Universidade do Minho
Escola de Engenharia

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Immobilized antimicrobial agents: potential development of microbial resistance

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ABSTRACT

With an aging society and the increasing use of medical implants and devices, the problem of biomaterial-associated infections (BAI) will increase in coming years. The development of materials able to prevent bacterial colonisation is a promising approach to deal with BAI and several strategies to confer biomaterials with antimicrobial properties are emerging. They have, however, some limitations to be solved, namely the potential development of microbial resistance to the antimicrobial agents immobilized on the functionalized surfaces.

This study aimed to evaluate the potential development of resistance by *Staphylococcus aureus* and *Staphylococcus epidermidis* adhered on polydimethylsiloxane (PDMS) functionalized with different antimicrobial compounds (vancomycin, rifampicin and benzalkonium chloride, BAC). PDMS functionalization was performed using a mussel-inspired coating strategy.

A preliminary optimization of antimicrobials immobilization to confirm their contact-killing activity was followed by leaching assays. The ability of antimicrobial surfaces to prevent biofilm formation was further assessed by XTT assay. The potential development of resistance towards the immobilized antimicrobials was evaluated by continuously recovering the cells adhered on the surfaces and allowing them to adhere to new surfaces for a total of 10 passages. Results showed that antimicrobial surfaces exhibited contact-killing activity being the best performance achieved for a basic pH (8.5) and an overnight incubation. The leaching assays revealed that the increase of the antimicrobial concentration produced higher inhibition zones, hence more antimicrobial release from the functionalized surfaces. Antimicrobial surfaces were able to impair biofilm establishment, although complete biofilm eradiation was not achieved. The possible development of resistance of these remaining cells was then investigated and results showed that cells recovered from BAC-functionalized surfaces did not express propensity for developing resistance, as they have susceptibility patterns similar to the cells recovered from the unmodified surfaces. Conversely, cells recovered from the surfaces modified with antibiotic exhibited a higher MBC as compared to cells recovered from unmodified PDMS. Surfaces functionalized with rifampicin were not tested for potential development of resistance because its immobilization without leaching could not be achieved. This study highlighted the importance of evaluating the potential development of microbial resistance towards immobilized antimicrobials, namely when antibiotics are used to modify biomedical surfaces. Although it is required to test BAC cytotoxicity after its immobilization, overall results also emphasized its potential to be used in the design of materials able to prevent BAI without fostering bacterial resistance.

RESUMO

Com o envelhecimento da sociedade e o aumento do uso de implantes e dispositivos médicos, a problemática das infeções associadas a biomateriais (BAI) deverá aumentar nos próximos anos. A melhor forma de lidar com estas infeções consiste na modificação dos biomateriais conferindo-lhes propriedades antimicrobianas. Existem, contudo, limitações que precisam de ser solucionadas, nomeadamente o potencial desenvolvimento de resistência microbiana aos agentes antimicrobianos após a sua imobilização nas superfícies.

Este trabalho teve como objetivo avaliar o potencial desenvolvimento de resistência de *Staphylococcus aureus* e *Staphylococcus epidermidis* aderidos em superfícies de polidimetilsiloxano (PDMS) funcionalizadas com diferentes compostos antimicrobianos (vancomicina, ripampicina e cloreto de benzalcónio, BAC). A funcionalização do PDMS foi realizada utilizando uma estratégia de revestimento inspirada na adesão dos mexilhões.

Primeiro, procedeu-se à otimização da imobilização dos antimicrobianos para confirmar a sua capacidade de matar por contacto, seguindo-se os ensaios para avaliar a sua libertação da superfície. A capacidade das superfícies antimicrobianas para prevenir a formação de biofilme foi avaliada através de um ensaio de XTT. O potencial desenvolvimento de resistência aos agentes antimicrobianos imobilizados foi em seguida avaliado através da contínua recuperação das células aderidas a estas superfícies antimicrobianas, seguindo-se a sua adesão a novas superfícies, processo repetido num total de 10 passagens. Os resultados demonstraram que as superfícies preparadas apresentaram propriedades antimicrobianas, obtendo-se um melhor desempenho para um pH alcalino (8.5) e um tempo de incubação de 16-18 horas. Os ensaios de libertação revelaram que um aumento da concentração do agente produziu maiores zonas de inibição, logo uma maior libertação. Estas superfícies foram capazes de prejudicar o estabelecimento de biofilme, contudo, não foram capazes de o erradicar completamente. O potencial desenvolvimento de resistência destas células foi depois investigado e os resultados demonstraram que as células recuperadas das superfícies funcionalizadas com BAC não apresentaram tendência para induzir resistência uma vez que estas exibiram o mesmo perfil de suscetibilidade que as células aderidas às superfícies não modificadas. Pelo contrário, as células em contacto com as superfícies funcionalizadas com vancomicina, apresentaram uma MBC superior à das células recuperadas das superfícies não modificadas de PDMS. A imobilização da rifampicina não foi possível de efetuar sem libertação pelo que não foi testada nos ensaios de resistência.

Este estudo salienta a importância de estudar o potencial desenvolvimento de resistência por parte de antimicrobianos imobilizados, sobretudo quando antibióticos são usados para modificar a superfícies de biomateriais. Apesar de ser necessário testar a citotoxicidade do BAC após a sua imobilização, foi ainda salientado o potencial do BAC na produção de materiais para prevenir as BAI sem despoletar resistência bacteriana.

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LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
AMP	Antimicrobial peptide
BAC	Benzalkonium chloride
BAI	Biomaterial-associated infection(s)
CFU	Colony forming units
DDD	Defined daily dose
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
ECDC	European Centre for Disease Prevention and Control
EPS	Extracellular polymeric substances
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
MAP	Mussel adhesive protein(s)
MBC	Minimum bactericidal concentration
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
NaCl	Sodium chloride
OD	Optical density
PBS	Phosphate-buffered saline
pDA	Polydopamine
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
PMS	Phenazine methosulfate
QAC	Quaternary ammonium compounds
SCV	Small colony variant
SD	Standard deviation
SEM	Scanning electron microscopy
TSA	Tryptic Soy Agar

TSB	Tryptic Soy Broth
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt

SCOPE AND AIMS

The use of biomaterial implants and medical devices is an increasingly common procedure in modern healthcare. Despite their benefits, the problem of biomaterial-associated infections (BAI) has also been increasing. These infections generally involve microbial colonization and biofilm formation on biomaterials, which results in a higher antimicrobial and host immune system resistance. Several studies have been conducted to prevent the formation of these microbial biofilms. Immobilization of antimicrobials such as quaternary ammonium compounds (QAC) and antibiotics has been proposed as a promising strategy to confer antimicrobial properties to medical devices and, therefore, to reduce the risk of infection.

Despite the promising results reported in the literature, most of these strategies fail to proceed into clinical trials and important factors involved in the pathogenesis of these infections are often neglected, namely toxicity issues and the potential development of resistance towards antimicrobials immobilized. So, it is crucial to understand the fate of bacteria that manage to adhere to antimicrobial surfaces to develop effective antimicrobial coatings able to combat these infections.

The main purpose of the present thesis is to assess the potential development of resistance by *S. aureus* and *S. epidermidis* against three antimicrobials, currently under investigation for use in medical devices, after their immobilization. To achieve this goal, two antibiotics (vancomycin and rifampicin) and a QAC will be immobilized onto polydimethylsiloxane (PDMS) surfaces using a mussel-inspired coating strategy. Their immobilization will be then optimized so that PDMS exhibits antimicrobial properties but without antimicrobials release. Their ability to impair biofilm establishment will be also performed. Once obtained antimicrobial surfaces that meet these requirements, the potential development of microbial resistance towards immobilized antimicrobials will be finally investigated.

KEYWORDS: BACTERIAL RESISTANCE, ANTIMICROBIAL COATINGS, POLYDOPAMINE, BIOFILMS.

1. INTRODUCTION

1.1 Biomaterial-associated infections

The last decades have been characterized, as never before in human history, by the broadest application of medical devices in all areas of medicine (Campoccia et al., 2013). Every year, millions of patients improve their quality of life through surgical procedures that involve medical devices that are implanted or not (Khan et al., 2014). These devices play an important role in human life to support and restore function after wear, trauma, surgical intervention or even to improve appearance (Domingues et al., 2015; Zimmerli & Sendi, 2011). Devices like joint and vascular prostheses, catheters, lenses, dental implants and others are increasingly being used since the 90s (Moraes et al., 2013). As a result of the increased life expectancy and the increasing demand for medical care from the aging population, the number of age-related diseases also increased. Thus, the need to carry out new treatments arises, which may involve the use of implants and long-term pharmaceutical administration (Campoccia et al., 2013; Khan et al., 2014).

In 2007, it was estimated that, worldwide, the use of medical devices approached half a billion devices per year, with catheters alone acounting for about 400 million pieces (Campoccia et al., 2013). The trend is that medical devices are being used increasingly, continuously and simultaneously (Baio, 2011).

Despite its great benefits, it has been recognized for more than half a century that the presence of a biomaterial implant or device in host tissue strongly predisposes for infection (Zaat et al., 2010). These devices provide foreign surfaces to the human body, to which microorganisms can adhere and start forming biofilms (structured communities of microorganisms that adhere to one another on a living or abiotic surface and produce extracellular polymeric substances which protect them from the external environment) (Desrousseaux et al., 2013). Accordingly, in modern medicine, biomaterial-associated infections (BAI) are the number one cause of failure of biomaterial implants and devices, resulting in high costs to the health care system (Domingues et al., 2015; Moraes et al., 2013; Zaat et al., 2010). For example, in the USA, more than 5 million central venous catheters are implanted annually, of which, more than 80000 lead to catheter-related bacteremia

(Desrousseaux et al., 2013). A study conducted in four European countries showed that bloodstream infections related to catheters accounted for over 1000 deaths with associated costs of between € 35 and € 164 million annually and per country (Desrousseaux et al., 2013). Therefore, the occurrence of BAI is clearly recognized as a world problem.

1.1.1 Routes of infection

As aforementioned, the undesirable complication often associated to the use of implants or medical devices is the occurrence of an infection caused by microorganisms. Microorganisms can be acquired from several sources including the operating room environment, surgical equipment, clothing worn by medical professionals, resident bacteria on the patient's skin, and bacteria already in the body (Hetrick & Schoenfisch, 2006). The best documented route of infection is direct contamination of an implant during surgery (perioperative contamination), such as in orthopedic, cardiovascular, plastic reconstructive, general surgery, and neurosurgery (Domingues, 2013; Zimmerli & Sendi, 2011). These infections occur after invasive procedures in the superficial or deep layers of the incision or in the organ or space that was manipulated or traumatized, and can be diagnosed 30 days after the surgical procedure (Amaral et al., 2013; *WHO Guidelines for Safe Surgery*, 2009). This way of contamination implies that an implant becomes contaminated with microorganisms before or during implantation into the human body (Domingues, 2013).

Perioperative infection accounts for about 15 % of all healthcare-associated infections and about 37 % of the hospital-acquired infections associated to surgical patients. In Western countries, the frequency of these infections is 15-20 % of all cases and, generally, surgery is responsible for 2-15 %. In general, perioperative infections lead to an average increase in length of hospital stay of 4-7 days. More specifically, patients infected with these kind of infections are twice as likely to die and to spend time in an intensive care unit and five times more likely to be readmitted after discharge (*WHO Guidelines for Safe Surgery*, 2009).

Despite the preparation performed in the skin before surgery, bacteria are always present. Quantitatively, the risk of acquiring an infection in the surgical site is much greater if it is contaminated by more than 10^5 microorganisms per gram of tissue. However, in the presence of a foreign material, such as an implant, the amount of microorganisms to produce a necessary infection is much lower (Domingues, 2013; *WHO Guidelines for Safe Surgery*, 2009).

Although sterilization and the use of aseptic techniques greatly reduces the levels of bacteria found in hospital settings, pathogenic microorganisms are still found at the site of approximately 90 % of all implants, so it is crucial to continue preventing this way of contamination (Hetrick & Schoenfisch, 2006).

The second route of infection is called postoperative because it occurs after the surgery, during the hospitalization period (Domingues, 2013). After biomaterial implantation, there is a 6 h decisive period, which is critical for the long-term success of the implant. Over this period, an implant is particularly susceptible to surface colonization (Hetrick & Schoenfisch, 2006). This way of infection is mainly caused by direct contamination of open wounds or by the use of invasive devices like infusion tubes, catheters, or drains (Domingues, 2013).

Postoperative infection can be triggered by different variables, that encompasses procedural variables (including type and length of procedure) and patient variables (such as the general medical and physical condition of patients before surgery) (Peterson, 2006). The most important variable is the type of surgery performed. Some studies show that the duration of the surgery is related to the infection rate. Simple procedures with short operative times and minimal incisions generally result in lower rates of postoperative infection as opposed to the complex procedures with long operative times (Peersman et al., 2006; Peterson, 2006).

At last, biomaterials can also be infected by the hematogenous route. Although the risk of developing a BAI is higher for events related to surgery, there is a residual risk for the possibility of late infections caused by microorganisms from local infections elsewhere in the body that are spread through the blood (Campoccia et al., 2013; Domingues, 2013). Most hematogenous infections are caused by infected skin lesions that produce recurrent bacteremia. This is supported by the fact that the majority (56 %) of the infections suspected to be hematogenous are caused by staphylococci. Other examples, such as dental or other surgical interventions, bacteriuria, intestinal surgery and pneumonia, have also been suggested as possible causes of hematogenous spread of microorganisms, which can cause temporal or chronic bacteremia, leading to infections. (Domingues, 2013; Gottenbos et al., 2001).

Another possible mechanism for hematogenous spreading from the intestinal tract is bacterial translocation, when bacteria, mainly Gram-negative strains, escape through the intestinal wall. Thus, BAI due to hematogenous spreading of bacteria to an implant site may occur any time after implantation (Domingues, 2013; Gottenbos et al., 2001).

1.1.2 Main causative organisms and infections incidence

The most commonly isolated pathogens from infected biomaterial surfaces include Gram-positive *Staphylococcus epidermidis* and *Staphylococcus aureus*, causing up to 60 % of all prosthetic hip implant infections since 1980. Additionally, Gram-negative organisms such as *Escherichia coli* and *Pseudomonas aeruginosa* are also isolated (Bruellhoff et al., 2010; Domingues, 2013; Hetrick & Schoenfisch, 2006; Joo & Otto, 2012; Subbiahdoss et al., 2011). **Table *1*** presents the percentage of incidence of BAI associated to different biomedical implants and devices and the main causative organism.

Table 1. Incidences and causative organisms of infections associated with commonly used medical devices and implants. Incidence data refers to the lifetime of the implant or device. (Busscher et al., 2012; Roosjen et al., 2006).

Medical device	Microorganism	% of incidence
Mechanical Heart Valve	*S. aureus*	1.88
Intraocular lens	*P. aeruginosa, S. epidermidis*	0.1–0.5
Urinary catheters	*Escherichia coli*	10– 20
Prosthetic Hip	*S. aureus, S. epidermidis*	2.6
Prosthetic Knee	*S. epidermidis, S. aureus*	3– 4
Mammary prosthesis	*S. aureus*	1– 7
Central Venous Catheter	*S. epidermidis, S. aureus*	4– 12

S. aureus is both a commensal bacterium and a human pathogen and it is a dangerous and versatile microorganism that can cause a wide variety of diseases (Nair et al., 2014; Otto, 2014). *S. aureus* is responsible for approximately 23 % of infections associated with prosthetic joints and is the leading cause of bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue and respiratory tract infections (Otto, 2014; Subbiahdoss et al., 2011; Tong et al., 2015).

Skin infections caused by *S. aureus* are commonly community-acquired, whereas respiratory tract infections are predominantly nosocomial infections. Among the range of nosocomial pathogens, *S. aureus* is the most common and associated with high morbidity and mortality. In hospitalized patients with debilitated conditions, such as in patients suffering from immune

deficiencies or viral infections, *S. aureus* is often responsible for developing pneumonia (Otto, 2014).

S. epidermidis is a microorganism present in the epithelial surfaces of every human being. It can be widely found on the skin, where is part of the commensal bacterial microflora (Hetrick & Schoenfisch, 2006; O'Gara & Humphreys, 2001). In a similar way to *S. aureus*, *S. epidermidis* is a major cause of nosocomial infections (Cheung et al., 2010). Almost 50 % of the infections associated with catheters, artificial joints and heart valves are caused by this microorganism (Subbiahdoss et al., 2011).

Although *S. aureus* infections are characterized by progressing rapidly and are generally more severe than *S. epidermidis* infections, *S. epidermidis* has the capacity to breach the epithelial barrier and adhere to the surfaces of indwelling medical devices during device insertion and form biofilms, having been recognized as an important opportunistic pathogen (Cheung et al., 2010; Hetrick & Schoenfisch, 2006; Otto, 2012).

P.aeruginosa is a ubiquitous microorganism that grows in many environmental sites (Lovewell et al., 2014). This bacterium is frequently associated with hospital acquired infections and is responsible for acute infections commonly associated with burn wounds and invasive instrument procedures (Lanini et al., 2011; Lovewell et al., 2014). It can be encountered in chronic infections typically in patients with persistent lung disease and immunocompromised (Lovewell et al., 2014).

Patient-to-patient transmission through contaminated medical devices is a well-established mechanism of *P. aeruginosa* spreading in healthcare settings (Lanini et al., 2011). Furthermore, this microorganism, which is able to live in biofilm mode, is resistant to a variety of chemicals, including antibiotics, detergents and hospital disinfectants, which facilitates its long-term persistence in hospital settings and diffusion between patients (Høiby, 2011; Lanini et al., 2011).

1.1.3 Biofilm formation on biomaterial surfaces

The ability to form biofilms is a universal attribute of almost all bacteria. Bacteria are able to grow adhered to almost every surface, forming highly complex communities called biofilms. Biofilms are composed of cells that grow in multicellular aggregates which are embedded in an extracellular matrix produced by the bacteria themselves (López et al., 2010).

In human life, biofilms can be found in many contexts, such as natural, medical and industrial environment. The mechanisms used by bacteria to form biofilms differ because they often depend on the environmental conditions and attributes of the strain concerned (López et al., 2010).

Biofilms can be composed of single or multiple species, depending on the device and its duration of use in the patient. For example, urinary catheter biofilms may initially be composed of single species, but longer exposures inevitably leads to biofilms composed by multispecies (Donlan, 2001).

In biofilm composition, cells represent almost 10-25 % of biofilm volume while the matrix represents 75-90 % (Garrett et al., 2008; Moraes et al., 2013). Its formation follows sequential steps starting with bacterial adhesion to the substrate, followed by proliferation and accumulation of extracellular matrix in multiple layers, culminating in a bacterial community that remains in the produced matrix. From this community, some microorganisms will become detached and be transported to nearby areas, spreading over the surface of the biomaterial (Moraes et al., 2013). The different steps involved in biofilm formation are schematized in ***Figure 1***.

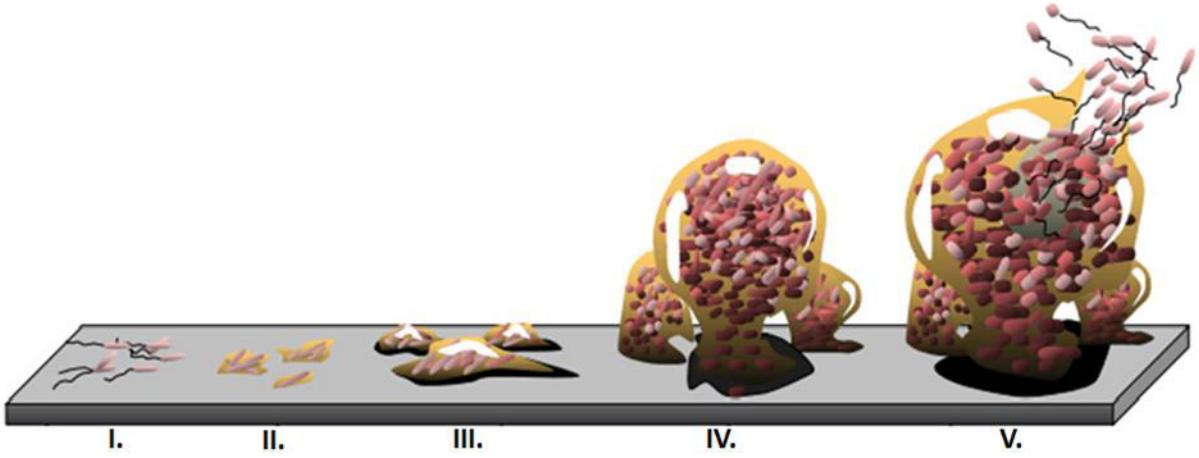

Figure 1. Schematic model representing the distinct developmental stages of microbial biofilms (Monroe, 2007). I. Conditioning film; II. Reversible attachment; III. Irreversible attachment; IV. Stronger adhesion between the bacteria and the surface; V. Dispersion of single cells from the biofilm matrix.

Immediately after insertion of a medical device in the body, the conditioning film occurs, in which proteins such as fibrinogen and immunoglobulins are deposited on the surface of the implant, facilitating the adherence of bacteria to the implant (Vickery et al., 2013).

The step II involves the weak adhesion of planktonic cells to a surface and the production of EPS. Planktonic bacterial cells can approach surfaces under bacterial motility, or under physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, surface electrostatic charge, and hydrophobic interactions (Kim et al., 2012; Kostakioti et al., 2013). Bacteria are attracted or repelled depending on the levels of nutrients, pH, ionic strength and temperature. The properties of the medium, along with the composition of the bacterial cell surface, affect the speed and direction of bacteria towards or away from the surface. After intercepting the surface, bacterial adhesion is mediated by additional extracellular adhesive appendices and secreted adhesins (Kostakioti et al., 2013). The initial attachment is dynamic and reversible, during which bacteria can detach if perturbed by repulsive forces, or in response to nutrient availability (Kostakioti et al., 2013). At this stage, as the adhesion is not final, the development of the biofilm and subsequent infection can be avoided by wash, antibiotics and host defences (Moraes et al., 2013). If conditions are favourable, bacteria reinforce the EPS production, consolidating the bacteria-surface bond (step III) (Garrett et al., 2008).

Step IV of biofilm development can be characterized by stronger adhesion between the bacteria and the foreign material, leading to cellular aggregation and the subsequent growth and maturation processes (Hetrick & Schoenfisch, 2006; Kim et al., 2012). Specific chemical reactions between compounds on the cell and substrate surfaces result in irreversible molecular bridging. Both polysaccharides and adhesin proteins within the bacterial membrane facilitate the attachment to substrate surfaces (Hetrick & Schoenfisch, 2006). Contact with the surface initiates responses that lead to changes in gene expression regulating factors favouring sessility, such as those involved in extracellular matrix formation (Kostakioti et al., 2013). Irreversible attachment is reached by bacteria that can resist the shear forces and maintain a constant grip on the surface and if provided with an appropriate supply of nutrients (Hetrick & Schoenfisch, 2006; Kostakioti et al., 2013).

Step V involves the dispersion of single cells from the biofilm matrix (Kim et al., 2012; Kostakioti et al., 2013). Within the mature biofilm there is a community that can sustain and maintain the biofilm architecture, providing a favourable environment for the resident bacteria. However, there may occur dispersion of cells caused by shear stresses. In addition, bacteria have developed ways to realize if the environment where they are, is favourable and may remain in the biofilm or return to planktonic mode. Biofilm dispersal can be triggered by

several cues, such as alterations in nutrient availability, oxygen fluctuations and increase of toxic products, or other stress-inducing conditions (Kostakioti et al., 2013).

Biofilms play an extremely important role in human health, because they protect bacteria from antibiotics and host immune responses. Biofilm formation is critical in the colonization of the implant surface, in the low efficiency of the host immune response, as well as in reducing the effectiveness of the antimicrobial treatment (Moraes et al., 2013).

In biofilm lifestyle, bacteria exhibit extreme resistance to antibiotics. In some cases, it has been found that killing bacteria in a biofilm requires approximately 1000 times the antibiotic dose necessary to achieve the same results in a suspension of planktonic cells (Hetrick & Schoenfisch, 2006).

The number of bacterial infections involving biofilms varies between 65 % and 80 % of all infections (Hetrick & Schoenfisch, 2006; Joo & Otto, 2012). Thus, biofilm development is the primary cause of BAI and because it is difficult to eliminate biofilm, removal of the contaminated device is often the only way to treat these infections (Desrousseaux et al., 2013; Hetrick & Schoenfisch, 2006; Moraes et al., 2013).

In this way, bacterial adhesion is often regarded as the most critical step to act in order to prevent BAI (Hetrick & Schoenfisch, 2006).

1.2 Strategies to fight BAI

1.2.1 Main treatment options

Infections associated with implanted biomaterials are a frequently occurring problem in modern healthcare (Engelsman et al., 2010). Treatment of these infections usually involves both medical and surgical measures, depending upon the cause and timing of the infection, and the condition of the host (Al-Mayahi et al., 2014).

The first treatment option is the administration of antimicrobial agents. Antibiotics are currently the preferred treatment strategy for bacterial infections (Al-Mayahi et al., 2014; Engelsman et al., 2010; Kostakioti et al., 2013). Conventional antibiotics work by preventing bacterial cell division (bacteriostatic) or killing the cell (bactericidal) (Kostakioti et al., 2013).

BAI are typically caused by commensal bacteria which adhere to the biomaterial surface and have the ability to form a biofilm on the implant surface. The extracellular matrix produced by cells, which hinders the diffusion of the antibiotic in the biofilm, along with the presence of metabolically inactive cells, contribute to fact that microorganisms become less susceptible to the action of the antimicrobial agent (Al-Mayahi et al., 2014; Engelsman et al., 2010; Kostakioti et al., 2013; Zaat et al., 2010; W. Zimmerli, 2014).

In the biofilm lifestyle, microorganisms are in a stationary phase of growth because oxygen and glucose are limited. Accordingly, successful treatment of BAI should consider this aspect. Some studies showed that most antimicrobial agents have a minimum bactericidal concentration (MBC), which is much higher during the stationary than the logarithmic phase of growth. The high stationary-phase MBC and the lack of efficacy against adherent bacteria are predictive of the failure of antibiotics in BAI (Zimmerli, 2014).

Other studies have showed that bacteria can also be located inside macrophages surrounding a biomaterial implant, where they remain protected against antibiotic treatment. Thus, both the biofilm mode of growth on the surface of a biomaterial implant as well as the bacterial localization in peri-implant tissues offer protection to the bacteria involved in BAI against routine antibiotic treatment, which may compromise the antibiotic efficacy (Engelsman et al., 2010).

BAI are usually treated with vancomycin, often in combination with rifampicin. Vancomycin has the ability to effectively penetrate the biofilm and to substantially reduce the number of viable bacteria (Engelsman et al., 2010). In a previous study, it was observed that treatment with rifampicin and vancomycin eradicated *S. epidermidis* from implanted biomaterial, but, despite the presence of rifampicin, bacteria in the surrounding tissue could survive. Thus, to eliminate bacteria in peri-implant tissue, alternative antibiotic combinations may be needed (Engelsman et al., 2010; Zaat et al., 2010; W. Zimmerli, 2014).

Although antibiotics have shown to be the best in the elimination of bacterial pathogens, high evidence indicates that they extensively damage the host microflora, create an environment where these pathogens can prevail, and they increase the selective pressure for resistance to antibiotics (Kostakioti et al., 2013; Vergidis & Patel, 2013; Wu et al., 2015).

The use of antibiotics is considered a pillar in the treatment of BAI, but it is often unsuccessful and insufficient to fight the infection because once antibiotics are removed, the biofilm is rapidly repopulated from cells that persisted during the treatment, resulting in recurrent

infections and chronic low-grade inflammation (Al-Mayahi et al., 2014; Engelsman et al., 2010; Kostakioti et al., 2013; Vickery et al., 2013).

The second treatment option is surgical therapy, when the infected device is removed but, as in antibiotics therapy, it is not fully effective if performed alone. Assays performed with antibiotic treatment in the presence of biofilm showed that antimicrobial therapy failed in all cases if it was instituted before device removal, whereas implant removal combined with antibiotic therapy was effective in all cases (Al-Mayahi et al., 2014; Campoccia et al., 2013; Engelsman et al., 2010; Kostakioti et al., 2013; Vickery et al., 2013).

Thus, the most effective solution for the treatment of BAI is to combine medical and surgical therapy (Vickery et al., 2013).

The typical procedure involves two stages in which the infected device is removed, the infection site is thoroughly cleaned, and antibiotics are systemically and locally delivered for a prolonged period of time. A new implant is then inserted when the infection is fully controlled and the surrounding tissue is not compromised (Busscher et al., 2012). However, the result of the insertion of a new device after BAI is uncertain, which increases patient pain and suffering, the length of hospital stay and consequent costs (Engelsman et al., 2010; Hetrick & Schoenfisch, 2006).

1.2.2 Preventing strategies

Bacterial biofilm contamination of surfaces in clinical workspaces is likely ubiquitous, and serves as a potential source of infection. The most common source of microorganisms is through the hands and skin. In a hospital setting, the transfer of these microorganisms can cause many infections, depending on the patient's condition. Therefore, the first and simplest strategy to prevent these infections is through the aseptic care (Xin, 2014).

Contamination of the environment surrounding the patient in the hospital is also an important source of bacteria. Typically, biofilms are found in wet areas, but studies have showed that bacteria can also exist on dry surfaces as biofilms which are protected from desiccation and have increased resistance to removal by detergents and the action of disinfectants. For these reasons, the risk of obtaining a nosocomial infection if the previous patient who has occupied the room had an infection caused by multi-resistant organism is increased. The existence of

multi-resistant bacteria may explain its permanency in the environment, despite the implementation of cleaning protocols increasingly rigorous (Vickery et al., 2013).

The same happens with the surgical instruments. Due to multi-resistant nature of the bacteria, cleaning and disinfection procedures are often ineffective in the decontamination of heat-sensitive instruments, such as endoscopes. Studies showed that approximately 1.8 % of endoscopes that were used remained infected with bacteria from the previous patient (Vickery et al., 2013).

Regarding biomaterial implants, the technically successful act of placing the device in the body does not guarantee the absence of an infection (Busscher et al., 2012). As aforementioned, an infection can occur by different routes (perioperative, postoperative and hematogenous contamination). Infections originated by the first two routes can be minimized through the disinfection and sterilization protocols, but no surgical site is truly sterile and pathogens are present in most operating rooms. These infections, when they occur, have a higher risk because bacteria adhered to the biomaterial surface can grow into a biofilm, becoming undetected by the immune system. Thus, an effective protection can only be offered by the integration of the biomaterial in host so that it establishes a normal immune response into the implant site (Busscher et al., 2012).

Facing this problem, there was the need to find different solutions that would improve the compatibility and integration of biomaterial implants and medical devices. These strategies include modifying the surface of the biomaterial to avoid the colonization of microorganisms (Busscher et al., 2012; Desrousseaux et al., 2013).

Currently, surface modification is the most promising strategy and with the best results to reduce the incidence of BAI (Busscher et al., 2012; Desrousseaux et al., 2013)

1.2.3 Surface modification to prevent BAI

The colonization of surfaces by bacteria is known to affect the function of several specific interfaces, such as those found in medical devices (Hasan et al., 2013). To combat this problem, investigations have focused on creating various strategies to eliminate or substantially reduce the extent of bacterial attachment and subsequent biofilm formation on these surfaces, such as surface modification (Hasan et al., 2013; Xin, 2014).

The properties of the surface can greatly influence the adhesion of bacteria. For instance, smooth surfaces act against the attachment of bacteria, unlike rough surfaces that favour the attachment. In addition, the hydrophilicity decreases the adhesion of bacteria, in contrast to hydrophobic surfaces (Lorenzetti et al., 2015).

Nowadays, surface modification of medical devices has often been reported as the approach with more potential to prevent biofilm formation (Bazaka et al., 2012; Xin, 2014). A summary of the surface modification strategies is illustrated in ***Figure 2***.

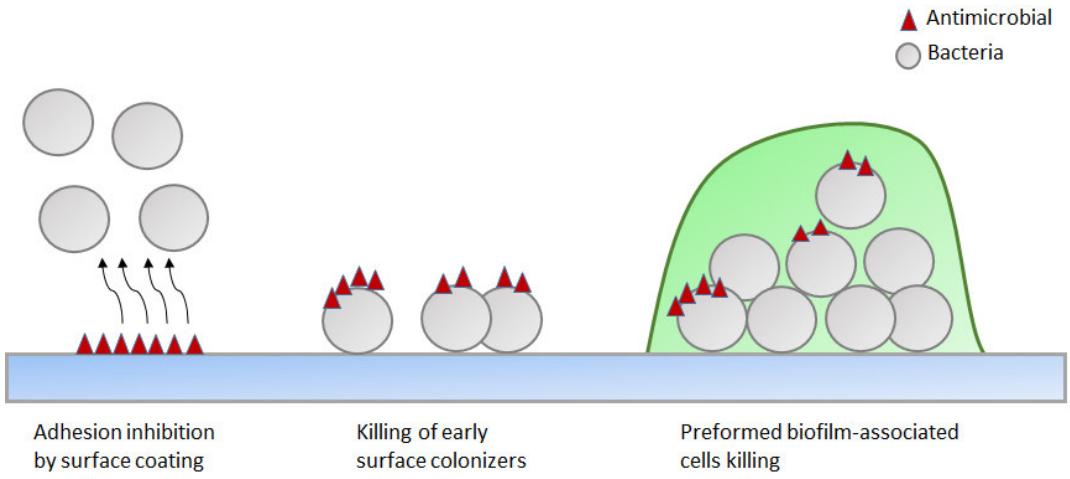

Figure 2. Antibacterial strategies developed to prevent biomaterial-associated infections. Some compounds can prevent the attachment of bacteria by coating of medical device surfaces. Biofilm development can be prevented by killing the early surface colonizers. Biofilm can be destroyed by agents which penetrate in biofilm matrix and kill biofilm-associated cells (di Luca et al., 2014).

Surface modification can be explored in two main areas. The first involves the use of biocides (antimicrobial coatings), in the development of coatings that may release these antimicrobial agents or kill microorganisms by contact. The other strategy is the development of anti-adhesive materials that prevent the attachment of bacteria (Campoccia et al., 2013; Chen et al., 2013; Desrousseaux et al., 2013; Sileika et al., 2011). A variety of anti-adhesive and antimicrobial coating strategies are strongly being explored to prevent BAI. ***Table 2*** describes some of the strategies that have been studied and their main disadvantages.

Table 2. Summary of mechanisms of action and main disadvantages associated to some antibacterial used in the development of antimicrobial and anti-adhesion coatings to prevent BAI.

Antibacterial compounds	Mechanism of action	Disadvantages	Refs
Antimicrobial peptides (AMP)	The target of AMP is mainly the bacterial membrane. Mechanisms depends on the type of AMP and include transmembrane pore formation, cell lysis and various metabolic inhibition mechanisms.	Short half-life and cytotoxicity when used in higher concentrations of soluble peptides.	(Brogden, 2005; Costa et al., 2011)
Antibiotics	Depends on the type of antibiotic and can act by different mechanisms (***Table 3***). For instance, the mechanism of action of vancomycin involves the breaking of cell wall peptidoglycan synthesis by binding to amino acids; Rifampicin acts by inhibition of transcription by binding to RNA polymerase.	Potential development of microbial resistance.	(Cloutier et al., 2015)
Silver	Silver has biocidal activity due primarily to the release of silver ions (Ag^{+}), which can interact with important enzymes of respiratory chain, deactivating them, and increase the frequency of DNA mutations.	Together with other elements, when used in high doses, have shown potential toxicity in human.	(Campoccia et al., 2013; Marambio-Jones & Hoek, 2010)
Quaternary ammonium compounds	QAC are detergents with antimicrobial effect. Its mechanism of action mainly involves an interaction with cell membranes, disruption of membrane integrity and leakage of cellular content.	Potential development of resistance.	(Buffet-Bataillonet al., 2012)

Table 2. Summary of mechanisms of action and main disadvantages associated to some antibacterial used in the development of antimicrobial and anti-adhesion coatings to prevent BAI (continuation).

Anti-adhesion compounds	Mechanism of action	Disadvantages	Refs
PEG-based coatings	When bacteria approach the PEG molecules, the compression of the PEG chains results in elastic repulsive force and the removal of water from hydrated PEG chains creates an unfavourable osmotic stress. This combination acts as repulsive forces preventing bacterial attachment.	Surface overwhelming by continuous protein attack and coating degradation (hydrolysis, chain cleavage, surface removal).	(Banerjee et al., 2011)
Heparin	It is an anticoagulant that possess a strong negative electrical charge (repelling bacteria negatively charged) and presents hydrophilic properties (forming a highly hydrated layer between the bacteria and the surface). These characteristics may prevent bacterial adhesion.	Due to their biodegradable nature, these coatings have a limited life time.	(Campoccia et al., 2013; Desrousseaux et al., 2013; Sin et al., 2009)
Zwitterionic polymers	Zwitterionic polymers are polymers composed of molecules containing both a positive and negative charge, conferring an overall neutral charge balance and makes the polymers ultra-hydrophilic. This combination can prevent not only the adsorption of proteins but also the adhesion of bacteria.	The usage of organic solvents which may affect the integrity of biomaterials.	(Mi & Jiang, 2014; Raynor et al., 2009)
Biosurfactants	Biosurfactants are amphiphilic compounds produced by microorganisms with distinct surface and emulsifying activities. The adsorption of biosurfactants to a surface modifies its hydrophobicity, interfering in the microbial adhesion, making them antiadhesive agents against pathogens.	Amounts of produced biosurfactant are very low.	(Gudiña et al., 2010; Rodrigues et al., 2006)

1.3 Development of resistance towards immobilized compounds

1.3.1 The emergence of multi-drug resistant strains

In recent decades, technological advancement along with the development of new drugs resulted in a significant reduction in mortality and increase of life expectancy. However, the overuse of antibiotics has led to the emergence of bacterial resistance, which is a natural phenomenon triggered by mutations in bacteria in order to protect themselves from antibacterial agents (Loureiro et al., 2016; Priyendu et al., 2015).

Bacterial resistance is a public health problem at global level and affects several areas such as medicine, production of animal food and agriculture, so it is very difficult to control and an inevitable event today. In hospital settings, antibiotics used to treat patients may enter the hospital sewer system, becoming a source of resistant organisms which spreads to other areas. Resistant strains can also arise from using antibiotics in sub-therapeutic concentrations (Priyendu et al., 2015). As a consequence, antibiotic resistance causes an elevated mortality and morbidity rate and an increase of treatment costs (Lin et al., 2015).

The consumption of antibiotics in Europe is very variable. According to the report from "European Centre for Disease Prevention and Control (ECDC) Surveillance Report: Surveillance of antimicrobial consumption in Europe, 2012" the country that has the highest consumption of antibiotics is Greece (31.9), while the Netherlands has the lowest consumption (11.3), expressed as defined daily dose (DDD) per 1000 inhabitants and per day. ***Figure 3*** shows the consumption of antibiotics in Europe.

Portugal reached the maximum consumption level in 2002 (26.5 DDD) reducing gradually until 2012 (22.7 DDD), but this is still higher than in other countries (ECDC, 2012).

It is believed that these significant differences in the consumption of antibiotics in different countries is due to incidence of infections acquired in the community, cultural and social determinants, structure of health care, available resources, knowledge about antibiotics, the pharmaceutical market and the practices of existent legislation (Ferech, 2006).

From the study of the relationship between the use of antimicrobials and antimicrobial resistance in Europe, it was found that the countries with a lower consumption of antibiotics, are also the countries where the level of resistance is lower. The opposite is also true and

Portugal is included on this side (Bronzwaer et al., 2002). For this reason the emergence of resistance strains is a matter of concern and deserves the best attention.

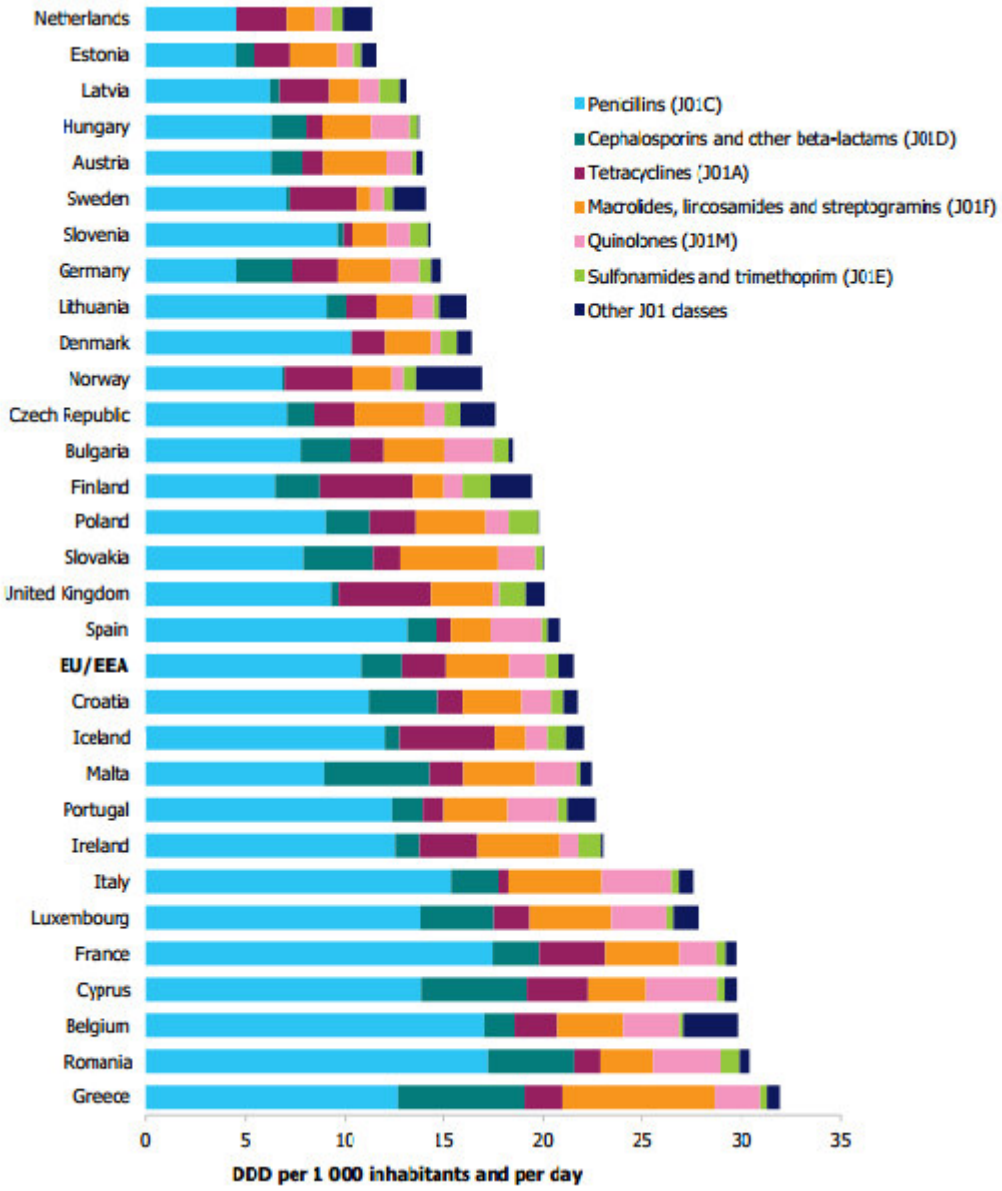

Figure 3. Consumption of antibiotics in Europe (ECDC, 2012).

1.3.2 Resistance mechanisms

For decades antibiotics were successfully used to treat patients with microbial infections. Over time, many infectious organisms, such as bacteria, were able to survive and develop resistance to specific antibiotics to which they once were susceptible, causing continuous infections. The rapid growth and evolution of microorganisms, such as bacteria, facilitates the development

of resistance to antimicrobials ("Antimicrobial (Drug) Resistance | NIH: National Institute of Allergy and Infectious Diseases," n.d.).

Nowadays, the concept of resistance is widely accepted and it is well known that bacteria are drastically more resistant to antibiotics, mainly in biofilms, as aforementioned.

This is a growing concern because species resistant to all known antibiotics have arisen and the emergence rate of antimicrobial resistance is unpredictable (Cooper et al., 2010).

Bacterial resistance may arise in different ways. Resistance can be intrinsic to the bacteria because of their genetic content and it is inherited from parents to progeny. Bacterial resistance can also be acquired or adaptive (due to the conditions of the surrounding environment) (Priyendu et al., 2015).

Acquired resistance

Acquired resistance may occur due to acquisition of a resistance gene by horizontal transfer of genes from resistant bacteria or by spontaneous mutations (Priyendu et al., 2015). In chronic infections bacteria are aggregated and very close, enabling the horizontal transfer of encoded genes for antibiotic resistance of a bacterium to another (Bjarnsholt et al., 2013). As this type of resistance is innate, bacteria maintain this state permanently (Priyendu et al., 2015).

Adaptive resistance

Adaptive resistance is developed according to the surrounding environmental conditions, such as the presence of antibiotics. This type of resistance involves a struggle for survival where organisms must adapt to the conditions of the environment faster than other organisms. Bacteria have an excellent ability to adapt to new conditions, thus leading to their survival (Priyendu et al., 2015).

This state of resistance is considered transient because when these conditions are removed it is reached the initial state (Fernández et al., 2011; Priyendu et al., 2015).

Bacteria can develop one or more mechanisms of resistance simultaneously against antibiotics (Priyendu et al., 2015; Silveira et al., 2006). Among the different resistance mechanisms, the most important are summarized in ***Table*** and their illustration are shown in ***Figure 4***.

Table 3. Summary of different mechanisms developed by bacteria to protect themselves from the antimicrobial agents.

Mechanism	Description	Reference
Destruction of the antibiotic	Bacteria may act on the antibiotic molecule by disrupting its structure or by catalyzing a reaction that chemically modifies it.	(Ebrahim, 2010; Kenneth & Ray, 2004)
Efflux of antibiotics	Mutant genes overexpress membrane transport proteins responsible for entry and exit of substances into the cytoplasm, which makes the output of the antibiotic to the extracellular medium be faster than its diffusion through the bacterial membrane while maintaining an insufficient concentration to act as a blocker of cellular functions.	(Ebrahim, 2010; Silveira et al., 2006)
Alteration of binding sites	Macromolecular antibiotic targets, such as ribosomes, proteins and cell wall constituents, are structurally modified from genes that express them, affecting the recognition of the drug to the target and decreasing their effectiveness.	(Ebrahim, 2010; Silveira et al., 2006)
Low cell permeability	The reduction in permeability of the antibiotic is due to structural changes in the number, selectivity or size of porins (outer membrane proteins), lowering the level of antibiotic in the bacteria.	(Ebrahim, 2010; Kenneth & Ray, 2004)

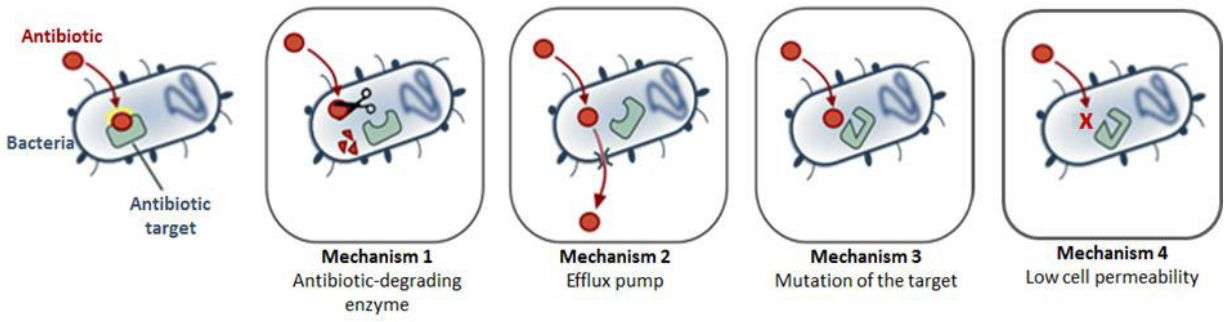

Figure 4. Schematic representation of different mechanisms of resistance developed by bacteria ("How does Bacterial Resistance happen? | Da Volterra," n.d.)

Using the mechanisms aforementioned, bacteria can overcome the action of antibiotics, even the most promising ones (Priyendu et al., 2015).

Facing this phenomenon of resistance, it is necessary to create new drugs able to overcome this ability of bacteria. However, there is always the possibility of emerging organisms resistant to these new agents (Cooper et al., 2010).

1.3.3 How to evaluate the potential development of bacterial resistance

In order to determine if bacteria are susceptible or resistant to an antimicrobial agent, it is necessary to evaluate their behaviour *in vitro*, pharmacological characteristics and later clinical trials (Kenneth & Ray, 2004).

The first step are *in vitro* tests where bacteria are exposed to the antimicrobial in a wide range of concentrations and the main parameter to be determined is the MIC (Kenneth & Ray, 2004). The results obtained from *in vitro* tests cannot be considered the only source of data. It should be taken into account pharmacological aspects of the antimicrobial agent and also information about the nature of disease and characteristics of the infection (Kenneth & Ray, 2004).

Currently, both in the development of new antibiotics such as the optimization of old antibiotics, predicting risk of resistance became, as never before, an increasingly important step in the drug development process for both pharmaceutical companies and researchers (Andersson, 2015).

Cooper et al. (2010) studied the behaviour of the bacteria when they are continuously exposed to honey. In ancient times honey was used to treat wounds. As with antibiotics, the extensive use of honey can trigger a selective pressure for the emergence of honey-resistant strains. To test the possible development of resistance two methods were tested. In the first method, initial bacteria were repeatedly cultivated (in fresh medium) in a sub-lethal concentration of the agent for 10 successive days. The MIC was determined on days 0 and 10. In the second method, bacteria were exposed to stepwise increasing concentrations of honey for 10 successive days. MIC of honey were determined before and after this period.

Overall, the results showed that, at the end of the training period, the MIC values remained very close to the initial values of MIC, confirming the potential of honey to treat wounds, without developing resistance.

In another study conducted by Tambe et al. (2001), the risk of development of resistance by *S. epidermidis* to antibiotics and antiseptics immobilized in central venous catheters was

evaluated. The used method involved the passage of the culture 10–20 times through sub-inhibitory concentrations of different antimicrobials, alone and in combination. The MIC of each antimicrobial, before and after passages, was determined and compared. The results of this study showed that the resistance develops more easily in the combination of antibiotics than for the antiseptics and more easily to rifampicin than to minocycline. It was also verified that catheters impregnated with antiseptics may have a low risk of colonization by bacteria resistant to antibiotics (Tambe et al., 2001).

Duran et al., (2012) evaluated the association between the antibiotic susceptibility patterns and the antibiotic resistance genes in staphylococcal isolates. Antimicrobial susceptibility was performed in a total of 298 staphylococci clinical isolates. For a rapid diagnosis of antibiotic resistance genes, a molecular method was performed. The genes implicated in resistance to oxacillin, gentamicin, erythromycin, tetracyclin and penicillin were amplified using multiplex PCR method, in which several different DNA sequences were simultaneously amplified, followed by an electrophoresis (Duran et al., 2012). For all antibiotics tested, resistance was found in at least one gene of the several tested and most of staphylococci tested possessed the same gene, the blaZ gene, which confers resistance to beta-lactams. The results showed that this study produced different results, once the phenotypic antibiotic susceptibility patterns were not similar to those obtained by genotyping done by multiplex PCR (Duran et al., 2012).

The presence of bacterial variants called small colony variants (SCV), originated by gene mutations in stress response, is another way to detect the potential development of resistance (Melter & Radojevič, 2010). These variants are not particularly virulent but have the ability to persist viable inside host cells and also exhibited resistance to various antibiotics and even to antiseptics (Kahl, 2014). The most evident feature of these SCV is their small colony size on conventional agar plates, their fastidious growth in pin-point colonies and homogeneous appearance (Kahl, 2014; Proctor et al., 2006).

The methods presented to study the possible development of resistance are simple and easy to apply. Furthermore, the fact that there are always emerging resistant organisms suggests that antimicrobial susceptibility should be monitored continuously.

2. MATERIALS AND METHODS

2.1 Microorganisms and culture conditions

2.1.1 Bacterial strains

In this work, two bacterial species, commonly associated to BAI infections were used:

- *Staphylococcus aureus* GB 2/1 isolated from explanted voice prostheses at the University Medical Centre of Groningen (the Netherlands) ;
- *Staphylococcus epidermidis* GB 9/6 also isolated from explanted voice prostheses at the University Medical Centre of Groningen (the Netherlands).

2.1.2 Media and growth conditions

During the accomplishment of this work different culture media were used and they were prepared according to the supplier instructions:

- TSB (Tryptic Soy Broth, 30 g/L, Liofilchem);
- TSA (TSB supplemented with Agar, 12,5 g/L, Liofilchem);
- MHB (Mueller Hinton Broth, 21 g/L, Liofilchem).

For inoculum preparation, initially, two colonies were inoculated in 20 mL of TSB medium and incubated overnight at 37 °C, 120 rpm. Thereafter, bacteria were collected by centrifugation (9000 g for 5 min at room temperature), washed and suspended in fresh medium to prepare a bacterial suspension. Cellular concentration was determined by measuring optical density (OD) at 640 nm, and adjusted using calibration curves previously prepared.

2.1.3 Bacteria preservation

All strains were stored at -80 °C in liquid medium with glycerol 20 % (v/v). For each experiment, these strains were rehabilitated in TSA plates and placed at 37 °C for 24 h. The plates were then stored at 4 °C up to one week.

2.2 Antimicrobial compounds

2.2.1 Benzalkonium chloride

Benzalkonium chloride (BAC), a quaternary ammonium compound, widely used in clinical disinfectant formulations, was purchased from Sigma. A stock solution was prepared and stored at 4 °C. Working solutions were prepared therefrom.

2.2.2 Vancomycin

Vancomycin, a glycopeptide antibiotic exerting a broad spectrum of activity against Gram-positive bacteria was obtained from Sigma (European Pharcopea). A stock solution was prepared and kept at -20 °C. From this solution, several aliquots were also prepared for further work.

2.2.3 Rifampicin

Rifampicin (AppliChem), belonging to rifamycin's class of antibiotics, is often associated to vancomycin to treat BAI. A stock solution was prepared and stored at -20 °C, from which, work solutions were then prepared.

2.2.4 MIC and MBC determination

A microdilution test was used to determine the minimal inhibitory (MIC) and bactericidal concentrations (MBC). Antimicrobials solutions were prepared in MHB and added to the wells of 96-well microtiter plate with round bottom (Orange, USA), with several concentrations being tested (ranging from 40 µg/mL to 0.16 µg/mL for BAC and from 64 µg/mL to 0.25 µg/mL for vancomycin). A bacterial inoculum, diluted to reach a final concentration of 5×10^5 CFU/mL, was added to the microtiter plate that was, then, incubated at 37 °C, 120 rpm for 24 h, making a total volume of 200 µL. In this assay, two controls were used, one without bacteria as a negative control and one without the antimicrobial compound as a positive control. MIC was determined by measuring the optical density at 640 nm, where clear wells (OD=negative control) were an indication of bacterial growth inhibition. MBC was determined by adding 10 µL from each well with no visible growth on a TSA plate and defined as the lowest

concentration where no colony growth was observed after 24 h at 37 °C. Three independent assays with six replicates for each condition were performed.

2.3 Surface modification

2.3.1 Polydimethylsiloxane preparation

The material used in this study was polydimethylsiloxane (PDMS), commonly referred to as silicone rubber (Dow Corning, USA). As it is known, silicone has been widely used in the biomedical field, such as in urinary catheters, contact lenses and medical/surgical implants (Zhang & Chiao, 2015). PDMS is a silicone elastomer with many attractive features for the development of biomedical applications such as biocompatibility, low toxicity, optical transparency, elastomeric properties, gas permeability, and low manufacturing costs. Furthermore, it is chemically inert, thermally stable and simple to handle and manipulate (Mata et al., 2005; Zhang & Chiao, 2015; Zhou et al., 2010). Once it is very flexible and stable, it has been used in a wide variety of prostheses such as breast implants, finger joints and heart valves and ear, nose and chin reconstruction (Ratner, 2004). Although PDMS has many desirable characteristics, it has also an innate hydrophobicity which can compromise the function of several medical devices because causes adsorption of many proteins leading to microbial adhesion and subsequent biofilm formation (Pinto et al., 2010; Zhang & Chiao, 2015; Zhou et al., 2010)

For the preparation of PDMS, a commercial kit (Sylgard 184 from Dow Corning, USA) was used, which contains two reagents, the liquid base of silicone rubber and a curing agent. The two reagents were mixed in a petri dish in a mass proportion of 10: 1 (liquid base / curing agent). After homogenization of the components, the mixture was kept at room temperature until polymerization (at least 2 days). Thus, was obtained a film having a thickness of about 3 mm which was subsequently cut into small circles with approximately 9 mm. Afterwards, the samples were immersed in a commercial detergent (Sonasol, Henkel Ibérica, Portugal) and sonicated for 5 minutes, followed by washing with distilled water, by sonication in methanol for 20 min and then another washing with distilled water. Finally, samples were sterilized at 121 °C for 15 min.

2.3.2 Polydopamine coating

Surface modification was performed by using a mussel-inspired coating strategy. Mussels secret adhesives proteins that allows them to adhere to marine surfaces, such as rocks, metal and polymer ship hulls, and wood structures, functioning as a glue able to resist adverse sea conditions. Mussel adhesive proteins (MAP) have an amino acid, 3,4-dihydroxyphenylalanine (DOPA) that is formed by modification of tyrosine, which contains a catechol group and confers adhesive and cohesive properties (Dalsin et al., 2002; Sileika et al., 2011; Waite, 2002). From the fact that MAP have large numbers of DOPA-Lysine tandem sequences, it was assumed that the coexistence of catechol (DOPA) and amine (lysine) groups are essential for the successful adhesion of these organisms. From these observations, Messersmith and co-workers (Lee et al., 2007) identified a small molecule that combines both functionalities, dopamine (Lynge et al., 2011). They reported that, in alkaline aqueous environment and in the presence of oxygen, dopamine polymerizes to form a thin layer called polydopamine (pDA), similar to adhesive layer produced by mussels (Sileika et al., 2011). Furthermore, the pDA-coated surfaces proved to be versatile substrates for further ad-layer deposition of several compounds, containing amine or thiol groups.

Dopamine coating was performed by placing 3 coupons of PDMS in each scintillation flask, with the aid of tweezers. Thereafter, a solution of dopamine was prepared (Sigma, St. Louis, Missouri; 2 mg/mL dopamine-HCl in 10 mM bicine buffer, pH 8.5) and sterilized by filtration. Then, 7 mL of solution were added per flask and incubated for 18 h at room temperature and 70 rpm. Coupons were then washed with sterile ultrapure water and finally placed in a 48-well plate until further immobilization of an antimicrobial compound. **Figure *5*** shows a schematic representation of this procedure.

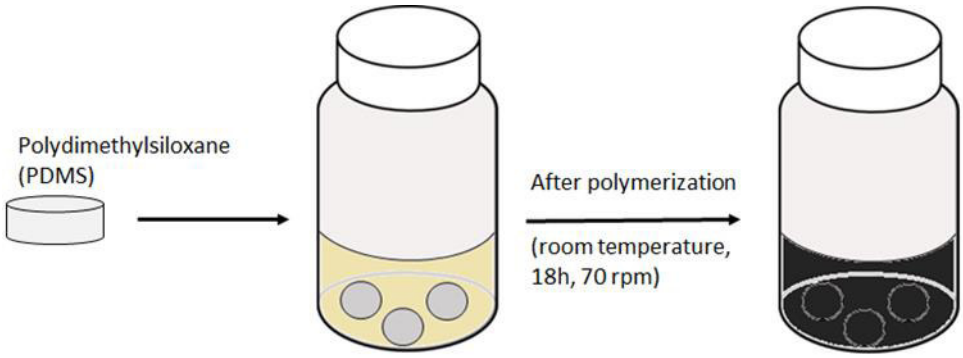

Figure 5. Schematic representation of immobilization strategy of pDA onto PDMS material. The coupons of PDMS were submerged in bicine buffer during 18 h.

2.3.3 Antimicrobial compounds immobilization

For antimicrobial compounds immobilization onto pDA-coated PDMS, solutions of these agents were prepared at different concentrations and in different buffer solutions (bicine, pH 8.5 or PBS, pH 7.4). Afterwards, 300 µL of these solutions were added to each well of a 48-well microtiter plate in which pDA-coated coupons were placed. The plate was kept at room temperature and 70 rpm for different periods of time. Afterwards, coupons were washed 3 times with ultrapure sterile water. Lastly, the coupons were placed to dry at 37 °C until being used. For optimization purposes, different concentrations, pH and incubation time were tested.

2.4 Antibacterial performance of modified surfaces

2.4.1 Contact-killing assay

With the purpose of testing the antimicrobial properties of the modified surfaces in contact, a previously reported qualitative test was performed with some modifications (Ding et al., 2012). Firstly, it was necessary to prepare a bacterial suspension in TSB medium adjusted to a final concentration of 1×10^6 CFU (colony forming units)/mL. Afterwards, the coupons, previously modified, were placed in sterile petri dishes and 20 µL of bacterial suspension was added to the surface of each coupon. The plates were incubated at 37 °C without shaking for 24 h. After this time, the coupons were transferred to TSA plates with the surface containing the bacterial suspension in contact with the medium. Finally, the plates were again incubated at 37 °C for 24h and the presence or absence of bacterial growth was observed. Bacterial

growth was tabulated as"+" and absence of growth as "-", as an indication of contact-killing activity. Three independent assays with three replicates for each condition were performed.

2.4.2 Leaching assay

The leaching assay aimed to verify if the immobilized compound was released from the coupon or not and it was performed as previously described (Asri et al., 2014). For this test, the compounds were initially immobilized as aforementioned. Next, a cell suspension was prepared in TSB medium with a concentration of 1×10^8 CFU/mL. The suspension was spread with a swab on TSA plates and afterwards the coupons were placed in these plates with the immobilized surface in contact with the bacteria. Finally, the plates were incubated at 37 °C for 72 h and the presence of a zone of inhibition around the coupons was evaluated. The absence of a zone of inhibition was taken as an indication that no antimicrobial compounds leached from the surfaces. Three independent assays with three replicates for each condition were performed.

2.4.3 Biofilm formation on the modified surfaces

After confirming the antimicrobial activity of the agents immobilized and their binding without release from the surfaces, the next step was to test their ability to prevent biofilm formation. The immobilization conditions (concentration, pH and incubation time) of each antimicrobial compound used in this assay were those that yielded modified surfaces with contact-killing activity and no compounds leaching. Biofilm cells metabolic activity was assessed using the XTT test. This test is based on the reduction of XTT by enzymes of metabolically active cells, producing a highly coloured formazan product which is water soluble. The reduced formazan can be quantified by spectrophotometry analysis, which is proportional to the bacterial metabolic activity (Roehm et al., 1991).

Firstly, a cellular suspension in TSB medium was prepared with a final concentration of 1×10^7 CFU/mL. Afterwards, the coupons previously modified were placed in a 48-well plate and 300 µL of cellular suspension was added to each coupon, followed by incubation at 37 °C and 120 rpm for 24 h. Passed 24 h, the coupons were washed 2 times with saline solution (0.9 % of NaCl) and 400 µL of XTT and PMS solution (150 µg/mL and 10 µg/mL respectively), were added, without light, followed by an incubation for 3 h at 37 °C and 120 rpm in the dark

(involved in aluminum foil). After that, 200 µL (in duplicate) of each well were transferred to a 96-well microtiter plate and the OD was measured at 490 nm using a microtiter plate reader (Tecan, Model Sunrise-basic Tecan, Austria). Three independent assays with three replicates for each condition were performed.

2.5 Evaluation of resistance development

The potential development of resistance was first evaluated for planktonic cells with antimicrobials in solution, following a procedure previously described (Cooper et al., 2010). Briefly, 40 µL of an overnight culture of *S. aureus* was inoculated in 20 mL of TSB medium containing a sub-inhibitory concentration (1/4 MIC) of BAC (0.3125 µg/mL) and vancomycin (0.125 µg/mL). The flask was then incubated at 37 °C and 120 rpm for 24 h. For 10 successive days, similar and freshly prepared flasks were inoculated with 40 µL of preceding days' culture. The MIC and MBC were determined on days 0 and 10.

In order to evaluate the potential development of resistance of cells adhered to the immobilized agents, an assay previously described was performed (Alves et al., 2016; Alves & Pereira, 2016). In this assay, cells adhered to the surfaces with and without modification are collected and placed in contact with new surfaces for 10 days. After incubating cells (1×10^7CFU/mL in TSB) for 24 h with these surfaces and wash them with saline solution (0.9 % of NaCl), each coupon was placed in an Eppendorf tube, to which was added 1 mL of TSB medium. Next, all coupons were sonicated for 3 min and stirred for 30 seconds in a vortex (optimization of these conditions are described in Supplementary material I). Hereafter, 300 µL of the collected cellular suspension were transferred to the wells of 48-well microtiter plate containing new coupons previously modified, followed by an incubation at 37 °C and 120 rpm for 24 h. Bacterial growth was monitored by CFU counting every day. This procedure was repeated during 10 days and in the last day the MIC and MBC of cells adhered to modified surfaces was performed and compared to the MIC and MBC of cells adhered to unmodified PDMS. Experiments were performed in triplicate.

2.6 Statistical analysis

Results were presented as mean ± standard deviation (SD). Statistical analysis was performed using Graph Pad Prism 5.01. First, data normality was checked using Kolmogorov-Smirnov test. After this analysis, parametric tests (one way Anova followed by Tukey's test) or non-parametric (Kruskal –Wallis test) were used depending on whether the samples were from normally distributed populations or not, respectively. Differences were plotted according to the following: * (p-values ≤ 0,05); ** (p-values ≤ 0,01); *** (p-values ≤ 0,001).

3. RESULTS AND DISCUSSION

Immobilization of antimicrobials, such as QAC and antibiotics onto a surface has been proposed as a promising approach to fight BAI. The main objective of this work was to evaluate the performance of two antimicrobial agents (the QAC BAC and the antibiotic vancomycin) in what concerns the possible development of resistance when they are immobilized on a surface to combat infections caused by *S. aureus* and *S. epidermidis.* Rifampicin was also tested against *S. epidermidis* as surfaces functionalized with vancomycin had no antimicrobial effect on this strain. Although it has been reported that BAC causes genotoxic effects in mammalian and plant cells at environmentally relevant concentrations (Deutschle et al., 2006) it is expected to reduce these effects with its immobilization as it has proved to happen for other QAC (Cavallaro et al., 2016).

3.1 Susceptibility of planktonic cultures to antimicrobials

The first step of this work was to evaluate the susceptibility of planktonic cultures of *S. aureus* and *S. epidermidis* to both antimicrobials, which was performed by determination of MIC and MBC. From ***Table 4*** it can be concluded that the strains investigated in this study exhibited different susceptibility patterns. Planktonic cells of *S. aureus* were more susceptible to vancomycin than to BAC. On the opposite, *S. epidermidis* was more susceptible to BAC than vancomycin. According to EUCAST, the MIC values of vancomycin indicate that both strains are not resistant to this antibiotic (EUCAST, 2015).

Table 4. MIC and MBC of antimicrobials BAC and vancomycin against planktonic cultures of *S. aureus* and *S. epidermidis*. MIC and MBC are expressed in µg/mL.

	Antimicrobial	MIC	MBC
S. aureus	BAC	1.25	5
	Vancomycin	0.50	0.50
S. epidermidis	BAC	0.31	0.625
	Vancomycin	4	4

3.2 Optimization of antimicrobials immobilization

Antimicrobials immobilization on PDMS surfaces coated with pDA was optimized by testing different concentrations of antimicrobial agent, incubation time and buffer pH. To confirm antimicrobials activity, a contact-killing assay was made, in which a small volume of bacterial suspension was dropped on the modified surfaces for 24 h. After transferring these surfaces to TSA plates, the presence or absence of bacterial growth was observed. Representative pictures of bacterial growth and contact-killing activity are presented in Supplementary Material II. The strain *S. aureus* was used as a model in this optimization phase because it is more virulent than *S. epidermidis* (Fey & Olson, 2010). So, it was assumed that the conditions chosen for *S. aureus* should also be effective for *S. epidermidis*. Results obtained from the optimization performed for *S. aureus* are shown in ***Table 5***.

Table 5. Optimization of BAC and vancomycin immobilization onto PDMS surfaces for *S. aureus* using polydopamine as an intermediate. Different concentrations, pH values and incubation periods of time were tested. Visible growth was used as an indicator of contact-killing activity and it was tabulated as "+" for growth and "-" for no visible growth.

Antimicrobial	Concentration (mg/mL)	pH	Incubation period	Bacterial growth
BAC	0.001	7.4	3 h	+
			Overnight	+
		8.5	3 h	+
			Overnight	+
	0.01	7.4	3 h	+
			Overnight	-
		8.5	3 h	-
			Overnight	-
	0.1	7.4	3 h	-
			Overnight	-
		8.5	3 h	-
			Overnight	-
Vancomycin	0.05	7.4	3 h	+
			Overnight	+
		8.5	3 h	+
			Overnight	+
	0.1	7.4	3 h	+
			Overnight	+
		8.5	3 h	+
			Overnight	+
	0.5	7.4	3 h	+
			Overnight	+
		8.5	3 h	+
			Overnight	-

The lowest concentration of BAC used (0.001 mg/mL), for any buffer pH or incubation period, was not able to inhibit bacterial growth. In contrast, the highest concentration tested (0.1 mg/mL) completely inhibited bacterial growth, irrespective of the conditions of pH and incubation time. For an intermediate concentration of BAC (0.01 mg/mL), results showed that an overnight immobilization in an alkaline buffer (bicine) were the best conditions to immobilize this agent on the surface.

Vancomycin demonstrated greater efficiency for a higher concentration (0.5 mg/mL) than BAC, but the remaining conditions were the same: alkaline pH and an overnight incubation.

Regarding the optimization of antimicrobials immobilization against *S. epidermidis*, antimicrobials were incubated in an alkaline pH overnight (the best conditions previously defined for *S. aureus*). The only parameter tested was antimicrobials concentration. On ***Table 6***, it is possible to consult the concentrations tested for BAC, vancomycin and rifampicin. Lower concentrations of BAC were tested because planktonic cultures of *S. epidermidis* previously proved to be more susceptible than *S. aureus.*

Table 6. Optimization of immobilization of BAC, vancomycin and rifampicin onto modified PDMS surfaces using polydopamine as an intermediate against *S. epidermidis.* Different concentrations were tested. Visible bacterial growth was used as an indicator of contact-killing activity and it was tabulated as"+" for bacterial growth and "-" for no visible growth.

Antimicrobial	Concentration (mg/mL)	Bacterial growth
BAC	0.0001	+ + +
	0.001	+ + +
	0.003	+ + +
	0.005	- - -
Vancomycin	0.5	+ + +
	1	+ + +
	2	+ + +
Rifampicin	1	- - -
	2	- - -

Both the lowest concentration of BAC (0.0001 mg/mL) as the concentration 10 times higher (0.001 mg/mL) did not inhibit the bacterial growth. The higher concentration tested (0.005 mg/mL) totally inhibited the bacterial growth. It was then tested an intermediate concentration (0.003 mg/mL) that only allowed the growth of some colonies.

When immobilized on a PDMS surface coated with pDA, vancomycin had no effect against *S. epidermidis*. A higher concentration than 2 mg/mL was not further tested, however, to avoid toxicity issues. The lack of vancomycin antimicrobial activity towards *S. epidermidis* after its immobilization, are in accordance with their planktonic susceptibility patterns, as *S. aureus* was more susceptible than *S. epidermidis* strain. However, results obtained in this study are not in accordance with a previously reported study, where vancomycin covalently bounded to titanium alloy surfaces could prevent colonization of both *S. aureus* and *S. epidermidis* strains (Jr et al., 2008). Differences found can be attributed to the different immobilization strategy. Furthermore, in this study a clinical isolate of *S. epidermidis* was used unlike in this other report where a reference strain was investigated. Clinical isolates, frequently exposed to stress conditions in a hospital environment can suffer a selection process that favours more pathogenic strains (Fraimow & Tsigrelis, 2011).

This antibiotic was, therefore, abandoned and another antibiotic, rifampicin, was then tested. Rifampicin was chosen because it is often used to fight biomaterial-associated infections, often combined with vancomycin (Niska et al., 2013). Results in ***Table 6*** shows that rifampicin immobilized under the same conditions of pH and incubation period, did not allow bacterial growth for any of the concentrations tested (2 mg/mL and 1 mg/mL). This compound does not have free amine or thiol groups and, therefore, the binding to polydopamine coating should not occur by covalent bonding as the others compounds used in this work. It is expected, then, that rifampicin will mostly binds by physical adsorption and, therefore, leaching should subsequently occur.

This preliminary optimization allowed to determine the best range of concentrations, pH of the buffer (bicine, pH 8.5) and the incubation time (overnight) for each antimicrobial agent and for each strain. It was also possible to conclude that, using this coating strategy with pDA, the antimicrobials conserved their antimicrobial activity after their immobilization, confirming the ability of polydopamine to bind to a wide range of molecules, especially with amine and/or thiol containing compounds (Lee et al., 2009).

3.3 Leaching assay

After defining the conditions which renders the surfaces with the best antimicrobial properties by contact, it was considered crucial to evaluate if the compound was limited to the coupon or if there was some leaching from the surface. This is important because bacteria exposure to sub-inhibitory concentrations has the potential to develop resistance (Bruenke et al., 2016).

Antimicrobials release from the surfaces was evaluated in terms of existence or not of a zone of inhibition surrounding the surfaces in contact with a bacterial lawn on a TSA plate. Different concentrations of BAC and vancomycin were tested (the concentrations that yielded surfaces with contact-killing activity) against *S. aureus* and are represented in **Figure *6***.

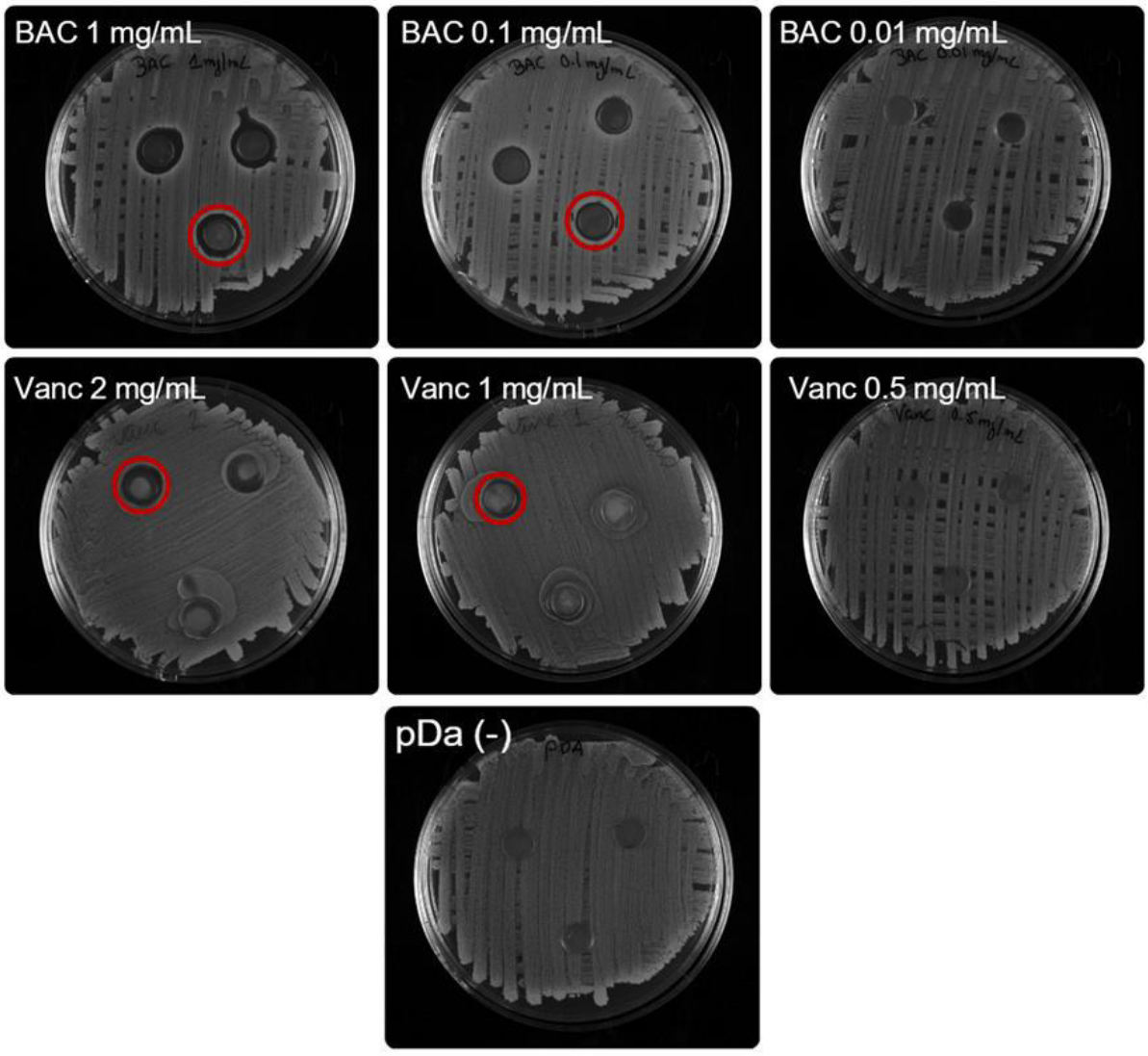

Figure 6. Absence or presence of an inhibition zone (denoted with a circle) around modified PDMS coupons on an agar plate, after 72 h incubation with S. aureus, indicating presence or absence of leaching of antimicrobial compounds.

Immobilization of BAC using the concentrations of 1 mg/mL and 0.1 mg/mL presented an inhibition zone that is proportional to the concentration, i.e. high concentrations caused greater growth inhibition zones. The concentration in which BAC leaching did not occur was 0.01 mg/mL. Vancomycin showed similar results. For the lowest concentration tested (0.5 mg/mL), it was not observed leaching from the surface, as opposed to the higher concentrations (2 mg/mL and 1 mg/mL). Results were compared to PDMS surfaces only coated with pDA in which an inhibition zone was not visible, serving, therefore, as the negative control.

The same procedure was performed for *S. epidermidis* strain and the results are presented in **Figure *7*.**

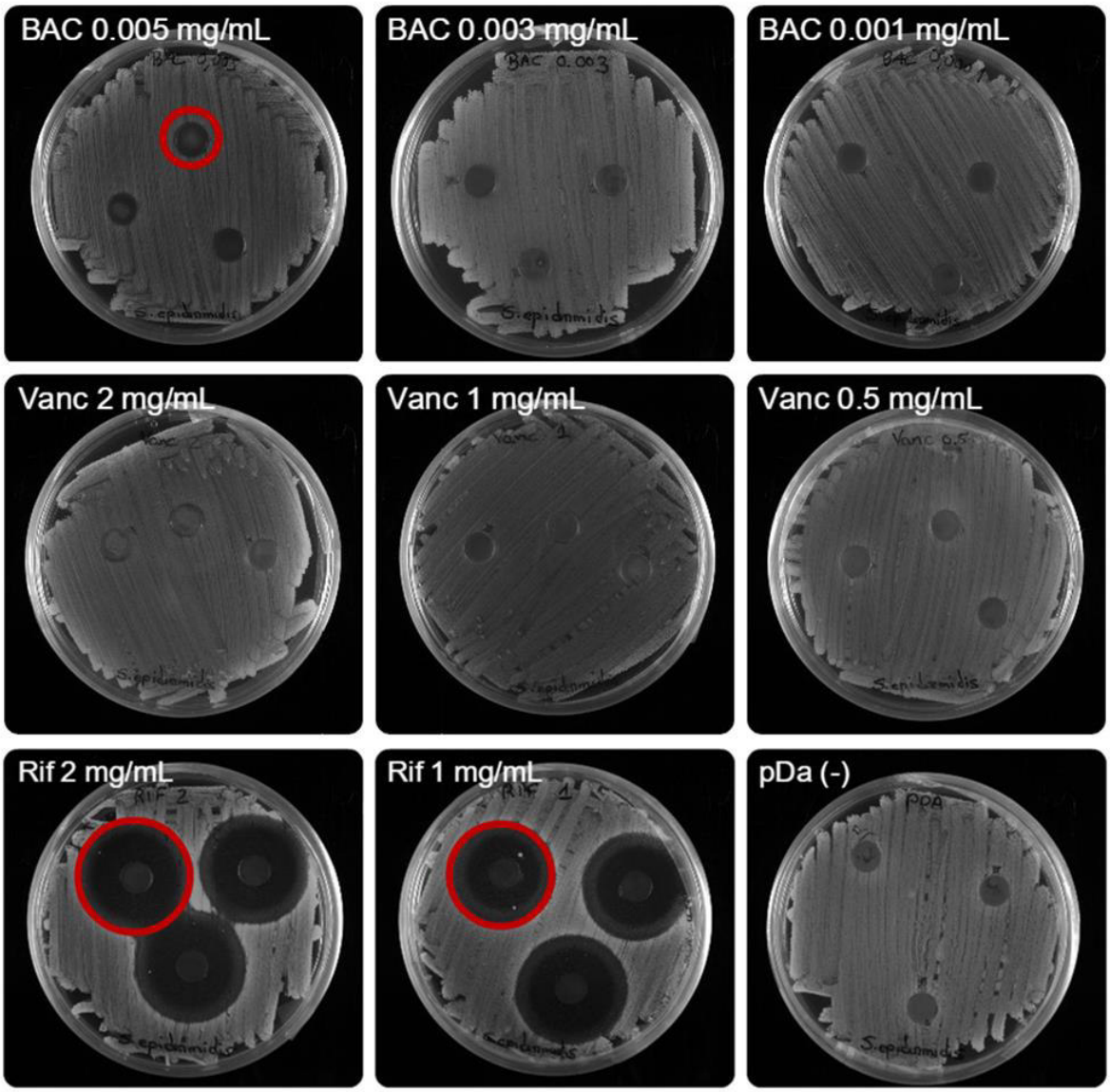

Figure 7. Absence or presence of an inhibition zone (denoted with a circle) around modified PDMS coupons on an agar plate, after 72 h incubation with S. epidermidis, indicating absence or leaching of antimicrobial compounds.

It is possible to verify that a minimal change in BAC immobilization concentration can lead to very different results. A slight release of BAC could be observed when an immobilization concentration of 0.005 mg/mL was used, as opposed to the very similar concentrations of 0.003 mg/mL and 0.001 mg/mL, where release did not occur.

Vancomycin release was also tested for this strain and showed that none of the concentrations used resulted in its leaching from the surface, as no inhibition zone could be observed. This results point out that the method used in this study for evaluating antimicrobials release from the surfaces depends on the strain susceptibility. Therefore, to

better confirm these results a quantitative method such as HPLC (high performance liquid chromatography) should be further performed.

Finally, for the antibiotic rifampicin it was possible to observe large zones of inhibition which indicates its release from the surface in higher quantities than for the others antimicrobials tested. These results were expected considering that this compound immobilization occurred mainly via physical absorption as it has no free amine or thiol groups to covalently bound to the surfaces using polydopamine as an intermediate.

From the overall results, it was possible to define the conditions for the immobilization of BAC and vancomycin to render the surfaces with antimicrobial properties but without release from the surfaces. For *S. aureus*, BAC and vancomycin were immobilized overnight, in bicine buffer at concentrations of 0.01 mg/mL and 0.5 mg/mL, respectively, whereas for *S. epidermidis*, BAC was immobilized at 0.003 mg/mL.

3.4 Biofilm formation on modified surfaces

To evaluate the anti-biofilm properties of antimicrobial coatings prepared, both bacterial strains were allowed to grow for 24 h on these surfaces and biofilm cells metabolic activity was evaluated using a XTT assay. In this assay, OD values are proportional to the number of metabolic active cells adhered on the surfaces.

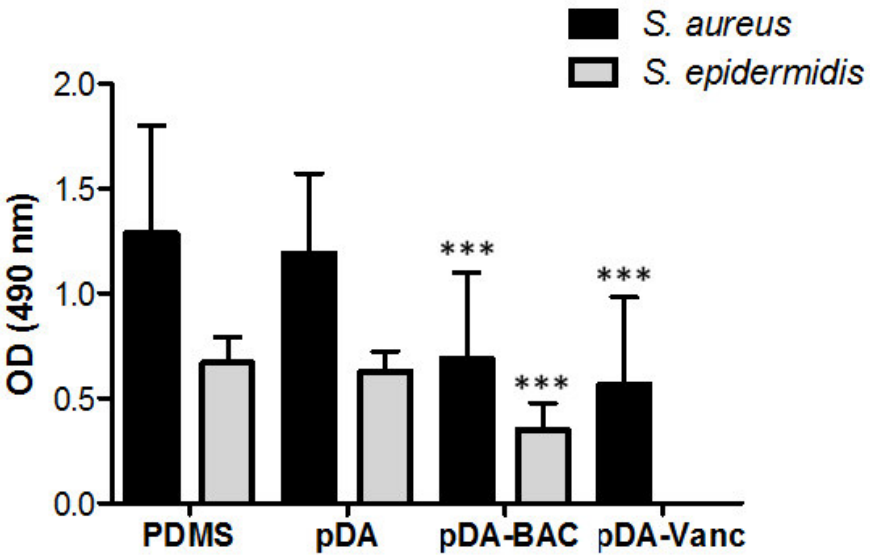

Figure 8. Metabolic activity of biofilm cells adhered to unmodified PDMS, pDA-coated PDMS, pDA-coated BAC and pDA-coated vancomycin. Significant differences were found for (***) $p < 0.001$, compared to PDMS control.

In **Figure *8*** it is possible to verify that, for both bacterial strains, unmodified PDMS as well as PDMS coated with a pDA layer exhibited higher values of OD, confirming their ability to be colonised by bacteria and consequent biofilm formation. Coatings with immobilized BAC (pDA-BAC) and vancomycin (pDA-Vanc) had effect on biofilm formation of *S. aureus*, reducing biofilm cells metabolic activity to approximately half, compared to the unmodified PDMS. For *S. epidermidis*, PDMS coated with BAC (pDA-BAC) also decreased biofilm cells metabolic activity, an indication that these surfaces could impair biofilm formation. Coatings with vancomycin were not tested against this strain, as it was not susceptible for none of the conditions investigated.

Despite the surface modification strategy adopted in this study has shown good results, providing antimicrobial and anti-biofilm properties, a complete eradication of the biofilm was not achieved, which has also been reported in previously reported studies (Nejadnik et al., 2008; Subbiahdoss et al., 2010).

3.5 Evaluation of development of resistance

It is well known that the excessive use and misuse of antimicrobials favours the selection of resistant strains (Russell, 2002). It is important, therefore, to evaluate the potential development of resistance towards the antimicrobials investigated in this study.

First, their potential development of resistance was investigated when in solution against planktonic cultures of *S. aureus*. For that, bacteria were repeatedly cultivated in a sub-inhibitory concentration (1/4xMIC) of the antimicrobial agent for a period of 10 days. MIC was determined on days 0 and 10.

Table 7. Antimicrobial susceptibility of planktonic cultures of *S. aureus* against BAC and vancomycin: MIC on day 0 and after 10 passages in a sub-inhibitory concentration. MIC and MBC are expressed in μg/mL.

Antimicrobial	Day 0		Day 10	
	MIC	MBC	MIC	MBC
BAC	1.25	5	1.25-2.5	5
Vancomycin	1	2	2	2

Table showed that, when *S. aureus* was cultured in a sub-inhibitory concentration of BAC or vancomycin for 10 consecutive days, a 2-fold increase in MIC was observed, indicating that the culture had become less susceptible to these antimicrobials. No changes on MBC were observed, however. To confirm if these phenotypical changes were permanent, *S. aureus* should be further sub-cultured in growth media without the antimicrobials.

To evaluate the potential development of bacterial resistance to antimicrobial surfaces previously optimized, a similar assay was adapted. Cells were put in contact with unmodified PDMS and modified surfaces for 24 h, for a period of 10 days. After this period, cells were recovered and used to determine the MIC and MBC of antimicrobials. As a control, the same procedure was performed for unmodified PDMS and pDA-coated PDMS. It is important to refer that, in this assay, resistance was not defined considering the clinical breakpoints, but a comparison between MIC values, before and after the modification of the surface. Results are shown in

Table .

Table 8. Antimicrobial susceptibility of adhered cells of *S. aureus* against BAC and vancomycin: MIC and MBC after 10 passages in contact with unmodified PDMS, pDA-coated PDMS and pDA-coated PDMS surfaces functionalized with antimicrobials. MIC and MBC are expressed in µg/mL.

Antimicrobial	**MIC**			**MBC**		
	PDMS	pDA	Antimicrobial	PDMS	pDA	Antimicrobial
BAC	2.5	2.5	2.5	5	5	5
Vancomycin	2	2	2	2	2	8

Result showed that cells adhered to modified surfaces with BAC revealed the same susceptibility as the cells adhered to the positive controls (PDMS and PDMS-pDA), which suggests that development of resistance to these antimicrobial agents did not occurred. However, when vancomycin was immobilized on pDA-coated PDMS surface, the values of MBC were higher (4-fold increase) compared to PDMS unmodified or coated with pDA.

Bacterial exposure to BAC in solution fostered the emergence of less susceptible bacteria but the same was not found after its immobilization. Antimicrobials immobilization offers, therefore, an alternative to minimize the risk of developing bacterial resistance.

In this work, the potential development of resistance by *S. aureus* to the antimicrobial surfaces for a longer period combined to the analysis of colony morphology was tried. However, due to several contaminations and consequent lack of time, it was not possible to conclude this study. Results were not accurate and, therefore, were not presented. Experiments for evaluating the potential development of resistance by *S. epidermidis* strain were not performed because the optimization of an antibiotic immobilization to render PDMS surfaces with antimicrobial properties against this strain and without leaching was not achieved within the realization of this work.

4. CONCLUSIONS

The problem of BAI is not recent and, with the increase of life expectancy and consequent increase of the use of implants and medical devices, it tends to get worse. These infections are serious because a large part of them are caused by bacteria capable of forming biofilms, impairing the performance of the antimicrobial agent. The ineffectiveness of treatment results in high mortality, morbidity and pain for patient. To date, the treatments used to combat these infections imply invasive procedures and/or antibiotic systemic administration which are subject to the microbial resistance phenomenon. So, the development of strategies, that work in the early stages of infection, is urgent. Currently there are several reported approaches to prevent BAI involving the development of materials that inhibit adhesion or kill microorganisms by contact. However, most of the current strategies presents some important limitations, including the emergence of multi-drug resistant bacteria and toxicity concerns.

In this study, a mussel-inspired coating strategy was successfully applied to immobilize antimicrobials agents (antibiotics and BAC) onto PDMS surfaces, imparting them with antimicrobial and anti-biofilm properties, with the final purpose of evaluating the potential development of resistance.

Results showed a better performance of antimicrobials when they are used in alkaline pH and an overnight incubation. Their release from the surface was proportional to the concentration used for their immobilization, as higher concentrations resulted in larger zones of inhibition, hence more leaching from the surfaces.

These surfaces did not completely eradicate the biofilm but disturbed its development. No propensity for developing resistance was found for immobilized BAC as the same susceptibility pattern was obtained for cells recovered from unmodified or modified surfaces. Cells recovered from the surfaces modified with vancomycin, exhibited a higher MBC as compared to cells recovered from unmodified PDMS.

This study alerts to the development of bacterial resistance and the risk associated to the immobilization of antibiotics, highlighting the great potential of BAC as antimicrobial coating for the prevention of BAI, once their cytotoxicity can be ruled out.

5. FUTURE WORK

To improve the performed work in the present thesis, it is proposed as future work the characterization of modified surfaces, determining their hydrophobicity using the sessile drop contact angle method; their surface morphology by scanning electron microscopy (SEM); and their surface roughness using atomic force microscopy (AFM).

Similarly to the several assays performed with *S. aureus*, it would be important to complete the same tests for *S. epidermidis*, namely find an antibiotic which immobilization renders the surfaces with antimicrobial properties but without leaching, so afterwards it could be assessed the potential development of resistance.

In this study, the evaluation of bacterial resistance was performed using a period of test of 10 days. To simulate the real conditions in which antimicrobials are often used for long periods of time, it would be important to increase periods of time to 30 days, for instance, for evaluation of resistance development.

During resistance assay, bacterial growth was monitored by CFU counting every day. It would be helpful to perform a study to evaluate gene expression and colonies morphology to detect the presence of SCV.

An experiment that would complete all this research would be to evaluate the activity of macrophages when they are brought into contact with these modified surfaces, so the role of immune system can also be taken into consideration. For this, animal models could be used. As found in this work, complete eradication of the biofilm was not achieved, therefore, in that environment are found live and dead bacteria. In this sense, it would be important to determine the fate of both live and dead bacteria when they are in contact with macrophages.

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SUPPLEMENTARY MATERIAL I – OPTIMIZATION OF SONICATION TIME

With the aim of determining the optimal sonication period of time able to remove the larger number of cells without compromising its viability, adhered cells to unmodified PDMS and PDMS-coated with pDA for 24 h, were detached by ultrasonic bath in a Sonicor SC-52 (Sonicor Instruments) operating at 50 kHz, during different periods of time followed by rapid vortex mixing for 30 s. Serial 10-fold dilutions were then performed and plated onto TSA plates that were incubated at 37 ºC.

The number of viable bacterial cells was expressed as CFU/mL in **Figure *9***. Results showed no significant differences between the tested times. So, in order to minimize the stress caused by sonication, 3 min of sonication was the chosen time.

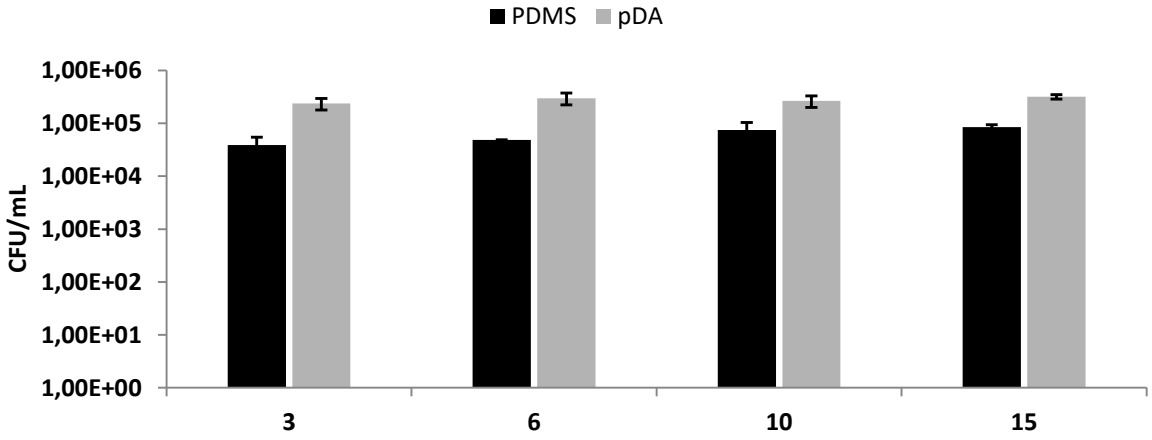

Figure 9. Optimization of sonication times (represented in minutes) to detach *S. aureus* adhered to PDMS and PDMS coated with pDA.

SUPPLEMENTARY MATERIAL II – CONTACT-KILLING

In order to evaluate compounds antimicrobial activity, contact-killing assays were performed. Antimicrobials were immobilized onto surfaces and, thereafter, a small volume of bacterial suspension was dropped on the surfaces. Representative images of results are in **Figure *10***.
As positive controls, growth was observed on PDMS and pDA-coated PDMS. On the contrary, absence of growth was an indication of contact-killing activity as shown on pDA-coated PDMS surfaces with growth media only (negative control). The higher concentration tested (BAC 1 mg/mL) did not allow bacterial growth.

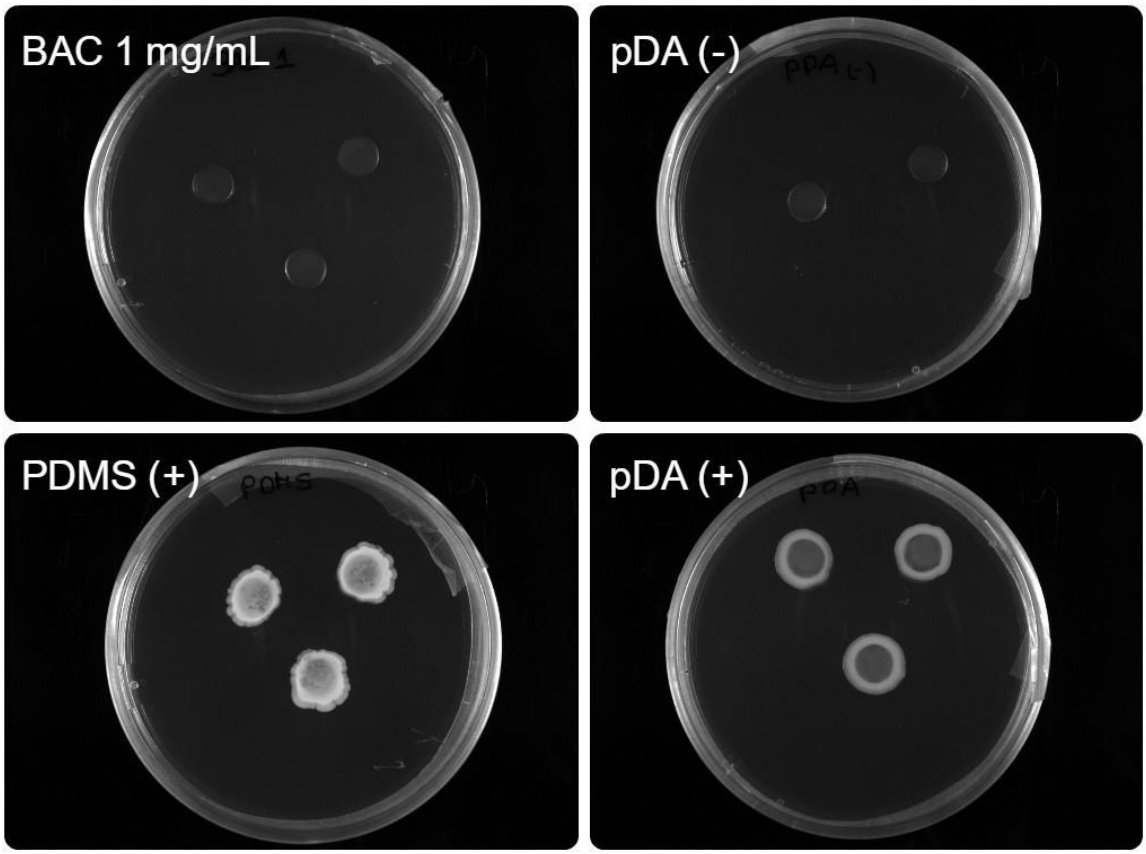

Figure 10. Representative images of contact-killing assay: bacterial growth can be observed on unmodified PDMS and PDMS-coated surfaces where a drop of a bacterial suspension of *S. aureus* was added. No growth was visible for surfaces immobilized with BAC (at a concentration of 1 mg/mL) as well as in the negative control (pDA-coated PDMS without bacteria).