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The use of phytochemicals with EDTA as catheter lock solutions

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July 14, 2017

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Abstract

Resistant bacteria are one of the most pressing healthcare challenges. Their ability to mutate and adapt against any compound that can affect them is threatening. Bacteria can also form biofilm, a community of microorganisms surrounded by polymeric substances, which improves their resistances and resilience on surfaces. These can develop in biomedical devices, such as hemodialysis catheters, and require treatment or even removal of the implanted device. It is necessary to develop effective molecules and strategies to control these biofilms.

The objective of this study was to develop a novel and effective antimicrobial formulation which can be used as a catheter lock solution for hemodialysis catheters. This formulation should be an alternative to antibiotics, and therefore, phytochemicals were selected as a source of new antimicrobial agents.

A review on some antimicrobial lock solutions tested in the literature, with an overview of their mode of action, is presented. To assess planktonic and sessile bacterial control, the minimum inhibitory concentration, minimum bactericidal concentration and fractional inhibitory concentration were determined and the reduction of culturable colony-forming units of biofilm cells was calculated. After these assays, the most promising formulation was tested against biofilm grown inside hemodialysis catheters. These experiments were performed with *Escherichia coli* and *Staphylococcus epidermidis* single and dual species biofilm.

Against planktonic bacteria, all the tested phytochemicals showed synergy with EDTA. Cuminaldehyde with EDTA reached the lowest FIC index, 0.32 and 0.63 against *Escherichia coli* and *Staphylococcus epidermidis* respectively. This combination was also the most capable of controlling biofilm. After a 24 h exposure period there were no CFU detected for any type of biofilm. The selected combination of cuminaldehyde with EDTA was then tested as a catheter lock solution, achieving log CFU reductions higher than 1 against *E. coli* and dual species biofilm grown inside a catheter.

The use of phytochemical substances, in particular cuminaldehyde, showed antimicrobial properties against the selected bacteria and their biofilms. These compounds are already known for being antioxidant and antifungal, and with the increased interest in them, other properties are likely to be found. As antimicrobials these may be able to replace antibiotics in many applications where their overuse is seen.

Resumo

O aparecimento de bactérias multi-resistentes constitui o maior desafio à saúde da espécie humana. A sua habilidade de mutar e de se adaptar aos compostos que as afetam é ameaçador. As bactérias também conseguem formar biofilmes, comunidades de microrganismos rodeadas de matéria polimérica que aumentam a resistência das bactérias e a sua resiliência nas superfícies. Estes biofilmes podem-se desenvolver em dispositivos biomédicos, como cateters de hemodiálise, e requerem o seu tratamento ou até a remoção do dispositivo. É preciso desenvolver novas moléculas e estratégias capazes de controlar estes biofilmes.

O objetivo deste estudo foi desenvolver uma formulação antimicrobiana nova e eficaz que pudesse ser usada como solução de bloqueio para cateters de hemodiálise. Esta formulação deveria servir de alternativa aos antibióticos, e, por esse motivo, fitoquímicos foram selecionados como fonte de novos agentes antimicrobianos.

Foi efetuada uma revisão da literatura relativamente às solução de bloqueio de cateters existentes e ao seu modo de ação. Para determinar a capacidade de controlar células planctónicas e sésseis, a concentração de inibição mínima, a concentração bactéricida mínima e a concentração de inibição fraccional foram determinadas, e a redução do número de unidades formadoras de colónias culturáveis foi calculada. Depois destes ensaios, a formulação mais promissora foi selecionada e testada em biofilmes desenvolvidos dentro de cateters de hemodiálise. Estas experiências foram efetuadas com *Escherichia coli* e *Staphylococcus epidermidis* nas suas formas simples e dupla em biofilme.

Contra células suspensas, todos os fitoquímicos testados mostraram sinergia com o EDTA. O cuminaldeído com EDTA atingiu o parâmetro FIC mínimo, 0.32 e 0.63 contra *Escherichia coli* e *Staphylococcus epidermidis* respetivamente. Esta combinação foi também a mais capaz de controlar biofilmes. Depois de 24 h de exposição não foram detetatas CFU para nenhum dos tipos de biofilme. A combinação selecionada de cuminaldeído e EDTA foi então testada como solução de bloqueio de cateter, conseguindo uma redução logarítmica de CFU superior a 1 contra *E. coli* e biofilme duplo num cateter de hemodiálise.

O uso de substâncias fitoquímicas, em particular o cuminaldeído, mostrou propriedades antimicrobianas contra as bactérias selecionadas e os seus respetivos biofilmes. Estes compostos são já conhecidos pelas suas propriedades antioxidantes e antifúngicas, e com o interesse acrescido neles, outras propriedades podem ser descobertas. Como agentes antimicrobianos estes podem substituir os antibióticos em aplicações onde se vê o seu uso excessivo.

Agradecimentos

Quero agradecer em primeiro lugar ao Doutor Manuel Simões e à Doutora Isabel João por me introduzirem ao tema e terem acompanhado o meu trabalho. À Teleflex que me forneceu os cateters de hemodiálise. À Carla, Paula e Sílvia pela ajuda no laboratório. À Cristina pela ajuda com as tabelas no Latex. À Rita, Catarina e Mariana, pelo apoio mesmo estando longe. Por último aos meus pais e irmã, que apesar de não perceberem muito do que eu fazia todos os dias, estavam sempre interessados em saber "como andavam as moléculas".

João Vale

Este trabalho foi em parte financiado pelos Projetos (i) POCI-01-0145-FEDER 006939 (Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, UID/EQU/00511/2013) financiado pelo Fundo Europeu de Desenvolvimento Regional (FEDER), através do COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) e por fundos nacionais através da Fundação para a Ciência e a Tecnologia I.P., (ii) NORTE-01-0145-FEDER-000005 – LEPABE-2-ECO INNOVATION, financiado pelo Fundo Europeu de Desenvolvimento Regional (FEDER), através do COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI) e Programa Operacional Regional do Norte (NORTE2020).

"Well, I never heard it before, but it sounds uncommon nonsense"

Lewis Carroll, Alice in Wonderland

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Abbreviations and Symbols

ALT	Antibiotic Lock Therapy
ANOVA	Analysis of Variance
C	Cuminaldehyde
CDC	Cocurrent Downflow Contactor
CFU	Colony-forming Unit
CIP	Ciprofloxacin
CRBSI	Catheter-related Bloodstream Infections
DHFR	Dihydrofolate Reductase
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetra-acetic Acid
EPS	Extracellular Polymeric Substances
FIC	Fractional Inhibitory Concentration
HIV	Human Immunodeficiency Virus
I3C	Indole-3-carbinol
LB	Luria-Bertani
LD	Live/Dead
MBC	Minimum Bactericidal Concentration
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD	Optical Density
PCA	Plate Count Agar
Q	Quercetin
QAC	Quaternary Ammonium Compound
RMA	Resistance-modifying Agent
RNA	Ribonucleic acid
rpm	Rotations Per Minute
SEM	Scanning Electron Microscopy
VA	Vanillic Acid
VRSA	Vancomycin-resistant *Staphylococcus aureus*

Chapter 1

Introduction

1.1 Context

Resistant microbial strains are a serious issue to the 21st century's population. Bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA) are already resistant to many classes of antibiotics and are able to survive months on hospital surfaces [7]. In the last 35 years, only 2 novel classes of antibiotics were discovered, which are only usable against Gram-positive bacteria [8]. The lack of new drugs and the development of resistances by microorganisms together pose a public health threat, and the misuse of antibiotics did amplify this problem [7, 8]. Other culprits include poor-quality antibiotics and inadequate surveillance and susceptibility assays [9].

There is therefore the need to develop new strategies to overcome this problem. This could include the development or discovery of new types of molecules that are able to control bacterial growth. Plant secondary metabolites, commonly referred as phytochemicals, can have antimicrobial properties and function differently from common antibiotics, thus surpassing usual bacterial resistance [10, 11]. Some can also modify a cell's resistance mechanisms, functioning as resistance modifying agent (RMA) and therefore improving the action of existing biocides [10].

The matter of bacterial colonization and resistance is noticeable in most medical scenarios. Dialysis patients have implanted catheters that can cause severe complications caused by microorganisms. Every year, in the United States of America, there are around 100 000 catheter-related bloodstream infections (CRBSI) with a mortality rate of 5 to 10% among hemodialysis patients [12]. It is estimated that 1 of every 20 central venous catheters inserted will be associated with at least one systemic infection [13] and that more than half of every catheter will be colonized at some point [14]. Although there are plenty of technologies and techniques used to attempt to lower these numbers, many of which will be described later in this study, current infection and mortality rates are still very high and bacteria still pose a great healthcare problem for these patients. This is the motivation for many studies on this subject over the last years.

1.2 Dissertation objectives and structure

The objective of this project was to develop a formulation with antimicrobial and antithrombatic properties to be used as a lock solution in hemodialysis catheters. The combination of an antimicrobial agent with a biofilm disruptor or resistance modifying agent, to improve the formulation's overall effectiveness against Gram-positive and negative bacteria, was screened. To avert the overuse of antibiotics and house-hold antimicrobials, phytochemicals were tested as the main biocide agent.

This field has gained traction in the last years, as the appearance of multi-resistant bacteria is getting more common. Therefore, there are plenty of studies in the literature that have similar goals to this one. A comprehensive table with antimicrobial and antibiotic lock solutions was compiled, to summarize and compare what has been studied up until this point.

The experiments performed involve an initial screening of multiple concentrations and combinations of substances, against both planktonic and sessile cells. To assess the spectrum of action, tests are made with Gram-negative and Gram-positive bacteria, and with a dual species culture of *Escherichia coli* and *Staphylococcus epidermidis*, two bacteria commonly associated with catheter-related infections. After selecting a promising lock solution candidate, biofilm were grown inside a hemodialysis commercial catheter, through the means of a bioreactor. The candidate's ability to control the biofilm was tested against a standard catheter lock solution, which serves as a positive control.

After this first Chapter where the thesis objectives and organization are presented, in Chapter 2 there is an overview of the state of the art regarding biofilms and their control in healthcare. Specifically the case of hemodialysis catheters is presented in more detail. Different technologies and antimicrobial solutions for catheters are reviewed, to understand what is currently used and which viable alternatives exist. In the third Chapter the experimental methods and protocols used in this thesis are described. In Chapter 4, the main results are presented and discussed. Finally, Chapter 5 includes the conclusions of the thesis and some directions and suggestions for future studies.

Chapter 2

Literature Review

2.1 Bacterial Resistance and Resistance-Modifying Agents

Evolution gave microorganisms powerful mechanisms against biocides. Less membrane permeability, which occurs if its composition is altered or if the transport proteins become more selective, will improve the cell's resistance to a substance. Moreover, efflux pumps also effectively remove unwanted compounds. Mechanisms such as enzymatic inactivation or target site mutation can also occur but are more common against antibiotics than against biocides [1, 15, 10]. *Pseudomonas aeruginosa* is considered to be responsible for 10-15% of the worldwide hospital infections. This is due to their natural toughness and ability to acquire resistances from other bacteria. Some strains have demonstrated virtually every known mutational mechanism of bacterial resistance [16]. Figure 2.1 presents a representation of these mechanisms and others against antibiotics.

The study and development of resistance-modifying agents can help mitigating this problem, as they are substances that improve the effectiveness of a known biocide or antibiotic. This effect may be due to greater solubility, resorption rate, bioavailability or interactions with the cell's resistance mechanisms. Due to that, it is said that RMAs are able to recycle antibiotics, which means that old antibiotics that are not used, because most strains are resistant, can be effective once again. This is often cheaper than developing new antimicrobials [10]. One of the most common examples is clavulanic acid, which is a RMA that binds to β-lactamases, turning penicillin-resistant bacteria susceptible to it [10]. The combination of RMAs and antibacterial substances with synergistic effects may be a strategic way of overcoming bacterial drug resistances.

2.2 Biofilms and biofilm control

Biofilms are communities of microorganisms attached to surfaces, and cells within it have improved resistances. In fact, concentrations required to eliminate sessile cells can be one thousand times higher than those necessary to kill their suspended equivalents [11, 17]. To produce biofilm, planktonic cells must deposit and adhere to a surface, which can be pre-conditioned by molecules. Because of its dynamic behaviour, there is growth of the biofilm, which includes both cellular

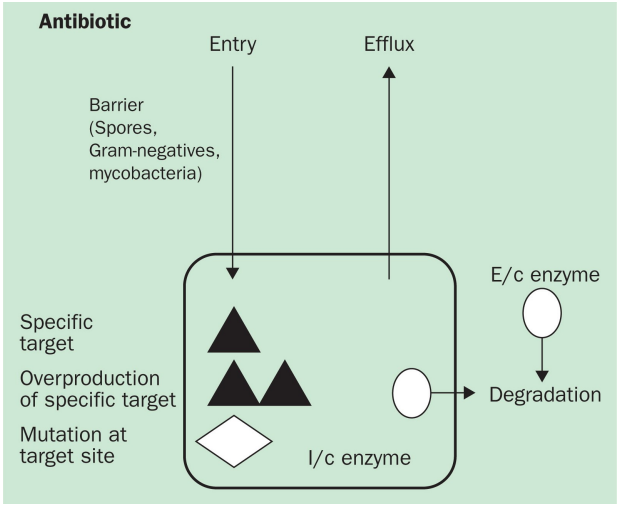

Figure 2.1: Most prevalent mechanisms of antibiotic resistance, adapted from Russel, 2003 [1]

division and the production of extracellular polymeric substances (EPS) that compose the matrix. Detachment can allow the biofilm to populate other areas of the surface [11, 18]. This process is shown in Figure 2.2. Lower mass transfer inside the polymeric matrix of the biofilm, which slows the flow of the antimicrobials, and reactional processes between the biofilm polymers and the antibiotics, are some of the mechanisms that explain the increased resistance to antibiotics [19]. These extracellular polymeric substances are mainly polysaccharides, but also include glycoproteins, glycolipids and even extracellular DNA, that allow facilitated horizontal gene transfer [20]. After an adverse condition, cells that survived within the biofilm can rapidly proliferate and regrow [11]. This so called persistent cell state is a recent explanation to the increased resistance. Most of the hospital-acquired infections are attributed to biofilms, and more than 80% of human infections are also related to them [21, 22].

Sterilization, disinfection and antisepsis are concepts regarding the different magnitude of microbial cleaning, from total elimination of microbial life and spores, sterilization, to killing most of the bacteria and preventing their growth on surfaces such as the skin, known as antisepsis [7, 23]. Different types of molecules are used to achieve these thresholds of cleaning, and they differ on their mechanism, effectiveness and range of action. Common household biocides include ethanol, sodium hypochlorite and quaternary ammonium compounds (QACs). These are extraordinarily effective against microorganisms but cannot be used, for instance, in biomedical devices, because of their low biocompatibility. Due to being globally used, strong selective pressure does cause resistant microorganism populations to appear [24]. Being environmentally unfriendly is also a disadvantage [1, 23, 25]. The main antibacterial substances used are still antibiotics, which have very distinct interactions inside the cells they target. This comes as both an advantage and a disadvantage. They are very effective and directed to their target, but a simple modification to the pathway that they effect will make them obsolete [1, 13, 26].

Phytochemicals may be an interesting alternative to these classic biocides. As they are plant

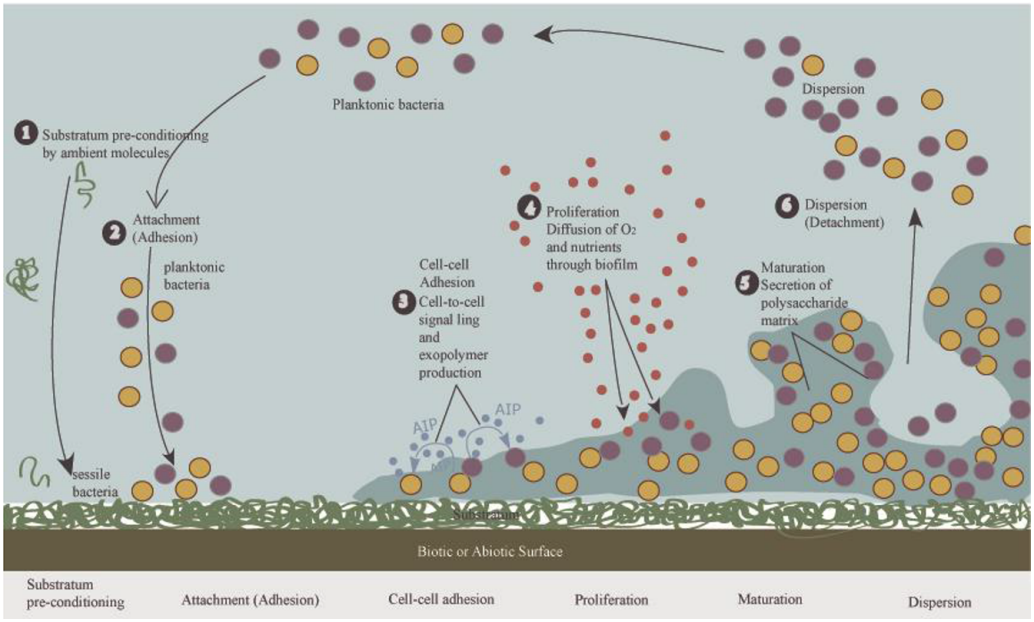

Figure 2.2: Representation of the biofilm formation process. Adapted from Kirmusaoglu, 2016 [2]

metabolites, phytochemicals are biocompatible at a great range of concentrations and mostly harmless against the environment. Studies show that some substances, such as ferulic and gallic acids, are promising as antimicrobials [21, 27], while others have potential as RMAs [10]. In these phenolic acids, the antimicrobial effect is due to membrane disruption and enzyme inhibition capabilities [11]. Essential oils are plant secondary metabolites that are used as food preservers, fragrances and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic or locally anesthesic drugs [28]. The carotenoids, or tetraterpenoids, are another class of phytochemical substances. β-carotene is the most recognized example of these substances, and its benefits as an antioxidant and potential anticarcinogen have been studied [29]. These properties make phytochemicals an attractive alternative to anthropogenic disinfectants.

Regarding biofilm control strategies, there are different approaches. Traditional treatments that are effective against suspended cells will slow down the initial attachment and early biofilm production. If an established biofilm is the target, mechanical forces can be used to physically remove it. Enzymes can target the EPS and effectively make sessile cells more susceptible. This is the case of alginate lyase, which helps antibiotic penetration in *P. aeruginosa* biofilms [30]. Since biofilms are essentially communities of microorganisms, disrupting the quorum sensing molecules is also a strategy. In *S. epidermidis*, the quorum sensing regulator agr is involved in biofilm detachment. The use of tobramycin in *P. aeruginosa* biofilms is also effective in the presence of quorum sensing inhibitor molecules [31]. Metal cations are involved in the biofilm structure matrix. Therefore, chelating agents may be able to destabilize it. Oxidizing agents and other dispersants can lead to cell detachment. An example is cis-2-decanoic acid, which was reported as able to induce dispersion in several bacterial biofilm and *Candida albicans* [32].

Finally, bacteriophage therapy may be able to prevent and control bacterial biofilms. A genetically engineered phage was able to eliminate sessile cells and reduce the EPS by the effect of a phage-associated depolymerase [6]. These and other strategies have promising results but further studies are needed to overcome limitations and expand the spectrum of action.

2.3 Biofilms in healthcare

Antonie Van Leeuwenhoek observed and was intrigued by the plaque of his own teeth in the 17th century. Centuries later this was considered the first report of a biofilm [33]. Since then, the presence of biofilm in the environment and in the human body has been studied. In general, biofilm formation can have a negative impact, causing infections, but also a protective and synergistic role. Bacteria attached to the human gut epithelial cells function as a barrier against foodborne pathogens [34]. The dental plaque is a multi-organism biofilm that forms in both healthy and unhealthy mouths. Teeth decay can be caused by the over development of some infectious strains, which are usually controlled by naturally occurring beneficial microorganisms that compete in that environment [35]. Periodontitis is caused primarily by *Porphyromonas gingivalis* and multi-organism biofilms that develop between the tooth root and the gum and cause inflammation [36]. Moreover, *P. aeruginosa* is known to cause pneumonia and lower respiratory tract infections to immunocompromised or cystic fibrosis patients [37]. Mycoses can be caused by *Candida* spp. and other fungal biofilms [30]. Biofilm formation can cause infections in wounds or burned-victims, and can often become chronic or non-healing [38]. Otitis media is a common disease in children and is caused by different microorganisms such as *Streptococcus pneumoniae* and *Haemophilus influenza* that colonize the middle ear [39].

If an indwelling or implanted medical device is contaminated, it either has to be removed or treated. Both have an associated cost and risk to the patient. Some devices that are susceptible to biofilm formation include artificial voice prostheses, replacement joints, prosthetic heart valves, cardiac pacemakers, cerebrospinal fluid shunts, endotracheal tubes, urinary catheters, peritoneal dialysis catheters, central venous catheters and contact lenses [30]. Figure 2.3 shows a microscopic analysis of pacemaker units with and without antimicrobial cover, and the biofilm that will develop on it if no antimicrobial measures are applied. The adhesion of bacteria to these exogenous devices can be promoted by proteins. The ability of *S. epidermidis* to colonize polystyrene is due to an autolyzin that can mediate the attachment. Moreover, a fibrinogen-binding protein known as clumping factor A (ClfA) is important to the binding of *S. aureus* to polyethylene and polyvinyl [40]. Infective endocarditis caused by *streptococci* or *staphylococci* biofilms are associated to patients with heart defects or prosthetic valves [33]. Catheter-related infections include colonization, localised infection (exit site, pocket, tunnel infection) and bloodstream infection. Different treatments are recommended for each site of contamination [41]. A study revealed that the percentage of patients undergoing indwelling urinary catheterization was 13.2% for hospital patients, 4.9% for nursing homes, and 3.9% for patients receiving home care in Denmark [42]. The colonization in these devices can cause their rapid obstruction, as the multi-species biofilm formed can produce

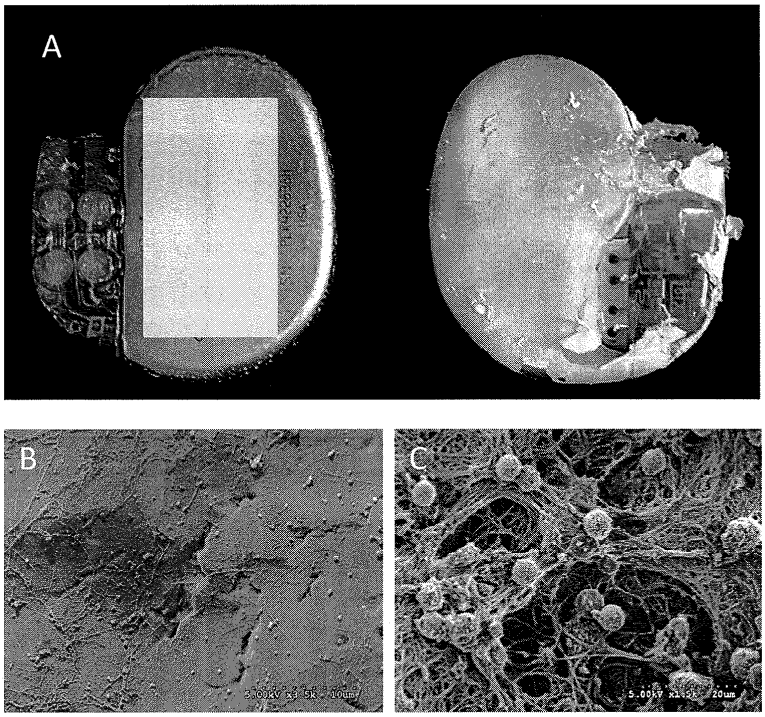

Figure 2.3: Biofilm formation in pacemakers, shown in both macroscopic and scanning electron microscopy analysis. (A) Two pacemakers are shown. The one on the left was implanted within an antimicrobial pouch, while the one on the right was not; (B) SEM of the pacemaker shown on the left in (A); (C) SEM of the pacemaker shown on the right in (A). Adapted from Citron et al, 2012 [3]

urease that will degrade urea, causing a rise of the pH and consequent precipitation of minerals [43]. Consequently, the development of devices and methods that decrease contamination rates and new biofilm control strategies are required to further lower mortality rates.

2.4 Colonization control in catheters

In hemodialysis catheters, biofilm formation is an ever-occurring source of infection. Although the number of catheter-related bloodstream infections have decreased in the last 15 years, they are still a major mortality cause [44]. Besides this, infection usually comes together with thrombosis [18]. The most common contaminants are *S. epidermidis*, *Staphylococcus aureus*, and *C. albicans* [45, 46]. Colonization usually occurs due to skin bacteria that are able penetrate the catheter implantation area and proliferate. Besides this, bacteria in the bloodstream can colonize a catheter segment that contacts with the blood and produce biofilm there. The catheter's lumen can also become colonized due to contaminated solutions that may be administrated [4]. These colonization sites are represented in Figure 2.4. For easy to replace devices such as short-term peripheral

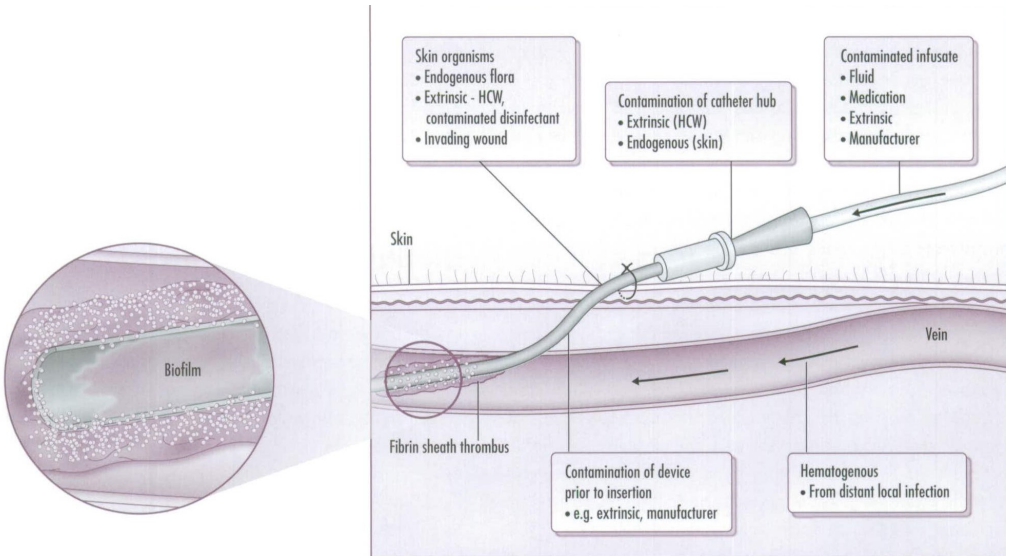

Figure 2.4: Representation of the multiple contamination sites the exist when implanting a catheter. Adapted from James et al, 2011 [4]

catheters, its removal is the optimal treatment. Catheters with a 48-72h persistent contamination or that reveal local skin infection should be replaced. Long-term or implanted devices should be treated with systemic antibiotics, as their removal and replacement can be dangerous [41]. Moreover, there are reports of a decreased antimicrobial efficacy when treating older biofilms [47]. Catheter-related infections also increase the overall cost of treatment from $6000 to over $90000 [48]. The main sources of contamination are the skin of the patient at the catheter insertion site, a contaminated catheter hub or malpractice when inserting the device [32].

In order to keep these devices sterile, there are two primary techniques which are used: the flushing technique, which is the manual insertion of normal saline solution inside the catheter to clean it, and the lock technique, which is the injection of a limited volume of an antimicrobial solution, following the flush, to prevent colonization and clot formation for the period of time that the catheter is not being used [49]. In 1987 this locking technique was first suggested as a method of preventing the backflow of blood into the catheter [50]. This was achieved by exerting pressure on the syringe plunger when injecting the last millimetres of solution. Nowadays these positive pressure techniques were replaced by specially design syringes (such as BD PosiFlush syringes ^{TM}). The lock solution remains inside the catheter between sessions, which for hemodialysis patients are usually 3 times per week.

The antibiotic lock therapy (ALT) is the use of antibiotics in high concentrations as a lock solution, often with an anticoagulant, to sterilize a catheter. This is used as a preventive measure in patients with history of catheter related infections and as treatment. To manage an infection, the antibiotic lock can be used as an adjunct to systemic antibiotics. This increases catheter salvage rates [51]. Treating a catheter-related infection with systemic antibiotics alone has a higher risk of causing serious complications such as endocarditis or epidural abscess [52]. Because of this,

the Stanford Hospital and Clinics Guidelines advises that antibiotic locks should only be used as treatment, and always with systemic therapy [53]. This is not always followed, and resistant strains appear, especially since antibiotics are used twice, both intravenously and inside the catheter [26, 54]. These guidelines also dictate that catheters infected with *S. aureus* or *Candida* spp. should be automatically removed, since salvage rates for these microorganisms with known treatments are low [53].

Ideally a lock solution has broad spectrum of activity, high biofilm penetration, biocompatibility, stability, low toxicity, low resistance and cost-effectiveness [51]. To achieve this ideal mixture, the constitution of lock solutions can be very distinct, from simple anticoagulants such as heparin [55] to combinations of citrate, methylene blue and parabens, which have strong antimicrobial effect [12]. Antibiotics are also used commonly in conjugation with anticoagulants, such as vancomycin and heparin [56] or minocycline with EDTA [57]. However, development of resistance to antibiotics and the possibility of toxicity caused by diffusion of the lock solution into the blood stream are disadvantages of these formulations [32, 55].

To test a lock solution's antibacterial capability, it is possible to perform *in vitro* and *in vivo* assays. *In vitro* testing involves quantifying the bacteria that grow in culture medium after contacting with the solution. Mathematically this is usually expressed by a logarithmic reduction of the number of colony forming units (CFU). The removal of the biofilm is another parameter that relates to antimicrobial effect [58]. *In vivo* tests are performed in infected catheters over the course of a long period, and are mathematically described by the reduction of the number of catheter related infections [59]. Table A.1 has several lock solutions and their effectiveness against the microorganisms listed, *in vitro* and *in vivo*.

2.5 Lock solutions components and modes of action

Heparin is the most used anticoagulant in lock solutions. It effectively reduces thrombosis in catheters and has been widely studied [60, 61] It is also generally used as an adjunctive anticoagulant in antimicrobial or antibiotic lock solutions. However, it can promote the growth of *S. aureus* biofilms by promoting cell-to-cell interactions [62] and there is also a risk of inducing bleeding [63]. For these reasons, alternative anticoagulants are often studied as heparin substitutes. Molecules that have the capacity to chelate, complexing with metallic ions, can function as anticoagulants. Sodium citrate is a common substitute. It functions as an anticoagulant, a preservative, and, at higher concentrations, as an antimicrobial capable of eliminating suspended bacteria such as *P. aeruginosa* or *E. coli* [64]. It is also extensively used in conjugation with other antibacterial substances in lock solutions. It is often regarded as better than heparin in locks, reducing the number of CRBSI [65]. High doses of citrate (47%) can, however, cause protein precipitation inside the catheter, paraesthesia and metallic taste. There is also one reported case of fatal cardiac arrhythmia [55]. These concentrations should therefore be avoided. Other anticoagulants include ethylenediamine tetra-acetic acid (EDTA), which is also a chelating agent and can single-handedly eradicate suspended and sessile bacteria in catheters [66, 67].

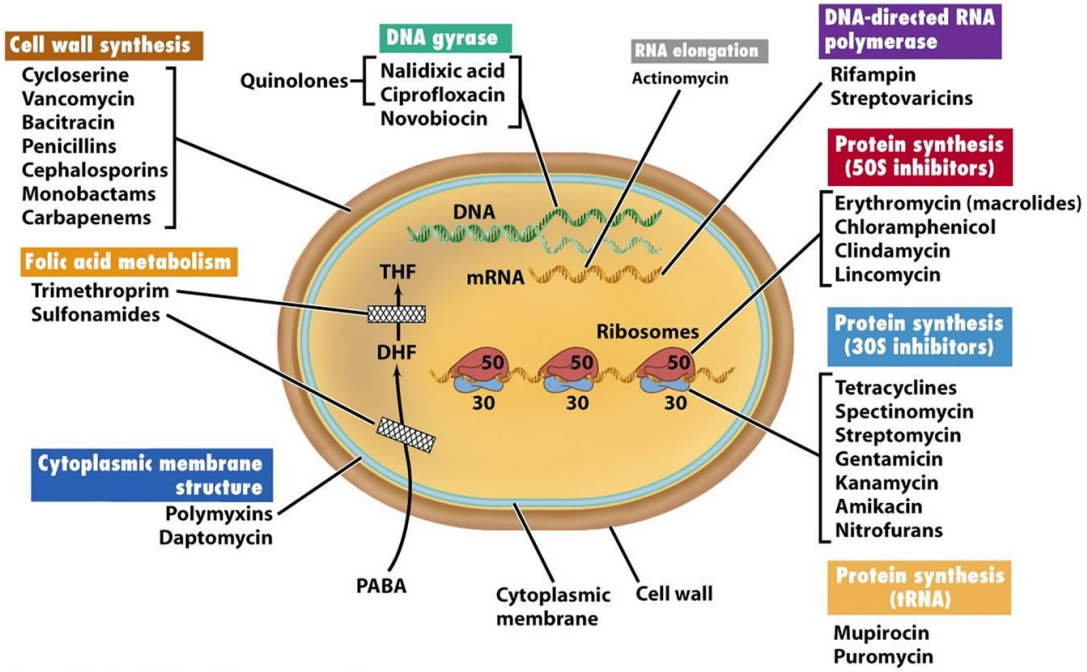

Figure 2.5: Representation of some bacterial sites that are targeted by different classes of antibiotics. Adapted from Bbosa et al, 2014 [5]

Ethanol can be effective in hemodialysis catheters, either in conjugation with another antimicrobial or not [68]. Concentrations up to 70% are used, eliminating formed biofilm of *S. aureus*, *E. coli*, *C. albicans* and others. Lower concentrations are also effective. The use of 4% citrate as an adjunctive anticoagulant to ethanol has also been studied. No colonies of *S. aureus, S. epidermidis, Pseudomonas aeruginosa* and *E. coli* were detected after 72h [69]. Urokinase, which is a protease, as treatment for occlusions in catheters is commonly used, and its capability as a lock or flush solutions has been already tested [70], reducing the probability of CRBSI in high-risk patients. The susceptibility of bacteria to other antimicrobials such as glyceryl trinitrate, in solution with ethanol and citrate has also been tested, being able to eliminate resistant strains of *staphylococci* [71]. Methylene blue is a dye with interesting antibacterial effects, specifically in conjugation with citrate and parabens as a lock solution, eradicating suspended cells with an exposure time of just one hour [12]. The use of glycerol at high concentrations (35% to 60%) has antibacterial properties, and that is also used as a catheter lock [72]. This effect comes from the bacteriostatic properties of this substance, also used as a cryopreservative, that eventually leads to cell death. Balancing it with sodium chlorine allows it to be hemocompatible, minimizing hemolysis and prolonging the clotting time [72]. The amino acid taurine can derive a substance called taurolidine, which is successfully used as an antimicrobial lock solution. It is biocompatible and greatly reduces the number of CRBSI [73].

Regarding antibiotics, their mode of action can differ greatly from one to another. Figure 2.5 presents an overview of the target site of multiple classes of antibiotics. Penicillin is a group of

β-lactam antibiotics. They have a broad spectrum of activity but bacteria become resistant to them if they produce β-lactamases, which are enzymes that can cleave the β-lactam ring, which is fundamental for the effectiveness of a penicillin [74]. These antibiotics inhibit the transpeptidase that cross-links peptidoglycan, the polymer that structures the cell wall, making the organism inviable [75]. They are used in conjugation with β-lactamase inhibitors, to improve their effectiveness and to be able to affect resistant cells. Their use in lock solutions is wide, often coupled with heparin. Another group of β-lactam antibiotics are the cephalosporins. They have a similar mode of action to penicillins and can be effective against both Gram-positive and Gram-negative species [76]. Carbapenems are also β-lactams, and have exceptional broad-spectrum range of action because of their efficient penetration in bacteria and stability to hydrolysis [77].

First generation quinolones were not the most used antibiotics. It was only in the early 1980's that, with the development of their second generation, they got more attention. The addition of a fluorine and a major ring substituent gave these antibiotics greater penetration into Gram-positive organisms and enhanced their pharmacokinetics and pharmacodynamics [78]. They act by binding to DNA gyrase and topoisomerase IV, two enzymes responsible for the relieve of strain of double-stranded DNA and its unlinking following the replication, respectively. Resistance is usually developed by mutations in these two enzymes or in porins, which are membrane transport proteins through which quinolones can penetrate [79].

Aminoglycosides are multifunctional hydrophilic sugars that have several amino and hydroxy functionalities. They are synergetic with many other antibiotics and have been used clinically for several decades. Their activity derives from their capacity to bind to specific types of RNA, such as prokaryotic rRNA or tRNAPHE [80]. For critically ill patients, glycopeptides can be used. These antibiotics have restricted use as they can be toxic. They are important against MRSA or in patients that have β-lactam hypersensitivity. In general, they have a narrow spectrum of action as they cannot penetrate some barriers such as the cerebrospinal fluid. They interact with the late stage synthesis of peptidoglycan, specifically the growth of the chain, by inhibiting transglycosylation. The target is not the enzyme responsible for this step. Instead, the antibiotic binds to the substract [81, 82]. Another class of antibiotics used as last resort are oxazolidinones, used to eliminate vancomycin resistant *Staphylococcus aureus* (VRSA) and other multi-drug resistant Gram-positive bacteria. They inhibit protein synthesis by binding to the 50S ribosomal subunit, preventing the formation of the initiation complex. Resistance can occur by target modification [83]. Daptomycin is also an important weapon against MRSA and VRSA. This lipopeptide has a distinct mechanism of action from most other antibiotics, thus being used against multi-drug resistance strains. It interacts directly with the bacterial cytoplasmatic membrane, causing rapid depolarization of its potential and posterior cell death [84].

The tetracyclines were discovered in the 1940s and, by binding to the 30S ribosomal subunit, can stop protein production in bacteria, leading to cell death. They are broad spectrum antibiotics but resistant bacteria have limited their effectiveness [85]. In response to this, glycylcyclines were developed. Their mode of action is similar to tetracyclines, but they are not affected by some resistance mechanisms developed by bacteria, such as the efflux pumps that expel tetracyclines

and ribosomal target modification, which they can overcome [86]. Antifolates can inhibiting the enzyme dihydrofolate reductase (DHFR) which is fundamental to the conversion of folic acid to reduced folate cofactors. This is especially important during DNA and RNA synthesis, which are processes whose rates are amplified in rapid dividing cells. Thus, cancer cells can be fought using these substances [87]. Another class of antibiotics are the polymyxins. They consist of a cyclic peptide with a hydrophobic tail, resembling a surfactant. These molecules bond with the phospholipidic layer, disrupting the inner and outer membrane [88].

As was discussed previously, although antibiotics have shown to be effective, there is the need for alternative antimicrobials. Included in the phytochemical class, indole-3-carbinol (I3C) is a constituent of cruciferous vegetables, such as broccoli, cauliflower, brussels sprouts, and cabbage. It has anticarcinogenic effects on animals and humans, and has been shown to inhibit the growth of some types of cancer cells [89], although there have been some reports about the toxicity of this substance [90]. As an antimicrobial it is mainly effective against Gram-positive bacteria, although its mode of action is still unknown [91].

Cuminaldehyde (C) is an essential oil which is a component of *Cuminum cyminum* seeds (cumin) and is commonly used as a spice or flavouring agent. As an antidiabetic drug, it has inhibitory effect against aldose reductase and α-glucosidase, and may be used as treatment in the future [92]. This substance has been shown to inhibit bacterial growth for several species [93]. Thymol, carvacrol and linalool are some of the many other essential oils that are known to inhibit bacterial growth against many species [93, 28].

Vanillin and vanillic acid (VA) extracted from the vanilla bean or plant are used as flavouring agents. Furthermore, they have antifungal properties, making these compounds interesting to the food industry. Their antimicrobial activity against multiple species has also been reported [94, 95].

In the flavonoids class, quercetin (Q) is an example of a substance with multiple positive activities. It was reported as antioxidant, anticancer, antithrombotic, anti-HIV and antimicrobial against multiple Gram-positive and Gram-negative species at high concentrations [96]. Some oxidation derivatives of quercetin, extracted from onions, appear to have even higher antioxidant and antimicrobial properties [97].

The development and discovery of novel molecules and synergistic combinations are fundamental to improving catheter salvage rates and to reduce the number of related-complications. The overuse of antibiotics has led to the appearance of many resistant strains, and therefore should not be taken for granted. Finding alternative solutions should be a focus.

2.6 Biofilm-producing reactor

When experimenting with biofilms, it is important to have a consistent and proper method for its development, as different strategies and conditions will affect reproducibility. Consequently, having an adequate model for a biofilm-producing reactor is a core part of experimenting with biofilm. Regarding biofilms produced inside catheters, there are plenty of studied setups that can simulate proper conditions.

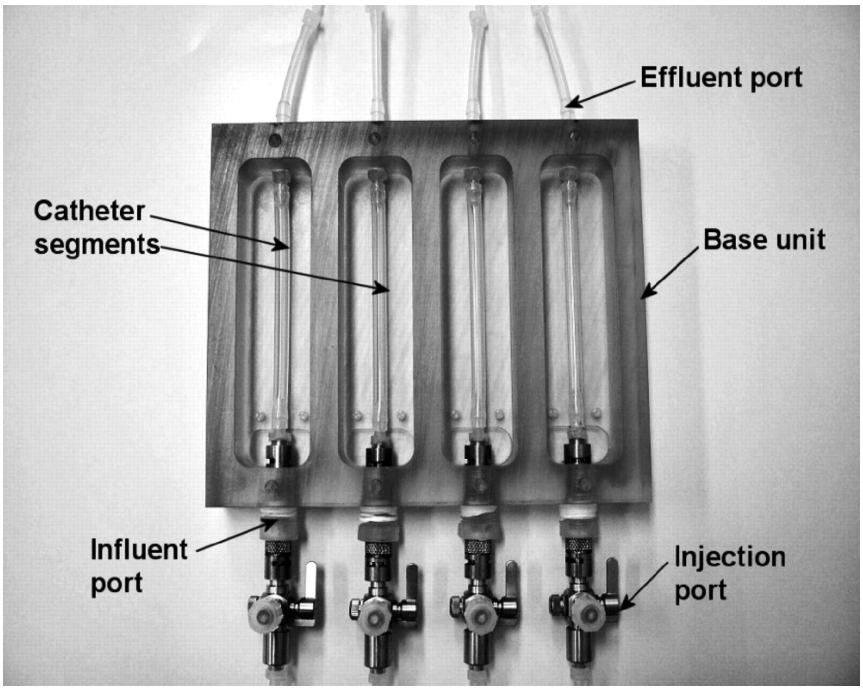

Figure 2.6: Drip flow reactor modified to flow through catheter segments, adapted from Curtin et al, 2006 [6]

The cocurrent downflow contactor (CDC) biofilm reactor is a continuous flow vessel that has a paddle which provides the appropriate shear forces to the bacteria developing in the coupons. The biofilm grows in the separate coupons and this setup mimics accurately and in a repeatable manner an environment with a renewable nutrient source and shear stress [98]. Drip flow reactors (Figure 2.6 are also used to study biofilm formation, allowing the biofilm to develop inside catheter segments at low flow rates [6]. The Calgary biofilm device is a modified microtiter plate that allows growth on the outer side of its 96 pegs [99]. Other devices used to grow biofilm include the modified Robbins device, disk reactors and perfused biofilm fermentator [33]. Factors that should be established when considering a model reactor are the temperature, presence of antimicrobials, identity and number of organisms, flow and shear rates, retention time, media composition and conditioning films that may exist [33].

In the specific case of biofilm growth inside a catheter, these and other devices can be adapted and used, as they all share the same required, yet trivial, premise: to develop biofilm you need to flow bacteria and growth media on the surface of the material. They have, however, differences in other variables, such as flow rate or size. Particularly in catheters, there are some considerations that need to be regarded. Its geometry, material and the presence of any coating are important variables when modelling. Silicone, polyurethane, polyethylene and polystyrene are used most of the times [100]. A temperature of 37°C should be used when incubating, as to mimic the human body. The organisms colonizing the catheter should be the most common contaminants of the type of catheter studied, such as, for instance, *S. epidermidis* for hemodialysis catheters and

Enterobacteriacae for urinary catheters, unless a particular microorganism is meant to be studied [101]. Regarding flow rate and retention time, there are two extreme situations. Between dialysis sessions there are low shear and flow rates as the catheter is essentially static. On the other hand, during a session, the blood flow rate that the dialysis machine provides is usually around 300-500 mL per minute [102]. Although the contamination does not develop significantly during this step, biofilm formation is generally increased in situations with higher flow rate, and therefore this can be used to model a worst-case scenario.

Regarding growth media, the environment inside a catheter is not suitable for experimenting and achieving reproducible results, since it does not have the nutritional properties for bacteria to develop properly. Therefore, a growth media is needed to allow proper development. This will not be representative of a real scenario, but will again be a worst-case approximation. Ringer's lactate solution is a replacement fluid in patients with low blood volume or low amount of electrolytes. It is used as media to mimic blood, as it has similar ion composition [103]. It can be used to study bacterial growth in hemodialysis catheters because of these similarities. Another example of a media that mimics blood is trypticase soy agar enriched with 5-10% sheep blood. This provides not only the nutrients that TSA inherently has, but also proteins important for bacterial growth [67]. It is also noteworthy that some proteins, such as fibrin and collagen, adhere to the catheters inner surface and potentiate bacterial colonization [101], and therefore the use of this media can help simulating this effect. Other media compositions, such as MHB or LB, can also be used [104]. Finally, regarding incubating and exposition times, since dialysis sessions usually occur every couple days, incubation periods of between 24 h to 72 h are appropriate. The incubation time chosen should take into consideration the microorganism growing, as different bacteria have different needs.

Having grown the microorganisms and applied the lock solution for the appropriate exposure time, the next step is to retrieve them. This involves cell and biofilm detachment, as suspended cells have already been flushed at this point. The primary used techniques are scrapping, vortexing and sonication. The first involves the detachment of biofilm using a microbrush on the inner surface of the tubing [104]. Although this is a simple method, it is hard to remove every bit of biomass using a brush and it is not reproducible, as different people will scrap differently. It is also possible to remove biofilm through vortexing. This is an easy and reproducible method, but may not remove all the sessile cells in the surface, depending on its shape. The alternative is sonication, which is the use of ultrasonic frequencies to detach the biofilm [105]. This method is more reliable and effective, but does require optimization of the frequency and the sonication time, as improper conditions may kill the bacteria instead of detaching them. The enumeration of the viable microorganisms that were retrieved is usually performed by CFU counting, which is relatively inexpensive. Other techniques include flow cytometry or microscopy methods.

Despite all the technology and research available regarding biofilm control, different approaches need to be taken in order to find alternative solutions for what is considered to be the one of the most pressing issues of our time, antimicrobial resistance.

Chapter 3

Materials and Methods

3.1 Bacterial strains and growth media

It was important to test the spectrum of action of the antimicrobials using both a Gram-negative and a Gram-positive bacteria. Taking into consideration the species that had been detected in biofilms in catheters [45, 46], *Escherichia coli* CECT 434 and *Staphylococcus epidermidis* ATCC 35984 were used in this study. These were incubated in Mueller-Hinton broth (MHB, Oxoid) and plated in Mueller-Hinton agar (MHA, Oxoid).

Since biofilms are often composed of different species, a multi-species biofilm with both these bacteria was also subject of testing. To grow dual species biofilm, the OD_{600} of each inoculum was adjusted to 0,1 and each well was filled with 100 μL of each bacterium. The CFUs were counted to ensure that the amount of CFUs of both species was the same for the initial bacterial inoculum. To confirm that both bacteria would develop in the biofilm, the colonies were compared by their colony shape and with a Live/Dead BacLight kit (Invitrogen) membrane integrity assay.

3.2 LD cell viability microscopy assay

The biofilm which was grown in microtitre plate wells was scrapped and diluted in 1 mL of saline solution. 300 μL of this bacterial suspension was again diluted in 1 mL of saline and then filtered through a Nucleopore (Whatman, UK) black polycarbonate membrane (pore size 0.22 μm). 250 μL of SYTO9 and 50 μL of propidium iodide were used as stains, and left to react for 7 minutes in darkness [106]. The membrane was mounted in a microscope slide and the preparation was observed using a epifluorescence microscope (LEICA DMLB2 microscope, with a Leica DFC300 FX camera, Leica Microsystems Ltd).

3.3 Antimicrobial agents

Quercetin (Q, Sigma Aldrich), indole-3-carbinol (I3C, Sigma Aldrich), vanilic acid (VA, Sigma Aldrich), cuminaldehyde (C, Sigma Aldrich), ethylenediamine tetraacetic acid (EDTA, VWR),

ciprofloxacin (CIP, Sigma Aldrich) were the selected antimicrobial agents that were subject to the initial screening. Ciprofloxacin was used as a positive control, since it is an effective antibiotic which is also used to treat contaminated catheters (Table A.1). Phytochemicals were dissolved in dimethyl sulfoxide (DMSO) while other antimicrobials were dissolved in distilled water. To dissolve EDTA, sodium hydroxide was added to the solution to increase its solubility. Hydrochloric acid were used for the same reason in the ciprofloxacin solutions.

3.4 Minimum inhibitory concentration and Minimum bactericidal concentration

Bacteria were incubated overnight at 37°C (FOC 225E Refrigerated Incubator, VELP Scientifica) in Mueller-Hinton broth at 160 rpm (IKA KS 130 Basic Orbital Shaker). The OD_{600} was adjusted to 0.1 (VWR V-1200 Spectrophotometer). 96-well polystyrene microtiter plates (Orange Scientific) were used to determine the minimum inhibitory concentration (MIC). The wells were filled with 180 μL of cells and 20 μL of antibacterial formulation at different concentrations and the plate was incubated at 37°C for 24 hours. By measuring the absorbance at 600nm (Spectrostar nano BMG Labtech), the MIC was considered the lowest concentration at which no growth was detected. The minimum bactericidal concentration (MBC) was determined by plating the supernatant of the wells with higher antimicrobial concentration than the MIC well on PCA plates. These were incubated at 37°C for 24 hours and the MBC was considered the concentration at which no growth was visible.

3.5 Fractional Inhibitory Concentration

To be able to compare synergism between multiple substances used in combination as antimicrobials, the Fractional Inhibitory Concentration (FIC) is calculated. This is an index number that is related to the type of interactions of the components, from antagonism to synergism. It is used primarily as a means of comparison. This parameter is defined as [107]:

$$FIC = \frac{C_{\mathrm{a}}}{MIC_{\mathrm{a}}} + \frac{C_{\mathrm{b}}}{MIC_{\mathrm{b}}} \tag{3.1}$$

For an antimicrobial formulation with 2 compounds, C_a is the concentration of substance a in the formulation and MIC_a is the minimum inhibitory composition of substance (a) when used by itself. The MIC is the lowest concentration at which there is no visible growth of suspended bacteria after the incubation period. Higher FICs imply antagonism, and lower show synergy. The range that corresponds to each type of interaction is not consensual, and depends on the number of compounds in the mixture. In a situation with two substances, it is common to consider synergism as FIC < 1 and antagonism as higher than that, while 1 is indifference [108, 109].

3.6 Culturable sessile cells after exposure to antimicrobials

Bacteria were grown overnight in MHB at 37°C and 160 rpm. The OD_{620} was adjusted to 0.04 and the wells of a microtiter plate were filled with 200 μL of bacterial suspension to produce biofilm. After a 24 h incubation period at 37°C and 160 rpm, the wells were washed with sterile saline and refilled with 180 μL and 20 μL of different concentrations of an antimicrobial agent. Following another 24 h incubation period, the wells were rewashed with saline solution and scraped three times using a micropipette tip (period of 1 minute each) with 200 μL of saline solution to resuspend sessile cells. The scraped content of each well was transfered to a microcentrifuge tube with 400 μL of saline (totalizing 1mL in each tube). After performing serial dilutions, 10 μL of each dilution was plated, in duplicates, on PCA plates using the drop plate method. CFUs were counted after 24 h at 37°C. Dilutions that presented CFU between 3-30 per 10 μL drop were considered. This is consistent with the spread plate method, which counts plates with 30-300 colonies with a spread volume of 100 μL [110].

3.7 Quantification of biofilm removal in hemodialysis catheters

After the initial screening by MIC, MBC and biofilm culturability, the best candidate was selected and tested in the removal of biofilm grown inside a catheter. A bioreactor closed system connected to a catheter segment was set up to mimic this scenario, shown in Figure 3.1 and Figure 3.2. The catheter used in the study is a commercial multi-lumen central venous catheter with antimicrobial surface (ARROWg^+ard Blue Plus®CS-45703-E). The catheter was cut into 3 cm segments in aseptic conditions, to allow for a higher number of assays with the available catheters. This length also allowed for easier biofilm removal, since the longer the segment, the harder it is to remove the cells in the middle of it.

Bacteria were incubated overnight at 37°C in MHB at 160 rpm and the OD_{600} was adjusted to 0.1. A volume of 200 mL of this bacterial suspension was pumped and recirculated through silicon tubing which had the catheter segment connected using a peristaltic pump (Fisher MCP 3000). Calibration curves are shown in Figures A.7 and A.8

An initial adhesion assay was performed, to test the surface's antimicrobial properties, with a flow rate of 1 mL/s, a growth time of 2 hours and exposition time to the antimicrobials of 30 minutes. This replicates a possible contamination during a dialysis session, which is characterized by high flow rates and short duration. Usual dialyser blood flow rate are around 300 mL/min [111, 112]

The period between dialysis treatments was also simulated. This experiment used a flow rate of 0.1 mL/s, which inside the catheter segments is equivalent to a velocity of 4,5 cm/s. After a 24 h growth period, the circulation was stopped and, using a sterile syringe, the control and selected formulation were inserted in the catheter segments. The exposition time was 24 h.

The solutions were removed and the segments were put into a tube with 10 mL of saline solution. Then, the cells were detached by vortexing at maximum velocity for 1 minute, twice

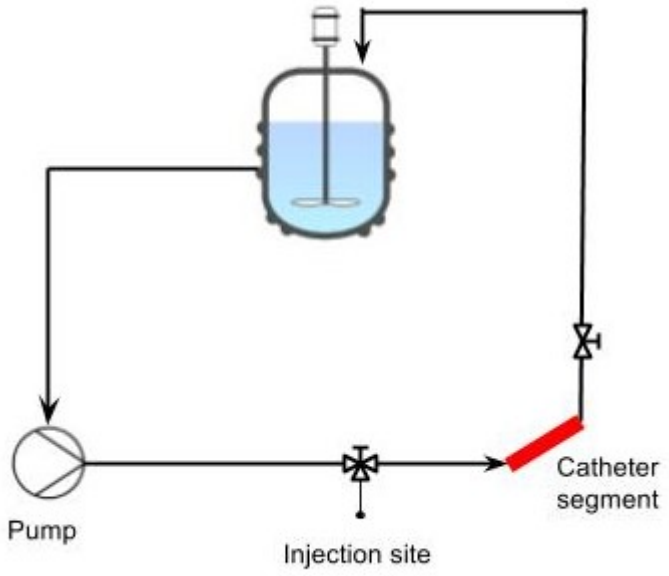

Figure 3.1: Simplified process diagram of the setup used in the hemodialysis biofilm experiment

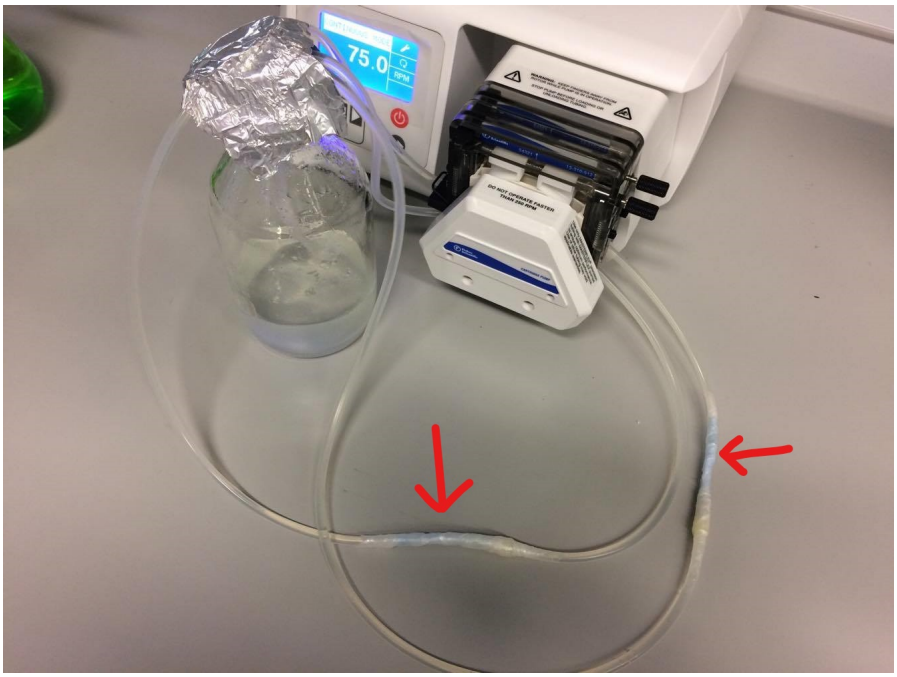

Figure 3.2: Photograph of the vessel connected to the pump and catheter segment

(VWR VV3 S40). By serial diluting these samples, and using the drop plate method, it was possible to enumerate the CFUs and calculate the biofilm percentage removal.

3.8 Data analysis

Statistical analysis was performed using MS Excel. Experiments were performed in triplicates with three repetitions and averages and standard deviations were calculated. The statistical significance of the results was calculated for a 95% confidence interval, $P < 0.05$, using ANOVA.

Chapter 4

Results and Discussion

4.1 Confirmation of the growth of a dual species biofilm

E. coli and *S. epidermidis* were the selected microorganisms for this study. Since one of the main objectives was to test the susceptibility of both single and dual species biofilm to antimicrobials, it was important to confirm if these two species would develop biofilm together or if one would dominate over the other.

Firstly, by serial-dilution, the number of CFUs of each species were counted (samples were adjusted to an absorbance of 0.1 at 600 nm). For *E. coli*, the number of CFUs was 2.7×10^7 and for *S. epidermidis* it was 2.6×10^7. These results are not statistically different. This was important to verify, since it was not intended for a species to be more prevalent than the other when developing the biofilm.

Figure 4.1 is a picture of the colonies formed after a 24 h incubation period of the scraped cells removed from a dual species biofilm. It is possible to distinctively differentiate the *E. coli* colonies (A), which are larger than the *S. epidermidis* colonies (B). This indicates that these bacteria can form biofilm together, which had been previously reported [113].

Further analysis with a dual species biofilm was performed using a membrane integrity fluorescence kit with epifluorescence microscopy. Figure 4.2 is the result of this assay. Two stains were used. SYTO 9 is a green stain that marks cells with intact membranes, while propidium iodide binds to cells that have damaged membranes, staining them red. The objective of this experiment was not to assess membrane integrity, but instead to visualize different bacterial shapes. It is possible to see (A) bacillus shaped and (B) coccus shaped cells. This is coherent with the bacteria that were used, *E. coli* and *S. epidermidis* respectively.

Having these confirmations on the development of a dual-species biofilm, although none of them are conclusive, it was possible to advance in the study with confidence that this type of biofilm was developing as expected.

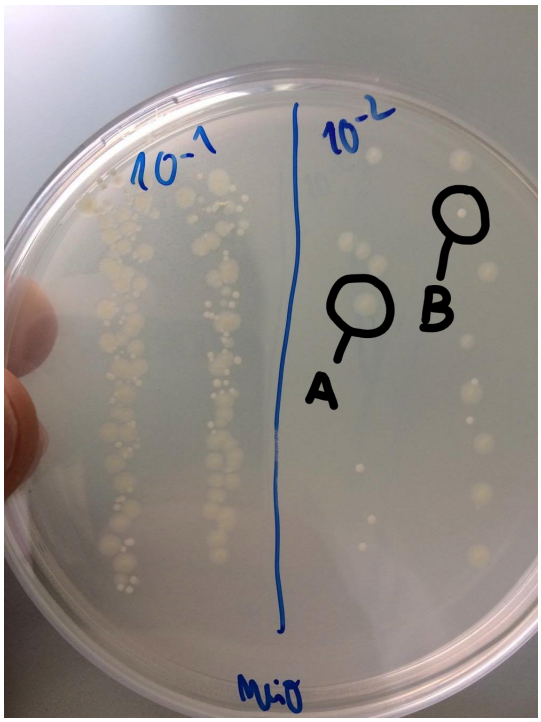

Figure 4.1: Photograph of the colonies grown from dual species biofilm to confirm that both species would develop and were distinguished from colony morphology

4.2 MIC and MBC of antimicrobial agents

To test the susceptibility of planktonic bacteria to the phytochemical compounds of interest, MIC and MBC assays were performed. Phytochemicals were used alone and also in conjugation with EDTA. This substance has shown biofilm control abilities and, as a chelating agent, may have synergy with other antimicrobials. Since the use of an anticoagulant is also a necessity for catheter lock solutions, this substance is of interest. The resulting MICs and MBCs are displayed in Table 4.1.

It should first be noted that the concentration of EDTA used in combinations should be sub-inhibitory, as it is not the primary antimicrobial agent. For that reason, the MIC of EDTA was determined. The MIC of this substance for *S. epidermidis* was quite lower than the MIC for *E. coli*. Therefore, the concentration of EDTA when used in conjugation with phytochemicals was adjusted accordingly. To keep the sub-inhibitory regime, 1 mM of EDTA was used with *S. epidermidis* and 5 mM was used with *E. coli*.

Using indole-3-carbinol it was not possible to determine a MIC. This parameter was determined by absorbance measurement of the samples before and after a 24 h incubation period. When this substance was used, the incubated samples had absorbances proportional to the I3C concentration, which was not expected. This could have happened, for example, due to a reactional process that was triggered in contact with bacteria, originating a product that absorbs at 600 nm. Because it was not possible to visualize a decrease of absorbance, the MIC was not deter-

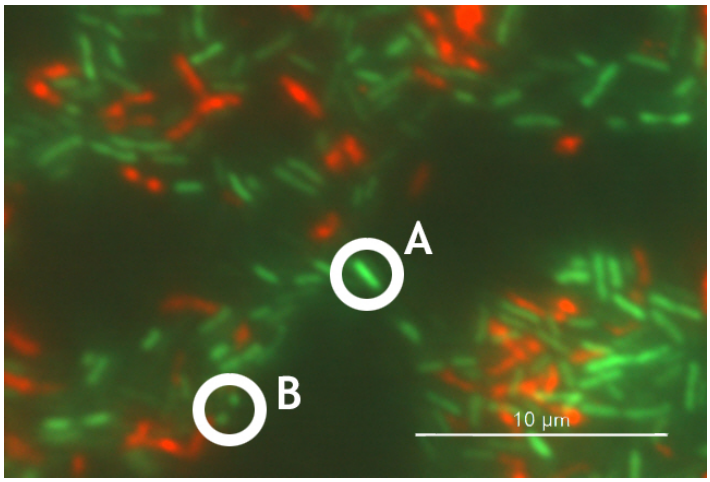

Figure 4.2: Microscopy image with (A) bacillus shaped cells and (B) coccus shaped cells, using LD BacLight kit with the LEICA epifluorescense microscope. Cells which are coloured green have an intact membrane, while red cells have lost membrane integrity.

mined and is referenced as (-) in Table 4.1. However, when the contents of the wells were plated in solid media, it was possible to determine a MBC and confirm the antimicrobial effectiveness of indole-3-carbinol.

Quercetin, at higher concentrations, would precipitate when contacting with water, either when preparing the dilutions or when it was put in the wells. For this reason, its use in catheters could be hazardous and dangerous.

When used by themselves, the phytochemicals tested did now show great antimicrobial ability, having MICs greater than 15 mM. However, EDTA was found to be active against suspended *S. epidermidis* by itself. It can also be seen that *E. coli* was more resistance than *S. epidermidis* against these substances. This could be because of the outer membrane that Gram-negative species have, which often provides increased resistance to biocides due to lower permeability [114]. Vanillic acid's MIC in other studies ranges from 2.5 mM to 5 mM for *E. coli* [94, 115] which is significantly lower than what was determined. When phytochemicals and EDTA were used in combination, this scenario was improved. Regarding cuminaldehyde, its usage with EDTA was able to eliminate bacteria against both species. It should be noted that the MIC and MBC for cuminaldehyde could not be determined at the concentrations tested (it was higher than the maximum concentration used, 25 mM). However, other studies report that the MIC for cuminaldehyde against *E. coli* is between 2.5 mM and 5 mM, which is much lower than what was determined [93, 116]. Quercetin and vanillic acid also showed improvements when used with EDTA. Although with I3C it was not possible to determine a MIC, the MBC when used with EDTA was lower for both species than when used alone. The determined MBC of I3C was slightly higher than what is reported in other studies, which is 11 mM compared to 15 mM in this study [117].

Regarding ciprofloxacin, which was used as a positive control, concentrations from 0.1 mg/mL to 1 mg/mL were tested, since this is a range of concentration that is commonly used to eliminate

Table 4.1: MICs and MBCs of formulations with and without EDTA against *E. coli* and *S. epidermidis*. The EDTA concentration is 5 mM for *E. coli* and 1 mM for *S. epidermidis*

Composition	Microorganism	MIC (mM)	MBC (mM)
C	*E. coli*	>25	>25
	S. epidermidis	>25	>25
I3C	*E. coli*	-	15
	S. epidermidis	-	5
Q	*E. coli*	15	>25
	S. epidermidis	15	>25
VA	*E. coli*	15	25
	S. epidermidis	15	20
EDTA	*E. coli*	>25	>25
	S. epidermidis	3	15
C + EDTA	*E. coli*	3	5
	S. epidermidis	7,5	25
I3C + EDTA	*E. coli*	-	4
	S. epidermidis	-	3
Q + EDTA	*E. coli*	5	>25
	S. epidermidis	7,5	>25
VA + EDTA	*E. coli*	10	25
	S. epidermidis	15	15

biofilm in catheters (Table A.1). The MIC and MBC were found to be lower than 0.1 mg/mL for both species. Further investigation regarding this was not pursued, as it was not the objective of the study. Other studies report the MIC of this antibiotic against some strains of *E. coli*, *S. aureus* and *P. aeruginosa* to be lower than 1 μg/mL [118]. This means that the concentrations tested, which are used to treat contaminated catheters, are up to one thousand times higher than the MIC.

Although the synergy between some of the combinations tested are noticeable, a numerical comparison is performed in the next section. This allows for a more objective screening process.

4.3 Fractional Inhibitory Concentration

To have a numerical comparison of the synergy of multiple combinations of substances, the FIC index is used. This parameter is not consensual. However, for this study, the index value for each combination itself is not important. Rather than that, the goal is to be able to compare them. For that objective, the FIC index is a suitable metric. Equation 3.1 shows that a lower FIC index is indicative of higher synergism. Table 4.2 includes the calculated FIC index for the tested combinations against both bacteria.

It is noteworthy that in some cases it was not possible to determine a MIC. That is the case of, for instance, I3C. In that case, the MBCs were used instead. It is not known if the MIC and MBC change in the same manner when combined with EDTA, and therefore the FIC for I3C and I3C with EDTA can not be correctly compared with the others. Another situation where it

Table 4.2: Resulting FIC index for the compositions tested against each bacterial species. FIC values marked with an asterisk (*) are indicative of synergy.

Composition	Microorganism	FIC
C + EDTA	*E. coli*	0,32*
	S. epidermidis	0,63*
I3C + EDTA	*E. coli*	0,47*
	S. epidermidis	0,93*
Q + EDTA	*E. coli*	0,53*
	S. epidermidis	0,83*
VA + EDTA	*E. coli*	0,87*
	S. epidermidis	1,33

was not possible to determine the MIC was with cuminaldehyde. The MIC is higher than the concentrations tested. However, the maximum value tested (25 mM) was used as the reference value when calculating the FIC. Mathematically this means that the actual value of its FIC is lower than the one presented in Table 4.2.

It is possible to see that every FIC for *E. coli* is lower than for *S. epidermidis*. At first sight this appears to go against the fact that *E. coli* showed more resistance than *S. epidermidis*. However, it should be reminded that the FIC index is not a metric for the resistance to a formulation. It only reflects synergy or antagonism. Since EDTA is effective against the second organism but not the first, when used alone in planktonic cells, this result is mathematically expected. If EDTA is not effective against *E. coli* when solely used, but is in combination with other antimicrobials, its synergy is greater. Comparing the index of different combinations, cuminaldehyde shows the highest synergy with EDTA. The real FIC value for C + EDTA is even smaller, as explained before. All the other formulations did, however, also show some degree of synergy.

The analysis of the MIC, MBC and FIC results allowed to choose which combinations would be tested in the following assays. Cuminaldehyde with EDTA and indole-3-carbinol with EDTA were chosen. The first was clearly the best candidate. The FIC index was the lowest for both bacteria and there were no issues when handling it that could make it not suitable for a catheter lock solution. Since quercetin appeared to precipitate when used in higher concentrations, its use as a lock solution is limited. It could deteriorate the catheter's surface, which by itself has antimicrobial properties, and could potentially cause occlusion. During the dialysis this could also cause diffusion of the substance into the bloodstream, as blood would flow through the precipitated quercetin. Because of this, the second formulation that was chosen in the planktonic cell screening was indole-3-carbinol with EDTA.

4.4 Culturable sessile cells after exposure to antimicrobials

The most promising combinations of phytochemicals with EDTA were tested for their ability to eliminate bacteria within a biofilm. Single and dual species biofilm were exposed to antimicrobials

for 6 h, 24 h and 72 h. The resulting percentage CFU reductions are presented as a heatmap in Table 4.3. Logarithmic reductions are shown as bar graphs for each set of conditions, from Figure 4.3 to Figure 4.11.

To choose which concentrations would be used, the results regarding MICs were observed. It was important to use similar concentrations for both phytochemicals to be able to compare their anti-biofilm properties without that variable. Because of this, 4 mM was the chosen concentration for phytochemicals, as it was an intermediary value from the MIC of cuminaldehyde and the MBC of indole-3-carbinol against both species. Regarding EDTA, the concentration was 5 mM. To have a better understanding of what a variation in concentrations would do to the CFU reduction, phytochemicals were also tested at twice the concentration, 8 mM, and EDTA at half the concentration, 2.5 mM.

The number of CFU per cm^2 grown in the microtiter wells as controls was significantly different for biofilm grown for the three time periods for *E. coli*, *S. epidermidis* and dual-species biofilm ($P < 0.05$). However, the growth of the different biofilms was not significantly different from each other ($P \geq 0.05$). This means that the number of CFU for the three types of biofilm are similar, but the number of CFU for 6 h, 24 h and 72 h biofilms are different. For these three times, on average, the number of CFU per cm^2 of *E. coli* was, respectively, $2.93 \times 10^6, 1.04 \times 10^5$ and 2.77×10^4. For *S. epidermidis* the number of CFU per cm^2 was $2.32 \times 10^6, 1.68 \times 10^6$ and 2.96×10^5 and for the dual-species biofilm the number of CFU per cm^2 was $2.40 \times 10^6, 1.87 \times 10^5$ and 4.21×10^4.

4.4.1 Overview of percentage CFU reduction with a heatmap

Table 4.3 has a visual representation of the percentage reduction after CFU counting. This type of table allows quick analysis of results by using a color scheme. From green to red cells, it is possible to visualize when no colonies were detected (green cells that correspond to 100% reduction) and when there was no reduction in the number of colonies (red cells with 0% reduction).

It is possible to see that, overall, *E. coli* cells were more resistant than the other biofilms in the first 6h of exposure, with only the highest concentrations achieving more than 90% CFU reduction. This higher natural resistance of *E. coli*, compared to *S. epidermidis* has been studied when both were subjected to other phytochemical substances, such as cranberry extracts [119]. This is not the case for EDTA, which did affect *E. coli* more than *S. epidermidis* by a considerable amount in the first 6 h. This is an interesting result, as this substance was not effective against *E. coli* suspended cells. As a quelating agent, EDTA complexes with metallic ions [120]. These may be more important for *E. coli* biofilm than for *S. epidermidis* biofilm, which would explain why it was more effective against it. It is noteworthy that the concentration tested for this substance is low compared to what is used as a catheter lock solution. A solution with 40 mg/mL of EDTA is used to successfully control contaminations from multiple species, with log reductions of 4-5 [66]. The concentration tested, however, only corresponds to about 1.46 mg/mL, which is significantly lower and therefore does not have the same effect. Nonetheless this is expected, as this substance was not intended to be the primary antimicrobial agent.

Cuminaldehyde was lackluster compared to the other formulations. A concentration of 4 mM was not able to reduce by more than 86,9% any biofilm after any exposure time. It can also be noted that CFU reductions for the 72 h period are lower than the 6 and 24 h assays for all the biofilms using this phytochemical alone. This could mean that even though this substance has some antimicrobial properties, in longer periods of exposure bacteria are able to regrow or adapt to it. A concentration of 8 mM does also show this regrowth in *E. coli* and dual-species biofilm.

The remaining combinations are shown to be effective in a 24 h period. There were no colonies detected after a 24 h exposure period in both cuminaldehyde concentrations when used with EDTA against all 3 biofilm types. Indole-3-carbinol was equally effective, but not against *E. coli*, where the reduction rates are only moderate. This matches with the results of the MBC testing, where the MBC for I3C was higher for *E. coli* than for *S. epidermidis*.

The dual species biofilm appears to be slightly more susceptible to cuminaldehyde than its single species counterparts, both with and without EDTA. This could have many explanations. For instance, a higher diffusion rate in the dual-species EPS, which could be more porous, would cause this. On the other hand, when using I3C, the CFU reductions for this biofilm type are in the range of single-species biofilms. This could mean that its mode of action is indifferent of biofilm type and its effect only depends on the bacteria itself. However, there was no statistical significance with a 95% confidence interval. Another set of independent repetitions for this experiment would be require to prove if this difference does really exist. There is, nonetheless, statistical significance for the different formulations using cuminaldehyde, for 6 h, 24 h and 72 h biofilms ($P < 0.05$).

Table 4.3: Heatmap of the CFU percentage reduction of single and dual-species biofilm exposed to the tested formulations.

	E. coli			*S. epidermidis*			Multi-species biofilm		
	6h	24h	72h	6h	24h	72h	6h	24h	72h
4 mM C	76	87	0	77	64	11	60	64	40
8 mM C	85	100	95	87	96	96	99	99	98
4 mM C + 5 mM EDTA	74	100	100	98	100	100	98	100	100
8 mM C + 5 mM EDTA	98	100	100	96	100	100	100	100	100
4 mM I3C	88	93	100	99	100	100	99	100	100
8 mM I3C	95	99	100	100	100	100	100	100	100
4 mM I3C + 5 mM EDTA	83	99	100	99	100	100	89	100	100
8 mM I3C + 5 mM EDTA	98	100	100	99	100	100	99	99	100
2,5 mM EDTA	81	100	100	70	93	100	80	68	100
5 mM EDTA	90	100	100	7	100	100	82	91	100

4.4.2 Logarithmic CFU reduction

Figure 4.3 to Figure 4.11 are bar graphs for the combination of the tested conditions, which include exposure time, type of biofilm and antimicrobial formulation. These results for different biocides have statistical significance within a 95% confidence interval. The CFU reduction is expressed as a logarithmic reduction, and, where there was no colonies detected, (ND) was put instead of a bar. This is because if no colonies are detected, the log CFU reduction tends to infinity mathematically. Standard deviations are represented as error bars. This representation allows for a more careful comparison of the effect of each formulation in a set of conditions.

Figures 4.3 to 4.5 represent the CFU reduction for *E. coli* biofilm. Figure 4.3 shows the log CFU reductions for a 6 h exposure time ($P < 0.05$).

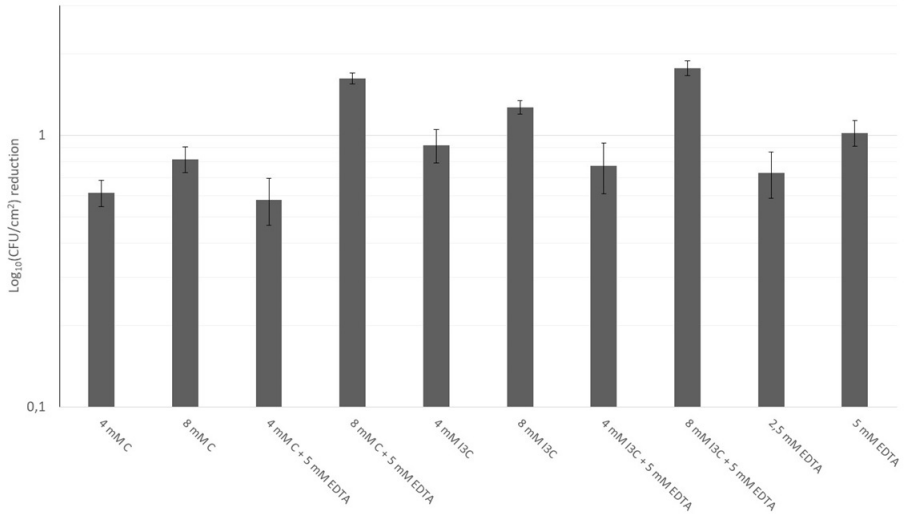

Figure 4.3: Log CFU reduction of *E. coli* biofilms after a 6h exposure period to the selected biocides and combinations.

A log reduction of 1.6 and 1.8 using 8 mM of cuminaldehyde and indole-3-carbinol with EDTA in a 6 h exposure period were achieved. This was expected, since these are the most concentrated formulations. In this exposure time it is, however, not possible to visualize synergy. The reduction caused by 5 mM EDTA is higher than when it is used with 4 mM of I3C or C, which indicates antagonism. It would be better to just use EDTA than to combine it with these concentrations of phytochemicals in these particular conditions.

Figure 4.4 represents the log CFU reductions for *E. coli* biofilms after a 24 h exposure to antimicrobials ($P < 0.05$).

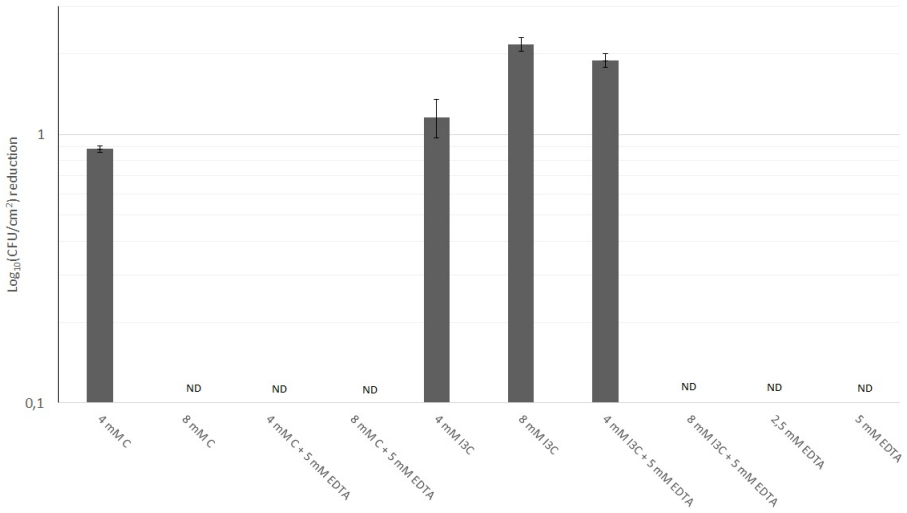

Figure 4.4: Log CFU reduction of *E. coli* biofilms after a 24 h exposure period to the selected biocides and combinations.

This figure reinforces that indole-3-carbinol may have antagonistic effects with EDTA against this type of bacterial biofilm using these concentrations. This is exemplified by the fact that 2,5 mM of EDTA caused an absence of colony growth, while 4 mM of I3C with 5 mM of EDTA did not. Synergy between cuminaldehyde and EDTA can not be seen in this figure as there were no CFU detected for both C + EDTA or EDTA when used alone. It shows, however, strong anti-biofilm activity against the species that was more resistant in the MIC and MBC experiments. The following figure 4.5 represents the CFU reduction after 72 h exposure ($P < 0.05$).

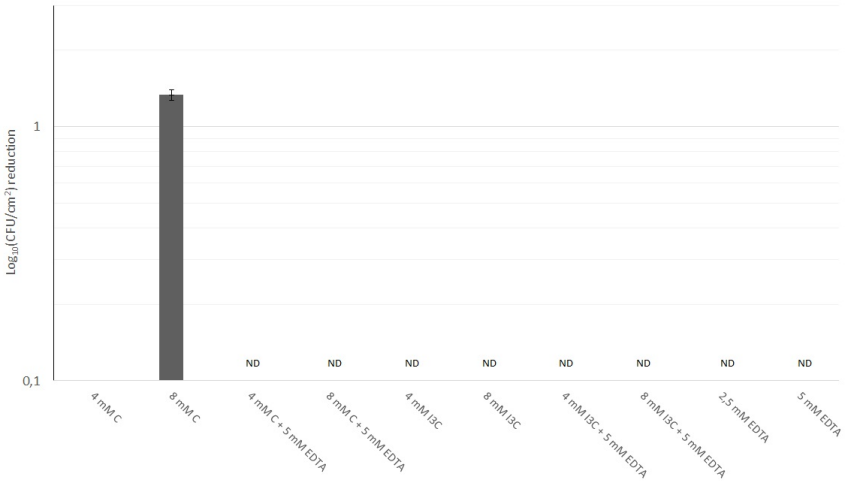

Figure 4.5: Log CFU reduction of *E. coli* biofilms after a 72h exposure period to the selected biocides and combinations.

In Figure 4.5, however, in every assay (except in the single use of cuminaldehyde), no colonies were detected. As can be seen in the MIC determination, the heatmap and throughout the bar graphs, cuminaldehyde alone does not have substantial antimicrobial or anti-biofilm effect. Other studies report that this substance can be antimicrobial against some bacteria, specially if it is used in combination with 10% NaCl [121]. There are, however, no studies regarding its anti-biofilm activity. Every other combination showed high activity for this exposure time. The CFU reduction after 72 h is also an important measure of the stability of these substances alone and in combination with EDTA, as it is important for a catheter lock solution to be stable. From this result, it is possible to assume that the formulations tested have antimicrobial activity at these conditions for 72 h. This was expected, as phytochemicals generally are stable for a long period of time [122]. Figure 4.6 represents the log CFU reduction of *S. epidermidis* biofilms after a 6 h exposure time ($P < 0.05$).

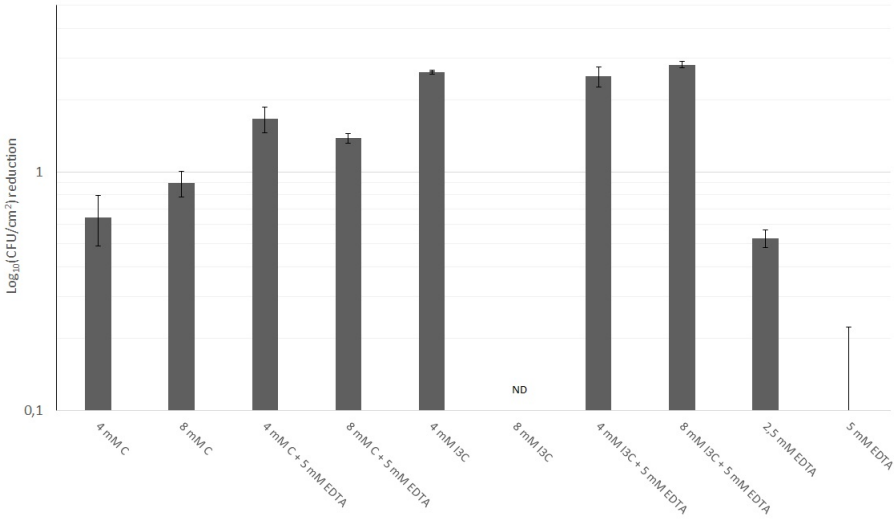

Figure 4.6: Log CFU reduction of *S. epidermidis* biofilms after a 6h exposure period to the selected biocides and combinations.

Regarding this microorganism, it is noticeable that I3C had substantial antimicrobial effect in the first 6 h of exposure, with log CFU reductions close to 3 in every formulations where colonies were detected. Cuminaldehyde with EDTA had moderate CFU reductions, while the use of EDTA alone showed low anti-biofilm activity, which is contrary to what was expected from the MIC and MBC determinations. Again the use of I3C alone showed better CFU reductions than when it is used with EDTA, which can reflect its antagonism when used against sessile cells. Figures 4.7 shows the reduction of CFU of this biofilm type after 24 h ($P < 0.05$).

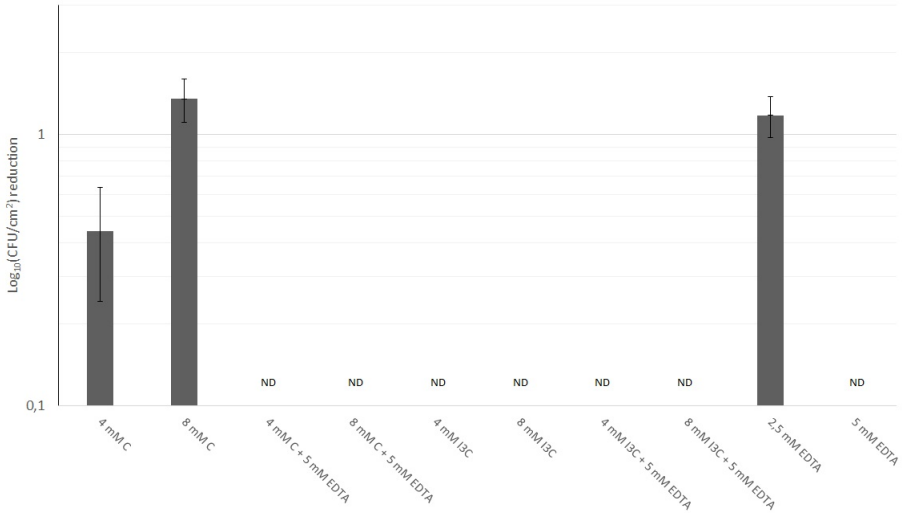

Figure 4.7: Log CFU reduction of *S. epidermidis* biofilms after a 24 h exposure period to the selected biocides and combinations.

It is noteworthy that 2.5 mM EDTA was still not able to fully eliminate sessile *S. epidermidis* in a 24 h exposure period. Because of this, the use of EDTA alone seems insufficient to use as a catheter lock solution in these concentrations. However, as stated previously, when this substance is used as a catheter lock solution, its concentration is more than 10 times higher than what was used in this study [66]. Figure 4.8 has the final CFU reductions for this type of biofilm, after 72 h of exposure ($P < 0.05$).

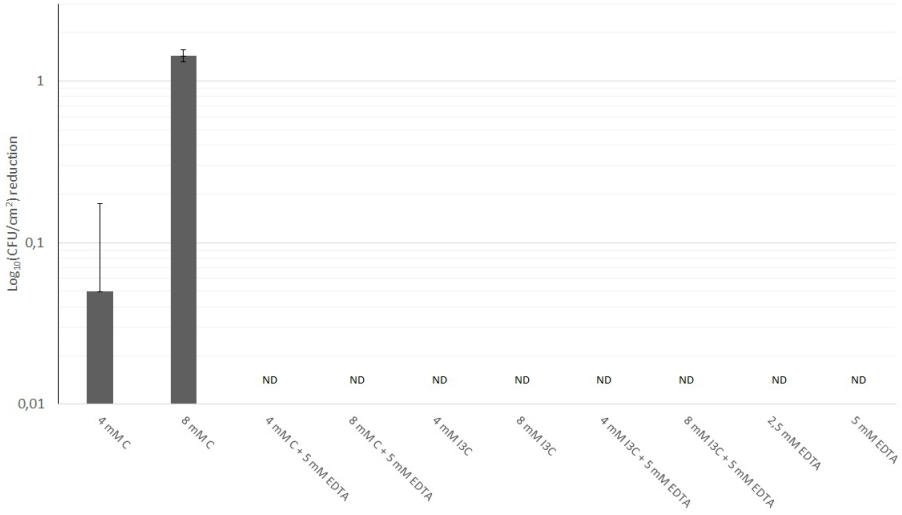

Figure 4.8: Log CFU reduction of *S. epidermidis* biofilms after a 72h exposure period to the selected biocides and combinations.

The log CFU reductions of *S. epidermidis* biofilm were similar to *E. coli*. CFU were only detected with cuminaldehyde alone. Finally, the log reduction of the dual-species biofilm is portrayed in Figures 4.9 to 4.11. Figure 4.9 refers to the log CFU reductions after a 6 h exposure period to the antimicrobials ($P < 0.05$).

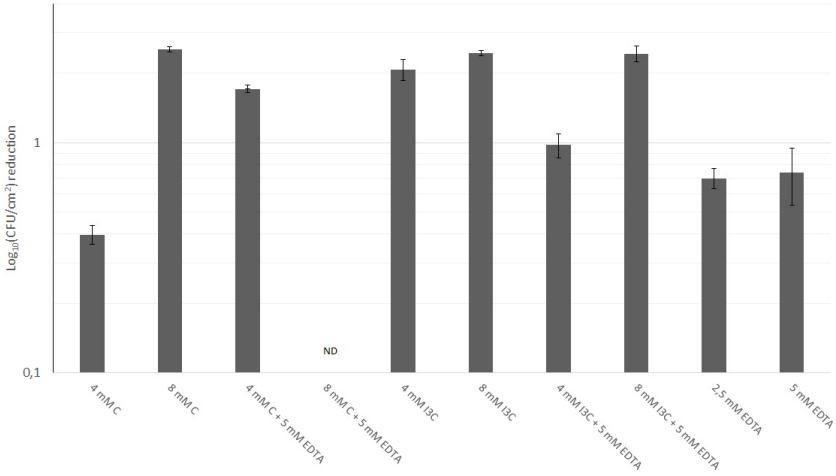

Figure 4.9: Log CFU reduction of a dual-species biofilms after a 6h exposure period to the selected biocides and combinations.

In this situation cuminaldehyde with EDTA was particularly effective. After only 6h there were no culturable CFUs detected by using 8 mM C with 5 mM EDTA. I3C was also effective, but its use with EDTA again showed antagonism. Figure 4.10 refers to the CFU reductions of the same type of biofilm exposed to antimicrobials for 24 h ($P < 0.05$).

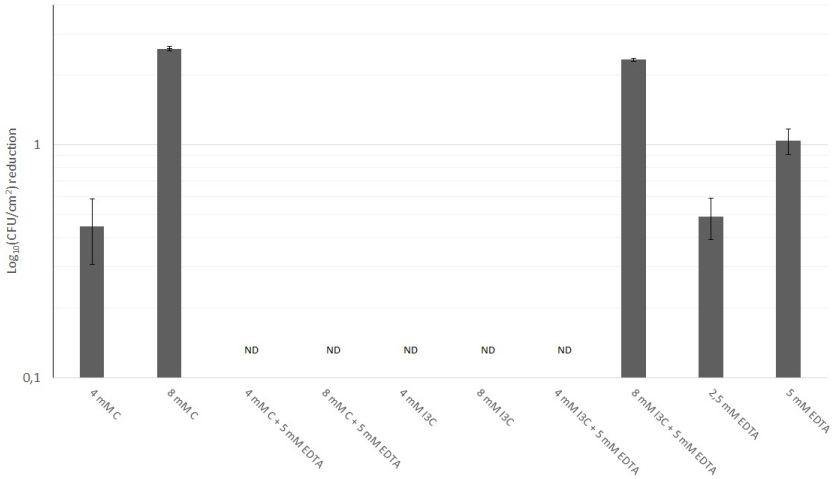

Figure 4.10: Log CFU reduction of a dual-species biofilms after a 24 h exposure period to the selected biocides and combinations.

With a 24 h exposure period, log reductions naturally increased, as happened in the single-species biofilms. However, EDTA was also not able to reduce the CFUs by more than 1 log until the 72h mark, which is represented in the Figure 4.11 ($P < 0.05$).

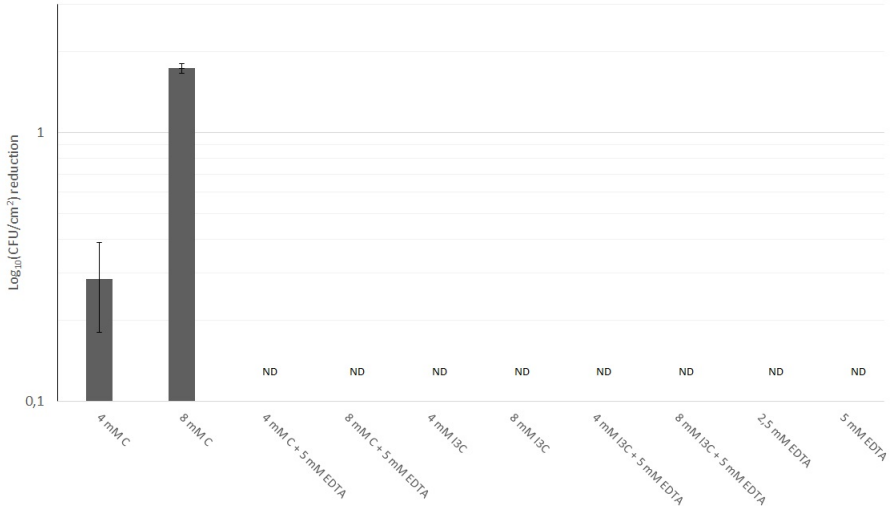

Figure 4.11: Log CFU reduction of a dual-species biofilms after a 72h exposure period to the selected biocides and combinations.

The CFU reduction of dual-species biofilm after 72 h of exposure is similar to its single-species counterparts. CFU were only present in biofilms treated with cuminaldehyde. It is noteworthy that some phytochemical substances have been reported as having only mild anti-biofilm activity against single and dual-species biofilm. This is the case of ferulic and salicylic acids when applied to 24 h aged biofilms [123]. The substances studied, however, appeared to have strong activity against single and dual-species biofilms, not only after 72 h but even after 24 h. Biofilm prevention was not studied in this work, since the activity against mature biofilms is a more extreme scenario to experiment with. However, it is known that many phytochemicals are able to control biofilms through its prevention, by mechanisms such as quorum sensing inhibiting or EPS production control [124]. It is not known if the substances studied have these kinds of interactions in the prevention of biofilms, and following work could expand upon this.

Since indole-3-carbinol showed, in these experiments, traces of antagonism with EDTA against the studied biofilms, it was not an interesting formulation for further testing. Its use with EDTA was counter-productive, although by itself this substance could have applications as an antimicrobial. Cuminaldehyde, however, was effective when combined with EDTA. After a 24 h period, there where no colonies detected of any biofilm type by using 4 mM of cuminaldehyde with 5 mM of EDTA. Moreover, these concentrations are similar to those used to eliminate planktonic cells. To control biofilms, concentrations are usually orders of magnitude higher than to eliminate suspended bacteria [11, 17]. This was not required for this combination, which makes it interesting.

Section A.2 has the resulting logarithmic CFU reductions of ciprofloxacin against the three types of biofilm. It is noticeable that this antibiotic was not very effective against *S. epidermidis* even after a 24 h exposure period. Compared to quinolones, fluoroquinolones are more effective against Gram-positive species, because of the addition of fluorine [78]. Nonetheless, it showed less activity than against *E. coli*. Moreover, the concentrations tested are two orders of magnitude higher than the reported MIC for this substance [118]. Comparing the activity of 4 mM of cuminaldehyde with 5 mM of EDTA with ciprofloxacin, the formulation with the phytochemical was as effective against *E. coli*, and more effective against *S. epidermidis*, as there was no development of CFU using it.

The combination of cuminaldehyde and EDTA therefore shows potential as an anti-biofilm formulation. Not only does it appear to be broad-spectrum, but it also is effective at the same concentrations against both planktonic and sessile bacteria. It also showed better anti-biofilm activity than ciprofloxacin at the tested concentrations, which is promising.

4.5 Quantification of biofilm reduction in hemodialysis catheters

The combination of 4 mM of cuminaldehyde with 5 mM of EDTA was the selected lock solution to be tested. This combination has an antimicrobial agent and an anticoagulant, and has been shown capable of controlling suspended and sessile cells of different species. A reactor inoculated with *E. coli*, *S. epidermidis* or a mixture of each was set up and bacteria passed through a hemodialysis catheter segment, where biofilm was allowed to develop.

It is noteworthy that the available catheters had triple lumen and were made of a polymer treated with an antimicrobial coating containing chlorhexidine, chlorhexidine acetate and silver sulfadiazine. Chlorhexidine is a commonly used antiseptic and biocide used to sterilize surfaces and medical equipment. It is a safe and effective antimicrobial compound, and, although it is generally considered to have a broad spectrum of activity, Gram-positive cocci appear to be more susceptible to it [125]. On the other hand, silver sulfadiazine is a topical antibiotic used primarily on burn wounds to prevent and treat bacterial infections [126]. The use of these substances has been studied in triple lumen catheters, and were shown effective at reducing colonization rates, especially against *staphylococci* up to 25 days [127].

An adhesion assay was conducted, to determine if bacteria adhered on the catheter surface at high velocities in a short period of time, 1 mL/s for 2 h. There was no growth detected using any of the two bacteria. This could indicate that a longer period of exposure is required to colonize this specific catheter type, as its antimicrobial coating may be enough to control short term contaminations.

Figure 4.12 shows the log CFU reductions determined experimentally for each biofilm type after a 24 h growth and 24 h exposure time with a low flow rate, 0.1 mL/s. It should be noted that there were no CFU detected in the *S. epidermidis* assays, either in the control or the experiment with the formulation. It is likely that because of the antimicrobial coating, this species was not able to colonize the segment. This means that for this particular case, the catheter was effective

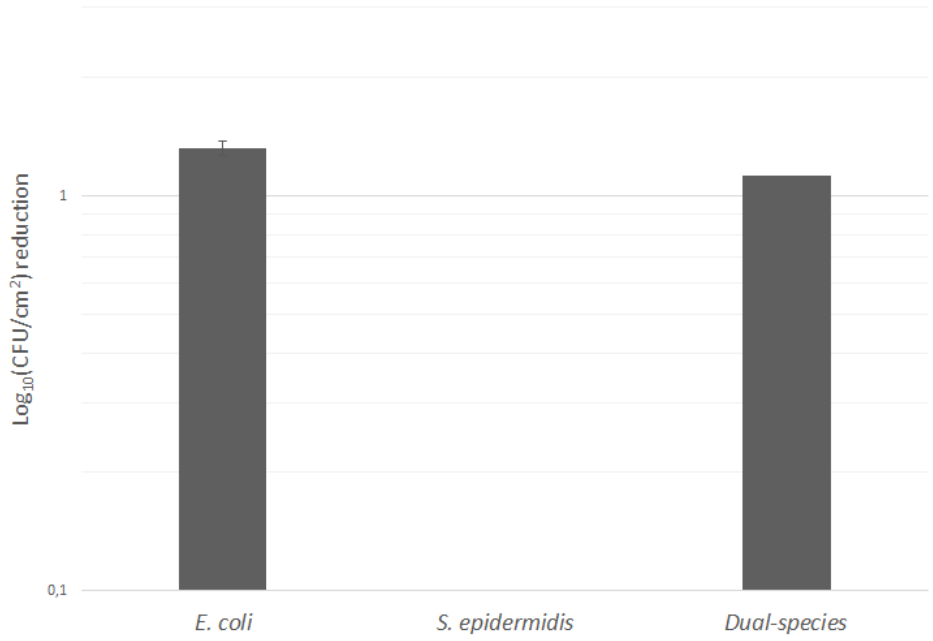

Figure 4.12: Log CFU reduction of biofilm after a 24 h exposure period inside a hemodialysis catheter

at stopping colonization. This is not the case for *E. coli* or the dual-species biofilm, which were able to develop. The number of CFU in the control assays were, however, of only approximately 100 CFU per cm^2 in the catheter segment. This could be because of a suboptimal removal of the attached cells, which were vortexed, or simply because the coating was able to control the growth to these levels. Moreover, it was possible to distinguish *S. epidermidis* colonies in the plates with the dilutions of the dual-species biofilms, although they did not grow as single-species biofilm. This could imply that, even though alone it was not able to establish biofilm in this catheter, an *E. coli* colonization facilitates the development of *S. epidermidis*. The Gram-negative species may be able to form a layer in contact with the coated polymer, while *S. epidermidis* may only adhere to that initial layer and not to the surface itself. Other studies reveal that some dual-species biofilm develop tower-like structures, instead of uniform distributions [128], which can explain the increased resistance of *S. epidermidis*, in this case, in the presence of the antimicrobial coating. The stage of the development of the dual-species biofilm also affects the predominance of the species that inhabit it in the presence of antimicrobials [129]. In the case here studied, it is likely that the early biofilm was composed mostly of *E. coli* that could develop in contact with the antimicrobial coating. Later on, *S. epidermidis* was able to develop on the early biofilm, where the concentration of antimicrobials was lower.

Significant CFU reduction was achieved against *E. coli* and dual-species biofilm by using cuminaldehyde and EDTA, as shown in Figure 4.12 ($P < 0.05$). This means that by using this

formulation as a lock solution, it is possible to further decrease the risk of infection and occlusion. The catheter's coating was not sufficient to completely nullify that risk.

As a positive control, 0.5 mg/mL of a ciprofloxacin solution was used in a similar experiment with all three types of biofilms. This concentration was chosen as it was effective at controlling sessile cells previously in this study and it is within the range of concentrations used in other studies as a antibiotic lock solution [66]. No CFU were detected after 24 h of exposure. As expected, this antibiotic was effective at controlling the existing biofilm. Comparing this result with the combination of phytochemical with EDTA, ciprofloxacin is overall more effective. However, the antibiotic itself does not have anticoagulant properties that are required in a lock solution. Besides this, the CFU reduction caused by cuminaldehyde with EDTA is high in the conditions tested and can be an interesting alternative to the use of antibiotics, since, as discussed previously, the use of these should not be trivial.

Overall, after all the presented and discussed results, cuminaldehyde and EDTA have potential as an antimicrobial lock solution. The substances have antimicrobial synergy, and show effectiveness against *E. coli* and *S. epidermidis*, both in planktonic and sessile forms, but also as a dual-species biofilm. Concentrations required to eliminate biofilms are similar to the ones used to eliminate suspended bacteria. This formulation performed better than ciprofloxacin, at the tested concentrations, against *S. epidermidis* biofilm. Regarding the control of biofilm inside a hemodialysis catheter, cuminaldehyde and EDTA showed high and statistical significant logarithmic CFU reductions. This formulation has the desired properties for an antimicrobial catheter lock, and the study of combinations using phytochemicals, such as this, should be urged, as they could be alternatives to antibiotics and toxic biocides in many fields.

Chapter 5

Conclusions and Future Directions

Currently there is a need for alternative antimicrobial agents that can successfully replace antibiotics. The objective of this study was to investigate possible synergistic effects between phytochemicals and EDTA, to use as an antimicrobial lock solution in hemodialysis catheters. *E. coli* and *S. epidermidis* were subjected to testing in planktonic and sessile form, both alone and as a dual-species biofilm.

Phytochemicals showed low antimicrobial activity when used alone against suspended bacteria. When used together with EDTA, synergy was found. The best indicative of this synergy was the FIC index, which was calculated. The most synergistic combination was cuminaldehyde with EDTA, with a FIC index of 0.32 for *E. coli* and 0.63 for *S. epidermidis*, with MICs of 3 mM and 7.5 mM, respectively, when used with EDTA.

E. coli biofilms appeared to be the most resistant against the tested formulations. Only the highest concentrations of cuminaldehyde and indole-3-carbinol with EDTA were able to reduce de number of CFUs by more than 90% after a 6h exposure period. EDTA showed higher CFU reduction against *E. coli* than against *S. epidermidis*, which was opposite of the effect seen in planktonic cells. Indole-3-carbinol showed less efficacy against *E. coli*. After an exposure period of 24h most combinations were moderately antimicrobial, but cuminaldehyde with EDTA stood out, being highly effective against all three types of biofilm, with log CFU reductions of at least 1.5.

This combination was selected as the most promising and was tested as a catheter lock solution. It was able to decrease the amount of CFUs of *E. coli* and dual-species biofilm by log CFU reductions of 1.3 and 1.1, respectively. *S. epidermidis* did not develop biofilm in the catheter, and therefore the ability for the formulation to control it could not be tested. This combination of 4 mM of cuminaldehyde with 5 mM of EDTA shows potential as a catheter lock solution, being able to control both planktonic cells and developed biofilms of the tested species.

To conclude, phytochemicals have properties that make them interesting antimicrobials. The study of their activity, spectrum and synergy with other substances may be a path to the discovery of alternatives to antibiotics. Cuminaldehyde with EDTA is an example of this.

Regarding future work and directions, there are some improvements that would strengthen this study. Firstly, using a catheter which does not have an antimicrobial coating would be better than what was used, since microorganisms would develop freely. *S. epidermidis* did not develop in the catheter used, most likely because of its coating or adhesion time. Also regarding the catheter, using sonication instead of vortexing could potentially increase biofilm detachment for CFU counts. This was not performed in this study because it would introduce another variable in the catheter experiments. It would be necessary to optimize the sonication frequency and time. Improper sonication would either not detach cells or kill them. Therefore, in studies with a larger duration, this is a technique that can be used.

Other than that, the formulation that was selected as most promising, 4 mM of cuminaldehyde with 5 mM of EDTA was not fully optimized. A future study could change these concentrations and find the sweet spot for antimicrobial activity of the formulation. It is not known if, for instance, 3 mM of EDTA would have the same antimicrobial effect as 5 mM in combination with 4 mM of cuminaldehyde. Testing this formulation with a wider variety of bacteria could also make it more attractive as an antimicrobial. Regarding comparison with other catheter lock solutions, it would be interesting to test it along side with other standard lock solutions such as heparin or sodium citrate, to compare performances.

Finally, it would be important to understand the mechanism of action of cuminaldehyde and to know why this improves with the presence of EDTA. This could lead to further optimization of the formulation.

Appendix A

Appendix

A.1 Catheter lock solutions

In the early stages of this study, to better understand catheter lock solutions, their components and activities, a comprehensive table with information about existing lock solutions was compiled. Table A.1 comprises an overview of lock solutions tested in multiple conditions. It has information on the solution's constituents, their purpose, the antimicrobial effect reported and which microorganisms were affected, when available. The information regarding antimicrobial effect may be about a reduction of the number of colonies or a reduction of the number of catheter-related bloodstream infections, if the study was *in vivo*.

Using this information it was possible to choose which constituents were important for a lock solution and which would be used in this study. Information contained in this table also serves as comparison of the results and as a standard of effectiveness. It could also help following studies to quickly gather information regarding a large number of lock solutions and antibiotic treatments.

Table A.1: Compilation of catheter lock solutions and their antimicrobial effects tested in the literature

Lock Solution	Constitution		Mode of Action	Effect	Time	Microorganism	Reference
Tetrasodium EDTA	**EDTA**	40 mg/mL	Chelator	4-5 Log Reduction	21h/25h	*S. epidermidis, P. aeruginosa, K. pneumoniae, E. coli, C. albicans*, MRSA	[66]
C/MB/P	**Citrate**	7%	Chelator	Erradicated suspended cells / 3-4 Log Reduction	1h of exposure	*S. epidermidis, E. faecalis, E. coli, P. aeruginosa, S. aureus, C. albicans*	[12]
	Methylene Blue	0,05%	Antimicrobial				
	Parabenos	0,17%	Preservative				
Ethanol Citrate	**Ethanol**	30%	Antimicrobial	Did not present formation of colonies or biofilm	1h of exposure and 72h incubation	*C. albicans, P. aeruginosa, E. coli*, MSSA, MRSE	[69]
	Sodium Citrate	4%	Preservative				
Sodium Citrate	**Sodium Citrate**	10%-47%	Preservative / Antimicrobial	5 Log Reduction	7 days / 21 days	*P. aeruginosa, E. coli, S. aureus*	[64]
Glycerol	**Glycerol**	35%-60%	Bactericidal	Did not present colony formation	24h and 48h	*A. baumannii; E. cloacae; P. aeruginosa*	[72]
	Sodium Chloride	0,5%-0,9%	Hemocompatibility				
Ethanol	**Ethanol**	70%	Antimicrobial	Erradicated Biofilm	>2h	*S. aureus, E. coli, B. cereus, P. aureginosa, C. albicans*	[68]

Table A.1: Compilation of catheter lock solutions and their antimicrobial effects tested in the literature (cont.)

Lock Solution	Constitution		Mode of Action	Effect	Time	Microorganism	Reference
Glyceryl Trinitrate	**Glyceryl trinitrate**	0,01%	Antimicrobial	Erradicated Biofilm	2h of exposure	*P. aureginosa, C. albicans*, MRSA, MRSE	[71]
	Ethanol	20%	Antimicrobial				
	Citrate	7%	Anticoagulant				
Minocycline EDTA Ethanol	**Minocicline**	3 mg/mL	Antibiotic	Did not present colony formation	1h of exposure	*C. parapsilosis*, MRSA	[57]
	EDTA	30 mg/mL	Chelator				
	Ethanol	30%	Antimicrobial				
Cefriaxone	**Cefriaxone**	83,3 mg/mL	Antibiotic	>4 Log Reduction	4 days of exposure	*S. epidermidis, S. aureus, E. aerogenes, K. pneumoniae*	[130]
Nafcillin	**Nafcillin**	83.3-166.6 mg/mL	Antibiotic	Did not present colony formation	4 days of exposure	*S. epidermidis, S. aureus*	[131]
Cefazolin	**Cefazolin**	0.5-10 mg/mL	Antibiotic	4 log red, 1 log red	24h of exposure	*P. aeruginosa*, MRSA, MSSA	[132, 133]
	Heparin	10-5000 units/mL	Anticoagulant				
Imipenem / Cilastatin	**Imipenem/Cilastatin**	50 mg/mL	Antibiotic	3 Log Reduction	24h of exposure	*S. epidermidis, S.aureus*	[134]
	Heparin		Anticoagulant				

Table A.1: Compilation of catheter lock solutions and their antimicrobial effects tested in the literature (cont.)

Lock Solution	Constitution		Mode of Action	Effect	Time	Microorganism	Reference
Teicoplanin	**Teicoplanin**	1-5 mg/mL	Antibiotic	Did not present formation of colonies	7 days	*S. epidermidis*	[135]
	Sodium Chloride						
Ciprofloxacin + Teicoplanin	**Ciprofloxacin**	0,8 mg/mL	Antibiotic	Did not present formation of colonies	48h	*P.aeruginosa*, MRSA, MSSA	[136]
	Teicoplanin	4 mg/mL	Antibiotic				
	Heparin	10000 units/mL	Anticoagulant				
Gentamicin	**Gentamicin**	0,1-5 mg/mL	Antibiotic	>4 Log Reduction	7 days of exposure	*S. epidermidis, S. aureus, E. aerogenes, K. pneumoniae*	[130, 136]
	Heparin	10-5000 units/mL	Anticoagulant				
Gentamicin + EDTA	**Gentamicin**	3 mg/mL	Antibiotic	>5 Log Reduction	24h of exposure	*P. aeruginosa*, MRSA, MSSA	[133]
	Daptomycin	1 mg/mL	Antibiotic				
	Citrate	28 mg/mL	Anticoagulant				
	EDTA	30 mg/mL	Chelator				
Aztreonam	**Aztreonam**	83,3 mg/mL	Antibiotic	Did not present colony formation	4 days of exposure	*E. aerogenes, K. pneumoniae*	[130]

Table A.1: Compilation of catheter lock solutions and their antimicrobial effects tested in the literature (cont.)

Lock Solution	Constitution		Mode of Action	Effect	Time	Microorganism	Reference
Daptomycin	**Daptomycin**	1-25 mg/mL	Antibiotic	1 Log Reduction	24h of exposure	MRSA, MSSA	[137]
	Heparin	100-10000 units/mL	Anticoagulant				
Daptomycin + Ethanol	**Daptomycin**	2,5 mg/mL	Antibiotic	5 Log Reduction	4h/day during 5 days	MRSA	[138]
	Ethanol	25%	Antimicrobial				
Daptomycin + Citrate	**Daptomycin**	5 mg/mL	Antibiotic	3 Log Reduction	72h of exposure	*S. aureus, S. epidermidis*	[139]
	Heparin		Anticoagulant				
	Calcium Chlorine	50 ug/mL					
Tigecycline	**Tigeclycline**	0,5 mg/mL	Antibiotic	>4 Log Reduction	24h of exposure	*P. aeruginosa*, MRSA, MSSA	[133, 140]
	EDTA	30 mg/mL	Chelator				
Colistin	**Colistin**	0,1 mg/mL	Antibiotic	Did not present colony formation	2 months	*S. aureus, E. faecium, P. aeruginosa, K. penumoniae*	[141]
	Vancomycin	0,1 mg/mL	Antibiotic				
	Heparin	100 units/mL	Anticoagulant				

Table A.1: Compilation of catheter lock solutions and their antimicrobial effects tested in the literature (cont.)

Lock Solution	Constitution		Mode of Action	Effect	Time	Microorganism	Reference
Taurolidin (Taurolock™)	**Taurolidin**	1,35%	Antimicrobial	Significative reduction of CRBSI		*Staphylococcus spp, Enterobacter spp, Bacillus spp, K. pneumoniae, E. coli*	[73]
	Citrate	4%	Preservative				
Heparin	**Heparin**	100-5000 units/mL	Anticoagulant	Promotes biofilm formation in S.aureus			[60]
Sodium Cloride	**Sodium Cloride**	0,85%	Hemocompatibility	-			[49]
Lepidurin	**Lepidurin**	400 ug	Anticoagulant	Similar to heparin			[142]
Urokinase			Enzimatic activity	Emergency cleaning of catheters			[70]
Amoxicillin	**Amoxicillin**	5 mg/mL	Antibiotic	Significative reduction of CRBSI		*Corynebacterium spp.*	[56]
	Heparin	2500 units/mL	Anticoagulant				
Penicillin G	**Penicillin G**	5000 units/mL	Antibiotic	Significative reduction of CRBSI		*P. acnes*	[56]
	Heparin	2500-5000 units/mL	Anticoagulant				

Table A.1: Compilation of catheter lock solutions and their antimicrobial effects tested in the literature (cont.)

Lock Solution	Constitution		Mode of Action	Effect	Time	Microorganism	Reference
Piperacillin	**Piperacillin**	100 mg/mL	Antibiotic	Significative reduction of CRBSI		*A. xylosodidans*	[56]
	Heparin	400 units/mL	Anticoagulant				
Vancomycin	**Vancomycin**	1-3 mg/mL	Antibiotic	Significative reduction of CRBSI / Did not present colony formation	Stability of 85 days	*Staphylococcus spp.*	[135, 143]
	Heparin	100-2500 units/mL	Anticoagulant				
Ampicillin	**Ampicillin**	2-5-10 mg/mL	Antibiotic	Inhibitory effect	Stability of 5 days	*S. epidermidis, E. faecalis*	[144]
	Heparin	10-5000 units/mL	Anticoagulant				
Piperacillin / Tazobactam	**Piperacillin / Tazobactam**	10 mg/mL	B lactamase inhibitor	88,8% Increase of sucess rate of treatments		*P. acnes, Corynebacterium spp, Gram-negative bacillus, E. faecium*	[145]
	Heparin	100 units/mL	Anticoagulant				
Cloxacillin	**Cloxacillin**	100 mg/mL	Antibiotic	Significative reduction of CRBSI		*S. epidermidis, S. aureus*	[146]

Table A.1: Compilation of catheter lock solutions and their antimicrobial effects tested in the literature (cont.)

Lock Solution	Constitution		Mode of Action	Effect	Time	Microorganism	Reference
Mezlocillin	**Mezlocillin**	2 mg/mL	Antibiotic	Effective treatment of infections		*E. cloacae*	[147]
Linezolid	**Linezolid**	0,2-2 mg/mL	Antibiotic	Treatment of infected catheters		*S. epidermidis*	[148]
	Heparin	10-10000 units/mL	Anticoagulant				
Cefazolin + Gentamicin	**Cefazolin**	5 mg/mL	Antibiotic	Significative reduction of CRBSI		*S. aureus*	[132, 149]
	Gentamicin	5 mg/mL	Antibiotic				
	Heparin	1000-5000 units/mL	Anticoagulant				
Ceftazidime	**Ceftazidime**	0,5-10 mg/mL	Antibiotic	Significative reduction of CRBSI		*S. epidermidis, S.aureus*	[150]
	Heparin	100-5000 units/mL	Anticoagulant				
Ceftazidime + Vancomycin	**Ceftazidime**	2,5 mg/mL	Antibiotic	Significative reduction of CRBSI		*S. epidermidis, S.aureus*	[150]
	Vancomycin	2,5 mg/mL	Antibiotic				
	Heparin	2500 units/mL	Anticoagulant				

Table A.1: Compilation of catheter lock solutions and their antimicrobial effects tested in the literature (cont.)

Lock Solution	Constitution		Mode of Action	Effect	Time	Microorganism	Reference
Ciprofloxacin	**Ciprofloxacin**	0,1-10 mg/mL	Antibiotic	Sterelization of infected catheters	18h	*S. aureus*	[61]
	Heparin	100-10000 units/mL	Anticoagulant				
Sulfamethoxazole / Trimethroprim	**Sulfamethoxazole / Trimethroprim**	10-16 mg/mL	Antibiotic	77,5% reduced infection risk		*Staphylococcus spp, Enterococcus spp, E. coli, Enterobacteriaceae*	[151]
	Heparin	100 units/mL	Anticoagulant				
Amikacin	**Amikacin**	0,02-40 mg/mL	Antibiotic	Effective treatment of infections	5 to 7 days of treatment	*E. faecali, K. pneumoniae, E. coli, S. aureus, S. epidermidis*	[152]
	Teicoplanin	0-10 mg/mL	Antibiotic				
	Heparin	0-10000 units/mL	Anticoagulant				
Gentamicin + Citrate	**Gentamicin**	0,32-2,5 mg/mL	Antibiotic	90% reduction of CRBSI		*S. aureus, S. epidermidis*	[153]]
	Citrate	40 mg/mL	Anticoagulant				

A.2 Culturable biofilm cells after exposure to ciprofloxacin

Subsection 4.4.2 describes the results of the logarithmic CFU reduction of biofilms when exposed to different phytochemicals with and without EDTA after 6h, 24h and 72h. As a positive control, ciprofloxacin was also tested in a similar manner. A concentration range of 0.1-1 mg/mL was chosen, as it was within the reported concentrations which are used as antibiotic catheter treatments, as of Table A.1. Results are present in Figures A.1 to A.6.

The antibiotic was reported as especially effective against Gram-negative bacteria, showing no growth in almost every concentration tested after 24 h against *E. coli*. However, against *S. epidermidis*, even after 24 h of exposure the reduction was only moderate. The dual-species biofilm showed traces of each of these behaviours, with a log CFU reduction of 3 when subjected to 0.6 mg/mL of ciprofloxacin and without detection of colonies with higher concentrations.

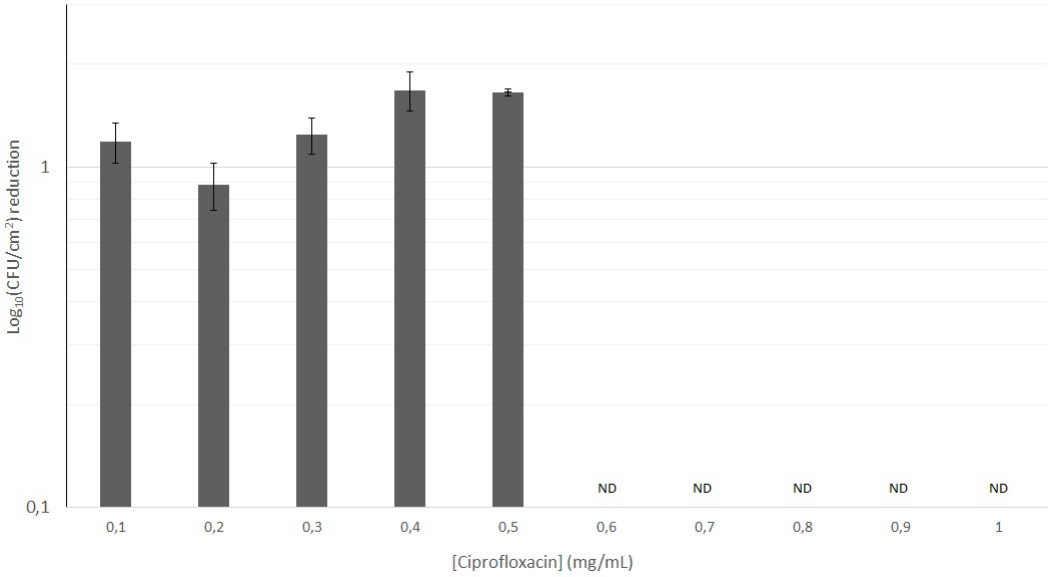

Figure A.1: Log CFU reduction of *E. coli* biofilms after a 6 h exposure period to CIP.

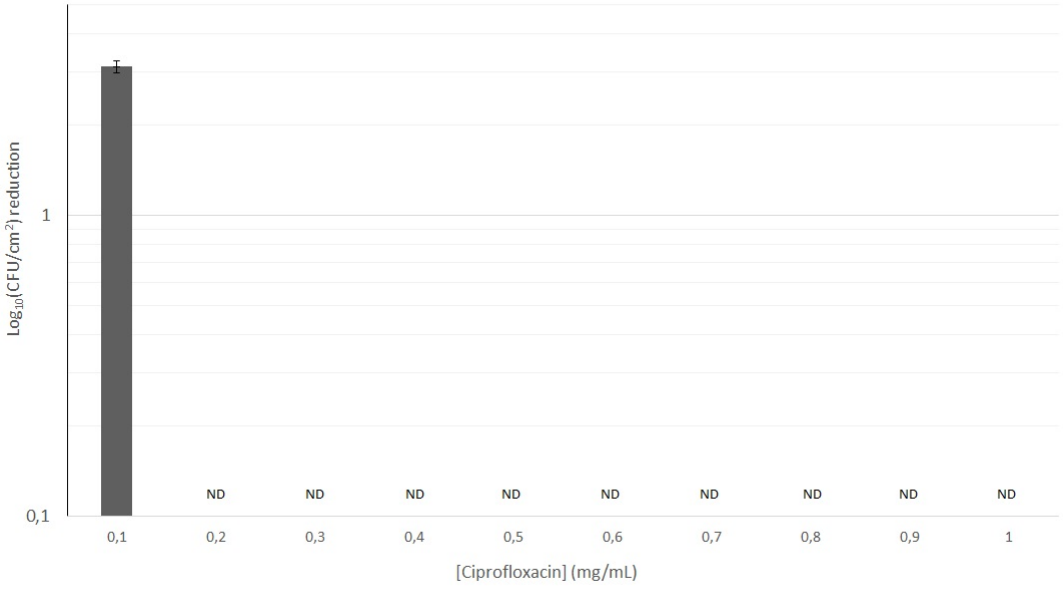

Figure A.2: Log CFU reduction of *E. coli* biofilms after a 24 h exposure period to CIP.

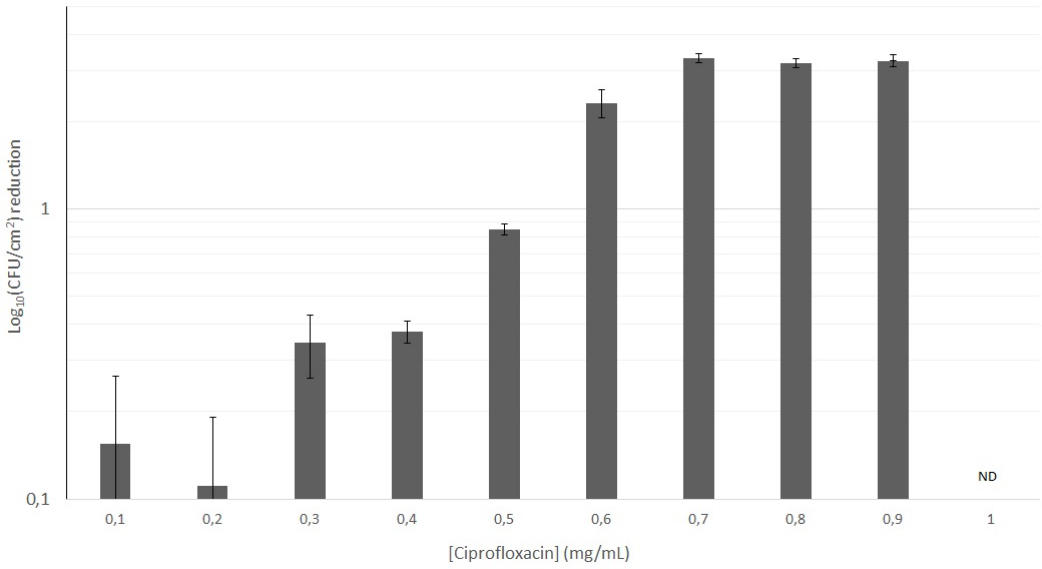

Figure A.3: Log CFU reduction of *S. epidermidis* biofilms after a 6 h exposure period to CIP.

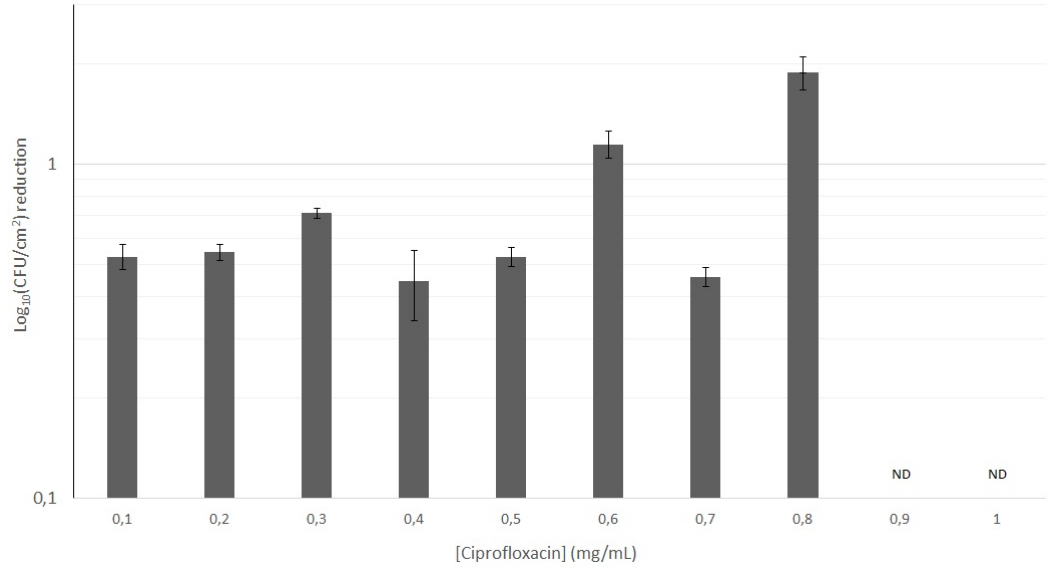

Figure A.4: Log CFU reduction of *S. epidermidis* biofilms after a 24 h exposure period to CIP.

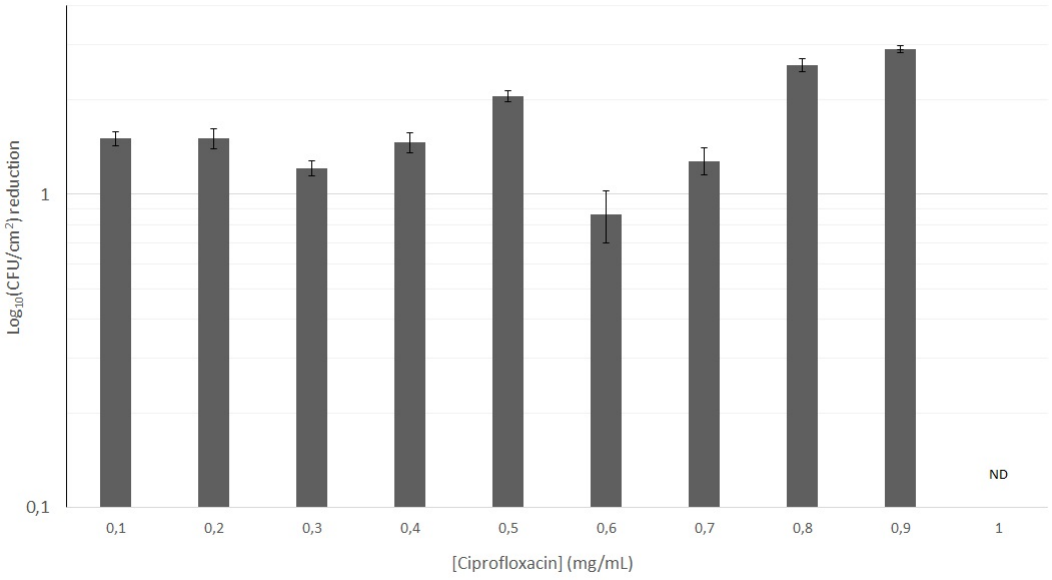

Figure A.5: Log CFU reduction of dual-species biofilms after a 6 h exposure period to CIP.

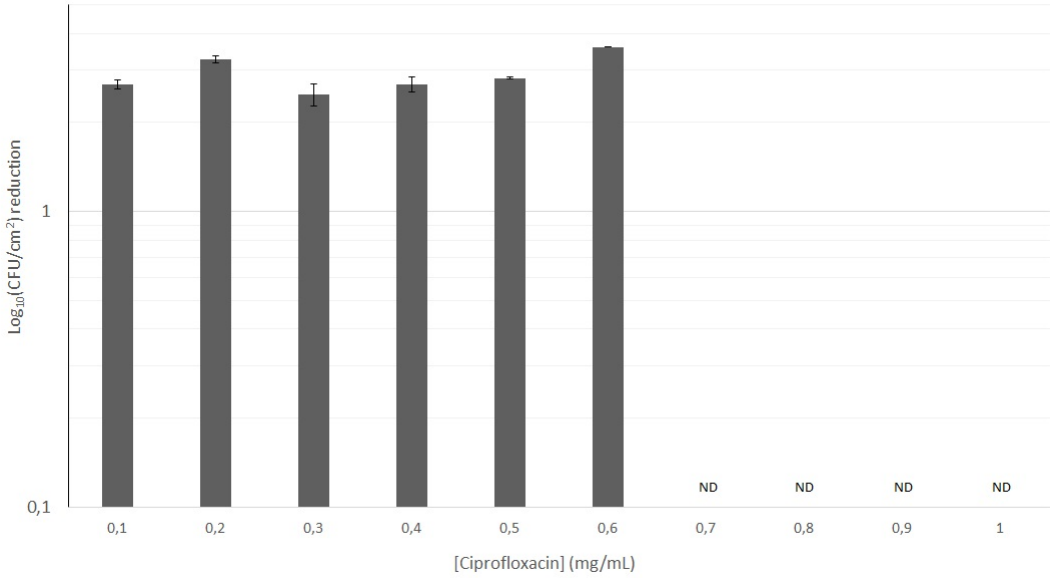

Figure A.6: Log CFU reduction of dual-species biofilms after a 24 h exposure period to CIP.

A.3 Pump calibration

The peristaltic pump used in the catheter assays was calibrated for the two different conditions, high and low flow rates. The two separate calibration curves are shown in Figures A.7 and A.8, and the respective equations that relate the flow rate with the pump's rotations per minute (rpm) are presented as equations A.1 and A.2. Linear regression was applied to experimental data points to define these lines. This was split into two parts, high flow rate and low flow rate, to improve the linear behaviour of the regression curve. Higher range of rotations per minute in the same data set would decrease the correlation coefficient.

$$Q = 0,0085 \times rpm + 0,1045 \tag{A.1}$$

$$Q = 0,0076 \times rpm + 0,0041 \tag{A.2}$$

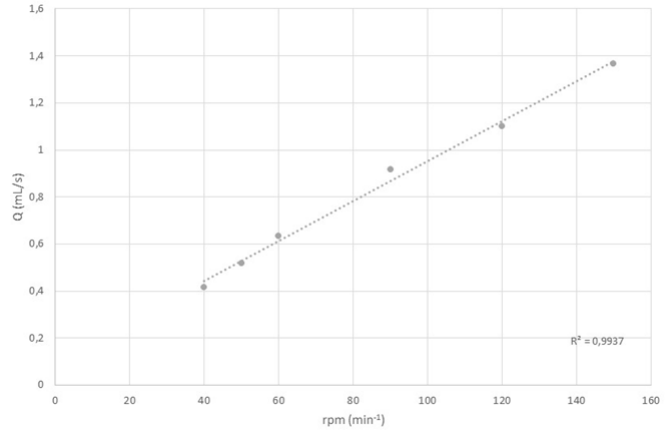

Figure A.7: Pump calibration curve for high rpm

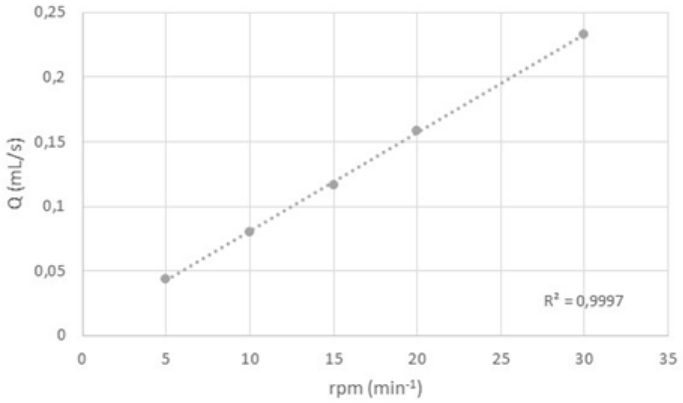

Figure A.8: Pump calibration curve for low rpm

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