Universidade do Minho
Escola de Engenharia

Mariana Roriz Abrantes

Influence of antibiotics on *Staphylococcus epidermidis* biofilms

outubro de 2013

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Professora Doutora Mariana Contente Rangel Henriques
Universidade do Minho
e do
Professor Doutor Johan Van Eldere
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DECLARAÇÃO

Nome: Mariana Roriz Abrantes

Endereço electrónico: a55521@alunos.uminho.pt Telefone: +351 916838465

Número do Bilhete de Identidade: 13768774

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Orientador(es):

Professora Doutora Mariana Contente Rangel Henriques

Professor Doutor Johan Van Eldere

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Influence of antibiotics on *Staphylococcus epidermidis* biofilms

Abstract

Staphylococcus epidermidis is a member of the coagulase-negative staphylococci and has gained substantial interest in recent years because it has become the most frequently cause of infections related to indwelling medical devices. Since *S. epidermidis* is resistant to some antibiotic treatments, it is of major importance to understand the mechanisms and the bacterial components involved in those mechanisms.

Therefore, the present dissertation aimed to understand the mechanisms and the bacterial components involved in the antibacterial resistance mechanisms. The first goal of this work was the evaluation of the activity of different groups of antibiotics, such as cell-wall and protein synthesis inhibitors, on *S. epidermidis* biofilms. Changes on *S. epidermidis* biofilms were evaluated regarding the total biofilms biomass, bacteria viability and biofilm's matrix changes after treatment. The second goal of this work was the evaluation of the influence of antibiotics on the matrix composition considering the contribution of PIA and extracellular DNA to the changes referred.

Antibiotics treatment with the glycopeptides, vancomycin and teicoplanin, had an effect on significantly increasing the total biofilm biomass on all strains. Even though these antibiotics were effective against biofilm associated bacteria assessed by XTT, by DMMB staining method it become obvious that they had an effect on increasing the amount of biofilms matrix. Thus imply that they promoted the membrane rupture, enhancing PIA's production and the formation of a rougher biofilm. Results found on the combinated treatment of DNase I plus antibiotics indicated that destruction of eDNA by DNase I leads to a decrease in the matrix, and as a result, antibacterial agents protein inhibitors, such as rifampicin and gentamicin, act more effectively to reduce the biofilm biomass.

In conclusion, it was possible to evaluate that antibiotic agents promoted alterations on *S. epidermidis* biofilms. It was also possible to conclude that *S. epidermidis* biofilms from different strains showed an enhanced resistance to the application of treatment with glycopeptide antibiotics. Those antibiotics are normally reserved for use against multi-resistant staphylococci, however from this study data, it is of major importance to have a better understanding of the resistance mechanisms and to find different alternatives.

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Abbreviations

agr – Accessory gene regulator

ANOVA – Analysis of Variance

AtlE – Autolysin E (E indicates its origin in *S. epidermidis*)

BSA - Bovine serum albumin

CoNS – Coagulase-negative *Staphylococcus* spp.

CV – Crystal violet

DMMB - 1,9-Dimethyl-Methylene Blue zinc chloride double salt

DNA – Deoxyribonucleic acid

eDNA – Extracellular Deoxyribonucleic acid

EDTA – Etgylenediaminetetraacetic acid

EPS – Extracellular polymeric substance

gDNA – Genomic Deoxyribonucleic acid

Genta – Gentamicin

h - Hours

HMDS – Hexamethyldisilazane

ica – Intercellular adhesion

MIC - Minimum inhibitory concentration

min – Minutes

OD – Optical density

Oxa – Oxacillin

P – Significance value

PBS - Phosphate buffered saline

PCR – Polymerase chain reaction

PIA - Polysaccharide intercellular adhesion

Rif – Rifampicin

s – Seconds

SD – Standard deviation

SDS - Sodium dodecyl sulphate

SEM - Scanning electron microscopy

TBS – Tris buffer saline

Teico – Teicoplanin

TSB – Tryptic soy broth

TTBS – Tris buffer saline with 0.4% Tween

Vanco – Vancomycin

Vs – Versus

WGA – Wheat germ agglutinin

XTT - 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

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Chapter 1 Introduction

1.1 Genus *Staphylococcus*

Staphylococci are members of the family *Micrococcaceae*. *Staphylococcus* spp. are characterized by round shaped cells, non-motile, non-spore forming, Gram-positive cocci, which are approximately 1 µm in diameter and that usually divide in multiple planes to form grape-like clusters (Figure 1) [1],[2].

The genus name Staphylococcus is derived from greek terms (*staphyle* and *kokkos*) that mean "a cluster of grapes", that is the most known microscopically aspect of these bacteria after gram-staining [3],[4].

(a) **(b)**

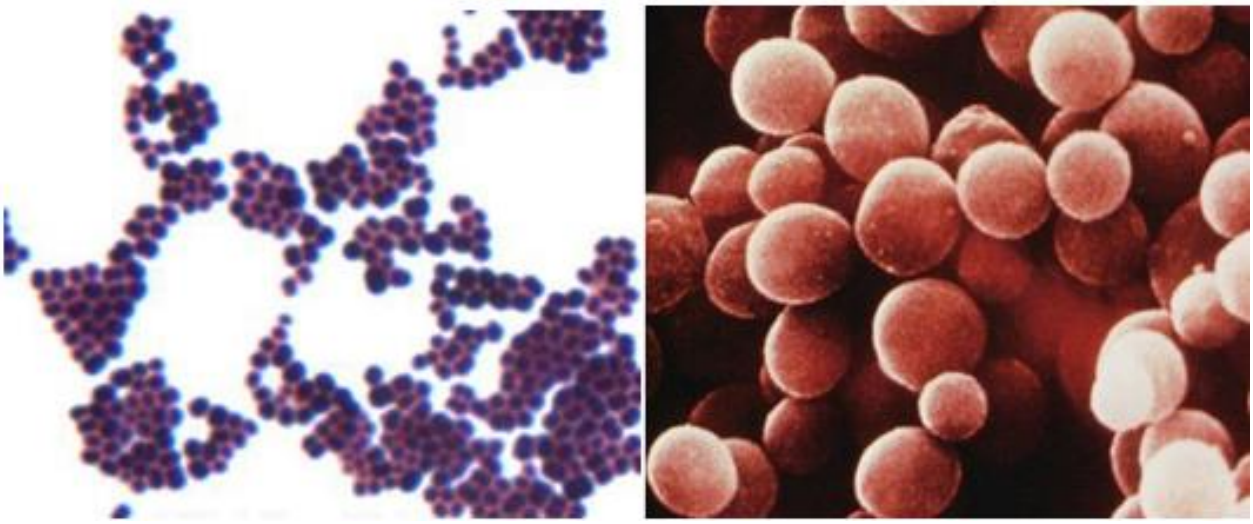

Figure 1 *Staphylococcus* (a) Gram-stained (adapted) [5]**. (b) Staphylococci organized like a grape cluster (adapted)** [6]**.**

More than 30 staphylococcal species have been identified [7], [8]. These species are characterized by a cell wall that consists of a thick layer of peptidoglycan with teichoic acids and lipoteichoic acids in between, which make them susceptible to lysis with lysostaphin but not with lysozyme [7]. Staphylococci are also oxidase-negative and ferment glucose anaerobically [9]. They grow either by anaerobic respiration or by fermentation that produces mostly lactic acid [10]. Catalase test is important to distinguishing streptococci from staphylococci, since all staphylococci can grow in the presence of bile salts and are catalase positive [4].

Staphylococci can be divided into two large groups based on the ability to produce the enzyme coagulase that coagulates plasma. The first group, known as coagulase-positive staphylococci, is mainly represented by *S. aureus* and *S. intermedius*, which can cause a variety of infections ranging from cutaneous to systemic infections. The second group is known as coagulase-negative staphylococci (CoNS) [11], [12]. They are normal inhabitants of the upper respiratory tract, skin, vagina and intestine, being among the most important bacteria that causes various infections in humans (e.g., folliculitis, scalded-skin syndrome and boils) [9].

CoNS are among the most commonly isolated organisms in the clinical microbiology laboratory. Owing to their ubiquitous nature and relative low virulence, CoNS have for a long time been considered to be clinically insignificant pathogens [1], [13]. Due to the widespread use of implanted medical devices, in recent decades, these bacteria are now considered as important causative agents of nosocomial bacteremias, infections of indwelling devices [14].

Staphylococcus epidermidis is currently the most significant member of the CoNS group. *S. epidermidis* is a Gram-positive bacteria, found on skin and mucous membranes of humans and other organisms, representing an important part of its normal microflora, and includes 65 to 90% of all staphylococci isolated from these environments [4], [15], [16].

Historically, *S. epidermidis* has been regarded as an innocuous commensal bacterium of the human skin. However, nowadays this bacterium is seen as an important pathogen and as emerged as one of the most important and frequent causes of nosocomial infection, mainly caused with implanted medical devices (Figure 2) [4], [15], [17]. In order to become a pathogen, *S. epidermidis* requires a predisposed host in order to change from a normal inhabitant of the human skin to an infectious agent, and therefore has to be described as opportunistic [15].

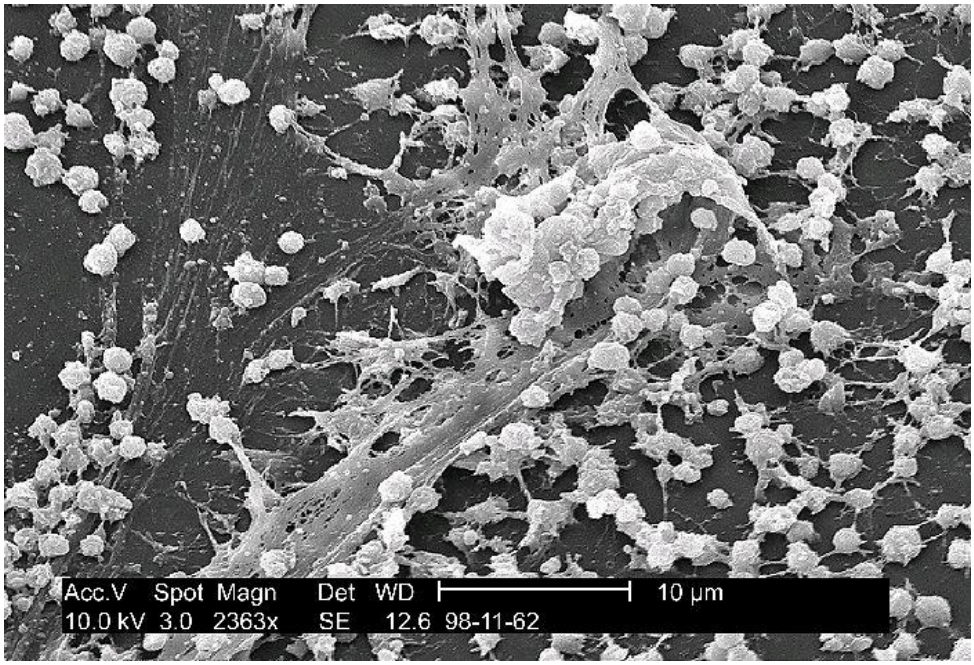

Figure 2 Scanning electron micrograph of a Staphylococcus biofilm on the inner surface of a needleless connector[75]**.**

1.2 Biofilm formation process

In 1987, Costerton *et al.* defined a biofilm as the accumulation of microorganisms and their extracellular products to form a highly structured bacterial community on a surface [18]. Biofilm formation as a multi-step process is considered the most important virulence factor of *S. epidermidis* [14]. Biofilm formation occurs in four distinct phases – attachment (adhesion), accumulation, maturation and detachment [19]. The process starts with the initial adhesion of cells to a surface and their subsequent maturation phase. Detachment of cells or cell clusters from an existing biofilm can lead to the dissemination of the infection (Figure 3).

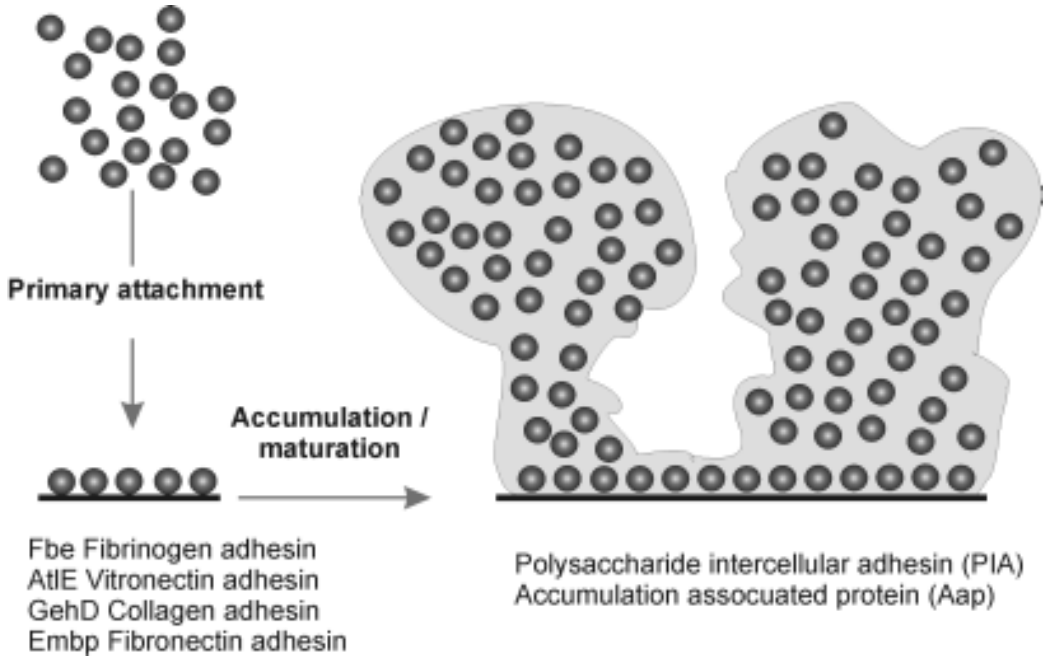

Figure 3 Schematic overview of the phases involved in *Staphylococcus epidermidis* biofilm formation on a surface [76]**.**

1.2.1 Adhesion

Initial bacterial adhesion is the first critical step in the development of implanted-associated infections. This process depends on bacterial cell surface characteristics and also on the nature of the polymer surface [20]. The initial step involves non- specific physicochemical forces (van der Waals), hydrophobic interactions and polarity changes [4], [21]. The colonization of an implanted device may occur by the microbial direct attachment to the polymer surface or by binding to the host matrix proteins which previously coated the biomaterial surface forming the "conditioning biofilm" [15].

The direct binding to the polymer surface is mediated by several surfaces structures of *S. epidermidis* biofilms, such as the fimbria-like proteins SSP-1 and SSP-2, the major autolysin AtlE and teichoic acids [22]–[24].

The two related staphylococcal surface proteins, SSP-1 and SSP-2, are fimbria-like polymers that were shown to contribute to the adherence to polystyrene surfaces [22]. The atlE gene encodes for autolysin and attachment to a polystyrene surface was demonstrated to be mediated, at least

in part, by the surface-associated autolysin AtlE. AtlE is active during the attachment to both conditioned and unconditioned polymer surfaces [25].

Aside from proteins, polysaccharide intercellular adhesin molecule (PIA), has been also associated with the initial adherence and slime production [25]. In fact, for a long time studies were prevalently centered on PIA of *S. epidermidis* biofilms, considered the major cell-to-cell connecting substance. However, recently the attention has been progressively focused also on extracellular DNA, another conspicuous component of the biofilm matrix [26]. The atlE gene, mentioned above, is necessary for both attachment and biofilm development. Since there is no evidence that AtlE protrudes from the bacterial cell surface, rather than a direct role in primary attachment, this autolysin may have an indirect role in *S. epidermidis* biofilm formation by releasing of eDNA. In concordance with this hypothesis is the finding that in cultures of atlE-defective *S. epidermidis* mutants the amount of eDNA is dramatically decreased when compared with its level in the wild-type strains [27].

Finally, also the host contributes to adhesion in device-related infections particularly with staphylococci, as mentioned above. Multiple specific receptors on the cell surface, called adhesins, bind to the host molecules, such as glycoprotein components in plasma or components of the host extracellular matrix. Many of these proteins belong to a family of microbial surface components that recognize adhesive matrix molecules (MSCRAMMs). MSCRAMMs mediate adhesion to various host cell types as well as to polymer surfaces coated with host plasma proteins [12].

1.2.2 Accumulation

Following microbial adherence to the implanted medical device, bacteria proliferate and accumulate as multilayered cell clusters, resulting in an extensive network of accumulated bacteria [19]. The biofilm accumulation involves intercellular aggregation that is mediated by intercellular adhesins. These include surface macromolecules such as exopolysaccharide, surface proteins, teichoic acids and extracellular DNA originating from lysed cells which are involved in formation of extracellular biofilm matrix [14].

In *Staphylococcus* spp., production of PIA by the genes in the intercellular adhesion (*ica*) operon, is nowadays the best-understood mechanism (*ica* or PIA-dependent mechanism) of biofilm formation [14], [28]. PIA is a major component of the extracellular staphylococcal carbohydrate

matrix of *S. epidermidis* [19]. Mack et al. described PIA as a major functional component involved in the intercellular adhesion, essential for the accumulation of multilayered *S. epidermidis* biofilms [29].

PIA is a linear β-1,6-linked glucosaminoglycan, composed of β-1,6-linked N-acetylglucosamine residues containing up to 15% de-N-acetylated amino groups and positively charged (Figure 4). These positive charges have been shown to have a major biological importance in biofilm formation, virulence and immune evasion [19], [30].

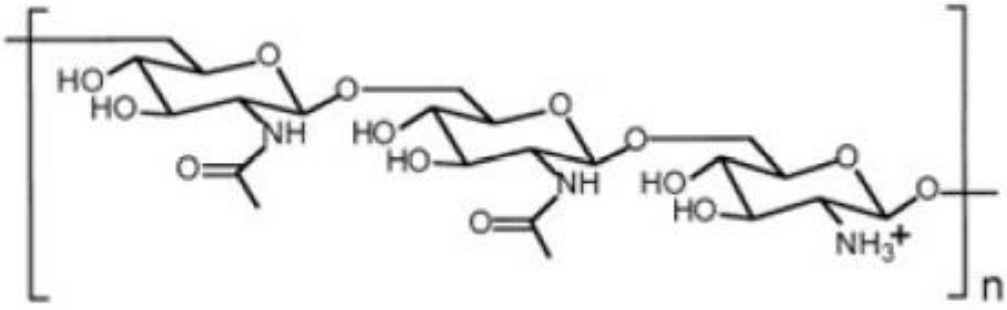

Figure 4 Structure of PIA [12]**.**

PIA is synthesized by enzymes encoded by the intercellular adhesion (ica) operon, which is more prevalent in colonizing *S. epidermidis* isolates [19]. The ica locus is composed of an operon, icaADBC, which encodes the structural genes required for PIA synthesis. The ica operon comprises 4 genes: *ica*A, *ica*D, *ica*B and *ica*C. A fifth gene, the divergently transcribed *ica*R gene, responsible for the transcription of *ica*ADBC is located upstream of the *ica*A start codon. IcaR gene is a member of the TetR family of transcriptional regulators and negatively regulates icaADBC expression [14]. The icaA gene product is responsible for N-acetylglucosaminyltransferase activity during PIA synthesis. IcaA adds N-acetylglucosamine from UPD-N-acetylglucosamine to the growing PIA chain, requiring the icaD gene product for optimal transferase activity. N-acetylglucosamine oligomers produced by icaAD reach a maximal length of 20 residues. Only when icaAD is coexpressed with icaC, longer oligomer chains are synthesized. The transmembrane protein IcaC plays a putative role in externalization, elongation and translocation of the growing polysaccharide to the cell surface and is responsible for the production of full-length PIA molecule [19]. The icaB gene product is a

surface-attached protein responsible for decacetylation of the poly-N-acetylglucosamine molecule (Figure 5).

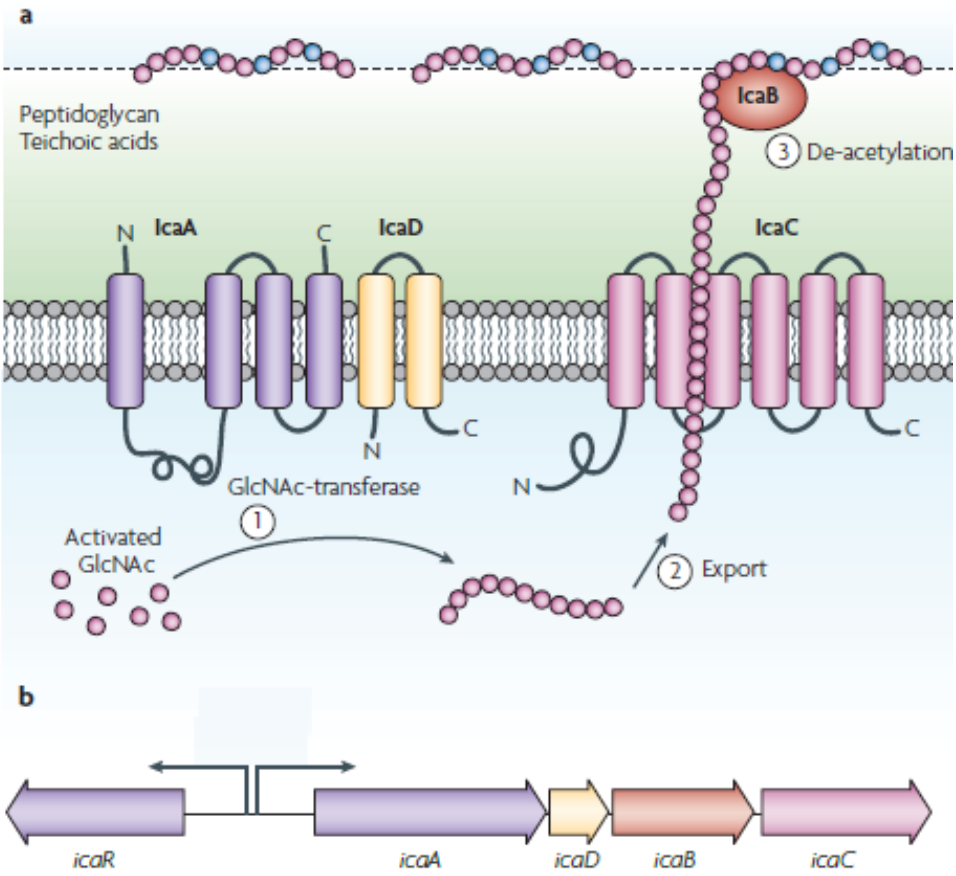

Figure 5 The exopolysaccharide poly-*N*-acetylglucosamine. a) The exopolysaccharide poly-N-acetylglucosamine (PIA), a partially de-acetylated β1-6-linked N-acetylglucosamine (GlcNAc) homopolymer involved in immune evasion and biofilm aggregation, is synthesized by the membrane-located GlcNAc transferase IcaA, which needs the accessory IcaD membrane protein for activity (step 1). The growing PNAG chain is probably exported by the IcaC membrane protein (step 2). After export, IcaB de-acetylase, located on the cell surface, removes some of the N-acetyl groups, giving the polymer a cationic character that is essential for surface attachment (step 3). **b)** The Ica proteins are encoded by the ica gene locus containing the icaADBC operon and the icaR gene, which encodes a regulatory protein. Expression of the icaADBC operon is regulated either directly at the icaA promoter or through expression of IcaR (adapted) [17].

The ica locus and biofilm formation are important parameters for staphylococcal colonization and survival on implanted medical devices. However, recent publications have revealed the emergence of biofilm-positive and ica-negative staphylococcal clinical isolates [31]. In cases of PIA-negative biofilm formation, adhesive proteins have been suggested to be involved in the accumulation phase. In some strains (ica-positive or negative), biofilm formation is mediated by specific surface proteins such as accumulation-associated protein (Aap), biofilm-associated protein

(Bap), or Bap homologue (Bhp) [19]. Biochemical and functional properties clearly differentiate Aap from other factors that have been implicated in biofilm formation. It was proposed that Aap has a role in the anchoring of PIA to cell surface [32].

1.2.3 Maturation and detachment

Maturation of the *S. epidermidis* biofilm is characterized by the generation of a slime glycocalix, which encases surface-bound organisms in a gelatinous matrix. The slime exopolysaccharide is not essential to the overall process of surface colonization, it is thought to increase the stability of the biofilm and therefore contribute to a more robust structure and making implanted medical devices colonized with slime-positive strains more difficult to treat.

A mature biofilm is seen as a very heterogeneous arrangement, with a basic community structure which comprises several layers, including the main bulk of the biofilm, a linking film, a conditioning film and the substratum to which the biofilm is attached [19], [33]. The mature structure reveals groups of micro colonies of bacterial cells encased in extracellular polymeric substance matrix [34]. The matrix is one of the most distinctive features of microbial biofilm where, in addition to PIA and protein, extracellular DNA has also been shown to be important in stabilizing the biofilm [35].

Mature biofilms form a three-dimensional, gel-like, highly hydrated and locally charged environment where microorganisms are largely immobilized. Matrix-enclosed micro colonies, sometimes described as mushroom-like forms are separated by fluid-filled channels [34]. Liquid flow in these channels, allowing diffusion of nutrients and oxygen to all cells in the biofilm [19].

At last, individual bacterial cells, capable of actively leaving a biofilm, can arise and spread from the surface film on the outer side of the mature biofilm to colonies in distant sites. The dispersion of virulent staphylococci has important implications from *S. epidermidis* biofilm infections. Even if cells actively detaching from the biofilm may colonize alternative sites, it can also contribute to the toxicology associated with acute infections [19].

In contrast to primary attachment and accumulation, detachment is not well understood. However, several factors have been proposed to be involved in biofilm detachment including: mechanical forces, changes in nutrient concentrations, cessation of production of biofilm building

material, and production of detachment factors controlled by quorum-sensing system accessory-gene regulator (agr) [19].

As mentioned, *S. epidermidis* virulence factors are mainly regulated by the agr locus [15]. The quorum-sensing phenomenon is known as the ability of bacteria to signal and sense the state of population density in order to change physiological needs under different environments [36]. Therefore, quorum-sensing is a strategy of cell-cell communication benefiting the biofilm community by controlling unnecessary overpopulation and competition for nutrients with important implications for the infectious process (Figure 6) [37]. Quorum sensing molecules have been recognized as important virulence regulators and demonstrated to be essential for biofilm formation. Vuong et al. (2003) reported that the agr system controls biofilm formation by regulation of biofilm factors such as AtlE and δ-toxin [38].

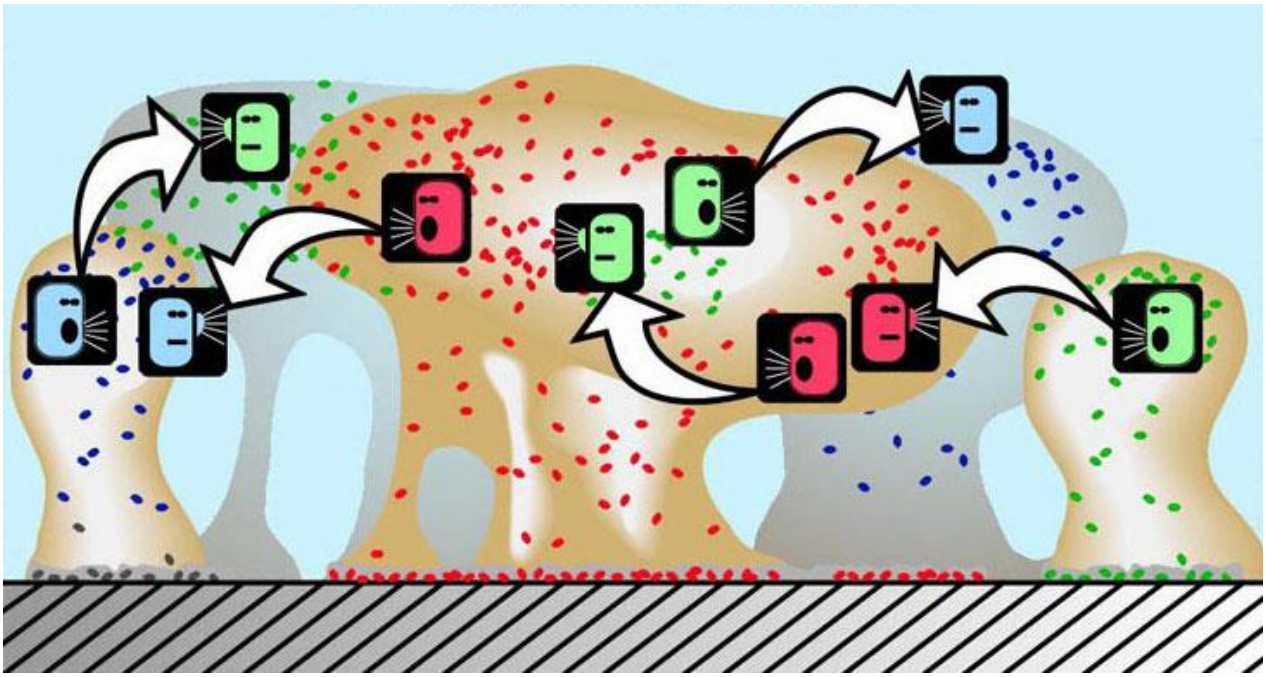

Figure 6 Quorum-sensing phenomenon. Cell to cell communication in a biofilm. Cartoon representing sessile cells "talking" to each other [77]**.**

1.3 Importance of *S. epidermidis* biofilms in clinical practice

In recent years, *S. epidermidis* has emerged as one of the most important and frequently causes of nosocomial infections, mainly associated with implanted medical devices [4], [15], [21]. Therefore and as referred above *S. epidermidis* species subsist both as commensal and as pathogenic, creating strategies for the purpose to transform the hospital environment in a new ecological niche. In fact, nearly 80 % of the cells involved in biomaterial associated infections are *S. epidermidis* related [12].

Infections caused by *S. epidermidis* are usually related to immunocompromised, immunosuppressed, long-term hospitalized and critically ill patients [19]. These individuals represent a very susceptible hosts with a less powerful immune system and that is why it is considered an opportunistic pathogen [39]. Physiological changes in *S. epidermidis* biofilms protect the bacteria from the host immune defense system by lowering the sensitivity towards harmful molecules. Such immune-evasion tactics enable the bacteria to persist during infection. Yao et al. (2005) described changes in *S. epidermidis* biofilm gene expression, including low metabolism, decreased transcription and translation and a change from aerobic production of energy to fermentation, resulting in non-aggressive and protected mode of growth that implies that *S. epidermidis* is less sensitive to antibiotics and host immune system and optimally suitable to long-term survival in the organism [19], [40]. So, early and acute infections are usually associated with *Staphylococcus aureus* biofilms, but *S. epidermidis* are typically responsible for chronic and profound infections, which occur months to years after the medical device implantation [4]. Furthermore, *S. epidermidis* infections are often persistent and relapsing and result from a rupture in the cutaneous surface, caused by trauma or insertion of a medical device [16]. That can be the channel in which bacteria enters the organism and becomes pathogenic [4].

The success of *S. epidermidis* as a pathogen can be explained by its highly adaptive nature, inherent genetic variability and intrinsic genetic flexibility, all of which enable it to resist to different external environments [40]. In addition to this factor, the increasing number of immunocompromised patients, the growing need of medical devices and the extensive use of antibiotics and disinfectants on hospital environments leading to antimicrobial resistance, provides conditions for a successful bacterial colonization of the implanted medical device [39].

In addition to implanted medical devices in immunocompromised patients, *S. epidermidis* is also responsible for native valve endocarditis in immunocompetent individuals, that results from the interaction between the vascular endothelium and bacteria circulating in the bloodstream [4], [21]. Furthermore, cases of wound infection, urinary tract infection, meningitis and pneumonia, associated to *S. epidermidis* have also been reported [4].

The indwelling medical devices mostly affected by *S. epidermidis* persistent infections include, prosthetic heart valves, urinary catheters and central venous catheters. Currently and in respect to *S. epidermidis* infections, catheter related infections are a major cause of patient morbidity and mortality, justifying most of the time the premature device removal and the increase costs and use of resources [4], [41].

In catheter related infections, in particular, the skin insertion site and the catheter hub are the two most important infection sources. In the case of short-term catheters, skin contamination is the most likely mechanism of pathogenesis, but for long-term catheters, hub contamination is more frequent [4]. After skin contamination, bacteria immediately migrate through the insertion site along the external surface of the catheter, colonizing the distal intravascular tip of the catheter, and making contact with the bloodstream. This ultimately leads to bloodstream infection [4], [41].

In long-term catheters the hub contamination is normal, due to the fact that such catheters are continuously and regularly manipulated and intercepted and so bacteria are usually more easily introduced into the hub by the hands of medical personnel. From the contaminated hub, microorganisms migrate through the internal surface of the catheter, where they can cause a bloodstream infection [4]. These devices are in direct contact with bloodstream, platelets, plasma and host tissue proteins are rapidly absorbed on the surface of intravenous catheters forming a conditioning film that enhances bacterial adherence to the medical device (Figure 7).

The virulence mechanisms referred for catheter related infections by *S. epidermidis* are in sort a way similar to the other implanted medical devices, such as urinary tract catheters, endotracheal tubes, contact lenses and voice prostheses.

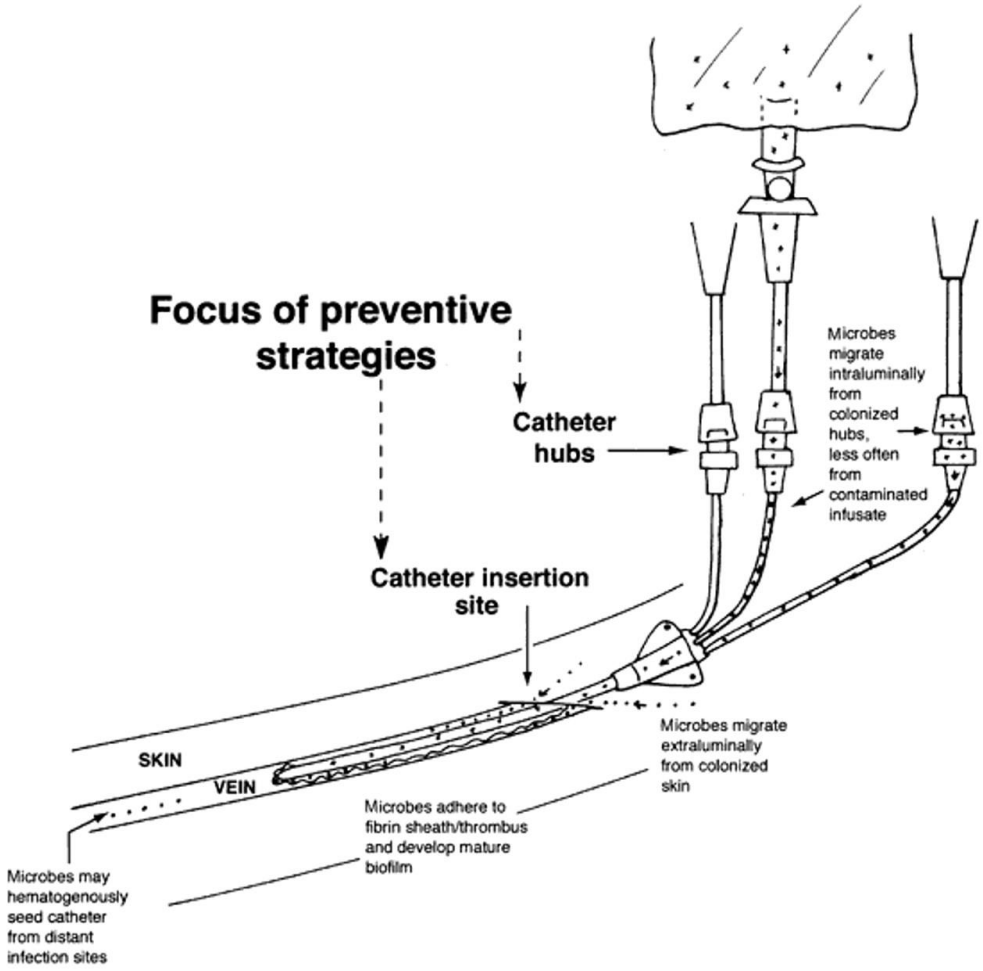

Figure 7 Catheter related bloodstream infections (scheme of the catheter insertion on the patient) [78]**.**

1.4 Treatment of biofilm related infections

Traditional treatment of *S. epidermidis* infections, including those caused by biofilms on implanted medical devices, involves the use of conventional antibiotic therapy directed against the known or the likely causative strain, being the final choice dependent on the microbiological, pharmacological and toxicological properties of the antibacterial agents. Accepted clinical practice often includes therapy of combination which of two or more antimicrobials. This approach comes with the clinical standard that a broader spectrum of activity of antibiotics is achieved and lower concentrations are required, and results in a more effective therapy and less resistance [19].

Administration of prophylactic antibiotic therapy to prevent colonization is also common practice during surgical insertion of most biomaterials [16]. However, infective complications often arise and it has been shown that even in the presence of antibiotics, adherence, colonization and the establishment of infection can occur on the surface of implanted medical devices. Unfortunately, implant-associated infections are recalcitrant to typical antimicrobial therapy and host defenses. These bacterial infections tend to be very difficult to eradicate and relapses occur frequently [17].

1.4.1 Antibiotic treatment

A number of factors conspire to render medical device-related infections resistant to standard antimicrobial treatment, including the distinct mode of growth displayed by biofilm populations, multi-drug bacterial resistance and the increasing prevalence of *S. epidermidis* as a nosocomial pathogen [17]. In Table 1 are shown some of the most used antibiotics on the clinical practice in order to eradicate *S. epidermidis* biofilm associated infections.

Table 1 Overview of used antibiotics on *Staphylococcus epidermidis* biofilms and their mechanism of action (adapted) [42], [43].

Antibiotic	Group	Mechanism of action
Vancomycin	Glycopeptide	Inhibits prolongation of peptidoglycan in cell wall
Teicoplanin		
Oxacillin	Penicillinase-resistant β-lactam	Inhibits cross-linking of peptidoglycan in cell wall
Rifampicin	Rifamycin	Inhibits protein synthesis by inhibiting RNA-polymerase
Gentamicin	Aminoglycoside	Inhibits protein synthesis by inhibiting the ribosomes

1.4.1.1 Cell wall active antibiotics

Vancomycin is a glycopeptide antibiotic used in the prophylaxis and treatment of infections caused by Gram-positive bacteria, like *S. epidermidis*. Vancomycin has been shown to be active against the majority of strains of this microorganism, both in vitro and in clinical infections. The bactericidal action of vancomycin results primarily from inhibition of cell-wall biosynthesis. Specifically, vancomycin prevents incorporation of N-acetylmuramic acid (NAM)- and N-acetylglucosamine (NAG)-peptide subunits from being incorporated into the peptidoglycan matrix which forms the major structural component of Gram-positive cell walls. In addition, vancomycin alters bacterial-cell-membrane permeability and RNA synthesis [43]–[45].

Teicoplanin is also a glycopeptide antibiotic with a spectrum of activity similar to vancomycin. Its mechanism of action is to inhibit bacterial cell wall synthesis. Teicoplanin inhibits peptidoglycan polymerization, resulting in inhibition of bacterial cell wall synthesis and cell death [46], [43].

Finally, oxacillin is a penicillin beta-lactam antibiotic. Oxacillin has in vitro activity against gram-positive and gram-negative, aerobic and anaerobic bacteria. The bactericidal activity of oxacillin results from the inhibition of cell wall synthesis and is mediated through oxacillin binding to penicillin binding proteins (PBPs). Oxacillin inhibits the third and last stage of bacterial cell wall synthesis [47], [48].

1.4.1.2 Protein synthesis inhibitors

Rifampicin is a bactericidal antibiotic drug of the rifamycin group. Rifampicin is an antibiotic that inhibits DNA-dependent RNA polymerase activity in susceptible cells. It is bactericidal and has a very broad spectrum of activity against most gram-positive and gram-negative organisms [43], [45].

Gentamycin is an aminoglycoside antibiotic. Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties for the treatment of life threatening infections. Aminoglycosides like gentamicin "irreversibly" bind to specific 30S-subunit proteins and 16S rRNA. Specifically gentamicin binds to four nucleotides of 16S rRNA and a single amino acid of protein S12. This interferes with decoding site in the vicinity of nucleotide 1400 in 16S rRNA of 30S subunit. This region interacts with the wobble base in the anticodon of tRNA. This leads to interference with the initiation complex, misreading of mRNA so incorrect amino acids are inserted into the polypeptide leading to nonfunctional or toxic peptides and the breakup of polysomes into nonfunctional monosomes [43], [45].

1.4.2 Antimicrobial resistance

The treatment of *S. epidermidis* infection is very difficult especially due to the increasing resistance to antibacterial agents. The frequent use of antibiotics, incorrect diagnosis, inappropriate prescribing and the preferential management of patients with broad-range antibiotics promoted the rapid spread of resistance even for modern antibacterial agents [49].

Antimicrobial resistance has a significant impact on patient outcome by enhancing virulence, delaying the administration of appropriate therapy, limiting available therapy and increasing hospitalization time and subsequent recovery, leading to increased morbidity and mortality [19]. A study carried out by Arciola et al. [48] on antibiotic resistance in exopolysaccharide-forming *S. epidermidis* strains from implant infections found that only 10% of the 342 clinical isolates tested were sensitive to all screened antibiotics. In that study, up to 80% of the isolates were ß-lactam resistant, 37% were methicillin resistant (MRSE) and 38% were resistant to imipenem. Aminoglycoside resistance was also observed in the clinical isolates with a frequency of 31–32%.

The glycopeptide antibiotics vancomycin and teicoplanin are normally reserved for use against multi-resistant staphylococci; however, due to increasing reliance on these agents, there are reports of reduced susceptibility of staphylococci to glycopeptides. The emergence of vancomycin-resistant CoNS is not surprising considering the genetic versatility of staphylococci and the overuse of growth inhibitory compounds that select for the development of resistant organisms [19], [50].

Most of the existing reports regarding the mechanism of glycopeptide resistance have focused on *S. aureus*. It appears due to the accumulation of mutations and not due to genetic exchange [19]. Cell-wall thickening associated with vancomycin resistance in S. *aureus* has been reported by a number of groups and it is thought to be a pre-requisite for vancomycin resistance in staphylococci. Nunes et al [46] characterized the glycopeptide susceptibility profiles and cell-wall ultrastructure of three clinical strains of CoNS with reduced susceptibility to glycopeptides, including *S. epidermidis*. It was highlighted that changes in cell-wall thickness were related to vancomycin minimum inhibitory concentrations (MICs), indicating that the bacterial cell ultrastructure plays an important role in glycopeptide resistance.

Resistance of biofilm populations to antimicrobials has been well documented. The properties of biofilms that result in antibiotic resistance may include slow growth, the presence of persisted cells, inactivation of antibiotics within the biofilm exopolysaccharide matrix, and limitations on antibiotic penetration imparted by the biofilm matrix (Figure 8) [51]. The biofilm environment promotes genetic exchange of antimicrobial resistance genes, increasing bacterial virulence and contributing to the development of multiresistance phenotypes [52], [19].

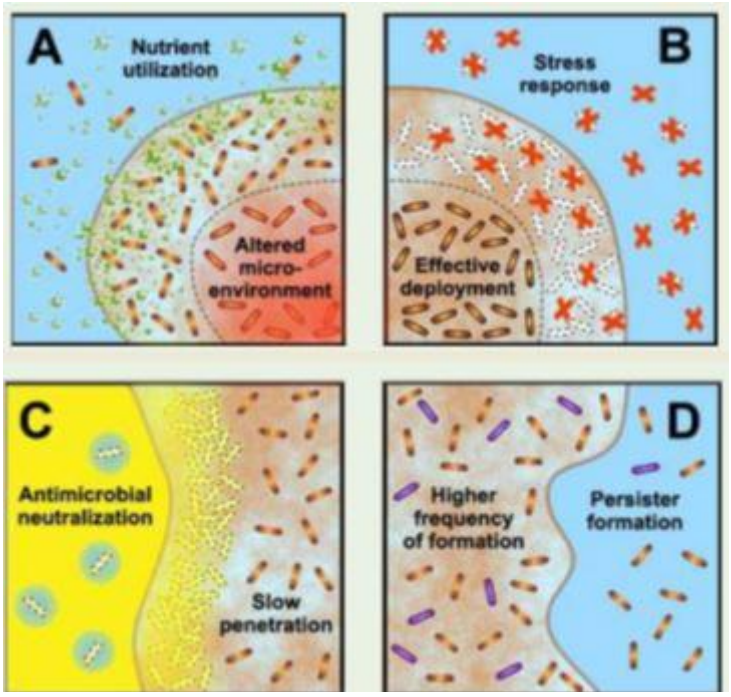

Figure 8 Antimicrobial agents resistance- schematic comparison between planktonic cell and biofilms (adapted). A- Fee-floating cells utilize nutrients but do not have sufficient metabolic activity to deplete substrates from the neighborhood of the cells. In contrast, the collective metabolic activity of groups of cells in the biofilm leads to substrate concentration gradients and localized chemical environments. Reduced metabolic activity may result in less susceptibility to antimicrobials. **B-** Free-floating cells carry the genetic code for numerous protective stress responses. Planktonic cells, however, are readily overwhelmed by a strong antimicrobial challenge. These cells die before stress responses can be activated. In contrast, stress responses are effectively implemented in some of the cells in a biofilm at the extense of other cells which are sacrificed. **C-** Free-floating cells neutralize the antimicrobial agent. The capacity of a lone cell, however, is insufficient to draw down the antimicrobial concentration in the neighborhood of the cell. In contrast, the collective neutralizing power of groups of cells leads to slow or incomplete penetration of the antimicrobial in the biofilm. **D-** Free-floating cells spawn protected persister cells. But under permissive growth conditions in a planktonic culture, persisters rapidily revert to a susceptible state. In contrast, persister cells accumulate in biofilms because they revert less and readily and are physically retained by the biofilm matrix [79]**.**

Treatment with antibiotics may kill planktonic bacteria shed from the biofilm surface; however, they fail to eradicate those embedded within the biofilm, which can then subsequently act as a local for a relapsing infection [34]. Following standard antibiotic treatment, a minority of drug-resistant bacteria exist that repopulate the biofilm. Subsequent retreatment of the repopulated biofilm results only in a reduction in bacterial number, indicating that the repopulated biofilm is much more resistant to treatment [19].

At present, conventional systemic therapies, using standard antimicrobial agents, represent the main strategy for the treatment and prevention of medical device associated infection. However, as detailed above, the available antibiotic therapies are usually ineffective because of the phenomenon of multidrug resistance and the resilient nature of adherent biofilm bacteria. As a result, effective eradication of the infection often necessitates the removal of the implant and its substitution. Importantly, major advances have been made, leading to a greater understanding of the complexities of biofilm formation of *S. epidermidis* and resulting in significant developments in the treatment and prevention of infections related to this member of the CoNS group [19], [51].

1.5 Aims and Objectives

S. epidermidis is currently the most significant member of the coagulase-negative staphylococci and constitutes the most widespread and persistent species found on skin and mucous membranes of the human body, representing an important part of its normal microflora. This pathogen has gained substantial interest in recent years because it has become the most frequently cause of infections related to indwelling medical devices, mainly due to its capability to adhere to surfaces and form multilayered, highly structured biofilms. In fact, the formation of biofilms has been considered the main virulence mechanism of *S. epidermidis* [15], [19].

Biofilms are very difficult to eradicate and are a source of many recalcitrant infections, with increased costs and used sources. Device-related biofilm infections with sessile populations are up to 1000-fold more resistant to antimicrobial agents than their planktonic counterparts [15].

Since *S. epidermidis* is resistant to some antibiotic treatments, it is of major importance to understand the mechanisms and the bacterial components involved in those mechanisms.

The first goal of this work is the evaluation of the influence of antibiotic treatment on *S. epidermidis* biofilms. Moreover, changes in matrix composition after treatment with antibiotics were considered. The contribution of PIA and extracellular DNA to those changes were analyzed.

Henceforth, the specific aims of this work are:

- Study of *S. epidermidis* biofilms treated with antibiotics (biofilm biomass, PIA's matrix and viable bacteria);
- Determination of PIA's contribution on biofilm formation by PIA dot blot;
- Determination of excretion of extracellular DNA after treatment with antibiotics by extracellular DNA extraction;
- Visualization of *S. epidermidis* biofilms by scanning electron microscopy.

Chapter 2 Materials and Methods

2.1 Bacterial strains and growth conditions

In this work, five *Staphylococcus epidermidis* strains were used: *S. epidermidis* 1457, *S. epidermidis* 1457ΔatlE, *S. epidermidis* 10b, *S. epidermidis* 567 and *S. epidermidis* 567-1. *S. epidermidis* 1457 was isolated from a catheter related bloodstream infection [53]. *S. epidermidis* 1457ΔatlE is derived from the strain 1457, and contains an erythromycin insertion mutation in the *atlE* gene [27]. *S. epidermidis* 10b is also a clinical isolate, from a central venous catheter infection [54]. *S. epidermidis* 567 is a clinical isolate from a urinary tract catheter infection. Strain 567-1 is the *agr*-mutated derivative from *S. epidermidis* 567 [55], which contains an erythromycin insertion mutation in the accessory gene regulator (Agr) quorum-sensing system. Bacteria were cultivated in tryptic soy broth (TSB, Oxoid, England), with erythromycin (10μg/ml; Sigma-Aldrich) added for *S. epidermidis* 1457ΔatlE and *S. epidermidis* 567-1.

2.2 Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) values of vancomycin (Sigma-Aldrich), teicoplanin (Sigma-Aldrich), oxacillin (Sigma-Aldrich), rifampicin (Sigma-Aldrich) and gentamicin (Sigma-Aldrich) were determined with the broth dilution methods, as recommended by European Committee on Antimicrobial Susceptibility Testing (EUCAST). Shortly, bacteria were grown overnight on blood agar plates. Several colonies were resuspended in physiologic saline (0.9% NaCl) to reach a density of 0.5 McFarland (Cobas Inocheck, Roche, Switzerland). The bacteria were diluted 1/100 in Mueller Hinton Broth II Cation Adjusted (CAMH, Oxoid England) and combined with an equal volume of the antibiotic solution, to reach a final concentration of 5×10^5 CFU/mL. After 18 h incubation at 37°C, bacterial growth was evaluated by measuring absorbance at 590 nm (Multilabel counter Victor3, PerkinElmer, USA). The MIC was determined as the lowest antibiotic concentration at which no bacterial growth was observed.

2.3 Biofilm formation

Biofilms were inoculated from overnight cultures on blood agar plates. Several colonies from the culture were resuspended in 0.9% NaCl to reach an optical density equal to 0.5 McFarland (Cobas Inocheck, Roche, Switzerland). Of these suspensions, 100 µl was added to 10 ml of the appropriate medium. *S. epidermidis* 1457 and *S. epidermidis* 10b were grown in 10 times diluted TSB (1/10 TSB). *S. epidermidis* 567 biofilms were grown in TSB with 4% NaCl (Sigma-Aldrich, Steinheim, Germany) and *S. epidermidis* 567-1 and *S. epidermidis* 1457ΔatlE biofilms were grown in TSB supplemented with 10 µg/mL erythromycin (Sigma-Aldrich). Then, 200 µl of the bacterial suspension was added in each well of a 96-well microtiter plate (Cellstar, Greiner Bio-One, Belgium). The plates were incubated statically at 37°C for 24 h.

2.4 Treatment of biofilms with antibiotics

2.4.1 Treatment

After biofilm formation for 24 h, the supernatants were discarded and the biofilms were washed once with phosphate-buffered saline (PBS, pH 7.4). The biofilms were treated with 200µl of antibiotic solutions, made in 100 times diluted TSB (1/100 TSB). A negative control was performed with a biofilm not exposed to the antibiotics. The tested antibiotics and concentrations were: vancomycin (40µg/mL, Sigma-Aldrich,), teicoplanin (50µg/mL, Sigma-Aldrich), oxacillin (10µg/mL, Sigma-Aldrich), rifampicin (10µg/mL, Sigma-Aldrich) and gentamicin (10µg/mL, Sigma-Aldrich). After adding the antibiotics, the plates were incubated at 37°C for 24 h.

2.4.2 Biofilm analysis

To assess the influence of the antibiotics on the preformed biofilms, they were stained with: crystal violet (CV, Sigma-Aldrich), XTT ([2,3 bis(2-Methoxy-4-nitro-5-sulfophenyl)-5-[(phenyalamino)carbonyl]-2H-tetrazolium hydroxide], Sigma-Aldrich,) or dymethylmethylene (DMMB, Sigma-Aldrich,).Crystal violet was used to determine the total biofilm biomass, XTT was used to assess the viability of the biofilm-associated bacteria and DMMB was used to stain biofilm's matrix.

2.4.2.1 Crystal violet assay

Supernatants were discarded and biofilms were gently washed twice with 250 µl PBS (pH 7.4). After fixation with 400 µl ethanol (95°) for 20 min, the plates were dried. The biofilms were stained with 230 µl of Hucker's CV during 15 min. The excess CV stain was removed by washing the plate under running tap water and the plates were dried. The bound CV was eluted by adding 150 µl acetic acid (5% v/v in ddH_2O) [56]. To measure the amount of CV that was bound, 100 µl of elute was transferred to a new 96-well microtitre plate and absorbance was read at 590nm using a spectrophotometer (Multilabel counter Victor3, PerkinElmer, USA). All samples were conducted in quadruplicate, and each experiment was repeated three times.

2.4.2.2 XTT assay

Biofilms were gently washed twice with 250 µl of PBS and then 250 µl of the XTT staining solution (containing 200 µg/mL XTT and 20 µg/ml phenazine methasulphate in 1/100 TSB) was added to each well. The microtiter plates were incubated in the dark for 2 h at 37 °C [55], [57]. The plates were then centrifuged for 5 min at 3000 rpm (Allegra X-22R Centrifuge, Belckman Coulter) and 100 µl from each well was transferred to a new microtiter plate and the absorbance read at 490nm.

2.4.2.3 DMMB assay

For the DMMB staining, two solutions were used: a staining solution and a decomplexation solution. Both solutions were prepared as described by Toté *et al* (2008) [58]. After discarding the supernatants, the plates were gently washed twice with 250 µl PBS. A volume of 250 µl of the DMMB staining solution was added to all wells and the plates were incubated at room temperature during 30 min, protected from light. Plates were then centrifuged at 2800 rpm for 20 min (Allegra X-22R Centrifuge, Belckman Coulter) and unbound DMMB was removed by rinsing with ddH_2O. To each well 100 µl decomplexation solution was added and plates were incubated for 30 min at room temperature and protected from light [58]. After which 100 µl of the decomplexation solution

was transferred to a new microtitre plate and absorbance was measured at 630nm (Multiskan Ascent, Thermo Electron Corporation, Finland).

2.5 Combined treatment of biofilms with DNase I and antibiotics

Biofilms were grown for 24 h at 37 °C, the supernatants were discarded and biofilms were washed once. Each well was filled with 200 µl of antibiotic in 1/100 TSB and DNaseI (Sigma-Aldrich) was added to a final concentration of 50 µg/mL [59]. Control wells were filled with antibiotic solutions in 1/100 TSB, without DNase I. Plates were incubated at 37°C for 24 h. Biofilms were washed twice with PBS and stained with CV as previously described.

2.6 Extraction of eDNA

Biofilm formation was carried out in a 6 well plate and 30 µl of a bacterial suspension of 0.5 McFarland was added to 3 ml of TSB. After 24 h of incubation at 37°C, biofilms were washed once with PBS and the supernatant from each well was gently aspirated. Each well was treated with antibiotics diluted in 1/100 TSB. Control wells were filled only with 1/100 TSB. The plates were incubated for 24 h at 37°C. A volume of 2 ml of the supernatant was aspirated. Biofilms were gently washed three times with PBS, without disturbing the adherent biofilm and then scraped from the wells in the presence of 1 ml of a two times concentrated proteinase k buffer (Qiagen, Belgium). Biofilm samples of the same condition from different wells were combined together to extract extracellular DNA from [60], [61].

The pooled biofilm samples were homogenized by vortexing during 10 min. After homogenization, samples were mixed with 10 µg/mL proteinase K (Qiagen, Belgium) and the mixtures were incubated at 37°C for 1 h. Afterwards biofilm samples were filtered through 0.2 µm polyethersulfone membranes (PALL Life Sciences, USA) [61]. The extracellular DNA was precipitated using cetyltrimethylammonium bromide (CTAB)-DNA precipitation method [62]. In brief, samples were incubated with 1 volume of CTAB solution (1% CTAB in 50 mM Tris-10mM EDTA, pH 8.0) at 65°C for 30 min and then centrifuged at 5000 × g for 10 min at 4°C. The pellets were resuspended in 500 µl of NAES buffer (50mM sodium acetate pH 5.1, 10mM EDTA pH 8.0 and 1% sodium dodecyl sulphate (SDS)). An equal volume of acidic phenol chloroform (pH 4.5, IAA (125:24:1), Ambion) was added to each preparation and then centrifuged for 5 min at 10.000 ×

g. The upper layer of the preparation was precipitated with 520 µl of isopropylalcohol and 35 µl of sodium acetate (NaOAc 3 M, Sigma). Samples were centrifuged for 15 min at 10.000 × g. Lastly, the pellet was washed two times with ethanol, dried under vacuum for 25 min and resuspended in MiliQ water [62]. The DNA concentration was measured by determining the absorbance at 260nm (A_{260}) and the purity of DNA was checked by determining the ratio of A_{260}/A_{280} using a spectrophotometer (NanoDrop 2000C, Thermo Fisher Scientific, Inc. Waltham, MA). The size of extracellular DNA was measured by gel electrophoresis.

2.7 PIA dot-blot

For this assay biofilms were grown on polyurethane catheter fragments of 0,7 cm in length (Multi-Lumen Central Venous Catheterization Set, Arrow International). The catheter fragments were suspended in 3 ml of the appropriated medium and 30 µl of 0.5 McFarland bacterial suspensions was added. Biofilms were grown at 37°C for 24 h, washed once and treated with antibiotics, again for 24 h at 37°C.

The PIA dot-blot was conducted as follows. Catheter fragments were washed twice with PBS and transferred to an eppendorf tube containing 500 µl 0.5 M EDTA (pH 8.0). Samples were incubated at 100°C for 5 min and then centrifuged at 13.200 rpm for 10 min. A volume of 250 µl of supernatant was treated with proteinase K (Qiagen, Belgium)at a final concentration of 2 mg/mL for 1 h at 60°C and afterwards for 30 min at 80°C to inactivate the proteinase K [63]. A series of twofold dilutions of PIA extract was prepared in Tris-buffered saline [TBS: 20 mM Tris/HCl (pH 7.4), 0.9% (w/v) NaCl]).

An Immobilon-P nitrocellulose membrane (Milipore Corporation, Ireland) was pretreated with methanol for 15 s, washed with ddH_2O for 2 min and then with TBS for 5 min. The membrane was put on a Whatson filter paper, wetted with TBS and put in the vacuum blotter machine (DotBlot System/Acryl, Schleichor Schuell, Germany). A volume of 200 µl was transferred to the membrane using a vacuum blotter. The membrane was dried at 55°C for 2 h and then blocked in 50 ml 5% (w/v) BSA (bovine serum albumin), in TTBS (TBS plus 0.05% Tween 20) for 30 min [64]. The membrane was washed three times for 10 min each in TTBS and incubated overnight with 50 ml of Wheat germ agglutinin-horsradish peroxidase conjugate (WGA-HRP, 130 ng/mL Sigma) in 1% (w/v) BSA- TTBS at 4 °C. The membrane was washed three times with TTBS for 10 min each and

ECL Western blotting detection agent Plus (GE Healthcare, Italy) was added for 1 min. PIA was detected using the Bio-Rad ChemiDoc™ XRS (Bio-Rad, USA).

2.7.1 gDNA extraction for detection of *icaA* operon

S. epidermidis 1457 and *S. epidermidis* 1457ΔatlE were cultured overnight in 5 ml TSB. Cultures were harvested by centrifugation for 2 min at 13.200 rpm and further DNA isolation was performed with InstaGene Matrix (Bio-Rad, USA) according to the instructions of the manufacturer.

Primers for the DNA amplification reaction are given in Table 1. Polymerase chain reaction (PCR) was performed on a GeneAmp PCR System 9700 (PE Applied Biosystems, USA). Cycling conditions were as follows: preheating for 5 min at 94°C, followed by 25 cycles of 30 s at 94°C, 60 s at 60°C and 60 s at 72°C [65]. The PCR reaction mixture had a volume of 25 µl, and consisted of 12.5 µl of PCR Master Mix (Promega, USA), 10 µmol/L of each primer, 5.5 µl of nuclease-free water (Promega, USA) and 2 µl of DNA template. In the PCR, a positive control and a negative control were included. The positive control for *icaA* detection was gDNA from *S. epidermidis* 1457 and the negative control was distilled water. Amplified products were analyzed by 2% agarose gel electrophoresis.

Table 2 Primers used in this study for icaA.

Gene	Sequence (5-3')	Primer	PCR product size (bp)
icaA	CACGTGCTCTATGCTGGATG	Forward	761
	CACGTGCTCTATGCTGGATG	Reverse	

2.8 Scanning electron microscopy

Scanning electron microscopy (SEM) was performed in order to check changes in biofilm structure and bacterial viability, after treatment with antibiotics. This experiment was only conducted for S. *epidermidis* 1457.

Biofilms were grown on a cover slip disc in a 12 well-plate, with TSB as culture medium. The inoculums was prepared as described in 2.3. After 24 h of growth, biofilms were rinsed with PBS and treated with antibiotics for 24 h at 37°C, in 1/100 TSB. After 24 h, samples were rinsed with 1 ml of PBS and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylatebuffer pH 7.4 during 2 h at room temperature. After primary fixation, samples were washed with 0.1 M cacodylatebuffer and postfixed for 2 h in 1% osmiumtetroxide. Biofilms were dehydrated by immersion in an ascending ethanol series 30%, 50%, 70% and 90% (v/v) for 5 min and 100% (v/v) ethanol for 3 x 5 min. Additionally, the samples were dried with hexamethyldisilazane (HMDS, Sigma-Aldrich) for 30 min with HMDS. [66]. The samples were then transferred to a vacuum desiccators for overnight drying. Samples were mounted on aluminum stubs with carbon tape, sputter-coated with platinum (Auto sputter coater, Agar Scientific) and observed with in a scanning electron microscope (Jeol, JSM 7401F).

In order to assess biofilm morphology in each sample, three fields were used for image analysis. Images were recorded at magnifications of 1.000 x, 4.000 x, 10.000 x and 25.000 x.

2.9 Statistical analysis

Results were compared with XLSTAT software (Addinsoft, France). Significant differences were determined via one-way analysis of variance (one-way ANOVA). All tests were performed with a confidence level of 95%.

Chapter 3 Results

3.1 MIC determination

The results of the antimicrobial susceptibility testing for all five *S. epidermidis* strains are summarized in Table 3. The results of the MIC determination were compared to the EUCAST clinical breakpoint tables for *staphylococci* to determine whether the strains were sensitive or resistant (Appendix 1: Clinical Breakpoints defined by EUCAST). All the strains were susceptible to the range of antibiotics used.

Table 3 Determination of antibiotic susceptibilities by Staphylococcus epidermidis used strains in planktonic cultures (MIC)

Strain	Antibiotics concentrations (µg/mL)				
	Vancomycin	**Teicoplanin**	**Oxacillin**	**Rifampicin**	**Gentamicin**
S. epidermidis 1457	2	4	>128	>128	>128
S. epidermidis 1457ΔatlE	1	>128	>128	>128	>128
S. epidermidis 10b	1	1	>128	>128	>128
S. epidermidis 567	1	1	>128	>128	>128
S. epidermidis 567-1	2	2	>128	>128	>128

3.2 Evaluation of activity of antibiotics on biofilms

Different methods were applied to evaluate the effects of the antibiotics treatment on the biofilm biomass, bacterial cell viability, changes on biofilms matrix and changes on biofilms structure. Antibiotics concentrations used corresponded to the serum peak concentrations.

Figure 9 presents the results for CV staining of the biofilms after treatment with antibiotics. CV staining was used to quantify the total biofilm biomass which includes both cells and exopolymeric matrix. It was evident that all *S. epidermidis* strains produced biofilms.

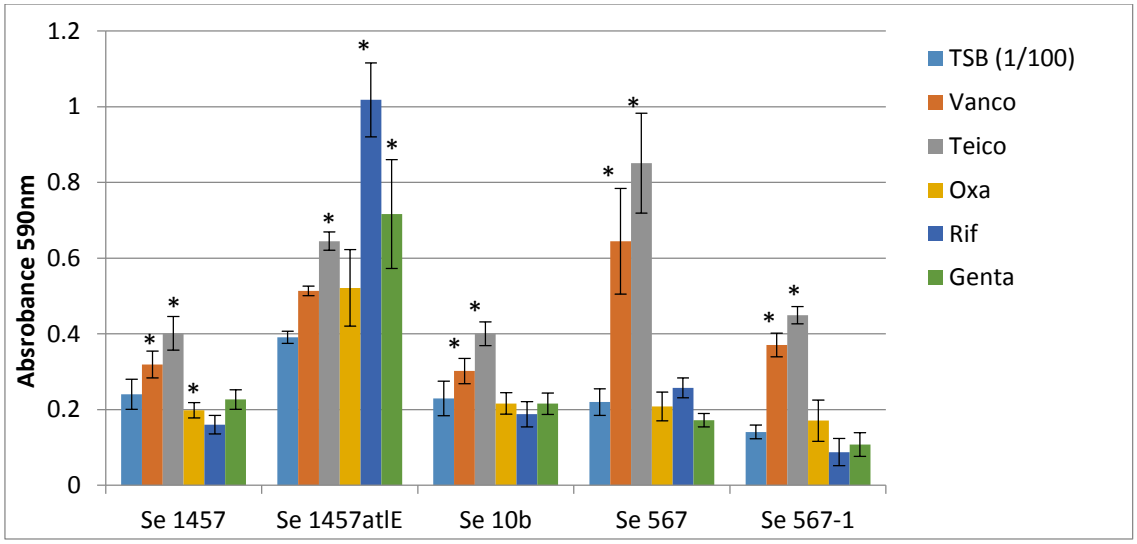

Figure 9 Absorbance values of *S. epidermidis (Se)* 1457, Se 1457atlE mutant, Se 10b, Se 567 and Se 567-1 biofilms stained with crystal violet after treatment with vancomycin (Vanco), teicoplanin (Teico), oxacillin (Oxa), rifampicin (Rif) and gentamycin (Genta). Absorbance was measured at 590nm. Error bars represent 95% confidence interval. Statistical differences compared to control (TSB 1/100) are marked with an asterisk (one-way ANOVA, p<0.05).

Treatment with rifampicin and gentamicin were not effective in reducing the total biofilm biomass significantly on all strains ($p>0.05$). Treatment with vancomycin and teicoplanin treatment resulted in a significant increase of total biofilm biomass in all strains ($p<0.05$). Treatment with oxacillin reduced significantly the total biofilm biomass only for *S. epidermidis* 1457 ($p<0.05$).

For *S. epidermidis* 1457ΔatlE, treatment with all antibiotics resulted in an increase in total biofilm biomass. The increase was significant for vancomycin, rifampicin and gentamicin ($p<0.05$).

Figure 10 presents the results of the XTT staining, showing changes in bacterial cell viability after treatment with antibiotics. Results show that all antibiotics applied reduced the bacterial cell viability.

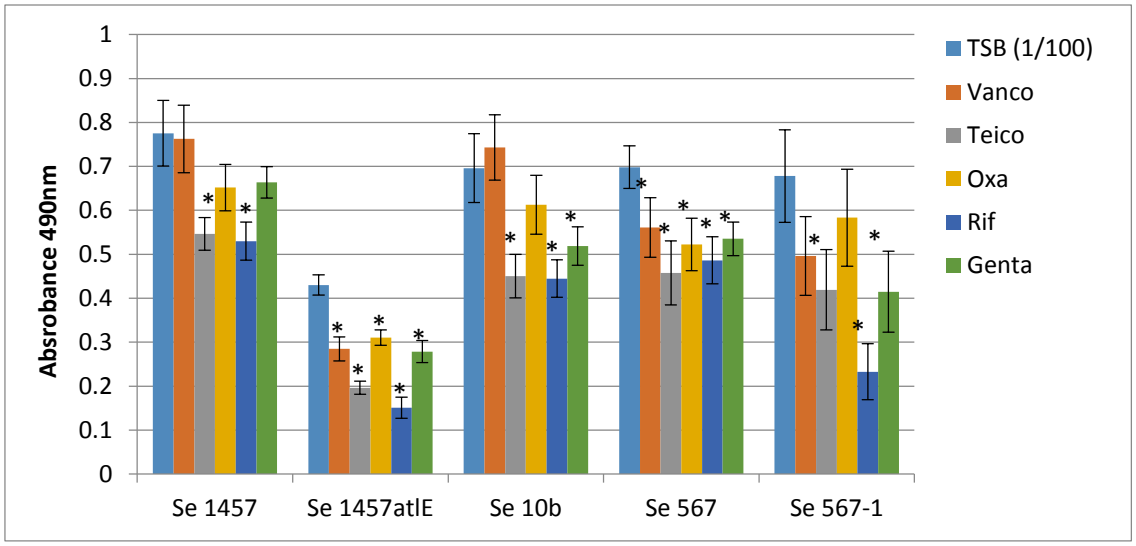

Figure 10 Absorbance values XTT solutions obtained from adhesion of *S. epidermidis (Se)* 1457, Se 1457atlE mutant, Se 10b, Se 567 and Se 567-1 strains after treatment with vancomycin (Vanco), teicoplanin (Teico), oxacillin (Oxa), rifampicin (Rif) and gentamycin (Genta). Absorbance was measured at 490 nm. Error bars represent 95% confidence interval. Statistical differences compared to control (TSB 1/100) are marked with an asterisk (one-way ANOVA,$p<0.05$).

According to Figure 10, cell viability reduction was significant in all *S. epidermidis* strains for teicoplanin, rifampicin and gentamycin, except for *S. epidermidis* 1457. For that strain the reduction was only significant for teicoplanin and rifampicin. Treatment with vancomycin and oxacillin only reduced significantly bacteria viability for *S. epidermidis* 1457ΔatlE and for *S. epidermidis* 567. In fact for those two strains, all the antibiotics reduced bacteria viability significantly.

Comparing the effect of antibiotics used against biofilm associated bacteria, it is shown that there are some significant differences between cell-wall active antibiotics (vancomycin, teicoplanin and oxacillin) and RNA and protein synthesis inhibitors (rifampicin and gentamicin, respectively).

Figure 11 summarizes the results of changes in the amount of biofilm matrix after treatment with antibiotics.

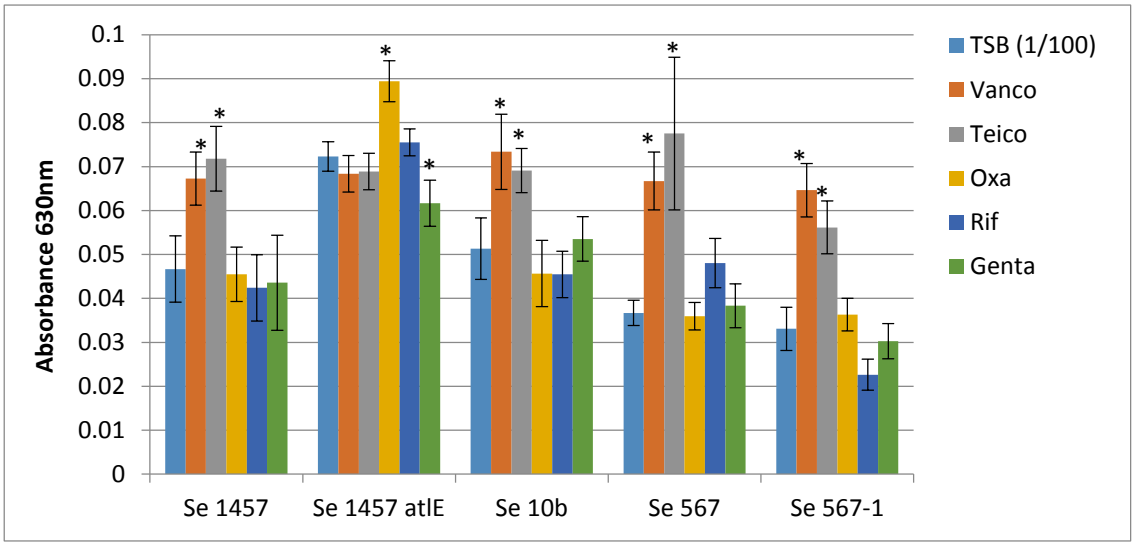

Figure 11 Absorbance values DMMB solutions obtained from adhesion of *S. epidermidis (Se)* 1457, Se 1457atlE mutant, Se 10b, Se 567 and Se 567-1 strains after treatment with vancomycin (Vanco), teicoplanin (Teico), oxacillin (Oxa), rifampicin (Rif) and gentamycin (Genta). Absorbance was measured at 630 nm. Error bars represent 95% confidence interval. Statistically different compared to control (TSB 1/100) (one-way ANOVA, $p<0.05$).

As it is possible to observe in Figure 11, vancomycin and teicoplanin significantly increased the amount of biofilms matrix present ($p<0.05$). For the other treatments (oxacillin, rifampicin and gentamycin), the increasing or reduction was not significantly shown. However, for *S. epidermidis* 1457ΔatlE, there is no increase in DMMB staining. For this strain it is remarkable that the amount of biofilms matrix increases after treatment with oxacillin and rifampicin, and reduces after treatment with gentamicin.

SEM analysis was performed to visualize changes in *S. epidermidis* 1457 biofilms morphology after treatment with antibiotics. SEM results are shown on Figure 12.

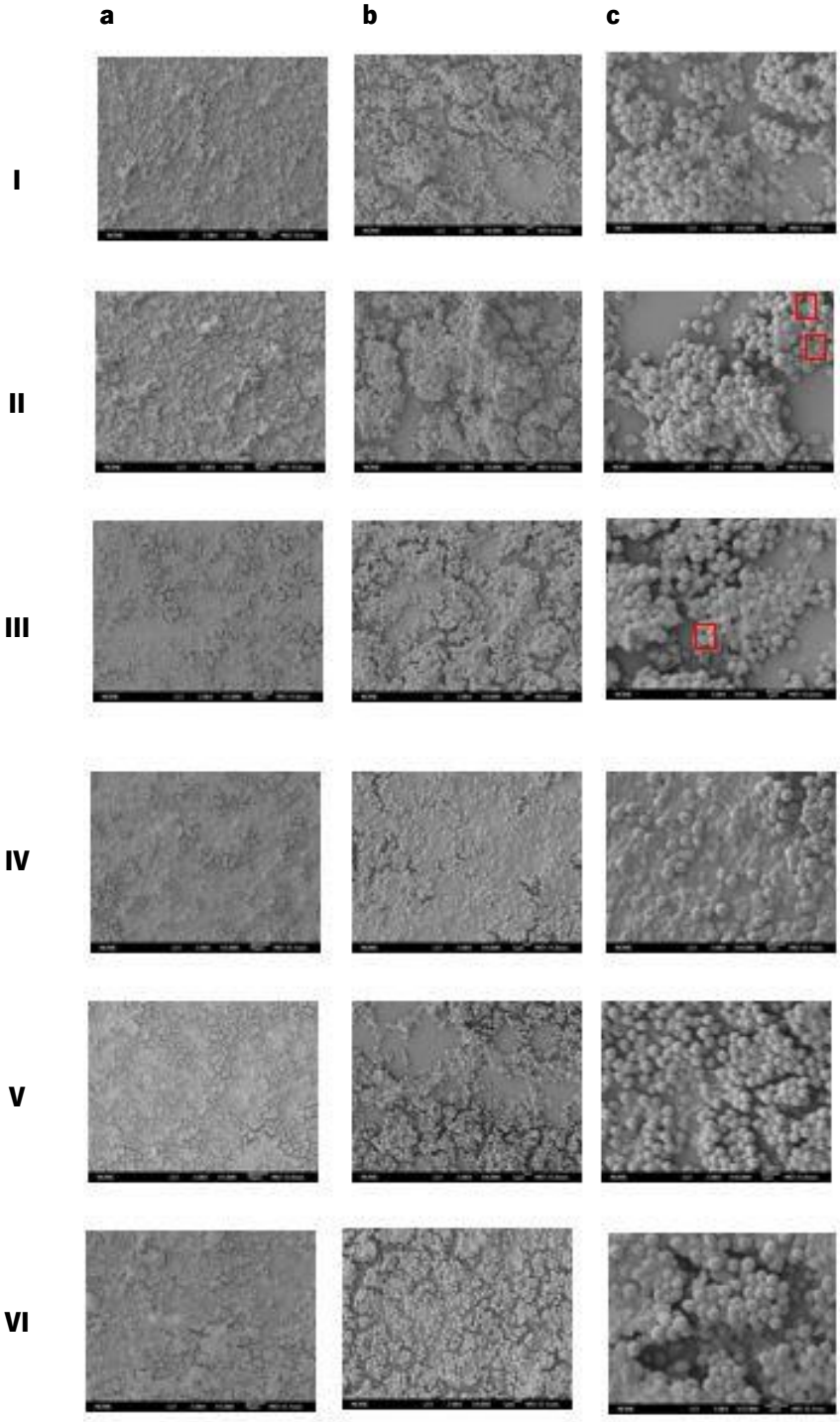

Figure 12 Representative scanning electronic microscopy photographs of *S. epidermidis* 1457 biofilms after treatment with: lane I- control (TSB 1/100); II- Vancomycin; III- Teicoplanin; IV- Oxacillin; V- Rifampicin and VI- Gentamicin. Experiments were performed in triplicate and biofilms were viewed at 1000x magnification (column a), 4000x magnification (column b) and 10000x magnification (column c).

Vancomycin treatment resulted in a rougher biofilm (Figure 12 IIa vs. Ia), with likely more bacteria attached (Figure 12 IIb vs Ib). Damaged bacteria can be identified from the creases (marked with red squares). Also treatment with teicoplanin resulted in damaged bacteria. The biofilms treated with teicoplanin look very similar to treatment with TSB 1/100 (Figure 12 IIIb vs Ib). Treatment with oxacillin (Figure 12 IV) made bacteria detach from the biofilm but the matrix was left behind. Also rifampicin treatment (Figure 12 V) resulted in bacterial detachment with matrix remaining, though to a lesser extent then oxacillin treatment. Gentamicin treatment finally (Figure 12 VI) gave a smooth biofilm containing the same amount of bacteria and matrix (Figure 12 VIb), or more (Figure 12 VIc) then the TSB 1/100 treated biofilms.

3.3 Influence of antibiotic treatment on biofilm matrix DNA and PIA

To explain the previous observations, antibiotic induced changes in the composition of the biofilm matrix were considered with a focus on extracellular DNA (eDNA) and PIA.

3.3.1 Extracellular DNA

Figure 13 shows the proportional reduction of the total biofilm biomass after treatment with DNaseI plus antibiotics, relative to the total biofilm biomass after treatment with only antibiotics.

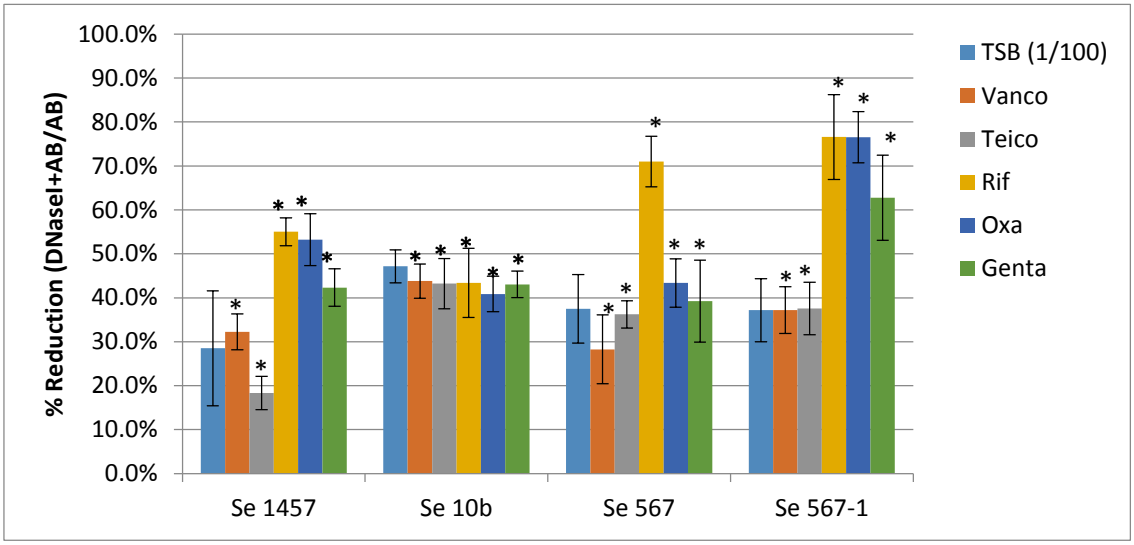

Figure 13 Percentual reduction of biofilm biomass assayed with CV staining. Absorbance reduction was compared between antibiotic treated (AB) and DNaseI combined with antibiotic treated biofilms (DNaseI+AB). Antibiotics (AB) used were Vanco – vancomycin; Teico- teicoplanin; Rif- rifampicin; Oxa- oxacillin and Genta- gentamicin.

In Figure 13 it is possible to observe that treatment of biofilms with DNaseI combined with antibiotics significantly reduced the total biofilms biomass of all strains and with all the tested antibiotics ($p<0.05$). That reduction is more notable for treatments with rifampicin for *S. epidermidis* 567 and with rifampicin, oxacillin and gentamycin for *S. epidermidis* 567-1. Those antibiotics allowed an up to 60 % reduction of biofilm biomass on the strains referred.

The effects of only antibiotic treatment on the excretion of DNA (eDNA) was quantified by extracting the eDNA out of the matrix. eDNA was quantified relatively by agarose gel electrophoresis and absolutely by spectrophotometric measurement.

Figure 14 shows a picture of the agarose gel electrophoresis of eDNA extracted from 24h old biofilms. That assay was done to check for the presence and amount of eDNA in all strains without any treatment.

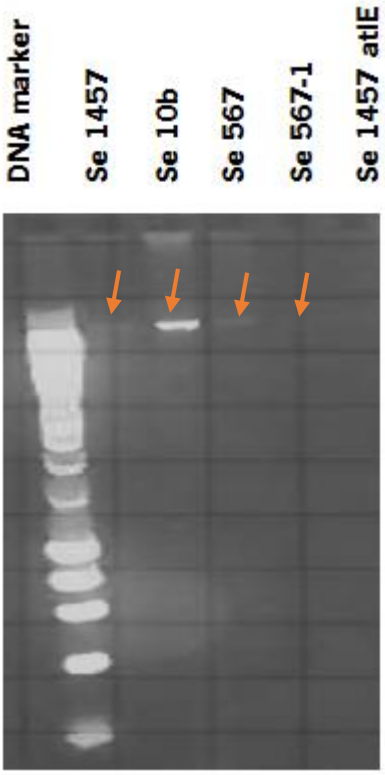

Figure 14 Gel electrophoresis of extracellular DNA extracted from all S. epidermidis strains used grown only in culture medium (Se – *S. epidermidis*).

It appeared that eDNA was present in biofilms of all strains except those produced by S. *epidermidis* 1457ΔatlE (marked with a red arrow). S. *epidermidis* 10b showed a bigger amount of eDNA, represented by a more intense band on the agarose gel.

Figure 15 shows the images of the electrophoreses of eDNA extracted from the biofilms of all strains after treatment with antibiotics.

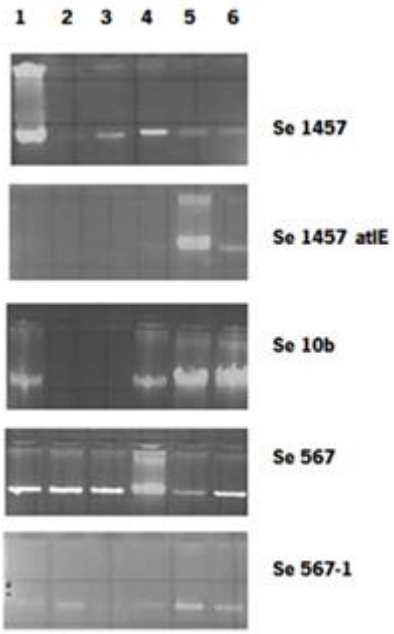

Figure 15 Gel electrophoresis of extracellular DNA extracted from different strains (Se – S. epidermidis). Lanes represent the treatments used: 1- TSB (1/100); 2- Vancomicin; 3- Teicoplanin; 4- Oxacillin; 5- Rifampicin; 6- Gentamicin.

According to Figure 15 treatment with antibiotics had an influence on eDNA excretion. Different treatments on the same strain produced different changes on the amounts of eDNA extracted. For *S. epidermidis* 1457 it is notable that treatment with teicoplanin and oxacillin renders an increase on the amount of eDNA extracted. For *S. epidermidis* 567 and *S. epidermidis* 567-1, the amount of eDNA extracted is really similar with and without treatment. Except for *S. epidermidis* 567 treated with oxacillin and rifampicin, that the amount of eDNA extracted is increased and decreased, respectively. For *S. epidermidis* 10b the amount of eDNA extracted is increased only in treatment with oxacillin, gentamycin and rifampicin, meaning that treatments with vancomycin and teicoplanin had no effect on the amount of eDNA excretion.

Lastly, for *S. epidermidis* 1457ΔatlE it was noticeable that no eDNA extracted was shown for not treated biofilms. Only for treatments with rifampicin and gentamycin it is possible to see an amount of eDNA excretion by the biofilm.

3.3.2 PIA (PIA dot-blot)

The effects of antibiotic treatments on the biofilm matrix with regard to the PIA content were assessed by PIA dot-blot.

Figure 16 shows the relative amounts of PIA in the biofilm matrix after treatment with antibiotics compared to the control (biofilms formed only in TSB (1/100)).

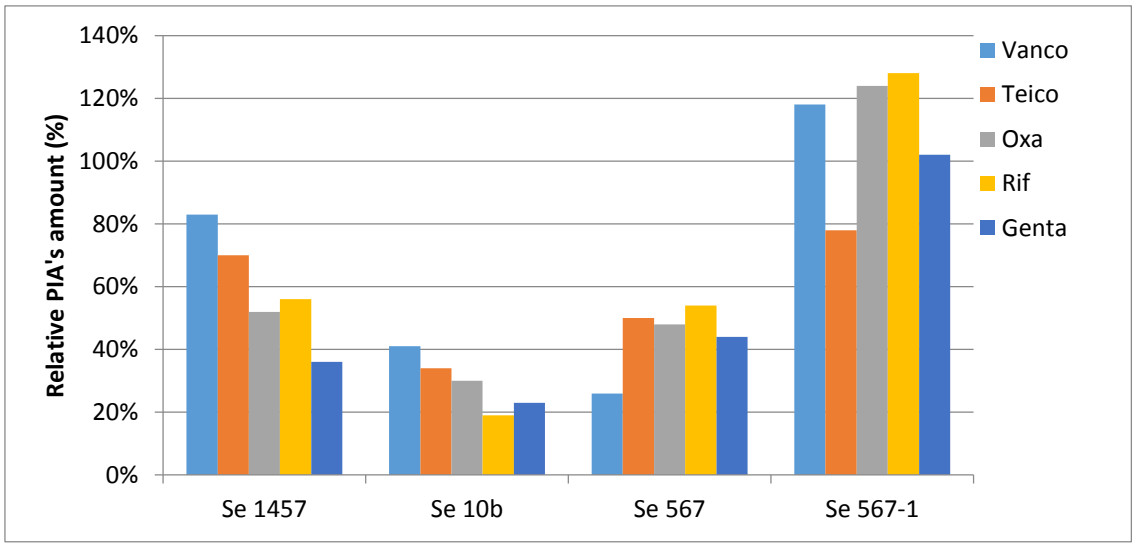

Figure 16 Relative amounts of PIA on biofilms were determined using a PIA dot blot assay after treatment with antibiotics: Vanco- Vancomycin; Teico- Teicoplanin; Oxa- Oxacillin; Rif- Rifampicin and Genta- Gentamicin. To determine the relative amounts of PIA, it was defined that the amount of PIA produced by each strain individually when treated with TSB (1/100) as 100% and expressed all other amounts as relative to the amount on that TSB (1/100) treatment.

Interestingly, for *S. epidermidis* 1457ΔatlE it was not possible to determine the relative amount of PIA because that strain did not show any PIA on the dot blot assay performed.

For *S. epidermidis* 567-1 treatment with vancomycin, oxacillin, rifampicin and gentamicin (Figure 16) there was an enhancement in the production of PIA up to more than 20% comparing with the absence of antibiotics treatment. For *S. epidermidis* 1457 and *S. epidermidis* 10b, vancomycin and teicoplanin treatment showed a higher amount of PIA when compared with treatment with the other antibiotics, although the difference is small. For *S. epidermidis* 567 the amount of PIA on vancomycin treatment is shown to be really reduced compared with the other treatments.

Figure 17 shows a representative dot blot assay for *S. epidermidis* 1457.

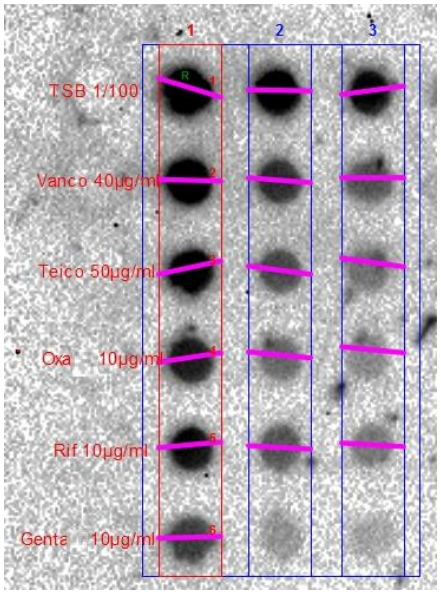

Figure 17 Representative *S. epidermidis* 1457 dot blot for determining spot volumes on each treatment: lane TSB (1/100)- control; Vanco- Vancomycin; Teico- Teicoplanin; Oxa- Oxacillin; Rif- Rifampicin and Genta- Gentamicin. Columns represent the different dilutions used: 1- 1/10; 2- 1/50; 3- 1/100.

In Figure 17 it is possible to see the differences in the amount of PIA between treatments. Results shown are comparable with those shown on Figure 16. Treatments with vancomycin and teicoplanin showed a higher amount of PIA, notable by the darker spot shown on 1/100 dilution (lane 3).

S. epidermidis 1457 ΔatlE was DMMB stained but did not show any PIA on dot-blot assay. Since PIA is the major component of the biofilms matrix and was not shown on PIA dot blot even though the mutant strain was DMMB stained, it was important to check if this strain contained the *ica* operon, needed for the production of PIA. For this purpose a PCR on *icaA* was conducted on the gDNA extracted from *S. epidermidis* 1457 ΔatlE and *S. epidermidis* 1457. Agarose gel electrophoresis of the PCR products was analysed (Figure 18).

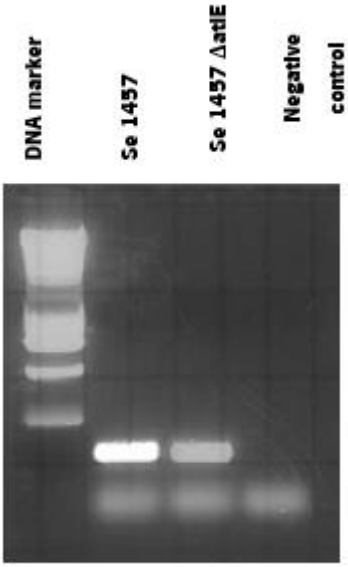

Figure 18 Gel electrophoresis result of *S. epidermidis* 1457 and *S. epidermidis* 1457 ΔatlE PCR on the icaA gene to check for its presence.

Comparing the results obtained for *S. epidermidis* 1457 and for *S. epidermidis* 1457 ΔatlE shown on Figure 18 it is possible to conclude that the mutant strain possesses the icaA operon. That result sustained the hypothesis that this strain should have the ability to produce PIA.

Chapter 4 Discussion

Staphylococcus epidermidis is the most frequently isolated species of the CoNS group. *S. epidermidis* is found on skin and mucous membranes of humans and other organisms, representing an important part of its normal microflora [16]. Previously regarded as relatively innocuous, has gained significant interest in recent years and has become one of the leading causes of infections related to implanted medical devices. Those infections have especial interest among immunocompromised, immunosuppressed and critically ill patients [15], [19], [39].

Treatment of *S. epidermidis* infections has become very difficult due to the increasing resistance to antibacterial agents. Antimicrobial resistance has a significant impact on patient outcome by enhancing virulence, delaying the administration of therapeutic medicines leading to increased morbidity and mortality [19]. It is a serious problem particularly notable in *S. epidermidis*, since many clinical isolates of this organism are resistant to up to eight different antibiotics. In addition to this problem is the fact that the major virulence factor of *S. epidermidis* is biofilm formation and that cells in biofilms are normally more resistant to antibiotics than planktonic cells, making drug resistance in a CoNS infection an even more serious problem [15], [16]. Therefore, it is crucial to understand the mechanisms of action and the changes promoted by antibiotics on biofilms, in order to better address this problem of antibiotic resistance.

4.1 Antibiotic susceptibility

Firstly, it became important to determine if the chosen antibiotics had an effect on the *S. epidermidis* strains used. Therefore, an assay to determine the MIC concentration for planktonic cells was carried out. Although standard antimicrobial susceptibility testing, which challenges planktonic (suspended) cells with an antimicrobial agent, will not accurately predict the efficacy of an agent against biofilm associated organisms, it is important in order to understand if the strain is naturally susceptible to the antibiotic or not.

To evaluate the efficacy of the antibiotics used in this study, a concentration range was chosen. These values were well defined in the standard [67] endpoints for antimicrobial susceptibility testing and thus enable evaluation of the efficacy of antibiotics against planktonic microorganisms. Results shown on Table 3 make it is possible to conclude that all the *S. epidermidis* strains used were susceptible to the antibiotics. It is important to note that the results are consistent with clinical breakpoints defined by EUCAST for *S. epidermidis* (Appendix 1: Clinical Breakpoints defined by EUCAST) [67].

Regarding the results for each strain separately, for *S. epidermidis* 1457 and *S. epidermidis* 10b the results are according to the expect comparing with the literature [68], [45]. MIC results for *S. epidermidis* 1457ΔatlE treated with vancomycin show to be different from the MIC for the wild-type strain. According to Hello et al. [69] that was not an expected result. In their study it was found that the altE mutant susceptibility to vancomycin was similar to the wild-type strain susceptibility. That difference found can maybe be explained by the fact that the mutant strain

4.2 Evaluation of antibiotics activity on biofilms

The pathogenesis of medical device infections associated with *S. epidermidis* is characterized by the microorganism's ability to colonize the surface of implanted medical devices by the formation of highly resistant biofilms [19]. Biofilm formation is, as mentioned, the major virulence factor in *S. epidermidis* infections and therefore it is of especial interest to address the mechanisms of action of the antimicrobial treatment applied. In this study, to evaluate the effect of antibiotics, it was necessary to discriminate between the biofilm matrix and the bacteria living within it. That is from a major importance because it is documented that when an antibiotic only acts on the slime layer, biofilm bacteria will start forming a new matrix almost immediately and if only biofilm bacteria are killed, the remaining matrix is predestined to become re-colonized [70].

The CV, XTT and DMMB assays were used in this study in order to determine the influence of antibiotic treatments on the biofilms, whether on the biofilm biomass, bacteria viability and biofilms matrix, respectively. The different antibiotics used and its different mechanisms of action are shown in Table 1 and explained in Chapter 1.5.1.

In particular, CV staining was used to evaluate the effect of antibiotics treatment on biofilms, regarding the changes on total biofilm biomass. This assay was important in order to conclude if the antibiotics had an effect on increasing or decreasing the total biofilm biomass formed compared to the control (cells growing only in the presence of TSB 1/100). Results obtained in the CV assay allowed to conclude that all *S. epidermidis* strains produced biofilms (Figure 9), as it was expected [15], [17].

Evaluating the strains capacity to form biofilms without any antibiotic treatment, it was possible to detect some changes in relation to the expectable results. For instances, *S. epidermidis* 1457 ΔatlE formed more biofilm biomass than *S. epidermidis* 1457 (its wild-type). Since the extracellular DNA is found as a major component required for initial bacterial attachment to surfaces, as well as for the subsequent early phase of biofilm development by *S. epidermidis*, it was expectable that the mutant strain formed less biofilm. That was the expectable result because the release of extracellular DNA is known to be mainly caused by the activity of the autolysin AtlE. Since *S. epidermidis* 1457 ΔatlE lacks on the atlE gene it was supposed to have less bacteria attached to the surface [27]. These results can maybe be explained by the media growth dilution used for the wild-type strain and not on the mutant strain, giving it more nutrients to colonize the surface of the

plate and reproduce. That difference in the growth media is related to the protocol for *S. epidermidis* 1457 ΔatlE biofilms cultivation referred by Qin et al. [27]

Results presented in Figure 9 also showed that biofilms of *S. epidermidis* 567 and *S. epidermidis* 567-1 have a very similar biomass. That result was not expectable, according to the literature, since the *agr* mutant, *S. epidermidis* 567-1, in previous studies exhibited a stronger biofilm formation [55]. Since the aim of this study was to evaluate the influence of antibiotics treatment on *S. epidermidis* biofilms, those results are of less importance and are only related to the control biofilms.

In fact, in the same strain the differences noted on the biofilm biomass were caused by the antibiotics treatments applied. Different antibiotics had different effects on each strain. For instances, by CV staining on Figure 9, it is noticeable that vancomycin and teicoplanin treatments had an effect, significantly increasing the total biofilm biomass on all strains. That result has already been documented by Hsu [44]. This vancomycin and teicoplanin tolerance of *S. epidermidis* was attributed to the poor activity of glycopeptides related to a diminished antimicrobial effect on *S. epidermidis* cells in biofilm already documented by Farber et al. [71].

The biofilm biomass enhancement caused by vancomycin and teicoplanin, in this study, can be explained by an increased excretion of extracellular DNA or and increased production of PIA caused by the action of those antibiotics on the cell wall. Increased excretion of eDNA enhance biofilm formation and adhesion cell-to-cell. The DNA in the extracellular space, released by the action of the antibiotics, can aggregate, therefore enhancing biofilms formation. The suggested increasing on PIA's production can be related to the rupture of the cell wall due to the antibiotics action. That can enhance PIA's production and creation of a linked connection between bacterial cells [44].

Only for *S. epidermidis* 1457 oxacillin treatment decreased significantly the total biofilm biomass formed. That can imply that this antibiotic has a bigger antimicrobial effect on reducing the total biofilm biomass formed compared to the other two cell wall active antibiotics, for this strain in particular.

S. epidermidis 1457 ΔatlE showed also more biofilm biomass comparing with all the other strains used in this work, except for *S. epidermidis* 567 treated with vancomycin and teicoplanin. Also in *S. epidermidis* 1457ΔatlE strain, treatment with teicoplanin, rifampicin and gentamycin had a significant increase on biofilm biomass. As this strain lacks on atlE gene, the biofilm formed

cannot be explained by the extracellular DNA release [27]. So, that may imply that the large amount of biofilm formed is due to the increased production of PIA.

The crystal violet assay has been extensively used, but likely because the estimated biomass does not distinguish between dead or living bacteria, it is not very informative when not complemented with a viability assay. Even with the increasing biofilms biomass detected it was important to determine if the antibiotics had an antimicrobial effect on the biofilm associated bacteria. That is why it was important to add the XTT assay. This method allows to stain only active bacteria cells in the formed biofilm, which means that it quantifies the bacteria in biofilms that have the capacity to continue to grow and proliferate the infection. According to Figure 10, it was expected all antibiotic treatments reduced bacteria viability for all strains since they were proven to be susceptible to the antibiotics in use by the MIC determination. Those results make it possible to conclude that all the antibiotics were successful on killing biofilm associated bacteria, suggesting that the increasing CV staining found on the biofilms, and therefore a large amount of biofilm biomass, had to be related to the increased amount of dead cells and biofilms matrix.

Comparing the effects on killing biofilm associated bacteria by different antibiotics, it is possible to conclude that for *S. epidermidis* 567 and *S. epidermidis* 10b, teicoplanin, rifampicin and gentamicin had a similar effect.

For *S. epidermidis* ΔatlE comparing to the wild-type, it is possible to see that the mutant strain was more susceptible to killing by antibiotics. In fact, it is possible to conclude that cell-wall active antibiotics had an increased effect on bacteria killing on that mutant strain. That result is supported by the literature that reported an implication of AtlE loss in the tolerance of *S. epidermidis* to cell wall active antibiotics [68].

To the CV and XTT staining methods, it was added the DMMB staining method. The aim of this staining method was the evaluation of the changes on the biofilms matrix in addition with the other two assays performed. DMMB is a cationic dye used for quantification of sulphated polysaccharides, more specifically glycosaminoglycans (GAGs) in biological samples. As there is structural similarity between PIA and GAGs, it was hypothesized by Toté et al. [58] that DMMB could be used for the specific detection of *S. aureus* biofilm matrix. Since PIA is also the major component of *S. epidermidis* biofilms matrix, it was also used in this study to detect PIA's content on the biofilms [72]. As seen before treatment, cell-wall active antibiotics, as vancomycin and

teicoplanin, had an effect on increasing biofilms' biomass. With the DMMB, Figure 11, it is possible to see that treatments with those antibiotics had an obvious effect on increasing the amount of biofilms matrix. Those cell-wall active antibiotics act on the bacteria cell-wall causing the membrane rupture and enhancing matrix production [73].

Besides the effects on biofilms biomass, bacteria viability and biofilms matrix, the changes promoted by antibiotics treatments in biofilms were visualized by scanning electron microscopy (SEM). The changes on *S. epidermidis* morphology were evaluated using 1457 *S. epidermidis* strain. This strain is known as a biofilm forming strain that produces a thick biofilm [53] and thus it was chosen to better illustrate the effects of the antibiotics on the biofilm structure, cell viability and the biofilm matrix through SEM analysis (Figure 12). According to the results, vancomycin treatment resulted in a rougher biofilm (Figure 12 IIa vs. Ia), with likely more bacteria attached (Figure 12 IIb vs Ib). It is possible to visualize some damaged bacteria but the main conclusion is that treatment with this antibiotic promoted a cell wall thickening. That result has been already documented by Hsu [44], that proved that treatment with vancomycin enhanced the biofilm thickness. That result for vancomycin treatment is also supported by the results obtained after CV, XTT and DMMB staining. This strain showed an higher biofilm biomass formed after CV staining, with a small effect on bacteria killing assayed by XTT staining. Even with DMMB staining, this strain showed a significant increase on the biofilm matrix after treatment with vancomycin.

Treatment with teicoplanin had an effect on increasing biofilms thickness even if in a less extent as vancomycin treatment. It also resulted in damaged bacteria. The biofilms treated with teicoplanin look very similar to treatment with TSB (1/100) (Figure 12 IIIb vs Ib). These SEM results are in agreement with the previous results obtained in CV, XTT and DMMB staining. It is noticeable the increased amount of biofilms biomass and biofilms matrix, as assayed by CV and DMMB staining methods.

Treatment with oxacillin (Figure 12 IV) and rifampicin (Figure 12 V) resulted in bacterial detachment with matrix remaining. Gentamicin treatment finally (Figure 12 VI) gave a smooth biofilm containing the same amount of bacteria and matrix or more then the TSB (1/100) treated biofilms. These results are in accordance with the ones obtained after the staining methods applied.

4.3 Influence of antibiotic treatment on biofilm matrix DNA and PIA

As mentioned by Qin et al. [27], in the process of biofilm formation, the cell-cell aggregation and the formation of a multilayered architecture, some matrix components such as eDNA and PIA are of utmost importance. In this study and to explain the changes on the biofilms after treatment with antibiotics it was important to assess the induced changes. Therefore it was evaluated if the changes on biofilms matrix regarding the extracellular DNA (eDNA) and the PIA's content.

The eDNA is found as a major component required for initial bacterial attachment to surfaces, as well as for the subsequent early phase of biofilm development. In antibiotic treatments it is important to understand if the eDNA release and consequent biofilm formation is affected. In this study, biofilms were treated with a combination of antibiotics plus DNase I and compared with the antibiotics treatment only. Previous studies showed that DNase I inhibits *S. epidermidis* biofilm formation, which suggests that eDNA can function as an adhesive component of the *S. epidermidis* biofilm matrix [27], [35]. Evidence suggests that eDNA is generated in *S. epidermidis* populations through AtlE-mediated lysis of a subpopulation of the bacteria, and the extracellular DNA promotes biofilm formation of the remaining population [27].

From results shown on Figure 13, it was found that DNase I plus antibiotics treatment efficiently inhibited biofilm formation by *S. epidermidis* 567 and 567-1 strains. For *S. epidermidis* 567 the inhibition was more noticeable for DNase I treatment plus rifampicin. For *S. epidermidis* 567-1 the inhibition was higher for the combined treatment of DNase I plus rifampicin, gentamicin and oxacillin. That data indicates that the destruction of eDNA by DNase I leads to a decrease in the matrix, and as a result, antibacterial agents act more effectively to reduce the biofilm biomass as it was previously proved Tetz et al. [59].

Although the percentual reduction was higher for the antibiotics referred above, also for the other strains and other treatments used, the percentual reduction was up to 30%. Only for S. *epidermidis* 1457 treated with teicoplanin and *S. epidermidis* 567 treated with vancomycin, the reduction was lower.

There are evidences that release of extracellular DNA is mainly caused by the activity of the autolysin atlE [27]. So, as it was expected, for S. epidermidis 1457ΔatlE there was no eDNA shown on Figure 14. The S. epidermidis 1457ΔatlE lacks on the atlE gene. In Figure 15, for S. epidermidis atlE mutant strain there is some eDNA shown on treatments with rifampicin and gentamicin. That

result can be explained because treatment with antibiotics renders the bacteria more sensitive to lysis during eDNA extraction procedure. Lysis of the bacteria, possibly during vortexing, can mean that it is not only eDNA that is being extracted but also intracellular DNA. That is the reason why the results from this method are not completely reliable.

Besides the activity on eDNA release, antibiotics treatment also promoted PIA's production. That can also be one of the explanations for the antibiotics activity on biofilms. PIA was found as the major component involved in intercellular adhesion, essential for the accumulation of the biofilms [19]. That is why it is so important to determine the effect of the antibiotics treatment on PIA's production because it is related to the capacity of the biofilms to accumulate on a surface.

In PIA dot-blot assay it was shown that for S. epidermidis 567-1, treatment with vancomycin, oxacillin, rifampicin and gentamicin (Figure 16) induced an enhancement in the production of PIA up to more than 20% comparing with the absence of antibiotics treatment. Those results can explain the increased amount of biofilm formed by that strain showed on Figure 9, especially for vancomycin treatment. In fact, these results allow to conclude that the amount of biofilm biomass formed was mainly due to the increased production of PIA, promoted by that cell-wall active antibiotic. Those results are in concordance with previous studies [44]. In a less extent difference, for S. epidermidis 1457 and S. epidermidis 10b, vancomycin and teicoplanin treatment showed a higher amount of PIA when compared with treatment with the others antibiotics. That result can be related with an enhancing production of PIA promoted by the action of those For S. epidemidis 567 the amount of PIA on vancomycin treatment is shown to be really reduced compared with the other treatments. That implies that the increased biofilm formation was due to an increased excretion of eDNA promoted by that antibiotic on 567 S. epidermidis strain.

In this assay, it was not possible to assess the amount of PIA on S. epidermidis 1457 ΔatlE. In fact, that strain was DMMB stained with an higher amount of matrix compared to the wild-type strain but did not show any PIA on dot-blot assay. For that reason it was important to check if this strain contained the icaA operon, needed for the production of PIA. Results shown on Figure 18 proved that the mutant strain possesses the icaA operon and therefore should be able to produce PIA. Even though the *icaA* operon is present it is possible that it is not expressed. As documented by Conlon et al. [74] the *icaR* gene can be the explanation for the regulation of *icaA.* IcaR product is a transcriptional repressor which plays an adaptive role in *S. epidermidis* biofilm formation by modulating the regulation of *ica* expression.

Another data is related to the fact that this mutant strain produced a large amount of biofilm biomass even in comparison with the other strains. That result had already suggested that the increased biofilm biomass formed assayed by CV staining, was related with the increased amount of PIA's production. It can be assumed that the strains is *icaADBC-* and so as a PIA-independent mechanism of biofilm formation. That result have already been documented for other *S. epidermidis* strains and Kogan et al. [11] suggested that in the biofilm formation it was involved the product of a limited proteolysis of the accumulation-associated protein (Aap).

Chapter 5 Conclusion and future perspectives

Staphylococcus epidermidis is the most frequently isolated species of the CoNS group. Previously regarded as relatively innocuous, has gained significant interest in recent years and has become one of the leading causes of infections related to implanted medical devices. Treatment of *S. epidermidis* infections has become very difficult due to the increasing resistance to antibacterial agents. It is a serious problem particularly notable in *S. epidermidis*, since many clinical isolates of this organism are resistant to up to eight different antibiotics. Therefore, it is crucial to understand the mechanisms of action and the changes promoted by different antibiotics on biofilms, in order to better address this problem of antibiotic resistance. So, this work aims to evaluate the influence of antibiotics on *S. epidermidis* biofilms in terms of changes in matrix composition after treatment, regarding the eDNA and the PIA content.

Regarding the MIC results, it was proven that all the *S. epidermidis* strains used were susceptible to all the antibiotics chosen for this study (vancomycin, teicoplanin, oxacillin, rifampicin and gentamicin). Those results for planktonic cells were also supported by the results obtained after XTT staining assay since all the antibiotics were successful on killing biofilm associated bacteria.

In relation to the biofilm biomass content and changes, results of crystal violet assay made it possible to conclude that all *S. epidermidis* strains produced biofilms and that the changes on biofilm biomass were promoted by antibiotics treatment. It was noticeable that vancomycin and teicoplanin treatments had an effect, significantly increasing the total biofilm biomass on all strains. Those glycopeptide had an effect on increasing biofilms biomass and by DMMB staining methods it become obvious that they had an effect on increasing the amount of biofilms matrix because they promote the membrane rupture and enhanced matrix production.

The changes promoted by antibiotics treatments in biofilms were visualized by scanning electron microscopy (SEM) on 1457 *S. epidermidis* strain. This strain was chosen because it forms a thicker biofilm and thus better illustrates the effects of the antibiotics on the biofilm structure, cell viability and the biofilm matrix through SEM analysis. According to the results, vancomycin and teicoplanin treatments resulted in a rougher biofilm, proving that treatment with vancomycin enhanced the biofilm thickness.

Concerning the differences promoted by antibiotics, the influence on matrix components such as eDNA and PIA were evaluated. From results it was found that DNase I plus rifampicin and gentamicin efficiently inhibited biofilm formation. That data indicated that the destruction of eDNA

by DNase I leads to a decrease in the matrix, and as a result, antibacterial agents act more effectively to reduce the biofilm biomass on protein synthesis inhibitor antibiotics.

Besides the activity on eDNA release, antibiotics treated biofilms were also tested by its PIA's production pattern changes after antibiotic treatment. The results obtained allowed the conclusion that the increased biofilm biomass formed was mainly due to the increased production of PIA, promoted by glycopeptide antibiotics, as vancomycin and teicoplanin.

In general, it was possible to conclude that *S. epidermidis* biofilms from different strains showed an enhanced resistance to the application of treatment with glycopeptide antibiotics. Those antibiotics are normally reserved for use against multi-resistant staphylococci, however from this study data and from previous studies, it is at major importance to have a better understanding of the resistance mechanisms and the look for different alternatives.

As future work, it would be interesting to perform an analysis with further precision to the *S. epidermidis* 1457 ΔatlE that lacks on the atlE gene, seen as an important component of biofilm formation. It would be as of major interest to better understand the mechanisms of biofilm formation regarding that strain and perform different and more precise analysis to determine what are the mechanisms that prevent PIA's production by that strain.

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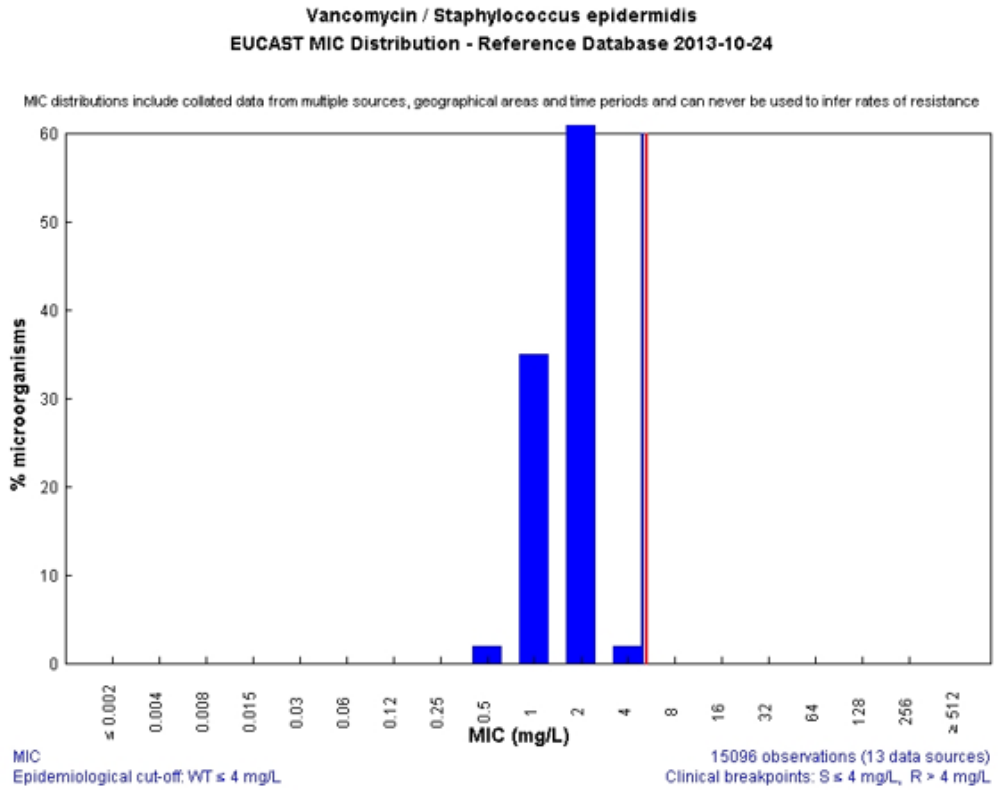
Appendix

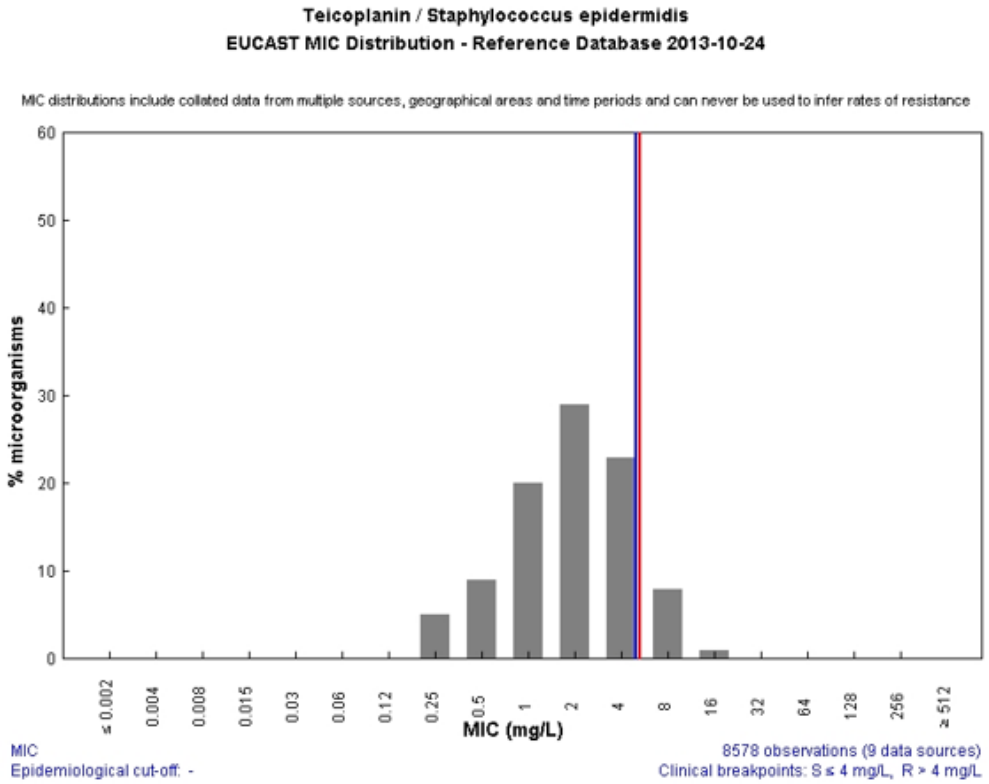
Appendix 1: Clinical Breakpoints defined by EUCAST

A.1 Clinical breakpoints for **a)** Vancomycin; **b)** Teicoplanin; **c)** Oxacillin; **d)** Rifampicin and **e)** Gentamicin, against *Staphylococcus epidermidis*.

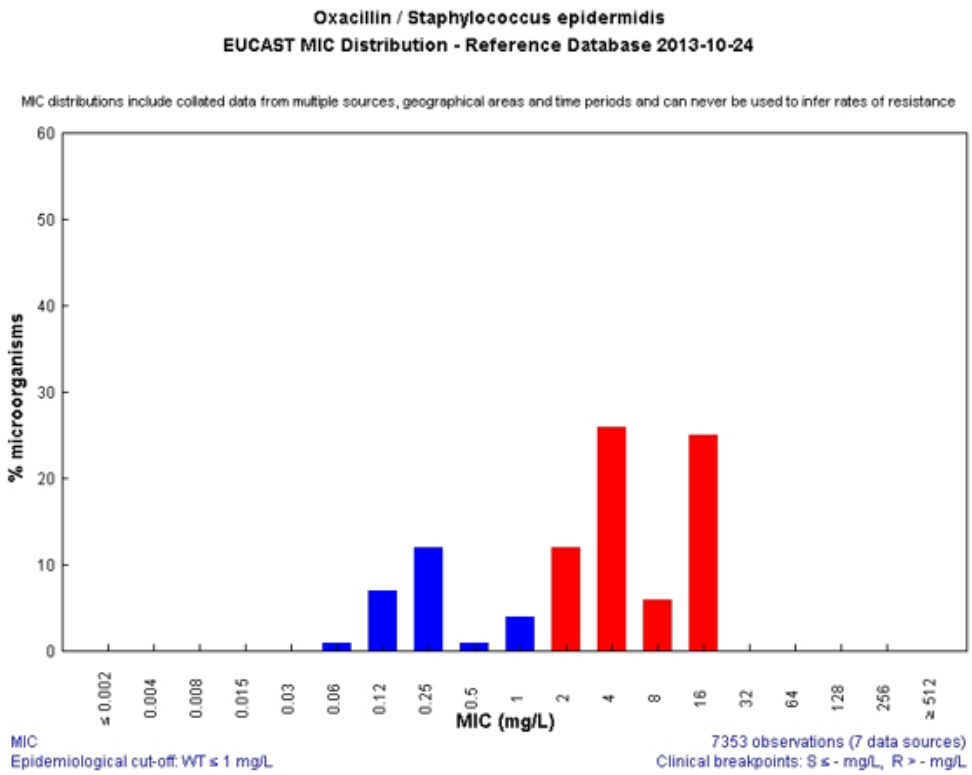
a)



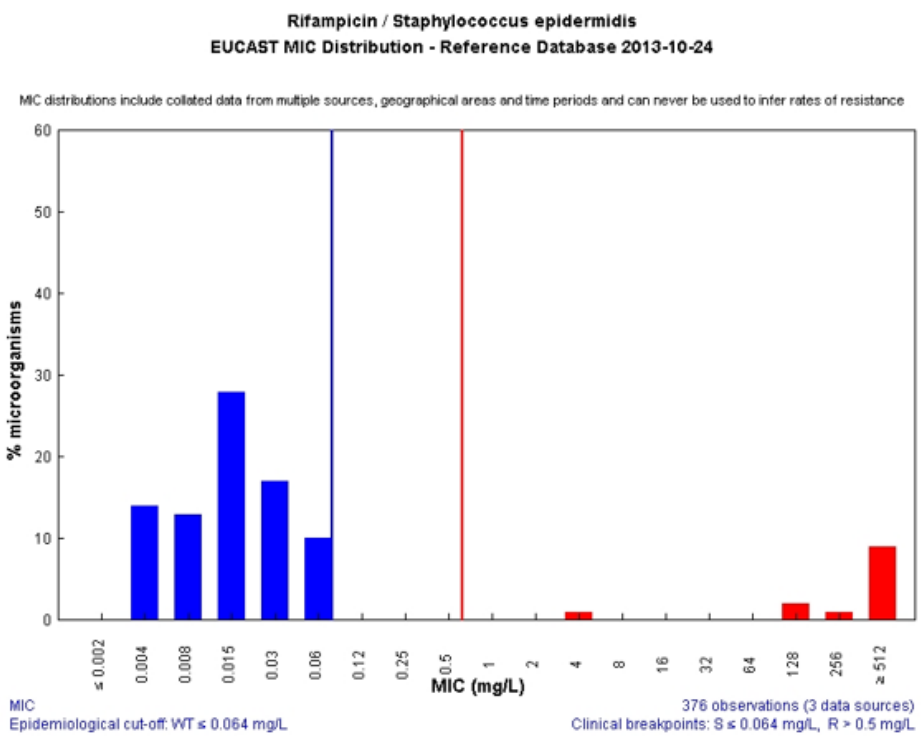
b)


Teicoplanin / Staphylococcus epidermidis
EUCAST MIC Distribution - Reference Database 2013-10-24
MIC distributions include collated data from multiple sources, geographical areas and time periods and can never be used to infer rates of resistance
% microorganisms
0
10
20
30
40
50
60
≤ 0.002
0.004
0.008
0.015
0.03
0.06
0.12
0.25
0.5
1
2
4
8
16
32
64
128
256
≥ 512
MIC (mg/L)
MIC
Epidemiological cut-off: -
8578 observations (9 data sources)
Clinical breakpoints: S ≤ 4 mg/L, R > 4 mg/L

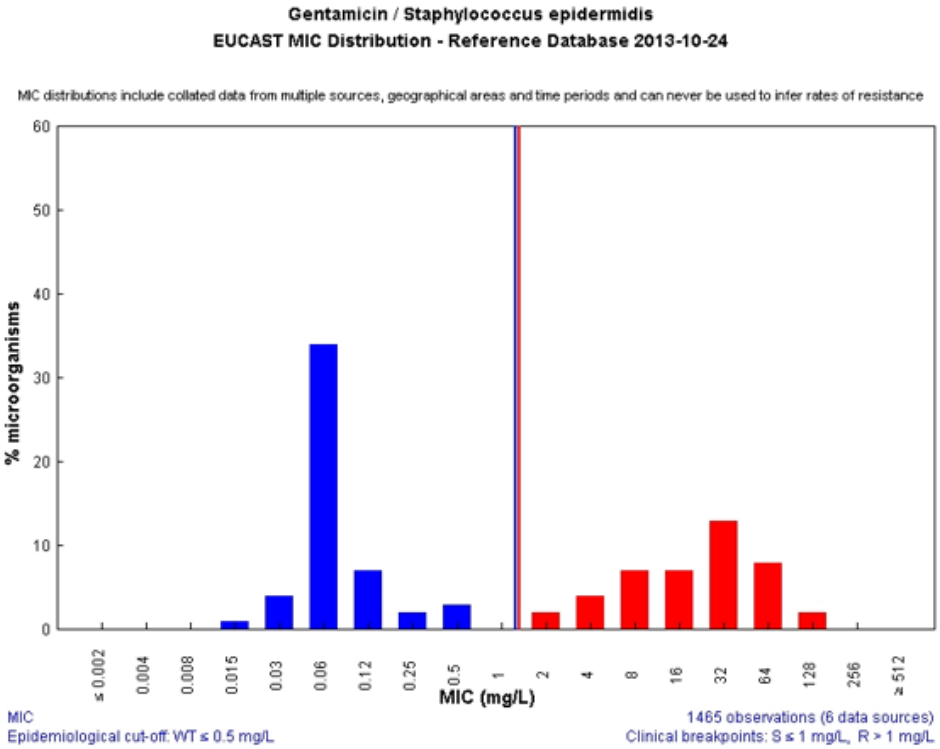
c)


Oxacillin / Staphylococcus epidermidis
EUCAST MIC Distribution - Reference Database 2013-10-24
MIC distributions include collated data from multiple sources, geographical areas and time periods and can never be used to infer rates of resistance
% microorganisms
60
50
40
30
20
10
0
≤ 0.002
0.004
0.008
0.015
0.03
0.06
0.12
0.25
0.5
1
2
4
8
16
32
64
128
256
≥ 512
MIC (mg/L)
MIC
Epidemiological cut-off: WT ≤ 1 mg/L
7353 observations (7 data sources)
Clinical breakpoints: S ≤ - mg/L, R > - mg/L

d)


Rifampicin / Staphylococcus epidermidis
EUCAST MIC Distribution - Reference Database 2013-10-24
MIC distributions include collated data from multiple sources, geographical areas and time periods and can never be used to infer rates of resistance
% microorganisms
MIC (mg/L)
≤ 0.002
0.004
0.008
0.015
0.03
0.06
0.12
0.25
0.5
1
2
4
8
16
32
64
128
256
≥ 512
MIC
Epidemiological cut-off: WT ≤ 0.064 mg/L
376 observations (3 data sources)
Clinical breakpoints: S ≤ 0.064 mg/L, R > 0.5 mg/L

e)


Gentamicin / Staphylococcus epidermidis
EUCAST MIC Distribution - Reference Database 2013-10-24
MIC distributions include collated data from multiple sources, geographical areas and time periods and can never be used to infer rates of resistance
% microorganisms
MIC (mg/L)
≤ 0.002
0.004
0.008
0.015
0.03
0.06
0.12
0.25
0.5
1
2
4
8
16
32
64
128
256
≥ 512
MIC
Epidemiological cut-off: WT ≤ 0.5 mg/L
1465 observations (6 data sources)
Clinical breakpoints: S ≤ 1 mg/L, R > 1 mg/L