibiotic solution with a seven times higher concentration was administered to the larvae.[17] Antibiotic toxicity was ruled out by tracking survival after single injections of high-dose concentrations. Each experiment contained five larvae per treatment group and was performed twice ($n = 10$), including PBS mock-infected and PBS mock-treated larvae. Larvae were incubated at 37°C and for each individual larva the survival and PSI (melanization index + activity index) (Figure 1) at 24, 48 and 72 h p.i. were noted. Kaplan–Meier survival curves and the mean PSI for each treatment group were generated.

Determination of haemolymph burden of E. faecalis in G. mellonella

After scoring at 72 h p.i., three larvae per treatment group were selected to determine the number of viable *E. faecalis* cells in the haemolymph. Both

Table 1. Clinical background and *in vitro* antibiotic susceptibility and interaction data of the three *E. faecalis* isolates analysed in this study

	Enterococcus faecalis strain		
	bk1653	va245	bk9367
Clinical data			
sex, age of patient (years)	female, 87	male, 76	female, 85
source of specimen	blood culture	swab of mitral valve	blood culture
focus of infection	urosepsis due to UTI	endocarditis	recurrent bacteraemia
MIC (mg/L)			
AMP	1	0.5	2
CPT	1	0.125	0.5
CRO	16	2	16
GEN	64	16	32
Antibiotic interaction *in vitro* (FICI)[a]			
AMP + CPT	synergistic (0.31)	no interaction (0.73)	synergistic (0.37)
AMP + CRO	synergistic (0.25)	synergistic (0.50)	synergistic (0.38)
AMP + GEN	synergistic (0.50)	no interaction (1.02)	no interaction (0.63)

The data are based on a previous study.[5] MICs were determined by the broth microdilution method. Antibiotic interactions were analysed by chequerboard assays resulting in FIC indices (FICIs). AMP, ampicillin; CPT, ceftaroline; CRO, ceftriaxone; GEN, gentamicin.
[a]The value shown in parentheses is the lowest observed FICI value.

(a)

Melanization	Score
no melanization, beige	0
dark aorta line, beige	1a
1-2 black spots without dark aorta line, beige	1b
dark aorta line plus black spots, beige to greyish	2
dark aorta line plus black spots, brown	3
black larvae	4
Activity	**Score**
move without stimulation	0
move when stimulated	1
minimal movement on stimulation	3
no movement	4

(b)

Figure 1. PSI system. After bacterial and antibiotic injections, each larva was assigned a modified melanization and activity index at 24, 48 and 72 h p.i.[6,28] These indices were added to obtain the PSI. (a) For both categories, the higher the index, the more the infection had progressed. For activity scoring, index 2 was left out to give more weight to sick larvae (score 3 or 4) in contrast to healthy larvae (score 0 or 1). (b) Images of the different stages of melanization of *G. mellonella* larvae. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

dead and viable larvae were selected at a ratio representing the current survival rate of the respective treatment group. In larvae that were already dead at 24 or 48 h p.i., viable bacteria in the haemolymph were quantified immediately after scoring at these timepoints. For collection of haemolymph, larvae were anaesthetized by placement on ice for 30 min and surface disinfected on the dorsal side with ethanol. The haemolymph was carefully drawn up dorsally into an insulin syringe (BD, Heidelberg, Germany), serially diluted and plated using the drop plate method.[18] TH agar plates supplemented with 8 mg/L tetracycline (bk1653 and bk9367) or 2 mg/L ciprofloxacin (va245) (both PanReac AppliChem, Darmstadt, Germany) were used to select the injected *E. faecalis* isolate from larval commensal enterococcal strains.[7,19] The plates were incubated at 37°C for 16 h, followed by determination of cfu/mL.

Pharmacokinetics of antibiotics in G. mellonella determined by agar plate diffusion assays

The pharmacokinetics of ampicillin, gentamicin, ceftaroline and ceftriaxone in the larvae were determined by agar plate diffusion assays as described by Hill *et al.*[20] with some modifications. Standard curves (antibiotic concentration versus radius of zone of inhibition) for each antibiotic were prepared with *E. faecalis* ATCC 29212 (for ampicillin) and *Escherichia coli* ATCC 25922 (for all other antibiotics) as indicator organisms, as previously described, except for the use of diffusion discs instead of manufactured agar wells. To determine the pharmacokinetics of the antibiotics in the larvae, antibiotics with a starting concentration of 2048 mg/L (ceftaroline, ceftriaxone and ampicillin) or 4096 mg/L (gentamicin) were injected into uninfected larvae. These concentrations corresponded theoretically to 68 mg/kg or 136 mg/kg dosages based on an average haemolymph volume of 50 µL and an average weight of 300 mg of the larvae. Haemolymph was then harvested from antibiotic- ($n = 3$) and PBS-treated ($n = 2$) larvae at different timepoints post-administration (20, 40, 60, 80, 100, 120, 240, 360 and 480 min) and agar diffusion assays were again performed as described by Hill *et al.*[20] The concentration of antibiotic present in the haemolymph at each timepoint was calculated based on the standard curves (Figure S3).

Statistical analysis

All analyses and graphical operations were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, USA). Kaplan–Meier survival curves were compared by the log-rank (Mantel–Cox) test and cfu/mL and PSI data were analysed by the Kruskal–Wallis test followed by Dunn's multiple comparison test, both with a confidence interval of 95%. *P* values of <0.05 were considered statistically significant.

Results

Efficacy of single administration of the antibiotic combinations on survival of larvae

Analyses of synergistic effects between ampicillin/gentamicin, ampicillin/ceftriaxone and ampicillin/ceftaroline were first performed by comparing survival rates of the larvae after infection followed by one-time dosing of single or combined antibiotic treatment (Figure 2). Consistent with the chequerboard assay setup, selected serial dilutions on the MIC scale, i.e. 2×, 4× and 8× MIC, of each of the individual antibiotics were administered and compared with combined concentrations of the antibiotics ($1\times MIC_A + 1\times MIC_B$ and $2\times MIC_A + 2\times MIC_B$). Survival rates of the larvae receiving dose combinations were statistically compared with (i) the survival rates of larvae receiving the dose equivalent to check for additive effects, e.g. $2\times MIC_{AMP}$ versus $1\times MIC_{AMP} + 1\times MIC_{CPT}$ (where AMP is ampicillin and CPT is ceftaroline); and (ii) with the survival rates after treatment with the next higher combined dose, as a true synergistic interaction yields the same or even stronger effect at lower doses of the combination constituents, e.g. $4\times MIC_{AMP}$ versus $1\times MIC_{AMP} + 1\times MIC_{CPT}$. The *E. faecalis* isolate bk1653 was selected because synergistic effects of all three antibiotic combinations had been observed *in vitro* (Table 1).

Except for ceftriaxone, all individual antibiotics showed a concentration-dependent effect on 72 h survival of the larvae, with the highest survival rates of 80% observed with 8× MIC of ampicillin or gentamicin (Figure 2a and d). Independent of the concentration, ceftriaxone treatment prolonged the survival only up to 24 h, but the survival rates reached close to zero at 72 h p.i. (Figure 2c). However, under combined treatment with ampicillin, nearly all larvae survived until the end of the observed time frame

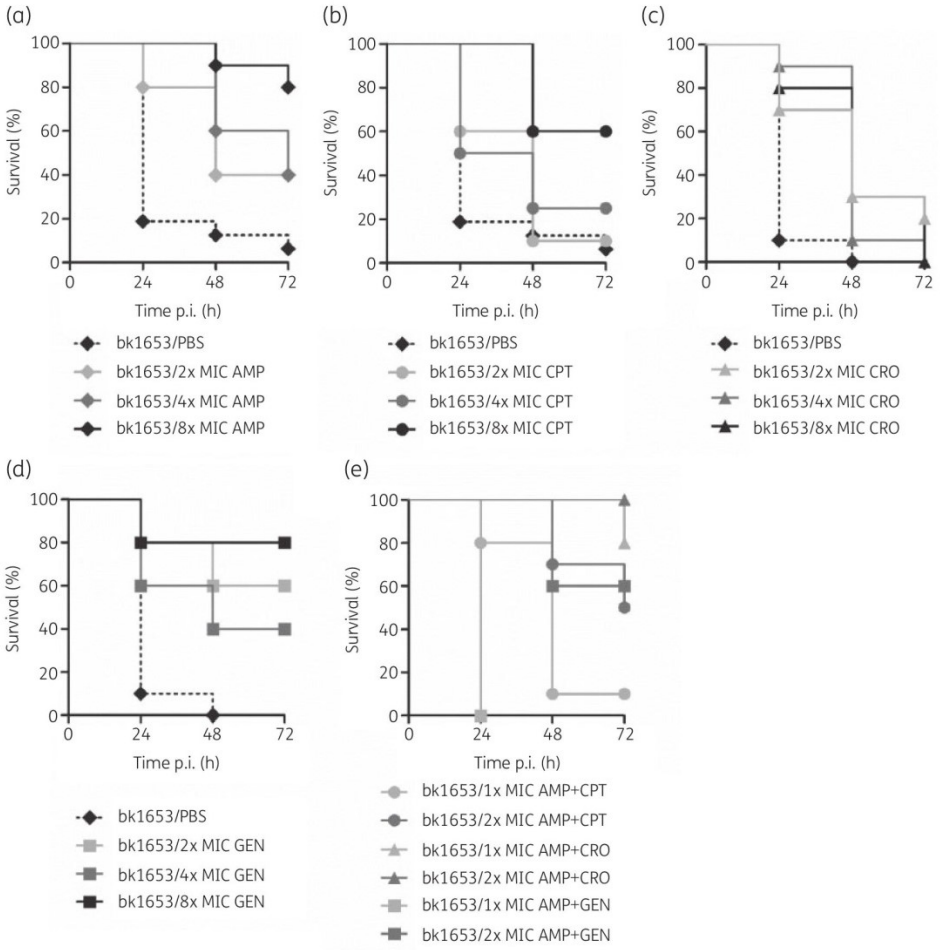

Figure 2. Effect of antibiotic treatment (dosing once) on the survival of larvae infected with *E. faecalis* bk1653. Larvae were infected with 10^6 cfu followed by antibiotic treatment 1 h p.i. The *in vitro* MICs of bk1653 were as follows: MIC_{AMP} = 1 mg/L, MIC_{CPT} = 1 mg/L, MIC_{CRO} = 16 mg/L and MIC_{GEN} = 64 mg/L. Each experiment was performed twice with five larvae per treatment group (n = 10), including PBS mock-infected larvae. Statistical analysis was performed by log-rank tests (Table 2).

at both dose combinations tested (Figure 2e). Significant differences between the survival curves of the combined ampicillin/ceftriaxone treatment and of the dose equivalents of the individual antibiotics were observed, indicating synergistic effects (Table 2). One-time dosing of 1× MIC_{AMP} + 1× MIC_{CRO} (where CRO is ceftriaxone) resulted in the same survival rate of 80% as one-time dosing of 8× MIC_{AMP}, confirming the synergistic interactions between ampicillin and ceftriaxone. In contrast, no synergistic interaction was observed between ampicillin and ceftaroline (Figure 2a, b, e; Table 2). For ampicillin/gentamicin, the dose combination of 1× MIC_{AMP} + 1× MIC_{GEN} (where GEN is gentamicin) showed a trend towards antagonism, with higher survival rates reached with monotreatment (2× MIC) with both antibiotics, while treatment with 2× MIC_{AMP} + 2× MIC_{GEN} led to additive effects (Figure 2a, d, e; Table 2).

Efficacy of single administration of the antibiotic combinations on the reduction in bacterial burden in the haemolymph

After using larval survival as a measure of additive or synergistic effects, bacterial survival in the haemolymph was assessed by comparing the cfu data of larvae treated with the dose combinations with those treated with the dose equivalents or the next higher combined dose (Figure 3). The effects of monotreatment versus combination treatment were first evaluated by a cut-off analysis based on a median reduction of 3 $\log_{10}$ cfu/mL at 72 h p.i. compared with PBS treatment to analyse bactericidal effects (Figure 3). Further, a statistical analysis was performed to check for significant differences between the corresponding treatment groups (Table 2).

Table 2. Statistical analysis of the larval survival rates, haemolymph bacterial load and larval PSIs after infection with *E. faecalis* isolate bk1653 followed by one dose of antibiotics

		Significance testing							
		dose equivalents				next higher doses			
Antibiotic combination	Dose combination	dose equivalents	survival rates	bacterial load (cfu/mL)	PSI	next higher doses	survival rates	bacterial load (cfu/mL)	PSI
AMP + CPT	1× MIC_{AMP} + 1× MIC_{CPT}	2× MIC_{AMP}	NS	NS	NS	4× MIC_{AMP}	*	NS	NS
		2× MIC_{CPT}	NS	NS	NS	4× MIC_{CPT}	NS	NS	NS
	2× MIC_{AMP} + 2× MIC_{CPT}	4× MIC_{AMP}	NS	NS	NS	8× MIC_{AMP}	NS	NS	NS
		4× MIC_{CPT}	NS	NS	NS	8× MIC_{CPT}	NS	NS	NS
AMP + CRO	1× MIC_{AMP} + 1× MIC_{CRO}	2× MIC_{AMP}	*	**	NS	4× MIC_{AMP}	NS	NS	NS
		2× MIC_{CRO}	**	NS	NS	4× MIC_{CRO}	***	NS	NS
	2× MIC_{AMP} + 2× MIC_{CRO}	4× MIC_{AMP}	*	NS	NS	8× MIC_{AMP}	NS	NS	NS
		4× MIC_{CRO}	***	NS	NS	8× MIC_{CRO}	***	NS	NS
AMP + GEN	1× MIC_{AMP} + 1× MIC_{GEN}	2× MIC_{AMP}	**	NS	*	4× MIC_{AMP}	***	NS	*
		2× MIC_{GEN}	**	NS	NS	4× MIC_{GEN}	*	NS	NS
	2× MIC_{AMP} + 2× MIC_{GEN}	4× MIC_{AMP}	NS	NS	NS	8× MIC_{AMP}	NS	NS	NS
		4× MIC_{GEN}	NS	NS	NS	8× MIC_{GEN}	NS	NS	NS

Combination treatments were compared with the dose equivalents and the next highest effective concentration. Survival rates of the respective treatment groups were compared by the log-rank (Mantel–Cox) test. Bacterial load and PSIs were analysed by the Kruskal–Wallis test followed by Dunn's multiple comparison test. For the PSI, analysis was performed at each timepoint (24, 48 and 72 h p.i.); only the results for 24 h p.i. are shown as no significant differences between the treatment groups could be observed at the other two timepoints.
***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS, not significant.

As a one-time monotreatment, all β-lactams at all concentrations tested failed to reduce the larval bacteria burden by at least 3 $\log_{10}$ cfu/mL, indicating bacteriostatic effects (Figure 3a). Ampicillin and ceftriaxone exhibited synergism by reducing the bacterial load by on average 3 $\log_{10}$ cfu/mL (1× MIC_{AMP} + 1× MIC_{CRO}) and 4 $\log_{10}$ cfu/mL (2× MIC_{AMP} + 2× MIC_{CRO}) (Figure 3b). However, due to the high variance in cfu/mL values, the significance testing of the ampicillin/ceftriaxone combination was not conclusive, except for 1× MIC_{AMP} + 1× MIC_{CRO} and the dose equivalent of 2× MIC_{AMP} indicating synergism (Figure 3a and b; Table 2). Ampicillin/ceftaroline showed only additive effects, with the same cfu/mL reduction reached as that reached with the dose equivalents of the individual agents (Figure 3a and b). Monotreatment with gentamicin at 8× MIC_{GEN} (512 mg/L) resulted in the highest observed median reduction by 6 $\log_{10}$ cfu/mL. However, this was not achieved with a lower dose combined with ampicillin, indicating no synergistic interaction between ampicillin and gentamicin.

Efficacy of single administration of the antibiotic combinations on the reduction in the PSIs

As a last parameter for determination of synergistic effects, a PSI was created by assessment of the degree of melanization and impairment of movement of the larvae (Figure 1). Mean PSIs were evaluated based on significant differences between PBS mock-treated larvae and the respective treatment group at different timepoints after infection (Figure 3c and d). Ampicillin and ceftaroline showed a significantly lower PSI compared with PBS only after 24 h p.i. and at the highest concentration tested (8× MIC). For the other concentrations, a visible but not significant trend to reduce the PSI in a concentration-dependent manner was observed. In contrast, ceftriaxone treatment led to a dose-dependent increase in PSI. For gentamicin, no significant difference in PSI was observed at any timepoint. For the antibiotic combinations, the highest and most significant reduction in the PSI compared with PBS was observed for ampicillin/ceftriaxone with both dose combinations. A significant reduction was also observed for 2× MIC_{AMP} combined with either 2× MIC_{CPT} or 2× MIC_{GEN} at 24 h p.i. Comparison with the dose equivalences, however, showed no significant differences (Table 2).

Pharmacokinetics of the antibiotics in G. mellonella

To correlate the efficiency of the antibiotics with their metabolism in the larvae and to determine the dosing interval for the multiple antibiotic treatments, the antibiotic clearance rates in the haemolymph were assessed. The antibiotic concentration–time profiles best fitted to sloping exponential curves with mean $R^2 = 0.84$ (Figure S4). The half-life of all the antibiotics ranged from 1 to 2 h (Table 3). Ceftriaxone showed the shortest half-life of 60 min, while ampicillin was degraded only half as fast, with a half-life of 126 min. Elimination rates were higher in the larvae than in humans, except for ampicillin (Table 3).

Efficiency of multiple dosing of the antibiotic combinations on larval survival rates and bacterial burden

To approximate the antibiotic dosing scheme for human conditions, multiple doses (at the same concentrations as those used

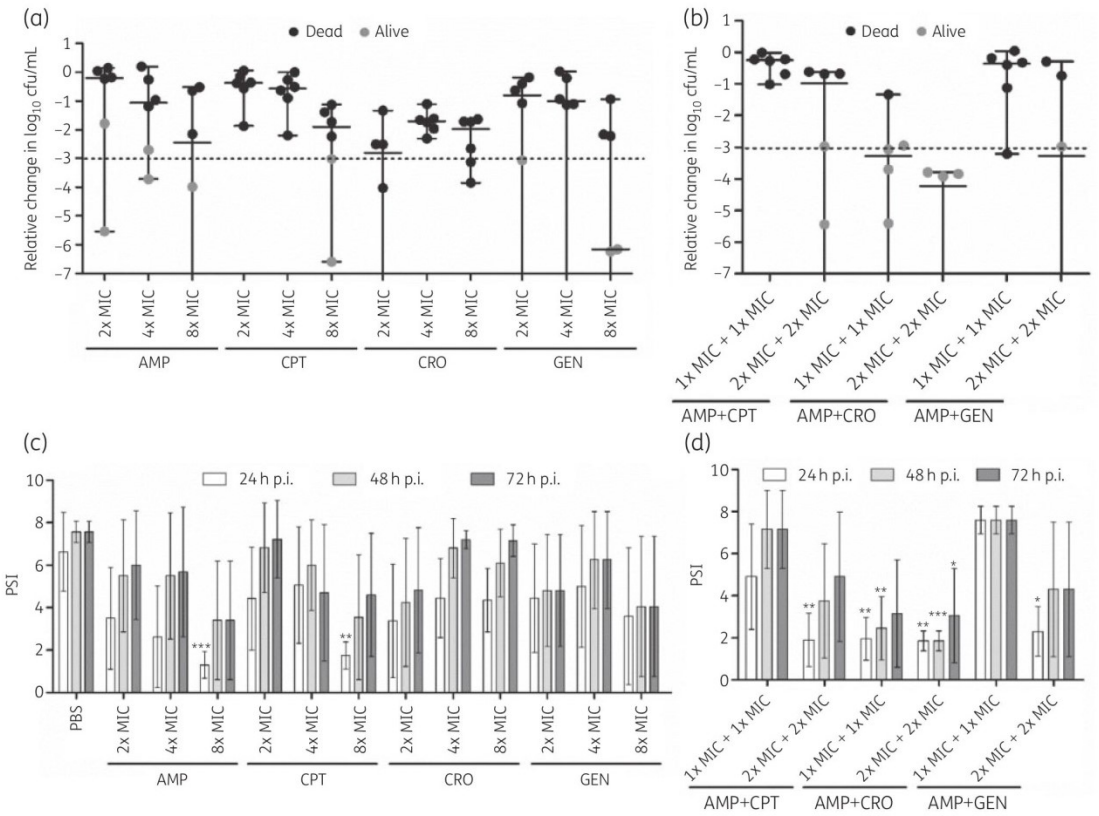

Figure 3. Haemolymph cfu burden and PSIs of *G. mellonella* after infection with *E. faecalis* isolate bk1653 followed by antibiotic treatment (dosing once). (a, b) cfu/mL values are shown for the single treatment groups in relation to the mean cfu/mL value for the PBS mock treatment (5.482×10^9), indicating the reduction in $\log_{10}$ cfu/mL due to treatment. Statistical analysis was performed by the Kruskal–Wallis test followed by Dunn's *post hoc* test (Table 2). Each experiment was performed twice with three larvae per treatment group ($n=6$). Data are depicted as column dotplots showing cfu values originating from alive (grey circles) and dead (black circles) larvae, with the overall median plus range. The horizontal dashed line represents the reduction threshold of 3 $\log_{10}$ cfu/mL compared with the PBS treatment. For evaluation, the median instead of the mean was used to correct for outliers due to the collection of the haemolymph from both dead and live larvae. (c, d) The mean PSIs are shown of the single (c) and combination (d) treatments of larvae. PSIs were assembled by addition of the melanization and activity indices of each larva at the respective time-point (Figure 1). Statistical analysis was performed by the Kruskal–Wallis test followed by Dunn's *post hoc* test, comparing the PSI of each treatment group with the PSI of the PBS mock treatment. ***$P<0.001$; **$P<0.01$; *$P<0.05$. The *in vitro* MICs of bk1653 were as follows: $MIC_{AMP}=1$ mg/L, $MIC_{CPT}=1$ mg/L, $MIC_{CRO}=16$ mg/L and $MIC_{GEN}=64$ mg/L. The results are based on $n=10$ (two experiments with five larvae each) per treatment group. The means ± SD are shown.

for one-time dosing) of the β-lactams were administered to the larvae to achieve a time-dependent bacterial killing effect. The dosing interval of a total of three doses every 2 h (1, 3 and 5 h p.i.) was based on the half-life of ampicillin (126 min) (Table 3), as ampicillin was the common component of all the antibiotic combinations. Gentamicin, exhibiting concentration-dependent killing, was administered once, both in monotreatment and combination treatment, to avoid the accumulation of toxic doses.

Compared with single dosing, larval survival was significantly enhanced for all monotreatments with β-lactams at all concentrations, especially ceftriaxone, which exhibited 60% survival after 72 h p.i. under triple 8× MIC_{CRO} dosing compared with 0% survival under one-time treatment (Figure 2c; Figure 4a–c). Similarly, increased survival rates with triple administration were observed for the antibiotic combinations; however, statistical analysis showed no clear differentiation between synergism and additivity (Figure 4d; Table S1). Three doses of β-lactam treatment led to a higher cfu/mL reduction than single treatment, with ampicillin (4× MIC_{AMP} and 8× MIC_{AMP}) and ceftriaxone (2× MIC_{CRO} and 8× MIC_{CRO}) reaching and exceeding, respectively, the reduction threshold of 3 $\log_{10}$ cfu/mL (Figure 4e). The same effect was observed for all triple-dosed combination treatments, especially at

Table 3. Pharmacokinetic parameters of the antibiotics in *G. mellonella*

Parameter	Ampicillin	Ceftaroline	Ceftriaxone	Gentamicin
Larval elimination rate (k_{el}) (min^{-1})	0.005499	0.008816	0.01153	0.01077
Larval elimination $t_{1/2}$ (min)	126	79	60	64
Human serum $t_{1/2}$ (min)	91	150	390	140

The concentrations of ampicillin, ceftaroline, ceftriaxone and gentamicin in the larval haemolymph were determined by agar diffusion assays (Figure S4). Pharmacokinetic analysis of antibiotic clearance in the larvae was performed using a one-phase decay model to determine the rate constant (k_{el}) and the $t_{1/2}$. Human serum $t_{1/2}$ values were taken from the literature and corresponded to single doses of 500 mg IV of ceftaroline fosamil and ceftriaxone, 1 g IV of ampicillin and 80 mg IV of gentamicin.[29–32]

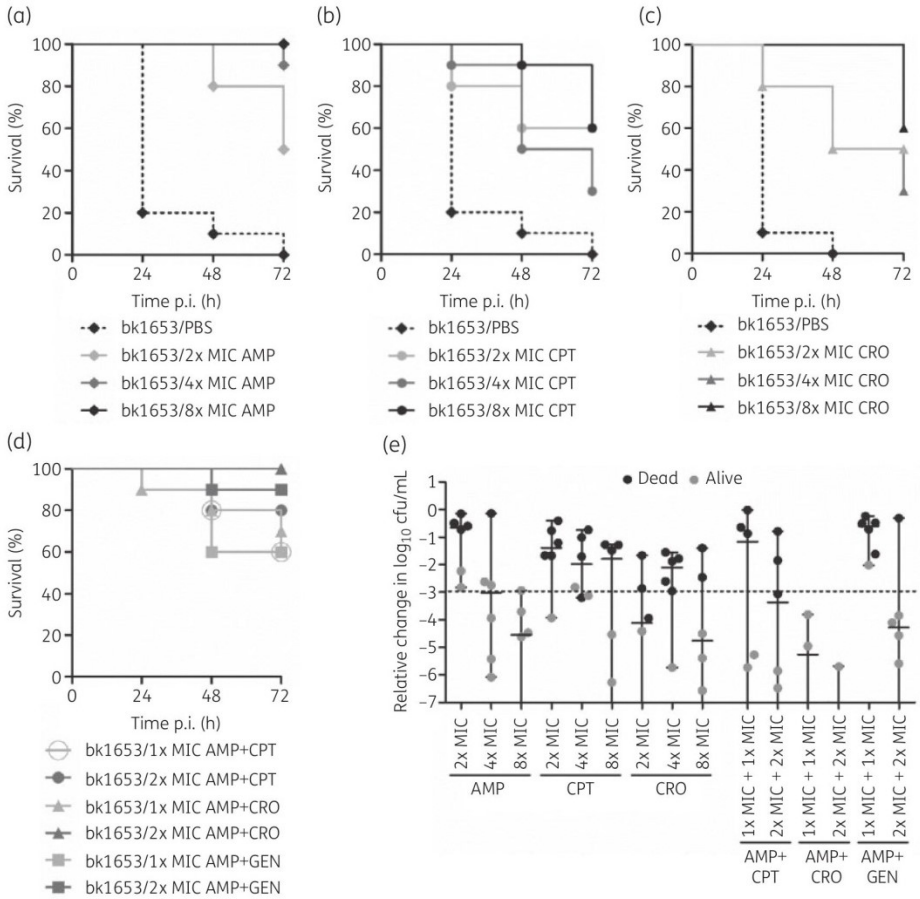

Figure 4. Survival curves and cfu haemolymph burden after three doses of antibiotics. Larvae were infected with *E. faecalis* isolate bk1653 followed by three β-lactam doses (1, 3 and 5 h p.i.), in both monotreatment and combined treatment. Gentamicin was administered only once (1 h p.i.) in the combination treatment. (a–d) The survival rates of two pooled experiments are shown ($n = 10$). Statistical analysis was performed by log-rank tests (Table S1). (e) The cfu/mL numbers of the single treatment groups are shown in relation to the mean cfu/mL value of PBS mock-treated larvae (4.815×10^9 cfu/mL). Data are depicted as column dotplots showing cfu values originating from alive (grey circles) and dead (black circles) larvae, with the overall median plus range ($n = 6$). The horizontal dashed line represents the reduction threshold of 3 $\log_{10}$ cfu/mL compared with the PBS treatment. Statistical analysis can be found in Table S1. The *in vitro* MICs of bk1653 were as follows: $MIC_{AMP} = 1$ mg/L, $MIC_{CPT} = 1$ mg/L, $MIC_{CRO} = 16$ mg/L and $MIC_{GEN} = 64$ mg/L.

the higher dose of 2× MIC + 2× MIC, with ampicillin/ceftriaxone leading to the highest observed mean reduction of 6 $\log_{10}$ cfu/mL (Figure 4e).

To analyse whether the synergistic effect between ampicillin and ceftriaxone was strain dependent, two additional *E. faecalis* isolates (Table 1) were tested for this combination in the larvae and compared with ampicillin/gentamicin. For isolate bk9367, analysis of both the survival curves and cfu/mL reduction showed additive effects for both antibiotic combinations, with the high efficiency of multiple ampicillin treatments compensating for the poor efficacy of treatment with ceftriaxone or gentamicin (Figure S5; Table S2). For isolate va245, both parameters indicated additive effects for ampicillin/ceftriaxone, whereas the survival curve analysis of ampicillin/gentamicin indicated even subadditive effects (Figure S6; Table S3).

Discussion

Three clinical *E. faecalis* isolates were analysed *in vivo* in a larval *G. mellonella* infection model for synergistic effects between ampicillin/gentamicin, ampicillin/ceftriaxone and ampicillin/ceftaroline. Our study suggests a scheme for the differentiation between additive and synergistic effects in larvae based on the three parameters of larval survival, bacterial load and change in melanization plus movement. This is the first study to apply a multiple antibiotic dosing scheme based on the half-lives of one of the tested antibiotics, namely ampicillin. According to these parameters, the antibiotic combinations after single-dose treatment showed a synergistic interaction between ampicillin and ceftriaxone against *E. faecalis* in the larvae, while the other two combinations showed no interaction of the individual compounds. The same results were obtained after adapting the dosing scheme to the half-life of ampicillin by three doses of antibiotic combination treatment. The synergistic effect between ampicillin and ceftriaxone seemed to be strain dependent, as the combination treatment demonstrated no significantly increased protection of the larvae after infection compared with monotherapy for two additional isolates.

These *in vivo* synergism results were only partially in accordance with those obtained *in vitro*. While ampicillin/gentamicin displayed mostly additive effects both *in vitro* and in the larvae, both ampicillin/cephalosporin combinations showed similar synergistic effects in several *in vitro* studies.[5,21,22] To exclude the possibility that the lack of synergism between ampicillin and ceftaroline in the larvae was due to compromised bioavailability of ceftaroline, we also analysed the effectiveness of the clinically applied prodrug ceftaroline fosamil (data not shown). No difference was noted between the pure substance and the prodrug. Furthermore, determination of ceftaroline pharmacokinetics suggested that sufficient levels of active ceftaroline were present in the larvae. The discrepancies between our *in vitro* and *in vivo* data might be explained by the different endpoints measured by the two methods; *in vitro* chequerboard assays evaluate growth inhibition, while the larval model assesses bacterial killing. Importantly, bacterial killing in the larvae was a result of both the antibiotics and the larval immune system.

The *in vitro* chequerboard analysis generally assigned more synergistic interactions to the antibiotic combinations, while our larval parameter analysis identified mainly additive effects. Of the three parameters, the haemolymph 3 $\log_{10}$ cfu/mL cut-off analysis allowed the most efficient grading of the effect size of the respective treatments. Synergistic effects were more obvious with single-dose antibiotic treatment after infection than with triple dosing, owing to the already high efficiency of the triple-dosed single β-lactams. Evaluation of the PSI was not sufficient to significantly differentiate between additive and synergistic effects, probably due to the short discriminative range of this PSI system. The applied PSI was created based on the observation of the melanization process in our experimental setup and might vary depending on the bacterial species or antibiotics used (e.g. ceftriaxone treatment led to strong pigmentation of the aorta line of infected larvae). Nevertheless, the PSI yielded results comparable to the survival rates and cfu/mL for the combination treatments when compared with PBS mock treatment, indicating that the PSI might be appropriate as an additional, but not primary, marker for the confirmation of synergistic effects. Moreover, the statistical power could be further enhanced by increasing the group size of larvae.

Pharmacokinetic analysis of the antibiotics revealed that the larval elimination rates did not match those of humans—a result that was consistent with other studies showing that some half-lives were comparable while others varied greatly between these two species.[20,23,24] The mechanisms by which drugs are eliminated in *G. mellonella* remain unknown. The larvae do not possess a liver or kidneys, but there are some structurally and functionally similar tissues, e.g. the Malpighian tubule system and the fat body.[25,26] The adaption of the dosing interval to the determined half-life of ampicillin led to an increased efficiency of the mono and combined antibiotic treatments. Although single-dose ceftriaxone treatment led to 100% larval death at 72 h p.i., it showed the highest cfu/mL reduction compared with the other β-lactam treatments. A possible toxic effect of ceftriaxone had been excluded in preliminary experiments, indicating that the bactericidal activity might cause hyperactivation of the larval immune system similar to sepsis. After triple dosing, ceftriaxone survival curves adjusted to those of ceftaroline, indicating that multiple doses of ceftriaxone are favourable.

Currently, there is insufficient evidence regarding whether *in vitro* and *in vivo* synergy testing results correlate with patient outcome.[27] While, in a retrospective study, ampicillin/ceftriaxone was suggested to be non-inferior to the standard therapy of ampicillin/gentamicin for *E. faecalis* IE,[4] larval results suggest a superior action of ampicillin/ceftriaxone for at least some strains. However, the additive effects between ampicillin/ceftriaxone for the other strains as well as for ampicillin/gentamicin raise the question of whether antibiotic combination therapy, with its high side-effect profile (e.g. gentamicin-related nephrotoxicity, cephalosporin-associated VRE colonization),[2] is superior to monotherapy.

G. mellonella larvae enable fast, low-cost and reproducible analysis of the synergistic effects of antimicrobials in an *in vivo* setting. Since synergism is not only determined by the mechanistic interaction of two drugs but depends on the dose of each drug in combination, the analysis of different antibiotic concentration combinations is useful. Further studies correlating larval antibiotic efficiency testing with clinical trials are needed to investigate whether the larval model is suitable for predicting tailored antibiotic therapies for *E. faecalis* IE.

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Transparency declarations

Pfizer supplied the ceftaroline powder but had no involvement in either the experimental design, the collection, analysis and interpretation of the data, or the decision to present these results. The authors have none to declare.

Supplementary data

Table S1 to S3 and Figures S1 to S6 are available as Supplementary data at *JAC* Online.

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Supplementary data

Figures

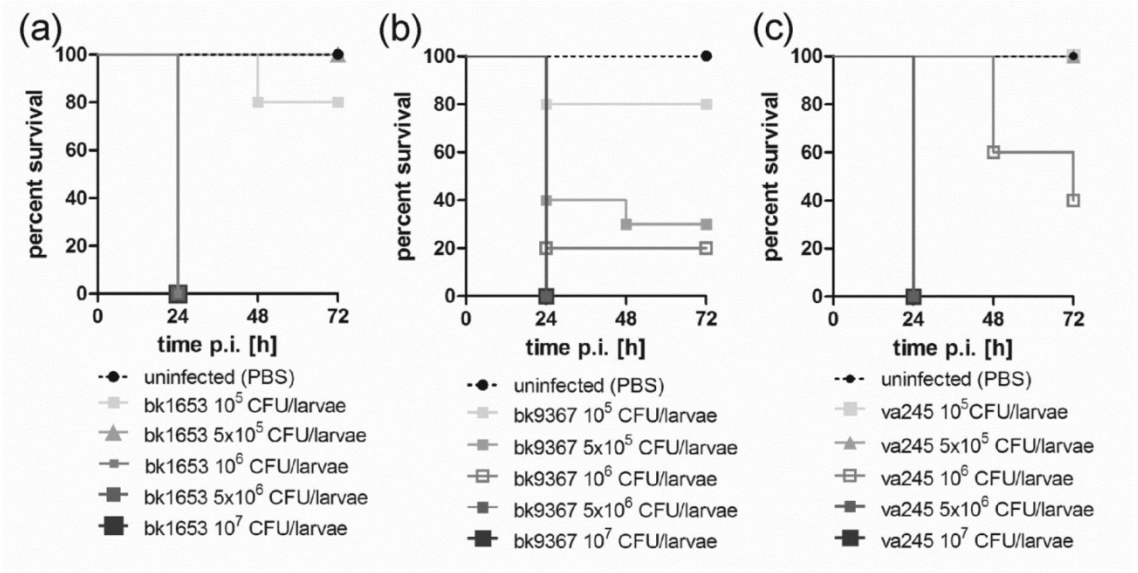

Figure S1: Bacterial titration curves of the *E. faecalis* isolates in the larvae. Larvae were infected via the proleg with 10-µL bacterial solutions with different cfu amounts of *E. faecalis* isolate bk1653 (a), bk9367 (b) or va245 (c). Cell density was determined by optical density measurement, and selected 10-fold dilutions of the inoculum suspensions were plated on TH agar to control for inoculum size. Each experiment was performed twice with 5 larvae per treatment group (n = 10), including PBS mock-infected larvae.

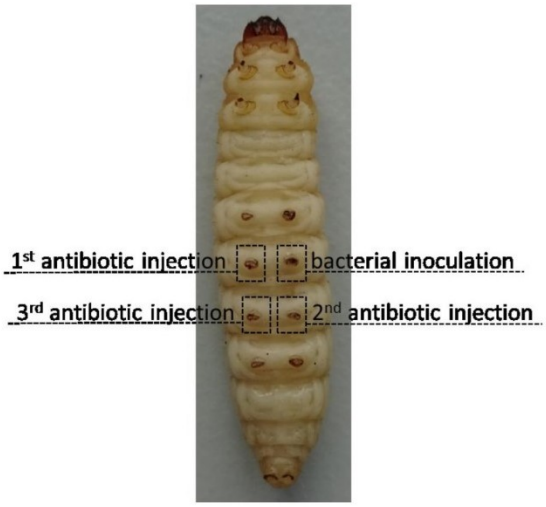

Figure S2: Different sites of injection of *G. mellonella* larvae used in this study. Injection was conducted in one of the depicted prolegs via a Hamilton syringe.

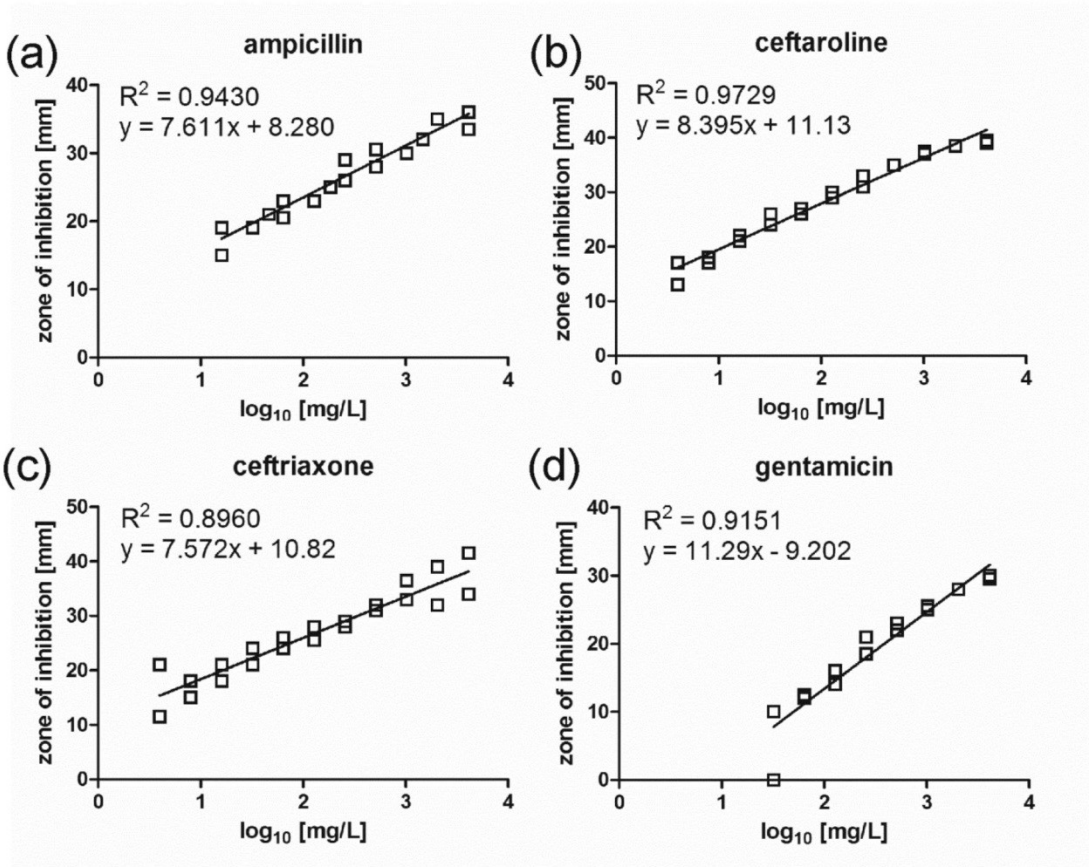

Figure S3: Antibiotic concentration standard curves for determination of pharmacokinetic parameters. Antibiotic standard curves showing the radius of the zone of inhibition (mm) versus known antibiotic concentrations in naïve *G. mellonella* haemolymph. Antibiotic-haemolymph solutions were pipetted on agar diffusion discs after inoculation of agar plates with *E. faecalis* ATCC 29212 (a) or *E. coli* ATCC 25922 (b-d) as an indicator organism. Linear regression analysis was performed and shown are the linear equation and coefficient of determination (R^2) for each antibiotic. Experiments were performed in duplicate.

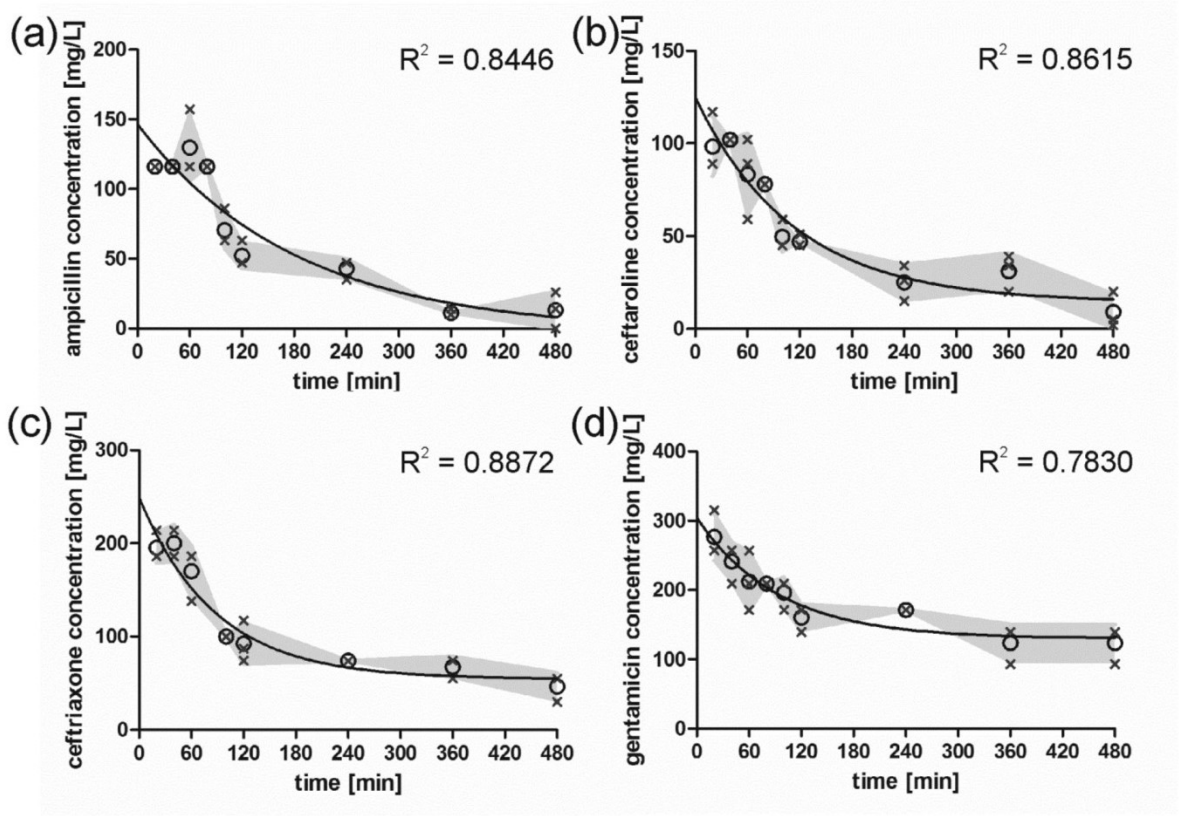

Figure S4: Concentration-time profiles of the antibiotics in *G. mellonella*. The concentrations of ampicillin (A), ceftaroline (B), ceftriaxone (C) and gentamicin (D) in the larval haemolymph were determined at intervals up to 8 h using an agar diffusion assay. Graphs show the mean value (circle) and replicates (cross) of each time point. The standard deviation is denoted by the shaded area.

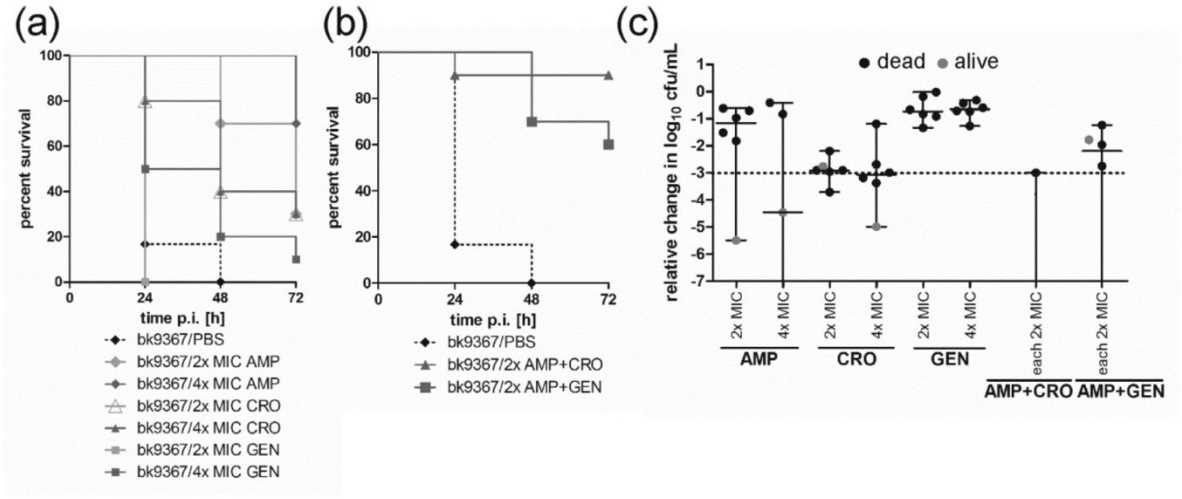

Figure S5: Survival curves and cfu haemolymph burden of *E. faecalis* isolate bk9367 after 3x dosing of antibiotics. Larvae were infected with *E. faecalis* isolate bk9367 followed by selected concentrations of three β-lactam doses (1 h, 3 h, 5 h p.i.), in both mono-treatment and combined treatment. Gentamicin was administered only once (1 h p.i.) in the combination treatment. A, B) Shown are the survival rates of two pooled experiments (n = 10). Statistical analysis was performed by log-rank tests (Table S2). C) cfu/mL values of the single treatment groups are shown in relation to the mean cfu/mL value of PBS mock-treated larvae (= 1.056 x 10^{10} cfu/mL). Data are depicted as column dotplots showing cfu values originating from alive (grey circles) and dead larvae (black circles), with the overall median plus range (n = 6). The horizontal dashed line represents the reduction threshold of 3 $\log_{10}$ cfu/mL compared to the PBS treatment. The statistical analysis can be found in Table S2. AMP = ampicillin, CRO = ceftriaxone, GEN = gentamicin. The *in vitro* MICs of bk9367 were as follows: MIC_{AMP} = 2 mg/L, MIC_{CRO} = 16 mg/L, MIC_{GEN} = 32 mg/L.

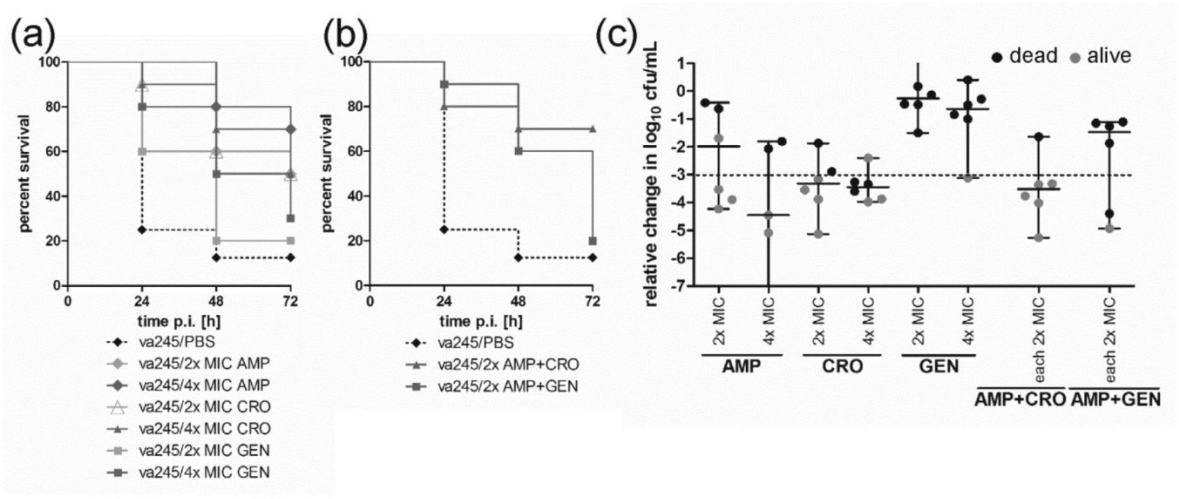

Figure S6: Survival curves and cfu haemolymph burden of *E. faecalis* isolate va245 after 3x dosing of antibiotics. Larvae were infected with *E. faecalis* isolate va245 followed by selected concentrations of three β-lactam doses (1 h, 3 h, 5 h p.i.), in both mono-treatment and combined treatment. Gentamicin was administered only once (1 h p.i.) in the combination treatment. A, B) Shown are the survival rates of two pooled experiments (n = 10). Statistical analysis was performed by log-rank tests (Table S3). C) cfu/mL values of the single treatment groups are shown in relation to the mean cfu/mL value of PBS mock-treated larvae (= 3.647 x 10^8 cfu/mL). Data are depicted as column dotplots showing cfu values originating from alive (grey circles) and dead larvae (black circles), with the overall median plus range (n = 6). The horizontal dashed line represents the reduction threshold of 3 $\log_{10}$ cfu/mL compared to the PBS treatment. Statistical analysis can be found in Table S3. AMP = ampicillin, CRO = ceftriaxone, GEN = gentamicin. The *in vitro* MICs of va245 were as follows: MIC_{AMP} = 0.5 mg/L, MIC_{CRO} = 2 mg/L, MIC_{GEN} = 16 mg/L.

Tables

Table S1: Statistical analysis of the larval survival rates and haemolymph bacterial load after infection with *E. faecalis* isolate bk1653 followed by 3x dosing of antibiotics (Figure 4). Combination treatments were compared to the dose equivalents and the next highest effective concentration. Survival rates of the respective treatments groups were compared by the log-rank (Mantel-Cox) test. Bacterial load was analysed by the Kruskal-Wallis test followed by Dunn's multiple comparison test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. ns = not significant. AMP = ampicillin, CPT = ceftaroline, CRO = ceftriaxone, GEN = gentamicin.

		significance testing dose equivalents			significance testing next higher doses		
antibiotic combination	**dose combination**	**dose equivalents**	**survival rates**	**bacterial load (cfu/mL)**	**next higher doses**	**survival rates**	**bacterial load (cfu/mL)**
AMP + CPT	1x MIC_{AMP} + 1x MIC_{CPT}	2x MIC_{AMP}	ns	ns	4x MIC_{AMP}	ns	ns
		2x MIC_{CPT}	ns	ns	4x MIC_{CPT}	ns	ns
	2x MIC_{AMP} + 2x MIC_{CPT}	4x MIC_{AMP}	ns	ns	8x MIC_{AMP}	ns	ns
		4x MIC_{CPT}	ns	ns	8x MIC_{CPT}	ns	ns
AMP + CRO	1x MIC_{AMP} + 1x MIC_{CRO}	2x MIC_{AMP}	ns	***	4x MIC_{AMP}	ns	ns
		2x MIC_{CRO}	ns	ns	4x MIC_{CRO}	ns	ns
	2x MIC_{AMP} + 2x MIC_{CRO}	4x MIC_{AMP}	ns	ns	8x MIC_{AMP}	ns	ns
		4x MIC_{CRO}	*	ns	8x MIC_{CRO}	ns	ns
AMP + GEN	1x MIC_{AMP} + 1x MIC_{GEN}	2x MIC_{AMP}	ns	ns	4x MIC_{AMP}	ns	ns
		2x MIC_{GEN}	ns	ns	4x MIC_{GEN}	ns	ns
	2x MIC_{AMP} + 2x MIC_{GEN}	4x MIC_{AMP}	ns	ns	8x MIC_{AMP}	ns	ns
		4x MIC_{GEN}	*	ns	8x MIC_{GEN}	ns	ns

Table S2: Statistical analysis of the larval survival rates and haemolymph bacterial load after infection with *E. faecalis* isolate bk9367 followed by 3x dosing of antibiotics (Figure S5). Combination treatments were compared to the dose equivalents and the next highest effective concentration. Survival rates of the respective treatments groups were compared by the log-rank (Mantel-Cox) test. Bacterial load was analysed by the Kruskal-Wallis test followed by Dunn's multiple comparison test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. ns = not significant. AMP = ampicillin, CPT = ceftaroline, CRO = ceftriaxone, GEN = gentamicin.

		significance testing dose equivalents		
antibiotic combination	**dose combination**	**dose equivalents**	**survival rates**	**bacterial load (cfu/mL)**
AMP + CRO	2x MIC_{AMP} + 2x MIC_{CRO}	4x MIC_{AMP}	ns	ns
		4x MIC_{CRO}	*	ns
AMP + GEN	2x MIC_{AMP} + 2x MIC_{GEN}	4x MIC_{AMP}	ns	ns
		4x MIC_{GEN}	**	ns

Table S3: Statistical analysis of the larval survival rates and haemolymph bacterial load after infection with *E. faecalis* isolate va245 followed by 3x dosing of antibiotics (Figure S6). Combination treatments were compared to the dose equivalents and the next highest effective concentration. Survival rates of the respective treatments groups were compared by the log-rank (Mantel-Cox) test. Bacterial load was analysed by the Kruskal-Wallis test followed by Dunn's multiple comparison test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. ns = not significant. AMP = ampicillin, CPT = ceftaroline, CRO = ceftriaxone, GEN = gentamicin.

		significance testing dose equivalents		
antibiotic combination	**dose combination**	**dose equivalents**	**survival rates**	**bacterial load (cfu/mL)**
AMP + CRO	2x MIC_{AMP} + 2x MIC_{CRO}	4x MIC_{AMP}	ns	ns
		4x MIC_{CRO}	ns	ns
AMP + GEN	2x MIC_{AMP} + 2x MIC_{GEN}	4x MIC_{AMP}	*	ns
		4x MIC_{GEN}	ns	ns

3.4 Manuscript in preparation:

A novel high-throughput method for biofilm quantification and measurement of antibiotic-induced tolerance in biofilms

Authors: **Lara Thieme**, Anita Hartung, Kristina Tramm, Julia Graf, Riccardo Spott, Oliwia Makarewicz, Mathias W. Pletz

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Abstract accepted for the article collection “"Biofilm-Biomaterial Interactions: Understanding, Preventing, and Eradicating Attachment in Infection” in Frontiers of Microbiology (impact factor 4.076).

Lara Thieme and Anita Hartung contributed equally to this manuscript (shared first authorship).

In this manuscript, a novel method aiming for high-throughput anti-biofilm testing is presented. Since biofilm-related infections such as infective endocarditis require individual biofilm susceptibility testing as performed for planktonic bacteria, the establishment of high-throughput biofilm quantification methods is of high importance for clinical routine diagnostics. Therefore, we adapted the recently published Start-Growth-Time (SGT) method to anti-biofilm testing, which indirectly measures CFU numbers of up to 96 samples by a culture-based method. Mature biofilms are disrupted after treatment, diluted and regrown as monitored by OD_{600nm} measurement. The time delay of the growth curves of treated samples thereby correlates with the amount of biofilm viable cell reduction compared to the untreated control. However, this correlation was interrupted by the appearance of altered growth kinetics induced by selected novel antibiotic treatments (i.e. dalbavancin). Here, instead of accurately quantifying the number of viable cells, the novel SGT method measured antibiotic-induced tolerance.

Abstract

Assessment of anti-biofilm activities of antibiotics is currently hampered by the lack of standardized, high-throughput biofilm susceptibility methods. Colony forming unit (CFU) determination by agar plating is still regarded the gold standard for biofilm quantification despite being time- and resource-consuming. Here, we propose an adaption of the novel high-throughput Start-Growth-Time (SGT) method to biofilm analysis, which indirectly quantifies CFU/mL numbers by evaluating regrowth curves of detached biofilms. The lag-phases of these growth curves depend upon the number of viable cells in the treated biofilm and therefore correlate with CFU numbers.

To validate the novel SGT method, the effect of dalbavancin, rifampicin and gentamicin treatment against mature biofilms of the model organisms *Staphylococcus aureus* and *Enterococcus faecium* was evaluated by the SGT method, conventional agar plating and the resazurin metabolic assay. The methods applied for biofilm quantification accessed different features of the viability status of the cells, i.e. the cultivability (CFU agar plating), growth behaviour (SGT) and the metabolic activity (resazurin assay). Biofilm bactericidal concentration (BBC) values were calculated for comparison of the methods corresponding to a 3 $\log_{10}$ CFU/mL biofilm reduction.

While the SGT data correlated well with the results obtained by the resazurin assay, they only partially correlated with the results obtained by conventional agar plating. Interestingly, instead of being parallel and lagged to the untreated growth control, dalbavancin treatment-derived growth curves showed a significantly slower increase with reduced cell doubling times and without distinct growth phases in a concentration-dependent manner. The altered growth kinetics of dalbavancin-treated biofilms were matched by reduced metabolic rates as determined by resazurin staining. However, no change in CFU numbers was observed by conventional agar plating. This lead to a mismatch of agar plating-derived BBCs and SGT-or resazurin-derived BBCs. For gentamicin treatment, all three methods showed comparable BBC values, while for rifampicin BBC values were more divergent but mostly within the method-related one-well dilution error.

In conclusion, the principle of lagged growth curves being proportional to the number of cells in the biofilm was only true for some antibiotic treatments, making the novel SGT method only suitable to a limited extent for biofilm quantification. Interestingly, while

dalbavancin was able to reduce the redox potential and change the growth behavior of the biofilm-embedded cells, these cells were still viable and cultivable. We hypothesized that the biofilms in order to survive increasing dalbavancin concentrations were switching to an antibiotic tolerant state by transiently slowing down essential bacterial processes. Once the antibiotic had been removed and the biofilms were re-transferred to the planktonic phase, the cells did recover, which was monitored by altered growth kinetics in the SGT method. Since this effect was only induced by specific antibiotic treatments, the SGT method might be well suited for detecting antibiotic-induced tolerance. Measurement of tolerance might influence clinical therapy guidelines since antibiotic-induced tolerance indicates the requirement of longer exposure times to the antibiotic, compared to higher concentrations necessary for treatment of antibiotic resistant cells. Overall, our data indicate that while several methods claim to accurately quantify the number of biofilm viable cells, the assessment of different features of the viability status of the cells, i.e. metabolic activity, growth behavior or cultivability, can lead to different quantification results. The method-of-choice should therefore be carefully adjusted to the specific research or clinical question.

Introduction

Microbial communities that are surrounded by a matrix of extracellular polymeric substance are commonly defined as biofilms (Hall-Stoodley et al. 2012). Bacterial biofilms represent the preferred life-form of microorganisms, following that they play a key role in many infectious diseases such as endocarditis, osteomyelitis, urinary tract infections and joint and soft tissue infections (Flemming et al. 2016). Increased antibiotic tolerance and/or resistance are one of the major hallmarks of biofilm-associated infections (BAI) (Stewart 2002). Since biofilm-embedded bacteria are usually genetically susceptible but phenotypically resistant, biofilm susceptibility is not predictable by the study of planktonic cells. So far biofilms are not addressed by microbiological routine diagnostics and treatment of BAI is guided by planktonic MIC testing, resulting in therapy failure and relapses. A multitude of biofilm susceptibility testing methods has been suggested, but none has so far reached a balance between the simplicity of a high-throughput (HTP) method and the complex representation of the *in vivo* biofilm situation (Azeredo et al. 2017, Coenye et al. 2018, Magana et al. 2018). Further, standardization of the existing methods, including consistent interpretation

of results and according recommendations, is lacking (Cruz et al. 2018, Thieme et al. 2019).

Colony forming unit (CFU) determination by agar plating is still regarded the gold standard among bacterial quantification methods, including biofilms (Azeredo et al. 2017) Since agar-plating is time- and resource-consuming, we aimed to indirectly depict CFU/mL numbers by a culture-based method. Therefore, we adapted the recently published Start-Growth-Time (SGT) method to anti-biofilm testing, which allows a rapid quantification of the absolute and relative number of live cells in a high throughput manner (Hazan et al. 2012). The principle is comparable to the methodology of quantitative PCR calculations. The biofilms after treatment are dissolved, diluted and regrown under continuous measurement of the optical density (OD) to obtain growth curves. The lag-phases of these growth curves are proportional to the number of cells in the initial biofilms, i.e. the more efficient the anti-biofilm treatment, the less CFU/mL, the longer the lag-phase. The SGT of each sample is defined as the time required to reach a defined OD threshold within the early log-phase of the culture (Hazan et al. 2012). The growth delay of the treated growth curves, i.e. the respective SGTs, can be correlated to the quantity of CFU/mL reduction in comparison to the untreated control by CFU-SGT-standard curves of the same untreated strain. This minimizes the standard agar-plating procedure to a limited number of plates while simultaneously allowing the indirect measurement of CFU reduction of up to 96 samples.

To compare the CFU reduction results obtained with the novel SGT method with those obtained by conventional agar plating and resazurin metabolic assay, we treated mature biofilms of the model organisms *Staphylococcus aureus* and *Enterococcus faecium* with serial concentrations of the antibiotics dalbavancin, rifampicin and gentamicin. Dalbavancin is a novel lipoglycopeptide with so far limited knowledge on biofilm eradication capability (Neudorfer et al. 2018), while the bactericidal antibiotics gentamicin and rifampicin were shown to exhibit anti-biofilm activity against Gram-positive biofilms (Coraça-Huber et al. 2012, Sandoe et al. 2006, Zimmerli and Sendi 2019). The methods applied for biofilm quantification accessed different features of the viability status of the cells, i.e. the cultivability (CFU agar plating), growth behaviour (SGT) and the metabolic activity (resazurin assay). Our data indicate that while all three methods are capable to

assess anti-biofilm effects, the method-of-choice needs to be carefully adjusted to the specific research or clinical question.

Methods

Bacterial strains and antibiotics

The clinical *S. aureus* strains (MSSA: bk4733 and va1642, MRSA: bk4002) and *E. faecium* strains (VSE: bk24498 and bk12713, VRE: bk17129) were obtained from the Institute of Medical Microbiology at Jena University Hospital, Germany. Test solutions of dalbavancin (Correvio GmbH, Bielefeld, Germany), rifampicin (Sigma Aldrich, St. Louis, USA) and gentamicin (TCI Europe, Zwijndrecht, Belgium) were prepared freshly for each experiment.

Biofilm formation and antibiotic treatment

For biofilm maturation, 200 µl of 0.5 McFarland bacterial cultures were incubated in 96-well microtitre plates for 48 h at 37 °C in a humidified chamber. *S. aureus* isolates were grown in Müller-Hinton (MH) broth and *E. faecium* isolates in Todd-Hewitt (TH) broth (both obtained from Sigma Aldrich, St. Louis, USA). For antibiotic treatment, the supernatants with planktonic cells were removed carefully, antibiotic solution with selected concentrations were prepared in the respective media and 200 µL per well were added to the biofilm. Pure media was used as growth control. Biofilms were incubated for additional 24 h at 37 °C. Then, supernatant was removed and biofilms were washed two times with 0.9 % NaCl before analysing the biofilm reduction with the different methods. Each experiment was done in triplicates. To compare the different quantification methods, the biofilm bactericidal concentration (BBC), which is the lowest concentration of an antibiotic reducing 99.9 % of biofilm-embedded bacteria (3 $\log_{10}$ reduction in CFU/mL) compared to the growth control,(Macia et al. 2014) was determined for each antibiotic and strain by each method.

CFU determination by agar plating

The washed biofilms were scraped off the wells and resuspended in fresh MH or TH broth. From each biofilm, a serial 10-fold dilution was made in media and 100 µl of selected dilutions were plated on MH or TH agar plates. After incubation of 18 h, colony forming

units (CFU_{AGAR}) were counted and bactericidal effects were calculated in relation to the untreated control biofilms.

Resazurin assay

Biofilm analysis by resazurin was adopted from van den Driessche et al. (Van den Driessche et al. 2014). 100 µl of the previously prepared 10^{-2} dilution were added to a new 96-well microtitre plate and mixed with 10 µl alamarBLUE cell viability reagent (Thermo Fisher Scientific, Dreieich, Germany). Fluorescence was measured every 10 min for 18 h with a microtitre plate reader (Infinite M200pro, Tecan, Switzerland). Measurements were done at 37 °C and with subsequently shaking for optimized growth conditions. For each isolate, a dilution series of a control biofilm was simultaneously measured to create a resazurin standard curve. The time to reach maximum fluorescence (t_{max}) was determined for each biofilm. The t_{max} and the CFU_{AGAR} of the tested dilution series were correlated by linear regression to set-up a standard curve and to determine the detection limit for each strain. From this standard curve, the CFU_{RESA} of the treated and untreated biofilms were calculated by the corresponding t_{max} and subtracted from each other (ΔCFU_{RESA}).

Start-Growth-Time method

Washed and resuspended biofilms were diluted 1:10 in fresh media and regrown in 96-well microtitre plates. The optical density was measured every 10 min at 600 nm for 18 h at 37 °C in a microtitre plate reader (Sunrise, Tecan, Switzerland). The SGT of each sample was defined as the time required to reach an OD_{600nm} threshold that was set at the start to midst of the logarithmic phase, depending on the resulting growth curves. For the relative comparison of treated and untreated samples, the absolute size of the OD_{600nm} threshold was not decisive but the unification for all samples. SGT values were normalized to the controls by the formula $\Delta SGT = SGT_{treated} - SGT_{control.}$ To assess the linearity between SGT and CFU_{AGAR} values and thereby the detection limit for each strain, a standard curve was performed on every run. Therefore, SGTs of a serial diluted control biofilm and, in parallel, CFU_{AGAR} counts were determined. The CFU_{SGT} reduction due to antibiotic treatment was calculated by the standard curve $\log_{10}$ CFU_{AGAR} (x-axis) versus SGT (y-axis), whereby the SGT time span correlating to 1 $\log_{10}$ CFU difference was given by the slope of the linear

regression. The resulting $\log_{10}$ CFU reduction was calculated by $\Delta\log_{10}$ CFU_{SGT} = ΔSGT / slope.

Results

To verify the new HTP method from Hazan et al. for biofilm quantification (Hazan et al. 2012), we recorded the growth curves of a dilution series of resuspended biofilms for each isolate. As on planktonic level time-lagged growth curves for biofilms could be observed in correlation to the CFU input (Figure 1). Comparable to a quantitative PCR, a specific OD threshold was defined within the midst of the exponential growth of the control biofilms. By this method, we received a linear correlation of SGT and CFU from 10^6 to 10^0 (Figure 1). The level of detection (LOD) reached up to 2 CFU/well for both species whereby the LOD and the detection range varied between experiments and isolates, especially for *E. faecium* (Supplement Material).

After calibration, we used the SGT method for analyzing the BBC of dalbavancin and gentamicin for *E. faecium* isolates (Figure 2). According to the SGT method, a CFU reduction was already achieved at low dalbavancin concentrations, with the BBC being reached at 128 mg/L (Figure 2A). By contrast, agar plating revealed only a CFU reduction of < 1 $\log_{10}$ CFU at the highest dalbavancin concentration tested, thereby not achieving the required 3 $\log_{10}$ CFU reduction for the BBC (Figure 2A).

On closer inspection, it was striking that the regrowth of the former dalbavancin-treated biofilms started at the same time like the controls but had a slower growth kinetic (Figure 2A, zoom). This changed growth behavior might be due to a reduced metabolism after antibiotic treatment. Therefore, we checked our results by resazurin assays as described by Driessche et al. (Van den Driessche et al. 2014). In parallel to the SGT method, a standard curve was performed within the experiment to calculate the CFU to the respective resazurin t_{max} value (Supplement material). The BBC determined by the SGT method (BBC_{SGT}) was consistent (± 1x BBC) with the BBC measured by the resazurin assay (BBC_{RESA}) for dalbavancin at 256 mg/L (Figure 2A, Table 1).

For gentamicin-treated *E. faecium* biofilms, the SGT method revealed no anti-biofilm effect for all tested concentrations and showed no change in growth behavior (Figure 2B). The CFU determination by agar plating and resazurin obtained the same results (Figure 2B, Table 1). For the other two *E. faecium* isolates, BBC results obtained by all three methods were in accordance as well (Table 1, Supplement material).

To test whether the changed growth behavior observed in the SGT method is strain- or antibiotic-dependent, we analyzed *S. aureus* biofilms by all three methods. Since gentamicin is ineffective against *S. aureus*, we used dalbavancin and rifampicin for testing. In contrast to *E. faecium*, dalbavancin-treated biofilms showed normal time-lagged growth curves or no growth at all (Figure 3A). By the SGT method, we calculated a 3 $\log_{10}$ CFU reduction already at 8 mg/L while with agar plating there was nearly no CFU reduction detectable for all concentrations (Figure 3A). However, the BBC_{RESA} was again in line with the BBC_{SGT} (Figure 3A, Table 1). Similar results were seen for rifampicin-treated *S. aureus* biofilms (Figure 3B, Table 1). The BBC_{SGT} and BBC_{RESA} were both reached at 4 mg/L of rifampicin, whereas no BBC could be determined by agar plating since none of the tested rifampicin concentrations obtained a 3 $\log_{10}$ CFU reduction (Figure 3B).

Discussion

In the presented study, a novel method termed SGT-method for HTP-analysis of anti-biofilm effects based on bacterial growth curves was suggested. To integrate the SGT-method into the pool of already established methods for quantification of biofilm-embedded cells, biofilm bactericidal effects of three different antibiotics against mature *S. aureus* and *E. faecium* biofilms were measured by resazurin staining, agar plating and the SGT method. All methods resulted in determination of CFU/mL values, either directly (agar plating) or indirectly via CFU/mL-calibrated standard curves (SGT, resazurin). While the SGT data correlated well with the results obtained by the resazurin assay, they only partially correlated with the results obtained by conventional agar plating. This lead to a partial mismatch between the SGT- or resazurin-derived BBCs (BBC_{SGT} and BBC_{RESA}), i.e. a 3 $\log_{10}$ CFU/mL reduction due to antibiotic treatment, and the current gold-standard, agar plating-derived BBCs (BBC_{AGAR}), questioning the utility of the novel SGT-method – and

of the well-established resazurin assay - for quantifying biofilm reducing effects.

Since for gentamicin-treated *E. faecium* biofilms both the BBC_{SGT} and BBC_{RESA} were in accordance with the BBC_{AGAR}, we arrived at the hypothesis that the SGT-method might only be well-suited for measuring the effect of bactericidal antibiotics, and not bacteriostatic ones. Our hypothesis was further supported by the fact that for the establishment of the original SGT-method for planktonic cells only bactericidal antibiotics had been used (Hazan et al. 2012). However, little to no consistency between $BBC_{SGT/RESA}$ and BBC_{AGAR} values of both rifampicin- and dalbavancin-treated biofilms – two further antibiotics classified as bactericidal (Nemeth et al. 2015) - could be observed in our study. Definitions of bactericidal/bacteriostatic antibiotics are usually based on the mode of action and the resulting bacterial killing efficiency, with bacteriostatic antibiotics arresting growth and bactericidal antibiotics achieving eradication. In microbiological *in vitro* experiments, this differentiation is reflected by a bacterial reduction threshold of ≥ 99.9 % being considered bactericidal. While the differentiation of bactericidal/bacteriostatic effects might apply for planktonic cells – although concerns have been raised that these two categories only exist under strict laboratory conditions and are inconsistent for a specific antibiotic against all bacteria (Pankey and Sabath 2004) – this categorization is not transferable to biofilm-embedded cells. Since biofilms resemble stationary-phase cultures with strongly reduced cell division rates, they are e.g. less susceptible to the on planktonic level bactericidal effects of cell-wall active agents such as dalbavancin. Therefore, while the SGT method might be well-suited for determining the efficiency of bactericidal but not bacteriostatic antibiotics on planktonic level, this is of little relevance for the quantification of biofilm bactericidal effects of antibiotics with still unknown biofilm activity, i.e. with biofilm inhibitory, bacteriostatic or biofilm eradicating, bactericidal effects.

Another possible explanation for the discrepancy between BBC_{SGT} and BBC_{AGAR} values might be due to the method-related comparison of two different time-points of bacterial growth. While the agar plating method assesses the number of CFUs in the stationary phase after 24 h of growth, the SGT method pictures the regrowth of the former biofilm-embedded cells over time, with the SGT values theoretically being calculated in the midst of the logarithmic phase. However, instead of being parallel and lagged to the untreated growth control as seen for gentamicin treatment, some antibiotic treatment-derived growth curves showed a significantly slower increase with reduced cell doubling times and without distinct

growth phases, as seen for dalbavancin-treated *E. faecium* biofilms. Here, the reduced growth kinetics of the cells led to higher SGT values since the OD_{600nm} threshold was reached later. As a consequence, the higher SGT values were falsely interpreted as lower number of cells leading to an overestimation of bactericidal efficiency by the SGT method. To analyse whether the recovery from antibiotic-induced stress is also monitored by a decreased CFU development on agar plates, we checked the number of CFUs formed on agar plates in 1 h intervals for a period of 14 h, with the hypothesis that the colonies of growth kinetic-altered samples appear later and might show an altered phenotype, e.g. formation of small colony variants, but finally result in the same amount of cells as the untreated control (data not shown). However, no difference in the time point of CFU appearance or shape, size and colour could be observed between treated and untreated samples, but changes in colony formation were generally hard to depict since both *E. faecium* and *S. aureus* form relatively small colonies. Nevertheless, the accordance of BBC_{SGT} and BBC_{RESA} values confirms that the high SGT values – and thereby putatively low CFU numbers - of some samples were an artefact due to decreased metabolic rates caused by antibiotic treatment. Resazurin is a stable redox indicator which's highly fluorescent reduction product resorufin can be easily and rapidly measured after 30-120 min of cell contact and is proportional to the number of metabolically active cells (Azeredo et al. 2017). Since the linear range between resorufin and CFU numbers is restricted to 10^6 - 10^8 CFU/biofilm, the conventional resazurin-based viability assay fails to depict a 3 $\log_{10}$ reduction required for BBC calculation (Sandberg et al. 2009). We therefore used an optimized method determining the time needed to reach the maximum fluorescence extending the linear range to 10^3 - 10^8 CFU/biofilm (Van den Driessche et al. 2014). While this new approach claims to accurately reflect CFU numbers as determined by agar plating, our results indicate that this was only true for some antibiotics. Since resazurin is being increasingly used to study microbial biofilms (Azeredo et al. 2017), researchers should be aware of a potential correlation bias and should validate effects by the conventional agar plating method.

As indicated by high SGT and low resazurin values, some antibiotics were able to slow down the bacterial metabolism, thereby reducing the redox potential of the cells and changing the growth behavior. However, these cells were still viable and cultivable as reflected by unchanged CFU_{AGAR} numbers. Bacteria use different strategies for survival

during exposure to antibiotics, namely resistance, tolerance and persistence (Brauner et al. 2016). Resistance describes the inherited ability of bacteria to grow, i.e. to proliferate, at high concentrations of an antibiotic irrespective of the duration of treatment due to molecular mutations. In contrast, tolerant cells survive high antibiotic concentrations by transiently slowing down essential bacterial processes at the cost of loss of cell proliferation. Once the transient trigger for tolerance is removed, cells do recover and growth can continue. While resistance and tolerance are attributes of the whole bacterial population, persistence is only attributable to a subpopulation (typically around 1 %) of clonal cells. Persistent cells can survive at high concentrations of antibiotics whereas the majority of the clonal bacterial population is rapidly killed (Harriott 2019). While antibiotic-resistant bacteria can form biofilms, the survival strategies characteristic for biofilms are antibiotic tolerance and persister cell formation (Stewart 2002). The altered growth kinetics observed in growth curves of dalbavancin-treated biofilms were therefore likely caused by the physiological rearrangements necessary to leave the tolerant state once the antibiotic had been removed and the biofilms were re-transferred to the planktonic phase. Persister cell formation was not directly measured by the SGT-method because no mixed growth patterns of persistent and non-persistent subpopulations were observed (Brauner et al. 2016). Since the changed growth behaviour was neither observed when the untreated biofilms were regrown nor for all antibiotics and concentrations, tolerance was only induced by specific antibiotic treatments. Importantly, the emergence of antibiotic-induced tolerance would have been missed if CFU determination would have only been performed by agar plating and not by the SGT or resazurin method. Measurement of tolerance might influence clinical therapy guidelines since antibiotic-induced tolerance indicates the requirement of longer exposure times to the antibiotic, compared to higher concentrations necessary for treatment of antibiotic resistant cells. Multiple treatments with dalbavancin might therefore lead to a significant reduction in CFU/mL values contrary to one-time treatment.

In conclusion, while the principle of lagged growth curves being proportional to the number of cells in the biofilms after antibiotic treatment was true for some antibiotics, the SGT method is rather not well suited for quantification of biofilm eradicating effects since it remains unclear for which classes of antibiotics CFU_{SGT} and CFU_{AGAR} values do match. However, the novel method might have value as a semi-quantitative method by detecting at which antibiotic concentration tolerance emerges without measuring the actual effect size,

i.e. how many log reduction in CFU/mL has been achieved. Here, a novel biofilm susceptibility parameter such as the biofilm effective concentration (BEC) could be established for evaluating the emergence of drug-induced tolerance in biofilms, but further experiments are necessary for validation. Overall, our data indicate that while several methods claim to accurately quantify the number of biofilm viable cells, the assessment of different features of the viability status of the cells, i.e. metabolic activity, growth behavior or cultivability, can lead to different quantification results. The method-of-choice should therefore be based on the specific research or clinical question.

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Transparency declarations

Correvio had no involvement in the experimental design, the collection, analysis and interpretation of the data, or the decision to present these results.

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Figures and Tables

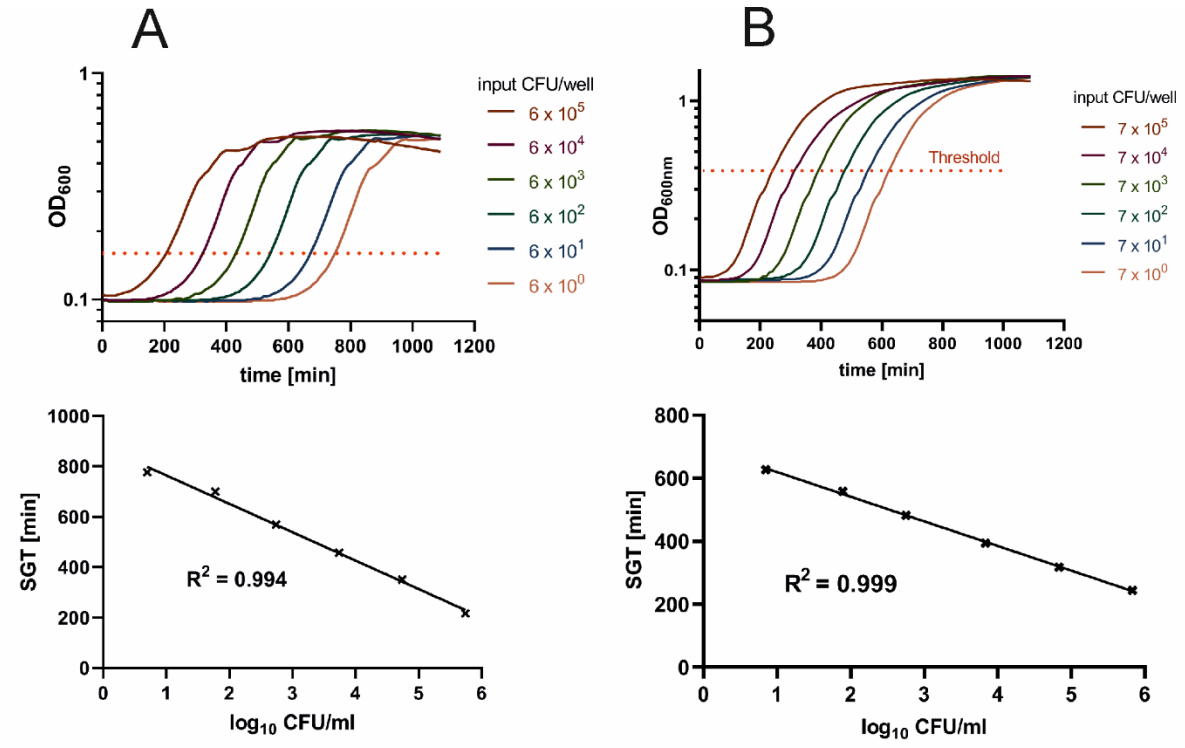

Figure 1: Standard curves determined by the SGT method and agar plating for exemplary isolates of *E. faecium* (column A) and *S. aureus* (column B) biofilms. The optical density at 600nm was recorded for 18 h for a dilution series of resuspended biofilms (48 h of growth). Simultaneously, CFU numbers were determined by agar plating.

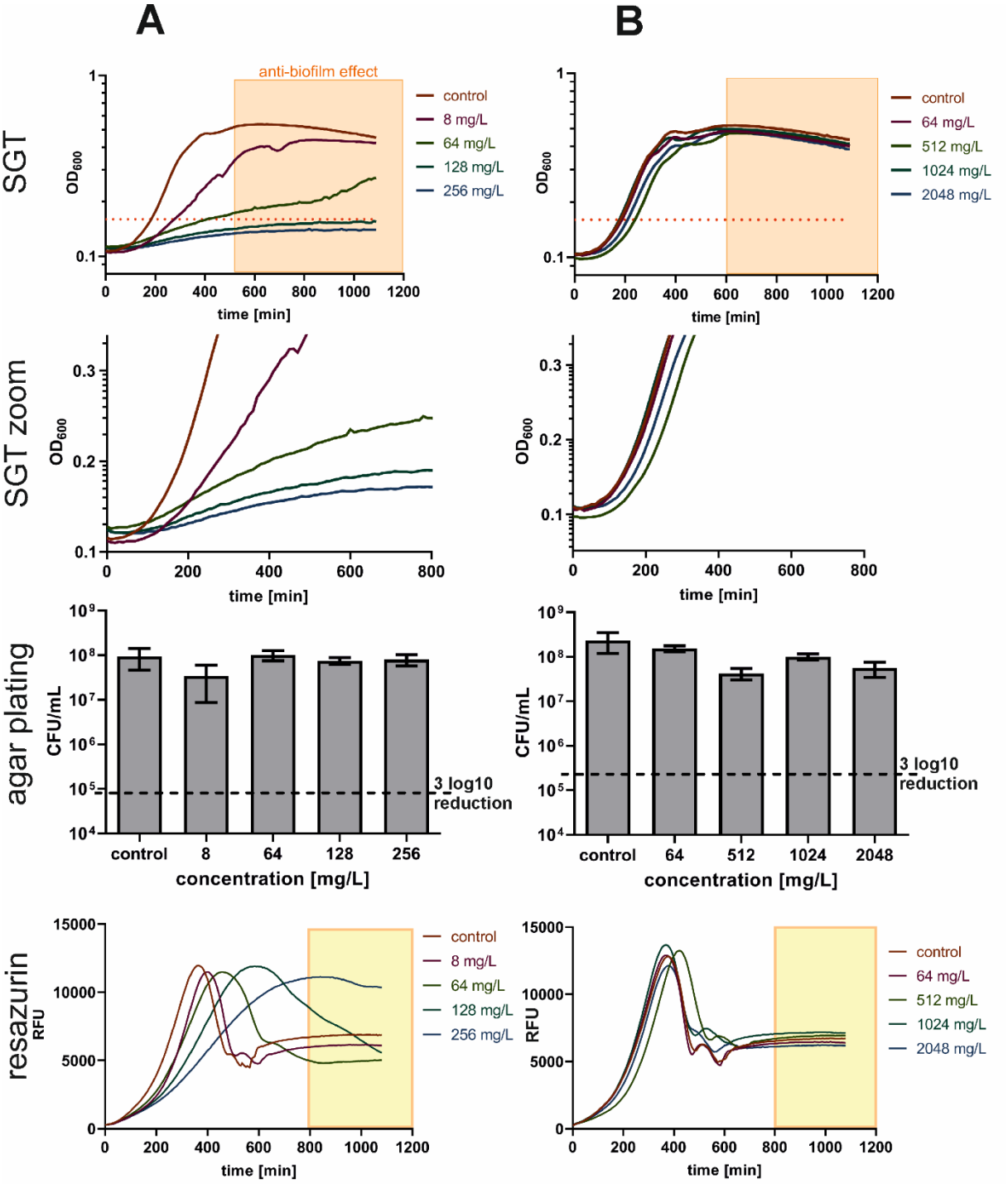

Figure 2: Determination of biofilm bactericidal effects with the three different methods in EF17129 biofilms treated with dalbavancin (column A) and gentamicin (column B). The yellow window ("anti-biofilm effect") indicates the area with an at least 3 $\log_{10}$ reduction in CFU compared to the untreated control.

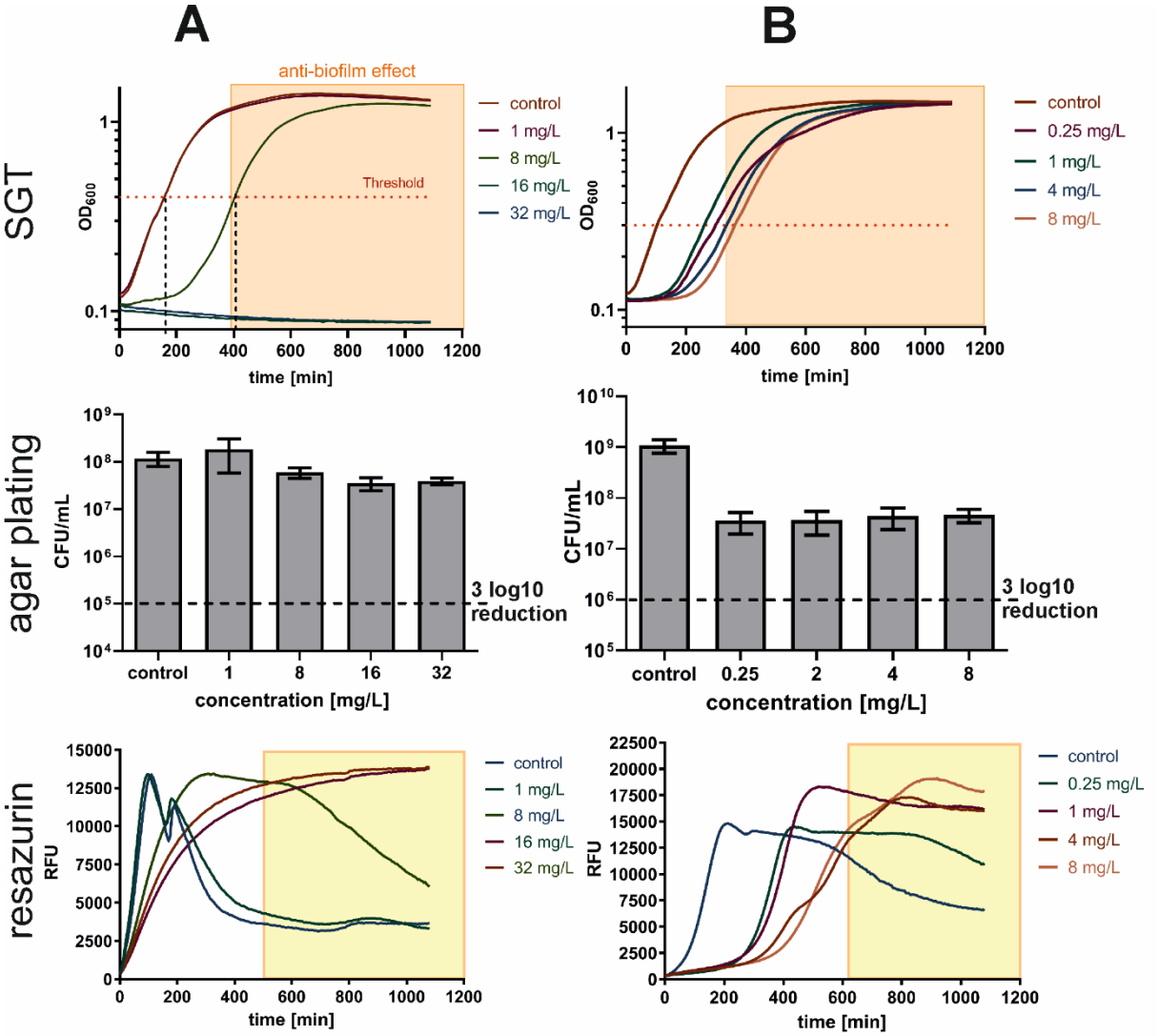

Figure 3: Determination of biofilm bactericidal effects with the three different methods in SA4002 biofilms treated with dalbavancin (column A) and rifampicin (column B). The yellow window ("anti-biofilm effect") indicates the area with an at least 3 $\log_{10}$ reduction in CFU compared to the untreated control.

Table 1: Comparison of biofilm bactericidal concentration (BBC) values obtained by different methods for biofilm quantification. The BBC is defined at the lowest concentration leading to 99.9 % eradication of the biofilm (= 3 $\log_{10}$ CFU/mL reduction). SGT = Start-Growth-Time.

Antibiotic	**Strain**		**calculated BBC [mg/L]**		
			SGT (=BBC_{SGT})	resazurin (=BBC_{RESA})	agar plating (=BBC_{AGAR})
Dalbavancin	*S. aureus*	4002	8	16	> 32
		4733	4	2	> 8
		1642/1	4	4	> 8
	E. faecium	24498	8	8	> 16
		12713	2	16	> 64
		17129	128	256	> 256
Rifampicin	*S. aureus*	4002	4	4	> 8
		4733	0.125	0.125	> 16
		1642/1	2	2	> 8
Gentamicin	*E. faecium*	24498	64	64	64
		12713	128	128	128
		17129	> 2048	> 2048	> 2048

Supplement Material

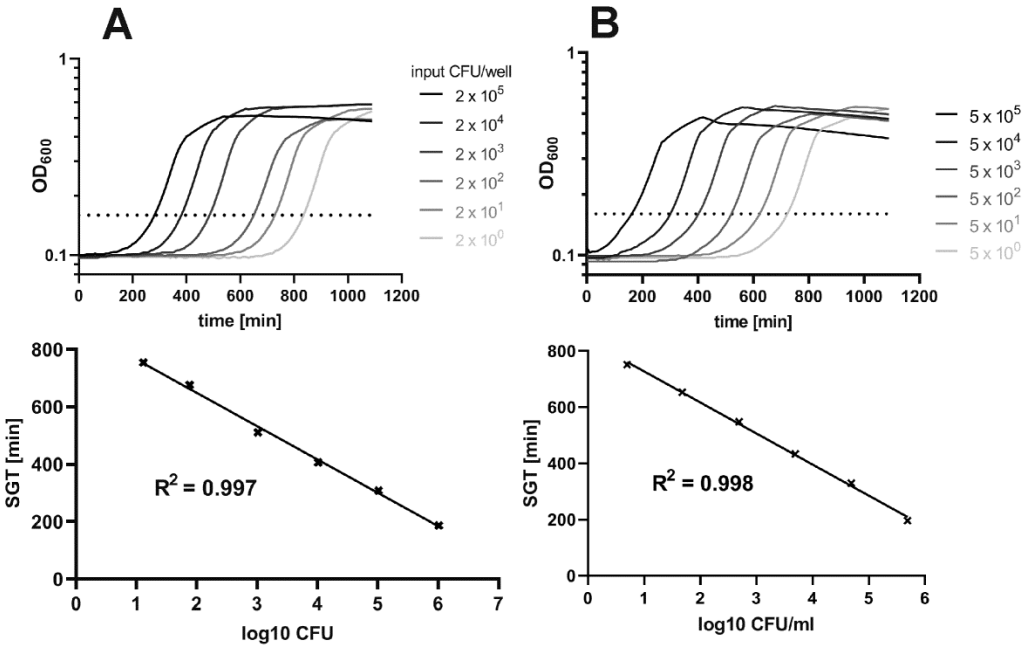

Figure S1: SGT standard curves for the other two *E. faecium* isolates (column A = EF24498 and column B = EF12713). The red dotted line indicates the threshold for SGT determination.

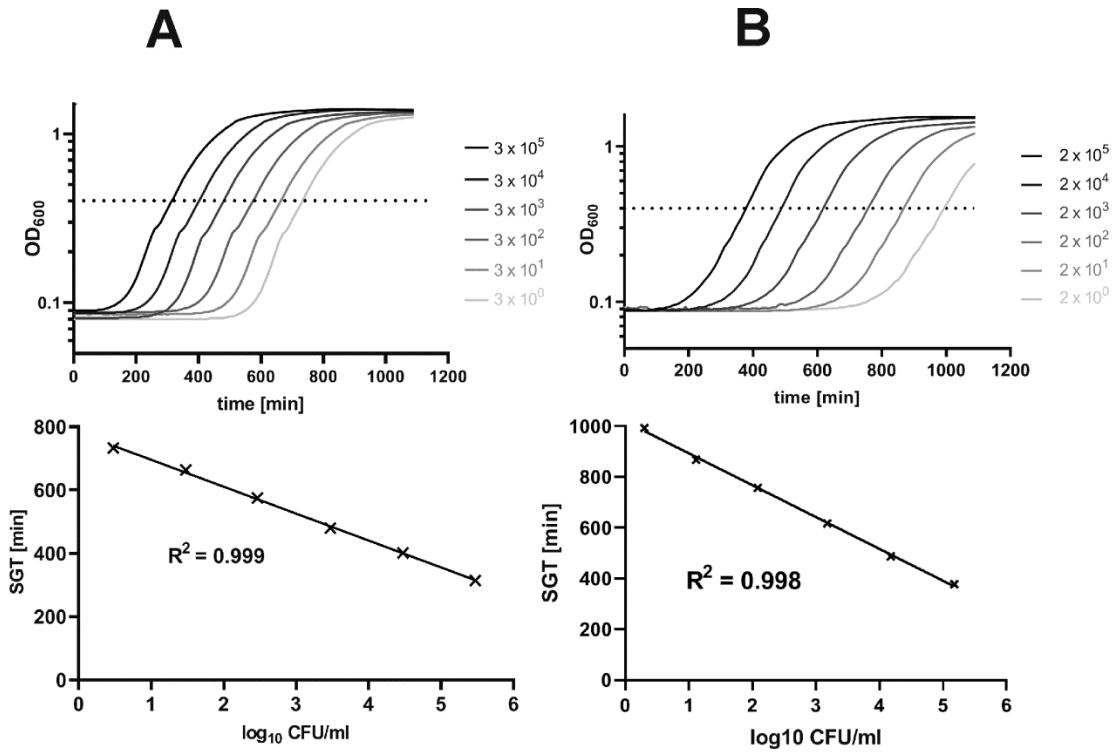

Figure S2: SGT standard curves for the residual *S. aureus* isolates SA4733 (column A) and SA1642 (column B). The red dotted line indicates the threshold for SGT determination.

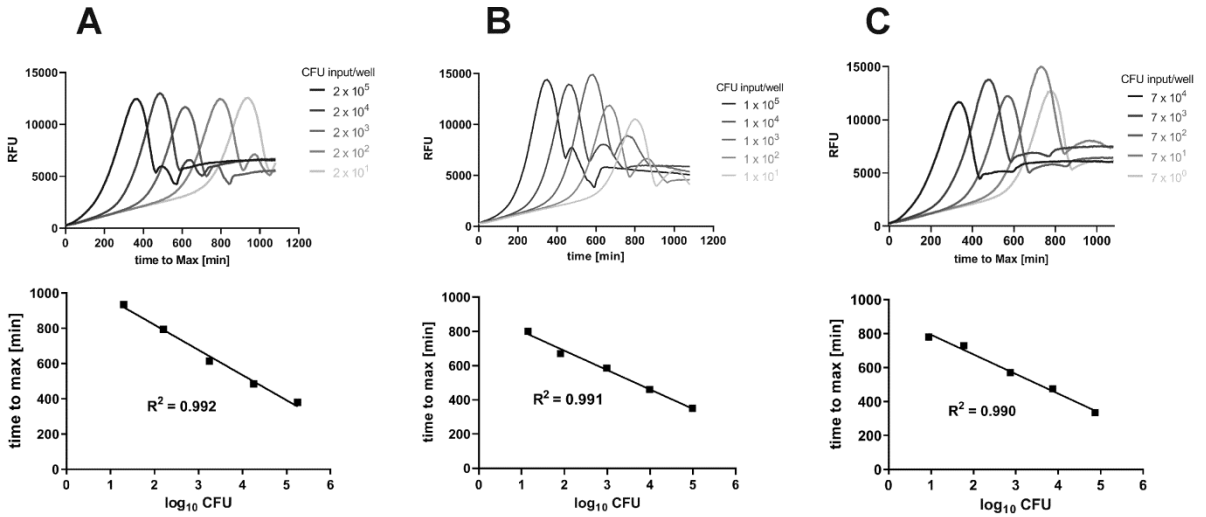

Figure S3: Resazurin standard curves for *E. faecium* EF17129 (column A), EF24498 (column B) and EF12713 (column C). Analysis of resazurin assay was done by time to RFU maximum determination.

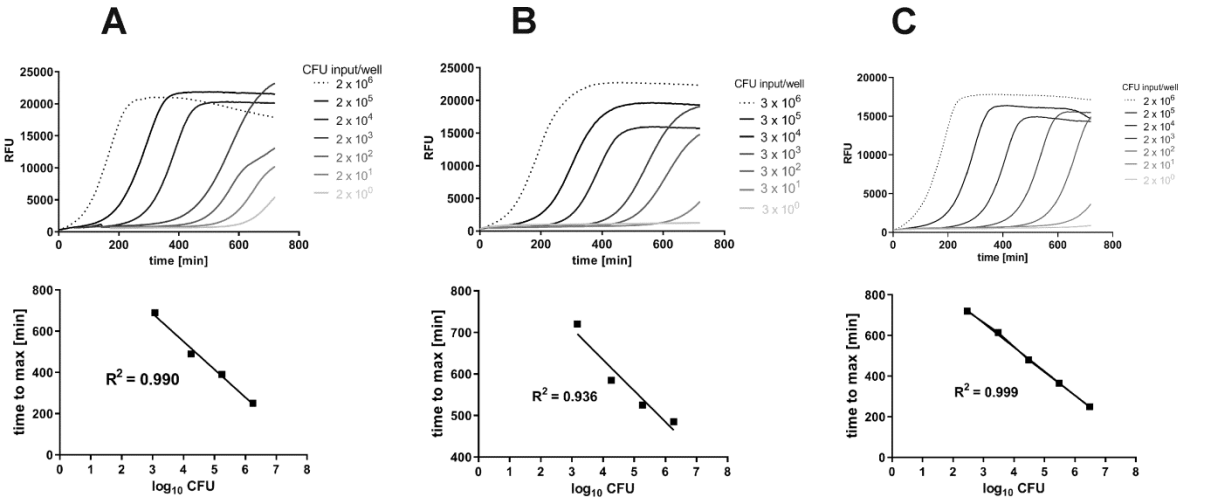

Figure S4: Resazurin standard curves for *S. aureus* SA4002 (column A), SA4733 (column B) and SA1642 (column C). Analysis of resazurin assay was done by time to RFU maximum determination.

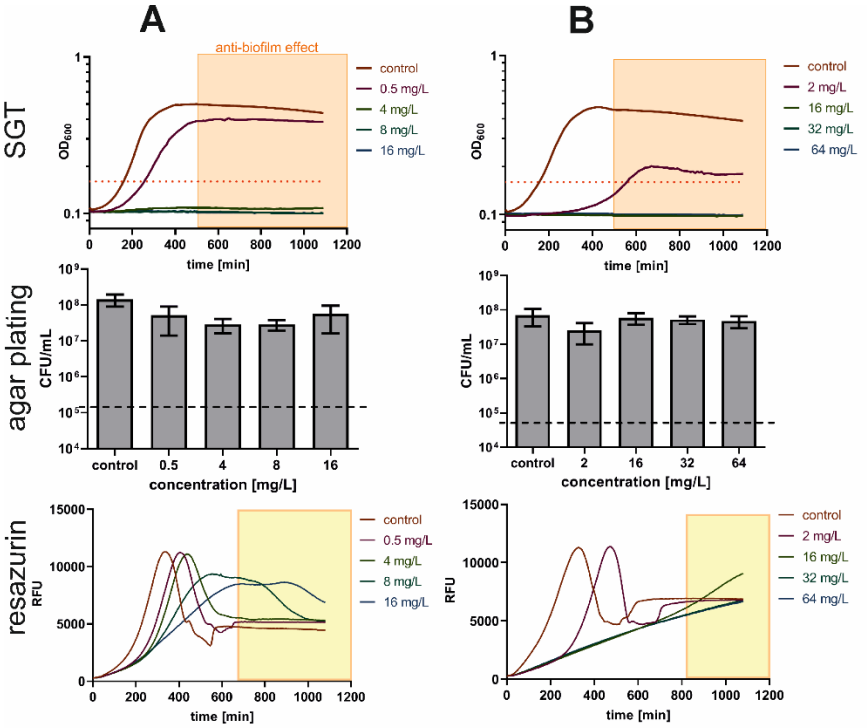

Figure S5: Analysis of **dalbavancin** treated *E. faecium* biofilms by SGT, agar plating and resazurin assay for EF24498 (column A) and EF12713 (column B). The orange window indicates a >3 $\log_{10}$ CFU reduction compared to untreated control. The red dotted line indicates the threshold.

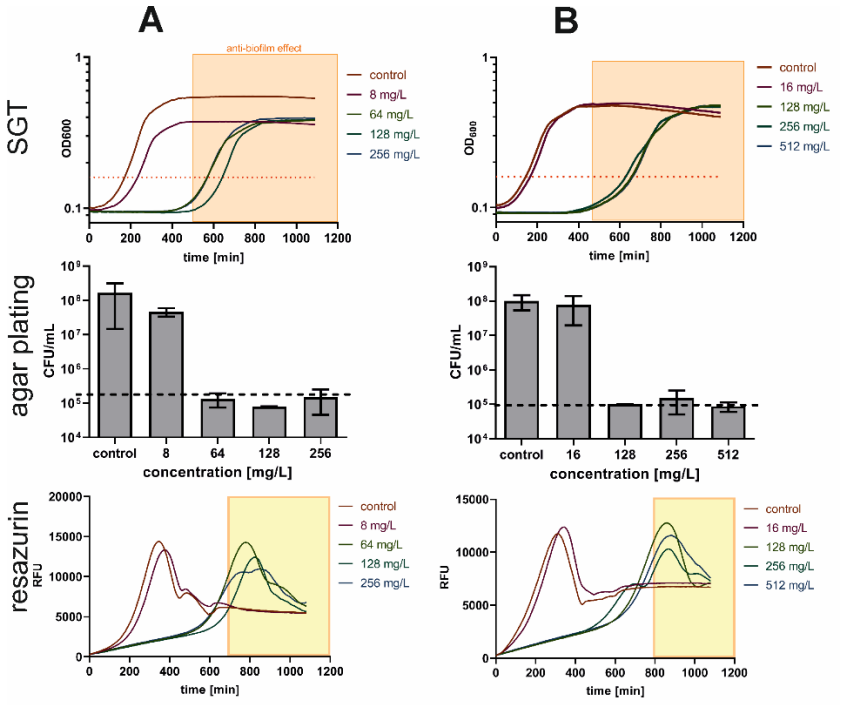

Figure S6: Analysis of **gentamicin** treated *E. faecium* biofilms by the three different methods for EF24498 (column A) and EF12713 (column B). The orange window indicates a >3 $\log_{10}$ CFU reduction compared to untreated control. The red dotted line indicates the threshold.

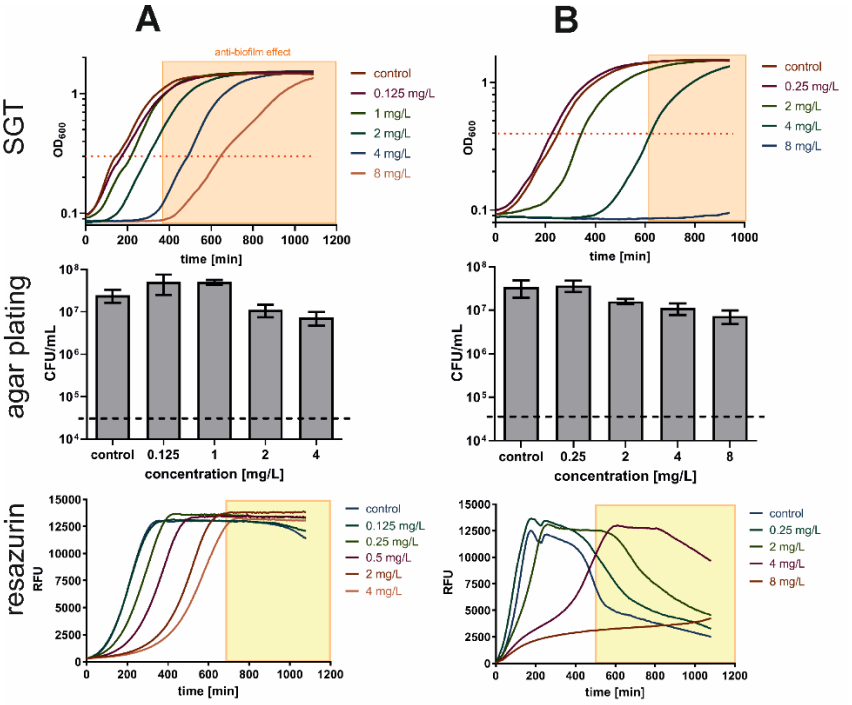

Figure S7: Analysis of **dalbavancin** treated *S.aureus* biofilms by SGT, agar plating and resazurin assay for SA4733 (column A) and SA1642 (column B). The orange window indicates a >3 $\log_{10}$ CFU reduction compared to untreated control. The red dotted line indicates the threshold.

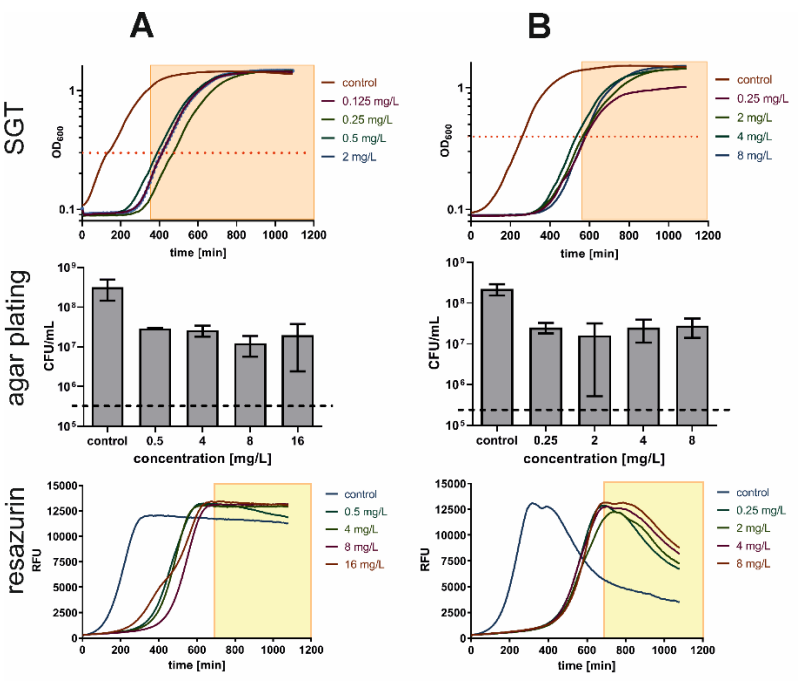

Figure S8: Analysis of **rifampicin** treated *S. aureus* biofilms by the three different methods for SA4733 (column A) and SA1642 (column B). The orange window indicates a >3 $\log_{10}$ CFU reduction compared to untreated control. The red dotted line indicates the threshold.

4 Discussion

4.1 Evaluation of synergy methodology

To compare the three antibiotic combinations ampicillin/gentamicin, ampicillin/ceftriaxone and ampicillin/ceftaroline for treatment of *E. faecalis* endocarditis, synergistic effects were assessed on planktonic level *in vitro* and *in vivo* by applying checkerboard analysis and the Gm infection model. *In vitro* checkerboard analysis has been intensively used to study the interaction of antimicrobial combinations for an array of pathogens, whereas this work suggested a first approach to differentiate between synergistic and additive effects *in vivo* in a larval insect model.

4.1.1 Critical analysis of the checkerboard method

While the methodological procedure of the checkerboard technique is well standardized, its interpretation varies among studies. Four different methods for interpreting the results of checkerboard assays have been described: the mean (or median) FICI, the lowest FICI, the full row and column FICIs and the two well method (Bonapace et al. 2002). All four methods are based on the same FICI formula but differ in their selection of the wells used for final FICI calculation, leading to widely different results and conclusions (Bonapace et al. 2002). While e.g., the mean or median FICI assesses the whole checkerboard assay by averaging all FICI values along the turbid/non-turbid interface, the minimal FICI method represents a one-point analysis by using the lowest of these FICI values for final checkerboard interpretation. The minimal FICI method neglects the one-well error occurring in MIC or checkerboard testing, meaning the minimal FICI may not be reproducible although the repeated checkerboard assay is in the one-well confidence interval range and therefore valid. While journals sometimes standardize the range of FICI values determining synergism, e.g., FICI ≤ 0.5 (Odds 2003), they fail to standardize by which of the four methods of checkerboard interpretation these ranges are supposed to be reached. This leads to a potential selection bias of e.g. using the minimal FICI to reach the synergistic FICI range. Often, the method of choice is not even stated by researchers (Bonapace et al. 2002). Besides the variability in the method of checkerboard interpretation, cut-off values of the FICI criteria “synergy”, “additivity” and “indifference” are lacking standardization (Hsieh et al. 1993). Some researchers define FICI values ≤ 0.5 as

synergistic, whilst others take FICI ≤ 0.8 as a threshold (den Hollander et al. 1998, Horrevorts et al. 1987, Stein et al. 2015). In the presented study, the minimal FICI and therefore the stricter synergy threshold of FICI ≤ 0.5 was used, which lead to almost identical results as using the median FICI with a synergy threshold of FICI ≤ 0.8 (Thieme et al. 2018). Especially the range and thereby definition of additive effects varies between publications: some differentiate between additive (FICI between 0.5 and 1) and indifferent (FICI between 1 and 4) effects (Doern 2014, Lewis et al. 2002), others refrain from fine-scale interpretations and define FICI values between 0.5 and 4 as “no interaction” between the antimicrobials (Odds 2003, Bremmer et al. 2016, Thieme et al. 2018). Above pitfalls of checkerboard interpretation illustrate that the very same checkerboard assay can be interpreted as showing synergistic, additive, indifferent or even antagonistic interactions between the two tested substances.

A further problem in synergy methodology is the fact that the different *in vitro* synergy methods available lead to different results. Several studies have evaluated the comparability of checkerboard assays, Etests and TKAs and found various degree of agreement (Lewis et al. 2002, Orhan et al. 2005, Sopirala et al. 2010, White et al. 1996). Further, it is unknown which synergy test produces the most clinically relevant results. There is a general lack of clinical correlation studies linking the mass of synergy *in vitro* data to direct treatment outcome. Outcome-based *in vitro* synergy studies have not been addressed in IE, but synergy testing has been used to predict patients’ outcomes in CF patients having a *P. aeruginosa* infection (Aaron et al. 2005). No difference between conventional versus synergy susceptibility testing (modified checkerboard analysis) could be identified regarding treatment failure rate, changes in lung function or bacterial density. Therefore, the 2009 Cystic Fibrosis Foundation practice guidelines specifically stated that synergy testing should not be done in CF patients (Aaron 2007, Flume et al. 2009).

Taken together, it remains open whether synergy testing as a whole – regardless of the method – can be recommended for routine use to guide patient care in infectious diseases such as IE.

4.1.2 Optimization of synergy testing in the *G. mellonella* larvae model

The *in vivo* analysis of the antibiotic combinations was based on a partial transfer of the checkerboard assay set-up into the Gm infection model. Selected concentrations

of both the single and combined antibiotics were based on the MIC effect scale and allowed for a comparison of the antibiotic dose combination with each the dose equivalent and the next higher combined dose of the single antibiotics (Thieme et al. 2020). For example, the dose combination 1x MIC_A + 1x MIC_B was compared to 2x MIC_A and 2x MIC_B (dose equivalences) and 4x $MIC_{A/B}$ (higher doses) since a synergistic interaction i) creates a stronger effect than its' dose equivalent (= expected additive effect), ii) yields at least the same effect at lower doses of the combination partners compared to the higher-dosed single agents. Comparison was done by examining larval survival, bacterial survival (haemolymph CFU load) and PSI data for significant differences between these treatment groups. This set-up enabled a differentiation between synergistic and additive effects likewise in checkerboard analysis, a distinction often neglected by other *in vitro* synergy methods and previous larvae synergy studies (Krezdorn et al. 2014, Luther et al. 2014, Skinner et al. 2017). The term "*in vivo* synergism" is generally avoided in literature and replaced by "*in vivo* efficacy" since most study designs do not differentiate between synergistic and additive effects in animal models (Fantin and Carbon 1992).

However, it is important to emphasize that the *in vitro* MIC effect scale and not an absolute effect size in the larvae, e.g., 50 % survival, was used to define the dose equivalences of the antibiotic combinations. The MIC scale did not necessarily correlate with larval survival, e.g. 1x MIC did not correlate with 100 % survival. Hence, this resulted in a mixture of an *in vitro* and *in vivo* approach to differentiate between synergistic and additive effects. To follow a complete *in vivo* approach the selection of the antibiotic dose combinations should have been based on the individual dose-effect curves of the single antibiotics in the larvae, and not on the effect reached *in vitro*, namely the MIC. Therefore, adequately obtained dose-effect curves in the larvae are needed, requiring the resource-consuming analysis of more antibiotic concentrations than done in the presented study, where only three doses (2x, 4x, and 8x MIC) were assessed. Further, specific effects for all three larval parameters likewise the *in vitro* MIC need to be established and statistically validated, such as 50 % survival, 3 log_{10} cfu/mL reduction or a half-fold reduction of the control PSI. Definition of such cut-off values would also enable the calculation of FICIs in the larvae but requires a much higher experimental set-up with a lot of retesting whether the determined cut-off value including its confidence interval (in checkerboard analysis ±1x MIC) is useful for larval

FICI calculation. Establishing sophisticated methods such as FICI scores for evaluation of synergy testing in insect larvae and other animal models is therefore a rigorous approach, wherefore *in vivo* synergism is still an undefined concept (Fantin and Carbon 1992).

While the Gm infection model allowed for a relatively fast *in vivo* synergy analysis of the EFIE-associated antibiotic combinations, the larvae show anatomic, pharmacodynamic and immunological limitations as a model organism for IE. Gm larvae possess a dorsal blood vessel representing a tubular heart, which produces peristaltic waves via contraction moving the haemolymph to the different organs. However, their heart structure lacks heart valves and chambers, therefore failing to adequately represent the bacterial attachment and biofilm formation taking place in IE. Researchers have started to establish a Gm biofilm toothbrush bristle implantation model, which could be modified to mimic an implant-associated (prosthetic valve, pacemaker) IE (Campos-Silva et al. 2019). Further, larval antibiotic elimination rates were only partly in accordance with those observed after human iv administration. The larvae possess tissue structures resembling detoxification organs, such as the Malpighian tubule system and the fat body forming the functional analogues to kidneys and liver (Arrese and Soulages 2010, Bresler et al. 1990). The exact organ system and mechanisms of drug elimination in Gm larvae have not been investigated, though (Cutuli et al. 2019). While the similarities to the mammalian innate immune system predestine Gm larvae to study specific innate immune responses, the lack of an adaptive immune system disables the Gm model to adequately reflect complex immune reactions taking place in IE.

Despite the limitations coming along with simple insect models, Gm larvae are well-suited for high throughput and proof-of-concept screens. Following the 3Rs framework in animal research (Replacement, Reduction, Refinement), introducing larval models as a bridge between *in vitro* and mammalian experiments leads to the reduction of experiments in ethically less desirable animal models. Studies in which Gm data correlate with mice data support the use of larvae as an alternative host model (Borman 2018, Brennan et al. 2002, Jander et al. 2000). Further studies linking larval data to outcome-based clinical studies are needed.

4.2 Evaluation of biofilm susceptibility testing

Besides synergism testing, a further criterion for comparison of ampicillin/gentamicin, ampicillin/ceftriaxone and ampicillin/ceftaroline for treatment of EFIE was their anti-biofilm effectiveness. However, no standardized biofilm susceptibility assay has been introduced into clinical practice until now. In the presented anti-biofilm study, a simple *in vitro* microtitre plate biofilm model with only one-time application of antibiotics was used, which is a strong oversimplification of the *in vivo* biofilm situation in IE (Thieme et al. 2018). The current lack of better suited biofilm susceptibility assays can be mainly attributed to the still poor representation of the *in vivo* biofilm setting in *in vitro* assays. Since biofilms form as an adaption strategy to stressful environmental conditions, e.g., nutrient depletion or attacks from the host immune system or antibiotics, their structure is highly dependent on the host surrounding (Jefferson 2004). The very same enterococcal isolate will form a structurally and compositionally different biofilm on e.g., heart valves versus wound bed, since different host materials, different flow and sheer force expositions (continuous blood flow depending on biofilm location on heart versus static wound exudate) and different immunological surroundings dominate both niches of infection (Dunny et al. 2014). Different environmental surroundings lead to the formation of phenotypically distinct biofilm subpopulations such as extracellular matrix producers, adhesive fiber producers, motile bacteria and metabolically dormant bacteria (Magana et al. 2018). Further, while biofilms found in EFIE are believed to be monospecies biofilms, most *E. faecalis* biofilm-associated infections originate from polymicrobial biofilms, e.g., UTIs or wound infections (Ch'ng et al. 2019). To adequately reflect this heterogeneity in a simple HTP assay suited for routine diagnostics is one of the main challenges in the management of biofilm-associated infections. While *in vitro* biofilms grown in microtitre plates or Calgary devices in an HTP set-up are usually surface-attached, *in vivo* biofilms typically occur as small aggregates deeply embedded in host material (Bjarnsholt et al. 2013). An antibiotic directly added to the *in vitro* biofilm may mimic the drug access to a biofilm implant-related infection but may fail to mimic antibiotic penetration in deep-seated tissue biofilm infections. Studies have demonstrated a link between architecture and antibiotic resistance in *E. faecalis* biofilms (Dale et al. 2017), making it crucial to mimic the *in vivo* biofilm structure in biofilm susceptibility assays to achieve a clinically meaningful assay readout.

A first step towards translation of anti-biofilm testing in clinical practice is the urgently needed standardization by official agencies such as the EUCAST. Although there might not yet exist the ideal biofilm method, standardized methods with precise definitions of duration of biofilm growth, duration of antimicrobial treatment and biofilm susceptibility endpoint parameters would lead to a reduction of contradicting findings between different anti-biofilm studies (Thieme et al. 2019). Since biofilms undergo differential steps in their maturation, the biofilm susceptibility results of an antibiotic differ greatly between nascent biofilms without nutrient and oxygen gradients and old, oxygen-limited, stationary phase biofilms, making unification in testing necessary. Simple microtitre plate HTP tests might be further adapted to the host environment by adding niche characteristic supplements during biofilm growth, e.g., collagen and fibrin for mimicking biofilms in EFIE. The addition of representative host material might also improve the novel Start-Growth-Time (SGT) biofilm quantification method presented in this study (manuscript in preparation). While the use of regrowth curves - whose lag-phases depend upon the number of living cells in the treated biofilm and therefore correlate with CFU numbers – replaces the time-consuming analysis of conventional CFU plating as an output parameter, this method does not yet overcome the poor mimicking of *in vivo* biofilm conditions. Independent of the modification of existing biofilm methods or the development of novel ones, clinical trials correlating these tests with treatment outcomes of biofilm-associated infections are urgently needed to gain evidence if a personalized biofilm diagnostic may improve patient outcome. Individual determination of biofilm-susceptible antibiotics for each clinical bacterial biofilm seems necessary since broad categorizations of antibiotics into biofilm-effective are not appropriate. For example, ceftaroline was shown to exhibit anti-biofilm activity against mature *S. aureus* biofilms (Barber et al. 2015, Lázaro-Díez et al. 2016), but not against *E. faecalis* biofilms, although both are often categorized into ‘Gram-positive’ biofilms. A bacterial genus-based classification of biofilm-effective antibiotics is also inappropriate since *E. faecalis* and *E. faecium* show contrary capabilities to form biofilms, with high and low rates of biofilm formation, respectively (Ch’ng et al. 2019). Even the individual *E. faecalis* isolates have shown different morphologies in their *in vitro* biofilms grown under the same conditions (Thieme et al. 2018). Therefore, biofilm-associated infections equal to planktonic infections likely require individual diagnostic testing and therapy. Similar as for synergism testing, only two clinical trials addressing

this issue for *P. aeruginosa* infections in CF patients have been conducted (Moskowitz et al. 2011, Yau et al. 2015). Both concluded that state-of-the-art biofilm susceptibility testing is non-superior to conventional susceptibility testing. A retrospective case control study using the SGT-MBEC-method for analysis of antibiotic combination therapies used for the treatment of complicated *S. aureus* bacteraemia has been initiated at the Institute of Infectious Diseases and Infection Control, Jena. Such studies might also contribute to answering how many percent or log reductions in biofilm-embedded bacteria are required to resolve symptoms of infections or whether the host could tolerate a certain amount of biofilm bacteria (Coenye et al. 2020).

The future of biofilm methodology will likely involve a combination of different technologies to adequately analyze the varied subpopulations occurring in biofilms, such as microscopic imaging, label-free chemical profiling of the EPS matrix or automated machine learning analysis algorithms (Coenye et al. 2020). To select the appropriate model for a specific question, the development of a decision tree including criteria for the choice of simple versus more complex models, parameters and *in situ* relevance of the biofilm model was discussed (Coenye et al. 2020). Until standardized biofilm susceptibility assays with a proven clinical validity have been developed, it remains questionable how *in vitro* generated anti-biofilm testing data correlate to the *in vivo* EFIE situation.

4.3 Evaluation of synergy results in terms of their clinical applicability

While both the synergy and biofilm methodology need massive improvement in terms of their standardization and clinical validity, the obtained results in the presented studies allow for an evaluation and comparison of the antibiotic combinations for treatment of *E. faecalis* IE. Clinically, combination therapy is employed for mainly three reasons: i) to broaden the empiric coverage by applying antibiotics with usually different spectra of activity, ii) to exploit the synergistic effects observed *in vitro*, leading to enhanced bactericidal effects of the combination compared to the individual antibiotics, or iii) to reduce the risk of selection pressure and emergence of resistance during antimicrobial therapy (Tamma et al. 2012). A differentiation between additive and synergistic interactions can be helpful for assessment of the risk-benefit ratio of combination over monotherapy. For treatment of *E. faecalis* IE, the central question is whether the prolonged addition of an aminoglycoside or cephalosporin to ampicillin is

worth the risk of side effects, e.g., nephrotoxicity, *C. difficile* colitis or VRE colonization, if the antibiotic combination shows only additive interactions. To date, it remains unclear if the –according to a few retrospective studies – similar efficacy of ceftriaxone/ampicillin versus gentamicin/ampicillin is mainly a result of true synergy or explained by the substantially decreased toxicity of ceftriaxone/ampicillin compared to gentamicin/ampicillin.

The synergy data presented in this thesis illustrate that the similar treatment outcome of ceftriaxone/ampicillin and gentamicin/ampicillin is likely not only due to lower rates of adverse events, i.e., renal impairment, but due to true synergistic effects of ceftriaxone/ampicillin. *In vitro* checkerboard analysis revealed that both cephalosporin-based ampicillin combinations – ceftriaxone/ampicillin and ceftaroline/ampicillin - showed comparable synergistic activity in most of the tested isolates on planktonic level (Thieme et al. 2018). In contrast, larvae synergy experiments showed synergistic effects for ceftriaxone/ampicillin in only one out of three tested strains, while ceftaroline/ampicillin even exhibited no interaction (Thieme et al. 2020). The synergistic effect of both dual β-lactam combinations is proposed to be based on complementary inhibition of PBP homologues, resulting in impairment of cell wall synthesis (Gavalda et al. 1999). While complete inhibition of the essential PBPs 4 and 5 by ampicillin alone leads to a bactericidal effect, partial saturation of PBP 4 and 5 by lower ampicillin concentrations coupled with complete saturation of non-essential PBPs 2 and 3 by cephalosporins shows equal activity (Mainardi et al. 1995). Very low concentrations of both ceftriaxone and ceftaroline are sufficient to block PBPs 2 and 3 due to their high affinity to these PBP homologues (Arbeloa et al. 2004). Ceftaroline additionally shows high affinity to PBP 5 (Henry et al. 2013), therefore at higher concentrations ceftaroline may compete with ampicillin for binding of PBP 5, potentially diminishing the synergistic effect. This behaviour may explain the lacking synergistic effect of ceftaroline/ampicillin in the larvae as well as the observed synergy inversion in *in vitro* biofilm preventive experiments at higher combined concentrations of both antibiotics. Further, it illustrates how synergistic mechanistic behaviours are closely linked to the concentrations of the single agents in the combination, which can be challenging to transfer to the clinical patient. The combination of ceftaroline/ampicillin has only been addressed by a limited number of *in vitro* synergy studies so far, suggesting equal effectiveness to ceftriaxone/ampicillin but the advantage of a

potentially safer side effect profile (Luther et al. 2016, Werth and Abbott 2015, Werth and Shireman 2017). A recent retrospective clinical trial evaluating ceftaroline fosamil – the prodrug form of ceftaroline – for Gram-positive IE included one case of left-sided EFIE showing successful treatment with ceftaroline/ampicillin (Destache et al. 2019). While seemingly safer than gentamicin as a combination partner, ceftriaxone has been associated with *C. difficile* and VRE infections, probably due to the excretion via the bile duct, as well as gastrointestinal colonization of VREs (McKinnell et al. 2012, Owens et al. 2008, Rice et al. 2004). Other cephalosporins with primarily renal excretion, including ceftaroline, seem not to promote VRE colonization although data are limited (Beganovic et al. 2018, Panagiotidis et al. 2010). Further, *in vitro* biofilm-preventive experiments indicated the selection of small colony variants (SCV) under high cephalosporin concentrations, although with different intensities and in a strain-dependent manner (Thieme et al. 2018). In larvae experiments, formation of SCVs was not observed upon any antibiotic treatment at any concentration (Thieme et al. 2020). Bacterial SCVs show a slow growth rate, resulting in atypical pinpoint-sized colonies, unusual biochemical characteristics and reduced antibiotic susceptibility (Proctor et al. 2006). They have been reported for a wide range of bacterial species, mostly staphylococci, whereas formation of SCVs in enterococci has not been thoroughly investigated (Wellinghausen et al. 2009). Since SCVs facilitate persistent and recurrent infections and have been linked with an *E. faecalis* IE relapse (Benes et al. 2013), further studies to assess the risk of SCV selection in *E. faecalis* isolates upon differential cephalosporin administration are mandatory.

Both *in vitro* and *in vivo* synergy experiments have shown inferiority of gentamicin/ampicillin compared to the cephalosporin-based combinations, with additive instead of synergistic effects being noted (Thieme et al. 2020, Thieme et al. 2018). Enterococci are intrinsically resistant to gentamicin (MIC_{GEN} < 128 mg/L) due to the low cell wall permeability of aminoglycosides, making aminoglycoside monotherapy ineffective (Hollenbeck and Rice 2012). Originally, ampicillin was combined with gentamicin to enhance intracellular uptake of aminoglycosides and thereby complement the lacking bactericidal activity of β-lactams (Beganovic et al. 2018). However, the increasing use of aminoglycosides has prompted the acquisition of aminoglycoside-modifying enzymes, resulting in the development of HLAR enterococci (MIC_{GEN} > 128 mg/L). The presence of HLAR abolishes the synergism with

ampicillin, thus combined gentamicin/ampicillin treatment is only beneficial for non-HLAR *E. faecalis* isolates (Nigo et al. 2014). However, in the presented checkerboard study only one out of 13 tested non-HLAR isolates showed synergistic effects between gentamicin and ampicillin, while all other isolates exhibited additive effects (Thieme et al. 2018). It is not clear whether this discrepancy is due to methodological issues, i.e. differential synergy methods or checkerboard interpretation compared to previous studies, or further genetic mutations of current clinical *E. faecalis* isolates. Nevertheless, it illustrates that broad statements about synergistic combinations for specific organisms are often inappropriate and need to be replaced by case-to-case testing. As this is not achievable with the currently available methods for synergism determination in clinical routine, synergy testing could e.g., be conducted in patients who do not tolerate gentamicin and therefore receive ceftriaxone/ampicillin but experience side effects due to ceftriaxone (e.g., biliary sludge). In these patients, confirmation or exclusion of synergy may help in the decision-making process to continue or stop ceftriaxone, i.e. to switch from combination to ampicillin monotherapy.

4.4 Evaluation of biofilm results in terms of their clinical applicability

Evaluation of the antibiotic combinations for treatment of EFIE requires not only synergy testing but also the analysis of their anti-biofilm effectiveness. The presented biofilm eradication studies showed that neither a cephalosporin- nor aminoglycoside-based combination in concentrations up to 1000x MIC was able to dissolve the biofilm structure of mature, one-day old *in vitro E. faecalis* biofilm sheets (Thieme et al. 2018). The missing anti-biofilm effectiveness cannot be attributed to a lack of synergistic effects since the single agents also showed no diminishing effect on biofilm thickness and titres. Enterococcal biofilm formation, structure and constitution is less well investigated and understood than for other biofilm-forming model organisms such as *P. aeruginosa* (Ch'ng et al. 2019). Drawing conclusions on antibiotic's effectiveness in enterococcal matrix penetration and biofilm eradication is therefore based on general conceptions about bacterial biofilms. Biofilm-specific antimicrobial tolerance mechanisms such as reduced antibiotic penetration, metabolic dormancy/persister phenomenon or oxygen gradients throughout the biofilm render most antibiotics insufficient for treatment of biofilm-associated infections (Macia et al. 2014). Only antibiotics targeting eradication of dormant cells are proposed to be efficient, e.g.,

antibiotics acting on transcriptional level, on bacterial topoisomerases or directly perforating the cell membrane (Aslam 2008). In *P. aeruginosa* biofilms, β-lactams and aminoglycosides only affected the outer, oxygen-rich part of the biofilm because they are inactive in anaerobic conditions (Borriello et al. 2004). Further, since both antibiotic classes interfere with processes of actively dividing cells, i.e. cell division and protein biosynthesis, complete eradication of mature biofilms, especially the subpopulation of metabolically quiescent bacteria and SCVs, seems mechanistic-wise unlikely. Other antibiotic classes with enhanced likelihood of anti-biofilm activity should be considered for treatment of EFIE, such as lipopeptides, e.g., daptomycin. Recent *in vitro* data as well as case reports show successful application of daptomycin and β-lactam combination treatment in EFIE but specific anti-biofilm activities have not been addressed (Sakoulas et al. 2013, Sierra-Hoffman et al. 2012). Dalbavancin – a second generation lipoglycopeptide approved for the treatment of acute bacterial skin and skin structure infections caused by Gram-positive bacteria - might be a promising option for completing treatment on an outpatient parenteral antibiotic therapy (OPAT) basis due its prolonged elimination half-life (Fernandez-Hidalgo et al. 2020). Further studies are needed to confirm its activity against enterococcal biofilms. Combinations of ciprofloxacin or linezolid with rifampicin were shown *in vitro* to reduce *E. faecalis* biofilms originating from prosthetic hip and joint infections (Holmberg et al. 2012).

The question remains why both ceftriaxone/ampicillin and gentamicin/ampicillin show treatment success in biofilm-burdened EFIE patients if from a theoretical and experimental point-of-view no anti-biofilm activity is expected. As discussed above in more detail, current high throughput anti-biofilm assays show poor clinical validity (Coenye et al. 2018). Especially the lack of host immune components in the presented anti-biofilm assay might explain the contradictive results between negative experiments and positive patient outcomes, indicating that the host's immune system interacts with the antibiotics for biofilm eradication. However, also the two clinical trials conducted for comparison of gentamicin/ampicillin and ceftriaxone/ampicillin for treatment of EFIE show several limitations, such as i) retrospective collection without randomization, ii) multicentre-design without uniform treatment guidelines and iii) inconsistent gentamicin dosing and therapeutic drug monitoring (Munita et al. 2013, Solla 2013). The biggest confounder for adequate comparison of both combinations is

the conduction of cardiac surgery during the observation period: 33 % in the ceftriaxone/ampicillin and 40 % in the gentamicin/ampicillin treatment arm underwent surgery during the active phase of infection (Fernandez-Hidalgo et al. 2013). Surgical removal of colonized heart valves eliminates most parts of the mature biofilm, thereby attributing significantly to treatment success since the antibiotics likely eradicate only leftover planktonic bacteria or microcolonies. Relapsing EFIE cases - approximately 3 % in each treatment arm (Fernandez-Hidalgo et al. 2013) - further indicate no complete eradication of enterococcal biofilms due to neither β-lactams nor aminoglycosides.

It remains unclear which patients can be treated successfully with double antibiotic combinations and whether treatment success can be attributed to an anti-biofilm effect. There is currently no differentiation between acute and subacute EFIE with regard to diagnosis and treatment guidelines (McDonald 2009). The slow development over time of subacute or chronic IE highly suggests the formation of mature biofilms, which in their dispersal state may trigger the onset of disease. In contrast, the sudden onset of acute IE may rather be associated with planktonic bacteria or early microcolony formation, possibly explaining why the antibiotic combinations show treatment success in most cases. A biofilm-staging study correlating the degree of biofilm formation in IE, i.e. planktonic - microcolonies - mature biofilm, with clinical symptoms and treatment outcome is currently under development (personal communication Dr. Annette Moter, 6th Joined Conference of the DGHM & VAAM, 9th March 2020).

5 Conclusion and outlook

From a microbiological perspective, treatment of EFIE with synergistic antibiotic combinations should be based on a differentiation in planktonic and biofilm-embedded bacteria. However, current clinical practice and routine antibiotic susceptibility testing methods do not allow a diagnostic fine-scale differentiation according to the life form of bacteria in IE, i.e. planktonic/free-living, early attachment, microcolony state or mature, dispersing biofilm. A differential treatment of EFIE based on the stage of the disease and the severity of symptoms may be considered: while EFIE patients with initial proven bacteraemia may benefit from synergistic antibiotic combination therapy, shortened combination or even less toxic monotherapy may be sufficient once initial bacteraemia has been treated and blood cultures have become sterile. However, both shortened ceftriaxone/ampicillin and gentamicin/ampicillin treatment, i.e. 4 instead of 6 weeks, have been associated with increased rates of relapses in a recent clinical trial of uncomplicated native valve EFIE (Pericas et al. 2018). Treatment of subacute, non-bacteraemic EFIE cases may also include the shift from intravenous to oral therapy. The recent Danish POET-trial (Partial Oral Treatment of Endocarditis) concluded that for stable IE patients changing to oral therapy was non-inferior to continued iv antibiotic treatment (Iversen et al. 2019). However, both ceftriaxone and gentamicin cannot be administered orally due to low reabsorption rates, creating the need for other antibiotics with sufficient bioavailability.

The synergy testing data on planktonic level in this thesis support the recent statement that aminoglycosides should be abandoned or at least drastically reduced in EFIE treatment (Lebeaux et al. 2019). For acute bacteraemia (i.e. planktonic cells), a cephalosporin-based combination seems to be superior to an aminoglycoside-based combination since both ceftriaxone/ampicillin and ceftaroline/ampicillin showed superior synergistic effects to gentamicin/ampicillin. However, replacing a 3rd with a 5th generation cephalosporin, i.e. ceftriaxone with ceftaroline, in combination treatment does not seem to provide further benefit because ceftaroline/ampicillin showed equal to inferior activity to ceftriaxone/ampicillin in terms of synergistic effects. Further studies weighing the clinical side effect profile of both cephalosporins, e.g., reduced VRE colonization, are advisable.

There is no evidence that antibiotic combination therapy is superior to monotherapy regarding the biofilm aspect of EFIE. None of the antibiotic combinations showed

bactericidal effects against *E. faecalis* biofilms, questioning the prolonged use over 4-6 weeks of cephalosporin- or gentamicin-based combination therapy for treatment of EFIE. However, it is important to highlight that current *in vitro* biofilm susceptibility assays are poorly predictive of the *in vivo* situation (Coenye et al. 2018). Depending on biofilm progression in the EFIE patient, subgroups of patients may benefit from directed, non-antibiotic biofilm treatment. An array of potential anti-biofilm strategies, such as quorum sensing inhibitors, phage therapy or antimicrobial peptides has been development in the last years with partially great success *in vitro* but unknown applicability *in vivo* (Ch'ng et al. 2019, Khalifa et al. 2015, Verderosa et al. 2019). No specific biofilm eradication strategies have been analysed for *E. faecalis* biofilms formed on heart valves so far. Research on oral, dental root-associated *E. faecalis* biofilms has suggested the degradation of the EPS, i.e. eDNA, followed by antibiotic therapy as a potential biofilm remediation strategy (Torelli et al. 2017). It remains questionable whether such biofilm eradication strategies can be transferred to IE-associated biofilms. Future investigations of treatment options of EFIE should therefore focus on both antibiotics and non-antibiotics with anti-biofilm activity against *E. faecalis* biofilms formed on both prosthetic and native heart valves.

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7 Appendix

7.1 List of figures

7.2 Danksagung

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7.3 Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Dr. Mathias W. Pletz, Dr. Oliwia Makarewicz.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Ort, Datum Unterschrift des Verfassers