Aus dem Institut Berlin-Brandenburger Centrum für Regenerative Therapien der
Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Antibiofilm approaches to combat *Streptococcus* and related species implicated in implant-associated infections

zur Erlangung des akademischen Grades
Doctor of Philosophy (PhD)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

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Datum der Promotion: 05.03.2021

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Abbreviations

λ – Lag phase
AD – *Abiotrophia defectiva*
AST – Antimicrobial susceptibility testing
ATCC – American Type Culture Collection
BMD – Broth microdilution assay
CAMH – Mueller Hinton II Broth cation adjusted
CFUs – Colony forming units
CLI – Clindamycin
CLSI – Clinical Laboratory Standards Institute
CLSM – Confocal laser scanning microscopy
DAP – Daptomycin
DNA – Deoxyribonucleic acid
EPS – Extracellular polymeric substances
ERY – Erythromycin
EUCAST – European Committee on Antimicrobial Susceptibility Testing
FICI – Fractional inhibitory concentration index
FOF – Fosfomycin
G6P – Glucose 6-phosphate
GC – Growth control
GE – *Granulicatella elegans*
GEN – Gentamicin
h – Hours
H_0 – Null hypothesis
IMC – Isothermal microcalorimetry
k – Growth rate constant
LEV – Levofloxacin
MBBC – Minimum biofilm bactericidal concentration
MBEC – Minimum biofilm eradication concentration
MHIC – Minimum heat inhibiting concentration
MIC – Minimal inhibitory concentration
min – Minutes
NG – Negative control
NVS – Nutritionally variant streptococci
PEN – Benzylpenicillin
PJI – Prosthetic joint infection
PK/PD – Pharmacokinetic/Pharmacodynamic
ProtK – Proteinase K
R – Resistant
RIF – Rifampicin
SA – *Streptococcus agalactiae*
sec – Seconds
SEM – Scanning electron microscopy
SO – *Streptococcus oralis*
SP – *Streptococcus pyogenes*
spp - Species
TSA/TSB – Tryptic soy agar / Tryptic soy broth

Zusammenfassung

Der zunehmende Einsatz von Implantaten ist in der modernen klinischen Praxis unerlässlich geworden und hat einen enorm positiven Einfluss auf die menschliche Gesundheit. Als Folge davon nimmt auch die Prävalenz von implantatassoziierten Infektionen, insbesondere von Infektionen der Gelenkprothesen, zu und bleibt eine der größten Komplikationen, mit denen Wissenschaftlern und Ärzte heutzutage in der Orthopädie konfrontiert sind.

Die Biofilmbildung spielt eine zentrale Rolle bei implantatassoziierten Infektionen. Aufgrund der erhöhten Resistenz von Biofilmen in der Antibiotikatherapie ist die Behandlung von Biofilm-Infektionen besonders anspruchsvoll und führt zu einer weltweiten Morbidität. Während die Infektionshäufigkeit nach orthopädischen Eingriffen gering ist, sind die Reinfektionsraten nach einer Infektion hoch. Zur Vermeidung eines Infektionsrückfalls ist die vollständige Beseitigung des Biofilms von größter Wichtigkeit, was ein evidenz-basiertes, umfangreiches Wissen zur richtigen Antibiotikaauswahl voraussetzt. Routinemäßige Suszeptibilitätstests zur Bestimmung der Antibiotikasuszeptibilität spiegeln jedoch nicht die Fähigkeit des Antibiotikums wider, Bakterien abzutöten, wenn diese in einem Biofilm wachsen. Viele *in vitro* Biofilm-Modelle, die auf Färbe-, Molekular- oder Mikroskopie-Techniken basieren, wurden im letzten Jahrzehnt zur Bewertung von Anti-Biofilm-Strategien entwickelt, jedoch hat sich noch keine Standardmethode etabliert.

Grampositive Bakterien im Mundraum, wie Streptokokken, *Abiotrophia* und *Granulicatella* Spezies, sind meist für hämatogene implantatassoziierte Infektionen verantwortlich, die durch Zahnmanipulation oder Ferninfektionen ausgelöst werden. Obwohl diese Infektionen seltener sind, stellt ihre Behandlung eine große Herausforderung dar. Die geringere Wirksamkeit der derzeitigen Therapien gegen diese Bakterienarten gibt der Forschung den Anstoß zur Identifizierung optimierter Therapien mit Antibiofilmwirkung.

Ziel dieser Arbeit war es, neue Erkenntnisse über die aktivsten Antibiotika gegen Biofilme von *Streptococcus*, *Abiotrophia* und *Granulicatella* Spezies durch die Etablierung einer zuverlässigen und hochsensiblen *in vitro* Methode auf der Basis der isothermen Mikrokalorimetrie (IMC) zu erlangen.

Unter Verwendung von porösen Glasperlen als Oberfläche für das Biofilmwachstum, konnten wir zeigen das die IMC als ein geeignetes analytisches Verfahren, zur Untersuchung von biofilmbildenden Stämmen, einschließlich anspruchsvoller Spezies, und ihrer Empfindlichkeit gegenüber antimikrobiellen Substanzen, ist. Die Auswertung der antimikrobiellen Aktivität gegen Streptokokken-Biofilme ergab eine schlechte Antibiofilmaktivität von Benzylpenicillin und Rifampicin (als Monotherapie oder in Kombination), was die Hypothese widerlegt, dass die Zugabe von Rifampicin zu einem β-Lactam-Antibiotikum eine synergistische Wirkung bei Streptokokken-Biofilmen hat. Ein besseres Ergebnis wurde bei Zwei-Paar-Antibiotika-Kombinationen gefunden, bei denen eines der Antibiotika einen mutmaßlich tödlichen Effekt auf „Persisters“ hat, wie z. B. Daptomycin oder Gentamicin. Unsere Ergebnisse deuten auch darauf hin, dass die Kombination eines Dispersionsmittels mit herkömmlichen Antibiotika den Zugang zu den Bakterien innerhalb des Biofilms erleichtern kann.

Insgesamt stellt diese Arbeit ein zuverlässiges *in vitro* Modell für die Biofilmprüfung dar und liefert neue – bisher unbekannte – Daten, die für die Behandlung von implantatassoziierten Infektionen durch Streptokokken und verwandten Spezies relevant sind, wobei vor allem auch betont wird, wie wichtig die Wahl der bestmöglichen Therapie für jede Art von bakterieller Infektion ist.

Abstract

The increasing use of indwelling foreign bodies has become essential in modern day clinical practice and it entails an enormously positive impact on human health. In consequence, the development of device-related infection, in particular prosthetic join infections, is also increasing and remains one of the major complications that scientists and clinicians face within orthopaedics nowadays.

Biofilm formation play a pivotal role in implant-associated infections. Due to the increased tolerability of biofilms to antibiotic therapy, the treatment of biofilm infections is particularly challenging causing worldwide morbidity. While the frequency of infection following orthopaedic surgery is low, once infection occurs, the rates of reinfection are high. To avoid infection relapse, the complete eradication of the biofilm is of paramount importance, which requires high-quality evidence on the choice of antibiotics. However, routine susceptibility tests to determine antibiotic susceptibility do not reflect the ability of the antibiotic to kill bacteria when growing in a biofilm. Many *in vitro* biofilm models based on staining, molecular or microscopy techniques have been developed during the last decade for the evaluation of antibiofilm strategies, however no standard method have been established yet.

Gram-positive bacteria present in the oral cavity, such as streptococci, *Abiotrophia* and *Granulicatella* species are mostly responsible for haematogenous device-infections, triggered by dental manipulation and remote infections. While these infections are rarer, their treatment presents a major challenge. The reduced effectiveness of current therapies against these bacterial species spurs research for the identification of optimized therapies with antibiofilm action.

The aim of this work was to gain new insights about the most active antibiotics against biofilms from *Streptococcus*, *Abiotrophia* and *Granulicatella* species through the establishment of a reliable and highly sensitive *in vitro* method based on isothermal microcalorimetry (IMC).

Using porous glass beads as a surface to grow biofilm on, we presented IMC as a suitable analytical tool for the investigation of biofilm-forming strains, including fastidious species, and their susceptibility to antimicrobials. Evaluation of the antimicrobial activity on streptococcal biofilms revealed a poor antibiofilm activity of benzylpenicillin and rifampicin (as monotherapy or in combination), rejecting the hypothesis that the addition of rifampicin to a β-lactam-antibiotic has a synergistic effect on streptococcal biofilms. A better outcome was found with two-pair antibiotic combinations where one of the antibiotics presented a putative killing effect on persiters, such as daptomycin or gentamicin. Our results also suggest that coupling a dispersal agent with conventional antibiotics may facilitate their access to the bacteria within the biofilm.

Overall, this work provides a reliable *in vitro* model for biofilm testing and generates new data – previously unknown – relevant for the treatment of implant-associated infections caused by *Streptococcus* and related species, emphasizing the importance of choosing the best possible therapy for each type of bacterial infection.

1. Introduction

1.1 Streptococci and related species

Streptococci were first described in Germany in 1868 by Billroth, being in the successive years associated to a variety of diseases.[1] They are members of the normal flora – predominantly in the oral and upper respiratory tract flora or the urogenital flora[2] – and opportunistic pathogens able to cause diverse clinical disease manifestations that range from subacute to acute or even chronic.[3] Significant human diseases are pneumonia, endocarditis, osteomyelitis, meningoencephalitis or urinary tract infections, along with severe infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome.[4] The genus *Streptococcus* is constituted by a heterogeneous group of bacteria with more than 50 species and ongoing adjustments in their nomenclature and taxonomy.[5] Indeed, one of the most recent changes was the establishment of two new genera, *Abiotrophia* and *Granulicatella*, split off the *Streptococcus* genus by genetic and phenotypic information.[6]

Abiotrophia and *Granulicatella* species were first described in 1961 by Frenkel & Hirsch as a new type of viridans group streptococcus and later on referred as nutritionally variant streptococci (NVS) due to their fastidious nutritional requirements.[7] These organisms hardly grow in sheep blood agar where streptococci usually grow, but require from supplementation with L-cysteine or pyridoxal hydrochloride.[8] They are found as normal inhabitants of the oral cavity, the throat flora and the urogenital and intestinal flora[9, 10] and alike some streptococci, they are frequently found as opportunistic agents of infection.[11, 12] *Abiotrophia* and *Granulicatella* species have emerged as causative pathogens for serious systemic infections such as infective endocarditis[13] and other infections including those associated to indwelling medical devices.[14-16]

1.2 Bacterial biofilms and clinical implications

Bacterial biofilms are built of planktonic cells (single-cells) attached to each other and/or a surface and frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS), which generally consist of lipopolysaccharides, proteins, lipids, glycolipids, and nucleic acids.[17] Biofilm formation is a protected mode of growth for bacteria to survive in diverse environments and disperse to colonize new niches.[18]

Generally, biofilm formation involve a complex process that begins with a first step called reversible attachment, in which planktonic cells have transient interactions with a surface, leading eventually to an irreversible attachment stage.[19] Following irreversible attachment, the adhered cells multiply and start secreting the EPS that encloses the cells and conforms the biofilm matrix, allowing the formation of small aggregates of bacteria called microcolonies.[20] As the biofilm matures, these microcolonies develop into large cellular aggregates encased by the matrix. Finally, in response to changing environmental conditions, bacteria within biofilms can escape the biofilm and disseminate to new locations.[21]

The process of biofilm formation typically results in an alteration in the bacterial phenotype in terms of growth rate and gene transcription of biofilm-embedded cells compared with that of their planktonic counterparts.[22] Biofilm-associated bacteria exhibit dramatically decreased susceptibility to antimicrobial agents and to the host immune system.[23] There are several mechanisms that have been proposed to contribute to biofilm phenotypic resistance such as a reduced diffusion of polar and charged antibiotics through the exopolysaccharide matrix, a more effective horizontal gene transfer between bacteria within biofilms supporting the development of resistance to antibiotics or an intrinsically increased mutagenesis in biofilms that contributes to adaptation processes including antibiotic resistance.[24] Besides, the existence of a subpopulation of antibiotic tolerant phenotype in the biofilm called “persisters” seems to be a significant factor in the resistance of biofilms to antimicrobials.[18]

Unlike resistant bacterial cells, where resistance mechanisms arise from genetic changes that block antibiotic activity, persisters present a transient non-heritable phenotype that is thought to be less sensitive to antibiotics because the cells are not undergoing cellular activities that antibiotics can corrupt, which results in tolerance. In contrast to resistant cells, persisters do not lead to an increase in the MIC compared with susceptible bacteria and cannot replicate in the presence of the antibiotic, but they are able to survive the antibiotic treatment and regrow once the level of antibiotics drops.[25]

Bacterial biofilms have become a crucial speciality in medicine and medical science due to its impact in the development and treatment of chronic infections. Researchers have estimated that 60 to 80 percent of microbial infections in the human body are caused by bacteria growing as a biofilm, including those related to endocarditis, cystic fibrosis, periodontitis, rhinosinusitis, osteomyelitis, non-healing chronic wounds, meningitis, urinary tract infections or prosthesis and implantable device- related infections.[26-28] Due to the increased tolerability of biofilms to antibiotic therapy, the treatment of biofilm infections is particularly challenging causing worldwide morbidity.[20]

1.3 Prosthetic joint infections

The increasing use of indwelling foreign bodies has become essential in modern day clinical practice and it entails an enormously positive impact on human health. Joint replacement is a life-enhancing procedure for millions of people worldwide each year.[29] However, the development of device-related infection by bacterial pathogens leads to delayed healing, compromised function, pain, prolonged treatment and great socio-economic costs.[30]

Prosthetic joint infection (PJI) is a rare but devastating surgical complication, which occurs in approximately 1-2% of all hip, shoulder or knee arthroplasties[31] and in between 1.9-10.3% of elbow replacements.[32] Most PJI cases are caused through intra-operative inoculation of microbes. Nevertheless, there is a risk for a haematogenous infection from a distant primary focus at any time after implantation.[33] PJI can manifest either as an early infection (within 4 weeks after implantation), predominantly caused by high-virulent pathogens (e.g. *Staphylococcus* aureus, streptococci, enterococci), or as a delayed infection (typically between 3 and 72 months after implantation) caused by low-virulent pathogens (e.g. coagulase-negative staphylococci or *Cutibacterium* species).[33]

Current treatment of implant-associated infections includes the delivery of a high dose of antibiotics according to the severity of the infection, and if symptoms persist, then surgical removal of the infected implant must take place.[20] Nevertheless, debridement, antibiotics, and implant retention may be attempted in some acute cases, especially in PJI. Although prophylactic administration of antibiotics prior to surgery has been shown to be highly successful in reducing infection rate, it has minimal or no protective effects in surgeries involving prosthetic implants.[34]

Over the last years, extensive work has been done in order to find new treatment procedures and therapeutic solutions with the focus on PJIs caused by staphylococcal species, the most common pathogen for these infections.[29] However, little has been published about other Gram positive bacteria causing prosthetic infections such as *Streptococcus*, *Abiotrophia* or *Granulicatella* species, while being less common, their treatment presents a major challenge. The reduced effectiveness of current therapies against these bacterial species spurs research for the identification of optimized therapies with antimicrobial activities of antibiofilm action. Indeed, even with surgical approach, the use of antibiofilm active agents remains of utmost importance and therapeutic decisions requires careful consideration.[35]

Streptococcus species are the second most frequent microorganisms isolated from indwelling medical devices, accounting for between 4% and 12% of the cases of PJI.[36] The optimal antimicrobial treatment for streptococcal PJI is unknown. Current guidelines recommend the use of intravenous benzylpenicillin or ceftriaxone (often for 2 weeks) before a shift to a high dose of oral amoxicillin,[37] but these antibiotics may not be good antibiofilm agents.[38, 39] Some authors recommend addition of rifampicin to amoxicillin for the treatment of rifampicin-susceptible streptococcal PJI.[37] The role of other antibiotics with a better antibiofilm profile has not been consistently explored in clinical studies.

Abiotrophia and *Granulicatella* species are mostly responsible for haematogenous infections, triggered by dental manipulation and remote infections (Figure 1). Infections by these species lead to high rates of complications and treatment failure.[40] These are mainly due to the difficulty to recover and identify isolates from clinical specimens, determining a delay in the beginning of the appropriate antimicrobial therapeutic regimen.[41, 42] There is no standardized therapeutic approach for the non-endocarditis infections caused by *Abiotrophia* and *Granulicatella* species. Moreover, only a few studies have investigated the antimicrobial susceptibilities of these species in planktonic bacteria[43-45] and, prior our study, no experimental data were available regarding the activity of antibiotics against biofilms.

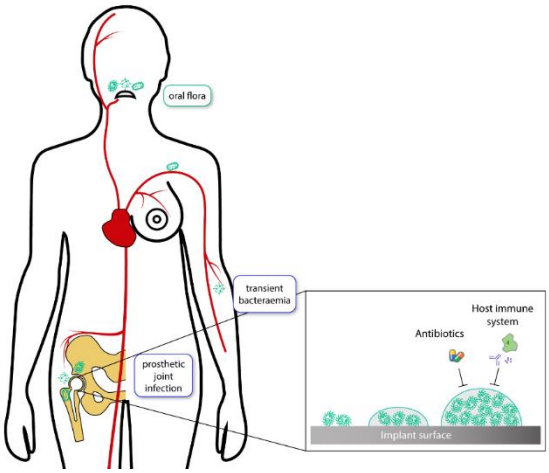

Figure 1. Schematic illustration of the most typical route of infection for NVS causing prosthetic joint infections. Oral bacteria gain access into the bloodstream through the tooth-tissue interface, causing transient bacteraemia, and finally reaching and colonizing indwelling medical devices. Following colonization, the biofilm formation on the implant surface takes place. Mature biofilms become highly tolerant to antibiotics and to the host immune system.

Limited evidence derived from clinical case reports shows favourable results in certain subgroups of patients when treated with certain antibiotic combinations.[46] Based on experimental studies, rifampicin combination treatment has been established for staphylococcal implant-associated infections, whereas an optimal treatment for species, such as streptococci, has not yet been determined.[35]

Emerging treatment approaches targeting the EPS of biofilms are getting increasing attention in the treatment of biofilm infections.[47] Despite the fact that the EPS composition varies among different bacterial species and environmental conditions, essential components including exopolysaccharides, proteins and extracellular DNA are part of virtually all biofilm structures.[48] Thus, a proposed strategy is to target specifically the protein fraction of the biofilm matrix by applying proteases, a class of enzymes that catalyse the cleavage of the peptide bonds in proteins.[49] The application of proteases to bacterial cultures have been shown to reduce the formation of biofilm[50] or to disperse established biofilms.[51]

1.4 Clinical relevance of antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is a laboratory procedure to identify which antimicrobial regimen is specifically effective against the corresponding microorganism being tested. The currently available international standard methods for AST have been developed by the Clinical Laboratory Standards Institute (CLSI)

and the European Committee on Antimicrobial Susceptibility Testing (EUCAST), providing also breakpoints for reporting the results of these methods. Several other commercial test assays have been developed to facilitate AST, such as Etest and disk diffusion testing, providing easy-to-read zones of inhibition, but presenting different degrees of agreement compared to the reference methods.[52-54] Besides, at present, there are no specific recommendations on susceptibility testing and interpretation of the minimal inhibitory concentration (MIC) for *Abiotrophia* and *Granulicatella* species provided by EUCAST, whereas the CLSI guidelines give indications on susceptibility testing and suggest interpretative criteria for this class of microorganisms, being therefore the representative guideline in most of the current available data.[8]

The aim of any AST is to accurately estimate the activity of single or combined antimicrobial agents against the pathogen of interest, allowing also the detection of resistance development. However, classic AST methods providing the susceptibility breakpoints and the PK/PD parameters that predict therapeutic success are performed with planktonically growing bacteria. Since biofilm-growing microorganisms are significantly more tolerant to antibiotics than planktonic cells, the classic susceptibility tests cannot be used to predict the therapeutic success for biofilm infections.[46] Indeed, a poor correlation between *in vitro* susceptibility data and clinical outcome in patients with biofilm-related infections after antibiotic therapy has been demonstrated.[41, 55, 56]

There is a lack of standard methods for AST of microbial biofilms that could help guide decisions about treatment in the clinical practice. Numerous approaches have been developed in the past years to evaluate the efficacy of antibiofilm strategies based on assays that identify the minimum biofilm eradication concentrations (MBEC) through *in vitro* models.[57] Some examples of these alternative techniques are based on the assessment of biofilm biomass, most typically in 96-well microtiter plates, such as the Calgary biofilm device, using staining methods (e.g. crystal violet or Syto9 staining) and a detection system like optical density (OD) or confocal laser scanning microscopy (CLSM) to detect live/dead cells.[58] Enumeration of viable cells through plate counts to calculate antimicrobial efficacy have also been described. [59, 60]

The biggest challenge of all these *in vitro* methods to analyse biofilms is to provide results that are highly representative of the antimicrobial efficacy against biofilms *in vivo*. There is no evidence yet that the introduction of standard biofilm susceptibility tests in clinical practice would improve the patient outcome, but such methods represent a valuable tool for research purposes and in the process of regulatory approval.[57]

1.5 Isothermal microcalorimetry

Isothermal microcalorimetry (IMC) is a sensitive technique used to monitor in the microwatt range any exothermic or endothermic reaction related to physical and chemical process in the tested samples. In the field of clinical microbiology, IMC enables a precise real-time monitoring of the heat flow related to the microbial metabolism, allowing the evaluation of microbial viability in the presence or absence of antimicrobial compounds at any time point. Quantitative data regarding the antimicrobial effect against different bacterial species can be collected by monitoring the thermokinetic parameters related to microbial cell replication and by measuring deviations in the heat flow of the treated samples compared to control samples.[61]

Measurements are performed at a constant temperature and the heat production is detected with a sensitivity on the order of 0.2 μW, which makes it possible for a small concentration of active microorganisms (between 2.5×10^4 and 1×10^5 CFU/mL) to produce a detectable signal.[62]

In contrast to other methods for the analysis of antibiofilm treatments, IMC is quite simple and does not require the use of dying reagents or time-consuming procedures for sample preparation and allows the use of the

samples after measurement for further analysis with other techniques. In IMC, generally, samples are placed in glass ampoules, which are sealed for air tightness, and introduced into one of the measuring channels, where heat flow can be measured for the desired time period (e.g. from hours to days).[63]

IMC has been extensively used for the evaluation of metabolism and growth of living cells in culture in medical and environmental microbiology and it appears as a useful tool as well for the study of different approaches related to biofilms, such as biofilm co-aggregation,[64] investigation of multispecies biofilms,[65] antimicrobial efficacy against biofilms[66] or more recently, for the detection of the presence of persisters in biofilms.[67]

2. Objectives of the work and working hypotheses

Globally, the aim of the study was to investigate the antimicrobial susceptibility of *Streptococcus* and the fastidious *Abiotrophia* and *Granulicatella* (formerly NVS) species to different classes of antibiotics – typically used for implant-associated infections – with a special focus on the antimicrobial activity towards biofilms.

Due to the lack of a universally accepted standardized measure of antimicrobial susceptibility in bacterial biofilms, the objective of the first original paper included in this cumulative dissertation was to determine the reliability and accuracy of isothermal microcalorimetry for the *in vitro* assessment of biofilm susceptibility to antimicrobials. To this end, *Streptococcus pyogenes* ATCC 19615 was used as reference bacteria. Additionally, the capability of both *Abiotrophia defectiva* and *Granulicatella elegans* to form biofilm *in vitro* using porous glass beads was investigated by scanning electron microscopy.

The objective of the second original work was to determine the antimicrobial susceptibilities of streptococcal species most frequently found in PJI. Through the antimicrobial analysis of different classes of antibiotics, alone and in combination against planktonic and biofilm-growing bacteria, we aimed to elucidate the most promising therapeutic approach against streptococcal biofilms. The null hypothesis (H_0) to be tested stated that the addition of rifampicin to a β-lactam-antibiotic has a synergistic effect in eradicating the biofilm of the three tested streptococcal species.

In addition, the effect of protease activity on streptococcal biofilms was investigated. The self-produced extracellular polymeric matrix of biofilms is believed to limit the penetration of antimicrobials to cells deeply embedded within the biofilm. Thus, disruption of such essential components could revert the physical tolerance of the bacteria in the biofilm to antimicrobials. To test this hypothesis, biofilms of *S. oralis*, *S. agalactiae* and *S. pyogenes* were exposed to different concentrations of proteinase K and then, the susceptibility to antibiotics was evaluated.

The third original paper had as objective the evaluation of the efficacy of single and combined antibiotics on *A. defectiva* and *G. elegans* biofilms, and to investigate the antibiofilm activity of gentamicin towards blood culture isolates from both species. The H_0 to be investigated – based on the results obtained with the ATCC standard laboratory strains – stated that biofilms from *A. defectiva* strains are more tolerant to the activity of gentamicin compared to *G. elegans* strains.

Overall, from a clinical point of view, the objective of this work was to provide the first insights for the best treatment strategy in terms of optimizing antimicrobial therapy to the narrowest and most active agents against *Streptococcus*, *Abiotrophia* and *Granulicatella* species mostly involved in haematogenous device-infections.

3. Material and Methods

3.1 Bacterial strains and antimicrobial agents

Five ATCC laboratory standard strains, namely *Streptococcus agalactiae* (ATCC 13813), *Streptococcus oralis* (ATCC 35037), *Streptococcus pyogenes* (ATCC 19615), *Abiotrophia defectiva* (ATCC 49176) and *Granulicatella elegans* (ATCC 700633) were used in this work. Moreover, ten *A. defectiva* and six *G. elegans* blood culture isolates, provided by the UCLA Department of Pathology and Laboratory Medicine, were employed for gentamicin testing. Bacteria were stored on a cryovial bead preservation system at -80 °C.

Fosfomycin was provided as purified powder by the manufacturer (InfectoPharm, Heppenheim, Germany). Daptomycin for injection was supplied by the manufacturer (Novartis Pharma Schweiz, Bern, Switzerland). Benzylpenicillin (Penicillin G 1 Mega) was purchased from Grünenthal Pharma AG (Mitlödi, Switzerland). Levofloxacin hemihydrate injectable solution (5 mg/mL; Sanofi Aventis Pharma AG, Zurich, Switzerland), gentamicin injectable solution (40 mg/mL; Ratiopharm GmbH, Ulm, Germany), clindamycin injectable solution (150 mg/mL; Fresenius Kabi GmbH, Bad Homburg, Germany) and rifampicin powder (Sandoz AG, Steinhausen, Switzerland) were purchased from the respective manufacturers. Stocks from each antibiotic at the desired concentration were prepared, aliquoted and stored at -20 °C until further use.

Proteinase K was purchased (P8107S, New England Biolabs, Ipswich, MA) and stored at -20 °C.

3.2 Culture media

Bacteria were cultivated on solid medium by using Tryptic Soy Agar (TSA) supplemented with 5% v/v defibrinated sheep blood and, in the case of *A. defectiva* and *G. elegans* strains, 10 mg/L pyridoxal hydrochloride were incorporated to the medium. Liquid bacterial cultures were prepared in Tryptic Soy Broth (supplemented TSB) and Mueller Hinton II Broth cation adjusted (supplemented CAMH), both supplemented with 2.5% v/v lysed horse blood and, if applicable, 10 mg/L pyridoxal hydrochloride.

The growth medium for testing daptomycin and fosfomycin was supplemented with 50 mg/mL calcium chloride and 25 mg/L glucose 6-phosphate (G6P), respectively. To exert its bactericidal activity, fosfomycin must reach the bacterial cytoplasm via the hexose monophosphate transport system, which is induced by G6P,[68] whereas in the case of daptomycin, it undergoes a structural transition to insert into the cell membrane, a process that appears to be facilitated by calcium ions.[69]

3.3 *In vitro* model for biofilm formation

Current *in vitro* standardized antimicrobial testing protocols employ predominantly planktonic bacteria. However, such microbiological tests cannot predict treatment outcome in device-related infections.[56] To overcome this limitation, in this study we applied an optimized *in vitro* assay using porous sintered glass beads.

In 1992, a study by Vergères and Blaser described a complex *in vitro* method to determine bactericidal activity of antibiotics on biofilms of bacteria adhering to glass beads.[70] Subsequently, Zimmerli and co-authors simplified the assay by using only selected antibiotic concentrations instead of the pharmacokinetic model and showed the capability of this *in vitro* model to predict the cure rate in an experimental device-related infections model in guinea pigs.[56]

For our study, we followed the assay described by Zimmerli *et al.*[56] with some modifications. The porous glass beads (diameter, 4 mm; pore size, 60 µm; porosity, 0.2 m^2/g) in this model served as support material for

bacterial adhesion. In order to allow biofilm formation on the glass beads, a microbial inoculum was prepared in a 50 mL polypropylene tube according to a McFarland standard turbidity of 1.0 and subsequently diluted 1:10 in supplemented TSB. The tube containing 1 mL medium per incubated bead (with a maximum of 10 beads per tube) was incubated for 24 h at 37 °C. After incubation, the medium was aspirated, and beads were washed three times with saline in order to avoid a carry-over of bacteria suspended in the incubation medium. The number of bacteria adhering on the glass beads was determined by sonication and colony counting (see section 3.5.4). Moreover, the biofilm formed in the beads was visualized by scanning electron microscopy (SEM).

3.4 Scanning Electron Microscopy

Direct observation of the bacterial adhesion and biofilm formation on abiotic surfaces represents a highly informative approach to better understand the development of biofilms.

SEM has been shown to be a suitable tool for detailed observation of individual cells and their morphology in the biofilm.[71] Thanks to its level of magnification and resolution, it enables the observation of the overall shape of microorganisms composing the biofilm, as well as their spatial organization within the extracellular polymeric matrix and/or the interface with the abiotic surface.

For SEM imaging, biofilms formed on porous glass beads as previously described (see section 3.3) were chemically fixed, dehydrated in ethanol percent series and then dried at the critical point. Samples were mounted on aluminium stubs, coated with 20 nm layer of gold-palladium, and then observed in the microscope.

3.5 Antimicrobial Assays

A total of seven antibiotics from different classes were selected to be tested against the bacterial species under study. The antibiotic selection was based on inclusion of different mechanisms of action, as depicted in figure 2, as well as on the affordability of the selected antibiotics in the clinical setting. Benzylpenicillin and rifampicin, as the current recommended antibiotic treatment in the management of streptococcal PJI (see "1.3 Prosthetic joint infections" at the introduction section), were included in our study and compared to the activity of fosfomycin, daptomycin, gentamicin, clindamycin or levofloxacin.

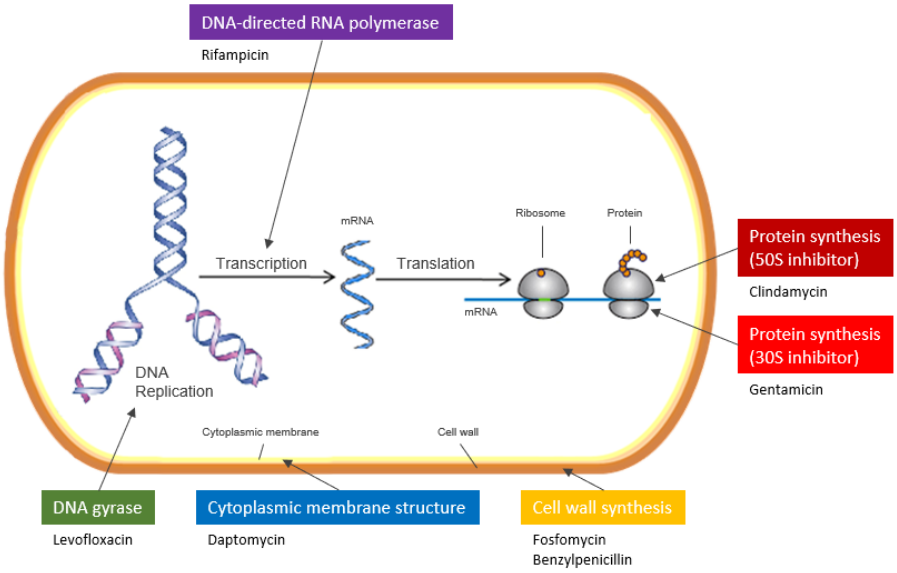

Figure 2. Cellular targets within the bacterial cell of the antibiotics under study. Image adapted from Medicine, Published 2016, DOI:10.5772/61327. Available from: https://www.intechopen.com/books/smart-drug-delivery-system/antibiotic-drug-delivery-systems-for-the-intracellular-targeting-of-bacterial-pathogens

3.5.1 Etest

The susceptibility of the bacterial strains to the action of each antibiotic *in vitro* was determined by Etest, a common test used by laboratories to determine the MIC. Etest was performed following the manufacturer's instructions. MIC_{Etest} was determined as the concentration value where the inhibition ellipse intersected the scale of the strip.

3.5.2 Broth microdilution assay

Antimicrobial susceptibility testing was performed using the standard broth microdilution assay (BMD) in supplemented CAMH according to the CLSI.[72] MIC_{BMD} was defined as the lowest concentration of antibiotic that prevented visible growth of bacteria in a broth dilution susceptibility test. The EUCAST and CLSI breakpoint recommendations were used to categorize the tested strains as susceptible, intermediate or resistant to each antibiotic. According to this criterion, a bacterial strain is said to be (a) "susceptible" to a given antibiotic when it is inhibited *in vitro* by a concentration of the drug that is associated with a high likelihood of therapeutic success, or (b) "intermediate" when it is inhibited *in vitro* by a concentration of the drug that is associated with an uncertain therapeutic effect or (c) "resistant" when it is inhibited *in vitro* by a concentration of this drug that is associated with a high likelihood of therapeutic failure.[73]

3.5.3 Isothermal Microcalorimetry

The *in vitro* activities of different classes of antibiotics against planktonic and biofilm-embedded cells was determined by IMC, using a TAM III-48 microcalorimeter with a detection limit of heat production of 0.2 µW and equipped with 48 minicalorimeter channels.

IMC is able to measure the heat flow of biological processes, allowing the real-time monitoring of heat produced by metabolically active bacterial cells in culture.[74] Hence, IMC was applied in our study to assess the effect of antimicrobials on bacterial growth by evaluating the impact caused on the heat-flow curve, such as reduction and/or delay of the heat production, compared to the growth control (not exposed to antimicrobials). IMC data were converted into microbiologically relevant information such as growth rate constant (k, h^{-1}) and lag phase (λ, h) by applying mathematical growth models, as previously reported.[75-77]

The effect of antibiotics on planktonic bacteria was evaluated co-incubating the antibiotic with the tested strain and measuring the inhibition of heat production. Bactericidal activity on biofilms was examined by monitoring the heat production related to the presence of viable/replicating bacteria in treated samples after removal of the antimicrobial agent.

According to this, the minimum heat inhibiting concentration (MHIC) was defined as the minimum concentration of antibiotic able to suppress the metabolic heat production of planktonic bacteria and the minimum biofilm bactericidal concentration (MBBC) as the lowest concentration that strongly reduced the cell viability within the biofilm leading to undetectable heat flow values.

3.5.4 Sonication and colony counting

To evaluate the reduction/eradication of biofilm-embedded cells after antibiotic treatment, samples were subjected to sonication. Sonication acts applying sound energy to agitate particles in a sample, in this study with the purpose of disaggregating the extracellular polymeric matrix of the biofilms and extract the embedded cells from the glass beads for colony counting of viable bacteria.

The number of bacteria cells adhering on the glass beads was determined by transferring washed beads to Eppendorf tubes filled with 1 mL Phosphate Buffered Saline (PBS; pH 7.4, 10mM). Bacteria adhering to the beads were removed by vortexed for 30 sec, followed by 1 min sonication in a bath sonication instrument at 40 kHz and 0.2 W/cm^2 and finally vortexed for 30 sec. Fifty microlitres of ten-fold serial dilutions of the sonication fluid were plated on supplemented TSA and colonies were counted after 18-24 h incubation at 37 °C under 5% CO_2 atmosphere and expressed as CFUs/mL.

The minimum biofilm eradicating concentration (MBEC) was defined as the lowest concentration of antibiotic required to kill all sessile cells resulting in the appearance of no colony after plating the sonication fluid (detection limit: < 20 CFU/mL).

3.5.5 Fractional inhibitory concentration index (FICI)

The FICI was used in our study to evaluate the potential synergistic effects of two-pair antibiotic combinations tested against planktonic cells or biofilms. The two antibiotics in combination exerted a synergistic effect if FICI was ≤0.5, an indifferent effect when FICI was >0.5 – 4 or an antagonistic effect if FICI was >4.[78]

Thus, the obtained MHICs, MBBC and MBEC values in our study were used to calculate the FICI of antibiotic combinations related to: (i) inhibition of planktonic bacteria (FICI); (ii) bactericidal activity against biofilms ($FICI_{MBBC}$); and (iii) eradicating activity against biofilms ($FICI_{MBEC}$).

FICI for all the combinations was determined as [(minimum concentration of drug A in combination) / (minimum concentration of drug A alone)] + [(minimum concentration of drug B in combination) / (minimum concentration of drug B alone)].

3.5.6 Biofilm treatment with proteinase K

The effect of proteinase-mediated biofilm disruption by proteinase K and the subsequent exposure to antibiotics against different streptococcal species was investigated. The aim was to enhance the microbial susceptibility of already established bacterial biofilms towards antibiotics.

To emphasize the effect of proteinase K, protease pre-treated biofilms were exposed to those antibiotics having poor antibiofilm activity against the tested bacterial species, namely fosfomycin, rifampicin, benzylpenicillin and levofloxacin.

Twenty-four hours old biofilms were incubated first with proteinase K. Following 1 h of incubation, increasing concentrations of antibiotics were added and the samples were incubated for a further 24 h. Then microbial viability was monitored during 48 h by IMC. Additionally, *S. oralis* biofilms were incubated with the highest tested concentration of proteinase K (100 mg/L) as control to ensure that the enzymatic treatment does not affect bacterial viability but acts in essence on the biofilm matrix.

4. Results

4.1 Validation of the *in vitro* experimental testing model

The effect of levofloxacin on planktonic and biofilm growing *S. pyogenes* cells, along with the determination of the concentration of antibiotic needed to prevent biofilm formation on the glass beads, was efficiently determined by IMC.

Exposure of *S. pyogenes* to levofloxacin (from 0.125 to 1 mg/L) resulted in a dose-dependent reduction of the heat produced by planktonic cells, where increasing antibiotic concentrations leaded to lower values in the heat flow and to a gradual decrease of the growth rate constants (*k*). The inhibitory ratios of levofloxacin against planktonic *S. pyogenes* ranged from a 24.72% inhibition, when exposed to 0.125 mg/L of antibiotic, to a 100% inhibition after exposure to 1 mg/L of antibiotic.

Similarly, the treatment of biofilms with increasing concentrations of levofloxacin (from 128 to 512 mg/L) caused a gradual increase in the lag phases (λ) of the treated samples compared to the untreated biofilm (Fig. 3), possibly due to a gradual decrease in viable bacteria on treated samples. Indeed, longer lag phases – obtained by the analysis of IMC data – were associated with a higher bactericidal effect of the drug – determined by a decreased number of bacterial colonies counted after plating of the sonication fluids – showing a good correlation between both methods, IMC and sonication and colony counting.

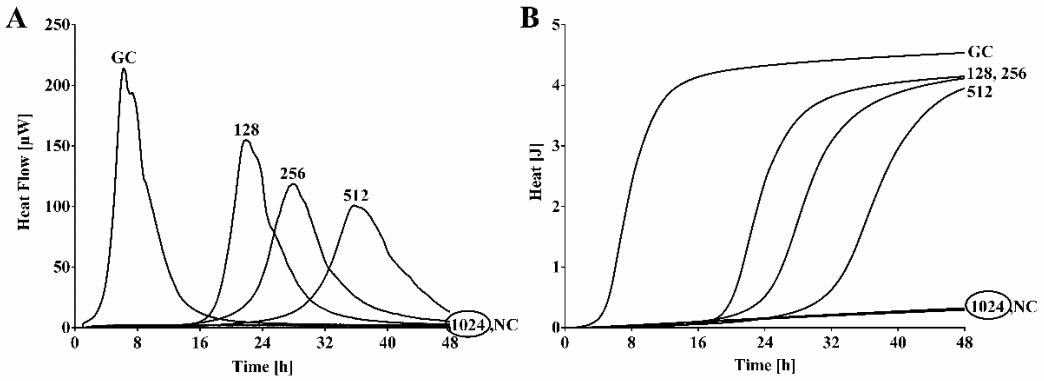

Figure 3. Microcalorimetric analysis of *S. pyogenes* (ATCC 19615) biofilm treated with different concentrations of levofloxacin. Plot A shows the heat flow (µW) produced by viable bacteria present in the biofilm after 24 h of antibiotic treatment at increasing doses (128 – 1024 mg/L) or no treatment (GC). Plot B shows the cumulative amount of heat (J) produced by each sample at any time point of the experiment. Numbers represent concentrations of levofloxacin (mg/L). Circled value represents the MBBC. GC growth control, NC negative control. Image adapted from https://doi.org/10.1007/5584_2018_291

The eradication of biofilm was achieved at a considerable higher concentration of antibiotic (1024 mg/L) compared to the inhibition of planktonic cells (1 mg/L). Furthermore, a concentration of levofloxacin up to 256 mg/L was not able to prevent biofilm formation. Hence, based on these results, levofloxacin does not seem suitable for the treatment of *S. pyogenes* biofilms despite its showed efficacy against planktonic cells.

As outcome from our study, we were able to define three parameters, namely MHICb, MBBC and MBEC, related to the antibiofilm activity of an antimicrobial compound. These parameters were implemented in our study for the examination of the antibiofilm activities of different antibiotics against other *Streptococcus* species, as well as against *A. defectiva* and *G. elegans* strains.

4.2 Visualization of biofilms by SEM

Ideally, many *in vitro* biofilm models aim to predict the performance of novel antimicrobials or antimicrobial materials *in vivo* as accurate as possible.[79] Our model, based on the growth on a three-dimensional abiotic surface, aims to simulate biofilm formation on surfaces of medical devices. Using this setup, we obtained dense and well-structured biofilms encased in extracellular matrix with high tolerance to antibiotic treatment.

A. defectiva and *G. elegans* showed the capability to adhere and form biofilm on porous glass beads after 24 h of incubation, as observed by SEM. In the microscopy images (Fig. 4), differences in the biofilm composition between both species could be visualized, where lower abundance of EPS was observed on *G. elegans* biofilm (Fig. 4B) compared to *A. defectiva* (Fig. 4D). The biofilms observed at the higher magnification showed organization of bacteria into clusters with presence of empty spaces, which most likely might become water channels.[80]

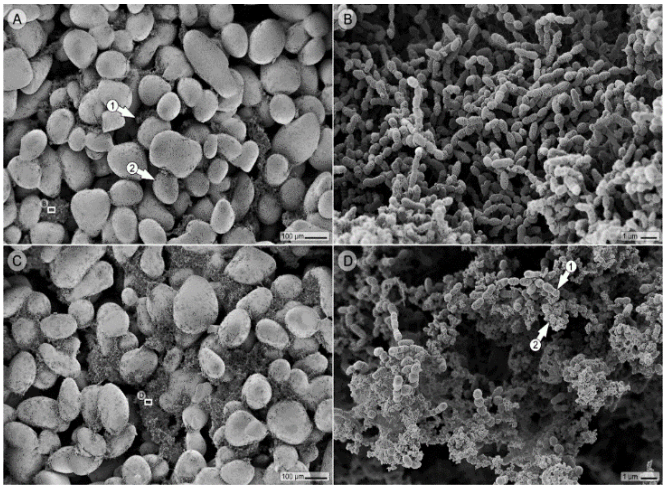

Figure 4. Biofilm formation of *G. elegans* ATCC 700633 (A and B) and *A. defectiva* ATCC 49176 (C and D) grown on porous glass beads. Images B and D are close-ups from A and C. Image A, arrows 1 and 2 indicate the biofilm and the sintered glass particles respectively. Image D, the arrows 1 and 2 point out a bacterium and the extracellular polymeric matrix of the biofilm respectively. The latter is significantly more abundant in the *A. defectiva* biofilm.

4.3 Activity of antibiotics against planktonic bacteria

4.3.1 Standard laboratory strains

Analogous susceptibility patterns towards most tested antibiotics were observed between the five ATCC strains used in this study. Rifampicin was the most active antibiotic against planktonic bacteria from all species followed by benzylpenicillin and levofloxacin, whereas fosfomycin was, with difference, the less active one. No antibiotic resistance was observed, except for *S. oralis*, which resulted resistant to daptomycin (assuming a susceptibility breakpoint of 1 mg/L) when tested with IMC, as well as *A. defectiva* and *G. elegans* strains, which both resulted resistant to clindamycin according to the CLSI breakpoints.

A scarce consistency was observed between Etest and microcalorimetry/BMD techniques when testing either clindamycin or daptomycin against *A. defectiva* and *G. elegans* strains. Low-level of agreement between BMD and Etest has being previously reported for other microorganism as well,[81] where lower MIC values for clindamycin were observed by Etest compared to BMD. Similarly, in the case of daptomycin, discrepancies in the results were also shown by Riedel *et al.*[82] who found that daptomycin MICs were 1–2 log concentrations lower by Etest compared with BMD in enterococcal isolates. In the case of *Abiotrophia* and *Granulicatella* species, Etest has been shown to be a poor measure of antimicrobial susceptibility.[54]

In our study, we present isothermal microcalorimetry as a reliable alternative, and consistent with the standard methods (BMD), also when testing fastidious microorganisms.

4.3.2 Blood culture isolates

In table 1, the results for the susceptibility of planktonic cells to gentamicin from all the tested blood culture isolates are shown. MIC/MHIC values ranged from 0.125 mg/L to 4 mg/L, correlating with data reported in other study, where gentamicin MIC > 4 mg/L was not observed.[45]

Table 1. Gentamicin susceptibilities of *A. defectiva* and *G. elegans* blood culture isolates evaluated by Etest, broth microdilution, microcalorimetry and sonication. Concentration values are expressed in mg/L.

Isolate	Identification	Gentamicin			
		MIC_{Etest}	MIC_{BMD}	MHIC	MBEC
A_01 (PEN-R)	*A. defectiva*	1.5	0.5	0.5	1
A_02 (PEN-R)	*A. defectiva*	1.5	1	1	4
A_03 (PEN-R)	*A. defectiva*	1	1	1	1
A_04 (PEN-R)	*A. defectiva*	8	2	2	8
A_05 (ERY-R)	*A. defectiva*	6	4	4	16
A_06	*A. defectiva*	6	2	2	2
A_07 (ERY-R)	*A. defectiva*	6	4	4	16
A_08 (ERY-R, CLI-R)	*A. defectiva*	2	2	2	8
A_09	*A. defectiva*	4	2	2	2
A_10	*A. defectiva*	6	2	1	4
G_01	*G. elegans*	1	0.125	0.25	0.25
G_02	*G. elegans*	4	1	1	0.5
G_03	*G. elegans*	1	0.125	0.125	0.125
G_04	*G. elegans*	1	1	1	1
G_05	*G. elegans*	2	0.5	0.5	0.5
G_06	*G. elegans*	0.75	0.5	0.25	1

PEN= benzylpenicillin; ERY= erythromycin; CLI= clindamycin; R= resistant; MIC, minimal inhibitory concentration BMD, broth microdilution assay; MHIC, minimal heat inhibitory concentration; MBEC, minimum biofilm eradicating concentration. Source: https://doi.org/10.1093/jac/dkz174 (Table 2)

A. defectiva strains presented generally higher MIC/MHIC values compared to *G. elegans* strains. Here as well, the MIC values obtained by Etest usually resulted higher than the ones obtained by BMD and calorimeter.

4.4 Activity of antibiotics against bacterial biofilms

4.4.1 Standard laboratory strains

The antimicrobial activity of different antibiotics against biofilms was evaluated by IMC and by colony counting of sonicated fluids (as described in sections 3.5.3 and 3.5.4).

The three streptococcal strains were susceptible only to higher concentrations of the tested antibiotics (mostly ranging from 16 to 1024 mg/L) when grown as biofilms if compared with the MHIC value obtained for planktonic bacteria (Table 2), except for gentamicin, where the MBBC values were within ± 1 doubling dilution of the MHIC. Hence, gentamicin represented the most active antibiotic against streptococcal biofilms.

Similarly, *A. defectiva* was susceptible to higher concentrations of most of the tested antibiotics (ranging from 8 to 1024 mg/L) when grown as biofilm compared to planktonic cells, whereas the biofilm formed by *G. elegans* resulted more susceptible to benzylpenicillin, clindamycin, daptomycin, gentamicin and levofloxacin compared to *A. defectiva* (Table 2). Notably, rifampicin was the most active antibiotic against *A. defectiva* biofilm, whereas *G. elegans* biofilm was susceptible in equal concentrations to benzylpenicillin and gentamicin.

Table 2. Antimicrobial susceptibility of planktonic (MHIC) and biofilm (MBBC) ATCC strains from *Streptococcus* spp., *A. defectiva* and *G. elegans* evaluated by microcalorimetry.

Antibiotic	MHIC (mg/L)					MBBC (mg/L)				
	SA	*SP*	*SO*	*AD*	*GE*	*SA*	*SP*	*SO*	*AD*	*GE*
FOF	64	128	128	64	32	>1024	>1024	>1024	>1024	>1024
RIF	0.128	0.064	0.128	0.016	0.016	1024	256	512	8	8
PEN	0.064	0.016	0.064	0.032	0.256	64	32	64	32	1
DAP	0.5	0.125	2	4	2	64	16	1024	16	4
GEN	4	4	8	4	1	8	4	16	32	1
LEV	1	0.5	2	0.256	0.256	1024	1024	1024	512	64

MHIC, minimum heat inhibitory concentration; MBBC, minimum biofilm bactericidal concentration; *SA, Streptococcus agalactiae* ATCC 13813*; SP, Streptococcus pyogenes* ATCC 19615*; SO, Streptococcus oralis* ATCC 35037*; AD, Abiotrophia defectiva* ATCC 49176*; GE, Granulicatella elegans* ATCC 700633; FOF, fosfomycin; RIF, rifampicin; PEN, benzylpenicillin; DAP, daptomycin; GEN, gentamicin; LEV, levofloxacin. Table adapted from Table 1 in https://doi.org/10.1093/jac/dkx265 and Table 1 in https://doi.org/10.1093/jac/dkz174

4.4.2 Blood culture isolates

As shown in Table 2, the MBBC of gentamicin for *A. defectiva* ATCC 49176 was 32 times higher than that for *G. elegans* ATCC 700633. To elucidate whether this difference in the activity of gentamicin observed between the two ATCC strains is shared by other strains, gentamicin was tested against the biofilm formed by 16 different blood culture isolates and assessed by plating of the sonication fluid after treatment.

Gentamicin tested against *G. elegans* clinical strains resulted in rather low MBEC values, ranging from 0.125 to 1 mg/L. Higher MBEC values of gentamicin, ranging from 1 to 16 mg/L, were observed when the antibiotic activity was assayed against *A. defectiva* strains (Table 1). 6 out of 10 *A. defectiva* strains had MBEC values 4–128 times greater compared with the MBEC values determined for *G. elegans* strains. These results seem to confirm our H_0.

4.5 Synergistic effect of different combinations of antibiotics

Due to the increased tolerability of biofilms to antibiotics, combinatorial therapies using more than one antibiotic for the treatment of biofilm infections are recommended.[46] The aim of a combinatorial therapy is to join the action of synergistic antibiotics to combat biofilms more efficiently while reducing the risk of antibiotic resistance. For instance, *in vitro* studies in *Pseudomonas aeruginosa* biofilms have revealed that antibiotics such as ciprofloxacin or β-lactams are effective only against metabolically active biofilm cells in outer layers, whereas colistin preferentially kill biofilm cells less metabolically active present in the inner layers,[83] thus supporting the use of a combined antimicrobial treatment. In our study, different classes of antibiotics with distinct mechanisms of action were tested in two-paired combinations against the biofilm of the five ATCC strains by IMC and by sonication and plating.

Gentamicin in combination with either benzylpenicillin or rifampicin showed a synergistic effect against the biofilm of all three tested streptococcal species, where a complete eradication of the biofilm could be achieved at a $FICI_{MBEC} \leq 0.258$, corresponding to concentrations of antibiotics 4 to 512 times lower compared to the monotherapy. In contrast, benzylpenicillin/rifampicin combination was synergistic against planktonic cells but not against biofilms, rejecting our null hypothesis.

The addition of daptomycin to either benzylpenicillin, rifampicin or gentamicin against *A. defectiva* biofilm resulted in a synergistic effect and a complete eradication of the biofilm ($FICI_{MBEC} = 0.5$) at a concentration of the antibiotics at least twice as low as those required by single antibiotics. A different combination of antibiotics,

namely gentamicin in combination with rifampicin or levofloxacin, lead to a synergistic effect against the biofilm of *G. elegans*, where biofilm eradication could be achieved at a $FICI_{MBEC}$ = 0.375.

4.6 Antibiotic activity after treatment with proteinase K

Several enzymes such as glycosidases, proteases and DNases have the potential to degrade the extracellular matrix of biofilms and, as consequence, the released planktonic cells become more accessible to antimicrobials.[84] A major constituent of biofilms are proteins, which make proteases to be considered the most potential enzymes for biofilm removal.[84] In our study, we explored the potential of proteinase K in degrading streptococcal biofilms and determined the combinatorial effect with antibiotics.

The exposure of biofilms for 1 h to 25, 50 and 100 mg/L proteinase K resulted in an increased susceptibility of the biofilms to antibiotics. As depicted in figure 4, pre-treating biofilms with 100 mg/L of proteinase K enable a complete suppression of heat production after exposure to fosfomycin, rifampicin, benzylpenicillin and levofloxacin at concentrations as low as 32, 0.256, 0.064 and 2 mg/L, respectively. Antibiotics at these concentrations displayed a minor effect against biofilms not treated with proteinase K (Fig. 4, continuous lines). Control experiments performed by incubating biofilms with proteinase K at the maximum tested concentration (100 mg/L) showed no effect on bacterial viability.

A much lower concentration of proteinase K (25 mg/L) led also to a total inhibition of heat production after treatment with 128 mg/L fosfomycin and 4 mg/L levofloxacin, concentrations similar to those found inhibiting planktonic cells (Table 2).

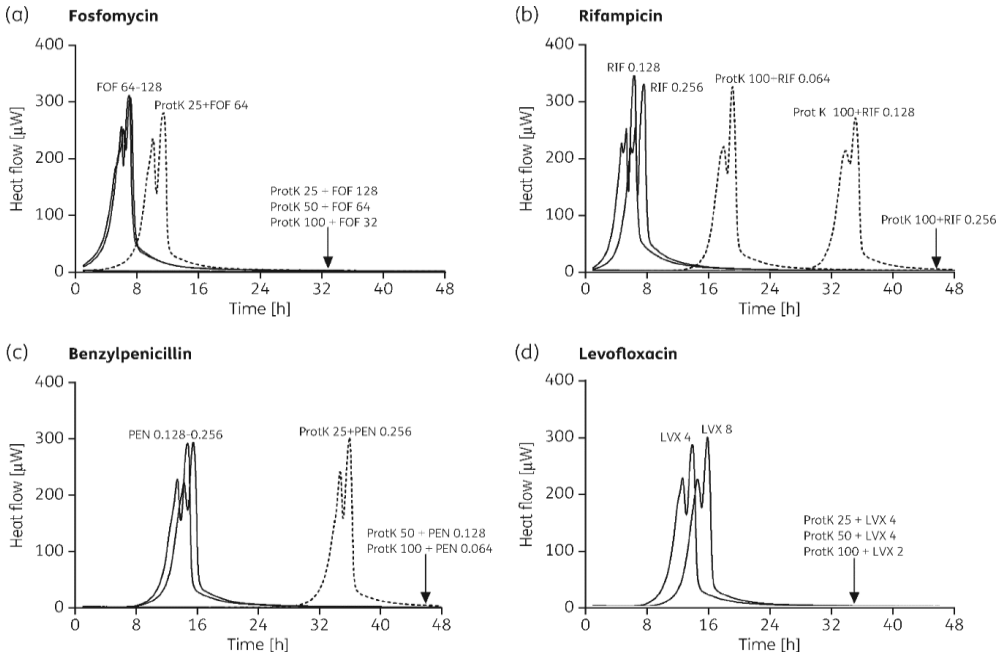

Figure 4. Evaluation of antimicrobial activity on enzymatically treated *S. oralis* biofilms by microcalorimetry. Plots show heat flow curves corresponding to biofilms pre-treated with proteinase K (protK) at different concentrations (25 mg/L, 50 mg/L and 100 mg/L) (dashed lines) or to biofilms not pre-treated enzymatically (continuous line) after treatment with sub-inhibitory concentrations of (a) fosfomycin, FOF; (b) rifampicin, RIF; (c) benzylpenicillin, PEN; or (d) levofloxacin, LEV. Numbers represent concentrations (in mg/L). Source: https://doi.org/10.1093/jac/dkx265

5. Discussion

A correct antibiotic regimen in implant-associated infections is a critical part of therapy. Tornero *et al*.[85] ratified suboptimal antibiotic choice as the most important predictor of treatment failure in cases of implant retention. The lack of evidence for an effective antimicrobial therapy against biofilms formed by streptococci, *Abiotrophia* or *Granulicatella* species makes more challenging the management of implant-associated infections caused by these microorganisms and spurs more research on this issue.

Conventionally, the parameters that predict the therapeutic success of antibiotics are determined using planktonic bacteria, thus failing to consider important factors that modulate antibiotic activity in biofilms.[57] Furthermore, some standard test, such as Etest, has been shown to be a poor measure of antimicrobial susceptibility concerning *A. defectiva* and *Granulicatella* species,[54] as shown also in our study. The analysis of antimicrobial susceptibility in biofilm-growing cells is a crucial step to establish an effective therapy.[86] Therefore, a high-throughput system for either screening the antimicrobial efficacy of different molecules or evaluating the minimal concentration able to kill or reduce biofilms is desirable. Many *in vitro* biofilm models based on staining, molecular or microscopy techniques have been developed during the last decade. Most of these techniques are destructive and only provide endpoint measurements.[87]

In our study, we investigated the suitability of IMC as an analytical tool for a fast and reliable investigation of biofilm-forming strains and their susceptibility to antimicrobials. Combined with an optimized *in vitro* biofilm model using porous glass beads as a surface to grow biofilm on, IMC allowed the evaluation of the metabolism and the dynamic behaviour of biofilm-growing cells during or after antimicrobial exposure for all bacterial species under study, including fastidious strains. Specifically, our study showed the scarce efficacy of levofloxacin against *S. pyogenes* biofilm despite its good performance against planktonic cells, supporting the idea that outcomes obtained on planktonic cells cannot be transferred to biofilms. The ability of IMC to precisely monitor in real-time the heat flow produced by a sample enables an easier identification – to some extent – of sample contamination based on the shape of the heat-flow "fingerprint", characteristic for each bacterial species, on the one hand and, on the other hand, through the fast detection of the metabolic heat of bacteria in negative samples.[88] Detection of the presence of a contaminant microorganism with other susceptibility tests is more difficult,[89] which might ultimately alter the assay results. However, IMC has also some limitations, since the measurement of the growth of surviving biofilm cells in liquid medium is rather indirect as the calorimeter cannot distinguish on these surviving cells growing as biofilm or as planktonic cells. Furthermore, IMC does not allow a direct quantification of non-replicating cells in the biofilm or of the total biomass, but as a non-destructive method, IMC-samples can be further analysed with other techniques, such as sonication or SEM, obtaining additional information complementary to calorimetric data. In our study, we could quantify the decrease of viable bacteria cells within treated biofilms by subjecting the samples to sonication.

Alternatively, flow-through-systems, where biofilms grow on tubes, slides, or membranes inside chambers under the flow of medium, have been established for the study of biofilms.[87] The medium flowing through the system flushes the non-adherent (i.e., planktonic) cells out of the system, so that the effect of specific carbon sources, antimicrobials or other compounds of interest can be directly observed on the biofilm and does not rely on the ability of biofilm cell regrowth, as is the case in our model. Besides, these systems can overcome limitations such as oxygen depletion and accumulation of metabolic waste products that occur in sealed ampoules, especially after long incubation times.[90] Unfortunately, common drawbacks of flow-through-systems are low throughput and the difficulties to sterilize some of these systems.

In the context of PJI, where not only the evaluation of antimicrobial compounds against biofilm is relevant but also to study the ability of different biomaterials or (coated) surfaces with antimicrobial properties in biofilm prevention, IMC might be of advantage as analytical tool over other popular techniques such as microscopy, mostly usable on flat and/or transparent materials. Braissant *et al.*, attested the use of IMC for the assessment of the antimicrobial properties of implant coatings obtaining good results at a minimal workload.[91] They presented a new experimental setting where the biofilm could develop within a solid media and in direct contact with the surface of interest, so that biofilm development could be continuously monitored, and the antimicrobial effect of the coated materials could be measured in terms of growth delay or growth inhibition.

Despite all the progress made in developing laboratory biofilm models, there is still a lack of standardization to assess the efficacy of antimicrobials against biofilms, as well as limited evidence regarding the ability of these *in vitro* tests to predict the *in vivo* situation and help guide clinical therapy.[57] Typically, the minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradication concentration (MBEC) are used to evaluate antibiofilm activity. In our study, we adapted these parameters ($MHIC_b$, MBBC and MBEC) to the applied tools (microcalorimetry and sonication/colony-counting) for the evaluation of the antibiofilm activity of different antibiotics in an attempt to provide results comparable to those obtained with other frequently used *in vitro* methods.

β-Lactams are the first choice antibiotics to treat streptococcal-PJI,[92] whereas in the case of *A. defectiva* or *G. elegans* there are no guidelines for managing PJI but the recommended treatment for endocarditis infections caused by these microorganisms is usually ampicillin or penicillin plus gentamicin.[93] The outcome of our study revealed a poor antibiofilm activity of benzylpenicillin and rifampicin, tested as monotherapy or in combination, against streptococcal biofilms showing consistency with the retrospective study of Akgün *et al.*,[94] in which the treatment outcome of biofilm-related PJIs caused by streptococci showed no differences when the patient's treatment regimen either included or did not include rifampicin. Although the use of rifampicin-based combinations was described in a few case reports related to PJIs caused by streptococci,[95, 96] with no clinical evidence, the effect of the use of this antibiotic in combination remains unclear. Translate into the clinical practice, the lack of antibiofilm activity observed with benzylpenicillin and rifampicin against streptococci could also explain infection relapses leading to higher failure rates in PJI caused by these microorganisms.[36, 95] As preferred therapy for streptococcal infections, β-lactams might be effective antibiotics for the initial planktonic phase of these infections, but their application in later phases or when biofilm formation is suspected should be questioned. Antibiotics with a mechanism of action dependent on cell wall synthesis, like β-lactams, might become less effective against biofilm-embedded bacteria or might even promote biofilm formation when administered at subinhibitory concentrations.[97] Furthermore, the high frequency of β-lactam resistance among *Abiotrophia* and *Granulicatella* species makes it imperative to search for alternative antibiotics with antibiofilm activity in order to find more effective combinatorial treatments.[43-45]

Persisters can remain viable over the course of drug exposure and repopulate the biofilm when the levels of antibiotic drop, causing the relapse of the infection.[98] In order to avoid infection relapse, treatment including antibiotics capable to target persisters appears as a promising approach. Lipopeptides such as daptomycin are able to cause bacterial cell death by permeabilization and depolarization of the bacterial cell membrane,[99] so that its bactericidal action is independent of the metabolic state of the bacteria, supporting its potential as anti-persisters therapy. Daptomycin have shown good antimicrobial activity against planktonic cells from a broad spectrum of Gram-positive bacteria, including most streptococci,[100] as well as *Abiotrophia* and *Granulicatella* species.[45]

To the best of our knowledge, our work provides the first insights on the antibiofilm activity of daptomycin – alone or in combinations – against the bacterial species under study. A synergistic effect was observed when daptomycin was combined with benzylpenicillin or rifampicin against *A. defectiva* biofilms but not against *G. elegans* or *S. oralis*. A possible synergistic effect of daptomycin combinations on *S. agalactiae* and *S. pyogenes* remains to be investigated. The synergism between daptomycin and benzylpenicillin could be explained by a mechanistic effect, where the initial repulsion of daptomycin by the cell net positive surface charge could be reduced by the β-lactam (mostly negatively charged),[101] favouring the binding of daptomycin to the cell surface. On the other hand, synergism between daptomycin and rifampicin has been associated in various *in vitro* and *in vivo* pharmacodynamic models of biofilm infection with preventing the emergence of daptomycin-non-susceptible strains.[102]

The ability of aminoglycosides to kill persisters has been previously demonstrated in *Escherichia coli* and *S. aureus*.[103] Moreover, some authors proposed an additional lethal effect of gentamicin, besides the inhibition of protein synthesis, by surface perturbation on *P. aeruginosa*.[104] Gentamicin has been also shown to present synergistic effect in combination with other antibiotics against biofilm from numerous bacterial species.[60, 105] In our study, gentamicin combinations showed an improved antibiofilm activity against all three streptococcal species as well as against *G. elegans*. The exact mechanism underlying the synergism of gentamicin with benzylpenicillin or rifampicin against streptococci, in addition to gentamicin/levofloxacin on *G. elegans*, remains to be elucidated. Taken into consideration that gentamicin acts mainly by inhibiting the bacterial protein synthesis, in order to be able to handle otherwise sublethal doses of the other antibiotic, the bacteria must induce certain stress responses through *de novo* protein biosynthesis.[106] Thus, the failure in timely synthesis of those stress-defence proteins could be the cause of the significant loss in cell viability observed in biofilms exposed to antibiotics in combination with gentamicin in our study. In enterococci, for instance, the gentamicin/benzylpenicillin synergistic combination has been proposed to be due to an easier intracellular uptake of the aminoglycoside facilitated by the β-lactam.[107]

A combinatorial therapy including antibiotics and dispersal agents seems a promising strategy to treat biofilm-forming infections. As seen with other enzymes,[108-110] our results showed an increased susceptibility of enzymatically treated biofilm to the subsequent action of four different antibiotics, decreasing the MBBCs to values that were comparable to the MHICs obtained for planktonic bacteria. This outcome seems to confirm the hypothesis that bacteria detached from the matrix re-enter in a planktonic state and regain normal antibiotic susceptibility. Even though the use of proteinase K as an adjuvant in the treatment of biofilm-related infections remains to be evaluated, especially in terms of safeness and effectiveness, our results could be seen as a proof of concept encouraging the use of this experimental setting to further expand studies to different potential dispersal agents, in order to find a combination treatment that could be effective and safe at the same time. The selection of the most appropriate enzyme will depend on the strain-specific composition of the biofilm matrix.[20]

From our study testing different blood-isolated bacterial strains, we could observe higher divergence in susceptibility to gentamicin among *A. defectiva* strains in comparison with *G. elegans* strains. Most *A. defectiva* strains had greater MBEC values compared to *G. elegans* strains, supporting our hypothesis that biofilms from *A. defectiva* strains are generally more tolerant to gentamicin. However, a higher number of clinical isolates would be necessary to validate our hypothesis. Considering the widespread use of gentamicin in both endocarditis[41] and non-endocarditis[111] infections and the higher frequency of *A. defectiva* causing endocarditis[112] in relation to *Granulicatella* spp., our results might suggest the need to review the current guidelines for the treatment of infections caused by *A. defectiva*.

In summary, this dissertation emphasizes the significance of biofilms in the development and treatment of implant-associated infections and underlines the importance of finding the most suitable therapy for each type of bacterial infection. As outcomes from our study, we provided potential antibiotic combinations with antibiofilm activity against the different bacterial species under investigation, including alternatives to the use of β-lactams if antibiotic resistance is suspected. Despite our efforts to expand the current knowledge regarding the antibiotic activity against biofilms of *Streptococcus*, *Abiotrophia* and *Granulicatella* species, when trying to draw conclusions or make clinical extrapolations, it is important to consider that the majority of the analysis in our study were performed using ATCC laboratory standard strains. Future studies including clinical isolates from PJI patients would certainly be much more informative and could reveal differences in the outcome between the laboratory ATCC strains and the clinical isolates, as shown in a recent study.[60]

Given the relatively high treatment failure rates and the difficulties in treating biofilm-related infections, alternative strategies to prevent and treat these infections should be considered. Current promising approaches include the development of coating implants with antibacterial and antibiofilm agents to inhibit the initial attachment of planktonic cells on the implant surface.[113] Bacteriophages – viruses that infect and replicate within bacteria – are also re-emerging as therapeutic agents, particularly in difficult-to-treat infections where successful outcomes have been recently published in patients with severe musculoskeletal infections.[114]

In the past years, several studies have been performed to demonstrate the potential and benefits of IMC for clinical applications. However, calorimetry methods are not yet fully integrated into microbiological routine procedures. The current cost of the instrument is still quite high for cost-efficient clinical laboratory tests. However, the non-invasive and non-destructive character of calorimetry, as well as the simplicity in sample preparation, qualifies it as a valuable tool for the evaluation of antimicrobial inhibitory profiles, including the evaluation of biofilm growth on surfaces.

6. References

1. Wilson LG. The early recognition of streptococci as causes of disease. *Med Hist* 1987; **31**: 403-14.
2. Davis CP. Chapter 6: Normal Flora. In: th, Baron S, eds. *Medical Microbiology*. Galveston (TX): University of Texas Medical Branch at Galveston, 1996.
3. Patterson MJ. Chapter 13 Streptococcus. In: th, Baron S, eds. *Medical Microbiology*. Galveston (TX): University of Texas Medical Branch at Galveston, The University of Texas Medical Branch at Galveston., 1996.
4. Sykes JE. Chapter 34 - Streptococcal and Enterococcal Infections. In: Sykes JE, ed. *Canine and Feline Infectious Diseases*. Saint Louis: W.B. Saunders, 2014; https://doi.org/10.1016/B978-1-4377-0795-3.00034-X.
5. Haslam DB, St. Geme JW. 117 - Classification of Streptococci. In: Long SS, Prober CG, Fischer M, eds. *Principles and Practice of Pediatric Infectious Diseases (Fifth Edition)*: Elsevier, 2018; https://doi.org/10.1016/B978-0-323-40181-4.00117-1.
6. Facklam R. What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clinical microbiology reviews* 2002; **15**: 613-30.
7. Collins MD, Lawson PA. The genus Abiotrophia (Kawamura et al.) is not monophyletic: proposal of Granulicatella gen. nov., Granulicatella adiacens comb. nov., Granulicatella elegans comb. nov. and Granulicatella balaenopterae comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 2000; **50**: 365-9.
8. Puzzolante C, Cuomo G, Meschiari M, Bedini A, Bonazza A, Venturelli C, Sarti M, Mussini C. Granulicatella adiacens and Abiotrophia defectiva Native Vertebral Osteomyelitis: Three Cases and Literature Review of Clinical Characteristics and Treatment Approach. *Case Rep Infect Dis* 2019; **2019**: 5038563.
9. Bouvet A, Grimont F, Grimont PAD. Streptococcus defectivus sp. nov. and Streptococcus adjacens sp. nov., Nutritionally Variant Streptococci from Human Clinical Specimens. *International Journal of Systematic and Evolutionary Microbiology* 1989; **39**: 290-4.
10. Ruoff KL. Nutritionally variant streptococci. *Clinical microbiology reviews* 1991; **4**: 184-90.
11. Hung W-C, Tseng S-P, Chen H-J, Tsai J-C, Chang C-H, Lee T-F, Hsueh P-R, Teng L-J. Use of groESL as a Target for Identification of Abiotrophia, Granulicatella, and Gemella Species. *Journal of Clinical Microbiology* 2010; **48**: 3532-8.
12. Ruoff KL. Chapter 22: Aerococcus, Abiotrophia, and Other Aerobic Catalase-Negative, Gram-Positive Cocci. In: Versalovic J, Carroll K, Funke G et al., eds. *Manual of Clinical Microbiology, 10th Edition*. Washington, DC: ASM Press, 2011; 10.1128/9781555816728.

13. Subedi S, Jennings Z, Chen SC. Laboratory Approach to the Diagnosis of Culture-Negative Infective Endocarditis. *Heart, lung & circulation* 2017; **26**: 763-71.
14. Cargill JS, Scott KS, Gascoyne-Binzi D, Sandoe JA. Granulicatella infection: diagnosis and management. *Journal of medical microbiology* 2012; **61**: 755-61.
15. Cassir N, Grillo JC, Argenson JN, Drancourt M, Levy PY. Abiotrophia defectiva knee prosthesis infection: A case report. *Journal of medical case reports* 2011; **5**: 438.
16. Rozemeijer W, Jiya TU, Rijnsburger M, Heddema E, Savelkoul P, Ang W. Abiotrophia defectiva infection of a total hip arthroplasty diagnosed by 16S rRNA gene sequencing. *Diagnostic microbiology and infectious disease* 2011; **70**: 142-4.
17. Czaczyk K, Myszka K. Biosynthesis of Extracellular Polymeric Substances (EPS) and Its Role in Microbial Biofilm Formation. *Polish Journal of Environmental Studies* 2007; **16**: 799-806.
18. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the Natural environment to infectious diseases. *Nature Reviews Microbiology* 2004; **2**: 95-108.
19. Armbruster CR, Parsek MR. New insight into the early stages of biofilm formation. *Proceedings of the National Academy of Sciences of the United States of America* 2018; **115**: 4317-9.
20. Khatoon Z, McTiernan CD, Suuronen EJ, Mah T-F, Alarcon EI. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon* 2018; **4**: e01067-e.
21. Petrova OE, Sauer K. Escaping the biofilm in more than one way: desorption, detachment or dispersion. *Curr Opin Microbiol* 2016; **30**: 67-78.
22. Donlan RM. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases* 2001; **33**: 1387-92.
23. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends in microbiology* 2001; **9**: 34-9.
24. Macia MD, Rojo-Molinero E, Oliver A. Antimicrobial susceptibility testing in biofilm-growing bacteria. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2014; **20**: 981-90.
25. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, Brynildsen MP, Bumann D, Camilli A, Collins JJ, Dehio C, Fortune S, Ghigo J-M, Hardt W-D, Harms A, Heinemann M, Hung DT, Jenal U, Levin BR, Michiels J, Storz G, Tan M-W, Tenson T, Van Melderen L, Zinkernagel A. Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology* 2019; **17**: 441-8.
26. Joo H-S, Otto M. Molecular basis of in vivo biofilm formation by bacterial pathogens. *Chem Biol* 2012; **19**: 1503-13.
27. Sharma D, Misba L, Khan AU. Antibiotics versus biofilm: an emerging battleground in microbial communities. *Antimicrobial Resistance & Infection Control* 2019; **8**: 76.
28. Lewis K. Riddle of biofilm resistance. *Antimicrobial agents and chemotherapy* 2001; **45**: 999-1007.
29. Tande AJ, Patel R. Prosthetic joint infection. *Clinical microbiology reviews* 2014; **27**: 302-45.
30. Poultsides LA, Liaropoulos LL, Malizos KN. The Socioeconomic Impact of Musculoskeletal Infections. *JBJS* 2010; **92**: e13.
31. Abad CL, Haleem A. Prosthetic Joint Infections: an Update. *Current Infectious Disease Reports* 2018; **20**: 15.
32. Li C, Renz N, Trampuz A. Management of Periprosthetic Joint Infection. *Hip Pelvis* 2018; **30**: 138-46.
33. Izakovicova P, Borens O, Trampuz A. Periprosthetic joint infection: current concepts and outlook. *EFORT open reviews* 2019; **4**: 482-94.
34. Secinti KD, Özalp H, Attar A, Sargon MF. Nanoparticle silver ion coatings inhibit biofilm formation on titanium implants. *Journal of Clinical Neuroscience* 2011; **18**: 391-5.
35. Moriarty TF, Kuehl R, Coenye T, Metsemakers WJ, Morgenstern M, Schwarz EM, Riool M, Zaat SAJ, Khana N, Kates SL, Richards RG. Orthopaedic device-related infection: current and future interventions for improved prevention and treatment. *EFORT Open Reviews* 2016; **1**: 89-99.
36. Lora-Tamayo J, Senneville E, Ribera A, Bernard L, Dupon M, Zeller V, Li HK, Arvieux C, Clauss M, Uckay I, Vigante D, Ferry T, Iribarren JA, Peel TN, Sendi P, Miksic NG, Rodriguez-Pardo D, Del Toro MD, Fernandez-Sampedro M, Dapunt U, Huotari K, Davis JS, Palomino J, Neut D, Clark BM, Gottlieb T, Trebse R, Soriano A, Bahamonde A, Guio L, Rico A, Salles MJC, Pais MJG, Benito N, Riera M, Gomez L, Aboltins CA, Esteban J, Horcajada JP, O'Connell K, Ferrari M, Skaliczki G, Juan RS, Cobo J, Sanchez-Somolinos M, Ramos A, Giannitsioti E, Jover-Saenz A, Baraia-Etxaburu JM, Barbero JM, Choong PFM, Asseray N, Ansart S, Moal GL, Zimmerli W, Ariza J. The Not-So-Good Prognosis of Streptococcal Periprosthetic Joint Infection Managed by Implant Retention: The Results of a Large Multicenter Study. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2017; **64**: 1742-52.
37. Peel T, Buising K, Dowsey M, Choong P. Management of Prosthetic Infection According to Organism. In: Kinov P, ed. *Arthroplasty - Update*. Rijeka: InTech, 2013; 10.5772/53244.
38. Conley J, Olson ME, Cook LS, Ceri H, Phan V, Davies HD. Biofilm Formation by Group A Streptococci: Is There a Relationship with Treatment Failure? *Journal of Clinical Microbiology* 2003; **41**: 4043-8.
39. Olson ME, Ceri H, Morck DW, Buret AG, Read RR. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Canadian Journal of Veterinary Research* 2002; **66**: 86-92.

40. Giannakopoulos K, Zompolou C, Behnes M, Elmas E, Borggrefe M, Akin I. Infective endocarditis - A word of caution on non-typical bacteria. *European review for medical and pharmacological sciences* 2016; **20**: 4782-5.
41. Bozkurt I, Coksevim M, Cerik IB, Gulel O, Tanyel E, Leblebicioglu H. Infective endocarditis with atypical clinical feature and relapse by Abiotrophia defectiva. *Journal of the Saudi Heart Association* 2017; **29**: 136-8.
42. Rhodes HM, Hirigoyen D, Shabnam L, Williams DN, Hansen GT. Infective endocarditis due to Abiotrophia defectiva and Granulicatella spp. complicated by infectious intracranial cerebral aneurysms: a report of three cases and review of the literature. *Journal of medical microbiology* 2016; **65**: 493-9.
43. Alberti MO, Hindler JA, Humphries RM. Antimicrobial Susceptibilities of Abiotrophia defectiva, Granulicatella adiacens, and Granulicatella elegans. *Antimicrobial agents and chemotherapy* 2015; **60**: 1411-20.
44. Mushtaq A, Greenwood-Quaintance KE, Cole NC, Kohner PC, Ihde SM, Strand GJ, Harper LW, Virk A, Patel R. Differential Antimicrobial Susceptibilities of Granulicatella adiacens and Abiotrophia defectiva. *Antimicrobial agents and chemotherapy* 2016; **60**: 5036-9.
45. Prasidthrathsint K, Fisher MA. Antimicrobial Susceptibility Patterns among a Large, Nationwide Cohort of Abiotrophia and Granulicatella Clinical Isolates. *J Clin Microbiol* 2017; **55**: 1025-31.
46. Ciofu O, Rojo-Molinero E, Macià MD, Oliver A. Antibiotic treatment of biofilm infections. *APMIS* 2017; **125**: 304-19.
47. Koo H, Allan RN, Howlin RP, Stoodley P, Hall-Stoodley L. Targeting microbial biofilms: current and prospective therapeutic strategies. *Nature reviews Microbiology* 2017; **15**: 740-55.
48. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future medicinal chemistry* 2015; **7**: 493-512.
49. Mukherji R, Patil A and Prabhune A. Role of Extracellular Proteases in Biofilm Disruption of Gram Positive Bacteria with Special Emphasis on Staphylococcus aureus Biofilms. *Enzyme Engineering* 2015; **4**: 126.
50. Chaignon P, Sadovskaya I, Ragunah C, Ramasubbu N, Kaplan JB, Jabbouri S. Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Applied microbiology and biotechnology* 2007; **75**: 125-32.
51. Fleming D, Rumbaugh KP. Approaches to Dispersing Medical Biofilms. *Microorganisms* 2017; **5**: 15.
52. Di Bonaventura G, D'Antonio D, Catamo G, Ballone E, Piccolomini R. Comparison of Etest, agar dilution, broth microdilution and disk diffusion methods for testing in vitro activity of levofloxacin against Staphylococcus spp. isolated from neutropenic cancer patients. *International Journal of Antimicrobial Agents* 2002; **19**: 147-54.
53. van der Heijden IM, Levin AS, De Pedri EH, Fung L, Rossi F, Duboc G, Barone AA, Costa SF. Comparison of disc diffusion, Etest and broth microdilution for testing susceptibility of carbapenem-resistant P. aeruginosa to polymyxins. *Annals of Clinical Microbiology and Antimicrobials* 2007; **6**: 8.
54. Alberti MO, Hindler JA, Humphries RM. Performance of Etest for Antimicrobial Susceptibility Testing of Abiotrophia defectiva and Granulicatella Species. *J Clin Microbiol* 2016; **54**: 2194-6.
55. Döring G, Flume P, Heijerman H, Elborn JS. Treatment of lung infection in patients with cystic fibrosis: Current and future strategies. *Journal of Cystic Fibrosis* 2012; **11**: 461-79.
56. Zimmerli W, Frei R, Widmer AF, Rajacic Z. Microbiological tests to predict treatment outcome in experimental device-related infections due to Staphylococcus aureus. *The Journal of antimicrobial chemotherapy* 1994; **33**: 959-67.
57. Coenye T, Goeres D, Van Bambeke F, Bjarnsholt T. Should standardized susceptibility testing for microbial biofilms be introduced in clinical practice? *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2018; **24**: 570-2.
58. Malone M, Goeres DM, Gosbell I, Vickery K, Jensen S, Stoodley P. Approaches to biofilm-associated infections: the need for standardized and relevant biofilm methods for clinical applications. *Expert Review of Anti-infective Therapy* 2017; **15**: 147-56.
59. Harrison JJ, Stremick CA, Turner RJ, Allan ND, Olson ME, Ceri H. Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nature Protocols* 2010; **5**: 1236-54.
60. Wang L, Di Luca M, Tkhilaishvili T, Trampuz A, Gonzalez Moreno M. Synergistic Activity of Fosfomycin, Ciprofloxacin, and Gentamicin Against Escherichia coli and Pseudomonas aeruginosa Biofilms. *Front Microbiol* 2019; **10**: 2522-.
61. Butini ME, Moreno MG, Czuban M, Koliszak A, Tkhilaishvili T, Trampuz A, Di Luca M. Real-time antimicrobial susceptibility assay of planktonic and biofilm bacteria by isothermal microcalorimetry. 2018.
62. Braissant O, Wirz D, Göpfert B, Daniels AU. Use of isothermal microcalorimetry to monitor microbial activities. *FEMS Microbiology Letters* 2010; **303**: 1-8.
63. Butini ME, Gonzalez Moreno M, Czuban M, Koliszak A, Tkhilaishvili T, Trampuz A, Di Luca M. Real-Time Antimicrobial Susceptibility Assay of Planktonic and Biofilm Bacteria by Isothermal Microcalorimetry. *Advances in experimental medicine and biology* 2019; **1214**: 61-77.
64. Postollec F, Norde W, van der Mei H, Busscher H. Enthalpy of interaction between coaggregating and non-coaggregating oral bacterial pairs - A microcalorimetric study. *Journal of microbiological methods* 2003; **55**: 241-7.
65. Astasov-Frauenhoffer M, Braissant O, Hauser-Gerspach I, Daniels AU, Weiger R, Waltimo T. Isothermal microcalorimetry provides new insights into biofilm variability and dynamics. *FEMS Microbiol Lett* 2012; **337**: 31-7.
66. von Rège H, Sand W. Evaluation of biocide efficacy by microcalorimetric determination of microbial activity in biofilms. *Journal of Microbiological Methods* 1998; **33**: 227-35.
67. Butini ME, Abbandonato G, Di Rienzo C, Trampuz A, Di Luca M. Isothermal Microcalorimetry Detects the Presence of Persister Cells in a Staphylococcus aureus Biofilm After Vancomycin Treatment. *Front Microbiol* 2019; **10**: 332.
68. Dijkmans AC, Zacarias NVO, Burggraaf J, Mouton JW, Wilms EB, van Nieuwkoop C, Touw DJ, Stevens J, Kamerling IMC. Fosfomycin: Pharmacological, Clinical and Future Perspectives. *Antibiotics (Basel, Switzerland)* 2017; **6**.
69. Miller WR, Bayer AS, Arias CA. Mechanism of Action and Resistance to Daptomycin in Staphylococcus aureus and Enterococci. *Cold Spring Harb Perspect Med* 2016; **6**: a026997.

70. Verg, xe, res P, Blaser J, xfc, rg. Amikacin, Ceftazidime, and Flucloxacillin against Suspended and Adherent Pseudomonas aeruginosa and Staphylococcus epidermidis in an in vitro Model of Infection. *The Journal of infectious diseases* 1992; **165**: 281-9.
71. Gomes LC, Mergulhão FJ. SEM Analysis of Surface Impact on Biofilm Antibiotic Treatment. *Scanning* 2017; **2017**: 2960194.
72. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing; 27th Edition. CLSIM100-S27*. Clinical and Laboratory Standards Institute., Wayne, PA, USA, 2016.
73. Rodloff A, Bauer T, Ewig S, Kujath P, Müller E. Susceptible, intermediate, and resistant - the intensity of antibiotic action. *Dtsch Arztebl Int* 2008; **105**: 657-62.
74. Astasov-Frauenhoffer M, Braissant O, Hauser-Gerspach I, Daniels AU, Weiger R, Waltimo T. Isothermal microcalorimetry provides new insights into biofilm variability and dynamics. *FEMS Microbiology Letters* 2012; **337**: 31-7.
75. Yang L, Qiu S, Xu F, Sun L-X, Zhao Z, Liang J, Song C. Microcalorimetric investigation of the growth of the Escherichia coli DH5α in different antibiotics. *Journal of Thermal Analysis and Calorimetry* 2007; **89**: 875-9.
76. Howell M, Wirz D, Daniels AU, Braissant O. Application of a microcalorimetric method for determining drug susceptibility in mycobacterium species. *Journal of clinical microbiology* 2012; **50**: 16-20.
77. Braissant O, Bonkat G, Wirz D, Bachmann A. Microbial growth and isothermal microcalorimetry: Growth models and their application to microcalorimetric data. *Thermochimica Acta* 2013; **555**: 64-71.
78. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. *Journal of Antimicrobial Chemotherapy* 2003; **52**: 1.
79. Roberts AEL, Kragh KN, Bjarnsholt T, Diggle SP. The limitations of *in vitro* experimentation in understanding biofilms and chronic infection. *bioRxiv* 2015: 032987.
80. Stoodley P, Debeer D, Lewandowski Z. Liquid flow in biofilm systems. *Appl Environ Microbiol* 1994; **60**: 2711-6.
81. Mayrhofer S, Domig KJ, Mair C, Zitz U, Huys G, Kneifel W. Comparison of broth microdilution, Etest, and agar disk diffusion methods for antimicrobial susceptibility testing of Lactobacillus acidophilus group members. *Appl Environ Microbiol* 2008; **74**: 3745-8.
82. Riedel S, Neoh KM, Eisinger SW, Dam LM, Tekle T, Carroll KC. Comparison of commercial antimicrobial susceptibility test methods for testing of Staphylococcus aureus and Enterococci against vancomycin, daptomycin, and linezolid. *Journal of clinical microbiology* 2014; **52**: 2216-22.
83. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes. *Molecular Microbiology* 2008; **68**: 223-40.
84. Saggu SK, Jha G, Mishra PC. Enzymatic Degradation of Biofilm by Metalloprotease From Microbacterium sp. SKS10. *Frontiers in Bioengineering and Biotechnology* 2019; **7**.
85. Tornero E, Morata L, Martinez-Pastor JC, Angulo S, Combalia A, Bori G, Garcia-Ramiro S, Bosch J, Mensa J, Soriano A. Importance of selection and duration of antibiotic regimen in prosthetic joint infections treated with debridement and implant retention. *The Journal of antimicrobial chemotherapy* 2016; **71**: 1395-401.
86. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis* 2016; **6**: 71-9.
87. Bahamondez-Canas TF, Heersema LA, Smyth HDC. Current Status of In Vitro Models and Assays for Susceptibility Testing for Wound Biofilm Infections. *Biomedicines* 2019; **7**: 34.
88. Fricke C, Harms H, Maskow T. Rapid Calorimetric Detection of Bacterial Contamination: Influence of the Cultivation Technique. *Front Microbiol* 2019; **10**.
89. Mah T-F. Establishing the minimal bactericidal concentration of an antimicrobial agent for planktonic cells (MBC-P) and biofilm cells (MBC-B). *Journal of visualized experiments : JoVE* 2014: e50854-e.
90. Maskow T, Morais FM, Rosa LFM, Qian YG, Harnisch F. Insufficient oxygen diffusion leads to distortions of microbial growth parameters assessed by isothermal microcalorimetry. *RSC Advances* 2014; **4**: 32730-7.
91. Braissant O, Chavanne P, de Wild M, Pieles U, Stevanovic S, Schumacher R, Straumann L, Wirz D, Gruner P, Bachmann A, Bonkat G. Novel microcalorimetric assay for antibacterial activity of implant coatings: The cases of silver-doped hydroxyapatite and calcium hydroxide. *Journal of biomedical materials research Part B, Applied biomaterials* 2015; **103**: 1161-7.
92. Peel T, Buising K, Dowsey M, Choong P. Management of Prosthetic Infection According to Organism. In: Arthroplasty Update, ed. Plamen Kinov *Medical University of Sofia*, Bulgaria 2013. DOI: 10.5772/53244
93. Madison G, Golamari R, Bhattacharya P. Endocarditis Caused by Abiotrophia and Granulicatella Species. In: Firstenberg MS, ed. *Advanced Concepts in Endocarditis*: IntechOpen, 2018; DOI: 10.5772/intechopen.74252.
94. Akgün D, Trampuz A, Perka C, Renz N. High failure rates in treatment of streptococcal periprosthetic joint infection: results from a seven-year retrospective cohort study. *The bone & joint journal* 2017; **99-b**: 653-9.
95. Fiaux E, Titecat M, Robineau O, Lora-Tamayo J, El Samad Y, Etienne M, Frebourg N, Blondiaux N, Brunschweiler B, Dujardin F, Beltrand E, Loiez C, Cattoir V, Canarelli JP, Hulet C, Valette M, Nguyen S, Caron F, Migaud H, Senneville E. Outcome of patients with streptococcal prosthetic joint infections with special reference to rifampicin combinations. *BMC infectious diseases* 2016; **16**: 568.
96. Sendi P, Christensson B, Uckay I, Trampuz A, Achermann Y, Boggian K, Svensson D, Widerstrom M, Zimmerli W. Group B streptococcus in prosthetic hip and knee joint-associated infections. *The Journal of hospital infection* 2011; **79**: 64-9.
97. Yu W, Hallinen KM, Wood KB. Interplay between Antibiotic Efficacy and Drug-Induced Lysis Underlies Enhanced Biofilm Formation at Subinhibitory Drug Concentrations. *Antimicrobial agents and chemotherapy* 2017; **62**: e01603-17.
98. Fisher RA, Gollan B, Helaine S. Persistent bacterial infections and persister cells. *Nature reviews Microbiology* 2017; **15**: 453-64.
99. Taylor SD, Palmer M. The action mechanism of daptomycin. *Bioorganic & Medicinal Chemistry* 2016; **24**: 6253-68.

100. Streit JM, Jones RN, Sader HS. Daptomycin activity and spectrum: a worldwide sample of 6737 clinical Gram-positive organisms. *Journal of Antimicrobial Chemotherapy* 2004; **53**: 669-74.
101. Smith PW, Zuccotto F, Bates RH, Martinez-Martinez MS, Read KD, Peet C, Epemolu O. Pharmacokinetics of β-Lactam Antibiotics: Clues from the Past To Help Discover Long-Acting Oral Drugs in the Future. *ACS Infect Dis* 2018; **4**: 1439-47.
102. Rose WE, Berti AD, Hatch JB, Maki DG. Relationship of *In Vitro* Synergy and Treatment Outcome with Daptomycin plus Rifampin in Patients with Invasive Methicillin-Resistant *Staphylococcus aureus* Infections. *Antimicrobial agents and chemotherapy* 2013; **57**: 3450-2.
103. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* 2011; **473**: 216-20.
104. Kadurugamuwa JL, Clarke AJ, Beveridge TJ. Surface action of gentamicin on Pseudomonas aeruginosa. *Journal of bacteriology* 1993; **175**: 5798-805.
105. Ruppen C, Hemphill A, Sendi P. In vitro activity of gentamicin as an adjunct to penicillin against biofilm group B Streptococcus. *The Journal of antimicrobial chemotherapy* 2017; **72**: 444-7.
106. Zhu M, Dai X. Maintenance of translational elongation rate underlies the survival of Escherichia coli during oxidative stress. *Nucleic Acids Res* 2019; **47**: 7592-604.
107. Moellering RC, Jr., Wennersten C, Weinberg AN. Synergy of Penicillin and Gentamicin against Enterococci. *The Journal of infectious diseases* 1971; **124**: S207-S13.
108. Kaplan JB, LoVetri K, Cardona ST, Madhyastha S, Sadovskaya I, Jabbouri S, Izano EA. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. *The Journal of antibiotics* 2012; **65**: 73-7.
109. Kumar Shukla S, Rao TS. Dispersal of Bap-mediated Staphylococcus aureus biofilm by proteinase K. *The Journal of antibiotics* 2013; **66**: 55-60.
110. Lauderdale KJ, Malone CL, Boles BR, Morcuende J, Horswill AR. Biofilm dispersal of community-associated methicillin-resistant Staphylococcus aureus on orthopedic implant material. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2010; **28**: 55-61.
111. Cerceo E, Christie JD, Nachamkin I, Lautenbach E. Central nervous system infections due to Abiotrophia and Granulicatella species: an emerging challenge? *Diagnostic microbiology and infectious disease* 2004; **48**: 161-5.
112. Madison G, Golamari R, Bhattacharya P. *Endocarditis Caused by Abiotrophia and Granulicatella Species*, 2018.
113. P., S.V.V.S.N. and P., S.V.V.S.. A Review on Surface Modifications and Coatings on Implants to Prevent Biofilm. *Regenerative Engineering and Translational Medicine* 2019; https://doi.org/10.1007/s40883-019-00116-3.
114. Onsea J, Soentjens P, Djebara S, Merabishvili M, Depypere M, Spriet I, De Munter P, Debaveye Y, Nijs S, Vanderschot P, Wagemans J, Pirnay J-P, Lavigne R, Metsemakers W-J. Bacteriophage Application for Difficult-to-treat Musculoskeletal Infections: Development of a Standardized Multidisciplinary Treatment Protocol. *Viruses* 2019; **11**: 891.

Statutory Declaration

"I, Mercedes González Moreno, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Antibiofilm approaches to combat *Streptococcus* and related species implicated in implant-associated infections", independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines.

The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.

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Date Signature

Declaration of contribution to the listed publications

Mercedes González Moreno contributed the following to the below listed publications:

Publication 1: Butini ME, **Gonzalez Moreno M**, Czuban M, Koliszak A, Tkhilaishvili T, Trampuz A, Di Luca M. Real-time antimicrobial susceptibility assay of planktonic and biofilm bacteria by isothermal microcalorimetry. Advances in experimental medicine and biology (2018); 1-17, doi: https://doi.org/10.1007/5584_2018_291.

Contribution in detail:

- Planning and organisation of experiments in agreement with all co-authors.
- Conducting a part of the experiments, in detail: Performance of the antimicrobial assays against planktonic and biofilm bacteria by real-time IMC and evaluation of biofilm eradication by sonication of beads for colony counting.
- Evaluation of the collected data and interpretation of all results. Performance of the data analysis and its graphical representation. The figures 1, 2 and 3 as well as the tables 1, 2 and 3 are the result of my analysis.
- Participation in the preparation of the manuscript and the review process.

Publication 2: Gonzalez Moreno M, Trampuz A, Di Luca M. Synergistic antibiotic activity against planktonic and biofilm-embedded *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Streptococcus oralis*. J Antimicrob Chemother (2017); 72: 3085–92. doi:10.1093/jac/dkx265

Contribution in detail:

- Planning and organisation of experiments in agreement with all co-authors.
- Independent execution of all experiments, in detail: Performance of antimicrobial assays by Etest, microcalorimetry and sonication of beads for colony counting. Evaluation of antimicrobial activity against biofilms pre-treated with proteinase K.
- Processing and evaluation of all collected data, interpretation of all results and preparation of all figures and tables.
- Preparation of the manuscript and participation in the review process.

Publication 3: Gonzalez Moreno M, Wang L, De Masi M, Winkler T, Trampuz A, Di Luca M. *In vitro* antimicrobial activity against *Abiotrophia defectiva* and *Granulicatella elegans* biofilms. J Antimicrob Chemother (2019). pii: dkz174. doi: 10.1093/jac/dkz174.

Contribution in detail:

- Planning and organisation of experiments in agreement with PD Dr. Trampuz and Dr. Mariagrazia Di Luca.
- Execution of all experiments, in detail: Evaluation of the antimicrobial activity by broth microdilution assays, Etest assays, microcalorimetry assays and sonication of beads for colony counting.
- Processing and evaluation of all collected data, interpretation of all the results and preparation of all figures and tables.
- Preparation of the manuscript and participation in the review process.

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Signature of the doctoral candidate

Printed copies of selected publications

Publication 1

Real-time antimicrobial suseptibility assay of planktonic and biofilm bacteria by isothermal microcalorimetry

Butini ME, **Gonzalez Moreno M**, Czuban M, Koliszak A, Tkhilaishvili T, Trampuz A, Di Luca M

Advances in Experimental Medicine and Biology - Advances in Microbiology, Infectious Diseases and Public Health (2018)

doi: https://doi.org/10.1007/5584_2018_291

Impact factor (2019): 2.450

Publication 2

Synergistic antibiotic activity against planktonic and biofilm-embedded *Streptococcus agalactiae*, *Streptococcus pyogenes* and *Streptococcus oralis*.

Gonzalez Moreno M, Trampuz A, Di Luca M.

Journal of Antimicrobial Chemotherapy (2017)

doi: https://doi.org/10.1093/jac/dkx265

Impact factor (2019): 5.439

Publication 3

In vitro antimicrobial activity against *Abiotrophia defectiva* and *Granulicatella elegans* biofilms.

Gonzalez Moreno M, Wang L, De Masi M, Winkler T, Trampuz A, Di Luca M.

Journal of Antimicrobial Chemotherapy (2019)

doi: https://doi.org/10.1093/jac/dkz174

Impact factor (2019): 5.439

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Complete list of publications

Peer-reviewed articles

Moreno MG, Lombardi L, Di Luca M, Antimicrobial peptides for the control of biofilm formation. Current Topics in Medicinal Chemistry (2017); 17:17. doi: 10.2174/1568026617666170105144830
Impact factor (2019): 3.233

Gonzalez Moreno M, Trampuz A, Di Luca M. Synergistic antibiotic activity against planktonic and biofilm-embedded *Streptococcus agalactiae, Streptococcus pyogenes* and *Streptococcus oralis*. Journal of Antimicrobial Chemotherapy (2017); 72: 3085–92. doi: https://doi.org/10.1093/jac/dkx265
Impact factor (2019): 5.439

Butini ME, **Gonzalez Moreno M**, Czuban M, Koliszak A, Tkhilaishvili T, Trampuz A, Di Luca M. Real-time antimicrobial susceptibility assay of planktonic and biofilm bacteria by isothermal microcalorimetry. Advances in Experimental Medicine and Biology - Advances in Microbiology, Infectious Diseases and Public Health (2018); 1-17. doi: https://doi.org/10.1007/5584_2018_291
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Gonzalez Moreno M, Wang L, De Masi M, Winkler T, Trampuz A, Di Luca M. *In vitro* antimicrobial activity against *Abiotrophia defectiva* and *Granulicatella elegans* biofilms. Journal of Antimicrobial Chemotherapy (2019). pii: dkz174. doi: 10.1093/jac/dkz174.
Impact factor (2019): 5.439

Wang L, Di Luca M, Tkhilaishvili T, Trampuz A, **Gonzalez Moreno M**. Synergistic Activity of Fosfomycin, Ciprofloxacin, and Gentamicin Against *Escherichia coli* and *Pseudomonas aeruginosa* Biofilms. Frontiers in Microbiology (2019); 10:2522. doi:10.3389/fmicb.2019.02522.
Impact factor (2019): 4.076

Mercedes Gonzalez Moreno, Maria Eugenia Butini, Elena Maryka Maiolo, Laura Sessa, Andrej Trampuz. Antimicrobial activity of bioactive glass S53P4 against representative microorganisms causing osteomyelitis – real-time assessment by isothermal microcalorimetry. Colloids and Surfaces B: Biointerfaces (2020); 189:110853, doi: https://doi.org/10.1016/j.colsurfb.2020.110853. Impact factor (2019): 4.389

Tamta Tkhilaishvili, Lei Wang, Carsten Perka, Andrej Trampuz, **Mercedes Gonzalez Moreno.** Using bacteriophages as a trojan horse to the killing of dual-species biofilm formed by *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus*. Frontiers in Microbiology (2020); 11:695 doi: https://www.frontiersin.org/article/10.3389/fmicb.2020.00695. Impact Factor (2019): 4.076

Onsea J, Wagemans J, Pirnay JP, Di Luca M, **Gonzalez-Moreno M**, Lavigne R, Trampuz A, Moriarty TF, Metsemakers WJ. Bacteriophage therapy as a treatment strategy for orthopaedic-device-related infections: Where do we stand? European Cells and Materials (2020); 5;39:193-210. doi: 10.22203/eCM.v039a13. PMID: 32368785. Impact Factor (2019): 3.741

Stéphane Corvec, Eva Seiler, Lei Wang, **Mercedes Gonzalez Moreno**, Andrej Trampuz. Characterization of medical relevant anaerobic microorganisms by isothermal microcalorimetry. Anaerobe (2020); 66:102282. doi: https://doi.org/10.1016/j.anaerobe.2020.102282. Impact Factor (2019): 2.709

Wang L, Tkhilaishvili T, Trampuz A, **Gonzalez-Moreno M**. Evaluation of staphylococcal bacteriophage Sb-1 as an adjunctive agent to antibiotics against rifampin-resistant *Staphylococcus aureus* biofilms. Frontiers in Microbiology (2020); 11:2700. Doi:10.3389/fmicb.2020.602057. Impact factor (2019): 4.076

Wang L, Tkhilaishvili T, Bernal-Andres B, Trampuz A, **Gonzalez-Moreno M**. Bacteriophage-antibiotic combinations against ciprofloxacin/ceftriaxone-resistant *Escherichia coli in vitro* and in an experimental *Galleria mellonella* model. International Journal Antimicrobial Agents (2020); 56:6. doi: https://doi.org/10.1016/j.ijantimicag.2020.106200. Impact Factor (2019): 4.621

Abstracts at international conferences

Mercedes González-Moreno, Elena Maryka Maiolo, Mariagrazia Di Luca, Andrej Trampuz. Activity of fosfomycin, rifampin and combination of both against *Streptococcus* biofilm.
26th European Congress of Clinical Microbiology and Infectious Diseases, 2016 Amsterdam. Poster P1705
36th Annual Meeting of the European Bone and Joint Infection Society, 2017 Nantes. Poster 159

Mercedes González Moreno, Andrej Trampuz, Mariagrazia Di Luca. Addition of gentamicin to different antibiotics enhances the eradication of biofilm-embedded *Abiotrophia defectiva* and *Granulicatella elegans*.
28th European Congress of Clinical Microbiology and Infectious Diseases, 2018 Madrid. Poster P1448

L. Hörndler, **M. Gonzalez-Moreno**, M. Czuban, A. Trampuz, M. Di Luca. Characterisation and antimicrobial susceptibility of *Staphylococcus epidermidis* persister cells generated *in vitro* both by chemical induction and by natural isolation. 28th European Congress of Clinical Microbiology and Infectious Diseases, 2018 Madrid. Poster P1828

Mercedes Gonzalez-Moreno, Tamta Tkhilaishvili, Andrej Trampuz, Mariagrazia Di Luca. Phenotypic characterisation of pan-drug/multidrug-resistant *Pseudomonas aeruginosa* clinical isolates and their susceptibility to antimicrobial peptides.
29th European Congress of Clinical Microbiology and Infectious Diseases, 2019 Amsterdam. Mini-Oral ePoster O1072
38th Annual Meeting of the European Bone and Joint Infection Society, 2019 Antwerpen. Poster P17

Mercedes Gonzalez-Moreno, Lei Wang, Margherita De Masi, Andrej Trampuz, Mariagrazia Di Luca. *In vitro* activity of gentamicin against biofilms of *Abiotrophia defectiva* and *Granulicatella elegans* blood isolates.
29th European Congress of Clinical Microbiology and Infectious Diseases, 2019 Amsterdam. Poster P0571
38th Annual Meeting of the European Bone and Joint Infection Society, 2019 Antwerpen. Poster P18

Lei Wang, **Mercedes Gonzalez-Moreno**, Tamta Tkhilaishvili, Mariagrazia Di Luca, Andrej Trampuz. *In vitro* activity of fosfomycin, ciprofloxacin, gentamicin and their combinations against *Escherichia coli* and *Pseudomonas aeruginosa* biofilms.
29th European Congress of Clinical Microbiology and Infectious Diseases, 2019 Amsterdam. Poster P0572
38th Annual Meeting of the European Bone and Joint Infection Society, 2019 Antwerpen. Free Paper C FP17

Lei Wang, Mariagrazia Di Luca, Tamta Tkhilaishvili, **Mercedes Gonzalez-Moreno**, Andrej Trampuz. *In vitro* synergistic activity of fosfomycin, ciprofloxacin and gentamicin combinations against *Escherichia coli* biofilms. 29th European Congress of Clinical Microbiology and Infectious Diseases, 2019 Amsterdam. Poster P0575

Acknowledgements

To my Spanish and German family for providing unconditional support and to F.B. for being always aside me during this path.

AG Trampuz - Centrum für Muskuloskeletale Chirurgie, Charité – Universitätsmedizin Berlin and Berlin-Brandemburg Center for Regenerative Therapies (BCRT)

Guidance and supervision: PD Dr. med. Andrej Trampuz, PD Dr. med. univ. etscient. med. Tobias Winkler and Dr. Mariagrazia Di Luca

Colleagues: M. Czuban, A. Koliszak, T. Tkhilaishvili and L. Wang

A special thank for all the support, motivation and inspiration. For all the good conversations and constructive discussions, for the trustiness and for the numerous opportunities to grow scientific- and professionally.

Funding

The PRO-IMPLANT Foundation for making it possible to carry out this research work and for further educational support.

Berlin-Brandenburg School for Regenerative Therapies (BSRT) for providing excellent opportunities to develop my personal and professional skills and for providing travel grants for the attendance to scientific conferences/courses.

Scientific Collaboration Institutions

The Advanced Light and Electron Microscopy Department from the Robert Koch Institute (Berlin), especially to Dr. Christoph Schaudinn, who performed the SEM analysis and contributed in the preparation of figure 4 “Biofilm formation of *G. elegans* ATCC 700633 (A and B) and *A. defectiva* ATCC 49176 (C and D) grown on porous glass beads” in this dissertation.

The UCLA Department of Pathology and Laboratory Medicine for providing us with the blood isolates used in our study.

Other Institutions

Dahlem Research School

SPARK-Berlin mentoring program