Universidade do Minho
Escola de Engenharia

Vânia da Silva Gaio

Study of the susceptibility to antibiotics of cells released from Staphylococcus epidermidis biofilms

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DECLARAÇÃO

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ABSTRACT

Worldwide, *Staphylococcus epidermidis* has been recognized as a leading cause of several clinically relevant infections, primarily associated with its notable ability to colonize surfaces and form biofilms, especially in the surface of medical indwelling devices. The formation of bacterial biofilms, which is a major concern in health care systems due to their high tolerance to antibiotics, may be divided in three mains stages: 1) adhesion, 2) maturation and 3) biofilm disassembly. During the last stage, cells are released from the biofilm to the surrounding environment by both active and passive mechanisms, often being associated with the development of serious complications as bacteremia and embolic events of endocarditis. Despite the clinical relevance of biofilm-released cells (Brc), disassembly remains the least studied stage of the biofilm lifecycle and little is known concerning the phenotypic changes that these cells undergo after being released from the biofilm. Thus, this study aimed to provide a better characterization of *S. epidermidis* Brc phenotype, in particular its susceptibility to different classes of antibiotics (cell wall, nucleic acids and protein synthesis inhibitors). By directly quantifying the susceptibility of Brc and comparing to that of biofilm and stationary planktonic cells, this study allowed to demonstrate that Brc exhibit a distinct antibiotic tolerance profile. Moreover, it was found that Brc seem to have a transient phenotype, strengthening the vision of a biofilm lifecycle with individual cell physiology changing overtime. Overall, this study provided some clinically relevant outcomes in the pathogenesis of biofilm-related infections, demonstrating that the metabolic state of *S. epidermidis* cells has an important impact on antimicrobial susceptibility, and this is not only related to the distinct features of intact biofilms and planktonic cells. A better characterization of the Brc phenotype may help in the development of more efficient therapeutic measures against *S. epidermidis* biofilm-related infections.

KEYWORDS: *STAPHYLOCOCCUS EPIDERMIDIS*, BIOFILM DISASSEMBLY, BIOFILM-RELEASED CELLS, ANTIBIOTIC TOLERANCE

SUMÁRIO

A espécie *Staphylococcus epidermidis* tem sido reconhecida, a nível mundial, como uma das principais causas de infeções clinicamente relevantes, principalmente devido à sua capacidade eminente para colonizar superfícies e formar biofilmes, especialmente em dispositivos médicos invasivos. A formação de biofilmes bacterianos, que está associada a um aumento da tolerância a antibióticos, pode ser dividida em três etapas: 1) adesão, 2) maturação e 3) dispersão do biofilme. Durante a última etapa, as células são libertadas do biofilme para o ambiente envolvente por mecanismos ativos e passivos, sendo frequentemente associadas ao desenvolvimento de complicações sérias como bacteriemia e eventos embólicos relacionados com endocardite. Apesar da relevância clínica da dispersão das células libertadas do biofilme (Brc), esta etapa continua a ser a menos estudada do ciclo de vida do biofilme e pouco é sabido acerca das alterações fenotípicas das Brc. Assim, este estudo teve como objetivo proporcionar uma melhor compreensão acerca do fenótipo das Brc de *S. epidermidis,* em particular a sua suscetibilidade a diferentes classes de antibióticos (inibidores da síntese da parede celular, de ácidos nucleicos e de proteínas). Ao quantificar diretamente a suscetibilidade das Brc em comparação à das células do biofilme e planctónicas estacionárias, este estudo permitiu demonstrar que as Brc exibem um perfil distinto de tolerância aos antibióticos. Adicionalmente, foi verificado que as Brc parecem apresentar um fenótipo transiente, reforçando a ideia de um ciclo de vida do biofilme com uma particular fisiologia das células que é alterada ao longo do tempo. De uma forma geral, este estudo forneceu conclusões clinicamente relevantes acerca da patogénese de infeções associadas aos biofilmes, demonstrando que o estado metabólico das células de *S. epidermidis* tem um impacto importante na suscetibilidade a antimicrobianos, facto que não está apenas relacionado com as caraterísticas distintas dos biofilmes intactos e das células planctónicas. Uma melhor caracterização do fenótipo das Brc pode auxiliar no desenvolvimento de medidas terapêuticas mais eficientes contra infeções relacionadas com biofilmes de *S. epidermidis.*

PALAVRAS-CHAVE: *STAPHYLOCOCCUS EPIDERMIDIS*, DISPERSÃO DO BIOFILME, CÉLULAS LIBERTADAS DO BIOFILME, TOLERÂNCIA A ANTIBIÓTICOS

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List of Abbreviations

Agr. Accessory gene regulator

ANOVA. Analysis of variance

Brc. Biofilm-released cells

BSAC. British Society for Antimicrobial Chemotherapy

CFU. Colony forming units

CLSI. Clinical and Laboratory Standards Institute

CoNS. Coagulase-negative staphylococci

DNA. Deoxyribonucleic acid

EPS. Extracellular polymeric substances

EUCAST. European Committee on Antimicrobial Susceptibility Testing

I. Clinically intermediate

MIC. Minimum inhibitory concentration

NCCLS. National Committee for Clinical Laboratory Standards

OD. Optical density

PIA. Polysaccharide intercellular adhesin

PNAG. Poly-N-acetyl-glucosamine

PSC. Peak serum concentration

PSMs. Phenol-soluble modulins

QS. Quorum-sensing

R. Clinically resistant

RNA. Ribonucleic acid

S. Clinically susceptible

SEM. Scanning electron microscopy

TSA. Tryptic soy agar

TSB. Tryptic soy broth

TSBG. Tryptic soy broth supplemented with 0.4 % (v/v) glucose

List of Publications

Abstracts and Posters

Gaio V, Acúrcio V, França A, Cerca N. (2016). Preliminary studies on the susceptibility of *Staphylococcus epidermidis* biofilm-released cells to antibiotics and ability to survive in the presence of human blood. In **Biofilms 7.** No. P3: 83, Porto, Portugal, June 26-28, 2016

França A, Gaio V, Carvalhais V, Perez-Cabezas B, Correia A, Pier GB, Vilanova M, Cerca, N. (2016). *S. epidermidis* biofilm-released cells: the final frontier? In **3rd International Conference Pathophysiology of Staphylococci**, Tubingen, Germany, September 15-17, 2016

1. Introduction

1.1 *Staphyloccus* genus

Staphylococcus genus belongs to the Staphylococcaceae family and contains around 50 species and more than 20 subspecies, many of which can be found in humans and other mammals [1,2]. Staphylococci are gram-positive bacteria, characterised by their spherical shape, with a diameter generally ranging from 0.5 to 1.5 μm [3]. Their tendency to be arranged in clusters that reminds clusters of grapes is a distinguish feature of these bacteria (Figure 1.1), owing the name from the Greek *staphylé* that means "bunch of grapes" [3,4].

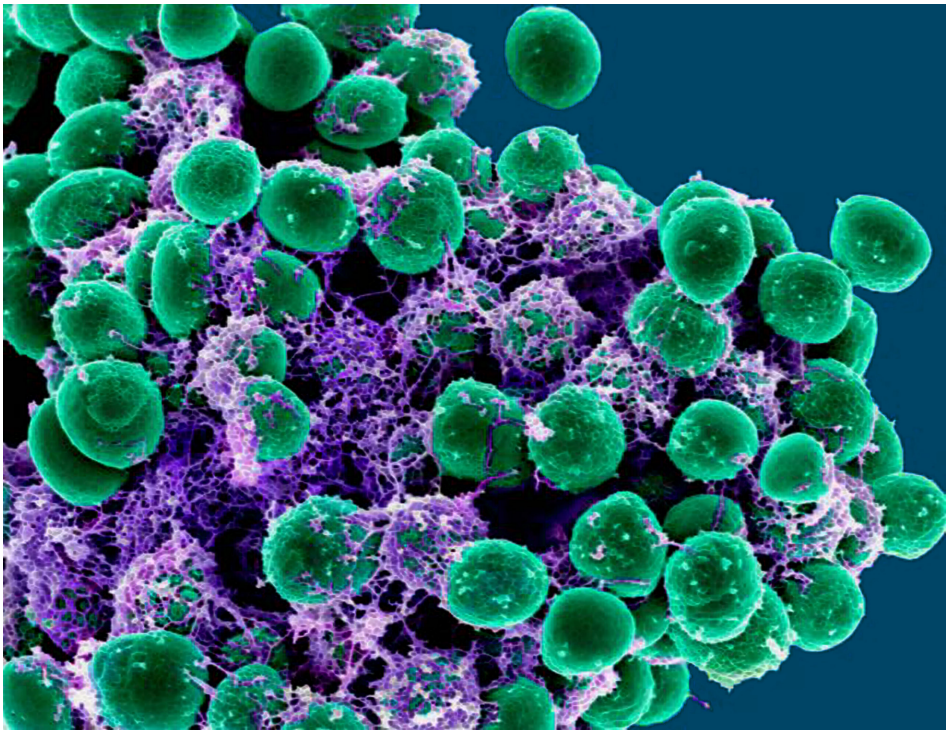

Figure 1.1 - Scanning electron microscopy (SEM) image of a grape-like cluster of *S. epidermidis.* Adapted from [5].

Gram-positive cocci are known for being very heterogeneous and, concerning catalase activity, *Staphylococcus* spp. are classified as catalase-positive, i.e., they produce catalase, an enzyme responsible for the catabolization of peroxide hydrogen into water and oxygen gas [3,6].

Along with other bacterial species, staphylococci are important pathogens of several mammals, including humans, and are responsible for a wide spectrum of infections, commonly termed "Staph infections", including a variety of life-threatening systemic diseases [3,7]. Skin and urinary tract infections, as well as infections of the soft tissues and bones, are common examples of injuries caused by several staphylococci, including by some opportunistic *Staphylococcus* species [3,7]. Opportunistic staphylococci owe the designation to their interactions with the host tissues, since these commensal

bacteria usually interact with the host in a probiotic way and, despite taking benefits from the host, they are not considered harmful to the same. Usually, these microorganisms only cause disease under specific circumstances, taking advantage of opportunities that are not generally available, as compromised physical barriers and compromised immune systems, generally in patients with predisposing factors [8,9].

1.1.1 *Staphylococcus epidermidis*

Staphylococcus epidermidis is part of the wide range of bacteria from the *Staphylococcus* genus and can be found on the skin and mucous membranes of humans [3,6]. These bacteria are able to grow and possibly cause disease in a great variety of conditions, as they have a remarkable ability to propagate in mediums with high levels of salts, besides being facultative anaerobic and being able to grow in a wide range of temperatures, from 18 to 40 °C [3,6].

Colonization by *S. epidermidis* is considered frequent and can be harmful to humans, however, this species is known to perform an important role in the maintenance of a healthy skin flora by competing with similar microorganisms which can be considerably more harmful, for instance *S. aureus* [10,11]. Being a common inhabitant of the skin, *S. epidermidis* can easily invade this physical barrier through wounds and follicles. This happens mainly when the skin barrier is compromised, for example due to medical practices as the insertion and removal of catheters and other medical devices, or upon fissures on the skin resulting from surgical procedures [7].

It has been argued that *S. epidermidis* is an accidental pathogen, based on diverse characteristics of the non-infectious lifestyle of this bacterium, for instance, this microorganism presents a benign relationship with the host and acts on a probiotic way to prevent the colonization by more harmful bacteria [12]. Hereupon, the occurrence of some chronic infections and diseases can be justified by the facility of this staphylococcal species to overcome some physiological barriers, as the skin, and to evade antimicrobial therapies and form biofilms that can lead to severe and recurrent infections [12–14].

S. epidermidis is a pathogen with some virulence factors in which interest has been increasing since these bacteria are pointed as one of the leading causes of nosocomial infections [11,15]. This species can be distinguished from *S. aureus*, one of the most pathogenic and well-known staphylococcal bacteria, due to its inability to produce coagulase, an enzyme that coagulates fibrin in blood, since *S. aureus* is coagulase-positive and *S. epidermidis* has a lack in the production of this

enzyme, being part of the coagulase-negative staphylococci (CoNS), which are usually less virulent and pathogenic than coagulase-positive species [6, 7].

Prosthesis, medical implants, catheters and shunts are some examples of indwelling medical devices that are becoming more and more common on medical practices, due to their great ability to improve the quality of life of many persons. These medical devices are becoming increasingly sophisticated, however, that does not prevent them from being colonized by several microorganisms. As a result, the surface of these biomedical devices often serves as a microbial reservoir and may lead to several infections, contributing to the increased number of biofilm-related infections [17,18]. Once an indwelling medical device is introduced into the human body, a variety of molecules will quickly coat the biomedical device, forming a film on its surface [17]. Fibronectin, vitronectin, albumin and immunoglobulins are some of the proteins and glycoproteins produced by the human body that, in the presence of a medical device, will allow the attachment of cells, potentially facilitating the formation of biofilms in the surface of the indwelling devices [19].

Although the majority of staphylococcal infections are local, they can evolve to systemic diseases, especially due to the release of bacteria from the infection sites and their consequent entrance in the bloodstream, being able to damage a diversity of organs [4,7]. A great deal of bloodstream infections related to the insertion of catheters, vascular grafts and other indwelling medical devices, since these surgical procedures enhance the exposure of patients to a large amount of bacteria [20]. Staphylococcal bacteremia is one of the most common systemic staphylococcal infections, being one of the major causes of mortality in hospitalized patients with chronic diseases, representing an increased concern due to the lack of effective ways of treatment [21,22]. Moreover, several antimicrobial therapies target *S. epidermidis* bacterium, since this is one of the most frequent microorganisms causing primary bacteremia and infections on indwelling medical devices, especially in ill patients and neonates [20,23].

S. epidermidis has some virulence factors that allow these bacteria to infect the human tissues and promote the occurrence of infections and diseases, being a major threat to immunocompromised patients [11,24]. Among these virulence factors, the capacity to form biofilms is highlighted, since bacteria within biofilms present some interesting particularities, including higher tolerance to several antimicrobial therapies [11,25]. Furthermore, a few studies have also shown that *S. epidermidis* biofilms present a higher tolerance to mechanisms of host defense, contributing to the evasion of the immune system and persistence of infections [26,27].

1.2 Biofilms

Biofilms are recognized as ubiquitous in nature, being the most common form of organization of several microorganisms, overcoming the number of microorganisms living in a planktonic form [28].

There are a few definitions of the term biofilm, yet, one of the most popular was given by Costerton *et al.*, defining biofilm as an aggregation of microorganisms and their extracellular products, forming a well structured population, generally attached to a surface [29]. An organic film, alternative designation for biofilms, can also be briefly described as an agglomeration of adhered microorganisms surrounded by a macromolecular matrix [19].

Human health can be deeply affected by the development of biofilms, not only because of the high tolerance towards antimicrobial therapies, but also because biofilms can serve as a continuous reservoir of several opportunistic bacteria that are able to colonize different surfaces [19, 31].

Although prevention is the main strategy referring to biofilm infection control, it is not always possible to avoid contamination of medical devices inserted in the human body, despite all the aseptic care in surgical interventions [6]. As a result, it is considerably frequent that contaminations by *S. epidermidis* occur after a surgical procedure [31].

Formation of bacterial biofilms is accepted as a survival strategy of bacteria and occurs in a spontaneous way, being accounted as responsible for several chronic and acute infections, from which can be pointed out bacterial wound infections, endocarditis and respiratory tract infections [18,32]. The existence of a polymeric matrix surrounding bacteria has some benefits in protecting bacteria towards environmental changes, as pH or temperature, and also protecting them from being removed from the surface, by washing or scraping [28,33]. Besides the contribution to the survival of the biofilm under assorted environmental adverse conditions, as the lack of nutrients, the biofilm matrix is also fundamental for the maintenance of the tridimensional structure of the biofilm [14,34]. Other benefits of residing within a polymeric matrix are the highest protection against exposure to antimicrobial therapies, compared to bacteria in the planktonic state, and the improved assess to nutrients [35,36].

Biofilms are very common in nature and present a crucial role in what refers to the occurrence and persistence of infectious diseases, since these biofilms can prosper on medical implants (Figure 1.2), as well as in the tissue of a large number of mammals [15,37].

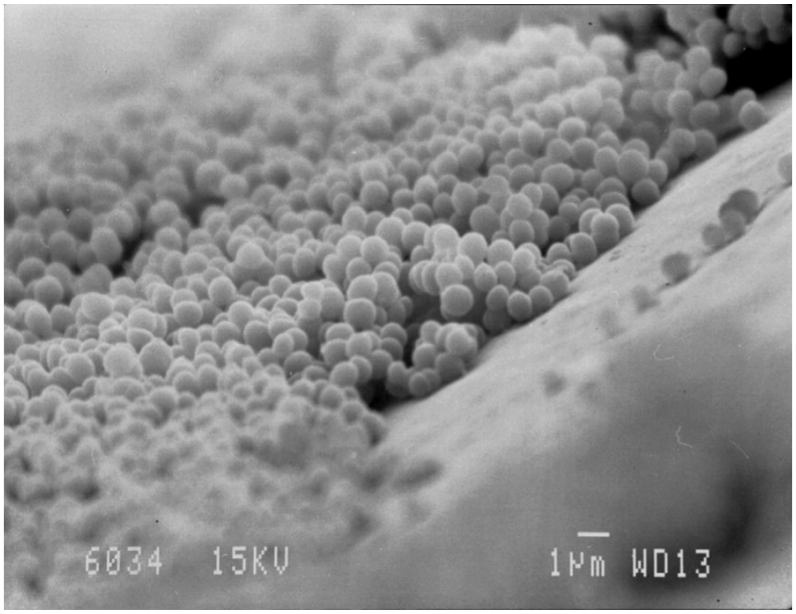

Figure 1.2 - Scanning electron microscopy (SEM) of a *Staphylococcus epidermidis* biofilm. Adapted from [38].

1.2.1 *Staphylococcus epidermidis* biofilms

The process of biofilm formation is the result of a controlled process that comprises multiple steps, being commonly divided in three main phases: attachment, maturation, and disassembly [33,39]. It is important to take into consideration that some authors divide the biofilm formation process in more than three phases, once they subdivide attachment and maturation into multiple stages, however these stages are interconnected and can overlap, being irrelevant to clinically distinguish these multiple stages [40].

Structural and metabolic heterogeneity is common among biofilms, with *S. epidermidis* biofilms being formed by very heterogeneous populations of cells, in which are involved live, dead, dormant, and persistent bacteria [27,41,42].

S. epidermidis biofilms are formed according to the general process of biofilm formation, presenting some particular molecules involved in the different stages of biofilm formation [12], as demonstrated in Figure 1.3. The biofilm formation process of this species is regulated by a system of cell-to-cell communication known as staphylococcal accessory gene regulator (*agr*) [43], as will be further described.

The first phase of biofilm development is generally termed initial adhesion or attachment and comprises bacterial adhesion to surfaces as a result of the contact of bacteria with those surfaces [28]. Non-specific interactions, as hydrophobic and electrostatic interactions, generally command this primary attachment to inert surfaces, in which bacteria adhere straightly to the surface of medical

devices in the body [28]. However, bacteria can also adhere to films of host-derived matrix molecules coating a surface, as the surface of biomedical devices, and, in this case, the surface proteins will mediate the adhesion of bacteria to the coated surface of the medical devices [15,28].

The AtlE autolysin is part of the specific proteins that mediate primary attachment, facilitating the adhesion of bacteria to surfaces or to previously attached host matrix proteins [15,45]. Moreover, Bap/Bhp protein is also involved in the first stage of biofilm formation, by increasing the hydrophobicity of the cell surface that facilitate the initial adhesion process [47].

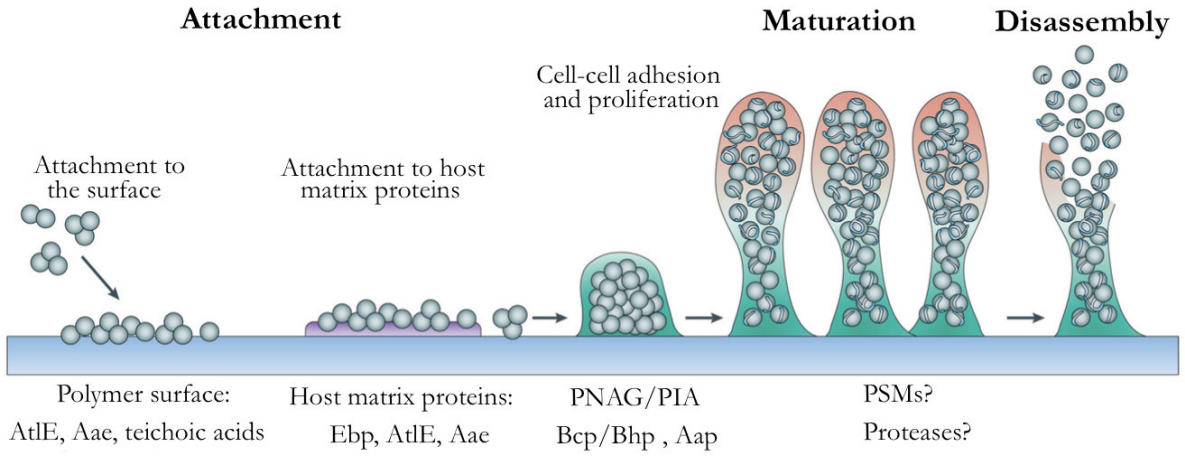

Figure 1.3 - Representation of *S. epidermidis* biofilm cycle and some of the molecules involved in the different phases of biofilm formation and disassembly. The process begins with the initial attachment to the surface, followed by the adhesion of cells to each other, forming clusters. Maturation of the biofilm is achieved by the growth of the bacteria clusters and production of the polymeric matrix by those aggregates, which will accumulate and surround bacteria. Lastly, a mature biofilm is obtained and bacteria can detach and disperse from this biofilm and colonize other surfaces. [33,44] Adapted from [12].

Succeeding stages of biofilm formation require specific interactions and molecules to allow the growth of bacteria into clusters [44,45]. For this reason, not all of the bacteria that initially adhere to a surface will be able to develop a biofilm. Some of the bacteria will detach from the surface, while only a part of those will enter the next phase and be able to form the biofilm, by influence of specific molecules, as intercellular adhesins and autolysins [39,48].

The second phase, called maturation, refers to the accumulation of several bacteria and formation of the hydrated polymeric matrix that surrounds cells in biofilms [12, 45]. During this phase, multicellular structures, namely clusters, are formed due to the aggregation of cells [12]. Therefore, some molecules, for instance adhesive and exopolysaccharide macromolecules, are secreted to enhance cell-to-cell communication and aggregation [12,44].

The *icaADBC* operon is often present in *S. epidermidis* bacteria and accomplish an important function in the aggregation of bacteria into clusters [50,51]. These proteins produce a polymer of N-acetyl glucosamine (PNAG) [50]. PNAG is commonly defined as a polysaccharide intercellular adhesin (PIA), which is pointed as the major responsible for the biofilm development of this species since it mediates the intercellular adhesion [50,52].

The development of the biofilm continues with the maturation of these agglomerates that grow and produce the extracellular polymeric substances (EPS) which will lodge between cells [33,53]. The major components of these polymeric substances are polysaccharides, proteins and nucleic acids that result from cellular metabolism and/or cell death process, however the composition of the matrix varies among different biofilms [39,54]. This complex extracellular matrix surrounds the bacteria attached to the surface and to each other [53], as shown in Figure 1.4.

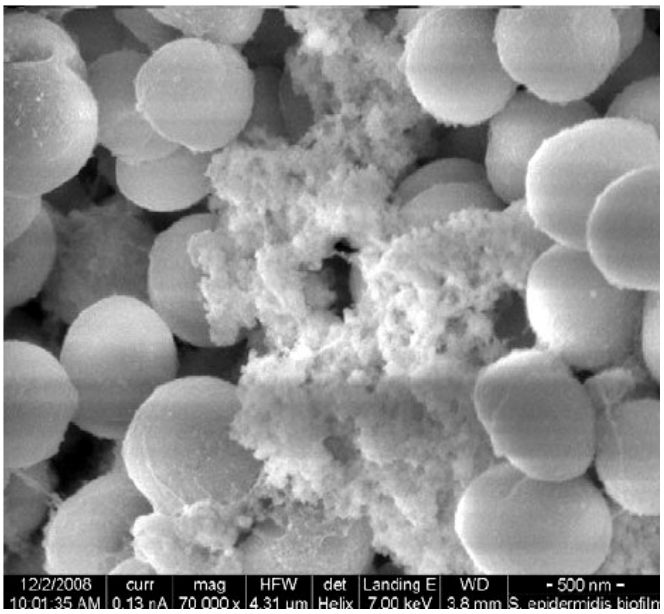

Figure 1.4 - Low vacuum secondary electron image of a *S. epidermidis* biofilm, with evidence to the polymeric matrix surrounding bacteria. Adapted from [55].

During maturation of the biofilm, the increasing number of bacteria and the production of the polymeric matrix lead to the expansion of the biofilm thickness [18,56]. However, the thickness of the biofilm does not increase infinitely and a disassembly process may occur in order to regulate the cell density of the biofilm [57,58]. The availability of nutrients and oxygen [59,60] and environmental parameters, as the pH, temperature and nature of the surfaces to which bacteria are attached [56, 59], cause an active release of biofilm cells, known as dispersion, that contributes to the regulation of the biofilm cell density. Furthermore, a passive release process may also contribute to the regulation of the extent of the biofilm, since shear forces are able to induce the detachment of biofilm cells [61,62].

The active and passive events of release may occur throughout the entire biofilm cycle, nevertheless, the remaining cells of the biofilm undergo further stages of maturation. It is important to have in consideration that biofilm infections are clinically relevant not only when they reach a mature state, but they can also be threatening in previous phases of the biofilm cycle. This may happen, for instance, because some of the clusters that are formed during maturation process may detach from the surface and enter blood circulation, introducing a potential danger of causing thromboembolisms that can, ultimately, culminate in patient death [63].

Later in this phase, a biofilm structure containing channels is formed. This event is dependent on adhesive and disruptive forces and allow the communication of cells with the exterior, enabling the circulation of nutrients and oxygen into the deeper layers of the biofilm [33,64].

Finally, the mature biofilm, characterized by a thicker film of bacteria and a more protuberant matrix, reaches a state that no longer allow the growth and division of cells due to nutritional and physicochemical limitations and, thereby, biofilm cells undergo a final disassembly process in a greater extent, by active or passive processes, as previously explained [44,57]. The previously formed channel-containing structure facilitates the evolution of the biofilm to achieve the disassembly phase [39,64]. The cells disassembled from the biofilm may be designated as biofilm-released cells (Brc) [58] and have the ability to colonize other sites, contributing to the spreading of infections among the host and to the occurrence of inflammation processes [65,66].

Disassembly remains the least understood phase of biofilm lifecycle and, therefore, some of its molecular mechanisms are not completely established [66]. Although the promoters of the disassembly are not entirely known, it is currently accepted that shear forces, associated with detachment, and/or specific gene expression, related to disassembly, can be the cause of the release of these new colonizers, leading to the propagation of the infection and to an increasing number of biofilms [57]. Furthermore, proteases and PSMs (phenol-soluble modulins) are thought to participate in the degradation of *S. epidermidis* biofilm matrix, contributing to the disassembly process, being modulated by a quorum-sensing mechanism that will be succeeding described [57,67].

It is known that disassembly involves some alterations in the biofilm, as the degradation of the extracellular polymeric matrix, as well as some physiological changes that allow the preparation of Brc to the environmental conditions outside the biofilm [57,68]. Therefore, Brc are believed to present distinct phenotypic features from both biofilm and planktonic cells [65], as will be further addressed.

1.2.2 Quorum-sensing

Despite a certain lack of knowledge about the mechanisms of maturation and detachment of the biofilm, it is known that there are several mechanisms of intercellular signalling among bacteria that result from the ability of microorganisms to produce molecules that can be recognized by specific receptors [44,69]. Quorum-sensing (QS) is an example of those mechanisms, though to be responsible for the transition of planktonic to biofilm lifestyle in bacteria and can be defined as a regulatory mechanism that exists in microorganisms to control gene expression, being dependent on cellular density [56]. This system allows cell-to-cell communication and mediates the secretion of molecules that act as signals to control the synchronization of gene expression and functional coordination among populations of microorganisms, as biofilms [57, 64].

The initiation of biofilm formation is triggered when, by quorum-sensing signalling, bacteria sense unfavourable or stress conditions, as the lack of nutrients and alterations in environmental parameters [56,71]. Due to different signalling by QS, biofilm formation and development differs according to distinct environmental conditions, as different temperatures, pH, and nutritional availability, among others [56,69]. Moreover, quorum-sensing mechanisms are involved in the monitoring and regulation of biofilm density, acting as a control to promote either the maturation of the biofilm, to increase its extent and thickness, or the inhibition of biofilm formation and stimulation of the dispersion phase, leading to a decrease in the amount of bacteria residing within the biofilm structure [56,70].

Similarly to what happens with other species, the formation and regulation of staphylococcal biofilms is a complex process, influenced by the environmental conditions and by the genotype of the microorganisms [72]. In *S. epidermidis*, biofilm formation is controlled by a system named *agr* (accessory gene regulator) [43], wherein the expression of targets regulated by *agr* is dependent on the density of cells, as it is characteristic of QS mechanisms [73]. *agr* was once viewed as a regulator of virulence factors, however, findings on the existence of this gene in non-pathogenic species lead to the assumption that this system is a quorum-sensing regulator, which includes the control of some virulence factors in pathogenic species, playing an important role in the species pathogenesis [43], but, as well, the control and regulation of other non-virulent mechanisms [74,75]. Consequently, *agr* is involved in the invasiveness of bacteria, by upregulating the expression of virulence factors and downregulating the production of surface proteins, contributing to the invasiveness of bacteria on the hosts [43].

When there is few *agr* activity, as a result of a low density of cells, the quorum-sensing mechanisms emit signals to increase the expression of surface proteins that allow bacterial colonization, so that they can divide and increase cellular density [76,77]. As a result of augmented cellular density, *agr* activity raises and the secretion of surface proteins decreases, reestablishing the balance by a mechanism of negative regulation [76,77]. However, the aging of the biofilm leads to the loss of viability of bacteria and to a reduction in the expression of *agr*, affecting the chronicity of the infections since the decrease in the production of signaling molecules will compromise the regulation mechanisms and have a negative effect on the balance of the number of bacteria [72,78].

It is believed that, besides the previously described functions, QS also performs a considerably important role in the release of cells from the biofilms and may influence the resistance to some antimicrobial drugs [43,71]. Some studies have already reported that *agr* expression is involved in the dispersion of staphylococci biofilms [58,78]. Furthermore, *agr* expression has been associated with decreased antibiotic susceptibility for staphylococcal biofilms [71], which may, as well, influence the tolerance of biofilm cells and Brc to antibiotics.

Quorum-sensing mechanisms, namely *agr* in what refers to staphylococci, have been pointed as potential targets for prophylaxis and therapy. An approach that have been suggested is the inhibition of genes directly involved in QS, since this would reduce the pathogenicity of several bacteria as a result of the attenuation of the expression of virulence factors commanded by QS [43,70]. However, the upregulation of adhesion mechanisms caused by *agr* inhibition may enhance cell adhesion and lead to a higher persistence and chronicity of biofilm infections, increasing biofilm formation [43,71,72]. For that reason, it is still unknown whether the advantages of the inhibition of QS would overlap the disadvantages, so that further studies need to be accomplished in this matter.

1.2.3 Biofilm tolerance to antibiotics

Biofilm infections are very threatening and the decrease in the susceptibility to antibiotics is a very concerning issue [15,31]. This increased tolerance often leads to situations where it is unsuitable to treat infections with common antibiotic therapies, since the concentration of antibiotic needed to kill bacteria within biofilms is higher than the peak serum concentration (PSC), which is the maximum concentration of antibiotic that the human body can endure after administration [79–81]. Different mechanisms that attempt to explain this feature will be presented ahead.

Biofilms are thought to admit a higher tolerance to antibiotics by a diversity of factors, from which can be highlighted the diffusional barrier to antibiotics, the existence of a more resistant

phenotype and a slow growth-rate of cells within the biofilm [25,36], as represented in Figure 1.5. Moreover, the existence of persister cells, with an increased tolerance to antibiotics, can also partially explain the inefficacy of antibiotic treatments in biofilms [82].

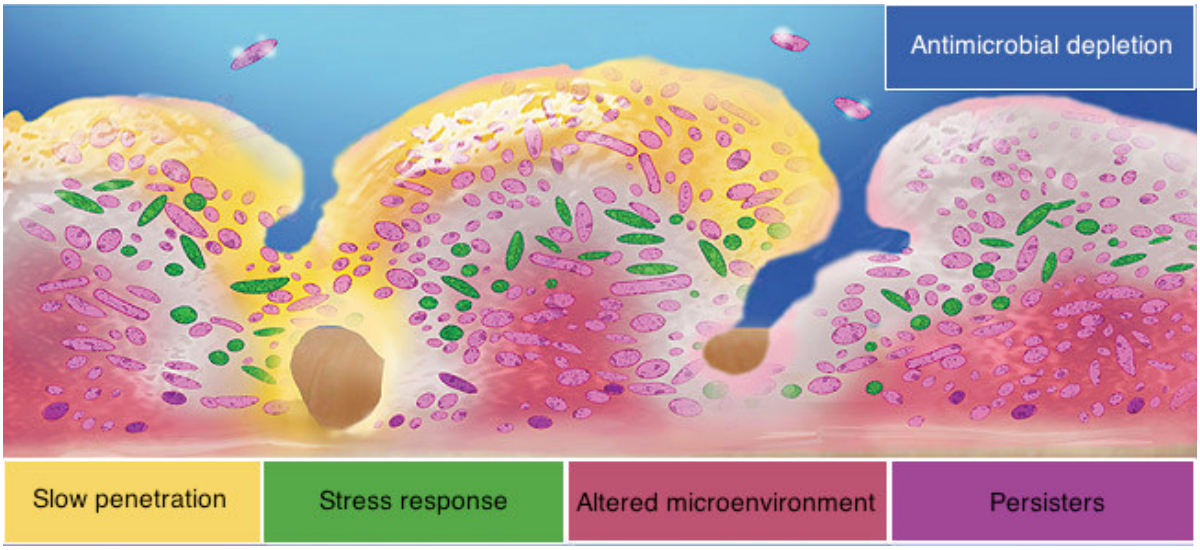

Figure 1.5 - Some hypothesis that attempt to explain the decreased susceptibility of biofilm cells to antibiotics. Adapted from [83].

The structure of the extracellular polymeric matrix acts as a physical diffusional barrier reducing and/or delaying the penetration of antibiotics into the biofilm, whereby antibiotics can no longer reach a great amount of bacterial cells [14,81]. For the same reason, the increased number of bacteria in biofilms, which result from cell division, contribute to the expansion of the thickness of the biofilm and, consequently, hinders the penetration of antimicrobial substances into the deeper layers of the biofilm [38,81].

Furthermore, the negatively charged polymeric matrix may also behave as a chemical barrier to the positively charged antimicrobial agents, since these agents tend to bind to the matrix and, thus, the amount of antimicrobial drugs that successfully reach biofilm cells is limited [18,84]. Moreover, some of the polysaccharides and proteins that constitute the matrix perform an important role in the protection of bacterial biofilm cells against antimicrobial therapies by acting as a protective barrier and/or inactivating some antibiotics [14,82,85].

Among the modifications that bacteria experience upon adaptation to biofilm mode, phenotypic changes are one of the most important, considering they may influence the susceptibility to antibiotics within the biofilm environment [14,81]. It is now accepted that bacteria residing within the biofilm are phenotypically different from free-floating bacteria, whereby some bacteria may experience a

differentiation process which leads to a resistant phenotype, contributing to the higher tolerance of biofilms against antimicrobials [65,81].

A slower growth-rate of bacteria is found in altered environment zones, since in the deeper layers of the biofilm the concentrations of oxygen and nutrients are reduced, leading to distinct growth conditions [76, 79], as represented in Figure 1.6.

The reduced bacterial growth-rate, as well as the resulting alteration of metabolic processes and reduced metabolic activity, present a limitation to the action of some antibiotic classes in biofilm cells, since it increases their tolerance to these chemical agents [25,82,86].

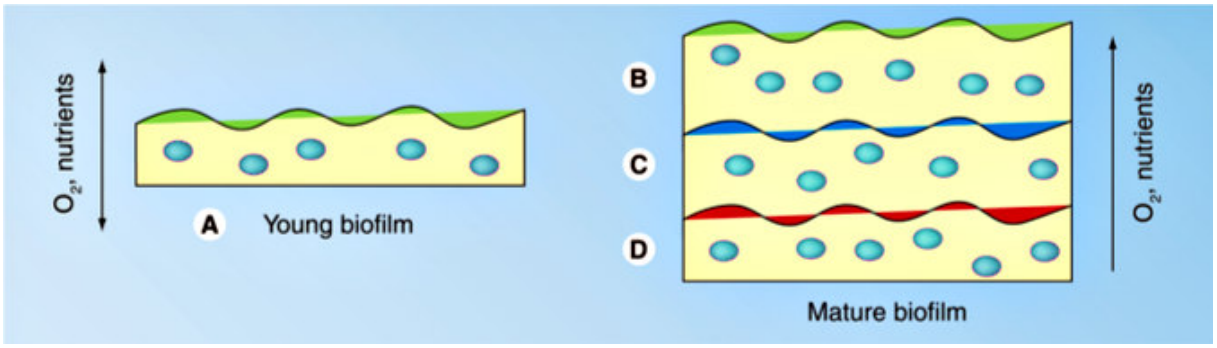

Figure 1.6 - Schematization of the heterogeneity of *Staphylococcus epidermidis* biofilms over the depth. Young biofilms (A) provide a high availability of nutrients and oxygen (O_2) to all the bacteria, while mature biofilms are characterized by deeper layers (D) with a small amount of nutrients and O_2, and upper layers (B) with a great accessibility of nutrients and oxygen. Adapted from [40].

The heterogeneity of cells within biofilms, from which can be highlighted the wide range of metabolic activities between cells [87], also contributes to the increased tolerance to antimicrobials [81,82]. Dormant and persister cells are characterized for becoming metabolically less active than other cells, mainly upon facing stressful conditions, and for presenting an increased tolerance to antibiotics, contributing to recalcitrant infections [88–90].

Dormant cells exist in a non-replicative state that is reversible, i.e., these cells are in a temporary dormancy state where they slowdown metabolic processes and are not able to replicate [89,90]. On the other hand, persistence refers to a state in which some bacteria survive antimicrobial treatments [89,91]. Thus, persisters are often defined as a sub-population of cells that entered a spontaneous dormant state in which they do not proliferate, presenting a substantial tolerance to antibiotics, being, however, able to restore their function when inoculated into fresh medium without antimicrobial substances [81,92]. Therefore, 9ipersistence may not be directly associated with dormancy, which means that not all dormant cells are persisters, especially taking into account that

persistence is mainly associated with antibiotic stress and dormancy often occurs in response to unfavorable environmental conditions rather than to antimicrobial therapies [91,93].

Although persister cells can exist in planktonic state, their frequency is higher in slow-growing biofilms, partially explaining the higher tolerance of biofilms against antibiotics, compared to planktonic cells [82]. These cells can be pointed as a cause of relapsing biofilms after antimicrobial treatments, since in the persistent state these cells survive antimicrobial drugs (resistant variants) and, afterwards, are able to proliferate and lead to the growth of the biofilm, culminating in a mature biofilm [81,82,94], as represented in Figure 1.7.

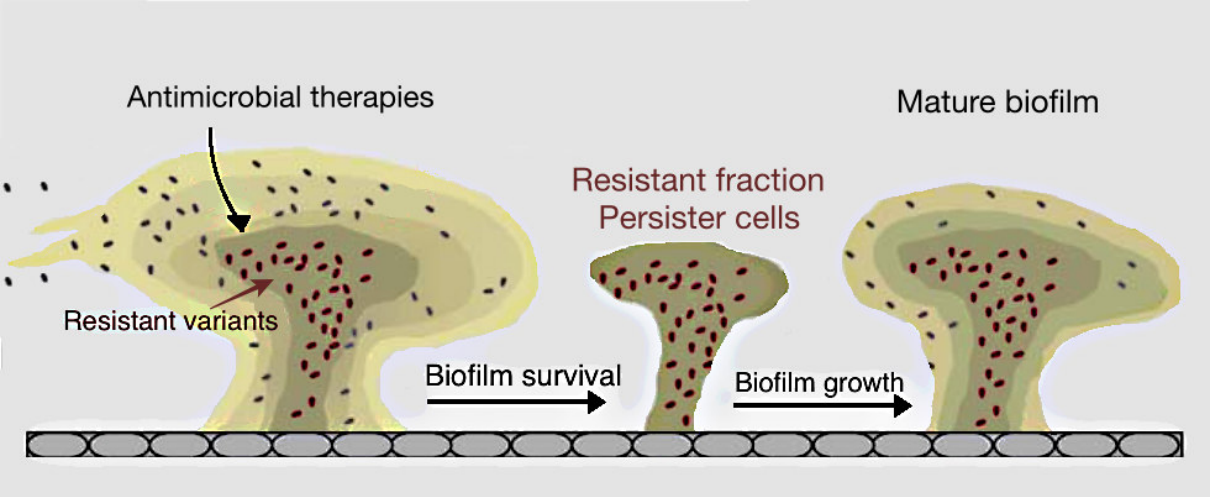

Figure 1.7 - Schematization of the resistance mechanism due to resistant/persister cells. Although antimicrobial therapies can eradicate part of the biofilm cells, some resistant variants are not affected by the antimicrobial drugs and are able to persist and maintain the biofilm survival. After antimicrobial therapy discontinuation, the resistant fraction is able to develop a new biofilm that will grow and reach maturation. Adapted from [94].

As a consequence of the reduced susceptibility of biofilms to common antibiotics, it is often necessary to use a combination of different antibiotics and substances capable of degrading the matrix that envelops bacteria, in order to expose cells to the antibiotics [80, 78]. However, due to the inefficacy of several therapies, the treatment of medical devices-related infections may result in failure and, in those cases, the removal of the infected medical devices is required, resulting in high health costs and great inconvenience to patients [96,97].

1.2.4 Biofilm-released cells (Brc)

Brc are cells that suffered disassembly from the biofilm, by either dispersion (active process) or detachment (passive process) during its lifecycle, being capable to trigger inflammatory events [65,66]. These cells may also act as new colonizers and are able to form biofilms in different loci after being released from the biofilm [65,66].

Surprisingly, little is known about the phenotypic alterations that these cells undergo, as well as about the impact of these alterations in the clinical field [67]. It was primarily thought that, soon after being released from the biofilm, Brc would revert the phenotypic alterations and become similar to planktonic cells again [65,81]. However, some studies have reported that cells released from the biofilms were different from both biofilm and planktonic bacteria, denying the previous assumption of immediate phenotype reversion [57, 58, 65, 99].

Recently, studies published by França *et al.* confirmed suspicions about phenotypic differences of Brc comparing to the biofilm and planktonic counterparts, regarding the inflammatory response and the reaction to antimicrobial therapies, that help explain the relapsing nature of infections of *S. epidermidis* biofilm-related infections [58,98]. These researchers have shown that *S. epidermidis* Brc may be more effective in the activation of the inflammatory response, since Brc induced a particular gene expression on mouse splenocytes, with an increased expression of several genes related to cell death, and induced a higher stimulation of pro-inflammatory cytokines [98]. They also showed that Brc present a higher tolerance than their planktonic counterparts against some antibiotics, retaining their tolerance when growing in the presence of the originating biofilm [58]. However, their transient phenotype was reverted when these bacteria proliferated planktonically in the absence of the originating biofilm [58].

This specific bacterial population merits special attention, as the disassembly of cells from the biofilm may provide a pathway to the occurrence of diverse injurious events and to the spreading of biofilm infection, particularly since these cells present a different behaviour against antimicrobials [58]. The determination of the antibiotic profile of Brc would provide significant insights to the pathophysiology of biofilm infections and facilitate the development of effective strategies to the control of infections related to biofilm disassembly [67]. Undoubtedly, a depth investigation on the properties of Brc should be performed in order to proficiently target, prevent and treat *Staphylococcus epidermidis* biofilm-related infections.

1.3 Aims and objectives

The aim of the present work was to determine the antibiotic tolerance profile of clinical strains of *S. epidermidis* Brc. To accomplish this goal, the work was divided into three main tasks.

The first task consisted in the study of the antibiotic susceptibility of cells released from 48-hour mature biofilms (Brc_{48H}) of *S. epidermidis* 9142. The main objective of this task was the comparison of the antibiotic effects in Brc_{48H} with the effects in 48 hour-biofilm cells and in stationary planktonic cells.

The aim of the second phase was to determine if cells released from biofilms with different stages of maturation presented distinct susceptibilities to antibiotics.

Finally, the purpose of the last phase of this work was to determine if the results of antibiotic susceptibility in the three different bacterial populations of different *S. epidermidis* isolates were consistent with the results obtained in the previous phases, for the control strain 9142. It was assumed that the results of this study would help to understand if the phenomenon of antibiotic tolerance of Brc is common to distinct isolates of this species.

2. Materials and Methods

2.1 Isolates and growth conditions

Staphylococcus epidermidis 9142, a blood clinical isolate known by its strong ability to form biofilms and generally used as a biofilm positive control [99,100], was the isolate selected for the majority of the experiments of this thesis. Furthermore, other clinical isolates (see Table 2.1) were used in order to compare the antibiotic susceptibility among different *S. epidermidis* and assess if the pattern behaviour remains constant in all the isolates tested.

Table 2.1 - Origin of the *Staphylococcus epidermidis* isolates used in this study

Isolates	Isolated from	Country of origin
9142 [100]	Blood culture	Germany
IE186 [101]	Infective endocarditis	United States of America
PT12003 [58]	Central catheter of a patient with gastric disease	Portugal
MEX60 [102]	Unknown	Mexico
DEN69 [103]	Unknown	Denmark
ICE09 [103]	Unknown	Iceland
URU23 [102]	Unknown	Uruguay

2.1.1 Biofilm formation and biofilm-released cells collection

An inoculum was done by adding one *S. epidermidis* colony into 2 mL of Tryptic Soy Broth (TSB) (Liofilchem, Teramo, Italy) and incubated in an orbital shaker overnight at 37 °C and with agitation at 120 rpm. Later, the overnight cells were diluted in TSB medium until an optical density at 640 nm (OD_{640}) of 0,250 ± 0,05 was reached, corresponding to an approximate concentration of 2 × 10^8 CFU (colony forming units) / mL [104]. Biofilms were formed through the inoculation of 15 μL of the adjusted suspension into a 24-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium), with 1 mL of TSB medium supplemented with 0.4 % (v/v) glucose (TSBG) to induce biofilm formation, being incubated at 37 °C with shaking at 120 rpm for as long as 72 (±1) hours in an orbital shaker. After each 24 (± 1) hours of incubation, spent medium was carefully removed and the biofilms were washed twice with a saline solution (0.9 % (m/v) NaCl in distilled water) in order to remove unattached cells, followed by the careful addition of 1 mL of fresh TSBG and subsequent incubation in the same

conditions. Finally, biofilms were washed twice with the saline solution, suspended in 1 mL of the same by scraping the cells from the plastic surface, and bacteria from either 24, 28, 48 or 72-hour biofilms were collected into a flask, pooling together at least 4 different biofilms to decrease the variability inherent to biofilm formation [105].

Biofilm-released cells (Brc) were collected, from at least 4 different wells, by careful aspiration, at different timepoints, from the biofilm bulk fluid of 28 or 48-hour biofilms, depending on the study concerned, and stored into a flask, as described previously [58].

2.1.2 Planktonic growth

From an overnight inoculum grown in the same temperature and agitation conditions previously mentioned (section 2.1.1), a dilution with TSB medium was performed in order to adjust the optical density to a cellular concentration of 2×10^8 CFU/mL. Following, 150 μL of this suspension were inoculated into a 25 mL Erlenmeyer containing 10 mL of TSBG and incubated at 37 °C with agitation at 120 rpm during 24 (± 1) hours. Stationary planktonic cells were, then, collected into a flask.

2.1.3 Cell homogenization

The three suspensions (disrupted biofilm cells, Brc and stationary planktonic cells) were submitted to a pulse of 5 seconds of sonication with 40 % amplitude (Ultrasonic Processor Model CP-750, Cole-Parmer, Illinois, U.S.A.) in order to homogenize the suspensions and disassociate possible existing clusters. As previously demonstrated [106], this sonication cycle did not have a significant effect on cell viability.

2.2 Characterization of the antimicrobial profile of planktonic *S. epidermidis*

A total of 10 antibiotics (see Table 2.2) with different mechanisms of action were used to assess the susceptibility of the three cell populations under study. A preliminary study was performed to characterize the antimicrobial profile of the *S. epidermidis* isolates under study, through the determination of the minimum inhibitory concentration (MIC) for each antibiotic.

Inocula from all the populations were diluted into TSB to obtain a concentration of about 2×10^8 CFU/mL, by measuring the $OD_{640,}$ after calibrating for CFU/mL [104]. Following, 2 μL of each suspension were added to different wells containing 200 μL of TSB medium with antibiotics, whereas different gradients of concentrations were used according to each antibiotic. Simultaneously, a positive control was performed by inoculating the same quantity of suspension into 200 μL of TSB without antibiotics. The MIC was determined as the lowest concentration of antibiotic that inhibited a visual growth of bacteria and the determination was based on at least two consistent replicates.

Table 2.2 - Mechanism of action and peak serum concentration (PSC) in mg/L of the ten antibiotics used in this study

Mechanism of action[a]	Antibiotic	PSC (mg/L)
Cell wall synthesis inhibitor	Dicloxacillin	59 [107]
	Imipenem	32 [108]
	Teicoplanin	50 [109]
	Vancomycin	40 [110]
Nucleic acids synthesis inhibitor	Ciprofloxacin	4.5 [111]
	Rifampicin	10 [110]
Protein synthesis inhibitor	Erythromycin	10 [112]
	Gentamicin	10 [113]
	Linezolid	18 [114]
	Tetracycline	16 [110]

[a] The mechanism of action of the antibiotics was determined by the information sheet provided by the antibiotics manufacturer.

2.3 Comparison of the antimicrobial susceptibility of the distinct *S. epidermidis* populations

Following the treatment and homogenization of the different cell populations, according to the previously described process (section 2.1), the suspensions were diluted in TSB medium in order to reach a concentration of about 2×10^8 CFU/mL. Next, 200 μL of the adjusted suspensions were inoculated into TSB medium, in a total of 2 ml, achieving a concentration of approximately 2×10^7 CFU/mL. Then, each antibiotic was added to the previous suspensions, at the peak concentration, and the tubes were incubated at 37 °C and 120 rpm agitation for a period up to 6 hours. Simultaneously, controls were performed by the inoculation of the same suspensions in TSB medium, without the addition of any antibiotic, and further incubation under the same conditions. All the tubes were prepared in duplicate, for all the conditions tested.

After 2 and 6 hours of incubation, one mL of each tube was collected and centrifuged at 4 °C and 16,000 g for 10 minutes. Next, the supernatant was carefully discarded and the pellet was resuspended in 1 mL of 0.9 % NaCl solution, with the aid of a pulse of 5 seconds of sonication at 40 % amplitude.

Finally, 10-fold serial dilutions were performed, vortexing each sample before each dilution, and plated onto Trypticase Soy Agar (TSA), which was prepared by the addition of 30 g/L of TSB (Liofilchem) and 15 g/L Agar (Liofilchem). The plates for CFU counting were incubated at 37 °C until the colonies were grown enough to allow the counting.

3. RESULTS AND DISCUSSION

3.1 Study of the antibiotic susceptibility of cells released from *Staphylococcus epidermidis* 9142 biofilms with 48 hours of maturation (Brc_{48H})

Biofilms, communities of bacteria embedded in a polymeric matrix, follow a lifecycle with three main stages: attachment, maturation and disassembly [12,29]. Over the disassembly stage, the biofilm release cells to the surrounding environment, namely biofilm-released cells (Brc), which are thought to be responsible for serious complications as, for instance, bacteremia [20]. Although several studies have been performed to compare the antibiotic susceptibility of bacteria in biofilms with their planktonic counterparts, little is know regarding the tolerance of Brc to antibiotics.

To overcome the lack of knowledge on the efficiency of antibiotics against Brc, the first studies of this thesis consisted in the determination of the susceptibility to antibiotics of biofilm-released cells from 48-hours mature biofilms (Brc_{48H}) and the comparison with both 48-hours mature biofilm cells and planktonic cells in the stationary phase, grown for 24 hours.

3.1.1 Preliminary MIC assay

First, a preliminary assay was performed by determining the minimum inhibitory concentrations (MIC) of all the antibiotics against *S. epidermidis* 9142, in order to verify if this control strain would be susceptible to the antibiotics under study. A standard MIC assay was conducted as previously described (section 2.2) and the results are presented in Table 3.1.

According to EUCAST, bacteria may be considered as clinically susceptible (S), clinically resistant (R) or clinically intermediate (I) to an antibiotic. When the MIC value is equal to or below the lower breakpoint value, bacteria are considered susceptible, meaning that the level of antimicrobial activity is associated with a significant chance of therapeutic success, while when the MIC value is higher than the upper breakpoint value, bacteria as defined as resistant, what is an evidence that there is a high probability of therapeutic failure with the antibiotic concerned. However, in some cases, the MIC range is in the middle of the breakpoint values, which may include the breakpoint limits, and bacteria are considered intermediate, meaning that the therapeutic effect is uncertain [115].

The results obtained in the MIC assays were compared with the clinical breakpoints for *S. epidermidis* described in the literature by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[116] for the majority of the antibiotics, and by the Clinical and Laboratory Standards

Institute (CLSI)[117] and British Society for Antimicrobial Chemotherapy (BSAC)[118] for dicloxacillin and imipenem, respectively, since EUCAST did not provide the MIC breakpoints for these antibiotics.

Table 3.1 - Determination of the MIC ranges in mg/L of ten antibiotics against *S. epidermidis* 9142 and evaluation, by EUCAST, CLSI and BSAC standards, of the susceptibility to the antibiotics tested

Antibiotic	**MIC range (mg/L)**	**Clinical breakpoint (mg/L)**		**Evaluation**
		S ≤	**R >**	
Dicloxacillin	0.125-0.25	0.25	0.5	Susceptible
Imipenem	0.125	4	8	Susceptible
Teicoplanin	2-4	4	4	Susceptible
Vancomycin	1-2	4	4	Susceptible
Ciprofloxacin	8-16	1	1	**Resistant**
Rifampicin	0.004-0.008	0.064	0.5	Susceptible
Erythromycin	1	1	2	Susceptible
Gentamicin	1-2	1	1	**Intermediate**
Linezolid	8	4	4	**Resistant**
Tetracycline	0.5	1	2	Susceptible

From the analysis of Table 3.1 and according to the CLSI breakpoints, 9142 was classified as susceptible to dicloxacillin. Similar, comparing the results to the BSAC breakpoints, this strain was found to be susceptible to imipenem. Through the comparison with the EUCAST clinical breakpoints, *S. epidermidis* 9142 was classified as susceptible to teicoplanin, vancomycin, rifampicin, erythromycin and tetracycline. On the other hand, this strain is thought to be resistant to ciprofloxacin and linezolid. Moreover, the MIC range obtained with gentamicin comprised both the susceptible and resistant limits of the clinical breakpoints, thus, this strain was classified as clinically intermediate to this antibiotic.

3.1.2 Susceptibility assays

To assess the effect of antibiotics among the three different populations of cells, the suspensions were simultaneously incubated under the same conditions with and without antibiotics (control), in order to evaluate the changes on the cultivability of the suspensions after having contacted with the antibiotics. The concentration chosen to accomplish these comparisons was the PSC for each antibiotic, which is thought to be the concentration that presents the highest relevance from the clinical point of view, since it is an estimation of the maximum concentration of antibiotic reached in the human bloodstream [79,110]. Furthermore, all the suspensions were adjusted to the same concentration prior to the incubation with the antibiotics, allowing to accomplish a more accurate comparison between the susceptibility of the distinct populations of cells, as previously described [79]. Since the antibiotics are frequently dependent on the cellular density of the population, the initial adjustment of the optical density is advantageous in what refers to a suitable comparison between populations, yet if the number of cells is too high or too low in comparison with the ideal range of action, the antibiotic may not be able to act as expected and present a lower efficacy of killing [119]. It is, however, important to take into consideration that the measurement of the OD only provides an estimation of the number of cells, since the extracellular products may also affect the OD value, meaning that the number of cells of the adjusted suspensions may continue to present some variability.

The evaluation of the different susceptibilities of the three populations over time was performed with two different times of incubation with PSC of antibiotics, namely 2 and 6 hours, and the results are represented in Figure 3.1 and Figure 3.2, respectively. Based on the MIC results, it is predicted that *S. epidermidis* 9142 will experience a significant reduction on the cultivability upon exposure to seven out of ten of the antibiotics, to which showed to be susceptible, and a smaller or negligible cultivability decrease with the three antibiotics to which was considered intermediate or resistant. However, it is important to recall that the MIC assay was performed with planktonic cells, whereby the conclusions may not be applied to biofilm cells and Brc, meaning that these populations may present a different reaction upon contacting with the antibiotics, as shown before with a limited number of antibiotics [58].

To confirm those earlier findings, this study was conducted with ten antibiotics with different mechanisms of action, namely cell wall synthesis inhibitors (dicloxacillin, imipenem, teicoplanin and vancomycin), nucleic acids synthesis inhibitors (ciprofloxacin and rifampicin) and antibiotics that act as inhibitors of protein synthesis (erythromycin, gentamicin, linezolid and tetracycline), being expected that different classes of antibiotics could generate different responses in the populations of bacteria tested [120]. Moreover, antibiotics with the same mechanism of action may also produce different effects on

the viability and/or cultivability of bacteria since they interact by several different ways with the cells [121,122].

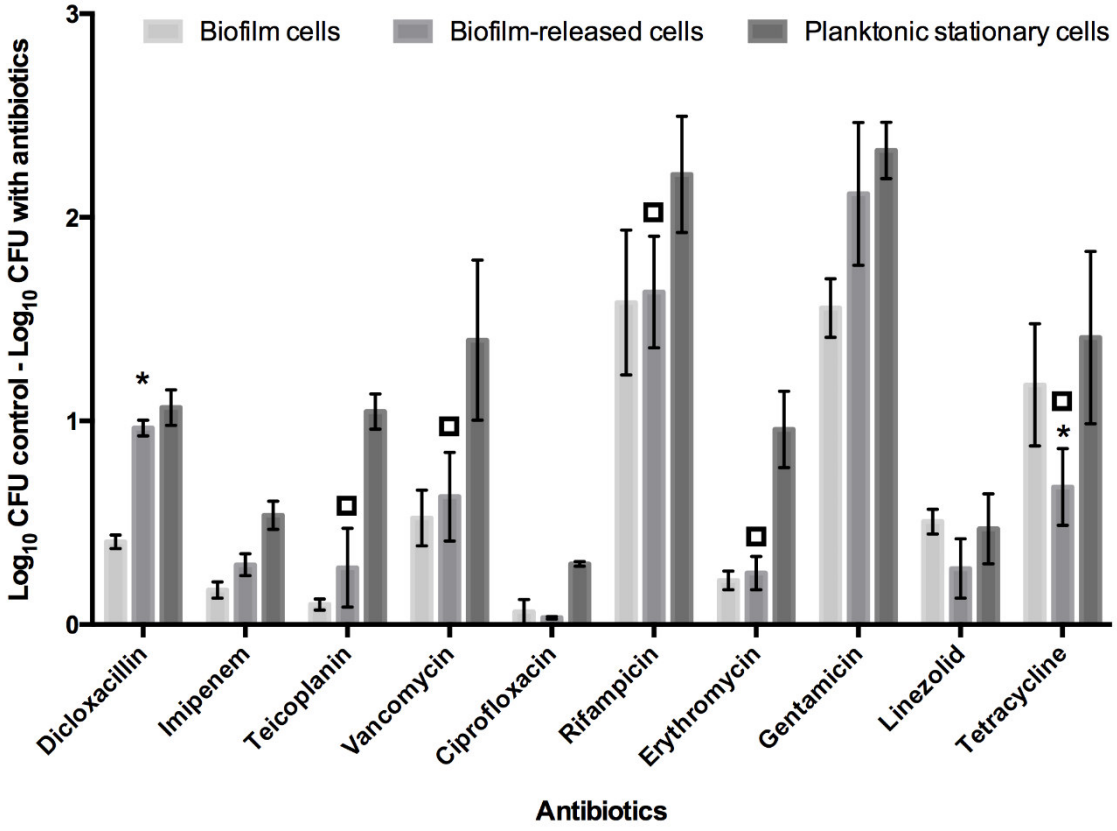

Figure 3.1 - Base 10 logarithmic CFU/mL reduction of *S. epidermidis* 9142 populations upon 2 hours of incubation with peak serum concentrations of distinct antibiotics. The columns represent the mean plus or minus standard error deviation, of at least three independent experiments. Statistical differences between groups were analysed with one-way ANOVA multiple comparisons, with * representing statistically significant differences (p <0.05) between biofilm cells and Brc and □ between Brc and their planktonic counterparts.

Analysing the results represented in Figure 3.1 it is noticeable that the majority of the antibiotics was substantially more effective against planktonic cells than against biofilm cells, and promoted an intermediary effect in Brc, being easily observable a higher occurrence of differences among Brc and planktonic cells (□) rather than between Brc and biofilm cells (*). Although some antibiotics were able to promote a decrease of about 2 $\log_{10}$ CFU/mL in some populations, the majority promoted a decrease on the cultivability of about 1 $\log_{10}$ CFU/mL and under. Thus, it was hypothesized that 2 hours of incubation may not be enough to promote a significant drop on the cultivability of *S. epidermidis* populations.

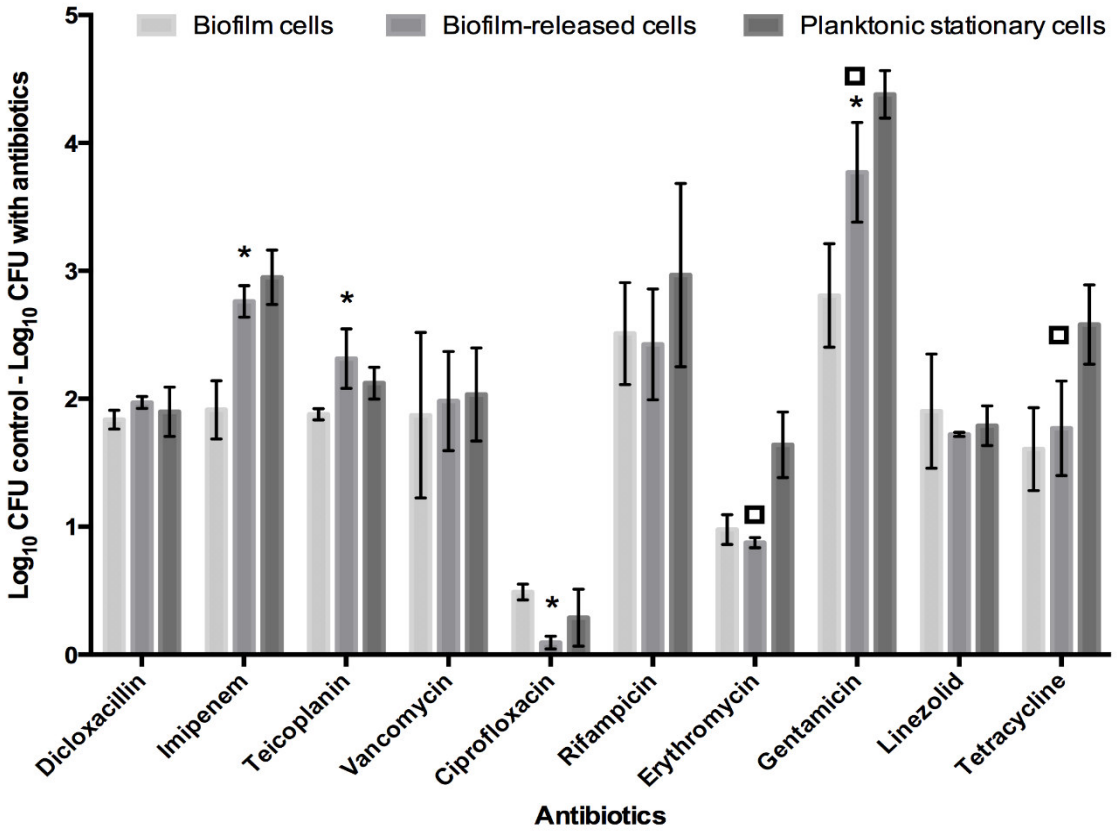

Figure 3.2 - Base 10 logarithmic CFU/mL reduction of *S. epidermidis* 9142 populations upon 6 hours of incubation with peak serum concentrations of distinct antibiotics. The columns represent the mean plus or minus standard error deviation, of at least three independent experiments. Statistical differences between groups were analysed with one-way ANOVA multiple comparisons, with * representing statistically significant differences (p <0.05) between biofilm cells and Brc and □ between Brc and their planktonic counterparts.

On the other hand, it is readily observed that after 6 hours of incubation (Figure 3.2) the decrease on the cultivability was more pronounced for all the populations and antibiotics, in comparison with the decrease of the shorter incubation period (Figure 3.1). While 2 hours of incubation promoted mostly reductions surrounding 1 $\log_{10}$ CFU/mL, with 4 more hours of incubation the medium reductions were close to 2 $\log_{10}$ CFU/mL for the majority of the antibiotics and populations. The fact that a longer incubation period led to higher decreases on the cultivability is in accordance with previous studies [58,79], and can be attributed to the fact that the populations of bacteria have the proper conditions to grow and multiply, allowing the antibiotics to be more effective in targeting the cell wall, and the protein and nucleic acids synthesis.

However, the extended period of incubation presents a technical limitation: although a significant increase in the reduction of the cultivability was found from 2 to 6 hours of incubation for all the populations tested, it is also clear that the longer period of incubation with the same antibiotics

demonstrated less differences on the susceptibility of the three populations studied, suggesting that the metabolism of the cells is changing overtime, reaching a more active state resembling exponential planktonic cells. Particularly regarding Brc, these findings seem to be related with a transient phenotype, in which these cells are more similar to biofilm cells moments after the disassembly process, but change the phenotype upon growing in the absence of the biofilm and become more similar to planktonic cells, by adapting their phenotype, as previously discussed in previous studies [58] and for distinct bacterial species [65], a phenomenon pointed to be related with quorum-sensing mechanisms [58,123].

From the analysis of the results presented in Figure 3.1, it is also possible to conclude that cell wall synthesis inhibitor antibiotics were substantially more effective in the reduction of the cultivability of planktonic cells comparing to the decrease in biofilm cells after 2 hours of incubation, as reported in previous studies [79,124] and had an intermediary effect on the cultivability of Brc. On the other hand, after 6 hours of incubation (Figure 3.2) small differences were found between the different populations under study. Among the antibiotics in this class, only dicloxacillin led to significant differences between biofilm cells and Brc, while both teicoplanin and vancomycin led to significant differences between Brc and planktonic cells, regarding the shorter incubation period. On the other hand, the longer incubation time led to significant differences between Brc and biofilm cells with both imipenem and teicoplanin, which is in accordance with the transient phenotype of Brc in the absence of the biofilm. Concerning Brc susceptibility to this class of antibiotics, dicloxacillin was the most effective in the reduction of the cultivability of the cells released from the biofilm after 2 hours of incubation, with a decrease of almost 1 $\log_{10}$ CFU/mL in comparison to the control suspension, while imipenem was the most effective after 6 hours of incubation, with a cultivability decrease around 2.8 $\log_{10}$ CFU/mL.

Regarding the susceptibility of the distinct populations with antibiotics acting on the inhibition of the nucleic acids synthesis, it is noticeable that only rifampicin was effective against this isolate with both the incubation periods tested. With 2 hours of incubation, the stationary planktonic suspension was significantly more affected by this antibiotic than Brc, while no differences were found among the three populations with the longest incubation period. This antibiotic promoted a reduction on the cultivability of Brc of 1.6 $\log_{10}$ CFU/mL after 2 hours of incubation, and of 2.4 $\log_{10}$ CFU/mL with 4 more hours of incubation. As can be seen in Figure 3.1 and Figure 3.2, *S. epidermidis* 9142 had a significantly lower decrease on the cultivability of all the populations with ciprofloxacin in comparison with any of the other antibiotics. This is not surprisingly, since the MIC values were above the PSC used in this particular circumstance. The fact that this isolate is very tolerant to ciprofloxacin is not surprising,

since some authors have previously reported that some *S. epidermidis* isolates may develop resistance against this antibiotic with some facility, in which the MIC values are higher than the clinical breakpoint established by EUCAST [125].

Lastly, the behaviour of the three populations of cells upon incubation with protein synthesis inhibitors was analysed and was found to vary according to each antibiotic. After 2 hours of incubation, erythromycin and linezolid were the least effective in decreasing the cultivability of both biofilm cells and Brc, with the first antibiotic having a significant higher effect in planktonic cells than in Brc. In turn, gentamicin showed no differences on the effect in Brc comparing with any of the two other populations of cells, and was the most effective antibiotic of this class against all the populations, with both the times of incubation tested. This antibiotic was the most effective, of all the antibiotics studied, reducing the cultivability of Brc, promoting a decrease of about 2.1 $\log_{10}$ CFU/mL and of nearly 3.8 $\log_{10}$ CFU/mL after 2 and 6 hours of incubation, respectively. These results may, at a first glance, seem surprising since, according to the preliminary MIC assay (Table 3.1), this isolate was classified as intermediate susceptible to gentamicin and resistant to linezolid. However, since the PSC of these antibiotics (10 mg/L and 18 mg/L, respectively) are higher than the MIC ranges found experimentally, the concentration used in the assay were sufficient to promote a notable reduction on the cultivability of the control strain. On the other hand, after 2 hours contacting with tetracycline, Brc experienced a significant lower decrease on the cultivability in comparison with both of the other populations under study, being the only antibiotic that promoted a significant higher reduction in biofilm cells than in Brc. However, the same was not observed after 6 hours of incubation, since Brc became as susceptible as biofilm cells, registering significant differences only in comparison with the planktonic population that experienced the highest reduction on the cultivability.

Although stationary planktonic cells are not as susceptible as in the exponential phase, it is not surprising that, in general, this population was the most susceptible to the antibiotics while biofilm cells were the most tolerant [79], and Brc had an intermediary behaviour.

Although some extracellular components of the biofilm may affect the efficacy of the antibiotics in the suspensions containing the biofilm cells and Brc [25,36], the adjustment of the density of the suspensions, by significantly diluting them, and the sonication and homogenization of the same, which reduced the quantity of polymeric substances mixed with the cells, minimized this effect.

All the classes of antibiotics studied are known to be more effective against actively growing cells, although both protein and synthesis inhibitors are able to act in cells that are not dividing but that still experience some metabolic activity [121,122]. This feature can, somehow, explain the decreased

susceptibility of biofilm cells against the majority of the antibiotics, as biofilm cells experience reduced growth rates, not multiplying as often as their planktonic counterparts [33,92]. Similarly, this evidence may explain some of the results obtained for Brc, since they have been proposed to be less active than log phase planktonic cells [33,65], something that should be further investigated.

It is important to notice that the previous results, regarding the evaluation of the cultivability, were based on the counting on plates of colony forming units (CFU), a method that allow the estimation of the number of cells with capacity to grow and replicate in a culture medium. Counting of CFU on agar plates presents some limitations, as it does not provide information on the amount of viable cells, since some of the viable cells can be uncultivable due, for instance, to a dormancy state [89]. Besides displaying some variability and presenting a limited detection regarding the quantification of the colonies, another limitation is related to the fact that it is assumed that each colony is formed by a single bacterium, however some colonies may be formed by more than a bacterium [126].

3.2 Study of the antibiotic susceptibility of cells released from biofilms with different stages of maturation

Biofilms undergo a lifecycle in which they grow, produce polymeric substances and release cells, going through different stages and becoming increasingly mature [33]. Disorders due to biofilm-infections may be caused by biofilms in different stages of maturation, in which the biofilm displays different properties, as the density of cells and the composition of the extracellular matrix, among others [33,39]. Consequently, it is important to evaluate if different stages of the biofilm growth affect the physiology of Brc, in particular its susceptibility to antibiotics.

To conduct this study, cells released from biofilms with 28, 48 and 72 hours of maturation (Brc_{28H}, Brc_{48H} and Brc_{72H}) were evaluated in terms of cultivability upon 2 hours of exposure to some antibiotics. According to the previous results, it was decided to only test the five antibiotics in which differences were found between Brc and the planktonic population after 2 hours of incubation, namely teicoplanin, vancomycin, rifampicin, erythromycin and tetracycline. The results of this experiment are presented in Figure 3.3.

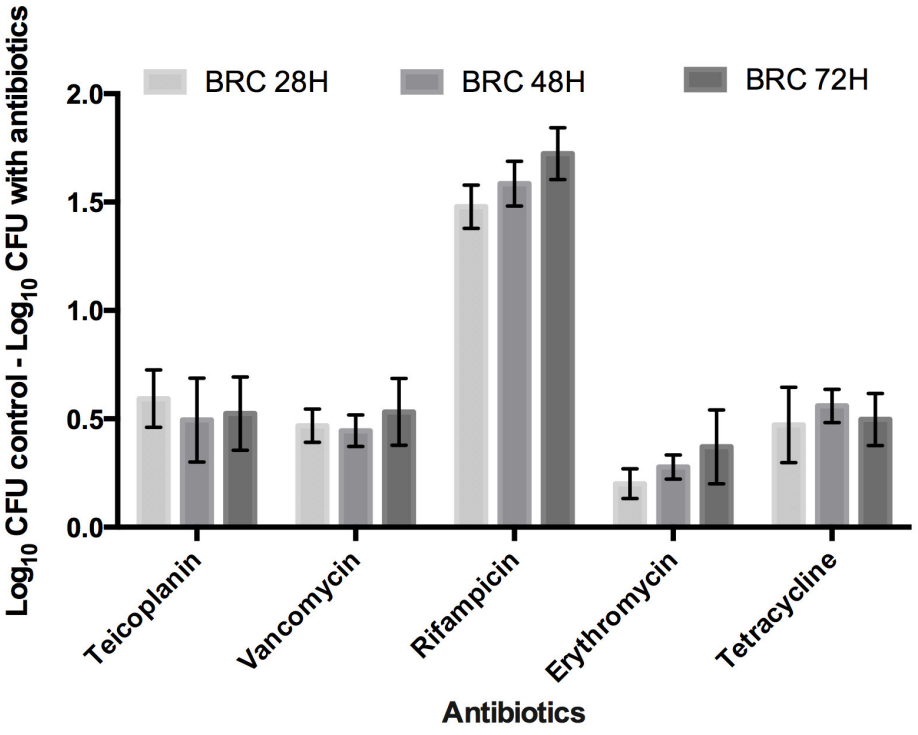

Figure 3.3 - Base 10 logarithmic CFU/mL reduction of *S. epidermidis* 9142 different Brc populations upon 2 hours of incubation with peak serum concentrations of five distinct antibiotics. The columns represent the mean plus or minus standard error deviation, of at least three independent experiments. Statistical differences between groups were analysed with one-way ANOVA multiple comparisons and no significant differences (p <0.05) were found among the different populations.

Although small differences were found among the cells released from the biofilms with different stages of maturation with all the antibiotics, as can be seen in Figure 3.3, none of these was considered significant from the statistical point of view, meaning that the aging of the biofilm had not a significant impact in the phenotype of Brc regarding antibiotic susceptibility.

Combining these data with the previously presented on section 3.1, it is reasonable to conclude that the phenotype of the cells that are released from the biofilm only suffers adaptions after growing in the absence of the originating biofilm. It was previously demonstrated that Brc would follow different phenotypical adaptations upon growing in the presence or absence of the biofilm, after being released [58], thereby, it is not surprising that Brc maintained their phenotype while being in the presence of the biofilm, despite the growth and maturation of the same.

3.3 Study of the antibiotic susceptibility of cells released from different *Staphylococcus epidermidis* isolates with 28 hours of maturation (BRC_{28H})

Staphylococcus epidermidis is known by its strong ability to form biofilms and is pointed as a major nosocomial pathogen associated with serious and recurrent infections [12,15]. Since this species is an inhabitant of the skin flora, it may easily invade the skin through wounds caused, for instance, due to medical practices as the insertion of catheters and medical devices [7,11]. Worldwide, a great number of *S. epidermidis* isolates were collected from different loci, as from blood cultures [100] and from central catheters [101], and the phenotypical differences that these isolates present may influence their susceptibility to antimicrobials [79].

Thus, the final phase of this project comprised the study of different *S. epidermidis* isolates and the comparison with the control strain (9142), in order to determine if the phenomena previously observed could be confirmed in clinical isolates. Analogously to the previous stage, only a fraction of the antibiotics was selected to assess the susceptibility of the distinct isolates, based on the significant different results found among Brc and the planktonic population obtained with the control strain used.

3.3.1 Preliminary MIC assay

Similar to the earliest experiments, a preliminary assay was performed in order to determine the MIC ranges of the selected antibiotics against the six *S. epidermidis* isolates chosen to this task. As previously, the determination of the susceptibility of the isolates to each antibiotic (S, R or I) was estimated based on the clinical breakpoints defined by EUCAST, when existing, and by CLSI and BSAC for the antibiotics to which no breakpoint limits were defined by EUCAST (see Table 3.1). The results of this standard MIC assay are presented in Table 3.2, in which MIC ranges above the clinical breakpoint (R) were identified in bold, and MIC ranges that classify the isolates as intermediary susceptible (I) were identified in bold followed by an asterisk.

Table 3.2 - Determination of the MIC ranges in mg/L of five antibiotics against six different *S. epidermidis* isolates

***S. epidermidis* strains**	**Teicoplanin**	**Vancomycin**	**Rifampicin**	**Erythromycin**	**Tetracycline**
IE186	4	2	0.004-0.016	1	**>32**
PT12003	0.25-1	1-2	0.002-0.008	1	0.25-1
MEX60	0.5-2	2	0.002-0.016	**>4**	**2-8 ***
DEN69	1-4	1-2	0.002-0.016	0.5-1	**>32**
ICE09	**2-8 ***	1-4	0.002-0.016	**>4**	**1-4 ***
URU23	**4-16 ***	2	0.002-0.016	**>4**	**0.5-2 ***

From the analysis of the results of the MIC assay (Table 3.2) it is possible to conclude that none of the isolates was classified as resistant to teicoplanin, however two of them (ICE09 and URU23) showed to be intermediate to this antibiotic and the remaining four were considered susceptible. Regarding vancomycin and rifampicin, all the isolates showed to be susceptible to these antibiotics. In turn, half of the isolates were susceptible to erythromycin (IE186, PT12003 and DEN69), while the other half was considered resistant. Lastly, two isolates were resistant to tetracycline, namely *S. epidermidis* IE186 and DEN69, and three isolates were classified as intermediate susceptible to this antibiotic, namely MEX60, ICE09 and URU23.

Vancomycin was the antibiotic that promoted the most similar results in all the isolates tested, since all the MIC ranges comprised values from 1 to 4 mg/L, seeming that the phenotypical differences existing between these isolates do not interfere significantly with the efficacy of this particular antibiotic. On the other hand, with other antibiotics, as tetracycline and teicoplanin, a great variability in the MIC ranges for different isolates was found, what is an evidence that the singular phenotypical features they experience may have a considerable influence on their reaction to some antibiotics. The fact that some antibiotics led to different results in distinct isolates of the same species is not surprising and it is somewhat frequent to find some isolates that are resistant to an antibiotic to which others of the same species are susceptible [79,127], as happened, for instance, with tetracycline.

3.3.2 Study of the biofilm formation ability of the six different *S. epidermidis* isolates selected

Inter- and intra-strain variability may be found in *S. epidermidis* biofilms, affecting the extent of the biofilm and the quantity of cells and extracellular matrix produced by the same [127]. The distinct densities of the *in vivo* biofilms may influence the efficacy of the antibiotics fighting infections, as thick biofilms, with a larger number of cells and EPS, are generally more tolerant to the antibiotics than weak biofilms [25,128].

It was recently demonstrated that, after carefully washing twice a robust biofilm, adding fresh medium to the same and allowing the biofilm to grow for at least 3 hours, the cells present in the bulk fluid were mainly cells resulting from the disassembly of the biofilm (Brc) and not the result of planktonic growth, a conclusion based on the comparison of the growth of the same suspensions in the presence or absence of the originating biofilm [58]. However, since these conclusions were drawn based on an isolate with a strong ability to form biofilms, the same may not happen with poor biofilm-forming isolates. Therefore, the assessment of the biofilm formation ability of the isolates selected was needed.

For a normalized quantification of the susceptibility testing, the density of the populations of cells was adjusted for the main experiments of this thesis, undervaluing the influence of the thickness of the biofilm and concentration of cells inside the biofilm and dispersed from the same. However, the amount of biofilm formed by the isolates, which is directly related to the concentration of cells inside the biofilm and intensity of QS signals, may influence the phenotype of Brc. To assess if the isolates selected would fit the previously described model of Brc development [58], the optical density (640 nm) of the biofilm and of the biofilm bulk fluid was measured at different points of the lifecycle, namely at 24, 28, 48 and 72 hours for the biofilm and at 28 and 48 hours for the biofilm bulk fluid, as illustrated in Figure 3.4.

Since the experiments were conducted under a fed-batch model, the replacement of the spent medium by fresh medium and the washing of the biofilm in between are essential to remove the unattached bacteria and to provide nutrients to the remaining bacteria in the biofilm, allowing the same to continue developing [129]. However, as biofilms are not rigid structures, they can easily break and/or release cells due to shear forces exerted by the addition of solutions. For that reason, washing of the biofilms must be carefully performed to maintain the biofilm structure as intact as possible, removing the majority of the cells that are loosely or not attached to the biofilm, thus, minimizing the number of cells that are detached from the biofilm by shear forces [58,130].

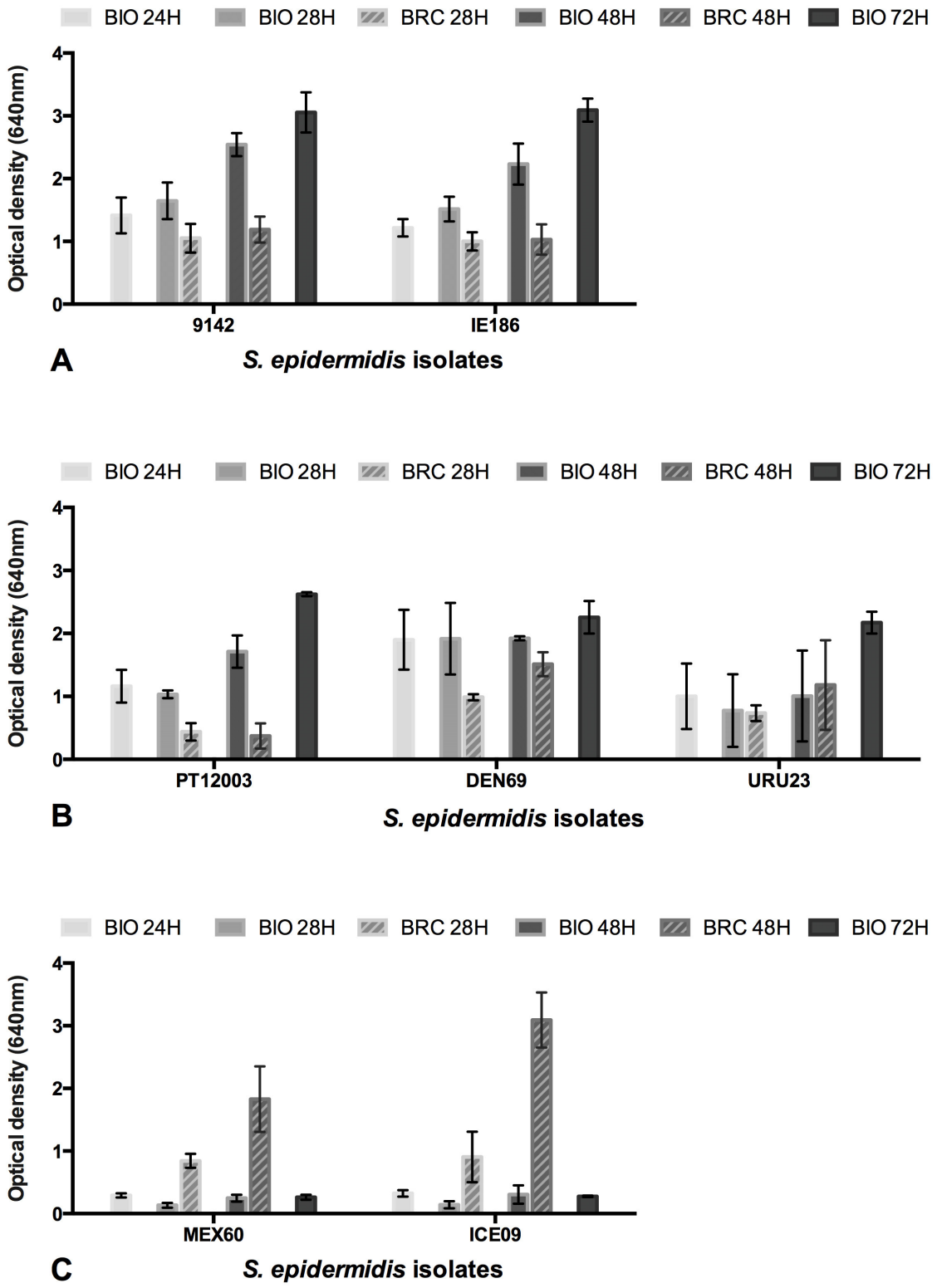

Figure 3.4 - Optical density (OD) of 24, 28, 48 and 72 hour-old mature biofilms (BIO) and 28 and 48-hours bulk fluid containing Brc (BRC) of seven *S. epidermidis* isolates, measured at 640 nm. (A) OD of high biofilm producing isolates; (B) OD of medium biofilm producing isolates; and (C) OD of low biofilm producing isolates. The columns represent the mean plus or minus standard error deviation, of at least three independent experiments

During its lifecycle, especially over the maturation phase, the biofilm develops due to the division of cells and production of EPS, enhancing the thickness of the biofilm and, consequently, increasing its optical density [33]. As can be seen in Figure 3.4 (A) and (B), the OD of the biofilm increased significantly over the time and the OD of the 72 hour-old biofilm was considerably high in the isolates represented in (A) and medium in the ones in (B), therefore these isolates were classified as good and medium biofilm formers, respectively. On the other hand, isolates represented in Figure 3.4 (C) showed a weak ability to form biofilm, being noticeable that the biofilms did not greatly developed until 72 hours of growth, thus being classified as low biofilm forming isolates.

A ratio of Brc/biofilm cells was calculated in order to compare the OD of cells that developed inside the biofilm *versus* the quantity of cells that was found in the biofilm bulk liquid. As can be seen in the figure above, good (Figure 3.4 (A)) and medium (Figure 3.4 (B)) biofilm forming isolates presented a ratio smaller than one at 28 hours, meaning that the amount of cells in the biofilm was higher than the amount of cells in the biofilm bulk fluid, while low biofilm formers (Figure 3.4 (C)) presented a ratio significantly higher, with approximately six times more cells in the bulk fluid than within the biofilm. At 48 hours, *S. epidermidis* 9142, IE186, PT12003 and DEN69 continued to present a ratio lower than one, while URU23 presented a ratio very close to one

On the other hand, the 48-hours ratio of the MEX60 and ICE09 isolates (Figure 3.4 (C)) was significantly higher than the ratio found at 28 hours. As illustrated in Figure 3.4 (C), the optical density of the biofilm bulk fluid significantly increased in that period, while the OD of the biofilm remained identical, against what was verified with the other isolates tested, meaning that, for these specific isolates, it is not possible to assure if the cells in the bulk fluid derived from the biofilm or were a result of planktonic multiplication. According to these findings, the previously described model for Brc obtention [58] may not be suitable for low biofilm forming isolates, and, therefore, Brc present in the biofilm bulk fluid may possibly present a distinct phenotype than Brc released from isolates with a stronger biofilm.

Analysing the results illustrated in Figure 3.4 it can also be verified that 9142 and IE186 (A) produced the thickest biofilms and were the only isolates in which a small increase in the OD was found from the 24 to the 28 hour-old biofilm, against what happened with all the other isolates where the 28-hours biofilm had the same or less extent than the 24-hours biofilm. The fact that these isolates present a high capacity of biofilm formation may explain the slight increase of cells and/or extracellular products in the 28-hours biofilm, while in isolates with a weaker biofilm formation ability no noticeable increase in the amount of biofilm cells or extracellular products is found in the same 4-hour period.

3.3.3 Vancomycin susceptibility of different *S. epidermidis* isolates and populations

Since no significant differences were found between the cells released from biofilms in different stages of maturation (Section 3.2), Brc chosen to perform this assay were the cells released from biofilms with 28 hours of maturation (BRC_{28H}). Using the Brc from the less mature biofilms reduced the experimental time, allowing to include more isolates in this experiment.

Here, the aim was to find how the three populations of cells of different isolates behave against the same antibiotic. The main focus of this study was to explore if the phenotypical differences of distinct *S. epidermidis* isolates affect their susceptibility to antibiotics and if the main differences found among the three populations of the control *S. epidermidis* are transversal to other isolates.

The preliminary MIC assay allowed determining if the selected isolates were susceptible to a range of five antibiotics. Based on these results (Table 3.2), vancomycin and rifampicin raised interest, since all the isolates tested were found to be susceptible to these antibiotics. Moreover, vancomycin led to very similar results of the MIC range for all the isolates, while there was more variability with rifampicin, thus, to reduce inherent susceptibility variability, vancomycin was the antibiotic chosen to perform this experiment. Therefore, separated suspensions containing biofilm cells, Brc and stationary planktonic cells of each isolate were incubated for 2 hours with PSC of vancomycin, and the results are presented in Figure 3.5, organized according to the ability of the isolates to form biofilms.

Analysing these results, it is promptly noticeable that all the isolates presented statistically significant differences between Brc and planktonic cells regarding the susceptibility to vancomycin, as represented with □ in Figure 3.5, confirming the observations with the control isolate. Furthermore, three isolates (PT12003, DEN69 and MEX60) also showed differences between the biofilm and Brc populations that were considered significant from the statistical point of view.

As previously reported, in *S. epidermidis* 9142 the decrease on the cultivability of Brc was very similar to the registered with the biofilm population, while planktonic cells were significantly more affected by the antibiotic. The stationary planktonic cells of IE186 also experienced a very high decrease on the cultivability, resembling the behaviour of the control isolate, although Brc were slightly more tolerant to the antibiotic than biofilm cells (not statistically significant). The results found with 9142 and IE186 may be explained by the fact that both of these isolates are strong biofilms formers, suggesting that Brc are supposedly more influenced by QS signalling from the thick biofilms produced.

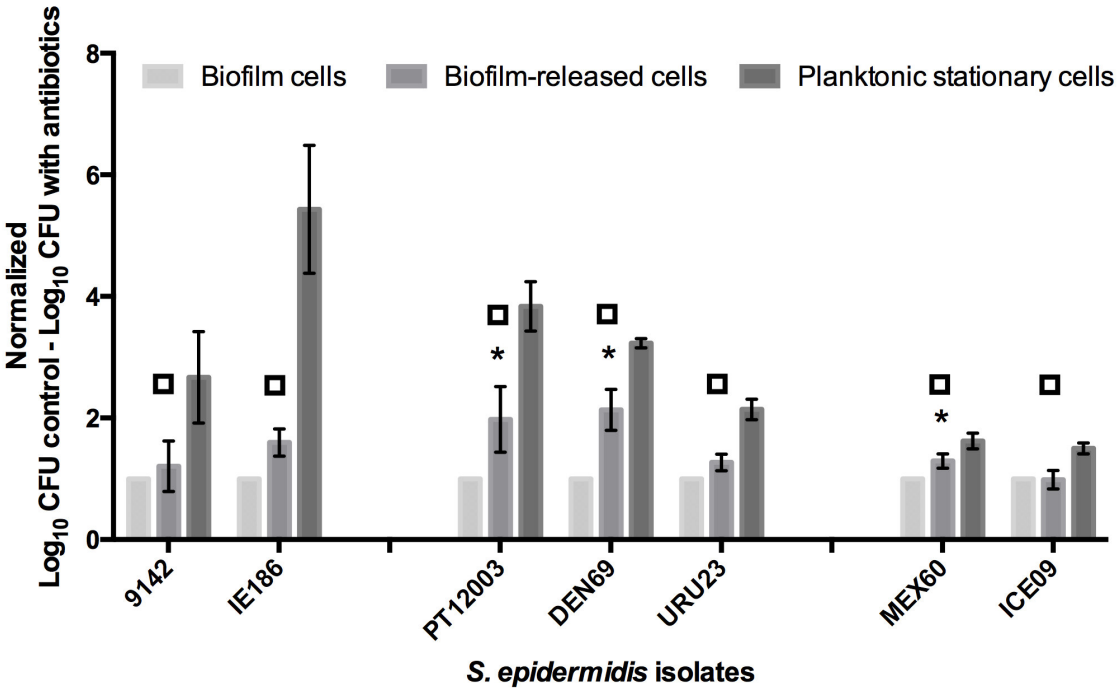

Figure 3.5 - Normalized base 10 logarithmic CFU/mL reduction of three populations of several *S. epidermidis* isolates upon 2 hours of incubation with peak serum concentrations of vancomycin. The results were normalized according to the results obtained for the biofilm population for each isolate, where the biofilm cultivability decrease was considered equal to 1 $\log_{10}$ CFU/mL. The second and third columns represent the mean plus or minus standard error deviation, of at least three independent experiments. Statistical differences between groups were analysed with one-way ANOVA multiple comparisons, with * representing statistically significant differences (p <0.05) between biofilm cells and Brc and □ between Brc and their planktonic counterparts.

Regarding isolates with a medium ability to form biofilms (PT12003, DEN69 and URU23), all led to results that resembled 9142 regarding the high decrease in the number of cultivable planktonic cells in comparison to the decrease in the cells from the biofilm. Although the behaviour pattern of these isolates presented some similarities with the control one, the decrease on Brc cultivability on PT12003 and DEN69 was considerably higher than the one registered in the biofilm, against what happened with 9142, what seems to be related by a lower influence of the biofilm QS signalling, as these isolates produce biofilm in a lesser extent than the control one.

Lastly, MEX60 and ICE09 were the isolates in which the cultivability reduction of the planktonic cells was more close to the value obtained with the biofilm. Although statistical significant differences were found among Brc and planktonic cells, the differences between these populations were considerably lower than the ones registered with all the other isolates. These results are in accordance with the fact that these isolates are poor biofilm formers and present an extremely high Brc/biofilm

ratio, since the biofilm bulk fluid may contain a larger number of cells growing planktonically mixed with Brc. As such, in a critical analysis, it is possible that, on these cases, the established experimental model is not able to produce a real distinct Brc population.

Based on the previous discussion, it seems that a greater ability to form biofilm is associated with a higher tolerance of Brc towards the antibiotics [70,72]. Moreover, it may be suggested that both strong and medium biofilm formers are very similar to the control strain, which is also characterized by a strong ability to produce biofilms, and produce a similar Brc population. On the other hand, weaker biofilm formers were not very similar to the control *S. epidermidis* used, and further investigation should be performed to assess if the main cells of the bulk fluids of these biofilms were Brc with a distinct phenotype or cells growing planktonically that do not fit the Brc development model.

4. Conclusions and Future work

4.1 Main conclusions

Staphylococcus epidermidis biofilm disassembly and, therefore Brc, has been associated with the emergence of several infections and serious complications, as bacteremia and infective endocarditis [131]. However, little information existed regarding the phenotypical particularities of this population, in particular Brc susceptibility towards antibiotics.

The characterization of Brc interaction with a range of antibiotics clinically used to treat *S. epidermidis* infections is fundamental to prevent serious complications associated with the systemic release of cells from the biofilm loci. Thus, this study aimed to overcome the lack of knowledge regarding the efficacy of these antibiotics acting against cells released from *S. epidermidis* biofilms.

This study highlights that, under the specific experimental conditions used in this thesis, *S. epidermidis* Brc presented phenotypic features that influenced their tolerance to antibiotics, presenting a behaviour that could be distinguished from both biofilm cells and from their planktonic counterparts. Moreover, considering the differences in the results found from 2 to 6 hour-exposure to antibiotics, it was concluded that Brc present a transient phenotype, being more similar to the biofilm phenotype soon after being released, but becoming increasingly more similar to planktonic cells when growing in the absence of the biofilm, confirming previous results demonstrated with a limited number of antibiotics [58], enhancing the idea of a particular cell population physiology changing overtime.

No obvious pattern was found among antibiotics with the same mechanism of action, what is not necessarily surprising since antibiotics with the same general mechanism of action also endue different specific mechanisms of interacting with bacteria [121,122]. For instance, nucleic acids synthesis inhibitors may act on the inhibition of DNA or RNA and even antibiotics acting on the same type of nucleic acids may present diverse modes of action against bacteria, e.g., DNA synthesis inhibitors may bind to and inhibit DNA gyrase or act on the inhibition of DNA polymerase [132]. According to the results obtained and to the fact that no pattern was found among all the antibiotics of each class tested, it was not possible to determine if any of the mechanisms of action was particularly more efficient in targeting the control *S. epidermidis* populations than others.

Although biofilms features can change over the time, such as the cell density and the composition of the extracellular matrix [33], the results obtained in the study comprising Brc released at different timepoints of the biofilm lifecycle demonstrated that there are no significant differences in the susceptibility to teicoplanin, vancomycin, rifampicin, erythromycin and tetracycline of Brc from 28, 48 or 72-hour mature biofilms.

Since Brc presented an increased resistance to the majority of the antibiotics tested, in comparison to the planktonic populations, the efficiency of the antibiotics used to combat these infections may be compromised, allowing Brc to disperse over the host inducing inflammation and infection proliferation.

It is commonly known that different isolates present some variability, as differences in the ability to form biofilms, that may affect their susceptibility to antibiotics [127], as this was confirmed experimentally, since different isolates presented distinct results upon being incubated with several antibiotics. However, the significant differences found between Brc and planktonic cells tolerance to vancomycin reported with the control strain were also found with all the other isolates tested.

Based on the results, it was also found that the phenotypical differences in the poor biofilm forming isolates led to minor differences between Brc and planktonic cells susceptibility, being suggested that the biofilm bulk fluid of these isolates may not represent a distinct Brc population.

Although data presented in this thesis have provided important insights in the pathogenesis of *S. epidermidis* biofilm-related infections, prophylactic and therapeutic approaches should be further investigated to combat spreading of infections and occurrence of inflammation due to the release of cells from the biofilm.

4.2 Suggestions for future work

For a better understanding of the *S. epidermidis* Brc phenotype and how it affects the susceptibility to antibiotics, further investigations should be done in this field, to better assess how Brc contribute to the failure of therapeutic measures.

Since a small number of strains was tested, it was not possible to draw conclusions related to the feasibility to use vancomycin against *S. epidermidis* infections, whereby a more significant number of isolates, as well as a wider range of antibiotics, should be tested in order to provide a better characterization of the Brc phenotype regarding their susceptibility to antibiotics and, therefore, contribute to the establishment of more efficient therapeutic measures against these biofilm-related infections.

The assessment of the viability of the distinct populations of cells, for instance by flow cytometry or by microscopic techniques, would be advantageous, since CFU counting only evaluates the cultivability of the cells, meaning that some bacteria may be affected by the antibiotics to the extent of not being able to replicate in plates, being, however, viable.

Moreover, since significant differences between Brc and their biofilm and planktonic counterparts were found, further investigation should be done concerning differences in the gene expression of the three populations, in order to assess if the up and/or downregulation of specific genes is associated with the particular phenotype found in Brc.

Future work in the combat of staphylococcal biofilm infections may include the development of therapeutic and prophylactic strategies targeting quorum-sensing signalling, since QS is pointed as a possible responsible for the release and phenotype of Brc. Hence, QS mechanisms and the expression of genes involved in QS signalling should be deeply analysed to elucidate the role of this mechanism in the Brc phenotype.

The main drawbacks of *in-vitro* experimental studies are the differences to the *in vivo* situations, as the presence/absence of biological substances and variations of the environmental conditions (nutrition, pH, temperature, among others) that influence bacterial phenotypes. Thus, it would be of major interest to study the phenotype of Brc in their normal biological context, performing *in vivo* or *ex vivo* experiments to predict the susceptibility of this population of cells to a wide range of antibiotics under real circumstances of biofilm-related infections.

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