The microbiome and microbial interactions in *in vitro* and *in vivo* endotracheal tube biofilms

Het microbioom en microbiële interacties in *in vitro* en *in vivo* endotracheale tube biofilms

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1.1 General introduction

1.1.1 Microbial biofilms: let's stick together

1.1.1.1 Definition

Biofilms can be defined as organized, surface associated microbial consortia embedded in a (self-produced) extracellular matrix[1,2] and are considered the most common mode of growth among micro-organisms[1-3]. Growth in a biofilm is advantageous to the inhabitants, as the extracellular matrix forms a protective barrier form harmful factors such as antibiotics, shear stress and desiccation[1-3]. Furthermore, nutrients can be trapped and oxygen levels are variable, creating specific micro-environmental niches[1]. The biofilm consortium most often consists of multiple microbial species, which all communicate and interact with each other[4].

1.1.1.2 Biofilms develop in stages

Biofilm formation typically occurs in five stages: reversible attachment, irreversible attachment, accumulation, maturation and detachment[1,2,5]. The exact mechanisms involved in these stages vary among microbial species or the type of biofilm.

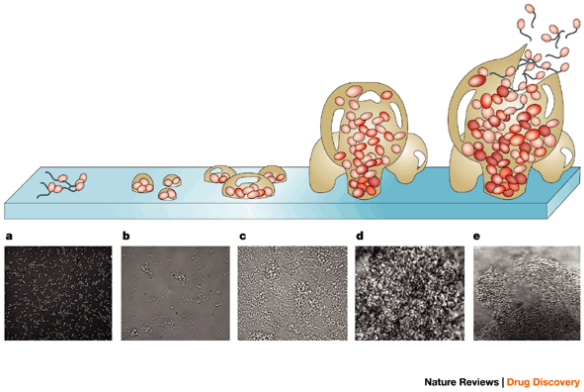

Figure 1: The stages of biofilm formation.

Reversible attachment

Biofilm formation starts with the reversible attachment of bacteria to a surface. Adhesive molecules on the bacterial cell wall are able to recognize certain surfaces and bind to specific sites, such as indwelling medical devices or industrial pipes[6]. In this stage the attachment is unstable and the cells can revert to the planktonic state or re-adhere until transcriptional changes are induced and they become irreversibly attached[2].

Irreversible attachment

Adhesion to a surface triggers a set of transcriptional changes and the bacterial cell enters the biofilm stage, which is phenotypically different from the planktonic mode of growth[2]. Binding to the surface becomes stronger and irreversible and the biofilm enters the next stage[2].

Accumulation

Irreversibly attached cells start to accumulate into a three-dimensional structure[2]. At this stage, cell-cell interactions become more important. For example, different bacterial species might recognize molecular factors of the initial colonizing species and join them in the biofilm[7]. Cells start to produce extracellular matrix consisting of polysaccharides, proteins, extracellular DNA, lipids, compounds from lysed cells, some species specific molecules and environmental factors[8]. This extracellular matrix forms a protective barrier for harmful environmental stimuli such as antibiotics and desiccation[8]. Furthermore, when the cell

density increases, small extracellular molecules, which are continuously excreted, accumulate as well and when they reach a certain threshold, other bacteria will be able to sense these molecules and adapt their expression levels accordingly[9]. This density-dependent communication is also known as quorum sensing and the excreted small molecules are called quorum sensing molecules[9]. The maturation of the biofilm is initiated.

Maturation

As the biofilm matures, a nutrient and oxygen gradient is created with the lowest amount in the deepest layers of the biofilm[10]. This micro-environment supports the growth of anaerobes and might lead to the development of persister cells[10]. Persister cells are in a dormant state and insensitive to oxygen and nutrient depletion or antibiotics that act on actively dividing cells[10]. When the inhabitants of a biofilm are eradicated by unfavorable conditions like nutrient loss or decontamination, persister cells can be re-activated when the situation is more favorable again, ensuring survival of the biofilm[10].

Detachment

In the final stage of biofilm formation a plateau of cell density is reached and space and nutrients become scarce[11]. At this point cells start to produce signals for dispersal, a process that is quorum sensing regulated[11]. Dispersed cells convert back to the planktonic mode of growth until they reach another (more favorable) surface to colonize[11].

1.1.2 Biofilms in the human body: the good, the bad and the recurrent

Bacterial biofilms exist in the environment as well as in the human body. One of the most famous examples of a biofilm in the human body is dental plaque, where a large variety of bacteria and fungi reside, some of which are responsible for oral diseases like dental caries or periodontitis[12]. Bacterial biofilms are also found the intestinal mucosa, playing a crucial role in digestion and offering protection from harmful organisms[13]. A particular problem is encountered when a biofilm exists on indwelling medical devices like catheters, prosthetic heart valves or artificial joints. Bacteria in the biofilm can cause chronic inflammation and dislodged pathogenic bacteria can access other parts of the human body and cause chronic and recurrent infections[14].

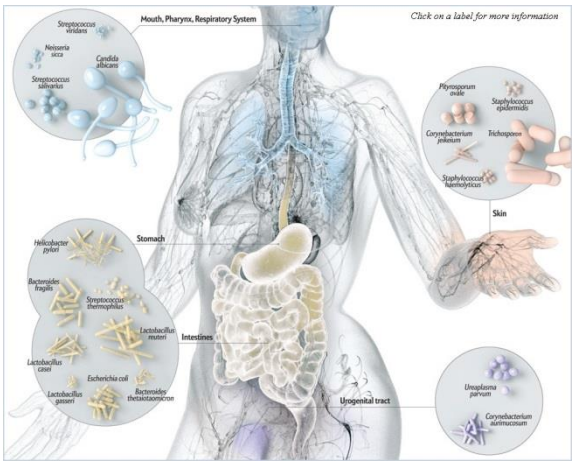

Figure 2: Bacteria in different parts of the human body.

Biofilms are a crucial part of the human body

The amount of bacterial cells in the human body is about ten times the amount of human cells and the human microbiome, the entire bacterial consortium in the human body, is often referred to as an invisible but crucial organ[15]. Most bacteria in the human microbiome exist as specialized multispecies biofilms, with different structures, functions and compositions. The composition of the microbiome is variable between individuals, body sites and even time, making it extremely difficult to identify a 'core human microbiome'[16-18]. Nevertheless, scientists worldwide are combining their knowledge to map the microbiomes of different human body sites and their function in the prestigious 'human microbiome project' (HMP)[17,18]. As a result, a core, healthy microbiome was identified at different body sites, some of which were even considered sterile for a long time[16,19].

The appreciation of the importance of the microbiome in vital physiological processes is rapidly increasing with the amount of information available. This is especially true for the gastro-intestinal tract, where bacteria are responsible for the production of vital vitamins, the metabolism of fat and other nutrients, competition with pathogens and even homeostasis of the immune system[15]. The latter is also modulated by the skin microbiome, which continuously interacts with keratinocytes and modulates an adequate innate immune response during injury[16,20]. Furthermore, the members of the commensal skin flora provide protection against pathogenic bacteria by competition[16]. Many more specialized microbiomes exist, each with their own niche, composition and function and many of which have yet to be discovered.

Most bacteria in the human body exist in a multispecies biofilm consisting of different bacteria and even fungi[21,22]. In this mode of growth, interspecies communication is crucial and often occurs by quorum sensing[23]. In addition, there is a growing body of evidence that the human microbiome communicates with the human host and even has a role in neurological and psychological development[15]. The exact mechanisms, however, remain unknown and future research is needed to further investigate this fascinating relationship.

Biofilm-related infections pose a serious healthcare threat

Despite the numerous benefits of the indigenous biofilms to the human body, biofilm-related infections pose a serious health threat. Various types of diseases are biofilm-related, including cystic fibrosis lung detoriation, chronic wounds and medical device related infections[14,24,25]. The latter is especially a problem in hospitals and intensive care units, where patients rely on many medical devices like central venous catheters, urinary tract catheters, parental feeding tubes and endotracheal tubes[6,26]. Biofilms are formed on the surface of these devices, protected from mechanical and biocidal stress[6]. When bacteria are dispersed from these biofilms, they have easy access to different body sites of the often immunocompromised patient where they may cause life-threatening infections[6]. Furthermore, pathogenic biofilms can form on human surfaces like the thick mucus of cystic fibrosis patients or a wound bed, where they cause chronic inflammation resulting in deterioration of the lung function or prevent wound healing[25,27]. Therefore, studying

(multispecies) biofilms and developing strategies to prevent their formation is crucial in the battle against (healthcare associated) infections.

All biofilms in the human body share one common property: they are nearly impossible to eradicate

Biofilms are among the earliest and most successful forms of live. Where individual, planktonic bacteria are vulnerable to all kinds of hostile factors, they are protected when they clump together as a biofilm[28]. One particular hostile factor is antibiotics, which sometimes easily eradicate bacteria in the planktonic mode of growth but are unable to kill all cells in a biofilm. This antibiotic-resistant phenotype can be caused by several mechanisms. First, the extracellular matrix provides a mechanical barrier inhibiting the penetration of antibodies or antibiotics[6,28]. Second, when the antibiotics are able to enter the biofilm, sensitive organisms might be protected by the antibiotic-modifying extracellular enzymes produced by their resistant co-inhabitants[6,28]. Third, as the biofilm matures, some cells shift to a dormant, viable but non-culturable state called persistence[6,28]. These persister cells are not dividing and resistant to bacteriostatic antibiotics. When the therapy is stopped, they can revert to an active state and re-infect the patient[28].

Besides protection against antimicrobial compounds, the biofilm matrix also provides structural stability in environments with high shear stress, like catheters, tooth surfaces or the intestine[8]. Complete removal of the biofilm from a certain surface can be extremely challenging and often requires a combination of antimicrobial compounds, mechanical forces and in some cases even matrix digesting compounds[25]. Furthermore, structural stability also protects against phagocytosis, either by immune cells or protozoan grazing[8]. The exact function and composition of the matrix is dependent on the environment and species present but certainly contributes to the survival of the biofilm inhabitants[8].

However, high stress resistance is not necessarily bad, as the commensal biofilms also are capable of surviving the shear stress, enzymes, changing conditions and immune system of the human body[15]. Moreover, resilience of the microbial ecosystem in ever changing conditions helps to keep a balance which is crucial for a good health, as colonization by pathogens and damage to the host is prevented. When this resilience is lost, changes in the environment might disturb the delicate balance and pathogens could take over the biofilm, a phenomenon called dysbiosis[22,29].

1.1.3 Ventilator associated pneumonia: a major cause of death

Hospital-acquired pneumonia is a serious threat for ICU patients all over the world and is the second most common hospital-acquired infection (the first being sepsis). Mortality rates are around 27% but can increase to 76% depending on the species involved and the underlying condition of the patient[30]. Hospital-acquired pneumonia is, in contrary to community-acquired pneumonia, often caused by opportunistic pathogens, that is, microorganisms that do not cause an infection in healthy people but can colonize and infect the critically ill. Mechanical ventilation of more than 48h increases the risk of developing pneumonia for up to twenty-fold, making mechanical ventilation the most important cause of hospital acquired pneumonia[31].

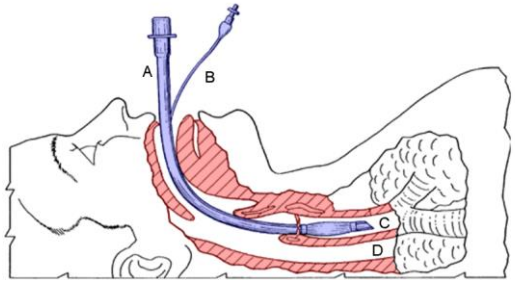

Figure 3: Intubated patient. A: endotracheal tube; B: cuff inflation tube with pilot balloon; C: trachea; D: aesophagus; E: cuff; Patient material and micro-organisms can easily accumulate in the lower part of the ETT around the cuff.

In addition to the underlying condition of the patient, the endotracheal tube (ETT) is believed to increase the risk to develop ventilator associated pneumonia (VAP), although the exact mechanisms are not yet understood[32]. As the ET tube passes through the respiratory system, it provides a bridge passing the natural defense mechanisms of the lungs, such as the anatomic airway barrier and the mucociliary clearance. Furthermore, bacterial growth on the ETT is detected within 24h of intubation and a biofilm is rapidly formed[32,33]. Micro-aspirates containing potentially harmful organisms might be detached from such biofilms and can cause infection in the lower respiratory tract[32]. Biofilm formation is believed to be facilitated by the presence of nutrient-rich patient material which accumulates in the lower part of the ETT, especially when the patient is in a horizontal position.

VAP can be caused by a large set of microorganisms, with *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* as the most important Gram-negative and *Staphylococcus aureus* as most important Gram-positive organisms. Furthermore, *S. epidermidis* was for a long time considered to be part of the non-pathogenic commensal flora but more recently has been isolated from, and thus associated with, a large number of nosocomial infections most of which associated with indwelling medical devices, including ETTs[34].

1.1.4 *Pseudomonas aeruginosa*: The perfect pathogen?

P. aeruginosa is a ubiquitous Gram-negative bacterium commonly found in soil, water and animals. Usually it does not cause problems in healthy individuals but it can become a dangerous pathogen when there is a breach in the natural barriers of the immune system. *P. aeruginosa* is a major cause of chronic infections in various wounds, like surgical wounds, burns and diabetic foot ulcers[35,36]. Furthermore, people suffering from the heritable disease cystic fibrosis all become colonized with *P. aeruginosa* at later age, leading to chronic respiratory inflammation and deterioration of the lung function[37]. Moreover, *P. aeruginosa* is a leading cause of death in hospital acquired infections, especially ventilator associated

pneumonia[38]. The success of *P. aeruginosa* as a pathogen can be explained by its high versatility and the capacity of rapid adaptation to changes in its environment. In addition, it possesses intrinsic resistance to many antibiotics and has the capacity to form strong, slimy biofilms, making it very difficult to treat infections caused by this pathogen[39].

1.1.4.1 P. aeruginosa biofilm formation

Everything comes down to good communication

P. aeruginosa is often used as a model organism for biofilm formation in Gram-negatives and therefore its biofilm formation is well-studied[39]. Quorum sensing is a sophisticated way of cell density dependent bacterial communication and plays a crucial role in *P. aeruginosa* biofilm formation. During quorum sensing, self-produced, low-molecular weight molecules (autoinducers) are excreted and their concentration increases with the cell density. When a threshold concentration is reached, the target genes are activated or repressed, creating a positive feedback loop[40,41]. A wide range of quorum sensing molecules exist and their production and effect is species or even strain dependent[41]. Cross-reactivity of these molecules produced by different species exists as some bacteria/individuales are able to sense and respond to molecules produced by other members of the same biofilm[42]. Moreover, in a population of quorum sensing producers, a subpopulation of non-producers might emerge that still benefits from the social goods but does not produce these costly molecules, a phenomenon called social cheating[43].

Until now, three main quorum sensing systems have been identified in *P. aeruginosa*: Las, Rhl and PQS[41]. The Las and Rhl system regulate together approximately 10% of the *P. aeruginosa* genome using N-Acylhomoserine lactones (AHLs), which are the most important quorum sensing molecules of *P. aeruginosa* and Gram-negatives in general as effector molecules[40,44]. Both systems consist of two components: an autoinducer synthase (LasI or RhlI) which induces the production of AHLs and a transcriptional regulator (LasR or RhlR) which binds to the respective quorum sensing molecules and activates the target signals when the threshold concentration is reached[9]. In *P. aeruginosa,* the Las system is dominant and regulates the Rhl System[44]. The Las system is responsible for the regulation of several *P. aeruginosa* virulence factors involved in acute infection and host cell damage, like elastase, extracellular toxins and proteases[44]. The Rhl system is involved in the secretion of rhamnolipids, biosurfactants capable of inducing biofilm dispersal in several bacteria and act as antimicrobials[44]. Furthermore, it represses the expression of the type 3 secretion system, a major virulence factor of *P. aeruginosa*. Both Las as Rhl contribute to maturation and sustaining of the biofilm. The third quorum sensing system, pseudomonas quinolone signal (PQS), is less well understood and uses many different types of quinolones as signal molecules, the most important being alkyl quinolones[45]. The PQS consists of several compounds, called the *pqsABCDE* operon and is involved in the response to iron starvation and bacterial interactions[40,44,46]. Furthermore, PQS is involved in the release of membrane vesicles, autolysis at high cell densities and the production of several virulence factors, including pyocyanin[40].

Bacterial communication is present in many species and often proofs to be involved in bacterial survival, competition and virulence. Disturbing bacterial communication can be a useful strategy to fight biofilm-related infections where traditional methods fail[47]. Indeed, the potential of quorum sensing inhibitors to prevent biofilm formation or enhance antimicrobial susceptibility is investigated for both clinical and environmental purposes[42]. There is, however, still a lot of work remaining before these compounds are ready to be used in the real world.

Exopolysaccharides provide structural support

Biofilm formation is partially dependent on the production and composition of extracellular matrix. The *P. aeruginosa* biofilm matrix contains, among other components, three important exopolysaccharides: Pel, Psl and alginate[48,49]. Strains may harbor only one or multiple polysaccharides and still produce biofilm as the functions are complementary[48,49]. The total biofilm structure is dependent on the combination of all three. The Pel and Psl polysaccharides are important in non-mucoid strains whereas alginate overproduction is characteristic of mucoid strains[48]. Psl is a galactose- and mannose-rich polysaccharide and has a major role in initial adherence[48,50]. In more mature biofilms it provides structural stability by cross-linking[49]. The Pel polysaccharide is a glucose-rich polymer responsible for initiating and maintaining cell-cell interactions and plays a role in later biofilm stages[51]. When both Pel and Psl are intact, Psl seems to be the dominant polysaccharide in the biofilm matrix. Where Psl favors stable and elastic biofilms, Pel is responsible for a visco-elastic and loose structure[49]. Alginate is the third exopolysaccharide and is often found in clinical isolates colonizing cystic fibrosis lungs[48]. Adaptation to the patient often involves the accumulation of many mutations leading to conversion to a mucoid phenotype due to alginate overproduction[52]. Alginate is involved in water retention and protects environmental biofilms against desiccation[48]. Furthermore, alginate overproduction creates a protective barrier for antibiotics and helps evading the immune system[48].

P. aeruginosa is a main player in biofilm-related infections

P. aeruginosa biofilms play a prominent role in various diseases, leading to highly resistant, recurrent and lethal infections like VAP and cystic fibrosis[38]. Furthermore, biofilms can cause problems in chronic wounds, like burn wounds, surgical site wounds, and diabetic foot ulcers[36]. The often mixed species biofilms reside in the wound bed protected from the host immune system and antibiotics, delaying the wound healing[53,54]. *P. aeruginosa* is a frequent inhabitant of such biofilms together with *Staphylococcus aureus* or coagulase negative *Staphylococci*[53]. Last, *P. aeruginosa* biofilms are a major challenge in medical device associated infections like VAP, as described above.

1.1.4.2 Several survival mechanisms explain the success of *P. aeruginosa* as a pathogen

P. aeruginosa possesses a high genomic and metabolic versatility

P. aeruginosa is a very successful nosocomial pathogen, as it is responsible for a wide range of hospital acquired infections[55]. The success of an organism or a certain clone is often the sum of several individual properties. *P. aeruginosa* is able to survive in many different and

harsh circumstances, like in the hospital setting, due to its high adaptability to changes in the environment[56,57]. One of the properties explaining this adaptability is the high plasticity of a considerably large genome (>6Mbp)[56]. The *P. aeruginosa* genome consists of a common 'core' which is conserved among isolates and an 'accessory' gene collection obtained from extrachromosomal elements and different among clones or isolates[56,57]. The accessory genome is constantly changing as new mobile genetic elements like plasmids, phages or transposons are taken up from the environment and incorporated into the genome when they provide an advantage, like antibiotic resistance. These elements might get lost again when there is a fitness cost and a selection pressure is absent. Furthermore, *P. aeruginosa* possesses high metabolic versatility due to the presence of numerous gene regulatory systems. These systems allow to sense the environment and to generate an appropriate response if necessary. *P. aeruginosa* is known to survive in several nutrient-poor conditions, like the surfaces of hospital equipment[58]. These kind of phenotypic changes are reversible as the conditions might also not be stable. During chronic infection, point mutations might accumulate and a stable, phenotypically different subpopulation might emerge[59]. Some strains have a defective DNA mismatch repair system and mutations will accumulate faster or recombination might occur more frequently[60]. These so called 'mutator' strains are often found in the clinical setting, especially in chronic infections[60,61]. The ability to rapidly generate potentially beneficial mutations might provide an advantage under antimicrobial or immune pressure and leads to the emergence of strains that are better adapted to the host[61]. The genomic and metabolic flexibility of *P. aeruginosa* contribute to the survival in unfavorable and stressful environments. However, these traits are not the only ones explaining the success of *P. aeruginosa*, as it has many more tricks in its' sleeve to avoid being killed. Moreover, it has developed many mechanisms to attack other bacteria or host cells, making it a feared pathogen in immunocompromised patients.

P. aeruginosa has a high frequency of antibiotic resistance

P. aeruginosa is different from most other Gram-negative bacteria due to a high intrinsic antibiotic resistance and the ability to develop additional acquired resistance to almost all antibiotics[44,62]. The intrinsic resistance of *P. aeruginosa* to many commonly used antibiotics is a combination of several factors. First, the outer cell membrane has a low permeability, making it more difficult for the antibiotics to accumulate to an active intracellular concentration[62,63]. Small hydrophilic molecules like β-lactam antibiotics and quinolones can only pass through aqueous porins and the diffusion of large molecules is prevented[63]. Since this barrier only slows down the effect of antibiotics, resistance is the result of several mechanisms combined. The intracellular accumulation of antibiotics is further limited by the presence of efflux pumps, which are capable of excluding all antibiotics except polymyxins[62,63]. Second, *P. aeruginosa* possesses mechanisms to modify and inactivate antibiotics. This is especially a problem for β-lactam antibiotics, which are rapidly inactivated by several classes of β-lactamases[63]. The addition of β-lactamase inhibitors like clavulanic acid might increase the susceptibility in some cases, but inhibitor-resistant enzymes are reported.

When antibiotic usage is high, *P. aeruginosa* can rapidly develop resistance to one or more antibiotics due to its flexible nature. Spontaneous mutations in antibiotic targets provide an

evolutionary advantage under antibiotic pressure as these mutants might be less susceptible [63]. Another way to acquire resistance is horizontal gene transfer of resistance elements originating from other bacteria. These genetic elements include plasmids, transposons, integrons and phages and can be transferred by conjugation, transduction or transformation[64]. *P. aeruginosa* might also transfer these resistance elements further on to other species[64]. Therefore overuse of antibiotics can rapidly give rise to the emergence of multidrug resistant isolates, not only of *P. aeruginosa* but also of other important pathogens like *Klebsiella pneumoniae* or *Escherichia coli*[65].

In addition, *P. aeruginosa* also possesses an inducible or adaptive response. Constant expression of antibiotic modifying enzymes or efflux pumps is costly for the cell and *P. aeruginosa* possesses a complex regulatory system to coordinate its metabolism. Sometimes resistance is only induced by environmental factors like the presence of the antibiotic or certain nutrients and therefore *in vitro* and *in vivo* susceptibility might be different[62]. Moreover, *P. aeruginosa* often exists as a biofilm *in vivo*, which provides a natural barrier against antibiotics as described earlier and leads to higher inhibitory concentrations of antibiotics as compared to *in vitro*. The high frequency of antibiotic resistance poses a serious challenge to treat *P. aeruginosa* infections. Early detection and appropriate antibiotic therapy are crucial to optimize the patient outcome. Moreover, the use of a wrong antibiotic or sub-inhibitory concentrations are even worse than no treatment at all, as the chance for the emergence of resistant clones increases and the chance of patient survival decreases, especially in chronic situations like cystic fibrosis[66].

1.1.5 Staphylococcus epidermidis: The commensal gone rogue

The Gram-positive *S. epidermidis* is the most common among the coagulase-negative *Staphylococci* and frequently found in the environment as well as on humans and animals[67-69]. It is considered part of the commensal human skin flora and harmless for immunocompetent people[67-69]. Moreover, it provides protection from colonization by the more virulent coagulase-positive *Staphylococcus aureus* by occupying the same niche[70]. In some cases, however, *S. epidermidis* can cause infection and forms a major healthcare-associated problem[68,69,71].

1.1.5.1 *S. epidermidis* as the 'accidental' pathogen

S. epidermidis is less virulent and invasive compared to its close relative *S. aureus*, which is a common cause of a large variety of infections[72]. As it is a common colonizer of the human skin and mucosa, it is well adapted to this hostile environment with large fluctuations in salt concentration and osmotic pressure[68]. Furthermore, *S. epidermidis* has evolved several mechanisms to prevent killing by the immune system, a trait that is necessary for its survival on the human host[69]. Although the relationship with the human host is usually benign, the balance can rapidly change when there is a breach in the immune system or epithelial barrier[67]. Moreover, *S. epidermidis* is the leading cause of indwelling medical device related infections in the ICU[34]. As *S. epidermidis* is present on the skin, it can easily be transferred to the medical device passing the first line of defense, the epithelial barrier[68]. *S. epidermidis* is well adapted to the commensal life in the human host and often misdiagnosed as

contamination in clinical samples. This in combination with the development of a highly diverse subpopulation makes it a dangerous pathogen in predisposed hosts.

S. epidermidis is a flexible survivor

Since *S. epidermidis* does not produce as many toxins or tissue-damaging factors as the pathogen *S. aureus*, its success as an opportunistic pathogen depends on other factors. Furthermore, many infections caused by *S. epidermidis* are subacute or chronic, indicating that it possesses sophisticated survival mechanisms rather than aggressive virulence factors[68]. Like *P. aeruginosa*, *S. epidermidis* is highly versatile capable of rapid adaptation to environmental changes[71,73]. The genome of *S. epidermidis* has high plasticity and contains many insertion sequences and mobile genetic elements[74]. The *Staphylococcal* chromosomal cassette (SCC) is a very important mobile molecular vector often associated with the methicillin resistance marker *mecA,* forming the SCCmec complex[71]. SCCmecs are frequently found in *Staphylococci* and carry several mobility genes allowing them to integrate into conserved regions of the *Staphylococcus* genome[73]. SCCmec is believed to be transferred form coagulase negative *Staphylococci* to *S. aureus* as methicillin resistance is extremely high in the first[73]. Furthermore, it has been shown that SCCmec elements from commensal *Staphylococci* originating from healthcare personnel were the same as in methicillin resistant *S. aureus* (MRSA) in the same hospital[71,74]. Therefore, *S. epidermidis* colonizers might serve as a reservoir of methicillin resistance for the far more dangerous *S. aureus*[74]. Furthermore, isolates with reduced susceptibility to vancomycin and linezolid are reported in the hospital setting[67]. Exposure of the commensal strains to antibiotics further selects for colonization by multidrug resistance strains and differentiation between infective and commensal strains remains challenging[67].

As a commensal organism, *S. epidermidis* needs to avoid being killed by the human immune system and developed several strategies. First, *S. epidermidis* developed the capacity to sense antimicrobial compounds produced by the host and to upregulate defense mechanisms[68]. Furthermore, *S. epidermidis* induces a mild inflammatory reaction compared to *S. aureus* and reduces the phagocytic activity of macrophages[69]. When it is taken up by a macrophage, it has the capacity to persist in a low metabolic state which is not killed by the oxidative burst and remains invisible for the immune system[69].

1.1.5.2 Biofilm formation of *S. epidermidis*

The ability to form biofilms is a key property for survival in the hospital environment and on the human host. Moreover, *S. epidermidis* biofilms are among the most common causes of medical device associated infections such as central venous catheter (CVCs) and prosthetic joint related infections[34]. When such devices are implanted, *S. epidermidis* originating from the skin of the patient, surgeon or nurse can enter the wound and form a biofilm on the implanted device[68]. *S. epidermidis* cells might bind directly to the synthetic surface of the medical device or on the human serum proteins that are coating this surface soon after insertion[34]. Adhesion to medical devices coated with human material is facilitated through Microbial Surface Compounds Recognizing Adhesive Matrix Molecules (MSCRAMMS) which have a high affinity for human plasma proteins like fibrinogen or fibronectin[75]. Once *S.*

epidermidis is attached to a synthetic surface, Polysaccharide Intercellular Adhesin (PIA) plays an important role in biofilm formation[76]. PIA or poly-N-acetylglucosamine (PNAG) is one of the key components of the extracellular matrix of *Staphylococcal* biofilms. After de-acetylation of the N-acetyl glucosamine residues of PIA, it becomes positively charged and serves as a glue sticking together the negatively charged *Staphylococci*.

Mature *Staphylococcus* biofilms are organized in a 3D-structure. As in *P. aeruginosa, S. epidermidis* biofilm formation and maturation is regulated by quorum sensing. However, the molecules involved in *Staphylococcal* communication are different. In *S. epidermidis,* auto-inducer 2 (AI-2) and phenol soluble modulins (PSM) are major regulators of biofilm formation[77]. AI-2 is sometimes referred to as the general language of bacteria since a wide range of Gram-positive and Gram-negative bacteria sense and respond to this molecule, even though they are non-producers[78]. In *S. epidermidis*, AI-2 stimulates the biofilm formation by enhancing the *ica* operon and *bhp*, a major biofilm protein[79]. PSM regulate biofilm detachment. Mutant strains with defective *agr* system, the major regulator of PSM, show very extensive biofilm formation, which could be an advantage in some types of infections.

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1.3 Aims

Pneumonia is a common complication in mechanically ventilated patients in intensive care units and is associated with high mortality and morbidity rates. The endotracheal tube (ETT) tends to be colonized with micro-organisms forming a biofilm within 24-48h of intubation. Biofilms on the ETT of mechanically ventilated patients are shown to increase the risk of developing pneumonia, also defined as ventilator associated pneumonia (VAP). Moreover, the microbial composition of these biofilms could increase the risk of developing VAP. The Gram-negative respiratory pathogen *Pseudomonas aeruginosa* is a common causative organism of VAP, often associated with high antibiotic resistance and mortality. In addition, *Staphylococcus epidermidis* is often isolated from ETTs and respiratory samples, and its involvement in pneumonia becomes more appreciated.

All above mentioned organisms are able to form biofilms and are frequently involved in biofilm-related infections. In previous studies it is shown that *P. aeruginosa* is able to inhibit *S. epidermidis* biofilm formation and to induce *S. epidermidis* biofilm dispersal in a strain dependent manner. *S. epidermidis*, on the other hand, is described to have very little effect on *P. aeruginosa* biofilm formation, although it could, along with other Gram-positives, increase the virulence of *P. aeruginosa,* resulting in worse patient outcome. Furthermore, in this thesis we identified an association of *S. epidermidis* with the Gram-negative pathogen *Klebsiella pneumoniae* in the ETT microbiome of mechanically ventilated patients that developed VAP. Very little is known about microbial interactions between *S. epidermidis* and *K. pneumoniae*. Since *S. epidermidis* is part of the normal commensal flora and shares the same habitat with (respiratory) pathogens, they are likely to interact, in either a positive or negative manner.

The aims of this thesis are:

1. To consolidate the available data on interactions between *P. aeruginosa* and *Staphylococci* and explore the link to clinical data. (Chapter 2)
2. To identify microbial interactions between clinical isolates of *P. aeruginosa* and *S. epidermidis,* including co-isolated strains. (Chapter 3)
3. To identify the microbiome present on the ETTs that are culture positive for *P. aeruginosa* or *S. epidermidis* and link these findings to patient parameters. (Chapter 4)
4. To identify microbial interactions between clinical isolates of *S. epidermidis* and *K. pneumoniae* during biofilm formation. (Chapter 5)

Chapter 2: *In vivo* and *in vitro* interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp.

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2.1 Abstract

The significance of polymicrobial infections is increasingly being recognized especially in a biofilm context wherein multiple bacterial species – including both potential pathogens and members of the commensal flora – communicate, cooperate and compete with each other. Two important bacterial pathogens that have developed a complex network of evasion, counter-inhibition and subjugation in their battle for space and nutrients are *Pseudomonas aeruginosa* and *Staphylococcus aureus.* Their strain- and environment-specific interactions, for instance in the cystic fibrosis lung or in wound infections, show severe competition that is generally linked to worse patient outcomes. For instance, the extracellular factors secreted by *P. aeruginosa* have been shown to subjugate *S. aureus* to persist as small colony variants. On the other hand, data also exist where *S. aureus* inhibits biofilm formation by *P. aeruginosa* but also protects the pathogen by inhibiting its phagocytosis. Interestingly, such interspecies interactions differ between the planktonic and biofilm phenotype, with the extracellular matrix components of the latter likely being a key, and largely underexplored, influence. This review attempts to understand the complex relationship between *P. aeruginosa* and *Staphylococcus* spp., focusing on *S. aureus*, that not only is interesting from the bacterial evolution point of view, but also has important consequences for our understanding of the disease pathogenesis for better patient management.

2.2 Introduction

Over the past decade there is a growing appreciation that the biofilm mode of growth is the most common lifestyle adopted by bacteria[1,2]. Biofilms can be defined as surface-associated, structured bacterial communities embedded in an extracellular matrix[2]. Living in a biofilm provides protection in a stressful environment where mechanical stress, desiccation and biocides are common threats[3,4]. Multiple species frequently exist together in a single biofilm, where they either improve the fitness of one another or compete for space and nutrients[1,5-7]. Most bacteria have developed interaction strategies to communicate within and between species in a cell density-dependent manner, for example, by using small diffusible molecules in a process called quorum-sensing[8,9]. Furthermore, many bacteria excrete antimicrobial components, also often regulated by quorum-sensing, to eliminate competitors[8,9]. Indeed, these multispecies interactions within the biofilm are important for the inhabiting bacteria and, given the increasing evidence of the link between biofilm-associated pathogens and disease, also from a clinical point of view[3,9].

S. aureus and *P. aeruginosa* are important pathogens causing a wide variety of infections, including pneumonia in cystic fibrosis (CF) patients, healthcare associated pneumonia and chronic wounds[10-12]. Initially, only an antagonistic relationship between both organisms was described as the presence of one is associated with the absence of the other in CF and both are rarely found in close association in chronic wounds. *S. aureus* mostly resides on the wound surface whereas *P. aeruginosa* is found in the deep layers[12,13]. We also recently showed a negative correlation between presence of *P. aeruginosa* and the total species diversity in *in vivo* endotracheal tube biofilms and a low co-occurrence of *P. aeruginosa* with *Staphylococcus epidermidis*[14]. Nonetheless, recent studies have also co-isolated *P. aeruginosa* and Gram-positive bacteria, including *S. aureus*, from the same infection site where increased virulence and/or antibiotic resistance is described[12,13,15-17]. After describing first *in vivo* observations occurring in human diseases, we will discuss and summarize *in vitro* data from the current literature on potential mechanisms of interactions between *P. aeruginosa* and *Staphylococcus* spp., primarily *S. aureus*.

2.3 Co-occurrence of *P. aeruginosa* and *S. aureus in vivo* is linked to worse disease outcomes

CF is a typical example of a biofilm-related infection wherein *P. aeruginosa* and *S. aureus* are frequently isolated from the lungs of these patients[10,18,19]. While, *S. aureus* is mostly acquired during childhood, the presence of *P. aeruginosa* is associated with increasing age and worsening patient prognosis[18-20]. An increasing incidence of *P. aeruginosa* with age has been shown to coincide with a decreasing *S. aureus* incidence in CF patients[10,18], data that primarily indicates an antagonistic relationship between the two pathogens. However, in cases where *P. aeruginosa* and *S. aureus* have been co-isolated, both pathogens seem to contribute independently and additively to the disease severity[19,20], presenting as increased lung inflammation and consequently increased lung damage compared to infection with a single pathogen[20]. Furthermore, due to repeated antibiotic therapy, CF patients also carry higher levels of methicillin-resistant *S. aureus* (MRSA) that is associated with a worse lung

function compared to methicillin- sensitive *S. aureus* (MSSA) but only in combination with *P. aeruginosa*[21].

Chronic wounds are another example of biofilm-related infections wherein co-presence of *P. aeruginosa* and *S. aureus* has been shown to result in delayed wound healing compared to single species infections[17,22,23]. In a pig wound model, infections initiated by *in vitro* preformed dual species biofilm caused a significant suppression of keratinocyte growth factor 1 (KGF1), which is responsible for re-epithelialization and wound closure[22]. In a rabbit ear-wound model, mixed species infection of *S. aureus* and *P. aeruginosa* caused an increased expression of the pro-inflammatory cytokines IL-1β and TNF-α, indicating a higher inflammatory response compared to single species infection[23]. Moreover, *S. aureus* and *P. aeruginosa* reached an equilibrium after 12 days of infection, with *P. aeruginosa* being the dominant pathogen[23]. In a mouse chronic wound model infected with *in vitro* preformed four-species biofilm and monitored up to 12 days, presence of multiple species was found to significantly delay wound healing only at 8 days post-infection[17]. However, polymicrobial infections showed increased antimicrobial tolerance compared to single species infection with *P. aeruginosa* in this study[17]. These studies suggest that, despite the constraints of different host backgrounds, multispecies infections can lead to delayed wound healing, increased inflammation and increased antibiotic tolerance, which all add to a worse patient outcome. *P. aeruginosa* is often the dominant pathogen due to its wide array of mechanisms to adapt to changing hostile environments, which allows colonization in a variety of niches. When *P. aeruginosa* encounters other bacteria like *S. aureus*, it can co-exist or take over the biofilm through production of various quorum-sensing regulated factors. Section 3 discusses *in vivo* animal studies exploring production of *P. aeruginosa* virulence factors in the presence of *S. aureus*.

2.4 Extracellular products of *Staphylococcus spp*. impact *P. aeruginosa* virulence *in vivo*

P. aeruginosa possesses a wide range of extracellular factors to survive and invade human tissues, often by modulating the immune system. The complex interplay between biofilms and the host immune response are reviewed in detail by Watters et al.[24]. Here, we discuss four molecules in particular which are upregulated in the presence of Gram-positive bacteria: LasB elastase, rhamnolipids, exotoxins and phenazines (**Figure 1**). LasB elastase is an extracellular protease capable of digesting the lung surfactant, the pulmonary antimicrobial enzyme lysozyme and transferrin, as well as slowing down the ciliary movement[25]. In addition, LasB impairs uptake of *P. aeruginosa* by macrophages and its protease activity leads to lung tissue damage, thereby decreasing pulmonary function and facilitating dissemination into the bloodstream[26]. Similar to LasB, rhamnolipids are glycolipidic biosurfactants that interfere with the lung surfactant activity by solubilizing the phospholipids and with airway immune response by disrupting the polymorphonuclear leucocyte chemotaxis and macrophage function, and also inhibit ciliary beating[27,28]. Furthermore, rhamnolipids increase inflammation by stimulating the release of the pro-inflammatory cytokines IL-6 and IL-8 by the airway epithelium[27]. An excessive inflammatory response and associated tissue damage is also induced by the release of exotoxins ExoT, ExoS and ExoY by the type III secretion system. The type III secretion system is a needle-like structure directly injecting exotoxins into other

bacteria, macrophages and epithelial cells, thereby killing them[25]. The fourth type of molecules, phenazines, are pigments produced by a large number of *Pseudomonas* spp., and have been shown to be involved in mediating microbial interactions as well as in CF disease progression. Pyocyanin is the most important phenazine and its production in the CF lung was shown to lead to goblet cell hyperplasia, airway fibrosis and alveolar airspace destruction[26,29]. Pyocyanin causes an imbalance between the T helper type 1 (Th1) and type 2 (Th2) cytokines, leading to overproduction of Th2 cytokines IL-4 and IL-13 and increased macrophage infiltration[29]. Essentially, release of these extracellular molecules by *P. aeruginosa*, partly in response to the presence of *S. aureus*, leads to increased tissue damage due to their cytotoxic and immune-modulatory effects, which also helps *P. aeruginosa* survival. Interestingly, not only *S. aureus* but Gram-positive commensals (coagulase negative staphylococci and viridans streptococci) can also alter the virulence of *P. aeruginosa* in a similar fashion[16]. Such immune modulation and evasion by collective bacterial species might underlie the worsened patient prognosis observed in multispecies infections (**Figure 1**). Sections 3.1 and 3.2 discuss the known mechanisms of how extracellular products of staphylococci modulate the four major virulence factors *P. aeruginosa* that were discussed above.

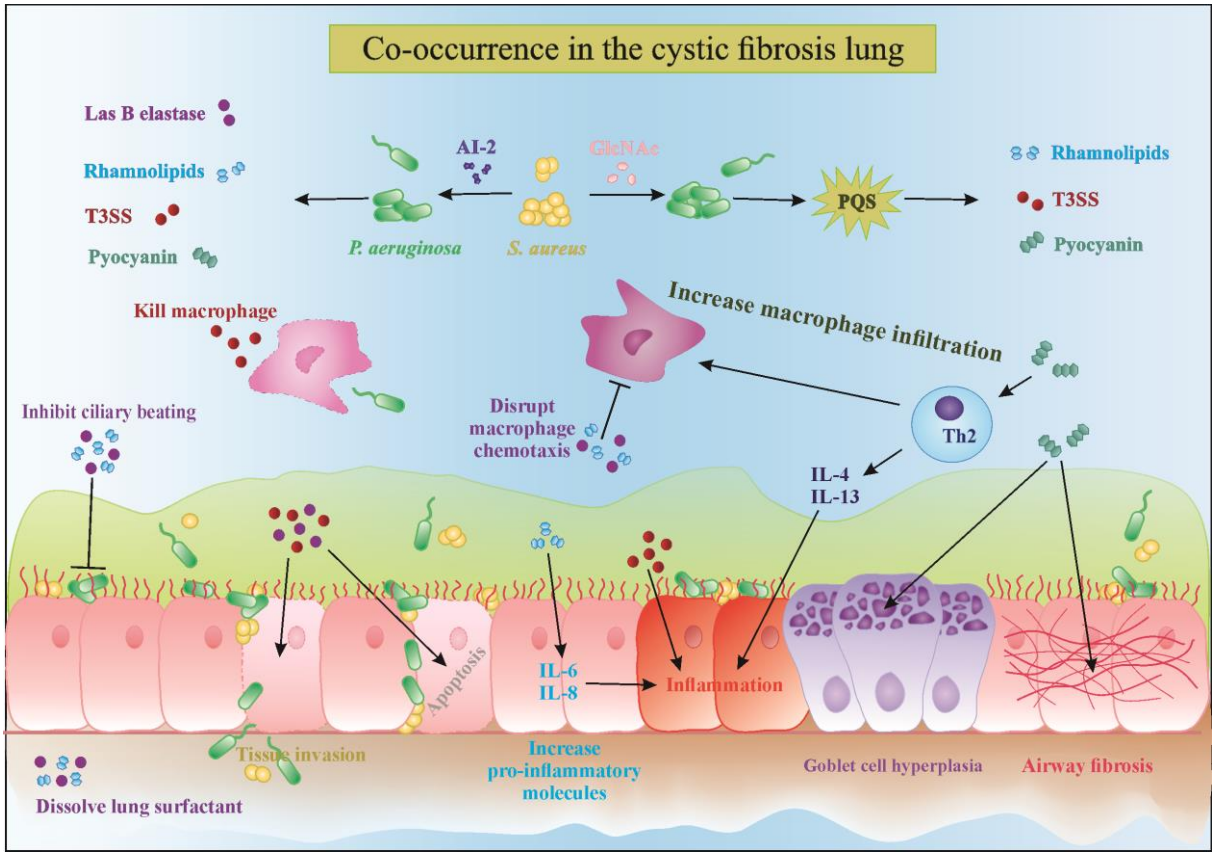

Figure 1: Interactions in the cystic fibrosis lung. The complex interplay between microbial competition and the human immune system results in increased secretion of pro-inflammatory cytokines, microbial virulence factors and consequently tissue damage and bacterial invasion of the epithelial barrier. Mixed species infections lead to a stronger decrease in ciliary beating and increased goblet cell hyperplasia and tissue fibrosis, which are characteristic of cystic fibrosis disease progression. PQS: Pseudomonas quinolone signal; T3SS: type 3 secretion system; AI-2: autoinducer 2.

2.4.1 AI-2 in the CF lung increases *P. aeruginosa* virulence and might be an important therapeutic target

Autoinducer-2 (AI-2) is a small diffusible quorum-sensing molecule produced by several bacteria, including staphylococci, and has been shown to cause upregulation of several major virulence genes of *P. aeruginosa* discussed above, including extracellular protease (*lasB*), rhamnosyltransferase involved in rhamnolipid synthesis (*rhlA*), exotoxins (*exoT, exoS, exoY*) and phenazines (*phzA1* and *phzA2*)[30]. Induction of *P. aeruginosa* virulence by AI-2 was shown both *in vitro* after screening of a random *lux* reporter-based promotor library and *in vivo* in rat lung infection and *Drosophila* chronic infection models[16,30,31]. AI-2 mediated quorum-sensing is now recognized as a universal language of interspecies communication regulating a wide variety of genes involved in virulence and biofilm formation in a cell-density dependent manner in a number of micro-organisms, including non-producers like *P. aeruginosa*[32]. Furthermore, AI-2 has been detected in substantial amounts in the sputum of CF patients and in infected rats[16], raising the possibility of interruption of AI-2 signalling to either slow down disease progression or hasten the healing process.

A promising approach is the use of AI-2 analogues like D-ribose that block the AI-2 pathway and inhibit *P. aeruginosa* virulence. Wang et al showed in a rat model of mechanical ventilation that co-inoculation of *P. aeruginosa* and *Streptococcus mitis* resulted in increased biomass, lung damage and rat mortality compared to infection with only *P. aeruginosa*[33]. Treatment with D-ribose of both the single and dual species infections showed a significant decrease in biomass and lowering of rat mortality in the latter group due to interference with AI-2 signalling[33]. Inhibition of *P. aeruginosa* virulence is not only beneficial because the *P. aeruginosa*-mediated damage is reduced but also because the immune system is less stimulated (**Figure 1**). Further studies showing the benefits of non-toxic biofilm inhibitors such as D-ribose in patient populations are awaited.

2.4.2 N-acetyl glucosamine sensing enhances the production of *P. aeruginosa* extracellular virulence factors

Another molecule that increases the virulence of *P. aeruginosa* and is commonly found in the CF lung is N-acetyl glucosamine (GlcNAc). GlcNAc is part of the Gram-positive cell wall polymer peptidoglycan and induces the virulence of *P. aeruginosa* by enhancing the *Pseudomonas* quinolone signal (PQS), which controls the production of extracellular virulence factors like pyocyanin, elastase, rhamnolipids and HQNO (discussed in section 4.1)[15,34-36] (**Figures 1 and 2**). The PQS, with 2-heptyl-3-hydroxy-4-quilonone as the main effector molecule, is one of the three quorum-sensing systems present in *P. aeruginosa*. PQS is positively regulated by LasR and negatively regulated by RhlR, the two other quorum-sensing systems of *P. aeruginosa* with N-acylhomoserine lactone as main effector molecule[35]. *P. aeruginosa* has the ability to sense the peptidoglycan shed by the Gram-positive commensal flora and in response increase the production of antimicrobials. The enhanced virulence in the presence of GlcNAc from Gram-positive bacteria was demonstrated *in vivo* in a *Drosophila* and *Galleria mellonella* infection model[15,37]. Both GlcNAc and AI-2 sensing are examples where *P. aeruginosa* can sense its environment and generate the appropriate response to

eliminate competitors by producing several virulence factors, which also has a negative impact on the host.

2.5 *P. aeruginosa* produces a wide variety of molecules that inhibit *S. aureus in vitro*

P. aeruginosa produces many molecules to compete with other microorganisms for space and nutrients. The number of molecules, quantities produced and even the structure of these molecules vary between different strains of *P. aeruginosa* as well as between different growth conditions (planktonic vs biofilm; the presence of host factors, antibiotics etc.). In order to achieve a better understanding of complex interplay of the different compounds in *in vivo* biofilm-related infections, it is necessary to dissect this complex system into individual subsystems and investigate each compound individually. In the following sections, we discuss the effect of different molecules produced by *P. aeruginosa* on *S. aureus in vitro*. An overview of the extracellular molecules produced by *P. aeruginosa* and their effect on *S. aureus* is shown in **Figure 2**.

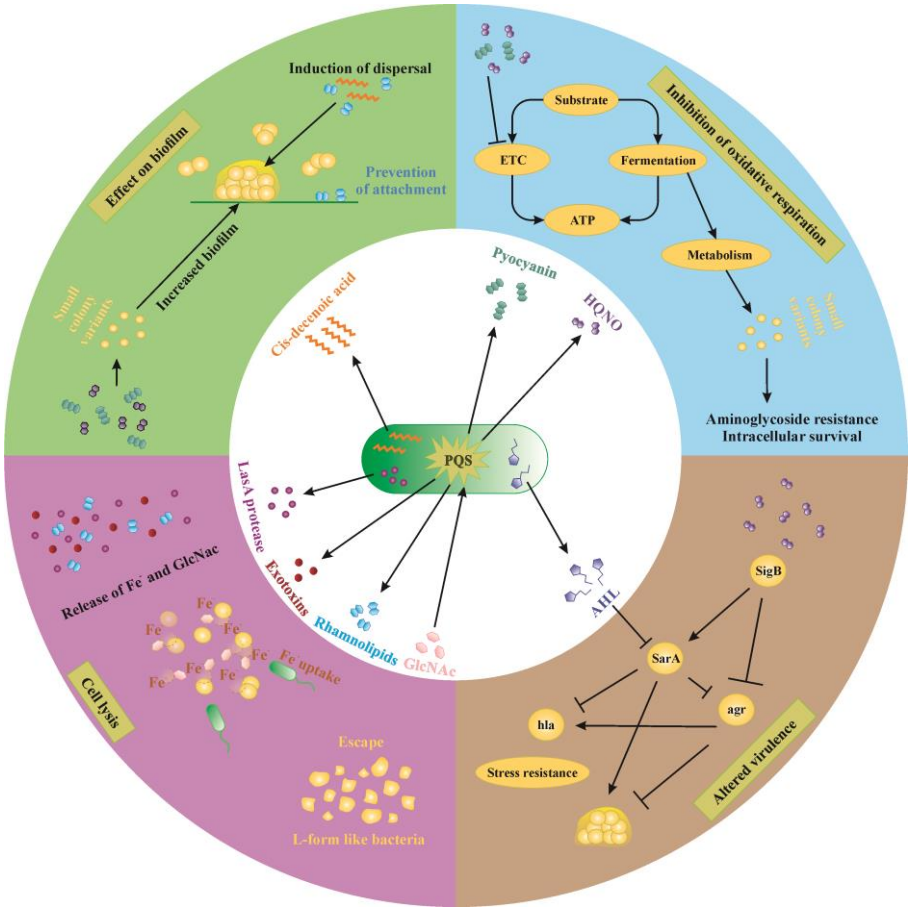

Figure 2: Small molecules secreted by *P. aeruginosa* and *S. aureus*. Extracellular factors produced by *P. aeruginosa* affect biofilm formation, oxidative respiration, cell lysis and virulence of *S. aureus*. Lysis of *S. aureus* leads to increased extracellular iron and N-acetyl glucosamine (GlcNac), which are sensed by *P. aeruginosa*. AHL: N-acyl homoserine lactone; HQNO: 4-hydroxy-2-heptylquinoline N-oxide; PQS: Pseudomonas quinolone signal; GlcNac: N-acetyl glucosamine.

2.5.1 *Pseudomonas* quinolone signal regulates the production of anti-staphylococcal 4-hydroxy-2-heptylquinoline N-oxide (HQNO)

P. aeruginosa strongly reduces or completely outcompetes *S. aureus* during co-culture in many *in vitro* model systems, both planktonic and biofilm[6,38,39]. This anti-staphylococcal activity of *P. aeruginosa* was first described in 1956 by Lightbown et al., who identified 4-hydroxy-2-heptylquinoline N-oxide (HQNO) as a major compound produced by *P. aeruginosa* that inhibited the cytochrome systems of some bacteria, including *S. aureus*[40]. The same phenomenon was again described by Machan et al. in 1991 by testing the culture supernatant of fifty *P. aeruginosa* clinical isolates on 261 staphylococci[41]. The growth of all staphylococci was reduced by each of the *P. aeruginosa* strains, although the extent of inhibition was strain-dependent. The factor responsible for this phenomenon was again identified as HQNO[42]. HQNO is the major compound produced by the *pqsABCDE* operon, which is regulated by the quorum-sensing system PQS. Although HQNO is described as an antistaphylococcal compound, it has no lytic activity against *S. aureus* itself but rather slows down the growth by inhibiting oxidative respiration (**Figure 2, right upper panel**)[36]. Exposure to an HQNO source suppresses the growth of *S. aureus,* resulting in small colonies which are easily missed in diagnostic cultures[43]. These so-called small colony variants represent a different phenotype with specific characteristics and will be discussed later in this review. Furthermore, HQNO can be detected at active concentrations in the sputum of CF patients infected with *P. aeruginosa*, suggesting that HQNO has the same effect in the lungs of CF patients as it has *in vitro*[43]. Although HQNO is one of the most important and well-studied antistaphylococcal compounds, it is not the only factor slowing down the growth and inhibiting oxidative respiration in staphylococci.

2.5.2 Pyocyanin inhibits oxidative respiration in *S. aureus*

Pyocyanin is one of the numerous pigmented phenazines produced by *P. aeruginosa* and an important virulence factor. Pyocyanin is produced during *Pseudomonas* biofilm formation, has a role in acute and chronic airway infections, enables anaerobic survival and serves as a redox-active antimicrobial compound[29,44]. Furthermore, by its inter-and intracellular signaling, pyocyanin enables *P. aeruginosa* to successfully compete with other bacteria and even fungi[45-47]. Like HQNO, pyocyanin also blocks the oxidative respiration and inhibits growth of *S. aureus,* also selecting for the small colony variant phenotype[44] (**Figure 2, right upper panel**). The production of pyocyanin can be observed after 8h of culture, around the same time when a strong reduction of *S. aureus* cells occurs during co-culture[44,45]. Furthermore, the presence of Gram-positive organisms, including some *Staphylococcus spp*., can induce pyocyanin production in *P. aeruginosa* by stimulating the PQS system[37,48] (**Figure 2, middle panel**). In addition, exposure to pyocyanin in the airways leads to pulmonary damage and contributes to CF pathogenesis[29]. Therefore, pyocyanin seems to be an antagonistic compound secreted to provide a competitive advantage to *P. aeruginosa* by harming *S. aureus,* other Gram-positive bacteria as well as the host.

2.5.3 LasA protease or staphylolysin effectively lyses *S. aureus* cells

P. aeruginosa secretes a staphylolytic endopeptidase called LasA protease or staphylolysin, which degrading pentaglycine in the cell wall of *S. aureus* causing cell lysis[49]. *P. aeruginosa* might use LasA protease to compete with staphylococci but also to acquire iron from *S. aureus* [50] (**Figure 2, left lower panel**). Because freely available iron is often limited, *P. aeruginosa* has developed several strategies to scavenge iron, like the synthesis of iron chelating siderophores, pyoverdin and pyochelin[51]. Transcription patterns of iron-regulated genes of *P. aeruginosa* in the presence of *S. aureus in vivo* are the same as in high-iron conditions *in vitro*, suggesting that *S. aureus* might be an iron source for *P. aeruginosa*[50]. This type of interaction is, however, only important when both species are located close together, like in multi-species biofilms[50]. The specificity of the LasA protease for staphylococci makes it a potential therapeutic candidate against staphylococcal infections especially those cause by antibiotic resistant MRSA strains as shown in a rat model of endophthalmitis[52]. However, *S. aureus* can survive LasA by the emergence of L-form-like colonies, which lack a cell wall[53], although the role of L-form like colonies in disease remains rather vague.

2.5.4 Cis-2-decenoic acid induces biofilm dispersal in a broad range of organisms including *P. aeruginosa*

Interspecies competition in biofilms not only occurs by inhibiting or killing the other species but also by inducing its dispersal. Biofilm dispersal is mainly induced when the environment becomes less favourable, like in case of nutrient depletion, and is extensively reviewed by Petrova et al.[54]. The exact mechanisms that induce biofilm dispersal are currently unknown, although several factors have been investigated[55,56]. Since most bacteria reside in a biofilm consisting of multiple species *in vivo*, the dispersal signal must be recognized by a wide range of species[55]. One class of such molecules are the *cis*-monosaturated fatty acids, which are small extracellular messenger molecules with broad inter-phylum and even inter-kingdom activities[55]. *P. aeruginosa* produces *cis*-2-decenoic acid, which induces a dispersion response in biofilms formed by a range of Gram-negative and Gram-positive bacteria, including *S. aureus* (**Figure 2, left upper panel**), yeast as well as in *P. aeruginosa*[55]. Interestingly, Davies et al showed that dispersion was only induced when the microcolonies reached a minimum of 40µm diameter and 10µm of thickness, indicating that a certain threshold concentration is needed for *cis*-2-decenoic acid to become active[55]. This molecule could possibly be employed to disrupt biofilms on surfaces, followed by disinfectants that can successfully clear planktonic bacteria.

2.5.5 Rhamnolipids promote biofilm dispersal and inhibit adhesion

Most *P. aeruginosa* strains produce rhamnolipids, biosurfactants consisting of one or two rhamnose molecules linked to one or two fatty acids[27]. While *cis*-2-decenoic acid is mainly used as a common signal for dispersion at the final biofilm stages, rhamnolipids are used to dislodge competing bacteria from the biofilm. Many different rhamnolipid homologues are produced, depending on the *Pseudomonas* strain and carbon source, and their synthesis is quorum-sensing regulated[27]. Rhamnolipids were shown to reduce the surface tension and to have an anti-adhesive and antimicrobial effect on many micro-organisms[57-59]. The

amphiphilic nature of rhamnolipids enables them to intercalate into the cell membranes of different microorganisms and form complexes, thereby permeabilizing the membranes and causing leakage of intracellular material[60]. Gram-positive organisms seem more susceptible to rhamnolipid permeabilization than Gram-negative because the presence of lipopolysaccharides protects the cell membranes of the latter against the effect of surfactants[27]. Furthermore, rhamnolipids were shown to promote biofilm dispersal in many different microorganisms, including *P. aeruginosa* itself, although this effect is strain dependent[58]. For *S. aureus* and *S. epidermidis*, rhamnolipids were shown to induce biofilm dispersal and to inhibit adhesion in a dose-dependent manner[58,59,61] (**Figure 2, left upper panel**).

2.5.6 Long-chain AHLs reduce growth and virulence of *S. aureus*

The N-acylhomoserine lactone (AHL) system is the most important and most extensively studied quorum-sensing system in *P. aeruginosa*. Many diverse AHLs are produced by various Gram- negative bacteria, all consisting of a homoserine lactone ring that is N-acylated with a fatty acyl group[35]. The length of the acyl chains may vary from 4 to 18 carbons and *P. aeruginosa* mainly produces a short-chain C4-HSL and a long-chain 3-oxo-C12-HSL, although other lengths might also occur[35]. The production of many virulence factors is regulated by AHL, including that of pyocyanin and rhamnolipids[35]. Although it is currently not described that Gram-positive bacteria produce AHLs, they might still be influenced by them[62]. For example, growth of *S. aureus* is inhibited by several long chain 3-oxo-AHLs (including C8, C10, C12 and C14 chains) in a concentration dependent manner, with C12 and C14 being the most effective[62]. At concentrations below growth inhibition, the function of staphylococcal accessory regulator *sarA* and accessory gene regulator *agr* are strongly reduced (see section 5.1), and consequently their dependent virulence factors like hemolysins, TSST-1, protein A and fibronectin-binding proteins (**Figure 2, right lower panel**)[62]. Moreover, inhibition of *agr* might lead to more biofilm formation in *S. aureus* due to reduced detachment, although this study only tested planktonic conditions and requires further research[62]. The short chain AHL produced by *P. aeruginosa* seems to have no effect on growth and *agr* expression of *S. aureus* [62]. Furthermore, long-chain AHLs produced by other Gram-negative bacteria are likely to have similar effects in *S. aureus,* although, again, more studies are required here.

2.5.7 *P. aeruginosa* might also cause increased expression of *S. aureus* virulence factors

As described before, wounds infected with both *S. aureus* and *P. aeruginosa* generally show delayed closure compared to the single species infected wounds[17,23]. In addition to host related factors, one of the reasons for this phenomenon might be the upregulation of *S. aureus* virulence factors during co-infection, as was demonstrated for the MRSA strain USA300[22]. Interestingly, although the growth of USA300 was strongly inhibited by *P. aeruginosa in vitro*, this effect was much weaker *in vivo*[22]. Furthermore, co-infection of wounds in a pig model induced *S. aureus* virulence factors *hla* and *pvl*, encoding α-hemolysin and Panton-Valentine leucocidin (**Figure 2, right lower panel**)[22]. Another example of increased virulence of *S. aureus* in the presence of *P. aeruginosa* is the induction of staphyloxanthin production observed in a white *S. aureus variant* isolated from a soft tissue wound[63]. This strain

possessed an intact and functional *crtOPQMN* operon, which is essential for production of the staphyloxanthin pigment, but was unable to induce pigment production on its own. Interestingly, staphyloxanthin production was induced by a *P. aeruginosa* co-isolate[63]. Furthermore, the pigment production in a characteristically golden-yellow *S. aureus* strain, which was co-isolated with the *Pseudomonas* strain and the white *S. aureus* variant, remained unaffected. However, this contradicts other studies that report an inhibition of *S. aureus* pigment production by pyocyanin and pyoverdin produced by *P. aeruginosa*[44]. Another result of this study that is in contradiction with other studies is that there was an unchanged expression of *sigB* that encodes the alternative transcription factor sigma B and which is previously reported to be upregulated in the presence of *P. aeruginosa*[64]. The discrepancies in results between different studies indicate the importance of co-evolution and adaptation of the different isolates to each other and their environment. Adaptation of *S. aureus* to *P. aeruginosa* might lead to an expression pattern that is similar to a stress-resistant phenotype.

2.6 *S. aureus* survives in the presence of *P. aeruginosa* as the small colony variant phenotype

As a defence mechanism, *S. aureus* has also devised strategies to survive in the presence of *P. aeruginosa*. One of these is the switch to the small colony variant (SCV), a well-characterized phenotype detected in various diseases, including CF and device-related infections[65]. SCVs appear as small, smooth colonies on a culture plate and grow significantly slower compared to wild type colonies. The SCV phenotype might appear naturally and is caused by a defective or inhibited electron transport pathway that switches *S. aureus* to a fermentative growth state. In addition to a decreased growth rate, SCVs also demonstrate decreased ATP yield, decreased pigmentation and often hemin or menadione auxotrophy[44,65]. Remarkably, the switch to a SCV phenotype increases survival of S. aureus in unfavorable conditions as it exhibits an increased aminoglycoside resistance, biofilm formation and intracellular survival[43,44,65,66]. Prolonged co-culture with *P. aeruginosa* or exposure to pure HQNO leads to a high proportion of stable *S. aureus* SCVs, an effect that is increased by the presence of aminoglycosides[43]. It has also been proposed that the reason why *S. aureus* and *P. aeruginosa* are not frequently detected together in diagnostic cultures of sputum of CF patients is because of the existence of *S. aureus* as SCVs that are more difficult to detect due to their small size and fastidious growth requirements[65,66].

2.6.1 SCV induction by *P. aeruginosa* is sigma B dependent

After the induction of the SCV phenotype during exposure to HQNO, the expression of three main regulatory mechanisms of virulence and biofilm formation is altered. First, the alternative transcription factor sigma B (SigB) is upregulated[64]. SigB regulates the general stress response of Gram-positive bacteria, repressing the expression of most exoenzymes and toxins, stimulating the expression of adhesins and promoting the persistence of *S. aureus* in host cells[66,67]. Second, stimulation of SigB was shown to stimulate the expression of the Staphylococcal accessory regulator SarA, which modulates the expression of the pore-forming toxin α-hemolysin (*hla*) and increases biofilm formation[64,68,69]. In addition, SigB represses a third important regulator of *S. aureus* biofilm formation, the accessory gene regulator (*agr*) system, which induces biofilm dispersal thereby decreasing the total biomass

and increases the expression of *hla*[64,66,70]. In conclusion, HQNO reduces the production of the toxin *hla* by increasing the expression of SigB, leading to an increased expression of SarA and a decreased expression of *agr*. Reduced expression of toxins helps *S. aureus* to remain intracellular and thus increase its chances of survival in the human host. The stimulation of sarA by upregulation of SigB might be counteracted by long chain AHLs, which inhibit sarA (**Figure 2, right lower panel**). The net effect is probably dependent on the *Pseudomonas* strain involved since the production of both factors might be variable between isolates[62,71].

2.7 *P. aeruginosa* and *S. aureus* in dual species biofilms

During dual species biofilm formation, the balance of attacking, evading and counter-attacking is even more important and the properties of each strain as well as some environmental factors will determine if a dual species biofilm will be formed. For example, the presence of environmental selection pressure, like antibiotics or the host immune system, stimulates a more synergistic relationship and biofilm formation as the tolerance of *S. aureus* to antibiotics is significantly higher during co-culture with *P. aeruginosa*[6,72]. In the following paragraphs we describe how certain extracellular factors influence the structure, characteristics and composition of the dual species biofilms (**Figure 3**).

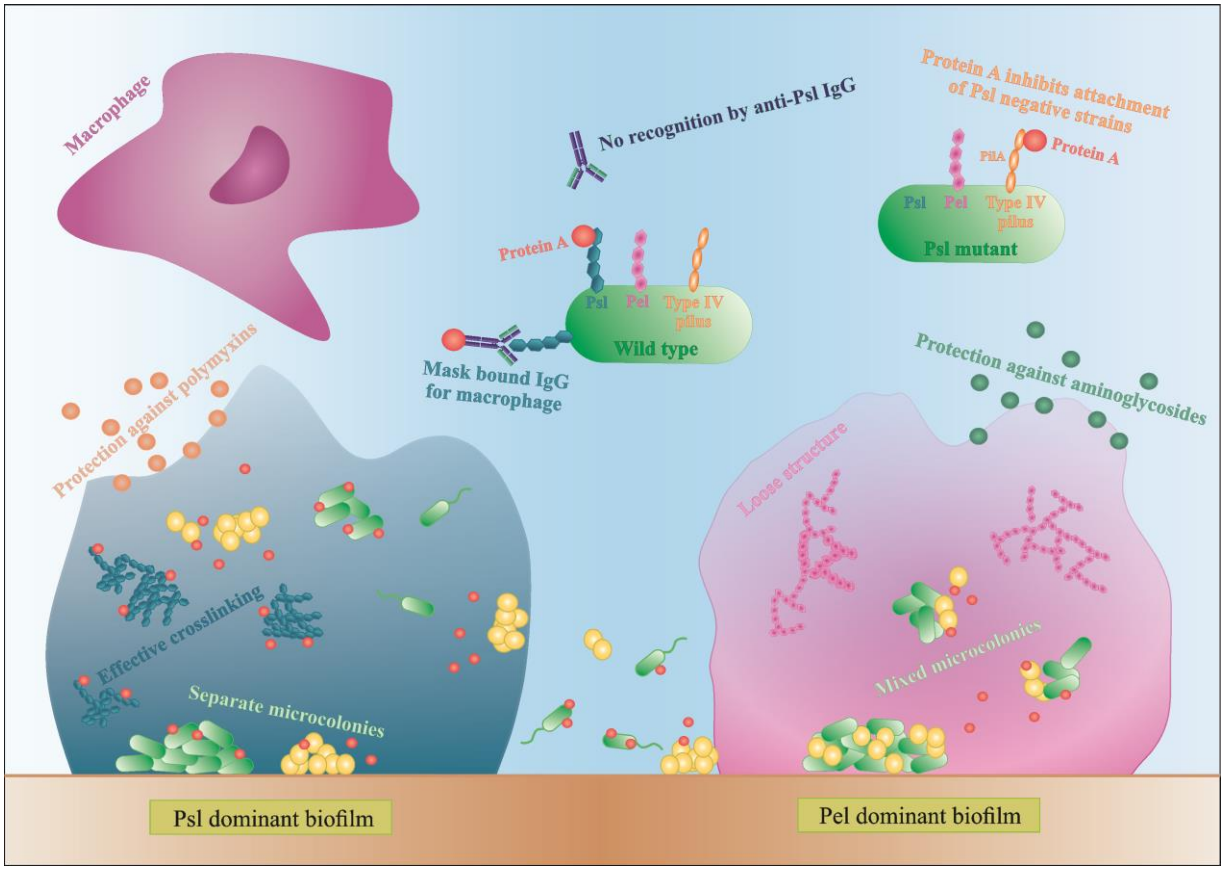

Figure 3: Interactions in mixed species biofilms. Differences in biofilm formation by *S. aureus* with a Psl- or Pel-dominant *P. aeruginosa* strain. IgG: immunoglobulin G.

2.7.1 *P. aeruginosa* extracellular polysaccharides are important in the formation of multi-species biofilms

P. aeruginosa produces three main exopolysaccharides (EPS): alginate, Pel and Psl, which form the extracellular matrix in the biofilm and have a structural and protective function[73-76]. Pel and Psl are the main EPS in non-mucoid strains[75]. The Psl polysaccharide is recently identified as repeating units of glucose-, mannose-, and rhamnose sugars and is mainly produced during the attachment phase of the biofilm[75]. Psl-positive strains have an elastic matrix with highly effective cross-linking of the matrix components[77]. Pel is glucose-rich and is mainly involved in pellicle formation and later stages of biofilm formation[75]. Contrary to Psl, Pel dominant strains form loose biofilm structures since Pel reduces the effective cross-linking in the matrix network (**Figure 3**)[77]. Consequently, Pel-mediated loosening of the *P. aeruginosa* biofilm allows *S. aureus* to infiltrate into the biofilm and form multi-species biofilms[77]. In contrast, the role of Psl in multi-species biofilm formation is not very clear. Chew et al showed that co-culture of a Psl-positive strain (PAO1) and *S. aureus* resulted in separated microcolonies without much association between both species[77]. Billings et al, on the other hand, showed that *S. aureus* was incorporated in the air-liquid interface of a Psl producing *P. aeruginosa* biofilm. Both studies use the same biofilm assay and the same *P. aeruginosa* strains, PAO1 and mutants derived from PAO1, but different *S. aureus* strains. Because the biofilm structure and dual species interactions are dependent on both *P. aeruginosa* and the *Staphylococcus* strains, this might explain the discrepancy between the studies. Nonetheless, both studies concluded that the EPS provides protection against antibiotics to all inhabitants of the biofilm, even the non-producers, although the biofilm as a whole is weakened[5]. More specifically, Psl functions as a protective barrier against the antibiotics colistin and polymyxin B, whereas Pel offers a protective barrier against aminoglycosides (**Figure 3**)[5,74]. These findings indicate that a minimum amount of EPS per cell present in the biofilm is needed for optimal protection against antibiotics[5]. These data suggests, if *S. aureus* is able to survive killing by *P. aeruginosa* and to co-exist in a multi-species biofilm, it benefits from the antimicrobial barrier formed by the *P. aeruginosa* matrix components. However, the third EPS, alginate, was not shown to have an effect on *S. aureus* and *S. epidermidis* biofilm formation. Alginate is mainly associated with chronic infections as its overproduction leads to the mucoid phenotype that frequently arises during long-term CF lung infection[73]. The switch to a mucoid phenotype contributes to the establishment of chronic colonization since alginate offers structural protection against uptake by macrophages and antimicrobials by forming a barrier limiting the penetration of antimicrobials, macrophages and macrophage-derived products, such as the pro-phagocytic cytokine IFN-γ[73,76]. Moreover, Leid et al suggest that alginate might cause the transition from acute to chronic infection by limiting IFN-mediated clearance by macrophages, which is the main mechanism of bacterial clearance during acute infection[76].

2.7.2 *S. aureus* protein A binds to Psl and type IV pili of *P. aeruginosa*

In addition to EPS, other extracellular factors are important in dual species biofilm formation. Yang et al showed that only *P. aeruginosa* strains producing type IV pili co-aggregate with *S. aureus* in microcolonies[78]. Type IV pili probably facilitate biofilm formation by binding to extracellular DNA (eDNA), which is derived from dead bacteria and part of the

biofilm matrix [78]. *P. aeruginosa* strains defective for the production of type IV pili or even treating the biofilm with DNAse I was shown to reduce the growth of mixed-species microcolonies[78]. Furthermore, using a single *S. aureus* laboratory strain, Armbruster et al showed inhibition of surface attachment of some *P. aeruginosa* clinical isolates due to the secretion of staphylococcal protein A (SpA) by *S. aureus*. SpA is a cell-wall associated extracellular adhesive protein of *S. aureus* that mediates biofilm formation and disrupts phagocytosis (by binding to the Fc portion of IgG antibodies) and its secretion was shown to be increased in artificial sputum[79]. Secreted SpA was shown to specifically bind both Psl and type IV pili of *P. aeruginosa*, stressing the importance of these two molecules in multispecies interactions (**Figure 3** [79]. In a Psl producing *P. aeruginosa*, all SpA seem to bind to the Psl, leaving the type IV pili free to mediate biofilm formation. In absence of Psl, SpA binds to the PilA component of type IV pili and inhibits adhesion of *P. aeruginosa*[79]. Furthermore, SpA seems to protect *P. aeruginosa* from phagocytosis, as the Psl-SpA complex is no longer recognized by anti-Psl IgG antibodies. SpA can also bind to the Fc domain of anti-Psl IgG antibodies and prevent recognition by neutrophils (**Figure 3**)[79].

2.8 *P. aeruginosa* and other staphylococci

2.8.1 *P. aeruginosa* induces biofilm dispersal in *S. epidermidis*

The antistaphylococcal molecules produced by *P. aeruginosa* are also active against other staphylococci, including *S. epidermidis,* although some are more resistant to killing compared to *S. aureus*. *P. aeruginosa* was shown to effectively inhibit and disrupt established *S. epidermidis* biofilms and induce detachment without killing during dual species biofilm formation[80-82]. After co-inoculation in equal proportions, *P. aeruginosa* and *S. epidermidis* could coexist for up to 18h. After this time point, the *S. epidermidis* cells in the biofilm are lysed by *P. aeruginosa*[82]. These data suggest that there are two stages in interactions between *P. aeruginosa* and *S. epidermidis*, the first includes the induction of detachment of viable *S. epidermidis* cells from the biofilm, while in the second stage cell lysis causes the total detachment[82]. Similar to *S. aureus*, the effect of *P. aeruginosa* on *S. epidermidis* is strain dependent as some *P. aeruginosa* strains have a more pronounced effect on some *S. epidermidis* strains while others are more resistant to *P. aeruginosa*[81]. Nevertheless, extracellular products that prevent initial attachment of some *S. epidermidis* strains to surfaces might be an interesting option for the development of coatings for indwelling medical devices, like peritoneal dialysis catheters[61]. Moreover, in this model, *P. aeruginosa* supernatant components replaced serum proteins on the catheter surface and reduced *S. epidermidis* attachment[61]. In addition, exposure of a *S. epidermidis* biofilm on a catheter to *P. aeruginosa* supernatant also caused dispersal of *S. epidermidis*[61]. The dispersed cells are, however, not killed making it less suitable as a treatment option and only interesting as a prevention strategy.

2.8.2 Yayurea A and B from the *S. intermedius* group are quorum-quenching molecules which provide protection against Gram-negative bacteria

P. aeruginosa is originally an environmental bacterium and shares a niche with many other, non-pathogenic staphylococci like the S*taphylococcus intermedius* group consisting of *S.*

delphini, S. intermedius, S. lutrae, S. pseudointermedius and *S. schleiferi*. All are common colonizers of various animals and rarely occur in humans[83,84]. This group of staphylococci produces two low molecular weight compounds, yayurea A and B, that inhibit the production of quorum-sensing regulated products in Gram-negative bacteria and provide protection against extracellular compounds produced by *P. aeruginosa*[85]. For example, the growth of *S. delphini* is not suppressed by respiratory toxins during co-culture with *P. aeruginosa.* Moreover, *S. delphini* is able to completely inhibit the production of pyocyanin[85]. The quorum-quenching effect of yayurea A and B covers a broad spectrum of Gram-negative bacteria, including *P. aeruginosa, Serratia marcescens, Vibrio harveyi* and *Chromobacterium subtsugae*[85]. Quenching of the quorum-sensing system of these Gram-negative bacteria does not kill them but rather maintains their physiological state as if the cell density is low, even though density is in fact high. This increases the chances of survival of the staphylococci since toxin production usually begins at high cell density[85]. Surprisingly, other staphylococci seem to be resistant to both molecules, even though they are not producers[85]. Interestingly, *S. aureus* is protected from killing by *P. aeruginosa* when yayurea A and B are added to the medium without the former having to undergo physiological changes (SCV formation), and represent promising candidates for inhibition studies of P. aeruginosa virulence and biofilm formation.

2.8.3 Variations in CydAB from *S. carnosus* provides protection against killing by *P. aeruginosa*

In addition to the *S. intermedius* group, several other non-pathogenic staphylococci (*S. carnosus*, *S. piscifermentans* and *S. simulans*) seem to be resistant to respiratory toxins secreted by *P. aeruginosa* due to alterations in the cydAB genes. These genes encode the two subunits of cytochrome bd quinol oxidase, of which homologues are also present in the genomes of *S. aureus* and *S. epidermidis*[86]. However, only the cytochrome bd quinol oxidase of the first group is resistant to the respiratory toxin, pyocyanin[86]. Furthermore, cloning of the *S. carnosus* cydAB cluster into *S. aureus* confers resistance to respiratory inhibitors produced by *P. aeruginosa*[86]. Further research showed that, whereas the CydA subunit is more conserved in staphylococci, CydB underwent a microevolution with relatively higher identity within than between the groups of pathogenic and non-pathogenic staphylococci[86]. This asymmetric evolution of CydB could be explained by the fact that the non-pathogenic staphylococci frequently inhabit the same environment as *Pseudomonas* spp. and were therefore selected for a higher resistance to respiratory toxins.

2.9 Concluding remarks

Both *P. aeruginosa* and staphylococci are highly versatile organisms, which readily adapt to a wide variety of environments and stress factors. In the first glance, these bacteria seem to have an antagonistic relationship as *P. aeruginosa* produces a wide variety of molecules inhibiting staphylococci and frequently outcompetes *S. aureus* and *S. epidermidis* during co-culture. This antagonistic behaviour is mainly shown during planktonic growth and under traditional culture conditions, where no host factors or antibiotics are present. However, under some *in vitro* and *in vivo* circumstances, both bacteria are able to co-exist and form dual species biofilms. These circumstances are dependent on a combination of strain-

dependent properties of both species and the presence or absence of certain environmental factors like antibiotics or host factors, the sum of which might tip the balance towards either killing or co-existence. The presence of some sort of selection pressure or presence of a preformed matrix seems to favour dual species biofilm formation whereas planktonic co-culture without selection pressure leads to domination of *P. aeruginosa*. Interspecies competition often leads to an increased production of virulence factors in both *P. aeruginosa* and *S. aureus*, which are also harmful to the human host. In addition, escaping the antistaphylococcal compounds results in a more stress-resistant phenotype of *S. aureus*, which is more difficult to be cleared by the immune system, to be eradicated by antibiotics and to be detected in diagnostic cultures. Furthermore, the presence of an extracellular matrix was shown to be beneficial for all biofilm inhabitants, providing protection against classical antibiotics and the host immune system, although the exact composition might be variable depending on the species present and the environment. The role of these matrix components (exopolysaccharides, eDNA, matrix proteins, host-derived factors etc.) in interspecies interactions and their role in disease pathogenesis provides an exciting opportunity for future research towards better patient care. When strains are co-existing for a longer time, they might evolve to a phenotype that is better adapted to the presence of the other. For example, non-pathogenic staphylococci that are frequently encountering *P. aeruginosa* have developed strategies to continue growing in the presence of *P. aeruginosa* antistaphylcoccal compounds, indicating parallel evolution. Moreover, *S. aureus* and *P. aeruginosa* strains isolated from the same chronic CF lung infection are less sensitive to, and produce less, HQNO, respectively. This strain adaptation and the underestimation of the co-existence of *P. aeruginosa* and *S. aureus* might still have a large impact on the clinical outcome of a patient and therefore should be a subject of continuing investigation.

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Chapter 3: Identification of microbial interactions between clinical isolates of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*

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3.1 Abstract

Here, we investigated the effect of the biofilm supernatant of *Pseudomonas aeruginosa* clinical isolates on the biofilm formation of *Staphylococcus epidermidis* clinical isolates and several *Staphylococcus aureus* reference strains. The biofilm supernatant of two *P. aeruginosa* strains inhibited and two strains stimulated the growth of *S. epidermidis* clinical isolates. However, coating with this supernatant reduced the adherence of most *S. epidermidis* clinical isolates and *S. aureus* reference strains with an average loss range of 10-100% compared to the control (coating with plain medium) after 6h and 24h. The same effect was observed during biofilm formation under biofilm supernatant pressure of all four *P. aeruginosa* strains tested after 6h of growth and for the supernatant of three of the four *P. aeruginosa* strains after 24h. Remarkably, the biofilm supernatant of one *P. aeruginosa* increased the biofilm formation of most *S. epidermidis* isolates after 24h of growth with an average increase range of 10-95%. We found that the *P. aeruginosa* strains with a stimulating effect on *S. epidermidis* growth and biofilm produced a lower amount of the virulence factors pyocyanin and pyoverdin. The importance of these factors in interspecies interactions is confirmed by the increased expression by *P. aeruginosa* under the pressure of *S. epidermidis* biofilm supernatant. Furthermore, the biofilm supernatant of *S. epidermidis* clinical isolates also stimulated the growth and biofilm formation of some *P. aeruginosa* strains although coating had no significant effect. During co-culture, *P. aeruginosa* reduced the colony forming units of *S. epidermidis*, confirming the mainly antagonistic effect.

3.2 Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen causing a wide range of (healthcare-associated) infections, including ventilator associated pneumonia (VAP)[1,2]. The success of this pathogen can be explained by its high intrinsic antibiotic resistance, production of various extracellular virulence factors and strong biofilm formation[3,4]. Biofilms are surface- attached communities of micro-organisms surrounded by an extracellular matrix, which protects the inhabitants from antimicrobials, mechanical removal and the host immune system[5]. In mechanically ventilated patients, a biofilm is formed on the endotracheal tube (ETT) within 24h of intubation and dislodged particles of these biofilms have easy access to the lower airways, potentially causing VAP[6,7].

Furthermore, most naturally occurring biofilms consist of multiple species, which are likely to interact with each other[8]. In the ETT, *P. aeruginosa* encounters members of the human commensal flora like *Staphylococci* or oral *Streptococci*, which are also frequently isolated from respiratory samples of mechanically ventilated patients and might cause VAP[9,10]. *P. aeruginosa* competes with these bacteria by excreting a wide range of extracellular factors such as pyocyanin, rhamnolipids and protease, which are also harmful for the patient and the presence of Gram-positive bacteria was shown to increase the virulence of *P. aeruginosa in vivo*[11-15]. In previous research, we showed a correlation between the bacterial consortium present in ETT biofilms and the patient prognosis, with the presence of *P. aeruginosa* being associated with lower species diversity and worse patient prognosis[16]. Furthermore, *P. aeruginosa* was shown to inhibit *S. epidermidis* biofilm formation and to induce *S. epidermidis* biofilm dispersal of life cells in a strain dependent manner[17-19]. *S. epidermidis*, on the other hand, was described to have very little effect on *P. aeruginosa* biofilm formation during dual species culture[17,18]. Currently, most studies focus on microbial interactions between *P. aeruginosa* and *Staphylococcus aureus*, a close relative of *S. epidermidis* which produces more virulence factors and shows a complicated relationship with *P. aeruginosa* in a wide range of infections[20]. Additionally, it was shown that co-isolated strains of *P. aeruginosa* and *Staphylococcus aureus* from chronic infections interacted differently compared to single isolates, suggesting co-adaptation of these strains[21].

Previous studies investigating microbial interactions between *P. aeruginosa* and *S. epidermidis* only use reference strains or a limited amount of clinical and environmental isolates obtained from various sources. Therefore, in this study we aim to investigate microbial interactions between clinical isolates of *P. aeruginosa* and *S. epidermidis* obtained from ETTs, including co-isolated strains. To do so, we will investigate the effect of extracellular factors produced during biofilm formation on the growth and early or late biofilm formation of the other. Furthermore, we will also investigate the effect of the biofilm supernatant and dual species culture on the biofilm structure using spinning disc confocal microscopy. A better understanding of interspecies interactions between common pathogens and commensals might lead to new potential targets for prevention and eradication strategies and to better predict the patient prognosis.

3.3 Methods

3.3.1 Bacterial strains and culture conditions

All clinical isolates used in this study were cultured from the endotracheal tubes (ETTs) of mechanically ventilated patients admitted to the intensive care unit of the University Hospital of Antwerp, Belgium and processed as described before[16]. Briefly, the lower 15 cm of the ETT was collected upon extubation and a slice above and below the balloon was tapped on Colombia blood agar base (Oxoid, UK) supplemented with 5% horse blood for culture (37°C, 5% CO2, 48h). Over a period of two years, 203 ETTs were collected, from which 36 *P. aeruginosa* and 51 *S. epidermidis* strains were isolated. Seven ETTs contained both organisms. For this study, we selected 22 *P. aeruginosa* and 15 *S. epidermidis* isolates, including five *P. aeruginosa* and four *S. epidermidis* co-isolated strains (PA095 and SE095, PA011 and SE011, PA4-609 and SE4-609, PA4-613 and SE4-613, PA129, **Tables 1 and 2**). In addition, two *P. aeruginosa* (PAO1 (ATCC 15692) and PA14 (UBPPC-PA14)) and one *S. epidermidis* (*S. epidermidis* 1457 (SE1457)) reference strains were used. Furthermore, we included a set of seven *S. aureus* reference strains to extrapolate the results to a broader range of *Staphylococci* (ATCC 29213, ATCC 6538, JE2 (USA300), ATCC 25923, NCTC 8325, EMERSA_15, USA300_FPR3757). All bacteria were cultured overnight on Colombia blood agar base (Oxoid, UK) supplemented with 5% horse blood for solid cultures or in trypticase soy broth (TSB, Becton, Dickinson and company, France) for liquid cultures at 37°C under static conditions unless stated otherwise.

Table 1: Strain information of the *S. epidermidis* used in this study. CVC: central venous catheter; ETT: endotracheal tube; MLST: multi locus sequence type; nt: not typable.

Strain	MLST type	SCCmec type	ACME element	Clinical data	Biofilm formation	Source	Co-isolates
***S. epidermidis* 1457**		/		/	reference	CVC	/
SE068	425	IV	/	pneumonia	strong	ETT	S. aureus C. freundii S. parasanguinis A. odontolyticus C. albicans C. dubliniensis S. pneumoniae N. perflava
SE4-613	2	IV	/	pneumonia	moderate	ETT	**P. aeruginosa** C. albicans C. dubliniensis
SE128	2	II	/	pneumonia	moderate	ETT	E. faecium C. glabrata
SE4-609	2	III	/	pneumonia	weak	ETT	**P. aeruginosa** L. paracasei
SE3-405	2	IV	type I	no pneumonia	strong	ETT	C. ablicans C. glabrata
SE5-803	2	nt	/	no pneumonia	moderate	ETT	S. pneumoniae C. albicans
SE5-811	2	IV	/	no pneumonia	weak	ETT	E. coli C. glabrata
SE3-410	2	II	type I	no pneumonia	strong	ETT	M. osloensis C. dubliniensis E. faecalis
SE011	2	IV	/	pneumonia	weak	ETT	**P. aeruginosa** E. faecalis
SE095	2	V/VII	/	no pneumonia	moderate	ETT	**P. aeruginosa** S. pneumoniae
SE3-419	2	III	type III	no pneumonia	moderate	ETT	C. albicans
SE105	2	III	/	pneumonia	moderate	ETT	S. maltophilia
SE108	2	IV	/	pneumonia	moderate	ETT	C. albicans
SE112	2	III	type II	pneumonia	moderate	ETT	C. albicans
SE045	54	IV		pneumonia	weak	ETT	/
SE078	2	V/VII	type III	pneumonia	weak	ETT	K. pneumoniae C. albicans

Table 2: Strain information of the *P. aeruginosa* used in this study. ETT: endotracheal tube; MLST: multi locus sequence type; nt: not typable.

strain	MLST type	Clinical data	Biofilm formation	Source	Co-isolates
PAO1		/	reference	Wound	/
PA14	253	/	reference	Burn wound	/
PA011	111	pneumonia	moderate	ETT	**S. epidermidis** E. faecalis
PA010	111	no pneumonia	strong	ETT	S. marcescens S. pneumoniae
PA060	111	pneumonia	moderate	ETT	P. mirabilis
PA2-201	111	pneumonia	strong	ETT	C. freundii E. faecium
PA065	111	pneumonia	moderate	ETT	P. mirabilis
PA4-606	244	pneumonia	strong	ETT	/
PA4-609	244	pneumonia	moderate	ETT	**S. epidermidis** L. pracasei
PA3-413	244	no pneumonia	moderate	ETT	E. faecium C. albicans Chryseobacterium sp.
PA012	1076	pneumonia	moderate	ETT	M. luteus A. gnomospecies 3
PA016	640	pneumonia	strong	ETT	/
PA4-613	640	pneumonia	moderate	ETT	**S. epidermidis** C. albicans C. dubliniensis
PA066	589	no pneumonia	moderate	ETT	/
PA1-009	253	no pneumonia	strong	ETT	E. faecium C. albicans
PA047	253	no pneumonia	moderate	ETT	C. albicans
PA129	253	no pneumonia	strong	ETT	**S. epidermidis** E. cloacae S. maltophilia S. haemolyticus
PA009	253	pneumonia	moderate	ETT	S. marcescens S. pneumoniae
PA095	560	no pneumonia	strong	ETT	**S. epidermidis** S. peroris
PA116	591	no pneumonia	moderate	ETT	/
PA124	597	no pneumonia	weak	ETT	C. glabrata
PA133	nt	no pneumonia	strong	ETT	S. marcescens C. glabrata
PA4-604	nt	no pneumonia	strong	ETT	K. pneumoniae C. albicans C. glabrata
PA031	nt	pneumonia	moderate		/

3.3.2 Preparation of cell-free biofilm supernatant

Biofilms were generated by incubating 100 ml of a bacterial suspension in TSB with a final concentration of 0.05 McFarland (McF) in a 145 x 20 mm polystyrene petri dish (Greiner bio-one, Germany) for 24h at 37°C. The biofilm supernatant was collected with a pipette, centrifuged at 4°C for 30 min at 4495xg (Multifuge 3 S-R, Heraeus) and filtered through a sterile 0.2 µm filter (MiniSart, Sartorius, Germany) or autoclaved (15 min, 120 psi). Biofilm supernatant was prepared fresh for every experiment and 10 µl was plated to confirm sterility. Experiments were conducted in three technical replicates and at three independent time points.

3.3.3 Coating of surfaces with biofilm supernatant

The surface of polystyrene 96-well plates (Greiner bio-one, Germany) was pre-coated by incubating 200 µl biofilm supernatant of four *P. aeruginosa* (PAO1, PA095, PA4-613 and PA124) or plain TSB as a control for 3h followed by emptying with a pipette and air drying. To determine the effect on biofilm formation, 20 µl inoculum (0.5 McF) of 16 *S. epidermidis* and seven *S. aureus* strains were inoculated in 180 µl of TSB in the pre-coated 96-well plates for 6h and 24h at 37°C. After 6h or 24h, the plates were washed three times with sterile phosphate buffered saline (PBS, Thermo Fisher Scientific, Lithuania) and fixed with methanol (Merck, Germany) for 20 min. The biomass was stained with 1% crystal violet and absorption was measured at 492nm (Multiskan, Thermo scientific, Germany). The effect of the supernatant of four *S. epidermidis* strains (SE4-613, SE4-609, SE095 and SE011) on 16 strains of *P. aeruginosa* was investigated in a similar manner in a 96-well plate with polystyrene peg lid (445497, Thermo Fisher Scientific, Denmark). The change in biomass was calculated for all strains as a percentage of the biofilm formation in the control well of the same strain.

3.3.4 Biofilm formation and growth in the presence of biofilm supernatant

Biofilm supernatant prepared as above was diluted 1:1 with double concentrated TSB (2x TSB) to restore the nutrient concentration. As a control, 2x TSB diluted 1:1 with sterile ultrapure water was used. Similar to above, 20 µl of 0.5 McF suspension was inoculated in 180 µl of 2x TSB with the biofilm supernatant of the other species or ultrapure water. The flat-bottom 96-well (*S. epidermidis*) and peg plates (*P. aeruginosa*) were incubated for 6h or 24h at 37°C and biomass was quantified by crystal violet staining as described above and the change in biomass was calculated as a percentage of the biofilm formation in the control well of each strain. To determine the effect of the supernatant the growth of the other, bacterial suspensions were prepared as above in 2x TSB with supernatant of the other species or ultrapure water in a 96-well plate for 24h at 37°C and the final OD600nm was measured for each well.

3.3.5 Pyocyanin and pyoverdin production by *P. aeruginosa*

The effect of *S. epidermidis* supernatant on the virulence of *P. aeruginosa* was investigated by the quantification of two major virulence factors, pyocyanin and pyoverdin. *P. aeruginosa* biofilms were prepared in 2x TSB with *S. epidermidis* supernatant or sterile ultrapure water as a control as above. The production of pyoverdin and pyocyanin was measured as described

by Filloux and Ramos[22]. Briefly, the cultures were diluted 1:10 in 50 mM pyridine-acetic acid buffer (pH 5). For pyoverdin, the wavelength range from 350-600 nm was measured in a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Germany). The pyoverdin concentration correlates with the absorption according to the beer-lambert law and when it is present in the sample, a specific band with maximum absorption at 380nm appears. The pyocyanin concentration is calculated at a wavelength of 691 nm using the following equation: [Pyocyanin (mM)] = $A_{Pyocyanin} cm^{-1}/4.31\ mM^{-1}cm^{-1}$. The production of both virulence factors in the presence of *S. epidermidis* supernatant was compared to the control well of each *P. aeruginosa* strains and all experiments were conducted in three technical replicates at three independent time points.

3.3.6 Biosurfactant production by *P. aeruginosa*

To investigate the production of biosurfactants by four *P. aeruginosa* strains (PAO1, PA095, PA4-613 and PA124), the cell-free biofilm supernatant was collected as above and a drop collapse assay was performed as described by Caiazza et al. with some modifications [23]. Briefly, a two-fold serial dilution of the supernatant was prepared and 30µl droplets were spotted on the surface of a polystyrene petri dish (Greiner Bio-one, Germany) and visually inspected for droplet formation followed by positioning the petri dish in a vertical position at a 90° angle for 10s. A high amount of biosurfactants resulted in a collapse of the droplets in a horizontal position and a smear of the droplet in a vertical position.

3.3.7 Dual species culture and competition

To determine interspecies competition of a selection of strains, 0.5 McF solutions of both species (eight *P. aeruginosa*: PAO1, PA14, PA4-613, PA124, PA011, PA065, PA066 and PA095 and five *S. epidermidis*: SE1457, SE4-613, SE011 and SE078) were mixed in equal proportions and diluted in 180 µl of TSB as above. After 24h, the content of each well was serially diluted in PBS and a colony count (CFU) was performed on Mannitol Salt Agar to quantify *S. epidermidis* (BD, France) and MacConkey agar to quantify *P. aeruginosa* (BD, France). The competitive index was calculated as the CFU count of *P. aeruginosa* divided by the CFU count of *S. epidermidis* and these values were log transformed. A competitive index = 0 indicates an equal competition, a competitive index > 0 indicates a competitive advantage of *P. aeruginosa* and a competitive index < 0 indicates a competitive advantage for *S. epidermidis*.

3.3.8 Spinning disc confocal microscopy

The effect of the biofilm supernatant as well as dual species culture on the biofilm structure of a selection of strains was evaluated by spinning disc confocal microscopy in µClear thin bottom 96-well plates (Greiner bio-one, Germany). The biofilm formation of the *S. epidermidis* strains SE1457, SE011 and SE095 was investigated with the supernatant of the *P. aeruginosa* strains PAO1, PA095, PA124 and PA4-613. Biofilm supernatant and bacterial inocula were prepared as above. After 24h of growth, the remaining medium was carefully removed with a pipette and the plate was washed once with sterile 0.9% saline and stained with Syto9 (10 µM, Life sciences, USA). The biofilm was visualized by spinning disc confocal microscopy (UltraVIEW VoX, Perkin Elmer) and the total biofilm volume was determined with Volocity 6.3 (Perkin Elmer).

3.3.9 Statistical analysis

All results were compared to their respective controls by a two sample t-test in excel. P-values below 0.05 were considered significant. A list with all individual p-values is provided in the **Supplementary data**

3.4 Results

3.4.1 The clinical isolates of *P. aeruginosa* and *S. epidermidis* show differences in growth and biofilm formation

Both *S. epidermidis* and *P. aeruginosa* isolates showed large differences in their final OD600nm and their ability to form biofilms, although a high OD600nm was not necessarily correlating to strong biofilm formation (**Figure 1**). The biofilm formation was determined by crystal violet staining and is presented here as a percentage of the control strains *P. aeruginosa* PAO1 and *S. epidermidis* 1457. Strains forming >75% biomass compared to the control were classified as strong biofilm formers, a biomass between 75% and 25% was considered moderate and <25% were weak biofilm forming strains. For *P. aeruginosa*, we identified 15 strong biofilm forming strains, seven with a moderate and two with a weak biofilm forming capacity. For *S. epidermidis,* four strains were classified as strong biofilm formers and 11 as moderate biofilm forming strains. Of these 11 moderate strains, seven were on the borderline of weak biofilm formation (25 -35% of the control).

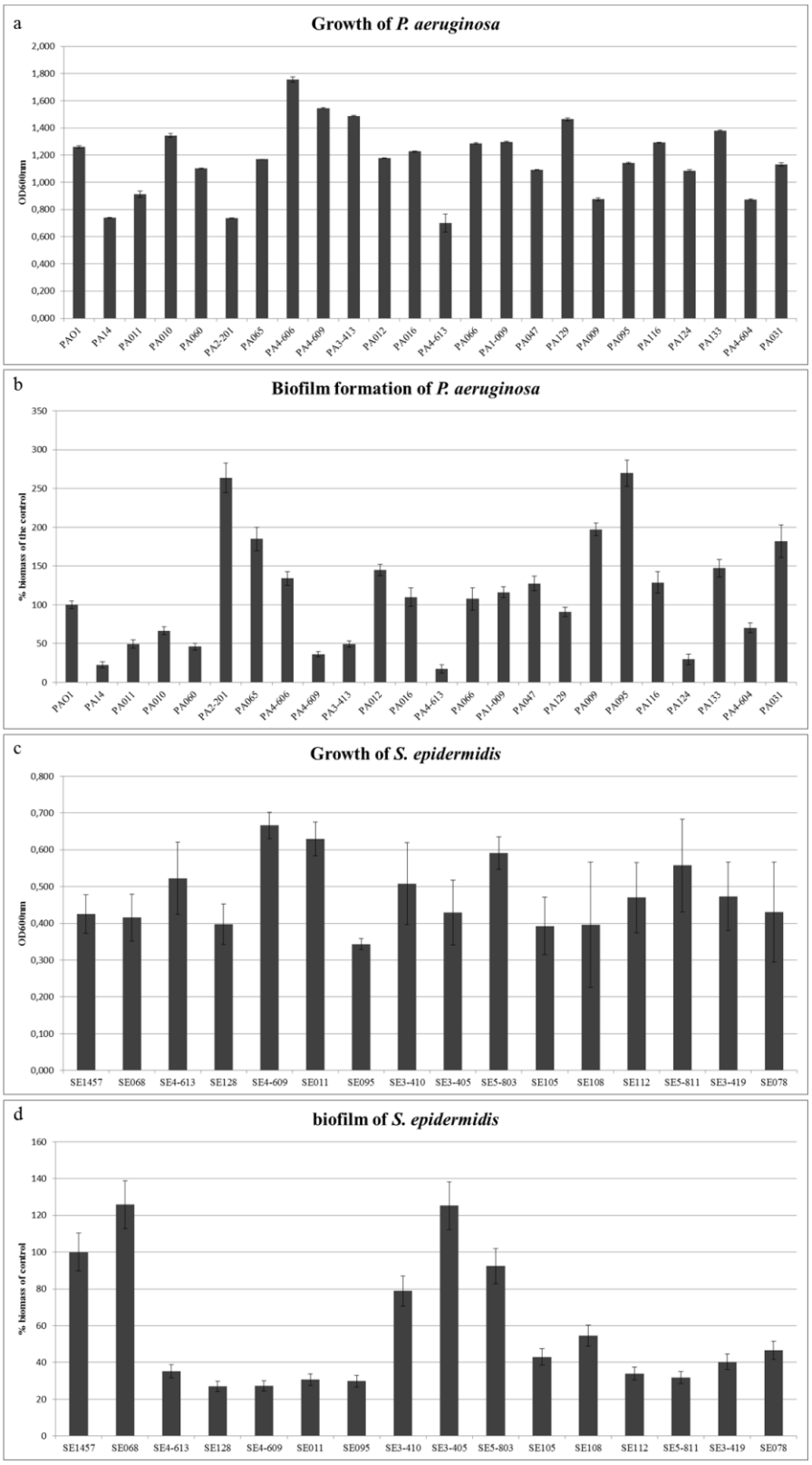

Figure 1: The growth (a and c) and biofilm formation (b and d) of the clinical isolates. The growth rates were measured with the end OD600nm and the biomass is presented as a percentage of the respective controls, PAO1 and *S. epidermidis* 1457 (SE1457). >75% of the control = strong biofilm formation; 75- 25% = moderate biofilm formation; <25% = weak biofilm formation.

3.4.2 The biofilm supernatants of two *P. aeruginosa* strains inhibit and of two strains stimulate *S. epidermidis* growth

First we determined the effect of the supernatant of four *P. aeruginosa* (PAO1, PA4-613, PA095 and PA124) on the growth of 16 *S. epidermidis* strains. The effect on the growth was calculated as a percentage of the change in the final OD600 in the presence of *P. aeruginosa* biofilm supernatant compared to the control (2x TSB with sterile ultrapure water, **Figure 2**). The reference strain PAO1 and one *P. aeruginosa* (PA095) co-isolated with *S. epidermidis* showed an antagonistic effect as they significantly reduced the growth of all *S. epidermidis* strains with an average of 52% and 64%, respectively (95% CI: 45-61% and 59-70%, respectively, $p<0.05$). Remarkably, the supernatant of the two other *P. aeruginosa* strains showed a synergistic effect since PA124 enhanced the growth of 11 *S. epidermidis* strains with an average increase of 39% (95% CI: 23-56%, $p<0.05$) and the co-isolated strain PA4-613 enhanced the growth of ten *S. epidermidis* strains with an average of 32% (95% CI: 24-41%, $p<0.05$). The growth of the remaining five *S. epidermidis* isolates was not significantly increased by these strains (average increase: 10%, 95% CI: 6-24%, $p>0.05$).

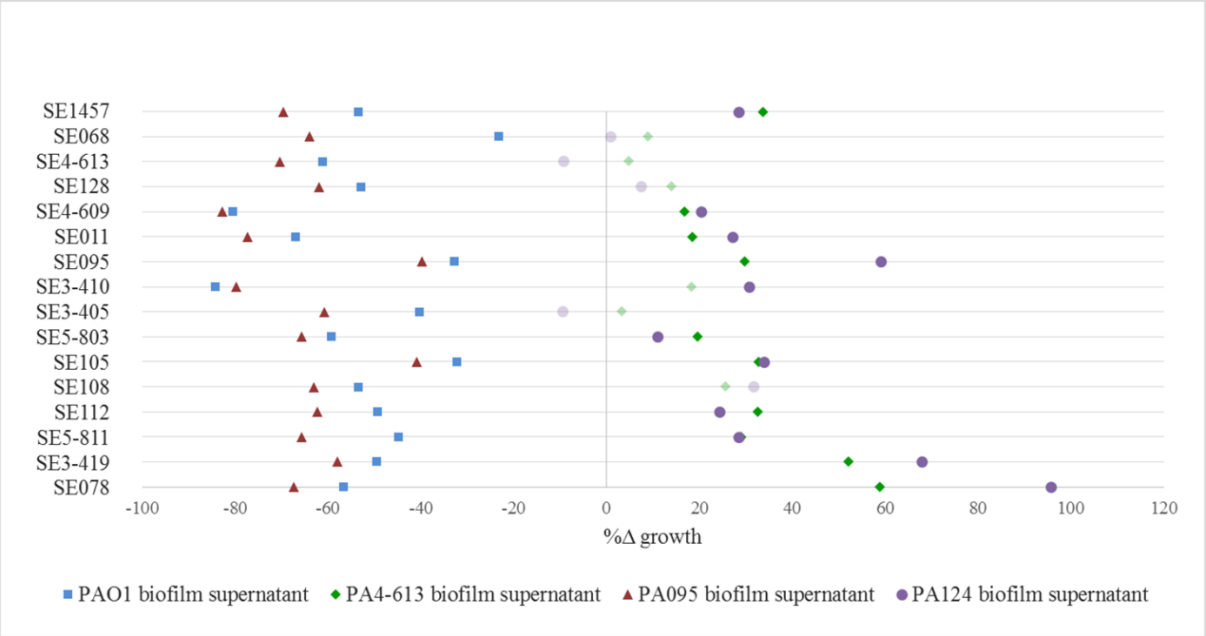

Figure 2: The changes in growth of the *S. epidermidis* strains in the presence of the biofilm supernatant of four selected *P. aeruginosa* strains. The values are presented as a percentage of the change compared to the control (Δ% growth). The transparent values are not significant ($p<0.05$).

3.4.3 Coating with *P. aeruginosa* biofilm supernatant reduces adherence of S. *epidermidis* and *S. aureus*

Second we investigated the effect of coating of supernatant of the four *P. aeruginosa* strains on the attachment of 16 *S. epidermidis* and seven *S. aureus* after 6h and 24h representing early and late biofilm stages, respectively. Coating with plain TSB was used as a control and the biofilm formation of each strain was compared to its biomass production in the control wells. After 6h of incubation, coating with the supernatant of PAO1 and PA095 again showed a strong antagonistic effect by reducing the biofilm formation of all *S.*

epidermidis with an average of 77% and 80%, respectively (95% CI: 73-81% and 95% CI:78-83%, p<0.05, **Figure 3a**) and of five and six of the seven *S. aureus* strains with an average of 72% and 67%, respectively (95% CI: 60-85% and 95% CI: 52-82%, p<0.05). Coating with the supernatant of PA124 and PA4-613 showed a less pronounced antagonistic effect as the biofilm of 15 and eight *S. epidermidis* strains was reduced with an average of 62% and 28%, respectively (95% CI: 58-65% and 95% CI: 20-37%, p<0.05). Furthermore, coating with the supernatant of PA124 reduced the biofilm of two *S. aureus* strains with an average of 85% (95% CI: 33-84%, p<0.05) whereas the supernatant coating of PA4-613 showed no significant effect on *S. aureus* biofilm formation after 6h. Additionally, one of the *S. aureus* strains, EMERSA_15, showed an increased biofilm production (91%) in the presence of the supernatant coating with PA124 supernatant (**Figure 3a**).

After 24h of growth, five *S. epidermidis* strains showed a similar biomass production as the control after PAO1 supernatant coating whereas the biomass of 11 strains was significantly reduced with an average of 83% (95% CI: 75-92%, p<0.05, **Figure 3b**) and the supernatant of PA095 reduced the biomass of 14 strains with an average of 72% (95% CI: 58-86%, p<0.05). Coating with PA124 supernatant reduced the biomass of 14 *S. epidermidis* with an average of 58% (95% CI: 49-66%, p<0.05) and coating with PA4-613 supernatant significantly reduced the biomass of six *S. epidermidis* with an average of 32% (95% CI: 20-44%, p<0.05). The effect of supernatant coating on *S. aureus* was variable after 24h and was dependent on both the *S. aureus* and *P. aeruginosa* strain. Coating with PAO1 supernatant reduced the biofilm formation of all *S. aureus* strains with an average of 60% (95% CI: 45-76%, p<0.05), coating with PA095 supernatant reduced the biomass of five strains with an average of 71% (95% CI: 60-83%, p<0.05), coating with PA124 supernatant reduced the biofilm production of three strains with an average of 41% (95% CI: 26-55%, p<0.05) and coating with PA4-613 supernatant reduced the biofilm formation of two *S. aureus* with an average of 46% (95% CI: 23-69%, p<0.05). Furthermore, the biofilm of two *S. aureus* was increased by PA124 by 133% and 54% (**Figure 3b**).

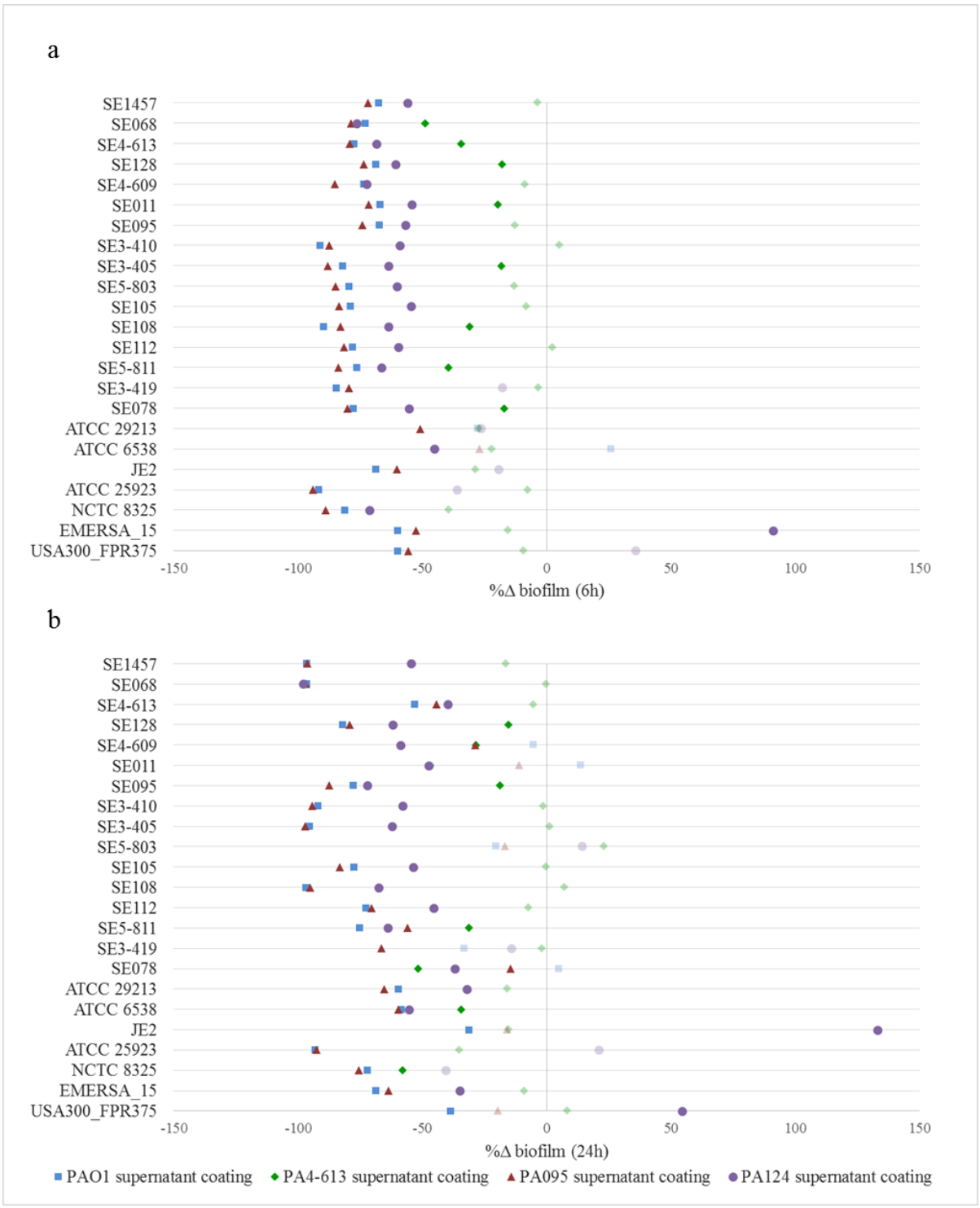

Figure 3: Changes in biofilm formation by the different *Staphylococcus* strains on a surface coated with biofilm supernatant of four different *P. aeruginosa* strains after 6h (a) and 24h (b). The values are presented as a percentage of the change compared to the control (%Δ biofilm). The transparent values are not significant ($p<0.05$).

3.4.4 *P. aeruginosa* supernatant inhibits *S. epidermidis* and *S. aureus* biofilm formation

Coating of the surface with biofilm supernatant affects mainly the initial attachment by forming a barrier masking the surface for *S. epidermidis*. Therefore, we also investigated the effect of *P. aeruginosa* biofilm supernatant added to the medium on the biofilm formation of *S. epidermidis* and *S. aureus* for 6h and 24h. After 6h, the biofilm formation of all *S. epidermidis* strains was reduced by PAO1 and PA095 supernatant with an average of 85% and 93%, respectively (95% CI: 81-89% and 95% CI: 91-95%, $p<0.05$, **Figure 4a**), the biomass of 14 *S. epidermidis* was reduced by PA4-613 supernatant with an average of 41% (95% CI: 38-45%, $p<0.05$) and the biomass of 12 *S. epidermidis* was reduced by PA124 supernatant with an average of 37% (95% CI: 91%-95%, $p<0.05$). The biomass of all *S. aureus* strains was reduced by the supernatant of PAO1, PA095 and PA4-613 with an average of 88%, 94% and 55%, respectively (95% CI: 81-95%; 95% CI: 90-98% and 95% CI: 45-65%, $p<0.05$) and the biofilm of five *S. aureus* was reduced by the supernatant of PA124 with an average of 50% (95% CI: 38-61%, $p<0.05$).

After 24h of incubation, PAO1 and PA095 supernatant inhibited 13 *S. epidermidis* with an average of 55% and 63%, respectively (95% CI: 41-68% and 95% CI: 52-74%, $p<0.05$, **Figure 4b**) and of all *S. aureus* strains with an average of 82% and 90%, respectively (95% CI: 72-92% and 95% CI: 79-100%, $p<0.05$). Furthermore, the supernatant of PA4-613 inhibited the biofilm formation of nine *S. epidermidis* and two *S. aureus* strains with an average of 53% and 62%, respectively (95% CI: 34-71% and 95% CI: 40-83%, $p<0.05$) and PA124 supernatant inhibited two *S. epidermidis* and two *S. aureus* strains with an average of 62% and 56%, respectively (95% CI: 36-88% and 95% CI: 31-80%, $p<0.05$). Remarkably the supernatant of PA124 also stimulated the biofilm formation of 11 *S. epidermidis* strains with an average of 60% (95% CI: 46-74%, $p<0.05$) and of one *S. aureus* with an average of 86%. The biomass of the other strains was not significantly increased or decreased compared to the control (**Figure 4b**). In addition, the biofilm formation of the reference strain SE1457 and two co-isolates *S. epidermidis* strains, SE011 and SE095, in the presence of the *P. aeruginosa* biofilm supernatant was investigated by confocal microscopy. These results also showed a strong decrease of the total biomass with 82-89% in the presence of the supernatant of PAO1 and PA095, a decrease of 23% in the presence of PA124 and of 44% in the presence of PA4-613 (**Figure 4c).**

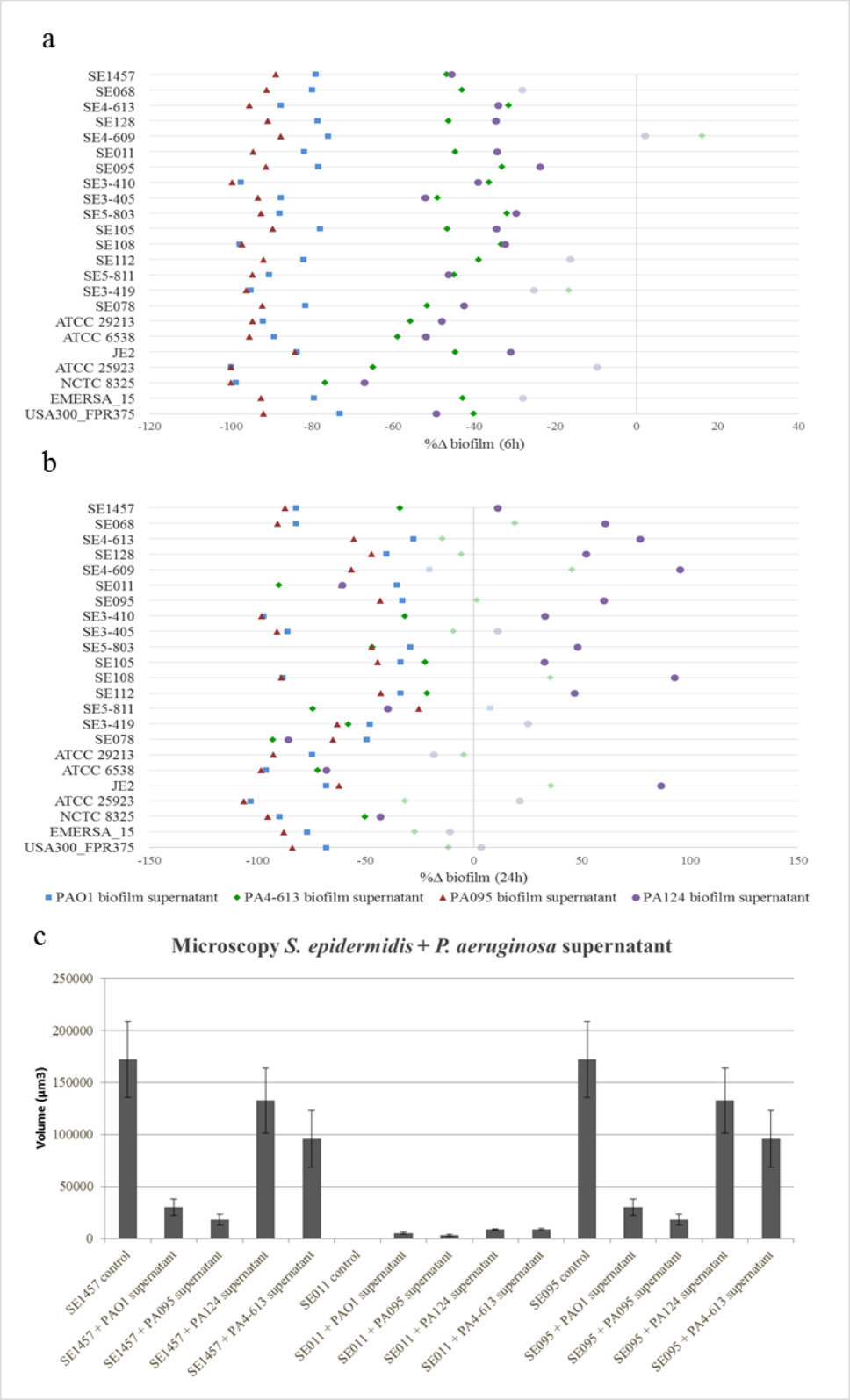

Figure 4: Changes in biofilm formation by the different *Staphylococcus* strains in the presence of the biofilm supernatant of four different *P. aeruginosa* strains after 6h (a) and 24h (b). The values are presented as a percentage of the change compared to the control (%Δ biofilm). The transparent values are not significant ($p<0.05$). (c) Total biomass volume of a selection of strains stained with Syto9.

3.4.5 *P. aeruginosa* shows a competitive advantage over *S. epidermidis* during dual species culture

Dual species cultures were initiated with a selection of eight *P. aeruginosa* and five *S. epidermidis* strains to investigate the effect on competition. We selected the reference strains PAO1, PA14 and *S. epidermidis* 1457, the co-isolated strains PA4-613, SE4-613, PA011, SE011, PA095 and SE095, the *P. aeruginosa* isolate (PA124) of which the supernatant stimulated growth and biofilm formation of *S. epidermidis*, a *S. epidermidis* isolate (SE078) of which the biofilm formation was reduced by PA124 and two additional *P. aeruginosa* isolates, PA066 and PA065. Both species were mixed in equal proportions and diluted 1:10 in fresh medium and cultured for 24h. After 24h, a quantitative culture on selective media was performed to determine the colony forming units (CFUs) of each species. Six of the eight *P. aeruginosa* strains showed a strong competitive advantage over all *S. epidermidis* strains with a competition index above 0 (**Table 3**). The two *P. aeruginosa* strains of which the biofilm supernatant showed a synergistic effect in the previous experiments also had a competitive index close to 0 during dual species culture with all *S. epidermidis* tested, indicating equal competition.

Table 3: Competition index during dual species culture. All values are log transformed and calculated as the CFU count of *P. aeruginosa* divided by the CFU count of *S. epidermidis*. Competition index = 0: equal competition; Competition index > 0: advantage for *P. aeruginosa*; Competition index < 0: advantage for *S. epidermidis.*

Stains	Competition index
PAO1 and SE1457	1.80
PAO1 and SE4-613	2.07
PAO1 and SE095	1.28
PAO1 and SE011	1.24
PAO1 and SE078	0.92
PA14 and SE1457	1.56
PA14 and SE4-613	1.72
PA14 and SE095	1.46
PA14 and SE011	1.06
PA14 and SE078	1.58
PA4-613 and SE1457	0.35
PA4-613 and SE4-613	0.44
PA4-613 and SE095	0.17
PA4-613 and SE011	0.03
PA4-613 and SE078	0.26
PA124 and SE1457	0.34
PA124 and SE4-613	0.11
PA124 and SE095	-0.04
PA124 and SE011	-0.03
PA124 and SE078	-0.15
PA011 and SE1457	1.99
PA011 and SE4-613	2.19
PA011 and SE095	2.11
PA011 and SE011	1.73
PA011 and SE078	2.10
PA065 and SE1457	0.85
PA065 and SE4-613	1.51
PA065 and SE095	1.28
PA065 and SE011	0.99
PA065 and SE078	0.94
PA066 and SE1457	1.01
PA066 and SE4-613	0.98
PA066 and SE095	1.53
PA066 and SE011	1.29
PA066 and SE078	1.06
PA095 and SE1457	1.77
PA095 and SE4-613	2.06
PA095 and SE095	2.15
PA095 and SE011	1.71
PA095 and SE078	1.83

3.4.6 The *P. aeruginosa* strains stimulating growth and biofilm of *S. epidermidis* produce low pyocyanin, pyoverdin and biosurfactant

The previous results suggest a mainly antagonistic relationship between the *P. aeruginosa* strains PAO1 and PA095 and *Staphylococci*, whereas the other two *P. aeruginosa* strains, PA4-613 and PA124 showed a less antagonistic or a synergistic effect on *S. epidermidis*. As the production of pyocyanin was associated with an inhibition of *S. epidermidis* biofilm and pyoverdin was shown to inhibit the growth of cystic fibrosis isolates[17,24], we also compared the production of these virulence factors by all *P. aeruginosa* isolates in absence of *S. epidermidis*. The production of virulence factors was highly variable among the clinical isolates, and the antagonistic strains PAO1 and PA095 produced significantly higher amounts of both virulence factors compared to the synergistic strains PA4-613 and PA124, which produced the lowest amount of pyocyanin and pyoverdin of all clinical isolates ($p<0.001$, **Figure 5**). In addition, rhamnolipids are biosurfactant molecules frequently produced by *P. aeruginosa* which play an important role in both biofilm formation and interspecies competition in Gram-positive as well as Gram-negative bacteria[25,26]. Therefore, we also determined biosurfactant production by the same four *P. aeruginosa* strains used in the supernatant experiments. Here, we found that the clinical isolate PA095 produced the highest amount of biosurfactant, followed by PAO1. The clinical isolates showing a synergistic effect on *S. epidermidis* produced very low amounts of biosurfactant shown by no droplet collapse or smear of the undiluted supernatant after 10s.

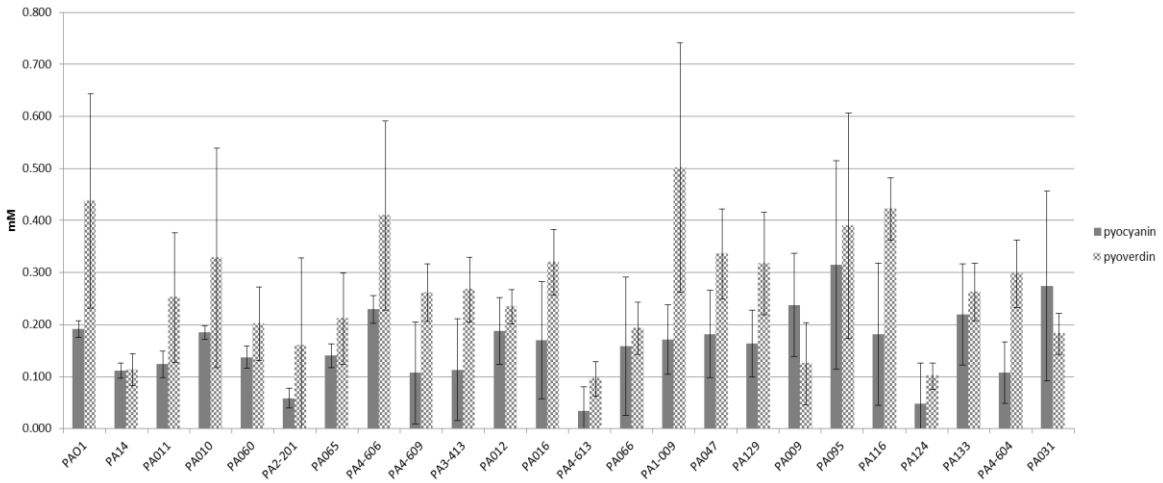

Figure 5: The pyocyanin and pyoverdin production by all *P. aeruginosa* isolates in the absence of *S. epidermidis*.

3.4.7 *S. epidermidis* biofilm supernatants increase the production of pyoverdin and pyocyanin of some *P. aeruginosa* clinical isolates

To investigate the effect of extracellular factors produced by *S. epidermidis* during biofilm formation on the virulence of *P. aeruginosa*, we quantified the production of pyocyanin and pyoverdin by all *P. aeruginosa* strains in the presence of the biofilm supernatant of four *S. epidermidis* clinical isolates (SE4-613, SE095, SE011 and SE4-609). The supernatant of all four *S. epidermidis* isolates significantly increased the production of pyocyanin in five and of pyoverdin in six *P. aeruginosa* strains (**Figure 6a and b**). Furthermore, 12 and 10 *P. aeruginosa* strains showed a respective increased production of pyocyanin and pyoverdin with at least one *S. epidermidis* and two *P. aeruginosa* strains, PA065 and PA060, increased the production of both factors in the presence of all *S. epidermidis* strains. The virulence of the other strains showed no significant change or was even reduced for some strain combinations (**Figure 6a and b**).

Figure 6: Changes in the pyocyanin (a) and pyoverdin (b) production by the different *P. aeruginosa* strains in the presence of the biofilm supernatant of four different *S. epidermidis* strains. The values are presented as a percentage of the change compared to the control (%Δ pyocyanin and %Δ pyoverdin). The transparent values are not significant ($p<0.05$).

3.4.8 *S. epidermidis* supernatants enhance the biofilm formation and growth of *P. aeruginosa*

We also aimed to investigate the effect of the biofilm supernatant of four *S. epidermidis* strains (SE4-613, SE095, SE011 and SE4-609), which were co-isolated with *P. aeruginosa*, on the growth and biofilm formation of 24 *P. aeruginosa* strains by measuring the final OD600nm after 24h or by staining the biomass with crystal violet. The change in OD600nm and biomass in the presence of *S. epidermidis* supernatant was calculated as a percentage of the control, 2x TSB with ultrapure water. In general, the biofilm supernatant of *S. epidermidis* seemed to have a synergistic or no effect on *P. aeruginosa*. The supernatant of SE4-613 and SE095 significantly increased the growth of 13 *P. aeruginosa* strains with an average of 33% and 28%, respectively (95% CI: 23-43% and 95% CI: 19-37%, $p<0.05$, **Figure 7a**) and the supernatant of SE4-609 and SE011 increased the growth of 11 *P. aeruginosa* with an average of 31% and 25%, respectively (95% CI: 20-43% and 95% CI: 16-34%, $p<0.05$). Furthermore, the supernatant of SE011 reduced the growth of three *P. aeruginosa* strains with an average of 19% (95% CI: 14-24%, $p<0.05$) and the supernatant of SE095 reduced the growth of one *P. aeruginosa* by 23%. The growth of the other *P. aeruginosa* strains was not significantly enhanced or reduced (**Figure 7a**).

The biofilm formation of five *P. aeruginosa* strains was consistently enhanced in the presence of the supernatant of all *S. epidermidis* strains with an average of 112% (SE4-613, 95% CI: 55-168%), 97% (SE4-609, 95% CI: 51-144%), 84% (SE095, 95% CI: 50-119%) and 100% (SE011, 95% CI: 55-145%, $p<0.05$ in all cases, **Figure 7b**). In addition, the biofilm formation of five *P. aeruginosa* strains was enhanced by at least one *S. epidermidis* strain and three *P. aeruginosa* strains formed less biofilm in the presence of the supernatant of one or two *S. epidermidis* isolates (41%, SE4-613; 36%, SE4-609; 31%, SE095). The biofilm formation of the remaining 12 strains, including the two reference strains, was not significantly increased or decreased by *S. epidermidis* supernatant (**Figure 7b**).

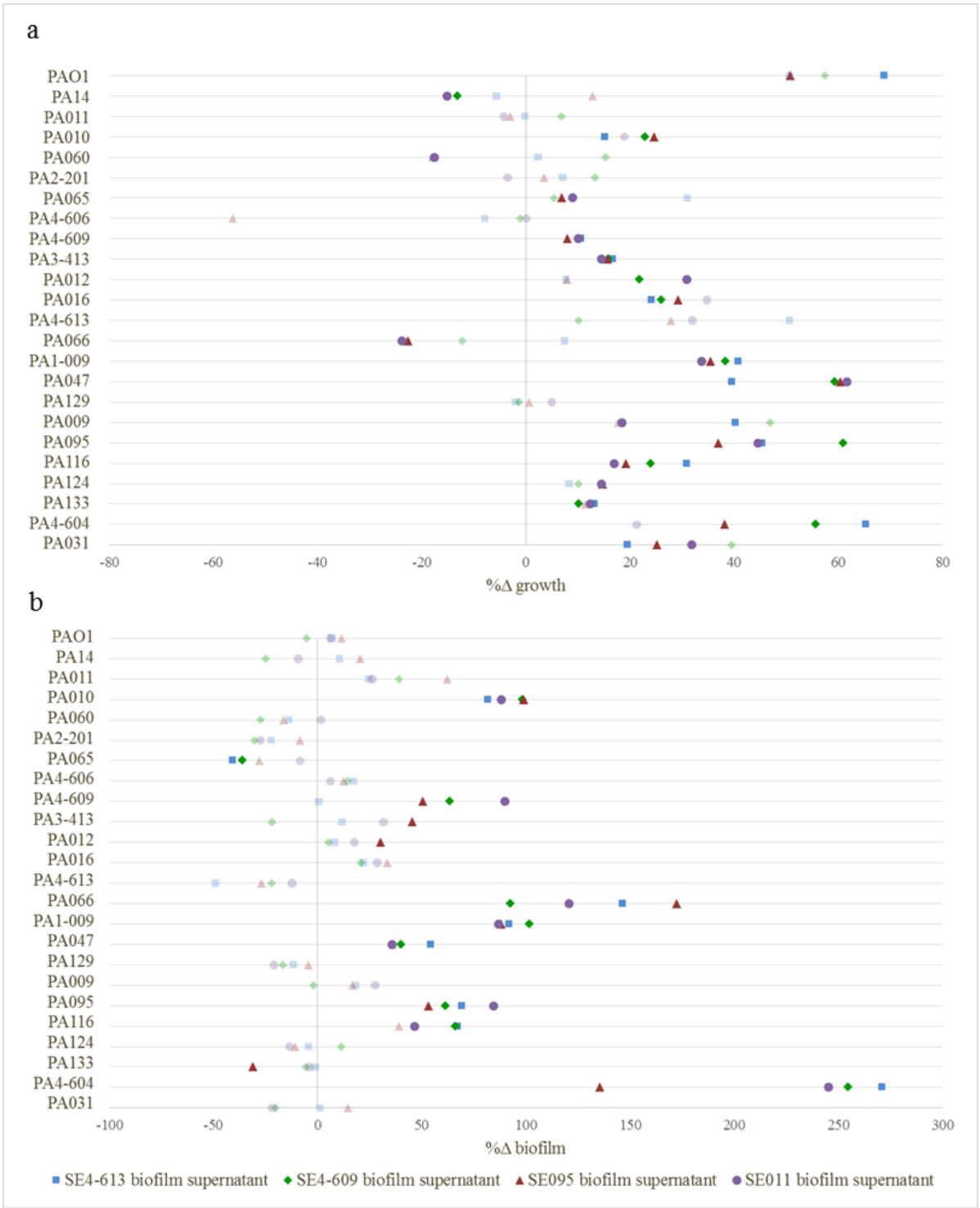

Figure 7: Changes in growth (a) and biofilm formation (b) after 24h by the different *P. aeruginosa* strains in the presence of the biofilm supernatant of four different *S. epidermidis* strains. The values are presented as a percentage of the change compared to the control (%Δ growth and %Δ biofilm). The transparent values are not significant ($p<0.05$).

3.4.9 Coating with *S. epidermidis* biofilm supernatants has no effect on the biofilm formation of most *P. aeruginosa* strains

To investigate the effect of coating of a surface with the *S. epidermidis* supernatant of four strains (SE4-613, SE095, SE011 and SE4-609) on the biofilm formation of 24 *P. aeruginosa* isolates, 96-well plates with peg lids were used. After 6h of growth, the biofilm production of most *P. aeruginosa* strains was unchanged, although the biofilm formation of four *P. aeruginosa* was significantly stimulated by the supernatant coating of SE095 with an average of 53% (95% CI: 39-43%, $p<0.05$, **Figure 8a**), the biofilm formation of three *P. aeruginosa* was stimulated by the supernatant of SE011 with an average of 73% (95% CI: 19-127%, $p<0.05$) and the biofilm formation of two was increased by SE4-613 coating with an average of 41% (95% CI: 39-43%, $p<0.05$). The *S. epidermidis* SE4-609, which was co-isolated with *P. aeruginosa*, had no significant effect on any *P. aeruginosa* isolate. In addition, one *P. aeruginosa*, PA133, showed a reduced adherence in the presence of the coated biofilm supernatant *S. epidermidis* SE095 (28%).

After 24h of growth, the biofilm formation of three *P. aeruginosa* strains was significantly increased by supernatant coating of SE4-609, SE095 and SE011 with an average of 65%, 76% and 90%, respectively (95% CI: 32-97%, 95% CI: 45-107% and 95% CI: 55-124%, $p<0.05$, **Figure 8b**). Furthermore, coating with the supernatant of SE4-613 increased the biofilm formation of one *P. aeruginosa* with 41%. Additionally, SE011 supernatant reduced the biofilm production of three *P. aeruginosa* with an average of 44% (95% CI: 27-61%; $p<0.05$) and coating with SE095 supernatant reduced the biomass of one *P. aeruginosa* with 45%. There was no significant change in the other *P. aeruginosa* strains in the presence of *S. epidermidis* supernatant coating (**Figure 8b**).

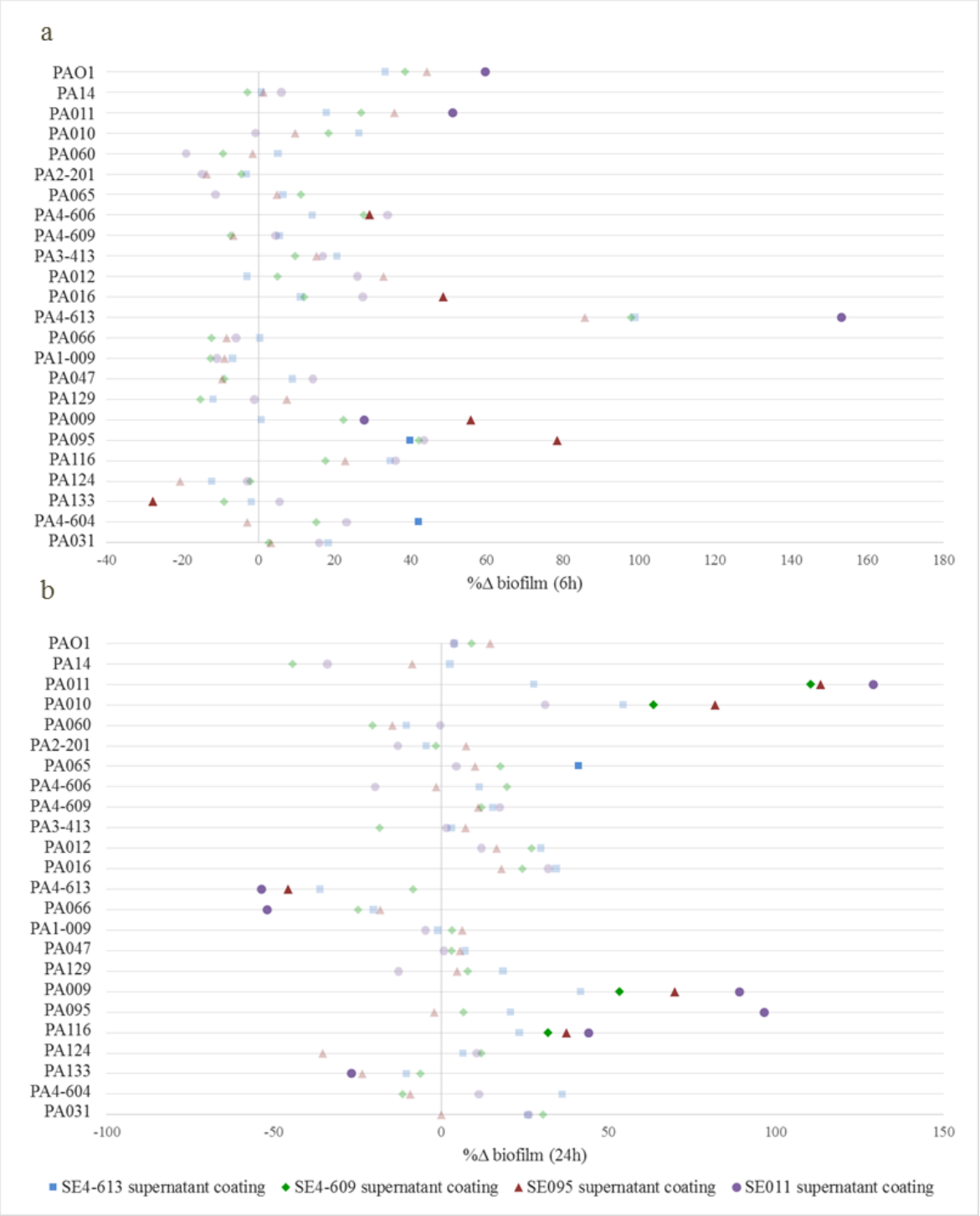

Figure 8: Changes in biofilm formation by the different *P. aeruginosa* strains on a surface coated with biofilm supernatant of four different *S. epidermidis* strains after 6h (a) and 24h (b). The values are presented as a percentage of the change compared to the control (%Δ biofilm). The transparent values are not significant ($p<0.05$).

3.5 Discussion

In this study we investigated the effects of extracellular factors produced by clinical isolates of *P. aeruginosa* and *S. epidermidis* during biofilm formation on the growth and biofilm production of the other, by both pre-coating a surface with biofilm supernatant and by incubating the inoculum in the presence of the supernatant. An antagonistic effect of *P. aeruginosa* on growth and biofilm formation of *S. epidermidis* has already been described before and was confirmed in most situations by this study[17-19,23]. However, some contradictions exist among different studies about the causative compounds of this antagonism, potentially due to the use of different strains and experimental conditions[17-19,24]. As both *P. aeruginosa* and *S. epidermidis* are highly versatile organisms, one *P. aeruginosa* strain can have a different effect on several *S. epidermidis* strains or the same *S. epidermidis* strain can react differently to different *P. aeruginosa* strains[17,18]. This phenomenon was clearly observed during interactions between *P. aeruginosa* and *S. aureus* isolated from cystic fibrosis patients, where *S. aureus* strains co-isolated with *P. aeruginosa* were less sensitive to an extracellular factor produced by *P. aeruginosa*, 2-heptyl-4-hydroxy quinolone N-oxide (HQNO), and *P. aeruginosa* strains co-isolated with *S. aureus* produced lower levels of this molecule compared to strains that were not co-isolated, suggesting co-evolution[21]. In the study by Qin et al, only reference strains were used whereas Pihl et al used a combination of reference strains and four *P. aeruginosa* and six *S. epidermidis* isolates from different sources[17-19,24]. Therefore, in this study we used reference strains and several clinical isolates from ETTs, including strains co-isolated from the same patient.

In general, the biofilm supernatant of *P. aeruginosa* strains co-isolated with *S. epidermidis* did not show a different effect on *S. epidermidis* growth and biofilm formation. There was, however, a much stronger antagonistic effect of the reference strain PAO1 and one of the co-isolated strains, PA095, compared to the other two. Moreover, these two strains even stimulated the growth of *S. epidermidis* and one stimulated the biofilm formation of a few strains after 24h. The difference between the strains with a synergistic and antagonistic effect is the production of the virulence factors pyocyanin and pyoverdin, which was significantly lower in the strains showing a synergistic effect. Pyocyanin is a redox-active antimicrobial compound, leading to a metabolic shift towards fermentation and a delayed growth in Gram-positive organisms[13]. Furthermore, it was shown to reduce the growth and enhance the biofilm formation in *S. aureus*, stressing the importance of this compound in interspecies interactions[13]. However, the study of Pihl et al showed the opposite of this study, a stronger inhibition of *S. epidermidis* biofilm formation by the *P. aeruginosa* with a defective pyocyanin production[18]. Additionally, this study also stated that *P. aeruginosa* had no effect on *S. epidermidis* growth, which is also in contraction with our study and the study of Qin et al.[18,19]. However, we are the first to report an increased growth of *S. epidermidis* in the presence of *P. aeruginosa* supernatant.

Most studies describe that *S. epidermidis* has no effect on the growth and biofilm formation of *P. aeruginosa*, which was also true for most combinations in this study, although the biofilm formation of some *P. aeruginosa* isolates was increased by the clinical isolates of *S. epidermidis*. Furthermore, here we showed that the supernatant of *S. epidermidis* biofilms increased the production of the virulence factors pyocyanin and pyoverdin of some *P.*

aeruginosa strains. These results are also in accordance with previous studies showing an increased virulence of *P. aeruginosa* as a response to N-acetylglucosamine and autoinducer-2 secreted by Gram-positive organisms[11,12,25,26]. Potential discrepancies between this study and previous studies might be explained by the way the supernatant was harvested. Here, we used the biofilm supernatant of mature biofilms of *P. aeruginosa* and *S. epidermidis*, including all quorum sensing regulated factors produced at high cell densities whereas Phil et al. and Qin et al. used the supernatant of planktonic cultures[17-19].

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Chapter 4: The endotracheal tube microbiome associated with *Pseudomonas aeruginosa* or *Staphylococcus epidermidis*

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4.1 Abstract

Ventilator-associated pneumonia (VAP) is one of the commonest hospital-acquired infections associated with high mortality. VAP pathogenesis is closely linked to organisms colonizing the endotracheal tube (ETT) such as *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, the former a common commensal with pathogenic potential and the latter a known VAP pathogen. However, recent gut microbiome studies show that pathogens rarely function alone. Hence, we determined the ETT microbial consortium co-colonizing with *S. epidermidis* or *P. aeruginosa* to understand its importance in the development of VAP and for patient prognosis. Using bacterial 16S rRNA and fungal ITS-II sequencing on ETT biomass showing presence of *P. aeruginosa* and/or *S. epidermidis* on culture, we found that presence of *P. aeruginosa* correlated inversely with patient survival and with bacterial species diversity. A decision tree, using 16S rRNA and patient parameters, to predict patient survival was generated. Patients with a relative abundance of *Pseudomonadaceae* <4.6% and of *Staphylococcaceae* <70.8% had the highest chance of survival. When *Pseudomonadaceae* were >4.6%, age of patient <66.5 years was the most important predictor of patient survival. These data indicate that the composition of the ETT microbiome correlates with patient prognosis, and presence of *P. aeruginosa* is an important predictor of patient outcome.

4.2 Introduction

Assisted ventilation is performed through an endotracheal tube (ETT), that is readily colonized by bacteria within 24 hours after intubation[1,2]. These microorganisms, migrating along the ETT cuff and inside the lumen of the ETT, form a so called biofilm, together with a network of secretions. Biofilm formation is indeed facilitated by the presence of nutrient-rich patient material which accumulates in the lower part of the ETT[3].

Ventilator associated pneumonia (VAP) occurs in approximately 9-27% of all mechanical ventilated patients after at least 48h of ventilation and is associated with high mortality and morbidity[4,5]. The exact mechanisms leading to the development of VAP are not understood, although a strong link exists with the microbial consortium on the ETT[1,6,7]. Endotracheal secretions often mirror the microbial consortium present in the ETT, at least at the resolution achieved by culture methods[8,9]. For this reason it is believed that micro-aspiration of detached pieces of biofilm containing harmful organisms might cause infection in the lower respiratory tract, and are believed to significantly contribute to the occurrence of (VAP)[1]. Regular screening of endotracheal secretions facilitates early diagnosis of the microorganisms linked to VAP and has been shown to impact on patient treatment and survival[10].

Pseudomonas aeruginosa is one of the most common causes of VAP followed by Gram-positive organisms like *Staphylococcus aureus*[5]. In some cases, however, no clear pathogen can be detected by conventional culture methods[11], while in other cases a significant growth of oro-pharyngeal or cutaneous commensals including *Staphylococcus epidermidis* can be observed, suggesting that these organisms are not as harmless as frequently believed[12]. Recently, the presence of oro-pharyngeal microorganisms was demonstrated on the ETTs of mechanically ventilated patients[6] and in respiratory samples[11], however, the presence of any of these organisms on the ETT did not always lead to VAP with these bacteria[7,12], indicating a contribution from host factors and the general health status of the patient. In fact, an important role for the entire bacterial consortium on the ETT in the development of VAP has been suggested[6] and involves interspecies interactions that provide nutrient sources or produce growth inhibiting molecules[13,14]. While the presence of specific bacteria leading to dysbiosis has been shown for diseases like periodontitis and cystic fibrosis[15,16], there is a lack of knowledge of such relationships within ETT biofilms and their potential role in the development of VAP.

Next generation sequencing technology is a powerful molecular tool to detect a wide variety of species, including the unculturable fraction. By screening a large collection of ETTs obtained from mechanically ventilated patients at the Antwerp University Hospital over a three-year period, we selected 39 ETTs that showed positive culture results for *P. aeruginosa*, a major VAP pathogen, and *S. epidermidis*, the most frequently cultured bacterium in this study. We aimed (i) to study organism and species diversity in ETT biofilms using a culture independent metagenomic approach by bacterial 16S rRNA and fungal internal transcribed spacer (ITS) amplicon sequencing, (ii) to determine the core microbiomes on the ETTs which were culture positive for the key organisms *P. aeruginosa* and *S. epidermidis* and (iii) to link

these findings with patient parameters in an attempt to identify markers of disease or of patient outcome.

4.3 Results

4.3.1 Abundance of ETT biomass does not correlate with VAP aetiology or patient prognosis

To study if the ETT biomass quantity or composition correlate to the development of VAP or to patient prognosis, we prospectively collected 203 ETTs from mechanically ventilated patients admitted to the intensive care unit (ICU) during a 3-year period, from April 2011 until December 2013, at the University hospital Antwerp, Belgium. Of these 203 patients, 44 progressed to pneumonia (VAP). Previous studies have shown that *P. aeruginosa* is a common VAP pathogen associated with high mortality and morbidity[5] whereas *S. epidermidis* is the most frequently isolated bacterium in this study (51/203 ETT) that under specific circumstances can become a nosocomial pathogen[12]. *P. aeruginosa* was cultured from 36/203 ETT and was the second most common bacterium (**Figure 1a)**. To further study co-carriage with *P. aeruginosa or S. epidermidis*, ETTs from 39 patients showing presence of *P. aeruginosa* (n=13), *S. epidermidis* (n=21), or both (n=5) on culture were selected for further analysis. Fifteen of these 39 patients progressed to pneumonia (VAP). In the ETTs showing presence of *P. aeruginosa*, this organism was also identified as the aetiology of the 4 VAP cases in broncho-alveolar lavage (BAL) cultures of these patients. However, in ETTs showing presence of *S. epidermidis*, other organism(s) were identified as the aetiological agent of VAP (9/11) or as a non-*aureus Staphylococcus* (1/11) or remained unidentified (1/11). Summary of the data from all 39 patients is shown in **Table 1** and details on patients progressing to VAP in **Supplementary Table S1**.

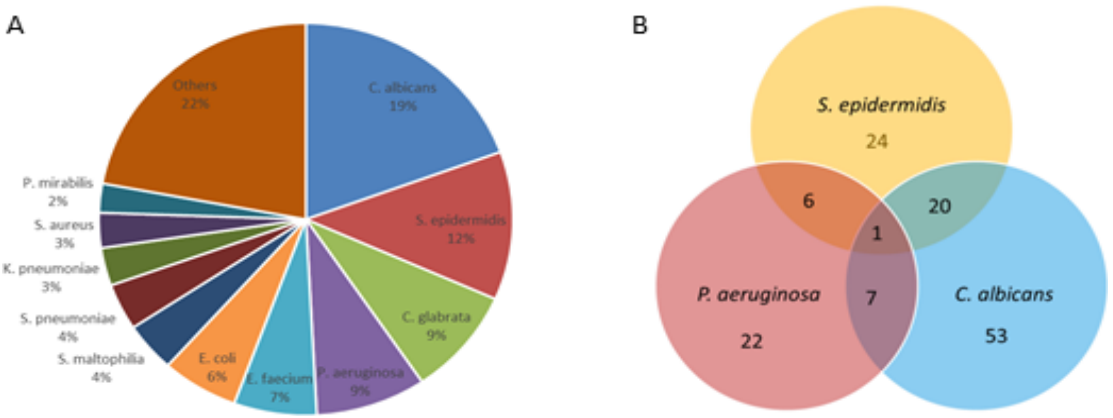

Figure 1: Culture results of 203 ETTs collected during the study (a) and of ETTs showing presence of *P. aeruginosa, S. epidermidis* and *C. albicans* (b).

Biomass was visually scored under a 5x microscope as present or absent and validated by histology (**Figure 2)**. H&E staining revealed a typical laminar patterning in the ETT biomass associated with small clusters of bacteria or yeast hyphae. We also found many lacunae in the structures that harboured human cells. Similar structures have been previously observed by

Inglis et al.,[17] who performed light and confocal scanning laser microscopy on ETT biofilms. Light and fluorescence microscopy allow the preservation of the ETT biofilm structures, in contrast to scanning electron microscopy used in most studies[2,9,18] were the original structure is lost.

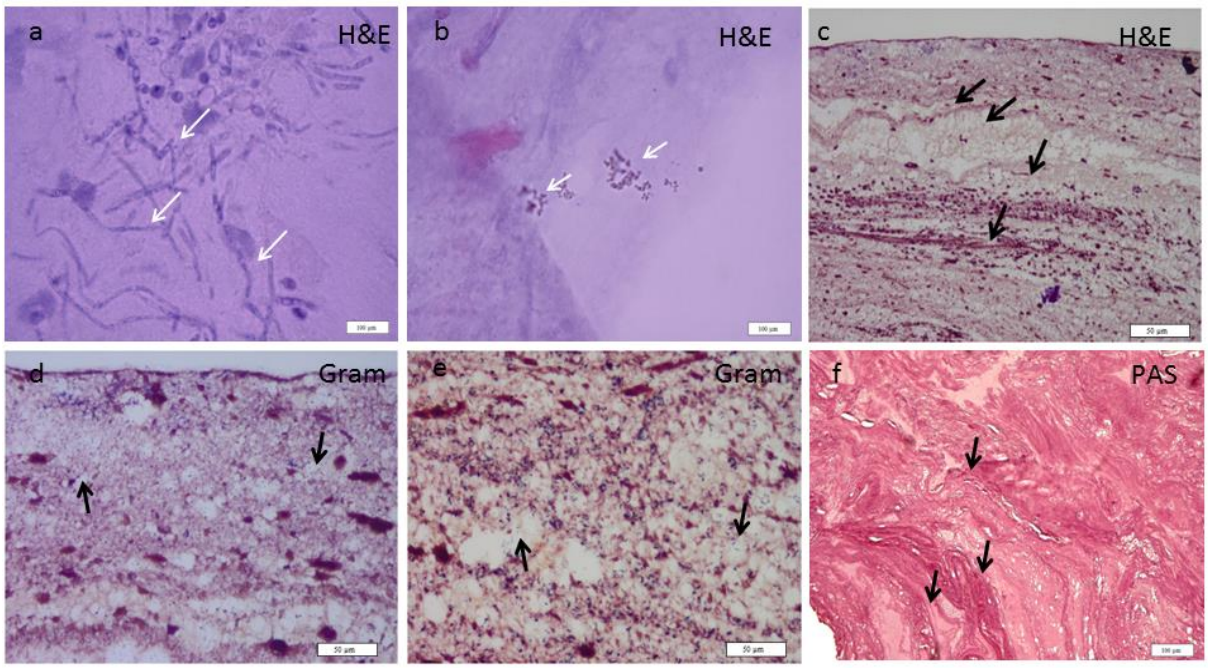

Figure 2: Histology of typical ETT biofilms. H&E staining revealed the presence of candida hyphae, white arrows (a); bacterial clusters, white arrows (b); and a clearly layered structure, black arrows (c); lacunae that harbored human cells are shown in the Gram-stained slices (d and e, black arrows); PAS stain also showed the layered structure (black arrows) and many cavities (f).

In our study, the presence of visible biomass in the ETT lumen did not correlate with development of VAP (P = 0.104) or patient survival (P = 0.100), indicating that the amount of ETT biofilm is not a major effector of VAP or patient prognosis. During mechanical ventilation, endotracheal secretions are removed on a regular basis with a suction catheter to prevent biofilm formation and VAP[19]. Furthermore, the ETT biofilm mainly consists of patient material in addition to bacteria and the exact amount of secretions produced is patient dependent[17]. These findings suggest that the presence of biomass as such is not necessarily bad for the patient prognosis and composition might play a role as well.

In addition, the APACHE II scores also did not significantly differ among the patients that developed VAP or not (P = 0.943) or survived or not (P = 0.196), indicating that the physiological condition was the same at the time of intubation. This result is confirmed by k-means clustering and logistic regression analysis which showed no correlation between patient survival and the reason of admission to the ICU. These data raised the question what other effectors, like the microbial consortium on the ETT or other patient related parameters are likely to play a role in VAP aetiology and patient prognosis.

Table 1: Overview of patient and ETT culture data. 39 ETTs were collected from same number of patients. 15 patients developed VAP during their intubation period. P*. aeruginosa* and/or *S. epidermidis* identified from ETT cultures and subsequent MALDI-TOF. Patients who developed VAP in each group are indicated.

	Total N° ETT: 39
Male	26
Female	11
Age	33-83 (median 61)
Days of intubation	2-43 (median 11)
VAP cases	15
Culture *P. aeruginosa*	13 (4 VAP)
Culture *S. epidermidis*	21 (9 VAP)
Culture *P. aeruginosa* and *S. epidermidis*	5 (2 VAP)

4.3.2 A two-step cluster analysis reveals three natural clusters with patient survival and development of VAP as the two main cluster predictors

To investigate patient-related parameters, the 39 patients were subdivided by a two-step cluster analysis, which facilitates the discovery of natural clusters of individuals with similar characteristics within a larger population[20]. The 39 patients were divided into three clusters with patient survival upon extubation (predictor importance 1) and development of VAP (predictor importance 0.55) being the most important cluster predictors (**Table 2**). All patients in clusters 2 and 3 (n=16 and 12, respectively) survived whereas all in cluster 1 (n=11) died. Details of the patients are shown in **Supplementary Table S1**. Clusters 2 and 3 are differentiated by the development of VAP; 13 of the 16 patients (81.2%) in cluster 2 and none of the 12 in cluster 3 progressed to VAP. On the other hand, only 2 patients from cluster 1 (18.2%), wherein 100% mortality was observed, developed VAP. Remarkably, all patients in cluster 2, who showed the highest incidence of VAP, survived. Because the development of VAP is usually known to be associated with high mortality[5], a combination of other factors might determine survival in this subset of patients. The third most important predictor of clustering was the presence of a visible biomass on the ETT upon extubation (predictor importance 0.25). All ETTs of cluster 3 and 9/11 (81.8%) ETTs of cluster 1 showed a visible biomass, while in only half of the ETTs of cluster 2 a biomass was visible.

As a fourth predictor, *P. aeruginosa* was more frequently isolated from ETTs in cluster 1 (7/11), while *S. epidermidis* was more frequent in cluster 2 (9/16 alone and 5/16 with *P.*

aeruginosa) and cluster 3 (8/12) (predictor importance 0.175). Average APACHE II scores (predictor importance 0.170) were similar for cluster 1 (26.55) and cluster 2 (25.5) and lower for cluster 3 (18.50), where all patients survived and none developed VAP. A least significant difference (LSD) post-hoc analysis showed that survivors in cluster 2 were on average 10 years younger than the patients who died in cluster 1 (P = 0.043), while there was no significant difference in age compared to cluster 3.

Table 2: Two-step cluster analysis of patient data. Variables are listed in order of importance.

Variables	**Cluster 1 (n=11)**	**Cluster 2 (n=16)**	**Cluster 3 (n=12)**
Patient survival (%)	0%	100%	100%
Patients developing VAP (n,%)	2, 18.2%	13, 81.8%	0, 0%
Biomass visible on ETT (n,%)	9, 81.8%	8, 50%	12, 100%
ETT culture results (n,%)	*P. aeruginosa (*7, 63.6%) *S. epidermidis* (4, 36.4%)	*P. aeruginosa* (2, 12.5%) *S. epidermidis* (9, 56.25%) Both (5, 31.25%)	*P. aeruginosa* (4, 33.3%) *S. epidermidis* (8, 66.7%)
APACHE II scores (average)	26.55	25.5	18.5
Age (average in years)	67.82	57.69	60.5
Duration of intubation in days (average)	12.45	13.69	11.42

4.3.3 The microbiome present on the ETTs correlates with patient prognosis

Next, we questioned whether the microbial composition of the ETT biomass might correlate to patient prognosis. Therefore, 16S rRNA analysis was performed to identify the ETT microbial consortium co-existing with *P. aeruginosa* or/and *S. epidermidis* and subjected these data together with the patient parameters to a multivariate analysis. First, we generated a decision tree that identified a low relative abundance of family *Pseudomonadaceae* as the key predictor of patient survival (**Figure 3**). A relative abundance of *Pseudomonadaceae* below 4.6% and of *Staphylococcaceae* below 70.8% correlated with a high chance of patient survival. When the relative abundance of *Pseudomonadaceae* was above 4.6%, patient age below 66.5 years also correlated with a higher chance of survival, confirming the importance of the organisms present on the ETTs and age of the patient (**Figure 3**).

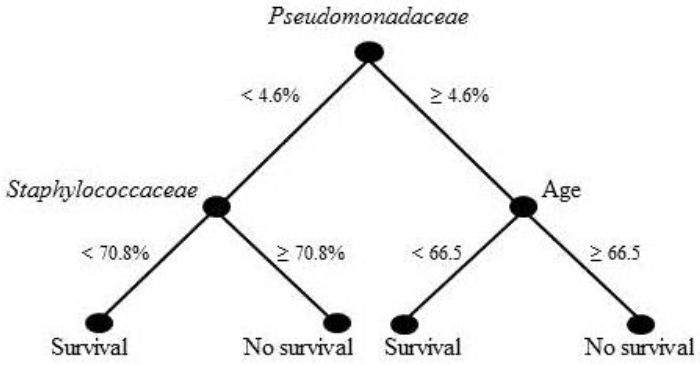

Figure 3: Decision tree predicting patient survival.

Second, we investigated whether other members of the ETT microbiome could be associated with patient survival using the online binomial tool LefSe dedicated for biomarker discovery[21]. This tool considers frequency as well as relative microbial abundance and performs a linear discriminant analysis (LDA) to calculate the effect size of each parameter[21]. An LDA score above 2 was considered significant. LefSe analysis showed that ETTs of patients who survived (n=28) were more likely to harbour the phylum *Actinobacteria* (**Figure 4a**, n=11, LDA score of 4.8). Within this phylum, *Actinomyces* and *Corynebacterium* were more frequently identified in the ETTs of the surviving patients (18/28 and 15/28, respectively) compared to the patients that did not survive (1/11 and 4/11, respectively; LDA score >3.6). In addition, *Bifidobacterium adolescentis* was identified in 9/28 ETTs of the survivors while it was completely absent in the ETTs of the patients that died (LDA score > 2.4). Furthermore, the ETTs of patients that died showed a close association with the genus *Pseudomonas*, including *P. aeruginosa* (8/11)*, P. fluorescens* (3/11) and uncultured *Pseudomonas spp*. (6/11). Also noteworthy was the presence of *Morganella morganii* (4/11), *Burkholderia cepacia* (2/11), *Proteus mirabilis* (2/11), *Marinomonas* sp. MED121 (2/11), and *Xylella fastidosa* (2/11) on the ETT of the patients that died. While these were found in only a fraction of the ETTs from the non-surviving patients, their frequency and abundance was significant in this group compared to the ETTs of the survivors (*Morganella morganii* 2/28, others absent, LDA score >2).

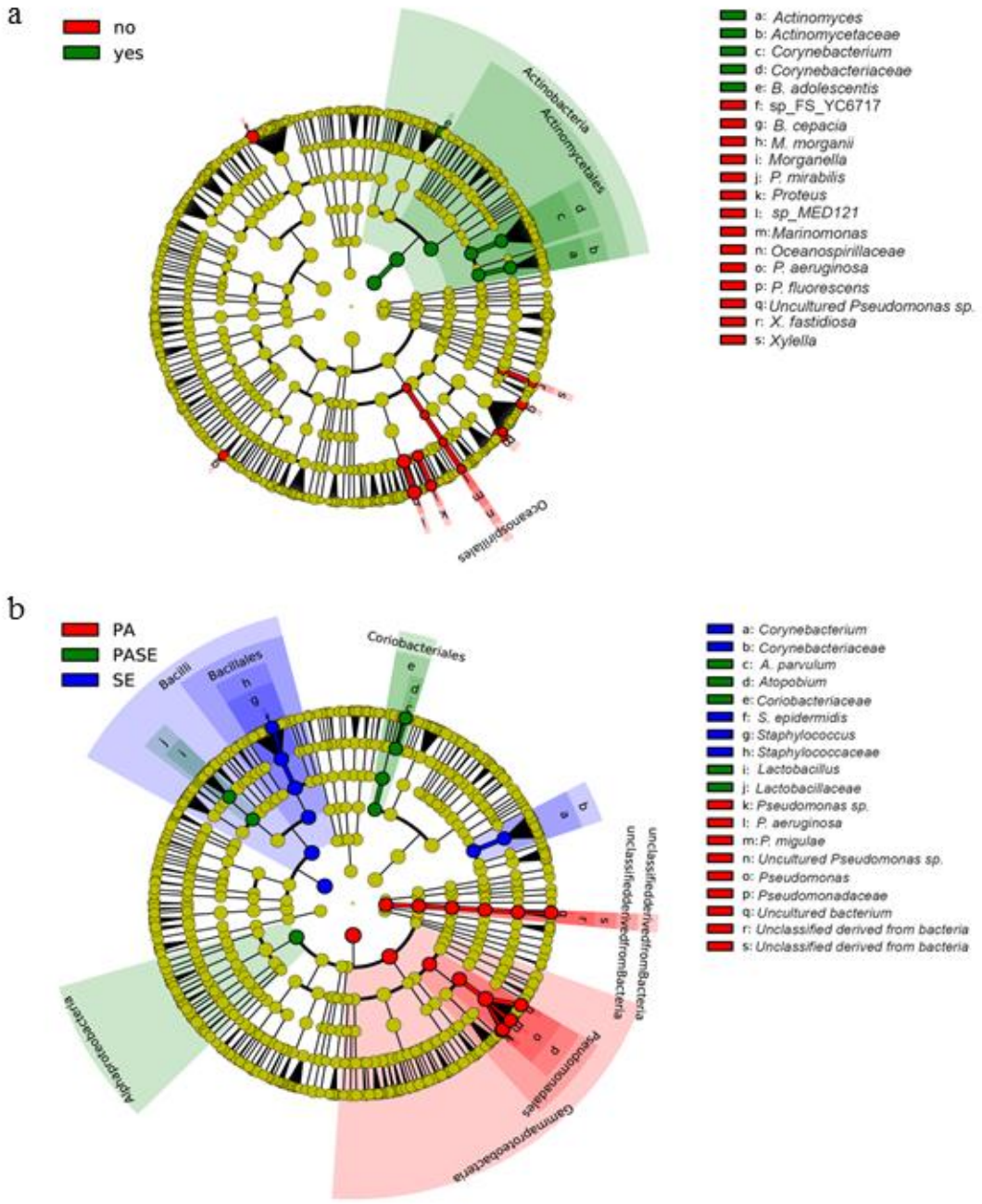

Figure 4: Cladogram showing the association of distinct ETT microbial components (OTUs) with patient survival (a) or with the 3 groups of ETTs defined by culture (b). The inner circles represent the highest taxonomic level and the coloured bands contain the taxonomic levels which are significantly more associated with one group of patients (LDA score >2). The cladogram was generated using the LefSe galaxy tool. A. Red: patients that did not survive upon extubation; Green: patients that survived upon extubation. B. Red: ETT with positive culture for *P. aeruginosa*; blue: ETT with positive culture for *S. epidermidis*; green: ETT with positive culture for both *P. aeruginosa* and *S. epidermidis.*

4.3.4 Distinct predominant microbiomes are associated with the presence of *P. aeruginosa* and *S. epidermidis*

After we identified significant associations between microbial composition and patient prognosis, we also questioned whether a microbial fingerprint might be linked to development of VAP. As the aetiology of VAP differed between the three groups of ETTs identified by culture (**Supplementary Table S1**), we aimed to identify the differential microbiomes of these three groups in correlation to VAP as well as a common ETT core microbiome.

Firstly, classification of all 16S sequences by alignment to the SILVA reference database showed a total of 8 different phyla that were associated with the ETTs analysed in this study. Within these phyla, 69 different families harbouring 354 different species were identified. The majority (61%) of the sequences belonged to three phyla: *Proteobacteria* (34%), *Firmicutes* (18%) and *Actinobacteria* (9%) as shown in **Figure 5a**. Of note, 39% of the sequences remained unclassified when compared to existing reference sequences in the Greengenes, SILVA and RDP databases. An additional BLAST against the NCBI nr database revealed that the majority (89%) of these were of the *Proteobacteria* family.

Secondly, we aimed to identify which families were present on the majority of ETTs across the three groups regardless of their relative abundance. *Enterobacteriaceae* and *Phyllobacteriaceae* could be identified on the majority of the ETTs (77% and 86%, respectively) (**Figure 5c**). However, at the level of the genus and species, the *Enterobacteriaceae* family showed a large diversity. The genera *Escherichia* and *Klebsiella* were the most and second-most commonly identified with *E. coli*, *E. albertii* and *K. pneumoniae* as the main representatives. However, none of these species were present in more than 50% of all ETTs. In contrast to the high variability of the *Enterobacteriaceae*, the *Phyllobacteriaceae* had only one representative, *Phyllobacterium myrsinacearum*, which was present at low abundance in 86% of all ETTs. More details on the species distribution are given in **Supplementary Table S2**.

Thirdly, we identified the phylum distribution and species diversity in the three groups of ETTs (**Figure 5b**). In the ETTs of the *P. aeruginosa* group, the phylum *Proteobacteria* was most abundant (98%), while *Firmicutes* and *Actinobacteria* (1% each) were present in a minority. In contrast, ETTs belonging to the *S. epidermidis* group showed a more even distribution over the three phyla: *Proteobacteria* (56%), *Firmicutes* (30%) and *Actinobacteria* (12%). In the third group, the majority of sequences were also assigned to the *Proteobacteria* (72%), followed by *Actinobacteria* (19%) and *Firmicutes* (9%). At lower taxonomic levels, ETTs belonging to the *S. epidermidis* group showed the maximum species diversity (290 species in total) and the highest average number of species per tube (35 species, 3357 reads). This was in contrast to the ETTs of the *P. aeruginosa* group that harboured on average 20 different species (3791 reads) per tube and in total 116 different species ($P = 0.029$) (**Supplementary Table S3**).

Lastly, we used the LefSe tool to discover distinct microbial signatures associated with each of the three ETT groups (**Figure 4b)**. In the *P. aeruginosa* group, only bacteria belonging

to the family *Pseudomonadaceae* were identified and these included various *Pseudomonas spp.* These data provide *in vivo* evidence for previous laboratory based studies describing the production of antibacterial factors and inhibition of competitive colonizers (non-pseudomonads) both on co-cultures and in a biofilm phenotype by *P. aeruginosa*[22-27]. In the *S. epidermidis* group, *Corynebacteriaceae* were identified in 17/21 (80%) of the ETTs (**Figure 5c**). Since the VAP aetiology was variable in this group, differences in the ETT microbial signatures of patients developing VAP or not were sought. Interestingly, presence of *Klebsiella pneumoniae* (7/9 VAP, 2/12 non-VAP) and *Serratia marcescens (*8/9 VAP, absent in non-VAP) among the *S. epidermidis* group that developed VAP was highly significant (LDA >4). Finally, in the group of ETTs containing both *P. aeruginosa* and *S. epidermidis* (n=5), *Lactobacillaceae* (4 ETTs) and *Coriobacteriaceae* (3 ETTs) were more prevalent (**Figures 4b and 5c**). The family of the *Lactobacillaceae* showed mainly *Lactobacillus* but sequences could not be identified to the species level. *Atopobium parvulum* was the main representative of the *Coriobacteriaceae.* We can conclude that the microbial composition of the ETTs consists of two parts: a common (up to family level) microbiome shared by all ETTs and a clearly distinct microbiome at least for the ETTs harbouring *P. aeruginosa* or *S. epidermidis*. Importantly, our finding that presence of *P. aeruginosa* (and lower species diversity) correlates strongly with worse patient outcome underlines the importance of detecting its presence as a marker of patient prognosis.

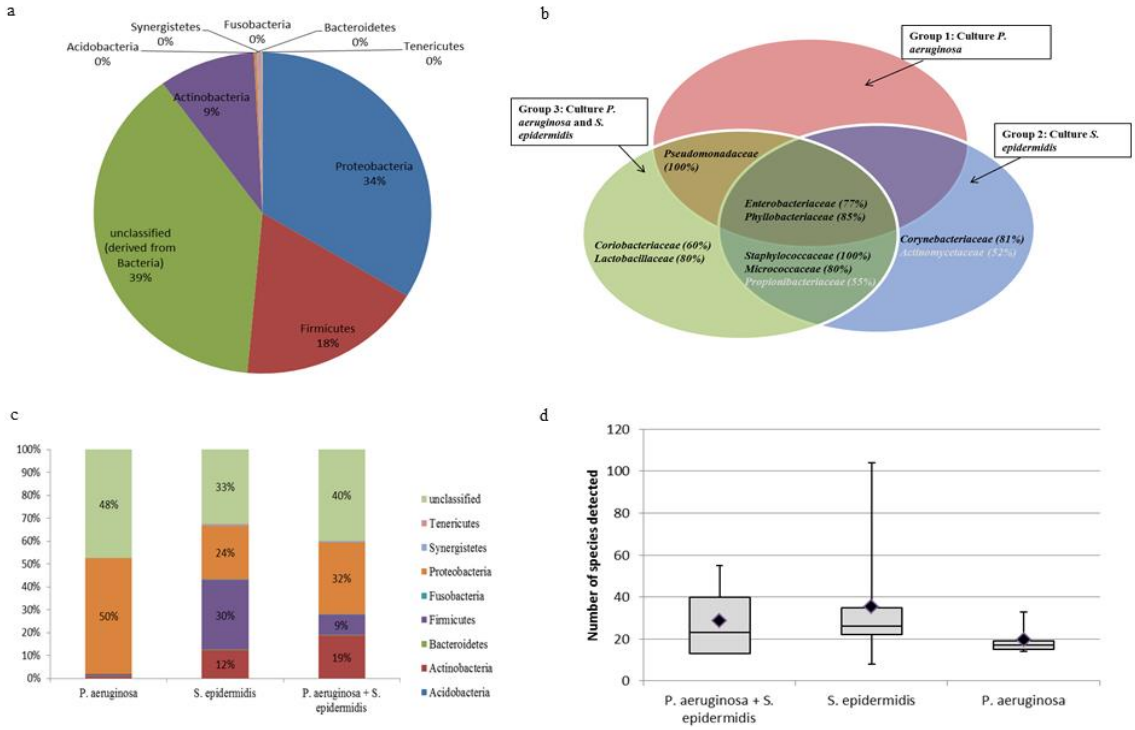

Figure 5: Distribution of the relative abundance of the major phyla. The phyla Proteobacteria and Firmicutes were the most abundant. BLAST analysis revealed that approximately 90% of the unclassified sequences also belonged to Proteobacteria (a). OTUs defined on the family level found to be associated with the 3 groups of ETTs. Families present in at least 50% of the ETTs in a certain group were assigned to the microbiome of that group. (b). Distribution of OTUs, defined at the phylum level, within the 3 groups of ETTs (c). Species diversity in the three groups of ETTs. Black squares: average number of species (d).

4.3.5 *Candida spp.* are the most common fungi in the ETT microbiome

During culture of the ETTs, *Candida albicans* and *Candida glabrata* were the most and third most common organisms isolated, with an incidence of 81/203 and 37/203 ETTs, respectively (**Figure 1a and b**). In the selection of ETTs used for this study, *Candida* spp. were present in 17/39 tubes, of which 11 also showed the presence of *S. epidermidis*, 4 of *P. aeruginosa* and 2 of both. ITS sequencing identified 55 different fungal species, of which the majority (70%) belonged to the phylum *Ascomycota* and a smaller proportion (27%) to *Basidiomycota*. All 17 samples harboured at least one and in most cases two of the following *Candida* species: *C. albicans* (11 ETTs), *C. glabrata* (13 ETTs) or *C. tropicalis* (14 ETTs). Additional *Candida* species identified include *C. catenulata* (8 ETTs), *C. dubliniensis* (2 ETTs) and *C. parapsilosis* (1 ETT) (**Supplementary Table S3**).

Furthermore, LefSe analysis assigned the family *Onygenaceae* to the group of ETTs from VAP patients with an LDA score of 2.5, however, this association was only present at order and family level, probably due to the low number of VAP patients in this subgroup (4 ETTs). Of note, relative abundance of strict and facultative anaerobes was significantly higher in the ETTs harbouring *Candida spp.* compared to the ETTs without *Candida* (54% versus 43%

relative abundance, respectively; strict anaerobes 13% versus 5%; facultative anaerobes 41% versus 38%, respectively) (Z-test, $P < 0.001$). These differences between the two groups were primarily due to relative proportions rather than species composition except for the genus *Prevotella*, which was found to be significantly associated with presence of *Candida* by LefSe analysis (LDA<2). Prior studies have shown that fungi are important for maintaining oral microbiome homeostasis but their role in the ETT biofilm remains unclear[28,29]. Remarkably, in our samples, *Candida* spp. were more frequent in the ETTs harbouring *S. epidermidis* and were only rarely co-isolated with *P. aeruginosa* (**Figure 1b)**. These results support earlier findings that *P. aeruginosa* and *C. albicans* share an antagonistic relationship[30] whereas *Staphylococci* and *C. albicans* are thriving well together[31].

4.1 Discussion

This study, to our knowledge, is the largest to date analysing the ETT biofilm in ventilated patients. Instead of a random molecular profiling of the bacterial community in the ETT, we opted to study the accessory microbiome co-colonizing with *P. aeruginosa* or *S. epidermidis*, the former is one of the most common causes of VAP and the latter one of the most common commensals with a known pathogenic potential. Both are the most common bacteria cultured from the ETTs in this study. Our aim was to decipher consistent associations between presence of *P. aeruginosa* or *S. epidermidis* and other organisms in the ETT biofilm in correlation with multiple patient parameters. Finally, this study provides a proof-of-concept of utilization of 16S microbiome data and patient parameters to generate a prognostic algorithm to predict patient survival.

We found that *P. aeruginosa* was consistently present in patients that did not survive upon extubation, suggesting that it might be an indicator of a worse patient outcome. Multivariate analysis of 16S data and patient parameters showed that relative abundance of *Pseudomonadaceae* was the most important predictor of patient survival followed by age and relative abundance of *Staphylococcaceae*. Our identified best-case scenario for patient survival was an abundance of *Pseudomonadaceae* below 4.6% and of *Staphylococcaceae* below 70.8%. When the relative abundance of the *Pseudomonadaceae* was above 4.6%, a patient age <66.5 years became the most important predictor of patient survival.

Although this algorithm is based on ETT microbiome data and represents a later stage of microbial colonization, previous research has shown consistent correlations between organisms isolated from ETTs and earlier stage respiratory samples like endotracheal aspirates and BALs[8,18]. In the present study, for patients who developed VAP due to *P. aeruginosa* (BAL cultures and clinical diagnosis), we were also able to identify the pathogen in the ETT of all patients using cultures and 16S analysis. Therefore this algorithm would be a useful addition to current ICU protocols in hospitals performing routine endotracheal aspirate sentinel cultures as well as for clinical trials pre-enriching for patients colonized with *Pseudomonas aeruginosa* and likely to develop VAP.

In addition to *P. aeruginosa*, the microbiome of patients who did not survive harboured other potentially pathogenic bacterial genera such as *Morganella*, *Proteus*, *Burkholderia* etc (**Figure 4a**). LefSe analysis showed that not all genera were consistently present in all

patients who died, however, there was a clear difference with the microbiome of surviving patients. The ETT microbiome of the latter group of patients tended to harbour the phylum *Actinobacteria,* which include *Bifidobacteria*, commonly used as probiotics, and non-diptheriae *Corynebacteriaceae*. Interestingly, a recent study analysing the benefits of probiotic *Bifidobacterium longum* administration in patients undergoing surgery, showed that an increase in relative abundance of *Actinobacteria* in the gut correlated inversely with blood inflammatory parameters[32]. These data support our findings that surviving patients tend to harbour a 'healthy' microbiome. Whether microbial shifts or dysbiosis in the endotracheal microbiome are the cause or the consequence of a worse patient outcome remains to be investigated and would require sequential sampling of the endotracheal microbiota in order to obtain better insight on the community dynamics.

Loss of microbial diversity has been previously identified as a consistent marker of a diseased microbiome[33,34]. In our study, while the incidence of VAP was indeed lower in the *S. epidermidis* colonized group, the patients who did develop VAP in this group did not show any loss of microbial diversity compared to others in this group (p=0.464). In fact, we found a significant loss of microbial diversity to be associated with *P. aeruginosa* colonization. This competitive exclusion is a hallmark of *P. aeruginosa* colonization and has also been previously observed in cystic fibrosis[26]. These data are also supported by *in vitro* studies demonstrating the predominant nature of *P. aeruginosa* and its inhibition of other potential pathogens or colonizers[13,22,25,26]. Of note, unlike the *P. aeruginosa* colonized group, the high microbial diversity in the *S. epidermidis* colonized group was also reflected in the concurrent difficulty in identifying a clear VAP pathogen in BAL cultures. While the possibility exists that the causative pathogen is a nonculturable (in the culture conditions utilized) organism, VAP might also be caused by a

a microbial consortium without a predominant pathogen[14]. Finally, lack of a clear VAP pathogen in BAL might also be due to the fact that microbiology laboratories conventionally report only the predominant organism (in most cases a *Staphylococcus* in the *S. epidermidis* group), while a low abundance pathogen might be missed on culture. In the *S. epidermidis* group, we found a consistent co-presence of *K. pneumoniae* and *S. marcescens*, present in relatively low numbers (median: 10.5 and 5 reads respectively) in patients who developed VAP whereas both were absent in the patients that did not develop VAP. Of note, ETT cultures did not grow *K. pneumoniae* and *S. marcescens* and their presence was only detected upon 16S sequencing. Since the ETT reflects the end-stage microbiome, the potential role of these correlations remain to be validated *in vitro* and in earlier stage patient samples.

Finally, *Candida spp.*, which are known nosocomial pathogens, were more often isolated from ETTs of the *S. epidermidis* group (13/17) These results corroborate prior studies demonstrating the antifungal activity of *P. aeruginosa*[31,35,36], and the frequent co-isolation of *S. epidermidis* and *C. albicans* in oral polymicrobial infections[13,31,37]. Fungi are also part of the healthy oral microbiota, supporting the growth of oral anaerobes and increasing the resilience of the consortium[28,38]. Indeed, we also found a remarkably higher relative abundance, but not differing species, of anaerobes in ETTs harbouring *Candida* versus those that did not.

In conclusion, this study represents one of the first instances/attempts of utilization of the patient microbiome and clinical parameters to develop a prediction model of patient prognosis. The next steps would involve analysis of sequential respiratory samples in a larger cohort of intubated patients to understand the endotracheal biofilm dynamics and to develop models based on presence or relative abundances of early microbial markers that are predictive of patient prognosis and of the risk of developing VAP.

4.2 Materials and Methods

4.3.6 Study design and sample collection

ETTs from 203 mechanically ventilated patients admitted to the intensive care unit (ICU) were collected prospectively during 2011-2013 at the Antwerp University Hospital in accordance with relevant regulations and guidelines. Inclusion criteria were minimum 48 hours of ventilation, non-pregnant and age above 18 years. This study was approved by the ethical commission of the Antwerp University Hospital (12/12/112) and informed consent to use these data for research purpose was obtained. The lower 15 cm of the collected ETTs was processed immediately. ETTs were divided into 5 parts, A-E (**Supplementary figure S1**). Parts A and E were cultured by gently tapping the ETT pieces on Colombia blood agar and Sabouraud's dextrose agar. Visually distinct colonies were identified using MALDI-TOF (Bruker Daltonics, Bremen, Germany). Thirty-nine ETTs that yielded *P. aeruginosa* (n=13) or *S. epidermidis* (n=21) or both (n=5) were studied further. From each of the 39 ETTs, parts below the cuff (D, E and E1) were utilized for sequencing (D and E) and for microscopy (E1) (**Supplementary figure S1**).

4.3.7 Microscopic analysis

Part E1 of ETT was fixed in 4% paraformaldehyde for 24h. When a biomass was visible on the internal lumen of the fixed E1 slices, the mass was removed by scalpel and prepared for standard paraffin embedding as done previously[39]. Histology was performed on 5µm thick sections stained with Gram, haematoxylin and eosin (H&E), and Periodic acid Schiff (PAS) stains. All light microscopy images were captured on a Zeiss lab.A1 (Heidelberg, Germany) microscope equipped with a UC30 colour camera (Olympus, Antwerp, Belgium).

4.3.8 DNA extraction and multiplexed pyrosequencing

Adherent material on parts D and E of the ETT was removed by sonication and vigorous vortexing (5 min. each). Samples were centrifuged (5 min. at 17949 x g, 5430 R centrifuge, Eppendorf, Rotselaar, Belgium) and reduced to a volume of approximately 0.5 ml. DNA was extracted using the Masterpure complete DNA and RNA purification kit (Epicentre, Leusden, The Netherlands) as recommended with the following modification: 1µl Ready-lyse lysozyme solution (Epicentre, Leusden, The Netherlands) was added to 150µl sample followed by incubation at 37°C for 30 minutes prior to the extraction. Primers V345_341F (CCT ACG GGR SGC AGC AG) and V345_909R (TTT CAG YCT TGC GRC CGT AC) were used for 16S amplification of the V3-V5 region and primers (ITS-F3): GCATCGATGAAGAACGCAGC and (ITS-R4): TCCTCCGCTTATTGATATGC for amplifying the fungal ITS-II region using multiplexed Roche 454 pyrosequencing[40-42]. Samples were purified by bead-beating prior to

amplification. Purified and quantified PCR products were pooled in equimolar amounts and uni-directional sequencing on ½ run was performed on 454 GS FLX Sequencer (Roche, Basel, Switzerland) using Titanium FLX reagents, resulting in 5000 reads with a 400-600 bp read length on average per sample (Microsynth, Balgach, Switzerland). Sequence analysis was performed using the online MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST)[43].

4.3.9 Sequence analysis

Sequences were sorted based on their multiplex identifier tag and the template-specific primers were trimmed. Sequences shorter than 200 bp were eliminated, and others were aligned against the SILVA database (release 115) using the mothur tool[44,45]. A second-round quality filtering eliminated non-aligned reads with < 95% alignment and chimera sequences were removed using the UCHIME[46] tool implemented in mothur. The taxonomic classification was performed using mothur based on a Bayesian method[47] with K-mer size of 8bp and 1000 replicates. An additional parallel analysis was performed using the online analysis server MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST)[43]. After pre-processing of the raw reads with default parameters, the 16S sequences were processed similar as described above. Processed reads were searched against the SILVA database using BLAT[48] for rRNA identification with 97% identity to cluster the sequences, where after the longest sequence of each cluster was used as a representative. BLAT similarity search with default parameters was performed against the M5rna database with the max e-value cut-off set to 1×10^5, the min % identity cut-off to 97% and a minimum alignment length cut-off of 15.

4.3.10 Analysis of microbial composition of the ETT biofilms

ETTs were grouped into those showing presence of *P. aeruginosa*, *S. epidermidis* or both organisms by culture and their microbial composition was analysed to the family level. Presence/absence of a family in each of the three groups was determined and assigned to the microbiome of a certain group with at a cut-off value of present in 50% of the ETT from a certain group. Differences in relative abundance on all taxonomic levels between the three ETT groups were identified by the binomial disclaim tool linear discriminant analysis effect size (LefSe)[21]. First, LefSe determines features (organisms, clades, operational taxonomic units, genes, or functions) most likely to explain differences between groups by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance. Second, linear discriminant analysis (LDA) is used to rank the features differing between the groups based on their effect sizes[21]. All taxonomic levels showing an LDA score above 2 were considered significant. Statistical significance was set at 0.05 and the strategy for multi-class analysis was set all against one.

4.3.11 Definitions

VAP was identified according to the classical definition as a bacterial pneumonia present in patients with mechanical ventilation for at least 48 hours combined with a new infiltrate onthe chest X-ray, signs of infection and detection of a bacterial causative agent[4,49]. The severity of illness in the ICU was scored according to the validated Apache II score[39].

4.3.12 Statistical analyses

Statistical analyses were performed in IBM SPSS statistics v2.0. Normality was tested using the Shapiro-Wilk test for normality. One-way ANOVA was used for normally distributed data and the Mann-Whitney U test for not normally distributed data. Two-step cluster analysis was performed to discover natural clusters in the dataset using the Schwarz's Bayesian Criterion (BIC) and outlier treatment was applied using default parameters. Statistical differences between each variable in the clusters were determined using one-way ANOVA for continuous data and $\chi 2$ for categorical data in a post-hoc least significant difference (LSD) analysis. Significance level was 0.05 in all statistical analyses. Logistic regression and K-means clustering of the 16S and patient data were performed in Matlab R2015a (MathWorks, Eindhoven, The Netherlands) and R platform v0.99.47 (RStudio, Boston, USA), respectively. Results of the two-step cluster analysis were confirmed by the k-means clustering algorithm on the R platform.

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4.3.13 Supplementary figures and tables

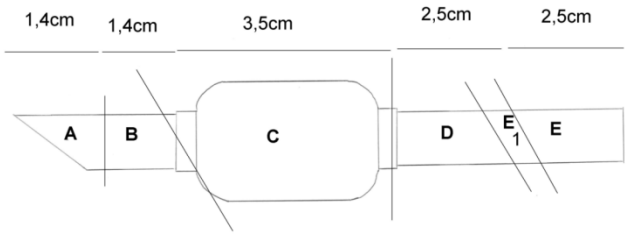

Figure S1: Schematic overview of a typical ETT. Parts A and E were cultured immediately and part D was used for 16S analysis in this study. Part B was stored at -80°C and part E1 was used for microscopy.

Table S1: Clinical data and ETT culture results of all patients. The causative agents were identified in the broncho-alveolar lavage (BAL) samples. If BAL was contra-indicated, an endotracheal aspirate was used.

Patients	Age (years)	APACHE II	Duration of ventilation (days)	Reason of admission	Patient survival	Organisms involved in VAP	ETT culture results
Patient 1	65	14	8	surgical	yes	no VAP	*S. epidermidis* *M. osloensis* *C. dubliniensis* *E. faecalis*
Patient 2	66	11	11	surgical	yes	no VAP	*S. epidermidis* *S. pneumoniae* *C. albicans*
Patient 3	62	14	5	surgical	yes	no VAP	*P. aeruginosa*
Patient 4	43	17	11	surgical	yes	no VAP	*S. epidermidis* *C. albicans* *C. glabrata*
Patient 5	63	21	2	internal	yes	no VAP	*S. epidermidis* *E. coli*
Patient 6	61	29	8	internal	yes	no VAP	*P. aeruginosa* *E. faecium* *Chryseobacterium sp.* *C. albicans*
Patient 7	60	29	4	internal	yes	no VAP	*S. epidermidis* *E. faecium* *S. mutans* *S. gordonii* *S. oralis*
Patient 8	82	48	5	internal	yes	no VAP	*P. aeruginosa* *S. epidermidis*
Patient 9	74	11	24	internal	yes	no VAP	*S. epidermidis* *S. paucimobilis* *C. albicans*
Patient 10	59	35	19	internal	yes	no VAP	*S. epidermidis* *E. faecium* *E. corrodens* *C. albicans* *C. glabrata* *C. robusta*
Patient 11	70	18	4	internal	yes	no VAP	*S. epidermidis* *A. odontolyticus* *L gasseri* *S. pneumoniae* *C. albicans*
Patient 12	69	21	2	internal	yes	no VAP	*S. epidermidis* *S. maltophilia* *C. albicans*
Patient 13	40	10	4	internal	yes	no VAP	*P. aeruginosa*
Patient 14	63	20	4	trauma	yes	no VAP	*P. aeruginosa*
Patient 15	33	30	15	trauma	yes	no VAP	*P. aeruginosa* *S. epidermidis*
Patient 16	67	24	4	surgical	yes	*E. coli*	*S. epidermidis* *E. coli*
Patient 17	69	25	20	surgical	yes	*P. aeruginosa*	*S. epidermidis* *E. faecium* *C. glabrata*
Patient 18	61	22	19	surgical	yes	*Acinetobacter* sp. and *Staphylococcus* sp. (not *aureus)*	*S. epidermidis*
Patient 19	55	7	23	internal	yes	*P. aeruginosa*	*P. aeruginosa* *C. freundii* *E. faecium*
Patient 20	68	31	22	internal	yes	*Staphylococcus* sp. (not *aureus*)	*S. epidermidis* *C. albicans*

Table S1: Continued

Patients	Age (years)	APACHE II	Duration of ventilation (days)	Reason of admission	Patient survival	Organisms involved in VAP	ETT culture results
Patient 21	58	24	12	internal	yes	*P. aeruginosa*	*P. aeruginosa*
Patient 22	39	20	19	internal	yes	*P. aeruginosa*	*P. aeruginosa* *S. epidermidis* *L. pracasei*
Patient 23	42	21	6	internal	yes	*S. aureus*	*S. epidermidis* *S. aureus* *S. oralis* *P. avidum* *C. glabrata*
Patient 24	55	35	15	internal	yes	*P. aeruginosa*	*P. aeruginosa*
Patient 25	47	22	8	internal	yes	*C. freundii*	*S. epidermidis* *C. freundii* *S. aureus* *S. parasanguinis* *A. odontolyticus* *S. penumoniae* *N. perflava* *C. albicans* *C. dubliniensis*
Patient 26	49	23	23	internal	yes	*K. pneumoniae*	*S. epidermidis* *K. pneumoniae* *C. albicans*
Patient 27	74	30	8	internal	yes	*K. pneumoniae* and *S. aureus*	*S. epidermidis* *E. faecium*
Patient 28	59	17	16	trauma	yes	VAP with sterile samples	*P. aeruginosa* *S. epidermidis* *C. albicans* *C. dubliniensis*
Patient 29	40	22	43	surgical	no	no VAP	*P. aeruginosa* *S. marcescens* *P. mirabilis*
Patient 30	70	35	6	surgical	no	no VAP	*P. aeruginosa* *E. faecium* *S. maltophilia* *M. luteus*
Patient 31	82	25	11	internal	no	no VAP	*P. aeruginosa* *C. albicans*
Patient 32	83	32	5	internal	no	no VAP	*P. aeruginosa* *K. pneumoniae* *C. albicans* *C. glabrata*
Patient 33	72	40	10	internal	no	no VAP	*P. aeruginosa* *S. aureus* *C. albicans* *C. glabrata*
Patient 34	80	27	17	internal	no	no VAP	*P. aeruginosa*
Patient 35	74	27	19	internal	no	no VAP	*S. epidermidis* *C. albicans*
Patient 36	60	17	12	internal	no	no VAP	*S. epidermidis* *E. faecium*
Patient 37	60	22	37	internal	no	no VAP	*S. epidermidis* *E. coli* *C. albicans*
Patient 38	58	14	7	surgical	no	*C. albicans* and commensal flora	*S. epidermids* *S. capitis*
Patient 39	58	32	5	internal	no	*P. aeruginosa* and *E. coli*	*P. aeruginosa* *P. mirabilis*

Table S2: Average relative abundance of the families in the core microbiomes and the percentage of ETTs harbouring them. The ETT are divided in three groups based on culture results.

Family	*P. aeruginosa* (n=13)			*S. epidermidis* (n=21)			*P. aeruginosa* + *S. epidermidis* (n=5)		
	% of tubes	Avg abundance	stdev	% of tubes	Avg abundance	stdev	% of tubes	Avg abundance	stdev
Enterobacteriaceae	76.92%	8.73%	20.87%	80.95%	12.77%	24.47%	60.00%	14.18%	0.22
Phyllobacteriaceae	84.62%	0.40%	0.65%	80.95%	0.53%	0.84%	100.00%	7.46%	0.14
Micrococcaceae	38.46%	0.20%	0.31%	80.95%	2.22%	5.05%	80.00%	5.62%	0.05
Staphylococcaceae	46.15%	0.07%	0.11%	95.24%	20.53%	33.43%	100.00%	5.31%	0.06
Propionibacteriaceae	23.08%	0.01%	0.03%	47.62%	0.11%	0.30%	60.00%	1.59%	0.02
Coriobacteriaceae	0.00%	0.00%	0.00%	42.86%	2.29%	4.63%	60.00%	0.22%	0.00
Lactobacillaceae	7.69%	0.01%	0.02%	42.86%	2.29%	8.32%	80.00%	0.95%	0.02
Corynebacteriaceae	23.08%	0.32%	1.00%	80.95%	2.30%	5.15%	40.00%	3.85%	0.08
Actinomycetaceae	30.77%	0.05%	0.09%	52.38%	1.47%	2.90%	20.00%	6.20%	0.13
Pseudomonadaceae	100.00%	38.89%	38.53%	28.57%	0.06%	0.18%	60.00%[a]	1.74%	0.02

[a]In the mixed group of *P. aeruginosa* and *S. epidermidis* the family of the *Pseudomonadaceae* could only be detected in 60% of the ETT. The other tubes contained sequences which were assigned to 'uncultured gammaproteobacterium' which could not be identified with BLAST.

Chapter 5: Identification of interspecies interactions between *Staphylococcus epidermidis* and *Klebsiella pneumoniae* during biofilm formation

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Manuscript in preparation

5.1 Abstract

The microbiome and corresponding microbial interactions therein might influence the transition from colonization to infection. An indication of potential microbial interactions is the frequent co-isolation *Klebsiella pneumoniae* and *Staphylococcus epidermidis* from respiratory samples and endotracheal tube biofilms of mechanically ventilated patients. As such, we attempt to understand the influence of extracellular factors produced during biofilm formation as well as the impact of co-culture on the biomass produced by either organism. The biofilm supernatant of one species was pre-coated on a 96-well plate or dissolved in fresh medium and incubated with the other species. In addition, *K. pneumoniae* and *S. epidermidis* were co-cultured to determine competition, colony forming units and total biomass. The biomass was quantified by crystal violet staining or confocal microscopy. Coating with *K. pneumoniae* supernatant reduced the adhesion of most *S. epidermidis* whereas *K. pneumoniae* supernatant in solution increased the biomass of most *S. epidermidis* by >50%. Coating with *S. epidermidis* supernatant reduced the biomass of 5/16 *K. pneumoniae* by 10-40%. *S. epidermidis* supernatant reduced the biomass of 5/16 *K. pneumoniae* strains by 20-60%. During co-culture, *K. pneumoniae* and *S. epidermidis* could co-exist although the biofilm structure was altered. These data indicate the relationship between *S. epidermidis* and *K. pneumoniae* can be either synergistic or antagonistic, depending on the particular strains and conditions. The adhesion of *S. epidermidis* was reduced in the presence of live *K. pneumoniae* cells as well as coated supernatant. This effect was lost in *K. pneumoniae* supernatant. *S. epidermidis* supernatant reduced the adherence of some *K. pneumoniae* strains.

5.2 Introduction

The importance of microbial biofilms and interactions therein has become widely accepted for various diseases, including medical device associated infections[1,2]. Biofilms on indwelling medical devices rarely consist of a single species and the microbial composition is a key factor determining the patient outcome[2,3]. Biofilms on the endotracheal tubes of mechanically ventilated patients were shown to increase the risk of developing pneumonia, also defined as ventilator associated pneumonia (VAP)[4-6]. Moreover, the specific microbial composition within these biofilms can increase the risk of developing VAP or predict the patient outcome[7,8]. On the one hand, VAP can be caused by notorious nosocomial pathogens such as *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Staphylococcus aureus*, which are associated with high antibiotic resistance and mortality[9,10]. However, the role of the commensal bacterium *Staphylococcus epidermidis* as a nosocomial pathogen is becoming more and more appreciated, as it is often isolated from medical devices and respiratory samples[4,11].

In a previous study, we demonstrated a relationship between the presence of *S. epidermidis* and *K. pneumoniae* on the endotracheal tubes of patients with VAP[8]. Very little is known about microbial interactions between *S. epidermidis* and *K. pneumoniae*, although they frequently share the same habitat[12]. Therefore, interactions between these two microorganisms, either positive or negative, can have important implications for virulence and patient outcome and are important sources for potential alternative treatment strategies. To date, only one paper described the effect of *K. pneumoniae* on *S. epidermidis* biofilm formation, where *K. pneumoniae* supernatants was found to inhibit *S. epidermidis* biofilm formation[13]. However, only four *S. epidermidis* strains were tested, including two reference strains and two clinical isolates. Moreover, the effect of *S. epidermidis* biofilm supernatant on the biofilm formation of *K. pneumoniae* or dual species biofilm formation were not tested.

In this paper we aim to identify potential microbial interactions between *S. epidermidis* and *K. pneumoniae* occurring during biofilm formation using growth in dual species biofilms to investigate the effect on growth, biofilm formation and competition. Furthermore, the effect of extracellular factors produced by one species on the biofilm formation of the other was investigated by incubating one with the biofilm supernatant from the other. Additionally, the effect of the biofilm supernatant on the biofilm structure of the other as well as the biofilm structure during dual species culture were investigated using spinning disc confocal microscopy. Here, we showed a synergistic and antagonistic relationship between both species during biofilm formation, which was strain- and condition- dependent.

5.1 Methods

5.1.1 Bacterial strains and culture conditions

Clinical isolates of *S. epidermidis* and *K. pneumoniae* were cultured from endotracheal tubes (ETTs) obtained from mechanically ventilated patients upon extubation at the intensive care unit of the University hospital of Antwerp[8]. Thirteen *K. pneumoniae* and 51 *S. epidermidis* were isolated from 203 ETTs collected between 2011 and 2013. In one ETT, both *K. pneumoniae* (KP078) and *S. epidermidis* (SE078) were recovered (**Tables 1 and 2**). Of the 64 strains isolated, all *K. pneumoniae* and 15 *S. epidermidis* were included in this study (**Tables 1 and 2**). When considering the clinical data, two *K. pneumoniae* and ten *S. epidermidis* were isolated from the ETT of patients that developed pneumonia. Additionally, two colistin resistant *K. pneumoniae* isolated from blood and urine of two patients at the Tzaneio General Hospital (Athens, Greece) were included. *K. pneumoniae* ATCC 700603 and ATCC 700721 and *S. epidermidis* 1457 (SE1457) were used as positive controls for biofilm formation[14]. Of the *K. pneumoniae* clinical isolates, two (KP078 and KP31CR) showed a hypermucoid phenotype, as determined by the string test[15], which changed the further flow-through as described below. All isolates were cultured overnight on Columbia blood agar base (Oxoid, UK) supplemented with 5% horse blood at 37°C.

5.1.2 Preparation of cell-free biofilm supernatant

Biofilms were grown in trypticase soy broth (TSB, Becton-Dickinson and Co., France) in a 145 x 20 mm polystyrene petri dish (Greiner bio-one, Germany) for 24h at 37°C under static conditions. The biofilm supernatant was collected with a pipette, centrifuged at 4°C for 30 min at 4495xg (Multifuge 3 S-R, Heraeus) and filtered through a sterile 0.2 µm filter (MiniSart, Sartorius, Germany) or autoclaved for 20 min at 15 PSI. Biofilm supernatant or sterile ultrapure water as a control were prepared fresh for every experiment and diluted 1:1 with double concentrated TSB to restore the nutrient concentration. All experiments were conducted in three technical replicates and at three independent time-points. Because of the hypermucoid phenotype of KP078, the biofilm supernatant was too viscous to be filter sterilized. Therefore, the KP078 supernatant was centrifuged and autoclaved in all experiments.

5.1.3 Biofilm formation in the presence of cell-free supernatant in a static 96-well plate assay

Four *K. pneumoniae* strains (ATCC 700603, ATCC 700721, KP019 and KP078) were utilized to study the impact of the biofilm supernatant on biofilm formation by 16 *S. epidermidis* strains during 24h (**Table 2**). Briefly, 20 µl of 0.5 McFarland solutions of all *S. epidermidis* strains were diluted in 180 µl *K. pneumoniae* supernatant in a flat-bottom, polystyrene 96-well plate (Greiner bio-one, Germany), and incubated at 37°C for 24h. Plates were washed three times with sterile phosphate buffered saline (PBS, Thermo Fisher Scientific, Lithuania) and fixed with 100% methanol (Merck, Germany) for 20 min. The biomass was stained with 0.1% crystal violet and absorption was measured at 492nm (Multiskan FC, Thermo scientific, Germany). The change in biomass of each strain was

calculated as a percentage of the biomass formed in double concentrated TSB with ultrapure water (1:1). Similar experiments were performed to study the impact of the biofilm supernatant of four *S. epidermidis* strains (SE1457, SE078, SE045 and SE3-405) on biofilm formation by 16 *K. pneumoniae* isolates (**Table 1**).

5.1.4 Pre-coating of 96-well plates with biofilm supernatant

To determine the effects of the *K. pneumoniae* biofilm supernatant on initial adherence by *S. epidermidis*, the bottom of a 96-well plate was pre-coated with the former. Plates were incubated with 200µl biofilm supernatant for 3h under static conditions at room temperature. After incubation, the supernatant was removed with a pipette and the plates were dried overnight in a flow cabinet. Biofilm formation was then quantified as described above after 6h and 24h. As a control, wells were coated with plain TSB and dried and seeded with the test *K. pneumoniae* or *S. epidermidis* strains. The change in biomass in the test wells was calculated as a percentage of biomass in the plain TSB-coated control wells.

5.1.5 Dual species biofilm formation

Two *S. epidermidis* strains (SE1457 and SE078) and three *K. pneumoniae* (ATCC 700603, KP019 and KP078) were selected for dual species biofilm formation based on the results of the supernatant experiments. Inocula of 0.5 McF were prepared and inoculated in equal proportions as above and incubated at 37°C for 24h. The total biomass was determined by crystal violet staining as above or by staining with Syto9 followed by Spinning disc confocal microscopy as described below.

5.1.6 Spinning disc confocal microscopy

For microscopy, a selection of *K. pneumoniae* (ATCC 700603, KP019 and KP078) and *S. epidermidis* (SE1457, SE011 and SE078) was based on the previous results and biofilms were cultivated in biofilm supernatant or as dual species biofilms in µClear thin bottom 96-well plates (655090, Greiner bio-one, Germany) for 24h. The medium was carefully removed with a pipette and the biomass was washed with sterile 0.9% saline solution and stained with Syto9 (10 µM, life sciences, USA). To determine the effect on the biofilm structure, one field in each well was visualized with a spinning disc confocal microscope (UltraVIEW VoX, Perkin Elmer) and three-dimensional reconstructed images were analysed with Imaris 8.3 (Bitplane) to determine the volume of the biofilm. Volocity 6.3 (Perkin Elmer) was used to capture and edit the images for display.

5.1.7 Statistical analysis

All effects were compared to the control using the two sample t-test in excel. P-values below 0.05 were considered significant.

5.2 Results

5.2.1 *K. pneumoniae* biofilm supernatant showed a predominant increase of biofilm formation by *S. epidermidis*

To investigate the effect of extracellular factors, 16 *S. epidermidis* strains were incubated for 24h in the biofilm supernatant of four *K. pneumoniae* strains was diluted with double-concentrated medium to restore the nutrient concentration or in double concentrated medium diluted with ultrapure water as a control. The change in biomass was calculated as the percent difference compared to the control of each individual strain. The *K. pneumoniae* reference strains ATCC 700603 and ATCC 700721 increased the biofilm production of 11 and 12 of the 16 *S. epidermidis* strains with an average of 144% and 167%, respectively (95% CI: 53-235% and 67-268%, $p<0.001$, **Fig 1a**). Furthermore, both strains reduced the biomass of three *S. epidermidis* with an average loss of 97% and 76%, respectively (95% CI: 96-98% and 71-80%, $p<0.001$). The clinical isolate KP019 increased the biomass of 11 *S. epidermidis* with an average of 162% (95% CI: 65-258%, $p<0.001$) and decreased the biofilm production of three *S. epidermidis* strains with an average loss of 97% (95% CI: 96-98%, $p<0.001$). The other clinical isolate (KP078), which was co-isolated with *S. epidermidis*, significantly increased the biomass of eight *S. epidermidis* with an average of 89% (95% CI: 42-135%, $p<0.001$) and decreased the biomass of 6 strains with on average 52% (95% CI: 28-76%, $p<0.001$), indicating that these effects are dependent on both the *S. epidermidis* and the *K. pneumoniae* strain. The biofilm formation of one *S. epidermidis* strain (SE3-410) was not significantly increased by any *K. pneumoniae* biofilm supernatant (average increase: 34%, $p>0.05$, **Fig 1a**). In addition, these effects were still present after autoclaving, suggesting the factor(s) responsible for the effects is (are) heat stable. Of note, the biofilm formation of three *S. epidermidis* isolates (SE078, SE011 and SE045) was consistently inhibited by the supernatant of all *K. pneumoniae* strains, with an average loss of 76% to 97%, depending on the *K. pneumoniae* and with the co-isolated strain being the least effective (average loss 76%). One of these isolates, SE078, was co-isolated with *K. pneumoniae*. All three *S. epidermidis* strains were weak biofilm formers and isolated from the ETT of patients that developed pneumonia during mechanical ventilation. The effect of the *K. pneumoniae* supernatant of three strains (ATCC 700603, KP019 and KP078) on the total biomass of SE1457 and SE011 was also determined by Syto9 staining, which stains life cells as well as extracellular DNA, followed by spinning disc confocal microscopy. Here, the total volume of the SE011 biomass was reduced by all three *K. pneumoniae* with 90% (ATCC 700603 and KP019) or with 100% (KP078) and the total volume of the SE1457 biomass was reduced by KP019 and KP078 with 68% and 92%, respectively (**Figure 1b**). The total volume of the SE1457 biofilm was not significantly altered in the presence of the supernatant of ATCC 700603 (increased with 7%).

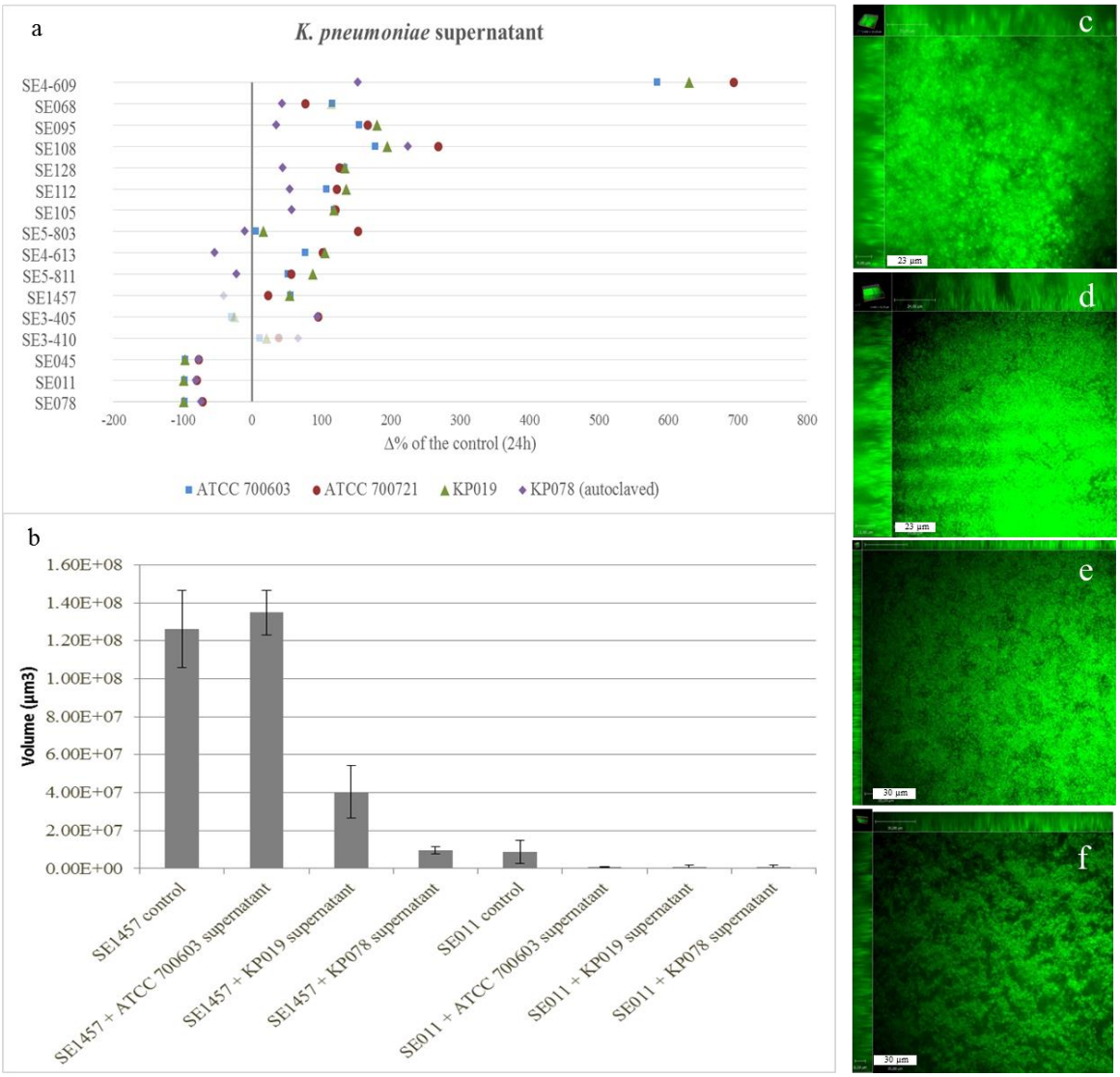

Figure 1: The effect of *K. pneumoniae* biofilm supernatant biofilm formation of *S. epidermidis*. (a) Biomass determined by crystal violet staining. All values are presented as the change in OD492nm calculated as the percentage of the control. The transparent markers are not significant. (b) Biomass determined by Syto9 staining. (c-f) Representative pictures of SE1457 as a control (c), with the supernatant of ATCC 700603 (d), KP019 (e) and KP078 (d). Bars 23 µm in (c) and (d) and 30 µm in (e) and (f).

5.2.2 Pre-coated *K. pneumoniae* biofilm supernatant reduced initial adhesion of *S. epidermidis*

To determine the effect of extracellular factors produced by *K. pneumoniae* biofilms on *S. epidermidis* adhesion, we pre-coated a 96-well plate with *K. pneumoniae* biofilm supernatant of four strains and quantified the biomass after 6h or 24h of growth to determine the effect on early and mature biofilm formation, respectively. Pre-coating with plain medium was used as a control and the change in biomass was calculated as a percentage of the control for each strain. After 6h incubation with KP078 supernatant, all 16 *S. epidermidis* showed a significant reduction in biomass compared to the biomass of the control with an average of 64% (95%

CI: 58-70%, p<0.001, **Fig 2a**). The clinical KP019 supernatant reduced the biomass of 11 *S. epidermidis* with an average of 23% (95% CI: 17-29%, p<0.001), while the ATCC 700603 reduced biomass in 11 *S. epidermidis* strains with an average of 20% (95% CI: 16-24%, p<0.05). Pre-coating with ATCC 700721 did not result in a significant reduction in biomass in any of the 16 *S. epidermidis* (average loss in biomass: 4%, 95% CI: 1-8%; p>0.05). After 24h, we observed a smaller loss of biomass in a smaller amount of *S. epidermidis* strains by KP078 as the biomass of 13 *S. epidermidis* strains was reduced with an average loss of 51% (95% CI: 42-60%, p<0.05, **Fig 2b**). KP019 reduced the biomass of 14 strains with an average of 43% (95% CI: 35-51%, p<0.05) whereas ATCC 700603 reduced the biomass of 9 strains with an average of 22% (95% CI: 10-34%, p<0.05). The strain ATCC 700721, which showed no significant effect after 6h, caused a reduction in the biomass of five *S. epidermidis* strains with an average of 21% after 24h (95% CI: 15-27%, p<0.05).

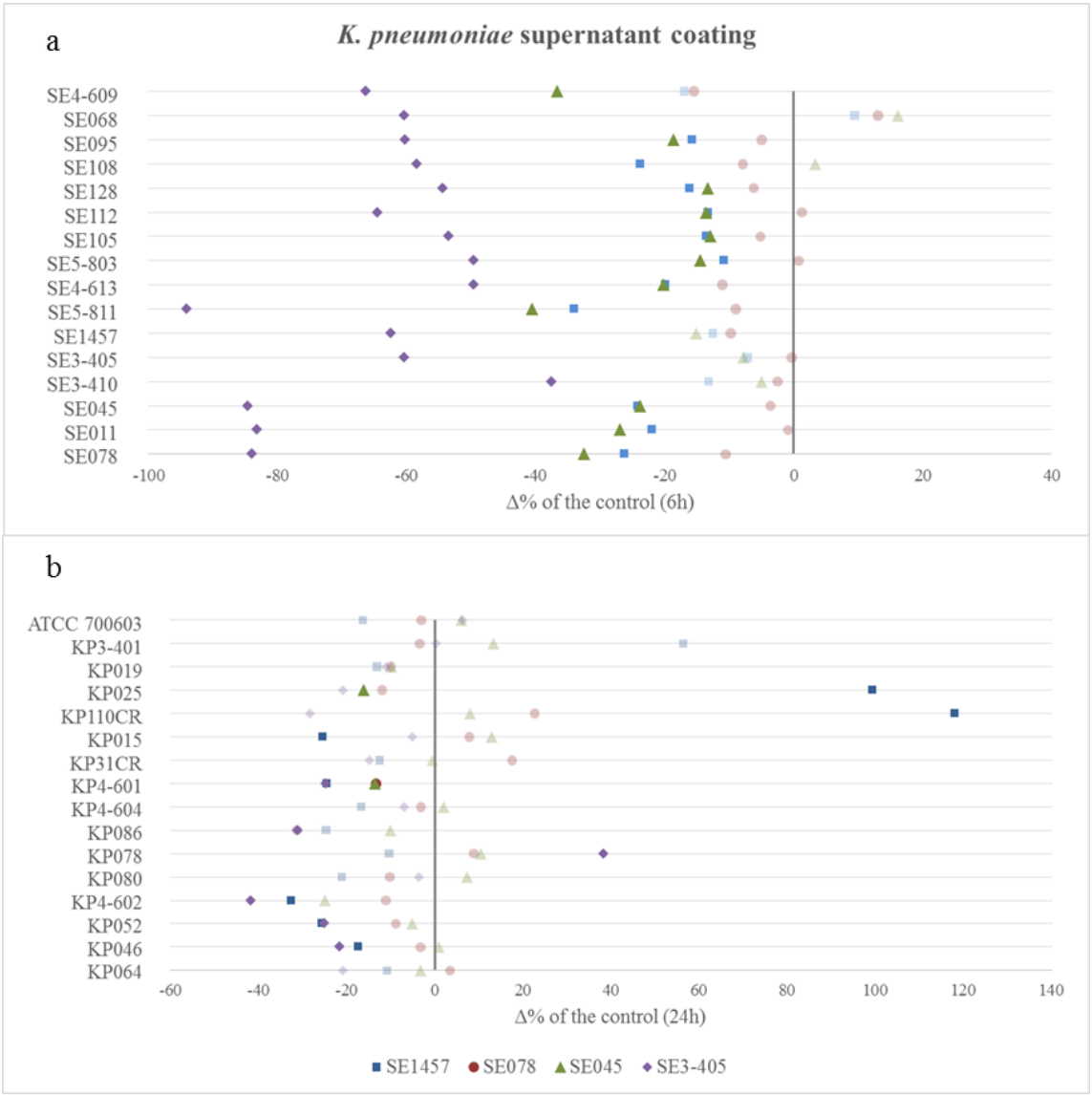

Figure 2: The effect of *K. pneumoniae* biofilm supernatant coating on early (a) and mature (b) biofilm formation of *S. epidermidis*. All values are presented as the change in OD492nm calculated as the percentage of the control. The transparent markers are not significant.

5.2.3 *S. epidermidis* supernatant consistently inhibited the biofilm formation of five *K. pneumoniae* strains

Next we studies whether the biofilm supernatant of four *S. epidermidis* strains (SE1457, SE078, SE3-405 and SE045) displayed an effect on the biofilm formation of 16 *K. pneumoniae* strains. Here, we showed a consistent reduction in the biomass of five *K. pneumoniae* strains by the supernatant of all *S. epidermidis* strains tested. The *S. epidermidis* reference strain SE1457 significantly reduced the biomass of seven *K. pneumoniae* strains with an average of 54% (95% CI: 43-64%, p<0.001, **Fig 3a**). The clinical isolates SE078 and SE3-405 significantly reduced the biofilm production of eight *K. pneumoniae* with an average of 28% and 44%, respectively (95% CI: 21-36%, p<0.05 and 35-45%, p<0.001). The clinical isolate SE045 was the least effective as it reduced the biomass of five *K. pneumoniae* strains with an average of only 32% (95% CI: 26-39%, p<0.01). In addition, the biomass of five *K. pneumoniae*, including both hypermucoid strains, was not significantly increased or decreased by any *S. epidermidis* tested (**Fig 3a**). The effect of the *S. epidermidis* supernatant of two strains (SE1457 and SE078) on the total volume of life cells and extracellular DNA of ATCC 700603 and KP078 was also determined by Syto9 staining followed by spinning disc confocal microscopy. The total biomass of ATCC 700603 was only reduced by the supernatant of SE078 by 78% (**Fig 3b**) and the total biomass of KP078 was reduced by the supernatant of both SE1457 and SE078 by 55% and 91%, respectively.

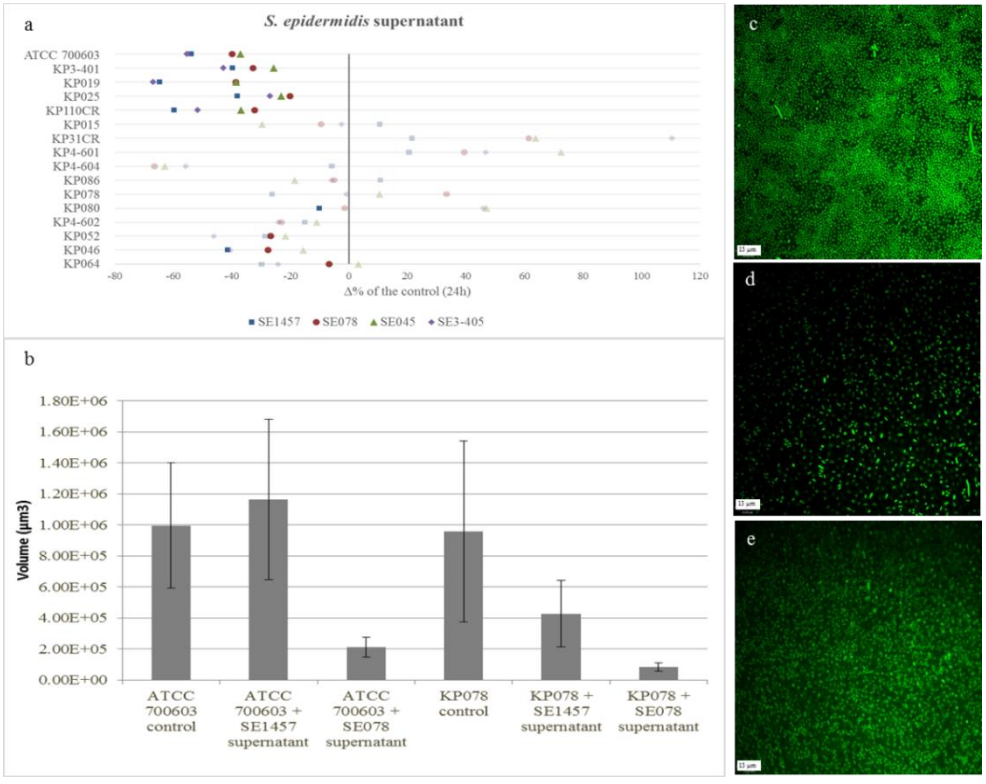

Figure 3: The effect of *S. epidermidis* biofilm supernatant biofilm formation of *K. pneumoniae*. (a) Biomass determined by crystal violet staining. All values are presented as the change in OD492nm calculated as the percentage of the control. The transparent markers are not significant. (b) Biomass determined by Syto9 staining. (c-d) Representative pictures of ATCC 700603 as a control (c), with the supernatant of SE1457 (d) and the supernatant of SE078 (e), bars (c-e) 13 µm.

5.2.4 Coating with *S. epidermidis* biofilm supernatant inhibited the adhesion of some *K. pneumoniae*

To determine the effect of *S. epidermidis* extracellular factors on the adhesion of *K. pneumoniae*, the surface of a 96-well plate was coated with *S. epidermidis* biofilm supernatant of four strains as above. After 6h of incubation, the biofilm supernatant of two *S. epidermidis* strains, SE1457 and SE3-405, significantly reduced the adhesion of seven and five *K. pneumoniae* strains with an average of 24% and 25%, respectively (95% CI: 19-29% and 95% CI: 15-34%, $p<0.05$, **Fig 4a**). Coating with SE078 supernatant only significantly inhibited the biofilm formation of the reference strain ATCC 700603 by 20% ($p<0.05$) and coating with SE045 supernatant inhibited only KP4-601 by 14% ($p<0.05$). The biofilm formation of the other *K. pneumoniae* strains, including the co-isolated strain KP078, was not significantly increased or decreased (**Fig 4a**). After 24h, coating with SE1457 and SE3-405 supernatant inhibited the biofilm formation of five and six *K. pneumoniae* strains with an average of 25% and 28%, respectively (95% CI: 20-30% and 95% CI: 19-37%, $p<0.05$). Furthermore, the reference strain SE1457 enhanced the biofilm formation of two *K. pneumoniae* strains with an average of 109% (95% CI: 90-127%; $p<0.05$), whereas SE3-405 enhanced the biofilm formation of one *K. pneumoniae* with 38% ($p<0.05$). Coating with SE078 and SE045 supernatant only significantly reduced the biofilm formation of KP4-601 by 13% and 14% ($p<0.05$, **Fig 4b**). The biofilm formation of the other strains was not significantly increased or decreased by coating with *S. epidermidis* supernatant (**Fig 4b**). The co-isolated strain KP078 was only significantly increased by SE3-405 supernatant (38%, $p<0.05$) and not significantly decreased with SE1457 supernatant (10%, $p>0.05$).

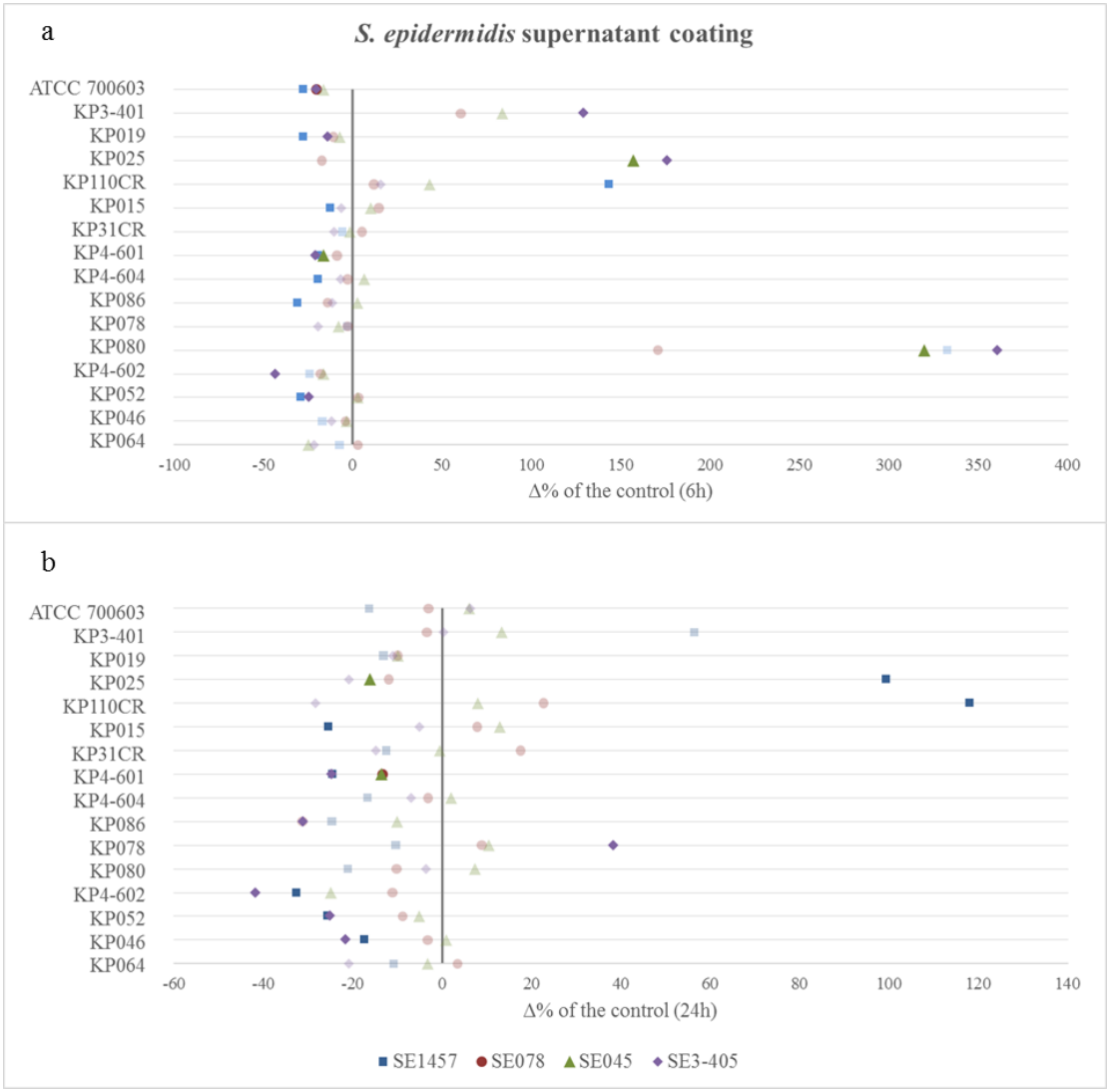

Figure 4: The effect of *S. epidermidis* biofilm supernatant coating on early (a) and mature (b) biofilm formation of *K. pneumoniae*. All values are presented as the change in OD492nm calculated as the percentage of the control. The transparent markers are not significant.

5.2.5 *S. epidermidis* biofilm structure was altered during dual species biofilm formation

To determine the effect of dual species biofilm on the total biofilm structure, *S. epidermidis* and *K. pneumoniae* inoculum of a selection of strains (SE1457, SE078, ATCC 700603 and KP078) was prepared as described above and incubated in µClear thin bottom 96-well plates for 24h in equal proportions. The biofilm was stained or with crystal violet to determine the total amount of biomass or with Syto9 to visualize the DNA in the bacteria and the extracellular matrix. For *S. epidermidis,* we selected the reference strain SE1457, which was stimulated by three of the four *K. pneumoniae* and the clinical isolate SE078, which was inhibited by all *K. pneumoniae* strains and co-isolated with KP078. *S. epidermidis* SE1457 was the strongest biofilm former, making a thick biofilm over the entire surface whereas the *K. pneumoniae* strains formed a single-cell layer at the bottom of the plate (**Fig 5a, c, f and g**).

During dual species biofilm formation, the total amount of biomass produced was in all cases close to the total amount of biofilm produced by the respective *K. pneumoniae* mono-species biofilm (**Fig 5a**). Microscopic analysis of these biofilms showed that *S. epidermidis* formed small clusters of biofilm with *K. pneumoniae* cells spread between them (**Fig 5d and e**). After quantification the volume to the total biomass was more closely to that of the *K. pneumoniae* mono-species biofilm (**Fig 5b**).

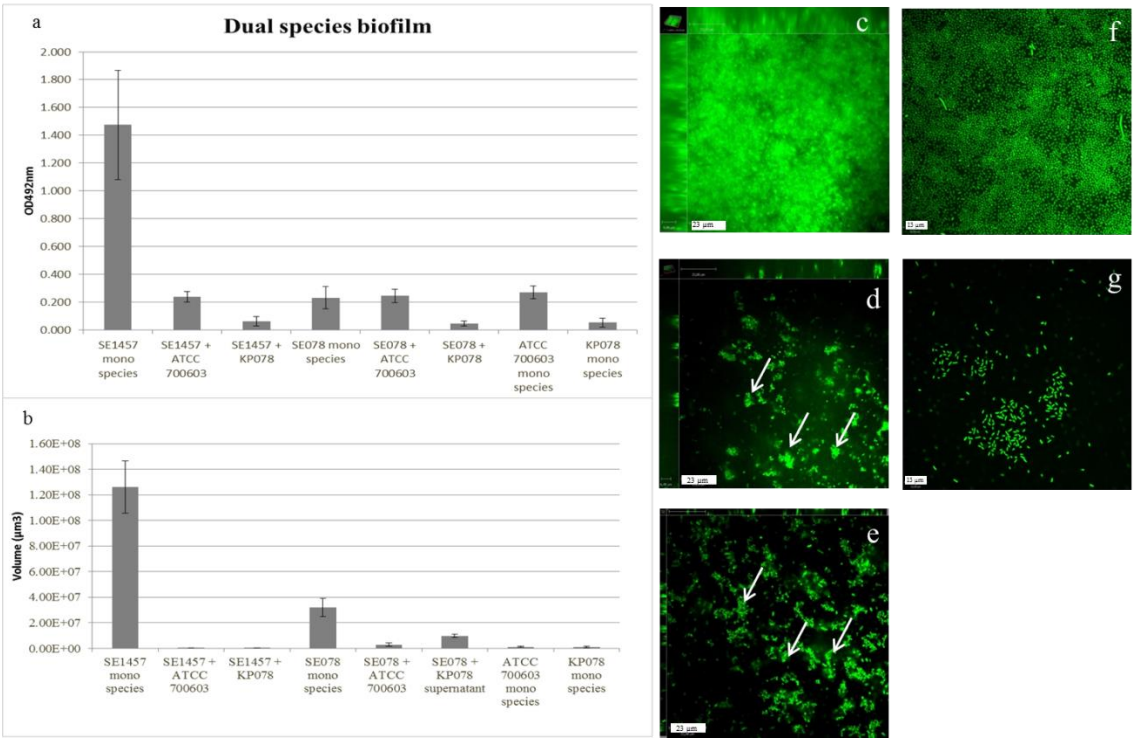

Figure 5: Dual species biofilm formation of *K. pneumoniae* and *S. epidermidis*. The total biomass was determined by crystal violet staining (a) or Syto9 staining followed by spinning disc confocal microscopy (b). Representative pictures of SE1457 mono-species biofilm (c), SE1457 and ATCC 700603 dual species biofilm (d), SE1457 and KP078 dual species biofilm (e), ATCC 700603 mono-species biofilm (f) and KP078 mono-species biofilm (g). Bars 23µm in (c), (d) and (e) and 13µm in (f) and (g). White arrows: *S. epidermidis* cell clusters.

5.3 Discussion

The biofilm mode of growth provides protection against many environmental factors, including mechanical stress and the administration of antimicrobials[16,17]. Furthermore, biofilms rarely consist of a single species and potential cooperation or competition between the different inhabitants can sometimes increase the virulence or stress resistance of some bacteria[18,19]. Therefore, (multispecies) biofilm formation on indwelling medical devices poses a serious threat to hospitalized patients[20,21]. Studying the effect of one species on the biofilm formation of another is a first step in understanding multispecies interactions and in identifying potential eradication strategies. In this study, we investigated the effect of biofilm supernatant produced by *K. pneumoniae* on the biofilm phenotype of *S. epidermidis* and vice versa.

First we showed that coating of the 96-well surface with *K. pneumoniae* biofilm supernatant could reduce the attachment of most *S. epidermidis* strains up to 24h. Remarkably, for most *S. epidermidis* strains the opposite was true in the presence of *K. pneumoniae* supernatant in the medium, where only the biofilm of three *S. epidermidis* strains was consistently inhibited while the biofilm of the others was stimulated. This inhibitory effect on the biofilm of *S. epidermidis* could be explained by an important biofilm molecule of *K. pneumoniae*, the capsular polysaccharide, which was already suggested by Goncalves et al.[13]. Various extracellular polysaccharides are described to exhibit anti-adhesion properties against a wide range of Gram-positive as well as Gram-negative bacteria[22,23]. The anti-biofilm activity of polysaccharides is obtained by several modes of action. First, some polysaccharides have biosurfactant activity, modifying both biotic and abiotic surfaces and thereby preventing surface adhesion as well as auto-aggregation[22]. Second, extracellular polysaccharides might act as signalling molecules downregulating adhesion molecules or induce dispersion[22]. Third, competitive binding of polysaccharides to biofilm molecules on the cell wall, like adhesins or type IV pili prevents attachment[22].

The importance of capsular polysaccharides is also visible in our results, where the most potent biofilm inhibiting *K. pneumoniae* strain was hypermucoid, a phenotype characterized by capsular polysaccharide overproduction[15]. Furthermore, Rendueles et al. identified a novel anti-adhesion polysaccharide, Ec300, produced by some *E. coli* and *K. pneumoniae* strains, which impairs the initial adhesion of Gram-positives by increasing the surface hydrophilicity[23]. The strain and assay specific discrepancies in our results suggest that there might be a surface modification inhibiting initial adhesion but this might not be the only mode of action. In conclusion, the net effect on the biomass production of *S. epidermidis* is probably a combination of the factors in the biofilm supernatant, the specific properties of the *S. epidermidis* strain and the culture conditions used.

Second, we showed that the presence of *S. epidermidis* supernatant could consistently reduce the biofilm production of five of the 16 *K. pneumoniae* strains, regardless of the *S. epidermidis* strain used. Additionally, some *K. pneumoniae* strains were inhibited by some *S. epidermidis* strains, suggesting multiple modes of action or multiple extracellular molecules, depending on the strains. Previous research indicated an antagonistic relationship between *S. epidermidis* and several human pathogens, including *S. aureus* and *Propionibacterium acnes*[24,25]. Moreover, the presence of *S. epidermidis* could protect against colonization by the more virulent *S. aureus*[25,26]. However, no antagonistic effect on *K. pneumoniae* has been described thus far and further research is needed.

Third, we found that the biofilm architecture of *S. epidermidis* was altered by the presence of live *K. pneumoniae* cells. Although both bacteria could be detected in the same well, they did not co-aggregate into a dual species biofilm but rather formed mono species clusters. These results indicate a more antagonistic relationship where *K. pneumoniae* produces factors inhibiting biofilm formation of *S. epidermidis* without killing. Furthermore, the presence of *S. epidermidis* seems to have little effect on the biofilm formation of *K. pneumoniae*, as the biofilm structure and total biomass always was close to the *K. pneumoniae* mono-species biofilm. However, the biofilm structure of *S. epidermidis* was altered in the presence of the supernatant of *K. pneumoniae*, resulting in a thinner structure. These results

are different compared to the stimulating effect shown by crystal violet staining. A possible explanation for this observation is the staining methods used in both assays. Crystal violet stains the entire biomass, including bacteria and extracellular matrix whereas Syto9 used for microscopy only takes the cells and extracellular DNA into account, leading to a potential underestimation of the total biomass. In addition, during the crystal violet staining the biomass formed over the entire surface of the wells in measured, including a substantial part on the air-liquid interface whereas microscopic examination only involves the bottom of the wells.

In conclusion, *S. epidermidis* and *K. pneumoniae* showed a complicated, strain dependent relationship. Both were able to co-exist in the same environment but the adhesion of *S. epidermidis* was reduced during dual species culture with live *K. pneumoniae* cells. Pre-coating a surface with *K. pneumoniae* biofilm supernatant could reduce the adherence of *S. epidermidis* for up to 24h. This effect was lost when *S. epidermidis* was incubated with *K. pneumoniae* biofilm supernatant dissolved in fresh medium. In this condition the biomass of most strains was even increased, except for a few strains. *S. epidermidis* showed a limited effect on *K. pneumoniae* biofilm production, although some strains were inhibited. Further research is needed to elucidate the exact mechanisms involved in these interspecies interactions, detecting the identity of the molecules produced by one species as well as the differential response of the members of the other and how to use these interactions to our own benefit.

5.4 References

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5.3 Supplementary information

Table 1: Strain information of the *S. epidermidis* strains used in this study. *S. epidermidis* 1457 is a strong biofilm forming reference strain. Strains were classified as strong (>75% of the reference), moderate (75%-25% of the reference) or weak (<25% of the reference) biofilm forming strains. ETT: endotracheal tube; CVC: central venous catheter.

S. epidermidis strains	*MLST type*	*Clinical information*	*Biofilm formation*	*Source*	*Co-isolated strains*
SE1457	/	/	strong	CVC	/
SE068	425	pneumonia	strong	ETT	*S. aureus* *C. freundii* *S. parasanguinis* *A. odontolyticus* *C. albicans* *C. dubliniensis* *S. pneumoniae* *N. perflava*
SE4-613	2	pneumonia	moderate	ETT	*P. aeruginosa* *C. albicans* *C. dubliniensis*
SE128	2	pneumonia	moderate	ETT	*E. faecium* *C. glabrata*
SE4-609	2	pneumonia	weak	ETT	*P. aeruginosa* *L. paracasei*
SE3-405	2	no pneumonia	strong	ETT	*C. ablicans* *C. glabrata*
SE5-803	2	no pneumonia	moderate	ETT	*S. pneumoniae* *C. albicans*
SE5-811	2	no pneumonia	weak	ETT	*E. coli* *C. glabrata*
SE3-410	2	no pneumonia	strong	ETT	*M. osloensis* *C. dubliniensis* *E. faecalis*
SE011	2	pneumonia	weak	ETT	*P. aeruginosa* *E. faecalis*
SE095	2	no pneumonia	moderate	ETT	*P. aeruginosa* *S. pneumoniae*
SE3-419	2	no pneumonia	moderate	ETT	*C. albicans*
SE105	2	pneumonia	moderate	ETT	*S. maltophilia*
SE108	2	pneumonia	moderate	ETT	*C. albicans*
SE112	2	pneumonia	moderate	ETT	*C. albicans*
SE045	54	pneumonia	weak	ETT	/
SE078	2	pneumonia	weak	ETT	***K. pneumoniae*** *C. albicans*

Table 2: Strain information of the *K. pneumoniae* strains used in this study. ATCC 700603 is a strong biofilm forming reference strain. Strains were classified as strong (>75% of the reference), moderate (75%-25% of the reference) or weak (<25% of the reference) biofilm forming strains. ETT: endotracheal tube;

K. pneumoniae strains	*MLST type*	*Clinical information*	*Biofilm formation*	*Source*	*Co-isolated strains*
ATCC 700603	/	/	strong	urine	/
KP3-401	1013	no pneumonia	strong	ETT	*N. flavescens* *C. albicans*
KP4-601	101	no pneumonia	strong	ETT	*K. oxytoca*
KP4-602	815	pneumonia	strong	ETT	*E. faecalis* *S. pettencoferi* *C. glabrata*
KP 4-604	101	no pneumonia	weak	ETT	*P. aeruginosa* *C. albicans* *C. glabrata*
KP015	101	no pneumonia	weak	ETT	*S. maltophilia* *B. weihenstephanensis*
KP 019	323	no pneumonia	strong	ETT	*C. parapsilosis* *S. haemolyticus* *E. feacalis* *C. glabrata*
KP025	25	no pneumonia	strong	ETT	*E. coli*
KP046	35	no pneumonia	strong	ETT	/
KP064		no pneumonia	strong	ETT	*E. coli*
KP078	1049	pneumonia	weak	ETT	***S. epidermidis*** *C. albicans*
KP080	1677	no pneumonia	weak	ETT	*E. faecium* *C. albicans*
KP086	700	no pneumonia	weak	ETT	*S. warneri* *K. rhizophilia* *T. otitidis*
KP052	/	no pneumonia		ETT	*E. coli* *C. albicans* *C. glabrata*
KP110CR	383	/	strong	blood	/
KP31CR	147	/	strong	urine	/

Chapter 6: Summary and future perspectives

6.1 Summary

A general description of the main topics in this work is provided in **chapter 1**: biofilm formation, ventilator associated pneumonia and the pathogens *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Biofilms are complex, structured communities of micro-organisms attached to a surface and surrounded by an extracellular matrix. The inhabitants of these biofilms are protected by this matrix against main stress factors like mechanical stress, desiccation, the immune system or antimicrobial products. Therefore, the biofilm mode of growth is the most common and natural mode of growth among bacteria. Most biofilms consist of multiple species, which constantly communicate and interact with each other. The composition of these biofilms is especially important in the human body, where the good, commensal flora helps with basic functions such as digestion and provides protection against potential pathogens by occupying the same niche. However, biofilm formation on indwelling medical devices like prosthetic joints or catheters poses a serious healthcare threat since pathogenic bacteria can dislodge from these biofilms and cause infection. Biofilm-related infections are very difficult to treat due to their high antibiotic- and other stress resistance, leading to chronic infection and relapse.

Ventilator associated pneumonia (VAP) is an important healthcare-associated infection caused by particles dislodged from the biofilm on the endotracheal tubes (ETTs) of mechanically ventilated patients, increasing mortality, morbidity and healthcare costs. These biofilms are formed within 24h of intubation in the lumen and around the cuff of the ETT, which provides direct entry to the lungs by omitting the cough reflex and mucociliary clearance. The Gram-negative bacterium *Pseudomonas aeruginosa* is an important pathogen causing VAP and is associated with high mortality due to a high intrinsic antibiotic resistance, a vast amount of virulence factors and the ability to form strong biofilms. In addition, *P. aeruginosa* has a highly flexible metabolism and genome, facilitating mutation and adaptation to changes in the environment, thereby complicating eradication.

Staphylococcus epidermidis is a commensal and usually harmless Gram-positive bacterium. However, it can cause serious infection in immunocompromised patients and is one of the leading causes of catheter related bloodstream infections. Remarkably, the adaptations to the commensal lifestyle also make *S. epidermidis* a dangerous pathogen, as it is able to avoid killing by the immune system and is adapted to sudden changes in its environment, like changing salt concentrations. Furthermore, like *P. aeruginosa*, *S. epidermidis* has a high intrinsic antibiotic resistance, flexible genome and the capacity to form strong biofilms. However, *S. epidermidis* does not produce many virulence factors unlike its close relative *Staphylococcus aureus*, which is also an important nosocomial pathogen. *P. aeruginosa* has a complex relationship with both *Staphylococcus* spp. and mixed species infections have a worse patient outcome compared to single species infections.

Therefore, the first aim of this work was to map the microbial interactions between *P. aeruginosa* and *Staphylococcus* spp., focusing on *S. aureus*, and their effect on the host. This is described in **chapter 2** as an extensive review of literature. *P. aeruginosa* and *S. aureus* have developed a complex network of evasion, counter-inhibition and subjugation in their battle for space and nutrients. For instance, in the cystic fibrosis lung and chronic wound infections,

their strain- and environment- specific interactions show severe competition generally linked to increased virulence, host inflammation and associated tissue damage. Additionally, *P. aeruginosa* secretes extracellular factors to kill *Staphylococci*, leading to cell lysis or inhibition of the oxidative respiration. *S. aureus* evades these mechanisms by growing as L-form like colonies or small colony variants, which are more resistant to antibiotics and have higher intracellular survival, allowing *S. aureus* to persist in the human host. On the other hand, *P. aeruginosa* is able to sense the presence of *Staphylococci* and as a response increase the production of virulence factors, thereby also causing damage to the host. Furthermore, data also exist that *S. aureus* excretes extracellular factors inhibiting *P. aeruginosa* biofilm formation but also protecting this pathogen by inhibiting its phagocytosis. In this chapter we attempt to understand this complex relationship, which is not only interesting from a bacterial evolution point of view, but also has important consequences for our understanding of the disease pathogenesis for better patient management.

The second aim of this study was to investigate the biofilm interactions between clinical isolates of *P. aeruginosa* and *S. epidermidis* and this is addressed in **chapter 3**. In addition, we also investigated the effect of the biofilm supernatant of some *P. aeruginosa* clinical isolates on the biofilm formation of several *S. aureus* reference strains. Here, we found that the biofilm supernatant of two *P. aeruginosa* strains inhibited and two strains stimulated the growth of *S. epidermidis* clinical isolates. However, coating with this supernatant reduced the adherence of most *S. epidermidis* clinical isolates and *S. aureus* reference strains with an average loss range of 10-100% compared to the control (coating with plain medium) after 6h and 24h. The same effect was observed during biofilm formation under biofilm supernatant pressure of all four *P. aeruginosa* strains tested after 6h of growth and for the supernatant of three of the four *P. aeruginosa* strains after 24h. Remarkably, the biofilm supernatant of one *P. aeruginosa* increased the biofilm formation of most *S. epidermidis* isolates after 24h of growth with an average increase range of 10-95%. We found that the *P. aeruginosa* strains with a stimulating effect on *S. epidermidis* growth and biofilm produced a lower amount of the virulence factors pyocyanin and pyoverdin. The importance of these factors in interspecies interactions is confirmed by the increased expression by *P. aeruginosa* under the pressure of *S. epidermidis* biofilm supernatant. Furthermore, the biofilm supernatant of *S. epidermidis* clinical isolates also stimulated the growth and biofilm formation of some *P. aeruginosa* strains although coating had no significant effect. During co-culture, *P. aeruginosa* reduced the colony forming units of *S. epidermidis*, confirming the mainly antagonistic effect.

The third aim is described in **chapter 4** and includes the analysis of the endotracheal tube microbiome associated with *P. aeruginosa* and *S. epidermidis* using 16S rRNA sequencing. We showed an inverse correlation between the presence of *P. aeruginosa* and the species diversity and patient survival. Furthermore, we generated a decision tree predicting patient survival using 16S rRNA data and clinical parameters. Patients with a relative abundance of *Pseudomonadaceae* <4.6% and *Staphylococcaceae* <70.8% had the highest chance of survival during mechanical ventilation. When the relative abundance of *Pseudomonadaceae* was >4.6%, the age of the patient (<66 years) was the most important factor predicting survival. In addition, this study showed consistent associations between presence of *S. epidermidis* and the respiratory pathogen, *Klebsiella pneumoniae* on the ETT of patients who developed

pneumonia. Therefore, we also aimed to identify *in vitro* interspecies interactions between clinical ETT isolates of *K. pneumoniae* and *S. epidermidis*, described in **chapter 5**.

In chapter 5 we attempted to understand the influence of extracellular factors produced by both *K. pneumoniae* and *S. epidermidis* during biofilm formation on the other organism as well as the biofilm structure in dual-species biofilms. We found that pre-coating of a 96-well plate with the biofilm supernatant of *K. pneumoniae* biofilms reduced adhesion of *S. epidermidis*. However, direct incubation in *K. pneumoniae* supernatant increased the biofilm formation of *S. epidermidis* by >50% compared to the control. Coating of a 96-well plate with *S. epidermidis* biofilm supernatant also reduced the adhesion of some *K. pneumoniae* with an average loss range of 10-40% and direct incubation with biofilm supernatant reduced the biomass with an average loss range of 20-60% compared to the control containing plain medium. During co-culture, both organisms could be isolated from the same well although the biofilm structure of *S. epidermidis* was less confluent. These data indicate that coating with the biofilm supernatant of one species can inhibit the attachment of the other. However, direct incubation with the supernatant increased the biofilm formation of most *S. epidermidis* and inhibited the biofilm formation of some *K. pneumoniae* strains.

6.2 General discussion and future perspectives

Biofilm related infections still remain a major complication in intensive care units, despite numerous efforts to combat them. The fast progress in medicine leaves a large population of immunocompromised patients harboring a large amount of indwelling medical devices, thereby increasing the risk of biofilm related infections caused by opportunistic pathogens such as *P. aeruginosa* and *S. epidermidis*. For instance, many patients in the intensive care unit require mechanical ventilation and the insertion of an ETT is almost immediately followed by bacterial colonization[1,2]. Many methods have been developed to prevent or minimize biofilm formation on the ETT, such as the use of different materials, different cuff shapes, luminal coatings or mechanical suctioning[3,4]. However, complete prevention of removal of these biofilms remains challenging and a combination of strategies is required[4]. The difficulties in biofilm eradication have led to the search for alternative strategies, such as, for example, the use of bispecific antibodies targeting both virulence and biofilm formation of *P. aeruginosa* to minimize host damage by inhibiting the excretion of exotoxins and to minimize adherence by targeting the exopolysaccharides[5]. Although this strategy is promising, it is only useful to protect patients colonized by *P. aeruginosa* and rapid and sensitive screening methods are necessary for this preventive measure to be effective. Furthermore, these measurements are only useful for the prevention of virulence and more research is needed develop the optimal combination of several strategies, like the use of rapid diagnostic tests followed by treatment with a combination of specific antibodies and the appropriate antibiotics to completely eradicate the biofilm and minimize the development of antibiotic resistance.

In recent years the importance of polymicrobial infections is increasingly being recognized. Biofilms rarely consist of a single species and the composition of biofilms in the human body can determine health and disease[6]. Distortion of the normal 'healthy' microbial composition (dysbiosis) might cause disease, like the incorporation of *Porphyromonas gingivalis* into a healthy oral biofilm causing a cascade of events leading to periodontitis[7]. In

this work, we first investigated the microbial interactions between *P. aeruginosa* and *Staphylococcus* spp. by an extensive literature search as well as experimentally using clinical isolates. The main focus of the current literature is on microbial interactions of *P. aeruginosa* with *S. aureus*. It was shown that both pathogens had a mainly antagonistic relationship during *in vitro* planktonic experiments but showed synergistic effects during biofilm formation or *in vivo*, where the battle between both organisms leads to increased virulence, antibiotic resistance, persistence in the human host and increased inflammation and associated damage[8]. The investigation of these interactions can lead to novel prevention strategies, which, like the bispecific antibody, do not target the eradication of the bacteria themselves but rather the biofilm formation or virulence. A promising candidate is D-ribose, which is an analogue of the general quorum sensing molecule autoinducer-2, excreted by many organisms and responsible for an increased virulence of *P. aeruginosa*[9]. D-ribose binds to the autoinducer-2 receptors, thereby blocking them and preventing increased virulence and biofilm formation by *P. aeruginosa*. However, polymicrobial interactions and their role in disease progression is only recently being studied and much more research is needed to use them to the patient's advantage.

An increased virulence of *P. aeruginosa* clinical isolates was observed in the presence of *S. epidermidis* extracellular factors produced during biofilm formation, as described in chapter 3. Additionally, some *P. aeruginosa* strains also showed an increased growth and biofilm formation in the presence of *S. epidermidis* biofilm supernatant *in vitro*. However, the investigation of the ETT microbiome associated with *P. aeruginosa* and *S. epidermidis* showed a correlation of the presence of *P. aeruginosa* with the absence of *S. epidermidis*, indicating an antagonistic relationship *in vivo*[10]. This antagonistic relationship was also shown in endotracheal aspirates, where the presence of *P. aeruginosa* was associated with a reduced species diversity under antibiotic pressure and this was linked to the development of pneumonia[11]. More research is needed to elucidate the mechanisms behind these interactions, why some strains are more susceptible to increased virulence and biofilm compared to others and what is the effect of these interactions *in vivo*. Identification of these mechanisms can improve existing prevention strategies and provide targets for new strategies. Another potential approach to reduce patient mortality is the use of the microbiome composition to predict the risk for complications such as pneumonia. It was already shown that the presence of *P. aeruginosa* can be linked to worse patient prognosis in chronic airway infections and during mechanical ventilation[10,12]. These data indicate that screening for the presence of *P. aeruginosa* and subsequent measures to reduce the virulence and transition to the biofilm phenotype can be beneficial for the patient prognosis in intensive care units. Better understanding of the microbiome and interspecies interactions therein can also lead to measures to maintain a healthy microbiome, for example by adequate antibiotic therapy or the use of probiotics.

6.3 Nederlandstalige samenvatting

Hoofdstuk 1 van dit werk geeft een algemene inleiding over de belangrijkste thema's in dit werk: biofilm vorming, ventilator geassocieerde pneumonie en de pathogenen *Pseudomonas aeruginosa* en *Staphylococcus epidermidis*. Een biofilm is een complexe en gestructureerde gemeenschap van micro-organismen die zijn aangehecht aan een oppervlak en omgeven door een extracellulaire matrix. De bacteriën in deze biofilm zijn beschermd tegen allerlei stressfactoren zoals mechanische stress, uitdroging, het immuunsysteem of antibacteriële producten. De meeste bacteriën groeien van nature als een biofilm, vaak bestaande uit meerdere species die met elkaar communiceren en interageren. Biofilms komen zowel voor in het milieu als in het menselijk lichaam en kunnen daar zowel een positieve als negatieve invloed hebben. Zo zorgen de goede bacteriën van de natuurlijke darmflora voor een betere vertering en verhinderen ze de groei van pathogenen. Biofilms kunnen echter ook ernstige schade berokkenen als ze groeien op medische apparatuur, implantaten of katheters. Biofilm-gerelateerde infecties zijn zeer moeilijk te behandelen en vaak chronisch van aard, wat leidt tot een hogere mortaliteit en morbiditeit, een langer verblijf in het ziekenhuis en een hoge gezondheidskost. De uitkomst van (al dan niet biofilm-gerelateerde) infecties is voor een deel afhankelijk van de bacterie die deze infectie veroorzaakt en infecties met meerdere species hebben vaak een slechtere uitkomst.

Een belangrijke complicatie voor patiënten in intensieve zorgeenheden is ventilator-geassocieerde pneumonie (VAP) veroorzaakt door bacteriën uit een biofilm op de endotracheale tube. Wanneer deeltjes met bacteriën van deze biofilm losraken, worden ze meteen in de luchtwegen geblazen en kunnen ze infectie veroorzaken. De Gram-negatieve bacterie *Pseudomonas aeruginosa* is een belangrijke oorzaak van VAP en is gelinkt aan een hoge mortaliteit en morbiditeit. Dit komt vooral door de hoge intrinsieke resistentie tegen aan groot aantal antibiotica, de aanwezigheid van verschillende virulentiefactoren en de sterke biofilm vorming. Daarenboven heeft *P. aeruginosa* een flexibel metabolisme en genoom, waardoor het gemakkelijk kan muteren en zich aan een veranderende omgeving aanpassen. Een ander belangrijk pathogeen van ziekenhuis-gerelateerde infecties is de Gram-positieve bacterie *Staphylococcus epidermidis*. Deze bacterie is onder normale omstandigheden een onschadelijk lid van de commensale flora maar kan ernstige infecties veroorzaken bij immuun gecompromitteerde patiënten. Net als *P. aeruginosa* heeft ook *S. epidermidis* een intrinsieke resistentie voor veel antibiotica, een sterke biofilm vorming en een hoge flexibiliteit, waardoor een infectie moeilijk te behandelen is. *S. epidermidis* heeft echter weinig echte virulentie factoren in tegenstelling tot zijn neef *Staphylococcus aureus*, ook een belangrijk nosocomiaal pathogeen. *P. aeruginosa* heeft een complexe relatie met beide *Staphylococcus* spp. en gemengde infecties hebben vaak een negatieve uitkomst voor de patiënt.

Een eerste doel van dit werk is het in kaart brengen van de microbiële interacties tussen *P. aeruginosa* en *Staphylococcus* spp., met de focus op *S. aureus*, en hun effect op de mens door een grondige literatuurstudie, **beschreven in hoofdstuk 2**. Beide pathogenen hebben een complex netwerk van inhibitie en ontsnappen ontwikkeld en zijn in een constant gevecht verwikkeld voor ruimte en nutriënten. De aanwezigheid van zowel *P. aeruginosa* en *S. aureus* in een infectie is gelinkt aan een slechtere uitkomst voor de patiënt in vergelijking met de

aanwezigheid van één van beiden. Interspecies interacties leiden vaak tot een verhoogde virulentie, inflammatie en daarmee gepaarde weefselschade, zoals bijvoorbeeld in de longen van mucoviscidose patiënten of in chronische wond infecties. *P. aeruginosa* produceert extracellulaire factoren om *Staphylococci* te doden door middel van cel lysis of inhibitie van de oxidatieve respiratie. *S. aureus* kan hieraan ontsnappen door te groeien als L-form kolonies of small colony variants, die tegelijkertijd ook meer resistent zijn tegen antibiotica en een betere intracellulaire overleving hebben. Aan de andere kant kan *P. aeruginosa* ook de aanwezigheid van Gram-positieve bacteriën voelen en als antwoord de productie van virulentiefactoren verhogen, wat ook leidt tot schade aan de gastheer. Verder zijn er ook data bekend waar *S. aureus* extracellulaire factoren produceert die de biofilm vorming van *P. aeruginosa* verhinderen maar tegelijkertijd dit pathogeen ook beschermen tegen fagocytose. In dit hoofdstuk proberen we de complexe relatie tussen beide pathogenen in kaart te brengen. Dit is niet alleen belangrijk vanuit het perspectief van bacteriële evolutie maar heeft ook belangrijke consequenties voor het begrijpen van de ziekte pathogenese en patiënten behandeling.

Het tweede doel van dit werk is beschreven in **hoofdstuk 3** en hier onderzoeken we biofilm interacties tussen klinische isolaten van *P. aeruginosa* en *S. epidermidis* uit endotracheale tube biofilms. We vonden dat het biofilm supernatant van twee *P. aeruginosa* stammen de groei van *S. epidermidis* klinische isolaten verhinderde en twee stammen de groei stimuleerden. Coating van een 96-well plaat met dit supernatant reduceerde de aanhechting van de meeste *S. epidermidis* klinische isolaten en *S. aureus* referentiestammen met een gemiddelde verlies range van 10-100% vergeleken met de controle (coating met gewoon medium) na 6h en 24h incubatie. Het zelfde was ook te zien onder druk van het biofilm supernatant van alle *P. aeruginosa* stammen na 6h incubatie en van drie van de vier stammen na 24h incubatie. Opmerkelijk was dat het biofilm supernatant van één *P. aeruginosa* stam de biofilm van de meeste *S. epidermidis* isolaten stimuleerde na 24h groei met een gemiddelde verhogingsrange van 10-95%. De *P. aeruginosa* stammen met een stimulerend effect op de groei en biofilm van *S. epidermidis* produceerden een lage hoeveelheid van de virulentiefactoren pyocyanine en pyoverdine. Het belang van deze virulentiefactoren in interspecies interacties blijkt ook uit de verhoogde productie van beide factoren in de aanwezigheid van *S. epidermidis* biofilm supernatant. Verder was ook de groei en biofilm vorming van sommige *P. aeruginosa* stammen gestimuleerd in de aanwezigheid van *S. epidermidis* biofilm supernatant maar coating had geen significant effect. Tijdens co-cultuur van beide bacteriën reduceerde *P. aeruginosa* de hoeveelheid kolonie vormende eenheden van *S. epidermidis*, wat de overwegend antagonistische relatie bevestigd.

Het derde doel van dit werk is beschreven in **Hoofdstuk 4** en omvat de analyse van het microbioom op endotracheale tubes van kunstmatig beademde patiënten die positief zijn voor *P. aeruginosa* en *S. epidermidis* met behulp van 16S rRNA sequencing. In deze studie vonden we een inverse correlatie tussen de aanwezigheid van *P. aeruginosa* met species diversiteit en de overleving van de patiënt. Ook konden we een beslissingsboom opstellen met 16S rRNA data en patiënt parameters om overleving te voorspellen. Patiënten met een relatieve hoeveelheid *Pseudomonadaceae* <4.6% en *Staphylococcaceae* <70.8% hadden de hoogste kans op overleving tijdens intubatie. Bij een relatieve hoeveelheid *Pseudomonadaceae* >4.6% was de leeftijd van de patiënt (<66.5 jaar)de belangrijkste factor

om overleving te voorspellen. Verder bleek dat het DNA van een ander Gram-negatief pathogeen, *Klebsiella pneumoniae*, vaak gevonden werd op de endotracheale tube die positief waren voor *S. epidermidis* en waarbij de patiënt een VAP ontwikkelde. Daarom hebben we ook de microbiële interacties tussen klinische isolaten van *K. pneumoniae* en *S. epidermidis* onderzocht , zoals beschreven in **hoofdstuk 5**.

In hoofdstuk 5 proberen we de invloed van extracellulaire factoren geproduceerd tijdens biofilm vorming door zowel *K. pneumoniae* als *S. epidermidis* op de biofilm vorming van de ander te achterhalen. We vonden dat een voor-gecoate laag biofilm supernatant van *K. pneumoniae* op een 96-well plaat de biofilm vorming van *S. epidermidis* verminderde met een gemiddelde verlies range van 30-60% in vergelijking met de controle (coating met blanco medium). Directe incubatie in *K. pneumoniae* biofilm supernatant leidde tot een verhoogde biofilm vorming van *S. epidermidis* met >50% in vergelijking met de controle (groei in normaal medium). Coating met *S. epidermidis* biofilm supernatant zorgde ook voor een verminderde aanhechting bij 5/16 *K. pneumoniae* stammen met een gemiddelde verlies range van 10-40% in vergelijking met de controle en directe incubatie met *S. epidermidis* supernatant zorgde voor een gemiddelde verlies range van 20-60% in vergelijking met de controle. Tijdens co-cultuur van beide species konden beide uit dezelfde well worden geïsoleerd maar was de structuur van de *S. epidermidis* biofilm veranderd in een minder confluente laag. Deze data wijzen op een stam- en conditie- afhankelijk effect van extracellulaire factoren op de biofilm vorming. Coating met het biofilm supernatant van beide species kan de aanhechting van de andere verhinderen en directe incubatie met biofilm supernatant stimuleert de biofilm vorming van de meeste *S. epidermidis* stammen maar verhindert de biofilm van sommige *K. pneumoniae.*

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