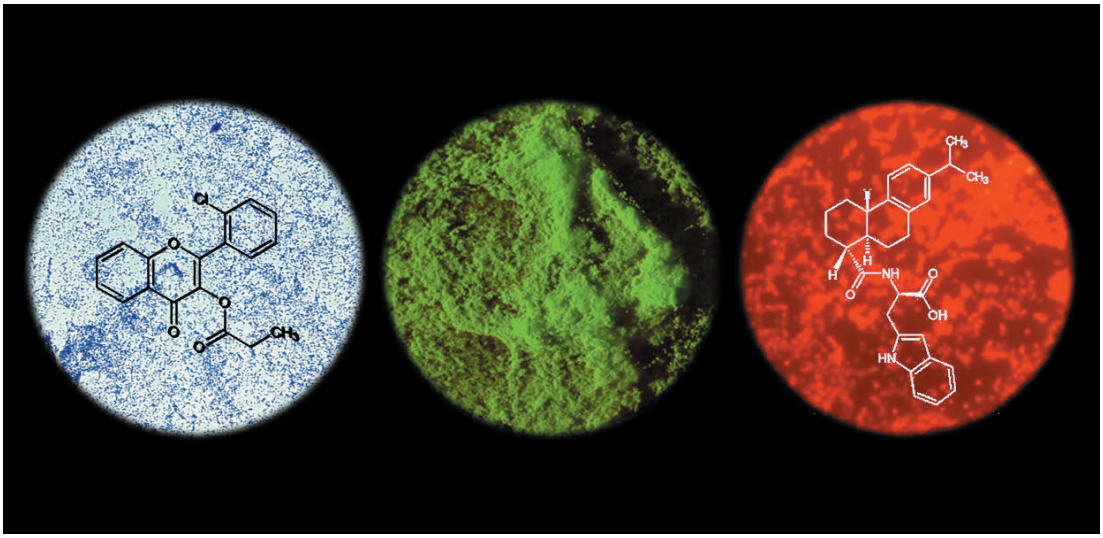
Åbo Akademi University

Suvi Manner

Early discovery approaches of biofilm inhibitors from naturally-inspired sources and insights into biofilm models

A study in Pharmaceutical Sciences



Suvi Manner

Born 1979 in Turku, Finland

Obtained her MSc degree in Biosciences (Pharmacy) at Åbo Akademi University in 2013

Cover: From left, light microscopy image of *P aeruginosa* biofilm treated with compound 2117, CLSM image of *S. aureus* biofilm formed in the drip flow reactor, and fluorescence microscopy image of *S. aureus* biofilm treated with compound 9b.

Early discovery approaches of biofilm inhibitors from naturally-inspired sources and insights into biofilm models

A study in Pharmaceutical Sciences

Suvi Manner

Pharmaceutical Sciences Laboratory
Faculty of Science and Engineering
Åbo Akademi University
Åbo, Finland, 2017

Supervised by

Docent Adyary Fallarero
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki, Finland

Professor Pia Vuorela
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki, Finland

Reviewed by

Docent Kirsi Savijoki
Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki, Finland

Associate Professor Helle Wangensteen
Department of Pharmaceutical Chemistry
School of Pharmacy
University of Oslo, Norway

Opponent

Professor Paul Cos
Laboratory for Microbiology, Parasitology and Hygiene
Faculty of Pharmaceutical, Biomedical and Veterinary Sciences
University of Antwerp, Belgium

ISBN 978-952-12-3625-9 (Print)
ISBN 978-952-12-3626-6 (Pdf)
Painosalama Oy – Turku, Finland, 2017

Table of Contents

Abstract

Biofilm formation complicates diagnosis and treatment of bacterial infections. Bacterial biofilms can be defined as structurally organized communities of bacterial cells embedded in a matrix of extracellular polymeric matrix (EPS). The majority of bacteria exist as biofilms in most natural environments. Biofilm bacteria are highly tolerant to antimicrobials and host immune responses. Conventional antibiotics are inefficient in the treatment of biofilm-associated infections, especially those occurring in hospitalized patients and associated with the use of medical devices. Moreover, *in vitro* laboratory methods that have been designed for growing of planktonic bacteria and evaluation of antimicrobials against them are not applicable for biofilms. Therefore, alternative methods and models have been developed for investigation of biofilms and testing of antimicrobials against biofilm-growing bacteria. However, so far, the repertoire of existing anti-biofilm agents is extremely limited and thus, there is a great need for the discovery and development of novel anti-biofilm compounds. In that context, the primary aim of this thesis project was to identify biofilm inhibitors from naturally-inspired sources. Towards this goal, 3570 compounds were screened for biofilm inhibition.

Screening campaigns were designed to explore different strategies aimed at the discovery of anti-biofilm leads with bactericidal or non-bactericidal effects. In one direction, two synthetic flavan derivatives as well as the D-tryptophan and the β-cyclohexyl-L-alanine derivatives of (+)-dehydroabietic acid (DHA) were identified as anti-biofilm leads. These leads were characterized as desirable antimicrobials that displayed both antibacterial and anti-biofilm activity in contrast to conventional antibiotics. They were able to prevent biofilm formation and eradicate pre-formed biofilms at micromolar concentrations. A second discovery strategy allowed the identification of two flavone derivatives as Quorum Sensing Inhibitors (QSIs). As opposed to the leads identified by the first strategy, these leads did not display any bactericidal activity but interfered with biofilm formation and maturation.

Furthermore, given the relevance of biofilm models for drug discovery, a comparative methodological study was also performed. Efficacy testing of conventional antibiotics in prevention of biofilm formation was conducted in two distinct biofilm models, microtiter well plates (MWP) and drip flow reactor (DFR), classified as closed and open systems, respectively. The goal was to investigate if the choice of model affects the experimental outcome. The comparative study revealed that biofilms grown under continuous flow

of nutrients displayed significantly higher antimicrobial tolerance than those grown in the absence of flow.

Altogether, this thesis project led to the identification of anti-biofilm leads, which can serve as starting points for further optimization towards more potent biofilm inhibitors that can be used either as alternatives to conventional antibiotics or as adjunctive agents in combination with conventional antibiotics or other antimicrobials. Given the complexity of biofilms, it is increasingly understood that no single strategy will be sufficient for biofilm control. Thus, complementary strategies aimed at interfering with biofilms in different mechanisms could offer a promising solution. Further, when selecting the best anti-biofilm compounds, activity of the most promising compounds needs to be confirmed using different biofilm models, as the choice of biofilm model was shown to have a profound impact on the experimental outcome.

Svensk sammanfattning

Att bakterier bildar biofilmer komplicerar diagnos och behandling av bakteriella infektioner. Bakteriella biofilmer kan definieras som komplexa bakteriesamhällen inbäddade i en matris av extracellulära polymera substanser (EPS), som består av polysackarider, proteiner och extracellulärt DNA. De facto är biofilmer den dominerande livsformen av bakterier i naturen. Bakterier kan fästa vid och bilda biofilmer på en mängd olika ytor, t.ex. på tänderna i form av plack, i vävnader, på proteser och även på fartygsskrov och stenar. Matrisen skyddar bakterierna från antibiotika, desinfektionsmedel och kroppens immunförsvar. Dessutom skiljer sig biofilmbakterierna markant från de bakterier som existerar som planktoniska, enstaka celler och växer i flytande medier, särskilt med avseende på tillväxt, stresstålighet och genuttryck. Detta ytterligare ökar deras motståndskraft och leder till uppkomsten av kroniska och svårbehandlade infektioner. Biofilmsalstrande bakterier står för upp till 80 procent av alla bakteriella infektioner, speciellt dem associerade med användningen av främmande material, t.ex. i form av katetrar och implantat, och hos hospitaliserade patienter. Trots detta riktar antibiotika in sig på att döda endast planktoniska bakterier. Hittills har inget antibiotikum speciellt mot bakteriella biofilmer godkänts av myndigheter.

Naturprodukter har varit en viktig källa i läkemedelsutvecklingen, speciellt i samband av upptäckten av antibiotika. I detta sammanhang var syftet med denna avhandling att identifiera bioaktiva substanser mot bakteriella biofilmer från substansbibliotek, innehållande föreningar som antingen är naturprodukter eller syntetiserade derivat, som alternativ till konventionell antibiotika. För detta ändamål utnyttjades olika sållningsmetoder vid sållningen av sammanlagt 3 570 substanser. Därtill utfördes en jämförande studie med antibiotika i två olika experimentella system som används för biofilmstudier för att undersöka inverkan av systemet på experimentella resultat.

Under projektets gång identifierades och karaktäriserades ett antal naturinspirerade substanser med olika verkningsmekanismer. Två syntetiska flavanderivat från ett stort substansbibliotek med flavonoider och D-tryptofan och β-cyklohexyl-L-alaninderivat av dihydroabietinsyra identifierades som ledtrådsmolekyler med hjälp av fenotypisk sållning. Till skillnad från antibiotika, uppvisade dessa molekyler antimikrobiell effekt både på biofilmbakterier och planktoniska celler. Målbaserad sållning av ett substansbibliotek med 3 040 naturproduktderivat ledde till identifikation av

substanser som inhiberade biofilmbildning genom att hämma bakteriell kommunikation, quorum sensing-signalering. Två derivat av flavonklass karaktäriserades som mest aktiva. Dessa substanser uppvisade biofilmreducerande effekt utan att bakterieväxten påverkades. Slutligen påvisade den jämförande studien att valet av experimentellt system påverkar antibiotikakänsligheten hos biofilmer. Biofilmer som hade växt i ett flödessystem, droppflödesreaktor (eng. drip flow reactor), var betydligt motståndskraftigare mot antibiotika än de som växte i mikrotiterplattor.

List of original publications

I. **Manner S**, Skogman M, Goeres D, Vuorela P, Fallarero A. Systematic exploration of natural and synthetic flavonoids for the inhibition of *Staphylococcus aureus* biofilms. *Int. J. Mol. Sci.* 2013, 14: 19434-19451.

II. **Manner S***, Vahermo M*, Skogman ME, Krogerus S, Vuorela PM, Yli-Kauhaluoma J, Fallarero A, Moreira VM. New derivatives of dehydroabietic acid target planktonic and biofilm bacteria in *Staphylococcus aureus* and effectively disrupt bacterial membrane integrity. *Eur. J. Med. Chem*. 2015, 102: 68-79.

III. **Manner S**, Fallarero A. Screening of natural product derivatives for quorum sensing inhibition identifies two structurally related flavonoid derivatives as potent quorum sensing inhibitors against Gram-negative bacteria (*manuscript*).

IV. **Manner S**, Goeres DM, Skogman M, Vuorela P, Fallarero A. Prevention of *Staphylococcus aureus* biofilm formation by antibiotics in 96-Microtiter Well Plates and Drip Flow Reactors: critical factors influencing outcomes. *Sci. Rep*. 2017, 7:43854.

* Equal contribution

Supporting publications

i. Oja T, San Martin Galindo P, Taguchi T, **Manner S**, Vuorela, PM, Ichinose K, Metsä-Ketelä M, Fallarero A. Effective anti-biofilm polyketides against *Staphylococcus aureus* from the pyranonaphthoquinone biosynthetic pathways of Streptomyces species. *Antimicrob. Agents Chemother*. 2015, 59(10):6046-52.

ii. Skogman ME, Kanerva S, **Manner S**, Vuorela PM, Fallarero A. Flavones as Quorum Sensing Inhibitors Identified by a Newly Optimized Screening Platform Using *Chromobacterium violaceum* as Reporter Bacteria. *Molecules.* 2016, 21(9). pii: E1211.

Contribution of the author

I. All the experimental work and data analysis were performed by the author. The manuscript was written together with the supervisors.

II. Biofilm experiments except for imaging were performed by the author. Author contributed to the writing of the manuscript.

III. All the experimental work and data analysis were conducted by the author. The first draft of the manuscript was written by the author.

IV. All the experimental work was performed by the author, except for the initial susceptibility testing of antibiotics in microtiter well plates and the confocal imaging. All data analysis was performed by the author. The first draft of the manuscript was written by the author.

Abbreviations

ADME	Absorption, distribution, metabolism and excretion
AHL	Acyl homoserine lactone
AI	Autoinducer
AI-2	Autoinducer 2
ALT	Antimicrobial lock therapy
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ASTM	American Society for Testing and Materials
ATCC	American type culture collection
ATP	Adenosine triphosphate
BAP	Biofilm-associated protein
BRA	Benefit-risk assessment
BZK	Benzalkonium chloride
c-di-GMP	Bis-(3′-5′)-cyclic dimeric guanosine monophosphate
CAUTI	Catheter-associated urinary tract infection
CBD	Calgary Biofilm Device
CRBSI	Catheter-related bloodstream infection
CDC	Center for Disease Control
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
CoNS	Coagulase negative Staphylococci
CRE	Carbapenem-resistant Enterobacteriaceae
CSTR	Continuous flow stirred-tank reactor
CV	Coefficient of variation
CVC	Central venous catheter
DFR	Drip flow feactor
DHA	(+)-Dehydroabietic acid
$DiBAC_{4(3)}$	Bis-(1,3-dibutylbarbituric acid)trimethine oxonol
DMMB	Dimethylmethylene blue
DMSO	Dimethyl sulfoxide
DOS	Diversity-oriented synthesis
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Fluorescent fluorescein diacetate

HTS	High-throughput screening
IC_{50}	Half-maximal inhibitory concentration
IC_{90}	90% inhibitory concentration
IUD	Intra uterine device
LB	Luria-Bertani
LBY	LB media supplemented with yeast extract
LC-MS	Liquid chromatography–mass spectrometry
LD	Log density
LR	Log reduction
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibitory concentration
MHB	Mueller-Hinton broth
MIABiE	Minimum information about a biofilm experiment
MIC	Minimum inhibitory concentration
MQ	Milli-Q
MRAB	Multidrug-resistant *Acinetobacter baumannii*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MW	Molecular weight
MWP	Microtiter well plate
NMR	Nuclear magnetic resonance
NP	Natural product
OD	Optical density
PBS	Phosphate buffer saline
PFR	Plug flow reactor
PIA	Polysaccharide intercellular adhesin
PNAG	Poly-N-acetyl glucosamine
QQ	Quorum quencher
QS	Quorum sensing
QSI	Quorum sensing inhibitor
RO5	Lipinski's rule of five
SAM	S-adenosylmethionine
S/B	Signal-to-background
S/N	Signal-to-noise
SA/V	Surface-area-to-volume
SAR	Structure-activity relationships
SD	Standard deviation

TOS	Target-oriented synthesis
TSB	Tryptic soy broth
TSA	Tryptic soy agar
UTI	Urinary tract infection
VRE	Vancomycin-resistant *Enterococcus faecium*
WGA	Wheat germ agglutinin
Z′ factor	Signal window coefficient

1 Introduction

Bacteria can switch between two lifestyles, and exist as free-floating, planktonic cells or as surface-associated communities encased in a matrix of extracellular polymeric substances, also known as biofilms. In fact, up to 99% of all bacteria are found in the form of biofilm at various stages of growth (Davey and O'Toole 2000). Biofilm bacteria exhibit distinct phenotype from planktonic counterparts, particularly with respect to gene expression and growth rate, and are significantly more tolerant to antimicrobials and host immune responses (Stewart and Franklin 2008). Several features connected to the biofilm mode of growth contribute to the tolerance of biofilms. First, biofilm matrix protects cells from external insults and environmental stresses. Secondly, within the biofilm, bacteria exist as a heterogeneous population of cells that are in various physiological states due to oxygen and nutrient gradients. Further, the non-dividing, dormant population of bacteria, persister cells, is in well-protected mode and highly tolerant antibiotics. Consequently, cells embedded in a biofilm are typically up to 1000 times more tolerant to antimicrobials than the planktonic cells of the same species (Olson et al. 2002).

Biofilms have a significant impact on human health, as it has been estimated that up to 80% of all human bacterial infections can be attributed to biofilm formation (Bjarnsholt 2013). Acute infections are generally curable with conventional antibiotics. However, when an infection develops into chronic state involving a biofilm, it may be impossible to eradicate. Despite this, antimicrobial drug discovery has conventionally focused on planktonic bacteria. Until now, all the marketed antibiotics are targeting the growth of dividing, planktonic cells (Fey 2010), and thereby, fail in the treatment of biofilm-associated infections. Since biofilms differ from planktonic cells by phenotype and morphology, several biofilm models have been developed for growing biofilms and evaluating antimicrobials against them (Lebeaux et al. 2013). However, to date, no antimicrobial agent has been approved by the regulatory authorities to be specifically used against biofilm-growing bacteria. Thus, novel anti-biofilm agents and approaches to treat and prevent biofilm formation are in great demand.

The majority of all marketed antimicrobials are based on natural compounds or synthetic derivatives of natural compounds (Newman and Cragg 2016). Therefore, natural compounds that have coevolved with bacteria in nature provide a reasonable starting point for exploration of anti-biofilm

compounds. As the eradication of existing biofilms is challenging, prevention of biofilm formation is an attractive approach to biofilm control. Further, since conventional antibiotics are ineffective against pre-formed biofilms, compounds that can eradicate pre-formed biofilms are of great importance. In this thesis, the search for compounds that act as efficient biofilm inhibitors was performed.

However, it has been argued that bactericidal compounds, while effective in the short-term, are also prone to the development of resistance. Therefore, searching for compounds that interfere with the biofilm lifecycle without bactericidal activity, for example via inhibition of quorum sensing (QS), is increasingly regarded as a valuable therapeutic alternative (Brackman and Coenye 2015). This strategy was also explored in this thesis project.

Finally, there is significant evidence suggesting that the choice of the biofilm model can have a significant impact on the experimental outcome (Buckingham-Meyer, Goeres and Hamilton 2007, Coenye and Nelis 2010). Understanding the differences between biofilm models is an essential aspect of biofilm research, and it greatly impacts drug discovery strategies. Thus, the evaluation of antimicrobials against biofilms in various models was also a focus in this thesis project.

2 Literature review

2.1 Bacterial biofilms

Bacterial biofilms are defined as surface-associated, organized communities of bacterial cells embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Donlan 2002). The biofilm phenomenon was first described in the 17th century by Antonie van Leeuwenhoek, who discovered bacteria in the form of plaque from his teeth and, using a microscope, observed that the "animalculi" had produced a community (Costerton, Stewart and Greenberg 1999). Today, it is well recognized that bacteria exist predominantly as biofilms rather than as free-floating cells in most environments (Watnick and Kolter 2000, Flemming et al. 2016). The biofilm mode of growth enables bacteria to resist environmental stresses; it protects bacteria from desiccation and external insults, including antimicrobials, host defenses and predators (Dunne 2002, López, Vlamakis and Kolter 2010). Biofilms can be formed virtually everywhere and have important consequences, as they can provide both beneficial and detrimental effects (Kolter and Losick 1998). Examples of beneficial biofilms include those in natural ecosystems, such as in streams, soil and plant rhizosphere allowing mutualistic symbioses, and commensal biofilms in the human body (Battin et al. 2016, Burmølle et al. 2010). However, in medical and industrial settings biofilms cause severe problems, i.e. chronic and persistent infections as well as biofouling and corrosion (Bjarnsholt 2013, Dobretsov, Abed and Teplitski 2013). This latter aspect has established biofilm control strategies as an attractive and important research area.

2.1.1 Biofilm lifecycle

The switch between planktonic and biofilm mode of growth occurs in response to environmental changes. This transition involves a number of physical, chemical and biological regulatory mechanisms and components that greatly differ between bacterial species, and even among strains of the same species (O'Toole, Kaplan and Kolter 2000, López et al. 2010). The best characterized molecular mechanism beyond biofilm formation is extracellular quorum sensing (QS), which coordinates expression of biofilm-specific genes in a cell density-dependent manner (Rutherford and Bassler 2012).

Biofilm formation is strongly influenced by the environmental conditions (Tolker-Nielsen and Molin 2000). For example, in the case of *Pseudomonas*

aeruginosa, environments rich in nutrients promote biofilm formation, whereas nutrient depletion leads to dispersal (Delaquis et al. 1989, Hunt et al. 2004). Contrary to *P. aeruginosa*, biofilm formation of *Escherichia coli* and *Staphylococcus aureus* is enhanced in the absence of nutrients (Ryu, Kim and Beuchat 2004, Pagedar, Singh and Batish 2010). Despite differences in regulatory components and molecular mechanisms, bacterial biofilm formation in general involves three consecutive steps; attachment, maturation and dispersal, and requires three essential components; bacteria, matrix and surface (Joo and Otto 2012). Biofilm formation is schematically shown in **Figure 1**.

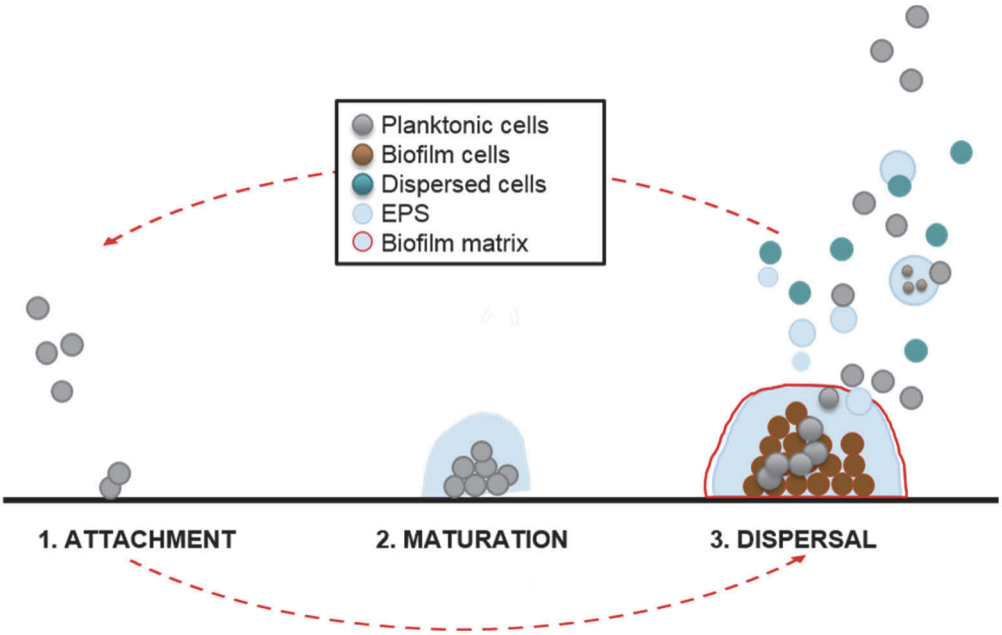


***Figure 1**. The biofilm lifecycle. The transition between the growth modes is a dynamic process*.

Several studies have shown that the initial attachment of bacteria to the surfaces typically occurs within minutes, and depending on the species and environmental conditions, microcolonies are formed within one to four hours after the irreversible attachment (Fallarero et al. 2013, Costerton 1984). Bacteria start to produce EPS within 6-12 hours and reach the highest cell density within 48 to 96 hours (Shafique et al. 2017, Anwar, Strap and Costerton 1992).

2.1.1.1 Attachment

Biofilm formation is initiated by dynamic surface attachment of planktonic bacteria. The attachment involves two steps, initial reversible attachment and irreversible attachment. Physical forces, including van der Waals forces, steric and electrostatic interactions along with extracellular bacterial surface structures, such as flagella and cell wall-attached adhesins, are involved in the initial adhesion step, while the expression of cell adhesion structures, such as pili and fimbriae, has been associated with the irreversibly binding (Pratt and Kolter 1998, Hall-Stoodley and Stoodley 2002). For instance, staphylococcal adherence to host tissues is mediated by specific adhesins, the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Heilmann 2011, Paharik and Horswill 2016), and also to abiotic surfaces when covered with the host plasma components (Cucarella et al. 2001). Also, autolysins (Heilmann et al. 1997), and the biofilm-associated protein (Bap), which is present in *S. aureus* isolates from animals with bovine mastitis but not in human isolates (Tormo et al. 2005), are involved in the adherence and biofilm formation of staphylococci. Furthermore, the polysaccharide intercellular adhesin (PIA) plays an important role (Arciola et al. 2015). In turn, flagella and type IV pili are essential factors mediating the initial attachment of *P. aeruginosa* and *E. coli* (Laverty, Gorman and Gilmore 2014).

2.1.1.2 Maturation

After the reversible attachment, bacterial cells start to communicate with each other by means of chemical signals (autoinducers), which result in the expression of biofilm-specific genes and the initiation of the biofilm maturation process (Davies et al. 1998). This stage involves aggregation of the cells into microcolonies and subsequent production of EPS followed by the growth and maturation of the adhered cells (Hall-Stoodley, Costerton and Stoodley 2004). Microcolonies are separated by water channels that enable the supply of nutrients and oxygen throughout the biofilm, as well as the draining of metabolic end products, respectively (Davey and O'Toole 2000, Lindsay and von Holy 2006). However, nutrients and oxygen are unevenly distributed within the biofilm, and pH and cell density vary as well. In response to the environmental conditions, cells differentiate and constitute a heterogeneous population within the biofilm (Stewart and Franklin 2008, Stoodley et al. 2002). The composition of biofilm matrix varies between species, but generally, the EPS is composed of proteins, polysaccharides and extracellular DNA (eDNA). For instance, three polysaccharides, Pel, Psl and alginate, have been shown to have an important role in biofilm formation and maintenance

of *P. aeruginosa* (Colvin et al. 2012). Poly-N-acetyl glucosamine (PNAG), in turn, is a major polysaccharide in the matrix of *Staphylococcus* spp. biofilms (Kropec et al. 2005). Importantly, the matrix acts as a stabilizing scaffold for the biofilm structure (Flemming and Wingender 2010).

2.1.1.3 Dispersal

The last stage, dispersal, involves the detachment of bacteria from outer layers of the biofilm and the colonization of new surfaces by the detached cells. The dispersal of cells also leads to dissemination of infections (Parsek and Singh 2003). Generally, it has been well recognized that cells switch to the planktonic mode and regain the susceptibility when they disperse from the biofilm (Singh et al. 2009, Kaplan 2010). However, recently a third phenotype of bacteria, namely dispersed cells, has been identified (McDougald et al. 2011). Dispersed cells with distinct physiology from the planktonic and biofilm cells have been characterized as highly motile. This characteristic allows fast colonization of new environments. Notably, dispersed cells of *P. aeruginosa* have been shown to be more virulent against macrophages compared to conventionally grown planktonic cells (Chua et al. 2014).

Dispersal can occur in two distinct ways, passively or actively. The passive detachment of cells, which occurs as a result of external influences, such as mechanical interruption or fluid shear, can be categorized into two sub-types, sloughing and erosion. Sloughing refers to rapid, massive loss of biofilm, while erosion happens continuously, leading to the detachment of single cells or small portions of the biofilm (Stoodley et al. 2001, Kaplan 2010). In contrast to passive dispersal, active dispersal refers to the events induced by the bacteria in response to a variety of environmental changes, including alteration in nutrient availability (Hunt et al. 2004), oxygen depletion (Thormann et al. 2005), toxic byproducts, bacteriophages and changes in temperature (Fleming and Rumbaugh 2017). In addition, production of EPS degrading enzymes can contribute to bacterial detachment of both gram-negative and gram-positive species (Hall-Stoodley and Stoodley 2005), as well as presence of fatty acids (Davies and Marques 2009), and QS-molecules (Boles and Horswill 2008, Lauderdale et al. 2010, Richards et al. 2008).

2.1.1.4 Quorum sensing (QS)

QS includes production, secretion and detection of chemical signals termed autoinducers (AIs) (Papenfort and Bassler 2016). As the cell density increases, the AI concentration increases simultaneously. Once a threshold concentration of AI is reached, transcription of the biofilm-specific genes

increases. The AIs have been demonstrated to coordinate several steps of biofilm formation, including attachment, maturation and dispersal. Additionally, QS controls various features, such as bioluminescence in *Vibrio fischeri* (Miyashiro and Ruby 2012), production of virulence factors in *P. aeruginosa* (Köhler, Buckling and van Delden 2009), and antibiotic production in *Chromobacterium violaceum* (McClean et al. 1997). Importantly, most of them are connected to the virulent phenotype.

Both gram-negative and gram-positive bacteria employ QS (Brackman and Coenye 2015). However, the QS mechanisms, as well as the signal molecules are diverse (**Figure 2**). Three main QS systems have been characterized; (i) the N-acyl homoserine lactone (AHL)-based system, (ii) the autoinducing peptide (AIP)-mediated system in gram-negative and gram-positive bacteria, respectively, and (iii) the autoinducer 2 (AI-2) system in both gram-negative and gram-positive bacteria. In gram-negative species, QS is mainly mediated by AHLs, which are synthesized from S-adenosylmethionine (SAM) and diffused through the bacterial membrane, and recognized and bound by cytoplasmic receptors. AHLs consist of a homoserine lactone ring bearing an acyl chain of C_4 to C_{18} in length (Fuqua, Parsek and Greenberg 2001). Regulation of the AHL-mediated QS is coordinated by two proteins, the transcriptional regulator R that is homologous to LuxR in *V. fischeri*, and the autoinducer synthase I corresponding to LuxI of *V. fischeri* (Fuqua, Winans and Greenberg 1994). For example, *P. aeruginosa* has two pairs of LuxI/LuxR homologs, LasI/LasR and RhlI/RhlR (de Kievit 2009), while in *C. violaceum*, the LuxI/LuxR homolog CviI/CviR is responsible for QS (McClean et al. 1997).

QS in gram-positive bacteria relies on secreted oligopeptides, also termed autoinducing peptides (AIPs), and a two-component regulatory system, which consists of a membrane-bound sensor and intracellular response regulator for controlling the gene expression (Miller and Bassler 2001). In the third QS system, LuxS regulates the production of AI-2 (Vendeville et al. 2005).

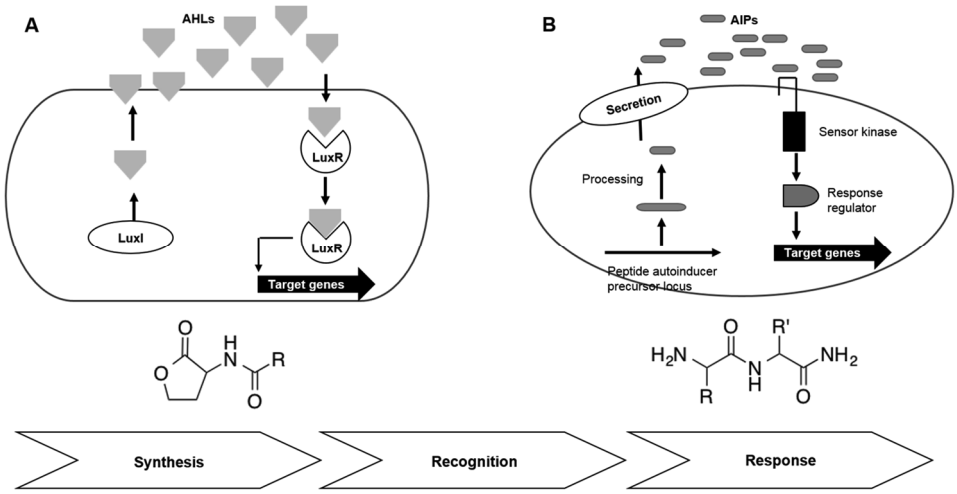


***Figure 2**. QS in gram-negative and gram-positive bacteria. Panel A depicts AHL-based QS in gram-negative bacteria, and panel B AIP-mediated QS system in gram-positive bacteria. Adapted from Bassler (2002).*

2.1.2 Specific features of biofilms

Biofilm mode of growth exhibits distinct phenotype from planktonic counterparts, particularly with respect to gene expression and protein production (Stewart and Franklin 2008). Bacteria within the biofilms are typically up to 1000-fold more tolerant to antimicrobials than their planktonic counterparts (Mah and O'Toole 2001, Ceri et al. 1999). Notably, development of antimicrobial tolerance occurs gradually over time. It has been shown that bacteria at early stages of biofilm development, as well as freshly formed biofilms, are substantially more susceptible to antibiotics than biofilms that have formed for 24-48 hours (Wolcott et al. 2010, Martens and Demain 2017). Biofilm bacteria may persist in the host due to increased evasion from host immune defenses, such as macrophages, which cannot penetrate the biofilm matrix (Moser et al. 2017). The conventional, heritable antibiotic resistance mechanisms include inactivation of drugs via hydrolysis (e.g., β-lactam resistance) (Munita, Bayer and Arias 2015) or modification of the drugs (e.g., aminoglycoside resistance) (Mingeot-Leclercq, Glupczynski and Tulkens 1999), alteration of the drug targets (e.g., fluoroquinolone resistance) (Redgrave et al. 2014), and efflux of drugs via membrane-bound efflux pumps (Webber and Piddock 2003). In contrast, the antimicrobial tolerance associated with biofilms is a transient, non-heritable phenotype that decreases the

susceptibility of bacteria (Stewart and Costerton 2001). Antibiotic tolerance of biofilms has been attributed to multiple causes, including the failure of antibiotics to target biofilm embedded cells, altered microenvironments within the biofilms that promote activation of stress responses, slow growth rate and persister cells.

The biofilm matrix provides protection from external insults and may represent a physical barrier to the diffusion of antibiotics, leading to retarded penetration (Bordi and de Bentzmann 2011). This feature, however, appears to be dependent on the physicochemical properties of the antimicrobials (Stewart 2015). For instance, rifampicin can penetrate *Staphylococcus epidermidis* biofilms (Zheng and Stewart 2002) and tetracycline *E. coli* biofilms (Stone et al. 2002), whereas transport of vancomycin through the matrix of *S. aureus* biofilms is retarded (Jefferson, Goldmann and Pier 2005). Similarly, hypochlorite penetration through *P. aeruginosa* and *Klebsiella pneumoniae* biofilms is delayed (Stewart et al. 2001). Moreover, the eDNA present in the matrix of *P. aeruginosa* biofilms has been proposed to act as a chelator of cationic antimicrobials, such as peptides, as well as to prevent penetration of aminoglycosides (Mulcahy, Charron-Mazenod and Lewenza 2008, Chiang et al. 2013). Additionally, enzymes, such as beta-lactamases that can degrade antibiotics are present in the matrix (Donné and Dewilde 2015). For that reason, penetration of ampicillin through *K. pneumoniae* biofilm is limited (Anderl, Franklin and Stewart 2000).

Nutrient and oxygen gradients within the biofilms contribute to the heterogeneity and differentiation of bacteria, which in turn affect the antimicrobial tolerance of biofilm-growing bacteria (Stewart 2002). Cells located at the surface are more metabolically active, while cells in the deeper layer of the biofilms are slow-growing due to lack of nutrients and oxygen suppression. As a result, cells in the different locations within a biofilm will respond differently to antimicrobial therapy (Nguyen et al. 2011, Poole 2012). Generally, antibiotics are less effective against stationary phase bacteria (Eng et al. 1991). For example, β-lactams target only exponentially growing bacteria (Tuomanen et al. 1986). Tetracycline and ciprofloxacin, in turn, have been reported to be effective in the killing of metabolically active cells in *P. aeruginosa* biofilms, whereas colistin was effective against the slow-growing fraction of the cells (Pamp et al. 2008). Further, a small fraction of the non-dividing, dormant population termed persister cells, contribute significantly to the antimicrobial tolerance of biofilms (Lewis 2008). The formation of persisters occurs in response to bacterial stress, as well as when biofilms reach

the mid-exponential growth phase (Dörr, Lewis and Vulić 2009). These cells survive even from prolonged antibiotic treatment, thus serving as reservoirs of infections. When the antibiotic exposure is over, the cells start to grow again leading to the relapse of infection (Lewis 2007, Mulcahy et al. 2010). The persister cells are not exclusively associated with biofilm bacteria, but more prevalent among biofilms than in planktonic cultures (Spoering and Lewis 2001).

Notably, even though the antimicrobial tolerance of biofilms is not connected to the conventional resistance mechanisms, mutation frequency and gene transmission are increased in biofilms due to proximity of bacteria (Høiby et al. 2010). The biofilm mode of growth has shown to promote horizontal gene transfer and adaptive mutations allowing bacteria to survive from environmental stresses, including antibiotic treatment and starvation (Savage, Chopra and O'Neill 2013, Driffield et al. 2008).

2.1.3 Clinical impact of biofilms

Biofilms are medically important, accounting for 65-80% of all bacterial infections according to the US Center for Disease Control (CDC) and the National Institutes of Health (NIH), respectively. Biofilm formation poses a significant economic burden for healthcare, as it is estimated that 17 million biofilm infections, leading to 550,000 deaths, occur in the United States every year (Worthington, Richards and Melander 2012). Moreover, biofilm formation complicates both diagnosis and treatment of infections. Biofilm-associated infections can occur both on host tissues, including wounds and lungs of cystic fibrosis patients, and on medical devices and foreign implanted material. Consequently, they can be divided into two groups: tissue- and device-related, depending on the surface involved in biofilm formation (Bjarnsholt 2013). Notably, the majority of nosocomial infections are biofilm-associated and related to the use of medical devices (Percival et al. 2015). Typical infections associated with biofilm formation are summarized in **Table 1**.

***Table 1**. Infections associated with biofilm formation and causative bacteria.*

Tissue-related infections	Bacterial species involved	Reference (ex)	Medical device-related infections	Bacterial species involved	Reference (ex)
Cystic fibrosis	*P. aeruginosa* *S. aureus* *H. influenzae*	*Høiby, Ciofu and Bjarnsholt 2010b, Ahlgren et al. 2015, Cardines et al. 2012*	Urinary tract catheters	*E. coli* *K. pneumoniae* *P. aeruginosa* *Acinetobacter* Coagulase negative Staphylococci (CoNS) Enterococci	*Niveditha et al. 2012*
Wounds	*S. aureus* *P. aeruginosa* *K. pneumoniae* *E. faecalis*	*Metcalf and Bowler 2013, Gjødsbøl et al. 2006*	Central-venous-catheters (CVCs)	*S. aureus* *S. epidermidis* *P. aeruginosa* *K. pneumoniae* *A. baumanii*	*Gahlot et al. 2014, Dasgupta 2002*
Chronic otitis media	Staphylococci *H. influenza* *S. pneumoniae* *M. catarrhalis*	*Wessman et al. 2015, Thornton et al. 2011*	Orthopedic implants, prosthetic joints	*S. epidermidis* *S. aureus* *Propionibacterium acnes*	*McConoughey et al. 2014, Achermann et al. 2014*
Urinary tract infection	*E.coli* *P. mirabilis* *P. aeruginosa* *K. pneumonia* Staphylococci	*Hashemizadeh, Kalantar-Neyestanaki and Mansouri 2017, Delcaru et al. 2016*	Prosthetic valves and pacemakers	*S. aureus* CoNS *S. pneumoniae* *P. acnes*	*Cahill and Prendergast 2016, Natsheh et al. 2014, Achermann et al. 2014*
Osteomyelitis	Staphylococci Enterococci Streptococci *P. aeruginosa* *H. influenza*	*Brady et al. 2008, Mohamed and Huang 2007*	Endotracheal tubes	*P. aeruginosa* *A. baumannii*	*Gil-Perotin et al. 2012*
Endocarditis	Staphylococci *S. pneumoniae*	*Agarwal, Singh and Jain 2010*	Peripheral vascular catheters	CoNS *S. aureus*	*Zhang et al. 2016*
Chronic sinusitis	*S. aureus* *P. aeruginosa* *H. influenza* *S. pneumoniae* *M. catarrhalis*	*Tikhomirova and Kidd 2013, Tajudeen, Schwartz and Palmer 2016*	Intra uterine devices (IUDs)	CoNS *G. vaginalis* Klebsiella spp. Staphylococci	*Abdel-Hafeez et al. 2014*
Dental caries	*S. mutans* *A. naeslundii*	*Huang, Li and Gregory 2011, Marsh 2006*	Contact lenses	*P. aeruginosa* *Achromobacter* *Stenotrophomonas* *Delftia*	*Wiley et al. 2012, Robertson et al. 2011*
Periodontitis	*P. gingivalis* *A. actinomycetemcomitans* *F. nucleatum*	*Teles et al. 2013*	Tissue fillers	*S. epidermidis* *P. acnes*	*Christensen et al. 2013*
Vaginosis	*G. vaginalis*	*Patterson et al. 2010*	Breast implants	*S. aureus* *S. epidermidis* *P. acnes*	*Chessa et al. 2016, Rieger et al. 2013*
Kidney or bladder stones	*P. mirabilis*	*Schaffer and Pearson 2015*	Urological prosthetics	*S. epidermidis*	*Faller and Kohler 2017*

Several bacterial species are involved in biofilm-associated infections that can be caused by single- or multi-species biofilms (Martin et al. 2015). The source of infection involves commensal species, such as staphylococci and *Propionibacterium acnes* as well as environmental pathogens, such as *P. aeruginosa* (Joo and Otto 2012). The major pathogens involved in biofilm-associated infections include both gram-positive, such as *S. aureus* and *S. epidermidis*, and gram-negative species, including *P. aeruginosa* and *E. coli*. Staphylococci are the most frequent cause of nosocomial infections, especially those associated with the use of catheters, orthopaedic materials, and implants, such as prosthesis and breast implants (Gomes, Teixeira and Oliveira 2014). The PIA produced by staphylococci has been shown to promote staphylococcal biofilm formation on foreign body materials (Olson et al. 2006). *S. aureus* is regarded as the most virulent representative, which infects both hospitalized patients and healthy immunocompromised individuals. In addition to the medical device-related infections, *S. aureus* is typically involved in osteomyelitis, endocarditis, chronic otitis media and sinusitis as well as in wound infections. Coagulase-negative staphylococci (CoNS), such as *S. epidermidis*, in turn, are the leading cause of medical device-related infections (Vuong et al. 2003, Wu et al. 2015). Together, *S. aureus* and CoNS account for 50% of the prosthesis-related infections (Song et al. 2013). Of these infections, those occurring within the three first months after the surgery are typically caused by *S. aureus*. By contrast, delayed infections occurring postoperatively, are usually caused by CoNS. Late infections (24 months after the surgery), in turn, are typically caused by *S. aureus* (Gbejuade, Lovering and Webb 2015). Moreover, *S. aureus* and CoNS are responsible for 75% of osteomyelitis cases (Brady et al. 2008), and further staphylococcal biofilms along with streptococci and enterococci account for over 80% of infective endocarditis cases (Elgharably et al. 2016).

P. aeruginosa is implicated in a variety of chronic biofilm infections, including cystic fibrosis, wound infections, sinusitis, otitis media, UTIs and keratitis. This bacterial species is the main causative bacteria of cystic fibrosis. Moreover, it is most frequently associated with the chronic pulmonary infections, such as pneumonia, particularly in cystic fibrosis patients (Høiby et al. 2010b, Moskowitz et al. 2004). In chronic wounds, *P. aeruginosa* is often found along with *S. aureus*, and particularly in the deeper layers of the wounds (Kirketerp-Møller et al. 2008). Typically, chronic wounds which are infected by *P. aeruginosa* are also deeper and larger in size than others (Gjødsbøl et al. 2006).

The leading cause of urinary tract infections (UTIs) is *E. coli*. These infections are also the most common cause of nosocomial infections, accounting for over 40% of all nosocomial infections (Jacobsen et al. 2008). Of these infections, 80% can be attributed to the use of urinary tract catheters (Sabir et al. 2017). In total, 10-20% of the hospitalized patients undergo catheterization, and among them, 10-50% develop a UTI. As a consequence, catheter-associated urinary tract infections (CAUTIs) cause one million additional hospital stays in the US every year (Hancock, Dahl and Klemm 2010, Foxman 2002).

2.1.4 Treatment of biofilm infections

As mentioned earlier, the biofilm mode of growth contributes to the antimicrobial tolerance of bacteria. Thus, preventative strategies are advantageous to available treatment strategies. Further, bacteria in early stages of biofilm formation are still susceptible to antimicrobial therapy (Høiby et al. 2010a), and therefore, eradication of younger biofilms using conventional antibiotics may be possible. Unfortunately, early diagnosis of biofilm infections is usually difficult. No detection methods for the diagnosis of biofilm infections are currently available in the clinical settings (Percival et al. 2015). As a result, the majority of biofilm-associated infections involve mature biofilms, which are difficult or even impossible to eradicate. Two distinct approaches for the treatment of biofilm infections exist, preventative strategies aimed at prevention of biofilm formation, and treatment of established biofilm infections.

2.1.4.1 Preventative approaches

Various strategies have been utilized for prevention of biofilm formation on medical devices. These include modification of the surface, incorporation of antimicrobials into materials, and a combination of anti-adhesive and antimicrobial properties to inhibit the bacterial attachment to the surfaces (Arciola et al. 2012). For instance, antimicrobial-impregnated central venous catheters (CVCs) have been shown to effectively decrease the risk of infections (Raad et al. 2008). In clinical settings, CVCs impregnated with minocycline and rifampicin, marketed as the Cook Spectrum™ catheter, have been shown to be the most effective impregnations (Lai et al. 2016). Such catheters have been demonstrated to significantly decrease the number of catheter-related bloodstream infections (CRBSIs) compared to other catheters, such as chlorhexidine and silver-sulfadiazine-impregnated. Also, polyurethane

catheters (Hydrocath®) coated with a hydrophilic polymer, polyvinylpyrrolidone, and impregnated with benzalkonium chloride (BZK), a quaternary ammonium antimicrobial agent, prevent effectively risk of catheter-associated infections (Tebbs and Elliott 1994). On the contrary, marketed urinary tract catheters, such as silver alloy-coated latex catheters and nitrofurazone-coated silicon catheters, have only shown to postpone, not prevent, the emergence of CAUTIs (Francolini and Donelli 2010). However, the coated catheters have been demonstrated to be effective in short-term catheterization lasting less than 30 days.

Antimicrobial lock therapy (ALT) is another important approach for preventing biofilm formation on CVCs. A combination of an antibiotic and heparin used for flushing or locking the line has shown to prevent catheter-related (gram-positive mediated) infections by 50% (van de Wetering, van Woensel and Lawrie 2013). Antibiotic concentration used for ALT should be 100- to 1000-fold higher than the planktonic minimum inhibitory concentration (MIC) (Høiby et al. 2015). The use of ALT is supported by the fact that antibiotics can help to minimize the dispersal of planktonic cells from the biofilms formed on the catheters (Wu et al. 2015). Additionally, conventional antibiotics can be employed prophylactically, which means that the antibiotic reaches the site of a potential infection before the causative microorganism (Høiby et al. 2015).

2.1.4.2 Treatment using conventional antibiotics

Selection of the right antibiotic is crucial when it comes to treatment of biofilm-associated infections. An antibiotic needs to be effective and well-penetrating in order to obtain sufficient antibiotic concentration at the site of infection. Unfortunately, the MIC and the minimum bactericidal concentration (MBC), which are regarded as gold standards for determination of antimicrobial susceptibility of planktonic microorganisms, cannot be used to guide the antibiotic selection for biofilm-associated infections, as they hardly reflect the *in vivo* situation (Andrews 2001, Olson et al. 2002, Hengzhuang, Høiby and Ciofu 2014). Particularly, the MIC serves as an important reference for treatment of acute bacterial infections *in vivo* (Ceri et al. 1999). As biofilms are up to 1000-fold more tolerant to antibiotics than planktonic cells even *in vitro*, significantly higher concentrations than the MIC are needed for treatment of biofilm infections, and the recommended doses are only empirical (Høiby et al. 2015).

In general, long-term antibiotic therapy with high doses is used for the tissue-related infections (Wu et al. 2015). Moreover, combination therapy is

typically recommended over monotherapy to obtain clinically effective concentrations (Raad et al. 2007). For the device-related infections, in turn, treatment involves removal of the infected device and surgical debridement in addition to aggressive antibiotic therapy (Percival et al. 2015). Similarly, treatment of wound infections also requires a combination of non-antimicrobial and antimicrobial strategies. Typically, the mechanical debridement is combined with antimicrobial therapy involving two antibiotics from different mechanistic classes or a combination of an antibiotic and a local disinfectant (Høiby et al. 2015, Aslam 2008).

For cystic fibrosis, aggressive antibiotic therapy is the most important treatment strategy. Since the eradication of infection is rarely possible, the treatment is aimed at preventing biofilm formation and, thus, the progression of the infection into a chronic state (Ciofu et al. 2012). A combination therapy involving nebulised tobramycin and colistin or inhaled colistin and orally administered ciprofloxacin has shown to be effective in postponing the emergence of chronic *P. aeruginosa* infections (Høiby 2011). For treatment of osteomyelitis, β-lactams, such penicillin G and oxacillin, which exhibit time-dependent action, can be used against *S. aureus* (Archer et al. 2011). Moreover, polymeric-based materials, such as polymethylmethacrylate-gentamicin bone cement and beads (Septocal®), can be used for the treatment of bone and soft tissue infections (von Eiff et al. 2005). Vancomycin is the most commonly used antibiotic for the treatment of *S. aureus* biofilm infections (Liu et al. 2011). However, rifampicin and daptomycin have been shown to be more effective than vancomycin against *S. aureus* biofilms (Smith et al. 2009, Kiedrowski and Horswill 2011). Typically, an effective combination therapy against *S. aureus* biofilm infections consists of vancomycin and rifampicin or daptomycin and rifampicin, as rifampicin has shown to enhance the efficacy of glycopeptide and lipopeptide antibiotics (Olson et al. 2010). Additionally, rifampicin remains the only antibiotic that is highly effective against staphylococcal biofilm infections. Importantly, when used in combination with other antibiotics, rifampicin represents the best current treatment along with surgical debridement for the treatment of prosthetic joint infections (Gómez et al. 2011).

2.1.4.3 Treatment using enzyme-based products

Poly-N-acetylglucosamine (PNAG), an exopolysaccharide produced by staphylococci, is an essential component of the biofilm matrix, which can be degraded by β-*N*-acetylglucosaminidase, Dispersin B, which is an enzyme produced by *Aggregatibacter actinomycetemcomitans* (Itoh et al. 2005). Dispersin

B has shown to disperse *S. epidermidis* and *E. coli* biofilms (Izano et al. 2008, Itoh et al. 2005). By contrast, dispersin B did not induce dispersal of *S. aureus* biofilms (Izano et al. 2008). Additionally, dispersin B has demonstrated synergistic activity with conventional antibiotics, leading to effective biofilm removal and killing the bacteria (Donelli et al. 2007, Darouiche et al. 2009). Dispersin B is commercially available as wound care gel and spray, and medical device coatings (Gawande, Leung and Madhyastha 2014, Miller et al. 2014). Notably, these applications are not registered as drugs but as medical devices. However, they have been shown to enhance the clinical outcome of the chronic wound treatment (Wolcott and Rhoads 2008).

Another enzyme-based marketed product is nebulized DNase, Pulmozyme (Dentice and Elkins 2016). The dornase alfa solution of recombinant human deoxyribonuclease I (rhDNase) is used as a mucolytic drug to promote improved clearance of secretions in the treatment of pulmonary disease in cystic fibrosis patients.

2.2 Antimicrobial drug discovery

In addition to the widespread presence of difficult-to-treat infections due to biofilm formation, the emergence of antimicrobial resistance (AMR) poses a significant threat to public health. Today, infectious diseases are the second leading cause of death worldwide and also, the third leading cause of death in developed countries (Fauci 2001). Drug-resistant bacteria cause poor clinical outcomes leading to increased health care costs and mortality. According to an estimate given by the CDC, more than two million antibiotic-resistant infections, which are responsible for 23,000 deaths, occur in the US every year. Further, infectious diseases are associated with an economic burden of more than 120 billion dollars. In the European Union, antibiotic-resistant infections lead to 25,000 annual deaths (Prestinaci, Pezzotti and Pantosti 2015). Both gram-positive, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE) and drug-resistant *Streptococcus pneumoniae*, and gram-negative bacteria, namely multidrug-resistant *Acinetobacter baumannii* (MRAB), carbapenem-resistant *Enterobacteriaceae* (CRE) and *P. aeruginosa* are resistant to multiple antibiotics and therefore of serious concern. For example, MRSA alone causes annually more deaths than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined (Ventola 2015, Pendleton, Gorman and Gilmore 2013).

Despite this, interests in antimicrobial drug discovery by pharmaceutical companies have declined. Antimicrobial drug discovery is considered as an economically unattractive investment (Projan 2003, Kresse, Belsey and Rovini 2007). Typically, antimicrobials, especially antibiotics, are used for short-term therapy, in contrast to the drugs intended for the treatment of chronic diseases, such as cardiovascular diseases and diabetes. Further, novel, recently approved antibiotics are saved for future use. Additionally, regulatory requirements for marketing approval in the US and EU generate an extra challenge, as they have been uncertain and prone to change (Renwick, Brogan and Mossialos 2016). Thus, there has been a substantial decrease in the number of approved antibiotics. Altogether, eight antibiotics, ceftaroline, fidaxomicin, bedaquiline, dalbavancin, tedizolid, oritavancin, ceftolozane–tazobactam, and ceftazidime–avibactam, have been approved by the FDA (the US Food and Drug Administration) between 2010 and 2015. Only one of them, bedaquiline, is a first-in-class antibiotic with a novel mechanism of action, different from those of previously approved (Deak et al. 2016).

2.2.1 General aspects

Drug discovery and development is a long and costly process (**Figure 3**). For a drug to be approved, it usually takes 12-15 years with a cost estimate of 2.6 billion dollars (Hughes et al. 2011, Avorn 2015). A majority of drug candidates fail in clinical trials, or even before, either due to lack of efficacy or adverse effects (Mullard 2017, Kola and Landis 2004). In fact, only one out of 5,000-10,000 lead compounds is approved and marketed as a drug (Balunas and Kinghorn 2005). Post-marketing safety monitoring conducted by the regulatory authorities after the approval can also lead to the withdrawn from the market in case of unfavourable benefit-risk assessment (BRAs) (Curtin and Schulz 2011). Antimicrobial drug discovery is not an exception with the estimated average of 8-12 years from discovery to market and, therefore, the discovery and development efforts need to be directed to meet the needs that will be present in 10 years (Thomson et al. 2004).

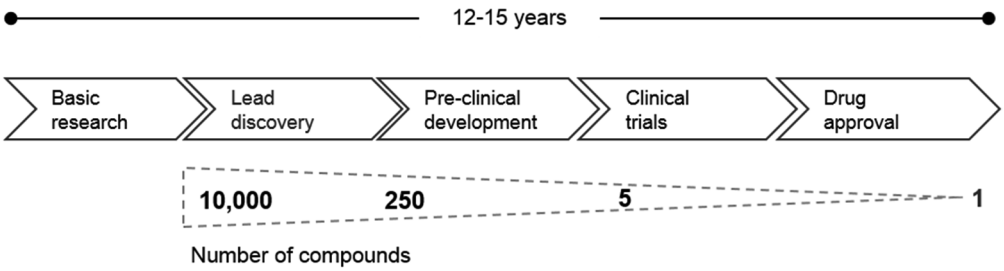


Figure 3. *Schematic representation of the drug discovery process. This thesis project focused on the lead discovery stage, schematically depicted in* **Figure 5**.

Even though the time span between studies varies, some similarities can be seen. The preclinical development before clinical trials takes approximately four years. The clinical trials including three phases typically take six years; one year for phase I involving 20–30 healthy volunteers in safety, tolerability, pharmacokinetic and dosage studies, 1.5 years for phase II involving 100–300 patient volunteers for investigation of the efficacy and side effects, and 3.5 years for phase III including 1000–5000 patient volunteers for monitoring the adverse effects related to long-term use. Finally, FDA review and approval takes one year as well as post-marketing monitoring (Martens and Demain 2017).

2.2.1.1 Lead discovery process

High-throughput screening (HTS), also referred to biomolecular screening, is an essential part of early drug discovery. It provides a practical way to identify hit compounds from large compound collections for further development. In the pharmaceutical industry, the standard antimicrobial bioassays, such as disk diffusion and broth or agar dilution, are not applicable in the antimicrobial drug discovery process in general, since assays need to be simple, rapid, inexpensive, and amenable for miniaturization (Swinney and Anthony 2011, Fallarero, Hanski and Vuorela 2014). The throughput of the standard methods is lower than that of HTS, and such methods are also more laborious and time-consuming compared to the HTS-based assays (von Nussbaum et al. 2006). Thus, the application of screening-compatible assays is essential for the efficient HTS of antimicrobials.

During the biomolecular screening, multiple compounds are screened for identification of those displaying desired bioactivity utilizing phenotypic or target-based screening approach (Terstappen et al. 2007, De La Fuente et al. 2006) (**Figure 4**). In phenotypic screening, compound libraries are typically screened using cell-based assays measuring effects on a particular

characteristic, such as cell proliferation in which several targets can be involved. In target-based screening, in turn, activity is measured against the pre-determined molecular targets, such as receptors or enzymes (Zheng, Thorne and McKew 2013).

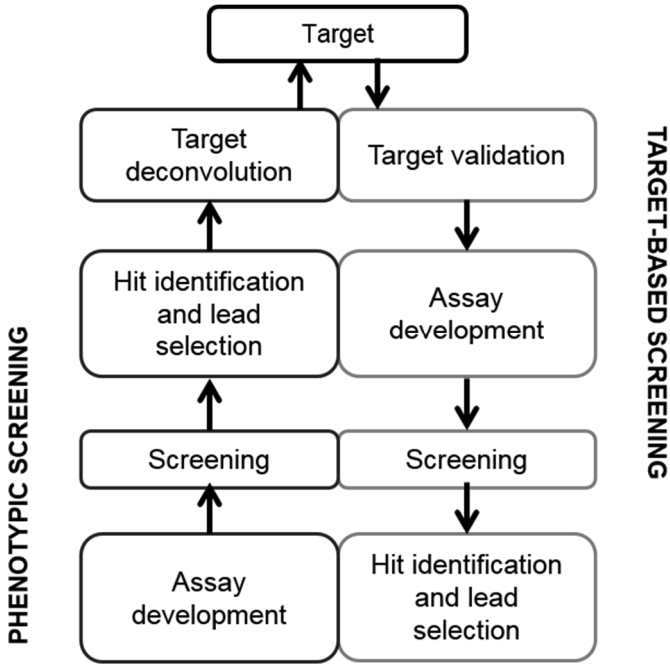


***Figure 4**. Phenotypic vs. target-based screening approach.*

Generally, target-based biochemical assays are more easily adaptable to HTS than cell-based phenotypic assays (Swinney 2013). However, target-based screens and design have not been as productive tools as phenotypic screening in the antimicrobial area (Katsuno et al. 2015, Swinney and Anthony 2011, Silver 2011), as phenotypic methods offer an unbiased mean to screen for targets in the complex biological systems. Moreover, phenotypic assays provide biologically relevant information, as the compounds are exposed to physiological conditions, such as cell membranes, cellular networks and different proteins. Thus, using the phenotypic approach, compounds affecting disease-related targets and signalling pathways can be identified (Schenone et al. 2013). However, the assays do not provide any information on the molecular mechanisms and targets of the compounds. Therefore, the subsequent identification of molecular targets responsible for the observed activity, termed target deconvolution, has to be performed (Hart 2005).

Several techniques are available for the target deconvolution. Proteomics and genomics-based approaches as well as high-throughput imaging platforms and computational methods can be applied to the identification of target proteins and pathways (Lee and Bogyo 2013).

HTS of a compound library results in the identification of primary hits with the desired bioactivity. Subsequently, the primary hits are tested in secondary assays to confirm the activity and to eliminate the false positives. The confirmed hits are further assayed to characterize their potency and selectivity, as well as the physicochemical properties (Keseru and Makara 2006). The ultimate goal of the hit-to-lead process (**Figure 5**) is to develop compounds with drug-like properties (Hughes et al. 2011). The lead development involving medicinal and combinatorial chemistry focuses on enhancing the pharmacokinetics and physicochemical properties aimed at improving the absorption, distribution, metabolism and excretion (ADME) and decreasing the toxicity. Notably, ADME properties are typically engineered at the expense of potency (Wassermann, Camargo and Auld 2014). The process is typically guided by Lipinski's rule of five (RO5) (Lipinski et al. 2001). According to the RO5, drug-like compounds shall fulfill the following criteria: molecular weight (MW) < 500 Da, octanol-water partition coefficient, log $P \leq 5$, number of hydrogen bond donors ≤ 5, and number of hydrogen bond acceptors ≤ 10. The rule does not predict the pharmacological activity of the compounds, but describes the physicochemical properties that contribute to good oral absorption. This step is of great importance for selection of the best leads for pre-clinical studies, and also, for reduction of the attrition rates in pre-clinical and clinical studies (Hughes et al. 2011, Manly et al. 2008, Bleicher et al. 2003).

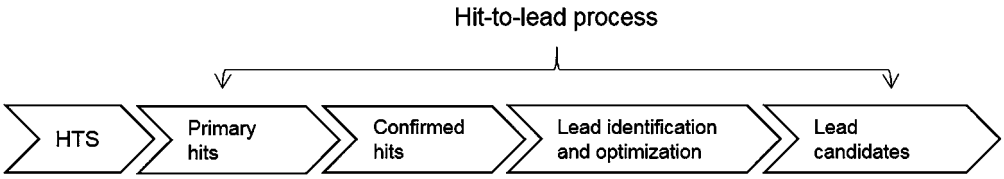


***Figure 5**. Schematic illustration of the lead discovery process during the early drug discovery process*.

Even though the RO5 has had a beneficial impact on drug discovery in general, it is not ideal for antimicrobial lead discovery, as antimicrobials (e.g. antibiotics, disinfectants and antifungals) do not follow the rule (Lipinski et

al. 2001, Lewis 2013). Thus, when discovering effective antimicrobial leads that benefit from the active transport into the target cells or tissues, and can penetrate into the bacterial membranes, the RO5 should not be strictly applied in the lead development (Macarron 2006).

2.2.1.2 Compound libraries

Compound libraries fall into two categories, natural or naturally-inspired and synthetic compound collections (Hong 2011). Synthetic libraries, which obey the RO5, represent the majority of compound collections, currently available (Serrano, Kombrink and Meesters 2015).

Natural products (NPs) differ from synthetic compounds in molecular properties. For example, NPs include more chiral centres and aromatic rings, and increased steric complexity, when compared to compounds obtained by synthesis. Moreover, NPs tend to contain more oxygen atoms than synthetic and semi-synthetic compounds, whereas synthetic compounds bear more nitrogen, sulphur and halogen atoms (Feher and Schmidt 2003, Koehn and Carter 2005). Additionally, natural compound libraries deviate from those designed according to the RO5, for example in terms of higher molecular mass, log *P*, and greater number of hydrogen bond donors and acceptors (Lipinski et al. 2001, Ortholand and Ganesan 2004). NP libraries can be composed of extracts (10-100 components) and partially purified fractions of microbial or plant origin (5-10 components) as well as of pure compounds (Shen 2015). Notably, NP libraries typically provide higher hit rates than synthetic libraries since natural scaffolds are evolutionarily optimized, complex drug-like compounds, which have evolved for optimal interactions with macromolecules in various biological systems. Moreover, many human protein targets include structural domains similar to the macromolecules (Li and Vederas 2009, Newman and Cragg 2016).

Combinatorial chemistry allows fast synthesis of a large number of synthetic compounds with drug-like properties (Kaiser et al. 2008). However, such collections typically display limited structural and chemical diversity as well as occupy a different chemical space when compared to NPs, leading to lower hit rates (Payne et al. 2007). To overcome this, diversity-oriented synthesis (DOS) can be utilized (Cordier et al. 2008). The diversity-oriented approach focuses on the creation of libraries including structurally complex compounds aimed at identifying simultaneously therapeutic protein targets and their regulators (Kaiser et al. 2008). The DOS libraries include biologically and chemically diverse compounds with optimal physicochemical properties. Generally, such libraries have a greater likelihood to produce hits and

potential leads, when phenotypic screening is utilized (Wassermann et al. 2014, Schreiber 2000). In contrast to DOS, target-oriented synthesis (TOS) generates smaller, focused libraries around specific scaffolds or chemotypes obtained either from biological screens or by virtual screening (Schreiber 2000). As focused libraries include several analogues of previously identified leads, they have a higher likelihood of being active towards the pre-determined target resulting in higher hit rates. However, the activity of such compounds is typically similar to that of the parent compound, and further, the TOS libraries occupy very narrow chemical space (Stockwell 2004, Kaiser et al. 2008).

2.2.1.3 Statistical analysis

To distinguish between active and inactive compounds, a threshold or a hit limit needs to be set. This can be either supported by statistical analysis or empirically set to obtain a reasonable number of hits for secondary screens (Walters and Namchuk 2003). Since bioactivity of the compounds in screening campaigns typically follows the normal distribution, the hit limit can be theoretically defined as standard deviations from the mean value of the compound collection signals. This way, the hit limit is set as 3 x SD (Zhang, Chung and Oldenburg 1999). High activity cutoffs lead to the identification of a few potent compounds for further optimization, and also, the diversity and chemical space occupied by the hits can be more limited. On the one hand, low activity cutoffs increase the diversity of the leads but, on the other hand, impede the further optimization of the leads (Zhu et al. 2013). Moreover, it is generally recognized that lower threshold for hits increases the number of false positive and false negative hits (Malo et al. 2006).

Additionally, the statistical analysis offers the means to monitor screening quality. To ensure that the screening campaigns provide valuable and consistent data, statistical parameters, such as signal window coefficient (Z' factor), signal-to-background (S/B) ratio (Zhang et al. 1999), signal-to-noise (S/N) ratio (Bollini et al. 2002), and coefficient of variation of the assay (CV_A) are defined (Iversen et al. 2006). The assay quality is typically determined using Z' both in the industry and academia (Inglese et al. 2007, Iversen et al. 2006). In turn, S/B ratio alone is regarded as an insufficient parameter to evaluate screening quality as it does not take into account the variability of the signals. By contrast, S/N ratio alone provides a good assessment of both signal window and variability of the signal. However, S/B and S/N ratios are typically used in conjunction with Z' (Zhang et al. 1999).

2.2.2 Drug discovery from natural products

Natural products have been the leading source of inspiration in drug discovery and development (Newman and Cragg 2016). About 50% of marketed drugs are NPs or NP-derived, with approximately one-third of the world top-selling drugs being NPs or their synthetic derivatives (Kingston 2011, Newman and Cragg 2012). Further, NPs, particularly from soil organisms, have provided the majority of lead structures for marketed anti-infectives (von Nussbaum et al. 2006, Silver 2011). This can be explained by the fact that neither NPs nor antimicrobials follow the RO5 and, thus, their physicochemical properties resemble each other (Payne et al. 2007). In fact, all but four classes of antibiotics are NPs or their derivatives (Wright 2012). Altogether 112 antibacterial small molecule drugs were approved between 1981 and 2014. Of these, NPs and NP-derivatives account for 73% (Newman and Cragg 2016). Similarly, most of the new antibiotics in late-stage development originate from NPs (Fernandes and Martens 2017).

Even though NPs have provided significant value for drug discovery, pharmaceutical companies have declined NP drug discovery, since it has been considered laborious and challenging. Traditional drug discovery approaches involving fermentation broths and extracts of microorganisms are time-consuming (Silver 2011). Also, when screening is conducted using crude or roughly fractioned extracts of NPs, additional steps, such as bioactivity-guided microfractionation, are required for the isolation and identification of the active component (Dandapani et al. 2012). Further, if the identified component is novel, a complete structural elucidation needs to be carried out. However, since NPs are usually complex in structure, identification of a pharmacophore and elucidation of structure-activity relationship (SAR) may be problematic (Ortholand and Ganesan 2004). Typically, the active component of an extract is a minor component and, therefore, inadequate for the further optimization and progression to the preclinical development (Balunas and Kinghorn 2005). Moreover, NP libraries, which consist of extracts and mixtures, are not compatible with current HTS platforms (Harvey 2008, Dandapani et al. 2012). In particular, the use of the target-based screening approach has prompted many pharmaceutical companies to use synthetic compound libraries instead of NP extract libraries (Koehn and Carter 2005). However, during the past decade, isolation and separation techniques, as well as structure elucidation methods, such as liquid chromatography–mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR), have significantly improved, allowing higher throughput of natural extracts (Singh and Barrett 2006). Likewise,

antimicrobial screening strategies have improved and moved away from the early phenotypic assays, in which compounds were identified without any hypothesis of the possible mechanism of action, to high-throughput, whole-cell, target-based assays (Payne et al. 2007). Additionally, the use of pure, pre-fractionated NP libraries has emerged as a means to screen NPs in HTS platforms (Dandapani et al. 2012).

2.2.2.1 Natural compound collections explored in this thesis

I Flavonoids (FL-500 Flavonoid derivatives, http://www.timtec.net/flavonoid-derivatives.html)

Flavonoids, plant and fungus secondary metabolites are among the most extensively studied classes of natural products with a polyphenolic nature, and widely reported for diverse antimicrobial activity, anti-biofilm activity included (Barbieri et al. 2017, Havsteen 2002, Friedman 2007, Ta and Arnason 2015). The core structure of flavonoids is comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring (c) in between (**Figure 6**). Altogether, more than 8000 flavonoids exist, and they are divided into 14 subclasses on the basis of oxidative status and substituents (Cushnie and Lamb 2011, Xie et al. 2015). Flavonoids exert anti-biofilm activity in various ways, namely by preventing biofilm formation, dispersing existing biofilms and interfering with the signaling processes involved in biofilm formation (Borges et al. 2016).

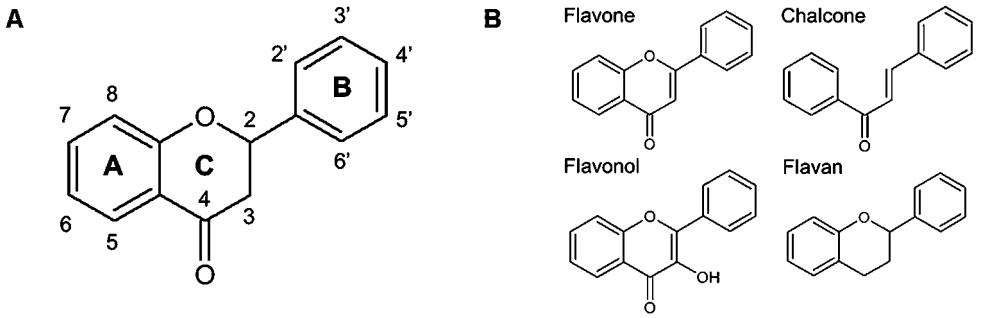


Figure 6. *Chemical structure of the flavonoid core (A) and structural backbones of the major subclasses included in the library (B).*

The compound library explored in the present thesis, consists of both naturally occurring flavonoids and their derivatives from nine different classes, including representatives of flavanones, flavones, chalcones, flavonols, dihydroflavonols, flavans, anthocyanins, isoflavonoids and neoflavonoids. This library is an example of libraries combining features of

natural products by combinatorial chemistry to increase the likelihood of activity (Baker et al. 2007).

II Amino acid derivatives of (+)-dehydroabietic acid (DHA)

The resin acid (+)-dehydroabietic acid (DHA) (**Figure 7**) is present in conifers, where it serves as a defensive compound against herbivores and microbial pathogens (González et al. 2010). Antimicrobial properties of resin acids and other naturally occurring diterpenoids have been widely studied (González 2015, Leandro et al. 2014, Sipponen and Laitinen 2011) and a few studies have also focused on their anti-biofilm properties (Fallarero et al. 2013, Kuźma et al. 2007, Ali et al. 2012). The previous identification of DHA as a potent inhibitor of *S. aureus* biofilms (Fallarero et al. 2013) prompted into synthesis and discovery of DHA-based anti-biofilm compounds. In this study, DHA was used as an inspirational core structure for TOS of 30 novel amino acid derivatives, since D-amino acids had been previously reported to induce biofilm dispersal (Hochbaum et al. 2011, Kolodkin-Gal et al. 2010). The design strategy focused on chemical modification of rings A and B in the diterpenoid core and, moreover, the synthesis was further supported by simultaneous bioactivity testing.

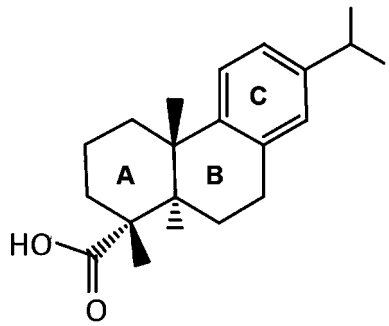


***Figure 7**. Chemical structure of (+)-dehydroabietic acid (DHA).*

III Natural product derivatives (NDL-3000, http://www.timtec.net/ndl-3000-natural-derivatives-library.html)

Natural product derivatives are typically synthesized using the concept of DOS, which relies on synthesis of compounds resembling natural products, also called mimics, or compounds that are based on natural product scaffolds, such as derivatives and synthetic analogues (Dias, Urban and Roessner 2012, Shang and Tan 2005). In the present thesis, a compound library consisting of 3040 compounds, covering several compound classes, such as alkaloids, natural phenols, nucleoside analogues, carbohydrates, purines, pyrimidines,

flavonoids, steroidal compounds and amino acids, was explored. The use of natural compounds as scaffolds for combinatorial chemistry enables generation of compounds with enhanced structural diversity and drug-like properties.

2.2.3 Anti-biofilm drug discovery

In addition to general challenges associated with antimicrobial drug discovery, anti-biofilm drug discovery is even more complicated due to the specific features of biofilms. This is further exemplified by the fact that no specific anti-biofilm agents have been approved by the regulatory authorities so far. To date, all the marketed antibiotics are developed against dividing planktonic cells (Fey 2010).

2.2.3.1 Targets and mechanisms of anti-biofilm agents

Three main strategies for the biofilm control have been proposed: prevention of biofilm formation, interference with biofilm maturation and disruption of pre-formed biofilms (Gupta et al. 2016) (**Figure 8**).

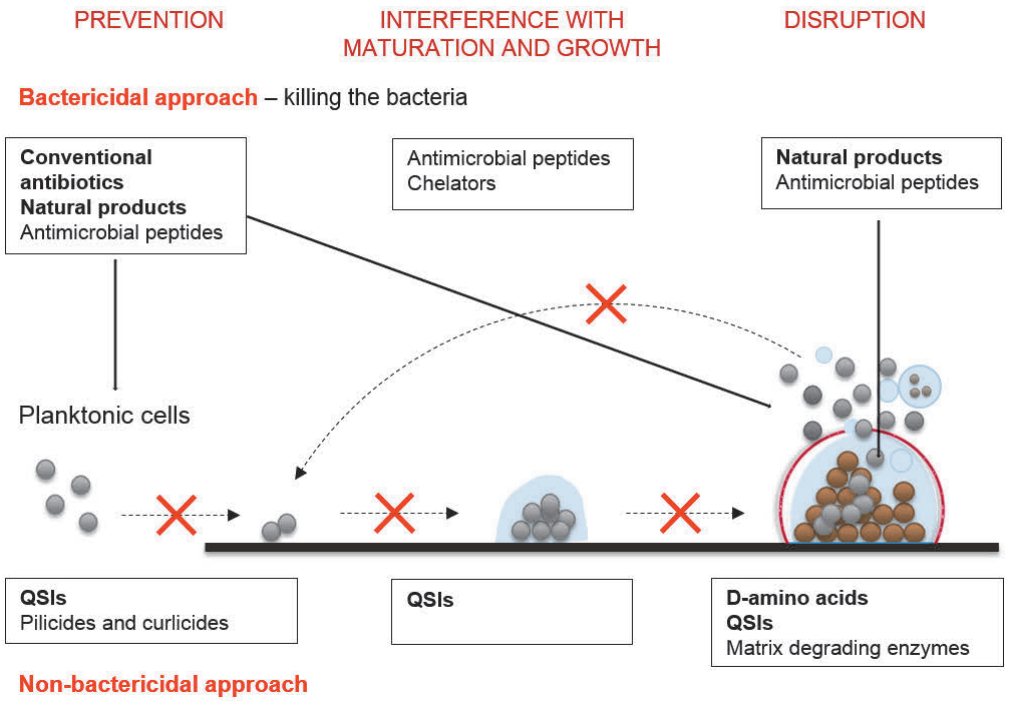


***Figure 8**. Strategies to control bacterial biofilms. Examples of both bactericidal and non-bactericidal approaches are shown in the boxes. Those marked in bold are included in this thesis.*

Most likely, a combination of these strategies will be the most effective solution. Many anti-biofilm compounds are non-bactericidal that restore bacteria to the planktonic mode of growth, in which cells are more susceptible to antibiotics and host immune clearance. Furthermore, bactericidal approaches aimed at killing the bacteria can be utilized in biofilm control (Bjarnsholt et al. 2013b).

The use of QSIs in biofilm control represents an attractive strategy, as QS is involved in several steps regulating biofilm formation, maturation and dispersal (Brackman and Coenye 2015). Moreover, QS is connected to the virulence and antibiotic susceptibility of bacteria (Brackman et al. 2011). Additionally, the QS systems provide several molecular targets, receptors involved in signal transduction, enzymes involved in signal molecule synthesis, and the signal itself (Reuter, Steinbach and Helms 2016). QSIs against both gram-negative and gram-positive bacteria have been identified both among natural and synthetic compounds, such as halogenated furanones (Hentzer et al. 2002), garlic (Smyth et al. 2010), patulin and penicillin acid (Rasmussen et al. 2005b), flavonoids, including quercetin and baicalein (Ouyang et al. 2016, Chen et al. 2016), indole derivatives (Biswas et al. 2015) and hammamelitannin analogues (Vermote et al. 2017). According to an estimate from 2008, QSIs could have reached the market in 2-7 years (Rogers, Carroll and Bruce 2012). However, even though several compounds have shown their QSI potential *in vitro* and *in vivo*, only a few clinical trials have been conducted or are being conducted (Reuter et al. 2016).

D-amino acids, which are involved in several biological functions, have also demonstrated their potential in biofilm control (Cava et al. 2011). Various D-amino acids have been shown to prevent biofilm formation and to induce biofilm dispersal in both gram-negative and gram-positive species (Brandenburg et al. 2013, Hochbaum et al. 2011, Kolodkin-Gal et al. 2010, Yu et al. 2016, Ramón-Peréz et al. 2014). Moreover, it has been demonstrated that amino acids enhance the efficacy of conventional antibiotics (Sanchez et al. 2014) and biocides (Jia et al. 2017) against biofilms. Further, *N*-acetylcysteine, a mucolytic agent has been shown to interfere with biofilm formation of *S. epidermidis* (Pérez-Giraldo et al. 1997).

Similarly, antimicrobial peptides (AMPs) have been shown to target several stages of biofilm development in several species. Additionally, AMPs have been reported to display synergistic activity with conventional antibiotics, and to modulate immune responses (Pletzer and Hancock 2016). AMPs target cell membranes and disrupt the cellular integrity leading to

translocation through the membranes (Fjell et al. 2011). One promising example of AMPs in biofilm control is human cathelicidin LL-37, which has been shown to inhibit *P. aeruginosa* biofilm formation at a concentration of 1/16 x MIC and to eradicate pre-formed biofilms at micromolar concentrations *in vitro*. Moreover, it has been shown that LL-37 can interfere with QS of *P. aeruginosa* (Overhage et al. 2008). Currently, a few AMPs are in preclinical phase, and according to an estimate given by Czaplewski et al. (2016), they could be marketed as anti-biofilm agents at the earliest in 2027.

Pilicides and curlicides, in turn, represent a non-bactericidal strategy to prevent biofilm formation. For instance, ring-fused 2-pyridones, such as FN075, have been shown to display anti-biofilm and anti-virulence activities by inhibiting the synthesis of curli and type 1 pili in uropathogenic *E. coli* both *in vitro* and *in vivo* (Cegelski et al. 2009, Guiton et al. 2012).

Since metallic cations, such as Ca^{2+}, Mg^{2+} and Fe^{2+}, contribute to biofilm formation and maturation, metallo-chelators have been proposed as anti-biofilm compounds (Abraham et al. 2012, Banin, Vasil and Greenberg 2005). Additionally, chelators have shown to potentiate the efficacy of conventional antibiotics in the killing of *P. aeruginosa* biofilms (Oglesby-Sherrouse et al. 2014). A couple of chelators, such as deferasirox (DSX), have been approved by the FDA and are used against *P. aeruginosa* (Moreau-Marquis, O'Toole and Stanton 2009).

The extracellular matrix components serve as an appealing target in biofilm control. For instance, matrix-degrading enzymes similar to dispersin B may act as effective dispersal inducers that enhance the efficacy of conventional antibiotics against biofilm-growing bacteria. Proteases, such as proteinase K and trypsin, which target matrix proteins, have shown to disperse *S. aureus* biofilms (Chaignon et al. 2007). Further, deoxyribonuclease, DNase has shown to induce dispersal of both gram-negative and gram-positive biofilms (Nijland, Hall and Burgess 2010). Additionally, diverse dispersal inducers have been identified. A fatty acid signalling molecule, *cis*-2-decenoic acid, produced by *P. aeruginosa* has been shown to induce biofilm dispersal of several species (Davies and Marques 2009), while nitric oxide has been demonstrated to promote dispersal of *P. aeruginosa* biofilms (Barraud et al. 2006).

Notably, because the molecular targets vary between species, anti-biofilm strategies are not universal but species- or even strain-specific (Bjarnsholt et al. 2013b). Moreover, the effect of a particular compound may vary even between strains of the same bacterial species (Abraham et al. 2012). Most of the current investigational strategies rely on compounds, which interfere with

biofilm lifecycle without displaying bactericidal activity. Approaches that are not targeting the bacterial viability are generally considered less prone to the development of resistance (Sperandio 2007). Moreover, such compounds do not harm the natural bacterial flora (Joseph et al. 2016). However, when using non-bactericidal strategies, remaining planktonic cells can initiate biofilm formation again when the compound concentration has decreased enough. Thus, combinatory strategies of non-bactericidal, such as QSIs, and bactericidal approaches, such as conventional antibiotics or new investigational drugs, would be ideal for biofilm control to ensure long-term biofilm removal. By using adjuvants, the carriage of antibiotics can be reduced, and their efficacy can be enhanced. Thus, the risk of emergence of antibiotic resistance can also be minimized (Rogers et al. 2012).

2.2.3.2 Models to study biofilms

Several *in vitro* models have been developed for antimicrobial drug discovery against biofilm-growing bacteria. Models can be divided into two groups, closed (static) and open (dynamic) systems depending on the flow of nutrient media and waste products (Lebeaux et al. 2013) (**Table 2, Figure 9**).

***Table 2**. Examples of in vitro models to study biofilms.*

	Closed system		**Open system**	
	Microtiter well plate (MWP)	Calgary Biofilm Device (CBD)	Drip flow reactor (DFR)	CDC reactor
Type	Batch	Batch	Plug flow reactor (PFR)	Continuous flow stirred-tank reactor (CSTR)
Mixing	Optional	Optional	Radial	Perfect
Fluid shear	No/Low	Gentle	Low	High
Flow	No	No	Yes, laminar	Yes, turbulent
Availability of a standardized protocol	No	Yes, ASTM E2799	Yes, ASTM E2647	Yes, ASTM E2562

MWP-based systems are closed (batch-reactor like) models, in which there is no flow in or out of the wells during the experiment. Consequently, the experimental conditions change because of nutrient depletion and accumulation of toxic products unless the growth media is regularly refreshed (Merritt, Kadouri and O'Toole 2005). However, MWP-based systems represent a versatile platform for anti-biofilm drug discovery, and they have been applied to anti-biofilm screening of natural compound libraries (Paytubi et al. 2017, Quave et al. 2008), susceptibility testing of antibiotics (Amorena et al. 1999, Ceri et al. 1999), efficacy testing of biocides (Pitts et al. 2003, Shakeri et al. 2007) and quantification of biofilm formation (O'Toole 2011). These devices are easy to handle and suitable for the use of multichannel pipettes, pipetting robots, and microplate readers (Duetz 2007). For HTS, the MWPs and the Calgary Biofilm Device (CBD) are the most frequently used (Bjarnsholt et al. 2013b). The CBD is a modified MWP, in which the polystyrene lid contains 96 pegs that can be fitted into the wells of a 96-MWP, allowing for biofilm formation on the pegs (Ali, Khambaty and Diachenko 2006, Harrison et al. 2010). The MWP-based systems are fairly cheap, and for the assays, only small volumes of reagents and media are needed (Coenye and Nelis 2010). When conducting the MWP-assays under low shear conditions (on shakers), they enable mimicking of the flow conditions and biofilm formation in veins or on urinary tract catheters (Moreira 2013). Besides the MWP-based systems, agar plate-based models are representatives of static biofilm systems (Gabrilska and Rumbaugh 2015). They enable investigation of bacterial adhesion (Oja et al. 2014), anti-biofilm efficacy (Hiltunen et al. 2016) and bacterial motility (Ha, Kuchma and O'Toole 2014).

In contrast to closed systems, open systems provide a continuous supply of nutrient media and bypass of waste products (Coenye and Nelis 2010). Such models, including Drip flow reactor (DFR) and the Center for Disease Control (CDC) biofilm reactor, are designed for in-depth investigations. The goal of the open systems is to better simulate natural environments by providing shear conditions and nutrients. However, such models are typically less cost-efficient and more difficult to use than the MWP-based ones. Moreover, the throughput of these models is lower compared to the MWP-based systems (Lebeaux et al. 2013).

The DFR is defined as a plug flow reactor, in which cell density and nutrient concentration change along the length of the coupon in reactor channels (Goeres et al. 2009). The nutrient media is continuously supplied by dripping, thus creating low shear conditions inside the reactor. In such

growth conditions, biofilms also develop streamers that can be found both in environmental and medical systems (Franklin et al. 2015). Consequently, DFR promotes formation of heterogeneous biofilms mimicking those formed at the air-liquid interface in natural environments. The CDC biofilm reactor, in turn, allows biofilm formation under a constant flow of nutrient media under high shear conditions, which leads to the formation of thick biofilms on the coupons on swirling paddles. Such biofilms are often found in nature (Goeres et al. 2005).

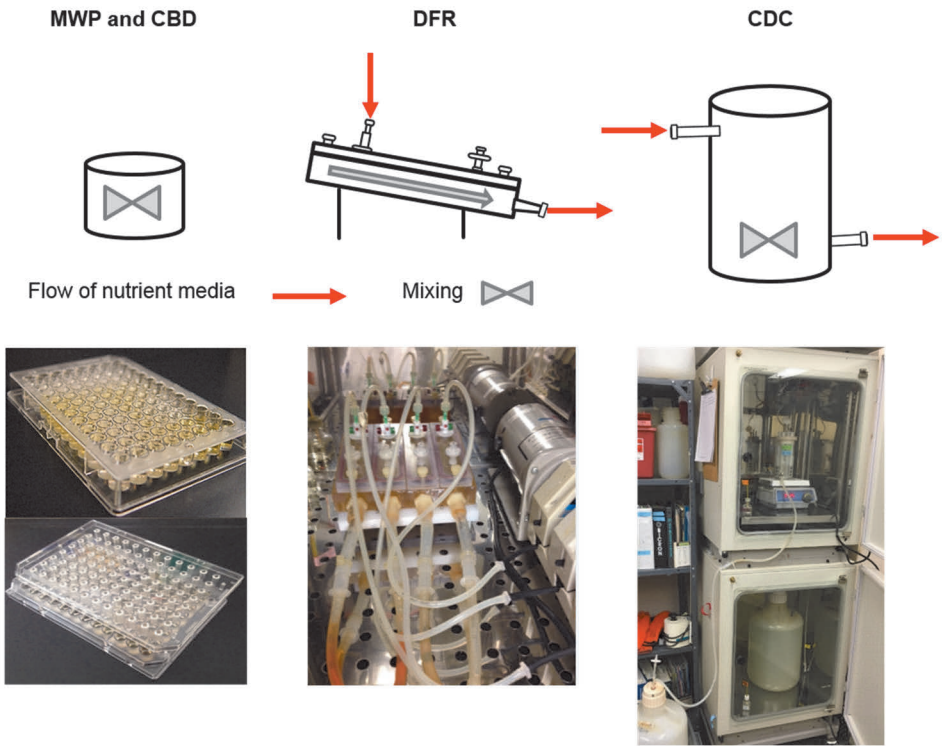


***Figure 9**. Experimental set-up of the biofilm models, MWP, CBD, DFR and CDC reactors from left to right. Supply of nutrients and mixing in each model is depicted in the top part of the figure, and images of the models are shown below. Picture of CDC reactor is courtesy of Darla Goeres. The upper part of the figure is adapted from Coenye and Nelis (2010).*

Coupons of various materials, such as silicone and ceramics, can be used in both reactors depending on the nature of the experiment and the conditions to be mimicked. Plug flow is typically found in pipes, tubing and catheters (Goeres et al. 2009). Moreover, the open systems can simulate infections that occur under the flow of fluids, such as in oral cavity (Adams et al. 2002),

wounds (Ammons, Ward and James 2011) and urinary tract (Curtin and Donlan 2006). Notably, as the hydrodynamic conditions and nutrient availability are crucial factors affecting the structure, density and thickness of the biofilms (Stoodley et al. 1998), the choice of the model system has been found to influence biofilm formation, and more importantly, antimicrobial susceptibility of the biofilms. Biofilms grown under turbulent flow in the CDC reactor have been found to be less susceptible than those grown under laminar flow in the DFR, and in the absence of flow in a static biofilm model (Buckingham-Meyer et al. 2007).

2.2.3.3 Challenges of anti-biofilm drug discovery

As previously mentioned, the antimicrobial research has traditionally focused on planktonic bacteria, and a wide variety of well-established *in vitro* methods exist (Balouiri, Sadiki and Ibnsouda 2016). Guidelines from the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) provide uniform testing procedures for antimicrobial susceptibility testing (AST) against planktonic bacteria. By contrast, no standardized methods have been approved by CLSI or EUCAST for evaluation of antimicrobial susceptibility of biofilms (Jacqueline and Caillon 2014). However, experimental standards for one anti-biofilm screening assay applicable for susceptibility testing are available (ASTM E2799), as set by the American Society for Testing of Materials (Harrison et al. 2010). Altogether, five standards for biofilms exist (ASTM E2196, ASTM E2647, ASTM E2562, ASTM E2799, and ASTM E2871), and they all are suitable as such only for *P. aeruginosa* (Malone et al. 2017).

Discrepancies between biofilm studies are frequently observed since the outcome is dependent on the experimental conditions, such as culture media, inoculum concentration and incubation conditions (Crémet et al. 2013, Stepanović et al. 2007). For the same reason, results from the *in vitro* studies may not correspond to the results obtained from *in vivo* studies (Barsoumian et al. 2015). Moreover, standardization is of great importance from the perspective of the regulatory authorities (Parker et al. 2014). Repeatability and reproducibility are essential attributes when considering the claims of anti-biofilm efficacy.

Similarly, conventional susceptibility breakpoints, including the MIC and the MBC, which are used to define the antimicrobial susceptibility of planktonic microorganisms (Andrews 2001, Olson et al. 2002) are not applicable for biofilms. To that end, the CBD was designed to assess the minimum biofilm inhibitory concentration (MBIC) (Moskowitz et al. 2004).

Furthermore, the CBD can be used for determination of the minimum biofilm eradication concentration (MBEC), which is defined as the lowest concentration of an antimicrobial agent required to eradicate existing biofilms (Olson et al. 2002). However, so far, results obtained using this device have not shown a breakthrough in the clinical settings (Waters and Ratjen 2015). This can be explained by the fact that biofilms formed on the pegs of the CBD reflect only partially the *in vivo* biofilms (Bjarnsholt et al. 2013a), as *in vitro*-grown biofilms in general. However, the MBIC and the MBEC may facilitate the development of effective anti-biofilm drugs.

To partially overcome the challenges associated with lack of standardization, the minimum information about a biofilm experiment (MIABiE) standards have been recently proposed (Lourenço et al. 2014). The MIABiE is not intended to provide specific standards for performing the biofilm experiments but to harmonize reporting of experimental data to enhance the reproducibility of experiments. Altogether, 15 modules that can impact the experimental outcome have been defined, for both data generation and characterization.

From the translational perspective, the MWP-based models are the most limited systems, since the nutrients become quickly depleted restricting the biofilm development in respect of microenvironments and morphology (Roberts et al. 2015). Moreover, most MWPs are of polystyrene (Kumar, Wittmann and Heinzle 2004). However, such surfaces are not typically utilized in medicine, thus making them not particularly relevant in mimicking infection conditions. Overall, the major drawback of the *in vitro* models is the lack of host immune responses, which are typically present at the site of infection (Roberts et al. 2015). However, when using co-culture biofilm models, *in vivo* conditions can be better mimicked (Subbiahdoss et al. 2011).

2.2.4 Anti-biofilm screening

The HTS in early anti-biofilm drug discovery needs to be based on simple, accurate and reproducible assays, preferably suitable for automation, as in general (Inglese et al. 2007). Similarly, both phenotypic and target-based screening approaches can be utilized for the identification of hit compounds. In phenotypic screening, assays measuring the desired endpoints are first developed and optimized, followed by screening and hit identification. Later on, subsequent assays with the selected leads are required to gain mechanistic information of the leads, and to identify the molecular targets inducing the observed phenotypic effects. By contrast, in case of target-based screening

approach, molecular targets are first identified and validated, following the assay development and screening to identify hits and leads that act on the predefined targets of interest (Swinney and Anthony 2011).

2.2.4.1 Phenotypic screening assays

Phenotypic assays, which rely on diverse staining methods are widely applied to the HTS for biofilm inhibitors from large compound libraries (Junker and Clardy 2007, Panmanee et al. 2013, Opperman et al. 2009). These assays, in general, are inexpensive and easy to perform, allowing straightforward identification of biofilm inhibitors. However, the molecular basis of the observed effects is not known, as the phenotypic effects are non-selective, measuring only the effects on selected characteristics according to the assay endpoint.

Various MWP-based assays exist for quantification of the biofilm viability, total biomass and the matrix (**Table 3**). These features can be measured using a plate reader, for example, through fluorescence or absorbance at certain wavelengths. The MWP-based assays are broadly applicable, and when optimized for a particular species, they provide highly repeatable data (Peeters, Nelis and Coenye 2008, O'Toole 2011).

***Table 3**. MWP-based assays used for biofilm quantification.*

Feature	Assay	Reference (ex)
Biofilm viability	Resazurin	Sandberg et al. 2009
	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT)	Pettit et al. 2005
	Fluorescent fluorescein diacetate (FDA)	Peeters et al. 2008
	Adenosine triphosphate (ATP)	Amorena et al. 1999
Biofilm biomass	Crystal violet	Stepanovic et al. 2000, Sandberg et al. 2008
	Congo Red staining	Stiefel et al. 2016
	Safranin Red	Patterson et al. 2010
Biofilm matrix	Dimethylmethylene blue (DMMB)	Toté et al. 2008
	Wheat germ agglutinin (WGA)	Skogman, Vuorela and Fallarero 2012

In the present thesis, resazurin and crystal violet staining assays were used to assess the anti-biofilm effects of compounds. Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide), also termed Alamar blue, is a non-invasive redox indicator used to monitor cellular viability. Resazurin is reduced into resorufin, a fluorescent, pink dye as a result of metabolically active cells, indicating the fraction of viable cells (O'Brien et al. 2000, Van den Driessche et al. 2014). Upon this reduction, the pink colour is further reduced to colourless state due to atmospheric oxygen (Mariscal et al. 2009). Crystal violet (hexamethyl pararosaniline chloride) is a dye that indifferently stains the entire biofilm, including viable bacteria, dead cells and the matrix, whereas abiotic surfaces remain colourless (Stepanovic et al. 2000). After the staining, the adsorbed dye is eluted in a solvent, and the amount of the solubilized dye is directly proportional to biofilm biomass (Pantanella et al. 2013). As the assay does not distinguish between viable or dead cells or the matrix, it provides inaccurate information on the antimicrobial activity (Pitts et al. 2003). By using a combination of two assays quantifying viability and total biomass, more comprehensive view of the antimicrobial activity of the compounds can be obtained, and moreover, it can be revealed whether the bacteria are removed or only killed (Stiefel et al. 2016, Toté et al. 2009).

2.2.4.2 Target-based screening

Contrary to phenotypic screening, target-based screening strategies focus on the identification of compounds that target a specific pathway or process essential for biofilm formation. Using this approach, specific biofilm inhibitors can be directly identified. QS represents a potential target, as it is involved in several stages of biofilm formation (Dickschat 2010), and an extensively used target in anti-biofilm screening (Hentzer et al. 2002, Jakobsen et al. 2012, Rasmussen et al. 2005a, Ding et al. 2011). In addition to the QS, specific amyloid proteins involved in biofilm formation, such as curli fibers of *E. coli* (Andersson and Chapman 2013, Cegelski et al. 2009), genes involved in nucleotide biosynthesis (Attila, Ueda and Wood 2009), and the intracellular c-di-GMP (Antoniani et al. 2010, Sambanthamoorthy et al. 2014) have been employed as targets in target-based screening approach for biofilm inhibitors.

In this thesis, QS system of *C. violaceum* was used as a target. In *C. violaceum*, production of the purple pigment violacein serves as a useful indicator of QS, whereas inhibition of QS leads to loss of the pigment (McClean et al. 1997). Compounds that inhibited violacein production were considered as QSIs.

3 Aims

Given the widespread presence of biofilms, scaffolds that have co-evolved in nature offer a reasonable starting point for exploration of potent biofilm inhibitors. The overall goal of this doctoral project was to identify naturally-inspired biofilm inhibitors from natural and naturally-derived compound libraries as alternatives to conventional antibiotics. A research strategy combining different screening approaches was utilized to identify bactericidal and non-bactericidal anti-biofilm leads, and further characterize their effects and mechanisms. In addition, a comparative efficacy study of conventional antibiotics was conducted in two different biofilm models.

The thesis is composed of four studies:

I. Flavonoids are one of the most widely studied classes of natural compounds with a variety of reported biological activities, including antimicrobial and anti-biofilm activities. The aim of this study was to develop a methodological workflow for screening of natural libraries with known antimicrobial properties, and apply it to the systematic exploration and characterization of biofilm inhibitors from a large flavonoids library.

II. (+)-Dehydroabietic acid (DHA) has been previously identified as a potent anti-biofilm agent against *S. aureus*. The aim of this study was to screen a small compound library consisting of amino acid derivatives of DHA for anti-biofilm activity, and to investigate if linking of amino acids to DHA enhances the specific anti-biofilm activity.

III. Quorum sensing (QS) controls biofilm formation of many bacteria. The aim of this study was to identify and characterize anti-biofilm scaffolds from a large compound library of natural product derivatives that act via inhibition of the QS without displaying bactericidal activity.

IV. Several models have been developed to study biofilms and to evaluate antimicrobials against them. The aim of this investigation was to conduct efficacy studies of selected antibiotics from various mechanistic classes using microtiter well plates (MWP) and a drip flow reactor (DFR) to investigate the impact of the model system on the experimental outcome.

4 Materials and methods

4.1 Materials

4.1.1 Bacteria and culturing conditions (studies I-IV)

The culturing conditions for each bacterial strain used in the present thesis are summarized in **Table 4**. Working stocks of all bacterial strains were prepared from the glycerol stocks (20% w/v at -70 °C) onto agar plates and incubated overnight. Agar plates were stored at +4 °C for a period lasting not longer than four weeks. To reach the optimal culturing conditions, *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa* were pre-cultured overnight. The actual cultures were prepared by diluting the pre-cultures 100-fold (*E. coli*, *P. aeruginosa*) and 1000-fold (*S. aureus*, *S. epidermidis*) in fresh media, and further incubating them (~4 h) to reach the exponential growth, until an optical density (OD_{595}) of 0.3-0.5 corresponding to 10^8 colony forming units per milliliter (CFU/mL). Cultures of *C. violaceum* were incubated overnight to an OD_{595} of 0.7 corresponding to 10^9 CFU/mL. In all cases, bacterial concentration was confirmed by serially diluting and plating the cultures on agar for determination of CFU/mL as follows:

$$CFU/mL = \frac{(Number\ of\ colony\ forming\ units\ x\ Dilution\ factor)}{Volume\ of\ culture\ plated}.$$

***Table 4**. Bacterial strains and growth conditions. TSA = tryptic soy agar, TSB = tryptic soy broth, LB = Luria-Bertani.*

Bacterium (strain)	**Agar**	**Pre-culture[1]**	**Culture media[2]**	**Growth conditions**	**Study**
***Staphylococcus aureus* (ATCC 25923)**	TSA	TSB	TSB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	I, II, IV
***Staphylococcus aureus* (Newman)**	TSA	TSB	TSB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	I, II, IV
***Staphylococcus epidermidis* (ATCC 35984)**	TSA	TSB	TSB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	II, IV
***Staphylococcus epidermidis* (ATCC 1228)**	TSA	TSB	TSB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	II
***Escherichia coli* (XL1 blue)**	TSA	TSB	TSB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	II
***Escherichia coli* (K-12)**	LB agar	LB broth	LB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	III
***Pseudomonas aeruginosa* (PA01)**	LB	LB	LB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	III
***Pseudomonas aeruginosa* (ATCC 15442)**	LB	LB	LB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	III
***Pseudomonas aeruginosa* (ATCC 9027)**	LB	LB	LB	Aerobic, +37 °C, shaking 220[1]/200[2] rpm	III
***Chromobacterium violaceum* (ATCC 31532)**	TSA	-	TSB	Aerobic, +27 °C, shaking 200 rpm	III
***Chromobacterium violaceum* (CV026)**	TSA	-	TSB	Aerobic, +27 °C, shaking 200 rpm	III

4.1.2 Cell lines, media and incubation conditions (studies II and III)

Mammalian cell lines were routinely cultured in 75 cm^2 cell culture flasks at +37 °C in 5% CO_2 in an air-ventilated humidified incubator to around 90% confluence. Harvesting was performed by trypsinization (0.25% (v/v)) for human lung cells or by physically scraping the adhered cells, for the RAW 264.7 cells. Cell suspensions (60 000 cells/well, 200 µL) were added into 96-microtiter well plates and the plates were incubated for 24 h prior to exposure to compounds. The culturing conditions for the two cell lines used in the study are summarized in **Table 5**.

***Table 5**. Cell lines and incubation conditions used in the study.*

Cell line	Media	Incubation conditions	Study
Human lung (HL) epithelial cells	RPMI 1640 supplemented with 10% inactivated fetal bovine serum (FBS), 2 mM L-glutamine and gentamycin 20 µg/mL	+37 °C, 5% CO_2	II, III
Mouse monocyte macrophage cells RAW 264.7	Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and gentamycin 20 µg/mL.	+37 °C, 5% CO_2	III

4.1.3 Compound libraries (studies I-III)

Details on the sources of the compounds included in the present thesis are summarized in **Table 6**. Three compound libraries containing NPs, NP-derivatives, semi-natural compounds and synthetic mimics were included in these studies. Libraries including 500 naturally occurring and natural derivatives of flavonoids (study I) and natural product derivatives (NDL-3000) (study III) were purchased from TimTec®. The NDL-3000 library consisted of 3040 compounds from various classes, such as alkaloids, natural

phenols, nucleoside analogues, carbohydrates, purines, pyrimidines, flavonoids, steroidal compounds and natural amino acids. The in-house library of 31 amino acid derivatives of (+)-dehydroabietic acid (DHA) (study II) was synthesized by the research group of Professor Jari Yli-Kauhaluoma (University of Helsinki, Finland). A panel of conventional antibiotics (study IV), was obtained from several companies (see supplementary table S7 in publication IV for details). The compound libraries were prepared in dimethyl sulfoxide (DMSO, minimum 99.9%, Sigma-Aldrich, St. Louis, MO, US) at a concentration of 20 mM, and stored in Matrix™ library storage tubes at –20 °C. For the screening assays, daughter plates with smaller aliquots of the compounds were prepared. Antibiotics were stored at –20 °C or +4 °C, and prepared either in Mueller-Hinton broth (MHB, Fluka Biochemika, Buchs, Switzerland) or DMSO depending on the solubility.

Table 6. *Compound libraries included in the study.*

Library	Number of compounds	Type	Source	Study
Flavonoids (FL-500)	500	commercial	TimTec® (Newark, US)	I
Amino acid derivatives of (+)-DHA	30	in-house	Group of Prof. J. Yli-Kauhaluoma (University of Helsinki)	II
Natural product derivatives (NDL-3000)	3040	commercial	TimTec® (Newark, US)	III
Antibiotics	27	commercial	Various suppliers (Supplementary table S7 in IV for details)	IV

4.1.4 Reference compounds (studies I-III)

Penicillin G potassium salt (Fluka Biochemika, Buchs, Switzerland) was used as positive control in screening assays (studies I and II), while vancomycin hydrochloride hydrate (Sigma Aldrich, St. Louis, MO, USA) was included as additional control antibiotic (study II). (+)-Dehydroabietic acid was used as reference compound in study II. In study III, azithromycin

(Cayman chemicals, Ann Arbor, MI, US) was included as viability control and quercetin dihydrate (Carl Roth GmbH, Karlsruhe, Germany) as positive control in violacein and motility assays. Usnic acid (Sigma Aldrich, St. Louis, MO, USA) was used as control compound in cell viability assays (studies II and III).

4.2 Methods

4.2.1 Biofilm formation (studies I-IV)

For biofilm formation, the exponentially grown bacteria were diluted to a starting concentration of ~10^6 CFU/mL, except for efficacy testing when biofilms were formed from diluted cultures of 10^7 CFU/mL (study IV). Information on the culture media used in biofilm formation is summarized in **Table 7**. The volume of the culture pipetted was 200 µL for 96-MWPs, 1.0 mL for 24-well plates, 1.5 mL for 12-well plates, and 5 mL for 6-well plates. The plates were routinely incubated at +37 °C, with shaking at 200 rpm for 18 h.

***Table 7**. Culture media used for biofilm formation.*

Bacterium (strain)	**Culture media**
Staphylococcus aureus **(ATCC 25923, Newman)**	TSB
Staphylococcus epidermidis **(ATCC 35984, ATCC 12228)**	TSB
Escherichia coli **(XL1 blue)**	TSB
Escherichia coli **(K-12)**	LB
Pseudomonas aeruginosa **(PA01, ATCC 15442, ATCC 9027)**	LB
Chromobacterium violaceum **(ATCC 31532)**	LB supplemented with 0.1% w/v yeast extract (LBY)
Chromobacterium violaceum **(CV026)**	LBY

4.2.2 Exposure to compounds in microtiter well plates (MWP)

4.2.2.1 Phenotypic screening (studies I and II) and antibiotic susceptibility testing (study IV)

Bacteria were exposed to compounds in two modes, prior to and post biofilm formation. In pre-exposure mode of the assay, compounds and bacteria were simultaneously added into the wells, and incubated for 18 h (+37 °C, 200 rpm), while in post-exposure mode, biofilms were first formed for 18 h, and thereafter, exposed to compounds for 24 h (+37 °C, 200 rpm). At the end of the incubation periods, effects of the compounds were quantified. The workflow for the performed phenotypic screens is schematically summarized in **Figure 10**.

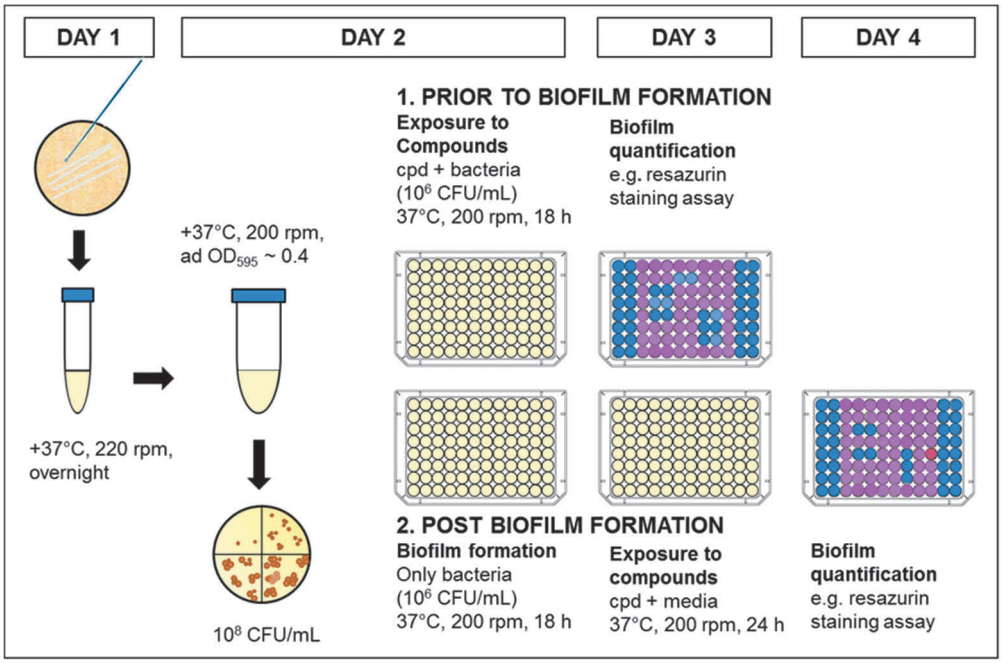


***Figure 10**. Biofilm formation, exposure to compounds and quantification of S. aureus (studies I, II and IV), S. epidermidis (studies II and IV) and E. coli XL1 blue (study II) biofilms.*

4.2.2.2 Target-based screening (study III) and efficacy testing (study IV)

Compounds and bacteria were simultaneously added into the wells and plates were incubated for 24 h (+27 °C, 200 rpm), or one hour (+37 °C, 200 rpm) in studies III and IV, respectively. In efficacy testing (study IV), bacterial suspensions were removed after one hour incubation with the tested antibiotics, fresh media was added and the plates were incubated for an

additional 24 h (+37 °C, 200 rpm). Effects of the compounds were determined at the end of the incubation periods.

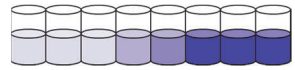
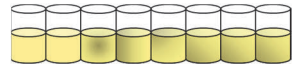
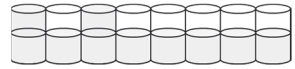
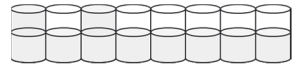
4.2.3 Bioassays

An overview of the bioassays applied to the study is provided here, details of the experimental procedures can be found in the original publications (studies I-IV).

4.2.3.1 Microtiter well plate (MWP)-based assays

The MWP-based assays used in the study are summarized in **Table 8**.

***Table 8**. Summary of the MWP-based assays.*

Assay	Readout	Endpoint	Visual outcome
Resazurin staining	Fluorescence	Viability, MBC, MBIC	
Crystal violet staining	Absorbance	Biomass, MBIC	
Turbidity assay (light scattering measurement)	Absorbance	Inhibition of bacterial growth, MIC	
Violacein reporter assay	Absorbance	Quorum Sensing (QS)	
ATP assay (CellTiter-Glo™)	Luminescence	Viability, ATP efflux	
$DiBAC_{4(3)}$	Fluorescence	Membrane depolarization	

4.2.3.1.1 Resazurin staining (studies I-IV)

Briefly, 400 µM resazurin (sodium salt, Sigma-Aldrich, Steinheim, Germany) solution was prepared in phosphate buffered saline (PBS, Lonza, Vievers, Belgium) and added into the wells at a final concentration of 20 µM after removing the media (biofilms and cells). For planktonic bacteria,

resazurin was directly added to the wells. The plates were incubated in darkness, at room temperature (RT) for different time periods, optimized for every measured system: 5 min (for planktonic bacteria), 20-45 min (for biofilm bacteria, depending on the species) and 2 hours (at +37 °C for mammalian cells). Fluorescence was read at excitation and emission wavelengths of 560 and 590 nm, respectively, using Varioskan Flash (Thermo Scientific, Vantaa, Finland) plate reader. The MBC was defined as the lowest concentration, which inhibited bacterial viability by 90% compared to untreated controls, and the MBIC was determined as the lowest concentration that inhibited biofilm formation by 90%, compared to untreated controls.

4.2.3.1.2 Crystal violet staining (studies I, III and IV)

Crystal violet staining was used to quantify the total biomass of biofilms. After removing the resazurin solution (studies I and IV) and the media (study III), the biofilms were stained with undiluted CV solution (2.3% (w/v) Sigma Aldrich, St. Louis, MO, USA) (studies I and IV) or diluted CV solution (0.23% (v/v, in MQ water) (study III) at RT for 5 min. After staining, the excess stain was washed off and the adsorbed dye was eluted in 96% ethanol. The amount of the solubilized dye, which is proportional to biofilm biomass, was measured spectrophotometrically at a wavelength of 595 nm after one hour, using Varioskan Flash multimode plate reader.

4.2.3.1.3 Turbidity measurement (studies I and II)

Bacterial light scattering (optical density) readings were used to determine the MIC against planktonic bacteria. For that purpose, at the end of overnight incubation, planktonic suspensions were transferred from the wells to sterile 96-MWPs. The lowest compound concentration required to inhibit the visible growth of suspended bacteria was recorded, and the scattered light was measured at 620 nm using Varioskan Flash multimode plate reader.

4.2.3.1.4 Violacein reporter assay (study III)

Violacein production was assayed for identification of QSIs in *C. violaceum* ATCC 31532 and CV026 strains. The culture of *C. violaceum* CV026 was supplemented with N-(β-ketocaproyl)-L-Homoserine lactone (3-O-C_6-(L)-HSL, Cayman chemicals, Ann Arbor, MI, USA) to specifically induce violacein production. After 24 hours incubation with compounds, violacein was extracted by centrifugation and mixing in ethanol and the absorbance was read at 595 nm using Varioskan Flash multimode plate reader.

4.2.3.1.5 ATP quantification assay (studies II and III)

Adenosine triphosphate (ATP) is widely used as an indicator of metabolically active cells (Weyermann, Lochmann and Zimmer 2005, Fan and Wood 2007). The bioluminescent detection of ATP utilizes luciferase, an enzyme that catalyzes the formation of light from ATP and luciferin, and the luminescent signal is proportional to ATP concentration. CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was used to detect ATP efflux from the *S. aureus* ATCC 25923 biofilm cells (study II) and to study cytotoxicity (study III). At the end of the exposure period to compounds, suspensions were filtered (0.22 μm pore size) and 10-fold diluted in PBS. Equal volumes of filtrates and CellTiter-Glo® reagent were added into a MWP (study II). In study III, following the incubation period, the media was removed from the plates, and sterile PBS and CellTiter-Glo® reagent, in equal volumes, were added into the wells. The plates were shaken for 2 min and incubated at RT for 10 min, and the luminescent signal was measured at 560 nm using Varioskan Flash multimode plate reader.

4.2.3.1.6 $DiBAC_{4(3)}$ bis-(1,3-dibutylbarbituric acid)trimethine oxonol assay (study II)

The membrane-potential-sensitive probe, $DiBAC_{4(3)}$ (Invitrogen™, Thermo Fisher Scientific CA, US) was prepared in PBS (5 μM) and used to monitor membrane depolarization of *S. aureus* ATCC 25923 biofilms. At the end of overnight incubation, the media was discarded and the biofilms were pre-incubated with the probe for 30 min. After pre-incubation, the biofilms were incubated with compounds prepared in $DiBAC_{4(3)}$ solution at RT for 1 h. After 1 h, the biofilms were washed twice with PBS and fluorescence was measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using Varioskan Flash multimode plate reader.

4.2.3.2 Agar plate-based assays

4.2.3.2.1 Viable plate counts (studies I-IV)

After the incubation periods, the biofilms were scraped off the substrates in the culture medium, disaggregated by sonication or by sonicating/vortexing, and the resulting suspensions were serially diluted and plated on agar plates (studies I, II and IV). In the case of planktonic bacteria, suspensions were removed by pipetting and homogenized by sonication, and serially diluted suspensions were plated on agar (studies I and III). Colony forming units (CFUs) were counted after overnight incubation, and the $\log_{10}$ density (LD) of viable cells per volume (studies I-III) or surface area (study

IV) was determined. Efficacy of the compounds was assessed by a logarithmic reduction (LR) assay (Pitts et al. 2003), in which LR was calculated from the difference in LD of untreated controls and treated bacteria (studies I, II and IV).

4.2.3.2.2 Motility assay (study III)

QS-regulated swimming and swarming motility of *P. aeruginosa* PA01 was determined on 0.3% (w/v) agar plates (**Figure 11**). Test compounds were pre-mixed with the agar and the solidified plates were point inoculated with *P. aeruginosa* (10^8 CFU/mL) and incubated at +37 °C for 18 h. The diameters of the swarming and swimming zones of the untreated and treated bacteria were measured after incubation.

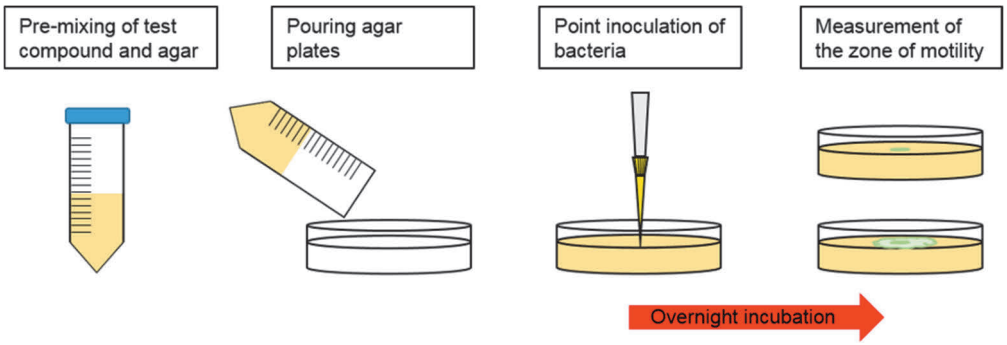


***Figure 11**. Schematic illustration of the motility assays.*

4.2.3.2.3 Drip flow reactor (DFR) assay (study IV)

Diluted bacterial cultures (10^7 CFU/mL) were pre-mixed with antibiotics and added at a final volume of 15 mL to three of the reactor channels, while 15 mL of bacterial culture was added to the control (fourth) channel. Biofilms in the presence or absence of antibiotics were formed on glass coupons placed in the channels by operating the reactor in batch phase (no flow) in a level position at +37 °C ± 2 °C for 1 h. After this batch phase, the reactor was drained and operated in continuous flow mode (flow rate 0.82 mL/min) at an angle of 10° for 24 h. At the end of this period, coupons were removed from the reactor channels and rinsed with sterile water. The biofilms were dislodged and disaggregated by sonication and vortexing, and the efficacy of antibiotics was quantified by performing viable plate counts, as described in 4.2.3.2.1.

4.2.4 Imaging of biofilms (studies II, III and IV)

4.2.4.1 Fluorescence microscopy (study II)

Biofilms were formed on coverslips placed on the bottom of 24-well plates, as described in 4.2.1. Planktonic suspension was removed and the biofilms were exposed to compounds for 1 h. At the end of the exposure period, the solutions were discarded, and bacterial biofilms were stained with LIVE/DEAD® BacLight™ stain (Invitrogen™, Thermo Fisher Scientific CA, US) for 15 min and imaged using an Inverted EVOS FL Epifluorescence Imaging System (Invitrogen™, Thermo Fisher Scientific CA, US) with a 40x coverslip-corrected objective.

4.2.4.2 Light microscopy (study III)

Biofilms were formed on coverslips placed on the bottom of 6-well plates, as described in 4.2.1, but only for 2 h. Thereafter, planktonic suspensions were removed, and compounds and fresh media were added to the wells and the plates were incubated for 22 h. At the end of the incubation period, coverslips were rinsed, air-dried and stained with 0.23% (v/v) crystal violet solution for 5 min. Imaging was carried out using EVOS® XL Imaging System (Invitrogen™, Thermo Fisher Scientific CA, US) at a magnification of 40x.

4.2.4.3 Confocal laser scanning microscopy (CLSM) (study IV)

For CLSM imaging, biofilms were formed in 12-well plates and DFR for 1 h. In MWP, planktonic suspensions were removed and fresh media was added into the wells followed by incubation for 24 h (+37 °C, 200 rpm). In DFR, biofilms were formed as described for control biofilms in 4.2.3.3. At the end of 25 h, the biofilms were stained with LIVE/DEAD® BacLight™ stain (Invitrogen™, Thermo Fisher Scientific CA and imaging performed using an upright Leica SP5 Confocal Scanning Laser Microscope using the 488 and 561 nm laser excitation lines.

4.2.5 Data analysis (studies I-IV)

Assay performance in screening assays was monitored by calculating Z′ factor, S/N and S/B (studies I-III). In the primary screening, all the compounds were tested in single wells (studies I and III), or in technical triplicates (study II), while during the follow-up studies, compounds were tested at least in three replicates and standard deviations (SDs) were calculated. For the half-maximal inhibitory concentrations (IC_{50}), 95% confidence intervals were calculated using GraphPad Prism software (version 5.0). In efficacy testing

(study IV), assay performance was evaluated in terms of coefficient of variation (CV) and repeatability standard deviation based on untreated control biofilms. Antibiotics were tested in duplicates, in at least three biological replicates (in MWPs) or five biological replicates (in DFR). SDs were calculated for each treatment. Statistical significance was determined using unpaired t-test with Welch's correction in studies III and IV, and *p*-values < 0.05 were considered as statistically significant.

Statistical parameters were calculated according to the following equations:

a) Signal window coefficient:

$$Z' = 1 - \left[\frac{3 * SD_{max} + 3 * SD_{min}}{|X_{max} - X_{min}|}\right]$$

b) Signal-to-noise (S/N):

$$\frac{S}{N} = \frac{X_{max} - X_{min}}{\sqrt{{SD_{max}}^2 + {SD_{min}}^2}}$$

c) Signal-to-background (S/B):

$$\frac{S}{B} = \frac{X_{max}}{X_{min}}$$

d) Coefficient of variation (CV):

$$CV = 100\% * \left[\frac{SD}{\bar{X}}\right]$$

in which SD_{min} and X_{min} correspond to the SD and mean value of the minimal signal, respectively, while SD_{max} and X_{max} refer to the SD and the mean of the maximal signal, respectively.

5 Results and discussion

5.1 Statistical analysis of the assay performance (studies I-IV)

Assay quality is an important aspect of the data analysis. To verify that the screening assays employed in the study performed well, and provided reliable and consistent data, assay performance was monitored by calculating statistical parameters: Z′ factor, S/B and S/N ratios (studies I-III), as originally described in Zhang et al. (1999) and Bollini et al. (2002). In general, Z′ is regarded as the preferred measure of assay performance for screening assays, and it shall be between 0.5 and 1 for an assay to be considered excellent (Zhang et al. 1999, Iversen et al. 2006). However, cell-based assays with Z′ values over 0.3-0.4 (Merten 2010, Iversen et al. 2006) are considered acceptable. The S/B ratio should be higher than 2, whereas for the S/N ratio, although no defined threshold value exists (Fallarero et al. 2014), higher S/N values indicate better assay performance (Zhang et al. 1999).

In study IV, assay performance was evaluated in terms of repeatability by calculating the repeatability SDs and the CV as in Pitts et al. (2001). Low values for these parameters indicate high assay repeatability.

5.1.1 Phenotypic screening (studies I and II)

Effects of the compounds on biofilm viability and biomass were assayed using resazurin and crystal violet staining, respectively. Overall, for resazurin staining, all the calculated parameters were higher than those for crystal violet assay. The Z′ value was always higher than 0.4, which is a reliable indicator of well performing cell-based assays (Fallarero et al. 2014). By contrast, Z′ values in crystal violet assay varied considerably between plates. However, most of the Z′ values were over 0.3.

As measured by means of Z′, the assay performance for resazurin staining in the primary screening is summarized in **Figure 12**.

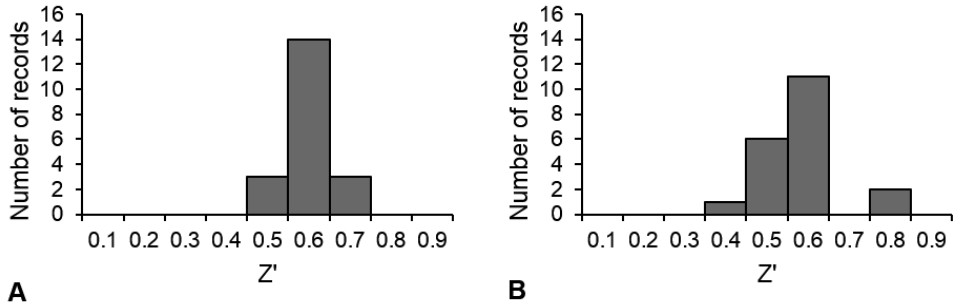


***Figure 12**. Frequency distribution of Z′ values for the viability (resazurin) assay performed in two exposure modes, prior to (A) and post (B) biofilm formation against S. aureus ATCC 25923 and S. aureus Newman during the primary screening of flavonoids and DHA-derivatives libraries. Z′ is calculated based on the maximal and minimum signals of a plate.*

In terms of Z′, the assay performance during the primary screening was good to excellent (Zhang et al. 1999). Moreover, the calculated mean Z′ of 0.64 ± 0.08, indicated a large separation band between the maximum and minimum signals demonstrating high quality of the screening. The calculated S/B and S/N ratios of 19.8 ± 4.5 and 9.1 ± 3.0, respectively, also demonstrated a wide window between the controls, and were also considered highly acceptable. Noteworthy, the S/B and S/N ratios were higher than 2.8 and 3.2, respectively, for crystal violet staining. In view of the higher throughput and better reproducibility of resazurin assay, only data obtained on this assay were used as basis for the identification of anti-biofilm hits. In addition, only resazurin staining assay was applied to primary screening in study II.

5.1.2 Target-based screening (study III)

A comparative analysis of the Z′ values (**Figure 13**) calculated for violacein reporter and resazurin staining assays revealed that both screening assays performed well. The vast majority of the Z′ values were ≥ 0.5, and the lowest Z′ values, 0.46 and 0.48 obtained on three assay plates, were very close to 0.5 and over 0.4, indicating the high signal robustness.

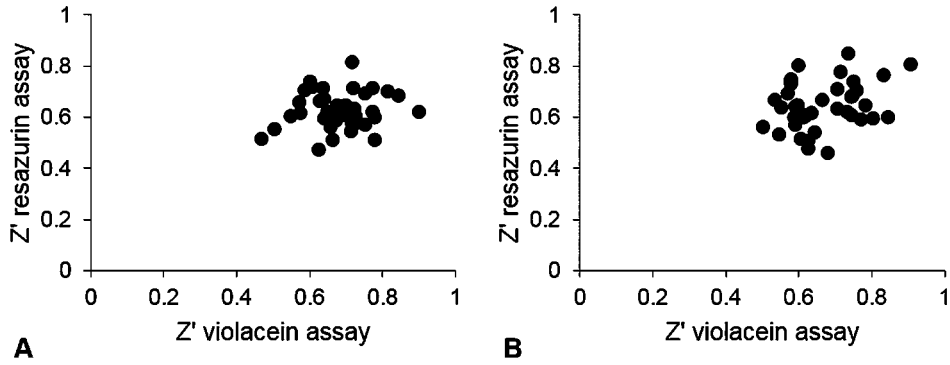


***Figure 13**. Correlation plot of Z' values obtained for the violacein and viability assays during the primary screening in study III to discover QSIs without bactericidal effects. In total, 76 plates were screened. Panel A refers to C. violaceum ATCC 31532 strain and panel B corresponds to C. violaceum CV026.*

The calculated mean Z' values for violacein extraction and resazurin assays performed on *C. violaceum* ATCC 31532 were 0.68 ± 0.09 and 0.63 ± 0.07, respectively. For *C. violaceum* CV026, the corresponding values were 0.67 ± 0.1 and 0.65 ± 0.09. The S/B and S/N ratios are summarized in **Table 9**. All the calculated parameters proved that the assays performed well.

***Table 9**. Statistical parameters calculated based on the primary screening.*

Parameter	Violacein extraction		Resazurin staining	
	ATCC 31532	CV026	ATCC 31532	CV026
S/N	12.0 ± 5.3	11.5 ± 6.8	8.7 ± 2.1	9.4 ± 3.2
S/B	6.8 ± 2.1	7.8 ± 2.6	8.3 ± 2.2	7.5 ± 2.3

5.1.3 Efficacy testing (study IV)

A comparative efficacy study of conventional antibiotics was performed in two distinct biofilm models, MWP and DFR. For both models, the repeatability SDs calculated based on the untreated control biofilms were low, 0.17 and 0.41 for MWP (n = 27) and DFR (n = 11), respectively, indicating high assay repeatability (Pitts et al. 2001). A repeatability SD of zero would indicate a complete (ideal) repeatability, while a large repeatability SD demonstrates that the outcome is not repeatable. Further, the calculated CVs were low, 2%

and 5% for MWP and DFR assays, respectively, reflecting low assay variability. A coefficient of variation of the signal <15% is considered acceptable (Fallarero et al. 2014). Thus, the assay produced reproducible data.

5.2 Screening for biofilm inhibitors (studies I-III)

Given the fact that biofilms are the predominant lifestyle of bacteria, scaffolds that have coevolved in nature offer a reasonable starting point for identifying anti-biofilm compounds with various mechanisms of action. A total of 3571 compounds from libraries containing both naturally occurring and naturally-inspired synthetic compounds were included in three studies. Of these, 531 compounds were screened for anti-biofilm activity against *S. aureus* ATCC 25923 and *S. aureus* Newman in two modes (prior to and post biofilm formation), and anti-biofilm activity was quantified based on biofilm viability using resazurin staining assay (studies I and II) as well as biomass using crystal violet staining assay (study I). This strategy was aimed at the discovery of anti-biofilm compounds with bactericidal effects. The second discovery strategy applied in this thesis focused on identification of non-bactericidal compounds targeting QS. To that end, 3040 compounds were screened against *C. violaceum* ATCC 31532 and CV026 for QSI activity and viability in parallel (study III). The workflow of screening processes and selection criteria applied to the exploration of biofilm inhibitors from compound libraries (studies I-III) is schematically shown in **Figure 14**.

The calculated hit rates, as defined based on the number of primary hits compared to the number of compounds included in the primary screening, were 2%, 80% and 0.3% for studies I, II and III, respectively. Typically, the hit rate during the primary screening is lower than 1% (Coma, Herranz and Martin 2009, Posner, Xi and Mills 2009). The higher hit rate in study I can be explained by the fact that flavonoids are reported for diverse antimicrobial activity (Havsteen 2002, Cushnie and Lamb 2005) and thus, expected to display also anti-biofilm properties. Further, natural compound libraries are known for higher hit rates than synthetic libraries, especially, when it comes to antimicrobial screening (Bérdy 2012). Moreover, phenotypic screening is generally considered more efficient than target-based screening in antibacterial discovery (Katsuno et al. 2015). The extremely high hit rate in study II was a result of the nature of the compound library. As the library synthesis was inspired by the previous identification of DHA as a potent inhibitor of *S. aureus* biofilms (Fallarero et al. 2013), and the DHA moiety was

included in each of the compounds in the library, the library was focused and also smaller in size.

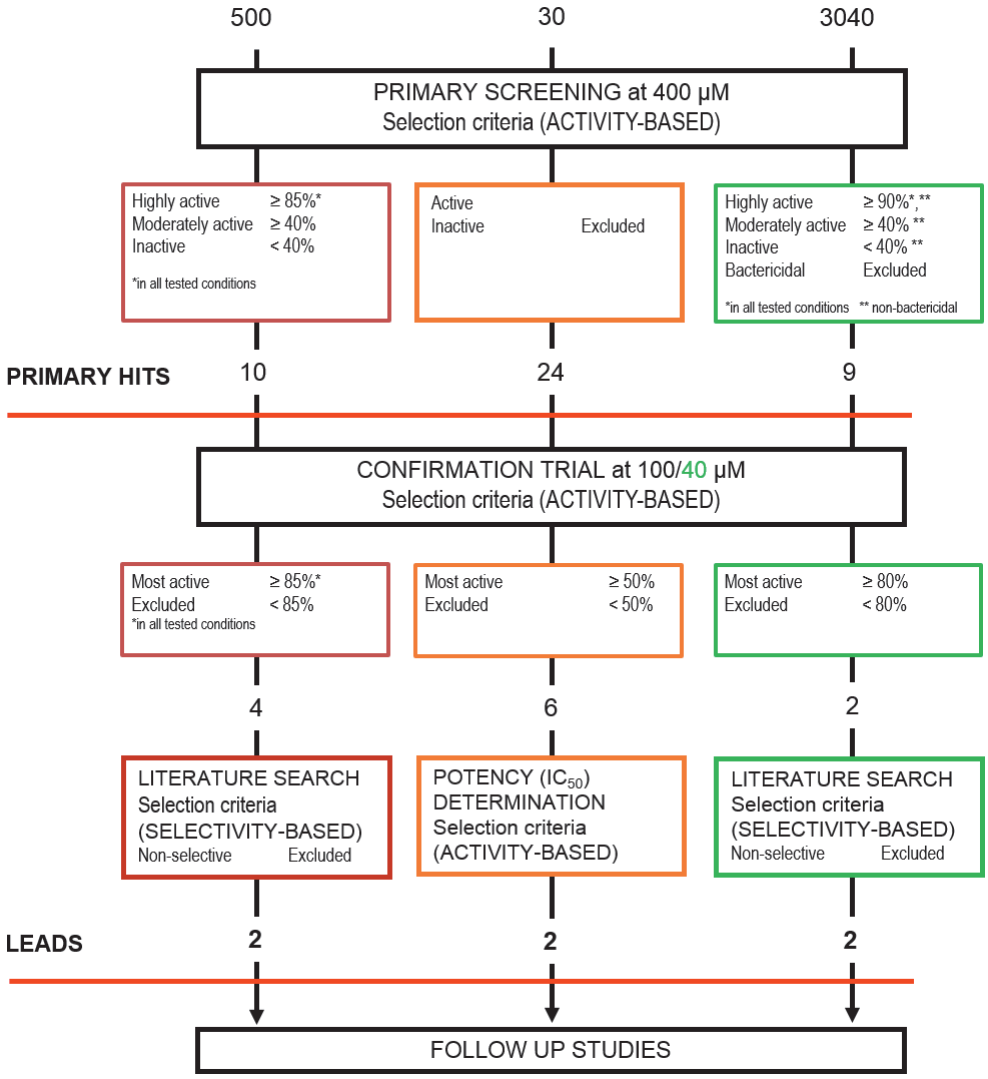


***Figure 14**. Schematic presentation of the screening process and selection criteria applied to the identification of biofilm inhibitors. Red boxes refer to study I, and orange and green to studies II and III, respectively.*

5.2.1 Screening results, classification of the compounds and lead selection (studies I-III)

5.2.1.1 Flavonoids collection (study I)

It has been shown that the experimental outcome of antimicrobial studies is strongly affected by the assay conditions and materials employed in the testing (Jenkins and Schuetz 2012). To gain a comparative and systematic view of the antimicrobial activity of flavonoids, and to minimize the sources of experimental variations, a compound library of 500 naturally occurring and synthetic flavonoids was screened against *S. aureus* under similar experimental conditions.

Primary screening conducted at 400 μM resulted in identification of ten primary hits, termed as highly actives. The hit limit was empirically set at 85% inhibition compared to untreated control biofilms on both strains and in both exposure modes, as quantified using resazurin staining. This allowed obtaining a reasonable number of potent hits that could be handled in the secondary assays. Further, 47 compounds, which displayed inhibitory activity ranging between 40 and 85% were classified as moderately actives. Finally, a total of 443 compounds had inhibitory activity less than 40%, and they were classified as inactives. Complete results of the primary screening based on resazurin staining are presented in Supplementary table 1 in study I. Anti-biofilm effects of the ten highly active compounds on biofilm viability, as measured using resazurin staining, are shown in **Figure 15**, and their structures are shown in Supplementary table 2 in study I.

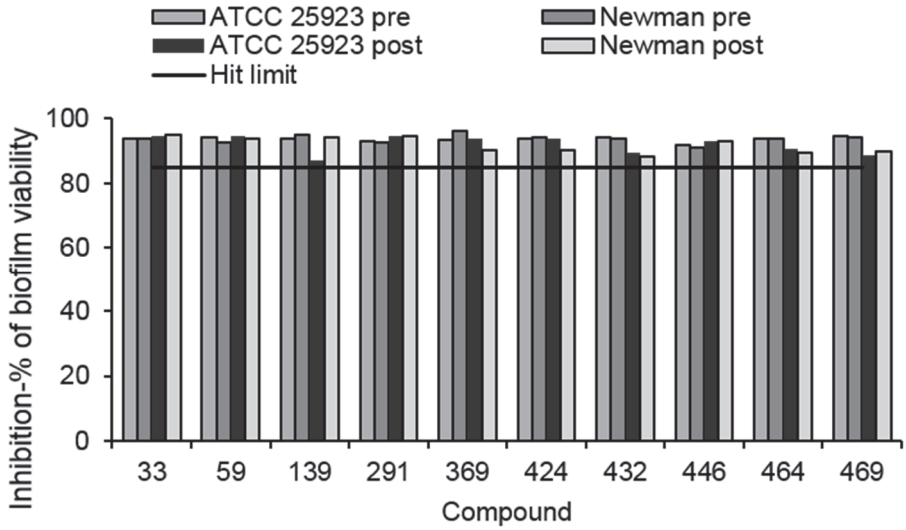


***Figure 15**. Anti-biofilm effects of the highly active flavonoids against S. aureus ATCC 25923 and S. aureus Newman, when added prior to (pre) or post biofilm formation. The hit limit was set at 85% inhibition compared to untreated control biofilms.*

A selection criterion combining activity and selectivity was utilized for identification of anti-biofilm leads. To that end, a secondary screen at 100 μM with the ten highly active compounds was first conducted against *S. aureus* ATCC 25923 in two exposure modes. The ATCC strain was selected since the resazurin assay utilized here has been optimized using that strain (Sandberg et al. 2009, Skogman et al. 2012). Among the ten highly active compounds, representatives from four distinct classes, namely isoflavones (1), flavans (3), chalcones (3) and flavanones (3) were present. Further, of these compounds, six were naturally occurring, while four were synthetic derivatives. Interestingly, none of these highly active compounds was previously reported for any anti-biofilm properties.

Upon confirmation testing at 100 μM, four compounds preserved the high activity (≥ 85% inhibition). A literature search using PubChem Bioassay project database (http://www.ncbi.nlm.nih.gov/pcassay) and Antimicrobial Index database (http://antibiotics.toku-e.com/) was performed. This search led to identification of two synthetic flavan derivatives, 6-chloro-4-(6- chloro-7-hydroxy-2,4,4-trimethylchroman-2-yl)benzene-1,3-diol (**291**) and 4-(6-hydroxyspiro[1,2,3,3a,9apentahydrocyclopenta[1,2-b]chromane-9,1′-cyclopentane]-3a-yl)benzene-1,3-diol (**369**) as leads. Structures of the lead

flavonoids are shown in **Figure 16**, and their anti-biofilm and antibacterial activity are discussed further in 5.3.1.

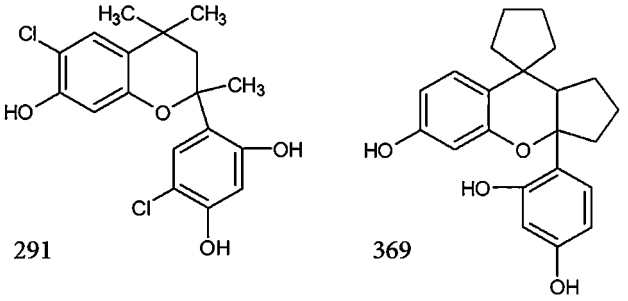


***Figure 16**. Chemical structures of the identified lead flavonoids.*

5.2.1.2 Amino acid derivatives of (+)-dehydroabietic acid (DHA) (study II)

Previously, DHA has been identified as the most potent anti-biofilm compound within the class of abietane-type diterpenoids (Fallarero et al. 2013). Here, various amino acids (L-, D- and unusual amino acids) were linked to DHA, and the library of 30 hybrid compounds was screened for anti-biofilm activity against *S. aureus*. Primary screening led to the identification of 24 primary hits. Following the retesting of these primary hits at 100 μM, compounds that inhibited biofilms by more than 50% were selected for further studies. Six compounds met this criterion, and anti-biofilm potencies (IC_{50}) against *S. aureus* ATCC 25923 were determined. Four compounds (**4e**, **9a**, **9b**, **11**) displayed potencies that were higher than or similar to the parent compound DHA (**Figure 17**). More importantly, all these compounds were more active than DHA in the post-exposure mode of the assay. Results from the entire testing are summarized in Supplementary tables 1 and 2 in study II. The two most potent compounds (**9b** and **11**) were selected as leads.

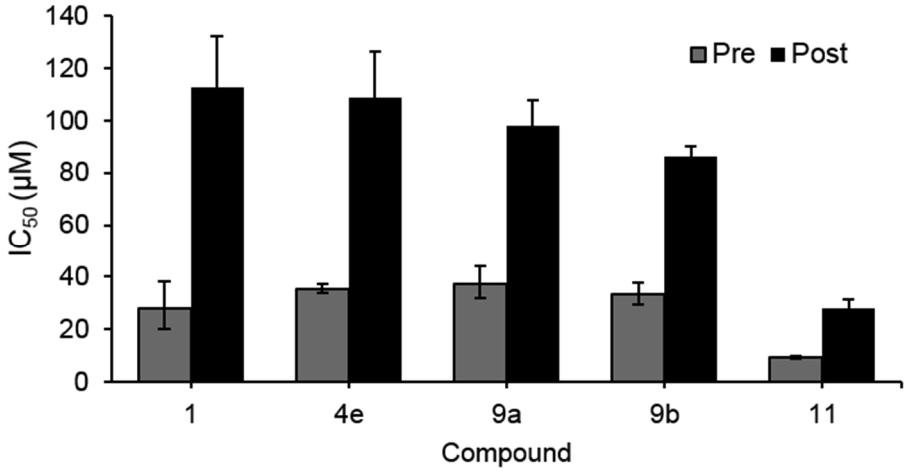


***Figure 17**. Anti-biofilm potencies of the most active amino acid derivatives of DHA measured in both exposure modes against S. aureus ATCC 25923. DHA was used as the reference compound and is coded as 1. Error bars represent the 95% confidence intervals.*

In general, successful hybrid compounds should exhibit better bioactivity than the fused parts separately (O'Connell et al. 2013). The D-tryptophan derivative (**9b**) and the β-cyclohexyl-L-alanine derivative (**11**), (**Figure 18**), which were selected as leads, were found to display significantly higher anti-biofilm activity than the reference compound DHA. Moreover, as compared to the anti-biofilm activities reported for D-amino acids (Hochbaum et al. 2011, Kolodkin-Gal et al. 2010), these derivatives are also more potent anti-biofilm compounds. Thus, linking of particular amino acids to DHA enhanced its specific anti-biofilm activity. The activity of these two leads (**9b** and **11**) is further discussed in 5.3.1.

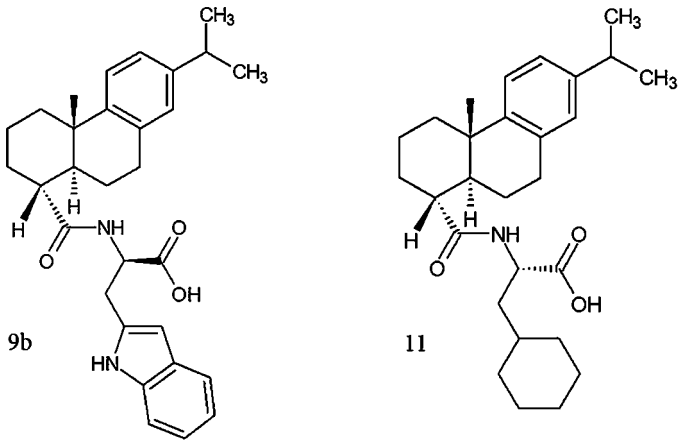


Figure 18*. Structures of the identified lead compounds.*

5.2.1.3 Natural product derivatives (study III)

Various naturally-derived compounds and their synthetic derivatives have been identified as QSIs (Nazzaro, Fratianni and Coppola 2013, Cady et al. 2012, Persson et al. 2005, Sintim et al. 2010). Compounds, such as alkaloids, phenols and carbohydrates, are involved in many biological processes and thus, thought to be a rich source of novel bioactive scaffolds (Cragg and Newman 2013, Koehn and Carter 2005). Here, a compound library of 3040 NP derivatives was screened for QSI using two strains of *C. violaceum* (ATCC 31532 and CV026) as reporter bacteria.

The threshold during the primary screening was set at 90% inhibition compared to untreated controls by means of violacein production in both strains. Moreover, all the compounds displaying bactericidal activity (≥ 40% inhibition on viability) were excluded, since QS is not affecting the bacterial growth. Therefore, inhibition of violacein production induced by bactericidal compounds cannot be considered as true QSI (Rasmussen and Givskov 2006). Based upon the primary screening at 400 μM, nine highly active hits were identified. Further, 328 non-bactericidal compounds were deemed as moderately active with an inhibitory activity between 40 and 90%, and the rest of the compounds were classified as inactives (n = 2062). QSI activity of the highly active compounds in the primary screening is shown in **Figure 19**, and the entire results from the primary screening are presented in Supplementary table 2 in study III.

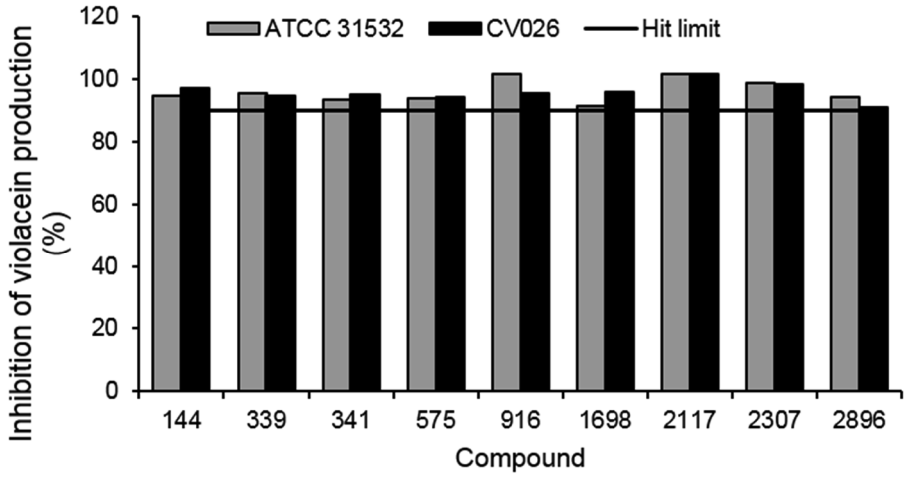


***Figure 19**. Screening for QSIs. Inhibition of violacein production in C. violaceum ATCC 31532 and CV026 by the highly active compounds. The hit limit was set at 90% inhibition compared to the untreated controls.*

Primary screening led to the identification of nine, structurally diverse highly active compounds from five distinct compound classes. Structures of these compounds are shown in Figure 3 in study III. This group comprised flavonoids (5), one alkaloid (1), a nucleoside analogue (1), an organosulfur compound (1) and a lignan (1). Given the chemical and structural diversity of the hits, no obvious structure-activity relationship seems to exist. Altogether, seven of them have not been previously reported for QSI activity against *C. violaceum*.

For the lead selection, secondary screens with the highly active compounds were performed at 40 μM against *C. violaceum* ATCC 31532. This strain was selected, as the violacein production in *C. violaceum* ATCC 31532 is not dependent on exogenous addition of AHLs, thus enabling identification of QSIs, which do not interfere with the signaling process by degrading the added AHLs (Skogman et al. 2016). In this screen, only one compound exceeded the threshold value (90%). Thus, the cut-off value was decreased to 80% to obtain several compounds for further studies. Finally, a literature search using PubChem Bioassay project database (http://www.ncbi.nlm.nih.gov/pcassay) was conducted. Following the literature search, two flavonoid derivatives of flavone class, 2-(2-chlorophenyl)-4-oxochromen-3-yl propanoate (**2117**) and 2-(4-methoxyphenyl)-4-oxochromen-3-yl decanoate (**2896**) were chosen as leads. Structures of the leads are shown in **Figure 20** and the lead compounds are discussed more in 5.3.2.

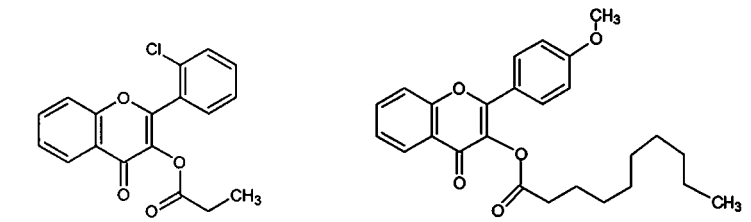


***Figure 20**. Chemical structures of the leads.*

5.3 Follow-up and mechanistic studies

5.3.1 Anti-biofilm potencies and antibacterial effects of the leads (studies I and II)

Anti-biofilm potencies (IC_{50} and IC_{90} values) against *S. aureus* ATCC 25923 were estimated based on at least 12 concentration points ranging from 0.01 to 400 μM (study I) and 0.001 to 400 μM (study II). The effects of the compounds were quantified using resazurin staining assay, and the potencies were calculated using a non-linear regression analysis. The MIC values corresponding to the concentrations that inhibited 90% of planktonic growth, and the MBC against planktonic bacteria were determined based on turbidity and using resazurin staining, respectively. Results are shown in **Figure 21**.

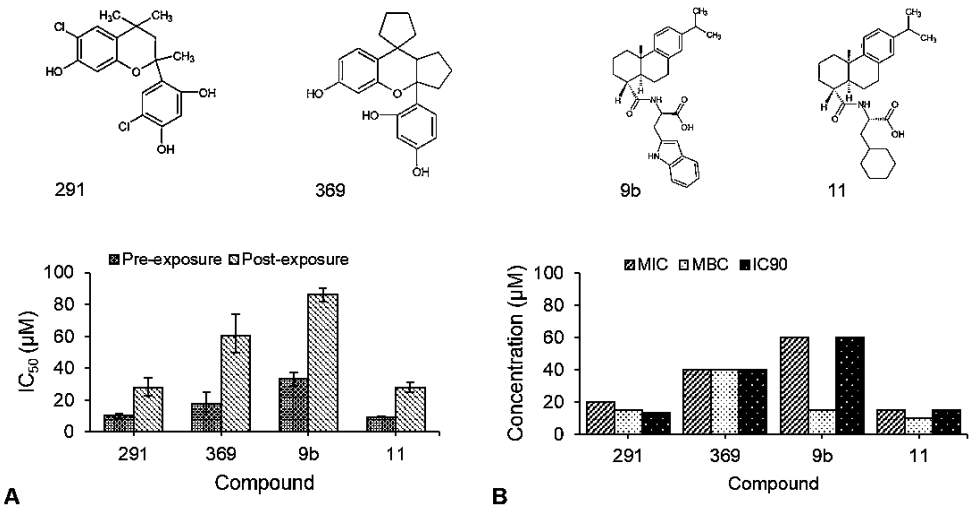


***Figure 21**. Structures, anti-biofilm potencies (A) and antibacterial effects (B) of the leads determined against S. aureus ATCC 25923. Error bars on the left represent the 95% confidence intervals.*

All the identified leads compounds, **291** and **369** (study I), and **9b** and **11** (study II), were shown to inhibit biofilm viability in a concentration-dependent manner, and the calculated potencies were at micromolar range. In the pre-exposure mode, the IC_{50} values ranged from 9.4 to 33.2 μM (**11** < **291** < **369** < **9b**) and IC_{90} values were between 13 and 60 μM (**291** < **11** < **369** < **9b**). Potency (IC_{50}) values recorded in post-exposure mode ranged from 27.8 to 86.2 μM (**11** < **291** < **369** < **9b**). Thus, concentrations of the leads required to eradicate the pre-formed biofilms were only approximately 3-fold higher than concentrations needed to prevent biofilm formation. Of note, neither penicillin G nor vancomycin, which were included as reference compounds in the studies, was equally effective in the eradication of pre-formed biofilms. At the highest test concentration (400 μM), an inhibition of 57% and 25% was registered for penicillin G and vancomycin, respectively. This finding demonstrates the superiority of the identified leads in comparison to conventional antibiotics when tested *in vitro* against biofilm-associated infections.

In addition to anti-biofilm activity, all the leads displayed antibacterial activity against planktonic cells. Overall, the antibacterial activity of the compounds was consistent with anti-biofilm activities in pre-exposure mode of the assay. The MIC values ranging from 15 to 60 μM (**11** < **291** < **369** < **9b**) were very similar to IC_{50} values determined against biofilm bacteria. Further, the determined MBC values between 10 and 40 μM (**11** < **291** < **369** < **9b**) were within the same range as the measured IC_{90} values. Consequently, the anti-biofilm activity of these compounds in the pre-exposure mode of the assay is most likely connected to their ability to kill planktonic cells before the surface attachment. In this way, biofilm formation is inhibited by reducing the number of bacteria involved in biofilm colonization. However, all the compounds were also active in the post-exposure mode of the assay, indicating that the compounds display anti-biofilm activity independent of the bactericidal activity.

Among the leads, compounds **291** and **11** were characterized as the most potent anti-biofilm compounds, while **11** was the most effective against planktonic bacteria. In general, compounds that inhibit the growth of microorganisms at concentrations below 10 μg/mL are considered as interesting antimicrobials (Ríos and Recio 2005). Of the hits, compounds **11** and **291** fulfilled this criterion with MIC values of 6.8 μg/mL and 7.4 μg/mL, respectively. Among plant-derived antimicrobials, the activity of these compounds can be considered as a high-level activity, since usually the

reported MIC values range between 100 μg/ml and 1000 μg/mL, and are orders of magnitude higher than of antibiotics derived from bacteria or fungi (Tegos et al. 2002). Furthermore, if the antibacterial activity of flavonoids is taken into consideration, compound **291** falls within the top ten most active flavonoids reported by Cushnie and Lamb (2011).

5.3.2 Killing efficacy of the anti-biofilm and antibacterial leads (studies I and II)

The Log Reduction (LR) is commonly used as a measure of the efficacy of antimicrobials, and the LR assay is considered as the gold standard when quantifying the efficacy of anti-biofilm compounds (Pitts et al. 2003). A LR higher than 2 (≥ 2-$\log_{10}$-unit reduction in the numbers of CFU/mL or CFU/cm^2 in comparison to the untreated controls) demonstrates a 99% reduction of bacterial burden, and further a LR of ≥ 3 is regarded as an indicator of a significant reduction of the number of bacteria resulting in the killing of 99.99% of the bacterial population. Furthermore, the killing of 3 $\log_{10}$ units is considered as a bactericidal mechanism (Traczewski et al. 2009), and importantly, LR of 3 indicates high anti-biofilm efficacy. The LR was determined by performing viable plate counts on the pre-formed biofilms after 24 hours exposure to the lead compounds (in the post-exposure mode).

In study I, the killing efficacy of the lead compounds was quantified both against planktonic and biofilm bacteria, as the leads were found to display both antibacterial and anti-biofilm activity (**Table 10**). Compound **291** resulted in a similar LR against both phases at all the test concentrations, thus confirming its equal antibacterial and anti-biofilm activities. Such compounds acting on both bacterial states at similar concentrations serve as promising starting points for novel, viable anti-biofilm compounds. Compound **369**, in turn, was found to be more effective against planktonic bacteria yielding a full log reduction (no countable colonies on agar after the incubation period) at the two highest test concentrations with lower efficacy against biofilm bacteria. Thus, compound **369** can be considered more as an antibacterial than an anti-biofilm compound.

***Table 10**. Killing efficacy of the lead flavonoids assessed against planktonic and biofilm bacteria of S. aureus ATCC 25923.*

Compound	Concentration (μM)	Log Reduction (LR)	
		Planktonic phase	Biofilm phase
291	20	0.1	0.6
	80	0.7	1.5
	200	3.5	3.5
	400	4.7	4.6
369	50	1.2	1.5
	100	4.1	3.9
	250	9.0	3.1
	400	9.0	3.9
Pen G	400	4.0	1.0

Further, to confirm the efficacy of the identified leads in biofilm removal, compounds **9b** and **11** were tested at 400 μM (**Figure 22**). The decrease in CFU ranged between 2.3 and 6.2 $\log_{10}$ units. The low efficacy of penicillin in biofilm removal was clearly demonstrated.

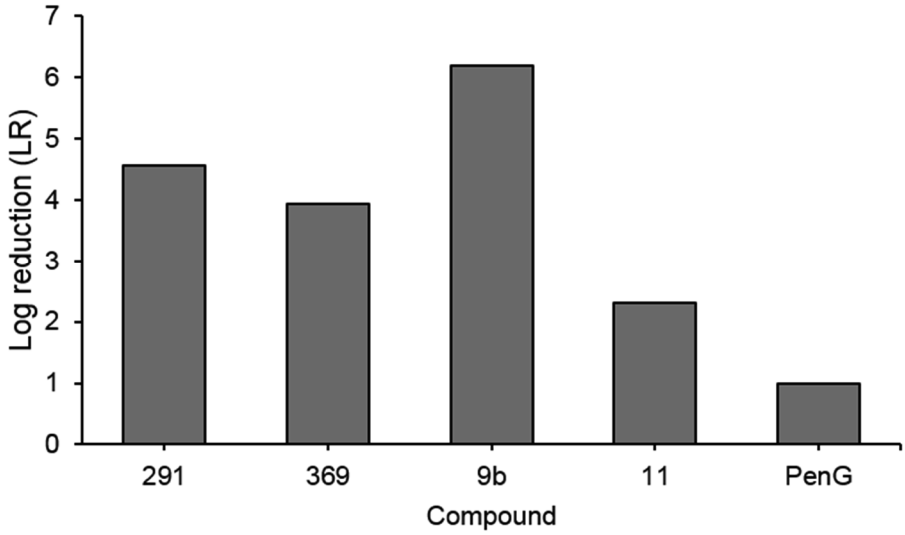


***Figure 22**. Log reduction (LR) values caused by the identified leads and the reference antibiotic when tested against 18 hours biofilms at 400 μM. SDs were ≤0.4 in all the cases and compounds 291 and 369 were tested only once.*

Killing efficacy studies also confirmed that none of the lead compounds can be regarded as a specific anti-biofilm compound. However, as the switch between two bacterial lifestyles, single cells and biofilms, is dynamic, the multifunctionality of these leads is advantageous compared to conventional antibiotics targeting only the dividing, planktonic cells. Such compounds are desirable antimicrobial compounds, as they can act both on planktonic and biofilm bacteria.

5.3.3 Mode of action (study II)

Since the leads were identified using a phenotypic screening approach, additional assays were performed to shed light on the underlying targets of the observed phenotypic effects (also known as target deconvolution) (Terstappen et al. 2007). First, time-kill kinetics of the leads were studied. As the leads (at 100 μM) were found to be fast-acting, causing more than 50% inhibition of biofilm viability after an exposure of one hour, their effects on the bacterial membrane were investigated. These studies revealed that the leads target the bacterial membrane, leading to membrane polarization and ATP efflux from the inner core of the biofilms (**Figure 23**). These findings were also confirmed by fluorescence imaging of the biofilms stained with live/dead stains SYTO 9 and propidium iodide.

Such mode of action resembles the activity of antimicrobial peptides (AMP) (Matsuzaki 2009). However, AMPs are larger in size and more susceptible to enzymatic proteolysis. In contrast, the compounds **9b** and **11**, bearing a D- and an unusual amino acid as a side chain, respectively, are more stable and less prone to enzymatic proteolysis.

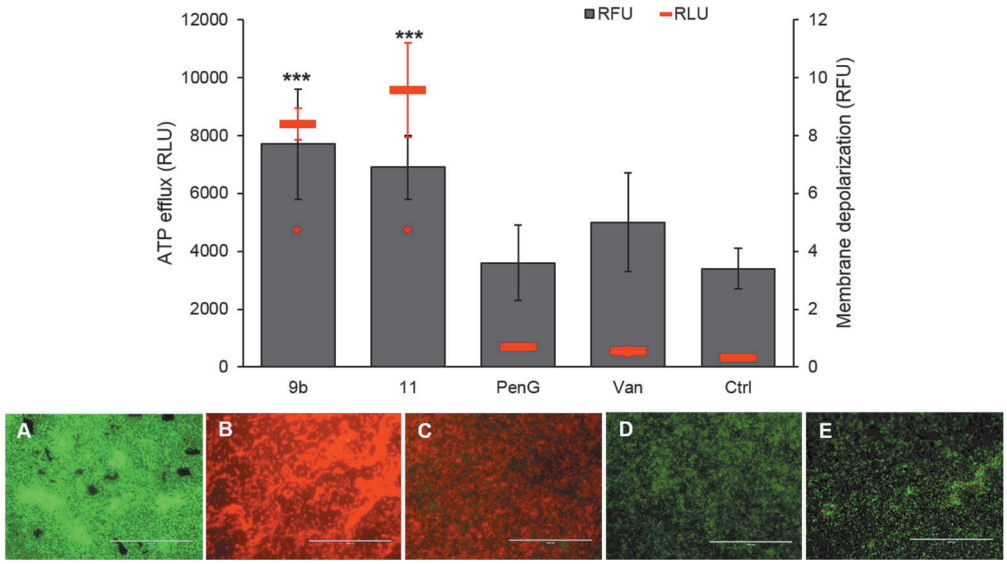


***Figure 23**. Events taking place after one hour exposure to the leads **9b** and **11** and reference antibiotics. *p < 0.05; ***p < 0.001 indicate statistically significant differences when compared to untreated biofilms. Fluorescence microscopy images of biofilms, from left, untreated biofilm (A), biofilms exposed to **9b** (B) and **11** (C) and reference antibiotics, penicillin G (D) and vancomycin (E). Scale bars correspond to 100 µm.*

5.3.4 Anti-QS activity (study III)

Virulence inhibition strategies have been considered as promising alternatives for traditional antimicrobials, especially in case of biofilms. In contrast to other anti-biofilm leads discussed above, the identified lead compounds **2117** and **2896** displayed anti-biofilm activity via inhibition of QS.

The N-acyl-homoserine lactone (AHL)-based QS system of *C. violaceum*, which consists of LuxI/LuxR homologs CviI/CviR (Stauff and Bassler 2011), was utilized as the target in primary screening. Following the initial testing against *C. violaceum*, which included primary and secondary screens along with potency assessment, activity of the identified leads was investigated against other gram-negative bacteria, including *P. aeruginosa*. In *P. aeruginosa*, LuxI/LuxR homologs RhlI/RhlR and LasI/LasR coordinate swarming motility and transition from microcolonies to fully formed biofilms, respectively (Köhler et al. 2009, Davies et al. 1998).

Results from the QSI studies for *C. violaceum* and *P. aeruginosa* PAO1 are summarized in **Figure 24**. The identified lead compounds were shown to affect several QS-mediated features involved in different stages of biofilm formation and bacterial virulence to varying degrees, but they were not

detrimental to bacterial growth. Even though the leads inhibited violacein production in *C. violaceum* at low micromolar concentrations with the IC_{50} values of 9.6 and 13.9 μM for **2117** and **2896**, respectively, their activity against *P. aeruginosa* was significantly lower even at the highest test concentration (400 μM). Such differences can be attributed to the species-specificity associated with QSIs over the system specificity (Jakobsen et al. 2012).

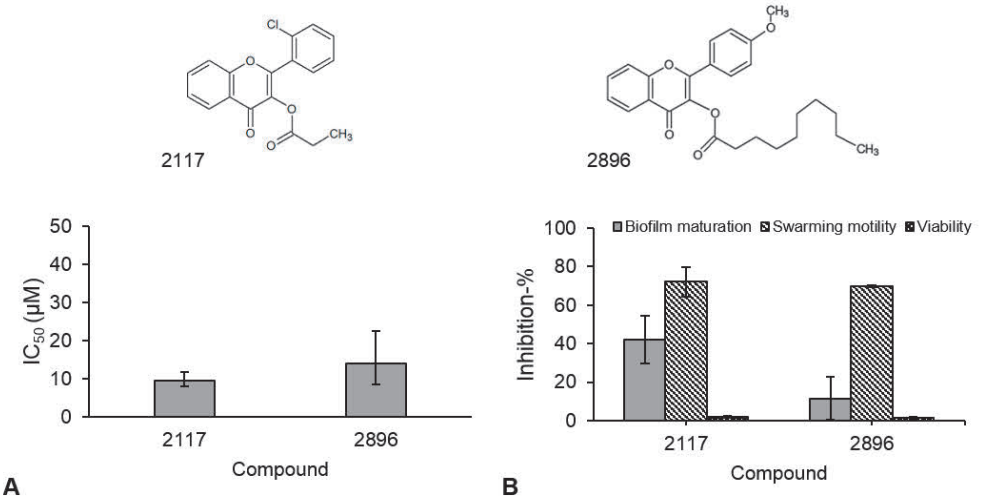


***Figure 24**. Structures and potencies (IC_{50} values) of the leads determined against C. violaceum ATCC 31532 (A), and effects on biofilm maturation, swarming motility and viability of P. aeruginosa PAO1, as assayed at 400 μM (B).*

Effects of the leads on biofilm maturation and architecture were also visualized using light microscopy (**Figure 25**). The biofilm density was significantly higher in the untreated control biofilm.

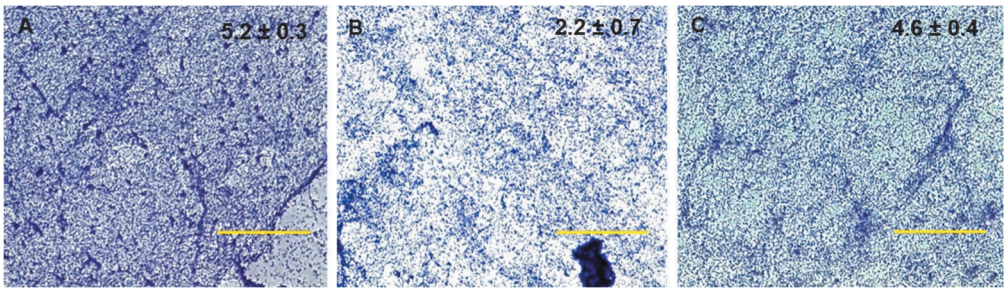


***Figure 25**. Light microscopy imaging of crystal violet stained P. aeruginosa PAO1 biofilms. From left, untreated control (A), microcolonies exposed to the lead compounds **2117** (B) and **2896** (C). The measured OD_{595} values after crystal violet staining are shown in each image. Scale bars correspond to 100 μm.*

5.3.5 Selectivity of the leads (studies I-IV)

Even though ATCC strains are well characterized and preferred to be used in antimicrobial testing (Cos et al. 2006), two strains of *S. aureus* (ATCC 25923 and Newman) were included in primary screening to avoid identification of strain specific-hits (studies I and II). Screening of the flavonoids library (study I) resulted in more strain-specific hits, while the activity of amino acid derivatives of DHA was generally similar against both strains (study II). Such characteristics have also previously been reported for flavonoids (Cushnie and Lamb 2011). For the same occasion, antibiotic susceptibility testing performed in study IV was conducted against two strains of *S. aureus* (ATCC 25923 and Newman) and one strain of *S. epidermidis* (ATCC 35984). In study III, the use of the ATCC strain and the violacein-deficient mutant strain CV026 in parallel, enabled distinction between real QSIs and quorum quenchers (QQs). The QSIs inhibited violacein production in both strains, while the QQs were active only against the mutant strain requiring the addition of exogenous AHLs to produce violacein.

In study II, during the follow-up studies, the activity of the six most active compounds and the parent compound DHA was assessed against *S. epidermidis* ATCC 12228 and ATCC 35984 in addition to *S. aureus* ATCC 25923 and Newman. Additionally, a gram-negative strain *E. coli* XL1 was included in the study to investigate, whether the identified hit compounds would display broad-spectrum activity (against both gram-positive and gram-negative species). Generally, broad-spectrum antimicrobials are esteemed as powerful tools to treat infections caused by a wide range of bacteria (Lynch 2012). No significant activity against the gram-negative representative was shown, indicating selective, narrow spectrum activity against gram-positive bacteria. However, all the compounds were active to varying degrees against other gram-positive strains when tested at the IC_{50} concentration determined against *S. aureus* ATCC 25923 in the pre-exposure mode of the assay, thus suggesting a broader anti-staphylococcal spectrum. Results for the lead compounds **9b** and **11**, as well as for penicillin G included as a control antibiotic, are shown in **Table 11**.

***Table 11**. Anti-biofilm activity of the DHA-derived leads against three other biofilm-forming strains measured at IC_{50} concentration determined against S. aureus ATCC 25923.*

Strain	Inhibition percentages of biofilm formation (%)		
	9b	**11**	**Pen G**
***S. epidermidis* ATCC 12228**	70.3 ± 5.6	42.3 ± 9.5	28.0 ± 2.3
***S. epidermidis* ATCC 35984**	66.9 ± 0.9	48.8 ± 4.8	15.5 ± 15.0
***E. coli* XL1 Blue**	8.7 ± 5.6	5.2 ± 11.3	9.6 ± 6.2

Antimicrobials with a narrow activity spectrum are considered as less harmful with lesser effects on beneficial bacteria (Silver 2011). Moreover, use of narrow-spectrum antibiotics that target only a specific pathogen can diminish the emergence of antibiotic-induced resistance (Levy and Marshall 2004).

5.3.6 Cytotoxicity of the leads (studies II and III)

An ideal anti-biofilm agent should be safe and effective as drugs in general. Typically, long-term treatment with antimicrobials at high doses is needed for treating biofilm infections (Beloin et al. 2014). Thus, anti-biofilm agents, as well as antibiotics in general, have an enhanced risk of toxicity due to high doses needed to achieve the desired efficacy (Lewis 2013). Furthermore, in contrast to many other therapeutic areas, in which lack of efficacy is the major cause of attrition, the clinical safety and toxicology are among the most critical factors for antimicrobials (von Nussbaum et al. 2006). Cytotoxicity testing on cell cultures predicts potential toxic effects in animals and humans (Riss and Moravec 2004, Mahto, Yoon and Rhee 2010). The identified lead compounds were tested against human lung (HL) epithelial cells (studies II and III) and mouse monocyte macrophage RAW 264.7 cells (study III) (**Table 12**). After 24 h exposure to compounds, compound **9b** was found to be cytotoxic (~80% inhibition of cell viability) at 100 μM, while compound **11** was shown to be slightly cytotoxic (~10% inhibition of cell viability) (study II). At the lower test concentrations, compound **9b** was also non-cytotoxic (0-10% inhibition of cell viability). In study III, compound **2117** was cytotoxic against RAW 264.7 cells at the highest test concentration, whereas against HL-cells it demonstrated minor cytotoxicity. Compound **2896**, in turn, did not exhibit any cytotoxic

effects even at the highest test concentration. In general, natural compounds are regarded as less toxic than synthetic compounds.

***Table 12**. Cell viability measured using resazurin staining assay (study II) and based on ATP levels (study III). N.A. = not analyzed.*

Compound	Concentration (μM)	Percent of cell viability (%)	
		HL	RAW 264.7
9b	1	95.7 ± 10.7	N.A.
	50	93.0 ± 8.1	N.A.
	100	23.0 ± 5.9	N.A.
11	1	98.5 ± 1.3	N.A.
	50	93.9 ± 6.4	N.A.
	100	92.6 ± 6.7	N.A.
2117	40	100.1 ± 0.8	101.1 ± 1.0
	100	88.8 ± 4.9	1.2 ± 0.5
2896	40	104.9 ± 3.7	104.0 ± 3.9
	100	102.6 ± 0.8	103.6 ± 4.8

5.4 Insights into biofilm models: MWP vs DFR (study IV)

Several *in vitro* models have been developed for biofilm research. In this study, efficacy of selected conventional antibiotics for biofilm prevention in the MWP and the DFR, representatives of closed and open systems, respectively, was assessed. The experiments were conducted utilizing a similar experimental set up to investigate the impact of the biofilm model on the experimental outcome and biofilm formation. Even though conventional antibiotics developed to kill or inhibit the growth of exponentially growing bacteria are generally inefficient against biofilm bacteria, they serve as a potential strategy for biofilm prevention (Kohanski, Dwyer and Collins 2010).

5.4.1 Selection process of antibiotics for efficacy testing

Initially, a susceptibility testing involving a panel of 27 clinically used antibiotics from various mechanistic classes was performed. Planktonic bacteria were exposed to two-fold dilution series of antibiotics at 22

concentrations, ranging from $4.88x10^{-4}$ mg/L to 1024 mg/L, and the MBIC values were quantified. Moreover, effects of the antibiotics at the same concentration range against pre-formed biofilms were evaluated. As expected, all the antibiotics were more effective in the pre-exposure mode of the assay. When assayed in the post-exposure mode of the assay, none of them was able to reduce biofilm viability by more than 60%. However, results from resazurin and crystal violet assays were fairly similar, indicating the bactericidal activity of the antibiotics against biofilm forming bacteria (Skogman et al. 2012). The entire results of the susceptibility testing are shown in Supplementary tables 1-4 in study III. Results of the ten most active antibiotics, when tested against *S. aureus* ATCC 25923, are summarized in **Table 13**. These antibiotics were selected further for the efficacy testing, first performed in the MWP.

***Table 13**. MBIC values determined using resazurin and crystal violet staining, respectively, and the effects on pre-formed S. aureus ATCC 25923 biofilms using resazurin staining assay. N/A = not available.*

	MBIC				**Effects on pre-formed biofilms**		
Antibiotic	Viability		Biomass		Viability		Inhibition-%
	mg/L	μM	mg/L	μM	mg/L	μM	
Penicillin	0.0156	0.04	N/A	N/A	≥0.0625	0.18	~60
Clindamycin	0.0313	0.07	0.0313	0.07	≥0.25	0.59	~50
Rifampicin	0.0313	0.04	0.0625	0.08	≥0.0313	0.04	~50
Doxycycline	0.125	0.12	0.25	0.24	≥4	3.90	~60
Tetracycline	0.25	0.52	0.125	0.26	≥2	4.16	~50
Oxacillin	0.25	0.52	0.125	0.30	0.125	0.30	~50
Ampicillin	0.25	0.62	0.125	0.31	≥0.5	1.24	~60
Dicloxacillin	0.5	0.98	0.5	0.98	≥0.125	0.24	~50
Levofloxacin	0.5	1.38	0.5	1.38	≥32	88.55	~50
Vancomycin	2	1.37	N/A	N/A	≥8	5.38	~50

5.4.2 Efficacy testing in MWP

The efficacy of the selected antibiotics in biofilm prevention was quantified using LR assay in MWP. Antibiotics were tested at four concentrations, 0.1, 1, 10 and 100 μM. The entire results are shown in Supplementary table 5 in III, and for the highest test concentration (100 μM) presented in **Figure 26**.

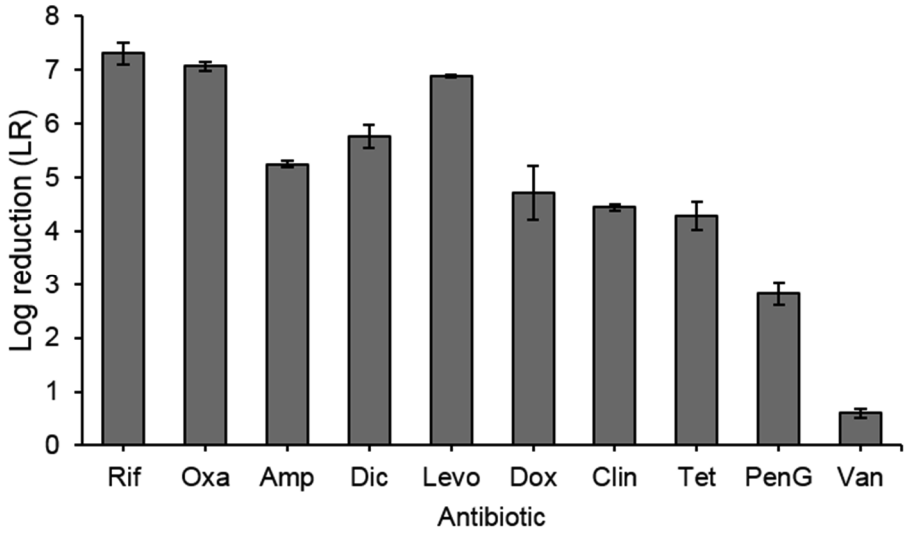


***Figure 26**. LR of ten selected antibiotics when tested at 100 μM in MWP. Rif = rifampicin, Oxa = oxacillin, Amp = ampicillin, Dic = dicloxacillin, Levo = levofloxacin, Dox = doxycycline, Clin = clindamycin, Tet = tetracycline, Pen G = penicillin G, Van = vancomycin.*

In this testing, penicillin G resulted in a LR of 2.91 ± 0.22 when assayed at 100 μM. In contrast, when killing efficacy was quantified against pre-formed biofilms in study I, penicillin G, used as the reference antibiotic, caused a LR of 1 at 400 μM upon an exposure of 24 h. These results demonstrate the antimicrobial tolerance associated with biofilms and notably, the fact that biofilm tolerance increases over time (Stewart 2015). Based upon the MWP testing, rifampicin, oxacillin and doxycycline were selected for DFR testing in order to include antibiotics with several targets and mechanisms of action.

5.4.3 Comparative efficacy study in MWP and DFR

In the DFR, rifampicin, oxacillin, and doxycycline were tested at 100 and 1000 μM. Results of the efficacy testing performed at 100 μM in the MWP and

the DFR are included in **Figure 27**. Biofilms grown in the DFR, when operated in continuous flow mode constantly providing fresh nutrients, were 7 to 26 times more tolerant to antibiotics than those grown in the absence of flow in the MWP, as quantified by means of Tolerance Factors (TF) (Stewart 2015). However, when the flow was eliminated from the DFR, the efficacies of the most active antibiotics rifampicin and doxycycline, when assayed at 100 μM, were almost similar to those measured in the MWP (**Figure 27**). Complete results from the efficacy testing are presented in Tables 1 and 4 in publication IV, and the TFs in Tables 2 and 5.

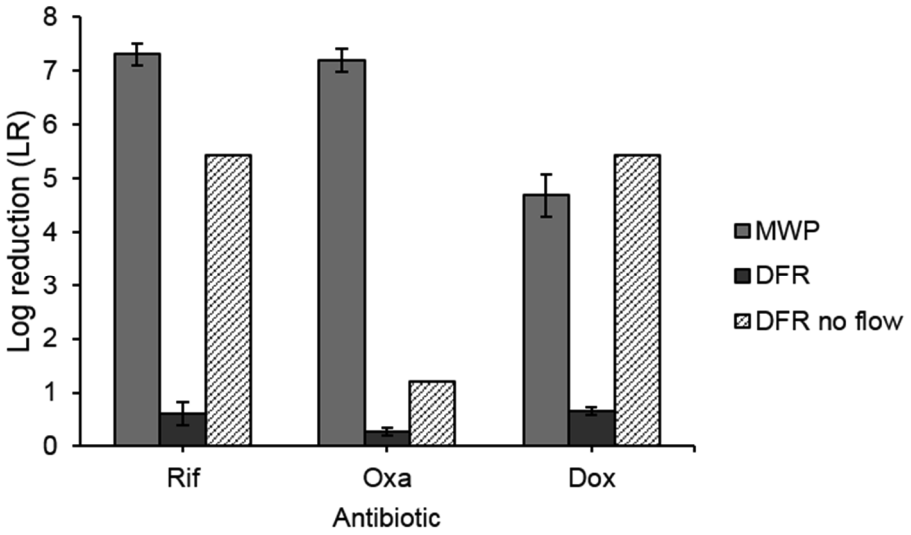


***Figure 27**. LR measured for the three selected antibiotics at 100 μM in both models. Experiments in the DFR were performed with and without the flow. Rif = rifampicin, Oxa = oxacillin, Dox = doxycycline.*

Several factors were analyzed to understand the differences in LRs between models. For that purpose, surface-area-to-volume (SA/V) ratios, which reflect the biofilm area exposed to antibiotics, were calculated. Further, the mean LD of control biofilms grown in both models, as well as the concentration of the planktonic inoculum used for biofilm formation, were compared (**Table 14**).

***Table 14**. Comparison of the models.*

Model	Surface-area-to-volume (SA/V) ratio	Control biofilm (log_{10} CFU/cm^2) mean ± SD	Planktonic inoculum (log_{10} CFU/mL) mean ± SD
MWP	7.961 cm^{-1}	8.07 ± 0.17	8.29 ± 0.21
DFR	2.708 cm^{-1}	7.60 ± 0.41	8.26 ± 0.25

Differences in the LR between the models could neither be attributed to differences in surface-area-to-volume (SA/V) ratios nor to bacterial density. Thus, the only clear factor that affected the differences was the nutrient flow (**Figure 27**). Moreover, biofilm architecture varied between the models, as visualized with CLSM (**Figure 28**). Biofilms grown in the DFR (C-D) were more heterogeneously distributed than the MWP-grown biofilms (A-B). Moreover, the DFR-grown biofilms were composed of larger clusters of bacteria.

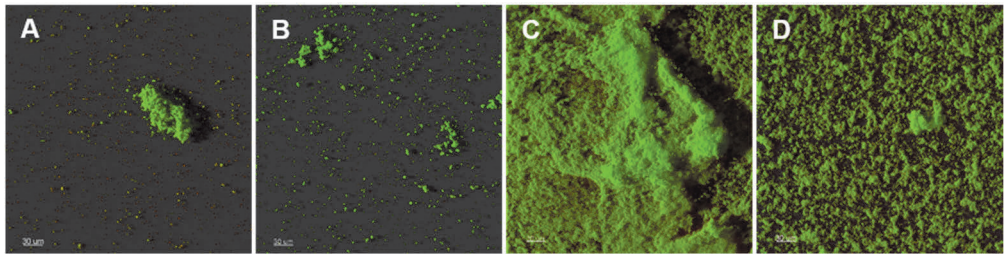


***Figure 28**. CLSM images of S. aureus ATCC 25923 biofilms grown in the MWP (A-B) and the DFR (C-D). Images are representatives taken from different positions of one well in a MWP and from different locations within one coupon in the DFR. Biofilms are stained with LIVE/DEAD BacLight kit. Scale bars correspond to 30 μm.*

Both models produced consistent and reproducible data, as shown in 5.1.3. However, they both fail in the complete reproduction of *in vivo* conditions, especially those bacteria encounter during chronic infections. Thus, none of the models can be judged as better than the other; one is only more suitable than another for a particular research question.

6 Conclusion and outlook

Currently, the discovery of strategies to cure biofilm-related infections is one of the most challenging and complicated tasks in antimicrobial drug discovery. Obviously, there has not been much success yet. To date, the best way to eliminate biofilm infection is by preventing it, that is, by killing the bacteria when they are in the planktonic mode of growth or by inhibiting the initial attachment. All the marketed antibiotics are developed against dividing planktonic cells, and the vast majority of existing standardized methodologies and *in vitro* breakpoints are applicable only for planktonic bacteria. Biofilm models intended for *in vitro* research, though there are several options available, are inadequate in mimicking conditions that occur in chronic biofilm infections. Moreover, the biofilm research suffers from the lack of standardization.

Conventional antibiotics alone are typically inefficient to overcome biofilm infections. Therefore, a combination of aggressive antibiotic therapy and surgery is often needed. An ideal anti-biofilm agent should preferentially affect the biofilm viability, biomass and the matrix. Thus, combinatory strategies involving both bactericidal and non-bactericidal approaches, i.e. QSIs or dispersal agents, would represent the optimal treatment strategy. The adjunctive compounds targeting bacterial virulence also help the host immune system to overcome infection.

The results obtained during this thesis project demonstrated the potential of natural compounds as biofilm inhibitors. Several compounds with various mechanisms of action from distinct compound classes were identified. Phenotypic screening, in studies I and II, led to the identification of multifunctional leads, which displayed both antibacterial and anti-biofilm properties in contrast to conventional antibiotics. Such compounds can be considered as desirable anti-biofilm compounds because they can first kill the planktonic population, thus, reducing the number of bacteria involved in biofilm formation, and thereafter, affect the biofilm lifecycle in another way. The identified leads (two synthetic flavan derivatives as well as the D-tryptophan and the β-cyclohexyl-L-alanine derivatives of DHA) inhibited biofilm formation and eradicated pre-formed biofilms at low micromolar concentrations. Additionally, two flavone derivatives were characterized as the non-bactericidal leads in study III, in which a large compound library of natural product derivatives was screened for QSI using a target-based screening approach. These flavone derivatives inhibited biofilm formation

and maturation by interfering with the QS-mediated processes, but they did not kill bacteria.

In the future, these compounds could be further optimized towards more potent anti-biofilm compounds or adjunctive agents. Especially flavonoids, which have been reported to display synergistic activity with conventional antibiotics, call for future testing in combinatory studies. Moreover, the identified QSIs would show the best potential when used as adjunctive agents, as they do not kill the planktonic bacteria and in this way, do not provide long-term anti-biofilm effect alone. Furthermore, additional testing of the identified leads against more pathogenic and resistant strains would be of great relevance, as the antimicrobial resistance poses a significant threat to global health. In that context, compounds that display their activity via non-bactericidal mechanisms could also help in minimizing the risk of resistance development.

Several models have been introduced for *in vitro* biofilm studies aiming to ease translation from *in vitro* to *in vivo* results, especially during drug discovery approaches. Efficacy testing of conventional antibiotics in biofilm prevention conducted in study IV, revealed that the experimental outcome is strongly dependent on the choice of model. These findings are in line with previous studies showing that the presence of flow substantially impacts the biofilm formation, and importantly, antimicrobial susceptibility of biofilms. As biofilms grown under flow were significantly more tolerant to antibiotics, the results suggest that the identified biofilm leads need to be tested in distinct biofilm models to facilitate the selection of the best compounds for *in vivo* studies. Notably, most biofilms outside the laboratory form under conditions in which flow is present. However, the final application of an anti-biofilm agent is the determinant for the model selection.

7 Acknowledgements

This thesis work was carried out at the Pharmaceutical Sciences Laboratory, Faculty of Science and Engineering, Åbo Akademi University during the years 2013-2017.

I would like to express my gratitude to my supervisors, Docent Adyary Fallarero and Prof. Pia Vuorela, for introducing me the interesting research field, bacterial biofilms, and for the opportunity to be part of the research group already as a master student. I am thankful for their encouragement, guidance and time during these years. Further, the distance supervision, after the research group moved to the University of Helsinki in 2014, has taught me a lot, especially to work independently.

Docent Kirsi Savijoki and Associate Professor Helle Wangensteen are acknowledged for taking the time to read my thesis and for their valuable feedback. I also want to thank Prof. Reko Leino and Docent Pekka Varmanen for being the members of my thesis committee.

I would wish to acknowledge all the co-authors and collaborators. I am very grateful to Associate Professor Darla Goeres for providing me the opportunity to work in her lab, the Standardized Biofilms Methods Laboratory, at the Center for Biofilm Engineering, Montana State University as well as for all the guidance and support during these years.

I am thankful to the members of Exploration of Anti-infectives Research group (AIR) at the University of Helsinki for all the help I have received and for traveling companion. To the PSL people, thank you for creating a nice working atmosphere even though we have not shared our research interests. I am grateful for all the scientific and non-scientific discussions, helpful advice and encouragement over these years.

A sincere thank you to my family and friends for their support and patience. You have always been there for me, listened and believed in me.

I would also like to thank the National Doctoral Programme in Informational and Structural Biology (ISB) for providing long-term funding. Additionally, financial support from Tor, Joe and Pentti Borgs minnesfond, Medicinska Understödsföreningen Liv och Hälsa rf, Turku Center for Systems Biology, The Finnish Pharmacists' Association, Åbo Akademi University and ISB is acknowledged.

Åbo, 2017
Suvi Manner

8 References

Abdel-Hafeez, M., N. El-Mehallaway, I. Khalil, F. Abdallah & A. Elnaggar (2014) Microbiological profile and biofilm formation on removed intrauterine contraceptive devices from a sample of Egyptian women. *J Obstet Gynaecol Res,* 40**,** 1770-6.

Abraham, N. M., S. Lamlertthon, V. G. Fowler & K. K. Jefferson (2012) Chelating agents exert distinct effects on biofilm formation in *Staphylococcus aureus* depending on strain background: role for clumping factor B. *J Med Microbiol,* 61**,** 1062-70.

Achermann, Y., E. J. Goldstein, T. Coenye & M. E. Shirtliff (2014) *Propionibacterium acnes*: from commensal to opportunistic biofilm-associated implant pathogen. *Clin Microbiol Rev,* 27**,** 419-40.

Adams, H., M. T. Winston, J. Heersink, K. A. Buckingham-Meyer, J. W. Costerton & P. Stoodley (2002) Development of a laboratory model to assess the removal of biofilm from interproximal spaces by powered tooth brushing. *Am J Dent,* 15 Spec No**,** 12B-17B.

Agarwal, A., K. P. Singh & A. Jain (2010) Medical significance and management of staphylococcal biofilm. *FEMS Immunol Med Microbiol,* 58**,** 147-60.

Ahlgren, H. G., A. Benedetti, J. S. Landry, J. Bernier, E. Matouk, D. Radzioch, L. C. Lands, S. Rousseau & D. Nguyen (2015) Clinical outcomes associated with *Staphylococcus aureus* and *Pseudomonas aeruginosa* airway infections in adult cystic fibrosis patients. *BMC Pulm Med,* 15**,** 67.

Ali, F., P. L. Sangwan, S. Koul, A. Pandey, S. Bani, S. T. Abdullah, P. R. Sharma, S. Kitchlu & I. A. Khan (2012) 4-epi-Pimaric acid: a phytomolecule as a potent antibacterial and anti-biofilm agent for oral cavity pathogens. *Eur J Clin Microbiol Infect Dis,* 31**,** 149-59.

Ali, L., F. Khambaty & G. Diachenko (2006) Investigating the suitability of the Calgary Biofilm Device for assessing the antimicrobial efficacy of new agents. *Bioresour Technol,* 97**,** 1887-93.

Ammons, M. C., L. S. Ward & G. A. James (2011) Anti-biofilm efficacy of a lactoferrin/xylitol wound hydrogel used in combination with silver wound dressings. *Int Wound J,* 8**,** 268-73.

Amorena, B., E. Gracia, M. Monzón, J. Leiva, C. Oteiza, M. Pérez, J. L. Alabart & J. Hernández-Yago (1999) Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed *in vitro*. *J Antimicrob Chemother,* 44**,** 43-55.

Anderl, J. N., M. J. Franklin & P. S. Stewart (2000) Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother,* 44**,** 1818-24.

Andersson, E. K. & M. Chapman (2013) Small molecule disruption of *B. subtilis* biofilms by targeting the amyloid matrix. *Chem Biol,* 20**,** 5-7.

Andrews, J. M. (2001) Determination of minimum inhibitory concentrations. *J Antimicrob Chemother,* 48 Suppl 1**,** 5-16.

Antoniani, D., P. Bocci, A. Maciag, N. Raffaelli & P. Landini (2010) Monitoring of diguanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. *Appl Microbiol Biotechnol,* 85**,** 1095-104.

Anwar, H., J. L. Strap & J. W. Costerton (1992) Establishment of aging biofilms: possible mechanism of bacterial

resistance to antimicrobial therapy. *Antimicrob Agents Chemother,* 36**,** 1347-51.

Archer, N. K., M. J. Mazaitis, J. W. Costerton, J. G. Leid, M. E. Powers & M. E. Shirtliff (2011) *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. *Virulence,* 2**,** 445-59.

Arciola, C. R., D. Campoccia, S. Ravaioli & L. Montanaro (2015) Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. *Front Cell Infect Microbiol,* 5**,** 7.

Arciola, C. R., D. Campoccia, P. Speziale, L. Montanaro & J. W. Costerton (2012) Biofilm formation in *Staphylococcus* implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. *Biomaterials,* 33**,** 5967-82.

Aslam, S. (2008) Effect of antibacterials on biofilms. *Am J Infect Control,* 36**,** S175.e9-11.

Attila, C., A. Ueda & T. K. Wood (2009) 5-Fluorouracil reduces biofilm formation in *Escherichia coli* K-12 through global regulator AriR as an antivirulence compound. *Appl Microbiol Biotechnol,* 82**,** 525-33.

Avorn, J. (2015) The $2.6 billion pill--methodologic and policy considerations. *N Engl J Med,* 372**,** 1877-9.

Baker, D. D., M. Chu, U. Oza & V. Rajgarhia (2007) The value of natural products to future pharmaceutical discovery. *Nat Prod Rep,* 24**,** 1225-44.

Balouiri, M., M. Sadiki & S. K. Ibnsouda (2016) Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis,* 6**,** 71-79.

Balunas, M. J. & A. D. Kinghorn (2005) Drug discovery from medicinal plants. *Life Sci,* 78**,** 431-41.

Banin, E., M. L. Vasil & E. P. Greenberg (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. *Proc Natl Acad Sci U S A,* 102**,** 11076-81.

Barbieri, R., E. Coppo, A. Marchese, M. Daglia, E. Sobarzo-Sánchez, S. F. Nabavi & S. M. Nabavi (2017) Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. *Microbiol Res,* 196**,** 44-68.

Barraud, N., D. J. Hassett, S. H. Hwang, S. A. Rice, S. Kjelleberg & J. S. Webb (2006) Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*. *J Bacteriol,* 188**,** 7344-53.

Barsoumian, A. E., K. Mende, C. J. Sanchez, M. L. Beckius, J. C. Wenke, C. K. Murray & K. S. Akers (2015) Clinical infectious outcomes associated with biofilm-related bacterial infections: a retrospective chart review. *BMC Infect Dis,* 15**,** 223.

Bassler, B. L. (2002) Small talk. Cell-to-cell communication in bacteria. *Cell,* 109**,** 421-4.

Battin, T. J., K. Besemer, M. M. Bengtsson, A. M. Romani & A. I. Packmann (2016) The ecology and biogeochemistry of stream biofilms. *Nat Rev Microbiol,* 14**,** 251-63.

Beloin, C., S. Renard, J. M. Ghigo & D. Lebeaux (2014) Novel approaches to combat bacterial biofilms. *Curr Opin Pharmacol,* 18**,** 61-8.

Biswas, N. N., S. K. Kutty, N. Barraud, G. M. Iskander, R. Griffith, S. A. Rice, M. Willcox, D. S. Black & N. Kumar (2015) Indole-based novel small molecules for the modulation of bacterial signalling pathways. *Org Biomol Chem,* 13**,** 925-37.

Bjarnsholt, T. (2013) The role of bacterial biofilms in chronic infections. *APMIS Suppl*, 1-51.

Bjarnsholt, T., M. Alhede, S. R. Eickhardt-Sørensen, C. Moser, M. Kühl, P. Jensen & N. Høiby (2013a) The *in vivo* biofilm. *Trends Microbiol,* 21, 466-74.

Bjarnsholt, T., O. Ciofu, S. Molin, M. Givskov & N. Høiby (2013b) Applying insights from biofilm biology to drug development - can a new approach be developed? *Nat Rev Drug Discov,* 12, 791-808.

Bleicher, K. H., H. J. Böhm, K. Müller & A. I. Alanine (2003) Hit and lead generation: beyond high-throughput screening. *Nat Rev Drug Discov,* 2, 369-78.

Boles, B. R. & A. R. Horswill (2008) Agr-mediated dispersal of *Staphylococcus aureus* biofilms. *PLoS Pathog,* 4, e1000052.

Bollini, S., J. J. Herbst, G. T. Gaughan, T. A. Verdoorn, J. Ditta, G. M. Dubowchik & A. Vinitsky (2002) High-throughput fluorescence polarization method for identification of FKBP12 ligands. *J Biomol Screen,* 7, 526-30.

Bordi, C. & S. de Bentzmann (2011) Hacking into bacterial biofilms: a new therapeutic challenge. *Ann Intensive Care,* 1, 19.

Borges, A., A. C. Abreu, C. Dias, M. J. Saavedra, F. Borges & M. Simões (2016) New perspectives on the use of phytochemicals as an emergent strategy to control bacterial infections including biofilms. *Molecules,* 21.

Brackman, G. & T. Coenye (2015) Quorum sensing inhibitors as anti-biofilm agents. *Curr Pharm Des,* 21, 5-11.

Brackman, G., P. Cos, L. Maes, H. J. Nelis & T. Coenye (2011) Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*. *Antimicrob Agents Chemother,* 55, 2655-61.

Brady, R. A., J. G. Leid, J. H. Calhoun, J. W. Costerton & M. E. Shirtliff (2008) Osteomyelitis and the role of biofilms in chronic infection. *FEMS Immunol Med Microbiol,* 52, 13-22.

Brandenburg, K. S., K. J. Rodriguez, J. F. McAnulty, C. J. Murphy, N. L. Abbott, M. J. Schurr & C. J. Czuprynski (2013) Tryptophan inhibits biofilm formation by *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother,* 57, 1921-5.

Buckingham-Meyer, K., D. M. Goeres & M. A. Hamilton (2007) Comparative evaluation of biofilm disinfectant efficacy tests. *J Microbiol Methods,* 70, 236-44.

Burmølle, M., T. R. Thomsen, M. Fazli, I. Dige, L. Christensen, P. Homøe, M. Tvede, B. Nyvad, T. Tolker-Nielsen, M. Givskov, C. Moser, K. Kirketerp-Møller, H. K. Johansen, N. Høiby, P. Jensen, S. J. Sørensen & T. Bjarnsholt (2010) Biofilms in chronic infections - a matter of opportunity - monospecies biofilms in multispecies infections. *FEMS Immunol Med Microbiol,* 59, 324-36.

Bérdy, J. (2012) Thoughts and facts about antibiotics: where we are now and where we are heading. *J Antibiot (Tokyo),* 65, 385-95.

Cady, N. C., K. A. McKean, J. Behnke, R. Kubec, A. P. Mosier, S. H. Kasper, D. S. Burz & R. A. Musah (2012) Inhibition of biofilm formation, quorum sensing and infection in *Pseudomonas aeruginosa* by natural products-inspired organosulfur compounds. *PLoS One,* 7, e38492.

Cahill, T. J. & B. D. Prendergast (2016) Infective endocarditis. *Lancet,* 387, 882-93.

Cardines, R., M. Giufrè, A. Pompilio, E. Fiscarelli, G. Ricciotti, G. Di

Bonaventura & M. Cerquetti (2012) *Haemophilus influenzae* in children with cystic fibrosis: antimicrobial susceptibility, molecular epidemiology, distribution of adhesins and biofilm formation. *Int J Med Microbiol,* 302**,** 45-52.

Cava, F., H. Lam, M. A. de Pedro & M. K. Waldor (2011) Emerging knowledge of regulatory roles of D-amino acids in bacteria. *Cell Mol Life Sci,* 68**,** 817-31.

Cegelski, L., J. S. Pinkner, N. D. Hammer, C. K. Cusumano, C. S. Hung, E. Chorell, V. Aberg, J. N. Walker, P. C. Seed, F. Almqvist, M. R. Chapman & S. J. Hultgren (2009) Small-molecule inhibitors target *Escherichia coli* amyloid biogenesis and biofilm formation. *Nat Chem Biol,* 5**,** 913-9.

Ceri, H., M. E. Olson, C. Stremick, R. R. Read, D. Morck & A. Buret (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol,* 37**,** 1771-6.

Chaignon, P., I. Sadovskaya, C. Ragunah, N. Ramasubbu, J. B. Kaplan & S. Jabbouri (2007) Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Appl Microbiol Biotechnol,* 75**,** 125-32.

Chen, Y., T. Liu, K. Wang, C. Hou, S. Cai, Y. Huang, Z. Du, H. Huang & J. Kong (2016) Baicalein inhibits *Staphylococcus aureus* biofilm formation and the quorum sensing system in vitro. *PLoS One,* 11**,** e0153468.

Chessa, D., G. Ganau, L. Spiga, A. Bulla, V. Mazzarello, G. V. Campus & S. Rubino (2016) *Staphylococcus aureus* and *Staphylococcus epidermidis* virulence strains as causative agents of persistent infections in breast implants. *PLoS One,* 11**,** e0146668.

Chiang, W. C., M. Nilsson, P. Jensen, N. Høiby, T. E. Nielsen, M. Givskov & T. Tolker-Nielsen (2013) Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother,* 57**,** 2352-61.

Christensen, L., V. Breiting, T. Bjarnsholt, S. Eickhardt, E. Høgdall, M. Janssen, N. Pallua & S. A. Zaat (2013) Bacterial infection as a likely cause of adverse reactions to polyacrylamide hydrogel fillers in cosmetic surgery. *Clin Infect Dis,* 56**,** 1438-44.

Chua, S. L., Y. Liu, J. K. Yam, Y. Chen, R. M. Vejborg, B. G. Tan, S. Kjelleberg, T. Tolker-Nielsen, M. Givskov & L. Yang (2014) Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nat Commun,* 5**,** 4462.

Ciofu, O., L. F. Mandsberg, H. Wang & N. Høiby (2012) Phenotypes selected during chronic lung infection in cystic fibrosis patients: implications for the treatment of *Pseudomonas aeruginosa* biofilm infections. *FEMS Immunol Med Microbiol,* 65**,** 215-25.

Coenye, T. & H. J. Nelis (2010) *In vitro* and *in vivo* model systems to study microbial biofilm formation. *J Microbiol Methods,* 83**,** 89-105.

Colvin, K. M., Y. Irie, C. S. Tart, R. Urbano, J. C. Whitney, C. Ryder, P. L. Howell, D. J. Wozniak & M. R. Parsek (2012) The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. *Environ Microbiol,* 14**,** 1913-28.

Coma, I., J. Herranz & J. Martin (2009) Statistics and decision making in high-throughput screening. *Methods Mol Biol,* 565**,** 69-106.

Cordier, C., D. Morton, S. Murrison, A. Nelson & C. O'Leary-Steele (2008) Natural products as an inspiration in the diversity-oriented synthesis of bioactive compound libraries. *Nat Prod Rep,* 25**,** 719-37.

Cos, P., A. J. Vlietinck, D. V. Berghe & L. Maes (2006) Anti-infective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. *J Ethnopharmacol,* 106**,** 290-302.

Costerton, J. W. (1984) The etiology and persistence of cryptic bacterial infections: a hypothesis. *Rev Infect Dis,* 6 Suppl 3**,** S608-16.

Costerton, J. W., P. S. Stewart & E. P. Greenberg (1999) Bacterial biofilms: a common cause of persistent infections. *Science,* 284**,** 1318-22.

Cragg, G. M. & D. J. Newman (2013) Natural products: A continuing source of novel drug leads. *Biochim Biophys Acta,* 1830**,** 3670-95.

Crémet, L., S. Corvec, E. Batard, M. Auger, I. Lopez, F. Pagniez, S. Dauvergne & N. Caroff (2013) Comparison of three methods to study biofilm formation by clinical strains of *Escherichia coli*. *Diagn Microbiol Infect Dis,* 75**,** 252-5.

Cucarella, C., C. Solano, J. Valle, B. Amorena, I. Lasa & J. R. Penadés (2001) Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol,* 183**,** 2888-96.

Curtin, F. & P. Schulz (2011) Assessing the benefit: risk ratio of a drug--randomized and naturalistic evidence. *Dialogues Clin Neurosci,* 13**,** 183-90.

Curtin, J. J. & R. M. Donlan (2006) Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*. *Antimicrob Agents Chemother,* 50**,** 1268-75.

Cushnie, T. P. & A. J. Lamb (2005) Antimicrobial activity of flavonoids. *Int J Antimicrob Agents,* 26**,** 343-56.

Cushnie, T. P. & A. J. Lamb (2011) Recent advances in understanding the antibacterial properties of flavonoids. *Int J Antimicrob Agents,* 38**,** 99-107.

Czaplewski, L., R. Bax, M. Clokie, M. Dawson, H. Fairhead, V. A. Fischetti, S. Foster, B. F. Gilmore, R. E. Hancock, D. Harper, I. R. Henderson, K. Hilpert, B. V. Jones, A. Kadioglu, D. Knowles, S. Ólafsdóttir, D. Payne, S. Projan, S. Shaunak, J. Silverman, C. M. Thomas, T. J. Trust, P. Warn & J. H. Rex (2016) Alternatives to antibiotics--a pipeline portfolio review. *Lancet Infect Dis,* 16**,** 239-51.

Dandapani, S., G. Rosse, N. Southall, J. M. Salvino & C. J. Thomas (2012) Selecting, acquiring, and using small molecule libraries for high-throughput screening. *Curr Protoc Chem Biol,* 4**,** 177-191.

Darouiche, R. O., M. D. Mansouri, P. V. Gawande & S. Madhyastha (2009) Antimicrobial and antibiofilm efficacy of triclosan and DispersinB combination. *J Antimicrob Chemother,* 64**,** 88-93.

Dasgupta, M. K. (2002) Biofilms and infection in dialysis patients. *Semin Dial,* 15**,** 338-46.

Davey, M. E. & G. A. O'Toole (2000) Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev,* 64**,** 847–867.

Davies, D. G. & C. N. Marques (2009) A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. *J Bacteriol,* 191**,** 1393-403.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton & E. P. Greenberg (1998) The involvement of cell-to-cell signals in the development

of a bacterial biofilm. *Science,* 280**,** 295-8.

de Kievit, T. R. (2009) Quorum sensing in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol,* 11**,** 279-88.

De La Fuente, R., N. D. Sonawane, D. Arumainayagam & A. S. Verkman (2006) Small molecules with antimicrobial activity against *E. coli* and *P. aeruginosa* identified by high-throughput screening. *Br J Pharmacol,* 149**,** 551-9.

Deak, D., K. Outterson, J. H. Powers & A. S. Kesselheim (2016) Progress in the fight against multidrug-resistant bacteria? A review of U.S. Food and Drug Administration-approved antibiotics, 2010-2015. *Ann Intern Med,* 165**,** 363-72.

Delaquis, P. J., D. E. Caldwell, J. R. Lawrence & A. R. McCurdy (1989) Detachment of *Pseudomonas fluorescens* from biofilms on glass surfaces in response to nutrient stress. *Microb Ecol,* 18**,** 199-210.

Delcaru, C., I. Alexandru, P. Podgoreanu, M. Grosu, E. Stavropoulos, M. C. Chifiriuc & V. Lazar (2016) Microbial biofilms in urinary tract infections and prostatitis: etiology, pathogenicity, and combating strategies. *Pathogens,* 5. pii: E65.

Dentice, R. & M. Elkins (2016) Timing of dornase alfa inhalation for cystic fibrosis. *Cochrane Database Syst Rev,* 7**,** CD007923.

Dias, D. A., S. Urban & U. Roessner (2012) A historical overview of natural products in drug discovery. *Metabolites,* 2**,** 303-36.

Dickschat, J. S. (2010) Quorum sensing and bacterial biofilms. *Nat Prod Rep,* 27**,** 343-69.

Ding, X., B. Yin, L. Qian, Z. Zeng, Z. Yang, H. Li, Y. Lu & S. Zhou (2011) Screening for novel quorum-sensing inhibitors to interfere with the formation of Pseudomonas aeruginosa biofilm. *J Med Microbiol,* 60**,** 1827-34.

Dobretsov, S., R. M. Abed & M. Teplitski (2013) Mini-review: Inhibition of biofouling by marine microorganisms. *Biofouling,* 29**,** 423-41.

Donelli, G., I. Francolini, D. Romoli, E. Guaglianone, A. Piozzi, C. Ragunath & J. B. Kaplan (2007) Synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth on polyurethanes. *Antimicrob Agents Chemother,* 51**,** 2733-40.

Donlan, R. M. (2002) Biofilms: microbial life on surfaces. *Emerg Infect Dis,* 8**,** 881-90.

Donné, J. & S. Dewilde (2015) The challenging world of biofilm physiology. *Adv Microb Physiol,* 67**,** 235-92.

Driffield, K., K. Miller, J. M. Bostock, A. J. O'Neill & I. Chopra (2008) Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J Antimicrob Chemother,* 61**,** 1053-6.

Duetz, W. A. (2007) Microtiter plates as mini-bioreactors: miniaturization of fermentation methods. *Trends Microbiol,* 15**,** 469-75.

Dunne, W. M. (2002) Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev,* 15**,** 155-66.

Dörr, T., K. Lewis & M. Vulić (2009) SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *PLoS Genet,* 5**,** e1000760.

Elgharably, H., S. T. Hussain, N. K. Shrestha, E. H. Blackstone & G. B. Pettersson (2016) Current hypotheses in cardiac surgery: biofilm in infective endocarditis. *Semin Thorac Cardiovasc Surg,* 28**,** 56-9.

Eng, R. H., F. T. Padberg, S. M. Smith, E. N. Tan & C. E. Cherubin (1991)

Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrob Agents Chemother,* 35**,** 1824-8.

Fallarero, A., L. Hanski & P. Vuorela (2014) How to translate a bioassay into a screening assay for natural products: general considerations and implementation of antimicrobial screens. *Planta Med,* 80**,** 1182-99.

Fallarero, A., M. Skogman, J. Kujala, M. Rajaratnam, V. M. Moreira, J. Yli-Kauhaluoma & P. Vuorela (2013) (+)-Dehydroabietic acid, an abietane-type diterpene, inhibits *Staphylococcus aureus* biofilms *in vitro*. *Int J Mol Sci,* 14**,** 12054-72.

Faller, M. & T. Kohler (2017) The status of biofilms in penile implants. *Microorganisms,* 5.

Fan, F. & K. V. Wood (2007) Bioluminescent assays for high-throughput screening. *Assay Drug Dev Technol,* 5**,** 127-36.

Fauci, A. S. (2001) Infectious diseases: considerations for the 21st century. *Clin Infect Dis,* 32**,** 675-85.

Feher, M. & J. M. Schmidt (2003) Property distributions: differences between drugs, natural products, and molecules from combinatorial chemistry. *J Chem Inf Comput Sci,* 43**,** 218-27.

Fernandes, P. & E. Martens (2017) Antibiotics in late clinical development. *Biochem Pharmacol,* 133**,** 152-163.

Fey, P. D. (2010) Modality of bacterial growth presents unique targets: how do we treat biofilm-mediated infections? *Curr Opin Microbiol,* 13**,** 610-5.

Fjell, C. D., J. A. Hiss, R. E. Hancock & G. Schneider (2011) Designing antimicrobial peptides: form follows function. *Nat Rev Drug Discov,* 11**,** 37-51.

Fleming, D. & K. P. Rumbaugh (2017) Approaches to dispersing medical biofilms. *Microorganisms,* 5.

Flemming, H. C. & J. Wingender (2010) The biofilm matrix. *Nat Rev Microbiol,* 8**,** 623-33.

Flemming, H. C., J. Wingender, U. Szewzyk, P. Steinberg, S. A. Rice & S. Kjelleberg (2016) Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol,* 14**,** 563-75.

Foxman, B. (2002) Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *Am J Med,* 113 Suppl 1A**,** 5S-13S.

Francolini, I. & G. Donelli (2010) Prevention and control of biofilm-based medical-device-related infections. *FEMS Immunol Med Microbiol,* 59**,** 227-38.

Franklin, M. J., C. Chang, T. Akiyama & B. Bothner (2015) New technologies for studying biofilms. *Microbiol Spectr,* 3.

Friedman, M. (2007) Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. *Mol Nutr Food Res,* 51**,** 116-34.

Fuqua, C., M. R. Parsek & E. P. Greenberg (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet,* 35**,** 439-68.

Fuqua, W. C., S. C. Winans & E. P. Greenberg (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol,* 176**,** 269-75.

Gabrilska, R. A. & K. P. Rumbaugh (2015) Biofilm models of polymicrobial infection. *Future Microbiol,* 10**,** 1997-2015.

Gahlot, R., C. Nigam, V. Kumar, G. Yadav & S. Anupurba (2014) Catheter-related bloodstream infections. *Int J Crit Illn Inj Sci,* 4**,** 162-7.

Gawande, P. V., K. P. Leung & S. Madhyastha (2014) Antibiofilm and antimicrobial efficacy of DispersinB®-KSL-W peptide-based wound gel against chronic wound infection associated bacteria. *Curr Microbiol,* 68**,** 635-41.

Gbejuade, H. O., A. M. Lovering & J. C. Webb (2015) The role of microbial biofilms in prosthetic joint infections. *Acta Orthop,* 86**,** 147-58.

Gil-Perotin, S., P. Ramirez, V. Marti, J. M. Sahuquillo, E. Gonzalez, I. Calleja, R. Menendez & J. Bonastre (2012) Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: a state of concept. *Crit Care,* 16**,** R93.

Gjødsbøl, K., J. J. Christensen, T. Karlsmark, B. Jørgensen, B. M. Klein & K. A. Krogfelt (2006) Multiple bacterial species reside in chronic wounds: a longitudinal study. *Int Wound J,* 3**,** 225-31.

Goeres, D. M., M. A. Hamilton, N. A. Beck, K. Buckingham-Meyer, J. D. Hilyard, L. R. Loetterle, L. A. Lorenz, D. K. Walker & P. S. Stewart (2009) A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor. *Nat Protoc,* 4**,** 783-8.

Goeres, D. M., L. R. Loetterle, M. A. Hamilton, R. Murga, D. W. Kirby & R. M. Donlan (2005) Statistical assessment of a laboratory method for growing biofilms. *Microbiology,* 151**,** 757-62.

Gomes, F., P. Teixeira & R. Oliveira (2014) Mini-review: *Staphylococcus epidermidis* as the most frequent cause of nosocomial infections: old and new fighting strategies. *Biofouling,* 30**,** 131-41.

González, M. A. (2015) Aromatic abietane diterpenoids: their biological activity and synthesis. *Nat Prod Rep,* 32**,** 684-704.

González, M. A., D. Pérez-Guaita, J. Correa-Royero, B. Zapata, L. Agudelo, A. Mesa-Arango & L. Betancur-Galvis (2010) Synthesis and biological evaluation of dehydroabietic acid derivatives. *Eur J Med Chem,* 45**,** 811-6.

Guiton, P. S., C. K. Cusumano, K. A. Kline, K. W. Dodson, Z. Han, J. W. Janetka, J. P. Henderson, M. G. Caparon & S. J. Hultgren (2012) Combinatorial small-molecule therapy prevents uropathogenic *Escherichia coli* catheter-associated urinary tract infections in mice. *Antimicrob Agents Chemother,* 56**,** 4738-45.

Gupta, P., S. Sarkar, B. Das, S. Bhattacharjee & P. Tribedi (2016) Biofilm, pathogenesis and prevention--a journey to break the wall: a review. *Arch Microbiol,* 198**,** 1-15.

Gómez, J., E. Canovas, V. Baños, L. Martínez, E. García, A. Hernández-Torres, M. Canteras, J. Ruiz, M. Medina, P. Martínez, A. Canovas, A. Soriano & M. Clavel (2011) Linezolid plus rifampin as a salvage therapy in prosthetic joint infections treated without removing the implant. *Antimicrob Agents Chemother,* 55**,** 4308-10.

Ha, D. G., S. L. Kuchma & G. A. O'Toole (2014) Plate-based assay for swimming motility in *Pseudomonas aeruginosa*. *Methods Mol Biol,* 1149**,** 59-65.

Hall-Stoodley, L., J. W. Costerton & P. Stoodley (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol,* 2**,** 95-108.

Hall-Stoodley, L. & P. Stoodley (2002) Developmental regulation of microbial biofilms. *Curr Opin Biotechnol,* 13**,** 228-33.

Hall-Stoodley, L. & P. Stoodley (2005) Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbiol,* 13**,** 7-10.

Hancock, V., M. Dahl & P. Klemm (2010) Abolition of biofilm formation in urinary tract *Escherichia coli* and *Klebsiella isolates* by metal interference through competition for fur. *Appl Environ Microbiol,* 76**,** 3836-41.

Harrison, J. J., C. A. Stremick, R. J. Turner, N. D. Allan, M. E. Olson & H. Ceri (2010) Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nat Protoc,* 5**,** 1236-54.

Hart, C. P. (2005) Finding the target after screening the phenotype. *Drug Discov Today,* 10**,** 513-9.

Harvey, A. L. (2008) Natural products in drug discovery. *Drug Discov Today,* 13**,** 894-901.

Hashemizadeh, Z., D. Kalantar-Neyestanaki & S. Mansouri (2017) Association between virulence profile, biofilm formation and phylogenetic groups of *Escherichia coli* causing urinary tract infection and the commensal gut microbiota: A comparative analysis. *Microb Pathog.*

Havsteen, B. H. (2002) The biochemistry and medical significance of the flavonoids. *Pharmacol Ther,* 96**,** 67-202.

Heilmann, C. (2011) Adhesion mechanisms of staphylococci. *Adv Exp Med Biol,* 715**,** 105-23.

Heilmann, C., M. Hussain, G. Peters & F. Götz (1997) Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Mol Microbiol,* 24**,** 1013-24.

Hengzhuang, W., N. Høiby & O. Ciofu (2014) Pharmacokinetics and pharmacodynamics of antibiotics in biofilm infections of *Pseudomonas aeruginosa in vitro* and *in vivo*. *Methods Mol Biol,* 1147**,** 239-54.

Hentzer, M., K. Riedel, T. B. Rasmussen, A. Heydorn, J. B. Andersen, M. R. Parsek, S. A. Rice, L. Eberl, S. Molin, N. Høiby, S. Kjelleberg & M. Givskov (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology,* 148**,** 87-102.

Hiltunen, A. K., M. E. Skogman, K. Rosenqvist, H. Juvonen, P. Ihalainen, J. Peltonen, A. Juppo & A. Fallarero (2016) Bioactive glass combined with bisphosphonates provides protection against biofilms formed by the periodontal pathogen *Aggregatibacter actinomycetemcomitans*. *Int J Pharm,* 501**,** 211-20.

Hochbaum, A. I., I. Kolodkin-Gal, L. Foulston, R. Kolter, J. Aizenberg & R. Losick (2011) Inhibitory effects of D-amino acids on *Staphylococcus aureus* biofilm development. *J Bacteriol,* 193**,** 5616-22.

Hong, J. (2011) Role of natural product diversity in chemical biology. *Curr Opin Chem Biol,* 15**,** 350-4.

Huang, R., M. Li & R. L. Gregory (2011) Bacterial interactions in dental biofilm. *Virulence,* 2**,** 435-44.

Hughes, J. P., S. Rees, S. B. Kalindjian & K. L. Philpott (2011) Principles of early drug discovery. *Br J Pharmacol,* 162**,** 1239-49.

Hunt, S. M., E. M. Werner, B. Huang, M. A. Hamilton & P. S. Stewart (2004) Hypothesis for the role of nutrient starvation in biofilm detachment. *Appl Environ Microbiol,* 70**,** 7418-25.

Høiby, N. (2011) Recent advances in the treatment of *Pseudomonas aeruginosa*

infections in cystic fibrosis. *BMC Med,* 9**,** 32.

Høiby, N., T. Bjarnsholt, M. Givskov, S. Molin & O. Ciofu (2010a) Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents,* 35**,** 322-32.

Høiby, N., T. Bjarnsholt, C. Moser, G. L. Bassi, T. Coenye, G. Donelli, L. Hall-Stoodley, V. Holá, C. Imbert, K. Kirketerp-Møller, D. Lebeaux, A. Oliver, A. J. Ullmann, C. Williams & E. S. G. f. B. a. C. E. E. W. Zimmerli (2015) ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. *Clin Microbiol Infect,* 21 Suppl 1**,** S1-25.

Høiby, N., O. Ciofu & T. Bjarnsholt (2010b) Pseudomonas aeruginosa biofilms in cystic fibrosis. *Future Microbiol,* 5**,** 1663-74.

Inglese, J., R. L. Johnson, A. Simeonov, M. Xia, W. Zheng, C. P. Austin & D. S. Auld (2007) High-throughput screening assays for the identification of chemical probes. *Nat Chem Biol,* 3**,** 466-79.

Itoh, Y., X. Wang, B. J. Hinnebusch, J. F. Preston & T. Romeo (2005) Depolymerization of beta-1,6-N-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. *J Bacteriol,* 187**,** 382-7.

Iversen, P. W., B. J. Eastwood, G. S. Sittampalam & K. L. Cox (2006) A comparison of assay performance measures in screening assays: signal window, Z' factor, and assay variability ratio. *J Biomol Screen,* 11**,** 247-52.

Izano, E. A., M. A. Amarante, W. B. Kher & J. B. Kaplan (2008) Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Appl Environ Microbiol,* 74**,** 470-6.

Jacobsen, S. M., D. J. Stickler, H. L. Mobley & M. E. Shirtliff (2008) Complicated catheter-associated urinary tract infections due to *Escherichia coli* and *Proteus mirabilis*. *Clin Microbiol Rev,* 21**,** 26-59.

Jacqueline, C. & J. Caillon (2014) Impact of bacterial biofilm on the treatment of prosthetic joint infections. *J Antimicrob Chemother,* 69 Suppl 1**,** i37-40.

Jakobsen, T. H., S. K. Bragason, R. K. Phipps, L. D. Christensen, M. van Gennip, M. Alhede, M. Skindersoe, T. O. Larsen, N. Høiby, T. Bjarnsholt & M. Givskov (2012) Food as a source for quorum sensing inhibitors: iberin from horseradish revealed as a quorum sensing inhibitor of *Pseudomonas aeruginosa*. *Appl Environ Microbiol,* 78**,** 2410-21.

Jefferson, K. K., D. A. Goldmann & G. B. Pier (2005) Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother,* 49**,** 2467-73.

Jenkins, S. G. & A. N. Schuetz (2012) Current concepts in laboratory testing to guide antimicrobial therapy. *Mayo Clin Proc,* 87**,** 290-308.

Jia, R., Y. Li, H. H. Al-Mahamedh & T. Gu (2017) Enhanced biocide treatments with D-amino acid mixtures against a biofilm consortium from a water cooling tower. *Front Microbiol,* 8**,** 1538.

Joo, H. S. & M. Otto (2012) Molecular basis of *in vivo* biofilm formation by bacterial pathogens. *Chem Biol,* 19**,** 1503-13.

Joseph, R., A. Naugolny, M. Feldman, I. M. Herzog, M. Fridman & Y. Cohen (2016) Cationic pillararenes potently inhibit biofilm formation without affecting bacterial growth and viability. *J Am Chem Soc,* 138**,** 754-7.

Junker, L. M. & J. Clardy (2007) High-throughput screens for small-molecule inhibitors of *Pseudomonas aeruginosa* biofilm development. *Antimicrob Agents Chemother,* 51**,** 3582-90.

Kaiser, M., S. Wetzel, K. Kumar & H. Waldmann (2008) Biology-inspired synthesis of compound libraries. *Cell Mol Life Sci,* 65**,** 1186-201.

Kaplan, J. B. (2010) Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J Dent Res,* 89**,** 205-18.

Katsuno, K., J. N. Burrows, K. Duncan, R. Hooft van Huijsduijnen, T. Kaneko, K. Kita, C. E. Mowbray, D. Schmatz, P. Warner & B. T. Slingsby (2015) Hit and lead criteria in drug discovery for infectious diseases of the developing world. *Nat Rev Drug Discov,* 14**,** 751-8.

Keseru, G. M. & G. M. Makara (2006) Hit discovery and hit-to-lead approaches. *Drug Discov Today,* 11**,** 741-8.

Kiedrowski, M. R. & A. R. Horswill (2011) New approaches for treating staphylococcal biofilm infections. *Ann N Y Acad Sci,* 1241**,** 104-21.

Kingston, D. G. (2011) Modern natural products drug discovery and its relevance to biodiversity conservation. *J Nat Prod,* 74**,** 496-511.

Kirketerp-Møller, K., P. Jensen, M. Fazli, K. G. Madsen, J. Pedersen, C. Moser, T. Tolker-Nielsen, N. Høiby, M. Givskov & T. Bjarnsholt (2008) Distribution, organization, and ecology of bacteria in chronic wounds. *J Clin Microbiol,* 46**,** 2717-22.

Koehn, F. E. & G. T. Carter (2005) Rediscovering natural products as a source of new drugs. *Discov Med,* 5**,** 159-64.

Kohanski, M. A., D. J. Dwyer & J. J. Collins (2010) How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol,* 8**,** 423-35.

Kola, I. & J. Landis (2004) Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov,* 3**,** 711-716.

Kolodkin-Gal, I., D. Romero, S. Cao, J. Clardy, R. Kolter & R. Losick (2010) D-amino acids trigger biofilm disassembly. *Science,* 328**,** 627-9.

Kolter, R. & R. Losick (1998) One for all and all for one. *Science,* 280**,** 226-7.

Kresse, H., M. J. Belsey & H. Rovini (2007) The antibacterial drugs market. *Nat Rev Drug Discov,* 6**,** 19-20.

Kropec, A., T. Maira-Litran, K. K. Jefferson, M. Grout, S. E. Cramton, F. Götz, D. A. Goldmann & G. B. Pier (2005) Poly-N-acetylglucosamine production in *Staphylococcus aureus* is essential for virulence in murine models of systemic infection. *Infect Immun,* 73**,** 6868-76.

Kumar, S., C. Wittmann & E. Heinzle (2004) Minibioreactors. *Biotechnol Lett,* 26**,** 1-10.

Kuźma, Ł., M. Rózalski, E. Walencka, B. Rózalska & H. Wysokińska (2007) Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea L.*: salvipisone as a potential anti-biofilm agent active against antibiotic resistant Staphylococci. *Phytomedicine,* 14**,** 31-5.

Köhler, T., A. Buckling & C. van Delden (2009) Cooperation and virulence of clinical *Pseudomonas aeruginosa* populations. *Proc Natl Acad Sci U S A,* 106**,** 6339-44.

Lai, N. M., N. Chaiyakunapruk, N. A. Lai, E. O'Riordan, W. S. Pau & S. Saint (2016) Catheter impregnation, coating or bonding for reducing central venous catheter-related infections in adults. *Cochrane Database Syst Rev,* 3**,** CD007878.

Lauderdale, K. J., C. L. Malone, B. R. Boles, J. Morcuende & A. R. Horswill (2010) Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. *J Orthop Res,* 28**,** 55-61.

Laverty, G., S. P. Gorman & B. F. Gilmore (2014) Biomolecular mechanisms of *Pseudomonas aeruginosa* and *Escherichia coli* biofilm formation. *Pathogens,* 3**,** 596-632.

Leandro, L. F., M. J. Cardoso, S. D. Silva, M. G. Souza, R. C. Veneziani, S. R. Ambrosio & C. H. Martins (2014) Antibacterial activity of *Pinus elliottii* and its major compound, dehydroabietic acid, against multidrug-resistant strains. *J Med Microbiol,* 63**,** 1649-53.

Lebeaux, D., A. Chauhan, O. Rendueles & C. Beloin (2013) From *in vitro* to *in vivo* models of bacterial biofilm-related infections. *Pathogens,* 2**,** 288-356.

Lee, J. & M. Bogyo (2013) Target deconvolution techniques in modern phenotypic profiling. *Curr Opin Chem Biol,* 17**,** 118-26.

Levy, S. B. & B. Marshall (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nat Med,* 10**,** S122-9.

Lewis, K. (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol,* 5**,** 48-56.

Lewis, K. (2008) Multidrug tolerance of biofilms and persister cells. *Curr Top Microbiol Immunol,* 322**,** 107-31.

Lewis, K. (2013) Platforms for antibiotic discovery. *Nat Rev Drug Discov,* 12**,** 371-87.

Li, J. W. & J. C. Vederas (2009) Drug discovery and natural products: end of an era or an endless frontier? *Science,* 325**,** 161-5.

Lindsay, D. & A. von Holy (2006) Bacterial biofilms within the clinical setting: what healthcare professionals should know. *J Hosp Infect,* 64**,** 313-25.

Lipinski, C. A., F. Lombardo, B. W. Dominy & P. J. Feeney (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev,* 46**,** 3-26.

Liu, C., A. Bayer, S. E. Cosgrove, R. S. Daum, S. K. Fridkin, R. J. Gorwitz, S. L. Kaplan, A. W. Karchmer, D. P. Levine, B. E. Murray, M. J Rybak, D. A. Talan, H. F. Chambers & I. D. S. o. America (2011) Clinical practice guidelines by the infectious diseases society of america for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children. *Clin Infect Dis,* 52**,** e18-55.

Lourenço, A., T. Coenye, D. M. Goeres, G. Donelli, A. S. Azevedo, H. Ceri, F. L. Coelho, H. C. Flemming, T. Juhna, S. P. Lopes, R. Oliveira, A. Oliver, M. E. Shirtliff, A. M. Sousa, P. Stoodley, M. O. Pereira & N. F. Azevedo (2014) Minimum information about a biofilm experiment (MIABiE): standards for reporting experiments and data on sessile microbial communities living at interfaces. *Pathog Dis,* 70**,** 250-6.

Lynch, T. J. (2012) Choosing optimal antimicrobial therapies. *Med Clin North Am,* 96**,** 1079-94.

López, D., H. Vlamakis & R. Kolter (2010) Biofilms. *Cold Spring Harb Perspect Biol,* 2**,** a000398.

Macarron, R. (2006) Critical review of the role of HTS in drug discovery. *Drug Discov Today,* 11**,** 277-9.

Mah, T. F. & G. A. O'Toole (2001) Mechanisms of biofilm resistance to

antimicrobial agents. *Trends Microbiol,* 9**,** 34-9.

Mahto, S. K., T. H. Yoon & S. W. Rhee (2010) A new perspective on *in vitro* assessment method for evaluating quantum dot toxicity by using microfluidics technology. *Biomicrofluidics,* 4.

Malo, N., J. A. Hanley, S. Cerquozzi, J. Pelletier & R. Nadon (2006) Statistical practice in high-throughput screening data analysis. *Nat Biotechnol,* 24**,** 167-75.

Malone, M., D. M. Goeres, I. Gosbell, K. Vickery, S. Jensen & P. Stoodley (2017) Approaches to biofilm-associated infections: the need for standardized and relevant biofilm methods for clinical applications. *Expert Rev Anti Infect Ther,* 15**,** 147-156.

Manly, C. J., J. Chandrasekhar, J. W. Ochterski, J. D. Hammer & B. B. Warfield (2008) Strategies and tactics for optimizing the hit-to-lead process and beyond-a computational chemistry perspective. *Drug Discov Today,* 13**,** 99-109.

Mariscal, A., R. M. Lopez-Gigosos, M. Carnero-Varo & J. Fernandez-Crehuet (2009) Fluorescent assay based on resazurin for detection of activity of disinfectants against bacterial biofilm. *Appl Microbiol Biotechnol,* 82**,** 773-83.

Marsh, P. D. (2006) Dental plaque as a biofilm and a microbial community - implications for health and disease. *BMC Oral Health,* 6 Suppl 1**,** S14.

Martens, E. & A. L. Demain (2017) The antibiotic resistance crisis, with a focus on the United States. *J Antibiot (Tokyo),* 70**,** 520-526.

Martin, C., W. L. Low, A. Gupta, M. C. Amin, I. Radecka, S. T. Britland, P. Raj & K. M. Kenward (2015) Strategies for antimicrobial drug delivery to biofilm. *Curr Pharm Des,* 21**,** 43-66.

Matsuzaki, K. (2009) Control of cell selectivity of antimicrobial peptides. *Biochim Biophys Acta,* 1788**,** 1687-92.

McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. Stewart & P. Williams (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology,* 143 (Pt 12)**,** 3703-11.

McConoughey, S. J., R. Howlin, J. F. Granger, M. M. Manring, J. H. Calhoun, M. Shirtliff, S. Kathju & P. Stoodley (2014) Biofilms in periprosthetic orthopedic infections. *Future Microbiol,* 9**,** 987-1007.

McDougald, D., S. A. Rice, N. Barraud, P. D. Steinberg & S. Kjelleberg (2011) Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol,* 10**,** 39-50.

Merritt, J. H., D. E. Kadouri & G. A. O'Toole (2005) Growing and analyzing static biofilms. *Curr Protoc Microbiol,* Chapter 1**,** Unit 1B.1.

Merten, C. A. (2010) Screening Europe 2010: an update about the latest technologies and applications in high-throughput screening. *Expert Rev Mol Diagn,* 10**,** 559-63.

Metcalf, D. G. & P. G. Bowler (2013) Biofilm delays wound healing: A review of the evidence. *Burns Trauma,* 1**,** 5-12.

Miller, K. G., P. L. Tran, C. L. Haley, C. Kruzek, J. A. Colmer-Hamood, M. Myntti & A. N. Hamood (2014) Next science wound gel technology, a novel agent that inhibits biofilm development by gram-positive and gram-negative wound pathogens.

Antimicrob Agents Chemother, 58**,** 3060-72.

Miller, M. B. & B. L. Bassler (2001) Quorum sensing in bacteria. *Annu Rev Microbiol,* 55**,** 165-99.

Mingeot-Leclercq, M. P., Y. Glupczynski & P. M. Tulkens (1999) Aminoglycosides: activity and resistance. *Antimicrob Agents Chemother,* 43**,** 727-37.

Miyashiro, T. & E. G. Ruby (2012) Shedding light on bioluminescence regulation in *Vibrio fischeri*. *Mol Microbiol,* 84**,** 795-806.

Mohamed, J. A. & D. B. Huang (2007) Biofilm formation by enterococci. *J Med Microbiol,* 56**,** 1581-8.

Moreau-Marquis, S., G. A. O'Toole & B. A. Stanton (2009) Tobramycin and FDA-approved iron chelators eliminate *Pseudomonas aeruginosa* biofilms on cystic fibrosis cells. *Am J Respir Cell Mol Biol,* 41**,** 305-13.

Moreira, J. M. R. G., Luciana C. Araújo, José D.P.Miranda, João M. Simões, Manuel Melo, Luís F. Mergulhão, Filipe J. 2013. The effect of glucose concentration and shaking conditions on *Escherichia coli* biofilm formation in microtiter plates. *Chem Eng Sci*, 94**,** 192-199.

Moser, C., H. T. Pedersen, C. J. Lerche, M. Kolpen, L. Line, K. Thomsen, N. Høiby & P. Jensen (2017) Biofilms and host response - helpful or harmful. *APMIS,* 125**,** 320-338.

Moskowitz, S. M., J. M. Foster, J. Emerson & J. L. Burns (2004) Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. *J Clin Microbiol,* 42**,** 1915-22.

Mulcahy, H., L. Charron-Mazenod & S. Lewenza (2008) Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog,* 4**,** e1000213.

Mulcahy, L. R., J. L. Burns, S. Lory & K. Lewis (2010) Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J Bacteriol,* 192**,** 6191-9.

Mullard, A. (2017) 2016 FDA drug approvals. *Nat Rev Drug Discov,* 16**,** 73-76.

Munita, J. M., A. S. Bayer & C. A. Arias (2015) Evolving resistance among gram-positive pathogens. *Clin Infect Dis,* 61 Suppl 2**,** S48-57.

Natsheh, A., M. Vidberg, R. Friedmann, E. Ben-Chetrit, A. M. Yinnon & S. Zevin (2014) Prosthetic valve endocarditis due to *Streptococcus pneumoniae*. *Springerplus,* 3**,** 375.

Nazzaro, F., F. Fratianni & R. Coppola (2013) Quorum sensing and phytochemicals. *Int J Mol Sci,* 14**,** 12607-19.

Newman, D. J. & G. M. Cragg (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod,* 75**,** 311-35.

Newman, D. J. & G. M. Cragg (2016) Natural products as sources of new drugs from 1981 to 2014. *J Nat Prod,* 79**,** 629-61.

Nguyen, D., A. Joshi-Datar, F. Lepine, E. Bauerle, O. Olakanmi, K. Beer, G. McKay, R. Siehnel, J. Schafhauser, Y. Wang, B. E. Britigan & P. K. Singh (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science,* 334**,** 982-6.

Nijland, R., M. J. Hall & J. G. Burgess (2010) Dispersal of biofilms by secreted, matrix degrading, bacterial DNase. *PLoS One,* 5**,** e15668.

Niveditha, S., S. Pramodhini, S. Umadevi, S. Kumar & S. Stephen (2012) The isolation and the biofilm formation of uropathogens in the patients with catheter associated urinary tract infections (UTIs). *J Clin Diagn Res,* 6**,** 1478-82.

O'Brien, J., I. Wilson, T. Orton & F. Pognan (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem,* 267**,** 5421-6.

O'Connell, K. M., J. T. Hodgkinson, H. F. Sore, M. Welch, G. P. Salmond & D. R. Spring (2013) Combating multidrug-resistant bacteria: current strategies for the discovery of novel antibacterials. *Angew Chem Int Ed Engl,* 52**,** 10706-33.

O'Toole, G. A. (2011) Microtiter dish biofilm formation assay. *J Vis Exp*.

Oglesby-Sherrouse, A. G., L. Djapgne, A. T. Nguyen, A. I. Vasil & M. L. Vasil (2014) The complex interplay of iron, biofilm formation, and mucoidy affecting antimicrobial resistance of *Pseudomonas aeruginosa*. *Pathog Dis,* 70**,** 307-20.

Oja, T., B. Blomqvist, K. Buckingham-Meyer, D. Goeres, P. Vuorela & A. Fallarero (2014) Revisiting an agar-based plate method: what the static biofilm method can offer for biofilm research. *J Microbiol Methods,* 107**,** 157-60.

Olson, M. E., H. Ceri, D. W. Morck, A. G. Buret & R. R. Read (2002) Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res,* 66**,** 86-92.

Olson, M. E., K. L. Garvin, P. D. Fey & M. E. Rupp (2006) Adherence of *Staphylococcus epidermidis* to biomaterials is augmented by PIA. *Clin Orthop Relat Res,* 451**,** 21-4.

Olson, M. E., S. R. Slater, M. E. Rupp & P. D. Fey (2010) Rifampicin enhances activity of daptomycin and vancomycin against both a polysaccharide intercellular adhesin (PIA)-dependent and -independent *Staphylococcus epidermidis* biofilm. *J Antimicrob Chemother,* 65**,** 2164-71.

Opperman, T. J., S. M. Kwasny, J. D. Williams, A. R. Khan, N. P. Peet, D. T. Moir & T. L. Bowlin (2009) Aryl rhodanines specifically inhibit staphylococcal and enterococcal biofilm formation. *Antimicrob Agents Chemother,* 53**,** 4357-67.

Ortholand, J. Y. & A. Ganesan (2004) Natural products and combinatorial chemistry: back to the future. *Curr Opin Chem Biol,* 8**,** 271-80.

Ouyang, J., F. Sun, W. Feng, Y. Sun, X. Qiu, L. Xiong, Y. Liu & Y. Chen (2016) Quercetin is an effective inhibitor of quorum sensing, biofilm formation and virulence factors in *Pseudomonas aeruginosa*. *J Appl Microbiol,* 120**,** 966-74.

Overhage, J., A. Campisano, M. Bains, E. C. Torfs, B. H. Rehm & R. E. Hancock (2008) Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect Immun,* 76**,** 4176-82.

O'Toole, G., H. B. Kaplan & R. Kolter (2000) Biofilm formation as microbial development. *Annu Rev Microbiol,* 54**,** 49-79.

Pagedar, A., J. Singh & V. K. Batish (2010) Surface hydrophobicity, nutritional contents affect *Staphylococcus aureus* biofilms and temperature influences its survival in preformed biofilms. *J Basic Microbiol,* 50 Suppl 1**,** S98-106.

Paharik, A. E. & A. R. Horswill (2016) The staphylococcal biofilm: adhesins, regulation, and host response. *Microbiol Spectr,* 4.

Pamp, S. J., M. Gjermansen, H. K. Johansen & T. Tolker-Nielsen (2008) 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. *Mol Microbiol,* 68**,** 223-40.

Panmanee, W., D. Taylor, C. J. Shea, H. Tang, S. Nelson, W. Seibel, R. Papoian, R. Kramer, D. J. Hassett & T. J. Lamkin (2013) High-throughput screening for small-molecule inhibitors of *Staphylococcus epidermidis* RP62a biofilms. *J Biomol Screen,* 18**,** 820-9.

Pantanella, F., P. Valenti, T. Natalizi, D. Passeri & F. Berlutti (2013) Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. *Ann Ig,* 25**,** 31-42.

Papenfort, K. & B. L. Bassler (2016) Quorum sensing signal-response systems in Gram-negative bacteria. *Nat Rev Microbiol,* 14**,** 576-88.

Parker, A. E., D. K. Walker, D. M. Goeres, N. Allan, M. E. Olson & A. Omar (2014) Ruggedness and reproducibility of the MBEC biofilm disinfectant efficacy test. *J Microbiol Methods,* 102**,** 55-64.

Parsek, M. R. & P. K. Singh (2003) Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol,* 57**,** 677-701.

Patterson, J. L., A. Stull-Lane, P. H. Girerd & K. K. Jefferson (2010) Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. *Microbiology,* 156**,** 392-9.

Payne, D. J., M. N. Gwynn, D. J. Holmes & D. L. Pompliano (2007) Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Discov,* 6**,** 29-40.

Paytubi, S., M. de La Cruz, J. R. Tormo, J. Martín, I. González, V. González-Menendez, O. Genilloud, F. Reyes, F. Vicente, C. Madrid & C. Balsalobre (2017) A high-throughput screening platform of microbial natural products for the discovery of molecules with antibiofilm properties against Salmonella. *Front Microbiol,* 8**,** 326.

Peeters, E., H. J. Nelis & T. Coenye (2008) Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J Microbiol Methods,* 72**,** 157-65.

Pendleton, J. N., S. P. Gorman & B. F. Gilmore (2013) Clinical relevance of the ESKAPE pathogens. *Expert Rev Anti Infect Ther,* 11**,** 297-308.

Percival, S. L., L. Suleman, C. Vuotto & G. Donelli (2015) Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. *J Med Microbiol,* 64**,** 323-34.

Persson, T., T. H. Hansen, T. B. Rasmussen, M. E. Skindersø, M. Givskov & J. Nielsen (2005) Rational design and synthesis of new quorum-sensing inhibitors derived from acylated homoserine lactones and natural products from garlic. *Org Biomol Chem,* 3**,** 253-62.

Pettit, R. K., C. A. Weber, M. J. Kean, H. Hoffmann, G. R. Pettit, R. Tan, K. S. Franks & M. L. Horton (2005) Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. *Antimicrob Agents Chemother,* 49**,** 2612-7.

Pitts, B., M. A. Hamilton, N. Zelver & P. S. Stewart (2003) A microtiter-plate screening method for biofilm disinfection and removal. *J Microbiol Methods,* 54**,** 269-76.

Pitts, B., A. Willse, G. A. McFeters, M. A. Hamilton, N. Zelver & P. S. Stewart (2001) A repeatable laboratory method for testing the efficacy of biocides

against toilet bowl biofilms. *J Appl Microbiol,* 91**,** 110-7.

Pletzer, D. & R. E. Hancock (2016) Antibiofilm peptides: potential as broad-spectrum agents. *J Bacteriol,* 198**,** 2572-8.

Poole, K. (2012) Bacterial stress responses as determinants of antimicrobial resistance. *J Antimicrob Chemother,* 67**,** 2069-89.

Posner, B. A., H. Xi & J. E. Mills (2009) Enhanced HTS hit selection via a local hit rate analysis. *J Chem Inf Model,* 49**,** 2202-10.

Pratt, L. A. & R. Kolter (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol,* 30**,** 285-93.

Prestinaci, F., P. Pezzotti & A. Pantosti (2015) Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Glob Health,* 109**,** 309-18.

Projan, S. J. 2003. Why is big Pharma getting out of antibacterial drug discovery? *Curr Opin Microbiol,* 6**,** 427-430.

Pérez-Giraldo, C., A. Rodríguez-Benito, F. J. Morán, C. Hurtado, M. T. Blanco & A. C. Gómez-García (1997) Influence of N-acetylcysteine on the formation of biofilm by *Staphylococcus epidermidis*. *J Antimicrob Chemother,* 39**,** 643-6.

Quave, C. L., L. R. Plano, T. Pantuso & B. C. Bennett (2008) Effects of extracts from Italian medicinal plants on planktonic growth, biofilm formation and adherence of methicillin-resistant *Staphylococcus aureus*. *J Ethnopharmacol,* 118**,** 418-28.

Raad, I., H. Hanna, Y. Jiang, T. Dvorak, R. Reitzel, G. Chaiban, R. Sherertz & R. Hachem (2007) Comparative activities of daptomycin, linezolid, and tigecycline against catheter-related methicillin-resistant *Staphylococcus* bacteremic isolates embedded in biofilm. *Antimicrob Agents Chemother,* 51**,** 1656-60.

Raad, I., R. Reitzel, Y. Jiang, R. F. Chemaly, T. Dvorak & R. Hachem (2008) Anti-adherence activity and antimicrobial durability of anti-infective-coated catheters against multidrug-resistant bacteria. *J Antimicrob Chemother,* 62**,** 746-50.

Ramón-Peréz, M. L., F. Diaz-Cedillo, J. A. Ibarra, A. Torales-Cardeña, S. Rodríguez-Martínez, J. Jan-Roblero, M. E. Cancino-Diaz & J. C. Cancino-Diaz (2014) D-Amino acids inhibit biofilm formation in *Staphylococcus epidermidis* strains from ocular infections. *J Med Microbiol,* 63**,** 1369-76.

Rasmussen, T. B., T. Bjarnsholt, M. E. Skindersoe, M. Hentzer, P. Kristoffersen, M. Köte, J. Nielsen, L. Eberl & M. Givskov (2005a) Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *J Bacteriol,* 187**,** 1799-814.

Rasmussen, T. B. & M. Givskov (2006) Quorum-sensing inhibitors as anti-pathogenic drugs. *Int J Med Microbiol,* 296**,** 149-61.

Rasmussen, T. B., M. E. Skindersoe, T. Bjarnsholt, R. K. Phipps, K. B. Christensen, P. O. Jensen, J. B. Andersen, B. Koch, T. O. Larsen, M. Hentzer, L. Eberl, N. Hoiby & M. Givskov (2005b) Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species. *Microbiology,* 151**,** 1325-40.

Redgrave, L. S., S. B. Sutton, M. A. Webber & L. J. Piddock (2014) Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. *Trends Microbiol,* 22**,** 438-45.

Renwick, M. J., D. M. Brogan & E. Mossialos (2016) A systematic review and critical assessment of incentive strategies for discovery and development of novel antibiotics. *J Antibiot (Tokyo),* 69**,** 73-88.

Reuter, K., A. Steinbach & V. Helms (2016) Interfering with bacterial quorum sensing. *Perspect Medicin Chem,* 8**,** 1-15.

Richards, J. J., T. E. Ballard, R. W. Huigens & C. Melander (2008) Synthesis and screening of an oroidin library against *Pseudomonas aeruginosa* biofilms. *Chembiochem,* 9**,** 1267-79.

Rieger, U. M., J. Mesina, D. F. Kalbermatten, M. Haug, H. P. Frey, R. Pico, R. Frei, G. Pierer, N. J. Lüscher & A. Trampuz (2013) Bacterial biofilms and capsular contracture in patients with breast implants. *Br J Surg,* 100**,** 768-74.

Riss, T. L. & R. A. Moravec (2004) Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay Drug Dev Technol,* 2**,** 51-62.

Roberts, A. E., K. N. Kragh, T. Bjarnsholt & S. P. Diggle (2015) The limitations of *in vitro* experimentation in understanding biofilms and chronic Infection. *J Mol Biol,* 427**,** 3646-61.

Robertson, D. M., Q. M. Parks, R. L. Young, J. Kret, K. R. Poch, K. C. Malcolm, D. P. Nichols, M. Nichols, M. Zhu, H. D. Cavanagh & J. A. Nick (2011) Disruption of contact lens-associated *Pseudomonas aeruginosa* biofilms formed in the presence of neutrophils. *Invest Ophthalmol Vis Sci,* 52**,** 2844-50.

Rogers, G. B., M. P. Carroll & K. D. Bruce (2012) Enhancing the utility of existing antibiotics by targeting bacterial behaviour? *Br J Pharmacol,* 165**,** 845-57.

Rutherford, S. T. & B. L. Bassler (2012) Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med,* 2.

Ryu, J. H., H. Kim & L. R. Beuchat (2004) Attachment and biofilm formation by *Escherichia coli* O157:H7 on stainless steel as influenced by exopolysaccharide production, nutrient availability, and temperature. *J Food Prot,* 67**,** 2123-31.

Ríos, J. L. & M. C. Recio (2005) Medicinal plants and antimicrobial activity. *J Ethnopharmacol,* 100**,** 80-4.

Sabir, N., A. Ikram, G. Zaman, L. Satti, A. Gardezi, A. Ahmed & P. Ahmed (2017) Bacterial biofilm-based catheter-associated urinary tract infections: causative pathogens and antibiotic resistance. *Am J Infect Control*.

Sambanthamoorthy, K., C. Luo, N. Pattabiraman, X. Feng, B. Koestler, C. M. Waters & T. J. Palys (2014) Identification of small molecules inhibiting diguanylate cyclases to control bacterial biofilm development. *Biofouling,* 30**,** 17-28.

Sanchez, C. J., K. S. Akers, D. R. Romano, R. L. Woodbury, S. K. Hardy, C. K. Murray & J. C. Wenke (2014) D-amino acids enhance the activity of antimicrobials against biofilms of clinical wound isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother,* 58**,** 4353-61.

Sandberg, M., A. Määttänen, J. Peltonen, P. M. Vuorela & A. Fallarero (2008) Automating a 96-well microtitre plate model for *Staphylococcus aureus* biofilms: an approach to screening of natural antimicrobial compounds. *Int J Antimicrob Agents,* 32**,** 233-40.

Sandberg, M. E., D. Schellmann, G. Brunhofer, T. Erker, I. Busygin, R.

Leino, P. M. Vuorela & A. Fallarero (2009) Pros and cons of using resazurin staining for quantification of viable *Staphylococcus aureus* biofilms in a screening assay. *J Microbiol Methods,* 78**,** 104-6.

Savage, V. J., I. Chopra & A. J. O'Neill (2013) *Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance. *Antimicrob Agents Chemother,* 57**,** 1968-70.

Schaffer, J. N. & M. M. Pearson (2015) *Proteus mirabilis* and urinary tract infections. *Microbiol Spectr,* 3.

Schenone, M., V. Dančík, B. K. Wagner & P. A. Clemons (2013) Target identification and mechanism of action in chemical biology and drug discovery. *Nat Chem Biol,* 9**,** 232-40.

Schreiber, S. L. (2000) Target-oriented and diversity-oriented organic synthesis in drug discovery. *Science,* 287**,** 1964-9.

Serrano, M., E. Kombrink & C. Meesters (2015) Considerations for designing chemical screening strategies in plant biology. *Front Plant Sci,* 6**,** 131.

Shafique, M., I. A. Alvi, Z. Abbas & S. Ur Rehman (2017) Assessment of biofilm removal capacity of a broad host range bacteriophage JHP against *Pseudomonas aeruginosa*. *APMIS,* 125**,** 579-584.

Shakeri, S., R. K. Kermanshahi, M. M. Moghaddam & G. Emtiazi (2007) Assessment of biofilm cell removal and killing and biocide efficacy using the microtiter plate test. *Biofouling,* 23**,** 79-86.

Shang, S. & D. S. Tan (2005) Advancing chemistry and biology through diversity-oriented synthesis of natural product-like libraries. *Curr Opin Chem Biol,* 9**,** 248-58.

Shen, B. (2015) A new golden age of natural products drug discovery. *Cell,* 163**,** 1297-300.

Silver, L. L. (2011) Challenges of antibacterial discovery. *Clin Microbiol Rev,* 24**,** 71-109.

Singh, R., P. Ray, A. Das & M. Sharma (2009) Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study. *J Med Microbiol,* 58**,** 1067-73.

Singh, S. B. & J. F. Barrett (2006) Empirical antibacterial drug discovery--foundation in natural products. *Biochem Pharmacol,* 71**,** 1006-15.

Sintim, H. O., J. A. Smith, J. Wang, S. Nakayama & L. Yan (2010) Paradigm shift in discovering next-generation anti-infective agents: targeting quorum sensing, c-di-GMP signaling and biofilm formation in bacteria with small molecules. *Future Med Chem,* 2**,** 1005-35.

Sipponen, A. & K. Laitinen (2011) Antimicrobial properties of natural coniferous rosin in the European Pharmacopoeia challenge test. *APMIS,* 119**,** 720-4.

Skogman, M. E., S. Kanerva, S. Manner, P. M. Vuorela & A. Fallarero (2016) Flavones as quorum sensing inhibitors identified by a newly optimized screening platform using *Chromobacterium violaceum* as reporter bacteria. *Molecules,* 21.

Skogman, M. E., P. M. Vuorela & A. Fallarero (2012) Combining biofilm matrix measurements with biomass and viability assays in susceptibility assessments of antimicrobials against *Staphylococcus aureus* biofilms. *J Antibiot (Tokyo),* 65**,** 453-9.

Smith, K., A. Perez, G. Ramage, C. G. Gemmell & S. Lang (2009) Comparison

of biofilm-associated cell survival following *in vitro* exposure of meticillin-resistant *Staphylococcus aureus* biofilms to the antibiotics clindamycin, daptomycin, linezolid, tigecycline and vancomycin. *Int J Antimicrob Agents,* 33**,** 374-8.

Smyth, A. R., P. M. Cifelli, C. A. Ortori, K. Righetti, S. Lewis, P. Erskine, E. D. Holland, M. Givskov, P. Williams, M. Cámara, D. A. Barrett & A. Knox (2010) Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis--a pilot randomized controlled trial. *Pediatr Pulmonol,* 45**,** 356-62.

Song, Z., L. Borgwardt, N. Høiby, H. Wu, T. S. Sørensen & A. Borgwardt (2013) Prosthesis infections after orthopedic joint replacement: the possible role of bacterial biofilms. *Orthop Rev (Pavia),* 5**,** 65-71.

Sperandio, V. (2007) Novel approaches to bacterial infection therapy by interfering with bacteria-to-bacteria signaling. *Expert Rev Anti Infect Ther,* 5**,** 271-6.

Spoering, A. L. & K. Lewis (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol,* 183**,** 6746-51.

Stauff, D. L. & B. L. Bassler (2011) Quorum sensing in *Chromobacterium violaceum*: DNA recognition and gene regulation by the CviR receptor. *J Bacteriol,* 193**,** 3871-8.

Stepanovic, S., D. Vukovic, I. Dakic, B. Savic & M. Svabic-Vlahovic (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods,* 40**,** 175-9.

Stepanović, S., D. Vuković, V. Hola, G. Di Bonaventura, S. Djukić, I. Cirković & F. Ruzicka (2007) Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS,* 115**,** 891-9.

Stewart, P. S. (2002) Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol,* 292**,** 107-13.

Stewart, P. S. (2015) Antimicrobial tolerance in biofilms. *Microbiol Spectr,* 3.

Stewart, P. S. & J. W. Costerton (2001) Antibiotic resistance of bacteria in biofilms. *Lancet,* 358**,** 135-8.

Stewart, P. S. & M. J. Franklin (2008) Physiological heterogeneity in biofilms. *Nat Rev Microbiol,* 6**,** 199-210.

Stewart, P. S., J. Rayner, F. Roe & W. M. Rees (2001) Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. *J Appl Microbiol,* 91**,** 525-32.

Stiefel, P., U. Rosenberg, J. Schneider, S. Mauerhofer, K. Maniura-Weber & Q. Ren (2016) Is biofilm removal properly assessed? Comparison of different quantification methods in a 96-well plate system. *Appl Microbiol Biotechnol,* 100**,** 4135-45.

Stockwell, B. R. (2004) Exploring biology with small organic molecules. *Nature,* 432**,** 846-54.

Stone, G., P. Wood, L. Dixon, M. Keyhan & A. Matin (2002) Tetracycline rapidly reaches all the constituent cells of uropathogenic *Escherichia coli* biofilms. *Antimicrob Agents Chemother,* 46**,** 2458-61.

Stoodley, P., I. Dodds, J. D. Boyle & H. M. Lappin-Scott (1998) Influence of hydrodynamics and nutrients on biofilm structure. *J Appl Microbiol,* 85 Suppl 1**,** 19S-28S.

Stoodley, P., K. Sauer, D. G. Davies & J. W. Costerton (2002) Biofilms as complex

differentiated communities. *Annu Rev Microbiol,* 56**,** 187-209.

Stoodley, P., S. Wilson, L. Hall-Stoodley, J. D. Boyle, H. M. Lappin-Scott & J. W. Costerton (2001) Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl Environ Microbiol,* 67**,** 5608-13.

Subbiahdoss, G., I. C. Fernández, J. F. Domingues, R. Kuijer, H. C. van der Mei & H. J. Busscher (2011) *In vitro* interactions between bacteria, osteoblast-like cells and macrophages in the pathogenesis of biomaterial-associated infections. *PLoS One,* 6**,** e24827.

Swinney, D. C. (2013) Phenotypic vs. target-based drug discovery for first-in-class medicines. *Clin Pharmacol Ther,* 93**,** 299-301.

Swinney, D. C. & J. Anthony (2011) How were new medicines discovered? *Nat Rev Drug Discov,* 10**,** 507-19.

Ta, C. A. & J. T. Arnason (2015) Mini review of phytochemicals and plant taxa with activity as microbial biofilm and quorum sensing inhibitors. *Molecules,* 21**,** E29.

Tajudeen, B. A., J. S. Schwartz & J. N. Palmer (2016) Understanding biofilms in chronic sinusitis. *Curr Allergy Asthma Rep,* 16**,** 10.

Tebbs, S. E. & T. S. Elliott (1994) Modification of central venous catheter polymers to prevent *in vitro* microbial colonisation. *Eur J Clin Microbiol Infect Dis,* 13**,** 111-7.

Tegos, G., F. R. Stermitz, O. Lomovskaya & K. Lewis (2002) Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrob Agents Chemother,* 46**,** 3133-41.

Teles, R., F. Teles, J. Frias-Lopez, B. Paster & A. Haffajee (2013) Lessons learned and unlearned in periodontal microbiology. *Periodontol 2000,* 62**,** 95-162.

Terstappen, G. C., C. Schlüpen, R. Raggiaschi & G. Gaviraghi (2007) Target deconvolution strategies in drug discovery. *Nat Rev Drug Discov,* 6**,** 891-903.

Thomson, C. J., E. Power, H. Ruebsamen-Waigmann & H. Labischinski (2004) Antibacterial research and development in the 21(st) Century--an industry perspective of the challenges. *Curr Opin Microbiol,* 7**,** 445-50.

Thormann, K. M., R. M. Saville, S. Shukla & A. M. Spormann (2005) Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. *J Bacteriol,* 187**,** 1014-21.

Thornton, R. B., P. J. Rigby, S. P. Wiertsema, P. Filion, J. Langlands, H. L. Coates, S. Vijayasekaran, A. D. Keil & P. C. Richmond (2011) Multi-species bacterial biofilm and intracellular infection in otitis media. *BMC Pediatr,* 11**,** 94.

Tikhomirova, A. & S. P. Kidd (2013) *Haemophilus influenzae* and *Streptococcus pneumoniae*: living together in a biofilm. *Pathog Dis,* 69**,** 114-26.

Tolker-Nielsen, T. & S. Molin (2000) Spatial organization of microbial biofilm communities. *Microb Ecol,* 40**,** 75-84.

Tormo, M. A., E. Knecht, F. Götz, I. Lasa & J. R. Penadés (2005) Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? *Microbiology,* 151**,** 2465-75.

Toté, K., D. V. Berghe, M. Deschacht, K. de Wit, L. Maes & P. Cos (2009) Inhibitory efficacy of various antibiotics on matrix and viable mass of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. *Int J Antimicrob Agents,* 33**,** 525-31.

Toté, K., D. Vanden Berghe, L. Maes & P. Cos (2008) A new colorimetric microtitre model for the detection of *Staphylococcus aureus* biofilms. *Lett Appl Microbiol,* 46**,** 249-54.

Traczewski, M. M., B. D. Katz, J. N. Steenbergen & S. D. Brown (2009) Inhibitory and bactericidal activities of daptomycin, vancomycin, and teicoplanin against methicillin-resistant *Staphylococcus aureus* isolates collected from 1985 to 2007. *Antimicrob Agents Chemother,* 53**,** 1735-8.

Tuomanen, E., R. Cozens, W. Tosch, O. Zak & A. Tomasz (1986) The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. *J Gen Microbiol,* 132**,** 1297-304.

van de Wetering, M. D., J. B. van Woensel & T. A. Lawrie (2013) Prophylactic antibiotics for preventing Gram positive infections associated with long-term central venous catheters in oncology patients. *Cochrane Database Syst Rev***,** CD003295.

Van den Driessche, F., P. Rigole, G. Brackman & T. Coenye (2014) Optimization of resazurin-based viability staining for quantification of microbial biofilms. *J Microbiol Methods,* 98**,** 31-4.

Vendeville, A., K. Winzer, K. Heurlier, C. M. Tang & K. R. Hardie (2005) Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria. *Nat Rev Microbiol,* 3**,** 383-96.

Ventola, C. L. (2015) The antibiotic resistance crisis: part 1: causes and threats. *P T,* 40**,** 277-83.

Vermote, A., G. Brackman, M. D. Risseeuw, T. Coenye & S. Van Calenbergh (2017) Novel hamamelitannin analogues for the treatment of biofilm related MRSA infections-A scaffold hopping approach. *Eur J Med Chem,* 127**,** 757-770.

von Eiff, C., B. Jansen, W. Kohnen & K. Becker. 2005. Infections associated with medical devices pathogenesis, management and prophylaxis. *Drugs*, 65**,** 179-214.

von Nussbaum, F., M. Brands, B. Hinzen, S. Weigand & D. Häbich (2006) Antibacterial natural products in medicinal chemistry--exodus or revival? *Angew Chem Int Ed Engl,* 45**,** 5072-129.

Vuong, C., C. Gerke, G. A. Somerville, E. R. Fischer & M. Otto (2003) Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. *J Infect Dis,* 188**,** 706-18.

Walters, W. P. & M. Namchuk (2003) Designing screens: how to make your hits a hit. *Nat Rev Drug Discov,* 2**,** 259-66.

Wassermann, A. M., L. M. Camargo & D. S. Auld (2014) Composition and applications of focus libraries to phenotypic assays. *Front Pharmacol,* 5**,** 164.

Waters, V. & F. Ratjen (2015) Standard versus biofilm antimicrobial susceptibility testing to guide antibiotic therapy in cystic fibrosis. *Cochrane Database Syst Rev***,** CD009528.

Watnick, P. & R. Kolter (2000) Biofilm, city of microbes. *J Bacteriol,* 182**,** 2675-9.

Webber, M. A. & L. J. Piddock (2003) The importance of efflux pumps in bacterial antibiotic resistance. *J Antimicrob Chemother,* 51**,** 9-11.

Wessman, M., T. Bjarnsholt, S. R. Eickhardt-Sørensen, H. K. Johansen & P. Homøe (2015) Mucosal biofilm detection in chronic otitis media: a study of middle ear biopsies from Greenlandic patients. *Eur Arch Otorhinolaryngol,* 272**,** 1079-85.

Weyermann, J., D. Lochmann & A. Zimmer (2005) A practical note on the use of cytotoxicity assays. *Int J Pharm,* 288**,** 369-76.

Wiley, L., D. R. Bridge, L. A. Wiley, J. V. Odom, T. Elliott & J. C. Olson (2012) Bacterial biofilm diversity in contact lens-related disease: emerging role of *Achromobacter*, *Stenotrophomonas,* and *Delftia*. *Invest Ophthalmol Vis Sci,* 53**,** 3896-905.

Wolcott, R. D. & D. D. Rhoads (2008) A study of biofilm-based wound management in subjects with critical limb ischaemia. *J Wound Care,* 17**,** 145-8, 150-2, 154-5.

Wolcott, R. D., K. P. Rumbaugh, G. James, G. Schultz, P. Phillips, Q. Yang, C. Watters, P. S. Stewart & S. E. Dowd (2010) Biofilm maturity studies indicate sharp debridement opens a time-dependent therapeutic window. *J Wound Care,* 19**,** 320-8.

Worthington, R. J., J. J. Richards & C. Melander (2012) Small molecule control of bacterial biofilms. *Org Biomol Chem,* 10**,** 7457-74.

Wright, G. D. (2012) Antibiotics: a new hope. *Chem Biol,* 19**,** 3-10.

Wu, H., C. Moser, H. Z. Wang, N. Høiby & Z. J. Song (2015) Strategies for combating bacterial biofilm infections. *Int J Oral Sci,* 7**,** 1-7.

Xie, Y., W. Yang, F. Tang, X. Chen & L. Ren (2015) Antibacterial activities of flavonoids: structure-activity relationship and mechanism. *Curr Med Chem,* 22**,** 132-49.

Yu, C., X. Li, N. Zhang, D. Wen, C. Liu & Q. Li (2016) Inhibition of biofilm formation by D-tyrosine: Effect of bacterial type and D-tyrosine concentration. *Water Res,* 92**,** 173-9.

Zhang, J.-H., T. D. Y. Chung & K. R. Oldenburg. 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen*, 4**,** 67-73.

Zhang, L., S. Cao, N. Marsh, G. Ray-Barruel, J. Flynn, E. Larsen & C. M. Rickard (2016) Infection risks associated with peripheral vascular catheters. *J Infect Prev,* 17**,** 207-213.

Zheng, W., N. Thorne & J. C. McKew (2013) Phenotypic screens as a renewed approach for drug discovery. *Drug Discov Today,* 18**,** 1067-73.

Zheng, Z. & P. S. Stewart (2002) Penetration of rifampin through *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother,* 46**,** 900-3.

Zhu, T., S. Cao, P. C. Su, R. Patel, D. Shah, H. B. Chokshi, R. Szukala, M. E. Johnson & K. E. Hevener (2013) Hit identification and optimization in virtual screening: practical recommendations based on a critical literature analysis. *J Med Chem,* 56**,** 6560-72.

ISBN 978-952-12-3626-6