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Abstract

Mechanical ventilation is a life-saving strategy for critically ill patients in the intensive care unit. However, this technique is not without risk, as VAP or ventilator-associated pneumonia is one of the most common nosocomial respiratory tract infections. Gram-negative organisms, including *Pseudomonas aeruginosa*, commonly cause VAP, followed by Gram-positive organisms like *Staphylococcus aureus*. The exact mechanisms of the development of VAP are, however, not yet understood.

For a long time, the presence of the commensal bacterium *Staphylococcus epidermidis* in diagnostic aspirates of patients with VAP was attributed to contamination of the sample. But, the growing importance of *S. epidermidis* as a causative agent of medical device-associated infections, suggests otherwise. The success of *S. epidermidis* as a causative pathogen of indwelling medical device-associated infections can be partly explained by its ability to form strong, drug resistant biofilms on the surface of indwelling medical devices, such as endotracheal (ET) tubes. However, the presence of this organism on the ET tube not always leads to VAP, suggesting a contribution from the bacterial consortium on the ET tube in the development of VAP. It is hypothesised that the presence, absence or relative abundance of certain species either directly triggers the development of VAP or affects predominant virulence factors associated with other species, for example *S. epidermidis,* possibly by means of altered gene expression. These interactions might also play an important role in the development of disease since the presence of species A might enhance or inhibit the growth of species B. Other pathogens might be the actual cause of VAP-infection and, in some cases, *S. epidermidis* might serve as a primary colonizing species on the endotracheal tube, providing a basis for other secondary pathogenic colonizers or even the other way around. The yeast *Candida albicans*, and the Gram-negative bacteria *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa,* were frequently found in the ET tube together with *S. epidermidis*.

This research project intends to characterize *S. epidermidis* isolates present on the ET tube of patients with and without VAP and to investigate specific dysbiotic interactions within the bacterial community in *in vitro* biofilm models. Clinical isolates of *S. epidermidis* were collected from 47 mechanically ventilated patients who did or did not develop VAP-infection. In general, *S. epidermidis* strains associated with VAP (n = 23) were compared with non-VAP (n = 24) associated strains in order to determine predominant causative bacterial factors for the development of VAP. To determine the evolutionary relationship between the different *S. epidermidis* isolates, a phylogenetic scheme was constructed using MLST typing. Susceptibility against frequently administered antibiotics in the hospital environment was tested in order to construct a resistance profile, which was analysed more in depth with *SCCmec* typing. In addition, both single- and multispecies biofilms of *S. epidermidis* were cultivated under both static and dynamic conditions in order to study the structure and interactions within the formed biofilms. Finally, biofilm-forming capacity of *S. epidermidis* was further studied by typing for presence of the ACME-element.

Throughout the research it became clear that the presence of *S. epidermidis* in patients is indeed not linked to the prevalence of VAP or non-VAP. It is, however, the dynamic balance of both positive and negative interactions with neighbouring bacteria that will play an essential role in determining whether certain virulence factors cause damage or not. This shows, in concordance with other reports, that more than one pathogen is needed to cause respiratory infections, demonstrating a polymicrobial aspect concerning the development VAP.

This research is aimed at increasing the understanding of antagonistic and synergistic interactions between *S. epidermidis* and pathogens like *S. marcescens* and *P. aeruginosa*, and their role in causing an imbalance within the consortium on the ET tube. This may prove to be a critical and fundamental aspect of VAP-infection and might have a significant impact on mortality, morbidity, and management of medical device-related infections associated with *S. epidermidis*.

Samenvatting

Kunstmatige beademing is vaak een levensreddende medische ingreep voor patiënten in de intensieve zorg. Doch, is deze techniek niet zonder risico, aangezien ventilatorgeassocieerde pneumonie (VAP) één van de meest voorkomende nosocomiale respiratorische infecties is. Gram-negatieve organismen zoals *Pseudomonas aeruginosa*, veroorzaken vaak VAP, gevolgd door Gram-positieve organismen zoals *Staphylococcus aureus*. Het exacte mechanisme waardoor VAP ontwikkeld is echter tot op heden nog niet bekend.
Gedurende een lange tijd werd de aanwezigheid van de bacteriële commensaal *Staphylococcus epidermidis* in de diagnostische stalen van patiënten met VAP geweten aan besmetting van het staal. Desondanks, suggereert het groeiende belang van *S. epidermidis* als de oorzaak van infecties gerelateerd met medische apparatuur anders. Het succes van *S. epidermidis* als oorzaak van infecties gerelateerd met medische beademingsapparatuur kan deels uitgelegd worden door de mogelijkheid om sterke, resistente biofilms te vormen op het oppervlak van zulke apparatuur. De aanwezigheid van dit organisme op de endotracheale (ET) tube veroorzaakt echter niet altijd VAP, wat een bijdrage suggereert van de bacteriële gemeenschap, die aanwezig is op de ET tube, in de algemene ontwikkeling van VAP. De aanwezigheid, afwezigheid of relatieve dichtheid van sommige soorten kan ofwel op directe wijze de ontwikkeling van VAP veroorzaken of kan enkele predominante virulentiefactoren van andere soorten, zoals *S. epidermidis,* mogelijk beïnvloeden door gewijzigde genexpressie. Deze interacties kunnen mogelijk een belangrijke rol spelen in de ontwikkeling van infectie aangezien de aanwezigheid van soort A de groei van soort B kan verhinderen of versterken. Derhalve, kunnen andere pathogenen de eigenlijke oorzaak van VAP-infectie vormen en *S. epidermidis* zou hier kunnen functioneren als een primaire koloniserende soort op de ET tube, die de basis legt voor andere secundaire pathogene koloniserende soorten, of omgekeerd. De gist *C. albicans*, en de Gram-negatieve bacteriën *K. pneumoniae*, *S. marcescens* en *P. aeruginosa*, werden frequent in aanwezigheid van *S. epidermidis* op de ET tube gevonden.

Dit onderzoeksproject heeft als doel *S. epidermidis* isolaten die aanwezig zijn op de ET tube van patiënten te karakteriseren en beoogt om specifieke dysbiotische interacties binnen de bacteriële gemeenschap te bestuderen in *in vitro* modellen. Klinische *S. epidermidis* isolaten werden verzameld van 47 kunstmatig beademde patiënten dewelke al dan niet VAP-infectie ontwikkeld hadden. *S. epidermidis* stammen geassocieerd met VAP-infectie (n=23) werden vergeleken met stammen van patiënten die geen VAP-infectie ontwikkelden (n=24). De evolutionaire verwantschap tussen de verschillende *S. epidermidis* isolaten werd onderzocht door middel van constructie van een fylogenetisch schema op basis van MLST typering. De gevoeligheid tegenover frequent toegediende antibiotica in het hospitaal werd onderzocht om een resistentieprofiel te kunnen opstellen, dat uitvoeriger werd geanalyseerd door middel van *SCCmec* typering. Zowel eensoortige als gemengde biofilms van onder meer *S. Epidermidis* werden opgekweekt onder statische en dynamische omstandigheden om de structuur en interacties in de gevormde biofilms te kunnen bestuderen. Ten slotte, zal de biofilm vormingscapaciteit van *S. epidermidis* uitvoeriger bestudeerd worden door typering voor aanwezigheid van het ACME-element.
Doorheen het onderzoek werd het duidelijk dat de aanwezigheid van *S. epidermidis* in patiënten inderdaad niet gerelateerd is aan het voorkomen van VAP of non-VAP. Het is echter de dynamische balans van zowel voordelige als nadelige interacties met nabije bacteriën die een essentiële rol speelt in het bepalen of zekere virulentie factoren al dan niet schade veroorzaken. Dit toont aan, in overeenstemming met andere rapporten, dat meer dan één pathogeen noodzakelijk is om respiratorische infecties te veroorzaken en wijst op een polymicrobieel aspect betreffende de ontwikkeling van VAP.

Dit onderzoek poogt om de antagonistische en synergistische interacties tussen *S. epidermidis* en pathogenen zoals *S. marcescens* en *P. aeruginosa*, en hun rol in het veroorzaken van een onevenwicht binnenin het consortium op de ET tube te bestuderen en te verhelderen. Dit kan een fundamenteel aspect blijken te zijn van VAP-infectie en zou een significante impact kunnen hebben op de mortaliteit, morbiditeit en het beheer van dergelijke infecties geassocieerd met *S. epidermidis*.

List of abbreviations

Aae	Autolysin/adhesion
Aap	Accumulation associated protein
ACME	Arginine catabolic mobile element
Agr	Accessory gene regulator
AHL	Acyl homoserine lactone
AI	Autoinducer
AIP	Autoinducer peptide
AMP	Antimicrobial protein
ANOVA	Analysis of variance
ArcC	Carbamate kinase
AroE	Shikimate dehydrogenase
ATCC	American type culture collection
AtlE	Major autolysin E of *S. epidermidis*
BAL	Bronchoalveolar lavage
Bap	Biofilm associated protein (*S. aureus*)
Bhp	Bap homologue protein (*S. epidermidis*)
CA	*Candida albicans*
CC	Clonal complex
Ccr	Cassette chromosome recombinase
CFU	Colony-forming unit
Cip	Ciprofloxacin
CLSI	Clinical and laboratory standards institute
CLSM	Confocal laser-scanning microscope
CoNS	Coagulase-negative *Staphylococcus* spp.
DLV	Double-locus variants
DPD	4,5-dihydroxy-2,3-pentadione
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EPS	Extrapolymeric substances
ET	Endotracheal tube
EUCAST	European committee on antimicrobial susceptibility testing
FBRI	Foreign body-related infections
Fox	Cefoxitin
Gtr	ABC transporter
Gm	Gentamicin
HGT	Horizontal gene transfer
HVR	Hyper-variable region
I	Intermediary
IBIVAP	Identification of predictive biomarkers of pneumonia in artificially ventilated patients
Ica	Intercellular adhesion genes
ICU	Intensive care unit
IS	Insertion sequence
KP	*Klebsiella pneumoniae*
LuxS	S-ribosylhomocysteinase
MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
MDR	Multi-drug resistant
MDP	Muramyl dipeptides
MEP	Mucoid exopolysaccharide
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing

MOSAR	Management of antimicrobial resistance in the hospital and the spread within the community
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-susceptible *S. aureus*
MSSE	Methicillin-susceptible *S. epidermidis*
MRSA	Methicillin-resistant *S. aureus*
MRSE	Methicillin-resistant *S. epidermidis*
MutS	DNA mismatch repair protein
OD	Optical density
PA	*Pseudomonas aeruginosa*
PBP	Penicillin-binding protein
PBP2a	Alternative penicillin-binding protein 2a
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pfs	5' methylthioadenosine/S-adenosylhomocystein nucleosidase
PGA	Poly-ɣ-dl glutamic acid
PI	Propidium iodide
PIA	Polysaccharide intercellular adhesin
PNA	Peptide nucleic acid
PNAG	Poly-N-acetylglucosamine
PNA FISH	Peptide nucleic acid fluorescence *in situ* hybridization
PSM	Phenol-soluble modulin
PVC	Polyvinylchloride
PyrR	Pyrimidine operon regulatory protein
QS	Quorum-sensing
R	Resistant
RH	S-ribosylhomocysteine
Rpm	Resolutions per minute
R-THMF	(2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran
S	Sensitive
SA	*Staphylococcus aureus*
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SCCmec	Staphylococcal cassette chromosome *mec*
SE	*Staphylococcus epidermidis*
SdrF, G, H	Serine-aspartate repeat protein family (F, G, H)
SDS	Sodium dodecyl sulphate
SLV	Single-locus variant
SM	*Serratia marcescens*
SPRI	Solid phase reversible immobilization
SRH	S-ribosylhomocysteine
ST	Sequence type
Stx	sulfamethoxazole with trimethoprim
TBE	Tris/Borate/EDTA
TLV	Triple-locus variant
TpiA	Triosephosphate isomerase
Tris	Tromethamine buffer
TSB	Tryptone soy broth
UPGMA	Unweighted pair group method with arimethic mean dendrogram
VAP	Ventilator-associated pneumonia
YqiL	Acetyl coenzyme A acetyltransferase

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Indwelling medical devices like intravenous catheters, prosthetic heart valves, joint prostheses, peritoneal dialysis catheters, cardiac pacemakers, cerebrospinal fluid shunts and endotracheal tubes save millions of lives every day, but constitute at the same time a high risk of foreign body-related infections (FBRI), which are notoriously difficult to treat [2]. VAP or ventilator-associated pneumonia is such an infection, complicating the course of mechanical ventilation in trauma patients in the intensive care unit (ICU).

1.1. Ventilator-associated pneumonia

1.1.1. The pathogenesis of VAP

VAP is the most common form of hospital-acquired pneumonia that develops in patients who have been ventilated for 48 hours or longer, and who had no symptoms of lower respiratory infection before the onset of mechanical ventilation [3]. Ventilator-associated pneumonia is the most frequent nosocomial infection in the ICU, occurring in 8-28% of mechanically ventilated patients. Mortality rates are high, ranging from 15-70%, and length of stay in the hospital is increased with 5 to 7 days, adding a cost of approximately €29 518 per patient [4-6]. VAP is defined as inflammation of the lung parenchyma by infectious agents, thereby causing shortness of breath, increasing respiratory rate, sputum production, chest pain, fever, fatigue, muscle ache and lack of appetite [7, 8]. A distinction can be made between early-onset VAP, which occurs during the first four days of mechanical ventilation, and late-onset VAP, which develops five or more days after initiation of mechanical ventilation [9]. For both conditions, not only the causative pathogens are different, but also the severity of the disease and prognosis. Early-onset VAP is predominantly caused by species such as *Haemophilus influenzae*, *Streptococcus pneumonia*, multi-drug susceptible *Staphylococcus aureus* (MSSA), and other Gram-positive bacteria. Late-onset VAP is mostly caused by bacterial species such as *Pseudomonas aeruginosa*, *Actinobacter* spp., multi-drug resistant *Staphylococcus aureus* (MRSA) and other multi-resistant Gram-negative bacilli [10]. Early-onset VAP is usually less severe and is characterized by significantly lower mortality and morbidity than late-onset VAP.

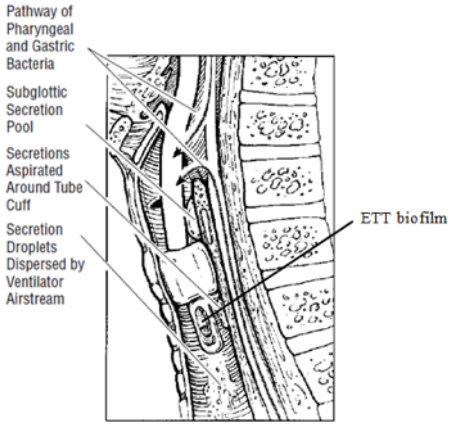

Figure 1: The development of VAP. Bacteria residing in the oropharynx and gastrointestinal tract can contaminate the subglottic secretion pool. Subglottic secretions above the ETT cuff are aspirated into the trachea and disseminated into the distal airways and lung parenchyma by ventilator force [1].

Pneumonia is caused by microbial invasion of the normally sterile lower respiratory tract and the lung parenchyma when the normal defence mechanisms of the lungs, such as; anatomic barriers, cough reflexes, mucociliary clearing, and innate and humoral immunity, are defect or challenged by an overwhelming inoculum of pathogenic bacteria [6]. A hospital-acquired infection is mostly caused by aspiration of potential pathogens that have colonized the mucosal surfaces of the oropharyngeal airways, but may also be caused by introduction of contaminated medical equipment inside the patient's body or by transmission of nosocomial pathogens from the hands of health-care workers [8, 11]. Additionally, teeth with dental plaques might also serve as a reservoir of infection [12], as does the stomach [13].

However, the presence of the endotracheal tube for ventilation is the most predominant factor for the development of VAP as it disrupts the cough reflex, promotes accumulation of tracheobronchial secretions and mucus, injures the tracheal epithelial surface, and provides a

direct conduit for pathogenic organisms to reach the lower respiratory tract through a hydrostatic gradient [14-16]. Endotracheal tube placement induces an abnormal continuum between the upper airway and the trachea, and establishes a subglottic reservoir of bacterial pathogens, which might become aspirated. Here, the inner surface of the endotracheal tube provides a unique restricted immunological environment of high shear forces and bi-directional flow [5]. Small numbers of organisms will initially lodge into irregularities along the endotracheal surface from which micro- and ultimately, macrocolonies and biofilms build up on the inner and outer lumen of the tube [17, 18]. On insertion, indwelling medical devices are rapidly coated with human nutrient-rich serum proteins, including fibronectin, collagen, fibrinogen and vitronectin [19]. As invading pathogens possess multiple adherence factors that can bind to these serum proteins, biofilm formation by bacteria is mainly favoured on the inner lumen of the endotracheal tube [20, 21] and can start within 24 hours of intubation [22]. Additionally, secretions in the subglottic pool are aspirated into the trachea and become part of these biofilms lining the endotracheal tube in the distal airways. Pieces of the biofilm can then be disseminated by the force of the ventilator [23]. Moreover, micro-aspirates containing organisms originating from these biofilms can reach the lung by means of pooling and leakage of these contaminated secretions around the endotracheal tube cuff, potentially leading to VAP [9, 14, 24-26].
By definition, biofilms are communities of microorganisms that stick to each other and/or to a surface, mostly by the production of a self-produced extracellular matrix. Biofilm formation can have significant implications for disease progression as these microbial communities are much more resistant to antibiotics than their planktonic counterparts [27], and can cause persistent infections that are often lethal [28].

1.1.2. The microbiology of VAP

Bacteria normally heavily colonize the aerodigestive tract above the vocal cords; however, the lower respiratory tract usually remains sterile [29]. The pulmonary microbial community is mainly constituted of the oral and nasopharyngeal microbiota, harbouring complex multispecies interactions, The communities can harbour more than 700 different species of which over 50% has not yet been identified [4]. The most predominant taxa that could be observed in the respiratory tract of healthy individuals are *Streptococcus*, *Corynebacterium*, *Neisseria*, *Prevotella*, *Fusobacterium*, *Haemophilus*, *Rothia* and *Veillonella* species [30]. Common species found in the mouth are *Porphyromonas gingivalis* and *Streptococcus mutans* [12]. But also *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacter cloacae* are frequently present [31]. Within the oral cavity, a diverse fungal microbiota can coexist [5]. The most frequently isolated species are *Candida* spp., followed by the genera *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, and *Aspergillus*. The microbiota found in the oropharynx consists out of the predominant phyla *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. The microbiota of the nostril contains the predominant phyla *Firmicutes* and *Actinobacteria*, including the bacterial families *Staphylococcaceae*, *Propionibacteriaceae*, and *Corynebacteriaceae* [32]. In correlation to VAP, *Corynebacteriaceae* are most commonly found on the ET tube associated with *S. epidermidis* [33]. Within each of these body areas, multiple species often compose microbial communities that interact negatively or positively with each other and their environment. In this way cooperation and competition between the constituents are keys to increase the fitness of the different species by means of intercellular interactions and communication [34-36]. In positive microbial interactions, a microorganism can be predisposed to infection through the creation of favorable conditions that allow other species to grow. In negative microbial interactions, a microorganism might preferentially grow through the elimination of favorable conditions, which are indispensable for the growth of other species [37].

In addition, unfavourable disruption of the dynamic balance between the host and the microbial community at local sites might lead to disease [38]. The presence of commensal pulmonary organisms within clinical samples of VAP-patients is often considered to be due to accidental contamination during the sampling and these bacteria are consequently not recognized as clinical significant pathogens [39]. It is, however, likely that many pathogens did not initially evolve as pathogens but as commensals, and simply take on this role as a result of a lack of ability of the host to maintain homeostasis. Homeostasis might become disrupted on admission in the hospital, where the "normal" microbiota is known to alter significantly within a few days after admission [40].
The composition of the pulmonary microbiome can also change significantly with diseased state of the host [34]. Disruption of mucociliary clearance, enhanced binding of bacteria to the epithelial cells and impairment of host immune responses will occur in hospitalized patients [41]. The normal oral microflora may represent pioneering colonising species and promote subsequent endotracheal tube biofilm development. The flora of the oropharynx and the anaerobic flora of the colon have a low virulence, but these bacteria can become overgrown by endogenous aerobic Gram-negative and Gram-positive bacilli that colonize the airway [34]. This might enhance the lingering virulence capacity of commensal strains and will form the origin of later disease. Overall, aerobic Gram-negative bacilli represent 58% of all VAP-infections, and Gram-positive cocci make up another 35% [9]. Besides bacterial causes, VAP can also occur as a complication of a wide range of factors (Table 30 in Addendum).

1.1.3. Multi-drug resistance in VAP

The absence of a golden standard for diagnosis of VAP keeps on complicating adequate management of the disease, resulting in inappropriate antibiotic therapy and emergence of multi-drug resistant strains [42, 43]. Additionally, medical device-associated infections are often resistant to the highest deliverable levels of antibiotics, resulting in significant tissue damage and long-term suffering for the patient [44].
A major goal of VAP management is to minimize the inappropriate use of antibiotics, yet at the same time, avoid under-treatment of this serious infection [45]. Therefore, several hospitals have defined a VAP-bundle containing guidelines to prevent development of VAP, rather than applying treatment [46]. Ventilator bundles are a group of interventions related to patients receiving mechanical ventilation that, when implemented together, result in better outcomes than when implemented individually. A number of these recommendations include a semi-recumbent body position of the patient (30°-45° elevation of the head above the line of the bed) to reduce pulmonary aspiration of gastric secretions and reflux fluids, a cuff pressure between 20 and 30cm H_2O to avoid leakage and passage of secretions to the lower airways, and maintenance of oral hygiene of the patient with clorhexidine (0.2%) to avoid contamination. Nonetheless, surgical removal and replacement of the device are often necessary and the only remaining option [47, 48].

1.2. Biofilm formation as a major virulence factor in VAP

1.2.1. What is a biofilm?

Bacteria are single-celled organisms that can exhibit two modes of behaviour; a planktonic state where cells are free-floating in a liquid medium, and a biofilm or attached state in which bacteria are closely packed and firmly attached to each other, usually also to a solid surface. Thus, a biofilm consists of three basic ingredients: microbes, glycocalyx and surface. Biofilms can be defined as highly structured multicellular matrix-enclosed consortia of microorganisms adherent to an inert or living surface with a characteristic three-dimensional structure and physiology [49, 50]. These biofilms show a basic organization in which cells grow in matrix-enclosed microcolonies separated by a network of open water and nutrient

channels, and are characterized by a sophisticated system of cell-cell signals, a high degree of cellular specialization and possibly even interspecies signalling [51].

Distinct stages in biofilm development include initiation, maturation, maintenance and dissolution or dispersion. First, a specific environmental signal induces a genetic program in planktonic cells, thereby initiating attachment to the surface. The surface is subsequently coated with an organic monolayer of polysaccharides or glycoproteins to which more planktonic cells can firmly attach. Once attached to the surface, bacteria become "cemented" to the substratum by sugary molecular strands called extracellular polymeric substances (EPS) in order to develop a mature biofilm. Once a microcolony has been established, the cells form a thick extracellular matrix of polysaccharide polymers and entrapped organic and inorganic materials. As the biofilm matures, the adherent bacteria and matrix takes on complex three-dimensional forms such as columns and streamers. Individual microcolonies may detach from the surface or may give rise to planktonic revertants that swim or float away from these matrix-enclosed structures, leaving hollow remnants of microcolonies or empty spaces that become parts of the water channels. Additionally, whole microcolonies may naturally escape from the biofilm. Detached cells are believed to return to the planktonic mode of growth, thus closing the biofilm developmental life cycle [225].

1.2.2. Multi-drug resistance associated with biofilm formation

Antibiotics may suppress infection cause by detached or planktonic bacteria, but do usually not eradicate biofilms, leading to recurrence following termination of treatment. Biofilms act as a pathogenic mechanism for microbial persistence and impaired response to treatment in VAP. In addition, mechanisms providing resistance to the biofilm are numerous and varied.

Most importantly, biofilms are enclosed within a protective extracellular polymeric substance matrix containing exopolysaccharides, proteins and nucleic acids. This matrix will act as a physical barrier restricting access. Second, slow growth of cells will contribute to decreased susceptibility for growth-rate dependent antimicrobials. A small fraction of bacteria maintain a 'viable but nonculturable state', enabling reconstitution of the biofilm once antibiotic therapy is discontinued and thereby causing a relapsing chronic infection [52]. These fractions of cells that neither grow nor die are called persister cells. Persister cells are a unique class of inactive but highly protected cells with an alternative phenotype that can withstand a wide range of antimicrobial agents, providing greater antimicrobial protection in a specific biofilm. Furthermore, biofilms will facilitate the spread of resistance genes against certain antibiotics by promoting horizontal gene transfer [2]. Biofilms can also evoke a stress response to hostile environmental conditions and overexpress certain resistance genes. Last, the extreme chemical microenvironmental conditions present within a biofilm, including altered pH, pO_2, pCO_2, and hydration level compromise the activity of certain antimicrobials such as aminoglycosides, macrolides and tetracyclines [53].

1.2.3. Multispecies biofilms

While monospecies biofilms are the most common lifestyle studied in laboratories, multispecies or mixed-species biofilms represent the most important lifestyle of microorganisms in hospital-acquired infections associated with indwelling medical devices [51, 54]. Once thought to occur exclusively between dental plaque bacteria, there are increasing reports of co-aggregation between bacteria from other biofilm communities in several diverse habitats. In these communities, microorganisms of different species will compete, cooperate and communicate with each other [55]. In order to survive and proliferate in such complex consortia, bacteria have developed a distinct interactivity in physical contact with their neighboring species, ranging from fierce competition for nutrients and chemical

warfare to collaborative cross-feeding and protective shielding [56, 57]. When a biofilm is composed of heterogeneous species, the metabolic by-products of one organism might serve to support the growth of another, while the adhesion of one species might provide ligands allowing the attachment of others (synergistic interactions). If two clusters of a population are in tight association with each other, one or both must be receiving a beneficial effect through co-metabolism or other forms of synergistic relationships [58], resulting in an overall higher biofilm volume [59]. Conversely, the competition for nutrients and accumulation of toxic by-products generated by primary colonizers can limit the species diversity within a biofilm (antagonistic interactions) [60]. Hereby, one species may completely dominate the biofilm [61].

It is believed that the universal quorum-sensing AI-2 signal and the AI-2 system play an important role in interspecies communication within the multispecies biofilm [62-64], and this is not only limited to interactions among different bacterial species, but also occurs between bacteria and fungi [65].

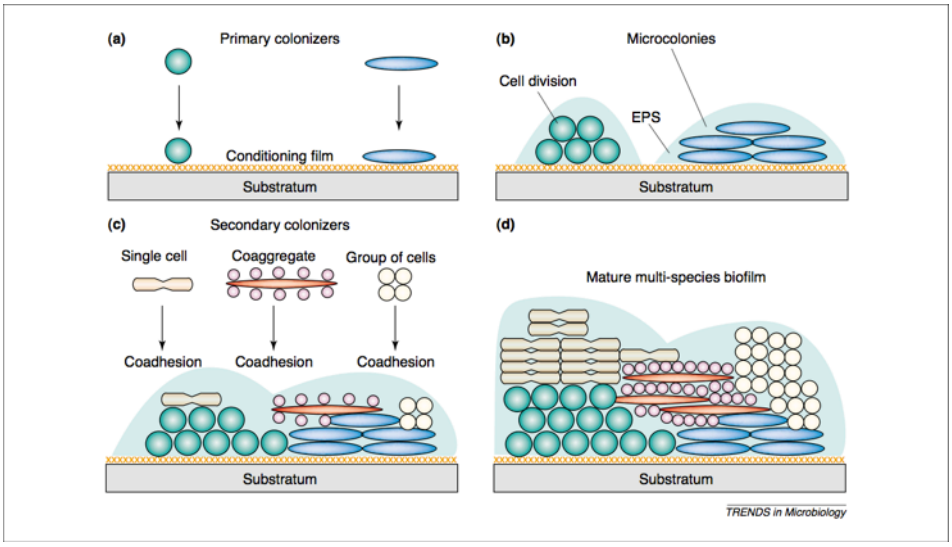

Figure 2: Figure illustrating co-aggregation in the development of multispecies biofilms. (a) Primary colonization of a substratum covered in a 'conditioning film' composed of polysaccharides and proteins; (b) cell growth, division and production of extracellular polysaccharide (EPS) leading to the development of microcolonies; (c) co-adhesion of single cells, co-aggregated cells and groups of identical cells into the young multispecies biofilm; and (d) maturation and formation of local clonal populations within the multispecies biofilm [54].

The formation and development of multispecies biofilms, illustrated in Figure 2, proceeds as a succession of adhesion and multiplication events. The first organisms to attach are the primary or early colonizers and primary colonization is mediated through physicochemical interactions with components of an adsorbed, organic conditioning film. If conditions are suitable, the primary colonizers can then multiply on the substratum to form microcolonies. As environmental conditions change within the young biofilm and the substratum becomes covered by bacteria, secondary or late colonizers are then able to attach to the primary colonizers and the biofilm begins to develop into a multispecies biofilm [54, 66, 67].

1.3. The role of *Staphylococcus epidermidis* as VAP pathogen

The skin commensal *Staphylococcus epidermidis*, belonging to the coagulase-negative *Staphylococci* (CoNS), is a member of the human commensal aerobic flora, and is becoming increasingly important in nosocomial and device-associated infections. In fact, *S. epidermidis* represents the most common source of infections on indwelling medical devices in health care [68]. This is because *S. epidermidis* is a permanent colonizer of the axillae, head and nares, resulting in a high probability of device contamination during insertion [69]. Epidemiological studies have demonstrated that healthy people carry between 10 and 24 different strains of *S.*

epidermidis at any one time. A human might benefit from *S. epidermidis* colonization since attachment of more virulent bacteria such as *S. aureus* is inhibited [22]. In contrast to *S. aureus*, *S. epidermidis* does not produce a great deal of aggressive virulence factors and hence infections by *S. epidermidis* are of a less acute and more long-lasting nature [70]. In addition, *S. epidermidis* has the ability to form sticky multi-layered bacterial aggregates, which are hard to remove and to eradicate [71]. Within this context, biofilm formation can be thought of as a major virulence factor, which contributes to the bacterium's ability to cause a nosocomial infection [72]. It is likely that most, if not all, 'virulence' factors have original functions in the commensal lifestyle of the bacterium [73]. Most of the pathogenicity of coagulase-negative *Staphylococci* appears to originate from molecular determinants, which evolved for a commensal life on the skin, but which may have risen to additional use during infection [19, 74-76]. Obviously, *S. epidermidis* lives on the edge between harmless commensalism and low-level virulence that most of all seems to aim at persistence rather than aggressively attacking the host [77].

1.3.1. Single-species biofilm of *S. epidermidis*

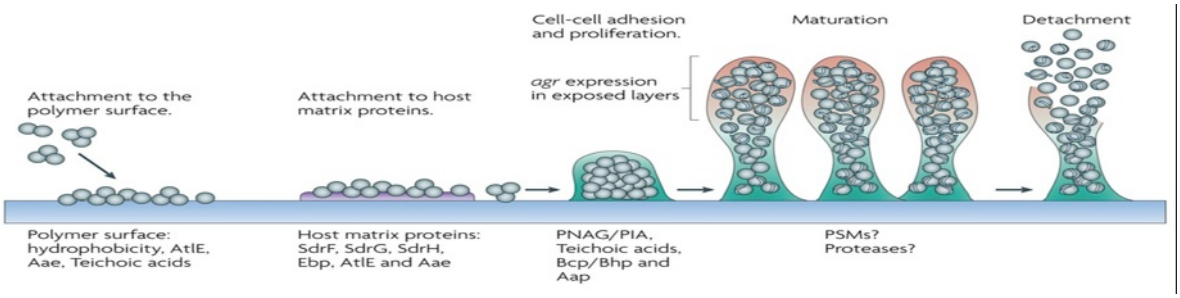

Figure 3: Biofilm formation of *S. epidermidis*. Attachment to uncoated material is mainly dependent on cell surface hydrophobicity, while surface proteins mediate adhesion to host-matrix covered devices. Afterwards, exopolysaccharides, specific proteins and accessory macromolecules provide intercellular integration. Biofilm maturation and structuring is regulated by quorum-sensing controlled expression of PSM's and proteolytic activity. Gene expression within the biofilm is significantly different from the planktonic mode of growth and includes overall down-regulation of basic cell processes [69].

In *S. epidermidis*, biofilm formation is regarded as its major virulence factor, and initial adherence to surfaces is mediated by hydrophobic interactions, electrostatic interactions, and van der Waals forces [50]. But adherence can also be mediated by bacterial surface structures and proteins such as teichoic acids and the autolysin/adhesion proteins AtlE and Aae [78]. On its surface, *S. epidermidis* also carries cell wall-associated proteins that interact with host matrix proteins that coat the endotracheal tube [79]. Matrix-binding proteins such as SdrF, SdrG, and others that bind to fibrinogen, collagen, vitronectin or fibronectin promote initial adherence of *S. epidermidis*. These particular surface proteins are called microbial surface components recognizing adhesive matrix molecules (MSCRAMM's) and can be used to evade the host immune system [75, 80, 81].

In many strains, the accumulative stage of *S. epidermidis* biofilm formation is accomplished by production of a polysaccharide adhesion (PIA), which represents a β-1, 6-linked glucose aminoglycan substituted with partly deacetylated residues [82]. PIA supposedly works like glue that sticks all the planktonic cells in the biofilm together by electrostatic interaction [83]. The enzyme complex responsible for PIA synthesis is encoded by the *ica*ADBC operon, which comprises an N-acetylglucosaminetransferase (*icaA* and *icaD*), a PIA deacetylase (*icaB*), a putative PIA exporter (*icaC*), and a regulatory gene (*icaR*) [84-86] (Figure 26 in Addendum). PIA is made up of sulfated polysaccharide, thereby allowing other bacteria to bind to the already existing biofilm, possibly creating a multilayer multispecies biofilm. Other mechanisms of biofilm formation include the accumulation-associated protein Aap that forms fibril-like, structures on the cell surface, and the biofilm-associated protein Bap/Bhp [22].

These specific surface proteins can mediate biofilm formation with PIA or exclusively on their own [19]. PIA-independent biofilms are considered "weaker" than the PIA-dependent biofilms; that means they often have a lower amount of extracellular matrix material. Additionally, teichoic acids and extracellular DNA are also part of the biofilm matrix and contribute to *S. epidermidis* biofilm formation as stabilizing factors through their polyanionic nature [87-89]. Once matured, the structure of the biofilm reveals groups of microcolonies, which are separated by fluid-filled channels that are thought to deliver nutrients and oxygen to all cells in the biofilm and facilitate the removal of metabolic waste [90, 91]. Finally, detachment of *S. epidermidis* biofilms is supposed to be associated with the activity of the quorum-sensing system *Agr* that controls a range of phenol-soluble modulins (PSMs) and proteases [70, 92]. The *S. epidermidis* δ-toxin is a PSM with a detergent-like function that is assumed to disrupt the polysaccharide-biofilm matrix, while *Agr*-controlled proteases are likely involved in the dispersion of protein-mediated biofilms [93, 94]. Additionally, phase variation of PIA, regulated by insertion sequence *IS*256, will induce reversible phenotypic and genotypic changes within the biofilm [95] resulting in dispersion towards new colonisable environments [96, 97].

1.3.2. Quorum-sensing in *S. epidermidis* biofilms

Bacteria produce small chemical-signal molecules (autoinducers or pheromones) that accumulate in the extracellular environment as they multiply within the maturating biofilm allowing them to engage in social behaviour [98]. Detection of these signals by bacterial cells enables bacteria to monitor the population density and adjust their gene expression accordingly [59]. This process of cell-cell communication is known as quorum sensing, and is a response to the signal molecules that is only initiated when a threshold level is reached. In general, Gram-negative bacteria use acylated homoserine lactones (AHL) as autoinducers, and Gram-positive bacteria use processed oligopeptides to communicate [99].
Two quorum-sensing systems could be identified and are characteristic for *Staphylococci*; the *LuxS* system with the universal *lux*S-encoded autoinducer 2 and the oligopeptide-two-component-type accessory gene regulator (*Agr*) system [100]. The *Agr* locus is believed to regulate a significant part (16%) of the chromosomal genes involving cell division, virulence and metabolic adaptation [101], and consists of two divergent transcription units, RNAII and RNAIII, controlled by the two promoters P2 and P3. RNAII contains four genes (*agrA*, *agrB*, *agrC* and *agrD*) that are transcribed from promoter P2 while P3 drives the transcription of RNAIII, known as the effector molecule of the *Agr* system. An AIP precursor peptide enzyme (*AgrB* for maturation and export of pheromones) and a two-component signal transduction system (*AgrC*, *AgrA*) is present [102] (Figure 27 in Addendum). In general, *Agr* will upregulate the expression of exoenzymes and toxins (δ-toxin), and will downregulate the expression of surface proteins (AtlE) [103]. However, it does not play a role in PIA synthesis. Conversely, the *LuxS* system regulates the transcription of *ica* genes and the subsequent synthesis of PIA by means of the 'universal' autoinducer-2 signal [104] (Figure 28 in Addendum). *LuxS*, the "universal language" for cross-species communication, is believed to have a dual functionality as either a cell-cell communication signal synthase or as an enzyme that is integral to central metabolism [105, 106]. The *LuxS* enzyme is a component of the S-adenosylhomocysteine (SAH) degradation pathway that reduces feedback inhibition of S-adenosylmethionine (SAM) dependent methylation. It converts S-ribosylhomocysteine (RH) to 4,5-dihydroxy-2,3- pentanedione (DPD) and homocysteine. In the presence of boron, outside the cell, DPD will form a furanosyl borate diester that has AI-2 activity [107].

Although both the *Agr* and *LuxS* quorum sensing systems are involved in the regulation of different factors needed for biofilm formation, they do share the same overall effect on

biofilm development; their activation represses biofilm growth, while disabling the regulatory system encourages biofilm formation [103, 108].

1.3.3. Multi-drug resistance in *Staphylococci*

Resistance to methicillin is widespread among hospital isolates of CoNS and in particular, *S. epidermidis* (methicillin-resistant *S. epidermidis*, MRSE), ranging globally from 75–90% [109]. Methicillin resistance in *Staphylococci* is due to the production of an alternative penicillin-binding protein (PBP2a) that has a low affinity for β-lactam antibiotics. Penicillin binding proteins (PBPs) are peptidase enzymes located in the bacterial membrane that catalyse the transpeptidase reactions of peptidoglycan during cell wall synthesis. β-lactams will covalently bind to these PBPs, thereby inactivating enzyme activity and ultimately contributing to bacterial death. Due to its low affinity, PBP2a can substitute for the enzymatic activity of these PBPs and allow completion of cell wall assembly [110]. The structural component of the *mec* gene, *mecA*, encodes the penicillin binding protein 2a. The *mecA* gene is located on a mobile genetic element called staphylococcal cassette chromosome *mec* (*SCCmec*). The *mec* gene complex comprises the *mecA* gene, its regulatory genes *mecI* and *mecR1*, hypervariable regions (HVR) and associated insertion sequences (IS). Another complex located on *SCCmec* is the cassette chromosome recombinase (*ccr*) gene complex. This gene encodes a recombinase that is responsible for the excision and integration of the *SCCmec* element. The most common *ccr* gene complexes are *ccrA* and *ccrB*. The regions around the *ccr* and the *mec* gene complexes are called J-regions, for "joining" or "junkyard" regions. These J-regions are considered as non-essential components, but may carry additional antimicrobial resistance determinants [111].

In close proximity to the *SCCmec* element and integrated at the *OrfX* site downstream, lies a genetic island called the arginine catabolic mobile element (ACME) [112, 113]. This mobile element contains one or two clusters of genes, *arcA* and/or *opp*-3. *ArcA* encodes a secondary arginine deiminase system that catalyses the conversion of arginine to ornithine, ammonia, and carbon dioxide, with the concomitant production of ATP, and is speculated to neutralize the acid environment on the human skin by producing ammonia. Additionally, ammonia synthesis has also proven to be important for reduction of pH stress in specific microniches that contain high concentrations of organic acids, such as biofilms [114, 115]. *Opp*-3 encodes an oligopeptidepermease system and is believed to facilitate the transport of quorum-sensing pheromones or antimicrobial peptides [19]. As the capacity to grow and survive within the host is an often-overlooked feature of bacterial pathogenicity, ACME might indirectly allow for enhanced transmission and thus increased pathogenicity [111, 116]. On the other hand, it might be possible that the presence of ACME benefits the nosocomial life-style, by promoting biofilm growth, allowing further adaptation and persistence in the hospital environment.

Both ACME and *SCCmec* are, however, not only integrated in the same chromosomal site *OrfX* [117], but are also both mobilized by the same SCC-encoded *ccr* recombinases, This suggests a linkage between colonization capacity and antibiotic resistance [118, 119].

Concluding remarks

Comprehending the bacterial interaction mechanisms by which *S. epidermidis* functions as a pioneering species for the development of VAP in mechanically ventilated patients, in relation to a VAP versus non-VAP patient status together with interactions therein, can lead to the identification of bacterial factors, which might play a role in the establishment of VAP. As such, a better understanding will lead to earlier diagnostics and preventive strategies and, consequently, to a lower mortality and incidence of hospital-acquired infections.

For a long time, *Staphylococcus epidermidis* was considered a harmless commensal found on the human skin and its presence in diagnostic aspirates of patients with VAP used to be attributed to contamination. However, the growing importance of *S. epidermidis* as a causative agent in indwelling medical device-associated nosocomial infections, suggests otherwise. It is possible that patients who are admitted in the hospital are soon colonized by biofilm-forming, multi-drug resistant *S. epidermidis* isolates and their microbiota will be drastically altered within days after admission, thereby causing disruption of homeostasis and affecting the normal inhabiting flora [4]. This newly acquired endogenous microbiota might represent the origin for a later infection, such as VAP. Strikingly, not all patients harbouring *S. epidermidis* proceed to VAP, although the mere presence of the endotracheal tube and the condition of the patient are important factors disturbing the normal pulmonary defence mechanisms. This suggests a role for other constituents in the bacterial consortium on the endotracheal tube. It is believed that the presence, absence or relative abundance of certain pathogens might directly trigger the development of VAP or might affect certain predominant virulence factors associated with the microbial flora present by means of altered gene expression.

Many normal flora residents likely did not initially evolve as pathogens, but simply took on this role as a result of the inability of the host to maintain homeostasis. While these flora residents express certain pathogenic factors, a dynamic balance of both synergistic and antagonistic interactions with their neighbouring bacteria plays an essential role in determining whether these pathogenic factors cause damage or not [120]. This theory is called the keystone-pathogen hypothesis. A keystone pathogen is an agent that remodels the commensal microbiota into a dysbiotic state by causing disruption of homeostasis upon admission in the hospital. This remodelling might occur through direct (for example, altering transcriptional profiles) or indirect effects (for example, manipulation of host signalling with impaired immunosurveillance) on the microbiota, or even by means of both mechanisms. Species that have disproportionately large effects on their communities, given their abundance, are thought to form the 'keystone' of the community's structure. These low-abundance microbial pathogens can then orchestrate inflammatory disease. Thereby, host homeostasis will be disrupted, leading to further dysbiosis. This is the change in the relative abundance of individual healthy microbiota components [121]. In other words, it is not merely the presence of a single organism in a complex community that determines the properties of a biofilm, but it are the interactions between the biofilm residents, which are crucial. The hypothesis was shown to be true for periodontitis and cystic fibrosis [31] (Figure 29 in addendum).

For this reason, it was hypothesised that a VAP-specific microbial community in the endotracheal tube characterizes patients with VAP, while patients without VAP will have a non-VAP specific microbial community. However, independent of whether or not disease will develop, there will be an overlap in both communities. It is believed that the skin commensal *S. epidermidis* is situated within this overlap between both communities, because its presence is not determining for VAP or non-VAP. It is likely that the ability of *S. epidermidis* to cause disease or not will be influenced by interactions with other bacterial species present. These species might be situated in the VAP or non-VAP community. When *S. epidermidis* has interactions with a species from the VAP community, homeostasis within the host will be disrupted and this will most likely trigger the expression of virulence genes associated with *S. epidermidis*. The most important virulence factor of *S. epidermidis* is biofilm formation, allowing the subsequent colonization of other possible pathogenic species, leading to a

vicious circle of increasing inflammation of the lungs by means of microbial interactions within this biofilm. The other way around, interactions of *S. epidermidis* with a non-pathogenic species, or in absence of pathogenic species, will prevent the expression of virulence factors and thus the formation of biofilms. In other words, a certain nosocomial pathogen could prove to be a keystone pathogen for the disease-provoking respiratory microbiota and more precisely, *S. epidermidis*.

The endotracheal tubes of mechanically ventilated ICU-patients with or without VAP were collected upon extubation in the university hospital of Antwerp (UZA). Samples included a time series of ET aspirates starting from the initiation of ventilation until extubation, but also encompass the collection of a bronchoalveolar lavage (BAL) sample upon development of VAP and the acquisition of the ET tube at extubation. When VAP was suspected, both ET aspirates and BAL were be cultured and the microorganisms were identified using mass spectrometry (MALDI-TOF). This revealed the presence of *S. epidermidis* in 47 ET tubes. Age of the patients was ranging from 2 to 82 years old (on average 60 years) and duration of their intubation ranged from 2 to 64 days (on average 11 days). Collected ET tubes were visually evaluated for biofilm formation prior to their division in several sections, which were either used for culture, microscopic analysis of biofilm structure or 16S sequencing. To reveal species diversity and relative abundance within the endotracheal tube associated with *S. epidermidis*, a 16S-based sequencing approach was used.

This research project intends to characterize clinical *S. epidermidis* isolates in the biofilm community present on the endotracheal tube of ICU patients. *S. epidermidis* strains associated with VAP (n = 23) will be compared with non-VAP (n = 24) associated strains. Understanding the composition of core communities associated with certain key pathogens and in relation to a VAP versus non-VAP patient status, together with interactions therein, can lead to the identification of bacterial factors, which might play a role in the development of VAP. The project consists out of three main objectives;

- The characterization of *S. epidermidis* isolates originating from endotracheal tubes from patients with or without VAP.

- The optimization of a monospecies static biofilm model system to study *S. epidermidis* biofilms.

- The optimization of a multispecies biofilm model system (both dynamic and static) to study interactions of *S. epidermidis* with cohabiting bacteria in a biofilm.

For a description of the most commonly used culture media, see Table 31 *in the Addendum.*

3.1. Bacterial strains

Table 1: Different bacterial strains used throughout the entire thesis project**.**

Species	Strain	Characteristics
Candida albicans	CA4-613 (Laboratory of Medical Microbiology), University of Antwerp.	Clinical sample, isolated from an endotracheal tube (sample nr. IBIVAP4-613), used for the multispecies biofilm assay.
Klebsiella pneumoniae	KP4-602 (Laboratory of Medical Microbiology), University of Antwerp.	Clinical sample, isolated from an endotracheal tube (sample nr. IBIVAP4-602), used for the multispecies biofilm assay.
Pseudomonas aeruginosa	PA133 (Laboratory of Medical Microbiology), University of Antwerp.	Clinical sample, isolated from an endotracheal tube (sample nr. IBIVAP0133), used for multispecies biofilm assay.
Serratia marcescens	SM5-807 (Laboratory of Medical Microbiology), University of Antwerp.	Clinical sample, isolated from an endotracheal tube (sample nr. IBIVAP5-807), used for the multispecies biofilm assay.
Staphylococcus epidermidis	SE ATCC 12228 (Winslow and Winslow) Evans, (LGC Standards), France.	Non-biofilm forming, non-infection associated commensal strain with a completely sequenced genome. Used as negative control for biofilm formation in the static biofilm assay [122].
	SE 1457 (Laboratory of Microbiology, Parasitology and Hygiene), University of Antwerp.	Strain capable of PIA-dependent biofilm formation. Used as positive control for biofilm formation in the static biofilm assay [96].
	Clinical isolates from endotracheal tubes[1] (Hospital UZA in Edegem).	
Staphylococcus aureus	SA ATCC 25923 (Laboratory of Medical Microbiology), University of Antwerp.	Recommended strain by CLSI guidelines to act as quality control for disk diffusion testing of antibiotics against *Staphylococci* [123].
	SA ATCC 29213 (Laboratory of Medical Microbiology), University of Antwerp.	Recommended strain by CLSI guidelines to act as quality control for microbroth dilution testing of antibiotics against *Staphylococci* [123].
	SA MOSAR-00003 (3813/04) (Laboratory of Medical Microbiology), University of Antwerp	Clinical isolate from project MOSAR ('Management of Antimicrobial Resistance in the hospital and the Spread within the Community, 2007-2012), and used as internal control for SCC*mec*-typing (type IV) (both *ccr* genes and *mec* gene complex class) [124].
	SA MOSAR-00004 (2233/97) (Laboratory of Medical Microbiology), University of Antwerp	Clinical isolate from project MOSAR and used as internal control for SCC*mec*-typing (type I) (both *ccr* genes and *mec* gene complex class) [124].
	SA MOSAR-00005 (2361/07) (Laboratory of Medical Microbiology), University of Antwerp	Clinical isolate from project MOSAR and used as internal control for SCCmec-typing (type III) (both *ccr* genes and *mec* gene complex class) [124].
	SA MOSAR-00011 (1033/05) (Laboratory of Medical Microbiology), University of Antwerp	Clinical isolate from project MOSAR and used as internal control for SCC*mec*-typing (type II) (both *ccr* genes and *mec* gene complex class) [124].
	SA MOSAR-00142 (07R1868) (Laboratory of Medical Microbiology), University of Antwerp	Clinical isolate from project MOSAR and used as internal control for SCC*mec*-typing (type V) (both *ccr* genes and *mec* gene complex class) [124].

[1]A complete overview of the patients from who the clinical isolate with corresponding strain number was obtained, their intubation time, age and personal concurrence of VAP or non-VAP is given in the Addendum (Table 32 in the Addendum).

	SA (MRSA)-S075 (Laboratory of Medical Microbiology), University of Antwerp	Clinical isolate used as internal control for ACME-typing (both *arcA* and *opp*-3 fragment typing) [119].

3.1.1. Matrix-assisted laser desorption/ionization time-of-flight

This technology generates characteristic mass spectral fingerprints, that are unique signatures for each microorganism and are thus ideal for an accurate microbial identification at the genus and species levels and has a potential to be used for strain typing and identification [125].

In MALDI analysis (VITEK® MS, bioMérieux), samples containing biomolecules (biopolymers such as DNA, proteins, peptides and sugars) and large organic molecules (polymers and other macromolecules) were prepared by mixing the samples with a α-cyano-4-hydroxycinnamic acid (αC) matrix (Max Ion™ Peptide/Protein MALDI Matrix Kit, Life Technologies™), which results in the crystallization of the sample within the matrix. The matrix is composed of small acid molecules that have a strong optical absorption in the range of the laser wavelength used. After the crystallization of the matrix and compound, the target was bombarded with brief laser pulses from usually a nitrogen laser. The matrix absorbs energy from the laser leading to desorption of the analytes that were then vaporized and ionized in the gas phase. This matrix-assisted desorption and ionization of the analytes lead to the formation of predominantly singly charged sample ions. The desorbed and ionized molecules were first accelerated through an electrostatic field and were then ejected through a metal flight tube that is subjected to a vacuum until they reach a detector, with smaller ions travelling faster than larger ions. Consequently, the time of ion flight differs according to the mass-to-charge ratio (m/z) value of the ion. Thus, bioanalytes separated according to their TOF created a mass spectrum that is composed by mass to charge ratio (m/z) peaks with varying intensities. A spectrum is thus a microbial signature that is compared with a database for the identification.

3.2. General characterization of *S. epidermidis*

3.2.1. Multilocus sequence typing

Characterization of isolates of bacterial pathogens on the basis of sequence variation is carried out by multilocus sequence typing (MLST), which generates a nucleotide sequence for internal fragments of seven housekeeping loci for each clinical isolate [126]. This method is a convenient and unambiguous description of the strain (or clone) [127]. Genomic differences between the *S. epidermidis* isolates are likely to reflect the evolutionary relationship amongst the strains and their phenotypic relatedness to VAP. Consequently, a phylogenetic scheme may be constructed [128]. Closely related isolates will have an identical sequence type, or sequence types that differ at only a few loci, whereas unrelated isolates will have completely different sequence types [129].

3.2.1.1. Solutions and buffers

Table 2: Chemical solutions used to determine the sequence types of the different *S. epidermidis* isolates via MLST, adapted from [130].

Solution	Concentration		References
Agarose gel	1.5%	Composed out of 2.4g Ultrapure™ Agarose, 16ml 5x TBE[2], 144ml miliQ water and 16ml GelRed™ Nucleic Acid Stain.	2014.
DMSO (Dimethyl sulfoxide)	100%	Used in PCR to inhibit the formation of secondary structures in the DNA template or the DNA primers.	2013.

[2] Made from 1l miliQ water, 54g 89mM Tris or tromethamine buffer, 27.5g 89mM boric acid and 20ml 2.5mM EDTA (ethylenediaminetetraacetic acid).

dNTP Mix[3] (Deoxynucleotide triphosphates)	1mM	Contains sodium salts of dATP, dCTP, dGTP and dTTP dissolved in water at pH 7.5. Form building blocks of the new DNA strand.	2014.
GelRed™ Nucleic Acid Stain	10 000x in water (0.5ml)	Fluorescent nucleic acid dye for staining of dsDNA, ssDNA or RNA in agarose gels.	Biotium Inc., 2014.
MassRuler DNA Ladder Mix (Ready-to-use, 80-10 000bp)	5µl ng	DNA ladder for accurate quantification and sizing of DNA fragments by agarose gel electrophoresis. Contains a mixture of individual chromography-purified DNA fragments.	Thermo Fisher Scientific Inc., 2014.
$MgCl_2$ (Magnesium dichloride)	50mM/μl	Acts as a cofactor for Platinum®*Taq* polymerase. Affects the annealing of the oligonucleotides to the template DNA by stabilizing the interactions, and also stabilize the replication complex of the polymerase with the template primer.	Invitrogen ™ by Life Technologies™ , 2014.

Table 3: Buffer solutions used to determine the sequence types of the different *S. epidermidis* isolates via MLST.

Buffer		References
Platinum® *Taq* buffer	Regulates optimal pH and salt concentration in PCR. Composed out of 20mM Tris-HCl (pH 8.0), 40mM NaCl, 2mM sodium phosphate, 0.1mM EDTA, 1mM DTT, stabilizers, and 50% (v/v) glycerol.	Invitrogen™ by Life Technologies™ , 2014.
Tichy buffer	Lysis buffer solution composed out of 200ml miliQ water, 0.50g sodium dodecyl sulphate (SDS) and 0.4M sodium hydroxide (NaOH).	2014.

3.2.1.2. Primers

For *S. epidermidis* the following 7 housekeeping genes will undergo typing: carbamate kinase, shikimate dehydrogenase, ABC transporter, DNA mismatch repair protein, pyrimidine operon regulatory protein, triphosphate isomerase, and acetyl coenzyme A acetyltransferase (Table 33 in the Addendum).

Table 4: PCR primers and their corresponding sequence (www.sepidermidis.mlst.net). Internal fragments of the seven loci can be amplified by PCR, using the primers listed beneath and by using chromosomal DNA as a template. Sequences for each locus must be obtained for both the forward and reverse strands, and must be 100% accurate, since even a single error can alter the allelic number obtained and may even convert a known allele into a novel allele and the other way around [131-133]. Primers were obtained from Invitrogen™ by Life Technologies™ , 2014.

Genes and function	Sequence (5'-3')	Amplicon size, bp.
Carbamate kinase (*ArcC*) (purine, glutamate, arginine and proline and nitrogen metabolism)		465
ArcC-F	TGTGATGAGCACGCTACCGTTAG	
ArcC-R	TCCAAGTAAACCCATCGGTCTG	
Shikimate dehydrogenase (*AroE*) (biosynthesis of aromatic amino acids)		420
AroE-F	CATTGGATTACCTCTTTGTTCAGC	
AroE-R	CAAGCGAAATCTGTTGGGG	
ABC transporter (*Gtr*) (transmembrane protein for translocation of substrates across membranes)		438
Gtr-F	CAGCCAATTCTTTTATGACTTTT	
Gtr-R	GTGATTAAAGGTATTGATTTGAAT	
DNA mismatch repair protein (*MutS*) (reparation of errors during DNA replication and DNA damage)		412
MutS-F3	GATATAAGAATAAGGGTTGTGAA	
MutS-R3	GTAATCGTCTCAGTTATCATGTT	
Pyrimidine operon regulatory protein (*PyrR*) (transcriptional attenuation of the pyrimidine nucleotide operon)		428
Pyr-F2	GTTACTAATACTTTTGCTGTGTTT	
Pyr-R4	GTAGAATGTAAAGAGACTAAAATGAA	
Triosephosphate isomerase (*TpiA*) (glycolysis and energy production)		424

[3] Made from a 4.0ml stock solution, containing 0.4ml 100mM dATP, 0.4ml 100mM dCTP, 0.4ml 100mM dGTP, 0.4ml 100mM dTTP, and 2.4ml 1mM Tris at pH 7.0.

Tpi-F2	ATCCAATTAGACGCTTTAGTAAC	
Tpi-R2	TTAATGATGCGCCACCTACA	
Acetyl coenzyme A acetyltransferase (*YqiL*) (fatty acid metabolism, degradation and synthesis of ketone bodies, ...)		416
YqiL-F2	CACGCATAGTATTAGCTGAAG	
YqiL-R2	CTAATGCCTTCATCTTGAGAAATAA	

3.2.1.3. Enzymes

Table 5: Enzyme used during PCR to determine the sequence types of the different *S. epidermidis* isolates via MLST.

Enzyme		References
Platinum® *Taq* DNA Polymerase (Automatic "hot start")	Concentration: 5 U/μl[4] A recombinant *Taq* DNA polymerase complexed with a proprietary antibody that blocks polymerase activity in ambient temperatures. Creates DNA molecules by assembling nucleotides.	Invitrogen™ by Life Technologies™ , 2013-2014.

3.2.1.4. Protocol

DNA of an overnight grown culture (Heratherm® incubator, Thermo Fisher Scientific Inc.) of the clinical isolates was extracted using the Tichy-method. In brief, 1 bacterial colony was suspended in 30μl Tichy-buffer. This solution was heated at 95°C for 15 minutes and then centrifuged at 16 000rpm or 39 210G for 1 minute (Heraeus® Biofuge® pico, DJB Labcare). Afterwards, the solution was diluted with 170μl miliQ water. 1μl of the isolated DNA was added to 24μl PCR-mix, containing for 1 reaction: 15.125μl miliQ water, 2.5μl dNTPs, 2.5μl Platinum® *Taq* buffer, 1μl primers for the corresponding housekeeping gene, 1μl DMSO, 0.75μl $MgCl_2$ and 0.125μl Platinum® *Taq* enzyme. PCR (Veriti® 96-well thermal cycler, Applied Biosystems® from Life Technologies™) involved an initial duration of 95°C for 3 min; denaturation during 34 cycles of 95°C for 30 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute; and a final elongation of 72°C for 10 minutes [133]. The PCR-product was then loaded on a 1.5% agarose gel with 5μl amplicon and 5μl gelloading buffer for one hour at 150V and 200mA. Massruler DNA ladder mix loading was used as marker. Because the genes of interest are housekeeping genes, thus indispensable for survival of the bacterium, each individual strain should inherently display a band on the gel and as such, no quality control was actually needed. Consequently, when a band between 400 and 500 base pairs became visible, DNA isolation and amplification had been successful. The obtained DNA then was sent for complete sequencing to the Genetic Service Facility, VIB. There, ExoSAP-IT® (Affymetrix™), a single-step enzymatic clean-up, was first added to remove contaminants like unconsumed dNTPs and primers from the PCR-product. First, sample ions were loaded onto a capillary by a process called electro kinetic injection. A high field voltage was applied between the sample and the anode end of the capillary, causing DNA ions to migrate into the capillary for sequencing. The 96-capillary 3730xl DNA Analyser (Applied Biosystems® from Life Technologies™) in combination with the ABI PRISM® BigDye™ (Applied Biosystems® from Life Technologies™) Terminator cycle sequencing kit with dichlororhodamine dye terminators was used for traditional DNA Sanger sequencing. This method is based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication [134]. Ultimately, the dye terminators were removed by the magnetic bead-based sequencing purification Agencourt® CleanSEQ system (Beckman Coulter™), based on Solid Phase Reversible Immobilization (SPRI®) technology. Here, magnetic particles coated with carboxyl groups

[4] One unit of Platinum®*Taq* DNA polymerase incorporates 10 nmol of deoxyribonucleotide into acid-precipitable material in 30 minutes at 74°C.

can reversibly bind to product DNA in the presence of polyethylene glycol (PEG) and salt [135], leaving contaminants in the solution. A magnetic field was used to pull the magnetic microparticles out of solution. Contaminants were aspirated and microparticles were thoroughly washed. Under aqueous conditions, purified nucleic acids were easily eluted from the magnetic microparticles. After this procedure, the acquired data was subjected to quality trimming using Seqman Pro™ software from DNAStar Lasergene®. Obtained gene sequences were then added to the MLST database for *S. epidermidis* (www.sepidermidis.mlst.net) to be compared to a reference sequence (see Table 33 in Addendum). An allelic number per locus was combined into an allelic profile, leading to a specific sequence type per strain.

This process eventually allowed to define clonal clusters and to visualize evolutionary relationships between the strains by using the algorithmic program eBURST® V3 from http://sepidermidis.mlst.net/eburst/. The program uses a simple model of bacterial evolution in which an ancestral (or founding) genotype increases in frequency in the population, and starts to diversify, producing a cluster of closely-related genotypes that all descend from the founding genotype [136]. The primary founder of a group was defined as the ST that differs from the largest number of other STs at only a single locus. Initial diversification of the founding genotype of a clonal complex would result in variants of the founder that differ at only one of the seven loci. These genotypes, which have allelic profiles that differ from that of the founder at only one of the seven MLST loci, were called single-locus variants (SLVs). Eventually, SLVs would diversify further, by producing variants that differ at two of the seven loci (double-locus variants [DLVs]), eventually leading to triple locus variants (TLVs) and so on. In larger eBURST groups there may be several STs besides the predicted primary founder that have a number of SLVs of their own. A ST that has diversified to produce multiple SLVs is called a subgroup founder. More precisely, in the eBURST diagram, a ST with at least two assigned descendent SLVs (i.e. three SLVs in total, as the link from the ST to its putative progenitor is also a SLV) is defined as a subgroup founder. The STs and their associated allelic profiles were used as input data. This data was divided into groups, where all members assigned to the same groups shared identical alleles at 6 of the 7 loci with at least one other member of the group. Isolates in the groups defined by eBURST were considered to belong to a single clonal complex. Clonal complexes are typically composed of a single predominant genotype with a number of much less common close relatives of this genotype.

3.2.2. Antibiotic susceptibility testing

High reoccurrence of VAP in ICU-patients is due to high antibiotic resistance in the pathogenic bacteria involved. This resistance could be due to obtained characteristics of the strain itself, such as the presence of the *mecA* gene, or due to protective properties associated with the biofilm mode of growth, such as presence of a glycocalyx (see '*1.2.2. Multi-drug resistance associated with biofilm formation'* and '*1.3.3. Multi-drug resistance in Staphylococci'*, Chapter I). As the type of resistance holds important clues for treatment and prevention, the phenotypic susceptibility of planktonic bacterial cells against specific antibiotics that are frequently administered in the hospital ICU was determined.

3.2.2.1. Antibiotics

Table 6: Antibiotics of different classes used for antibiotic susceptibility testing of the isolated clinical strains of *S. epidermidis*.

Antibiotic	Class	Disk Content	Reference

Cefoxitin	Cephalosporin[5] (disrupt the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity).	30µg	BD (Becton, Dickinson and Company) BBL™ SensiDisc™, 2015.
Ciprofloxacin	Fluoroquinolones (blocks DNA-replication by binding to the enzyme DNA-gyrase thereby causing two-sided fractures in the bacterial chromosome).	5µg	OXOID, part of Thermo Fisher Scientific Inc., 2014.
Gentamicin	Aminoglycosides (binding to the 30S subunit of the bacterial ribosome, thereby interrupting protein synthesis).	10µg	BD (Becton, Dickinson and Company) BBL™ SensiDisc™, 2017.
Sulfamethoxazole with Trimethoprim	Miscellaneous (inhibit successive steps in the folate synthesis pathway; Folic acid is an essential precursor in the *de novo* synthesis of the DNA/RNA nucleosides thymidine and uridine. inhibition of the enzyme starves the bacteria of two bases necessary for DNA replication and transcription).	23,75µg + 1,25µg	BD (Becton, Dickinson and Company) BBL™ SensiDisc™, 2016.
Vancomycin	Glycopeptides (prevents the synthesis of long polymers of N-acetylmuramic acid and N-acetylglucosamine of the bacterial cell wall).		Sigma-Aldrich®, 2014.

The choice of applied testing method (disk diffusion or microbroth dilution) was based on recommendations of CLSI (Clinical and Laboratory Standards Institute) [123] and EUCAST (European Committee on Antimicrobial Susceptibility Testing) [137] guidelines. These guidelines were used to assess whether a specific strain had developed resistance against a certain antibiotic or not, depending on the minimal inhibitory concentration (MIC) and the zone diameter of inhibited growth.

3.2.2.2. Protocol for disk diffusion testing

The technique of choice was the Kirby-Bauer or disk diffusion method, as this method is standardized and recommended in both EUCAST and CLSI guidelines. An inoculum of the strains of interest was incubated overnight at 37°C without CO_2 (Heratherm™ Advanced protocol Microbiological Incubator, Thermo Fisher Scientific Inc.). The next day, 100mm MHA petri dishes (Greiner Bio-One™) were inoculated with the organism under evaluation by moving a swab dipped in a 0.5 McFarland[6] solution. Four 6mm paper disks impregnated with a specific concentration of the antibiotic of choice were placed on the agar plate. Because the disk absorbs water from the agar, the antimicrobial diffuses into the surrounding agar. The rate of diffusion through the agar is not as rapid as the rate of extraction of the antimicrobial out of the disk, therefore the concentration of antimicrobial is highest near to the disk and a logarithmic reduction in concentration occurs as the distance from the disk increases. After overnight incubation at 37°C without CO_2, each antimicrobial had a unique breakpoint zone size indicating sensitivity to that particular antimicrobial compound. This zone corresponds with the lowest concentration of the particular antibiotic at which bacterial growth is still inhibited by lysis [138, 139]. This parameter was compared with CLSI guidelines and EUCAST guidelines, giving certain threshold values for particular antibiotics in order to investigate whether a specific bacterial strain is resistant (R), sensitive (S) or intermediary (I) to a particular antibiotic.

Table 7: Zone diameter interpretive criteria (in mm), according to CLSI guidelines. For example cefoxitin; strains were considered to be resistant if the diameter of inhibited growth was smaller than or equal to 24mm, whereas they were

[5]Cefoxitin is also representable for possible resistance to penicillins and carbapenems, reflecting methicillin-resistance.
[6] Equal to an inoculum of 1.5 x 10^8 colony-forming units per ml.

considered to be susceptible when the diameter was larger than or equal to 25mm. Everything in between these two values is considered to be intermediate.

Cefoxitin 30μg		Gentamycin 10μg		Trimethoprim-sulfa.1.25-23.75μg		Ciprofloxacin 5μg	
S	R	S	R	S	R	S	R
≥ 25	≤ 24	≥ 15	≤ 12	≥ 16	≤ 10	≥ 21	≤ 15

Table 8: Zone diameter interpretive criteria (in mm), according to EUCAST guidelines. For example cefoxitin; strains were considered to be resistant if the diameter of inhibited growth was smaller than or equal to 25mm, whereas they were considered to be susceptible when the diameter was larger than or equal to 25mm. Everything in between these two values is considered to be intermediate.

Cefoxitin 30μg		Gentamycin 10μg		Trimethoprim-sulfa. 1.25-23.75μg		Ciprofloxacin 5μg	
S	R	S	R	S	R	S	R
≥ 25	< 25	≥ 22	< 22	≥ 17	< 14	≥ 20	< 20

S. aureus ATCC 25923 was used a quality control, which should have the following disk diffusion ranges for a reliable result.

Table 9: Zone diameter interpretive criteria (in mm), according to CLSI guidelines, for the quality control *S. aureus* ATCC 25923.

Cefoxitin 30μg	Gentamycin 10μg	Trimethoprim-sulfa. 1.25-23.75μg	Ciprofloxacin 5μg
23-29	19-27	24-32	22-30

3.2.2.3. Protocol for microbroth dilution testing

The disk diffusion test does not differentiate vancomycin-susceptible isolates of *S. epidermidis* from vancomycin-intermediate isolates, nor does the test differentiate amongst vancomycin-susceptible and resistant isolates of CoNS, which will all give similar zones of inhibition. According to CLSI guidelines [140] microbroth dilution tests should be performed to determine the susceptibility of all *Staphylococcal* isolates to vancomycin.

First, an antibiotic stock of vancomycin had to be prepared. More than 100mg of the antibiotic powder was roughly weighted and used to calculate the volume of solvent (miliQ water) needed to obtain the final concentration of the stock solution (5120µg/ml). This was done according to the following formula;

$$volume\ solvent\ (ml) = \frac{actual\ weight\ of\ antibiotic\ powder\ (mg) \times potency\ of\ antibiotic\ (900\frac{\mu g}{mg})}{startconcentration\ of\ stock\ solution\ (5120\frac{\mu g}{ml})}$$

Next, a dilution scheme for the broth was made. The antibiotic stock solution was distributed over 12 separate flasks and diluted with cation-adjusted MHB-medium (pH between 7.2 – 7.4) (intermediate concentrations displayed in Table 10). This way, 12 antibiotic dilutions were prepared and 0.05ml of these dilutions was distributed into a 96-well microtiter plate (CELLSTAR® 96 Well Plate Flat Bottom (polystyrene), Greiner Bio-One™). Next, a standardized inoculum was prepared by suspending bacterial colonies directly in MHB-medium, to achieve 0.5 McFarland. Of this solution, a 10-fold and a 100-fold dilution were prepared for broth inoculation on the 96-well microtiter plate. Within 15 minutes after the inoculum had been standardized, 0.05ml inoculum needed to be added to each well, containing the antimicrobial agent of the corresponding dilution series. This ultimately resulted in a 1:2 dilution of each antimicrobial concentration and a 1:2 dilution of the inoculum (final concentrations displayed in Table 10). For each strain tested, a blank (0.05 ml MHB) and a growth control (inoculum without vancomycin) were included. These inoculated microdilution plates were incubated for 24h at 37°C without CO_2 (Heratherm™ Advanced protocol Microbiological Incubator, Thermo Fisher Scientific Inc.). The minimal inhibitory

concentration end points were determined by comparing the amount of growth in the wells containing the antimicrobial agents with the amount of growth in the growth-control wells. The MIC is the lowest concentration of an antimicrobial agent that completely inhibits growth of the organism in the microdilution wells as detected by the unaided eye. *S. aureus* ATCC 29213 was used as a reference strain, according recommendations of CLSI guidelines.

Table 10: Dilution scheme for microbroth dilution test of vancomycin against *S. epidermidis* isolates.

Step	Concentration (µg/ml)	Source	Volume (ml)	MHB (ml)	Intermediate concentration (µg/ml)	Final concentration (µg/ml)	Log_2
1	5120	Stock	2	18	512	256	9
2	512	Step 1	2	2	256	128	8
3	512	Step 1	2	6	128	64	7
4	512	Step 1	2	14	64	32	6
5	64	Step 4	2	2	32	16	5
6	64	Step 4	2	6	16	8	4
7	64	Step 4	2	14	8	4	3
8	8	Step 7	2	2	4	2	2
9	8	Step 7	2	6	2	1	1
10	8	Step 7	2	14	1	0.5	0
11	1	Step 10	2	2	0.5	0.25	-1
12	1	Step 10	2	6	0.25	0.125	-2

A 1000-fold dilution inoculum was also spiral plated on a MHA plate (Greiner Bio-One™) using Eddy jet 2® (Thermo Fisher Scientific Inc., IUL Instruments) to look for purity and concentration, and grown overnight at 37°C without CO_2 (Heratherm™ Advanced protocol Microbiological Incubator, Thermo Fisher Scientific Inc.). The presence of approximately 100 colonies indicated an inoculum of $1x10^6$ CFU/ml. According to CLSI guidelines, a final optimal inoculum of $5x10^5$ CFU/ml should be reached for optimal results.

Table 11: MIC breakpoint (in µg/ml), according to CLSI and EUCAST guidelines. For example vancomycin; strains were considered to be resistant if the MIC was larger than or equal to 32µg/ml, whereas they were considered to be susceptible when the MIC was smaller than or equal to 4µg/ml. Everything in between these two values is considered to be intermediate.

Vancomycin		
	S	R
CLSI	≤4	≥ 32
EUCAST	≤4	>4

3.2.3. *SCCmec*-typing

Typing of *S. epidermidis* isolates for *SCCmec* was performed by typing for two constituting fragments of the mobile genetic element, the *crr* genes and the *mec* gene complex class. This allows detection of particular types of *SCCmec* complex, which may play a profound role in antibiotic resistance, more precisely methicillin resistance, of the particular clinical isolates.

3.2.3.1. Solutions and buffers

For SCCmec-typing a multiplex PCR was performed. This required all the same chemical solutions that were used to determine the sequence types of the different S. epidermidis isolates via MLST, in Table 2 (see '*3.2.1. Multilocus sequence typing'*, Chapter III).

Table 12: Chemical solutions used to determine presence of *SCCmec* (both the *mec* gene complex class and the *ccr* genes) within the different *S. epidermidis* isolates.

Solution	Concentration		References
Agarose gel	1.0%	Composed out of 1.6g Ultrapure™ Agarose, 16ml 5x TBE, 144ml miliQ water and 16ml GelRed™ Nucleic Acid Stain.	2014.
GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10 000bp)	5 µl ng	Recommended for sizing and approximate quantification of a wide range of double-stranded DNA on agarose gels. The ladder is a mixture of chromatography-purified individual DNA fragments.	Thermo Fisher Scientific Inc., 2014.

3.2.3.2. Primers

Table 13: Primers used for *SCCmec*-typing of the different *S. epidermidis* isolates. Selection of primers was based on *Kondo, Y., et al.*, 2007 [124] Primers were obtained from Invitrogen™ by Life Technologies™ , 2012.

Primers for PCR		Sequence (5'-3')	Amplicon size, bp.
***Ccr* gene primers** (excision and integration of *SCCmec* element)			
	mA1 (mecA)	TGCTATCCACCCTCAAACAGG	286
	mA2 (mecA)	AACGTTGTAACCACCCCAAGA	
	α1 (ccrA1)	AACCTATATCATCAATCAGTACGT	695
	α2 (ccrA2)	TAAAGGCATCAATGCACAAACACT	937
	α3 (ccrA3)	AGCTCAAAAGCAAGCAATAGAAT	1791
	βc (ccrB1, ccrB2, ccrB3)	ATTGCCTTGATAATAGCCTTCT	
	α4.2 (ccrA4)	GTATCAATGCACCAGAACTT	1287
	β4.2 (ccrB4)	TTGCGACTCTCTTGGCGTTT	
	γR (ccrC)	CCTTTATAGACTGGATTATTCAAAATAT	518
	γF (ccrC)	CGTCTATTACAAGATGTTAAGGATAAT	
***Mec* gene complex class primers** (methicillin resistance)			
	mI6 (*mecI*)	CATAACTTCCCATTCTGCAGATG	1963
	IS7 (IS*1272*)	ATGCTTAATGATAGCATCCGAATG	2827
	IS2(iS-2) (IS*431*)	TGAGGTTATTCAGATATTTCGATGT	804
	mA7 (*mecA*)	ATATACCAAACCCGACAACTACA	

3.2.3.3. Enzymes

For SCCmec-typing a multiplex PCR was performed. This required the same enzyme that was used to determine the sequence types of different S. epidermidis isolates via MLST, Table 5 (see '*3.2.1. Multilocus sequence typing'*, Chapter III).

3.2.3.4. Protocol

Typing of *S. epidermidis* isolates for *SCCmec* was performed by typing for two constituting factors of *SCCmec*, the *ccr* genes and the *mec* gene complex class. This allows distinguishing between various types of *SCCmec*. DNA of the *S. epidermidis* clinical isolates was extracted using the Tichy-method, which was already explained (see '*3.2.1. Multilocus sequence typing'*, Chapter III). For the *ccr* genes, 2µl of the isolated DNA was added to 23µl PCR-mix, containing for 1 reaction: 3.80µl miliQ water, 5µl dNTPs, 2.5µl platinum *Taq* amplification buffer, 1µl primers for the *ccr* genes, 1.5µl $MgCl_2$ and 0.2µl platinum *Taq* DNA polymerase. Multiplex PCR (Veriti® 96-well thermal cycler, Applied Biosystems® from Life Technologies™) for the *ccr* genes involved an initial duration at 94°C for 2 min; 30 cycles with first, denaturation at 94°C during 15 sec, then, annealing at 55°C during 30 sec and extension at 68°C during 2 min; and a final elongation of 72°C for 2 min. The PCR-product was then loaded on a 1.0% agarose gel with 5µl amplicon and 5µl gelloading for 45 min at 150V and 200mA. For the *mec* gene complex class, 2µl of the isolated DNA was added to 23µl PCR-mix, containing for 1 reaction: 3.80µl miliQ water, 5µl dNTPs, 2.5µl platinum *Taq* amplification buffer, 1µl primers for the *mec* gene complex class, 1.5µl $MgCl_2$ and 0.2µl

platinum *Taq* DNA polymerase. Multiplex PCR (Veriti® 96-well thermal cycler, Applied Biosystems® from Life Technologies™) for the *mec* gene complex class involved an initial duration at 94°C for 3 min; 30 cycles with first, denaturation at 94°C during 15 sec, then, annealing at 55°C during 30 sec and extension at 68°C during 1 min; and a final elongation of 72°C for 2 min. The PCR-product was then loaded on a 1.0% agarose gel with 5μl amplicon and 5μl gelloading for 60 min at 150V and 200mA. In both protocols, Generuler 1kb DNA ladder loading was used as marker. MOSAR-00004, MOSAR-00011, MOSAR-00005, MOSAR-00003 and MOSAR-00142 functioned as positive controls for types I, II, III, IV and V in typing of both *ccr* genes and the *mec* gene complex class, respectively. Identification of both complexes was performed by comparing with reference fragment lengths, based on *Kondo, Y., et al,* 2007 [124].

Table 14:Reference fragment lengths for the identification of the corresponding *ccr* complex belonging to the *SCCmec* element.

Ccr complex	Fragment length
Type 1	695 bp.
Type 2	937 bp.
Type 3	1 791 bp.
Type 4	1 287 bp.
Type 5	518 bp.
***mec*A**	286 bp.

Table 15: Reference fragment lengths for the identification of the corresponding *mec* complex belonging to the *SCCmec* element.

Mec complex	Fragment length
Class A	1 963 or 1 797 bp.
Class B	2 827 bp.
Class C	804 bp.

3.2.4. Static biofilm assay

The static assay allows distinguishing weak from strong biofilm forming clinical strains of *S. epidermidis*, which might reflect in VAP or non-VAP pathogenesis of patients.

3.2.4.1. Solutions

Table 16: Acquired chemical solutions in order to perform the static biofilm assay to asses biofilm-forming capacity of the clinical isolates of *S. epidermidis*.

Name		Concentration	References
Crystal violet (Tris (4-(dimethylamino) phenyl) methylium chloride)	Basic dye that ionizes in water and stains the bacterial cell wall (peptidoglycan, polysaccharides, proteins, nucleic acids and other extracellular matrix components of the biofilm) [141, 142].	2% Absorption maximum at 590 nm	2013.
Glacial acetic acid	Amphiprotic solvent for nonaqueous titration of basic substances and organic compounds (release and resolubilization of bound crystal violet dye) [143].	33% 1:3 dilution of glacial acetic acid (96%)	Merck KgaA, 2006.
Methanol	Fixation of biofilm (reduction of solubility and reduction of hydrophobic interactions of tertiary proteins) [144].		Merck KgaA, 2005.
PBS (Phosphate buffered saline	Buffered, isotonic solution used for washing of the biofilm, to remove all planktonic cells [145].	1:10 dilution of 10x PBS[7], pH 7.4	Ambion® from Life Technologies™

[7]Composed out of 1.37M sodium chloride, 0.027M potassium chloride, 0.08M sodium phosphate dibasic, and 0.020M potassium phosphate monobasic.

solution)			, 2014.

3.2.4.2. Measurement of crystal violet absorption

The amount of biofilm formed by each particular strain was measured through colorimetry or the quantitative measurement of optical density. Colorimetry is used to measure the intensity of absorption of colored compounds (for example, the biofilm with crystal violet) over a narrow range of frequencies. A microtiter plate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific Inc.) measures the light absorption through the medium [141]. The amount of crystal violet bound in each well is proportional to the amount of biofilm formed and can be directly quantified by measuring light absorption. Unfortunately, absorbance is only measured in a small area of the assay well. Therefore, crystal violet must be eluted from the biofilm by adding glacial acetic acid for more accurate results [145].

3.2.4.3. Protocol

First, bacterial cultures had to be incubated overnight at 37°C without CO_2 (Heratherm™ Advanced protocol Microbiological Incubator, Thermo Fisher Scientific Inc.) in TSB-medium. The next day, cultures were diluted to 0.5 McFarland and 20µl of the inoculum was added to a 96-well microtiter plate (CELLSTAR® 96 Well Plate Flat Bottom (polystyrene), Sigma-Aldrich), containing 180µl fresh TSB-medium in each well. The edges of the plate were sealed off with parafilm to prevent evaporation of the medium and to stimulate biofilm formation by low oxygen conditions [146]. The plate was incubated for 48 hours at 37°C without CO_2. After incubation, the contents of the wells were discarded and each well was washed three times with 200µl sterile PBS. Next, the wells were fixed with 150µl methanol during twenty minutes. Afterwards, they were dried in an inverted position at 37°C. The plate was stained with 150µl crystal violet during fifteen minutes. The stain was removed by placing the plate under slowly running tap water until no dye was released anymore. After the plate had air-dried, the dye was eluted by adding 150µl 33% glacial acetic acid. Thirty minutes later, the plate was read on the microtiter plate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific Inc.) at a wavelength of 492nm.

The assay was performed on three distinct days, each time on three different plates and strains on the same plate were added in quadruplicate. *S. epidermidis* ATCC 12228 functioned as a negative control and *S. epidermidis* 1457 was used as a positive control with PIA-dependent biofilm formation capacity, as described in literature [147, 148].

3.2.5. ACME-typing

Typing of *S. epidermidis* isolates for ACME was performed by typing for two constituting fragments of ACME, *ArcA* and *Opp*-3. This allows detection of particular types of ACME elements, which may play a profound role in colonization capacity of the bacterium and allow a more elaborate insight in the biofilm-forming capacity particular clinical isolates due to its regulatory role of pH stress in microniches.

3.2.5.1. Solutions and buffers

For ACME-typing a singlex PCR was performed. This required all the same chemical solutions that were used to determine the sequence types of the different S. epidermidis isolates via MLST, in Table 2 (see *'3.2.1. Multilocus sequence typing'*, Chapter III). *Just like for SCCmec-typing, a 1.0 % agarose gel was used for gel electrophoresis.*

Table 17: Buffer solutions used to determine presence of ACME (both the *ArcA* fragment and the *Opp-3* cluster) within the different *S. epidermidis* isolates.

Buffer		References
Super *Taq* buffer (with 15mM $MgCl_2$)	Regulates optimal pH and salt concentration in PCR. Composed out of 100mM Tris-HCl, pH 9.0, 5mM $MgCl_2$, 500mM KCl, 1% Triton X-100 and 0.1% (w/v) stabilizer.	BV Sphaero Q, 2014.

3.2.5.2. Primers

Table 18: Primers used for ACME-typing of the different *S. epidermidis* isolates. Selection of primers was based on [119]. Primers were obtained from Invitrogen™ by Life Technologies™ , 2012.

Fragment and function		Sequence (5'-3')	Amplicon size, bp.
ArcA (arginine deiminase pathway)			1946
	AIPS.27 (forward)	CTAACACTGAACCCCAATG	
	AIPS.28 (reversed)	GAGCCAGAAGTACGCGAG	
***Opp*-3** (oligopeptide permease system)			1183
	AIPS.45 (forward)	GCAAATCTGTAAATGGTCTGTTC	
	AIPS.46 (reversed)	GAAGATTGGCAGCACAAAGTG	

3.2.5.3. Enzymes

Table 19: Enzyme used during PCR to determine presence of ACME (both the *ArcA* fragment and the *Opp*-3 element) within the different *S. epidermidis* isolates.

Enzyme		References
High concentration Super *Taq*™ DNA polymerase	Concentration: 15U/μl[8] Taq DNA polymerase with thermal stability and with a proofreading activity that reduces the error rate of *Taq* polymerase.	BV Sphaero Q, 2014.

3.2.5.4. Protocol

DNA of the *S. epidermidis* clinical isolates was extracted using the Tichy-method, which is already explained in (see '*3.1.2. Multilocus sequence typing'*, Chapter III). 1μl of the isolated DNA was added to 24μl PCR-mix, containing for 1 reaction: 5.65μl miliQ water, 10μl dNTPs, 2.5μl super *Taq* buffer, 2.5μl primers for the *ArcA* fragment or the *Opp*-3 cluster of ACME, 0.75μl $MgCl_2$ and 0.1μl high concentration super *Taq* enzyme. PCR (Veriti® 96-well thermal cycler, Applied Biosystems® from Life Technologies™) for the *ArcA* fragment involved an initial duration at 95°C for 3 min; 35 cycles with first, denaturation at 95°C during 1 min, then, annealing at 48°C during 2 min and extension at 71°C during 3 min; and a final elongation of 72°C for 10 minutes. PCR (Veriti® 96-well thermal cycler, Applied Biosystems® from Life Technologies™) for the *Opp*-3 cluster involved an initial duration at 95°C for 3 min; 35 cycles with first, denaturation at 95°C during 1 min, then, annealing at 57°C during 2 min and extension at 72°C during 2 min; and a final elongation of 72°C for 10 minutes. The PCR-product for both fragments was then loaded on a 1.0% agarose gel with 5μl amplicon and 5μl gelloading for 90 min at 150V and 200mA. Generuler 1kb DNA ladder loading was used as marker. MRSA-S075 functioned as a positive control for both fragments respectively.

3.2.6. Growth curves

Growth curves were constructed to determine the average growth rate per strain of *S. epidermidis* and to determine whether a biofilm forming strain expresses altered patterns of bacterial growth compared to non-biofilm forming strains.

3.2.6.1. Measurement of optical density

[8]One unit of Super *Taq*™ DNA polymerase incorporates 10 nmol of deoxynucleotides into acid insoluble form in 30 min at 74°C.

Growth of the bacterial strains was measured through optical density with a spectrophotometer (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific Inc.), using SkanIt™ software. Optical density (OD) measurement of bacterial cultures is based on the amount of light scattered by the culture rather than the amount of light absorbed [149]. The underlying principle is that most of the light scattered by the bacterial cells no longer reaches the photoelectric cell, so that the electric signal is weaker than with a cell-free sample [150]. The turbidity or optical density of a suspension of cells is directly related to cell mass or cell number [151].

3.2.6.2. Protocol

A bacterial swab was added to a TSB-medium containing glass tube. These tubes had to incubate overnight at 37°C without CO_2 (Heratherm™ Advanced protocol Microbiological Incubator, Thermo Fisher Scientific Inc.). The next day, growth curves were constructed by adding 20µl of the incubated inoculum (0.5 McFarland) to 180µl fresh TSB-medium in a 96-well microtiter plate (CELLSTAR® 96 Well Plate Flat Bottom (polystyrene), Greiner Bio-One). The optical density or cell density of each well was measured with a spectrophotometer (MultiSkan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific Inc.), using SkanIt™ software during a course of 24 hours at 37°C, each fifteen minutes, at 600nm. In total 96 measurements were made. The growing pattern of each strain was measured in quadruplicate.

3.3. Cultivation of a multispecies biofilm

Based on data generated by 16S sequencing [152], a core community, associated with *S. epidermidis* and non-VAP or VAP, was identified. The species *Candida albicans*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Serratia marcescens* were selected. For *S. epidermidis*, a moderate biofilm-forming strain (SE4-613) was chosen.

3.3.1. 16S-analysis and microbiome discovery

The 16SrRNA gene is used for phylogenetic studies as it is highly conserved between different species of bacteria and archaea. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. 16S rRNA genes from different microbes will have a few different nucleotides scattered throughout the sequences, those nucleotides at the very beginning or end of the gene are the same from organism to organism.
ET tubes with positive culture results for *S. epidermidis* were selected and the total DNA was extracted using the Masterpure™ complete DNA purification kit (Epicentre®, an Illumina® company). The 16S sequencing was outsourced. This means that the acquired DNA would undergo amplification by PCR using region of interest-specific primers. The V3-V5 regions of the 16S gene were amplified and sequenced (400 000 reads) using the Illumina platform (MiSeq® System, Illumina®), which enabled the detection of single bases as they are incorporated in growing DNA strands. Fluorescently labelled reversible terminators were incorporated, visualized and cleaved for the incorporation of the next one. Sequence reads were phylogenetically classified after quality trimming and revealed several species frequently associated with VAP.
To identify the microbiome specifically associated with the pathogen of interest, *S. epidermidis*, samples were identified on family level based on the presence or absence and the relative abundance of a certain family on the ET tube [33].

3.3.2. Static biofilm assay

This assay allows to quantify the amount of biofilm formed between two selected species (*S. epidermidis* with *C. albicans*, *K. pneumoniae*, *S. marcersens* or *P. aeruginosa*) and to visualize the structure of the formed biofilm together with possible interactions therein.

3.3.2.1. Protocol

With exception of the bacterial strains, the same material was required as for the static biofilm assay (see *'3.2.4. Static biofilm assay',* Chapter III). *In addition, staining was needed for fluorescence visualisation of the multispecies biofilm within the 96-well microtiter plate.*

The multispecies biofilm assay is performed similar to the static biofilm assay as described above, with an incubation time of 24h to prevent build-up of toxic waste products and starvation. The assay was performed on three distinct days, each time on four different plates and strains on the same plate were added in quadruplicate. Three plates were used for staining and one was used for fluorescence microscopy. However, in this assay, multiple concentrations of mixed bacterial species were added to the wells. For each combination of *S. epidermidis* and another species, five concentrations were tested. These dilutions were attained throughout all experiments involving the cultivation of multispecies biofilms.

Table 20: Concentrations of different species combinations to determine biofilm formation in the multispecies biofilm assay.

Strain			Concentration (µl)	
S. epidermidis	*C. albicans*	(1:1)	1	1
		(1:10)	1	9
		(10:1)	9	1
		(1:1000)	1	999
		(1000:1)	999	1
S. epidermidis	*K. pneumoniae*	(1:1)	1	1
		(1:10)	1	9
		(10:1)	9	1
		(1:1000)	1	999
		(1000:1)	999	1
S. epidermidis	*S. marcescens*	(1:1)	1	1
		(1:10)	1	9
		(10:1)	9	1
		(1:1000)	1	999
		(1000:1)	999	1
S. epidermidis	*P. aeruginosa*	(1:1)	1	1
		(1:10)	1	9
		(10:1)	9	1
		(1:1000)	1	999
		(1000:1)	999	1

3.3.2.2. Fluorescence microscopy

Table 21: Kit used for the visualization of the structure of a multispecies biofilm of *S. epidermidis* with *C. albicans*, *K. pneumonia*, *S. marcescens* or *P. aeruginosa* through fluorescence microscopy.

Kit		*Excitation/Emission (nm)*	*Reference*
SYTO®9 Green-Fluorescent Nucleic Acid Stain, L7012	SYTO 9 is a green-fluorescent nucleic acid stain, which binds to DNA and RNA of both viable and dead bacterial cells [153].	485/530	Invitrogen™ by Life Technologies™, 2014.

To gain insight in the structure of the different biofilms, additional fluorescence microscopy (SYTO® 9 Green-Fluorescent nucleic acid stain L7012, Invitrogen™ by Life Technologies™, belonging to the LIVE/ DEAD *Bac*Light Bacterial Viability Kit) was performed. With this kit, cells with a compromised membrane (which are considered to be dead) should be stained red by propidium iodide (PI), whereas cells with an intact membrane

(which are considered living) should be stained green by SYTO 9 [153, 154]. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. SYTO 9 is a green intercalating membrane permeant nucleic acid stain, which diffuses passively through cellular membranes and binds to DNA of both viable and dead cells, provided they contain nucleic acid. In contrast, PI penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence. PI is apparently excluded from cells with structurally intact cytoplasmic membranes because of the size and charge of the propidium molecule. Under the fluorescence microscope, the specimen is illuminated with light of a specific wavelength, which is absorbed by the fluorophores, causing them to emit light of longer wavelengths or a different color than the absorbed light. Illumination light is separated from the weaker emitted fluorescence through use of a spectral emission filter.

Plates for fluorescence microscopy were fixated with methanol but not stained with crystal violet. 20µl SYTO 9 dye was diluted in 10ml miliQ water and 150µl of this solution was added to each well. To avoid photobleaching the plate was kept in the dark. Because the number of dead cells was likely to be very limited after a fresh culture growth of 24h and PI is known to cause a lot of background fluorescence, only SYTO 9 staining was used. Imaging was performed with a Carl Zeiss™ microscope with high-end fluorescence imaging systems (Axio Observer® with Cell Observer SD, ApoTome.2, LSM 710) using the software ZEN (Zeiss Efficient Navigation®) pro 2012. Quantification of the obtained images was performed using the program ImageJ (Image Processing and Analysis in Java) downloaded from http://imagej.nih.gov.html. It allows counting and measuring objects in thresholded images by scanning the image or selection and filling the found objects to make them visible. Particle size could range between 3μm^2 and 'Infinity'. Finally, the particle count, total particle area, average particle size, area fraction and the mean of the analysed image were calculated.

3.3.3. Dynamic biofilm assay

This system allows growing biofilms under a wide range of physiological conditions that represent an *in vivo* or *in situ* community of *S. epidermidis* with *P. aeruginosa* and *S. epidermidis* with *S. marcescens* more closely than the static assay did. Each combination was tested *in duplo* at a dilution of 1000 times (Table 20, see *'3.3.2. Static biofilm assay'*, Chapter III) to distinguish interaction patterns between the species. Afterwards, multispecies biofilms with the moderate biofilm former of *S. epidermidis* were selected for further processing with fluorescence microscopy and confocal laser-scanning microscopy using live/dead SYTO® 9 staining (Table 21, see *'3.3.2. Static biofilm assay'*, Chapter III).

3.3.3.1. Protocol

Overnight grown cultures (Heratherm™ Advanced protocol Microbiological Incubator, Thermo Fisher Scientific Inc.) were diluted to an optical density of 0.5 McFarland. Multispecies combinations were then diluted 10 times in 1 ml of TSB-medium to obtain a value of 0.05 McFarland. First, a small amount of medium (150μl) was added into the input well of a 48-well Bioflux™ glass plate (Fluxion Biosciences Inc.), using reverse pipetting to avoid air bubbles. Flow was regulated from the input well to the output well at a rate of 5 dyne/m^2 with the Bioflux™ 200 pressure interface (Fluxion Biosciences Inc.). Afterwards, 85μl inoculum

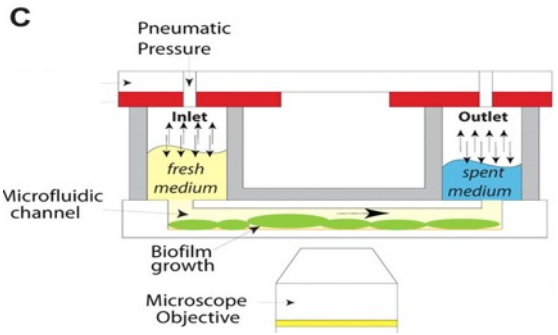

Figure 4: Observation area or viewing window of the 48-well Bioflux™ plate, where formed biofilms can be visualised, using microscopy.

was added in the output well and shear flow was regulated three seconds from the output well to the input well at a flow rate of 2 dyne/m². This allows locating the bacteria in the observation area where also the medium is, hereby avoiding contamination of fresh medium in the input wells. The bacteria were allowed to adhere to the glass surface for one hour without flow. Later, 1ml fresh medium was again added in the input well and the Bioflux™ plate (Fluxion Biosciences Inc.) was placed on the Bioflux™ Heating Stage (Fluxion Biosciences Inc.) at 37°C. After five hours, the flow cells were inspected for biofilm formation at the laminar flow microfluidic channels. Outside this region, the channel geometry contributes to a region of non-uniform shear as well as higher shear, providing inconsistent results. After growth, excess medium from output and input wells was removed and 400 μl of fresh medium was again added to the input wells. Then, 1 μl of SYTO® 9 green was pipetted into the input wells under a flow of 0.5 dyne/m² during 10 min. Because the number of dead cells was likely to be very limited after a fresh culture growth of 5h and PI is known to cause a lot of background fluorescence, only SYTO® 9 staining was used. Using both fluorescence and confocal laser-scanning microscopy, biofilms were visualised.

3.3.3.2. Visualisation of the biofilm

3.3.3.2.1. Fluorescence microscopy

Formed biofilms in the observation area were analysed using fluorescence microscopy with live staining and Brightfield microscopy (Axio Observer® with Cell Observer SD, ApoTome.2, LSM 710) using the software ZEN (Zeiss Efficient Navigation®) pro 2012. Quantification of obtained images was performed using the program ImageJ, as explained before (see *'3.3.2. Static biofilm assay'*, Chapter III). It should be mentioned here, that the program quantified the entire image, meaning also the edges of the viewing window where no cells adhered (black). As such, a total colonized fraction of 100% can never be obtained, however, the edges appeared in a comparable way on each image thereby reducing the impact on the obtained values.

3.3.3.2.2. Confocal laser-scanning microscopy

Confocal laser scanning microscopy (CLSM) (Inverted Research Microscope ECLIPSE Ti, Nikon®) allows 3D localization of labeled target molecules in cells. The basic key to the confocal approach is the use of spatial filtering techniques to eliminate out-of-focus light or glare in specimens whose thickness exceeds the immediate plane of focus [155]. Another key feature of confocal microscopy is its ability to acquire in-focus images from selected depths, a process known as optical sectioning. The CLSM produces optical sections by scanning the specimen point-by-point with a laser beam focused in the specimen, and using a spatial filter (usually a pinhole or a slit) to remove unwanted fluorescence from above and below the focal plane of interest. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically complex objects [156]. The fluorescence images, representing biofilm formation under dynamic conditions, were three-dimensionally reconstructed using Volocity® 6.3. 3D Image Analysis Software from PerkinElmer™ (http://cellularimaging.perkinelmer.com).
Biofilm structures were visualized with SYTO® 9 staining. However, this is not an optimal method to distinguish Gram-positive bacteria from Gram-negative bacteria as each species will have the same colour and shapes of cells are very hard, if at all, to distinguish within the assay. Better approaches are, for example, peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH), LIVE *Bac*Light™ bacterial gram staining or even genetic manipulation of the strains [157]. Unfortunately, these staining techniques had yet to be optimized, for which no time was left anymore before printing of this thesis.

3.4. Statistical analysis

Statistical analysis of the obtained results was performed using the program R 3.0.2., downloaded from http://cran.r-project.org/. P-values less than 0.05 were considered as significant. First, normality of the data was tested using the Shapiro-Wilk Normality Test. The F-test was used to compare the variances of two datasets (VAP and non-VAP). When the data was normally distributed and had an equal or unequal variance, a Two-Sample T-Test was used to compare the difference between the two datasets. When the data was not normally distributed and had an equal or unequal variance, a Mann-Whitney U-Test or Wilcoxon Rank Sum Test was used to compare the difference between the two datasets. These tests were performed for all assays. A Multiple Analysis of Variance Test was used to link the data of the different characterization assays together. When residuals of this test were normally distributed, a Tukey Multiple Comparisons of Means Test was performed to provide more in depth analysis of the data series. When data was not normally distributed, a Friedman Rank Sum Test was performed. For the fluorescence and confocal images obtained in '*Part 2 – Cultivation of a multispecies biofilm,* Chapter IV', no statistical analysis was applied, as the objective was to look only at biofilm structures on the obtained images.

Part 1 – General characterization of *S. epidermidis*

4.1.1. Identification based on sequence variation

Among all analysed samples (n=47), 18 different sequence types could be discovered. Twenty-seven strains (57%) belonged to sequence type 2, which was by far the most predominant sequence type in this analysis. Thirteen of these strains were associated with non-VAP patients and fourteen were associated with VAP patients. Within the non-VAP associated group, two strains belonged to sequence type 87 (4.25%), which was absent in the VAP associated group. Within the VAP associated group, two strains belonged to sequence type 54 (4.25%), which was absent in the non-VAP associated group. In both groups, one strain belonged to sequence type 88 (4.25%). The remaining strains (n=14, 29.8%) belonged to various different sequence types, which are displayed in Table 22. One newly identified sequence type was found and passed on to the MLST database. This unknown sequence type was not included for further analysis.

Table 22: Allelic profile and sequence type per *S. epidermidis* strain associated with VAP or non-VAP. Each colour represents a different sequence type. The sequence type with '?' refers to the newly identified sequence type.

non-VAP group			VAP-group		
strain	allelic profile	sequence type	strain	allelic profile	sequence type
SE 0094	7122411	2	SE 0105	7122411	2
SE 0095	7122411	2	SE 0108	7122411	2
SE 0117	7122411	2	SE 0112	7122411	2
SE 0092	7122411	2	SE 0118	7122411	2
SE3-410	7122411	2	SE3-403	7122411	2
SE2-202	7122411	2	SE4-609	7122411	2
SE3-405	7122411	2	SE4-613	7122411	2
SE3-419	7122411	2	SE 0119	7122411	2
SE5-803	7122411	2	SE 0128	7122411	2
SE5-811	7122411	2	MNP 008	7122411	2
MNP 001	7122411	2	SE 0011	7122411	2
SE 0040	7122411	2	SE 0026	7122411	2
SE 0043	7122411	2	SE 0078	7122411	2
SE 0069	1121217	88	SE 0085	7122411	2
SE 0127	7112211	87	SE 0020	1122411	54
SE 0017	7112211	87	SE 0045	1122411	54
SE 0109	7121331	23	SE 0107	1121217	88
SE3-408	2111211	59	SE 0100	1121111	14
SE 0096	11262116	60	SE 0030	1162211	17
SE 0113	12121110	83	SE 0035	2122411	35
SE 0061	1112111	130	SE 0070	1126216	294
SE4-605	1112511	190	SE 0068	11121110	425
SE 0129	12198658	193	SE 0041	1121218	454
SE5-808	11406214	?			

No significant difference was found between the VAP and non-VAP associated group as the VAP associated group contained 9 different sequence types and the non-VAP associated group contained 11 (p=0.6849). ST's and allelic profiles were implemented in the eBURST program to construct a model of the evolutionary history of *S. epidermidis* (Figure 5). All sequence types are connected with each other within the diagram, which means they all belong to the same worldwide predominant single clonal complex, CC2. According to *Miragaia, M., et a*l., 2008 [158] CC2 consists out of two domains; cluster I contains the predicted primary ancestor (ST2, blue circle) of the clonal complex and respective single-locus (SLV) and double-locus variants (DLV), while cluster II contains the remaining sequence types that include several subgroup founders. Sequence type 2, 54 and 35 belong to cluster I, whilst all other sequence types found belong to cluster II. It is remarkable that all three sequence types of cluster I, except for a large variety of ST2, belong to the VAP-

associated strains. These two groups of clusters within this CC have significant (p=2.652e-09) contrasting recombination rates, distinct phylogenetic congruence and different levels of complexity on descendence patterns. These results indicate that *S. epidermidis* is a highly diverse organism that has been subjected to considerable recombination events throughout history.

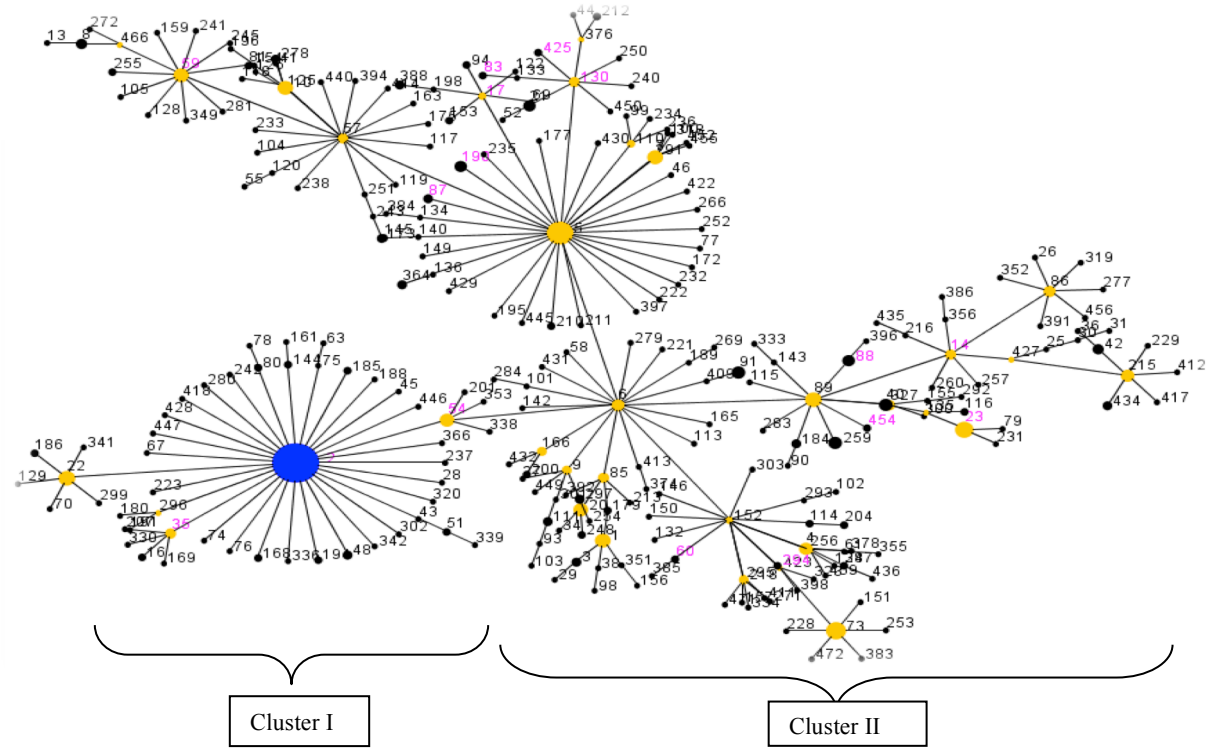

Figure 5: eBURST diagram representing evolutionary distance between the clinical isolates of *S. epidermidis*. The primary founder (ST2) of the group is coloured blue. Subgroup founders are coloured yellow on the eBURST diagram. All STs that are connected with each other can be considered to belong to the same clonal complex (CC2). The average distance of each ST to each other ST in the group is also shown, and is computed from the average number of differences in their allelic profiles. The numbers in pink represent the sequence types from this study, which were compared with every sequence type available in the MLST database (represented by numbers in black).

A dendrogram representing the evolutionary distance between different sequence types and strains of *S. epidermidis* is given in the addendum (Figure 30). Here, bootstrapping provides a level of support for the predicted primary founder and for subgroup founders.

4.1.2. Determination of an antibiotic resistance profile

4.1.2.1. Disk diffusion testing

After the disk diffusion test, forty-two out of forty-seven (89.4%) clinical isolates proved to be resistant against at least one antibiotic. Of these forty-one strains, all were resistant to cefoxitin, indicating a very high level of methicillin-resistance. Only five strains (10.6%) were fully susceptible for all antibiotics, two of which belonged to the VAP-group. For cefoxitin (fox), in total, forty-two strains were resistant (89.4%) and five were susceptible (10.6%). For ciprofloxacin (cip), twenty-four strains proved to be resistant (51%), fourteen strains were susceptible (29.8%) and nine strains were intermediate (19.2%). For gentamicin (gm), twenty-four strains were resistant (51%), twenty-two strains were susceptible (46.8%) and one was intermediate (2.2%). Last, for sulfamethoxazole with trimethoprim (stx), thirty strains were resistant (63.8%), fourteen were susceptible (29.8%) and three were intermediate (6.4%). Strains were divided in two groups to determine a possible difference between VAP (n = 23) and non-VAP associated strains (n = 24). Values of quality control *S. aureus* ATCC

25923 for cefoxitin, gentamycin, trimethoprim with sulfamethoxazole, and ciprofloxacin, were all within the predicted range given in the CLSI guidelines, indicating reliable results.

Table 23: List of susceptible and resistant strains of *S. epidermidis* according to CLSI guidelines, determined via disk diffusion susceptibility testing. Red squares refer to resistant strains, green to susceptible strains and yellow to intermediate strains. The inhibited zone diameter is given in mm.

non-VAP								
	Cefoxitine 30µg		Gentamycin 10µg		Trimethoprim-sulfa. 1.		Ciprofloxacin 5µg	
Strain	diameter	breakpoint	diameter	breakpoint	diameter	breakpoint	diameter	breakpoint
SE 0094	8	R	6	R	8	R	18	I
SE 0095	8	R	7	R	10	R	7	R
SE 0117	7	R	7	R	7	R	15	R
SE 0092	0	R	0	R	0	R	9	R
SE3-410	9	R	10	R	9	R	9	R
SE2-202	0	R	6	R	6	R	0	R
SE3-405	15	R	30	S	24	S	34	S
SE3-419	8	R	9	R	8	R	13	R
SE5-803	8	R	18	S	7	R	8	R
SE5-811	0	R	28	S	8	R	0	R
MNP 001	0	R	0	R	0	R	16	I
SE 0040	0	R	0	R	0	R	0	R
SE 0043	0	R	29	S	0	R	8	R
SE 0109	14	R	16	S	25	S	0	R
SE3-408	16	R	31	S	26	S	36	S
SE 0096	29	S	34	S	29	S	30	S
SE 0113	9	R	34	S	7	R	7	R
SE 0127	12	R	14	I	28	S	16	I
SE 0017	0	R	36	S	32	S	17	I
SE 0061	14	R	12	R	30	S	16	I
SE4-605	14	R	27	S	14	I	34	S
SE 0129	27	S	31	S	31	S	35	S
SE 0069	9	R	32	S	12	I	35	S
SE5-808	26	S	31	S	34	S	29	S

VAP								
	Cefoxitine 30µg		Gentamycin 10µg		Trimethoprim-sulfa. 1.		ciprofloxacin 5µg	
Strain	diameter	breakpoint	diameter	breakpoint	diameter	breakpoint	diameter	breakpoint
SE 0105	7	R	6	R	7	R	0	R
SE 0108	8	R	9	R	8	R	10	R
SE 0112	8	R	9	R	8	R	8	R
SE 0118	0	R	0	R	9	R	15	R
SE3-403	7	R	8	R	8	R	9	R
SE4-609	0	R	0	R	0	R	0	R
SE4-613	9	R	16	S	9	R	8	R
SE 0119	0	R	0	R	0	R	17	I
SE 0128	0	R	0	R	0	R	7	R
MNP 008	0	R	0	R	0	R	16	I
SE 0011	0	R	0	R	0	R	13	R
SE 0026	0	R	0	R	0	R	16	I
SE 0078	0	R	0	R	0	R	17	I
SE 0085	7	R	34	S	14	I	0	R
SE 0100	26	S	30	S	33	S	36	S
SE 0030	0	R	31	S	30	S	38	S
SE 0035	39	S	44	S	35	S	40	S
SE 0020	0	R	0	R	0	R	0	R
SE 0045	0	R	0	R	0	R	15	R
SE 0107	14	R	29	S	10	R	32	S
SE 0070	24	R	34	S	33	S	29	S
SE 0068	8	R	32	S	0	R	0	R
SE 0041	9	R	28	S	25	S	34	S

Data according to CLSI guidelines (Table 23) was compared. No significant difference in susceptibility for or resistance against cefoxitin ($p = 0.6541$) between the VAP and non-VAP group was observed. The same is true for gentamycin ($p = 0.4080$), trimethoprim with sulfamethoxazole ($p = 0.4129$) and ciprofloxacin ($p = 0.9858$).

Table 24: List of susceptible and resistant strains of *S. epidermidis* according to EUCAST guidelines, determined via disk diffusion susceptibility testing. Red squares refer to resistant strains, green to susceptible strains and yellow to intermediate strains. The inhibited zone diameter is given in mm.

non-VAP								
	Cefoxitine 30µg		Gentamycin 10µg		Trimethoprim-sulfa. 1.		Ciprofloxacin 5µg	
Strain	diameter	breakpoint	diameter	breakpoint	diameter	breakpoint	diameter	breakpoint
SE094	8	R	6	R	8	R	18	R
SE095	8	R	7	R	10	R	7	R
SE096	29	S	34	S	29	S	30	S
SE109	14	R	16	R	25	S	0	R
SE113	9	R	34	S	7	R	7	R
SE117	7	R	7	R	7	R	15	R
SE092	0	R	0	R	0	R	9	R
SE3-410	9	R	10	R	9	R	9	R
SE2-202	0	R	6	R	6	R	0	R
SE3-405	15	R	30	S	24	S	34	S
SE3-419	8	R	9	R	8	R	13	R
SE4-605	14	R	27	S	14	I	34	S
SE5-803	8	R	18	R	7	R	8	R
SE5-808	26	S	31	S	34	S	29	S
SE5-811	0	R	28	S	8	R	0	R
SE127	12	R	14	R	28	S	16	R
SE3-408	16	R	31	S	26	S	36	S
MNP 0001	0	R	0	R	0	R	16	R
SE017	0	R	36	S	32	S	17	R
SE040	0	R	0	R	0	R	0	R
SE043	0	R	29	S	0	R	8	R
SE061	14	R	12	R	30	S	16	R
SE069	9	R	32	S	12	R	35	S
SE 0085	27	S	31	S	31	S	35	S

VAP								
	Cefoxitine 30µg		Gentamycin 10µg		Trimethoprim-sulfa. 1.		ciprofloxacin 5µg	
Strain	diameter	breakpoint	diameter	breakpoint	diameter	breakpoint	diameter	breakpoint
SE100	26	S	30	S	33	S	36	S
SE105	7	R	6	R	7	R	0	R
SE107	14	R	29	S	10	R	32	S
SE108	8	R	9	R	8	R	10	R
SE112	8	R	9	R	8	R	8	R
SE118	0	R	0	R	9	R	15	R
SE3-403	7	R	8	R	8	R	9	R
SE4-609	0	R	0	R	0	R	0	R
SE4-613	9	R	16	R	9	R	8	R
SE119	0	R	0	R	0	R	17	R
SE 0128	0	R	0	R	0	R	7	R
MNP 008	0	R	0	R	0	R	16	R
SE 0011	0	R	0	R	0	R	13	R
SE020	0	R	0	R	0	R	0	R
SE026	0	R	0	R	0	R	16	R
SE030	0	R	31	S	30	S	38	S
SE035	39	S	44	S	35	S	40	S
SE041	9	R	28	S	25	S	34	S
SE045	0	R	0	R	0	R	15	R
SE068	8	R	32	S	0	R	0	R
SE070	24	S	34	S	33	S	29	S
SE078	0	R	0	R	0	R	17	R
SE085	7	R	34	S	14	I	0	R

Data according to EUCAST (Table 24) guidelines was compared. No significant difference in susceptibility for or resistance against cefoxitin ($p = 0.9706$) between the VAP and non-VAP group was observed. The same is true for gentamycin ($p = 0.4527$), trimethoprim with sulfamethoxazole ($p = 0.2431$) and ciprofloxacin ($p = 0.8261$).

Additionally, when the antibiotic susceptibility was compared amongst different sequence types that where obtained with MLST typing, a significant difference between ST2 and other sequence types was obtained for all antibiotics (ciprofloxacin: $p=0.0082$; cefoxitin: $p=0.0002$ and trimethoprim with sulfamethoxazole: $p=0.0021$), except gentamicin ($p=0.1079$). This indicates that ST2 strains, associated with both VAP and non-VAP, show a very high level of resistance, not only to methicillin (cefoxitin) but also, to a lesser extent, to the other antibiotics tested. ST54 strains were resistant to every tested antibiotic; but, because there were only two strains of this sequence type present in the total experiment, the obtained results cannot be considered as significant. Looking at the clusters, defined with MLST, a significant difference between cluster I and cluster II was obtained for resistance against aminoglycosides ($p=0.02667$), fluoroquinolones ($p=0.02751$), cephalosporins ($p=0.002035$) and the miscellaneous class of antibiotics ($p=0.00208$). However, this is likely due to the overwhelming presence of ST2 in cluster I. Resistance against one of the applied antibiotics could be linked to observed resistance to another applied antibiotic as a significant difference was found ($p=2.152e-06$), suggesting mechanisms of resistance might be linked or co-inherited within the same complex in the same highly selective environment.

4.1.2.2. Microbroth dilution testing

Almost all strains (n=44, 93.6%) displayed susceptibility towards vancomycin according to CLSI guidelines. Only three of them displayed intermediate susceptibility (6.4%) according to CLSI and resistance according to EUCAST guidelines. This indicates that resistance has not been widespread yet and that vancomycin should remain the main drug of choice in treating *S. epidermidis*-related VAP-infections. Moreover, when comparing the group of non-VAP associated strains (n=24) with VAP associated strains (n=23) no significant difference could be found ($p = 0.0744$). The three intermediate strains, belonged to sequence type ST2, the same sequence type that displayed most resistance against the other antibiotics tested. When looking at the other antibiotics tested, the intermediate strains proved to be multi-drug resistant. However, there are a lot of other ST2 strains present in the clinical sample investigated and none of the others displayed vancomycin-resistance. This indicates that intermediate susceptibility might be due to heteroresistance or other spontaneous mutations within the particular bacterial genome. No significant difference could be found between ST2 and the other sequence types present in the sample ($p=0.5474$). A significant difference was again found between cluster I and cluster II of the defined CC ($p=1.382e-07$).

Quality control, *S. aureus* ATCC 29213 should have, according to the CLSI guidelines, a MIC between approximately 0.5-2 μg/ml. The measured MIC of *S. aureus* in the experiment was 1 μg/ml, indicating that the experiment was performed correctly. The colonies on each MHA-plate were counted to make an estimation of the final inoculum of the bacteria. The number of colonies counted, was multiplied with the standard inoculum of $1x10^6$, giving the initial inoculum. This number was then divided by 100 (100 fold dilution of 0.5 McFarland solution in 10 ml saline) and again by 2 (1:2 dilution of each antimicrobial concentration and 1:2 dilution of the inoculum), giving the broth or final inoculum used in the experiment. Inoculum counts ranged from $4.61x10^5 – 6.26x10^5$, indicating reliable results.

Table 25: List of of *S. epidermidis* strains in relation to vancomycin susceptibility or resistance according to CLSI guidelines (left) and EUCAST guidelines (right), determined via microbroth dilution susceptibility testing. Red squares refer to resistant strains, green to susceptible strains and yellow to intermediate strains. The MIC is expressed in µg/ml.

non-VAP			VAP		
	Vancomycin			Vancomycin	
Strain	MIC		Strain	MIC	
SE 0094	4	S	SE 0100	2	S
SE 0095	4	S	SE 0105	2	S
SE 0117	4	S	SE 0107	8	I
SE 0092	4	S	SE 0108	16	I
SE3-410	2	S	SE 0112	8	I
SE2-202	2	S	SE 0118	2	S
SE3-405	2	S	SE3-403	2	S
SE3-419	2	S	SE4-609	2	S
SE5-803	2	S	SE4-613	2	S
SE5-811	4	S	SE 0119	4	S
MNP 001	2	S	SE 0128	2	S
SE 0040	2	S	MNP 008	2	S
SE 0043	2	S	SE 0011	2	S
SE 0109	2	S	SE 0026	2	S
SE3-408	2	S	SE 0078	2	S
SE 0096	4	S	SE 0085	2	S
SE 0113	2	S	SE 0030	2	S
SE 0127	4	S	SE 0035	2	S
SE 0017	2	S	SE 0020	2	S
SE 0061	2	S	SE 0045	2	S
SE4-605	2	S	SE 0070	2	S
SE 0129	2	S	SE 0068	2	S
SE 0069	2	S	SE 0041	2	S
SE5-808	2	S			

non-VAP			VAP		
	Vancomycin			Vancomycin	
Strain	MIC		Strain	MIC	
SE094	4	S	SE100	2	S
SE095	4	S	SE105	2	S
SE096	4	S	SE107	8	R
SE109	2	S	SE108	16	R
SE113	2	S	SE112	8	R
SE117	4	S	SE118	2	S
SE092	4	S	SE3-403	2	S
SE3-410	2	S	SE4-609	2	S
SE2-202	2	S	SE4-613	2	S
SE3-405	2	S	SE119	4	S
SE3-419	2	S	SE 0128	2	S
SE4-605	2	S	MNP 008	2	S
SE5-803	2	S	SE 0011	2	S
SE5-808	2	S	SE020	2	S
SE5-811	4	S	SE026	2	S
SE127	4	S	SE030	2	S
SE3-408	2	S	SE035	2	S
MNP 0001	2	S	SE041	2	S
SE017	2	S	SE045	2	S
SE040	2	S	SE068	2	S
SE043	2	S	SE070	2	S
SE061	2	S	SE078	2	S
SE069	2	S	SE085	2	S
SE 0085	2	S			

4.1.3. Determination of the presence of the *SCCmec*-element

Five classes of *SCCmec* were tested for *S. epidermidis*. General classification is based on presence of the *ccr* genes *A/B* and/or *C*, the *mec* gene (*A/B/C/D* or *E*) complex class and the J-regions that surround the *ccr* genes and the *mec* gene complex class (especially for subtypes of *SCCmec* IV). Eight major *SCCmec* types and five types of recombinase-encoding *ccr* gene complexes (types 1-4, or *ccrAB1* to *ccrAB4*, and type 5, or *ccrC*) have been identified up to date [159]. Additionally, four classes of the *mec* gene complexes are currently known: class A *mec*, consisting of *IS431mec-mecA-mecR1-mecI*; class B *mec*, consisting of *IS431mec-mecA-mecR1-IS1272*; class C *mec*, consisting of *IS431mec-mecA-mecR1-IS431*; and class D *mec*, consisting of *IS431mec-mecA-mecR1* with no insertion sequences downstream of *mecR1* [124]. There are two distinct class C *mec* gene complexes; in the class C1 *mec* gene complex, the IS431 upstream of *mecA* has the same orientation as the IS431 downstream of *mecA* (next to HVR), while in the class C2 *mec* gene complex, the orientation of IS431 upstream of *mecA* is reversed. C1 and C2 are regarded as different *mec* gene complexes since they have likely evolved independently [160]. Many different structures, including insertion sequences and transposons, have been identified among the major *SCCmec* types in regions other than *mec* gene complex and *ccr* gene complex; i.e. in the J-regions. However, researching this was beyond the scope of this thesis as only the presence or absence of the *SCCmec* element in function of methicillin-resistance was of particular importance. Consequently, *SCC* element types could be defined according to the combination of *ccr* type and *mec* class.

Table 26: General classification of *SCCmec* types based on the presence of the *mec* complex and the *ccr* complex for clinical isolates of *S. epidermidis*. Based on guidelines of the International Working Group on the Classification of *Staphylococcal* Cassette Chromosome Elements [160].

SCCmec type	*mec* complex	*ccr* complex
I	Class B	Type 1
II	Class A	Type 2
III	Class A	Type 3
Iva	Class B	Type 2
Ivb	Class B	Type 2

Ivc	Class B	Type 2
Ivd	Class B	Type 2
V	Class C2	Type 5
VI	Class B	Type 4
VII	Class C1	Type 5
VIII	Class A	Type 4

As mentioned in the literature review, the *mecA*-encoded alternative penicillin-binding protein PBP2a mediates resistance to β-lactams. Consequently, strains expressing the *mecA* gene are referred to as methicillin resistant [161]. Forty-one out of forty-seven strains (87%) displayed presence of the *mecA* gene, corresponding with methicillin-resistance assigned during antibiotic susceptibility testing. *SCCmec* IV was the one of the most frequent type (n=13, 28%) amongst the isolates, as was *SCCmec* type III (n=10, 21%), followed by *SCCmec* type II (n=5, 11%), *SCCmec* type V/VII (n=4, 9%) and *SCCmec* type I (n=2, 4%). Seven *S. epidermidis* strains (15%) were designated as untypeable as they displayed no distinct *ccr* complex or multiple *ccr* complexes. This might be the cause of partial transfer of the corresponding genetic material as they did display presence of the *mecA* gene and the *mec* complex. Six *S. epidermidis* strains (13%) did not possess a *SCCmec* element at all. Four of them were susceptible to methicillin according to guidelines of CLSI, and an additional two strains were susceptible to methicillin according to guidelines of EUCAST. In the methicillin-resistant CoNS, *SCCmec* displayed more polymorphous structures, with frequent *ccr-mec* combinations not described in MRSA, and multiple and/or untypeable *ccr* allotypes.

Table 27: Different *SCCmec* types present in the clinical isolates of *S. epidermidis*, based on the occurrence of the *mec* complex and the *ccr* complex.

non-VAP				VAP			
strain	*ccr* complex	*mec* complex	SCC*mec* type	strain	*ccr* complex	*mec* complex	SCC*mec* type
SE 0094	type 2	class A	II	**SE 0105**	type 3	class A	III
SE 0095	type 5	class C	V/VII	**SE 0108**	type 2	class B	IV
SE 0117	type 2	class B	IV	**SE 0112**	type 3	class A	III
SE 0092	type 3	class A	III	**SE 0118**	type 3	class A	III
SE3-410	type 2	class A	II	**SE3-403**	type 3	class A	III
SE2-202	type 2	class A	II	**SE4-609**	type 3	class A	III
SE3-405	type 2	class B	IV	**SE4-613**	type 2	class B	IV
SE3-419	type 3	class A	III	**SE 0119**		class A	untypeable
SE5-803		class A	untypeable	**SE 0128**	type 2	class A	II
SE5-811	type 2	class B	IV	**MNP 008**	type 1	class B	I
MNP 001		class A	untypeable	**SE 0011**	type 2	class B	IV
SE 0040	type 3	class A	III	**SE 0026**	type 2/3	class A	untypeable
SE 0043		class A	untypeable	**SE 0078**	type 5	class C	V/VII
SE 0109	type 2	class B	IV	**SE 0085**	type 3	class A	III
SE3-408	type 1	class B	I	**SE 0100**			susceptible
SE 0096			susceptible	**SE 0030**	type 2	class A	II
SE 0113	type 2	class B	IV	**SE 0035**			susceptible
SE 0127	type 2	class B	IV	**SE 0020**		class A	untypeable
SE 0017	type 5	class C	V/VII	**SE 0045**	type 2	class B	IV
SE 0061	type 2	class B	IV	**SE 0107**	type 2	class B	IV
SE4-605	type 5	class C	V/VII	**SE 0070**			susceptible
SE 0129			susceptible	**SE 0068**	type 2	class B	IV
SE 0069	type 3	class A	III	**SE 0041**	type 2/3	class A	untypeable
SE5-808			susceptible				

Distribution of *SCCmec* types did not differ significantly (p=0.5581) between clinical isolates from patients with or without VAP. Often, identical or closely related STs carried each of the *SCCmec* types (I, II, III, or IV) and that same *SCCmec* type could be found within multiple, mostly unrelated STs. A significant difference could be not found (p=0.0730) between ST2 and all the other sequence types. As expected, all susceptible phenotypes, strains without an *SCCmec* complex, were found to have a sequence type other than ST2. ST2 mainly displayed the common *SCCmec* complexes III (33%) and IV (19%). However, when looking at the SCC*mec* complex distribution across cluster 1 and cluster 2 according to the eBURST

diagram no significant difference (p=0.3849) was found between ST2, 35 and 54, and the other sequence types. A link could be found between the occurrence of the SCC*mec* complex and resistance against gentamicin (p=0.0.02753), ciprofloxacin (p=0.02571), and most importantly, cefoxitin (p=1.458e-06). These results suggest that additional resistance to other antibiotics may be carried on the same SCC*mec* element that confers methicillin resistance, but on a different location than the *mec* complex. More precisely, on the J-regions. However, no such connection could be demonstrated for sulfamethoxazole with trimethoprim (p=0.0.0522), and vancomycin (p=0.0.6961). The latter is logical since resistance against vancomycin has only recently been on the rise. Resistance to sulfamethoxazole with trimethoprim might be correlated to the presence of other antibiotic resistance inferring genes or mutations than the *SCCmec* element in the *Staphylococcal* genome.

4.1.4. Cultivation of a biofilm under static conditions

Biofilm-forming capacity of the strains was compared to the biofilm growth of a positive control (SE 1457) and a negative control (ATCC 12228) (Table 1, see *'3.1. Bacterial strains'*, Chapter III) to quantify the amount of biofilm formed and to distinguish strong biofilm formers from weak biofilm formers. Cut-off OD values for weak (OD_{492}<0.251), moderate (OD_{492}: 0.251-0.754) and strong (OD_{492}>0.754) biofilm formers were defined as 25% and 75% ratios of the average OD-value obtained for the positive control (OD_{492}=1.005), based on *Smith, K., et al.*, 2008 [162, 163].

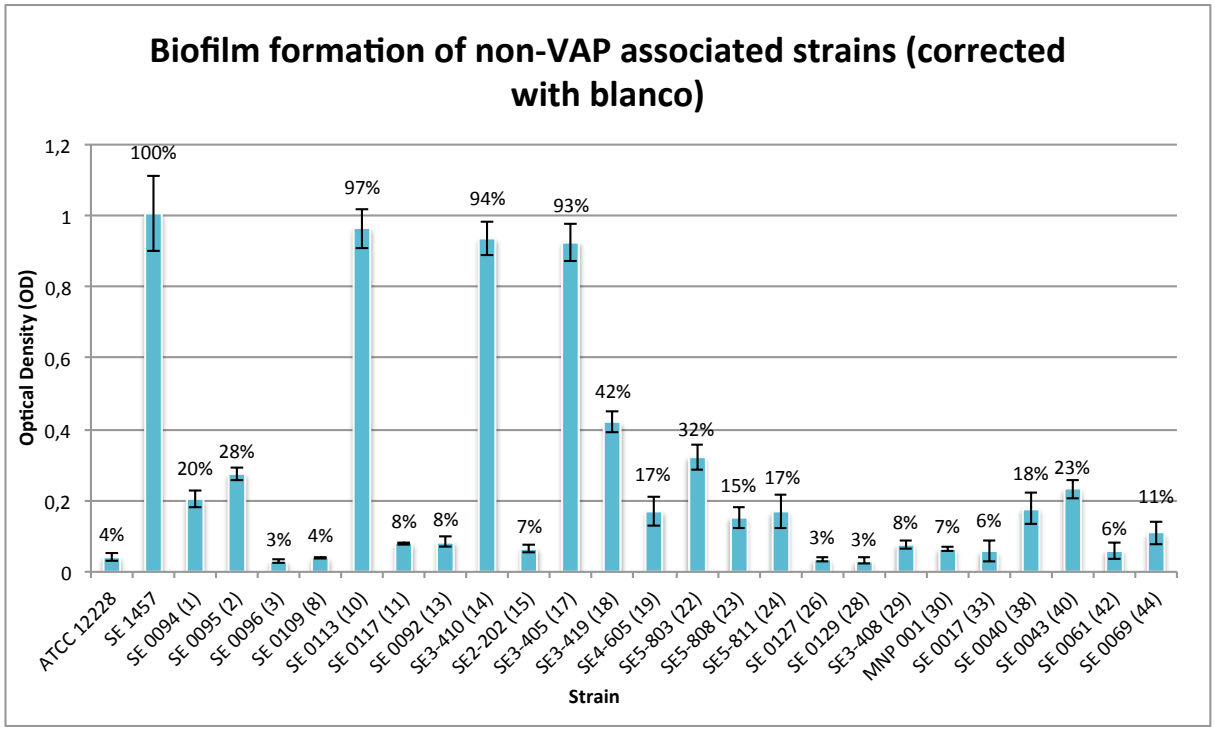

Figure 6: Biofilm-forming capacity of the different clinical isolates of *S. epidermidis*, associated with non-VAP, during the static biofilm assay. The amount of biofilm-formation was corrected with the background value of the blank (only TSB-medium) and expressed as a percentage compared to growth of the positive control. Error bars representing the standard deviation per strain were included. ATCC 12228 was used as a negative control, while SE 1457 was used as positive control.

Most of the clinical isolates (n=47) showed biofilm formation to some extent. Two VAP (n=23, 9%) and three non-VAP associated strains (n=24, 13%) could be quantified as strong biofilm-forming strains. Within the VAP associated group, six strains (26%) could be considered as moderate biofilm-formers, whereas three moderate biofilm-formers (13%) were identified in the non-VAP associated group. Fourteen strains (58%) of the non-VAP associated group were weak biofilm-formers, compared to thirteen strains (57%) of the VAP

associated group. Last, four strains (17%) of the non-VAP associated group could be considered as very weak biofilm-forming strains, as they had almost the same value or a lower value as the negative control, which is known to be biofilm-defective. Within the VAP associated group, this was the case for two strains (9%). No significant difference between the VAP and non-VAP associated group (p=0.5301) could be detected. Looking at the sequence type it became clear that, within the non-VAP associated group, almost all strong or moderate biofilm-formers (n=5), except for one strain, belonged to sequence type 2 (21%). Within the VAP associated group, no such relationship could be found as only three out of six moderate biofilm formers, belonged to sequence type 2 (13%). The other three moderate and two strong biofilm-formers were randomly distributed across the different sequence types. However, this result was not statistically significant (p=0.0922). No significant difference (p=0.1914) in biofilm formation could be found between the two evolutionary clusters. Three strains within the VAP-associated group, which displayed a moderate or strong biofilm-forming capacity, were susceptible to almost all the antibiotics tested, with exception for cefoxitin (methicillin-resistance) (13%). However, this pattern did not show in the rest of the data as no significant difference in antibiotic susceptibility according to biofilm formation capacity could be detected (gentamicin: p=0.2606; ciprofloxacin: p=0.1540; cefoxitin: p=0.2695; trimethoprim with sulfamethoxazole: p=0.5259; and vancomycin: p=0.5864). Last, the prevalence of the *SCCmec* complex could not be linked to biofilm forming capacity (p=0.4508).

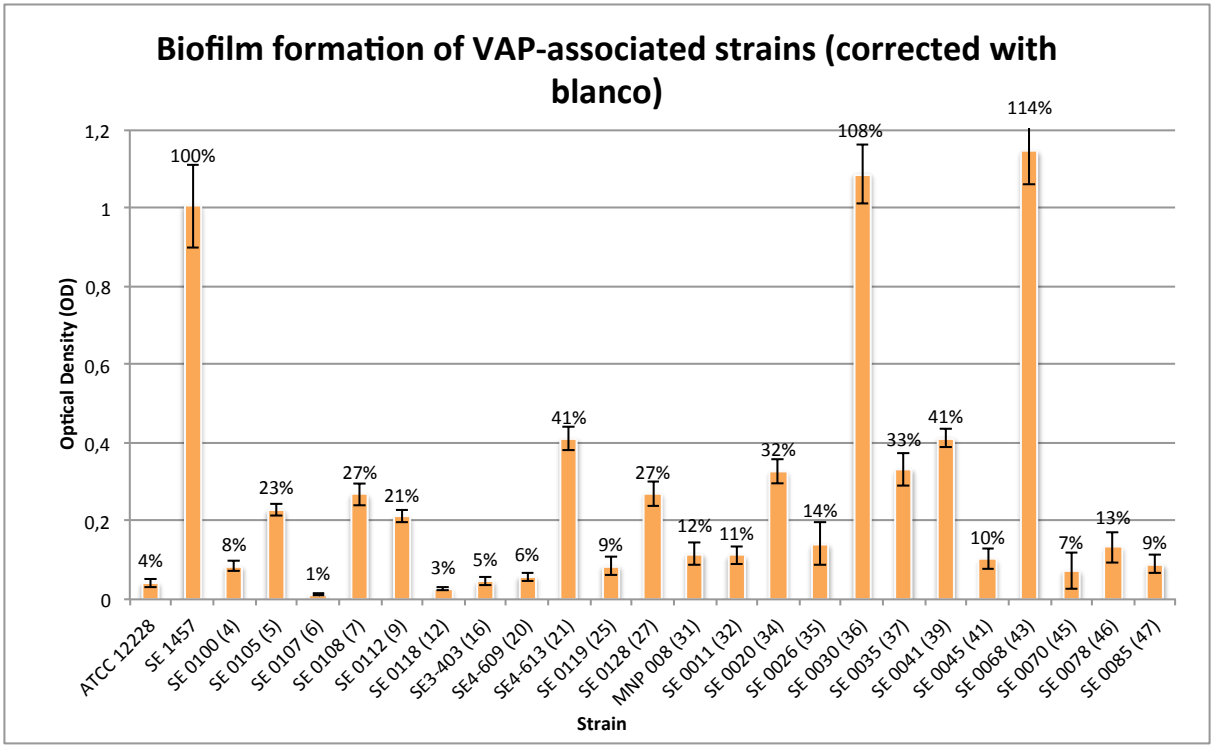

Figure 7: Biofilm-forming capacity of the different clinical isolates of *S. epidermidis*, associated with VAP, during the static biofilm assay. The amount of biofilm-formation was corrected with the background value of the blank (only TSB-medium) and expressed as a percentage compared to growth of the positive control. Error bars representing the standard deviation per strain were included. ATCC 12228 was used as a negative control, while SE 1457 was used as positive control.

4.1.5. Determination of the presence of the ACME-element

Up to date, three ACME allotypes have been identified. Type I consists out of the *ArcR/A/D/B* or *C* gene and the *Opp-3 (A/B/C/D or E)* cluster. Type II consists out of the *Arc* locus without the *Opp-3* cluster, while type III consists out of the *Opp-3* cluster without the *Arc* locus [112].

Table 28: General classification of ACME types based on the presence of the *ArcA* fragment and the *opp-3* cluster for clinical isolates of *S. epidermidis*. Based on *Diep, B. A., et al., 2008* [119].

ACME element	*ArcA* fragment	*Opp*-3 cluster
I	*ArcR/A/D/B* or *C*	*Opp*-3
II	*ArcR/A/D/B* or *C*	Absent
III	Absent	*Opp*-3

In total, only fourteen of forty-seven clinical *S. epidermidis* isolates (30%) displayed presence of the ACME-element. The most predominant ACME-elements are type I with five isolates (36%), and type III with five isolates (36%), followed by type II with four isolates (29%). Blanks in the table of results (Table 29) did not display any presence of the *ArcR/A/D/B* or *C* gene and the *Opp*-3 cluster (70%). To date, types II and III ACME have been identified only in *S. epidermidis*, and variants of ACME II, III, and I have also been identified in *S. epidermidis* [113]. Most striking, ACME-type I only occurred in the non-VAP group. However, sample size was not large enough to draw any reliable conclusions.

Table 29: Different ACME-element types present in the clinical isolates of *S. epidermidis*, based on the occurrence of the *Opp-3* cluster and the *ArcA* fragment.

non-VAP				VAP			
strain	*ArcA* fragment	*Opp*-3 cluster	ACME-element	strain	*ArcA* fragment	*Opp*-3 cluster	ACME-element
SE 0094				**SE 0105**			
SE 0095				**SE 0108**			
SE 0117				**SE 0112**	*ArcR/A/B/D* or *C*		type II
SE 0092				**SE 0118**			
SE3-410	*ArcR/A/B/D* or *C*	*Opp*-3	type I	**SE3-403**			
SE2-202				**SE4-609**			
SE3-405	*ArcR/A/B/D* or *C*	*Opp*-3	type I	**SE4-613**			
SE3-419		*Opp*-3	type III	**SE 0119**			
SE5-803				**SE 0128**			
SE5-811				**MNP 008**			
MNP 001				**SE 0011**			
SE 0040	*ArcR/A/B/D* or *C*	*Opp*-3	type I	**SE 0026**			
SE 0043				**SE 0078**		*Opp*-3	type III
SE 0109				**SE 0085**	*ArcR/A/B/D* or *C*		type II
SE3-408				**SE 0100**			
SE 0096				**SE 0030**		*Opp*-3	type III
SE 0113				**SE 0035**			
SE 0127				**SE 0020**			
SE 0017	*ArcR/A/B/D* or *C*	*Opp*-3	type I	**SE 0045**			
SE 0061	*ArcR/A/B/D* or *C*	*Opp*-3	type I	**SE 0107**			
SE4-605	*ArcR/A/B/D* or *C*		type II	**SE 0070**	*ArcR/A/B/D* or *C*		type II
SE 0129		*Opp*-3	type III	**SE 0068**			
SE 0069				**SE 0041**		*Opp*-3	type III
SE5-808	*ArcR/A/B/D* or *C*		type II				

A significant difference was found between the VAP and non-VAP group, showing occurrence of different types of ACME-elements (p=0.0241), with type I only occurring in strains associated with non-VAP. However, when only looking at the number of ACME-elements in the VAP and non-VAP group, no significant difference could be found (p=0.6799). It could be that the VAP-group isolates have evolved according to their pathogenic nature, and thereby gradually losing indicators of their commensal lifestyle as type II and III only carry the *ArcA* fragment or the *Opp*-3 cluster. This observation is supported by the fact that only seven ACME-carrying isolates, from the twenty-seven belonged to ST2, also belonged to this ST (26%). However, no significant difference could be found between ST2 isolates and the isolates carrying other sequence types (p=0.0881). Also, cluster I and cluster II isolates did not show a significant difference in prevalence of the ACME-element (p=0.8933). It could also be possible that only parts of the mobile elements were inherited, which is more likely. The presence of the ACME-element did not show a link with resistance to gentamicin (p=0.3704), ciprofloxacin (p=0.7124), cefoxitin (p=0.6737), vancomycin (p=0.5346) or sulfamethoxazole with trimethoprim (p=0.6722). Additionally, of the six

strains that display a susceptible phenotype for methicillin according to CLSI and EUCAST guidelines, three carried an ACME-element, either type II or type III. Presence of the ACME-element did not concur with occurrence of the *SCCmec* complex, showing a significant difference in distribution (p=2.919e-10). Additionally, presence of ACME-elements in the clinical isolates could not be linked to biofilm forming capacity (p=0.3163).

4.1.6. Construction of growth patterns

Cut-off OD values for weak (OD_{600}<0.769), and strong (OD_{600}>0.769) growing strains were defined as lower or higher ratios of the average OD-value obtained for all strains, both VAP and non-VAP together (OD_{600}=0.769). In addition, the steeper the exponential phase of a strain was, the faster it would grow. The growth curve for each bacterial strain (n = 47) showed characteristic phases of lag, exponential and stationary growth. All strains expressed almost the same growth pattern. Strains associated with VAP (n = 23) were compared to each other and placed in one graph (Figure 8), as where the non-VAP associated strains (n = 24) (Figure 9), in order to compare the growth patterns between both groups. In the non-VAP group only two strains (8%) were rather weak growers, while the rest (n=22, 91%) were strong growers. In the VAP group three strains were rather weak growers (13%), while the rest (n=20, 87%) were strong growers. For the VAP-associated group, there were two strains that showed a shift in their nutrient metabolism throughout their growth pattern (SE 0105 and SE 0112). The same was true for one strain of the non-VAP associated group (SE 0095). Presumably, these strains switch to another nutritional substrate with a less energy-efficient metabolic pathway to avoid build-up of toxic waste products and/or extracellular signalling molecules and acidification of the medium. Bacteria in planktonic cultures, and also in biofilms, can turn on stress-response genes and switch to more tolerant phenotypes upon environmental stresses, such as alterations in nutritional quality, cell density, temperature, pH or osmolarity [2].

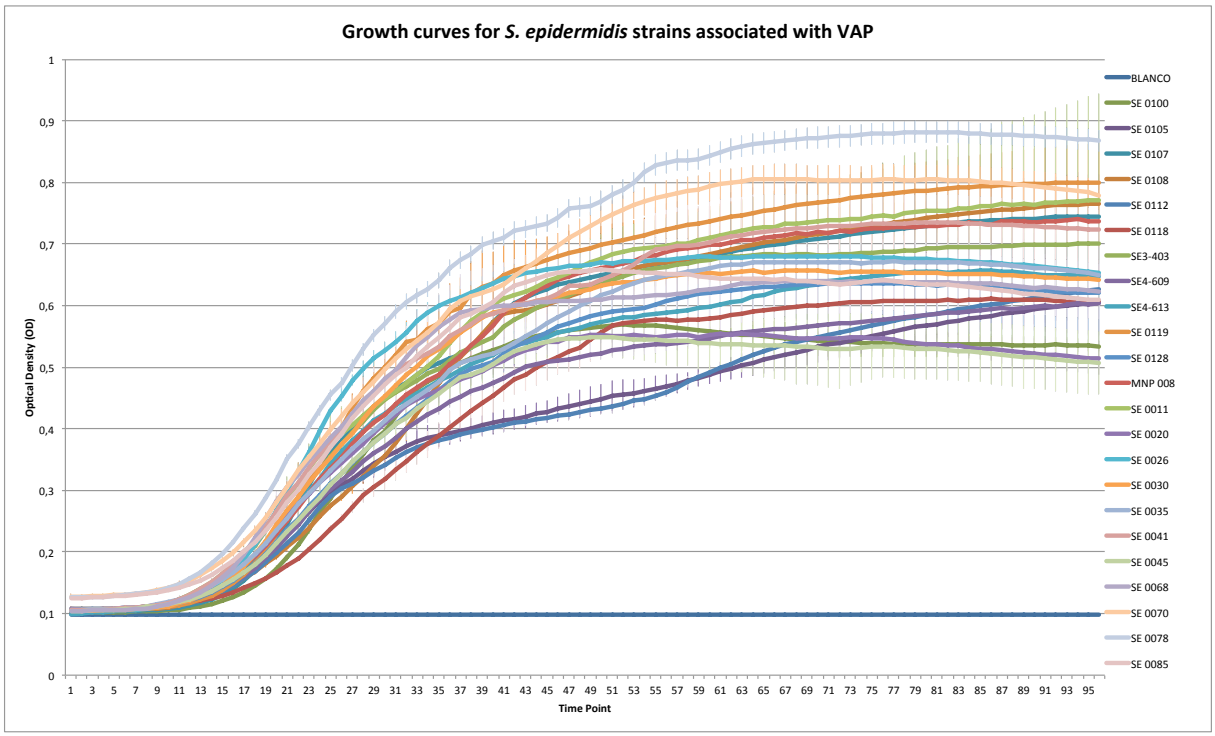

Figure 8: Growth curves for *S. epidermidis* strains associated with VAP. Error bars of the corresponding strain are highlighted in the same colour as the growth curve of that strain.

No significant difference (p = 0.3194) between VAP and non-VAP associated strains was found. However, there was considerable variance present (p=2.2E-16) between the growth

patterns of the individual strains, especially for the VAP-associated group. Growth patterns were randomly distributed amongst strains, independent of biofilm-forming capacity, thereby showing a significant difference when compared to each other (p=0.6.338e-11). This was especially true for strains SE 0078, SE 0045, SE3-403 and SE 0105. No distinct pattern could be found when the growth averages were compared between the different sequence types (p=0.2941). However, four out of five strong growers with the highest OD_{600} (22%) from the VAP group had sequence type 2. The strains with sequence type 54 both displayed intermediate growth rates, however, the sample number is too low for definitive conclusions. Other strains with high growth rates were randomly distributed. No significant difference was found between the two evolutionary clusters (p=0.6269). No pattern could be distinguished in correlation with antibiotic resistance of the corresponding strains (gentamicin: p=0.1282; ciprofloxacin: p=0.1639; cefoxitin: p=0.9568; trimethoprim with sulfamethoxazole: p=0.9224; and vancomycin: p=0.3678). Growth patterns did not show any connection to prevalence of ACME-elements in several isolates (p=7403). And also presence of *SCCmec* complexes in the clinical isolates could not be linked to growth (p=0.3542).

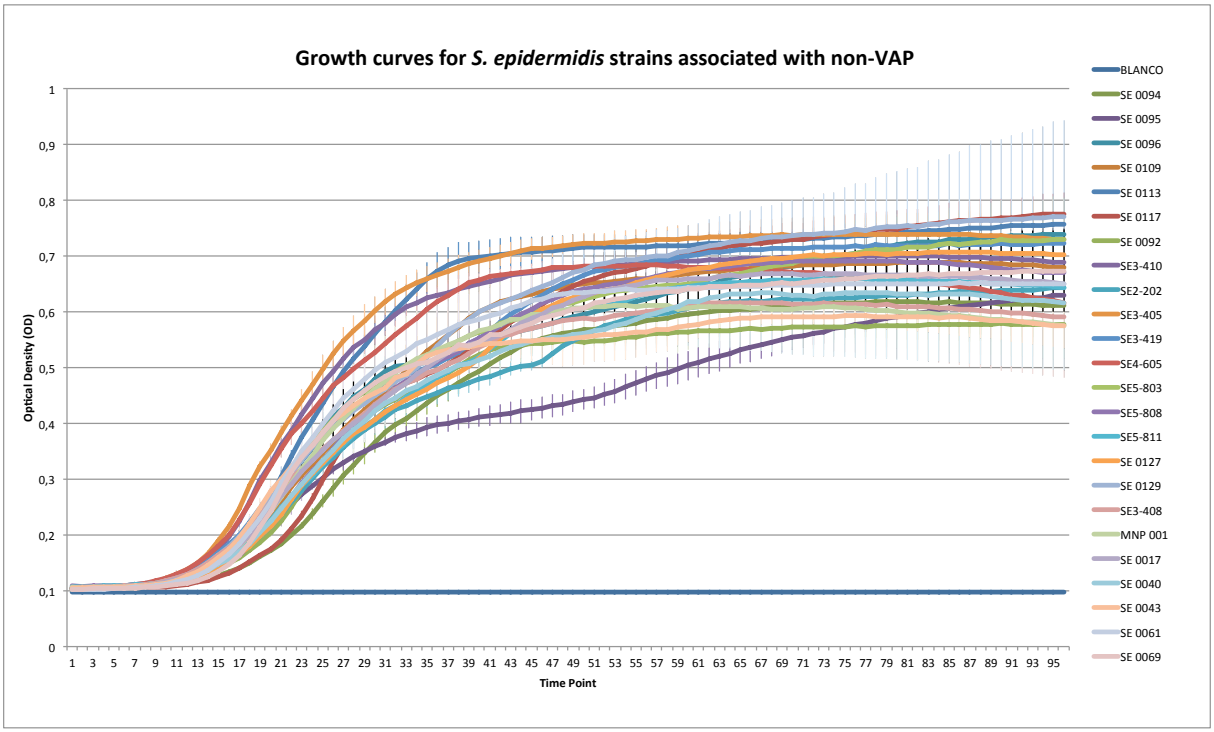

Figure 9: Growth curves for *S. epidermidis* strains associated with non-VAP. Error bars of the corresponding strain are highlighted in the same colour as the growth curve of that strain.

Part 2 – Cultivation of a multispecies biofilm

4.2.1. Cultivation of a biofilm under static conditions

The static assay provided an insight in the communication between *S. epidermidis* and *C. albicans*, *S. marcescens*, *P. aeruginosa* or *K. pneumonia*, selected by 16S analysis. To distinguish synergistic from antagonistic interactions, biofilm formation in multispecies biofilms consisting of different bacterial concentrations was determined and compared with individual biofilm formation of *S. epidermidis* (100%) and individual biofilm forming capacity of the corresponding strain (*C. albicans* (19%), *S. marcescens* (45%), *K. pneumonia* (257%) and *P. aeruginosa* (159%)). This means that all values will be relative to the value obtained for *S. epidermidis* as central organism in this research. Additional data concerning growth of all multispecies ratios can be found in the Addendum (Figure 31, Figure 32, Figure 33 and Figure 34).

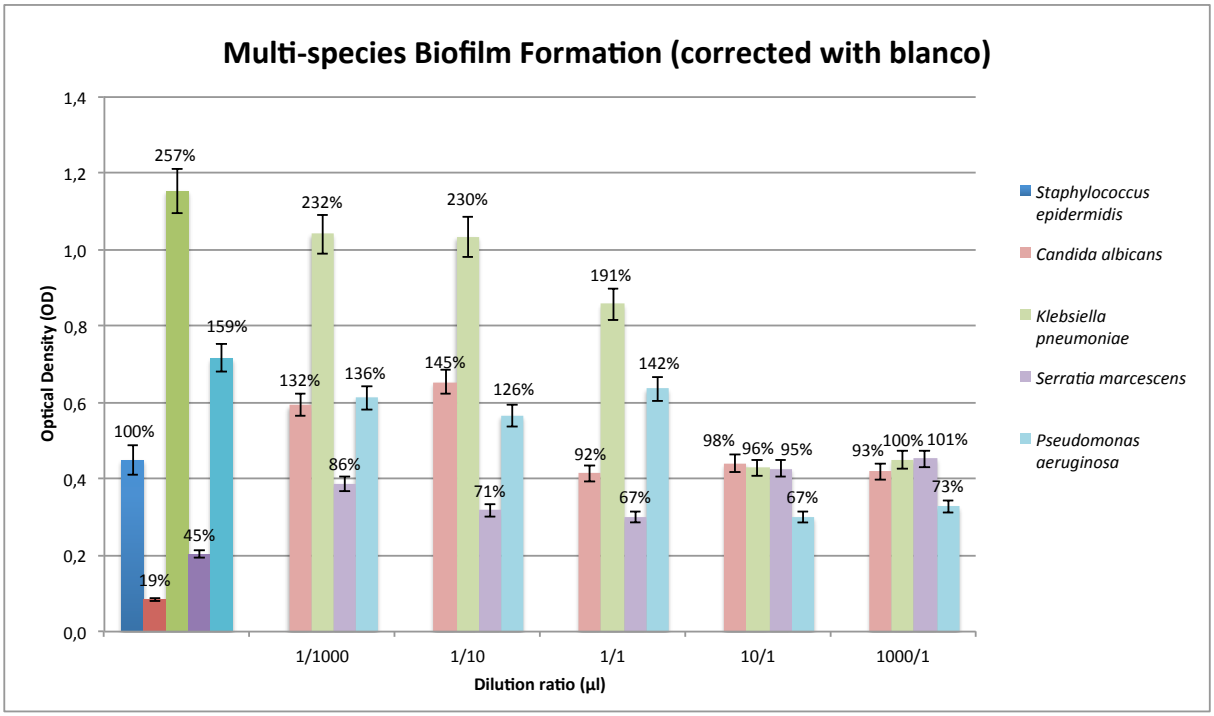

Figure 10: Multispecies biofilm formation between *S. epidermidis* and *C. albicans*, *S. marcescens*, *K. pneumoniae* or *P. aeruginosa*, during the static biofilm assay. Solid bars represent single-species biofilms, whereas faded bars represent multispecies biofilms between S. *epidermidis* and *C. albicans*, *S. marcescens*, *K. pneumoniae* or *P. aeruginosa*. The amount of biofilm-formation was corrected with the background value of the blank (only TSB-medium) and expressed as a percentage compared to individual growth of *S. epidermidis*. Error bars representing the standard deviation per strain were included. A 1/10 and 1/1000 dilution referred to a higher ratio of *C. albicans*, *S. marcescens*, *K. pneumoniae* or *P. aeruginosa* and a lower ratio of *S. epidermidis*. A 10/1 and 1000/1 dilution referred to a higher ratio of *S. epidermidis* and a lower ratio of *C. albicans*, *S. marcescens*, *K. pneumoniae* or *P. aeruginosa*. See **Table 20**, '*3.2.2. Static biofilm assay*', Chapter III for correct dilution quantities.

In the single-species biofilm, *C. albicans* (19%) formed significantly less biofilm than *S. epidermidis* (100%). However, when *C. albicans* was mixed with *S. epidermidis*, biofilm formation significantly increased. This is especially true for the concentrations containing more *C. albicans* than *S. epidermidis* (145% and 132%). For the concentrations where there was more *S. epidermidis* present values fluctuated around that of the single-species *S. epidermidis* biofilm. According to these results, *S. epidermidis* might be helping *C. albicans* in establishing biofilm formation and growth or the other way around.
K. pneumoniae (257%) formed significantly more biomass in the single-species biofilm than *S. epidermidis* (100%). Mixed biofilms of *S. epidermidis* and a high quantity of *K. pneumoniae* formed significantly more biofilm (190%, 230% and 232%) compared to *S. epidermidis* alone, but not as much as single-species *K. pneumoniae* biofilms (257%). This suggests competition for nutrients, resulting in intermediate biofilms, reflecting differences in growth rates and efficiency of nutrient use. In addition, biofilms with more *S. epidermidis* than *K. pneumoniae* fluctuated around the single-species *S. epidermidis* biofilm formation (96% and 100%), further supporting this hypothesis. *K. pneumoniae* grows much faster (R^2=0.97325) than *S. epidermidis* (R^2=0.85539) and, given resources are readily available, will probably eventually dominate dual-species biofilms of both species. Obtained results suggest an intermediary interaction between *S. epidermidis* and *K. pneumoniae*, thereby mainly reducing prevalence of *K. pneumoniae*.
S. marcescens (45%) on its own formed less biofilm on its own than *S. epidermidis* (100%) did. The biofilm forming capacity of *S. marcescens* was enhanced when it was mixed with *S. epidermidis*, as even dual-species biofilms where *S. marcescens* was present in an initially higher quantity than *S. epidermidis* formed more biofilm than the single-species biofilm of *S.*

marcescens (67%, 71% and 87%), while showing an inverse correlation compared to the numbers expected from their single-species biofilms. For the concentrations where *S. epidermidis* was present in a higher quantity, biofilm formation was comparable with individual biofilm forming capacity (95% and 101%). Based on these results, it is hypothesised that *S. marcescens* has no effect on *S. epidermidis* biofilm formation while, *S. epidermidis* likely stimulates biofilm formation and growth by *S. marcescens*.
Last, *P. aeruginosa* formed more biofilm on its own (159%) than *S. epidermidis* (100%). When the strains were mixed with each other, biofilm forming capacity decreased and the dual-species biofilms formed less biofilm mass than those of either single species (67% for a SE/PA ratio of 10:1 and 73% for a SE/PA ratio of 1000:1). When *P. aeruginosa* was in abundance, there was overall more biofilm formation, than when *S. epidermidis* was in abundance. These interactions clearly indicate an antagonistic interaction.

Fluorescence microscopy was performed in order to gain insights in the structure and spatial distribution of the different species within the multispecies consortium. As a reference, the individual biofilm structure of *S. epidermidis* and the individual biofilm structure of the corresponding species (*C. albicans*, *K. pneumoniae*, *S. marcescens* or *P. aeruginosa*) was compared to the structure of the multispecies biofilm.

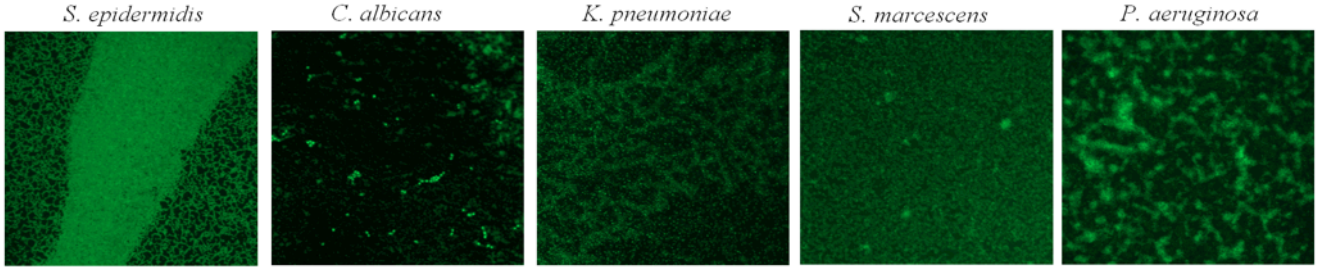

Figure 11: 24h single-species biofilm of *S. epidermidis*, *C. albicans*, *K. pneumoniae*, *S. marcescens* and *P. aeruginosa* formed under static conditions. Visualisation with SYTO 9 'live' staining at 20x magnification. *S. epidermidis*: colony count: 1291; total area: 767 752 000 mm²; fraction colonized of entire well: 53.2%. *C. albicans*: colony count: 1621; total area: 236 172 000 mm²; fraction colonized of entire well: 16.4%. *K. pneumoniae*: colony count: 4677; total area: 374 102 000 mm²; fraction colonized of the entire well: 25.9%. *S. marcescens*: colony count 7213; total area: 614 991 000 mm²; fraction colonized of entire well: 42.6%. *P. aeruginosa*: colony count 2369; total area: 402 934 000; fraction colonized of entire well: 27.9%.

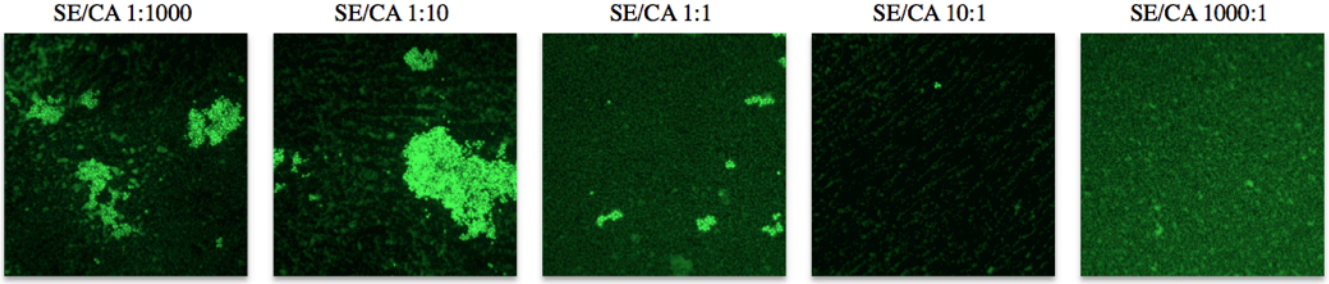

Figure 12: 24h multispecies biofilm between *S. epidermidis* and *C. albicans* in different concentrations and formed under static conditions. Visualisation with SYTO 9 'live' staining at 20x magnification. SE1/CA1: colony count: 3325; total area: 484 946 000 mm²; fraction colonized of entire well: 33.6%. SE1/CA10: colony count 1549; total area: 730 088 000 mm²; fraction colonized of entire well: 50.6%. SE10/CA1: colony count: 2874; total area: 252 725 000 mm², fraction colonized of entire well: 17.5%. SE1/CA1000: colony count: 2532; total area: 717 617 000 mm²; fraction colonized of entire well: 49.7%. SE1000/CA1: colony count: 2338; total area: 657 598 000 mm²; fraction colonized of entire well: 45.6%.

The images shown above demonstrate that *S. epidermidis* and *C. albicans* can co-exist together within the same biofilm and are able to grow in close proximity to each other. Biofilm structure is characterised by a mixture of the growth pattern of *C. albicans* (bright aggregations of cells) and *S. epidermidis* (less bright signal due to lower cell mass compared to *C. albicans*). When *S. epidermidis* in abundance, the bacteria would take over the biofilm, likely due to the faster growth rate and minimal presence of the fungus. When *S. epidermidis* numbers were lower, *C. albicans* may predominate and act as an anchor for subsequent

colonisation by *S. epidermidis* as was shown in literature [164]. The obtained results indicate a possible synergistic interaction between *C. albicans* and *S. epidermidis*, which is manifested by a higher biovolume of the mixed biofilm than by *C. albicans* grown as single species alone. However, more elaborate experiments are necessary to confirm these observations.

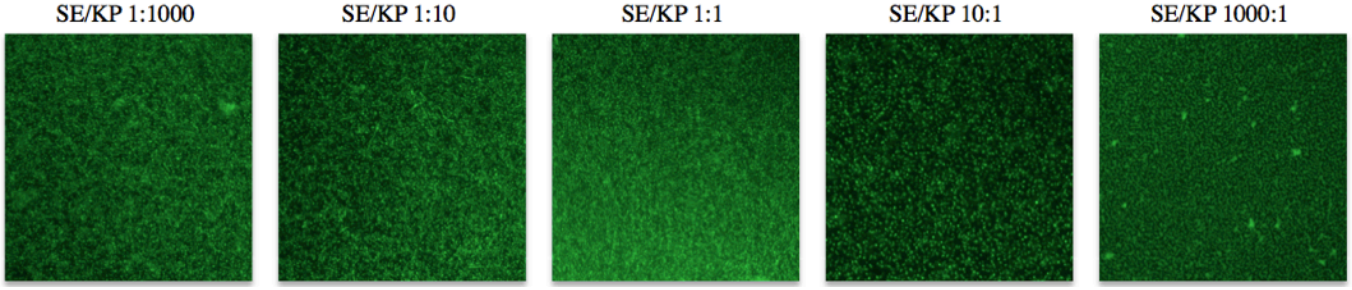

Figure 13: 24h multispecies biofilm between *S. epidermidis* and *K. pneumoniae* in different concentrations and formed under static conditions. Visualisation with SYTO 9 'live' staining at 20x magnification. SE1/KP1: colony count: 2538; total area: 234 202 000 mm²; fraction colonized of entire well: 33.1%. SE1/KP10: colony count: 2320; total area: 131 213 000 mm²; fraction colonized of entire well: 25.1%. SE10/KP1: colony count: 627; total area: 43 725 000 mm²; fraction colonized of entire well: 20.7%. SE1/KP1000: colony count: 2458; total area: 220 928 000 mm²; fraction colonized of entire well: 31.3%. SE1000/KP1: colony count: 2169; total area: 637 210 000 mm²; fraction colonized of entire well: 44.1%.

When bringing *K. pneumoniae* and *S. epidermidis* together in a dual-species biofilm, at any ratio, the biofilm structure is almost completely dominated by *K. pneumonia*, visible by its granular view. This quick surface covering is likely due to the fast growth rate of *K. pneumoniae* compared to the growth rate of *S. epidermidis,* as stated before. *K. pneumoniae* is capable of overgrowing and covering the surface, even in small quantities, leaving almost no room to attach for *S. epidermidis*. Only in the highest concentration, with a minimal presence of *K. pneumoniae* (1000:1), *S. epidermidis* will mainly colonize the surface as this image resembles the single-species image of *S. epidermidis*. However, *S. epidermidis* will not be completely outcompeted by *K. pneumoniae* as all the formed biofilms show an intermediate structure compared to the single-species biofilms. Both the 1:1000 and 1:10 images show more or less the same degree of surface colonization, mainly by *K. pneumoniae* with a limited amount of *S. epidermidis*. The 1:1 image shows an even higher colonization but the biofilm structure has changed slightly compared to the previous images, indicating that *S. epidermidis* can here colonize more surface, but still not much, because it is present in an equal amount as *K. pneumoniae*. Finally, the 10:1 image shows that *K. pneumoniae* can still colonize the surface even when *S. epidermidis* is in abundance. There is, however, less area colonized than in the previous images, showing that the presence of *S. epidermidis* in the biofilm definitely plays an important role and that *K. pneumoniae* will only gradually overcome *S. epidermidis*. This might suggest a limited degree of competition, without production of antimicrobials, as one species would then completely disappear from the biofilm.

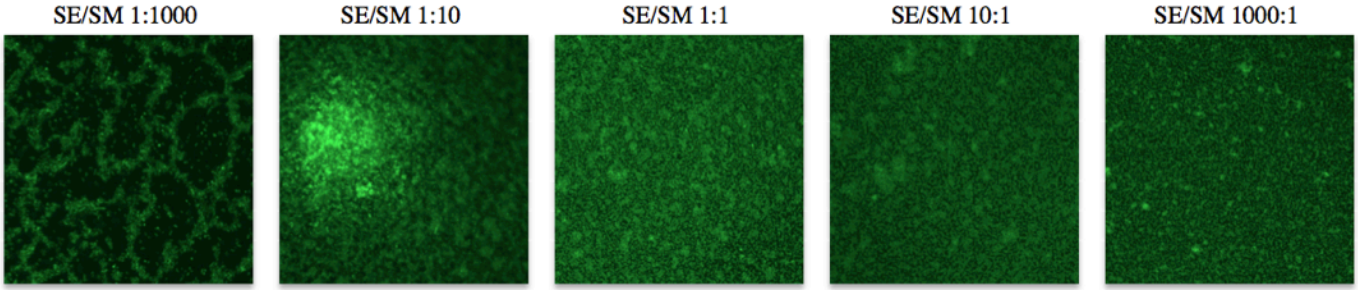

Figure 14: 24h multispecies biofilm between *S. epidermidis* and *S. marcescens* in different concentrations and formed under static conditions. Visualisation with SYTO 9 'live' staining at 20x magnification. SE1/SM1: colony count: 1817; total area: 712 215 000 mm²; fraction colonized of entire well: 49.3%. SE1/SM10: colony count: 1359; total area: 722 445 000 mm²; fraction colonized of entire well: 50%. SE10/SM1: colony count: 1162; total area: 724 156 000 mm²; fraction colonized of entire well: 50.2%. SE1/SM1000: colony count: 537; total area: 101 649 000 mm²; fraction colonized of entire well: 29.4%. SE1000/SM1: colony count: 2450; total area: 667 882 000 mm²; fraction colonized of entire well: 46.3%.

The multispecies biofilm of *S. epidermidis* and *S. marcescens* likely shows a mixed biofilm structure of both species, as the images obtained (1:10, 1:1 and 10:1) do not seem to resemble either of the single-species biofilm images. Referring to the results obtained for biofilm formation, it might very well be possible that *S. epidermidis* and *S. marcescens* will form a biofilm together in order to enhance biofilm formation and colonization. It is unfortunately, based on these images, difficult to say which species will serve as early colonizer for the other species. However, *S. marcescens* has shown to have a higher growth rate (R^2=0.97279) than *S. epidermidis* (R^2=0.85539), allowing to hypothesise that *S. marcescens* might first adhere to the polystyrene surface of the wells thereby inducing subsequent colonization and adherence of *S. epidermidis,* which in turn helps *S. marcescens* again to form biofilms. The 1000:1 image resembles the single-species morphology of *S. epidermidis* again. The 1:1000 image, however, does not even remotely resemble the single-species morphology of *S. marcescens*. The reason why it starts producing completely different biofilm structures is not clear based on these images.

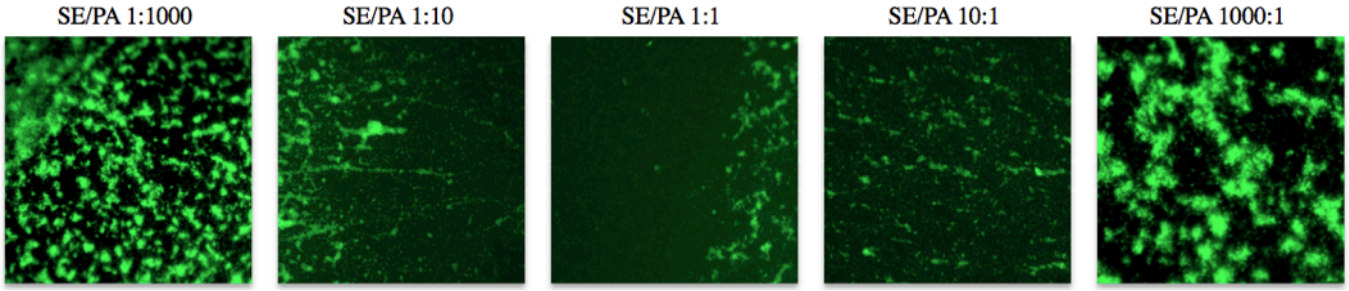

Figure 15: 24h multispecies biofilm between *S. epidermidis* and *P. aeruginosa* in different concentrations and formed under static conditions. Visualisation with SYTO 9 'live' staining at 20x magnification. SE1/PA1: colony count: 3915; total area: 495 465 000 mm²; fraction colonized of entire well: 34.3%. SE1/PA10: colony count: 2287; total area: 414 015 000 mm²; fraction colonized of entire well: 28.7%. SE10/PA1: colony count: 7500; total area: 444 156 000 mm²; fraction colonized of entire well: 30.8%. SE1000/PA1: colony count: 320; total area: 137 550 000 mm²; fraction colonized of entire well: 49.8%. SE1/PA1000: colony count: 1164; total area: 723 044 000; fraction colonized of entire well: 50.1%.

The 1:1000 and 1000:1 images clearly show the same morphology and structure as for the single-species biofilm of *P. aeruginosa*. Here, *P. aeruginosa* will dominate biofilm formation and thereby inhibit any growth of *S. epidermidis* suggesting the contribution of a variety of secreted antimicrobials. However, the other three images show an intermediate morphology that does not resemble either single-species morphology. Zooming in on the images shows that the formed structures do have a cloudy morphology, resembling *P. aeruginosa*. So, *P. aeruginosa* will here again completely outcompete *S. epidermidis,* but it might be that this is more difficult as in this ratio *S. epidermidis* is present in a larger quantity compared to the 1:1000 and 1000:1 ratio. Again, the observations point in the direction of an antagonistic relationship between both species.

4.2.3. Cultivation of a biofilm under dynamic conditions

4.2.3.1. Fluorescence microscopy

Based on the results obtained in the static dynamic multispecies assay, SE/SM and SE/PA combinations were selected for further research due to the synergistic and antagonistic interactions observed respectively. In addition, the antagonistic effect of *P. aeruginosa* on *S. epidermidis* might prove to have industrial implications, as the extracellular polysaccharides from *P. aeruginosa* may represent a novel target for the development of agents to control *S. epidermidis* biofilms at sites of infection. While the synergism between *S. epidermidis* and *S. marcescens* was chosen for further research as it might be correlated to their co-occurrence in VAP versus non-VAP as observed in the microbiome analysis (Figure 35 in the Addendum).

In order to make a comparison between the cultivated multispecies biofilms of *S. epidermidis*,

P. aeruginosa and *S. marcescens*, single-species biofilms were first visualised. Multispecies images were visualised in a 1:1000 dilution and a 1000:1 dilution.

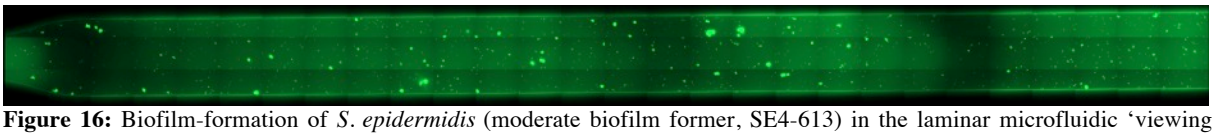

Figure 16: Biofilm-formation of *S. epidermidis* (moderate biofilm former, SE4-613) in the laminar microfluidic 'viewing window' channel after 5h growth. Visualisation with SYTO 9 'live' staining at 40x magnification with 2μm/pixel. Colony count: 762; total area: 26 152 000 mm^2; fraction colonized of entire channel: 1.9%.

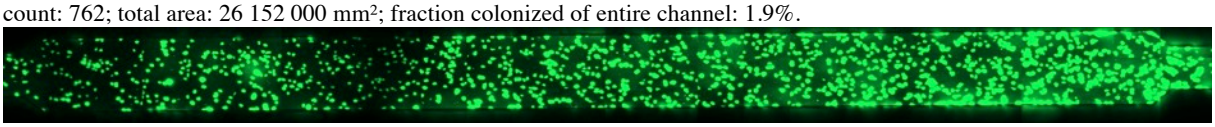

Figure 17: Biofilm-formation of *P. aeruginosa* in the laminar microfluidic 'viewing window' channel after 5h growth. Visualisation with SYTO 9 'live' staining at 40x magnification with 2μm/pixel. Colony count: 929; total area: 332 304 000 mm^2; fraction colonized of entire channel: 23%.

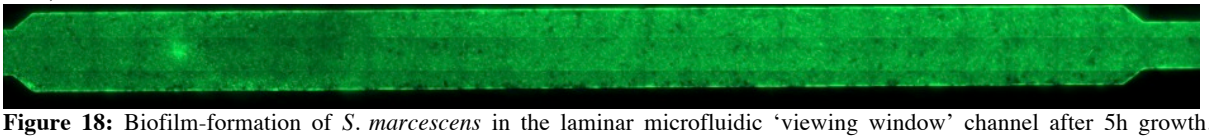

Figure 18: Biofilm-formation of *S. marcescens* in the laminar microfluidic 'viewing window' channel after 5h growth. Visualisation with SYTO 9 'live' staining at 40x magnification with 2μm/pixel. Colony count: 911; total area: 948 304 000 mm^2; fraction colonized of entire channel: 65.7%.

S. epidermidis single-species biofilms consisted of a layer of adhered cells, spotted with small developing microcolonies. *P. aeruginosa* single-species biofilms were characterised by the presence of numerous small microcolonies. And *S. marcescens* biofilms consisted of a confluent, thick layer of adhered cells without any sign of microcolonies.

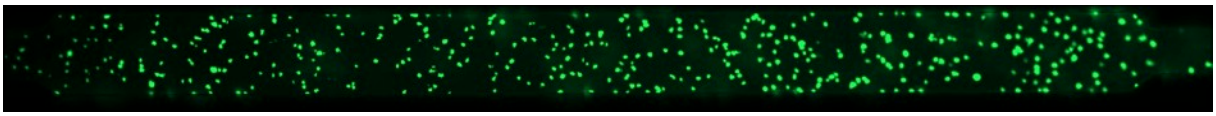

Figure 19: Biofilm-formation of *S. epidermidis* (SE4-613, moderate biofilm-forming strain) and *P. aeruginosa* in the laminar microfluidic 'viewing window' channel after 5h growth at a 1:1000 ratio. Visualisation with SYTO 9 'live' staining at 40x magnification with 2μm/pixel. Colony count: 371; total area: 73 483 000 mm^2; fraction colonized of entire channel: 5.2%.

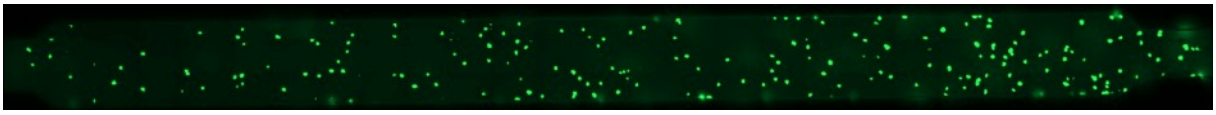

Figure 20: Biofilm-formation of *S. epidermidis* (SE4-613, moderate biofilm-forming strain) and *P. aeruginosa* in the laminar microfluidic 'viewing window' channel after 5h growth at a 1000:1 ratio. Visualisation with SYTO 9 'live' staining at 40x magnification with 2μm/pixel. Colony count: 282; total area: 53 198 000 mm^2; fraction colonized of entire channel: 3.8%.

When combined in both a 1:1000 and 1000:1 ratio, *P. aeruginosa* and *S. epidermidis* mixed-species biofilms showed characteristics of single-species *P. aeruginosa* biofilms. When *S. epidermidis* was, however, present in a larger quantity, *P. aeruginosa* formed significantly less microcolonies than it did on its own or in the 1:1000 ratio. In the 1:1000 ratio *P. aeruginosa* also formed less microcolonies than in the single-species biofilm. It has become clear throughout the experiments that *P. aeruginosa* will exert a negative effect on *S. epidermidis* when together in a mixed-species biofilm, completely removing *S. epidermidis* from the culture. But according to these results, inhibition of *S. epidermidis* might be accompanied with an energy cost for *P. aeruginosa* due to the production of antimicrobials, because of which it will start to form significantly less microcolonies in combination with *S. epidermidis*. Overall, the obtained results confirm the antagonistic relationship between *S. epidermidis* and *P. aeruginosa*, by inhibition of *S. epidermidis,* as shown in literature [165].

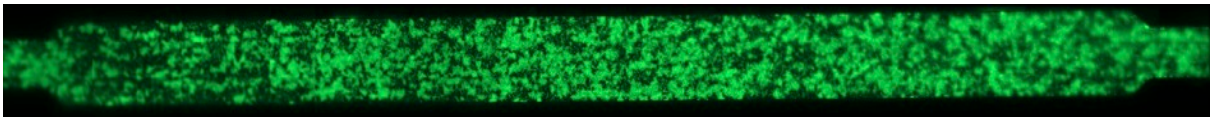

Figure 21: Biofilm-formation of *S. epidermidis* (SE4-613, moderate biofilm-forming strain) and *S. marcescens* in the laminar microfluidic 'viewing window' channel after 5h growth at a 1:1000 ratio. Visualisation with SYTO 9 'live' staining

at 40x magnification with 2μm/pixel. Colony count: 467; total area: 697 808 000 mm^2; fraction colonized of entire channel: 48%.

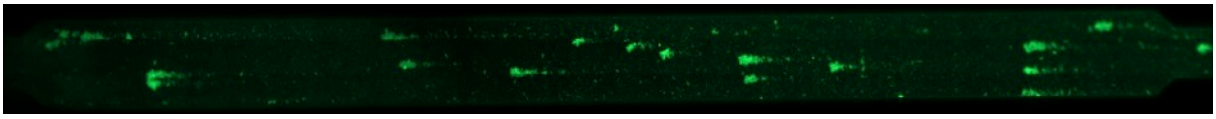

Figure 22: Biofilm-formation of *S. epidermidis* (SE4-613, moderate biofilm-forming strain) and *S. marcescens* in the laminar microfluidic 'viewing window' channel after 5h growth at a 1000:1 ratio. Visualisation with SYTO 9 'live' staining at 40x magnification with 2μm/pixel. Colony count: 2495; total area: 64 641 000 mm^2; fraction colonized of entire channel: 4.5%.

Mixed-species biofilms of *S. marcescens* and *S. epidermidis* again showed a very completely different morphology than the single-species biofilms of both species. *S. epidermidis* and *S. marcescens* dual-species biofilms showed the presence of a large amount of microcolonies when *S. marcescens* was the most abundant species, while only few microcolonies could be observed when *S. epidermidis* was the dominant species in the inoculum. Again, the 1000:1 ratio showed a completely different morphology than the other ratio. It might be possible that a high concentration of *S. epidermidis* induces stress on *S. marcescens*, which will in response shift to a different type of biofilm as has been proven in literature that biofilm formation of *S. marcescens* is dynamic [166]. Due to this observation it is difficult to state whether *S. epidermidis* and *S. marcescens* will have a synergistic or an antagonistic interaction with each other, as it appears to be beneficial up to a certain threshold point after which biofilm formation decreases.

4.2.3.2. Confocal laser-scanning microscopy

Biofilms formed between *S. epidermidis* and *P. aeruginosa* or *S. epidermidis* and *S. marcescens* were further analysed in order to distinguish the Gram-positive cocci of *S. epidermidis* from the Gram-negative rods of *P. aeruginosa* and *S. marcescens*, and to determine their individual positions within the biofilm.

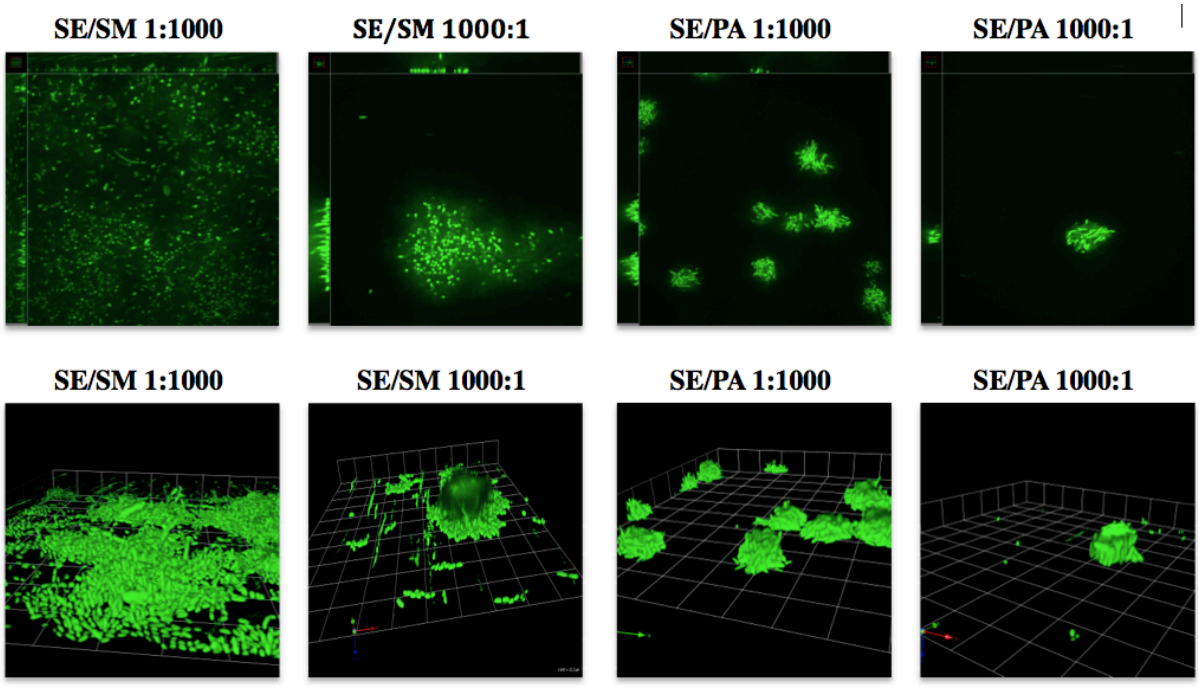

Figure 23: First row: 2D image of the multispecies biofilm of *S. epidermidis* and *P. aeruginosa,* or *S. epidermidis* and *S. marcescens* in the laminar microfluidic 'viewing window' channel after 5h growth. Visualisation with SYTO 9 'live' staining at 60x magnification.(*a*) Plan view, (*b)* and (*c)* Side views through the biofilm in the XZ and YZ planes, respectively. 1 unit is equal to 200µm. Second row: 3D image of the multispecies biofilm of *S. epidermidis*, and *P. aeruginosa,* or *S. epidermidis* and *S. marcescens* in the laminar microfluidic 'viewing window' channel after 5h growth. Visualisation with SYTO 9 'live' staining at 60x magnification. Structure of the biofilm is represented in X, Y and Z planes, respectively. 1 unit is equal to 13.3µm.

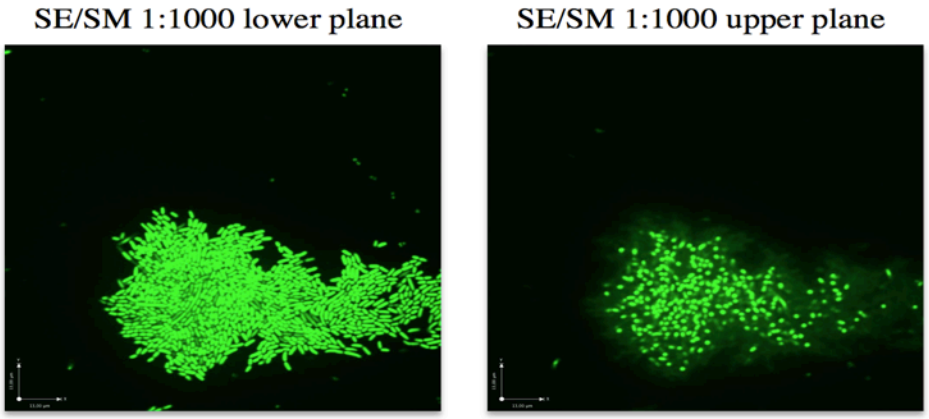

Figure 24: Plane section of the multispecies biofilm of *S. epidermidis* and *S. marcescens* in the laminar microfluidic 'viewing window' channel after 5h growth. Visualisation with SYTO 9 'live' staining at 60x magnification. 1 unit is equal to 200µm.

Looking at the 2D and 3D image for the multispecies biofilm between *S. epidermidis* and *S. marcescens*, it becomes clear that a small quantity of *S. epidermidis* will induce biofilm formation by *S. marcescens*, whereas a large quantity of *S. epidermidis* will inhibit biofilm formation and *S. marcescens* will produce a confluent layer of adhered cells as was seen for the single-species biofilm. Rod-shaped cells of *S. marcescens* dominate the biofilm. However, within the biofilm there are clearly some cocci present, showing that *S. epidermidis* might be a constituting part of the multispecies biofilm between *S. epidermidis* and *S. marcescens*. When looking at the plane sections of these images, it is clear that the basis of the biofilm will be formed by an aggregation of rod-shaped cells, the upper plane sections will consist out of cocci. The cocci, however, could also prove to be rods at an angle of 90°. Therefore, additional analysis is necessary.

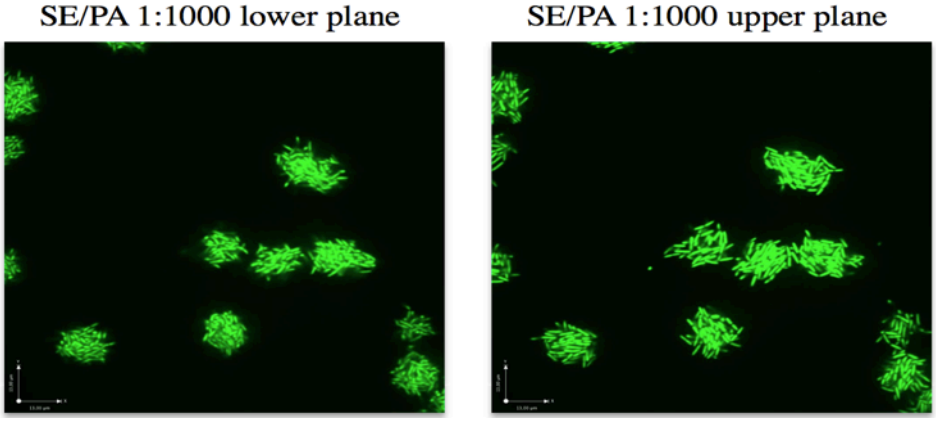

Figure 25: Plane section of the multispecies biofilm of *S. epidermidis* and *P. aeruginosa* in the laminar microfluidic 'viewing window' channel after 5h growth. Visualisation with SYTO 9 'live' staining at 60x magnification. 1 unit is equal to 200µm.

Looking at the 2D and 3D image for the multispecies biofilm between *S. epidermidis* and *P. aeruginosa* and their quantification, *P. aeruginosa* will form less microcolonies in a mixture with *S. epidermidis* than in a single-species culture. The multispecies culture between *P. aeruginosa* and *S. epidermidis* predominantly exists out of rod-shaped cells forming a microcolony. There is no sign of cocci in both cultures, strengthening the idea of complete inhibition of *S. epidermidis* by *P. aeruginosa*. Looking at the plane sections of these images, it is clear that both the basis of the microcolonies as well as the top are constructed out of all rod-shaped cells with no sign of cocci. It becomes clear that *P. aeruginosa* has an antagonistic effect on *S. epidermidis* as suggested many times in literature.

Part 1 – General characterization of *S. epidermidis*

5.1.1. Identification based on sequence variation

The idea behind MLST is that isolates of a particular strain, belonging to the same chain of transmission, are clonally related; they descend from a common ancestor. During their evolutionary history, isolates diversify through point mutations, recombination or the acquisition and/or deletion of mobile genetic elements, giving rise to extensive genomic and phenotypic diversity [158]. In a typical MLST approach, recombination is expected to occur with a much higher frequency than point mutations. Therefore, one does not look at the total sequence similarity between strains, but only at a few loci [167].

By using extensive multilocus sequence typing (MLST), it was shown that the population structure of *S. epidermidis* in hospital environments worldwide is composed of a major and highly diverse genetic lineage, i.e., clonal complex 2 (CC2) [168]. The majority of both commensal and nosocomial strains are known to belong to this complex [72]. However, in total, the nosocomial *S. epidermidis* population appears to be composed out of nine clonal lineages that were found to be spread worldwide [169]. It was suggested by *Miragaia, M., et al*., 2005 [117] that strains of CC2 appear to have an enhanced capacity for transfer of genetic material. The predominance of this lineage in the hospital environment may be connected to its suitability for frequent recombination that could have brought genomic plasticity to this *S. epidermidis* lineage and contributed to the acquisition and modulation of antibiotic resistance determinants. ST2 constitutes the founder sequence type of clonal complex 2 [71]. Moreover, strains belonging to ST2 (also known as ST27), the by far most frequently found clonal type of hospital-associated invasive *S. epidermidis*, are all *ica*- and IS256-positive [19, 170]. This means that they supposedly form multi-layered biofilms, which was not proven in the results, but that they also possess a high multiple antibiotic resistance profile [171], which renders this lineage extremely well adapted to the nosocomial environment [101].

5.1.2. Determination of an antibiotic resistance profile

The results clearly indicate a certain degree of acquired antibiotic resistance in planktonic *S. epidermidis* isolates before the initiation of biofilm formation associated with VAP. Approximately 70% of the *S. epidermidis* strains isolated in the hospital environment have acquired resistance to methicillin, and the majority of them are also resistant to almost all other classes of antimicrobial agents [158, 172]. The results indicate no clear difference in antibiotic susceptibility between VAP and non-VAP associated strains, as all isolates already showed a certain degree of resistance, and thus adaptation to antibiotic pressure in the hospital, before colonization of the recently admitted patient. Additional antibiotic resistance during VAP might arise due to biofilm formation on the endotracheal tube or due to characteristics of other constituting pathogens present.

Numerous bacterial antimicrobial resistance phenotypes result from the acquisition of external genes that may provide resistance to an entire class of antimicrobials. External genes are frequently associated with large transferable extrachromosomal DNA elements called plasmids, on which there may be other mobile DNA elements such as transposons, integrons and insertion sequences [110]. In resistant clinical isolates, the lion's share of resistances appears to be plasmid mediated [173]. Horizontal gene transfer (HGT) among multiple members of the *staphylococcal* species is a frequent event, allowing for adaptation to shifting host environments [174]. Moreover, they are very adept at acquiring the necessary genetic information to survive in highly selective environments. Due to the ubiquity of *S. epidermidis* as a human commensal microorganism this bacterium is an optimal carrier and reservoir for

antibiotic resistance genes [175]. Horizontal gene transfer both within and beyond species borders is likely favoured during biofilm formation, the major virulence factor of *S. epidermidis,* due to close aggregation of cells [116]. On top of that, additional non-specific resistance in biofilms associated with their general architecture and structure is of great concern in CoNS infections. Additionally, in a minority of cases acquired resistance may also arise due to a mutation in the genetic make-up of the bacterium. Occurring at the normal rate of mutation, with one sub-clone every 10^5-10^6 colonies [176], heteroresistance provides a tool for natural evolution to drug resistance. Such mutants may proliferate, thereby giving rise to a new resistant population with a higher MIC than that for the wild-type population [177]. Heteroresistance to methicillin and vancomycin has been shown to occur in CoNS [19].

Whether antibiotic resistance is intrinsic or acquired, the genetic determinants of resistance encode specific biochemical resistance mechanisms that may include enzymatic inactivation of the drug, alterations to the structure of the antibiotic target site, and changes that prevent access of an adequate concentration of the antimicrobial agent to the active site, such as efflux pumps and permeability barriers [110].

5.1.3. Determination of the presence of the ACME- and *SCCmec*-element

S. epidermidis is a highly diverse species that evolves mainly by recombination and readily acquires mobile genetic elements such as *SCCmec* cassettes and ACME-elements [71]. The data obtained suggest extensive intraspecies transfer of ACME, *SCCmec*, and other *SCC*-elements among *S. epidermidis*, perhaps owing to the fact that 81% of the *S. epidermidis* population carry *ccr* gene complexes [118]. A high rate of interspecies transfer is commonly observed within the CC2 lineage [119]. Both ACME and *SCCmec* are, however, not only integrated in the same chromosomal site *OrfX* [117], but are also both mobilized by the same *SCC*-encoded *ccr* recombinases, However, concurrence of both elements together could not be confirmed and a linkage between colonization capacity and antibiotic resistance seems unlikely according to the results.

ACME is a genomic island in *Staphylococci* which is mostly not associated with a higher capacity of causing disease, but it may be considered as an indicator of benign skin flora isolates because it contributes to optimal *staphylococcal* life on the acidic human skin by L-arginine catabolism, pH regulation and ATP production, hereby conferring a fitness advantage for colonization and transmission [112, 118]. ACME allotype I is the most prevalent among the most widely disseminated lineages of *S. epidermidis* (CC2). ACME was often found in association with different allotypes of *SCCmec* [118]. The high prevalence of ACME among *Staphylococcus* species that are common commensals of the human skin, e.g. *S. epidermidis*, suggest that this element is unlikely to contribute to the capacity of coagulase-negative *staphylococci* to cause disease in humans. As such, the hypothesis that ammonia has a function in controlling pH homeostasis during growth in acidic microniches such as biofilms, and thus that ACME might play a role in pathogenesis, could not be supported due to lack of difference between VAP and non-VAP groups. ACME is thought to be more common among commensal isolates and may represent a survival benefit for *S. epidermidis* colonising healthy individuals in the community [111]. However, the acquisition of ACME by *S. epidermidis* might have allowed for the expansion of its typical colonization niches, providing new opportunities for transmission, dissemination, and consequently, evolutionary adaptation to the hospital environment.
In addition, the hospital environment with highly selective pressures of antibiotics and disinfectants, and the coherent pathogenic lifestyle, seem to promote an increase in the frequency, diversification and amplification of the neighbouring *SCCmec* element by recombination between multiple acquired *SCCmec* genes and an increased expression of *ccr*

genes after antimicrobial exposure [178] as carriage of MRSE-*SCCmec* was found to be common in patients at hospital admission. Moreover, the main reason appears to be that in the hospital a large reservoir of *SCCmec* types exist in other CoNS, and this may be contributing to the overall genetic diversity observed in *S. epidermidis*. The *SCCmec* element is responsible for wide-observed methicillin resistance amongst a broad range of *Staphylococci*, as confirmed by the results. Whereas, additional antimicrobial resistance was likely inferred from the J-regions of the *SCCmec* complex that are known to carry additional insertion sequences [179]. The most common *SCCmec* type found in MRSE is *SCCmec* type IV [178], although variant isolates showed to harbour more than one type [180]. The fact that more than one type of *SCCmec* could be found in some isolates suggests that new variants may be present in CoNS [181]. The small size of the *SCCmec* complex may enhance its mobility and ease its current spread in the community, as in the hospital, with a possible impact of frequent vancomycin use in the latter setting [159, 181]. The fact that these isolates can survive in the hospital provides them with a higher capacity for dissemination and the accumulation of relevant genetic traits for survival. It is possible that this kind of clone is responsible for shuffling genetic traits, like *SCCmec* between the community and the hospital. In addition, the spread of *SCCmec* is even more promoted during biofilm formation, the most important virulence property of *S. epidermidis*, associated with hospital-acquired infections, resulting in a vicious circle of increasing methicillin-resistance and more aggressive pathogenicity for hospital-acquired infections [178, 182, 183].

5.1.4. Cultivation of a biofilm under static conditions

The formation of biofilms is the most important virulence factor of *S. epidermidis*. However, a single individual patient can carry many phenotypically different *S. epidermidis* strains with variable antibiotic resistance profiles, capacities to form biofilms and overall gene compositions [184]. While in the normal microflora only about 6% of the *S. epidermidis* strains possess the ability of slime production, in clinical isolates this propriety can be present in a 10 times higher percentage of strains [185]. The observed *S. epidermidis* isolates were able to grow moderate biofilms, so they could establish a basis for consecutive infections. A link between biofilm forming capacity and the prevalence of VAP could, however, not be established, allowing to conclude that other interactions within the microbial consortium or *in vivo* circumstances might trigger additional biofilm formation of *S. epidermidis* [186-189]. On its own, *S. epidermidis* appears unable to grow substantial biofilms and, thus, to cause VAP. Intermicrobial competition and natural variance might explain the presence of strong biofilm-formers in some patients, while others only display weak biofilm-forming capacity.

It has been suggested many times in literature that clinical strains harbour genetic information for biofilm formation (*ica*-operon) in addition to insertion sequences (IS256) and genes that mediate methicillin and aminoglycoside resistance [72]. The presence of multiple copies of IS256 renders the *S. epidermidis* genome flexible to adapt to varying environments and might affect the expression of certain genes that are associated with pathogenesis [190]. Within this context, the hospital environment may pave the way by promoting a shift in the commensal bacterial population towards strains with enhanced virulence [170]. Patients will become colonized with these strains via transmission by healthcare workers or other patients within days after admission [40]. This newly acquired endogenous microflora might then form the origin for later infection by altered gene expression through interactions with other pathogens. Invasive strains might be selected at the time of foreign body implantation from a large population of adapted strains, which are thought to be the prototype avirulent *S. epidermidis* subpopulation due to their biofilm-forming capacity. Some of the adapted strains may become invasive under favourable conditions and can cause infections like VAP, while others remain non-invasive under similar conditions [191, 192]. This indicates that hospitalization

represents a selection step that preferentially allows expansion of *icaADBC*-, *mecA*-, and IS*256*-positive *S. epidermidis* strains, a hypothesis that is supported by the finding that commensal *S. epidermidis* strains isolated from patients hospitalized on a surgical intensive care unit displayed a similar high prevalence of *icaADBC*, *mecA*, and IS*256* [170].

5.1.5. Construction of growth patterns

The growth of bacteria in culture is considered as the most straightforward *in vitro* model of infection. Because many bacteria replicate by binary fission, the plotting of culture growth versus incubation time allows seeing the effect of changing conditions on generation time and reveals several stages of growth [221]. However, a lot of interstrain variation was detected within the VAP group and in a lesser extent between the strains of the non-VAP group. Likely, PSMs, PIA and other biofilm factors all have original roles in establishing growth and allowing survival in microbial agglomerations on the skin, thereby resulting in the same phenotypic differences as seen with biofilm formation capacity [19]. VAP-associated strains will probably possess more elaborate strategies for foreign-body colonization and thus more virulence associated genes like *icaADBC*, *aap*, *atlE*, *bhp*, *fbe*, *mecA*, and IS256 that will influence the overall growth [170].

Part 2 – Cultivation of a multispecies biofilm

5.2.1. *Candida albicans*

Candida albicans is a commensal fungus that is a major pathogen of humans at the same time. *C. albicans* can form a bilayered structure comprising a mixture of yeasts, germ tubes and young hyphae [193]. In the host environment, *C. albicans* is often found to hold bacterial species in polymicrobial biofilms where extensive interspecies interactions are likely to take place that may impact the *C. albicans* transition between virulent and avirulent states [194, 195]. For example, airway colonization with *C. albicans* can impair the macrophage immune response and thus generate favorable conditions that increase susceptibility to pneumonia caused by *S. epidermidis*. Direct physical interactions between *Staphylococci* and fungi range from bacterial cell contact and aggregation with fungal hyphae or yeast cells to organized bacterial biofilms on the surface of fungal hyphae. *Staphylococcal* cells have shown to bind firmly to yeast and hyphal forms of the fungus [58, 164], which may act as a scaffold. Here, sugars of the cell wall protein Als3, expressed on the hyphae of the *C. albicans* cell, are involved in the interaction [55, 196, 197]. *Staphylococcal* factors such as muramyl dipeptides (MDP), subunits of the bacterial cell wall [198], can influence fungal growth or physiology, and, conversely, fungal factors have been shown to control bacterial behaviour and survival. Moreover, fungal cell surface proteins can modulate the action of antimicrobials, whilst bacteria can affect the activity of antifungals in mixed biofilms [199].

5.2.2. *Klebsiella pneumoniae*

Klebsiella pneumoniae is one of the most frequent causes of catheter-associated urinary tract infections. The ability of the bacterium to form biofilms on medical devices plays a major role in pathogenesis [200]. Unfortunately, a lot remains unknown about the biofilm-forming mechanism of *K. pneumoniae* [200-202]. Results indicate that *K. pneumoniae* and *S. epidermidis* cannot stably coexist in laboratory-grown biofilms due to differing rates of bacterial attachment and detachment of the two species. *K. pneumoniae* has shown to have a much higher growth rate than *S. epidermidis*, thereby mainly overgrowing and colonising the surface by means of type 1 and/or type 3 fimbrial adhesins, without giving *S. epidermidis* a chance. The type 3 fimbriae mediate binding to target tissue using the MrkD adhesion protein [203, 204]. Avoidance of initial colonization of competing strains by the rapid occupancy of all available adhesion sites by a physical bacterial barrier, is referred to as ‘surface

blanketing' [66]. This is mostly accompanied by rapid surface migration, which was not observed but might be visualized with better suitable techniques in future research. Although this simple and intuitive strategy is often mentioned as a possible competition mechanism, the interaction between *K. pneumoniae* and S. *epidermidis* cannot really be called antagonistic.

5.2.3. *Serratia marcescens*

Serratia marcescens is emerging as an important opportunistic pathogen, particularly for immunocompromised patients and ocular infections [205, 206]. The bacterium is known to form filamentous biofilms with cell chains, filaments and cell clusters [207, 208]. Despite the fact that *S. marcescens* was frequently isolated from the endotracheal tubes of clinical patients harbouring *S. epidermidis*, up till now, very few studies have explored the possibility of a mixed community between *S. epidermidis* and *S. marcescens*. The results obtained suggest that there might be synergistic interactions between the two species within the multispecies consortium, when *S. marcescens* might act as a primary colonizer due to its serrawettin-mediated swarming motility [209], facilitating and enhancing subsequent colonization and biofilm formation by *S. epidermidis*. However, colonization might also be the other way around, as it was difficult to observe the colonization rate with the performed methods. Additional testing is needed here. It could be, however, also be true that the observed morphological differences between the multispecies biofilm of *S. epidermidis* with *S. marcescens* and the single-species biofilm of both organisms are caused by stress induction due to the presence of *S. epidermidis*. *S. marcescens* can form two kinds of biofilms; a filamentous type and a microcolony-type, as a response to stress [166]. The bacterium will start to form microcolonies and show enhanced biofilm formation in response to a threshold concentration of secreted extracellular compounds by *S. epidermidis* or in response to nutrient-limiting conditions due to the presence of *S. epidermidis*. This will protect the sensitive population against the toxic substances and starvation, thus enabling, controversially, long-term coexistence with *S. epidermidis*. [166, 209]. The interaction between *S. marcescens* and *S. epidermidis* seems to be synergistic at first glance, but turns out to be based on antagonistic interactions that have an unforeseen positive effect.

5.2.4. *Pseudomonas aeruginosa*

P. aeruginosa is one of the most frequent causes of chronic nosocomial infections. It is believed that early infection with other pathogens like *S. epidermidis* primes the airway for later infection by *P. aeruginosa* [210], as this might create the opportunity for the bacterium to undergo additional "mucoid" phenotypical changes, which allows a more effective evasion of the host response by overproduction of alginate [211-213]. The presence of *S. epidermidis* can trigger the production of many virulence factors by *P. aeruginosa* [64]. Extracellular products of *P. aeruginosa* can inhibit *staphylococcal* growth and may disrupt established biofilms by *S. epidermidis* [165]. *P. aeruginosa* produces natural products such as antimicrobial quinolones, rhamnolipids, and *LasA* proteases (staphylolysins that target the pentaglycine bride of the staphylococcal cell wall) that make it impossible for *S. epidermidis* to activate its quorum-sensing systems [165]. This phenomenon is called 'quorum quenching' [198]. *P. aeruginosa* also excretes an arsenal of small respiratory inhibitors, like pyocyanin, hydrogen cyanide, or N-oxides, which will suppress the aerobic metabolism of *S. epidermidis*. The blockage of the electron transport pathway drives *S. epidermidis* to fermentative growth, which is accompanied by decreased ATP yield and finally results in the formation of smaller or no colonies at all [197, 214]. All these compounds are, however, costly to synthesize and might result in decreased biofilm formation by *P. aeruginosa* [215], which was proven in the dynamic biofilm model. The results suggest that *P. aeruginosa* can negatively counteract colonization by *S. epidermidis,* using a variety of antimicrobials and ensuring persistence and dominance in the host microhabitat [216, 217].

S. epidermidis has developed interesting strategies to conquer the hospital environment as a new ecological niche and to transform into a notorious pathogen. The ability to form persistent biofilms, resistance against many of today's most commonly administered antibiotics and the presence of multiple copies of mobile DNA elements such as ACME and SCC*mec* in the genome, have proven to be all characteristics that substantially differ some strains of *S. epidermidis* that are highly adapted to the hospital environment from commensal *S. epidermidis* strains in the community [218].
MLST typing of clinical isolates revealed that the population structure of *S. epidermidis* worldwide is composed of a major and highly diverse genetic lineage, i.e., clonal complex 2 (CC2) [168]. The majority of both commensal and nosocomial strains are known to belong to this complex [72]. Strains belonging to primary founder ST2, the by far most frequently found clonal type of hospital-associated invasive *S. epidermidis*, are all *ica*- and *IS*256-positive [171]. This way, ST2 strains are well adapted to the host and have the capacity to adapt to environments with distinct characteristics, such as the hospital environment with extensive use of antibiotics. In this context, the hospital environment may pave the way by promoting a shift in the commensal bacterial population towards strains with enhanced virulence [170].

Within the hospital, there are a large number of immunocompromised patients present as potential susceptible hosts, inserted medical devices represent a suitable habitat for colonization, and bacteria are subjected to high selective forces owing to the extensive use of antibiotics and disinfectants [218]. The skin flora of patients entering the hospital contains predominantly antibiotic-susceptible CoNS. However, once the patients are hospitalized, their skin flora becomes altered by the acquisition of antibiotic-resistant isolates from hospital personnel or by the selection of antibiotic-resistant bacteria from the patients' endogenous flora on account of systemic antibiotic administration or vaccinations. In either case, colonized skin serves as a huge reservoir for dissemination of multi-drug resistant CoNS throughout the hospital. A prosthetic device might become infected by the inoculation of small numbers of *Staphylococci*, from the skin of the patient into the surgical wound, during implantation of the medical device. After inoculation, the bacteria adhere to the indwelling medical device and proliferate in association with the foreign body [219]. A community of microorganisms colonizes these devices and become the source of pathogens involved in the infection. The formed biofilm will represent the origin for later infection.

S. epidermidis represents the most common source of infections on indwelling medical devices in health-care [68]. This is likely due to the fact that *S. epidermidis* is a permanent colonizer of the human skin, resulting in a high probability of device contamination during insertion [69]. Within the highly selective non-homeostatic environment, *S. epidermidis* undergoes transition from a commensal pathogen to a more aggressive opportunistic pathogen through the acquisition of additional virulence factors by interspecies and interstrain horizontal gene transfer [174] and through altered gene expression. It might be that invasive strains are selected at the time of foreign body implantation from a population of adapted strains, which are thought to be the prototype avirulent *S. epidermidis* subpopulation due to their biofilm-forming capacity. Some of the adapted strains may become invasive under favourable conditions and can cause infections like VAP, while others remain non-invasive under similar conditions [191], depending on the type of interaction.

Due to the lack of a clear difference between VAP and non-VAP associated strains and a great phenotypic variability regarding specific properties such as colony morphology, growth

rate, haemolysis, biofilm formation and antibiotic susceptibility, it is tempting to presume that other pathogens might be the actual cause of VAP-infection and that *S. epidermidis* 'just' serves as a primary or secondary colonizing species, which attaches to small irregularities along endotracheal tube surfaces [220] to form a basis for other secondary pathogenic colonizers. It became obvious that a number of known VAP-causative organisms, such as *Candida albicans*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa*, are frequently found in the presence of *S. epidermidis*.
It is known that *P. aeruginosa* and *S. epidermidis* commonly coexist in infected patients due to colonization of the surfaces of indwelling medical devices, but not necessarily within the same biofilm community. *S. epidermidis* presumably plays the role of a primary colonizer on the endotracheal tube, thereby priming the airway for subsequent infection of the respiratory tract by *P. aeruginosa*. This might create the opportunity for the bacterium to undergo additional "mucoid" phenotypical changes, which allows a more effective evasion of the host response by overproduction of alginate. In most cases, the cell density is always low when a bacterium starts to invade niches occupied by other species. Thus, the organism is unable to activate its quorum-sensing systems, and use quorum-sensing-regulated products to compete with other species. In the early stage of VAP, *P. aeruginosa* inhabits lung tissues, along with other species, including *Staphylococci*, and *P. aeruginosa* eventually becomes the only or the prominent pathogen within these patients as their disease progresses due to inhibitory competition. However, despite the fact that *S. epidermidis* and *P. aeruginosa* will maintain an antagonistic interaction with each other and *P. aeruginosa* acts like the well-renowned keystone pathogen in the respiratory microbiota, *S. epidermidis* will likely be indispensible for establishing the origin of VAP-infection.
In the case of *S. marcescens*, there is a completely different story to tell. Naked medical device surfaces exposed to bacteria will be colonized initially by only *S. epidermidis*. This might provide conditions that allow additional species to form a mixed biofilm. Subsequently, some attached pioneering bacteria will be recognized and serve as anchor for secondary colonizers such as *S. marcescens*. The presence of *S. epidermidis*, however, will induce a stress response by *S. marcescens*, leading to an increased mutation rate within the genome of the bacteria. This induces distinct morphological changes in the biofilm growth pattern of *S. marcescens*, resulting in the formation of microcolonies that will protect *S. marcescens* against possible secreted extracellular compounds from *S. epidermidis* or against nutrient-limiting conditions, and enhance the adaptive potential of the microbial population. This enables, controversially, long-term coexistence with *S. epidermidis*. *S. marcescens* biofilm formation likely increases the opportunities of this keystone pathogen to infect humans, but it needs 'help' from *S. epidermidis* in order to achieve that.

Throughout the research it became clear that the presence of *S. epidermidis* in patients is indeed not linked to prevalence of VAP or non-VAP. It is however, the dynamic balance of both synergistic and antagonistic interactions with neighbouring bacteria that will play an essential role in determining whether certain virulence factors cause damage or not. This shows, in concordance with other reports, that more than one pathogen is needed to cause respiratory infections, demonstrating a polymicrobial aspect, and confirming the fact that the keystone pathogen hypothesis is not only valid for diseases such as periodontitis and cystic fibrosis, but also for respiratory infections such as VAP.

Biofilm formation is often considered the underlying reason why treatment with an antimicrobial agent fails as an estimated 65–80% of all infections is thought to be biofilm-related. This presents a serious challenge in health care. In order to increase our knowledge concerning biofilm biology, biofilm model systems for the study of the often complex communities under controlled conditions are indispensable. In the future, there should be a stronger focus on the development of appropriate animal models. Such biofilm model systems are essential to gain a better understanding of the mechanisms involved in biofilm formation and resistance. Finally, perhaps the greatest challenge for the future will be the translation of findings from animal experiments to human medicine for the development of new diagnostic tools and treatment modalities targeting keystone pathogens in complex dysbiotic diseases.

Defining the role of a pathogen in pneumonia depends on many criteria such as its loads, the type of sample, the tools used for its detection, and the type of the pathogen. Moreover, clinical data are mostly combined with those criteria to interpret the real role of the microorganism. However, despite major advances in the research field, it still remains utterly difficult to draw conclusions from *in-vitro* biofilm research on *in-vivo* associated infections [221].

Since biofilm formation has proven to be a critical and fundamental aspect of VAP-infection, emerging new therapies should focus on antibiofilm agents that inhibit the microbial attachment process, thereby preventing subsequent colonization by pathogens. The identification of certain epidemic clonal lineages with distinct genetic markers such as antibiotic resistance, biofilm-mediating genes, and IS elements can help to facilitate decision-making about appropriate treatment. Also, the growing evidence for CoNS being reservoirs for the evolution and spread of antibiotic resistance genes in MRSA might have practical consequences for future infection control and hygiene measures in hospitals and ICU's.
Having a significant impact on mortality, morbidity, and both social and economic costs, prevention and management of medical device-related infections associated with *S. epidermidis* should remain a priority in future research.

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Appendix I: Tables

Table 30: Known and suspected microbiologic causes of ventilator-associated pneumonia (VAP). Ranging from bacteria to fungi and viruses. *Staphylococcus epidermidis*, the pathogen of interest for this project proposal belongs to the coagulase-negative Staphylococci.

Gram-positive cocci	Anaerobic bacteria
Staphylococcus aureus	Bacilli
Streptococcus pneumonia	*Bacteroides* species
Other *Streptococci*	*Fusobacterium* species
Coagulase-negative *Staphylococci*	*Prevotella* species
Enterococci	*Actinomyces* species
	Cocci
Gram-positive rods	*Veillonella* species
Corynebacterium species	Peptostreptococci
Listeria monocytogenes	
Nocardia species	Atypical bacteria
	Legionella species
Aerobic Gram-negative bacilli	*Legionella*-like amoebal pathogens
Haemophilus influenzae	*Mycoplasma pneumonia*
Lactose fermenting Gram-negative bacilli	*Chlamydia pneumonia*
Escherichia coli	
Klebsiella species	Fungi
Enterobacter species	*Candida* species and other yeasts
Proteus species	*Aspergillus* species and other molds
Serratia species	*Pneumocystis carinii*
Citrobacter species	
Hafnia alvei	Viruses
Non-lactose fermenting Gram-negative bacilli	Influenza and other respiratory viruses
Pseudomonas aeruginosa	Herpes simplex virus
Acinetobacter calcoaceticus and *baumannii*	Cytomegalovirus
Stenotrophomonas maltophilia	
Burkholderia cepacia	Miscellaneous causes
	Mycobacterium tuberculosis
Gram-negative cocci	*Strongyloides stercoralis*
Neisseria species	Others
Moraxella species	

Table 31: Different media used during the experiments for the cultivation of clinical isolates of *S. epidermidis*.

Medium		Formulation	Reference
Columbia Blood Agar	42.5g in 1L distilled water[9], 50mL defibrinated horse blood[10]	Pancreatic digest of casein 11.5g; peptic digest of animal tissue 5.0g; yeast extract 3.5g; pancreatic digest of heart muscle 3.0g; sodium chloride 5.0g; corn starch 1.0g, agar 17.0g.	BD (Becton, Dickinson and Company) BBL™, 2017
Mueller-Hinton Agar (MHA)	38g in 1L distilled water	Beef extract 2.0g; acid hydrolysate of casein 17.5g; starch 1.5g; agar 17.0g.	BD (Becton, Dickinson and Company) BBL™, 2017
Mueller-Hinton Broth (MHB)	22g in 1L distilled water	Beef extract 3.0g; acid hydrolysate of casein 17.5g; starch 1.5g.	BD (Becton, Dickinson and Company) BBL™, 2017
Tryptone Soy Broth (TSB)	30g in 1L distilled water	Sodium chloride 5.0g; enzymatic digest of soya bean 3.0g; glucose 2.5g; pancreatic digest of casein 17g; di-potassium hydrogen phosphate 2.5g.	BD (Becton, Dickinson and Company) BBL™, 2016

[9]Destilled water is pure water from which all anorganic salts and other organic substances have been removed through distillation. An alternative is MiliQ water, a trademark created by Milipore Corporation to describe 'ultrapure' water.

[10]Defibrinated horse blood is an essential nutritional supplement for microbiological culture media. During the blood harvesting procedure, a gentle rocking process will remove all the fibrin strands from the whole blood whilst re-oxygenating the red cells. Defibrinated horse blood is ideal for blood agar plate preparations.

Table 32: ICU-patients from who different clinical isolates of *S. epidermidis* where collected with corresponding age, intubation time and concurrence of VAP or non-VAP during hospital stay.

Patient	Strain	Age (years)	Intubation Time (hours)	VAP
1	SE 0094	74	24	no
2	SE 0095	82	6	no
3	SE 0096	74	6	no
4	SE 0100	42	6	yes
5	SE 0105	58	5	yes
6	SE 0107	72	6	yes
7	SE 0108	56	18	yes
8	SE 0109	70	4	no
9	SE 0112	68	22	yes
10	SE0113	54	6	no
11	SE 0117	74	19	no
12	SE 0118	74	8	yes
13	SE 0092	41	4	no
14	SE3-410	65	8	no
15	SE2-202	60	12	no
16	SE3-403	58	7	yes
17	SE3-405	43	11	no
18	SE3-419	60	18	no
19	SE4-605	63	2	no
20	SE4-609	39	19	yes
21	SE4-613	59	16	yes
22	SE5-803	57	11	no
23	SE5-808	60	4	no
24	SE5-811	60	37	no
25	SE 0119	52	64	yes
26	SE 0127	82	5	no
27	SE 0128	74	20	yes
28	SE 0129	33	15	no
29	SE3-408	69	2	no
30	MNP 001	75	32	no
31	MNP 008	72	11	yes
32	SE 0011	79	35	yes
33	SE 0017	71	16	no
34	SE 0020	55	34	yes
35	SE 0026	54	7	yes
36	SE 0030	63	3	yes
37	SE 0035	63	7	yes
38	SE 0040	76	7	no
39	SE 0041	18	6	yes
40	SE 0043	81	17	no
41	SE 0045	61	19	yes
42	SE 0061	12	15	no
43	SE 0068	47	8	yes
44	SE 0069	59	19	no
45	SE 0070	2	10	yes
46	SE 0078	49	23	yes
47	SE 0085	67	4	yes

Table 33: Reference sequences and names of 7 housekeeping genes used for MLST characterization of *S. epidermidis* isolates.

Housekeeping gene	Sequence
Carbamate kinase **(ArcC)**	TTCTACACTAAAGAAGAAGCAAATCGTATTCAACAGGAAAAAGGTTATCAATTTGTAGAAGA TGCTGGTCGAGGTTACCGTCGCGTTGTACCATCACCACAACCAATATCTATTATCGAACTGGA AAGTATTAAAACTCTAGTAGAAAATGACACACTCGTCATCGCTGCAGGTGGAGGTGGTATACC AGTCATTCGCGAACAGCATGATAGCTTTAAAGGTATAGATGCCGTCATCGATAAAGACAAAA CAAGTGCATTATTAGGTGCTGATATTCACTGTGATCAACTCATTATTTTAACAGCGATTGATTA TGTTTATATCAACTATCATACTGACCAACAACAAGCACTTAAAACAACAAATATAGATACGCT TAAAACATATATTGAAGAAGAACAATTTGCCAAAGGCAGCATGCTACCTAAAATCGAATCTG CCATCTCCTTTATTGAAAATAATCCT
Shikimate dehydrogenase **(AroE)**	GCGGTAAATACAGTTTTAGTTAAAGATGGTAAGTGGATTGGTTATAATACTGATGGAATTGGT TATGTTAATGGTTTAAAACAAATATATGAAGGTATAGAAGACGCTTATATATTAATTTTAGGT GCAGGTGGAGCAAGTAAAGGTATAGCAAATGAATTATATAAAATCGTTCGTCCGACTTTAACA GTTGCAAATAGAACGATGTCTCGTTTTAATAATTGGTCGTTAAATATTAACAAAATAAATTTA AGCCATGCAGAAAGCCATTTAGATGAATTTGATATTATAATAAACACTACACCTGCTGGTATG AACGGCAATACAGATTCTGTAATTTCTTTAAATCGTTTAGCTTCACATACTTTAGTAAGTGATA TTGTTTATAATCCATATAAAACACCAATACTAATAGAAGCT
ABC transporter **(Gtr)**	CCAATTCAGGATCAAGTGCAGATGTTGGCTCATCAAATAACATCACTTTAGGATTCATGGCTA ATGCACGTGCAATTGCGACACGTTGTTGTTGCCCACCTGATAAAGCATGTGGCCGTTGATCTTT AACATGTACCAATCCAACCTTAGCCAATAAATTCATTGCTTCTTCATTAGCCGTTGCTTTATTC ATCTTTTTAACTGTTATAAGACCTTCCATAACGTTTTCTAATGCAGATTTATGTGGAAATAAAT TATAATTTTGAAAAACCATTCCTGATTGTTGTCTTACTTTAATTTGAGATTTCTTATCTTTAGCA TTATATGTCATGCCATTGACATACACTGTACCTTCAGTAGGTATCTCTAAAGCATTAATCATTC TAAGTAAAGTCGTTTTACCTGAACCTGAACGTCCAATTAGTGTCACAACTTCAC
DNA mismatch repair protein **(MutS)**	TTTGAAATGAGTTACTTTGAGTTCTCCTGTAGAAACATCACAATAGCATAATCCAAATTCTTCA TTTTCGATAAAACTTAAAATATAATTATTTTTCTTTTCATCCATACCATTTTGATCCATAACAGT TCCTGGTGTGATGATTCTTACAACTTCTCTTCTAACCATTCCTTTTGTTTGCTTTGGATCTTCCA TTTGTTCACATATAGCGACCTTATACCCCTTATTAATCAATGTTTCAATGTAATTATCAGCAGA ATGATATGGTACGCCACACATCGGAATAGGATTTTCTTTTTTAGCATCTCTTTTCGTCAATGTT ATTTCAAGTACTCTTGATGCTTCTTTAGCATCATCAAAGAACATTTCATAGAAATCTCCGAGTC TAAAAAATAGCAAACAATCATCATAT
Pyrimidine operon regulatory protein **(PyrR)**	CGTACAATCACACGAATTGCTCATGAAATTCTAGAATATAACAAGGGAACTAAAGATTTAGTT CTATTAGGCATTAAAACAAGAGGTGCTTTTTTAGCACATCGTATACAAGATAAAATAAATTCA ATTGAACAACAATTAGTACCAACAGGTACTATCGATATCACGCATTTTCGAGATGACGTTGAT AAGGTAGTGCAACAAGCTGATCAATACGCTTTTGATATTAATGTAGATATTAATAACAAAGTG GTTGTTATCATTGACGATGTTTTGTATACCGGACGTACAGTAAGAGCCTCATTAGATGCGATTT TATTACATACAAGACCTATTAAAATAGGGCTTGCAGCACTTGTGGATCGTGGTCATCGTGAAC TCCCTATACGCGCAGATTTTGTAGGAAAAAATATACCTACAGCACGAGA
Triosephosphate isomerase **(TpiA)**	AATTGGAGCACAAAACGCTTACTTTGAAGAAAGCGGTGCTTATACTGGAGAAACTTCACCAGT AGCATTATCTGAATTAGGTGTTAAATATGTAGTGATTGGTCACTCAGAGCGTCGTGACTATTTC CACGAAACTGACGAAGAAGTAAACAAAAAAGCGCATGCTATCTTCAATCACGGTATGACACC TATTATTTGTGTAGGTGAATCTGATGAAGAACGTGAAGCTGGTAAAGCAAATAAAATCGTAGG TAATCAAGTGAAAAAAGCTGTCGAAGGTTTATCAGATGATCAACTTAAAGAAGTTGTTATTGC ATATGAACCAATTTGGGCTATCGGTACTGGTAAGTCATCTACATCTGAAGATGCAAATGAAAT GTGTGCTCACGTACGTCAAACATTAGCTGACTTATCTAGTCAAGAG
Acetyl coenzyme A acetyltransferase **(YqiL)**	TATTTAAGGATATACCTGCCTATGAACTAGGTGCAACAGTTATTCGTCAAATTTTAGAACATA GTCAAATAGATCCTAATGAAATCAATGAAGTTATTCTAGGAAACGTATTACAGGCAGGTCAAG GACAAAATCCTGCTCGTATTGCTGCGATTCATGGTGGTGTGCCAGAAGCGGTACCTTCTTTTAC TGTAAATAAAGTTTGCGGTTCTGGATTAAAAGCGATTCAACTTGCCTATCAATCTATTGTAGCG GGAGATAATGAGATTGTTATCGCTGGAGGCATGGAAAGTATGTCTCAATCTCCAATGCTTCTT AAAAATAGTCGTTTCGGTTTTAAAATGGGAAATCAAACTTTAGAAGATAGTATGATAGCTGAT GGTTTAACTGATAAGTTTAATGATTACCATATGGGT

Appendix II: Figures

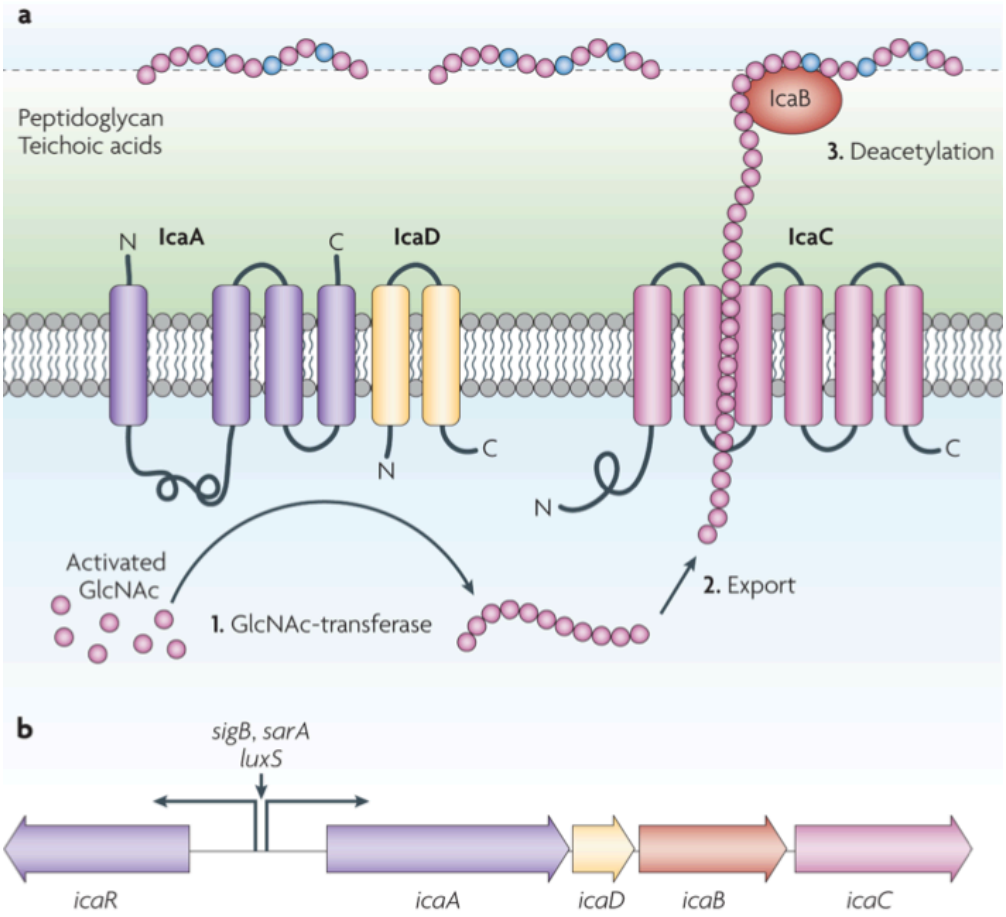

Figure 26: The exopolysaccharide PNAG/PIA. Pia is synthesized by the membrane located N-acetylglucosaminetransferase IcaA that needs the accessory IcaD membrane protein for activity. The growing PNAG/PIA chain is likely exported by the IcaC membrane protein. After export, the surface-located IcaB deacetylase removes some of the N-acetyl groups, giving the polymer a cationic character that is essential for surface attachment. The Ica proteins are encoded in the *ica* gene locus, which contains the *icaADBC* operon and the *icaR* gene encoding a regulatory protein. Expression of the *icaADBC* operon can be regulated directly or via global regulatory proteins[69].

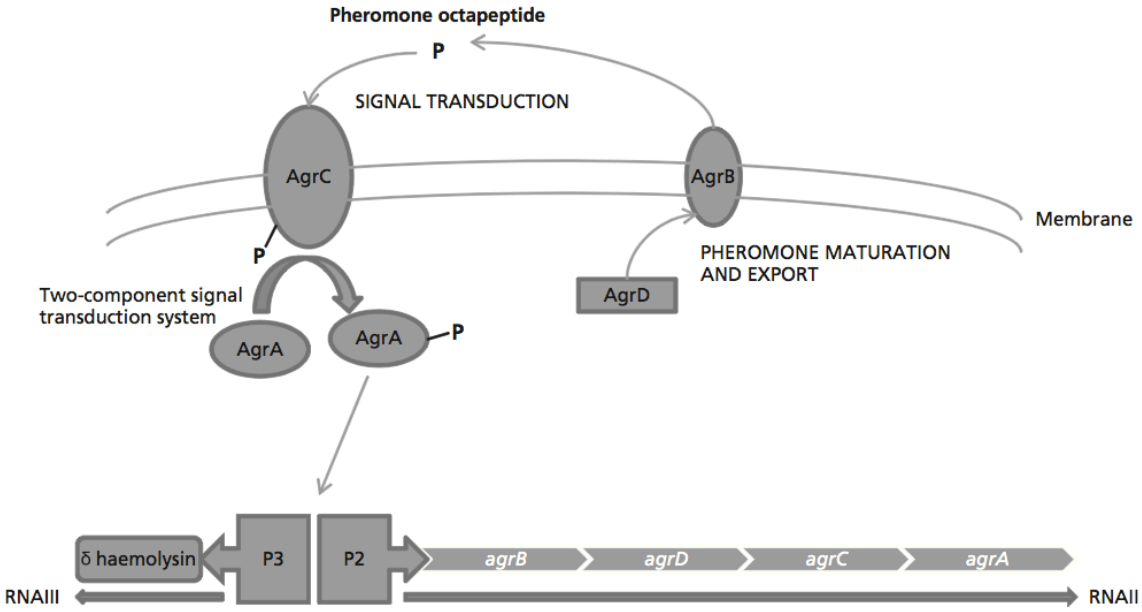

Figure 27: The *Staphylococcal Agr* quorum-sensing system. AgrD, the autoinducing pro-peptide, encoded by the *agrD* gene is posttranslationally modified by the endopeptidase, AgrB. The modified pheromone octapeptide binds to the transmembrane receptor, AgrC, activating the response regulator AgrA. This, in turn, induced transcription of RNAII and RNAIII via the P2 and P3 promotors [103, 222].

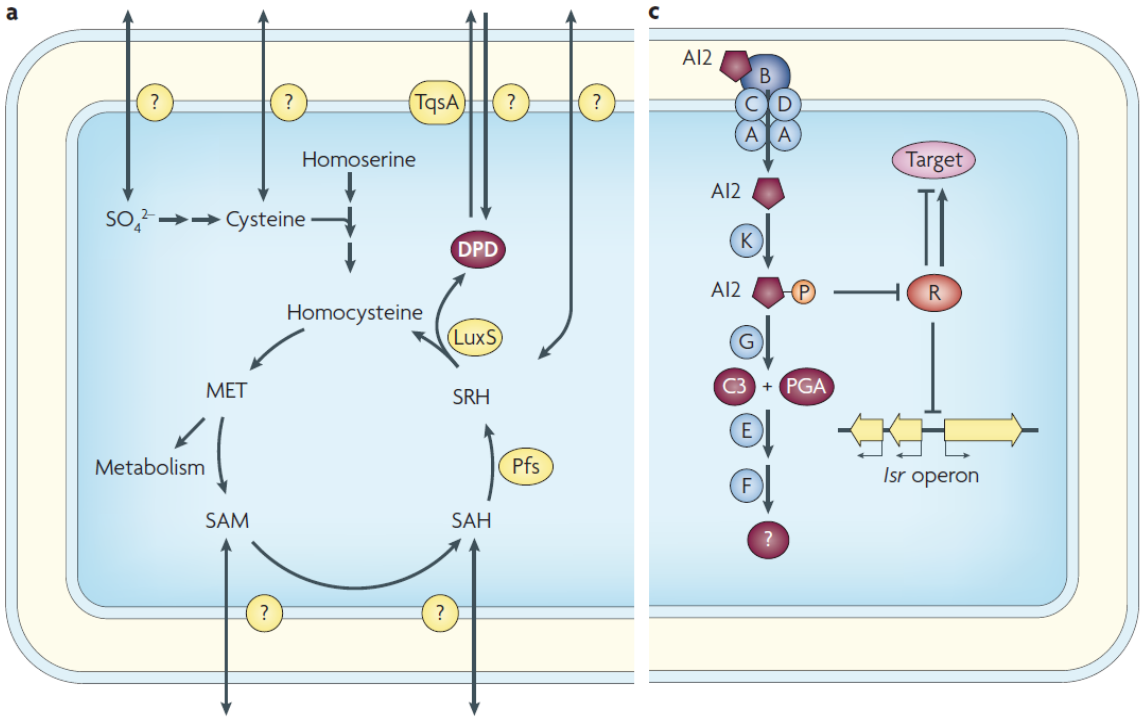

Figure 28: The *Staphylococcal LuxS* quorum-sensing system. AI-2 is produced from S-adenosylmethionine (SAM) in three enzymatic steps. SAM is an essential cofactor for processes such as DNA, RNA and protein synthesis. The use of SAM as a methyl donor in these and other metabolic processes produces the toxic intermediate S-adenosylhomocysteine (SAH), which is hydrolysed to S-ribosylhomocystein (SRH) and adenine by the nucleoside enzyme Pfs (5' methylthioadenosine/S-adenosylhomocystein nucleosidase). LuxS catalyses the cleavage of SRH to 4,5-dihydroxy 2,3-pentanedione (DPD) and homocysteine [223, 224]. In the presence of boron, DPD will form a furanosyl borate diester that has AI-2 activity. The subsequent accumulation of AI-2 may be damaging to the cellular DNA and, hence, AI-2 is extruded from the cell [107]. The cyclic derivative of DPD, (*2R,4S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) is found bound to a homologue of the periplasmic binding protein LsrB. LsrB is part of an ABC transporter (encoded by the *lsrACDBFGE* operon). This complex then docks with the Lsr ATP-binding-cassette importer, which causes AI-2 to be internalized. Direct phosphorylation and cleavage of AI-2 by LsrF, LrsG and LrsK follows, ultimately resulting in transcriptional regulation of the *lsr* operon [105].

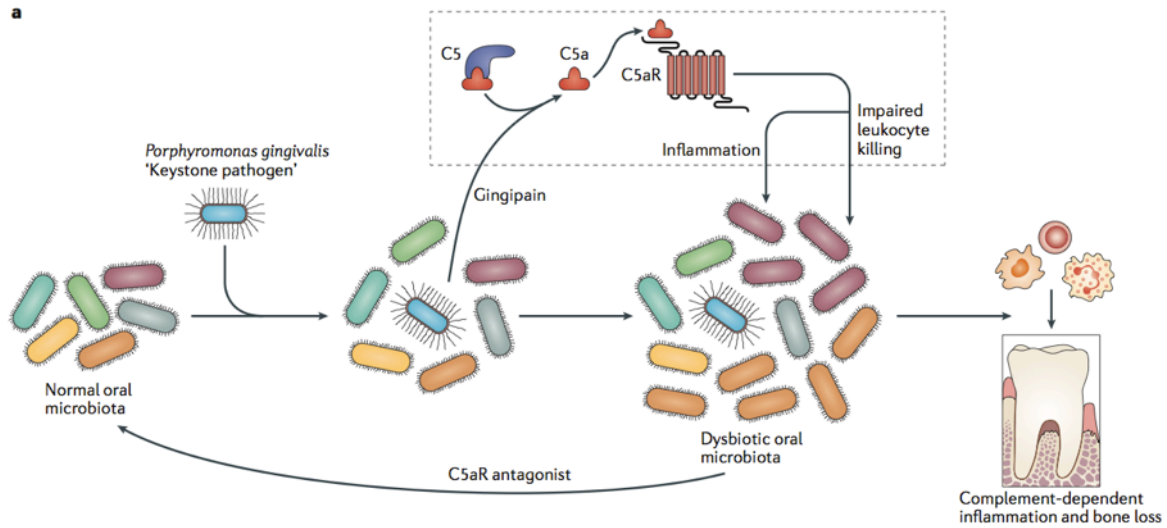

Figure 29: The keystone-pathogen hypothesis exemplified with *Porphyomonas gingivalis*, the cause of periodontitis. Despite its low-level colonization of the periodontium, *P. gingivalis* has evolved sophisticated strategies to evade or subvert components of the host immune system rather than acting directly as a pro-inflammatory bacterium. This way, the periodontal pathogen transforms the normally symbiotic microbiota into a dysbiotic state, which leads to a breakdown in the normal homeostatic relationship with the host. Accordingly, *P. gingivalis* impairs innate immunity in ways that alter the growth and development of the entire biofilm, triggering a destructive change in the normally homeostatic host-microbiota interplay in the periodontium. This required *P. gingivalis* gingipain, a C5 convertase-like enzyme that cleaves complement component C5, generating high levels of C5a locally. C5a-induced activation of C5a-receptor (C5aR) triggers inflammation but is also crucially involved in subversive crosstalk with Toll-like receptor 2, which impairs leukocyte killing. The ability of *P. gingivalis* to orchestrate inflammatory disease via community-wide effects while being a minor constituent of this community qualifies it as a keystone pathogen [121].

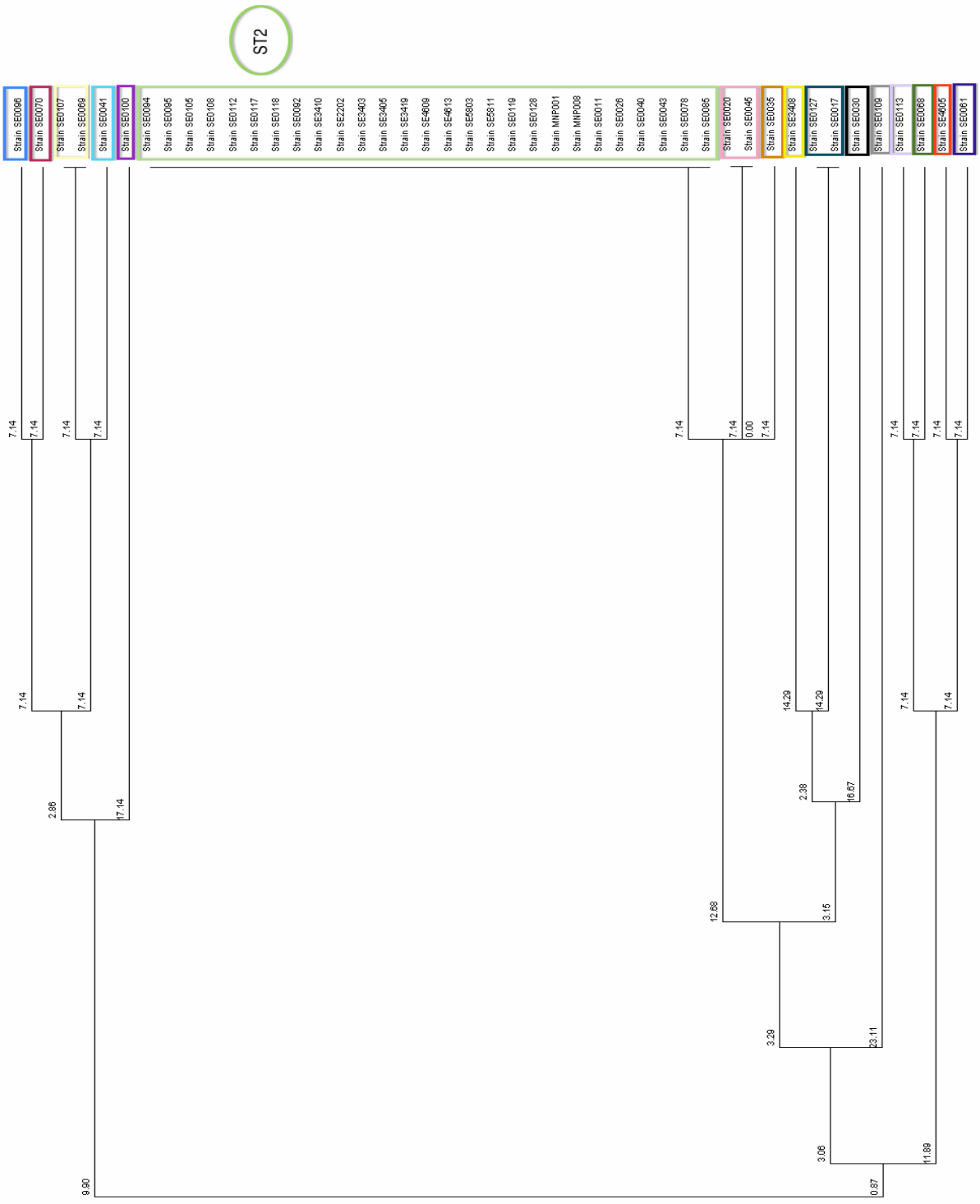

Figure 30: Dendrogram, or the unweighted pair-group method with arithmetic averages (UPGMA), representing the evolutionary distance between the different sequence types and strains of *S. epidermidis*. Each colour represents a different sequence type. The image is based on a matrix of pairwise differences in the allelic profiles of the isolates [136]. The level of confidence in the assignment of the primary founder of each group is tested by 're-sampling with replacement' for each ST type. For each re-sampling the predicted primary founder is computed, and a tally is kept of the number or times that each ST in the group is predicted to be the primary founder of the group. The bootstrap values shown for each ST are the percentage of times the ST was predicted to be the primary founder of the group in the bootstrap re-samplings. Bootstrapping provides the level of support for the predicted primary founder of the clonal complex and for subgroup founders. The bootstrapping value on each node represents the linkage distance between branches.

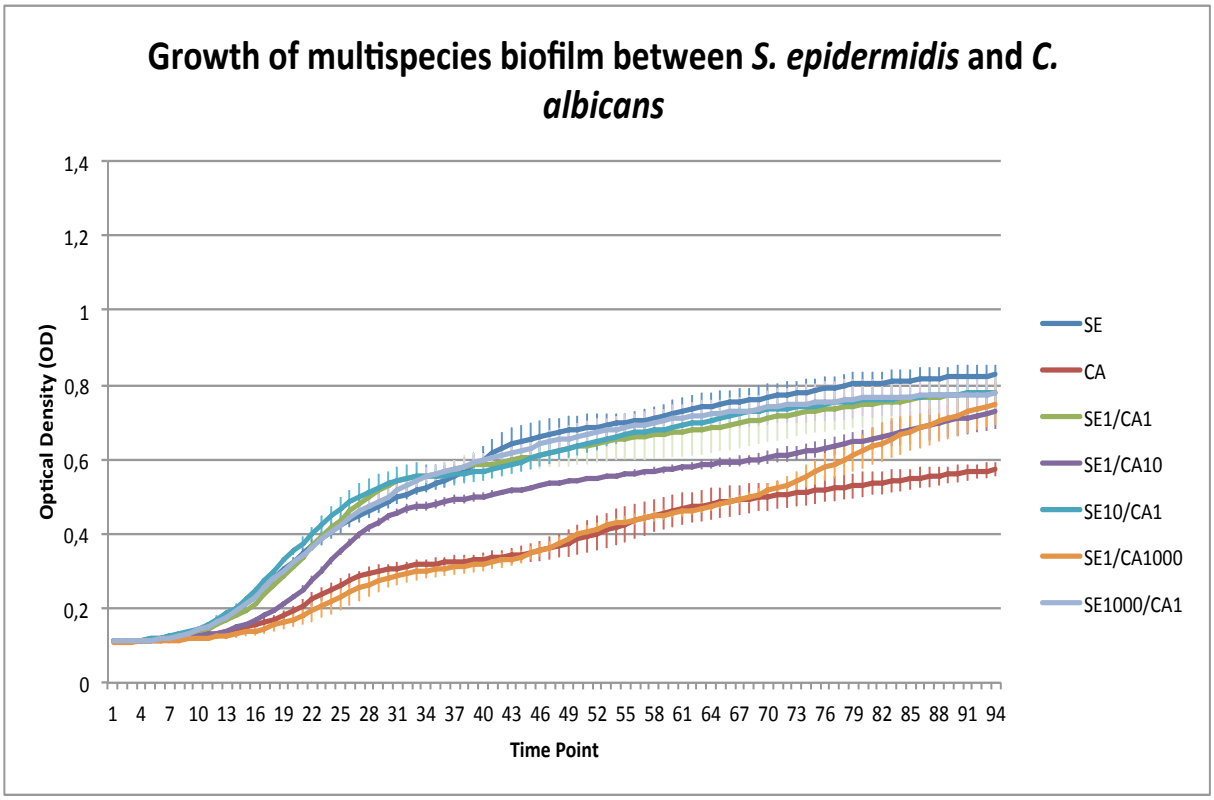

Figure 31: Growth of multispecies biofilm formed between *S. epidermidis* and *C. albicans*. Error bars of the corresponding strain are highlighted in the same colour as the growth curve of the corresponding concentration.

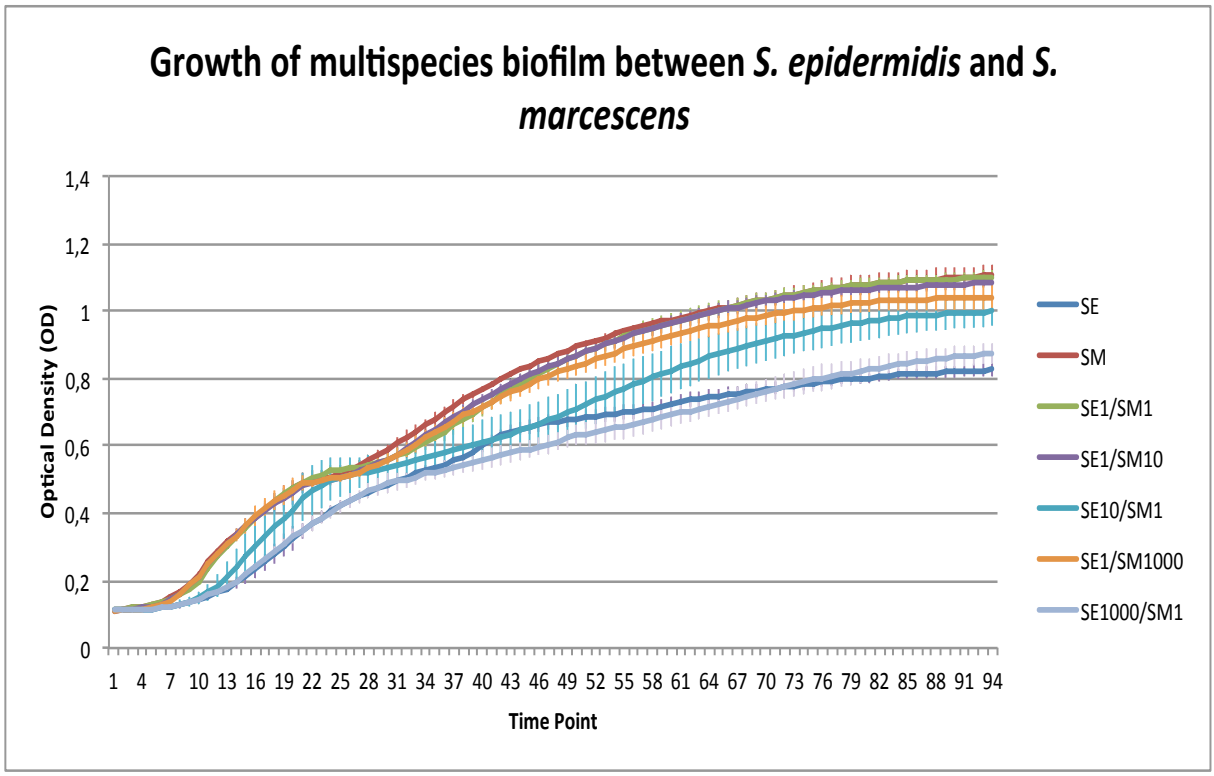

Figure 32: Growth of multispecies biofilm formed between *S. epidermidis* and *S. marcescens*. Error bars of the corresponding strain are highlighted in the same colour as the growth curve of the corresponding concentration.

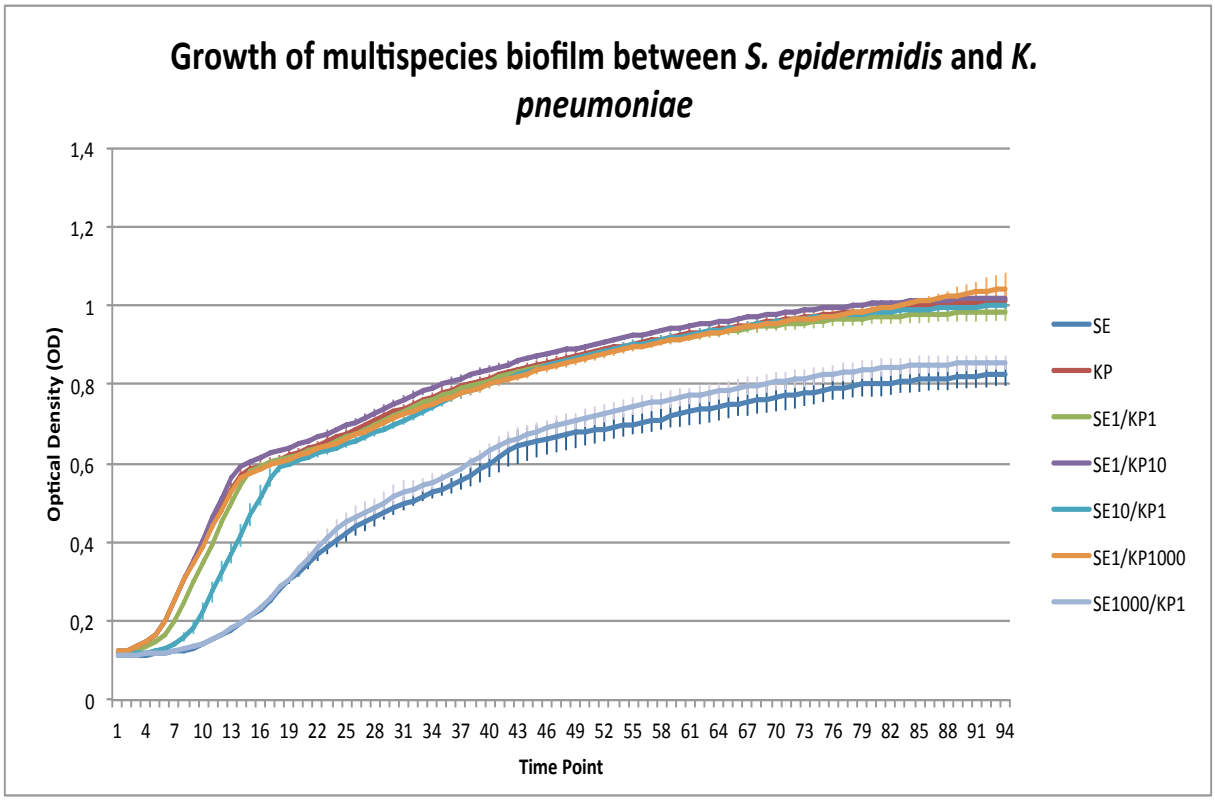

Figure 33: Growth of multispecies biofilm formed between *S. epidermidis* and *K. pneumoniae*. Error bars of the corresponding strain are highlighted in the same colour as the growth curve of the corresponding concentration.

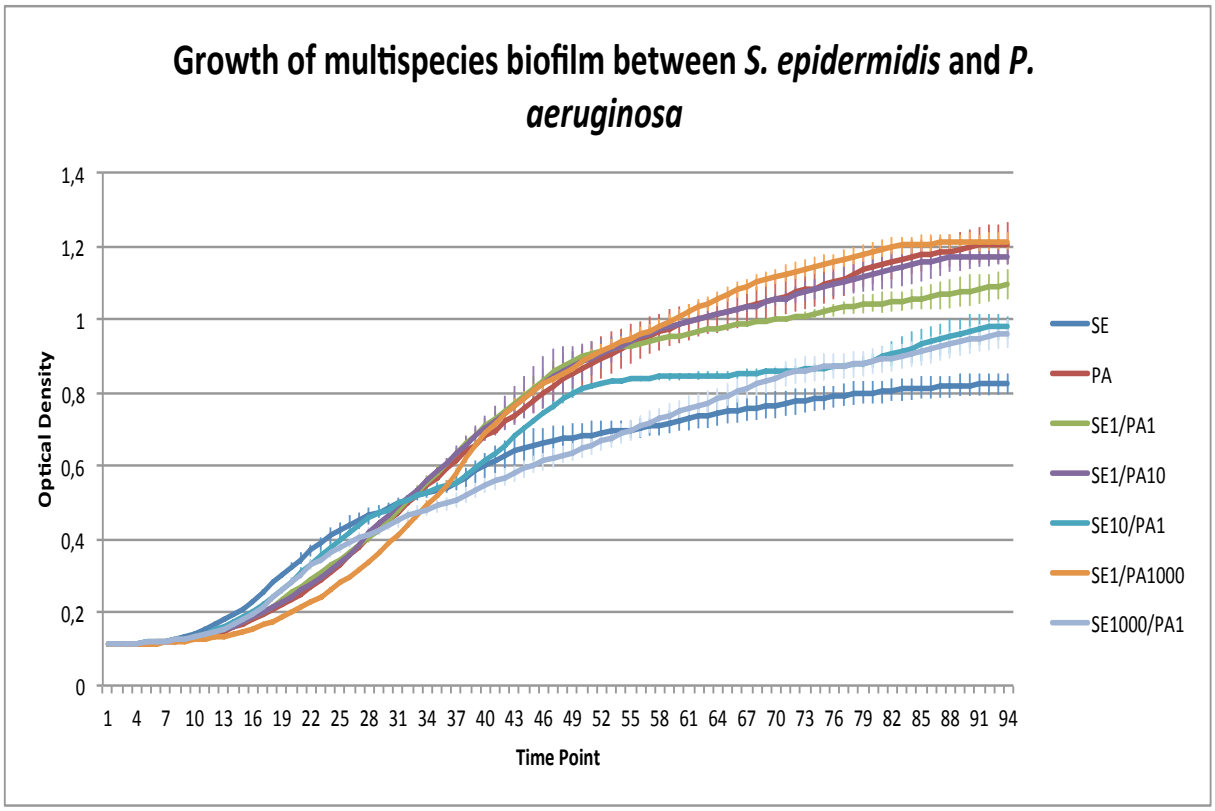

Figure 34: Growth of multispecies biofilm formed between *S. epidermidis* and *P. aeruginosa*. Error bars of the corresponding strain are highlighted in the same colour as the growth curve of the corresponding concentration.

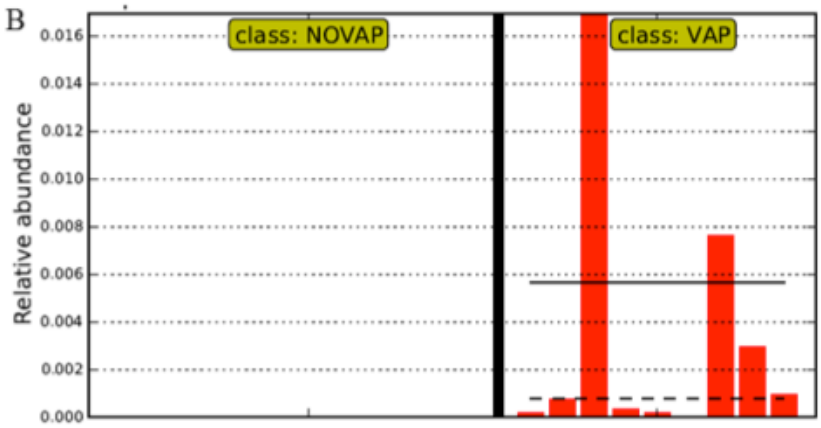

Figure 35: Relative abundance of *S. marcescens* in the VAP and non-VAP ET tubes which were positive for *S. epidermidis*. The full line represents the average and the intermittent line the median. Together with *K. pneumoniae*, *S. marcescens* was found to be the most important organism associated with *S. epidermidis* and VAP, while it was almost absent in non-VAP ET tubes. Results from *Hotterbeekx, A. et al.,* 2014 [33].

Appendix III: Glossary[11]

[11] Based on '*Henderson's dictionary of biology*' by *Lawrence, E. (2008)* [226] and '*Microbiology: an evolving science'* by *Slonczewski, J. L. & Foster, J. W. (2009)* [225].

Allele	An alternative form of a gene. A diploid organism carries two alleles for each gene locus, one on each homologous chromosome. The two alleles may be identical or different and it is the particular combination of alleles that determines the phenotype of the organism.
Allelic profile	Genotype or the different sequences at each locus that were assigned different allele numbers. The profile is defined by the alleles at seven loci of the housekeeping genes by eBURST.
Antagonism	The killing, injury or inhibition of one microorganism by a product of another. If two populations stay away from each other in the biofilm, or one species completely dominates morphology, this indicates that these populations are interacting competitively.
Autoinducer	Chemical signalling molecule or pheromone used by Gram-positive and Gram-negative bacteria to regulate density-dependent gene expression by a mechanism known as quorum sensing. Mostly species-specific, with exception for autoinducer 2 used in the *LuxS* system.
Biofilm	Highly structured matrix-enclosed community whose cells express genes in a pattern that differs profoundly from that of their planktonic counterparts. This film has a basic organization in which cells grow in matrix-enclosed microcolonies separated by a network of open water channels.
Bacteriocins	Proteinaceous secreted toxins, which may exert either specific or nonspecific effects on other bacteria within a consortium.
Breakpoint zone of inhibited growth	Corresponds with the lowest concentration of the particular antibiotic at which bacterial growth is still inhibited by lysis. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium.
Bronchoalveolar lavage	A safe technique and a practical method for obtaining cells and secretions from a large area of the lung that can be examined microscopically immediately after the procedure and are also suitable for culture by quantitative techniques. Bronchoscopic BAL specimens are generally obtained by advancing the bronchoscope distally into a medium-sized bronchus of the area of alveolar infiltrate identified on the chest X-ray, until the airway is occluded proximally. The lung segment is lavaged with 30-50 ml sterile isotonic saline and after 5-10s a sample of 5-10 ml obtained using gentle suction. The process is repeated several times and samples pooled for analysis.
Clonal complex	A group of multi-locus genotypes in which every genotype shares at least 5 loci in common with at least one other member of the group. Clonal complexes are thus mutually exclusive.
Co-aggregation	The process of adhesion between genetically distinct bacterial partners. Co-aggregation between pairs of bacteria is highly specific and typically mediated by a protein 'adhesin' on one cell type and a complementary saccharide 'receptor' on the other.
Colony-forming unit	Refers to individual colonies of bacteria, yeast or mold. A colony of bacteria or yeast refers to a mass of individual cells of same organism, growing

	together. Colony forming units are used as a measure of the number of microorganisms present in or on surface of a sample.
Commensal strain	Strain that lives together and shares food resources with a different species, while not harming the other and benefiting from the association.
Conjugation	Involves transfer of DNA via sexual pilus and requires cell –to-cell contact.
Consortium	Kind of symbiosis or assemblage involving two or more species in which all partners gain benefit from each other.
Double-locus variant	Genotypes which have allelic profiles that differ from that of the founder at two of the seven loci.
Dysbiosis	A change in the relative abundance of individual microbiota components compared with their abundance in healthy individuals.
Early-onset VAP	VAP occurring during the first four days of mechanical ventilation. The condition is less severe and has a better prognosis than late-onset VAP. Commonly caused by *H. influenzae*, *S. pneumoniae*, *S. aureus* and Gram-positive Enterobacteriaceae.
Extracellular matrix	Macromolecular ground substance of connective tissue, secreted by fibrinoblasts and other connective tissue cells, and which generally consists of proteins, polysaccharides and proteoglycans.
Fitness advantage	The ability of the organism or biofilm to persist or grow in a given environment or under a particular environmental stress.
Fixation	The treatment of specimens to preserve structure, for microscopy for example. Fixation can occur through heat, perfusion, immersion or chemical solutions.
Glycocalyx	General term for the carbohydrate-rich layer outside the cell wall of bacteria. Synonyms are capsule, cell coat or slime layer.
Heteroresistance	Resistance to certain antibiotics expressed by a subset of a microbial population that is generally considered to be susceptible to these antibiotics according to traditional in vitro susceptibility testing.
Homeostasis	Maintenance of the constancy of internal environment of the body or part of the body.
Housekeeping genes	Genes that are expressed in most cell types and which are concerned with basic metabolic activities common to all cells.
Horizontal gene transfer	The acquisition of genes by one species from another species (alt. lateral gene transfer). Many of the antibiotic resistance genes are carried on plasmids, transposons or integrons that can act as vectors that transfer these genes to other members of the same bacterial species, as well as to bacteria in another genus or species. Horizontal gene transfer may occur via three main mechanisms: transformation, transduction or conjugation.
Hospital-acquired pneumonia	(nosocomial pneumonia) Refers to any pneumonia contracted by a patient in a hospital at least 48-72 hours after being admitted. It is usually caused by a

bacterial infection, rather than a virus.

Insertion sequence A simple type of transposon found in bacteria, consisting of around 800-15000 bp. and carrying only the genetic functions for its own transposition. It consists out of a transposase gene flanked by short, inverted-repeat sequences that are the target of transposase. Insertion sequences are also found at the ends of some other transposons.

Integron Large mobile genetic element that contains numerous gene clusters and can capture genes. By itself, it is not mobile, but it can be carried by plasmids or transposons.

Keystone pathogen Species that have disproportional large effects on their communities, given their abundances, thereby causing inflammatory disease.

Late-onset VAP VAP that develops 5 or more days after initiation of mechanical ventilation. The condition has a high mortality and morbidity and is thought to be associated with prior antibiotic therapy. Commonly caused by *P. aeruginosa*, Actinobacter spp., *S. aureus* and multi-resistant Gram-negative bacteria.

Locus The site on a chromosome occupied by a given gene. In a diploid cell there are two copies of each locus, each occupied by an allele of the gene in question.

Macrocolony Fundamental unit of a biofilm, wherein close contacts of cells provides a perfect environment for the creation of nutrient gradients, genetic exchange and signalling. Visible with the unaided eye.

McFarland A reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing. Equal to an inoculum of 1.5×10^8 colony-forming units per ml.

Microbiota Organisms of microscopic size (bacteria, algae, protozoa, slime moulds and fungal mycelium and spores) in any ecosystem or habitat.

Microbiome The collective genomes of the microbes (composed of bacteria, bacteriophage, fungi, protozoa and viruses) that live inside and on the human body.

Microcolony Fundamental unit of a biofilm, wherein close contacts of cells provides a perfect environment for the creation of nutrient gradients, genetic exchange and signalling. Only visible with the aid of a microscope.

Minimal inhibitory concentration The lowest concentration of an antimicrobial agent that completely inhibits growth of the organism in the tubes or microdilution wells as detected by the unaided eye.

Mobile genetic element DNA such as plasmids or transposons that may be exchanged between bacteria by horizontal gene transfer, and which often carry virulence or antibiotic resistance genes.

Multilocus sequence typing Allelic variation at multiple housekeeping loci is indexed directly by nucleotide sequencing of internal fragments of approximately 450 base pairs and the resulting data is stored on a central database on the internet.

Multispecies biofilm	Biofilm composed out different bacterial species, functional consortia of cells that often possess a combined metabolic activity that is greater than that of the component species.
Natural selection	The process by which evolutionary change is chiefly driven according to Darwin's theory of evolution. Environmental factors such as climate, disease, competition from other organisms, and availability of certain types of food will lead to the preferential survival and reproduction of those members of a population genetically best fitted to deal with them. Continued selection will therefore lead to certain genes becoming more common in subsequent generations.
Nosocomial strain	Hospital-acquired strain that is optimally adapted to the clinical environment.
Operon	Cluster of co-regulated genes with related functions found commonly in bacterial genomes. The series of genes in an operon are transcribed as a single mRNA and consists of an upstream promoter and a downstream terminator. Genes transcribed in an operon are shown to be functionally related and are often engaged in regulating the same metabolic pathway.
Plasmid	Extrachromosomal molecule of DNA that replicates independently from the bacterial chromosome and are not essential for an organism's survival. They can carry resistance genes or fitness enhancing genes.
Photobleaching	Loss of colour by photosensitive pigments on exposure to light.
Point mutation	A mutation involving a change at a single base-pair (single nucleotide) in DNA.
Primary founder	The ST that differs from the largest number of other STs at only a single locus.
Quorum sensing	The ability of individual bacteria to sense the presence of other bacteria in a population via sensing the concentration of secreted signalling molecules called autoinducers.
Quorum quenching	A mechanism whereby microorganisms disrupt the quorum-sensing mechanisms of other species.
Recombination	The process by which a donor DNA molecule replaces a segment of a host genome or is inserted into a host genome. Any exchange between, or integration of, one DNA molecule into another, which may be reciprocal or non-reciprocal.
Sequence type	Allelic profile; the combination of alleles at each of the loci.
Single-locus variant	Genotypes which have allelic profiles that differ from that of the founder at only one of the seven MLST loci.
Single-species biofilm	Aggregate of microorganisms of a single species in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance adhere to each other and/or to a surface.
Subgroup founder	A ST that has diversified to produce multiple SLVs.

Synergism	The acting together of species, often to produce an effect greater than the sum of the two agents acting separately. if two clusters of a population are in tight association with each other, on or both must be receiving a beneficial effect through co-metabolism or other forms of synergistic relationships.
Transduction	Involves transfer of DNA from one bacterium into another via bacteriophages.
Transformation	Involves uptake of short fragments of naked DNA by naturally transformable bacteria.
Transposon	A small genetic unit that carries resistance genes, replication genes and genes that allow them to jump to other regions of the chromosome or plasmids.
Triple-locus variant	Genotypes which have allelic profiles that differ from that of the founder at only three of the seven MLST loci.
Van der Waals forces	Weak non-covalent interatomic attractive forces, of importance in forming and maintaining the three-dimensional structure of proteins and in interactions between proteins.
VAP-bundle	a group of interventions related to patients receiving mechanical ventilation that, when implemented together, result in better outcomes than when implemented individually.
Ventilator-associated pneumonia	A hospital-acquired pneumonia that develops in patients who have been treated with mechanical ventilation for 48 hours or longer, and who had no signs or symptoms of lower respiratory infection before they were intubated and treatment with mechanical ventilation began.
Virulence factor	Genes and proteins that facilitate the establishment and persistence of the organism in the human body.
16S rRNA gene	Gene comprised of conserved stretches of sequences that can be used to design universal primers to amplify the gene from the majority of known bacterial species. These regions are interspersed with variable sequence regions, the sequences of which can be used to assign identity and phylogeny of the organisms in a mixed community.