The effect of interspecies interactions on the antimicrobial susceptibility of multispecies biofilms

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DANKWOORD

Voor papa

“And as we wind on down the road,
our shadows taller than our soul.
There walks a lady we all know,
who shines white light and wants to show
how everything still turns to gold.”

(Led Zeppelin)

TABLE OF CONTENTS

List of abbreviations

2-AA	2-amino acetophenon
2-D	Two-dimensional
3-oxo-C_{12}-HSL	N-3-oxo-dodecanoyl-L-homoserine lactone
3-D	Three-dimensional
Agr	Accessory gene regulator
AHL	Acyl-homoserine lacton
AI-2	Autoinducer-2
AIP	Autoinducing peptides
Als3p	Agglutinin-like sequence 3
AQ	Alkyl quinolone
ATCC	American Type Culture Collection
Bcc	*Burkholderia cepacia complex*
BHI	Brain heart infusion
bp	Base pair
BSA	Bovine serum albumin
BzCl	Benzalkoniumchloride
CAT	Chloramphenicol acetyltransferase
CET	Cetrimide
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CHX	Chlorhexidine
CLSM	Confocal laser scanning microscopy
Cq	Cycle threshold value
DGE	Differential gene expression
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	double-stranded DNA
DSF	Diffusible signal factor
EDGE	Empirical analysis of DGE
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
EPM	Extracellular polymeric matrix
EPS	Extracellular polymeric substances
ET	Endotracheal tube
ETOH	Ethanol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDR	False discovery rate
HAC	Hospital antiseptic concentrate
H2O2	hydrogen peroxide
HHQ	4-hydroxy-2-heptyl-4-quinolone
HPF	high pressure freezing
HQNO	2-heptyl-4-hydroxyquinoline N-oxide
LMG	Laboratorium voor Microbiology, Faculty of Sciences Ghent University
LPS	Lipopolysaccharides

MIC	Minimum inhibitory concentration
MDC	Minimum duration for killing
MDR	Multidrug-resistant
MQ	MilliQ water
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTP	Microtiter plate
NaOCl	Sodium hypochlorite
ND	Not determined
OD	Optical density
PMA	Propidium monoazide
PCMX	chloroxylenol
PAI	*Pseudomonas* isolation agar
PMA	Propidium monoazide
PQS	*Pseudomonas* quinolone signal
PS	Physiological saline
PVP-I	Povidone-iodine
QPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPKM	Reads per kb per million
SAB	Sabouraud agar
SAM	S-adenosylmethionine
SCV	Small colony variants
SEM	Standard error mean
SMG	*Streptococcus milleri* group
SPC	Solid-phase cytometry
STDEV	Standard deviation
TEM	Transmission electron microscopy
TSA	Tryptic soy agar
UDP	Uridine-5'-triphosphate
VAP	Ventilator-associated pneumonia
VBNC	Viable but non-culturable

Chapter I: INTRODUCTION

1. Biofilms

1.1 Historical background of biofilms

More than 300 years ago, the presence of multispecies biofilms was observed for the first time. Antonie van Leeuwenhoek described this phenotype in his microscopic observations on particles scraped off his own teeth and tongue in 1684. [1, 2] However, the importance of his observations was not acknowledged at that time and for the next century, microbiological research was mainly focused on planktonically growing microorganisms and their properties. [2, 3] Gradually, awareness rose again that microbes typically occur in aggregates of microorganisms, adhered to surfaces and surrounded by a self-produced slime layer. [2, 4, 5] However, it was not until 1981 that a general theory on biofilms was proposed. Costerton [6, 7] described that microorganisms adhere to living and nonliving materials through polysaccharides or a glycocalyx, and he introduced the term 'biofilm'. By the beginning of the 1980s, over 120 peer-reviewed research papers describing co-aggregation between dental plaque bacteria were published and the significance of multispecies biofilms was acknowledged. [8] Later on, investigation of non-host environments, including soil, seawater and drinking water systems, also revealed the presence of multispecies biofilms. [9] Nowadays, multispecies biofilms are acknowledged to be highly important and widely distributed [10], but only recently the complexity of and interactions in multispecies biofilms have been the subject of thorough investigations [11].

1.2 Biofilms form structured and coordinated communities

1.2.1 Consecutive stages of biofilm formation

The definition of a biofilm, as formulated by Costerton [12], is: *"A biofilm is a multicellular community composed of prokaryotic and/or eukaryotic cells embedded in a matrix composed, at least partially, of material synthesized by the sessile cells in the community."* The biofilm matrix provides mechanical stability and protection against stress. [13] It consists of highly hydrated extracellular polymeric substances (EPS), mainly including polysaccharides, proteins, nucleic acids, and lipids. The EPS forms the scaffold for the three-dimensional structure of the biofilm by immobilizing the biofilm cells and bringing the cells in close proximity, which allows them to interact. [14]

Biofilm cells can attach nonspecifically to a variety of surfaces, including water system piping, and indwelling medical devices (e.g. implants and catheters). They can also be embedded in host-material (e.g. mucus) of living tissues such as in the lung or in chronic wounds. [2, 7, 15] In context of host tissue, attachment often occurs through highly specific interactions between host receptors and microbial cell surface structures. [16]

In general, biofilm formation is believed to include several stages, which occur continuously during biofilm formation [17]: (a) initial attachment to a surface, (b) formation of micro-colonies, (c) maturation, and (d) dispersal, as shown in Figure 1. [18]

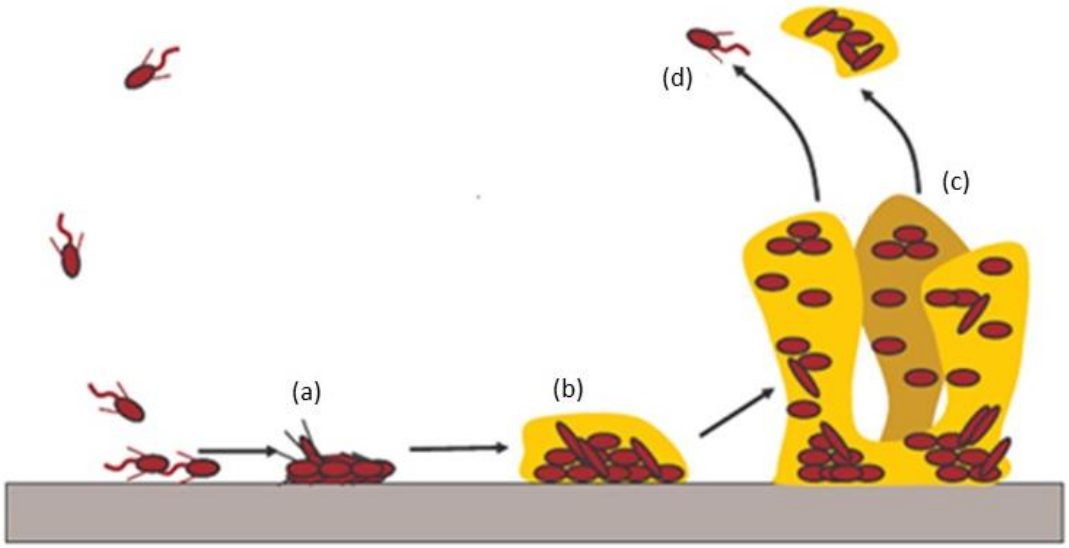

Figure 1: Consecutive stages of biofilm formation: (a) attachment to a surface; (b) formation of micro-colonies; (c) maturation; and (d) dispersal. [18]

(a) Initial attachment to a surface

In general, after exposure to an aqueous medium, a material surface will quickly become coated by polymers from the medium. The solid-liquid interface between this conditioned surface and the medium provides an ideal environment for the attachment of microorganisms. [19] Most studies indicate that in general, microorganisms attach more rapidly to hydrophobic, nonpolar surfaces (e.g. plastics and silicone), due to a hydrophobic interaction between the surface and the cell wall. [2] Several cell surface structures, e.g. proteins, capsules, fimbriae and flagella, are described to play a role in the initial attachment to hydrophobic surfaces, while lipopolysaccharides (LPS) would be more important in the attachment to hydrophilic surfaces. [2, 10] Besides cell properties, properties of the medium also play a major role. A certain degree of increase in temperature, nutrient concentration, or flow velocity, can lead to an increased attachment of cells. At this stage, microorganisms are still susceptible towards antimicrobial treatment. [10, 20, 21] Furthermore, upon attachment, down- and up-regulation of a number of genes is observed, for example, upregulation of *algC* and *algD* in *Pseudomonas aeruginosa,* genes that are involved in the synthesis of Psl and Pel, two important biofilm matrix exopolysaccharides of *P. aeruginosa* [22], or upregulation of genes involved in fermentation in *Staphylococcus aureus*, favoring the aerobic-anaerobic shift in metabolism in an oxygen limited developing biofilm. [23]

(b) Formation of micro-colonies

Following reversible attachment, cells will become irreversibly attached (i.e. they cannot be removed by gentle rinsing) by strong covalent and hydrogen bonds. [21] The cells will divide, they will form non-motile micro-colonies, and will secrete EPS. [20] EPS will hold the micro-colonies together and will firmly attach them to the surface. [24] The EPS is thought to be the first mechanism of resistance in biofilms. It can act as an adsorbent or reactant, reducing the amount of antimicrobial agent available to interact with biofilm cells, and it can physically reduce the penetration of antimicrobial agents through the biofilm. [25]

(c) Maturation

The biofilm maturation stage is characterized by continued multiplication of the primary attached cells and continued secretion of EPS leading to the formation of a thick biofilm matrix. [26] The matrix serves as a scaffold, contributing to cell-to-cell connection and interaction, and to the biofilm structure. [17] For some species, including *P. aeruginosa* [27] and *S. aureus* [28], it is described that cells will release eDNA, another important EPS component. [29] Furthermore, the presence of water channels has also been described for *Pseudomonas* spp. and *Staphylococcus* spp., amongst others. [30, 31] These water channels are interstitial voids that can separate the cell aggregates in a mature biofilm, and that allow the diffusion and uptake of nutrients and oxygen, and the removal of harmful metabolic products. [15, 21] As there is a nutrient and oxygen gradient in the biofilm, cells in the deeper layers will experience a nutrient and oxygen limitation. The microenvironment thus changes from aerobic to facultative anaerobic. [32]

(d) Dispersal

Dispersal can be divided into three phases: detachment of cells from the biofilm, translocation of the cells, and attachment of the cells in the new location. [24] Upon stressful conditions (starvation, fluid shear, human intervention), biofilm cells can detach and disperse by shedding single cells or small biofilm portions at low levels (erosion) or by sudden detachment of large portions (sloughing). [2, 10] Dispersal can also be induced by the bacteria themselves (‘genetically programmed dispersal’) [24]. In addition, cell lysis of bacteria within the biofilm releases enzymes (including glycosidases, proteases, and deoxyribonucleases) that can degrade the biofilm matrix and contribute to dispersal. [2] Detachment of biofilm aggregates contributes to biological dispersal and disease transmission. [24] In the human body, it may result in the production of emboli and urinary tract or bloodstream infections. [2, 15] Therefore, dispersal is not only a selective advantage when environmental conditions become unfavorable, but also when conditions are favorable. [24]

1.2.2 Role of quorum sensing in biofilm formation

Quorum sensing (QS), a bacterial cell-to-cell communication process, can contribute to cell attachment. [7, 33] QS involves the production, detection, and response to extracellular signaling molecules. [34] Gram-positive and Gram-negative bacteria use different types of QS molecules. Some Gram-positive bacteria communicate using auto-inducing peptides (AIPs), while some Gram-negative bacteria use acyl-homoserine lactones (AHLs). [34] In addition, some Gram-positive and Gram-negative bacteria can also use the autoinducer-2 (AI-2) system, of which the production depends on S-adenosylmethionine (SAM) as a substrate. SAM is converted to S-adenosylhomocysteine, thereby providing methylgroups to demethylated acceptor groups. S-adenosylhomocysteine is then converted to S-ribosylhomocysteine and in a next step to 4,5-dihydroxy-2,3-pentanedione, which is the precursor of AI-2. The reaction is catalyzed by LuxS. [35] Inside the cell, AI-2 is converted to phosphor-AI-2 which can bind to the repressor of the *lsr* operon, thereby releasing the *lsr* operon and increasing its own uptake. [34, 36] Fungi use small primary alcohols as signaling molecules, such as farnesol and tyrosol in *Candida albicans*. [37-39] When the cell population reaches a sufficient density, signal molecules in the environment reach concentrations required to alter gene expression of, amongst others, genes related to biofilm formation. [34, 37, 40] For example, Davies et al. [41] showed that the extracellular signal N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) is necessary for the development of *P. aeruginosa* biofilms. Furthermore, QS regulation of swarming motility, siderophores, and rhamnolipids, also contributes to *P. aeruginosa* biofilm formation. [34] For *S. aureus*, the opposite is true, the presence of active QS molecules impedes attachment and biofilm development, and contributes to dispersal and colonization of new sites. [42] In addition, QS has been described to play a role in the resistance of biofilm bacteria, for example in the resistance of *P. aeruginosa* biofilms towards tobramycin and hydrogen peroxide. [43] As a result, the addition of QS inhibitors might increase the success of antibiotic treatment by increasing the susceptibility of biofilms. [44, 45]

1.3 Biofilms show an altered antimicrobial susceptibility

Biofilms are heterogeneous structures that show an altered susceptibility towards antimicrobials compared to their planktonic counterparts, due to resistance, tolerance, or persistence (Figure 2). [15]

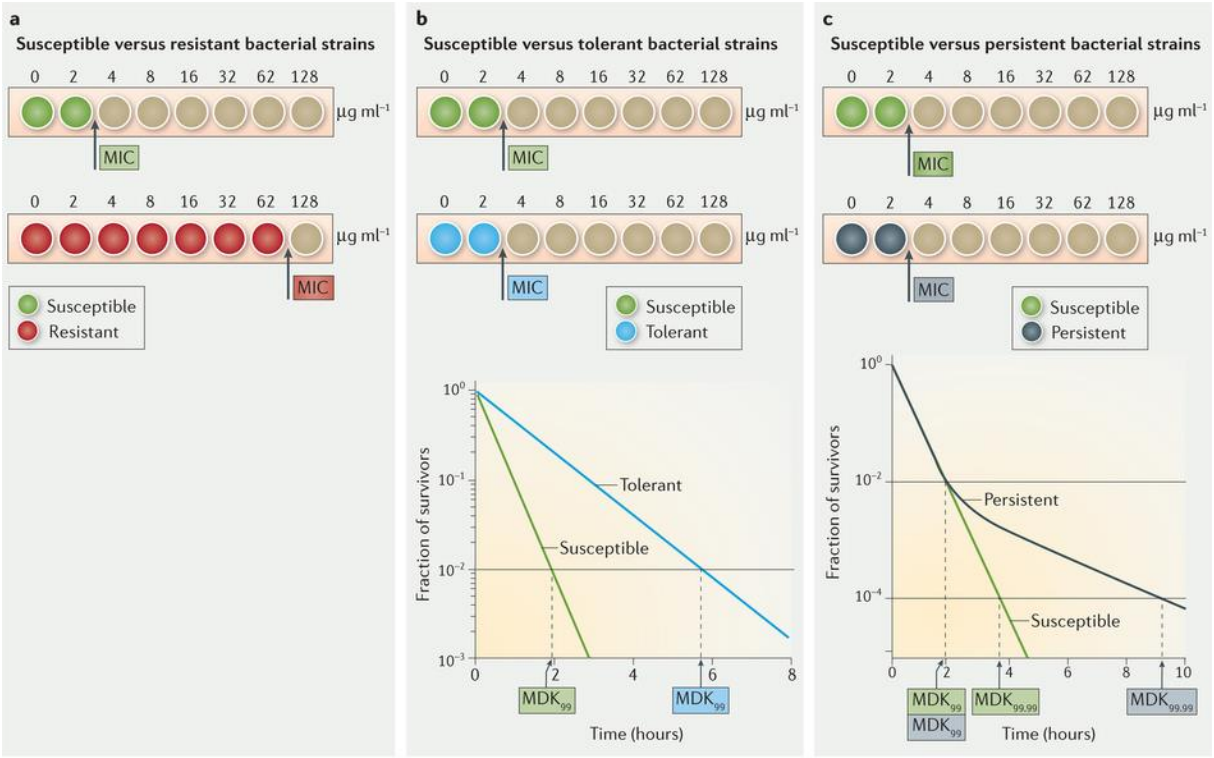

Figure 2: The survival strategies of resistance, tolerance and persistence: (a) the minimum inhibitory concentration (MIC) for a strain that is susceptible to an antibiotic is lower than the MIC for a resistant strain; (b) the MIC of a tolerant strain is similar to that of a susceptible strain, but the MDK_{99} (which is the minimum duration for killing (MDK) for 99% of bacterial cells) for a tolerant strain is higher than the MDK_{99} for a susceptible strain; (c) the MIC of a persistent strain is similar to that of a susceptible strain, but the $MDK_{99,99}$ is higher for a persistent strain than the $MDK_{99,99}$ for a susceptible strain. [46]

(a) Resistance

Antibiotic resistance can be subdivided into two types: acquired and intrinsic resistance. Both are stable and can be transferred vertically. [47, 48] Acquired resistance occurs when an initially susceptible subpopulation of microorganisms obtains the ability to withstand the action of the antimicrobial agent, and is able to proliferate under the selective pressure. It can result from mutations or horizontal gene transfer. [49] Biofilms provide an ideal niche for horizontal gene transfer, as the proximity of cells within or between the micro-colonies favors the exchange of plasmids by conjugation. [48] As a result, conjugation occurs at a much higher rate than seen in planktonic cultures. [50] Intrinsic resistance is the innate ability of microorganisms to resist antimicrobial agents through structural or functional characteristics [51, 52], for example, the low permeability of the semipermeable outer membrane of Gram-negative bacteria. [48] The expression

of efflux pumps has also been described as a resistance mechanism [53], especially in Gram-negative bacteria. [54, 55] Using efflux pumps, bacteria can remove toxic substances, including antimicrobial agents. [56] For example, for *P. aeruginosa*, upregulation of the expression of efflux pumps has been described to be involved in biofilm resistance against azithromycin [57], colistin [58, 59], tobramycin and ciprofloxacin [60]. Furthermore, in biofilms, the extracellular matrix can contribute to intrinsic resistance, through (i) diffusion-limitation and (ii) adsorptive loss. [61]

(i) Diffusion-limitation

The presence of a hydrated and charged extracellular matrix with hydrophobic pockets was thought to physically influence the access of antimicrobial agents to the bacterial cells, as antimicrobial agents must diffuse through the biofilm matrix to reach and inactivate the bacterial cells. [62, 63] However, it was already reported that a reduced diffusion would only provide a short-term protection, and would have no substantial role during long-term antimicrobial exposure. [7] Furthermore, Rani et al. [64] indicated that a diffusion-limitation hypothesis is probably incorrect, as their data could not demonstrate a physical penetration barrier of antimicrobial agents. In addition, Daddi Oubekka et al. [65] also demonstrated that therapeutic vancomycin concentrations were not hindered by the matrix of an *S. aureus* biofilm and that the antibiotic could reach all biofilm cells. Tetracycline was also shown to rapidly reach all *Escherichia coli* biofilm cells. [66] Experimental data regarding the diffusion of one antibiotic through the biofilm of one strain should be interpreted carefully as the results cannot be extrapolated to another antibiotic or another strain. [67]

(ii) Adsorptive loss

If antimicrobial agents are strongly charged or highly reactive, adsorption to matrix-associated charged sites or direct neutralization can chemically quench them during diffusion. [61] For example, highly hydrophilic, positively charged antimicrobial agents (e.g. aminoglycosides and quaternary ammonium compounds) can be subjected to adsorptive loss, as they can become bound to the anionic sites within the matrix (carboxyl, sulphate and phosphate groups, of nucleic acids and proteins). [61] In addition, extracellular DNA (eDNA) can be present in the matrix, which can chelate cations, leading to cation-limited conditions and subsequently LPS modifications and impaired self-promoted uptake of antibiotics such as aminoglycosides and antimicrobial peptides in Gram-negative organisms. [7] Furthermore, the matrix keeps extracellular enzymes close to the biofilm cells, and as a result, chemical neutralization of antimicrobial agents by extracellular enzymes can lead to degradation of antimicrobial agents (e.g. β-lactamases can inhibit β-lactam antibiotics) and protection of the biofilm cells. [14] However, the net effect of adsorptive loss once again is a delay of killing and not a complete prevention. [61]

(b) Tolerance & persistence

Tolerance is the ability of a whole bacterial population to survive transient exposure to high concentrations of antimicrobial agents (see Figure 2). [46] Tolerance can be acquired through a genetic mutation, conferred by environmental conditions, or induced by the antibiotic itself. [46] In a biofilm, due to depletion of nutrients and/or an oxygen gradient, microorganisms can show a reduced metabolic activity and growth rate, depending on their spatial location within the biofilm. [61, 68] As a result, some antibiotics will be only active against the outer, growing aerobic biofilm population (e.g. aminoglycosides, β-lactams, and fluoroquinolones). [7, 69] The term "persisters" refers to a subpopulation of tolerant microorganisms, able to survive very high concentrations of antibiotics. [51] Persistence is characterized by a biphasic time-kill curve (see Figure 2), as the majority of the population is rapidly killed whereas a subpopulation persists the treatment. [46, 70] Persistence is non-heritable: after removal of the antibiotic pressure, persister cells can resume growth and these growing cells will give rise to a heterogeneous population that is as susceptible as the original population, and again possesses a small proportion of persister cells. [46] Therefore, persister cells appear to be responsible for the recalcitrance of chronic infections. [70, 71]

2. Multispecies biofilms

2.1 Clinical relevance of multispecies biofilms

Early culture studies made it clear that not all species were able to grow in the culture media used. Therefore, the implementation of culture-independent techniques in the past decade had an impact on the ability to sample and identify microbiota as accurately as possible. [72, 73] These studies have revealed ever-increasing species richness and complexity, first thoroughly described for bacteria residing in the oral cavity. [74] Recently, the presence of multispecies biofilms has also been revealed, amongst others, in chronic wounds, in infections due to the use of (indwelling) medical devices, and in respiratory tract infections in cystic fibrosis (CF) patients. [20, 75]

Dermis infections, including burns, diabetic foot ulcers, and surgical-site infections are particularly prone to become chronic wounds. [76] Damaged tissues and proteins (collagen, fibronectin) present in chronic wounds can allow bacterial attachment [77, 78], as the bacterial glycocalyx binds to those tissues and proteins. [79] In combination with an impaired host immune response, bacterial attachment eventually leads to the formation of biofilms, resulting in non-healing wounds. [76, 80, 81] The predominant bacteria in chronic wounds include *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., *Pseudomonas* spp., *Enterococcus* spp., and various anaerobes (e.g. *Prevotella* spp.). [80, 81] Furthermore, fungi are also present, the most prevalent genus being *Candida*. [82] Usually two to five species reside together in a single ulcer. [77] Dalton et al. [83] found that wound closure was delayed in multispecies wound infections of *S. aureus* and *P. aeruginosa*, compared to in an infection with one species. Impaired wound healing could be the result of a down-regulation of the expression of keratinocyte growth factor 1 due to simultaneous infection with those two species, and increased expression of *S. aureus* virulence factors panton-valentine leukocidin and α-hemolysin in presence of *P. aeruginosa*, promoting pathogenicity and delayed wound healing. [84]

Contamination of abiotic surfaces (e.g. stethoscopes, keyboards, telephone handsets, bed rails, medical charts [85]) in the hospital environment with a number of bacterial pathogens and fungi, including methicillin-resistant *S. aureus* (MRSA), *Staphylococcus epidermidis*, β-hemolytic streptococci, *P. aeruginosa*, *Acinetobacter* spp., and *C. albicans* [85-87], forms a daily clinical challenge and a considerable healthcare risk. [85] In addition, the use of medical devices, for example an endotracheal tube (ET) for mechanical ventilation in critically ill patients, also holds a risk for multispecies biofilm formation. [88] These biofilms are hard to remove and form a source of chronic infection. [88] Species simultaneously recovered from ET are, among others, *S. aureus*, *P. aeruginosa, Candida* spp., *E. coli* and *S. epidermidis*. [89, 90] Furthermore, primary colonization could increase the risk for subsequent colonization by other microorganisms , e.g. primary colonization of burn wounds

with *S. aureus* is quickly followed by colonization with *P. aeruginosa*, and fungal infections in the later stages. [91] Flexible endoscopes have also been described to be associated with outbreaks of multiple infectious organisms [92], e.g. *P. aeruginosa* [93] and *E. coli* [94].

Clinically relevant multispecies biofilm communities are also found in the lungs of patients with CF. [95] Already in 1977, scientists began to realize that sessile bacteria were also directly related to lung disease when *P. aeruginosa* aggregates were found in sputum from CF patients. [96, 97] CF is the most common autosomal recessive disorder in Caucasian, affecting one in 2500 newborns. It is characterized by a defect in the cystic fibrosis transmembrane conductance regulator protein (CFTR) which results in defective or absent chloride channels. [98, 99] A healthy functioning lung will have a thin, hydrated, biphasic mucus layer above the cilia of the epithelial cells. The top layer is slightly more viscous and serves to trap bacteria and particles that enter the lungs, whereas the more fluid bottom layer allows cilia to beat within it, pushing the entire mucus layer up the lung for expectoration. As a result, the lungs will be cleared from bacteria and debris. [98] In CF patients, due to the defect in CFTR, there is an increased salt concentration in the airway surface fluid. This higher salt concentration inhibits the activity of antimicrobial peptides and proteins from the innate immune system. [100] As a consequence, bacteria are able to colonize the lungs. A high salt concentration will also lead to impaired mucociliary clearance, contributing to increased mucus levels. [98] Within this thick mucus, a steep oxygen gradient will form with hypoxic or anoxic regions [101] and clearance of bacteria that were able to colonize the lungs, will be impaired, stimulating the persistence of bacteria. [69] As a result, biofilm infections can survive for decades in CF lungs. These infections are characterized by the loss of virulence and a phenotypically adaptation towards chronic infection, due to the accumulation of mutations. [7]

The lung can be divided into two distinct parts, as shown in Figure 3: the smaller conductive zone and the larger respiratory zone. The conductive zone (150 ml, 5% of the lung volume) includes the upper part of the lung, including trachea, small and large bronchi and terminal bronchioles. This part has cilia and submucosal glands, producing mucus. The respiratory zone (3000 ml, 95% of the lung volume) includes the alveolar ducts, alveolar sacs, and respiratory bronchioles. This section has no cilia and no submucosal glands. [98, 102] Inhalation therapy will mainly target the conductive zone, whereas systemic antibiotic therapy mainly targets the respiratory zone. [103] Lower airway inflammation is considered as a hallmark of patients with CF, and will increase with age and disease progression. [101] In CF patients that underwent aggressive antibiotic therapy, lungs could not be cleared, and bacteria were mainly found in the conductive zone of the lungs. They were embedded aggregates in mucus, not adhering to the epithelial wall itself, and protected against antibiotics and host defenses. In the respiratory zone only small numbers of bacterial aggregates or planktonic cells

were found, indicating that the respiratory zone is protected from massive infection by the intensive treatment. In contrast, in chronically infected CF patients that were not intensively treated, bacterial aggregates were also observed in the respiratory zone. [80]

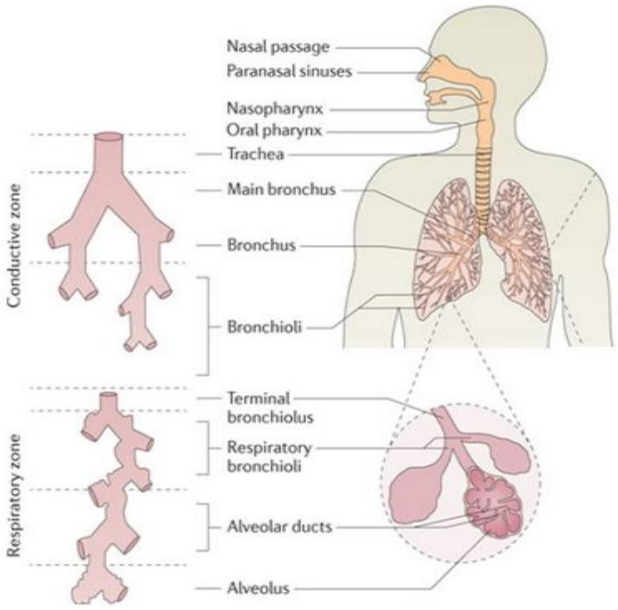

Figure 3: Anatomical regions from the human respiratory system, including the conductive zone and the alveolar region. [104]

Airway communities of young CF patients are highly diverse and dynamic. In CF lungs, bacterial clearance is impaired, stimulating the persistence of bacteria and the development of multispecies biofilms. [105, 106] At a later disease-stage, due to many factors including changes in nutrient availability and the host immune system, and interspecies interactions, bacterial diversity decreases (as shown in Figure 4) [107-109]. Over time, a decrease in bacterial diversity will correlate with progressive lung disease and decreased lung function. [101, 110] *S. aureus* was found to be the primary pathogen in pediatric patients, and possesses several virulence factors, including capsule formation, hemolysis, biofilm formation, and the prevalence of small colony variants (SCV). [101] During adulthood, *P. aeruginosa* will become the prevalent pathogen. [101] Colonization with *Burkholderia cepacia* complex (*Bcc*) is also observed in a smaller fraction of patients, and is associated with a worse prognosis and increased risk of death. [101, 111-113] When coinciding with a reduced colonization by *P. aeruginosa*, *S. aureus* in adults could be a marker for milder disease. [109] However, the frequency of *S. aureus* will steeply decline as *S. aureus* becomes displaced by *P. aeruginosa*. [107] Despite the evidence of effective early chronic suppressive treatment of *S. aureus*, this approach has been controversial. As a result of continuous anti-staphylococcal treatment, *S. aureus* colonization was reduced, but an earlier and more frequent occurrence of *P. aeruginosa* was observed. [114] In addition, there is increased local hypoxia in the lung due to CF specific increased epithelial O_2 consumption [115]. *P. aeruginosa* will respond to the hypoxic environment with

increased alginate production. As a result, local hypoxia is exacerbated and anaerobiosis results. [116] Furthermore, local hypoxia leads to an increased dependency on systems that can mediate the uptake of reduced, ferrous iron. [115] *P. aeruginosa* is able to switch from the pyoverdine/pyochelin siderophores to its FeO system using redox-cycling phenazines, and as a result is able to take up ferrous iron. [117] This could lead to an advantage of *P. aeruginosa* over other CF pathogens that are not as adapted at ferrous iron uptake. [109] By outcompeting *S. aureus*, *P. aeruginosa* can utilize iron released during the induced cell lysis of *S. aureus*, and thus *P. aeruginosa* benefits again. [118]

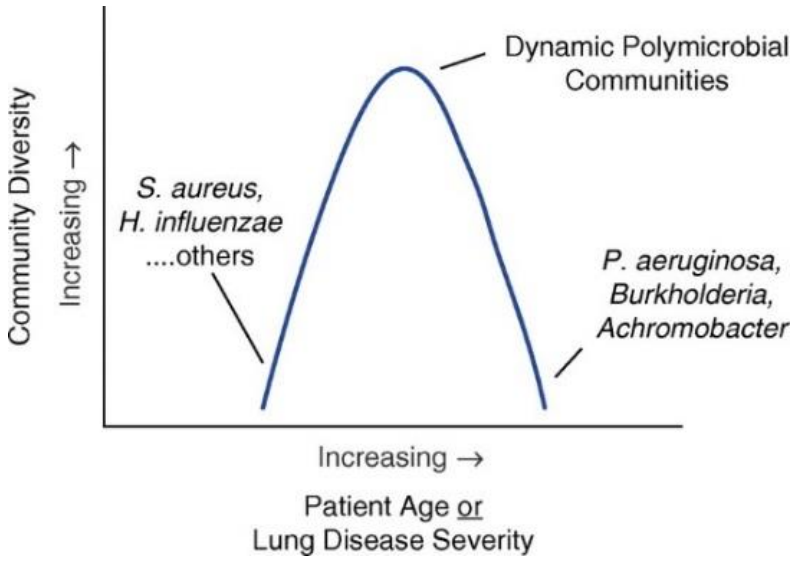

Figure 4: Bacterial community diversity versus patient age or lung disease severity. Initially, bacterial diversity increases with a peak in young adulthood. Afterwards, a decline in diversity is seen with advancing age and disease progression. [108]

Recently, the relevance of emerging pathogens likes species of the *Streptococcus milleri* Group (SMG), also known as the *Streptococcus anginosus* group, comprised of *Streptococcus anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius*, has been emphasized. [107, 119-123] Deep-sequencing studies identified species of the SMG as one of the three most abundant and prevalent organisms in CF sputum. [124] Species of the SMG have the capacity to trigger pulmonary exacerbations, as they are found to be numerically dominant at the onset of exacerbations. [122, 125, 126] Subsequent treatment of the SMG resulted in effective resolution of pulmonary exacerbations and a return to clinical stability. [125, 126] This suggests that SMG are relevant respiratory pathogens [127], both in children and adults. [119, 128, 129] Nevertheless, the mechanisms of SMG pathogenicity are not well understood. Hyaluronidases, deoxyribonucleases (DNases), ribonucleases, chondroitin sulfatase, gelatinase, and collagenase produced by SMG may contribute to tissue disruption and pathogenicity. However, the presence of species of the SMG is often underestimated, as these pathogens do not grow easily on routinely used laboratory media,

and if they do so, they are often dismissed as clinically irrelevant normal microbiota. [127] SMG are commonly isolated from mixed infections with *P. aeruginosa*. Interactions between *S. anginosus* and *P. aeruginosa in vitro* further suggest increased cell numbers of *S. anginosus* in co-culture with *P. aeruginosa*, together with an increased expression of virulence factors by *P. aeruginosa*. [130, 131] However, *P. aeruginosa* adaptation to chronic colonization of the CF lung is associated with a loss of virulence factors. [106] Nevertheless, CF patients chronically infected with *P. aeruginosa* still suffer from exacerbations and show a decline in lung function. This suggests that SMG may play a significant role in patients chronically infected with *P. aeruginosa*. [125] On the other hand, Filkins et al. [120] reported a positive correlation between the presence of the SMG and clinical stability, independent of the presence of *P. aeruginosa*. They further suggest that low SMG levels may increase the diversity of the CF lung, contributing to patient health, whereas excessive SMG levels may lead to increased pathogenicity and a clinical decline. Therefore, treatment not leading to a bacteriologic response in the perceived principal pathogens, could still lead to a clinical improvement during antibiotic therapy, as SMG may respond. [127]

In addition, the climax-attack model has been proposed for CF (Figure 5) [132], wherein *S. aureus*, non-mucoid *P. aeruginosa* and S*. anginosus* are described to be part of the attack communities. Attack communities are virulent, transient communities associated with exacerbations, that elicit strong immune responses and scarring. In the next step, climax communities (including mucoid *P aeruginosa*, chronic *S. aureus*, and *Achromobacter* spp.) will colonize the scar tissue and dominate during relatively stable periods. [132] However, *Achromobacter* spp. are also described to cause acute exacerbations. [133] Therefore, the grouping in the climax-attack model will depend on the patient population studied, and thus the climax-attack model cannot be seen as the golden standard.

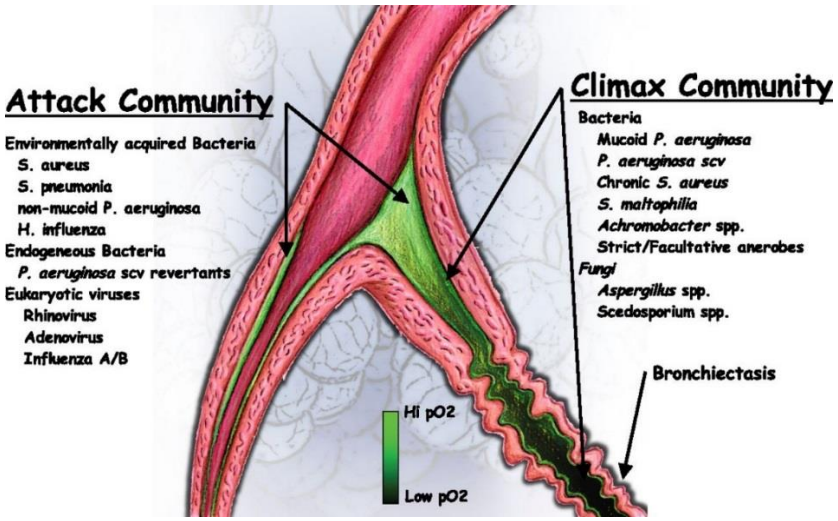

Figure 5: The Climax-Attack model. Community composition affects the surrounding environment, resulting in community stability during climax community growth and instability during attack community growth during exacerbation. [132]

Over the last decade, deep-sequencing results have identified dozens of other bacterial genera in one single sputum sample contributing to the complexity of the CF lung community [119, 123, 134, 135], including *Prevotella* spp., *Rothia* spp., *Neisseria* spp., *Stenotrophomonas* spp., *Achromobacter* spp., *Gemella* spp., and *Fusobacterium* spp., as well as diverse fungi, such as *Candida* spp. and *Aspergillus* spp. [107, 120, 136] Currently, the role of the microbiome in the pathogenesis of CF is being more and more investigated. [137] For example, anaerobic bacteria in the lower airways, common to both CF and healthy lungs, are recently described to contribute to the pathogenesis of lower airway disease in CF. [138] Furthermore, it was already reported that a lower community diversity correlates with worse lung function [119, 139], which might be due to the fact that the density of the remaining species increases to maintain a stable airway bacterial load.

2.2 Spatial organization in multispecies biofilms

2.2.1 Types of spatial organization

Three forms of spatial organization in multispecies biofilms can be observed, as shown in Figure 6. [140]

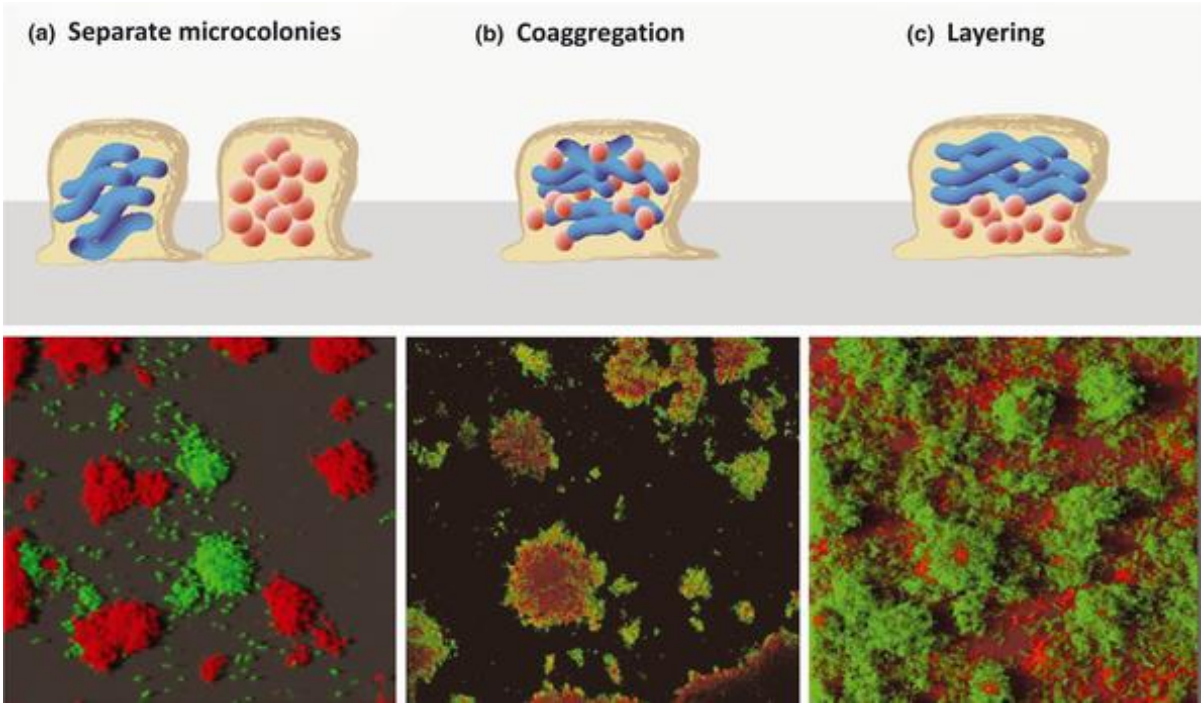

Figure 6: Spatial organization of multispecies biofilms: (a) separate micro-colonies, side by side; (b) co-aggregation; and (c) layering. [140]

(a) Separate micro-colonies

Separate micro-colonies (species segregation) are mostly seen in chronic infections. [141] For example, Burmolle et al. [80] confirmed that several bacterial species were present in the same chronic wound, but that the bacteria existed in monospecies aggregates within that wound. *S. aureus* was primarily located close to the wound surface, whereas *P. aeruginosa* was located deeper in the wound bed. [142]. In addition, Rudkjobing et al. [143] described almost complete segregation of the different bacterial species within CF lung sputum (Figure 7).

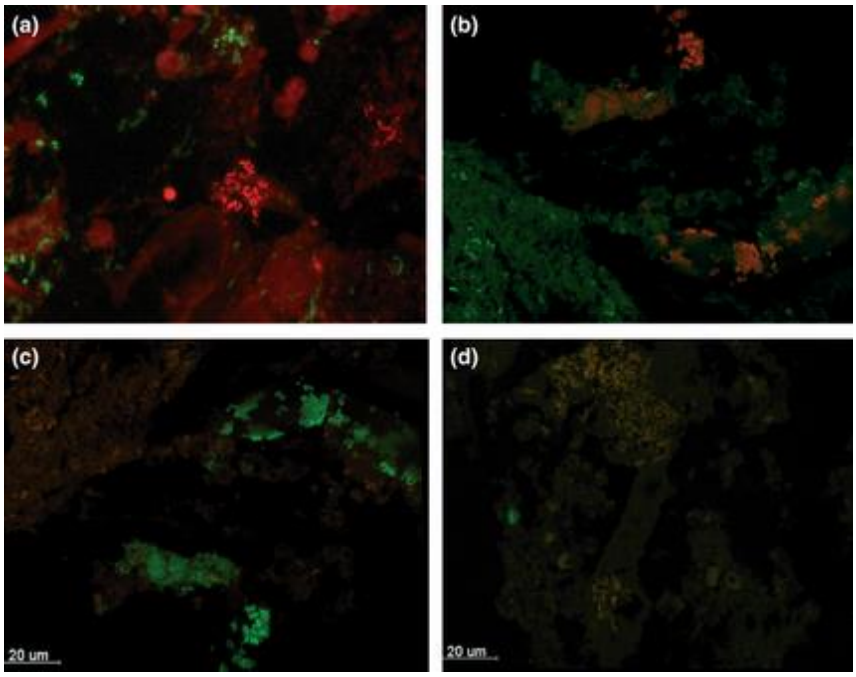

Figure 7: Microscopic visualization of bacterial aggregates/biofilms in expectorated sputum of CF patients. Frames (a), (c), and (d) show P. aeruginosa (red) and other bacterial species (green), and frame (b) shows S. aureus (red) and other bacterial species (green). The different species seem to be segregated from each other. [143]

Another example of separate micro-colonies *in vitro* can be seen in Figure 8, with a hospital isolate (isolated from an endoscope washer-disinfector) of *Bacillus subtilis* in red and *E. coli* SS2 in green. [144]

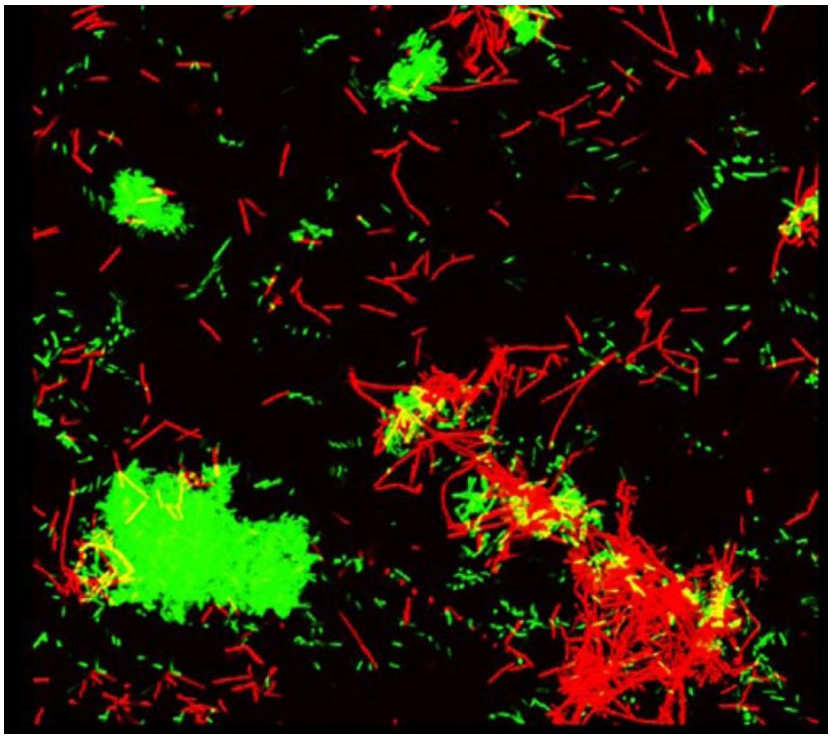

Figure 8: Separate micro-colonies of B. subtilis NDmed mCherry (red) and *E. coli* SS2 GFP (green), adapted from [144].

(b) Co-aggregation

In co-aggregation, the primary colonizer will migrate rapidly across the surface to colonize as much as possible through specific or non-specific physicochemical interactions. In suitable conditions, the primary colonizers will multiply and form micro-colonies. [8] Subsequently, these micro-colonies will be recognized by secondary colonizers, which will use adhesins located on the surface of the initial colonizers as an anchor to attach. [8, 145] Bacteria will intermix within the same aggregate, and live as co-aggregates in a multispecies biofilm. [80, 140] Co-aggregation is often seen in biofilms on medical devices and in dental biofilms. [141] An example of co-aggregation between *C. albicans* and Gram-negative and Gram-positive bacteria can be seen in Figure 9. [146]

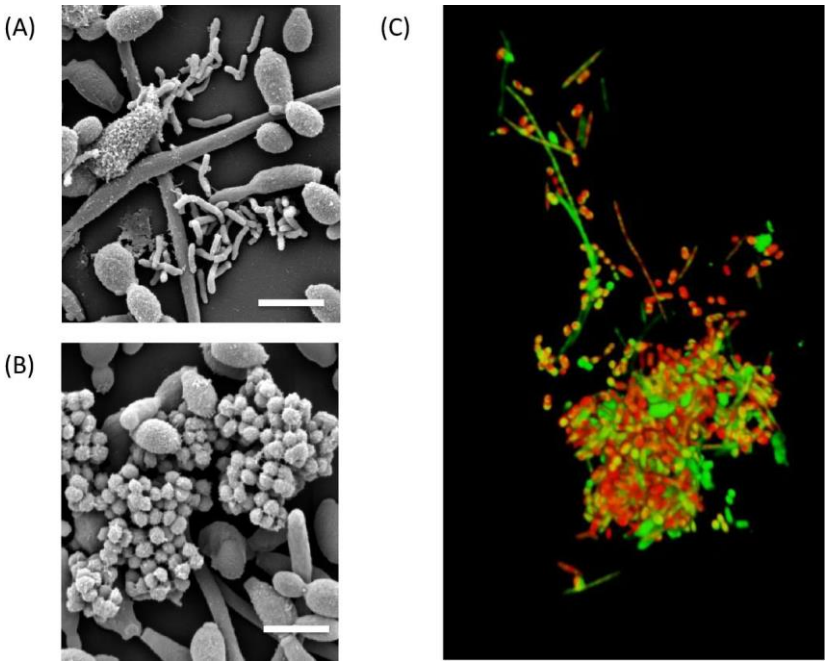

Figure 9: Spatial organization of C. albicans and bacteria, either (a) Gram-negative or (b) Gram-positive, or both (c). White scale bars = 20 μm. [146]

An increased production of EPS plays an important role in the adhesion process. The EPS envelop the attached cells, and can act as receptors for co-aggregation. [8] As a result of close contact, co-aggregation can lead to cooperative interaction with enhanced fitness, leading to increased growth. [16, 147] This form of spatial organization between dental plaque bacteria was already observed in the early 1970s. [8, 148] *Streptococcus gordonii* increases expression of genes involved in the synthesis of extracellular polymers that function as co-aggregation receptors for a surface adhesin expressed by *Porphyromonas gingivalis*. [140, 149] In addition, fimbriae of *P. gingivalis* play an important role in the mediation of co-aggregation with other bacteria in multispecies biofilm formation. [140] As a result, even species that do not form biofilms on their own, can attach in mixed species biofilms. [148] Furthermore, type IV pili of *P. aeruginosa* are shown to mediate multispecies biofilm formation with *S. aureus* by binding of these pili to eDNA of *S. aureus*. [148]

(c) Layering

In the third form, a layered structure can be observed. One species can be found in the upper layers of the biofilm, and another in the lower layers. [32, 140] This form of structural organization is for example seen with two populations that have different oxygen preferences [150]. It was described that an anaerobic *P. gingivalis* population was protected from oxygen by layering under a more aerotolerant *Treponema denticola* population. [151] The ability of species to provide an appropriate environment in a biofilm for another species thus enhances the establishment of a multispecies biofilm. [152] On the other hand, layering can also be a strategy to avoid competing bacteria, as seen

in 'surface blanketing'. [75] Here, one species will rapidly occupy all available adhesion sites, to prevent adhesion of another organism. [46] For example, a mucoid variant of *Pseudomonas fluorescens* overproduces EPS, and as a result, *P. fluorescens* will be on top of the biofilm to have more access to the oxygen, at the expense of competing strains. [153]

2.2.2 What is the reason behind the different spatial organizations of biofilms?

Local differences in selection pressure and nutrient availability could be an explanation for the observed spatial organization in multispecies biofilms. [141] For oral bacteria, loss of adhesion via co-aggregation would result in being swallowed due to shear forces. Therefore, there is a strong selective pressure for co-aggregation. [154] In environmental biofilms of freshwater strains, co-aggregation seemed to be subject to on- or off-switching by the microorganisms, as it was observed at various time points, but not continuously. This could indicate a form of environmental control of the expression of co-aggregation receptors and/or adhesins, upon starvation or stress. [8] Tolker-Nielsen et al. [33] showed that *P. aeruginosa* and *Pseudomonas putida* cells, when mixed, initially formed separate micro-colonies. However, with time, the cells migrated from one micro-colony to the other and intermixed. [2] These results suggest that the nutritional conditions present can influence structural development in a biofilm. [17] In addition, Nielsen et al. [155] studied *in vitro* mixed biofilm formation of *Pseudomonas knackmussi* and *Burkholderia xenovorans*. *B. xenovorans* produces chlorobenzoate, which can be metabolized by *P. knackmussi*. In medium enriched with chlorobiphenyl, which can only be metabolized by *P. knackmussi*, co-aggregates could be observed. In contrast, in medium with a high citrate concentration, which can be metabolized by both species, separate micro-colonies were present. The level of mixing (co-aggregation) will thus depend on the strength of interactions between the species, as reported by Estrela et al. [156]. Using an individual-based modelling framework, they described that weak metabolic interdependence leads to separate micro-colonies. [156] Furthermore, the motility of bacteria also plays a role. A lower motility, and as a result, lower cell migration and impaired intermixing, is observed when the viscosity is increased. For example, *S. aureus* can survive in wounds that also contain *P. aeruginosa*, possibly because the wound environment is highly viscous and restrictive of cell migration, preventing intermixing. [16] In addition, polysaccharides of the EPS can also influence the multispecies biofilm organization. [157] Pel and Psl, two major polysaccharides in *P. aeruginosa* play a distinct role in the organization of multispecies biofilms of *P. aeruginosa* and *S. aureus*. When both species are in close proximity, Pel is involved, whereas in a layered structure, with *P. aeruginosa* on top of *S. aureus*, Psl is involved. [157] Pel is shown to reduce effective cross-linking within the *P. aeruginosa* matrix. As a result of this increased matrix loosening, *S. aureus* might be able to infiltrate and to associate with *P. aeruginosa*, or vice versa. On the other hand, Psl is shown to increase the elasticity and effective cross-linking

within the *P. aeruginosa* matrix, which might confer a physical barrier that does not allow for interaction with *S. aureus.* [157]

Another explanation for the spatial organization in separate micro-colonies sometimes seen in chronic infections could be the restricted bacterial diversity due to the key challenge to survive the encounter with the host immune system. [141] The host immune system is activated by the primary pathogen, decreasing the chances of another pathogen to survive and to mix in-between the pre-existing chronic biofilm infection. [80] Furthermore, during chronic infection, a lot of dead cells are present, together with a constant blood supply. This results in a high nutrient availability and as a consequence, there is only a low selection pressure for co-aggregation, since it is not crucial for growth and proliferation of the colonizing single species. [141] Furthermore, living in separate micro-colonies can represent an advantage for bacteria as one species can be protected from disadvantageous interactions with another. [16] For example, prolonged co-existence is seen in the presence of a toxin-producing neighbor, as the population is better protected against these toxins in the separate micro-colony. [145, 158]

2.2.3 Role of bacterial growth rate in multispecies biofilm formation

Bacterial growth rate also plays a role in the formation of a multispecies biofilm. [145] In 1991, Banks and Bryers [159] stated that establishment of a second organism in an existing monospecies biofilm, depended on the relative growth rates, with the faster-growing organism, *P. putida*, becoming the dominant species, but allowing co-existence of the slower growing one, *Hyphomicrobium* sp.. Another example was reported by Komlos et al. [160] in 2005. They observed that in dual species biofilms of the aerobic *Burkholderia cenocepacia* and the facultative aerobic *Klebsiella oxytoca*, *B. cenocepacia* dominated at low substrate concentrations because of a higher growth rate, whereas in high substrate concentrations, *K. oxytoca* gained advantage as the increased biomass resulted in lower oxygen levels, disadvantageous for *B. cenocepacia*. [160] On the other hand, Stewart et al. [161] investigated mixed biofilms of *P. aeruginosa* and *Klebsiella pneumoniae* and reported that both species were able to co-exist, although *P. aeruginosa* growth rates were much slower in the mixed biofilm.

2.3 Role of QS in multispecies biofilms

2.3.1 Role of AI-2 QS molecules

Neighboring cells can interact with each other during biofilm formation, for example through QS. [140] Most of these QS systems promote intraspecies communication. [162, 163] AI-2 is described to be recognized by different bacterial species, indicating that AI-2 could be a more universal signal that could promote interspecies communication. [163, 164] However, Rezzonico and Duffy [165]

demonstrated that the presence of AI-2 receptors is primarily limited to a narrow group of bacteria, even though alternative receptors might be present, and that previous performed tests are insufficient to demonstrate the existence of an AI-2 based communication. In addition AI-2 is also known to directly contribute to the metabolism [165], as AI-2 is a byproduct of the activated methyl cycle, suggesting an alternative role for AI-2 than being a universal language for interspecies communication. [166]

Nevertheless, interspecies communication is described to have a positive effect on multispecies biofilm formation, for example, it was reported that biofilm formation of *Moraxella catarrhalis* is promoted by AI-2 QS signals produced by *Haemophilus influenzae*. [167] In addition, McNab et al. [168] described the promotion of biofilm formation of *P. gingivalis* by AI-2 signals produced by *S. gordonii*. On the other hand, a negative effect on biofilm formation through AI-2 is also described, for example, AI-2 produced by *Aggregatibacter actinomycetemcomitans* inhibits biofilm formation of *C. albicans*. [169] In addition, Jang et al. [170] reported that AI-2 of *Fusobacterium nucleatum* differentially regulated biofilm growth of two oral streptococci. Growth of *S. gordonii* was enhanced, whereas growth of *Streptococcus oralis* was decreased. [170]

In addition, not all relevant pathogens produce AI-2 molecules. *P. aeruginosa*, for example, does not produce AI-2 signal molecules [171], but might be able to react to the presence of AI-2, as described by Li et al. [172] They showed that the addition of synthetic AI-2 to *P. aeruginosa*, at low concentration, resulted in increased biofilm formation. In addition, they speculated that the presence of *Klebsiella* spp. and *Streptococcus* spp. (both AI-2 producers) in e.g. ventilator-associated pneumonia (VAP), could influence biofilm formation of *P. aeruginosa*, also a common cause of VAP, through AI-2. [172]

2.3.2 Role of AHL-molecules

Interspecies communication can also occur through other QS signals, for example, some *Bacillus* spp. are described to have AHL-degrading capacity, which can result in the inhibition of biofilm formation of other pathogenic species, such as *P. aeruginosa*. [144, 173] In addition, 3-oxo-C_{12}-HSL, an AHL produced by *P. aeruginosa* is also described to interfere with the *S. aureus* cytoplasmic membrane and to down-regulate *S. aureus* exotoxin production and expression of the accessory gene regulator (agr). [174] Furthermore, *B. cenocepacia* is capable of perceiving AHL signals produced by *P. aeruginosa*, whereas *P. aeruginosa* does not respond to *B. cenocepacia* AHLs. These results indicate that AHL-mediated cross-talk between these two pathogens, albeit unidirectional, can play an important role in the virulence and biofilm formation of a mixed community of *P. aeruginosa* and *B. cenocepacia*. [174]

2.3.3 Role of other QS molecules

In addition, a signal peptide produced under QS regulation by *Streptococcus mutans* was described to control the production of a bacteriocin inhibitory to *S. gordonii*. In reaction, *S. gordonii* interferes with the signal peptide production. [175] Furthermore, besides AI-2, the agr system of staphylococci is also suggested to be an inter-species communication system, as for staphylococci, it has been described that the *S. aureus agr* locus is highly responsive to AIP's of other staphylococcal spp., which cross-inhibit the agr system of *S. aureus*, resulting in a decreased virulence of *S. aureus*. [176] Furthermore, as a response to diffusible signal factor (DSF), a fatty acid signal molecule secreted by *B. cenocepacia*, *P. aeruginosa* reorganizes as filaments in a mixed biofilm. [16] In addition, another DSF-like signal of *B. cenocepacia*, cis-2-dodecenoic acid, was described to reduce hyphal growth of *C. albicans*. [177]

2.3.3 Role of QS in the cystic fibrosis environment

Duan et al. [171] described the detection of AI-2 in sputum of CF patients, produced by avirulent oropharyngeal flora, increasing the viability of *P. aeruginosa*, and enhancing lung damage caused by this pathogen. Furthermore, QS signals also play an important role in the relationship between *S. aureus* and *P. aeruginosa* within CF airways. [178] Through the agr QS system, *S. aureus* enhances the formation of *P. aeruginosa* SCVs in co-culture, genetic mutant strains with defects in their electron transport chain, but the precise mechanisms remain unclear. [109, 179] The presence of SCV of *P. aeruginosa* is associated with increased adhesion to respiratory cells, increased biofilm formation, higher antimicrobial resistance and increased damage to host cells. [180-183] On the other hand, the production by *P. aeruginosa* of pyoverdine, pyochelin, hydrogen cyanide, and 2-alkyl-4-(1H)-quinolones (AQs), including 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), were found to play a major role in the killing of *S. aureus*, as shown by using deletion mutants. [118, 184] Through the production of siderophores and HQNO, and due to the oxygen competition, *P. aeruginosa* inhibits the oxidation of cytochrome *b* and the reduction of cytochrome aa_3, and sequesters iron, necessary as a heme cofactor for cytochrome, and competes for the terminal electron acceptor. These events result in the inhibition of the electron transport chain of *S. aureus*. As a response, *S. aureus* will switch from acetate production (aerobic respiration) towards lactate fermentation. *P. aeruginosa* can benefit from lactate fermentation by S. aureus, by preferentially consuming the lactate. The reduced energy production by fermentation and the shift in pH due to lactate accumulation leads to a growth disadvantage for *S. aureus* and may increase the susceptibility of *S. aureus* towards antimicrobial factors produced by *P. aeruginosa*. [107] As this interaction requires siderophore-mediated iron uptake by *P. aeruginosa*, it demonstrates the importance of iron. [80, 109] In contrast, Fugere et al. [184] reported that production of HQNO could enhance biofilm formation by *S. aureus*. Increased

biofilm formation could then provide some pressure on *P. aeruginosa* to select for HQNO-deficient strains. Furthermore, HQNO and pyocyanin also select for *S. aureus* SCV, which rely on fermentation as a primary energy source, and are well adapted to the fermentative lifestyle, leading to increased persistence of *S. aureus* in chronic infections in the presence of *P. aeruginosa*. As SCV's are poorly detectable, the presence of *S. aureus* may be underestimated. [107, 118, 178, 185, 186] Qazi et al. [187] reported that *P. aeruginosa* AHL QS molecules also affect *S. aureus*, as long chain AHLs could reduce exotoxin production of *S. aureus* and *agr* and *sarA* expression, involved in biofilm formation. In addition, *Pseudomonas* quinolone signal (PQS) and its precursor 4-hydroxy-2-heptyl-4-quinolone (HHQ), show antimicrobial activity against *S. aureus*. [188-190] PQS is able to chelate iron, thus creating iron depletion and consequently leading to growth suppression of *S. aureus*, indicating again that iron has a major role in antimicrobial activity of *P. aeruginosa* against *S. aureus*. [190, 191] Furthermore, PQS-induced phenazine molecules of *P. aeruginosa* are also described to inhibit *E. coli* growth, likely due to the inhibition of cellular respiration, and due to the induction of reactive oxygen radicals in *E. coli* cells that are still able to grow aerobically. [192]

As CF lung disease progresses, *P. aeruginosa* adapts to the lung. It undergoes substantial changes, including the loss of virulence factors. [107, 190, 193, 194] *P. aeruginosa* strains isolated after several years of chronic lung infections seem to be deficient in HQNO production, which could be the result of the effect of the lung environment, or due to the production of 2-amino acetophenon (2-AA) by *P. aeruginosa*. [184, 195] 2-AA selects for the emergence of *las*R mutants [195], with reduced HQNO production [196]. Reduced HQNO production could also be due to the production of alginate by mucoid *P. aeruginosa* strains, as alginate has been shown to reduce the production of AHLs and PQS [197], leading to a lower expression of siderophores, HQNO and rhamnolipids [198], all required for efficient killing of *S. aureus* [199]. As a result, these *P. aeruginosa* strains show a reduced capacity to outcompete *S. aureus*, which is reflected in the fact that although *S. aureus* usually precedes colonization by *P. aeruginosa*, it is still found in cultures of 51% of the adult patients. [200]

Furthermore, *P. aeruginosa* and *C. albicans* are frequently co-isolated from CF patient sputum, even though the clinical relevance of their relationship is unclear. [201-203] *P. aeruginosa* was found to kill *C. albicans* hyphae, in part through production of phenazine derivatives which affect cell wall integrity, whereas *C. albicans* in the yeast form was resistant to killing. [204, 205] By doing so, *P. aeruginosa* will use *C. albicans* as a fungal scaffold upon which it can form a biofilm. [201] This was proven by *in vivo* rat experiments which demonstrated that primary colonization of lung tissue with *C. albicans* increased *P. aeruginosa* pneumonia rates, by the formation of a secondary biofilm leading to disease. [201] On the other hand, during the acute phase of infection, the *P. aeruginosa* QS signal 3-oxo-C_{12} inhibits the ability of *C. albicans* to switch from yeast to hyphae, important for *C. albicans*

biofilm maturation. [206-208] However, during chronic infection, *P. aeruginosa* 3-oxo-C_{12} levels drop, and *C. albicans* is able to switch to filamentous growth, and subsequently to form a mature biofilm with potential invasive growth. [209] In addition, the production of the QS molecule farnesol by *C. albicans* leads to a reduced signal production (resulting in e.g. reduced pyocyanin production) and swarming motility of *P. aeruginosa*, two of *P. aeruginosa* major virulence factors. [206] The reduction in swarming motility might be mediated via rhamnolipids. Rhamnolipids are required for swarming and are partly regulated by *P. aeruginosa* QS signals, downregulated by *C. albicans* farnesol. [209] This interaction has also been observed between *Acinetobacter baumanii* and *C. albicans*. Like *P. aeruginosa*, *A. baumanii* will also inhibit filamentation and biofilm formation, key virulence factors of *C. albicans*, leading to attenuated virulence of *C. albicans* and increased nematode survival after infection with both pathogens. [210] Conversely, farnesol produced by *C. albicans* inhibits *A. baumanii* growth, likely an evolutionary defense. [210]

2.4 Interactions within multispecies biofilms

Dynamic interactions in multispecies biofilms render organisms in the biofilm more robust to environmental fluctuations and efficient in the production or degradation of a variety of compounds, resulting in the maintenance of the stability of the multispecies community and the persistence of biofilms. [61, 75, 145, 211, 212] According to West et al. [213], based on the effect of social behavior on each population (actor and recipient) in a binary system, interactions can be roughly divided into competition or cooperation. When the recipient benefits, the interaction is termed cooperation, resulting in mutualism (beneficial for both actor and recipient), commensalism (beneficial for recipient, no effect on actor), and altruism (beneficial for recipient but costly to actor) (Table 1). [211, 212, 214] Foster et al. [215] applied a more stringent definition of between-species cooperation, namely that both species must increase their productivity in co-culture, i.e. that two strains must produce more than the sum of the biomass of the individual strains, to be cooperative. In contrast, when the recipient is negatively affected, the interaction is termed competition, resulting in selfishness (beneficial for actor) or spite (negative effect on actor) (Table 1). [9, 214] Examples of interspecies interactions will be described in the next paragraphs.

Table 1: Interactions between multiple species, selected for by natural selection. Adapted from [216].

	Effect on recipient	
Effect on actor	*+*	*-*
+	mutualism	selfishness
-	altruism	spite
neutral	commensalism	

2.4.1 Effect of interactions on metabolism and growth

2.4.1.1 Cooperation and cross-feeding

In general, interspecies metabolic interactions within a multispecies biofilm will affect biofilm development and structure. [217] Cooperation typically leads to an organization wherein all species are located close together (co-aggregation). [145] As a result of direct contact between cells, stronger reciprocity is seen, which facilitates the exchange of metabolites. [211]. During initial co-colonization, the degree of interactions will be minimal, but will increase as the community grows and different micro-colonies come into closer proximity. [156, 211] Spatial organization of a multispecies biofilm thus plays a major role in the outcome of interspecies interactions. [140] Lee et al. [217] demonstrated that by cooperating, when resources are limited, a multispecies biofilm community can maximize and optimize the use of nutrients to enhance its growth and persistence (mutualism), and thus has a physiological advantage compared to a monospecies biofilm. [217] In addition, when metabolites produced by one species, which are of no benefit to this producer, are released as waste products in the local biofilm environment, they can contribute to a change in local composition. These changes can subsequently lead to conditions favoring neighboring species, leading to a commensal interaction, known as metabolite "cross-feeding", and resulting in a change of the abundance of the population. [145, 218] For example, ammonia generated by *Fusobacterium nucleatum* and *Prevotella intermedia* increase the pH to a level enhancing growth of *P. gingivalis*. [219] Another example of cross-feeding is the formation of a dual species biofilm of *Acinetobacter* sp. strain C6 and *P. putida* R1 in limiting concentrations of benzyl alcohol. *Acinetobacter* strain C6 will establish in the upper layer of a biofilm, allowing leakage of excess benzoate into the lower layers of the biofilm, where benzoate is metabolized by *P. putida* R1. However, if both species were grown in a system where they could not establish a fixed position, competition for benzyl alcohol was seen, and *Acinetobacter* sp. strain C6 eventually out-competed *P. putida* R1. [220] Furthermore, "food for detoxification" is seen in the mutualistic relationship between *A. actinomycetemcomitans* and *S. gordonii*. *S. gordonii* produces lactate, which is used by *A. actinomycetemcomitans* ("cross-feeding"), and in exchange, *A. actinomycetemcomitans* detoxifies peroxide, a by-product of *S. gordonii* metabolism. Both species will co-aggregate, with *A. actinomycetemcomitans* surrounding *S. gordonii*, reaching higher burdens during co-infection than in mono-infection. Nevertheless, by sensing peroxide and subsequent production of dispersin B, *A. actinomycetemcomitans* will maintain a > 4 µm gap from *S. gordonii* to avoid growth inhibition by peroxide. [221] Similar to cross-feeding of *A. actinomycetemcomitans* by *S. gordonii*, Stacy et al. [222] described an example of "cross-respiration". *A. actinomycetemcomitans* shifted from a primarily fermentative to a respiratory metabolism that enhanced its growth yields and persistence, as a result of an enhanced

bioavailability of oxygen provided by *S. gordonii*. These results indicate that a commensal organism can provide electron acceptors that promote the respiratory growth and fitness of other pathogens. [222] Furthermore, the development of aerobic-anaerobic mixed biofilms is facilitated in the wound environment due to a low oxygen tension. [223] *S. aureus* and *K. pneumoniae* promote growth and virulence expression of vitamin K-dependent *Prevotella* spp., by providing hemin, vitamin K, and succinate. In the presence of glucose, especially in diabetes patients, succinate production by *K. pneumoniae* is enhanced, and as a result, an increased presence of *Prevotella* spp. is seen. Furthermore, Whiteson et al. [224] proposed a model of cross-feeding throughout the CF airways between *Streptococcus* spp. and phenazine producing *P. aeruginosa*. In low oxygen, low pH, and QS conditions, *Streptococcus* will activate its acetoin metabolism and produce 2,3-butanedione and 2,3-butanediol to avoid lethal acidification. As a result, phenazine producing *P. aeruginosa* strains will use these products as a carbon source and will form biofilms with architectures that increase surface area, leading to an increased access to oxygen. These *P. aeruginosa* strains will also increase phenazine production, subsequently increasing reactive oxygen species generation and providing additional electron acceptors. These data support the role of phenazine as an alternative electron acceptor in oxygen limited conditions. As a result, increasing 2,3-butanedione production and survival of *Streptococcus* is observed. [224]

In addition, cross-kingdom interactions leading to growth promotion are also described. [225-227] For example, *C. albicans* strains that are actively respiring will reduce oxygen levels and therefore provide an ideal growth environment for oral streptococci, whereas the latter will provide nutrients promoting fungal growth. [225] In contrast, fungal-bacterial communities can also inhibit growth of one community member, for example, commensal *Lactobacillus* spp. present in the female reproductive tract of 96% of healthy women, secrete organic acids and produce hydrogen peroxide, thereby inhibiting *C. albicans* growth and virulence. [227]

2.4.1.2 Exploitation competition

In contrast, when bacterial species are growing together in a multispecies biofilm, nutrients and space can become limited and interactions can become competitive. By consuming essential nutrients, the dominant species can inhibit growth of others, providing a competitive advantage. [46, 75] This form of competition, involving rapid utilization of the limited nutrients without direct interaction, is also called “exploitation competition” or “scramble competition” [228]. For example, *P. aeruginosa* inhibits growth of *E. coli* through the sequestration of iron in the environment by *P. aeruginosa* siderophores. [192] Inhibition of *S. aureus* due to iron sequestration by *P. aeruginosa* was described too (see Section 2.3). [190] Exploitation competition is also seen with “cheaters”. [229] Cheaters can emerge when a population benefits from the cooperative traits expressed by

neighbors (“common goods”), without providing the energy required to produce and secrete the resources themselves (‘‘selfish” behavior of cheaters, “altruistic” behavior of producers). [229, 230] For example, *Vibrio cholerae* secretes diffusible chitinases to digest chitin as primary food source. This chitinase secretion, which carries a fitness cost, was shown to be exploitable by mutants that did not secrete chitinases. [231] Cheating is tolerated to a certain extent, however, when too many cheaters are present in the population, cheating can result in the “tragedy of the commons” or depletion of the common goods, indicating that there is a delicate balance between cooperators and cheaters. [232] Cheating fits in the theory of kin selection, first proposed by W.D. Hamilton in 1964 [233, 234], which holds that altruistic cooperative behavior is favored as it helps relatives to do better. [235] If relatedness is high, cooperators are shown to interact with cooperators and to spread, whereas when relatedness is low, cooperators and cheaters interact, allowing the cheaters to exploit cooperators and to spread. [236, 237] Furthermore, Stacy et al. [16] described that different species (nutrient-producers and cheaters) often intermix upon high nutrient availability, however, in nutrient-depleted conditions, the number of dividing cells is reduced and the different species will segregate into distinct sectors. In this way, nutrient-producers could avoid and out-compete cheaters and the benefits will fall preferentially on the nutrient-producing cells. [238] Cheating is for example also seen in *P. aeruginosa* populations in chronically infected patients. Strains with mutations in *lasR*, which regulates extracellular functions including the production of antimicrobial molecules (e.g. hydrogen cyanide and pyocyanin), the cytochrome inhibitor HQNO, and proteases, spread in the population as they benefit from production of these products by their wildtype neighbors, avoiding the cost of production. [228] Recently, Morris et al. [239] proposed the “Black Queen Hypothesis”, suggesting that on one hand extracellular secreted products are unavoidably present in the environment, benefitting others, for example, the costly production of siderophores. [40] On the other hand, the hypothesis also considers that species have lost costly vital functions or pathways, if these functions or pathways are provided by the neighboring bacteria, which resulted in the enhancement of their fitness. As a consequence, dependency is developed, and these species are expected to perform better in a multispecies biofilm than in a monospecies biofilm. [9, 229]

2.4.2 Effect of interactions on antimicrobial susceptibility

2.4.2.1 Protection against antimicrobial agents

(a) Enzymatic antibiotic degradation

An important form of cooperation in multispecies communities with an effect on antimicrobial susceptibility is "indirect pathogenicity" (also termed "passive resistance"). [240, 241] Indirect pathogenicity is a protection at a distance against antimicrobial agents. [242] This protection is due to antibiotic degradation by a resistant species, which enables growth of a neighboring susceptible pathogen, that is the intended target of the antimicrobial treatment. [241-243] Several examples of indirect pathogenicity are described in literature and are shown in Table 2.

Table 2: Examples of indirect pathogenicity described in literature.

Species protected	**Protected**		**Produced by**	**Reference**
	against	**by**		
H. influenzae	Ampicillin	Penicillinases	Enterobacteriaceae	[244]
streptococci	Penicillin	Penicillinases	staphylococci	[245]
ampicillin-susceptible *S. aureus*	Ampicillin	Penicillinases	*P. aeruginosa*	[246]
H. influenzae	β-lactam antibiotics	β-lactamases	*M. catarrhalis*	[167]
S. pneumoniae	β-lactam antibiotics	β-lactamases	*M. catarrhalis*	[247]
P. aeruginosa	Imipenem, ceftazidime	β-lactamases	*S. maltophilia*	[248]
S. pneumoniae	Amoxicillin	β-lactamases	*H. influenzae*	[249]
E. coli, *K pneumoniae* and *P. aeruginosa*	Carbapenems	Carbapenem-hydrolyzing class D β-lactamases	*A. baumannii*	[250]
S. pneumoniae	Chloramphenicol	Chloramphenicol acetyltransferase	Resistant *S. aureus* /*S. pneumoniae*	[243]
K pneumoniae and *P. protegens*	Sodium dodecyl sulfate	Hydrolases	*P. aeruginosa*	[217]
S. aureus	Gentamicin	Aminoglycoside-modifying enzymes	*P. aeruginosa*	[76]

One of the best known examples of "indirect pathogenicity" is the protection of one species from killing by β-lactam antibiotics through β-lactamases produced by another species. Maddocks et al. [244] were the first to describe this phenomenon. In the past decades, several other cases have been described (Table 2). [16, 167, 245-248, 251, 252]

Even though protection of *Streptococcus pneumoniae* against amoxicillin treatment was observed through β-lactamases produced by *H. influenzae* [249], Westman et al. [253] could not observe a protective effect of β-lactamase produced by another *H. influenzae* strain on the susceptibility of *S. pneumoniae* towards amoxicillin, indicating that the effect may depend on the type of β-lactamases produced, and on the producing microorganism. [254]

Besides for β-lactam antibiotics, indirect pathogenicity is also described for other antimicrobial agents. For example, Sorg et al. [243] described that chloramphenicol resistant *S. pneumoniae* and *S. aureus* strains, expressing chloramphenicol acetyltransferase (CAT), were able to protect susceptible *S. pneumoniae* cells by intracellular antibiotic degradation of chloramphenicol. Strikingly, susceptible cells were able to outcompete resistant bacteria during antibiotic therapy when the fitness cost of CAT expression became too high, therefore, passive resistance is only sustainable when the expression comes at a modest fitness cost.

Interactions between *S. aureus* and *P. aeruginosa* have been extensively described in the context of CF. Very often, presence of *P. aeruginosa* has been correlated with a decrease in *S. aureus* cell number. [107, 190, 255-258] However, the presence of *P. aeruginosa* could also lead to protection of *S. aureus*. For example, Deleon et al. [76] determined the antibiotic susceptibility of *S. aureus* and *P. aeruginosa* when grown together in a chronic wound model. For *P. aeruginosa*, no difference was seen, whereas *S. aureus* showed enhanced tolerance towards gentamicin and ciprofloxacin. They proposed that aminoglycoside-modifying enzymes excreted by *P. aeruginosa* could have inactivated gentamicin. [76] In contrast, Nicoloff et al. [242] demonstrated passive resistance of *Micrococcus* spp. and *E. coli* towards macrolides, tetracycline and chloramphenicol, but not towards aminoglycoside antibiotics. β-lactamases are localized in the periplasm or excreted in the medium, and their hydrolysis mechanism, which involves water molecules, does not consume energy. On the other hand, aminoglycoside-modifying enzymes are located in the cytoplasm and their modification reactions are energy-consuming and involve complex co-factors. Therefore, efficient drug degradation, and as a consequence, passive resistance, might depend on the ability of the intracellular enzymes to reach and modify, or to destroy their antibiotic target. [242, 243]

(b) Decreased antimicrobial penetration

B. subtilis isolated from endoscope washer-disinfectors showed resistance against high concentrations of peracetic acid used in these devices. As a result, *B. subtilis* was able to protect *S. aureus* within a multispecies biofilm during treatment with this oxidizing agent. [92] A possible explanation could be the fact that *B.* subtilis produces an amphiphilic protein, BslA, which forms a protective hydrophobic coating at the interface with the air, preventing penetration of biocides and

protecting other species present in the biofilm. [259] Interactions between *P. aeruginosa* and *B. cenocepacia* have also been described to lead to altered antibiotic resistance. Polysaccharides produced by *B. cenocepacia* can interact with those produced by *P. aeruginosa* and lead to a firmer multispecies biofilm, leading to a decrease in diffusion of antibiotics, and consequently to an increased resistance. [145, 260]

In addition, De Brucker et al. [261] reported that β-1,3-glucan from *C. albicans* decreased ofloxacin susceptibility of *E. coli* in a multispecies biofilm. A decreased penetration rate of ofloxacin into the biofilm could be an explanation for the observed protection. On the other hand, *C. albicans* susceptibility towards amphotericin B and caspofungin was not altered in presence of *E. coli*. Furthermore, *S. aureus* was shown to be preferentially associated with the hyphae of *C. albicans* through binding to surface-associated adhesins. [262] More specifically, the agglutinin-like sequence 3 (Als3p), a *C. albicans* hyphae-specific adhesin, has been reported to be involved in the co-adherence process. [263] Co-colonization with *C. albicans* hyphae provides *S. aureus* with the means of epithelial penetration, as *C. albicans* adheres to and penetrates tissue via its invasive hyphae. This phenomenon is described as "microbial hitchhiking". [264] As a result, *S. aureus* becomes coated by extracellular matrix (and more particularly, β-1,3-glucan [265]) secreted by *C. albicans*, which results in an enhanced resistance of *S. aureus* to vancomycin. [262] Enhanced tolerance was also observed towards oxacillin and nafcillin. [265] For *C. albicans*, no difference in resistance to amphotericin B was seen when grown together with *S. aureus.* [266] "Microbial hitchhiking" has also been described by Pammi et al. [267] for *S. epidermidis* and *C. albicans*, as *S. epidermidis* can also adhere to both yeast and hyphae of *C. albicans*. As a result, *S. epidermidis*, formed significantly thicker biofilms with an increased cell number and an increased extracellular matrix, in presence of *C. albicans*. Consequently, *S. epidermidis* will be protected against vancomycin, and conversely, *S. epidermidis* will protect *C. albicans* against fluconazole through a decreased matrix penetration. [267-269]

Tolerance towards disinfectants through a decreased penetration is also reported. [144] For example, Behnke et al. [270] reported that the structured association of *B. cepacia* and *P. aeruginosa* led to an increased survival rate after exposure to chlorine. In a mixed biofilm, more cells were contained in large clusters and thus shielded from the disinfectant. In addition, the presence of an altered EPS composition and a higher viscosity, which delays penetration of the chlorine into the biofilm, also contributed to increased survival. [270] This increase in viscosity could be the consequence of chemical interactions between the polymers produced by each species present. [271] Another example is given by Simoes et al. [272], who described that multispecies biofilms formed by six drinking water-isolated bacterial species were more resistant towards treatment with sodium hypochlorite (NaOCl). The increased resistance could be partly explained by a higher cell

density in the multispecies biofilm relative to those of monospecies biofilms, or due to difficulty in penetration by the disinfectant of the matrix surrounding the multispecies biofilm.

(c) Protection through QS molecules

Even though HQNO produced by *P. aeruginosa* is described to kill *S. aureus*, it can also lead to enhanced tolerance of *S. aureus* towards aminoglycosides, such as tobramycin, by inhibiting electron transport and inducing the formation of SCV of *S. aureus*. [107] Furthermore, *M. catarrhalis* can utilize exogenous AI-2 provided by *H. influenza*, resulting in an increased *M. catarrhalis* biofilm density. [167] As a result, *M. catarrhalis* was protected from killing by clarithromycin. [167]

(d) Protection through an altered gene expression

Vandecandelaere et al. [273] described an upregulation of *S. epidermidis* genes encoding resistance towards oxacillin, erythromycin, and tobramycin. Subsequently, increased resistance of *S. epidermidis* could be observed when grown in a dual species biofilm with *S. aureus*. On the other hand, no difference in resistance of *S. aureus* could be observed. These results indicate that one species can influence the gene expression of another species, leading to an increased resistance.

(e) Influence of the model system

Important to note, is that the model used to study multispecies interactions can have an impact on the outcome. It was shown that a mixed biofilm of *P. fluorescens* and *Bacillus cereus*, grown in a rotating stainless steel device for 7 days, led to an increased tolerance of both species towards a surfactant and an aldehyde. In contrast, when both species were grown in a flow system for 16 hours, *B. cereus* was more susceptible towards chlorine. [144, 274]

2.4.2.2 Contest competition

Inhibitory compounds produced by one species, for example molecules that facilitate competitors' dispersal or antimicrobial compounds (e.g. toxins), can lead to out-competition of another species. [145, 228] For example, increased degradation of polysaccharides, nucleic acids, and protein components of one species by another species, results in compromised biofilm formation. [275] Direct antagonistic interactions between competitors are termed "contest competition" or "interference competition". [228]

(a) Production of antimicrobial compounds

The production of anti-competitor toxins is an example of "spite", because it results in direct fitness costs to both the actor and the recipient. [276] In *E. coli* for example, the cost of production of bacteriocins even results in suicide, as cell lysis is required to release these antimicrobial compounds. Bacteriocins are highly diffusible, the producing cell is thus unlikely to experience the benefit of

killing a competitor. [277] As a result, even if cell death is not required, the production is an inevitable metabolic cost that is likely to be greater than direct fitness benefits. [276]

Furthermore, some Gram-negative organisms release membrane vesicles (MVs) containing hydrolytic enzymes. These vesicles can fuse with other bacteria, resulting in lysis of these bacteria. [278] For example, *Myxococcus xanthus* MVs can lyse *E. coli*. [279] In addition, MVs formed by *P. aeruginosa* can attach to the surface of *S. aureus*, and release enzymes, including a 26 kDa peptidoglycan hydrolase (autolysin) and the LasA protease. [280] These *P. aeruginosa* enzymes contribute to lysis of *S. aureus* by cleaving the peptidoglycan pentaglycine interpeptides of the cell wall. [281]

S. epidermidis is described to inhibit biofilm formation of *S. aureus* through the production of a serine protease, Esp, which degrades specific biofilm matrix proteins and cell wall fractions, without affecting *S. aureus* growth rate. [282, 283] In addition, the *nuc1* gene product (i.e. staphylococcal nuclease) and recombinant NUC1 protein, of *S. aureus*, were found to prevent biofilm formation of *P. aeruginosa*, *Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis*. [284] Furthermore, *S. aureus* was described to secrete a large nuclease toxin, EsaD, that targets competitor bacteria. [285] In addition, an exo-β-D-fructosidase, FruA, from *Streptococcus salivarius*, can inhibit biofilm formation of *S. mutans*, by inducing the decrease of polysaccharide production. [286] Nevertheless, it cannot be excluded that the primary role of such molecules is to regulate biofilm formation by the producer, rather than antagonizing biofilm formation of other species. [275]

Furthermore, many streptococci generate waste products, including abundant hydrogen peroxide and lactate, causing oxidative stress and acid stress, respectively. These waste products are most concentrated in close proximity of the producing streptococci, affecting local competitors, e.g. *P. aeruginosa*, *H. influenzae* and *S. aureus*, corresponding to a loss in community diversity. [16, 287] *Streptococcus oligofermentans*, for example, has been described to correlate with a reduced incidence of *S. mutans*. *S. mutans* produces lactic acid that inhibits growth of other oral species. *S. oligofermentans* uses the *S. mutans*-produced lactic acid to generate hydrogen peroxide, which in turn inhibits *S. mutans*. [288] Besides competition through the production of hydrogen peroxide by some *Streptococcus* spp., these species are also described to prevent the attachment of competing microorganisms to the surface of airway cells, for example by the expression of neuraminidases that cut off cell surface-expressed sialic acids of *H. influenzae*. [289]

(b) Production of other molecules leading to competition

Species of the SMG have also shown to induce *P. aeruginosa* strains to increase expression of virulence factors, e.g. elastase and pyocyanin, by stimulating the reversion of the mucoid phenotype to the high pyocyanin expressing non-mucoid phenotype, contributing to CF lung disease

progression. [130, 290] In addition, these secondary metabolites produced by *P. aeruginosa* can also inhibit microbial growth [291, 292] For example, as a response to cell wall fragments shed by *S. aureus* during cell lysis, *P. aeruginosa* also increases its production of pyocyanin, leading to growth inhibition and killing of *S. aureus*. [293] However, *S. aureus* is able to survive in chronic wounds, possibly because of the high viscosity and subsequent restricted cell migration and mixing in chronic wounds, which consequently prevents further killing of *S. aureus*. [16] On the other hand, pyocyanin produced by *P. aeruginosa* protects *A. baumannii* from killing by amikacin and carbenicillin. [294] Pyocyanin leads to the generation of reactive oxygen species in *A. baumannii* cells, subsequently inducing the expression of catalase and superoxide dismutase in these cells, as a protection mechanisms against oxidative stress, thereby increasing *A. baumannii* persistence against amikacin and carbenicillin. This interaction allows both species to coexist. [294]

(c) Induction of biofilm dispersion

Other secondary metabolites of *P. aeruginosa* (e.g. rhamnolipids and cis-2-decenoic acid) are known to stimulate dispersal of established biofilms of other species. [295-299] As a result, when there is a decreased rhamnolipids production by *P. aeruginosa*, increased biofilm formation of another community member can be observed. For example, Price et al. [124] described an interspecies interaction between *P. aeruginosa* and *S. constellatus*. Upon addition of tobramycin, *P. aeruginosa* rhamnolipids production was reduced. As a result, *S. constellatus* showed enhanced biofilm formation. These data obtained *in vitro* could be linked with the clinical observations of a worsened disease *in vivo*, as the presence of *S. constellatus* has been correlated with the onset of pulmonary exacerbations in CF patients. [124, 127]

In addition, Filkins et al. [107] observed a biofilm dispersion phenotype of *S. aureus* in combination with both mucoid and non-mucoid *P. aeruginosa* strains. Park et al. [300] previously reported that *P. aeruginosa* proteases are involved in this biofilm dispersion phenomenon. Furthermore, Al-Bakri et al. [301] showed that *P. aeruginosa* strains producing *B. cenocepacia* growth inhibitory substances can displace an existing biofilm of *B. cenocepacia*. In addition, Kaplan et al. [302] reported that *A. actinomycetemcomitans* produces dispersin B, known to effectively disperse biofilms of *S. epidermidis* by degrading its polysaccharide intercellular adhesin. Another example is that of *Bacillus licheniformis* which produces DNases, and as a result is able to disperse competing biofilms of e.g. *E. coli* and *Pseudomonas* spp. [303] The production of nitric oxide (NO) under anaerobic conditions by bacteria growing in the deeper layers of the biofilm [275], also results in induction of dispersion of other members in the biofilm. [304]

(d) Alterations in gene expression

Matrix exopolysaccharides can also act as signaling molecules, inducing gene expression changes in competing bacteria. These changes can lead to antagonistic interactions, resulting in a decreased biofilm formation. [140, 275] For example, the probiotic bacterium *Lactobacillus acidophilus* produces polysaccharides which interfere with the expression of enterohemorrhagic *E. coli* (EHEC) surface adhesins, thereby decreasing the formation of biofilms by EHEC without affecting growth rate or QS. [305]

Chapter II: OBJECTIVES

Microbes do not live in isolation, instead they inhabit complex multispecies biofilm communities. [140, 306] However, most laboratory studies so far have focused on monospecies biofilms. [307, 308] In doing so, our knowledge of these biofilms through molecular and biochemical studies has greatly increased. However, the relevance of these findings to the characteristics of multispecies biofilm communities, and consequently, to the characteristics of chronic diseases and their treatment regimen, is unclear. This understanding is necessary to successfully prevent and treat multispecies biofilm infections. [140] Recently, it was appreciated that multispecies infections are much more common than previously thought, and that pathogens in a multispecies infection site display enhanced resistance compared to the same pathogen in a monospecies infection. As a result, there has been a switch from studying monospecies biofilms towards multispecies biofilms. [11, 83, 154, 218, 221, 309, 310] However, while a lot has been learned about well-known pathogens such as *P. aeruginosa* and *S. aureus*, our current understanding of other pathogens, including *S. anginosus*, is still very limited. [9, 73, 140] The main aim of this dissertation was to investigate whether living in a multispecies biofilm influences the antibiotic susceptibility of the different species present, and if so, which molecular mechanisms are involved.

In the first part, we wanted to investigate whether or not it was possible to reproducibly grow, treat, and quantify a multispecies biofilm in a 96-well microtiter plate (MTP) consisting of *S. aureus*, *P. aeruginosa*, and *C. albicans*, as these species are often isolated from multispecies biofilms on medical equipment.. We aimed to improve the growth medium to allow growth of *S. aureus* and *C. albicans* in presence of *P. aeruginosa*, as both species are reported to be killed by *P. aeruginosa in vitro* (but not necessarily *in vivo*) (Chapter III paper 1) [107, 227, 311]. Cell numbers were determined through selective plate counts. Using this model system, we evaluated the difference in susceptibility towards disinfectants between a mono- and multispecies biofilm.

Secondly, in chapter III paper 2, we wanted to determine the susceptibility of *P. aeruginosa*, *S. aureus* and *S. anginosus* towards other antimicrobial solutions. In order to quantify *S. aureus*, *S. anginosus*, and *P. aeruginosa* at the same time, we again used the selective plate count method. Bacterial susceptibility was determined towards a variety of antibiotics, both when grown in a mono- and multispecies biofilm.

Next, in chapter III paper 3, in order to elucidate molecular mechanisms responsible for the differences in susceptibility observed for *S. anginosus* in chapter III paper 2, we performed RNA-sequencing of *S. anginosus* in a mono- and multispecies biofilm. We confirmed the results obtained with RNA-sequencing by measuring cell wall thickness of *S. anginosus,* using transmission electron microscopy (TEM).

In chapter III paper 4, we aimed to develop a culture-independent quantification method, as the presence of viable but non-culturable (VBNC) bacteria often poses a risk to public health. These cells are characterized by a loss of culturability on routine agar, which can lead to an underestimation of total viable cell numbers. Using this culture-independent quantification method, based on PMA-qPCR, we wanted to quantify *P. aeruginosa* in a mono- and multispecies biofilm with *S. aureus*, *S. anginosus*, and *B. cenocepacia*. In a second part, we also wanted to evaluate the susceptibility of *P. aeruginosa* in these biofilms, again using antibiotic agents instead of disinfectants (Chapter III paper 4).

Chapter III:
EXPERIMENTAL WORK

Paper 1:

Adapted from:

Activity of disinfectants against multispecies biofilms formed by *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa*.

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ABSTRACT

Microbial biofilms are a serious threat to human health. Recent studies have indicated that many clinically-relevant biofilms are polymicrobial. In the present study, multispecies biofilms were grown in a reproducible manner in a 96 well microtiter plate. The efficacy of nine commercially available disinfectants against *Staphylococcus aureus*, *Candida albicans*, and *Pseudomonas aeruginosa* in multispecies biofilms was determined and compared. Results showed that *P. aeruginosa* was more susceptible towards sodium hypochlorite, chloroxylenol, hydrogen peroxide, cetrimide and hospital antiseptic concentrate (HAC) in a multispecies biofilm compared to in a monospecies biofilm. *S. aureus* was more susceptible towards chlorhexidine, cetrimide and HAC, but less susceptible to chloroxylenol. In general, *C. albicans* was more susceptible to disinfectants in a multispecies biofilm. For

P. aeruginosa, the involvement of ROS in the increased killing by hydrogen peroxide was further investigated. In conclusion, results showed that the direction and the magnitude of the effect in a multispecies biofilm depend on the strain and the disinfectant used and challenge the common belief that organisms in multispecies biofilms are always less susceptible than in monospecies biofilms.

INTRODUCTION

Multispecies biofilms, a common problem in clinical practice are frequently found in chronically infected wounds and on indwelling medical devices (eg prostheses, stents, implants, catheters, and endotracheal tubes) [266]. These persistent biofilm infections can lead to severe illness with a prolonged hospital stay, increased costs, and high mortality.

Multispecies biofilms are sessile communities in which cells of various microorganisms are present. These cells are attached to each other and are surrounded by a self-produced extracellular polymeric matrix (EPM) [20]. The EPM mainly consists of polysaccharides, DNA, proteins, and dead cells [312]. It may restrict the penetration of antimicrobial agents and lead to the accumulation of antibiotic-degrading enzymes. The EPM also affects pH, oxygen concentration, and nutrient availability in the deeper layers of the biofilm. The production of an EPM and the transfer of resistance genes favored by high cell density contribute to an increased resistance of biofilms [20, 313].

Multispecies biofilms are increasingly recognized as an important phenomenon. Nonetheless, their properties remain poorly studied, and there is little information on disinfectant susceptibility of multispecies biofilms. Simoes et al. assessed the susceptibility of mono- and multispecies biofilms formed by six bacterial species (including *Acinetobacter calcoaceticus*) to sodium hypochlorite (NaOCl) and found that a multispecies biofilm including all six bacteria had the highest resistance to

NaOCl, while a multispecies biofilm without *A. calcoaceticus* was more susceptible to NaOCl than the monospecies biofilms [314]. Peters et al. assessed the susceptibility of mono- and multispecies biofilms of *Candida albicans* and *Staphylococcus aureus* to ethanol and found that there was no difference in susceptibility between species in mono- and multispecies biofilms [315].

C. albicans is often found together with bacteria in infections [316-318]: multispecies biofilms of *C. albicans* and *S. aureus* and *C. albicans* and *P. aeruginosa* are described [266, 311, 319]. *C. albicans* is an eukaryotic, polymorphic, opportunistic pathogen which, in most individuals, is present as a harmless commensal in the oral cavity. However, in case of immune deficiency, it can cause severe superficial infections and even lead to life-threatening systemic infections. Known virulence factors include molecules associated with adhesion and host-cell invasion, morphological switching between yeast and hyphal forms, and the secretion of hydrolases [320]. The production of multiple virulence factors and the expression of resistance genes makes *S. aureus* a significant problem in the hospital [321]. *P. aeruginosa* is a prokaryotic, opportunistic, nosocomial pathogen showing a high infection rate in people with cystic fibrosis or immunocompromised patients. *P. aeruginosa* produces a variety of virulence factors, both cell-associated (eg lipopolysaccharides, flagella, pili) and extracellular (e.g. proteases, pyocyanin, exotoxins), the production of which is often regulated by quorum sensing [311, 322, 323].

In order to prevent the transmission of these and other species, guidelines for disinfection in healthcare facilities have been issued [324]. However, these guidelines are mostly based on susceptibilities of planktonic cells and do not take into account the decreased susceptibility of sessile cells.

In the present study multispecies biofilms were reproducibly grown, consisting of *S. aureus*, *P. aeruginosa* and *C. albicans* in a 96 well microtiter plate. The effect of disinfectants on each species in the biofilm was studied, in order to gain more insight into the role of biofilm composition on efficacy of surface decontamination. To rationalize possible differences in susceptibility to a selected disinfectant, ie H_2O_2, between mono- and multispecies biofilms, gene expression levels of oxidative stress-related genes of *P. aeruginosa* and *S. aureus* were compared in a mono- and multispecies biofilm.

MATERIAL & METHODS

Bacterial strains

S. aureus Mu50 (methicillin-resistant *S. aureus* [MRSA]), *C. albicans* SC 5314, and *P. aeruginosa* PAO1 were cultured overnight at 37°C in brain heart infusion (BHI) borth (Oxoid, Basingstoke, UK).

Disinfection procedures

The following disinfectants were tested: Hospital Antiseptic Concentrate (HAC; Regent Medical, Manchester, UK) (1.0% (v/v); 15 min) containing cetrimide (0.5%) and chlorhexidine (0.05% (v/v)), cetrimide (Certa, Braine-l'Alleud, Belgium) (0.15% (w/v); 15 min), chlorhexidine (Fagron, Waregem, Belgium) (0.4% (v/v); 15 min), chloroxylenol (Reckitt Benckiser, Brussels, Belgium), (5.0% (v/v); 5 min), ethanol (Certa) [70% (v/v); 2 min], hydrogen peroxide (Acros, Geel, Belgium) (1.5% (v/v); 30 min) and sodium hypochlorite (Forever, Courcelles, Belgium) (0.05% (v/v); 5 min). Concentrations and contact times were based on previous work from our research group [313]. All disinfectant solutions were prepared using water of standard hardness (WSH), filter-sterilized and stored at 4°C for up to 1 month.

Determination of the efficacy of disinfectants against planktonic and sessile cells

To assess the effect of the disinfectants against planktonic cells, a modified European Suspension Test (EST) was used as described previously: an overnight suspension in BHI (with approximately 10^8 CFU/ml of *S. aureus* or *P. aeruginosa* and approximately 10^7 CFU/ml of *C. albicans*) was added alone (a monospecies suspension) or in combination (a multispecies suspension) to a disinfectant solution at 20°C [313]. After a given contact time, the disinfectant was neutralized by a commercially available neutralizer (Dey-Engley Neutralizing Broth; DENB, Sparks, MD, USA). The efficacy of the neutralizer was evaluated for each disinfectant prior to testing. Each test was performed in triplicate.

For mono- and multispecies biofilm formation round-bottomed microtiter plates were used. Inoculum suspensions containing approximately 10^6 CFU/ml of *S. aureus*, 10^6 CFU/ml of *P. aeruginosa* and/or 10^5 CFU/ml of *C. albicans*, were made in BHI. To prevent killing of *S. aureus* and *C. albicans* by *P. aeruginosa* [311, 325], BHI was supplemented with bovine serum albumin (BSA) [326]. For each test condition, 12 wells of a round-bottomed polystyrene 96-well microtiter plate (TPP, Trasadingen, Zwitzerland) were filled with 100 µl of the suspension. Wells filled with sterile BHI served as blanks. After 4 hours of adhesion at 37°C, non-adhered cells were removed and each well was rinsed with 100 µl of physiological saline (0.9% (w/v) NaCl) (PS). 100 µl of fresh BHI supplemented with 5% BSA (w/v) was added and the plates were incubated for an additional 20 hours to allow biofilm maturation. The supernatant was then again removed and each well was

rinsed using 100 µl PS. To assess the effect of the disinfectants, 120 µl of the test solution was added to each well. 120 µl of WSH was added to control and blank. After the prescribed contact time, the disinfectant was neutralized with DENB, as described previously [313]. Quantification of the cells prior to and after treatment was done using plate counting. To this end, biofilms were detached by vortexing (5 min) and sonication (5 min), without affecting viability, as previously determined in our lab (data not shown) and as described by Kobayashi et al. [327], followed by collection of the content of the wells in a sterile tube. This procedure was repeated after the addition of 100 µl PS to each well. Plate counting was performed on selective growth media; i.e. tryptic soy agar supplemented with 7.5% NaCl (w/v) and amphotericin B (0.025 mg/ml) for *S. aureus*, Sabouraud dextrose agar supplemented with nitrofurantoin (0.100 mg/ml) and tobramycin (0.008 mg/ml) for *C. albicans*, and cetrimide agar for *P. aeruginosa*, respectively. All experiments were performed in six-fold.

For biofilms, growth recovery of an untreated biofilm on selective growth media was compared with growth recovery on a general medium (TSA for *S. aureus* and *P. aeruginosa* and SDA for *C. albicans*); both recoveries were the same for each species (data not shown). For planktonic cultures, growth recovery after treatment with hydrogen peroxide on selective media was compared with growth recovery on a general medium; both recoveries were again the same for each species (data not shown). The possibility that injured or stressed microorganisms are less prone to grow on a particular selective medium cannot be ruled out completely. Nevertheless, the experiment described above indicates that the use of selective media will only have a minimum impact on the growth recovery. Therefore, the plate method with selective growth media was used in this study.

Determination of residual hydrogen peroxide concentration

Mono- and multispecies biofilms were grown as described above and treated with 1.5% H_2O_2 (v/v) for 30 minutes. The residual concentration of H_2O_2 in the supernatant was determined titrimetrically with a 0.002M permanganate solution [328].

Accumulation of reactive oxygen species in biofilm cells

Mature mono- and multispecies biofilms of *P. aeruginosa* formed in round-bottomed microtiter plates were rinsed with 100 µl PS and treated for 30 minutes at room temperature with 120 µl of a 1.5% H_2O_2 solution (v/v). Controls were incubated under identical conditions without H_2O_2. Intracellular reactive oxygen species (ROS) accumulation was determined using a fluorometric assay with 2',7'-dichlorofluorescein diacetate (DCFHDA) (Invitrogen, Carlsbad, CA, USA). To this end, biofilms were incubated with 10µM DCFHDA, simultaneously with the H_2O_2. Fluorescence was measured after 30 minutes of incubation (excitation and emission wavelengths of 485 nm and 535 nm, respectively). The values obtained were corrected for background fluorescence and compared

with those obtained with control biofilms. *Since the conversion of this dye is proportional to the number of metabolically active cells in the biofilm, results were normalized to the total number of cultivable cells (total cultivable cell number of P. aeruginosa cells in a monospecies biofilm and total cultivable cell number of S. aureus, P. aeruginosa and C. albicans cells in a multispecies biofilm).* ROS levels were quantified in three biological replicates (each consisting of 24 technical replicates).

Expression of stress related genes in P. aeruginosa biofilm cells

Following treatment of *P. aeruginosa* biofilms with 1.5% H_2O_2 for 30 minutes and subsequent neutralization with DENB for 5 minutes, biofilms cells were collected as described above. Untreated biofilm cells served as a control. Total RNA extraction was performed with the SV Total RNA Isolation System according to the manufacturer's instructions (Promega, Fitchburg, WI, USA). The RNA concentration was determined with the BioDrop µLITE (BioDrop, Cambridge, UK). First strand cDNA was synthesized using the qScript cDNA SuperMix according to the manufacturer's instructions (Quanta Biosciences, Gaithersburg, MD, USA). Real-time PCR (CFX96 Real Time System; Bio-Rad, Hercules, CA, USA) was carried out with the PerfeCTa SYBR Green FastMix (Quanta Biociences). Primer sequences for *oxyR*, *fpvaI*, *ahpC*, *pvdS* and *sodM* were obtained from the literature [329-331]. Primers for *katA* and *katB* were designed using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using *P. aeruginosa* PAO1 sequences obtained from GenBank. The specificity of each pair of primers was evaluated by melting curve analysis. Expression levels were normalized using two reference genes [332] (*fabD* and *oprM*; primer sequences were obtained from literature [333]). Primers are listed in Table 1. Three independent biological repeats were carried out, each consisting of 72 technical repeats.

Expression of stress related genes in S. aureus biofilm cells

To quantify the expression of stress related genes in *S. aureus* biofilms, the same procedure was followed as described above. Primer sequences for *dps*, *katA*, *sodA* and *ldhA* were obtained from the literature [334]. Primers for *trxB* and *ahpF* were designed using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with *S. aureus* Mu50 sequences obtained from GenBank. Primers are listed in Table 1. Expression levels were normalized using two reference genes (*tpi* and *yqi;* primers were selected from the MLST database, http://saureus.mlst.net/misc/info.asp).

***Table 1**: Primers used for quantifying the expression of P. aeruginosa and S. aureus genes by qPCR.*

Target gene	Primer sequence (5'-3')		Ref.
	Forward primer	Reverse primer	
P. aeruginosa			
ahpC	GCAAGTTCATCGAGGTGACC	CTTTCTGGAACTCGCCGTAG	[330]
fabD	GCATCCCTCGCATTCGTCT	GGCGCTCTTCAGGACCATT	[333]
fpvaI	CTGCAGCAGTGCGATCAAGGGCAA	GTGGACGTCGCAGGTTCCAGTAGCTCT	[330]
katA	CCATGACATCACCCCCTACA	CGGTGTAGAAACGCATGGAG	This study
katB	TGCGATCAAGTTCCCGGATA	GGCACGTGGGAGAAGAAATC	This study
oprM	CCATGAGCCGCCAACTGTC	CCTGGAACGCCGTCTGGAT	[333]
oxyR	CTCACCGAACTGCGCTACA	CGAGTCGGCCAGCACTT	[329]
pvdS	GCGTTCTTCAGGCTCCAGTC	AGTTGATGTGCGAGGTTTCC	[329]
sodM	CCTTGCCTTACGCCTACGATG	TGCCGCAGCAGACTTTCCA	[329]
S. aureus			
ahpF	AATTGCTGCTTCAACCCAG	AAAGGTGTTGCATTCTGCCC	This study
dps	CACAAAGCTACACAATTTCCACTG	ATACATCATCGCCAGCATTACC	[334]
katA	ATGCGCAAAGATATCGATTAGGA	TGGTGGCTTTTTATATTCAGGTTG	[334]
ldhA	GCAACATGGAAATTCTCTGG	CAGTCAATACGGCATCTTCAT	[334]
sodA	CTGCTGTACGTAATAATGGCGG	ATGTAGTCAGGGCGTTTGTTTTG	[334]
tpi	TCGTTCATTCTGAACGTCGTGAA	TTTGCACCTTCTAACAATTGTAC	MLST database
trxB	GCTGCAGTATACGCATCACG	ATTAGCCATTTGACCGCCTG	This study
yqi	CAGCATACAGGACACCTATTGGC	CGTTGAGGAATCGATACTGGAAC	MLST database

Statistical data analysis

Statistical data analysis was performed using SPSS software, version 21 (SPSS, Chicago, IL, USA). The normal distribution of the data was verified using the Shapiro-Wilk test. Normally distributed data were analyzed using an independent sample t-test. Non-normally distributed data were analyzed using a Mann-Whitney test. Differences with a p-value < 0.05 were considered as significant. For the gene expression levels, only differences of more than a twofold up- or down-regulation and with a p-value ≤ 0.01 were considered as significant.

RESULTS

In vitro growth of multispecies biofilms

A multispecies inoculum suspension containing approximately 10^6 CFU/ml of *S. aureus* and *P. aeruginosa* and 10^5 CFU/ml of *C. albicans* in BHI was added to a 96-well microtiter plate. The mature biofilm consisted of approximately $2x10^2$ CFU/well *S. aureus*, 10^1 CFU/well *C. albicans*, and $2x10^6$ CFU/ml *P. aeruginosa*. The low numbers for *S. aureus* and *C. albicans* suggest that growth and/or biofilm formation of these species were inhibited by *P. aeruginosa*. In order to prevent this, BSA was added to the medium. Several concentrations of BSA (ranging from 0% to 5%) were tested for promoting growth of *S. aureus* and *C. albicans* in the presence of *P. aeruginosa*. The addition of 5% BSA gave the highest cell counts: *S. aureus* grew to approximately 10^7 CFU/well, *C. albicans* to approximately 10^5 CFU/well and *P. aeruginosa* to approximately 10^7 CFU/well. For *S. aureus* and *P. aeruginosa*, these cell numbers were the same as in a monospecies biofilm (approximately 10^7 CFU/well). For *C. albicans*, there was approximately a 1 log reduction compared to the cell numbers in a monospecies biofilm (approximately 10^6 CFU/well).

Effect of disinfectants on planktonic cultures

A modified EST was used to determine the survival of planktonic cells after treatment with a variety of disinfectants. All treatments resulted in a killing of all cells, except for NaOCl (0.05%; 5 min) and H_2O_2 (1.5%; 30 min), as shown in Table 2.

Effect of disinfectants on biofilm cultures

Mature mono- and multispecies biofilms were treated with different disinfectants and the number of surviving cells was determined by plate counting on a selective growth medium. The results are shown in Table 2. In general, *C. albicans* was more susceptible towards disinfectant treatment when grown in a multispecies biofilm, except for chloroxylenol and ethanol. *S. aureus* was more susceptible towards chlorhexidine, cetrimide and HAC, whereas it was less susceptible towards chloroxylenol. In addition, *P. aeruginosa* was more susceptible towards sodium hypochlorite, chloroxylenol, hydrogen peroxide, cetrimide, and HAC in a multispecies biofilm.

Residual concentration of hydrogen peroxide

For H_2O_2, a significant difference in killing of *P. aeruginosa* could be observed between a mono- and multispecies biofilm. In addition, for H_2O_2, several mechanisms have been described to quantify the molecule and the effect [313, 335]. Therefore, we decided to further explore what could cause the difference in the efficacy of this disinfectant. The concentration of H_2O_2 was determined in the supernatant of *P. aeruginosa* monospecies biofilms and in the supernatant of multispecies biofilms treated for 30 minutes with 1.5% H_2O_2. The residual concentration of H_2O_2 in the supernatant of a mono- and multispecies biofilm was 0.015 ± 0.007% (mean ± SEM) and 0.035 ± 0.027% (mean ± SEM), respectively. The mean values were not significantly different from each other or from the blank ($p>0.05$).

Accumulation of ROS in biofilms

DCFHDA was used to measure the accumulation of ROS following treatment with H_2O_2. Treatment of a monospecies P. aeruginosa biofilm and a multispecies biofilm both resulted in a significant increase in ROS accumulation ($p\leq0.05$) (Fig 1.). The accumulation of ROS after treatment of a monospecies *P. aeruginosa* biofilm was not significantly different from the accumulation of ROS after treatment of a multispecies biofilm ($p>0.05$). An untreated monospecies P. aeruginosa biofilm showed a significantly higher basal ROS level than an untreated multispecies biofilm ($p\leq0.05$). An untreated S. aureus biofilm showed a very low basal ROS level, while an untreated C. albicans biofilm had a similar basal ROS level as a P. aeruginosa biofilm (data not shown).

***Table 2**: Killing of planktonic and biofilm cells by disinfectants. The results are expressed as % killed and are shown as the average ± SEM (n=6). *Cells of this organism are significantly more killed in a multispecies biofilm than in the corresponding monospecies biofilm (p<0.05). [+]Cells of this organism are significantly more killed in a monospecies biofilm than in a multispecies biofilm (p<0.05). [#]NR no reduction observed.*

Disinfectant			***S. aureus***		***P. aeruginosa***		***C. albicans***	
	conc. (%)	time (min.)	mono	multi	mono	multi	mono	multi
Planktonic cells[•]								
NaOCl	0.05	5	99.991±22.81	99.992±35.72	99.986±14.01	99.838±15.54	99.975±32.24	99.991±33.54
H_2O_2	1.5	30	100.000±0	100.000±0	100.000±0	100.000±0	100.000±0	87.504±6.30
Biofilm cells								
NaOCl	0.05	5	90.982±13.81	96.024±9.72	[#]NR	98.840±15.86*	92.763±2.21	95.497±3.02*
BzCl	0.2	15	99.499±15.85	99.952±15.51	72.943±9.12	75.232±10.43	89.510±7.35	99.314±9.90*
PCMX	5.0	5	99.934±3.12[+]	96.459±4.23	85.487±8.27	99.916±4.76*	99.991±5.60[+]	99.247±3.41
ETOH	70	2	6.660±16.61	66.069±16.98	99.042±9.52	98.920±5.54	54.831±15.21	88.045±18.53
H_2O_2	1.5	30	71.430±8.91	88.220±9.88	[#]NR	96.944±9.12*	47.807±6.99	82.572±8.36*
CHX	0.4	15	95.498±3.29	100.000±0*	99.999±2.94	100.000±0*	99.915±2.61	100.000±0*
CET	0.15	15	46.349±16.61	95.938±17.02*	[#]NR	49.252±20.65*	[#]NR	53.377±12.02*
HAC	1.0	15	75.009±7.15	96.144±13.47*	[#]NR	45.466±13.14*	14.286±3.19	43.042±10.26*
PVP-I	7.5	15	99.999±9.78	100.000±0	100.000±0	99.721±8.31	98.835±7.51	100.000±0*

NaOCl, sodium hypochlorite. BzCl, benzalkoniumchloride. PCMX, chloroxylenol. ETOH, ethanol. H_2O_2, hydrogen peroxide. CHX, chlorhexidine. CET, cetrimide. HAC, hospital antiseptic concentrate. PVP-I, povidone-iodine. [•] Other treatments resulted in a complete reduction of cells in all condition

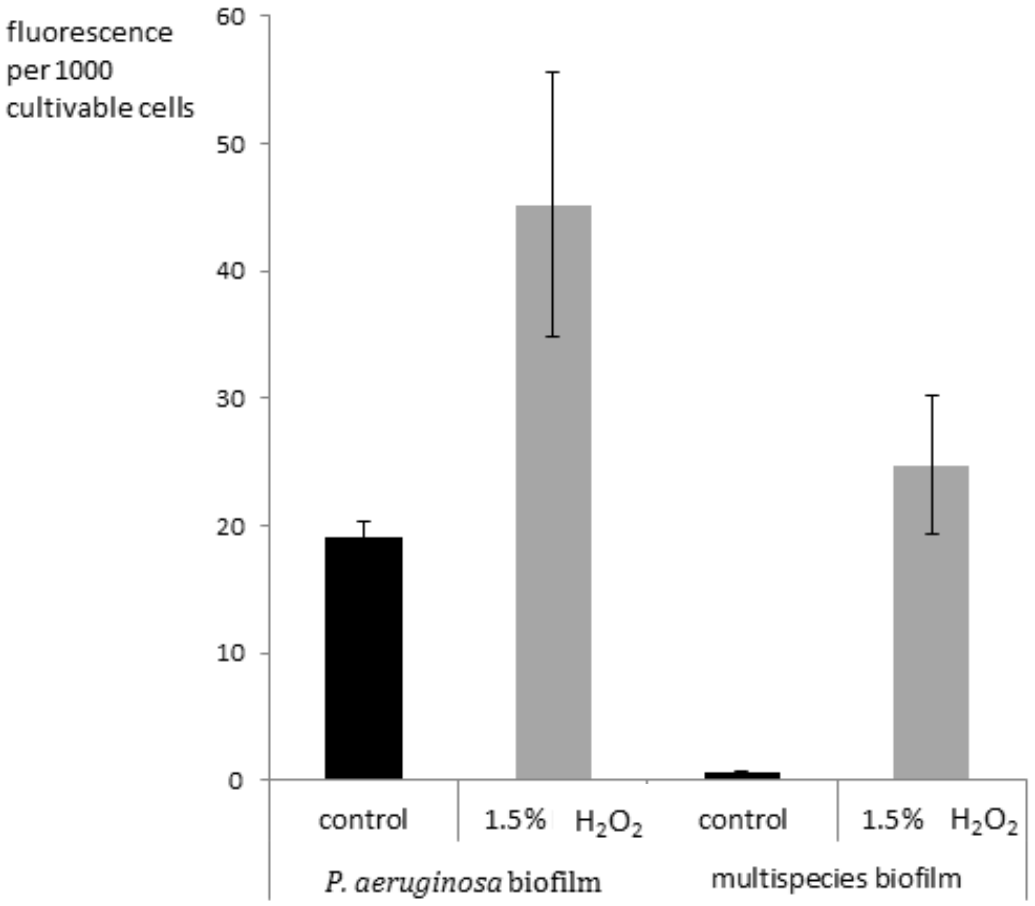

Figure 1: *Accumulation of ROS in mature P. aeruginosa monospecies biofilms and in multispecies biofilms following treatment with hydrogen peroxide. Data are expressed as fluorescence per 1000 cultivable cells (cultivable cells of P. aeruginosa for a monospecies biofilm and cultivable cells of S. aureus, P. aeruginosa, and C. albicans for a multispecies biofilm). Black bars represent untreated biofilms; grey bars represent biofilms treated with 1.5% hydrogen peroxide for 30 minutes. Data presented are the mean and SEM of three independent experiments on at least 24 biofilms. Statistical analysis with a Mann-Whitney test indicated a significant difference ($p \leq 0.05$) between a monospecies control biofilm and a multispecies control biofilm, between a monospecies control biofilm and a monospecies test biofilm, and between a multispecies control biofilm and a multispecies test biofilm. There was no significant difference ($p \leq 0.05$) in fluorescence after treatment between a mono- and a multispecies biofilm.*

Expression of stress related genes in P. aeruginosa and S. aureus

For P. aeruginosa, gene expression levels of oxidative stress-related genes (oxyR, katA, katB, ahpC, pvdS, fpvaI, and sodM) in a mono- and multispecies biofilm, after treatment with H_2O_2, were compared by qPCR. The same was done for S. aureus (dps, katA, sodA, ldhA, trxB and ahpF). There was no significant difference in gene expression between a mono- and multispecies biofilm for any of the genes investigated (data not shown).

DISCUSSION

Multispecies biofilms containing *S. aureus*, *P. aeruginosa* and *C. albicans* were reproducibly grown in 96 well microtiter plates, in the presence of 5% BSA, and were examined for their susceptibility against various disinfectants. The susceptibility of both planktonic and sessile cells of each species individually was determined.

Our results demonstrate that the tested disinfectants effectively kill planktonic cells, with almost all disinfectants resulting in >99.999% reduction in cell numbers. However, few disinfectants retained this high efficacy when applied to mono- or multispecies biofilms. Previous data suggest that multispecies biofilms are often less susceptible to antimicrobial agents than monospecies biofilms [178, 185, 262, 266, 314]. However, data obtained in the present study demonstrate that this is not always the case and that susceptibility depends on the nature of microbial species present and the disinfectant used. In a multispecies biofilm, *P. aeruginosa* is more susceptible to sodium hypochlorite, chloroxylenol, chlorhexidine, cetrimide, HAC and H_2O_2. *S. aureus* appears to be more susceptible to chlorhexidine, cetrimide and HAC, and less susceptible to chloroxylenol. For the other disinfectants, no difference in susceptibility of *S. aureus* was observed between a mono- and multispecies biofilm. *P. aeruginosa* is known to produce 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), which is thought to be associated with the higher resistance of *S. aureus* cells towards antimicrobial agents [178]. *C. albicans* is also known to have a protective effect on *S. aureus*, by coating the *S. aureus* cells with secreted matrix [266]. The mechanisms responsible for the observed higher susceptibility of *S. aureus* when grown in a multispecies biofilm are not clear yet and require further investigation. *C. albicans* is more susceptible to all disinfectants in a multispecies biofilm except to ethanol and chloroxylenol. The production of a phenazine toxin by *P. aeruginosa*, which enhances the production of reactive oxygen species, could be responsible for the 1 log reduction in *C. albicans* cell numbers observed in a control multispecies biofilm [311]. Whether or not this phenazine toxin also plays a role in the higher susceptibility of *C. albicans* in a multispecies biofilm is not clear. Other mechanisms could be responsible, but require further investigation. For example, it was described that LPS from *P. aeruginosa* have adverse effects on *C. albicans*, resulting in a decreased filamentation and metabolic activity of *C. albicans*. [336] In addition, downregulation of *C. albicans* iron-regulated proteins was also observed in response to *P. aeruginosa* presence. [337]

For H_2O_2, 96.94% of *P. aeruginosa* cells in a multispecies biofilm were killed, unlike *P. aeruginosa* cells in a monospecies biofilm, which were not affected. One of the mechanisms involved in this protection is thought to be a diffusion interaction: H_2O_2 is neutralized by catalases in the surface layers of the biofilm at a faster rate than the penetration of H_2O_2 into the biofilm [338]. In order to elucidate the mechanisms responsible for the change in susceptibility towards H_2O_2, the residual

fraction of H_2O_2 was determined, the accumulation of ROS, and the expression of oxidative-stress related genes in a mono- and multispecies biofilm.

The amount of H_2O_2 in the supernatant of a treated *P. aeruginosa* monospecies biofilm was not significantly different from that in the supernatant of a treated multispecies biofilm, nor from that in the untreated control. This indicates that all H_2O_2 had been degraded, both in mono- and in a multispecies biofilms.

Following treatment with H_2O_2, the ROS-production in a multispecies biofilm was not significantly different from the ROS-production in a monospecies *P. aeruginosa* biofilm. This suggests that there is no additional production of ROS in a multispecies biofilm, and, therefore that ROS production is probably not responsible for the observed increase in H_2O_2 susceptibility of *P. aeruginosa* in a multispecies biofilm. The basal ROS production in an untreated monospecies *P. aeruginosa* biofilm was significantly higher than the basal ROS production in an untreated multispecies biofilm: *S. aureus* has a much lower basal ROS production than *P. aeruginosa* and thus does not contribute to the ROS production in an untreated multispecies biofilm.

Finally, the expression of oxidative-stress related genes was measured in both *P. aeruginosa* and *S. aureus*. As *P. aeruginosa* becomes more sensitive to H_2O_2 when it is growing in a multispecies biofilm, we expected to see a decrease in the expression of oxidative-stress related *P. aeruginosa* genes in this condition. However, there was no significant difference between the expression of the genes in a mono- and multispecies biofilm. Nor was there a difference between the expression of oxidative-stress related genes in *S. aureus* in a mono- and multispecies biofilm. Future research is required to investigate whether other mechanisms (e.g. posttranslational regulation of gene expression) could be involved. In addition, differences in diffusion rate of H_2O_2 and other disinfectants between a mono-and multispecies biofilm, could also be responsible for the altered killing efficacy.

CONCLUSION

Using in vitro growth in 96 well microtiter plates, it was possible to evaluate the biofilm eradicating activity of various disinfectants. The data in the present study indicate that the effect of the presence of several species on the susceptibility of another species against disinfectants depends on the species present and the disinfectant used, and challenge the common belief that organisms in multispecies biofilms are always less susceptible than in monospecies biofilms.

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Paper 2:

Adapted from:

Community composition determines activity of antibiotics against multispecies biofilms

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ABSTRACT

In young cystic fibrosis (CF) patients , *Staphylococcus aureus* is typically the most prevalent organism, while in adults, *Pseudomonas aeruginosa* is the major pathogen. More recently, it was observed that also *Streptococcus anginosus* plays an important role in exacerbations of respiratory symptoms. These species are often co-isolated from CF lungs, yet little is known about whether antibiotic killing of one species is influenced by the presence of others. In the present study, we compared the activity of various antibiotics against *S. anginosus*, *S. aureus* and *P. aeruginosa* when grown in monospecies biofilms, with the activity observed in a multispecies biofilm. Our results show that differences in antibiotic activity against species grown in mono- and multispecies biofilms are species- and antibiotic-dependent. Less *S. anginosus* cells are killed by antibiotics that interfere with cell wall synthesis (amoxicillin+sulbactam, cefepime, imipenem, meropenem, and vancomycin) in presence of *S. aureus* and *P. aeruginosa*, while for ciprofloxacin, levofloxacin, and tobramycin, no difference was observed. In addition, we observed that the cell-free supernatant of *S. aureus*, but not that of *P. aeruginosa* biofilms, also caused this decrease in killing. Overall, *S. aureus* was more killed by antibiotic treatment in a multispecies biofilm, while for *P. aeruginosa*, no differences were observed between growth in mono- or multispecies biofilms.

The results of the present study suggest that it is important to take the community composition into account when evaluating the effect of antimicrobial treatments against certain species in mixed biofilms.

INTRODUCTION

Cystic fibrosis (CF) is one of the most common autosomal recessive diseases in Caucasians. [339] CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, eventually leading to thick airway mucus. [340] A major predictor of morbidity and mortality in CF patients is respiratory infection due to bacteria that form biofilms in this thick airway mucus. [341] In childhood, *Staphylococcus aureus* is the most prevalent species. [114] In adulthood, there is a shift resulting in *Pseudomonas aeruginosa* being the predominant species. In addition to these common pathogens, the CF lung microbiome contains numerous other microorganisms, including *Burkholderia* spp., *Prevotella* spp., *Rothia* spp., *Stenothrophomonas* spp., and/or *Streptococcus* spp. [120, 342] Members of the *Streptococcus milleri* group (SMG), including *Streptococcus anginosus*, can be found in children [119, 129] and adults [122, 128] and have recently been linked to acute pulmonary exacerbations in 39% of hospital admissions of adult CF patients. [127, 343] In their cross-sectional patient cohort study, Filkins et al. [120] showed that SMG species were also present in clinically stable patients, suggesting that modest levels of SMG species can contribute to patient health, while

excessive levels may lead to clinical decline. The biofilm-mode of growth of bacteria plays an important role in the persistence of lung infections in CF patients. [69] In a biofilm, bacterial cells are protected by a self-produced polymer matrix, show a decreased susceptibility to antibiotics, and are not efficiently cleared by the immune system. [68, 102]

Species in a multispecies biofilm can cooperate or compete with each other and this may impact the course, treatment and outcome of biofilm-related CF airway infections. [140] Most studies thus far have focused on interactions between *S. aureus* and *P. aeruginosa*. In early adulthood, *S. aureus* and *P. aeruginosa* initially can co-exist, however, at a later stage, *P. aeruginosa* will outcompete *S. aureus* and benefit from its presence by using *S. aureus* as an iron source. [118] Both 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) and siderophores produced by *P. aeruginosa* play an important role in this competition. These factors, in combination with oxygen utilization by *P. aeruginosa*, are known to result in inhibition of the electron transport chain in *S. aureus*, leading to a growth disadvantage for *S. aureus*. [107] However, suppression of the *S. aureus* respiration by *P. aeruginosa* has also been reported to protect *S. aureus* from killing by the aminoglycoside tobramycin. [178] In addition,

P. aeruginosa is known to form membrane vesicles (MVs) containing cell wall-degrading enzymes. These MVs can attach to the surface of *S. aureus*, release enzymes such as LasA protease, and consequently lead to the killing of *S. aureus*. [280]

Few studies have focused on the interactions between other bacterial species present in the CF lung microbiome. Recently, SMG species were found to increase expression of *P. aeruginosa* virulence factors, e.g. elastase and pyocyanin, contributing to CF lung disease progression. [130] In contrast, the generation of reactive nitrogenous intermediates by oral streptococci can inhibit *P. aeruginosa* growth. [344, 345] It has also been demonstrated that penicillinases produced by *S. aureus* protect *Streptococcus* spp. against penicillins. [251, 346] Still, little is known about the effect of interspecies interactions in multispecies biofilms on antibiotic killing of biofilm cells. Therefore, in the present study, we compared the killing of three commonly co-isolated bacteria, *S. anginosus*, *S. aureus* and *P. aeruginosa*, by various antibiotics, when grown as a mono- or multispecies biofilm.

MATERIALS & METHODS

Bacterial strains

S. anginosus LMG 14502 (isolated from human throat) and LMG 14696 (isolated from a respiratory infection), *P. aeruginosa* DK2 (isolated from CF sputum) and PAO1 (MH340 [347, 348], isolated from a wound), *S. aureus* LMG 10147 (isolated from a wound), *S. aureus* W2, W8, and W22 (three CF sputum isolated, kindly provided by Dr. Jozef Dingemans, MD), ATCC 25923 (clinical isolate), Mu50

(isolated from a wound), and USA300 (isolated from a soft tissue infection) were cultures overnight at 37°C in Brain Heart Infusion broth (BHI) (Oxoid, Basinstoke, UK).

Formation of biofilms in medium

Biofilm formation was performed as described previously. [349] Briefly, inoculum suspensions containing approximately 10^6 CFU/ml of *P. aeruginosa*, 10^6 CFU/ml of *S. aureus* and/or $5x10^6$ CFU/ml of *S. anginosus* were made in biofilm medium (BHI supplemented with 5% (w/v) BSA, 0.5% (w/v) mucin type II, and 0.3% (w/v) agar [350]). 96-well MTP (TTP, Trasadingen, Switzerland) were filled with the inoculum suspensions of all three species alone or together. After 4 h of adhesion at 37°C, the supernatant was removed and 100 µl fresh medium was added to the wells. After an additional 20 h, cells were rinsed with 100 µl physiological saline (PS) and 200 µl of an antibiotic solution in biofilm medium was added to the mature biofilm. To the control wells, 200 µl of biofilm medium was added. The plates were incubated for 24 h at 37°C. For each test condition, a minimum of three biological replicates with each two technical replicates was included.

Antibiotic solutions

The difference in susceptibility of all three species between growth in a mono- and multispecies biofilm was determined towards amoxicillin+sulbactam (both Sigma-Aldrich, Diegem, Belgium), aztreonam (TCI Europe, Zwijndrecht, Belgium), cefepime (Sigma-Aldrich), ceftazidime (Sigma-Aldrich), ciprofloxacin (Sigma-Aldrich), colistin (Sigma-Aldrich), imipenem (Sigma-Aldrich), levofloxacin (Sigma-Aldrich), meropenem (Hospira, Illinois, USA), tobramycin (TCI Europe), and vancomycin (Sigma-Aldrich) in biofilm medium. The concentrations used (partly based on concentrations that can be reached in serum or sputum) were the following: 5 µg/ml (amoxicillin) [351], 4 µg/ml (sulbactam), 500 µg/ml (aztreonam) [352], 500 µg/ml (cefepime) [353], 150 µg/ml (ceftazidime) [354], 150 µg/ml (ciprofloxacin) [355], 200 µg/ml (colistin) [356], 100 µg/ml (imipenem) [357], 500 µg/ml (levofloxacin) [358], 500 µg/ml (meropenem) [359], 200 µg/ml (tobramycin) [360], and 512 µg/ml (vancomycin) [361].

Generation of cell-free culture supernatant and formation of S. anginosus biofilms and planktonic cultures in cell-free culture supernatant

Monospecies biofilms of *S. aureus* or *P. aeruginosa* were formed in biofilm medium as described above. After 4 h of adhesion and 20 h of maturation, the culture supernatant was collected and centrifuged at 5000 rpm for 10 minutes. The supernatant was subsequently sterilized using 0.22 µm filters (Merck Millipore, Billerica, Massachusetts, USA), resulting in cell-free culture supernatant. To confirm complete removal of all microorganisms, 100 µl of the supernatant was spread on a BHI agar plate and incubated for 24 h at 370C. Supernatant was either used immediately or stored at -20°C for

a maximum of 48 h. For supernatant of planktonic *S. aureus* cultures, *S. aureus* was grown overnight in biofilm medium for 16 h, and the supernatant was collected as described above.

To evaluate the effect of growth in supernatant, firstly, growth curves of *S. anginosus* LMG 14502 were determined in biofilm medium, biofilm medium 1:1 diluted with supernatant of an *S. aureus* LMG 10147 biofilm, or 1:3 or 1:10 diluted with MQ water (Merck Millipore), using an Envision multilabel reader (PerkinElmer LAS, Boston, MA, USA) by plotting the OD at 590 nm versus incubation time. Next, monospecies biofilms of *S. anginosus* were grown and treated as described above in a 1:1 mixture of biofilm medium and biofilm supernatant of *S. aureus* or *P. aeruginosa*, or in biofilm medium 1:3 diluted with MQ. Cell numbers were determined by plate counting.

To determine the activity of biofilm supernatant on planktonic *S. anginosus* cultures, overnight *S. anginosus* cultures in BHI were put to OD_{605nm} 0.05 in biofilm medium or in biofilm medium 1:1 diluted in biofilm supernatant of *S. aureus*. After 24 h at 37°C and 250 rpm, tubed were centrifuged (5000 rpm, 5 min), supernatant was removed, and pellets were rinsed with PS. Vancomycin (2xMIC = 2µg/ml) in biofilm medium or in biofilm medium 1:1 diluted with supernatant was added to the test tubes for another 24 h (37°C, 250 rpm). Tubes were again centrifuged (5000 rpm, 5 min), supernatant was removed, and pellets were rinsed using PS. Cell numbers were determined using the plate count method as described below.

Quantification of biofilm cells

After 24 h of treatment, biofilms were washed with PS and cells were collected by sonication and vortexing as described previously [350]. Cell numbers were determined by the plate count method, using selective agar for *S. anginosus* (BHI agar supplemented with 1.25 mg/l triclosan (Sigma-Aldrich), *S. aureus* (tryptic soy agar supplemented with 7.5% NaCl), and *P. aeruginosa* (*Pseudomonas* isolation agar). *S. anginosus* plates were incubated anaerobically for 48 h at 37°C, while *S. aureus* and *P. aeruginosa* plates were incubated aerobically for 48 h at 37°C. Log CFU/ biofilm was calculated by subtracting the log surviving cells after treatment from the corresponding log control cells.

Determination of the MIC

MIC values of amoxicillin(+sulbactam), cefepime, imipenem, meropenem, and vancomycin towards *S. anginosus* LMG 14502 were determined in duplicate according to the EUCAST broth microdilution protocol in flat-bottom 96-well MTP (TTP). [362] Concentration ranging from 0.03125 to 25 µg/ml. The MIC was defined as the lowest concentration for which no significant difference in OD_{590nm} was observed between blank and inoculated wells after 24 h of growth at 37°C. [335] Results obtained in replicate experiments did not differ more than two fold. When a twofold difference was observed, the lowest concentration was recorded as the MIC.

Effects of DNase I on the antibiotic susceptibility of a 24 hours-old S. anginosus biofilm

Biofilms were formed as described above in cation-supplemented medium (0.015% (w/v) $CaCl_2$; 2.0 mM $MgCl_2$), essential for DNase I activity [363, 364]. After 24 h of incubation at 37°C, the supernatant was discarded, and biofilms were washed with 100 µl PS. 200 µl of an antibiotic solution together with 100 µg/ml DNase I (Sigma-Aldrich) was added to the wells. After 24 h of additional incubation at 37°C, cell numbers were determined by plate counting on BHI agar supplemented with 1.25 mg/l triclosan.

Quantification of extracellular DNA in the biofilm matrix

eDNA in the biofilm matrix was quantified as previously described [365]. Briefly, *S. anginosus* biofilms were formed in biofilm medium or biofilm supernatant of *S. aureus* as described above. Biofilm cells were washed with PS and collected by pipetting up and down in Eppendorf protein LoBind microcentrifuge tubes (1.5 ml) (Eppendorf AG, Hamburg, Germany). 100 µl of this solution was used for plate counting to determine the number of biofilm cells. Subsequently, biofilm cells were separated from the matrix by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant was aspirated and filtered through a 0.2 µm cellulose acetate filter (Whatman GmbH, Dassel, Germany). The amount of eDNA was quantified using the Quantifluor dsDNA System kit (Promega, Madison, WI, USA) and normalized to the number of biofilm cells (determined by plate counting). Five biological replicates were included.

Statistical data analysis

Statistical data analysis was performed using SPSS software, version 24 (SPSS, Chicago, Illinois, USA). The Normal distribution of the data was verified using the Shapiro-Wilk test. Normally distributed data were analyzed using an independent sample *t*-test. Non-Normally distributed data were analyzed using a Mann-Whitney test. Differences with a p-value ≤ 0.05 were considered significant.

RESULTS

P. aeruginosa influences biofilm formation of S. aureus and S. anginosus in a multispecies biofilm

S. anginosus, *S. aureus*, and *P. aeruginosa* were grown as mono-or multispecies biofilms in a 96-well microtiter plate (MTP). Medium containing bovine serum albumin (BSA) was used to allow for better growth of *S. aureus* in the presence of *P. aeruginosa*, as previously published. [350] *S. anginosus* LMG 14502 grew to a significantly lower cell number when co-cultured with *S. aureus* LMG 10147 and *P. aeruginosa* DK2 (difference of 0.61 ± 0.19 log, p≤ 0.05) (Figure 1). Similar results were obtained for another strain of *S. anginosus* (LMG 14696) (decrease in cell number of 0.50 ± 0.36 log, p ≤ 0.05). Also for *S. aureus* LMG 10147 a reduction in cell number was observed when grown in a multispecies biofilm with *S. anginosus* LMG 14502 and *P. aeruginosa* DK2 (1.12 ± 0.40 log, p ≤ 0.05). For *P. aeruginosa* DK2, no difference in cell numbers was observed.

When *S. aureus* LMG 10147 and *S. anginosus* LMG 14502 of LMG 14696 were grown together in a dual species biofilm, no significant difference in cell numbers could be observed compared to a monospecies biofilm, nor for *S. aureus* (difference of 0.02 ± 0.07 log and 0.31 ± 0.47 log when grown with *S. anginosus* LMG 14502 and LMG 14696, respectively, p > 0.05), neither for *S. anginosus* (difference of 0.01 ± 0.75 log and 0.24 ± 0.42 log, for LMG 14502 and LMG 14696, respectively, p > 0.05). These results indicate that *P. aeruginosa* is responsible for the decrease in cell numbers observed in a multispecies biofilm.

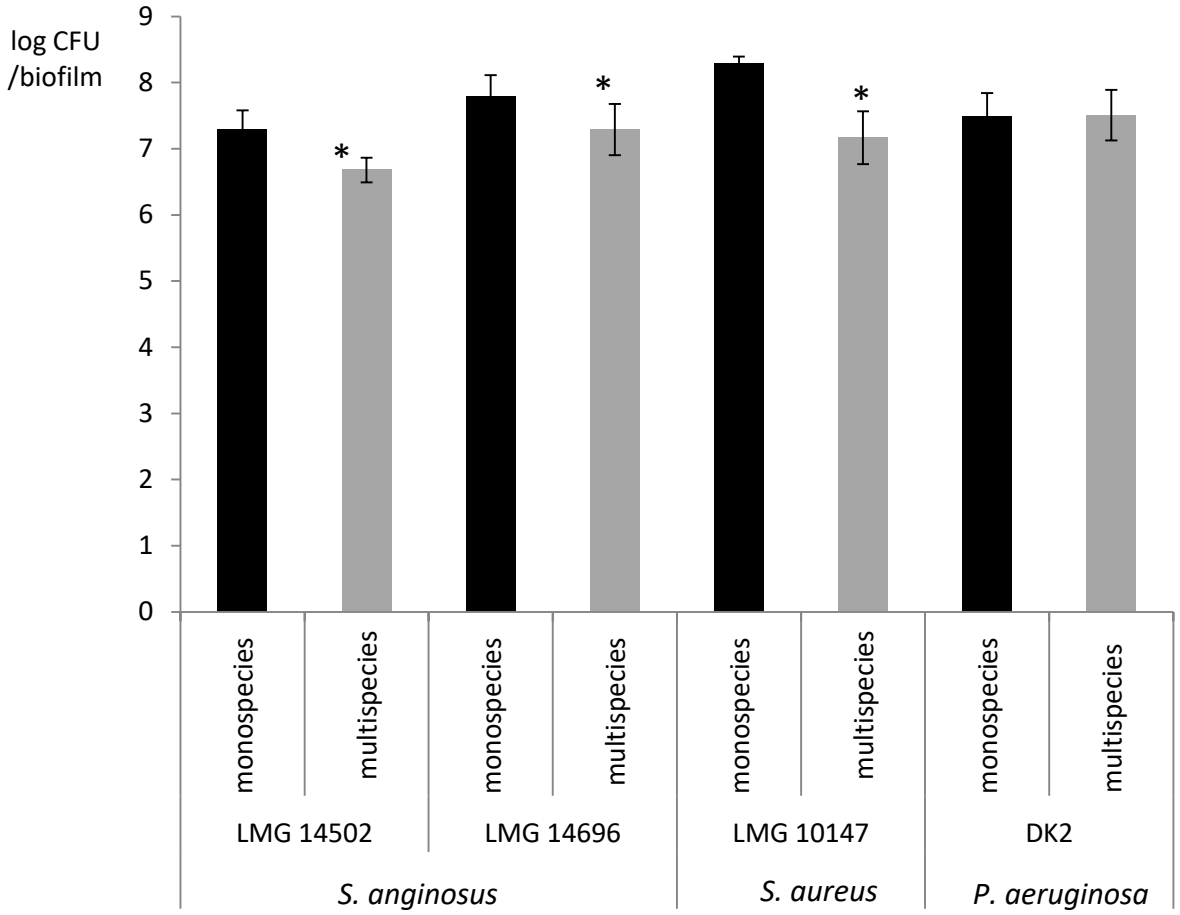

***Figure 1**: Average number of CFU of the different strains recovered from single and multispecies biofilm. Multispecies biofilms contained S. anginosus (LMG 14502 or LMG 14696), S. aureus LMG 10147 and P. aeruginosa DK2. Error bars represent standard deviations. n = 3 for P. aeruginosa, n = 6 for S. aureus* and *S. anginosus*. *significantly different from monospecies biofilm ($p \leq 0.05$). Inoculum suspensions contained approximately 10^6 CFU/ml of *P. aeruginosa*, 10^6 CFU/ml of *S. aureus* and/or $5x10^6$ CFU/ml of *S. anginosus*.

More S. aureus cells are killed by antibiotics in a multispecies biofilm while antibiotic killing of P. aeruginosa is not affected

Antibiotic-mediated killing of *P. aeruginosa* DK2 and *S. aureus* LMG 10147 in a mono-and multispecies biofilm was determined and the results are shown in Table 1. Our data show that for *S. aureus* LMG 10147 there was significantly more antibiotic killing in a multispecies biofilm with *S. anginosus* LMG 14502 and *P. aeruginosa* DK2 ($p \leq 0.05$) than in a monospecies biofilm, when exposed to amoxicillin+sulbactam, ceftazidime, ciprofloxacin, imipenem, levofloxacin, meropenem, tobramycin, or vancomycin. On the other hand, for *P. aeruginosa* DK2, no significant difference in killing between a mono- and multispecies biofilm could be observed for any of the antibiotics tested.

Antibiotic killing of S. anginosus in mono-versus multispecies biofilm

The log colony forming units (CFU) of *S. anginosus* LMG 14502 killed per biofilm after treatment with several antibiotics is shown in Table 1. A significantly decreased killing of *S. anginosus* LMG 14502 in a multispecies biofilm is observed for amoxicillin+sulbactam, imipenem, and vancomycin ($p \leq 0.05$). To determine whether or not this was dependent on the *P. aeruginosa* strain used, antibiotic killing of

S. anginosus in the presence of *S. aureus* LMG 10147 and *P. aeruginosa* PAO1 was evaluated as well (Table 1). While the presence of *P. aeruginosa* PAO1 had no significant effect on the *S. anginosus* cell number in the absence of treatment (6.73 ± 0.53 log CFU/biofilm in a multispecies biofilm compared to 7.29 ± 0.28 log CFU/biofilm in a monospecies biofilm, $p > 0.05$), after treatment, a significant decrease in killing by amoxicillin+sulbactam, imipenem, and vancomycin was again observed in the multispecies biofilm. Furthermore, when using PAO1, a significantly decreased killing was also observed for cefepime, ceftazidime, levofloxacin, and meropenem, but not for ciprofloxacin and tobramycin.

In order to determine if the decreased antibiotic killing is due to an extracellular factor produced by *S. aureus* and/or *P. aeruginosa*, antibiotic killing of *S. anginosus* LMG 14502 was assessed in the presence of supernatant (1:1 diluted in medium) obtained from a monospecies biofilm of *P. aeruginosa* DK2 or PAO1, or *S. aureus* LMG 10147 (Figure 2). Firstly, we assessed the biofilm formation of the strains under these condition, in the absence of antimicrobial agents. When grown in the biofilm supernatant of *P. aeruginosa* DK2, a significant reduction in biofilm formation was observed (0.52 ± 0.14 log, $p \leq 0.05$), while diluted biofilm supernatant of *P. aeruginosa* PAO1 or *S. aureus* LMG 10147 had no effect. Secondly, growth curves of *S. anginosus* LMG 14502 were evaluated. Results show that *S. anginosus* grew to a lower optical density (OD) in biofilm medium 1:1 diluted with *S. aureus* biofilm supernatant, compared to undiluted biofilm medium. Growing *S. anginosus* in biofilm medium diluted 1:3 with MilliQ (MQ) water could mimic growth profile in

biofilm supernatant, whereas almost no growth could be observed in a 1:10 dilution in MQ (Supplementary Figure S1). Therefore, to evaluate if an alteration in growth curve of *S. anginosus* in the presence of biofilm supernatant could be responsible for an altered antibiotic killing, killing of *S. anginosus* LMG 14502 was evaluated in biofilm medium 1:3 diluted with MQ. Biofilm cell numbers were asses in the absence of treatment, and after treatment with cefepime, imipenem, and vancomycin. No difference could be observed compared to growth in pure biofilm medium (Supplementary Table S2).

However, a significant decrease in killing by amoxicillin+sulbactam, cefepime, imipenem, meropenem, or vancomycin could be observed when *S. anginosus* was grown in biofilm supernatant of *S. aureus* LMG 10147, but not in the biofilm supernatant of *P. aeruginosa*. These data suggest that *S. aureus* is responsible for the observed decreased antibiotic killing of *S. anginosus* in a multispecies biofilm.

To evaluate if the decreased antibiotic killing observed in biofilm medium and in biofilm supernatant of *S. aureus* LMG 10147 is strain-dependent, a second *S. anginosus* strain was tested (LMG 14696). Again, a significant decrease in killing of *S. anginosus* by amoxicillin+sulbactam, cefepime, imipenem, meropenem, or vancomycin, but not by ciprofloxacin, levofloxacin, or tobramycin, could be observed when *S. anginosus* was grown in a multispecies biofilm (Table 1). When grown in biofilm supernatant of *S. aureus* LMG 10147, a decreased killing by amoxicillin+sulbactam, cefepime, imipenem, meropenem, and vancomycin was observed as well (Figure 2).

For vancomycin (512 µg/ml), experiments were repeated using supernatant of a planktonic overnight *S. aureus* LMG 10147 culture in biofilm medium. Again, a decreased killing of *S. anginosus* biofilm cells by vancomycin could be observed (a decrease of 0.55 ± 0.66 log was seen), indicating that the effect is not limited to biofilm supernatant of *S. aureus* LMG 10147, but that it is also observed with supernatant of planktonic *S. aureus* cultures.

Furthermore, experiments with vancomycin (2xMIC) were also repeated using shaking planktonic cultures of *S. anginosus* instead of biofilms, grown in pure biofilm medium, or in medium 1:1 diluted with biofilm supernatant of *S. aureus*. No viable planktonic cells could be recovered in any of the conditions, indicating that the protective effect of *S. aureus* biofilm supernatant is specific for *S. anginosus* biofilm cells.

***Table 1**: Biofilm cells (log ± stdev) of S. aureus LMG 10147, P. aeruginosa DK2, S. anginosus LMG 14502, and LMG 14696 killed after treatment with antibiotics, when grown in a mono- or multispecies biofilm. n≥3. *significantly different from monospecies biofilm (p ≤ 0.05). [a]ND: Not determined.*

Antibiotic solution (µg/ml)	***S. aureus* LMG 10147**		***P. aeruginosa* DK2**		***S. anginosus***				
	Monospecies	Multispecies (+ *S. anginosus* LMG 14502 + *P. aeruginosa D*K2)	Monospecies	Multispecies (+ *S. anginosus* LMG 14502 + *S. aureus* LMG 10147)	Monospecies LMG 14502	Multispecies LMG 14502 (+ *S. aureus* LMG 10147 + *P. aeruginosa* DK2)	Multispecies LMG 14502 (+ *S. aureus* LMG 10147 + *P. aeruginosa* PAO1)	Monospecies LMG 14696	Multispecies LMG 14696 (+ *S. aureus* LMG 10147 + *P. aeruginosa* DK2)
Amoxicillin (5) + sulbactam (4)	0.46 ± 0.18	1.86 ± 0.56*	0.23 ± 0.17	0.72 ± 0.19	3.65 ± 0.46	1.59 ± 0.57*	1.32 ± 0.60*	4.69 ± 0.72	1.74 ± 0.79*
Aztreonam (500)	0.47 ± 0.22	0.16 ± 0.22	0.82 ± 0.25	0.08 ± 0.22	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
Cefepime (500)	0.31 ± 0.04	1.48 ± 1.27	0.75 ± 0.19	1.59 ± 0.47	3.02 ± 0.61	2.56 ± 0.68	1.31 ± 0.32*	4.50 ± 0.38	1.49 ± 0.89*
Ceftazidime (150)	0.62 ± 0.12	2.62 ± 0.36*	1.24 ± 0.27	2.19 ± 0.13	1.76 ± 0.73	1.68 ± 0.59	0.14 ± 0.65*	4.03 ± 0.91	3.20 ± 0.50
Ciprofloxacin (150)	0.88 ± 0.07	2.00 ± 0.51*	4.48 ± 0.38	3.73 ± 0.43	3.73 ± 0.97	3.69 ± 1.00	2.96 ± 0.40	3.58 ± 0.91	3.62 ± 0.69
Colistin (200)	0.09 ± 0.07	-1.44 ± 0.19	3.68 ± 0.30	2.25 ± 0.29	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
Imipenem (100)	0.52 ± 0.08	1.70 ± 0.07*	1.55 ± 0.16	1.41 ± 0.37	3.13 ± 0.60	2.16 ± 0.27*	1.47 ± 0.56*	4.40 ± 0.30	1.22 ± 0.74*
Levofloxacin (500)	1.67 ± 0.55	3.90 ± 0.41*	4.37 ± 0.25	3.73 ± 0.37	3.83 ± 0.39	3.90 ± 0.41	2.89 ± 0.26*	3.30 ± 0.52	2.43 ± 1.15
Meropenem (500)	0.19 ± 0.23	3.14 ± 0.52*	2.07 ± 0.15	0.92 ± 0.24	3.48 ± 1.28	3.29 ± 0.74	1.70 ± 0.33*	4.11 ± 0.46	2.57 ± 0.59*
Tobramycin (200)	0.65 ± 0.40	2.67 ± 0.23*	1.29 ± 0.27	2.52 ± 0.48	4.77 ± 1.31	4.57 ± 1.64	3.29 ± 0.47	3.26 ± 0.25	4.42 ± 0.11*
Vancomycin (512)	0.46 ± 0.10	1.83 ± 0.47*	0.36 ± 0.19	0.05 ± 0.26	3.35 ± 0.52	1.99 ± 0.93*	1.15 ± 0.42*	3.36 ± 0.77	2.32 ± 0.84*

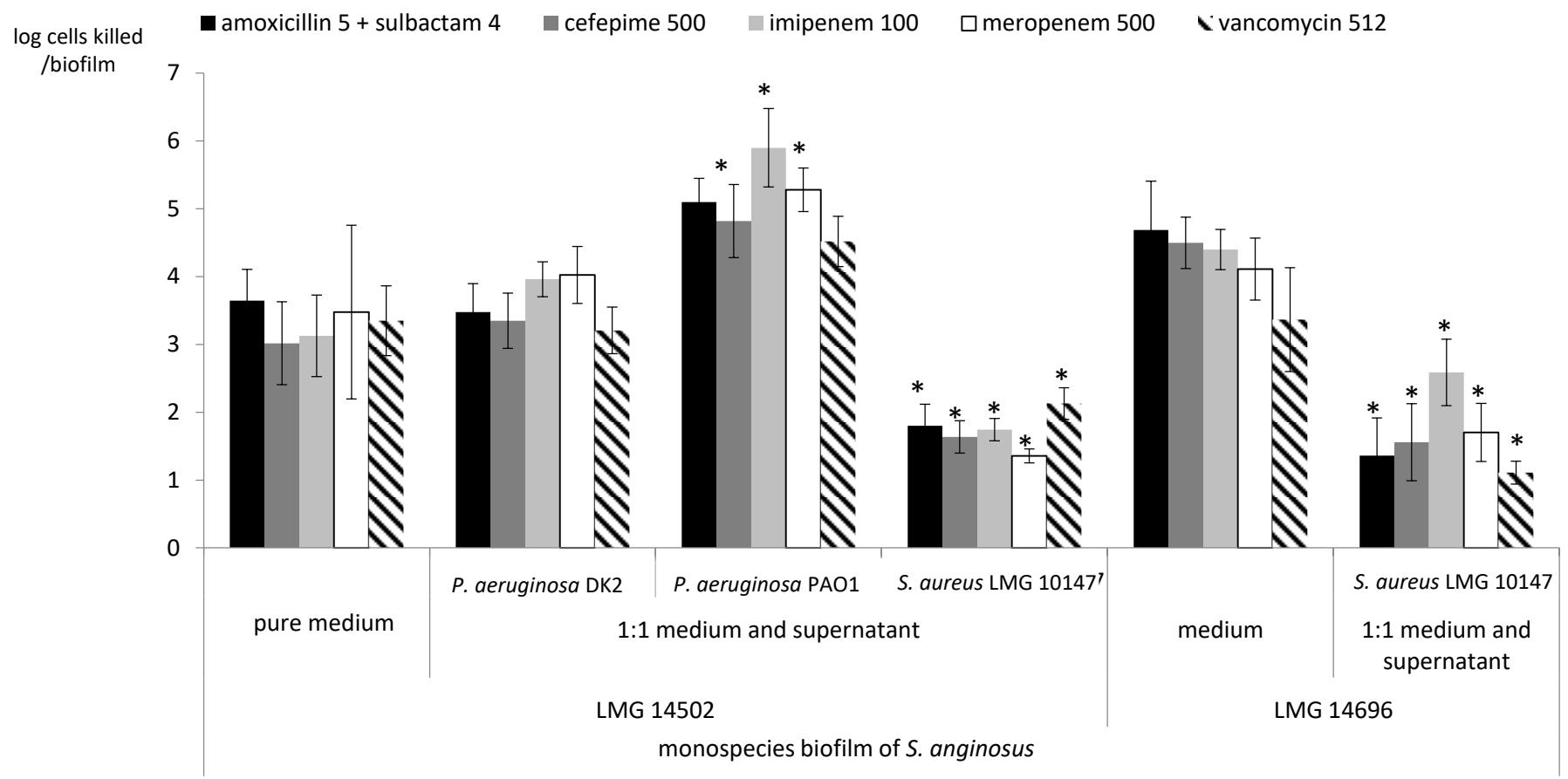

***Figure 2**: Log S. anginosus biofilm cells killed after treatment with antibiotic solutions (concentrations in µg/ml) in several conditions: (i) monospecies biofilm in pure medium (BHI supplemented with 5% BSA, 0.5% mucin type II, and 0.3% agar), (ii) monospecies biofilm in diluted biofilm supernatant of a monospecies biofilm of P. aeruginosa DK2, (iii) monospecies biofilm in diluted biofilm supernatant of a monospecies biofilm of P. aeruginosa PAO1, (iv) monospecies biofilm in diluted biofilm supernatant of a monospecies biofilm of S. aureus LMG10147. Error bars represent standard deviations. n≥3. *significantly different from monospecies biofilm (p ≤ 0.05).*

Other S. aureus strains also decrease susceptibility of S. anginosus

To evaluate whether other *S. aureus* strains also cause a decreased antibiotic killing, *S. anginosus* was grown and treated in biofilm supernatant of several other strains (Table 2). There was a significant decrease in killing of *S. anginosus* (for all antibiotics tested) when it was grown and treated in biofilm supernatant of *S. aureus* W8, W1, W22, and Mu50 ($p \leq 0.05$). When grown in the biofilm supernatant of USA300 and ATCC25923, there was also decreased killing of *S. anginosus*, except for cefepime (supernatant of USA300), and meropenem and vancomycin (supernatant of 25923). Killing by ciprofloxacin, levofloxacin, and tobramycin was also evaluated in the biofilm supernatant of *S. aureus* strains LMG 10147 and W8 (Table 3), but no difference could be observed. Similar results were obtained with *S. anginosus* LMG 14696 (Table 4).

Both for *S. anginosus* LMG 14502 and LMG 14696, experiments were repeated using dual species biofilms of *S. anginosus* and several *S. aureus* strains. Results show that *S. anginosus* (both LMG 14502 and LMG 14696) was also protected against antibiotic killing when grown together with *S. aureus* (see Supplementary Table S3 and S4).

***Table 2**: Biofilm cells (log ± stdev) of S. anginosus LMG 14502 killed after treatment with amoxicillin + sulbactam, cefepime, imipenem, meropenem, or vancomycin, when grown in biofilm medium or in diluted biofilm supernatant of several S. aureus strains. n≥3. *significantly different from growth in biofilm medium (p ≤ 0.05).*

	Log killed of a monospecies *S. anginosus* LMG 14502 biofilm, grown and treated in						
	Biofilm medium	**SN of monospecies biofilm of *S. aureus***					
Antibiotic solution (µg/ml)		**W8**	**W1**	**W22**	**USA300**	**ATCC 25923**	**Mu50**
Amoxicillin (5) + sulbactam (4)	3.65 ± 0.46	0.93* ± 0.40	0.37* ± 0.35	0.66* ± 0.41	0.55* ± 0.33	0.15* ± 0.44	0.54* ± 0.66
Cefepime (500)	3.02 ± 0.61	1.18* ± 0.43	0.73* ± 0.40	1.38* ± 1.85	3.18 ± 0.53	0.89* ± 0.48	0.39* ± 0.65
Imipenem (100)	3.13 ± 0.60	1.88* ± 0.15	1.02* ± 0.41	0.56* ± 0.10	1.32* ± 0.42	1.14* ± 0.61	0.53* ± 0.59
Meropenem (500)	3.48 ± 1.28	1.27* ± 0.44	2.38 *± 0.57	1.35* ± 0.82	0.70* ± 0.11	2.13 ± 1.31	1.13* ± 1.34
Vancomycin (512)	3.35 ± 0.52	2.89* ± 0.61	1.21* ± 0.42	1.62* ± 0.51	1.45* ± 1.35	3.55 ± 0.39	1.03* ± 1.04

***Table 3**: Biofilm cells (log ± stdev) of S. anginosus LMG 14502 killed after treatment with ciprofloxacin, levofloxacin, or tobramycin, when grown in biofilm medium or in diluted biofilm supernatant of S. aureus LMG 10147 or W8. n≥3. No significant differences could be observed (p > 0.05).*

	Log killed of a monospecies *S. anginosus* LMG 14502 biofilm, grown and treated in		
Antibiotic solution (µg/ml)	**Biofilm medium**	**Biofilm SN *S. aureus* LMG 10147**	**Biofilm SN *S. aureus* W8**
Ciprofloxacin (150)	3.73 ± 0.97	2.12 ± 0.75	3.52 ± 0.52
Levofloxacin (500)	3.83 ± 0.39	3.05 ± 0.62	2.83 ± 0.29
Tobramycin (200)	4.77 ± 1.31	4.43 ± 0.98	4.42 ± 0.25

***Table 4**: Biofilm cells (log ± stdev) of S. anginosus LMG 14696 killed after treatment with amoxicillin + sulbactam, cefepime, imipenem, meropenem, or ancomycin, when grown in biofilm medium or in diluted biofilm supernatant of S. aureus W22, USA300, ATCC 25923, or Mu50. n≥3. *significantly different from growth in biofilm medium (p ≤ 0.05).*

	Log killed of a monospecies *S. anginosus* LMG 14696 biofilm, grown and treated in				
	Biofilm medium	**SN of monospecies biofilm of *S. aureus***			
Antibiotic solution (µg/ml)		**W22**	**USA300**	**ATCC 25923**	**Mu50**
Amoxicillin (5) + sulbactam (4)	4.69 ± 0.72	0.86* ± 0.39	0.65* ± 0.32	0.81* ± 0.08	1.12* ± 0.52
Cefepime (500)	4.50 ± 0.38	0.78* ± 0.22	0.74* ± 0.09	0.33* ± 0.42	0.82* ± 0.52
Imipenem (100)	4.40 ± 0.30	1.04* ± 0.48	0.52* ± 0.44	0.36* ± 0.14	0.84* ± 0.13
Meropenem (500)	4.11 ± 0.46	0.71* ± 0.45	0.26* ± 0.42	0.25* ± 0.31	0.68* ± 0.46
Vancomycin (512)	3.36 ± 0.76	0.65* ± 0.85	0.48* ± 0.40	0.25* ± 0.34	0.70* ± 0.53

Cell-free cultures supernatant of S. aureus did not alter the minimal inhibitory concentrations of S. anginosus LMG 14502

To evaluate if cell-free supernatant of *S. aureus* biofilms could lead to a decrease in minimal inhibitory concentration (MIC) value, MIC values of amoxicillin, amoxicillin+sulbactam, meropenem, cefepime, imipenem, and vancomycin towards *S. anginosus* LMG 14502 were determined in biofilm medium and in biofilm supernatant of *S. aureus* LMG 10147 (1:1 diluted in medium) (Table 5). No difference in MIC was observed between both conditions, except for amoxicillin. In biofilm supernatant, the MIC of amoxicillin was 0.5 µg/ml, whereas the MIC in pure biofilm medium was 0.0625 µg/ml. Addition of a β-lactamase inhibitor again reduced the MIC in supernatant to the same value as in pure biofilm medium. MIC values of *S. anginosus* LMG 14696, *S. aureus* LMG 10147, and *P. aeruginosa* DK2 were also determined (see Supplementary Table S5).

***Table 5**: Minimal inhibitory concentration values (µg/ml) of S. anginosus LMG 14502 in biofilm medium, compared to grown in diluted biofilm supernatant of a monospecies S. aureus LMG 10147 biofilm.*

Antibiotic solution	**Biofilm medium**	**Biofilm medium 1:1 diluted with biofilm SN of *S. aureus* LMG 10147**
Amoxicillin	0.0625	0.5
Amoxicillin + 4µg/ml sulbactam	0.0625	0.0625
Meropenem	0.0625	0.125
Cefepime	0.25	0.25
Imipenem	0.015625	0.015625
Vancomycin	1	1

Extracellular DNA concentration is not altered in biofilms grown in the supernatant of S. aureus

To investigate the role of extracellular DNA (eDNA) in the reduced antibiotic efficacy, its concentration in biofilms grown in pure medium and in diluted biofilm supernatant was determined. No significant differences were observed (Table 6). To confirm the lack of a role for eDNA, we evaluated the activity of a selection of antibiotics (imipenem, meropenem, cefepime, and vancomycin) in the presence of DNase I. No difference in killing efficacy could be observed (Table 7).

Table 6: *eDNA concentration in ng/ml per 10^8 CFU ± stdev of S. anginosus LMG 14502 and LMG 14696 grown in biofilm medium or in diluted biofilm supernatant of S. aureus LMG 10147. n=5. No significant differences could be observed (p > 0.05).*

	eDNA concentration (ng/ml per 10^8 CFU)	
***S. anginosus* strain**	**Biofilm medium**	**Biofilm SN of *S. aureus* LMG 10147**
LMG 14502	71.28 ± 31.79	137.0 ± 65.91
LMG 14696	30.40 ± 11.23	36.24 ± 10.80

Table 7*: biofilm cells (log ± stdev) of S. anginosus LMG 14502 killed after treatment with cefepime, imipenem, meropenem, and vancomycin, with or without the addition of DNase I (100 µg/ml), when grown in diluted biofilm medium of S. aureus LMG 10147. n≥3. No significant differences could be observed (p > 0.05).*

	Biofilm medium 1:1 diluted with biofilm SN of *S. aureus* LMG 10147	
Antibiotic solution (µg/ml)	Without DNase I	With DNase I (100 µg/ml)
Cefepime 500	1.64 ± 0.24	1.57 ± 0.40
Imipenem 100	1.74 ± 0.16	1.68 ± 0.46
Meropenem 500	1.36 ± 0.10	1.09 ± 0.46
Vancomycin 512	2.13 ± 0.23	1.97 ± 0.51

DISCUSSION

Research on activity of antibiotics against biofilms has mainly focused on monospecies biofilms [366-368], while many biofilm-related infections are due to multiple species [80, 369]. Therefore, in the present study, we investigated the role of community composition on antibiotic-mediated killing in a multispecies biofilm.

Antibiotic killing of *S. anginosus*, *S. aureus* and *P. aeruginosa* was compared in mono- and multispecies biofilms. Our results show that the antibiotic killing of *P. aeruginosa* is not influenced by the presence of *S. aureus* and *S. anginosus*, which is in line with results observed by Price et al. [124]. They reported no significant difference in killing of *P. aeruginosa* by tobramycin when grown together with *S. constellatus*, also member of the SMG. DeLeon et al. [76] investigated the killing of *P. aeruginosa* by gentamicin and ciprofloxacin. Also in this study, no significant difference in killing of *P. aeruginosa* was observed when grown together with *S. aureus*. On the other hand, Michelsen et al. [179] suggested protection of *P. aeruginosa* in the presence of *S. aureus* on agar plates containing inhibitory levels of tobramycin, gentamicin, and ciprofloxacin, which could be explained by an overexpression of efflux mechanisms and lipopolysaccharide modification, induced by the presence of *S. aureus*. [179]

Furthermore, our results show that more *S. aureus* cells are killed by antibiotic treatment when grown together with *S. anginosus* and *P. aeruginosa*, both by antibiotics that interfere with cell wall synthesis and those that interfere with other cellular processes (e.g. RNA translation). For example, we observed an increased killing by ciprofloxacin and tobramycin. However, DeLeon et al. [76] observed no difference in killing by ciprofloxacin and even a protection of *S. aureus* against gentamicin in presence of *P. aeruginosa*. Aminoglycoside-modifying enzymes produced by *P. aeruginosa* were proposed to be involved. In addition, Hoffman et al. [178] showed that HQNO produced by *P. aeruginosa* protected *S. aureus* during co-culture from killing by tobramycin. The use of another growth medium and other bacterial strains, or the presence of *S. anginosus*, could be responsible for the observed differences in outcome between the present study and theirs. In contrast to *S. aureus*, less *S. anginosus* cells were killed in a multispecies biofilm, but this was only observed for antibiotics that interfere with cell wall synthesis, not with other antibiotics. However, the results for *S. anginosus* to some extent also depend on the *P. aeruginosa* strain present in the multispecies community (DK2 vs. PAO1).

When antibiotic-mediated killing of sessile *S. anginosus* cells was quantified in supernatant of several *S. aureus* strains (both supernatant of planktonic *S. aureus* cultures, and supernatant of *S. aureus* biofilm cells), a decreased killing of *S. anginosus* was again observed for antibiotics that interfere

with transpeptidation and transglycosylation in cell wall synthesis, but not for other classes. In contrast, in the biofilm supernatant of *P. aeruginosa* (both DK2 and PAO1), no decreased killing of sessile

S. anginosus cells could be observed for any of the antibiotics used. These results indicate that a yet unidentified factor produced by *S. aureus* is responsible for the reduced killing of *S. anginosus*. As the effect is less pronounced when grown in a multispecies biofilm compared to in the supernatant of *S. aureus*, the negative influence of *P. aeruginosa* on *S. aureus* or *S. anginosus* could lead to a lower protective effect of *S. aureus* on *S. anginosus*. These experiments were also carried out with planktonic *S. anginosus* cultures, but to our surprise, no protection was seen, suggesting the observed decreased killing of *S. anginosus* is biofilm-specific.

Next, we wanted to investigate which mechanism could be responsible for the decreased antibiotic killing of *S. anginosus* in the presence of biofilm supernatant of *S. aureus*. Some studies reported that eDNA in the biofilm matrix could chelate cations leading to antibiotic tolerance, e.g. to vancomycin. [267, 365, 370, 371] However, when quantifying the eDNA concentration in monospecies *S. anginosus* biofilms, we could not observe a difference between the eDNA concentration in biofilms grown with or without biofilm supernatant. We confirmed that eDNA is not a major player in this regards by showing that the addition of DNase I (described to enhance the effect of antibiotics in some conditions [365, 372]), did not affect the number of surviving *S. anginosus* cells recovered from biofilms treated with antibiotics. Furthermore, the effect observed is also not due to the presence of β-lactamases of *S. aureus*, as protection is also seen against vancomycin, against amoxicillin in the presence of a β-lactam inhibitor, and in the supernatant of a β-lactamase negative *S. aureus* strain (ATCC 25923). In addition, the effect also seems to be independent from growth inhibition, as growth is reduced by DK2 but not by PAO1, whereas DK2 has less effect than PAO1 on the reduction of antibiotic susceptibility of *S. anginosus*.

Our data ambiguously demonstrate that interactions between species in a multispecies biofilm not always lead to a change in antimicrobial susceptibility, but that these changes depend on the antibiotic and the species involved. Interestingly, sessile *S. anginosus* cells seem to be protected by one or more compounds secreted by *S. aureus*, as decreased killing is also observed when *S. anginosus* is grown in the cell-free supernatant of *S. aureus* planktonic or biofilm cultures. Further experiments will be needed to elucidate the mechanisms involved. As protection could only be observed when using antibiotics that interfere with cell wall synthesis, an alteration of cell wall thickness of *S. anginosus* might play a role. Another suggestion for further research, is the possibility of a different bioavailability of the antimicrobial agent in a mono- and multispecies biofilm, which might also impact the final antibiotic concentration that reaches the biofilm cells and that can

effectively kill them. Finally, the treatment of CF patients is often directed towards the major pathogens *P. aeruginosa* and/or *S. aureus*, and our data show that this can lead to an increased survival of *S. anginosus*, which in turn could lead to acute exacerbations and a decline in lung function [122, 126, 127, 343]

ACKNOWLEDGEMENTS

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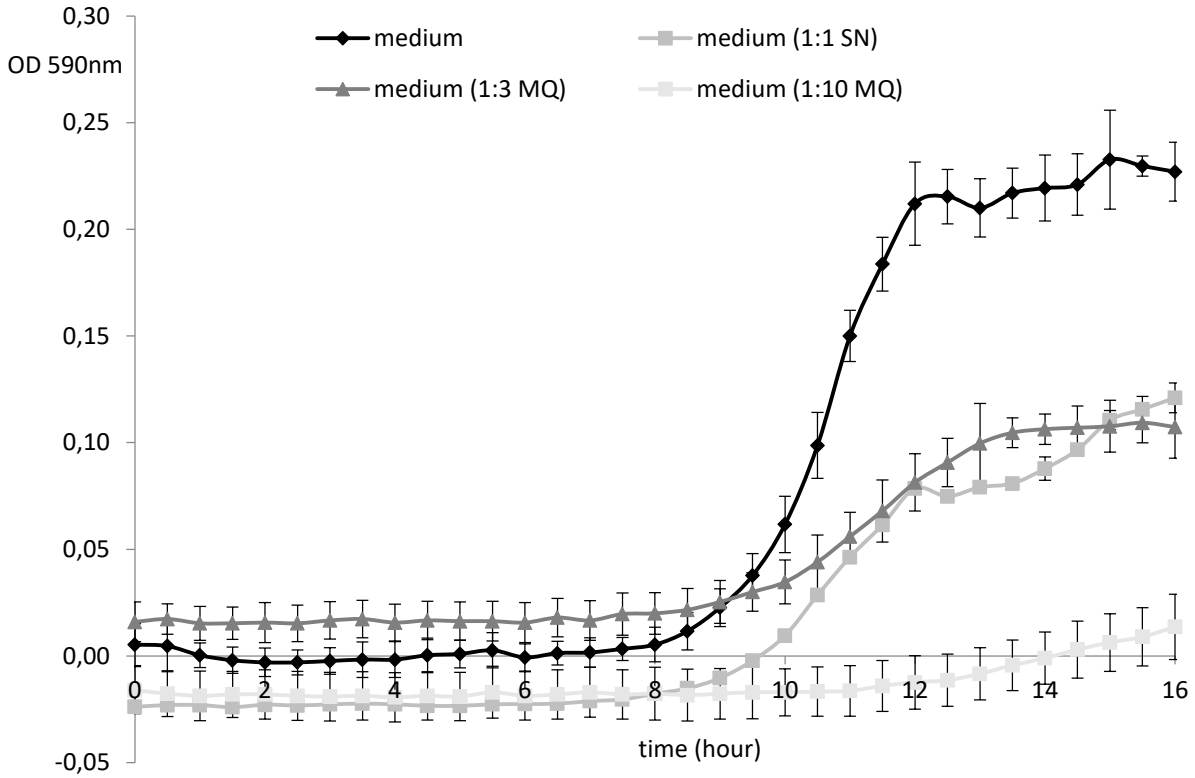
SUPPLEMENTARY DATA



***Supplementary Figure S1**: Growth curve experiment for S. anginosus LMG 14502, grown in medium, medium 1:1 diluted with supernatant, or 1:3 or 1:10 diluted with MQ. OD was plotted versus incubation time. Each data point is an average reading from three cultures. Error bars represent standard deviation.*

***Supplementary Table S2**: Biofilm cells (log ± stdev) of S. anginosus LMG 14502 killed after treatment with cefepime, imipenem, or vancomycin, when grown in biofilm medium or in 1/3 diluted biofilm medium. n=3. No significant differences could be observed (p > 0.05).*

Antibiotic solution (µg/ml)	**Biofilm medium**	**Biofilm medium 1:3 diluted with MQ**
Cefepime 500	3.02 ± 0.61	3.93 ± 0.78
Imipenem 100	3.13 ± 0.60	4.09 ± 0.65
Vancomycin 512	3.35 ± 0.52	2.47 ± 0.47

***Supplementary Table S3**: Biofilm cells (log ± stdev) of S. anginosus LMG 14502 killedafter treatment, when grown in biofilm medium together with S. aureus LMG 10147 or Mu50. n=3. *p ≤ 0.05.*

	Log killed of *S. anginosus* LMG 14502, grown and treated in		
	Biofilm medium (monospecies biofilm)	**a dualspecies biofilm with *S. aureus***	
Antibiotic solution (µg/ml)		**LMG 10147**	**Mu50**
Cefepime (500)	3.02 ± 0.61	0.83* ± 0.04	0.74* ± 0.22
Imipenem (100)	3.13 ± 0.60	1.13* ± 0.27	0.75* ± 0.52
Meropenem (500)	3.48 ± 1.28	0.56* ± 0.10	0.72* ± 0.50
Vancomycin (512)	3.35 ± 0.52	0.40* ± 0.05	0.53* ± 0.32

***Supplementary Table S4**: Biofilm cells (log ± stdev) of S. anginosus LMG 14696 killed after treatment, when grown in biofilm medium together with S. aureus W22, or Mu50. n=3. *p ≤ 0.05.*

	Log killed of *S. anginosus* LMG 14696, grown and treated in		
	Biofilm medium (monospecies biofilm)	**a dualspecies biofilm with *S. aureus***	
Antibiotic solution (µg/ml)		**W22**	**Mu50**
Cefepime (500)	4.50 ± 0.38	0.55* ± 0.68	0.26* ± 0.19
Imipenem (100)	4.40 ± 0.30	0.33* ± 0.14	0.17* ± 0.13
Meropenem (500)	4.11 ± 0.46	0.40* ± 0.18	0.13* ± 0.11
Vancomycin (512)	3.36 ± 0.76	0.22* ± 0.12	0.41* ± 0.10

***Supplementary Table S5**: Minimal inhibitory concentration values (µg/ml) of S. anginosus LMG 14696, S. aureus LMG 10147 and P. aeruginosa DK2, determined in duplicate according to the EUCAST broth microdilution protocol.*

	MIC values (µg/ml)		
Antibiotic solution	***S. anginosus* LMG 14696**	***S. aureus* LMG 10147**	***P. aeruginosa* DK2**
Amoxicillin	0.0625	2	> 512
Meropenem	0.015625	0.25	1
Cefepime	0.25	2-4	2-4
Imipenem	0.125	0.03125	4
Vancomycin	1	2	> 512

Paper 3:

Decreased susceptibility of *Streptococcus anginosus* to cell wall-acting antibiotics in multispecies biofilms is due to increased thickness of the cell wall.

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ABSTRACT

Streptococcus anginosus, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* are often co-isolated from sputum of cystic fibrosis (CF) patients. Little is currently known about how these bacteria influence each other's antibiotic susceptibility. It was recently shown that *S. anginosus* is protected from the activity of cell wall-acting antibiotics when it grows in a multispecies biofilm with *P. aeruginosa* and *S. aureus*. To elucidate the mechanism(s) responsible for this decreased killing of *S. anginosus*, we obtained a comprehensive overview of gene expression in *S. anginosus* cells grown in mono- and multispecies biofilms. Genes involved in cell wall synthesis were induced when *S. anginosus* is grown in a multispecies biofilm, suggesting that increased cell wall thickness plays a role in the reduced susceptibility. To confirm changes in the cell wall thickness of *S. anginosus*, transmission electron microscopy (TEM) was used. TEM data confirmed that *S. anginosus* indeed formed a thicker cell wall in a multispecies biofilm, leading to the observed decrease in susceptibility towards cell wall-acting antibiotics.

INTRODUCTION

In young cystic fibrosis (CF) patients, *Staphylococcus aureus* is the most commonly isolated pathogen [255], while in adults *Pseudomonas aeruginosa* is most frequently isolated [101]. Nevertheless, *S. aureus* and *P. aeruginosa* are co-isolated from approximately half of the adult patients [200]. *Streptococcus anginosus*, a member of the *Streptococcus milleri* group (SMG) is an emerging pathogen in patients with CF, and is frequently found to be the cause of acute exacerbations [120, 127, 343]. In CF sputum, *S. anginosus* is often co-isolated with *S. aureus* and *P. aeruginosa* [120, 342] but not much is known about how these three organisms influence each other's susceptibility in multispecies consortia. In the presence of *P. aeruginosa*, biofilm formation of *Streptococcus constellatus* (another member of the SMG), was increased after treatment with tobramycin [124]. In addition, *P. aeruginosa* was shown to protect *S. aureus* from killing by aminoglycosides [76, 178], but under certain conditions it can also inhibit growth of *S. aureus* [107, 118]. We previously observed there was less killing of *S. anginosus* by cell wall-acting antibiotics (amoxicillin + sulbactam, imipenem, vancomycin,) in a multispecies biofilm with *S. aureus* and *P. aeruginosa* than in a monospecies biofilm; this effect was not observed with other bactericidal antibiotics (tobramycin, ciprofloxacin) [373]. In contrast, antibiotics killed a larger fraction of *S. aureus* cells when these were grown in the presence of *S. anginosus* and *P. aeruginosa* [373].

The goal of the present study is to elucidate the mechanisms responsible for the previously observed decreased killing of *S. anginosus* in a multispecies biofilm [373].

MATERIALS & METHODS

Bacterial strains

S. anginosus LMG 14502, *P. aeruginosa* DK2, and *S. aureus* LMG 10147 were cultured overnight at 37°C in Brain Heart Infusion broth (BHI; Oxoid, Basingstoke, UK).

Biofilm formation

Biofilm formation was assessed as described previously [1]. Briefly, inoculum suspensions containing approximately $5x10^6$ CFU/ml of *S. anginosus*, 10^6 CFU/ml of *S. aureus*, or a combination of $5x10^6$ CFU/ml of *S. anginosus*, 10^6 CFU/ml of *P. aeruginosa* and 10^6 CFU/ml of *S. aureus*, were made in biofilm medium (BHI supplemented with 5% (w/v) bovine serum albumin (BSA), 0.5% (w/v) mucin type II, and 0.3% (w/v) agar). 96-well microtiter plates (MTP; TTP, Trasadingen, Switzerland) were filled with the inoculum suspensions. After 4 hours of adhesion at 37°C, the supernatant was discarded and 100 µl fresh biofilm medium was added. Cells were allowed to grow for an additional 20 h to form a mature biofilm.

Antibiotic treatment

After 24 h biofilm growth, biofilm supernatant was removed, and 200 µl of a 512 µg/ml vancomycin (Sigma-Aldrich, Diegem, Belgium) solution in biofilm medium was added for another 24 h.

Whole genome sequencing of S. anginosus LMG 14502

A pure culture of *S. anginosus* LMG 14502 on BHI agar was incubated anaerobically for 24h at 37°C. DNA was extracted as described by Pitcher *et al*. [374]. Lysis was increased by adding lysozyme (24 mg/ml) (Sigma-Aldrich), and DNA was RNase treated. A library was prepared using an adapted protocol of the NEBNext kit (New England Biolabs, Ipswich, USA). Samples were sequenced on the Illumina HiSeq 4000 platform (150 bp paired end reads), yielding approximately 750 Mb data (Oxford Genomics Center, University of Oxford, United Kingdom). Demultiplexed raw reads were imported into CLC Genomics Workbench 8.5.1 (CLC Bio, Aarhus, Denmark). *De novo* assembly with CLC Genomics Workbench generated 492 contigs. After initial quality control, only contigs with an average coverage of > 500 and a consensus length > 5000 were retained, resulting in a draft genome consisting of 1,859,791 bp in 11 contigs. The RAST server was used to annotate the consensus genome sequence [375]. The contigs were deposited in the NCBI Sequence Read Archive (accession number: SRP096309).

RNA-sequencing and data-analysis

Gene expression was quantified in single-species biofilms of *S. anginosus* LMG 14502 or in multispecies biofilms of *S. anginosus* together with *S. aureus* LMG 10147 and *P. aeruginosa* DK2. For each condition, three independent samples were obtained. After harvesting of the biofilm

cells by vortexing (5 min) and sonication (5 min) [376], RNA was extracted immediately using the Ambion RiboPure Bacteria Kit (Ambion, Austin, TX) according to the manufacturer's instructions. rRNA (on 2 µg total RNA) was depleted using the Ribo-Zero Magnetic Kit for bacteria (Epicentre, Illumina). Truseq stranded RNA library preparation kit (Illumina) was then used to create strand specific libraries. Quality control of the libraries (DNA 1000 chip, Agilent Technologies, Santa Clara, US) was performed and their concentration was determined according to recommendations provided by Illumina. The libraries were equimolarly pooled and sequenced using an Illumina NextSeq 500, generating 75 bp unpaired reads. Fastq files were deposited in ArrayExpress under the accession number E-MTAB-5426.

Using CLC Genomics Workbench, an initial quality control was performed, and quality filtered reads of the mono- and multispecies biofilm samples were mapped (length fraction 0.6 and similarity fraction 0.9) against the genomes and contigs of all three species simultaneously. For *S. aureus* LMG 10147, contigs from WGS project number LHUS01 were used [377]. For *P. aeruginosa* DK2, the NCBI reference sequence with accession number NC_018080 was used [378]. Reads mapping to *S. anginosus* were then extracted using the splitChromosome option of the bamUtil repository (https://github.com/statgen/bamUtil) and counted. On average, 6% of reads derived from mixed-species biofilms (on average 3.483.004 reads) mapped to *S. anginosus*. 6% of the reads derived from *S. anginosus* monospecies biofilms were then random sampled using CLC Genomics Workbench to match the number of reads mapping to *S. anginosus* derived from mixed-species biofilms (Supplementary Table S1). Reads from all six samples were then mapped to contigs of all three species and only reads mapping to *S. anginosus* were used for downstream analysis. RNA sequencing data were normalized on total counts and statistical analysis was performed using Empirical Analysis of DGE of the CLC genomics workbench software. The number of reads assigned to a transcript were divided by the transcript length and normalized to the number of mapped reads to obtain reads per kb per million (RPKM) expression values. Only genes that were significantly differentially regulated (FDR corrected p-value < 0.05) and with at least a 2-fold change compared to the monospecies biofilm, were considered.

Electron microscopy

Monospecies biofilms of *S. anginosus* and *S. aureus*, and multispecies biofilm of *S. anginosus*, *S. aureus* and *P. aeruginosa*, were grown and treated with vancomycin as described above. Biofilm cells were washed, collected by vortexing (5 min) and sonication (5 min). Next, biofilm cells were either submitted to chemical fixation or to high pressure freezing (HPF).

For chemical fixation, biofilm cells were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 and centrifuged at 1500 rpm. Low melting point-agarose was used to keep the cells concentrated for further processing. Cells were fixed for 4 hours at room temperature and then O/N at 4°C after replacing the fixative with a fresh solution. After washing in buffer, cells were post-fixed in 1% OsO_4 with 1.5% $K_3Fe(CN)_6$ in 0.1 M sodium cacodylate buffer at room temperature for 1 hour. After washing, cells were dehydrated through a graded ethanol series, including a bulk staining with 1% uranyl acetate at the 50% ethanol step. Dehydration was followed by embedding in Spurr's resin. The polymerization was performed at 70°C for 16 h.

For HPF, biofilm cells were frozen as a paste in a high-pressure freezer (Leica EM ICE; Leica Microsystems, Vienna, Austria). Freeze substitution was carried out using a Leica EM AFS (Leica Microsystems) in dry acetone containing 1% ddH_2O, 1% OsO_4 and 0.5% glutaraldehyde over a 4-day period as follows: -90°C for 24 h, 2°C per hour increase for 15 h, -60°C for 24 h, 2°C per hour increase for 15 h, and -30°C for 24 h. At -30°C, the carriers were rinsed 3 times with acetone for 20 min each time. Samples were warmed up slowly to 0-4°C and infiltrated stepwise over 3 days at 0-4°C with Spurr's resin and embedded in capsules. The polymerization was performed at 70°C for 16 h.

Next, for both chemical fixation and HPF, ultrathin sections with a gold interference color were cut using an ultra-microtome (Leica EM UC6), followed by a post-staining in a Leica EM AC20 for 40 min in uranyl acetate at 20°C and for 10 min in lead stain at 20°C. Sections were collected on Formvar-coated copper slot grids. Grids were viewed with a JEM 1400plus transmission electron microscope (JEOL, Tokyo, Japan) operating at 60 kV.

The thickness of the cell wall, in each condition after chemical fixation ($n = 38$ for *S. anginosus* and $n \geq 10$ for *S. aureus*) , was measured at nearly-equatorially cut surfaces using ImageJ software (http://imagej.nih.gov/ij/) [365]. To improve the accuracy, only cells that were cut in the middle and that had approximately the same size, were included in the analysis. Statistical data analysis was performed using SPSS software, version 24 (SPSS, Chicago, Illinois, USA). The normal distribution of the data was verified using the Shapiro-Wilk test. Non-normally distributed data were analyzed using a Mann-Whitney test. Differences with a p-value < 0.05 were considered significant.

RESULTS AND DISCUSSION

S. anginosus genes involved in cell wall synthesis are upregulated when grown in a multispecies biofilm

RNA sequencing was used to elucidate the molecular mechanisms responsible for the reduced susceptibility of *S. anginosus* towards cell wall-active antibiotics when grown in a mixed community. Therefore, we compared the transcriptome of *S. anginosus* grown in an untreated monospecies biofilm with that of the same *S. anginosus* strain grown in an untreated multispecies biofilm. 285 *S. anginosus* genes (15.4%) were significantly upregulated, and 103 genes (5.5%) were significantly downregulated ($p < 0.05$, fold change > 2) in the multispecies biofilm (Supplementary Table S2).

Homologs of several *S. aureus* genes that are involved in cell wall synthesis and cell wall recycling [365, 379-381], were differentially expressed in *S. anginosus* in the untreated multispecies biofilm (Table 1). Interestingly, these genes are also differentially expressed after treatment with cell wall-active antibiotics and have been associated with a reduced susceptibility to vancomycin [365, 381-383]. For example, UDP-N-acetylmuramoylalanyl-D-glutamate-L-lysine ligase (*murE*) is upregulated, as is observed in *S. aureus* upon vancomycin exposure. [365, 379, 384] In addition, genes involved in synthesis of peptidoglycan components, such as *dapA* and *dapB*, were also upregulated. Furthermore, the gene encoding the transpeptidase sortase A was also upregulated. Sortase A is known to play a role in the assembly of pili in Gram-positive bacteria. [385] These data strongly suggest that the decreased susceptibility towards antibiotics that interfere with cell wall synthesis, observed in *S. anginosus* in a multispecies biofilm, is due to thickening of the cell wall.

Furthermore, genes involved in cell division were also upregulated (Supplementary Table S2). Consequently, the upregulation of genes involved in cell wall recycling for example, of a gene encoding peptidoglycan hydrolase (Table 1), might be linked with the increased cell division. In *S. aureus,* a peptidoglycan hydrolase (*lytM*) was also observed to be upregulated after exposure to daptomycin [384], and is described to be involved in cell wall remodeling [384, 386, 387].

Table 1: *S. anginosus genes involved in cell wall biosynthesis and cell wall recycling upregulated in untreated multispecies biofilms (expressed as fold change compared to untreated monospecies biofilm).*

Annotation	**Fold change**	**FDR corrected p-value****	**Ref.**	**Protein_ID in reference genome *S. aureus* NCTC 8325**
N-acetylmuramoyl-L-alanine amidase *lytA*	14.30	2.67E-07	[388-390]	YP_501041.1
Foldase protein precursor *prsA* *	12.93	3.25E-10	[365, 379, 384]	YP_500469.1
Peptidoglycan N-acetylglucosamine deacetylase *pgdA*	11.43	1.44E-05	[391]	YP_499269.1
4-hydroxy-tetrahydrodipicolinate synthase *dapA**	11.06	0.04	[384]	YP_499923.1
Peptidoglycan hydrolase, autolysin 2*	9.58	2.01E-05	[365, 384, 392-395]	YP_500516.1
Sortase A *srtA*	9.37	1.83E-07	[385, 396]	YP_501293.1
holliday junction resolvase *recU* *	9.11	6.54E-07	[365, 384, 397]	YP_500229.1
4-hydroxy-tetrahydrodipicolinate reductase *dapB* *	9.06	1.43E-06	[384]	YP_499924.1
Poly(glycerophosphate chain)D-alanine transferprotein *dltD* *	6.79	0.0007	[383, 392, 398-400]	YP_499423.1
Methionyl-tRNA formyltransferase *fmt**	5.87	0.006	[365, 379, 384, 401-404]	YP_499722.1
UDP-N-acetylmuramoylamoylalanyl-D-glutamate-L-lysine ligase *murE* *	5.65	0.01	[365, 379, 384]	YP_499507.1
Multimodular transpeptidase-transglycosylase, similar to penicillin binding protein*	4.99	0.04	[365, 384]	YP_499687.1

The RNA sequence data are presented as the mean fold-change of 3 biological replicates. Only values > 2 fold change and p < 0.05 are included. *genes previously reported to be differentially expressed in *S. aureus* after treatment with cell wall active antibiotics. **as obtained through the EDGE test: single vs mix, tagwise dispersions

Transmission electron microscopy confirms an increased cell wall thickness of S. anginosus when grown in an untreated multispecies biofilm

Results from the transcriptome analysis suggest that growth in a multispecies biofilm with *P. aeruginosa* and *S. aureus* induces the expression of *S. anginosus* genes involved in cell wall biosynthesis and recycling. This might lead to changes in cell wall morphology which could explain the observed decreased susceptibility towards cell-wall acting antibiotics [1]. To confirm this we used TEM to measure cell wall thickness in different *S. anginosus* biofilms, in the presence or absence of vancomycin.

TEM images of cells from a monospecies *S. aureus* or *S. anginosus* biofilm allowed us to differentiate *S. aureus* and *S. anginosus* based on appearance, both after chemical fixation and HPF (Figure 1 and 2, respectively). *S. aureus* cells can be recognized as clearly contoured cells, with short fimbriae, whereas *S. anginosus* cells show a less defined cell contour and are surrounded by larger fimbriae.

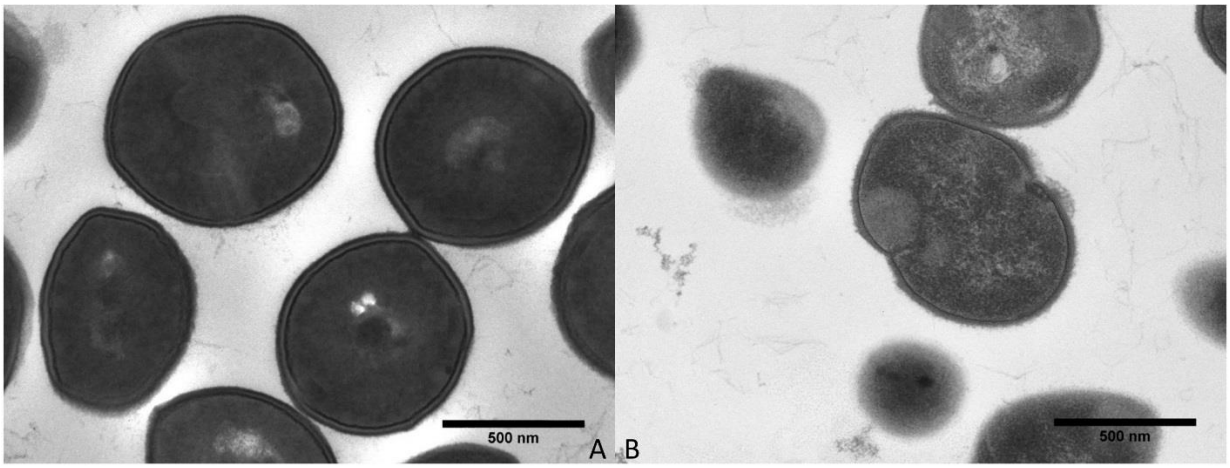

Figure 1: *Cells from a monospecies S. aureus (a) and S. anginosus (b) biofilm. TEM pictures obtained after chemical fixation. Scale bar = 500 nm.*

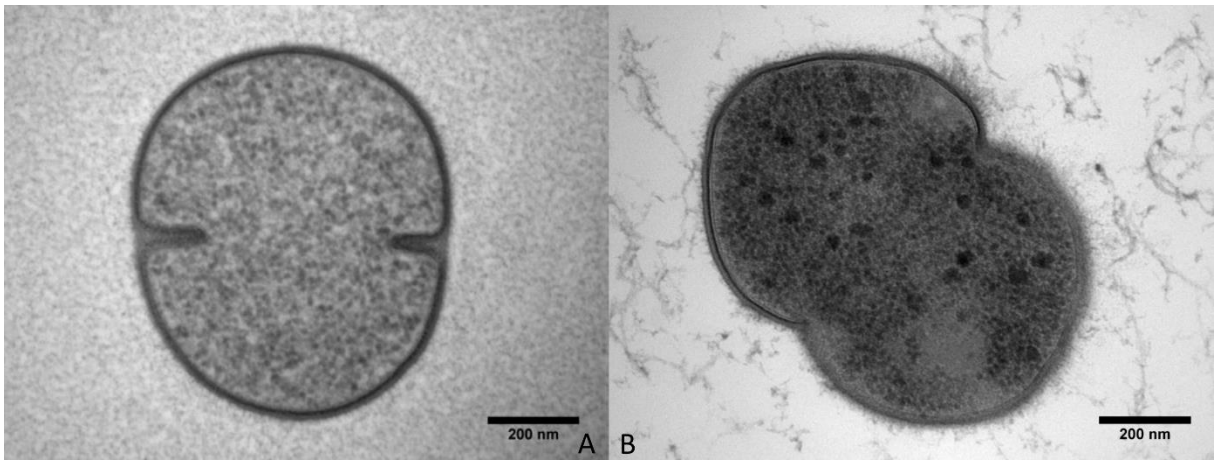

Figure 2: *Cells from a monospecies S. aureus (a) and S. anginosus (b) biofilm. TEM pictures obtained after HPF. Scale bar = 200 nm.*

S. anginosus cells grown together with *S. aureus* and *P. aeruginosa* in a multispecies biofilm (Figure 3a) have a significant thicker cell wall (including the layer of fimbriae surrounding the cell wall), than cells from a monospecies biofilm (Figure 3b). The average increase in cell wall/fimbriae layer thickness is 8.01 ± 3.09 nm ($p < 0.05$). This thicker layer is also present after treatment with vancomycin in a multispecies biofilm (Figure 3c). Cells from a monospecies *S. anginosus* biofilm treated with vancomycin (Figure 3d), show no significant difference in thickness of cell wall/fimbriae layer compared to untreated cells. The thickness of the cell wall/fimbriae layer of *S. anginosus* cells in the different conditions is shown in Figure 4. Images of *S. anginosus* cells after HPF in a mono- and multispecies biofilm, treated and untreated, are shown in Supplemental Figure S1. For both fixation methods, the same trend was observed, indicating that the method of fixation has no impact on the results.

The observed upregulation of peptide-cleaving carboxypeptidases and glycan-cleaving lytic transglycosylases, described to play a role in creating space within the peptidoglycan polymer to accommodate structures such as fimbriae [386, 405], likely contributes to this increased cell wall thickness. Similarly, the upregulation of the transpeptidase sortase A, known to be involved in the assembly of pili [385] is also likely to be involved.

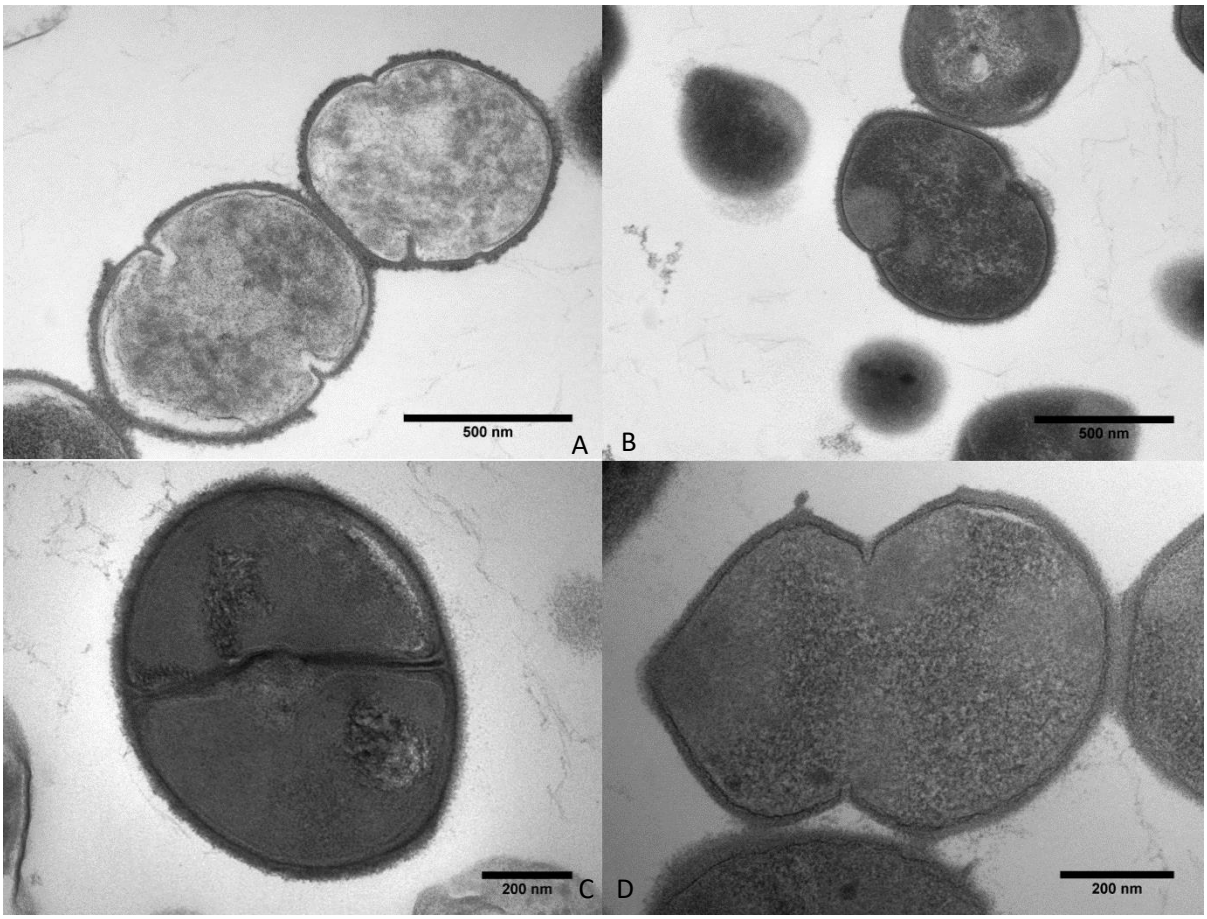

Figure 3: *S. anginosus cells* grown in several conditions. TEM pictures obtained after chemical fixation. (a) untreated multispecies biofilm (average cell wall thickness 30.22 ± 2.79 nm); (b) untreated monospecies biofilm (average cell wall thickness 21.97 ± 1.60 nm); (c) vancomycin (512 µg/ml) treated multispecies biofilm (average cell wall thickness 30.00 ± 2.66 nm); (d) vancomycin (512 µg/ml) treated monospecies biofilm (average cell wall thickness 20.90 ± 1.24 nm). Scale bar = 500nm or 200 nm.

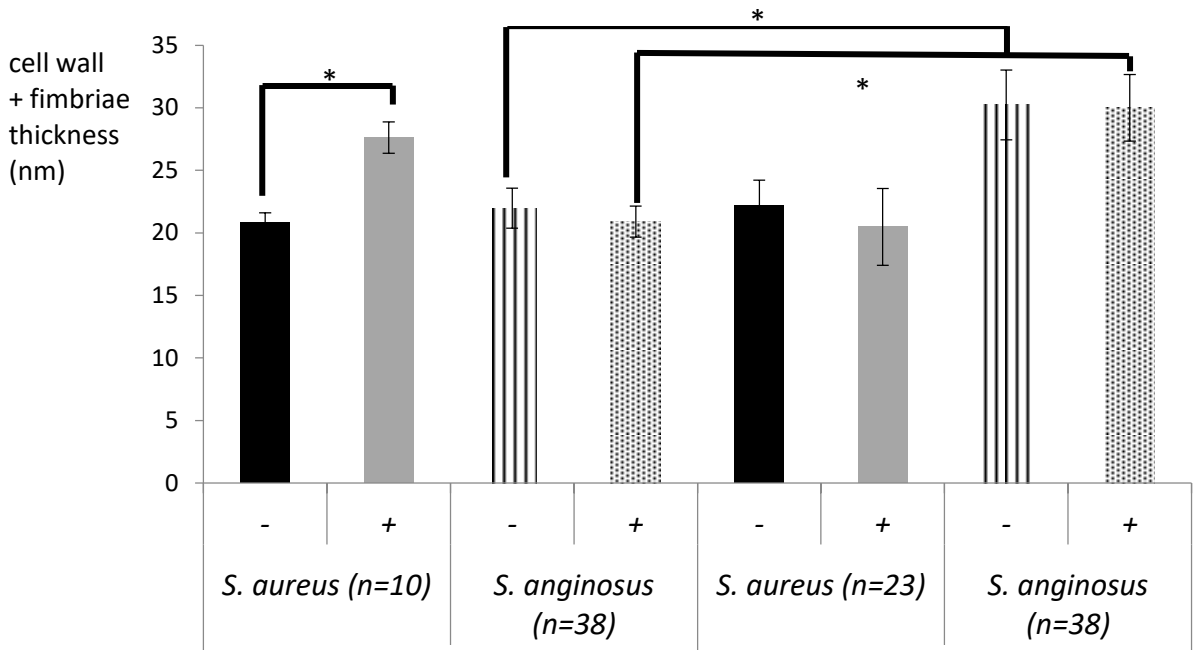

Figure 4: *Thickness (in nm) of S. anginosus and S. aureus cell wall (including layer of fimbriae) measured after chemical fixation. (-) untreated, (+) treated with vancomycin 512 µg/ml. *p < 0.05. Error bars represent standard deviations.*

While growth in a multispecies biofilm increased cell wall thickness of *S. anginosus* cells, cell wall thickness of untreated *S. aureus* biofilm cells did not differ between cells grown in a mono- or multispecies biofilm. Also, *S. aureus* cells in a multispecies biofilm treated with vancomycin showed no increased cell wall thickness compared to untreated *S. aureus* cells in a multispecies biofilm, in contrast to *S. aureus* cells in a monospecies biofilm (Figure 4). Images of *S. aureus* cells are shown in Supplemental Figure S2 and S3.

These results are in line with our recently published data, that *S. aureus* becomes more susceptible towards cell wall-active antibiotics when grown in a multispecies biofilm [1]. As cell wall thickening is described as a common feature of reduced vancomycin susceptibility in *S. aureus* [365, 381-383], the lack of cell wall thickening could be the reason behind the increased killing of *S. aureus* by vancomycin in a multispecies biofilm [1].

In conclusion, our data demonstrate that growth in multispecies biofilms can have an impact on the morphology of a particular bacterial species, with downstream consequences for the effectivity of antibiotics.

FUNDING

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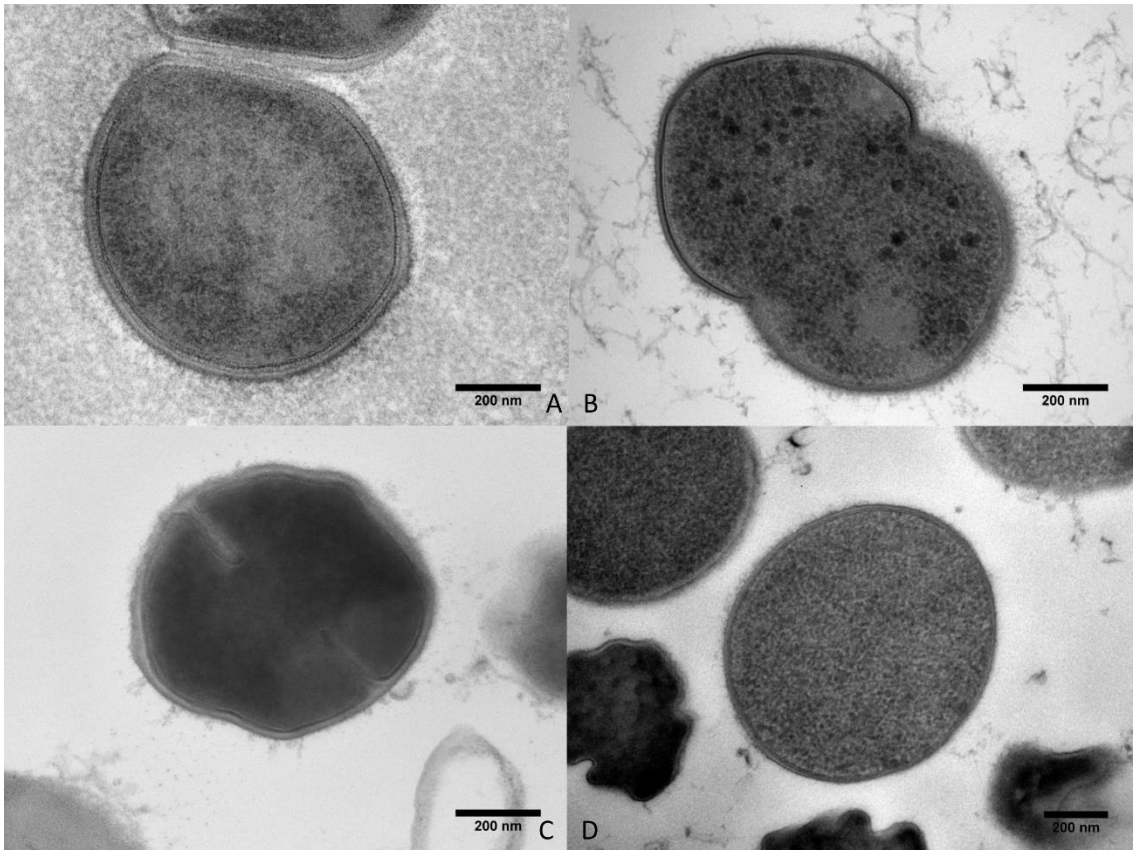
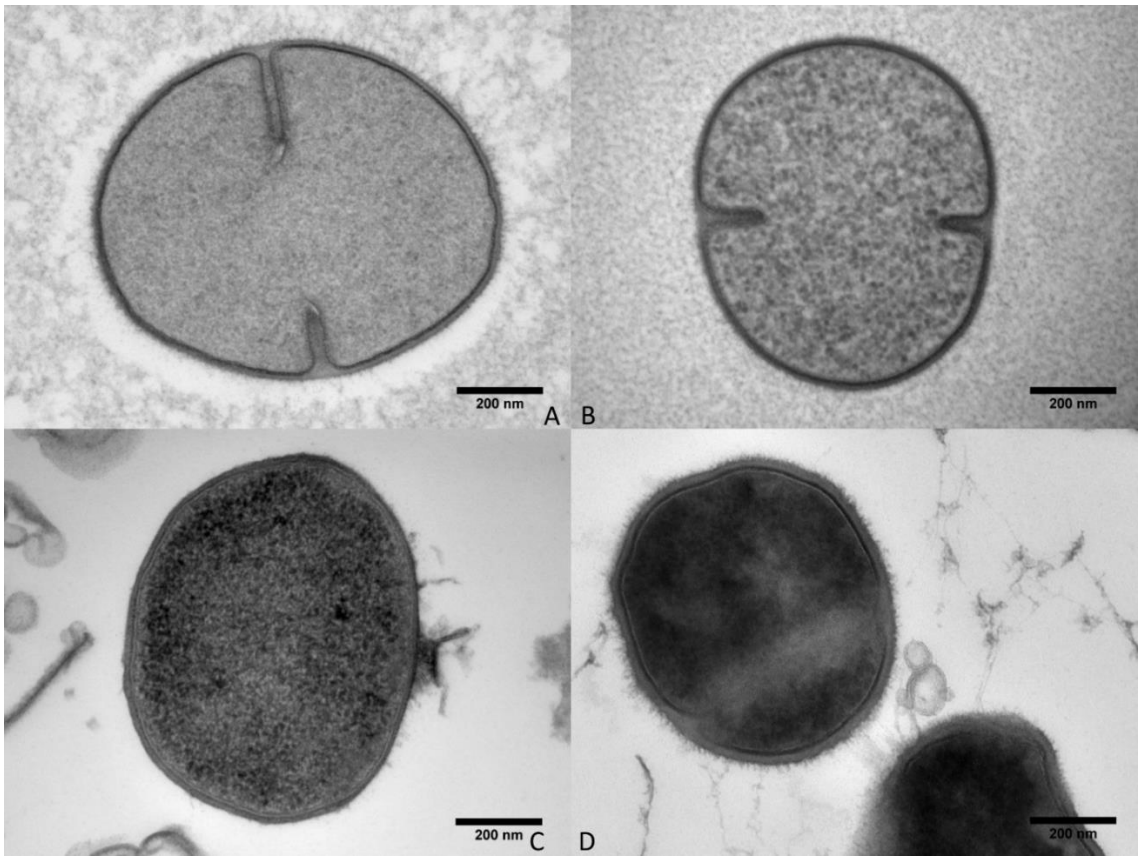
Transparency declarations

Nothing to declare.

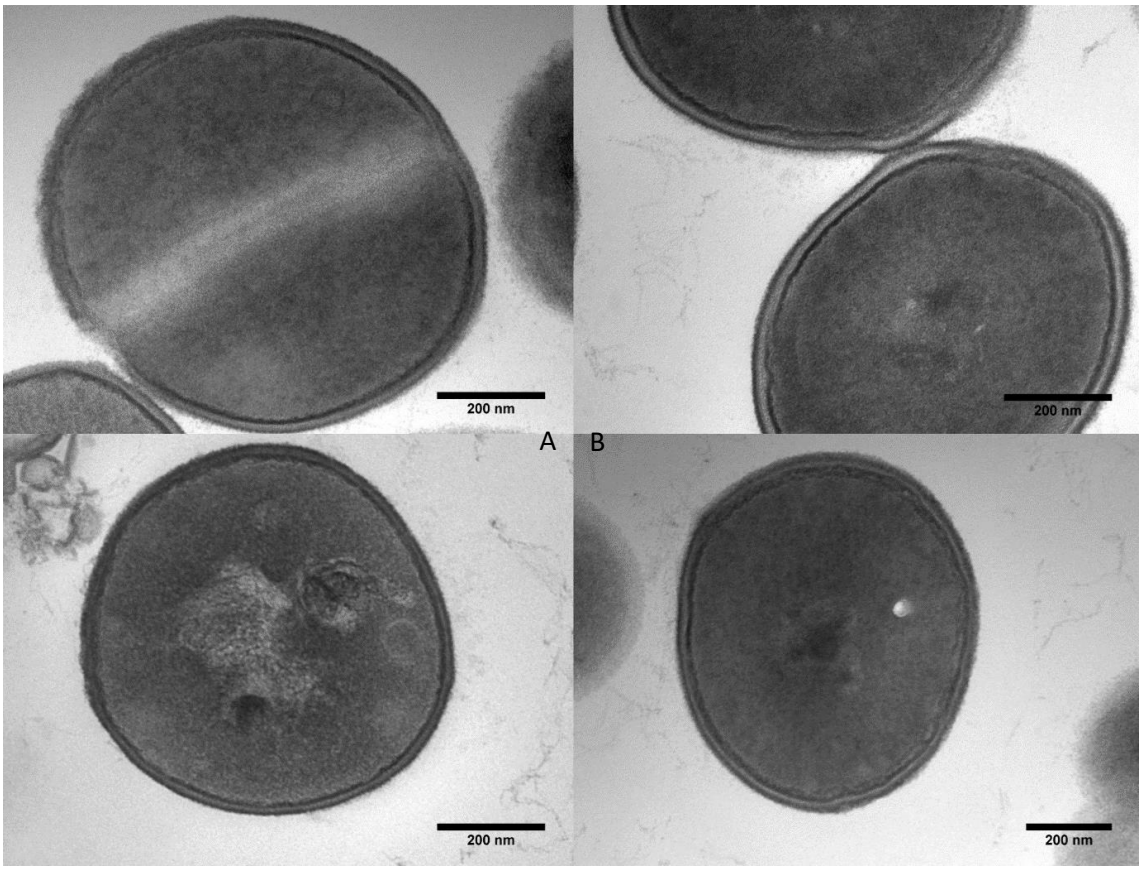
SUPPLEMENTARY DATA



Supplemental Figure S1: *TEM pictures of cells from S. anginosus* grown in several conditions after HPF. (a) untreated multispecies biofilm; (b) untreated monospecies biofilm; (c) vancomycin (512 µg/ml) treated multispecies biofilm; (d) vancomycin (512 µg/ml) treated monospecies biofilm. Length scale bar = 200 nm.



Supplemental Figure S2: *TEM pictures of cells from S. aureus* grown in several conditions after HPF. (a) untreated multispecies biofilm; (b) untreated monospecies biofilm; (c) vancomycin (512 µg/ml) treated multispecies biofilm; (d) vancomycin (512 µg/ml) treated monospecies biofilm. Length scale bar = 200 nm.



Supplemental Figure S3: *TEM pictures of cells from S. aureus grown in several conditions after chemical fixation: (a) untreated multispecies biofilm (average 22.16 ± 2.05 nm); (b) untreated monospecies biofilm (average 20.82 ± 0.78 nm); (c) vancomycin (512 µg/ml) treated multispecies biofilm (average 20.47 ± 3.07 nm); (d) vancomycin (512 µg/ml) treated monospecies biofilm (average 27.62 ± 1.26 nm). Length scale bar = 200 nm.*

***Supplementary Table S1**: Read numbers per sample, and after sampling using CLC Genomics Workbench, of multispecies biofilms of S. anginosus LMG 14502, S. aureus LMG 10147, and P. aeruginosa DK2, and of monospecies biofilms of S. anginosus LMG 14502. n/a: not applicable.*

Sample	Multispecies a	Multispecies b	Multispecies c	Monospecies a	Monospecies b	Monospecies c
Total reads after quality control	67340992	59339487	64831943	72279366	65404257	64258689
Number of reads mapping to all three genomes	45135965	48162789	50770622	n/a	n/a	n/a
Number of reads after random sampling	n/a	n/a	n/a	4336761	3924256	3855522
Number of reads mapping to *S. anginosus* contigs and used for gene expression analysis	3408855	5534580	1415577	3945017	3705419	3729040

Supplementary Table S2: Changes in S. anginosus LMG 14502 gene expression by RNA sequence analysis after 24h of growth in a mixed community together with S. aureus LMG 10147 and P. aeruginosa DK2 compared to growth in a monospecies biofilm. The RNA sequence data are presented as the mean fold-change (n = 3). Only values > 2 fold change and $p < 0.05$ are included.

Annotation	Experiment - Fold Change (normalized values)	EDGE test: single vs mix, tagwise dispersions - FDR p-value correction
hypothetical protein_53	1140.42	6.23E-13
6-phospho-beta-glucosidase (EC 3.2.1.86)_2	536.86	3.28E-16
PTS system, beta-glucoside-specific IIB component (EC 2.7.1.69) / PTS system, beta-glucoside-specific IIC component / PTS system, beta-glucoside-specific IIA component_3	189.94	1.23E-13
HMP-PP hydrolase (pyridoxal phosphatase) Cof, detected in genetic screen for thiamin metabolic genes (PMID:15292217)_4	170.20	1.14E-34
FIG01114907: hypothetical protein_2	96.69	0.001758
FIG00516476: hypothetical protein	83.23	0.045092
FIG00524451: hypothetical protein	80.34	0.045375
Hypothetical protein DUF454_1	78.20	0.000109
FIG01120440: hypothetical protein	40.37	3.41E-17
putative ComG operon protein 3	37.93	0.01417
hypothetical protein_47	32.83	8.42E-15
hypothetical protein_49	30.57	5.31E-10
Membrane-bound protease, CAAX family_1	30.55	0.001478

Ribonuclease III (EC 3.1.26.3)	30.49	1.61E-13
hypothetical protein_95	30.30	2.84E-07
DNA for glycosyltransferase, lytic transglycosylase, dTDP-4-rhamnose reductase	29.47	0.015947
Alcohol dehydrogenase (EC 1.1.1.1); Acetaldehyde dehydrogenase (EC 1.2.1.10)	29.26	4.74E-30
Late competence protein ComGG, FIG068335	29.11	0.020872
Late competence protein ComGF, access of DNA to ComEA, FIG012620	29.07	0.012244
hypothetical protein_115	26.39	0.000989
Substrate-specific component NiaX of predicted niacin ECF transporter	23.29	0.004358
Glycosyl transferase, family 2_2	21.95	2.26E-05
hypothetical protein_48	21.64	6.23E-08
Hypothetical protein DUF454_2	21.60	4.17E-05
Late competence protein ComGA, access of DNA to ComEA	21.07	0.022842
Zn-dependent hydrolase (beta-lactamase superfamily)	20.00	5.14E-21
DegV family protein_2	17.98	5.89E-09
Multidrug resistance transporter, Bcr/CflA family	17.88	0.048081
Peptide chain release factor 2; programmed frameshift-containing	17.60	1.34E-17
hydrolase, NUDIX family	17.28	0.005969
Trans-2,cis-3-Decenoyl-ACP isomerase	16.78	1.28E-08
FIG01118945: hypothetical protein	16.77	1.25E-08

FIG01114860: hypothetical protein	16.32	0.000134
Ribonuclease HII (EC 3.1.26.4)	16.27	2.54E-05
FIG01116656: hypothetical protein_2	16.24	4.21E-06
Acyl-phosphate:glycerol-3-phosphate O-acyltransferase PlsY	16.15	5.91E-06
L-serine dehydratase, alpha subunit (EC 4.3.1.17)	15.77	1.42E-11
FIG01115594: hypothetical protein	15.19	0.011244
hypothetical protein_50	15.18	0.016804
Purine nucleoside phosphorylase (EC 2.4.2.1)_1	15.18	6.44E-08
N-acetylmuramoyl-L-alanine amidase	14.30	2.67E-07
Immunodominant antigen A	14.22	2.04E-13
Cell division transporter, ATP-binding protein FtsE (TC 3.A.5.1.1)_2	14.13	1.05E-13
NAD-dependent protein deacetylase of SIR2 family	13.89	3.33E-06
Exopolysaccharide biosynthesis protein related to N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase	13.80	0.000276
FIG01117230: hypothetical protein	13.73	0.037878
Aspartyl-tRNA(Asn) amidotransferase subunit B (EC 6.3.5.6) Glutamyl-tRNA(Gln) amidotransferase subunit B (EC 6.3.5.7)	13.57	1.26E-12
hypothetical protein_5	13.55	0.019227
Chromosome partition protein smc	13.16	2.82E-08
ABC transporter, ATP-binding protein_4	13.10	4.91E-05
Foldase protein prsA precursor, putative(	12.93	3.25E-10

EC:5.2.1.8)		
1-acyl-sn-glycerol-3-phosphate acyltransferase (EC 2.3.1.51)	12.91	1.08E-07
N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)_1	12.70	7.73E-10
Tyrosine-protein kinase EpsD (EC 2.7.10.2)	12.37	4.09E-10
FIG01116141: hypothetical protein	12.30	0.002105
PTS system, cellobiose-specific IIA component (EC 2.7.1.69)_1	12.30	0.017171
Diphosphomevalonate decarboxylase (EC 4.1.1.33)_1	12.29	0.039448
Deoxyguanosinetriphosphate triphosphohydrolase (EC 3.1.5.1)	12.16	1.7E-05
Phage integrase (Site-specific recombinase)	12.14	0.001437
rRNA small subunit 7-methylguanosine (m7G) methyltransferase GidB	11.94	0.001089
oxidoreductase, Gfo/Idh/MocA family_1	11.89	0.003051
hypothetical protein_178	11.80	0.027262
FIG01114292: hypothetical protein	11.77	0.001142
CAAX amino terminal protease family	11.68	0.000374
ligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)_2	11.60	4.27E-06
HD domain protein	11.55	0.011594
Cadmium efflux system accessory protein	11.48	0.004262
FIG01119954: hypothetical protein	11.46	0.008711

Peptidoglycan N-acetylglucosamine deacetylase (EC 3.5.1.-)	11.43	1.44E-05
hypothetical protein_181	11.37	0.010999
Hydrolase (HAD superfamily)	11.10	0.002768
PhnO protein_2	11.07	2.04E-05
ABC transporter ATP-binding/membrane spanning protein - multidrug resistance	11.07	0.03704
4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7)_1	11.06	0.039698
Ribose 5-phosphate isomerase A (EC 5.3.1.6)	11.03	1.57E-06
FIG01119076: hypothetical protein	11.01	0.022735
membrane protein, putative_1	10.99	0.029514
Maltose O-acetyltransferase (EC 2.3.1.79)_2	10.83	0.00169
Alpha-D-GlcNAc alpha-1,2-L-rhamnosyltransferase (EC 2.4.1.-)_1	10.82	1.71E-08
MutT/nudix family protein_1	10.79	5.43E-05
FIG01113973: possible membrane protein	10.68	0.000494
Maltose O-acetyltransferase (EC 2.3.1.79)_1	10.67	5.82E-05
GTP-binding protein EngB	10.33	6.4E-06
FIG000605: protein co-occurring with transport systems (COG1739)	10.18	0.009464
Acetyltransferase, putative	10.10	0.039801
Lipid A export ATP-binding/permease protein MsbA_7	10.02	0.000128
Beta-glucoside bgl operon antiterminator, BglG	9.92	0.004255

family_2		
Phosphoglycerate mutase family	9.87	7.53E-05
Candidate zinc-binding lipoprotein ZinT	9.82	5.49E-07
Ribonucleotide reductase transcriptional regulator NrdR	9.78	0.000107
Purine nucleoside phosphorylase (EC 2.4.2.1)_2	9.72	7.97E-08
Trehalose operon transcriptional repressor	9.71	0.049097
Neutral endopeptidase O (EC 3.4.24.-)	9.63	1.6E-05
Peptidoglycan hydrolase, Autolysin2 (EC 3.5.1.28)	9.58	2.01E-05
ATP-dependent Clp protease ATP-binding subunit ClpX	9.58	1.98E-05
SatD	9.53	5.6E-05
Fumarate reductase, flavoprotein subunit precursor (EC 1.3.99.1)_1	9.49	2.03E-05
FIG001960: FtsZ-interacting protein related to cell division	9.44	1.4E-07
Glycosyltransferase	9.44	0.000727
tRNA-dependent lipid II--L-alanine ligase	9.38	6.85E-06
Sortase A, LPXTG specific_1	9.37	1.83E-07
FIG01114578: hypothetical protein	9.33	8.41E-06
Signal recognition particle receptor protein FtsY (=alpha subunit) (TC 3.A.5.1.1)	9.22	8.83E-05
Membrane protein involved in the export of O-antigen, teichoic acid lipoteichoic acids_1	9.21	0.02281
thiJ/pfpI family protein	9.18	0.008671

putative phosphomannomutase	9.14	9.5E-06
RecU Holliday junction resolvase	9.11	6.54E-07
Xaa-Pro dipeptidyl-peptidase (EC 3.4.14.11)	9.06	5.35E-05
4-hydroxy-tetrahydrodipicolinate reductase (EC 1.17.1.8)	9.06	1.43E-06
Multidrug resistance protein B	9.03	0.030159
Chromosome (plasmid) partitioning protein ParB	9.01	0.039791
hypothetical protein_45	8.94	0.018512
Zinc ABC transporter, inner membrane permease protein ZnuB	8.86	4.01E-06
Protein of unknown function DUF419	8.86	0.015496
Exopolysaccharide biosynthesis transcriptional activator EpsA	8.80	7.96E-06
Hypothetical similar to thiamin biosynthesis lipoprotein ApbE	8.79	0.000105
Two-component response regulator SA14-24_2	8.79	2.24E-07
Rod shape-determining protein MreD	8.75	0.010896
Undecaprenyl-phosphate galactosephosphotransferase (EC 2.7.8.6)	8.72	9.07E-07
Two-component sensor kinase SA14-24_2	8.71	4.59E-06
internalin, putative_1	8.69	0.002325
Substrate-specific component BioY of biotin ECF transporter	8.69	0.025705
fructose sensor histidine kinase	8.66	0.035201
Enoyl-[acyl-carrier-protein] reductase [FMN]	8.61	6.67E-05

(EC 1.3.1.9)_2		
Phosphoglycolate phosphatase (EC 3.1.3.18)	8.61	0.013861
Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily F1 (as in PMID19099556)	8.61	5.43E-05
Ribonucleotide reductase of class Ib (aerobic), alpha subunit (EC 1.17.4.1)	8.58	8.29E-05
membrane protein, putative_2	8.56	0.047032
Tyrosine-protein phosphatase CpsB (EC 3.1.3.48)	8.49	0.000392
hypothetical protein_60	8.48	0.01087
Cell division protein FtsX	8.42	3.9E-06
Low molecular weight protein tyrosine phosphatase (EC 3.1.3.48)	8.41	1.17E-05
3'-to-5' exoribonuclease RNase R	8.39	0.001158
Translation initiation factor 2	8.36	5.87E-06
Calcium-transporting ATPase (EC 3.6.3.8)	8.30	6.28E-05
ATP-dependent RNA helicase YfmL	8.28	0.000707
ABC transporter ATP-binding protein_4	8.08	0.005268
HMP-PP hydrolase (pyridoxal phosphatase) Cof, detected in genetic screen for thiamin metabolic genes (PMID:15292217)_2	8.03	0.000122
Tyrosine recombinase XerC	8.02	0.016456
Histidine triad (HIT) nucleotide-binding protein, similarity with At5g48545 and yeast YDL125C (HNT1)	8.01	0.001008

Alanyl-tRNA synthetase (EC 6.1.1.7)	8.01	4.26E-05
Competence protein CoiA	7.98	0.031731
ADP-ribose pyrophosphatase	7.96	0.003336
Phage tail length tape-measure protein	7.92	0.048521
hypothetical protein_79	7.89	0.013387
Prephenate dehydratase (EC 4.2.1.51)	7.86	0.001607
DNA replication intiation control protein YabA	7.82	0.003565
Glycosyltransferase involved in cell wall biogenesis (EC 2.4.-.-)	7.77	0.000831
Ribonucleotide reductase of class III (anaerobic), activating protein (EC 1.97.1.4)	7.76	0.000819
Dihydroorotase (EC 3.5.2.3)	7.74	0.002457
Aspartyl-tRNA synthetase (EC 6.1.1.12)_1	7.73	0.010125
Putative deoxyribonuclease YcfH	7.68	0.020311
Cell division protein FtsZ (EC 3.4.24.-)	7.66	1.49E-05
D-alanine--poly(phosphoribitol) ligase subunit 1 (EC 6.1.1.13)	7.65	5.41E-05
Malonyl CoA-acyl carrier protein transacylase (EC 2.3.1.39)	7.65	0.000515
Transcriptional regulator	7.64	0.002876
Cell division protein FtsK	7.59	0.00012
PhnO protein_1	7.57	0.015243
tmRNA-binding protein SmpB	7.55	0.008109
Cell division initiation protein DivIVA	7.46	1.54E-05
galactofuranose transferase	7.43	0.01058

fructose response regulator of fruA and EII fructose/mannose	7.40	0.031731
Mn-dependent transcriptional regulator MntR	7.34	0.000157
FIG001721: Predicted N6-adenine-specific DNA methylase	7.31	0.000324
Alpha/beta hydrolase fold (EC 3.8.1.5)_1	7.30	0.005439
Aspartokinase (EC 2.7.2.4)	7.28	0.015668
2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase (EC 2.3.1.89)	7.28	0.036396
Cold shock protein CspA	7.27	0.020319
Substrate-specific component YkoE of thiamin-regulated ECF transporter for HydroxyMethylPyrimidine	7.26	0.037343
Ribonuclease J1 (endonuclease and 5' exonuclease)	7.23	7.06E-05
3-oxoacyl-[acyl-carrier-protein] synthase, KASIII (EC 2.3.1.180)	7.21	0.000219
Lipid A export ATP-binding/permease protein MsbA_9	7.21	0.00143
tRNA nucleotidyltransferase (EC 2.7.7.21) (EC 2.7.7.25)	7.20	0.001528
hypothetical protein_163	7.19	0.000497
FOG: TPR repeat	7.18	0.042313
UDP-galactose:(galactosyl) LPS alpha1,2-galactosyltransferase WaaW (EC 2.4.1.-)	7.10	0.002146
Topoisomerase IV subunit A (EC 5.99.1.-)	7.10	0.013738

Serine protease, DegP/HtrA, do-like (EC 3.4.21.-)	7.09	0.000478
DNA topology modulation protein flar-related protein	7.08	0.021462
MORN motif family protein	7.06	8.92E-05
Transcriptional regulator of fatty acid biosynthesis FabT	7.05	9.59E-05
S1 RNA binding domain_1	7.04	0.008813
hypothetical protein_109	7.01	0.000495
LrgA-associated membrane protein LrgB	6.97	0.016656
Bacterial/Archaeal Transporter family protein	6.94	0.013861
Aldose 1-epimerase (EC 5.1.3.3)	6.93	0.000559
Helicase loader DnaB	6.90	0.000482
Putative FMN hydrolase (EC 3.1.3.-);5-Amino-6-(5'-phosphoribitylamino)uracil phosphatase	6.90	0.015668
HMP-PP hydrolase (pyridoxal phosphatase) Cof, detected in genetic screen for thiamin metabolic genes (PMID:15292217)_3	6.89	0.003045
Rod shape-determining protein MreC	6.88	0.005844
Competence-specific sigma factor ComX	6.86	0.03531
Ribosomal RNA small subunit methyltransferase B (EC 2.1.1.-)	6.85	0.000338
Two-component response regulator_2	6.81	0.002697
DNA gyrase subunit A (EC 5.99.1.3)	6.80	0.000707
hypothetical protein_140	6.79	0.02684

Poly(glycerophosphate chain) D-alanine transfer protein DltD	6.79	0.000682
Methylase involved in ubiquinone/menaquinone biosynthesis	6.78	0.018591
Transcriptional repressor AdcR for Zn(2+)-responsive expression	6.78	0.004538
hypothetical protein_40	6.77	0.031816
Anaerobic ribonucleoside-triphosphate reductase (EC 1.17.4.2)	6.77	0.007608
Phosphoglycerate mutase family 5	6.75	0.019649
Aspartyl-tRNA(Asn) amidotransferase subunit A (EC 6.3.5.6) @ Glutamyl-tRNA(Gln) amidotransferase subunit A (EC 6.3.5.7)	6.72	0.00022
DNA mismatch repair protein MutL	6.68	0.001329
Metal-dependent hydrolase YbeY, involved in rRNA and/or ribosome maturation and assembly	6.66	0.003274
Hypothetical protein VC0266 (sugar utilization related?)	6.65	0.003059
hypothetical protein_179	6.64	0.044803
HPr kinase/phosphorylase (EC 2.7.1.-) (EC 2.7.4.-)	6.61	0.005368
PTS system, maltose and glucose-specific IIC component (EC 2.7.1.69) / PTS system, maltose and glucose-specific IIB component (EC 2.7.1.69) / PTS system, maltose and glucose-specific IIA component (EC 2.7.1.69)	6.58	0.000377
GTP-binding protein Era	6.54	0.002785
RecD-like DNA helicase YrrC	6.53	0.000852

Lactoylglutathione lyase (EC 4.4.1.5)_3	6.53	0.03227
Mg(2+) transport ATPase, P-type (EC 3.6.3.2)	6.53	0.007044
putative esterase	6.52	0.010896
UDP-galactopyranose mutase (EC 5.4.99.9)	6.51	0.014244
3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)_2	6.51	0.003987
Zinc ABC transporter, ATP-binding protein ZnuC_3	6.46	0.001633
Ribosomal small subunit pseudouridine synthase A (EC 4.2.1.70)_1	6.44	0.018954
FIG01118502: hypothetical protein	6.44	0.049097
tRNA-dependent lipid II-Ala--L-alanine ligase_1	6.41	0.002061
Substrate-specific component PdxU2 of predicted pyridoxin-related ECF transporter	6.40	0.014438
Tyrosine-protein kinase transmembrane modulator EpsC	6.39	0.002562
Hydroxymethylpyrimidine phosphate kinase ThiD (EC 2.7.4.7)	6.37	0.005879
Isocitrate dehydrogenase [NADP] (EC 1.1.1.42)	6.36	0.022136
Oligopeptide transport ATP-binding protein OppD (TC 3.A.1.5.1)	6.36	0.002822
Promiscuous sugar phosphatase YidA, haloacid dehalogenase-like phosphatase family	6.36	0.007869
FIG01115027: hypothetical protein	6.31	0.038063
Deoxyuridine 5'-triphosphate nucleotidohydrolase (EC 3.6.1.23)	6.29	0.041266

Acyltransferase family_2	6.29	0.002014
Putative deoxyribose-specific ABC transporter, permease protein	6.27	0.001437
Alpha-L-Rha alpha-1,3-L-rhamnosyltransferase (EC 2.4.1.-)	6.24	0.004279
Mutator mutT protein (7,8-dihydro-8-oxoguanine-triphosphatase) (EC 3.6.1.-)_2	6.24	0.011223
putative Zn-dependent protease	6.17	0.004728
acetyltransferase, GNAT family_4	6.16	0.037446
Serine acetyltransferase (EC 2.3.1.30)	6.11	0.011421
Putative deoxyribose-specific ABC transporter, ATP-binding protein	6.11	0.003614
Membrane spanning protein	6.09	0.014106
Branched-chain amino acid transport ATP-binding protein LivF (TC 3.A.1.4.1)	6.03	0.010093
Uracil-DNA glycosylase, family 1	6.03	0.04183
dTDP-4-dehydrorhamnose reductase (EC 1.1.1.133)	6.03	0.004771
Serine/threonine protein kinase PrkC, regulator of stationary phase	6.02	0.001441
RecA protein	6.02	0.001383
Ribonucleotide reductase of class III (anaerobic), large subunit (EC 1.17.4.2)	6.01	0.003979
MutT/nudix family protein_2	5.99	0.015243
TPR-repeat-containing protein	5.98	0.022624

tRNA pseudouridine synthase A (EC 4.2.1.70)	5.97	0.0248
hypothetical protein_111	5.94	0.042015
Lon-like protease with PDZ domain	5.89	0.015098
Membrane-associated zinc metalloprotease	5.89	0.003545
Methionyl-tRNA formyltransferase (EC 2.1.2.9)	5.87	0.005879
Predicted hydrolase (HAD superfamily)	5.86	0.034693
general stress protein, putative	5.85	0.006174
putative endoglucanase precursor	5.85	0.007549
FIG01120040: hypothetical protein	5.83	0.012215
Phosphate transport system regulatory protein PhoU	5.81	0.02243
ribosomal protein L7Ae family protein	5.78	0.036636
COG0488: ATPase components of ABC transporters with duplicated ATPase domains	5.76	0.017294
Transposase_2	5.74	0.030906
hypothetical protein_18	5.70	0.02288
FIG009886: phosphoesterase	5.68	0.018995
UDP-N-acetylmuramoylalanyl-D-glutamate--L-lysine ligase (EC 6.3.2.7)	5.65	0.013423
CTP synthase (EC 6.3.4.2)	5.64	0.013382
Ribonucleotide reductase of class Ib (aerobic), beta subunit (EC 1.17.4.1)	5.63	0.027015
Chromosome replication initiation protein DnaD	5.62	0.03704
FIG01117679: hypothetical protein	5.59	0.019234
Oligopeptide ABC transporter, periplasmic oligopeptide-	5.58	0.019227

binding protein OppA (TC 3.A.1.5.1)_1		
Chaperone protein DnaJ	5.57	0.01146
2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I alpha (EC 2.5.1.54)	5.56	0.006368
Glutamate transport ATP-binding protein	5.50	0.022157
Muramoyltetrapeptide carboxypeptidase (EC 3.4.17.13)	5.49	0.031608
ABC transporter ATP-binding protein_7	5.48	0.017709
N-acetyl-L,L-diaminopimelate deacetylase (EC 3.5.1.47)	5.46	0.045978
hypothetical protein_19	5.43	0.045325
Transcriptional regulator OrfX	5.41	0.04465
3'->5' exoribonuclease Bsu YhaM	5.39	0.013423
Biotin carboxylase of acetyl-CoA carboxylase (EC 6.3.4.14)	5.27	0.018465
Branched-chain amino acid transport system permease protein LivM (TC 3.A.1.4.1)	5.26	0.045269
SSU ribosomal protein S1p	5.20	0.027913
3-oxoacyl-[acyl-carrier-protein] synthase, KASII (EC 2.3.1.179)	5.17	0.024318
DNA-directed RNA polymerase delta subunit (EC 2.7.7.6)	5.15	0.019518
Transcription termination protein NusA	5.13	0.035477
ADP-ribose pyrophosphatase (EC 3.6.1.13)	5.12	0.024955
Dihydrofolate synthase (EC 6.3.2.12) @	5.11	0.021932

Folylpolyglutamate synthase (EC 6.3.2.17)_1		
Multimodular transpeptidase-transglycosylase (EC 2.4.1.129) (EC 3.4.-.-)_1	4.99	0.047396
RNA-binding protein Jag	4.81	0.039374
Aggregation promoting factor	4.71	0.041992
Iron-sulfur cluster assembly ATPase protein SufC	-2.09	1.42E-18
Phosphoglucomutase (EC 5.4.2.2)	-2.09	1.27E-21
Conserved domain protein SP0160	-2.09	1.41E-13
LSU ribosomal protein L29p (L35e)	-2.19	4.03E-08
Glycerol dehydrogenase (EC 1.1.1.6)	-2.19	5.08E-11
Oxidoreductase ucpA (EC 1.-.-.-)	-2.19	9.15E-14
Integrase_3	-2.20	6.47E-13
hypothetical protein_24	-2.22	1.01E-10
Transcriptional regulators, LysR family	-2.24	2.26E-05
LSU ribosomal protein L36p	-2.28	3.91E-11
Iron-sulfur cluster assembly protein SufD	-2.30	1.88E-21
Regulator of the multidrug efflux pump pmrA	-2.34	9.3E-18
Phosphocarrier protein of PTS system	-2.38	0
SSU ribosomal protein S17p (S11e)	-2.46	1.42E-07
Transcriptional regulator SpxA1	-2.47	1.13E-08
hypothetical protein_77	-2.55	7.65E-18
FIG00256590: hypothetical protein	-2.56	1.14E-14
Transcriptional regulator, XRE family	-2.57	3.47E-12
Cell wall surface anchor family protein_2	-2.58	4.95E-09

Transcriptional repressor of the fructose operon, DeoR family	-2.60	1.07E-11
Predicted amino-acid acetyltransferase (EC 2.3.1.1) complementing ArgA function in Arginine Biosynthesis pathway	-2.60	1.65E-25
Nucleoside-binding protein	-2.63	6.27E-24
Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein MalE	-2.76	1.87E-25
putative ABC transporter, ATP-binding protein	-2.78	1.27E-20
FIG007491: hypothetical protein YeeN	-2.86	8.18E-25
NADH peroxidase (EC 1.11.1.1)	-2.90	1.1E-19
Probable L-ascorbate-6-phosphate lactonase UlaG (EC 3.1.1.-) (L-ascorbate utilization protein G)	-2.92	1.29E-16
FIG01117589: hypothetical protein_1	-2.92	8.7E-13
Ascorbate-specific PTS system, EIIB component (EC 2.7.1.69)	-2.94	3.94E-15
Manganese superoxide dismutase (EC 1.15.1.1)	-3.01	3.02E-16
FIG01114257: hypothetical protein	-3.04	2.45E-07
Heat shock protein 60 family chaperone GroEL	-3.04	1.09E-25
Pyruvate formate-lyase (EC 2.3.1.54)_1	-3.14	7.87E-17
L-lactate dehydrogenase (EC 1.1.1.27)_2	-3.19	4.27E-25
Lactose phosphotransferase system repressor	-3.21	1.44E-12
Pyruvate formate-lyase (EC 2.3.1.54)_2	-3.22	0
PTS system, galactosamine-specific IIC component (EC 2.7.1.69)	-3.29	3.63E-22
Glycogen biosynthesis protein GlgD, glucose-1-phosphate adenylyltransferase family	-3.30	1.84E-29

Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	-3.37	0
Protein yjgK	-3.38	1.65E-17
hypothetical protein_34	-3.61	0
histone acetyltransferase Gcn5, putative	-3.72	2.32E-24
Glucose-1-phosphate adenylyltransferase (EC 2.7.7.27)	-3.81	1.09E-32
Glutamate synthase [NADPH] large chain (EC 1.4.1.13)_2	-3.82	1.93E-18
Peptide methionine sulfoxide reductase MsrA (EC 1.8.4.11) / Peptide methionine sulfoxide reductase MsrB (EC 1.8.4.12)_1	-3.90	4.94E-16
PTS system, galactose-specific IIC component (EC 2.7.1.69)	-3.90	3.03E-24
hypothetical protein_156	-3.92	4.78E-09
Transcriptional regulator, Cro/CI family	-4.01	8.34E-18
Cell wall surface anchor family protein_1	-4.11	3.18E-12
PTS system, cellobiose-specific IIC component (EC 2.7.1.69)_2	-4.13	2.79E-18
Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.3) (EC 3.6.3.5); Copper-translocating P-type ATPase (EC 3.6.3.4)	-4.17	1.9E-31
PTS system, galactosamine-specific IIB component (EC 2.7.1.69)	-4.18	1.27E-20
hypothetical protein_129	-4.18	1.4E-12

Cytochrome c-type biogenesis protein CcdA homolog, associated with MetSO reductase	-4.20	1.53E-09
Fructose-bisphosphate aldolase class II (EC 4.1.2.13)	-4.20	0
Heat shock protein 60 family co-chaperone GroES	-4.28	5.29E-26
Glycerol uptake facilitator protein	-4.39	1.04E-24
Alkyl hydroperoxide reductase protein F (EC 1.6.4.-)	-4.40	9.68E-31
Glycerol kinase (EC 2.7.1.30)	-4.41	2.09E-22
Transaldolase (EC 2.2.1.2)	-4.50	2.82E-22
Phosphoenolpyruvate-dihydroxyacetone phosphotransferase (EC 2.7.1.121), dihydroxyacetone binding subunit DhaK	-4.53	2.83E-21
hypothetical protein_32	-4.58	1.14E-20
FIG01118633: hypothetical protein	-4.62	9.83E-27
Alkyl hydroperoxide reductase protein C (EC 1.6.4.-)	-4.73	6.37E-29
Universal stress protein family	-4.80	3.31E-39
PTS system, galactosamine-specific IID component (EC 2.7.1.69)	-5.00	2.18E-26
5'-nucleotidase (EC 3.1.3.5)	-5.26	2.1E-29
Arginine/ornithine antiporter ArcD_2	-5.29	6.6E-40
Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases_2	-5.36	3.87E-39

hypothetical protein_21	-5.37	0
PTS system, cellobiose-specific IIB component (EC 2.7.1.69)_2	-5.41	7.8E-23
1-phosphofructokinase (EC 2.7.1.56)	-5.51	2.75E-29
Pyruvate,phosphate dikinase (EC 2.7.9.1)	-5.58	1.65E-25
Phosphoenolpyruvate-dihydroxyacetone phosphotransferase (EC 2.7.1.121), ADP-binding subunit DhaL	-6.10	3.96E-24
PTS system, fructose-specific IIA component / PTS system, fructose-specific IIB component (EC 2.7.1.69) / PTS system, fructose-specific IIC component	-6.21	3.88E-36
hypothetical protein_38	-6.42	8.77E-29
PTS system, galactose-specific IIA component (EC 2.7.1.69)	-6.63	7.01E-22
Acid phosphatase (EC 3.1.3.2)	-6.97	2.25E-38
Carbamate kinase (EC 2.7.2.2)	-7.31	5.44E-35
Phosphoenolpyruvate-dihydroxyacetone phosphotransferase (EC 2.7.1.121), subunit DhaM; DHA-specific IIA component	-7.48	6.63E-27
Lactaldehyde dehydrogenase involved in fucose or rhamnose utilization (EC 1.2.1.22)	-7.59	2.29E-45
FIG01115091: hypothetical protein	-7.73	1.02E-39
Thiol:disulfide oxidoreductase associated with MetSO reductase	-7.77	1.16E-16
PTS system, galactose-specific IIB component	-8.25	2.15E-38

(EC 2.7.1.69)		
Predicted PTS system, galactosamine-specific IIA component (EC 2.7.1.69)	-8.43	3.9E-45
Copper chaperone	-8.97	1.15E-75
Putative Dihydrolipoamide dehydrogenase (EC 1.8.1.4); Mercuric ion reductase (EC 1.16.1.1); PF00070 family, FAD-dependent NAD(P)-disulphide oxidoreductase	-10.32	6.5E-46
ABC transporter ATP-binding protein_8	-10.47	2.77E-17
Thioredoxin_1	-10.67	1.51E-34
Ribosomal subunit interface protein	-10.86	4.12E-30
Acetoin dehydrogenase E1 component alpha-subunit (EC 1.2.4.-)	-10.97	1.1E-40
Pyruvate oxidase (EC 1.2.3.3)	-12.22	1.05E-70
Dihydrolipoamide dehydrogenase of acetoin dehydrogenase (EC 1.8.1.4)	-13.21	2.11E-42
Negative transcriptional regulator-copper transport operon	-14.99	2.64E-69
hypothetical protein_138	-15.00	1.15E-42
hypothetical protein_124	-15.24	3.17E-43
Lactate 2-monooxygenase (EC 1.13.12.4)	-15.56	1.02E-37
Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex (EC 2.3.1.-)	-16.56	1.1E-40
Acetoin dehydrogenase E1 component beta-subunit (EC 1.2.4.-)	-17.06	1.17E-47
RNA polymerase sigma factor, ECF subfamily	-17.83	2.71E-34

FIG01116295: hypothetical protein	-18.30	9.44E-52
Ornithine carbamoyltransferase (EC 2.1.3.3)	-19.94	7.4E-68
Arginine deiminase (EC 3.5.3.6)	-60.42	5.81E-33

Paper 4:

Adapted from:

Quantification of *Pseudomonas aeruginosa* in multispecies biofilms using PMA-qPCR

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ABSTRACT

Multispecies biofilms are an important healthcare problem and may lead to persistent infections. These infections are difficult to treat, as cells in a biofilm are highly resistant to antimicrobial agents. While increasingly being recognized as important, the properties of multispecies biofilms remain poorly studied. In order to do so, the quantification of the individual species is needed. The current cultivation-based approaches can lead to an underestimation of the actual cell number and are time-consuming. In the present study we set up a culture-independent approach based on propidium monoazide qPCR (PMA-qPCR) to quantify *Pseudomonas aeruginosa* in a multispecies biofilm within 24 hours but with minimal hands-on time. As a proof of concept, we explored the influence of the combined presence of *Staphylococcus aureus*, *Streptococcus anginosus* and *Burkholderia cenocepacia* on the antimicrobial susceptibility of *P. aeruginosa* using this PMA-qPCR approach. For colistin,

P. aeruginosa showed a higher susceptibility in a multispecies biofilm, whereas for levofloxacin, a lower susceptibility was observed. For tobramycin, no difference in susceptibility could be observed, using PMA-qPCR. These results indicate that PMA-qPCR can be used to quantify the number of bacterial cells surviving antibiotic treatment, as long as the antibiotic treatment impairs the membrane integrity and the surviving cell number is higher than 5 log.

INTRODUCTION

Specific quantification of the different members in a multispecies biofilm is a challenging task. Cultivation-based approaches are time-consuming and can lead to an underestimation of cell numbers due to the presence of viable but nonculturable bacteria (VBNC). VBNC bacteria will not grow on routinely-used microbiological media, but are nevertheless still viable and potentially virulent [406]. A promising alternative for cultivation-based methods is quantification based on qPCR. However, a major drawback of qPCR-based quantification is the overestimation of cell numbers due to the presence of extracellular DNA and DNA originating from dead cells, and adjustments are required to differentiate between viable and dead bacteria. Treatment of bacterial samples with propidium monoazide (PMA) prior to DNA extraction has been proposed as an effective method to avoid the detection of extracellular DNA and DNA from dead cells [407-409]. PMA only enters membrane-compromised cells, and once inside the cell, it intercalates into DNA between the bases (one PMA molecule per 4 to 5 base pairs DNA, with little or no sequence preference). Besides intercalating into DNA of membrane-compromised cells, PMA can also intercalate into extracellular DNA [410, 411]. After exposure to strong visible light, the photoreactive azido group of PMA is converted to a reactive nitrene radical. This nitrene radical forms a stable covalent nitrogen-carbon bond with the DNA, resulting in permanent DNA modification. The modified DNA is then lost

together with cells debris during genomic DNA extraction and will not be amplified during the qPCR reaction [412, 413]. Excess PMA is inactivated by reaction with water molecules in solution, prior to DNA extraction, and thus will not affect the DNA from viable cells after cell lysis [412]. Nevertheless, the use of PMA has some limitations. The discrimination between viable and dead cells is only based on membrane integrity, and the effect of antimicrobial therapies that do not target the cell membrane can thus not be monitored using PMA [414]. Secondly, viable cells with a slightly damaged cell membrane will not be quantified [415] and the presence of a high number of dead cells (>10^4 cells/ml) can affect the quantification of viable cells [416]. Finally, the presence of other compounds in the sample, e.g. environmental compounds that can bind to PMA, can subsequently prevent PMA to bind to DNA [417]. Nevertheless, PMA-qPCR can be used as a quick and reliable method to overcome the presence of VBNC. Therefore, we decided to use PMA-qPCR to selectively quantify *P. aeruginosa* in mono- and multispecies biofilms following exposure to various antibiotics that are used to treat respiratory infections in cystic fibrosis (CF).

MATERIALS & METHODS

Bacterial strains

P. aeruginosa ATCC9027, *Staphylococcus aureus* LMG10147, *Burkholderia cenocepacia* LMG16656, and *Streptococcus anginosus* LMG14502 were cultured overnight at 37°C in Brain Heart Infusion broth (BHI) (Oxoid, Basingstoke, UK).

Antibiotic solutions

The difference in susceptibility of *P. aeruginosa* in a mono- and multispecies biofilm towards colistin (Sigma-Aldrich, Diegem, Belgium), tobramycin (TCI Europe, Zwijndrecht, Belgium), and levofloxacin (Sigma-Aldrich) was determined. The concentration used for colistin and tobramycin was 200 µg/ml, for levofloxacin 100 µg/ml. These concentrations were based on the levels achievable in CF sputum by inhalation therapy [418]. All antibiotic solutions were prepared in physiological saline (0.9% [w/v] NaCl) (PS) [419]. Minimal inhibitory concentrations [420] of colistin, tobramycin and levofloxacin were determined in duplicate according to the EUCAST broth microdilution protocol using flat-bottom

96-well microtiter plates (TPP, Trasadingen, Switzerland) as previously described [313].

Quantification of *P. aeruginosa* cells in monospecies and multispecies biofilms

Formation of *P. aeruginosa* monospecies and multispecies biofilms

For formation of mono- and multispecies biofilms, round-bottomed 96-well microtiter plates (MTP) were used. Inoculum suspensions containing approximately 10^6 CFU/ml of *P. aeruginosa* alone or 10^6 CFU/ml of *P. aeruginosa* in combination with 10^6 CFU/ml of *S. aureus*, 10^7 CFU/ml of *B. cenocepacia*

and 10^7 CFU/ml of *S. anginosus*, were made in BHI. The inoculum cell numbers were based on preliminary optimization experiments, and led to biofilms with the highest cell numbers (data not shown). BHI was supplemented with 5% (w/v) bovine serum albumin (BSA) [350], 0.5% (w/v) mucine type II, and 0.3% (w/v) agar. Mucine and agar were added to mimic the composition of CF sputum and to increase the medium viscosity, respectively. Sterile medium served as blank and was included on each plate. After 4 hours of adhesion at 37°C, wells were rinsed with 100 µl PS to remove non-adhered cells. 100 µl of fresh medium was added to the wells and the plates were incubated for an additional 20 hours. After 20 hours, the supernatant was again removed, each well was rinsed using 100 µl PS and 100 µl of **t**he antibiotic solution (colistin, tobramycin or levofloxacin) was added to the mature biofilms. To the wells of the control biofilm plate, 100 µl PS was added. The plates were then again incubated at 37°C for 24 hours. For each test condition, 72 technical replicates were included. All experiments were performed on three different occasions. Confocal imaging was performed as described in Udine et al. [421]. The control cell numbers of *P. aeruginosa*, *S.* aureus and *B.* cenocepacia, respectively determined on cetrimide agar, mannitol salt agar and tryptic soy agar supplemented with tobramycin (4 µg/ml) and nitrofurantoin (25 µg/ml), increased after 24 hours, respectively with 1.30, 0.67 and 0.95 log cfu/biofilm, indicating that these species are actually growing in the multispecies biofilm. The control cell number of *S. anginosus,* determined on Mc Kay agar [343] did not change, indicating that this species is present in the multispecies biofilm.

Propidium monoazide cross-linking

After 24 hours of antibiotic treatment, the antibiotic solution in the test plate and the PS in the control plate was removed. The wells were rinsed with 100 µl PS. Next, biofilms were detached by vortexing (900 rpm) and sonication (both 5 min), followed by collection of the content of the wells in a sterile tube. The vortexing and sonication step was repeated after the addition of 100 µl PS to each well. The sterile tube was centrifuged (5 min at 5000 rpm), and the pellet was resuspended in 1.5 ml of PS. For each treatment, 2 wells of a 24-well plate were filled with 600 µl of the cell suspension. 1.5 µl of a 20 mM PMA solution in dH_2O (Biotium, Inc., California, USA) was added to the first well (final concentration of 50 µM). To the second well, 1.5 µl of MilliQ water (MQ water) (Millipore, Billerica, MA, VS**)** was added. The plates were vortexed (5 min, 300 rpm, room temperature) in the dark and exposed to light for 10 min, using a LED-lamp (Dark Reader transilluminator, Clare Chemical Research, US**)** (output wavelength 465-475 nm) [422].

Effect of PMA on P. aeruginosa cell viability

To analyze the effect of 50 µM PMA on cell viability, overnight grown planktonic *P. aeruginosa* cells (OD 1.0) were used. To 2 ml of this culture, 5 µl of a 20 mM PMA solution in dH_2O was added (final concentration of 50 µM)**.** As a control, 5 µl of MQ water was added instead of the PMA solution. The

plates were incubated in the dark (5 min, 300 rpm) and exposed to light for 10 min. Next, the cell numbers of control and test group were determined via the plate count method (on tryptic soy agar) and by solid-phase cytometry (SPC) (ChemScan RDI; AES-Chemunex, Ivry-sur Seine, France), as described previously [423].

Extraction of genomic DNA

After incubation with PMA, 500 µl of cell suspension from each well was transferred to a sterile Eppendorf tube. The samples were centrifuged (10 min, 13000 rpm) and DNA from Gram-negative organisms was extracted as described previously [374]. Briefly, the pellets were washed with 500 µl RS-buffer (0.15M NaCl [Sigma-Aldrich], 0.01M EDTA [VWR, Leuven, Belgium], pH 8.0) and resuspended in TE-buffer (1 mM EDTA, 10 mM Tris-HCl [Sigma-Aldrich]). 500 µl GES-buffer (60 % [w/v] guanidium thiocyanate [Sigma-Aldrich], 0.5 M EDTA, pH 8.0, 1 % [w/v] sarkosyl [Sigma-Aldrich]) was added and the samples were placed on ice for 10 min. After the addition of 250 µl cold ammonium acetate (7.5 M [VWR]), the samples were placed back on ice for 10 min. Subsequently, 500 µl cold chloroform/isoamylalcohol (24:1) (Roth, Karlsruhe, Germany) was added. Samples were mixed thoroughly and centrifuged for 20 min at 13000 rpm. Supernatant was then collected in a new tube and 0.54 volumes cold isopropanol (Sigma-Aldrich) were added to precipitate the DNA. Samples were then centrifuged (10 min, 13000 rpm), and supernatant was removed. 150 µl ethanol (Sigma-Aldrich) (70% [v/v]) was added and samples were centrifuged for 1 min. This step was repeated. The DNA pellet was air-dried, 30 µl TE-buffer was added, the samples were placed at 4°C for 24 hours and were then treated with RNase.

Following electrophoresis on 1% agarose gels, genomic DNA was visualized with GelRed (GelRed nucleic acid, Biotium) and the genomic DNA concentration was measured with Quantifluor dsDNA kit (Promega, Madison, US).

qPCR

Real-time PCR (CFX96 Real Time System; Bio-Rad, Hercules, CA, USA) was carried out with the PerfeCTa SYBR Green FastMix (Quanta Biosciences). Species-specific primer sequences for *gyrB* of *P. aeruginosa* were designed using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using

P. aeruginosa and *B. cenocepacia* sequences obtained from GenBank. The forward primer and the reverse primer were 5'-GGTGTTCGAGGTGGTGGATA-3' and 5'-TGGTGATGCTGATTTCGCTG-3', respectively. The specificity of the primers was evaluated by melting curve analysis.

To generate a standard curve, DNA extracted from serially-diluted and PMA-treated planktonic *P. aeruginosa* cultures was used for qPCR. The C_q-values obtained were plotted against the number

of viable cells determined by SPC. The serial dilutions were prepared from a *P. aeruginosa* overnight suspension (OD 0.1). Cells were diluted from 10^9 CFU/ml to 10^4 CFU/ml in PS. Six independent biological repeats were included.

Effect of PMA on C_q-values of defined ratios of viable and dead *P. aeruginosa* cells

Planktonic *P. aeruginosa* cells (OD 1.0) were killed by heating for 15 min at 95°C. Complete killing was confirmed by SPC. Mixtures of viable and dead cells were prepared, in which viable cells represented 0%, 0.1%, 1%, 10%, 50%, 75% and 100% of the total population. Four wells of a 24-well plate were filled with 600 µl of each mixture. PMA was added to 2 wells and MQ water was added to the other 2 wells (PMA-negative control). Cells were then treated as described above (2.3.2.) and C_q-values were determined via qPCR. Six independent biological repeats were carried out.

Statistical data analysis

Statistical data analysis was performed using SPSS software, version 22 (SPSS, Chicago, IL, USA). The normal distribution of the data was verified using the Shapiro-Wilk test. Non-normally distributed data were analyzed using a Mann-Whitney test. Normally distributed data were analyzed using an independent sample t-test. Differences with a p-value < 0.05 were considered as significant.

RESULTS AND DISCUSSION

Optimization of the PMA-qPCR

Treatment with PMA (50 µM) did not affect the number of viable cells as determined via SPC (Figure 1), so it can be concluded that PMA itself has no inhibitory effect on *P. aeruginosa*. Therefore, all experiments were conducted with a PMA concentration of 50 µM.

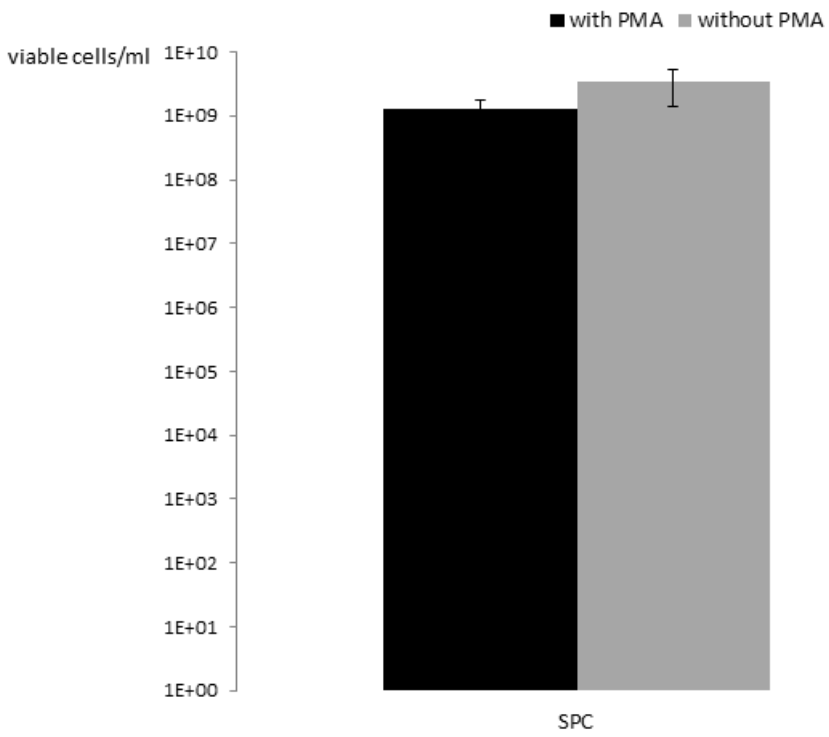

Figure 1: *Number of viable cells (determined using SPC) in PMA-treated (50 µM) and untreated samples. Treatment with PMA (50 µM) did not affect the number of viable cells (Mann-Whitney test, p > 0.05).*

Increasing the fraction of viable cells in the mixture led to an increase in the genomic DNA yield after PMA treatment (Figure 2A, 2B and Figure 3). As shown in Figure 2A, the DNA concentration increases with an increasing percentage of living cells, after PMA treatment. Without PMA treatment, the DNA concentration in all mixtures is comparable (Figure 2B). This indicates that the DNA of the heat-killed cells is still present in PMA-untreated mixtures. The correlation between the DNA concentration and the percentage of living cells is shown in Figure 3. The same trend can be seen as in Figure 2A and 2B: the DNA concentration in the PMA-treated mixtures is increasing with an increasing number of living cells, while the DNA concentration in the PMA-untreated mixtures is higher for a lower percentage of living cells and quickly reaches a plateau phase. This indicates that the DNA concentration (and subsequently the viable cell number) is less overestimated in PMA-treated samples.

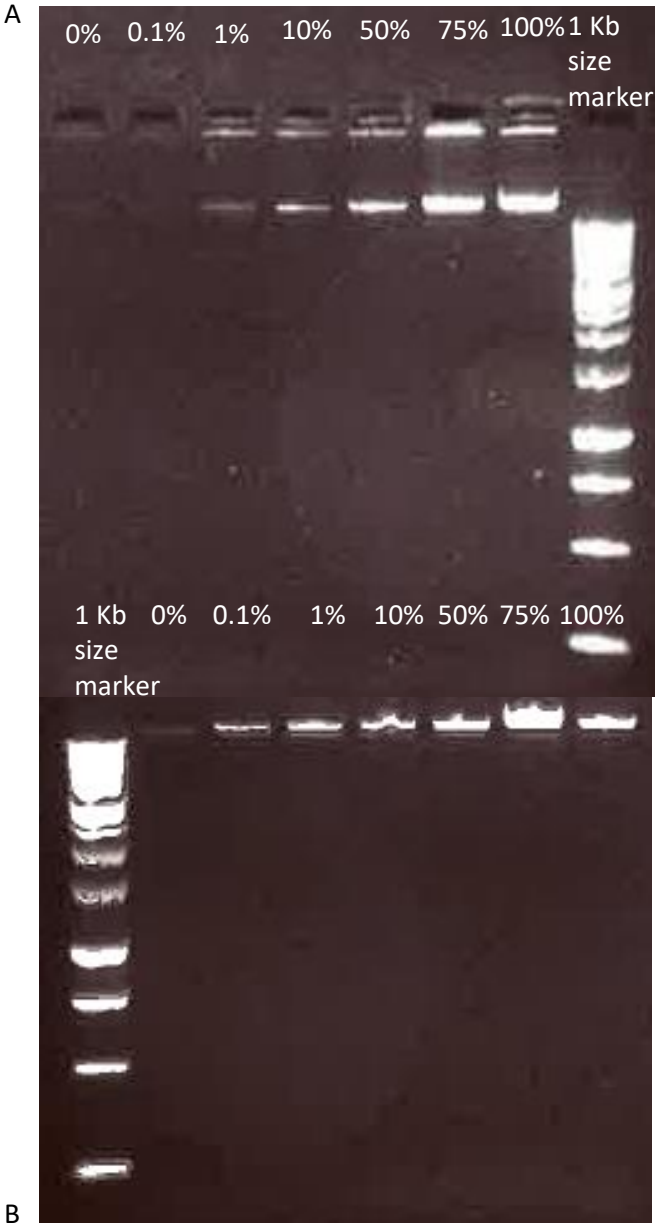

Figure 2: *Genomic DNA extracted from PMA-treated mixtures (A) and PMA-untreated mixtures (B), containing an increasing fraction of viable P. aeruginosa cells.*

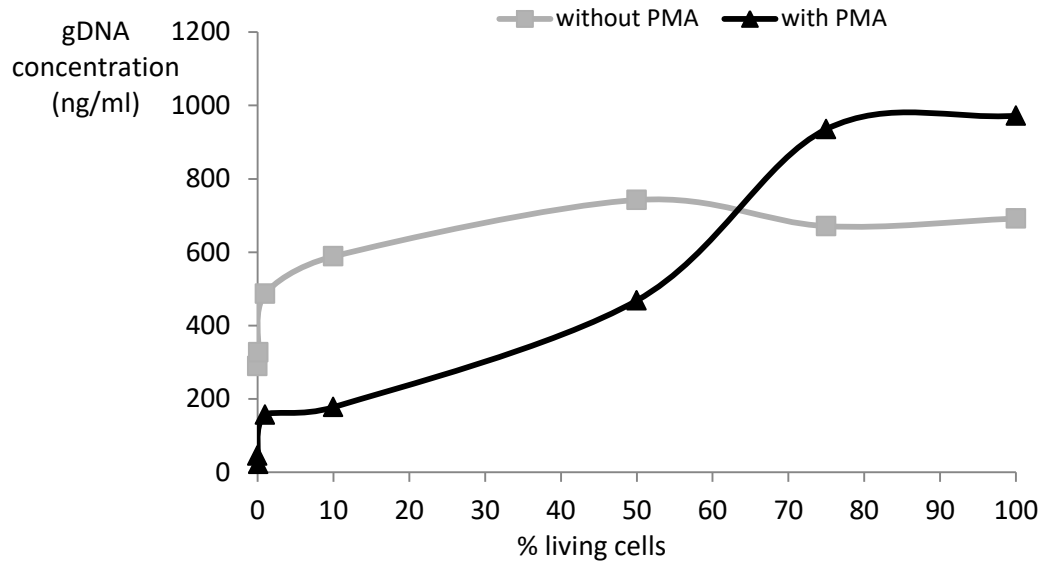

Figure 3: *gDNA concentration (ng/ml) corresponding with a percentage of living cells. Data were obtained with the same samples used in Figures 2A and 2B.*

Increasing the fraction of viable cells in the mixture also led to a significant decrease of the C_q-value determined via qPCR (Figure 4). The C_q-values obtained with PMA-treated mixtures were significantly higher than the C_q-values of corresponding PMA-untreated mixtures ($p<0.05$). The higher C_q-value after PMA treatment indicates that the qPCR amplification of DNA of heat-killed cells is efficiently inhibited by the addition of PMA. This is confirmed by the decrease in C_q-value after increasing the fraction of viable cells and was also described by Alvarez et al. [407].

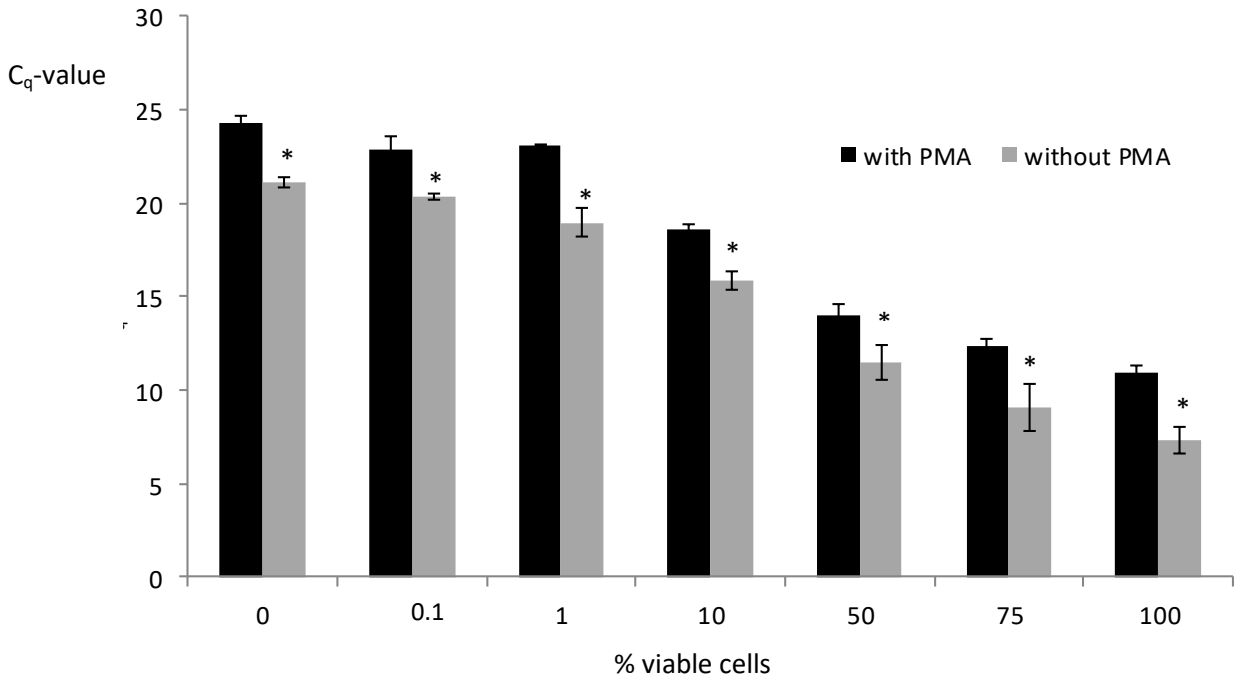

Figure 4: *Effect of PMA treatment on C_q-values obtained following qPCR using DNA extracted from mixtures of viable and heat-killed P. aeruginosa cells. The addition of PMA leads to a higher C_q-value. This indicates that the amplification of DNA of heat-killed cells was inhibited by PMA. Error bars represent the standard error mean (n = 6). (*: p<0.05, Mann-Whitney test).*

When plotting the log of the number of viable cells versus C_q-values obtained, a linear relationship was observed between both parameters (R^2 = 0.9685) (Figure 5). The linear range of this relationship is between 10^5 and 10^9 cells, indicating that the method used is limited to treatments that result in a number of surviving cells higher than 10^5. A viable cell number of 10^5 corresponds to a C_q-value of approximately 30. Nocker et al. [413] described that signals from killed cells could not be suppressed completely by PMA at very low ratios of live/killed cells, with corresponding C_q-values of 30 or higher. This could be due to the sensitivity of exponential amplification, and inherent to the method used, and could be a possible explanation for the lower limit of the linear range.

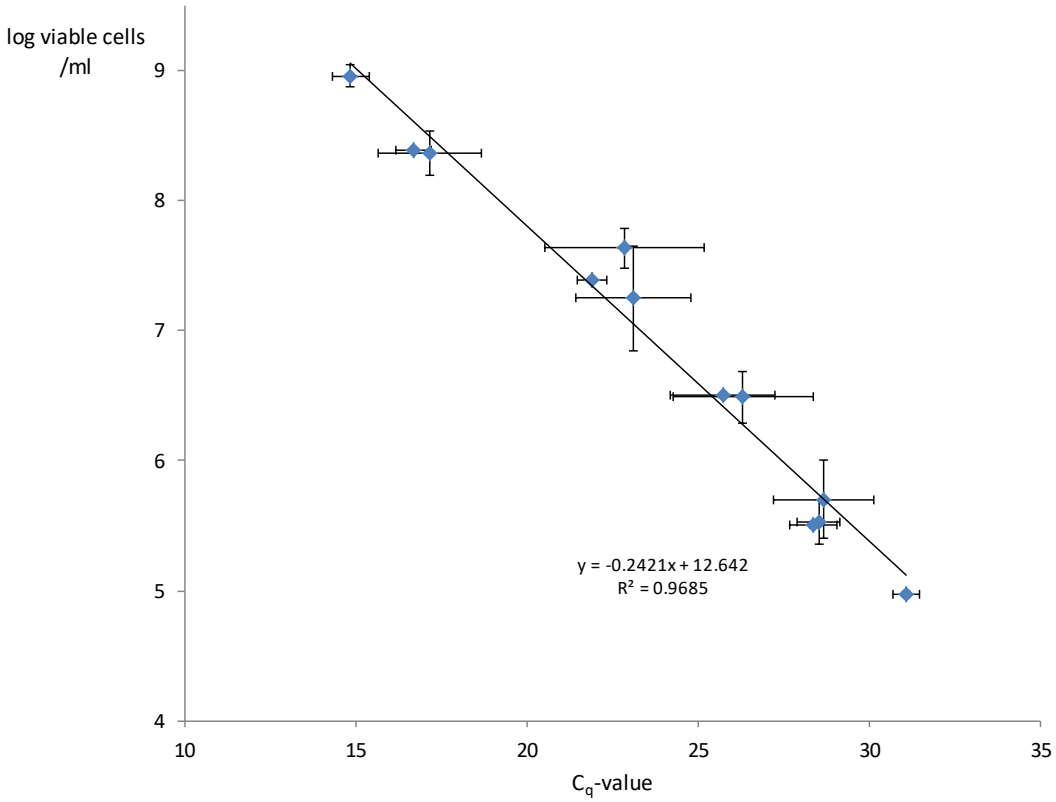

Figure 5a: *Correlation between log viable P. aeruginosa cells/ml determined via SPC and C_q-values determined via PMA-qPCR. The equation for the linear trendline is y=-0.2421x+12.642 with R^2 = 0.9685. Using this equation, the log viable cells/ml can be calculated from the C_q values obtained with PMA-qPCR. Since one biofilm represents a volume of 200 µl, the log viable cells/biofilm can be calculated by dividing the log viable cells/ml by five. Error bars represent standard deviations (n = 6).*

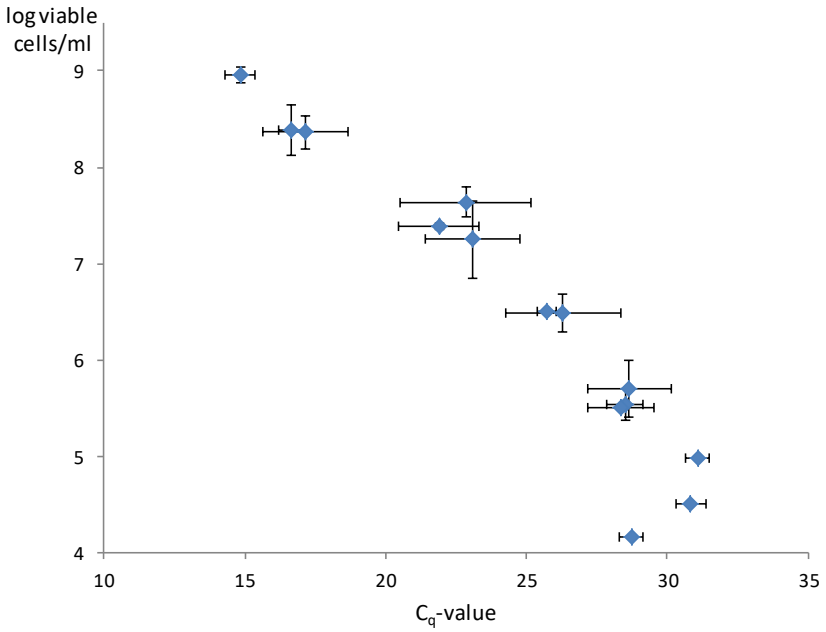

Figure 5b: *Correlation between log viable P. aeruginosa cells/ml determined via SPC and C_q-values determined via PMA-qPCR, including Cq values corresponding with log viable cells/ml values < 5 log. These data indicate that 5 log viable cells/ml is the lower limit of detection. Error bars represent standard deviations (n = 6).*

Susceptibility of planktonic and sessile *P. aeruginosa* cells to colistin, levofloxacin, and tobramycin

The MIC of tobramycin for *P. aeruginosa* ATCC 9027 planktonic cells was 0.5 µg/ml, the MIC of colistin was 2 µg/ml and the MIC of levofloxacin was 1 µg/ml. These concentrations are below the breakpoint for *P. aeruginosa* (National Committee for Clinical and Laboratory Standards. 2007), indicating that *P. aeruginosa* is sensitive to the antibiotics used.

A confocal image of the mature multispecies biofilm is shown in Figure 6. Both Gram-negative rods (*P. aeruginosa* and *B. cenocepacia*) and Gram-positive cocci (*S. aureus* and *S. anginosus*) are present.

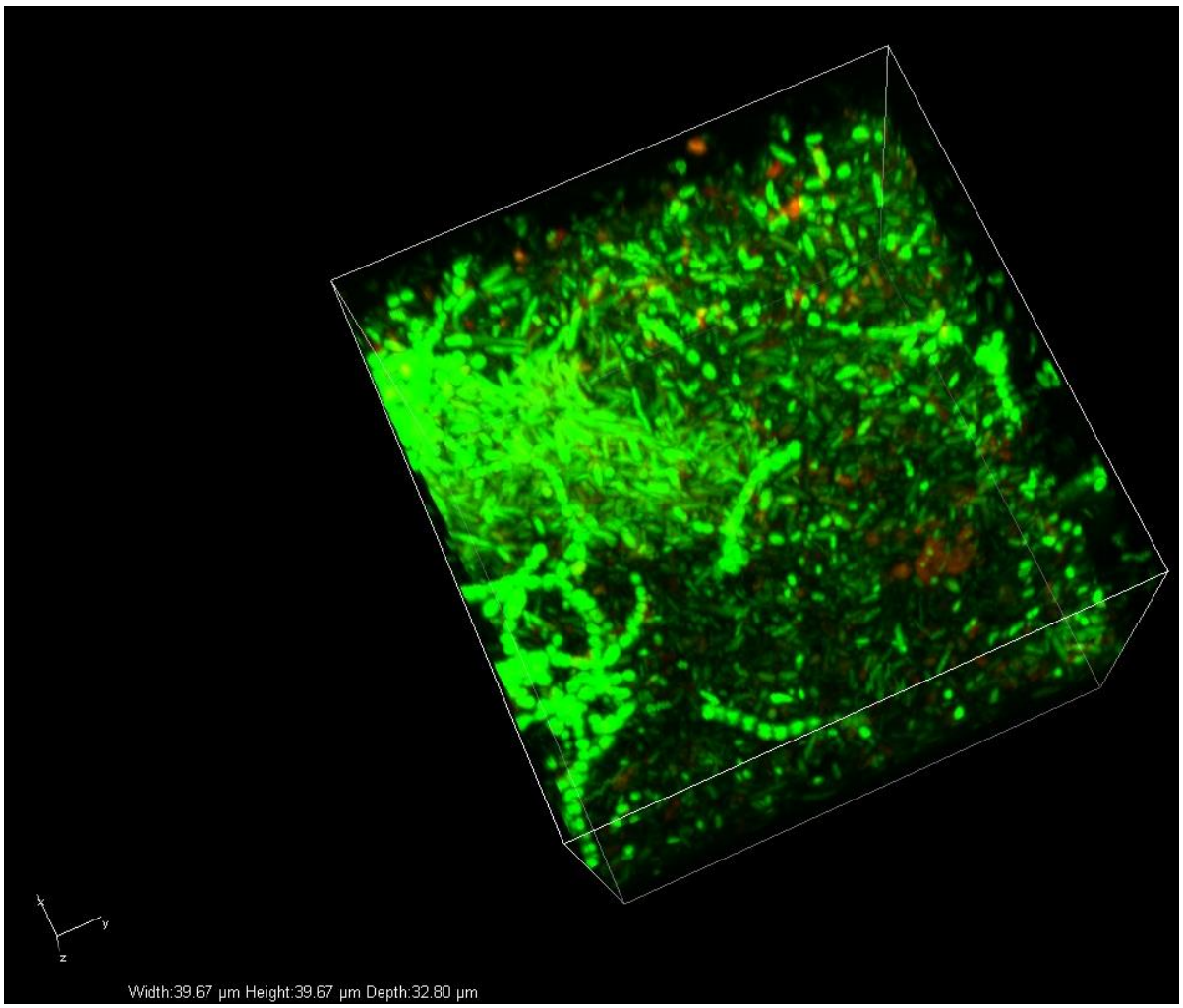

Figure 6: *A confocal image of a mature multispecies biofilm (Live/Dead staining). Gram negative rods and Gram-positive cocci are present.*

The susceptibility of sessile *P. aeruginosa* cells in mono- and multispecies biofilms was determined with PMA-qPCR. The reduction of the number of viable cells was calculated by using the equation for

the linear trendline describing the relationship between the log viable *P. aeruginosa* cells/ml and the C_q-value obtained with PMA-qPCR. Colistin treatment (200 µg/ml, 24 hours) led to a significant decrease ($p<0.05$) in the number of viable *P. aeruginosa* cells, both in mono- and multispecies biofilms (Figure 7, Table 1). In multispecies biofilms, this average reduction was 1.26 log. Using the plate count method, an average reduction of 1 log was observed in *P. aeruginosa* monospecies biofilms. However, based on PMA-qPCR, more viable cells were present, suggesting that the use of the plate count method leads to an underestimation of the surviving cell numbers. However, the opposite, that PMA-qPCR leads to an overestimation of the surviving cell numbers, cannot be ruled out completely. The results also show that *P. aeruginosa* is significantly more sensitive to colistin in a multispecies biofilm with *S. aureus*, *S. anginosus* and *B. cenocepacia* than in a monospecies biofilm ($p<0.05$). After treatment with levofloxacin (100 µg/ml, 24 hours), there was also a significant reduction in the number of viable *P. aeruginosa* cells, both in mono- and multispecies biofilms ($p<0.05$) (Figure 7). Based on the equation for the linear trendline describing the relationship between the log viable *P. aeruginosa* cells/ml and the C_q-value obtained with PMA-qPCR, a 1.57 log reduction was observed for *P. aeruginosa* in monospecies biofilms, while in multispecies biofilms, this average reduction was only 0.94 log ($p<0.05$) (Table 1). These results indicate that *P. aeruginosa* is less susceptible to levofloxacin in a multispecies biofilm.

For tobramycin (200 µg/ml, 24 hours), there was no significant increase in C_q-values after treatment ($p>0.05$) (Figure 7, Table 1). Nevertheless, experiments using the plate count method showed an average reduction of *P. aeruginosa* in monospecies biofilms of 2.35 log after treatment with tobramycin (Figure 8). A likely explanation is that tobramycin causes little or no loss of membrane integrity [424, 425]. Bacterial cells can be killed by tobramycin, but their DNA can still be amplified in the qPCR reaction, as PMA cannot bind to the genomic DNA of intact cells. DNA of dead cells is then extracted together with the DNA of living cells in the DNA extraction procedure and amplified during qPCR, resulting in a lower C_q-value and an overestimation of the number of viable cells. For levofloxacin, little indirect effect on the membrane integrity was described [426], which could explain why we do observe a difference in cell numbers between a mono- and multispecies biofilm, using PMA-qPCR.

Table 1: *C_q-values (± SEM) obtained with PMA-qPCR and calculated log values of viable P. aeruginosa biofilm cells (using the equation for the linear trendline), after treatment with colistin (200 µg/ml), levofloxacin (100 µg/ml) or tobramycin (200 µg/ml) for 24 hours and incubation with PMA. The difference in the calculated number of viable cells after treatment is significantly different between mono- and multispecies biofilms (p< 0.05).*

Biofilm type	Treatment	C_q-value (± SEM)	Calculated log viable cells/biofilm	Δlog
Multispecies	-	15.13 ± 0.28	8.27	1.26
	colistin	20.39 ± 0.64	7.01	
Monospecies	-	15.98 ± 0.35	8.07	0.53
	colistin	18.18 ± 0.23	7.54	
Multispecies	-	12.79 ± 0.38	8.85	0.95
	levofloxacin	16.71 ± 0.38	7.90	
Monospecies	-	14.80 ± 0.15	8.36	1.57
	levofloxacin	21.29 ± 0.46	6.79	
Multispecies	-	15.04 ± 0.44	8.30	-0.05
	tobramycin	14.84 ± 0.43	8.35	
Monospecies	-	14.80 ± 0.15	8.36	-0.02
	tobramycin	14.70 ± 0.18	8.38	

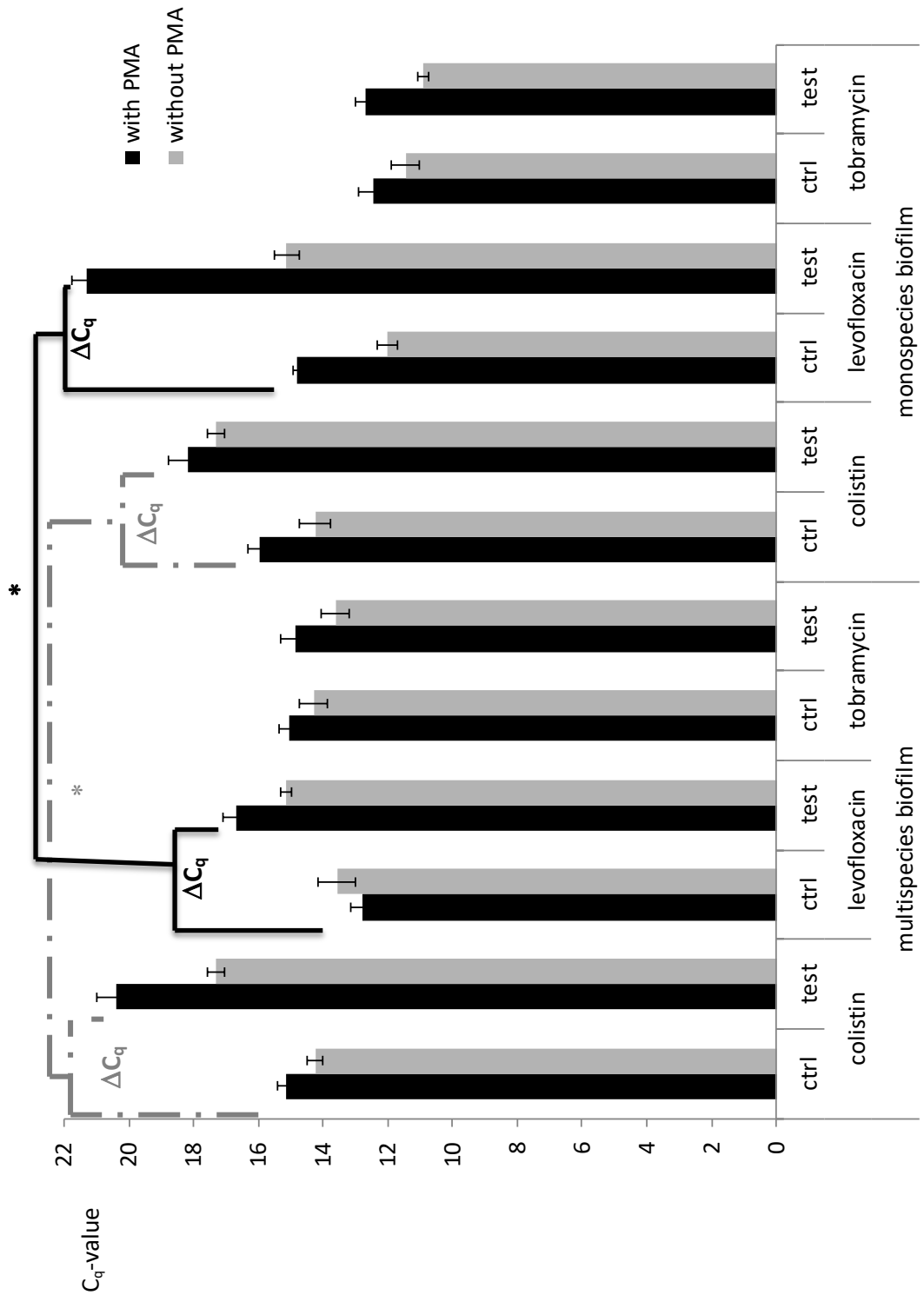

Figure 7: *C_q-values obtained with PMA-qPCR of DNA samples recovered from mono- or multispecies biofilms, after treatment with colistin (200 µg/ml), levofloxacin (100 µg/ml) and tobramycin (200 µg/ml) for 24 hours. Error bars represent standard error mean values (n = 3x2). (*: p<0.05)*

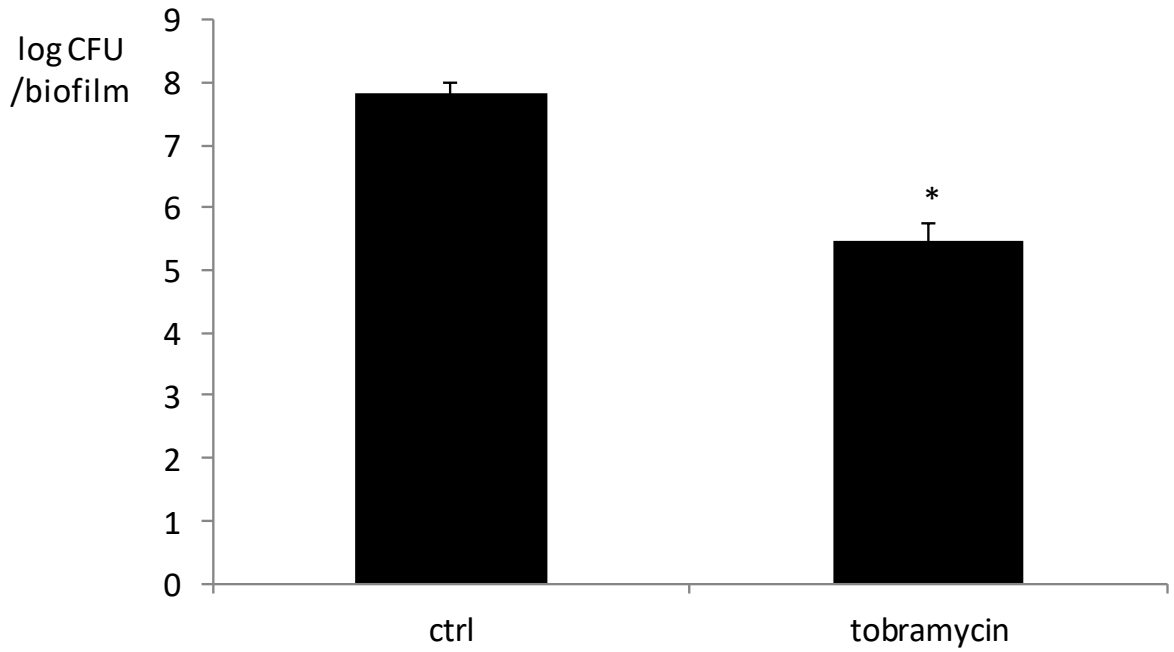

***Figure 8**: Log P. aeruginosa biofilm cells in a control biofilm and in a test biofilm, after treatment with tobramycin (200 µg/ml) for 24 hours, determined with the plate count method. Errors bars represent standard deviation values (n = 3x2) (p<0.05).*

CONCLUSION

The present study shows that PMA-qPCR is a useful alternative for the plate count method to quantify *P. aeruginosa* in mono- and multispecies biofilms, after treatment with a membrane-compromising agent. This method can thus be used to avoid underestimating the cell number due to the presence of VBNC. The use of PMA, able to inhibit amplification of DNA of dead cells, avoids an overestimation of the viable cell number seen with conventional qPCR. However, there are some limitations: the number of cells surviving after treatment should be higher than 10^5 cells/ml and the treatment should compromise the integrity of the membrane. Nevertheless, the PMA-qPCR method was successfully used to determine the difference in susceptibility of *P. aeruginosa* in a mono- and multispecies biofilm towards colistin and levofloxacin: *P. aeruginosa* grown in a multispecies biofilm appears to be less affected by levofloxacin, and more sensitive to colistin than when grown in a monospecies biofilm. These data indicate that the effect of the presence of different members in a biofilm on the susceptibility of *P. aeruginosa* depends on the antibiotic used, and that *P. aeruginosa* in a multispecies biofilm is not always less susceptible to antibiotics than in a monospecies biofilm

Chapter IV: BROADER INTERNATIONAL CONTEXT, RELEVANCE, AND FUTURE PERSPECTIVES

4.1. Why is there a need to study multispecies biofilms?

Biofilms are ubiquitous and the preferred mode of growth of micro-organisms. [427] Their role in a variety of infectious diseases is becoming more acknowledged as our understanding grows. [428] Until recently, researchers have mostly focused on monospecies biofilms. Extensive use of culture-dependent isolation and quantification methods and Koch's postulates associating one micro-organism with one disease have for long masked the presence of multiple species. However, due to the use of culture-independent methodologies, it is becoming clear that most infections are associated with multispecies biofilms. [429] For example, medical equipment [148], chronic wounds [430], and airways of CF patients [431], are colonized by multiple species. The clinical prognosis of an infection can be worse in case of infection with multiple species as interactions between them can contribute to an increased morbidity and mortality. For example, 100% mortality was seen in mice after dual infection of *C. albicans* together with *S. aureus*, whereas mice exhibited high resistance towards infection with either species alone. [432] Recently, Peters et al. [433] also demonstrated, using a murine model of peritonitis, that monospecies infection with *C. albicans* or *S. aureus* was nonlethal, and that co-infection led to a 40% mortality rate. In addition, Pammi et al. [434] reported that multispecies bloodstream infections were associated with more than a 3-fold increase in mortality in patients in the neonatal intensive care unit, and in an increase in duration of infection. Furthermore, Wang et al. [435] evaluated mortality rate in an endotracheal intubation rat model. They found that endotracheal tubes (ETTs) covered with dual-species biofilms of *S. mitis* and *P. aeruginosa* increased the mortality rate compared to ETTs covered with monospecies biofilms. Whiley et al. [130] reported that *P. aeruginosa* showed enhanced pathogenicity in a *Galleria mellonella* infection model, in the presence of oral commensal streptococci.

Furthermore, airways of CF patients harbor a unique microbiome (i.e. *"the catalog of the microbes present and their genes"* [436]) and have a reduced diversity compared to airways of healthy persons.[437] In CF patients, the most frequently isolated species are *Pseudomonas* spp. and *Streptococcus* spp. (Figure 1) [438], whereas in healthy persons, *Phenylobacterium* and *Prevotella* spp. are most commonly isolated. [437, 439] Therefore, not only the presence of multiple species plays a role, as in healthy persons multiple species are present in the airways as well, but the composition of the community is also very important, and will have a big impact on disease progression and outcome. [437]

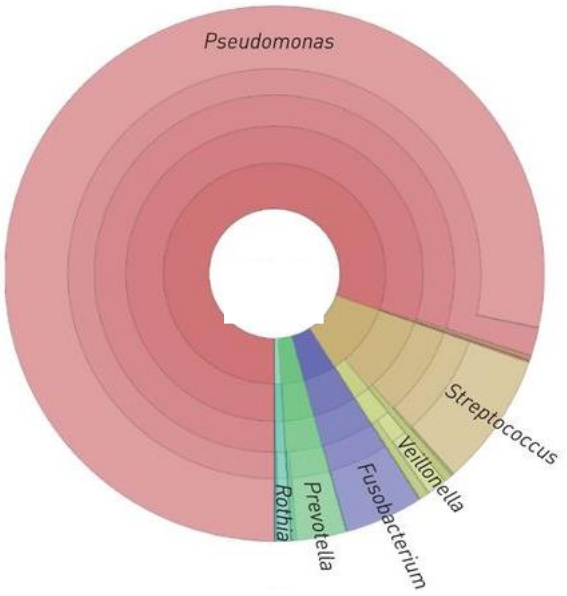

Figure 1: *Distribution of CF sputum microbiome, as determined through 16S pyrosequencing analysis. Adapted from [438].*

Therefore, results from laboratory tests obtained with monospecies biofilms, hardly mimic results that would be obtained using multispecies biofilms. There is an urgent need for a change in direction to understand why and how micro-organisms stick together in a multispecies biofilm, what their interspecies relationship is and how they interact, as well as what the implications are for the infections they cause. [440, 441]

Until now only little research has been focusing on the susceptibility and protective mechanisms of species in communities with more than two members. [241] To address this gap, and in order to understand how one species influences the other, we started with a small-scale community consisting of three or four different species. As we are aware that the study design might impact the study outcome [442], this will be discussed in the next paragraphs.

4.2. Impact of study design on study outcome

4.2.1 In vitro models used to study multispecies biofilms

In this dissertation, mono- and multispecies biofilms used to study differences in susceptibility, were formed on the bottom of 96 well MTPs, one of the simplest static methods for studying surface-attached biofilms. [141, 442, 443] Advantages of MTPs include their low cost, an easy set-up, and the possibility to perform high throughput screening (HTS). [442, 444] Minor limitations of MPTs are that it is an end-point measurement, and that it usually can be used for only short term experiments, whereas a major weakness of the MTPs is that, in a standard setting, the host microenvironment is not taken into account. [442] However, during infection, pathogens will encounter host factors that may be beneficial or detrimental for the initiation and development of the infection. [445, 446] The host microbiome can be defined as "*the collection of host microbiota, host biochemical cues and*

biophysical forces that pathogens might encounter during an infection" [445], and includes multiple host cell types (e.g. macrophages, epithelial cells, and neutrophils [447]), factors produced by these cells, resident microbiota and factors produced by these residents, ECM components, and physical forces. [445] Mucus, another host factor, also affects pathogenesis of for example *P. aeruginosa*, in CF patients. [447] A model system better mimicking the *in vivo* CF lung situation is an organotypic three-dimensional (3-D) human lung epithelial cell culture model, which reflects key aspects of the CF lung tissue, including 3-D architecture, secretion of mucins, barrier function, and multicellular complexity [445, 448, 449]. Crabbé et al. [445] compared the *P. aeruginosa* biofilm-inhibitory activity between this 3-D lung epithelial cell model and a plastic surface (MTP). They found that aminoglycoside antibiotics were more effective in inhibiting *P. aeruginosa* association with the 3-D cells than with plastic, possibly due to antimicrobial compounds produced by respiratory epithelial cells (e.g. defensins), which may act in synergy with the antibiotics. [445] In addition, Anderson et al. [450] compared gene expression of *P. aeruginosa* after tobramycin treatment when grown in a tissue culture system on CF-derived human airway cells with growth on a plastic surface. They observed a downregulation of genes involved in PQS biosynthesis in biofilms grown on CF airway cells versus plastic-grown biofilms, in combination with an upregulation of a gene involved in suppression of bacterial virulence. Interactions with the host might thus lead to an altered gene expression. [450]

Also for chronic wounds, the host microenvironment plays a major role. Sun et al. [429] developed an *in vitro* chronic wound biofilm model to study multispecies interaction, the Lubbock model. They incorporated plasma and red blood cells, typically found in the wound bed, and they utilized a chopped meat-based medium that mimics nutrients obtained through damaged tissue in early stages after wound debridement. [429] In addition, the presence of fibroblasts is important, as these cells regulate numerous, essential repair processes in chronic wounds, necessary for healing. [451] In a first approach, the involvement of fibroblasts has been studied in 2-D monolayer cultures of fibroblast cell lines. [452] However, cell-matrix interactions, cell-cell interactions, and disease-specific cells are not present and the relevance of this model is limited. [451] Over the last decade, 3-D tissue models, known as human skin equivalents [453], have been engineered and shown to more accurately mimic features of *in vivo* cell physiology. [454] Furthermore, protease activity, is also altered in chronic wounds [455], leading to degradation of proteins, which, in combination with excessive degradation of the ECM and growth factors, deprives cells of attachment sites, disrupting the wound healing process. [456, 457] Overall, the chronic wound environment consists of an excessive inflammatory tissue, which contributes to delayed wound healing, and should be taken into account when studying infections in chronic wounds. [456]

Not only do host factors influence the progression and the outcome of an infection caused by a mono- or multispecies biofilm, but they also play a role during interspecies interactions. A specific example is described by Pernet et al. [257]. They reported that *P. aeruginosa* induces type-IIA-secreted phospholipase A2 (sPLA2-IIA, a host enzyme with bactericidal activity) production through the secretion of toxin ExoS, resulting in levels sufficient to kill *S. aureus*, but with only a limited effect on *P. aeruginosa* itself. In addition, the levels of sPLA2-IIA increase with the age of CF patients. [257] These results indicate that a bacterium can eradicate another by manipulating the host innate immunity. [257] Furthermore, the human antimicrobial peptide LL-37 is expressed in epithelial cells of the skin and the respiratory tract, and in leukocytes, and can target bacterial division. [458, 459] Sorg et al. [243] showed that inhibition of susceptible bacteria due to the presence of bacteriostatic antibiotics, led to a reduced susceptibility of these bacteria to LL-37. Upon removal of the bacteriostatic antibiotic, there was an overgrowth of the antibiotic-susceptible bacteria, as they had become less sensitive to LL-37.

Furthermore, within-host evolution has also been described for *P. aeruginosa*, resulting in remodeling of regulatory networks and central metabolism, acquisition of antibiotic resistance genes and loss of extracellular virulence factors. [194] Therefore, in the future, experiments using model systems that involve host secreted factors and mimic more the host environment, are required, as the more closely *in vivo* host-pathogen interactions are reflected *in vitro*, the more relevant the outcome of susceptibility experiments will be to the patient. [445]

Furthermore, the pH of the environment can also have an impact. The CF lung has been described to be an acidic environment [460-462], with enhanced chemical stability of ferrous iron and far greater solubility of ferric iron [463], influencing the composition of the community. Lower pH levels of the thin layer of airway surface liquid (ASL) in CF patients also reduced antimicrobial activity of the ASL, thereby impairing the killing of bacteria that enter the lung of the newborn. [464] In addition, antibiotic effectivity can be decreased in a lower pH environment [465], as described for fidaxomicin [466], tobramycin [467], gentamicin and oxacillin [468].

Next, oxygen levels in the environment can also play an important role. Within the CF lung, the increased densitiy of the mucus makes it difficult for oxygen to diffuse across the mucus and into the blood. [469] As a result, there will be an oxygen gradient in the luminal mucus, that is severe enough in the basal layer to be termed microaerobic, or even in a more severe case, anaerobic. [469] [116, 470] Consequently, bacteria that are normally not found in the lung, can grow and develop in the low oxygen conditions. [469] *P. aeruginosa* can survive the reduced oxygen conditions by utilizing nitrate as an alternative electron acceptor. [470, 471] Furthermore, low oxygen levels are described to lead

to induced alginate production, increased biofilm formation combined with the loss of flagella, and even a higher antibiotic resistance of *P. aeruginosa*. [447] For example, penicillin and cephalosporin showed a decreased efficacy against *P. aeruginosa* under hypoxia [472], as well as tobramycin, ciprofloxacin, carbenicillin, ceftazidime, chloramphenicol, and tetracycline. [473] In addition, low oxygen conditions relevant to the CF lung are described to affect the competition between *P. aeruginosa* and *S. aureus*. In hypoxic conditions, *S. aureus* showed a higher survival rate. [474] This could be due to the fact that lower oxygen tension influences the expression of *P. aeruginosa* virulence genes. [475, 476]

Nevertheless, MTPs can be easily used to grow and to initially study multispecies biofilms. [141] However, to be able to compare results between different research centers, effort is needed to standardize experimental studies of multispecies biofilms using identical model systems, as differences between model systems can lead to different outcomes. [442] As mentioned in Chapter 1, *P. fluorescens* and *B. cereus* were less susceptible towards treatment when grown in a rotating stainless steel device for 7 days, compared to when grown in a flow system for 16 hours. [144]. Furthermore, there is only a scarce extrapolation from *in vitro* to *in vivo* models, and even more scarce to clinical studies. [444] Therefore, it might be recommended to also include *in vivo* model systems. For example, the nematode *Caenorhabditis elegans* can easily be used to compare the virulence of a mono- and multispecies infection. [477] In addition, the invertebrate *Galleria mellonella* infection assay can also be used to investigate differences in pathogenesis. [478, 479] More closely related to humans, is the use of vertebrate models like *Danio rerio* (zebrafish). [480] Bergeron et al. [481] recently published that *C. albicans* and *P. aeruginosa* are synergistically virulent, using a transparent zebrafish model. More specific in the context of CF, a mouse lung infection model systems can be included. [482]

Not only differences between model systems lead to different outcomes, but also differences within models, e.g. the growth medium, the bacterial strains, and the antibiotic concentration used, might lead to different research outcomes. [444]

4.2.2 Influence of the medium used

An important factor affecting the study outcome is the medium used to grow and to treat biofilms. Among others, *S. aureus* and *C. albicans* are reported to be killed by *P. aeruginosa* when grown in its presence. [107, 227, 311] However, they are often co-isolated *in vivo*, and therefore we adapted our multispecies biofilm growth medium by adding BSA to allow optimal growth of all species present. BSA was described to bind to *P. aeruginosa* 3-oxo-C_{12} QS molecules and to capture *P. aeruginosa* outer membrane proteins, thereby leading to a decreased virulence towards other species. [76, 483,

484] By adding BSA, we could increase the growth of *S. aureus* and *C. albicans* in a multispecies biofilm with *P. aeruginosa*. However, depending on the *P. aeruginosa* strain, small differences could be observed, indicating that a difference in virulence between *P. aeruginosa* strains also results in a different growth of neighboring strains (see 4.2.3). DeLeon et al. [76] also observed a difference in competitive behavior when *S. aureus* and *P. aeruginosa* were grown in traditional laboratory growth media, compared to in a wound-like environment with added bovine plasma and laked horse red blood cells. In the traditional growth medium, *S. aureus* was quickly eradicated, whereas it co-existed with *P. aeruginosa* in the wound-like medium, similar to our observations. On the other hand, fetal bovine serum has also shown to bind antibiotics and peptides. [485, 486] Albumin binding should thus be taken into consideration in studies using antimicrobial peptides as antimicrobial agents. [445]

For experiments performed in this dissertation, we also supplemented the medium with mucin. Mucin is the main component of secreted mucus, which also contains a large number of proteins, nucleic acids, carbohydrates, lipids, amino acids, and electrolytes. [487, 488] It forms a protective airway coating secreted in the healthy airway [489], with a concentration of approx. 0.3-0.6 mg/ml. [490, 491] In CF airways, mucin was suggested to serve as an attachment surface, and to impact biofilm development. [488] When grown in artificial sputum medium, *P. aeruginosa* was described to form tight micro-colonies suspended within the medium instead of being attached to a surface as in standard broth medium. [488, 492] In addition, Landry et al. [493] found that *P. aeruginosa* biofilm development occurred differently on surfaces coated with mucin compared with development on glass and surfaces coated with actin or DNA, due to inhibition of surface-associated motility. On mucin-coated surfaces, biofilms developed as large cellular aggregates, and these aggregates showed an increased tolerance towards tobramycin compared with biofilms grown on glass. [493] On the other hand, Caldara et al. [494] demonstrated that mucin biopolymers promoted the motility of planktonic bacteria, thereby preventing their adhesion to underlying surfaces, without killing or trapping the bacteria. Yeung et al. [495] also showed that mucin promoted surface motility in *P. aeruginosa*. At first sight, these findings are in contrast, however, the effects of mucin on motility might depend on the native 3D-structure of mucin and its biophysical properties (e.g. viscoelasticity and lubricity), preserved in native mucus [493], but not when adsorbed to a surface [495]. It is thus important to study mucins in their native 3D-form, as this will reveal information that cannot be captured by collapsed mucin monolayers. [494] Furthermore, the type of mucin and the mucin concentration will also influence whether or not dispersion or aggregation will occur. For example, the presence of salivary mucin MUC7 leads to aggregates of *S. gordonii* and the adherence of *S. gordonii* to surfaces whereas mucin MUC5B has no effect on aggregation or binding. [496] In addition, *P. aeruginosa* lipopolysaccharides were described to induce the production of reactive

oxygen intermediates, which subsequently cause the release of transforming growth factor α, which then up-regulates the expression of mucins by human epithelial airway cells. [488, 497] On the other hand, *P. aeruginosa* also produces extracellular serine proteases (LasB) that can reduce the amount of mucus. [498] Interaction between production and degradation of mucins will thus provide a net mucin concentration suitable for the full development of layered structures in the biofilm. Several groups [488, 492, 499], including ours, utilized a concentration of mucin of 0.5%. Haley et al. [488] also tested the effect of 0.25% and 1% mucin. They found that in the presence of both 0.25% and 1% mucin, the multilayered biofilm like structure was replaced with a structured consisting of small micro-colonies and individual cells, leading to an architecture that was more diffuse than seen with 0.5% mucin, with increased biovolume and thickness, but a decreased roughness. Using immunodetection, Henke et al. [489] showed that the concentrations of mucins is decreased in sputum from CF patients with stable disease (up to 89% less than normal mucus), whereas mucin concentrations are greatly increased during pulmonary exacerbations (up to levels similar to normal). During exacerbation, host inflammatory or immune mediators might stimulate mucin production and secretion as a protective response. [489] Furthermore, Henke et al. [489, 500] described that mucin is only a minor component in CF sputum, and that DNA is the dominant polymer. They found that DNA was present in significantly higher concentrations than in healthy samples, probably as a result of leukocyte necrosis, again indicating the importance of the presence of host factors in *in vitro* model systems. [489, 500] Henderson et al. [491] also found reduced mucin levels in CF sputum compared to normal sputum when using immunologically based quantification analysis. On the other hand, they also described that the average total mucin content of normal sputum samples was 2,710 µg/ml, whereas the average mucin concentration of sputum from CF was 6,454 µg/ml ($P = 0.001$), when using physical techniques to measure the concentration (size exclusion chromatography/differential refractometry techniques). [491] Thus, their data suggest that mucin concentrations in CF sputum are higher than in healthy samples, and that there will be a general problem with respect to antibody-dependent measurement of mucins in CF secretions due to the presence of free proteases that can cleave the antigenically exposed regions of mucins. [491]

The presence of mucus can also influence the composition of and interactions between the multispecies community. For example, Kavanaugh et al. [501] demonstrated that mucin could suppress the yeast-to-hyphae transition of *C. albicans*, thereby protecting against colonization by *P. aeruginosa*. Furthermore, Frenkel and Ribbeck [502] showed that mucin shifted cells from a competing mixed-species biofilm, consisting of *S. sanguinis* and *S. mutans*, into the planktonic form, thereby promoting co-existence of the two competing bacteria. By promoting a less competitive mode of growth, the presence of mucus could thus increase bacterial diversity. [502] In addition,

dispersed bacteria will be more susceptible towards antibiotic treatment. [488] It is thus very important to supplement the growth medium with mucin, however, it is not easy to determine in which form and in which concentration.

Furthermore iron is an essential nutrient for bacteria. Often, iron concentrations are limiting, especially in the context of infections. [503] In contrast, iron levels have been found to be high in CF sputum. [107, 504] In CF, the majority of the iron is associated with ferritin and in the ferric (Fe^{3+}) form with limited bioavailability. To be able to survive in the CF niche, CF pathogens have a range of mechanisms by which they can form usable ferrous (Fe^{2+}) iron. [505] Iron has also been described as the cause of persistent *P. aeruginosa* infection in CF patients. [506] Furthermore, *P. aeruginosa* has shown to use LasA protease to acquire iron from *S. aureus* after cell lysis of the latter. [118] In addition, Nguyen et al. [503] showed that iron depletion enhanced AQ-mediated antimicrobial activity of *P. aeruginosa* against *S. aureus*. Filkins et al. [107] used a CF bronchial epithelial co-culture model and demonstrated that *P. aeruginosa* requires both its major siderophores to kill *S. aureus*. Iron thus plays a central role in the modulation of interspecies interactions [505], and it should thus be taken into account that *in vitro* used iron levels will impact the study outcome. Therefore, it is recommended to use a minimal growth medium where the iron levels can be controlled, and wherein the influence of high and low iron levels on the study outcome can be evaluated.

Next, bacteria have shown to become less susceptible to antibiotics when nutrients are limited, as is the case in biofilm growth, occurring in many infections. [507] This could be due to passive effects of a growth arrest leading to the inactivity of antibiotic targets, or to an active response to starvation, controlled by the starvation-signaling stringent response, which leads to increased virulence and increasing antioxidant defenses. [507, 508] For *C. albicans*, a higher biofilm formation was observed in Roswell Park Memorial Institute 1640 medium compared to sabouraud dextrose broth or yeast nitrogen base. A difference in biofilm formation might lead to a difference in susceptibility, again indicating the importance of the medium used in the model. [509] In addition, de Queiroz et al. [510] also found an influence of the culture medium, when comparing activity of chlorhexidine on *S. mutans* biofilms. On the other hand, if only a weak biofilm is formed in minimal medium, antibiotics will be more effective in the minimal medium compared to in the rich medium. For example, Harrison et al. [511] compared growth of *P. aeruginosa* and *E. coli* monospecies biofilms in rich and in minimal medium. For *P. aeruginosa*, they did not observe a difference in biofilm formation, whereas *E. coli* was a poor biofilm former in the minimal medium. In line with these observations, Bernier et al. [512] reported that *E. coli* biofilms grown in rich medium were less susceptible towards ofloxacin and ticarcillin than those grown in minimal medium.

4.2.3 Influence of the consortium

Determining the relevant scale for a study is a very important step in the extension of an *in vitro* model, as this affects the needed complexity in multispecies biofilm experiments. [73] One of the most important aspects is choosing species and strains that are relevant for the study. [73] Typically, one of three approaches is applied *in vitro* to establish a multispecies communities: (i) the use of a pre-established strain collection, bringing together strains from various sources, (ii) the use of strains coexisting in the same environment, or (iii) using complex environmental samples without a cultivation step. [73] The easiest approach for studying interspecies interaction is to implement a low-diversity model community from well-characterized laboratory strains. However, in most cases, these strains are not co-isolated, which might compromise extrapolation from *in vitro* to *in vivo* results. [73] In addition, short-term coadaptation of two independently isolated strains was shown when they were grown together in a dual species biofilm, leading to a specialized association with each other. [513]

Many recent analyses describing the total microbial community are based on metagenomics. [431, 514-517] This approach leads to the discovery of the various species inhabiting a given environment. [73] In a next step, in order to reveal potential interaction between community members across spatial or temporal gradients, network analysis of significant co-occurrence patterns might be used. [518] These result can help in deciding which species to include [73], as often, minority members in the community can play essential roles in the community [519, 520]. For example, as shown in Figure 2, the presence of minority species, such as *Hafnia alvei* and *P. vulgaris*, can increase the severity of a chronic wound infection (deep wound). [521] Nevertheless, in both deep and superficial wounds, *S. aureus* is most frequently isolated (70%). [521] *P. aeruginosa* is another frequently isolated organism, with up to 10% occurrence, whereas in immunocompromised patients, or patients who have undergone abdominal surgery, species of the *Enterobacteriaceae* family are mostly identified. [521] Chellan et al. [522] found mixed fungal and bacterial infections in 21.4% of their patients population, while 5.8% had only fungal infection. *Candida* spp. were the most predominant fungal isolates. The same trend was described by Dowd et al. [523], indicating that fungi are also important wound pathogens. In addition, *Pseudomonas* spp. and *Staphylococcus* spp. are also often isolated in the hospital environment [524], as well as fungi. [525-527] Therefore, in Chapter III, paper I, we have selected *S. aureus*, *P. aeruginosa*, and *C. albicans* as members of our multispecies community.

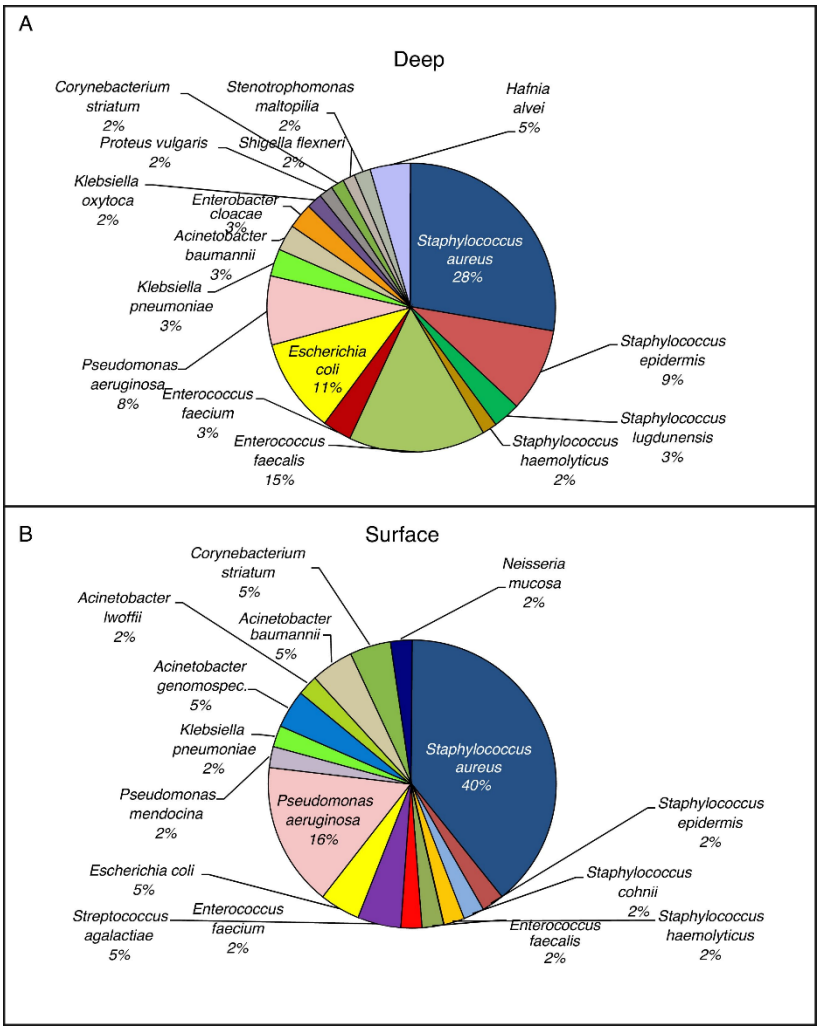

Figure 2: *Representation of microorganisms present in patients' wounds. Patients (n = 50) were divided into two groups, based on surgical wounds classification: (A) deep wounds and (B) surface wounds. [521]*

In CF patients, *S. aureus* is the most frequently isolated microorganism in young patients, whereas *P. aeruginosa* levels increase with patients' age (Figure 3). [528] Members of the *B. cepacia* complex are less frequently recovered, however, these micro-organisms are associated with a rapid decline in lung function. [99, 529] In addition, *Streptococcus* spp. are described as emerging pathogens in CF patients, responsible for acute exacerbations. [120, 122, 123] These species are often found to be present in the same sputum sample. [107, 431, 530] Therefore, in paper 2, 3 and 4 in Chapter III, we included *S. aureus*, *P. aeruginosa*, *S. anginosus* (and *B. cenocepacia*). However, it is important to note that chronic airway infections in CF patients are associated with genetic and phenotypic changes in the infecting species, well studied in *P. aeruginosa*. [104] *P. aeruginosa* isolates from chronic CF infections are characterized by lack of motility, loss of QS, a changed cell envelope, overproduction of alginate (leading to a mucoid phenotype), a slow growth, antibiotic resistance, and loss-of-function mutation in virulence genes, suggesting attenuation of virulence. [104, 200, 531, 532] These characteristics are suggested to be advantageous for survival *in vivo*, and to be the result of evolution

of *P. aeruginosa* within the host. [104] Furthermore, as a consequence of these altered characteristics, chronic *P. aeruginosa* isolates show a reduced capacity to outcompete *S. aureus*. [200] Inclusion of an early or late *P. aeruginosa* isolate might thus also have an influence on the outcome of an *in vitro* study. In our experiments, we used non-mucoid *P. aeruginosa* strains, therefore, we cannot extrapolate and generalize the results obtained in Chapter III to other *P. aeruginosa* strains.

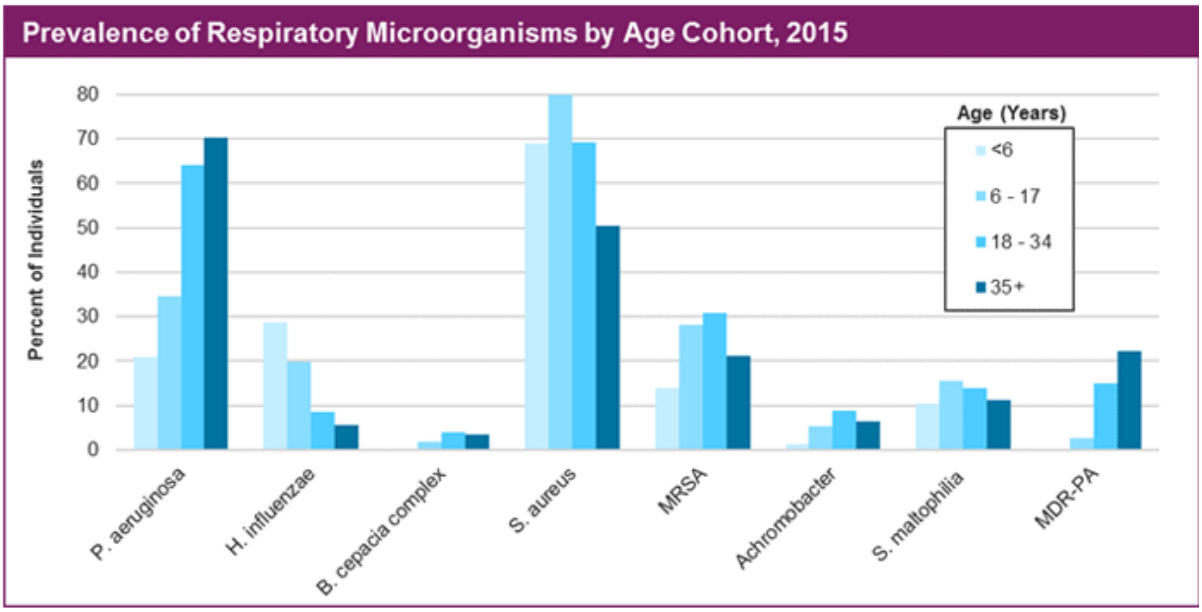

Figure 3: *Prevalence of respiratory microorganisms in CF patients by age cohort in 2015. MRSA: methicillin-resistant S. aureus; MDR-PA: multi-drug resistant P. aeruginosa. [528]*

Furthermore, in the hospital environment, in chronic wounds, and in CF lungs, many more bacteria are present than the species we included in our experiments. [119, 142, 533, 534] Consequently, adding or replacing members can change study outcomes. In addition, chronic wounds and CF lungs are often initially infected with *S. aureus*, followed by infection with *P. aeruginosa* at a later stage. However, in this dissertation, we inoculated all species at the same time. Again, this could have an influence on the observed study outcomes. In their study, DeLeon et al. [76] investigated whether *P. aeruginosa* could colonize secondarily to *S. aureus*. They observed a successful colonization of both species. However, they did not evaluate the effect on antibiotic susceptibility of secondary colonization.

Besides species and strain selection, it is also important to evaluate spatial organization in *in vitro* model systems, as spatial composition will influence the maintenance of diversity and interspecies interactions. Ideally, *in vitro* spatial organization should reflect the *in vivo* situation. [535] In recent years, visualization techniques have become a valuable tool for studying multispecies communities. Nondestructive CLSM, if possible in combination with fluorescent species-specific labelling, is often

used. [211] An alternative is fluorescence *in situ* hybridization (FISH), enabling examination of the spatial distribution in communities, and successfully used, although it requires destructive end-point analysis. [536-538] In this dissertation, we only have limited information about the spatial organization in our multispecies community. Again, study outcomes can thus not be generalized, as they might be dependent on the spatial organization within the *in vitro* model system.

4.2.4 Quantification of bacteria in a multispecies biofilm

One of the biggest evolutions contributing to our knowledge of multispecies communities is the development of culture-independent identification and quantification methods. For example, underestimation of the presence of species of the SMG group in CF lungs, due to insufficient microbiological detection using standard methods, might lead to an underestimation of the health risk and wrong treatment regimens. [539] In addition, there is the risk of an underestimation of the total cell number due to the presence of VBNC bacteria [406] Therefore, the technique used to quantify species in patient samples or to quantify species in *in vitro* model systems, will have a big impact on the outcome of the cell numbers. Culture-independent identification and quantification methods have been extensively developed over recent years. DNA extraction and subsequent qPCR can assess the individual ratio of members in the multispecies community. [520] Besides qPCR, flow cytometry has recently been proposed as a fast and precise alternative to quantify biofilms. [540] However, due to their need for optimization and cost, these techniques are still not widely used in routine laboratories. [541] In cases where the patient does not respond well to antimicrobial treatment, or when a biofilm is suspected to be present, these techniques might be considered as a second line diagnostic tool, to have a better estimation of which core community members to target. [542] In Chapter III article 4, we optimized the quantification of *P. aeruginosa* in a multispecies biofilm with *B. cenocepacia*, *S. anginosus* and *S. aureus*, using PMA-qPCR as proposed by Nocker et al. [543, 544] Using PMA, we were able to discriminate viable and non-viable cells, also after antibiotic treatment. This method was already used to quantify bacterial species, e.g. in a multispecies oral biofilm consisting of *S. gordonii*, *S. oralis*, *Veillonella parvula*, *Fusobacteriulm nucleatum*, and *Prevotella intermedia*. [407] Exterkate et al. [545] also showed enhanced differences in biofilm composition after treatment, when adding PMA. This indicates that PMA treatment might be a requirement to obtain the correct biofilm composition after antimicrobial treatment. [546] Recently, Nguyen et al. [542] analyzed the effect of PMA on the microbiota of CF patients during exacerbation. They found that the low abundance population diversity is impacted by PMA treatment, whereas abundant taxa were not affected. These data are in agreement with the cut off of 10^5 CFU/ml we proposed (Chapter III article 4) to obtain the most adequate effect of PMA treatment.

4.3 Do species in a multispecies biofilm show altered antibiotic resistance?

In Table 1, an overview of results is given of several articles that examined antibiotic killing in a multispecies biofilm of species that were also included in the *in vitro* model used in this dissertation. In the next paragraphs, differences between our studies and the other studies will be discussed. In general, it is clear that the study outcome will depend on the study design, and that there is no standard answer to the question whether or not species show an increased resistance in a multispecies biofilm.

Table 1: *Antibiotic susceptibility of several species in multispecies biofilms in other studies*

Microorganisms used	Antibiotics used	Medium used	Results	Reference
S. aureus; *C. albicans*	Vancomycin (≤ 1600 μg/ml); amphotericin B (≤ 40 μg/ml)	50% bovine serum, or brain heart infusion broth	*S. aureus* was killed less; no difference in killing of *C. albicans*	Harriott and Noverr [266]
S. aureus; *C. albicans*	Ethanol (5-50%)	Tryptic soy broth containing 0.2% glucose	Increased regrowth *of S. aureus*; *C. albicans* counts remained similar	Peters et al. [547]
S. aureus; *P. aeruginosa*	Gentamicin (200 μg/ml); tetracycline (20 μg/ml); ciprofloxacin (200 μg/ml)	Wound-like medium made up of 45% Bolton broth, 50% bovine plasma, and 5% laked horse red blood cells	*S. aureus* was killed less by gentamycin and tetracycline, no difference in killing by ciprofloxacin; for *P. aeruginosa*, no difference in killing for any of the antibiotics tested	DeLeon et al. [76]
S. aureus; *P. aeruginosa*	Ciprofloxacin (0.125 μg/ml and 0.500 μg/ml)	Tryptic soy broth	*S. aureus* was killed less; no difference in killing of *P. aeruginosa*	Magalhaes et al. [548]
S. aureus; *P. aeruginosa*	Agar plates with aminoglycosides (tobramycin (> 0.4 μg/ml), gentamicin, amikacin, kanamycin), β-lactams (carbenicillin, ceftazidime), macrolides (azithromycin) and chloramphenicol	Mueller–Hinton agar	*S. aureus* was killed less by aminoglycosides; no difference in killing by the other antibiotics	Hoffman et al. [178]
S. constellatus (member of SMG); *P. aeruginosa*	Tobramycin (5 μg/ml)	minimal essential medium supplemented with 2 mM L-glutamine and 0.4% arginine	*S. constellatus* was killed less	Price et al. [124]
B. cenocepacia; *P. aeruginosa*	Chlorine (30 ppm)	low nutrient sterile defined medium consisting of 0.1 g glucose, 0.018 g NH_4Cl, 3.93 g phosphate buffer, 2 ml 0.1 M $MgSO_4$	Both *B. cenocepacia* and *P. aeruginosa* were killed less	Behnke et al. [270]

4.3.1 Pseudomonas aeruginosa

In Chapter III article 4, *P. aeruginosa* was increasingly killed by colistin, but less by levofloxacin, when grown together with *B. cenocepacia*, *S. anginosus* and *S. aureus*, compared to in a monospecies biofilm. In contrast, when we grew *P. aeruginosa* together with *S. aureus* and *S. anginosus*, we could not observe any differences in susceptibility (Chapter III article 2). The absence of *B. cenocepacia* in the latter community might impact the susceptibility of *P. aeruginosa*. In addition, quantification in article 4 was done using PMA-qPCR, whereas the plate count method was used in article 2. This difference in quantification method could also play a role in the observed differences in susceptibility. Similarly, Deleon et al. [76] and Magalhaes et al. [548] grew *P. aeruginosa* in a dual species biofilm with *S. aureus*, and also did not observe any differences in susceptibility of *P. aeruginosa* towards gentamicin, ciprofloxacin, or tetracycline.

When using disinfectants, Behnke et al. [270] observed a decreased susceptibility of *P. aeruginosa* towards chlorine, in the presence of *B. cenocepacia*. In Chapter III article 1, we observed an increased susceptibility of *P. aeruginosa* towards NaOCl, PCMX, H_2O_2, CHX, HAC, and CET, when grown in the presence of *S. aureus* and *C. albicans*. These data indicate that susceptibility depends on the disinfectant used, and on the other species included in the community.

4.3.2 Staphylococcus aureus

Peters et al. [547] observed an increased regrowth of *S. aureus* after EtOH treatment when grown in a multispecies biofilm with *C. albicans*. In Chapter III article 1, our results did not show an increased survival of *S. aureus* after EtOH treatment in presence of *C. albicans* and *P. aeruginosa*, indicating that the presence of *P. aeruginosa* might lead to another outcome. Only after PCMX treatment, we could see a significant increased survival of *S. aureus* in the multispecies biofilm. On the other hand, *S. aureus* was killed more by CHX, CET, and HAC in a multispecies biofilm with *C. albicans* and *P. aeruginosa*. Again, results depend on the disinfectant used and other species included.

In addition, when treating *S. aureus* with antibiotic solutions in presence of *S. anginosus* and *P. aeruginosa*, an increased killing of *S. aureus* was observed for all antibiotics used (including tobramycin, vancomycin, and ciprofloxacin) (Chapter III article 2). Furthermore, TEM data (Chapter III article 3) suggested that *S. aureus* does not increase cell wall thickness in response to e.g. vancomycin when grown in presence of *S. anginosus* and *P. aeruginosa*, which might be the cause of the increased susceptibility of *S. aureus* in a multispecies biofilm, as observed in article 2. In contrast, DeLeon et al. [76] observed a protection of *S. aureus* against gentamicin and tetracycline treatment in presence of *P. aeruginosa*. Hoffman et al. [178] and Magalhaes et al. [548] also observed a

protection of *S. aureus* in presence of *P. aeruginosa* against aminoglycosides and ciprofloxacin, respectively.

Differences in *in vitro* model systems used in the studies might be the cause of the observed differences in susceptibility: other growth medium, other strains, incubation time, the presence or absence of *S. anginosus*, other antibiotic concentrations. The study design thus clearly has a major impact on the study outcome, therefore, it is very important to take every variable into consideration. As *S. anginosus* is often co-isolated with *P. aeruginosa* and *S. aureus* [120, 127], including *S. anginosus* in the *in vitro* model system might better reflect the CF community. However, studies represented in Table 1, as well as ours, did not include host factors. Thus, it cannot be predicted how *in vivo* susceptibility would resemble the observed *in vitro* susceptibility.

4.3.3 Streptococcus anginosus

In Chapter III article 2, we observed a protection of *S. anginosus* against antibiotics that interfere with cell wall synthesis when *S. anginosus* was grown together with *S. aureus* and *P. aeruginosa*. Further experiments revealed that *S. aureus* played a major role in this protection. Protection of *S. anginosus* against penicillins by β-lactamases produced by *S. aureus* was already described [251, 346]. In addition, Price et al. [124] observed an increased growth of *S. constellatus* in presence of *P. aeruginosa* after tobramycin treatment. However, in Chapter III article 2 we could not observe a difference in growth of *S. anginosus* in presence of *P. aeruginosa* and *S. aureus* after tobramycin treatment. Nevertheless, this result is not surprising, as Price et al. only saw this effect with one particular *S. constellatus* strain, and not with two other *S. constellatus* strains, nor with an *S. anginosus* or *S. intermedius* strain (all SMG members). This again emphasizes the role of the species and strain used, and the importance of testing multiple clinically relevant strains. Another clear example is the one described in Chapter I, wherein Weimer et al. [249] observed a protection of *S. pneumoniae* by *H. influenza* against killing by amoxicillin, whereas Westman et al. [249, 253] did not. Therefore, in Chapter III article 2, we evaluated the antibiotic effectivity against more than one bacterial strain to confirm our data and to have an indication whether or not the observed effect was strain-dependent.

4.3.4 Candida albicans

C. albicans did not show an increased regrowth in presence of *S. aureus* after treatment with EtOH, as reported by Peters et al. [547] These results are in line with our observations in Chapter III article 1, where we could not observe a difference in susceptibility of *C. albicans* towards EtOH when grown with or without *S. aureus* and *P. aeruginosa*. We do could observe an increased killing of *C. albicans*

by NaOCl, BzCl, H_2O_2, CHX, CET, HAC, and PVP-I, whereas a decreased killing by PCMX was observed in the presence of *S. aureus* and *P. aeruginosa*.

4.3.5 Mechanisms of altered resistance in our in vitro model system

As described in Chapter I (Section 2.4.2.1), indirect pathogenicity is a major contributor to altered resistance observed in multispecies communities. [241, 242] The production of aminoglycoside-modifying enzymes and β-lactamases has been described to lead to a decreased killing of neighboring community members. [76, 242, 250, 251] In Chapter III article 2, we also observed an increase in MIC of amoxicillin of *S. anginosus* when grown in the supernatant of an *S. aureus* biofilm. The addition of a β-lactamase inhibitor again reduced the MIC value, indicating that β-lactamases of *S. aureus* were responsible for the observed increase. Nevertheless, in Chapter III article 2, protection was also observed in the supernatant of a β-lactamase negative *S. aureus* strain. Clearly, other mechanisms than protection against β-lactam antibiotics through β-lactamases are active. However, the elucidation of these mechanisms is still at the beginning. The use of next-generation sequencing techniques allows to identify genes and proteins of which expression is affected by the presence of other strains. [9] Using next-generation sequencing (Chapter III article 3), *S. anginosus* showed to upregulate genes involved in cell wall thickening when grown together with *P. aeruginosa* and *S. aureus* in a multispecies biofilm, possibly leading to an increased resistance towards antibiotics that interfere with cell wall synthesis. In addition, when performing TEM, microscopy images confirmed that *S. anginosus* indeed altered its cell wall in presence of the other species. If these results could be confirmed in *in vivo* models, they should be taken into account when choosing a treatment regimen directed towards *S. anginosus*, as *S. anginosus* has been described to cause acute clinical exacerbations in CF patients. [126, 343] For example, the addition of lysozyme to the treatment regimen could increase the efficacy of antibiotics targeting the cell wall biosynthesis. [100] TEM images also revealed that *S. aureus* did not alter its cell wall thickness upon treatment with vancomycin when grown in presence of *S. anginosus* and *P. aeruginosa*, explaining the observed increase in susceptibility of *S. aureus* towards vancomycin in a multispecies biofilm seen in Chapter III article 2. As far as we know, this has not yet been described for *S. aureus* or *S. anginosus* by other authors. These results thus emphasize the importance of combining multiple techniques, i.e. selective quantification, next-generation sequencing, and microscopy to fully explore what causes alterations in susceptibility.

4.4 Recommendations for the future

4.4.1 Recommendations for researchers

Culture-(in)dependent methods allow to identify the species in a sample, by they cannot identify the spatial organization of each species in a sample, nor can they determine which species is contributing to pathogenesis. Even though microscopy fails to determine which species is the major contributor to pathogenesis, it can reveal the bacterial orientation and distribution, which also contributes to interspecies interactions (see Chapter I section 2.2.2). Future research should focus on the exploration of the spatial organization within the *in vitro* model used, and on the investigation of how adaptations of the model system, e.g. the use of another growth medium, impact that organization. In a next step, it can be investigated how alteration in antimicrobial susceptibility in a multispecies biofilm depends on the spatial organization, or vice versa. Furthermore, the inclusion of host factors into the model system should be taken into consideration. Thereby it is important to also include strains isolated from the human niche one wishes to investigate, as these strains have already been in contact with host factors and might have undergo adaptations. A better spatial and temporal understanding of interspecies interactions, and interactions with the host and the environment will lead to better management of human infections, not only focusing on antimicrobial treatment, but also focusing on altering the stability of mixed communities. [444]

New molecular techniques greatly enhanced our knowledge about multispecies communities. So far, most molecular studies focused on the identification of species not routinely isolated. However, the present and future challenge will be the determination of which species are responsible for the development of infection and how they respond to treatment. The amount of data obtained using molecular techniques will thus expand dramatically, therefore, an appropriate framework is required to analyze the complex interactions between micro-organisms, their environment, and the host. [549]

4.4.2 Recommendations for clinicians

In modern diagnostic microbiology, pure cultures of infectious agents are isolated using culture growth. Subsequently, antimicrobial therapy is directed towards any pathogen detected, after which individual samples are again collected and analyzed to confirm effective eradication. However, there are some major drawbacks [549]: (i) not all pathogens grow well on the culture media used, and are often overlooked using standard detection methods; (ii) sample collection currently used, e.g. swabs, often underestimate microbial diversity, e.g. using a swab in chronic wounds, *S. aureus* was detected, whereas *P. aeruginosa* was overlooked as it grew in the deeper regions [550]; (iii) as a result of the isolation method used, susceptibility testing is performed on pure cultures, however, *in vivo*, multiple species are present, and – as we and others have shown – diversity leads to the potential for

microbial interactions, which in turn plays a role in the behavior of each species present in the community, and in the relationship with the host and the respond to therapy. [549] Therefore, it is important to consider the susceptibility of the community as a whole and to take the presence of other micro-organisms into account to fully understand the impact of therapy. [549] In order to do this, much more research needs to focus on the development of model systems that can be used as 'a golden standard' to study interspecies interactions, and that can be easily implemented in the clinic. In addition, a number of other factors will also require consideration, including which clinical outcome to link with microbiological data (e.g. patient symptoms, radiographic scores, quality of life scores,…), and how to rank these clinical outcomes. [549, 551]

Chapter V: SUMMARY

One of the main causes of morbidity and mortality worldwide are bacterial infections, e.g. chronic wounds, respiratory infections in CF patients, and infections due to the use of medical devices. Often, these infections are due to the presence of biofilms. Consequently, there is only limited effectivity of available antimicrobial treatment, contributing to the persistence of biofilms and persistence of the infection. Furthermore, bacterial infections are often caused by multiple micro-organisms living together in a multispecies biofilm. As a result, antimicrobial susceptibility of co-infecting species can be altered in a multispecies biofilm compared to their susceptibility in a monospecies biofilm, leading to a decrease or an increase in resistance and a different disease progression compared to infections caused by monospecies biofilms. The main objective of this dissertation was to compare antimicrobial susceptibility of clinically important micro-organisms (*P. aeruginosa*, *S. aureus*, *S. anginosus*, and *C. albicans*) between growth in a mono- and multispecies biofilm.

In order to determine susceptibility of multispecies biofilms to antimicrobial agents, we first optimized an *in vitro* model system to allow growth of all species present. Supplementation of the medium with BSA allowed survival of *S. aureus* and *C. albicans* in presence of *P. aeruginosa*. This medium was then used to grow mature mono- and multispecies biofilms of *P. aeruginosa*, *S. aureus*, and *C. albicans*, hereby mimicking multispecies biofilms in the inanimate hospital environment (e.g. on surfaces and medical equipment). After treatment, using the European suspension test procedure, efficacy of several disinfectants towards multi- versus monospecies biofilms was quantified through selective plate count method. Our results suggested that the difference in susceptibility (either an increase, decrease, or no change) between a mono- and multispecies biofilm depended on the species and the disinfectant used and could not be generalized.

After the first part, secondly, we focused on species commonly co-isolated in sputum from CF patients. We evaluated antibiotic treatment of *P. aeruginosa*, in a mono- and multispecies biofilm with *S. aureus*, *S. anginosus*, and *B. cenocepacia*. The same medium as in the first part allowed us to reproducibly grow mature mono- and multispecies biofilms and to subsequently treat these biofilms for 24 h with colistin, tobramycin, or levofloxacin. In order to selectively quantify *P. aeruginosa* through a culture-independent method, we optimized, validated, and implemented a promising alternative quantification method, PMA-qPCR, enabling to take into account potential viable but not culturable bacteria. Through the prior treatment of the samples with PMA, we were able to distinguish live from dead cells and as a result, only live cells were quantified using qPCR. Furthermore, we observed differences in *P. aeruginosa* susceptibility in presence of the other species. However, differences depended on the antibiotic used.

Therefore, in the third part, we also evaluated the antimicrobial susceptibility of *S. aureus* and *S. anginosus* (an emerging CF pathogen causing acute exacerbations) in addition to that of *P. aeruginosa*, in biofilms comprised of these three bacteria. The efficacy of a range of antibiotics towards all species was determined, and species survival was quantified using selective plate counts for all three species. Again, only minor differences in antibiotic susceptibility of *P. aeruginosa* could be observed between growth in a mono- and multispecies biofilm. In contrast, *S. aureus* was more susceptible towards all antibiotics used when grown in a multispecies biofilm. For *S. anginosus*, the difference in susceptibility depended on the antibiotics used. A decrease in susceptibility could be observed after treatment with antibiotics that interfere with cell wall synthesis (e.g. vancomycin and imipenem), whereas no difference in susceptibility could be observed with antibiotics that interfere with other cellular processes (e.g. tobramycin and ciprofloxacin). Furthermore, we showed that *S. aureus* played a major role in this protective effect. In order to elucidate mechanisms responsible for the observed decreased susceptibility of *S. anginosus* to antibiotics that interfere with cell wall synthesis when grown in a multispecies biofilm, phenotypic and transcriptomic analyses were conducted. Our findings ruled out the involvement of altered *S. anginosus* growth rate or biofilm eDNA concentrations in mono- versus multispecies biofilms. In a next phase, we performed transcriptome analysis of *S. anginosus* in an untreated mono- and multispecies biofilm. Results showed that 285 genes (15.4%) were significantly up-regulated, and 103 genes (5.5%) were downregulated in *S. anginosus* when grown in a multispecies biofilm. Several genes reported to be upregulated in *S. aureus* after treatment with cell wall active antibiotics and to lead to resistance through an increase in cell wall thickness, were also found to be upregulated in *S. anginosus* upon growth in a multispecies biofilm, without any antibiotic treatment. In order to confirm the indication n that an alteration in cell wall could also play a role in the observed decrease in susceptibility of *S. anginosus* in a multispecies biofilm, we performed TEM to evaluate cell wall thickness of *S. anginosus* in a mono- and multispecies biofilm, untreated or treated with vancomycin. At the same time, cell wall thickness of *S. aureus* was also compared in these conditions. We could observe a thicker cell wall/fimbriae layer of *S. anginosus* when grown in an untreated multispecies biofilm compared to a monospecies biofilm. After treatment with vancomycin, the thicker cell wall/fimbriae layer could still be observed in the multispecies biofilm. These results indicate that *S. anginosus* alters its cell wall/fimbriae layer in the presence of *S aureus* and *P. aeruginosa*, which subsequently might play a role in the observed decreased susceptibility of *S. anginosus* towards cell wall active antibiotics in a multispecies biofilm. Furthermore, as expected, cell wall thickness of *S. aureus* in a monospecies biofilm increased upon exposure to vancomycin compared to an untreated monospecies biofilm. However, to our surprise, in a multispecies biofilm, *S. aureus* did not increase cell wall thickness upon exposure towards vancomycin, which could explain the observed increase in

susceptibility of *S. aureus* towards vancomycin in a multispecies biofilm. Furthermore, the effect in one micro-organism might be the opposite of the effect in another micro-organism, as shown for the alteration in cell wall thickness in *S. aureus* and *S. anginosus* in a multispecies biofilm.

To conclude, our data demonstrate that it is very hard to generalize how species in a multispecies biofilm will respond to antibiotic treatment. Whether they will become more or less susceptible, will depend on the community composition and on the antimicrobial treatment used. Hence, it is important to mimic the *in vivo* community composition as closely as possible when assessing the antibiotic susceptibility profiles of individual species. As a consequence, in the clinic, antibiotic susceptibility data obtained on single species will not necessarily be predictive of their susceptibility in the patient when multispecies communities are present. A clear and comprehensive view of which species are present and how they influence each other's antibiotic susceptibility will be necessary to help guiding the choice of treatment regimen.

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Tavernier S., Kart D., Van Acker H., Nelis HJ., Coenye T. 2014. Activity of disinfectants against multispecies bofilms formed by *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa*. Biofouling; 30(3):377-83.

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Tavernier S., Crabbé A., Tuysuz M., Stuer L., Henry S., Rigole P., Dhondt I., Coenye T. 2017. Community composition determines activity of antibiotics against multispecies biofilms. Antimicrobial agents and Chemotherapy. AAC.00302-17. doi: 10.1128/AAC.00302-17.

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CONFERENCES

2017	ASM Conference on Mechanisms of Interbacterial Cooperation And Competition, Washington, USA	poster
2016	Antimicrobial Resistance in Microbial Biofilms and Options for Treatment, Ghent, Belgium	poster
2016	39th European Cystic Fibrosis Conference, Basel, Switzerland	oral
2015	ESBG Eurobiofilm 2015, Brno, Czech Republic	oral
2015	Knowledge for Growth, Ghent, Belgium	poster
2014	1st ASM Conference on Polymicrobial Infections, Washington, USA	oral
2014	Summerschool 'Molecular and physiological regulation of medical and environmental microbial biofilms', Leuven, Belgium	oral
2013	ESBG Eurobiofilm 2013, Ghent, Belgium	oral
2012	Biofilms 5, Paris, France	poster

TRAININGS

Doctoral Schools UGent	Advanced Academic English, writing skills
	Grow your personal leadership – Grow your future career
	Effective presentation skills
	Communication skills
	Project Management
	Career training
	Clinical studies: study design, implementation and reporting
	P&G PhD seminar
Ghent University	"Crash course" in patents and IP