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Combined Antibiotic and Antifungal Therapy as a Potential Strategy to Control Bone-related Polymicrobial Biofilm

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Dissertation for the Master in Biomedical Engineering

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Abstract

Bone infections (osteomyelitis, joint infections or septic arthritis) are diseases triggered by the microorganisms (bacteria and/or fungus) penetration into the bone or adjacent tissues, resulting from trauma or surgical procedure. These infections can cause high morbidity rates due to bone loss and destruction, fractures and septicemia, making treatment challenging. Within bone infections, those caused by polymicrobial biofilms are of extreme importance at a clinical level, namely when microorganisms of different types coexist, such as the coexistence of bacteria (for example, *Staphylococcus aureus*) and fungi (for example, *Candida albicans*). The coexistence of these microorganisms may result in a synergistic or/inhibitory interaction and may enhance the pathogenicity and chronicity of the infection, as well as limiting therapeutic options. Thus, it is crucial to understand the contribution of each microbial population within the biofilm in order to select the most appropriate therapy for the treatment of this type of biofilm.

Alternative strategies have been explored, as traditional approaches, namely systemic and local administration of antibiotics, only focus on bacteria eradication. In addition, with the emergence of antibiotic-resistant bacteria, an emerging public health problem, new antimicrobial combinations are urgently needed.

The use of a combination therapy, that use at least two different antimicrobials for the treatment of polymicrobial biofilms, is a strategy that has grown in academic and clinical fields, since it can bring some advantages compared to conventional monotherapy, such as: (i) synergistic action, overcoming the main survival mechanisms of biofilm cells against antimicrobials; (ii) it may bolster drug-efficacy by lower doses of antimicrobials and, consequently lower drug toxicity; and (iii) possibility to treat polymicrobial biofilms, due to multi-target against different microorganisms within biofilms. However, further studies, namely of the interaction between different microorganism within the biofilm and the efficacy of antimicrobial compounds as a combined treatment, are needed for this therapy to become a common practice.

In this sense, this work has as its main purposes to study the growth kinetics of mono-*Staphylococcus aureus* and *Candida albicans* biofilms over 72 h and to study and understand the efficacy of ceftriaxone and amphotericin B-based mono- and combined-therapy against mono- and dual-species *Staphylococcus aureus* and *Candida albicans* biofilms.

Significant differences in biofilm densities between 48 and 72 h were observed for both mono-species biofilms studied, indicating that the biofilms stabilized at 48 h, reaching maximum values of 7.44 Log CFU/mL for *S. aureus* and 6.27 Log CFU/mL for *C. albicans*.

Regarding mono-species *Staphylococcus aureus*, it was observed that ceftriaxone reduced 99.9% (3 logs) of bacterial biofilm, indicating that 0.25 mg/mL of antibiotic has high anti-biofilm activity. The action of amphotericin B was also tested in order to check if 10 µg/mL of antifungal could have a toxic effect against *Staphylococcus aureus*. The results revealed that *Staphylococcus aureus* density and viability were not affected by antifungal, as expected. After combined treatment (i.e., antibiotic + antifungal), a reduction of 99.9 % (3 logs) of *Staphylococcus aureus* density was observed, indicating that the ceftriaxone activity was not affected by amphotericin B.

Concerning mono-species *Candida albicans* biofilm, a reduction of 90% (1 log) of fungal biofilm was observed, confirming the anti-biofilm activity of amphotericin B. The possible effect of ceftriaxone against *Candida albicans* was also evaluated, concluding that 0.25 mg/mL of ceftriaxone did not affect the *Candida albicans* biofilm. Furthermore, it was observed that amphotericin B in combined treatment had the same antifungal effect compared to mono treatment.

Regarding the activity of ceftriaxone in dual-species biofilm, it reduced bacterial density within dual-species biofilm by 99.8 % (2.81 Logs), indicating the effectiveness of ceftriaxone in targeting and reducing *Staphylococcus aureus* within dual-species biofilm. Besides, the obtained results suggest that the presence of *Candida albicans* did not affect the activity of ceftriaxone against *Staphylococcus aureus*

On what concerns the activity of amphotericin B in dual-species biofilm, it reduced fungal density within dual-species biofilm by 97.7 % (1.66 Logs). This reduction demonstrates the effectiveness of amphotericin B in targeting and reducing *Candida albicans* within dual-species biofilm. In addition, the provided results imply that the presence of *Staphylococcus aureus* did not affect amphotericin B's activity against *Candida albicans*.

Concerning the combination of ceftriaxone and amphotericin B, it significantly reduced bacterial density by 98.9 % (1.9 Logs), whereas it had no significant impact in fungal density, within dual-species biofilm. These results suggest that the presence of *Candida albicans* did not affect the activity of the combined therapy against *Staphylococcus aureus*. Conversely, the presence of *Staphylococcus aureus* seemed to affect the activity of the combined therapy against *Candida albicans*. This implies that the *Staphylococcus aureus* might somehow shield *Candida albicans*.

In summary, the combined therapy may not provide any additional benefits over using monotherapy in mono-species biofilms of *S. aureus* and *C. albicans*. However, the CT was able to reduce the overall density of a dual-*S. aureus* and *C. albicans* biofilm. Moreover, the results proved that the combination of two different kind of antimicrobials did not affect their antimicrobial activity.

Keywords: Polymicrobial biofilms, Combined therapy, Bone infections, Ceftriaxone, Amphotericin B

Resumo

Infeções ósseas (osteomielite, infeções nas articulações e artrite séptica) são patologias despoletadas pela penetração de microrganismos (bactérias e/ou fungos) no osso e tecidos adjacentes, resultantes de traumas ou procedimentos cirúrgicos. Estas infeções podem originar elevadas taxas de morbidade devido a perda e destruição óssea, fraturas e infeções sistémicas, sendo desafiante o seu tratamento. Dentro das infeções ósseas, as provocadas por biofilmes polimicrobianos são as mais preocupantes a nível clínico, nomeadamente quando coexistem microrganismos de diferentes tipos, como o caso da coexistência de bactérias (por exemplo, *Staphylococcus aureus*) e fungos (por exemplo, *Candida albicans*). A coexistência destes microrganismos pode promover interações sinérgicas ou inibidoras e, consequentemente, podem levar ao aumento da patogenicidade e cronicidade da infeção, bem como limitar as opções terapêuticas. Assim, é crucial perceber a contribuição de cada população microbiana presente no biofilme, de forma a selecionar a terapia mais adequada para o tratamento deste tipo de biofilmes.

Estratégias alternativas têm sido exploradas, uma vez que as abordagens tradicionais, nomeadamente administração sistémica ou local de antibióticos, apenas focam a erradicação de bactérias. Para além disto, com a emergência de bactérias resistentes a antibióticos, um problema emergente de saúde pública, novas combinações antimicrobianas são urgentemente necessárias.

O uso de uma terapia combinada, que usa pelo menos dois antimicrobianos diferentes para o tratamento de biofilmes polimicrobianos, é uma estratégia que tem crescido no seio académico e clínico, uma vez que pode acarretar algumas vantagens comparativamente à monoterapia convencional, tais como: (i) ação sinérgica, que permite ultrapassar os principais mecanismos de resistência antimicrobiana; (ii) poderá aumentar a eficácia do tratamento, diminuindo a dose de antimicrobianos e, consequentemente, diminuindo a toxicidade inerente; e (iii) possibilidade de tratar biofilmes polimicrobianos, devido à ação de diferentes agentes antimicrobianos em diferentes microrganismos presentes num biofilme. Contudo, estudos adicionais, nomeadamente da interação entre diferentes microrganismos dentro do biofilme e da a interação e eficácia de compostos antimicrobianos como um tratamento combinado, são necessários para que esta terapia se torne uma prática comum.

Neste sentido, este trabalho tem como objetivos principais estudar a cinética de crescimento de biofilmes simples de *Staphylococcus aureus* and *Candida albicans* ao longo de 72 h, e estudar e perceber a eficácia das terapias simples e combinada de ceftriaxona e

anfotericina B no tratamento de biofilmes simples e mistos de *Staphylococcus aureus* e *Candida albicans*.

Diferenças significativas nas densidades do biofilme entre 48 e 72 h foram observadas para ambos os biofilmes simples estudados, indicando que os biofilmes estabilizaram às 48 h, alcançando valores máximos de 7.44 Log CFU/mL para *Staphylococcus aureus* e 6.27 Log CFU/mL para *Candida albicans*.

No que diz respeito ao biofilme simples de *Staphylococcus aureus*, foi observado que a ceftriaxona reduziu 99.9% (3 logs) do biofilme bacteriano, indicando que 0.25 mg/mL de antibiótico tem uma grande atividade anti-biofilme. A ação da anfotericina B também foi testada para estudar se 10 µg/mL do antifúngico teria um efeito tóxico em *Staphylococcus aureus*. Os resultados revelaram que a densidade e viabilidade de *Staphylococcus aureus* não foram afetadas pelo antifúngico, tal como era expectável. Após o tratamento combinado (i.e., antibiótico + antifúngico), uma redução de 99.9 % (3 Logs) na densidade de *Staphylococcus aureus* foi observada, indicando que a atividade da ceftriaxona não foi afetada pela anfotericina B.

Relativamente ao biofilme simples de *Candida albicans*, uma redução de 90% (1 log) do biofilme fúngico foi observada, confirmado a atividade anti-biofilme da anfotericina B. O possível efeito da ceftriaxona em *Candida albicans* foi também avaliado, concluindo que 0.25 mg/mL de ceftriaxona não afetou o biofilme de *Candida albicans*. Para além disto, foi observado que a anfotericina B em tratamento combinado teve um efeito antifúngico semelhante face ao efeito observado no tratamento simples.

Relativamente à atividade da ceftriaxona em biofilmes de dupla espécie, ela reduziu a densidade bacteriana dentro do biofilme de dupla espécie em 99.8 % (2.81 Logs), indicando a eficácia da ceftriaxona em reduzir a *Staphylococcus aureus* dentro de um biofilme de dupla espécie. Para além disso, os resultados obtidos sugerem que a presença da *Candida albicans* não afetou a atividade da ceftriaxona contra a *Staphylococcus aureus*.

No que diz respeito à atividade da anfotericina B em biofilmes de dupla espécie, ele reduziu a densidade fúngica num biofilme de dupla espécie em 97.7% (1.66. Logs). Esta redução demonstra a eficácia da anfotericina B em reduzir a *Candida albicans* dentro de um biofilme de dupla espécie. Adicionalmente, os resultados obtidos sugerem que a presença da *Staphylococcus aureus* não afetou a atividade da anfotericina B contra a *Candida albicans*.

Relativamente à combinação da ceftriaxona e da anfotericina B, ela reduziu a densidade bacteriana em 98.9 % (1.9 Logs), enquanto que não teve impacto na densidade fúngica, dentro de um biofilme de dupla espécie. Estes resultados sugerem que a presença da *Candida albicans* não afetou a atividade da terapia combinada em *Staphylococcus aureus*. Pelo contrário, a presença da *Staphylococcus aureus* pareceu afetar à atividade da terapia combinada em *Candida albicans*. Isto sugere que a *Staphylococcus aureus* poderá de certa forma proteger a *Candida albicans*.

Em suma, a terapia combinada poderá não fornecer benefícios adicionais em relação ao uso de uma monoterapia em biofilmes simples de *Staphylococcus aureus* e *Candida albicans*. No entanto, a terapia combinada foi capaz de reduzir a densidade total do biofilme de dupla-espécie de *Staphylococcus aureus* e *Candida albicans*. Para além disso, os resultados provam que a combinação de duas classes diferentes de antimicrobianos não afetaram a atividade antimicrobiana dos mesmos.

Palavras-chaves: Biofilmes polimicrobianos, Terapia combinada, Infeções ósseas, Ceftriaxona, Anfotericina B

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“Do not go where the path may lead, go instead where there is no path and leave a trail”

Ralph Waldo Emerson

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Acronyms, Abbreviations and Symbols

AmB	Amphotericin B
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
CFU	Colony Forming Unit
CRO	Ceftriaxone
CT	Combined Therapy
ECM	Extracelular Matrix
EPS	Exopolyssacharides
FDA	Food and Drug Administration
IE	Infective Endocarditis
i3S	Instituto de Investigação e Inovação em Saúde
MBEC	Minimal Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
MIC90	Minimum Inhibitory Concentration at which 90% of isolates are inhibited~
MSA	Mannitol Salt Agar
PBP	Penincilin-Binding Proteins
QS	Quorum Sensing
SD	Standard Deviation
SDA	Sabouraud 2% Dextrose Agar
SDB	Sabouraud 2% Dextrose Broth
SEM	Scanning Electron Microscopy
TCP	Tissue Culture Plate
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WBC	White Blood Cells

Chapter 1

Introduction

Planktonic and sessile states are different forms of microbial life adopted depending on environmental stimuli, which can concede diverse advantages under specific conditions.

Planktonic is a unicellular life phase where cells are suspended (free-floating), which allows for microorganisms' dispersion and the colonization of new environment. Free-floating cells may have a raised metabolism, leading to extensive proliferation and they show great motility and capability of relocation. In contrast, a sessile state is a multicellular life phase in which microorganisms are organized in clusters enclosed by a self-secreted extracellular matrix (ECM). The sessile state, denominated "biofilm", allow sessile cells live in a coordinated, more permanent manner that favors their proliferation. Biofilms can anchor to the biotic or abiotic substratum and they are highly resistant to harsh conditions (e.g., antibiotic treatment) and allow cell-to-cell interactions, promoting lateral gene transfer and, therefore mutations and evolution.(1) For those reasons, biofilms are a common cause of hard-to-treat and life-threating infections.(2)

1.1 Biofilm: A Well-organized Community of Microorganisms

As previously referred, biofilms (Figure 1.1) are defined as a three-dimensional organized structure constituted by microorganism clusters (bacteria or fungi) adhered in either biotic (e.g., host tissue) or abiotic (e.g., biomaterial/implant) surface or interface and encased in a protective self-produced hydrated ECM composed mainly of proteins, polysaccharides, and extracellular DNA. These structures are heterogenous, both in space and time, and reveal "water channels" with colossal importance to the growth of cells within the biofilm, since allowing the passage of essential nutrients and oxygen.

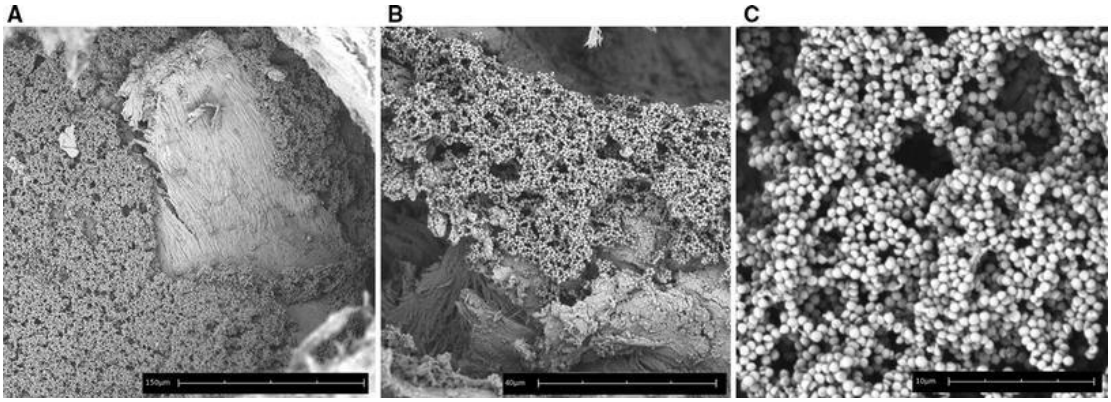

Figure 1.1 –*Staphylococcus aureus* 24-h-old biofilm developed on human bone. Images were depicted by Scanning Electron Microscopy (SEM), at scale bars: (A) 150 µm; (B) 40 µm; (C) 10 µm.(3)

Biofilms exhibit extreme resilience, insensitivity, or tolerance to severe conditions, including nutrient limitation (that is the main reason why the growth rate of biofilm-associated organisms is typically slower than planktonic organisms), antibiotic treatment (biofilms are up to 10-1000 times more resistant to antimicrobials than planktonic cells), UV radiation, pH stress, chemical exposure, dehydration, osmolarity, host immune response and mechanical and shear forces.(4, 5)

Biofilms can be divided into: (i) monomicrobial; (ii) polymicrobial (Figure 1.2). A monomicrobial biofilm (Figure 1.2A) encases a community of a single species of microorganisms, while a polymicrobial biofilm (Figure 1.2B) contains a diverse community of multispecies of microorganisms.(6) Due to complex interactions between different species, polymicrobial biofilms may have a different architecture that may enhances robustness and resilience to antimicrobials, compared to monomicrobial biofilms.(7, 8) The presence of different species within a biofilm may induce the growth and virulence gene expression, contributing for a higher production of extracellular polymeric substance (EPS) and, thus robustness and antimicrobial resistance. (7-15) Monomicrobial biofilms are frequently screened in burn wounds, pancreatic and abdominal wall infections, while polymicrobial biofilms are major causes of periodontitis, osteomyelitis, cystic fibrosis and urinary tract infections.(7, 13, 16-21)

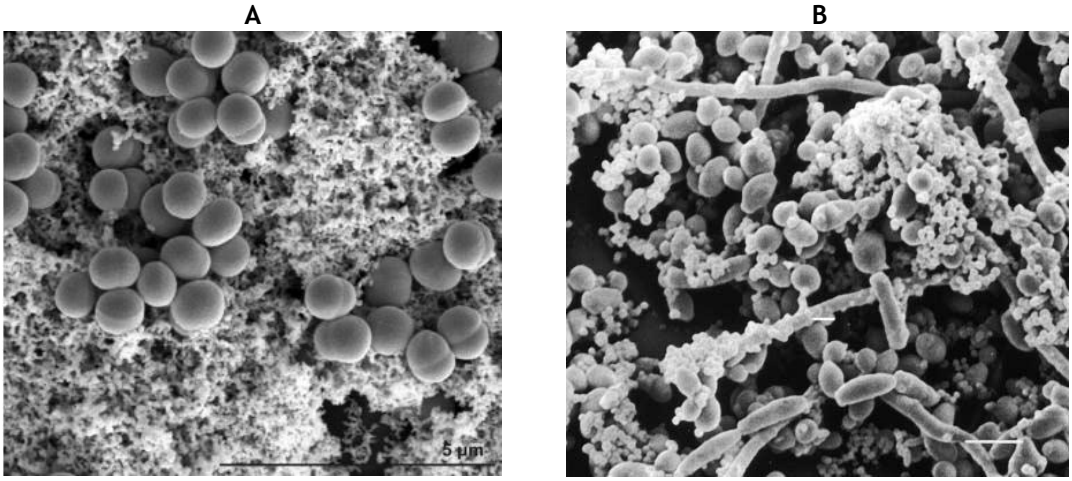

Figure 1.2 - Representation of a mono- and polymicrobial biofilm. (A) Single- *Staphylococcus epidermidis (S. epidermidis)* biofilm; (B) Dual-*S. epidermidis* and *Candida albicans* biofilm. Biofilms were depicted by SEM, at scale bars: (a)5 µm; and (b) 10 µm.(22, 23)

Regarding microorganisms' behavior within polymicrobial biofilms, the microorganisms can interact with each other in synergetic/cooperative or inhibitory/antagonistic interactions, producing metabolites that can interfere positively or negatively with growth and biofilm formation, and consequently affect the pathogenicity of infection.(24-26) The synergistic (or cooperative) effect can be manifested by: (i) passive resistance - protecting from antibiotic-killing activity; (ii) metabolic cooperation – producing adenosine triphosphate (ATP), carbon and nitrogen to synthesize metabolites and maintain redox balance; (iii) metabolic by-product –cooperative effect denoted metabolic cross-feeding (or syntrophy), generating metabolic byproducts capable of increase the growth of the surrounding species, and (iv) quorum sensing (QS) systems –signal exchange, allowing cell-to-cell communication and trigger a wide range of biological processes within the biofilm (e.g., detachment).(9-15) Conversely, antagonism (or inhibitory) effect can be due to: (i) the production of factors that kill or inhibit the growth of the surrounding species; (ii) the production of chemical signals, negatively affecting the behavior and physiology of the surrounding species; and (iii) limitation of nutrients, leading to starvation.(13)

In general, biofilm formation (Figure 1.3) is a dynamic process that involves the adhesion of microorganisms and clustering. In this sense, it can be described into four distinguished stages: (i) attachment– organic/inorganic molecules and microorganisms are adsorbed on a surface substrate (e.g., bone) through physical forces, such as electrostatics, electric double layer forces, hydrophobic and Van der Waals interactions, leading to irreversible microorganisms' attachment to the surface mediated by production of EPS matrix; (ii) formation of microcolonies – once the attachment is established, cells start to multiplicate and divide, leading to the formation of multicellular surface-attached communities; (iii) maturation – cell-to-cell interactions allow cells within biofilm to take specialized functions, secreting mature biofilm components, namely proteins, polysaccharides and extracellular DNA; and (iv) detachment - microorganisms/microcolonies from biofilm are dispersed and released into surrounding media as free-floating microorganisms, which further can spread and colonize another site, causing other surface contamination, systemic infections or septicemia.(4, 5, 27-29)

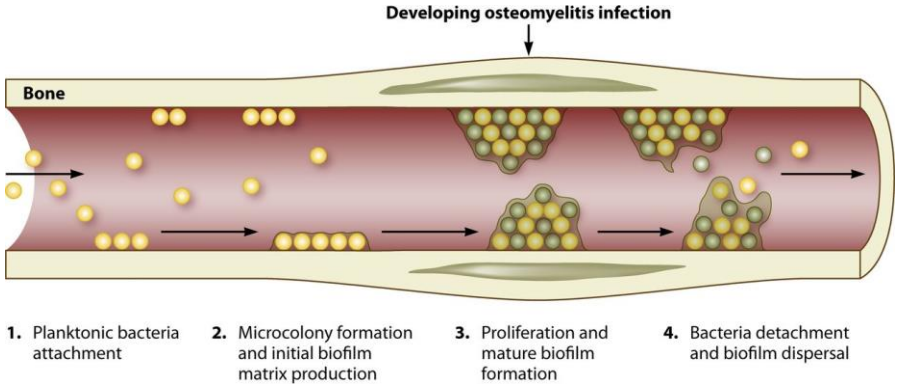

Figure 1.3 – Stages of biofilm formation (e.g., in bone). The process starts with the attachment of planktonic cells on a tissue surface (1). Thereafter, microorganisms' growth within multicellular-attached communities leads to the formation of microcolonies (2). During maturation, cells within the biofilm acquired specialized functions, secreting mature biofilm compounds, leading to an infection (3). At the detachment phase, biofilm may disperse microcolonies and planktonic into the surroundings (4).(30)

1.2.1 Biofilm-related Bone Infections

A significant proportion (60 – 80 %) of microbial infections are caused by biofilms. Those infections include tissues (e.g., bone) and medical-related infections (Figure 1.4), which represent a huge medical challenge, in terms of infection diagnosis and treatment.(31, 32)

Bone infections, such as osteomyelitis, joint infections or septic arthritis are diseases resulting from direct microorganisms (bacteria and/or fungi) penetration into the bone or adjacent tissues through the bloodstream, often after a trauma or a surgical procedure. When these microscopic entities reach the metaphysis of the bone, an infection may occur, leading to the recruitment of white blood cells (WBCs). WBCs secrete enzymes to phagocytize intruders, however they also may lyse the bone as they possess osteolytic properties, resulting in pus formation. The intraosseous pressure increases, hindering proper blood flow (Figure 1.5). Ultimately, serious morbidity due to bone loss and destruction, pathologic fractures and septicemia may occur, posing significant management challenges.(29, 33-35) Commonly due to their high recalcitrance to antimicrobial therapies and prolonged hospital stays, these infections are most of the time recurrent and untreatable.(4, 36-38)

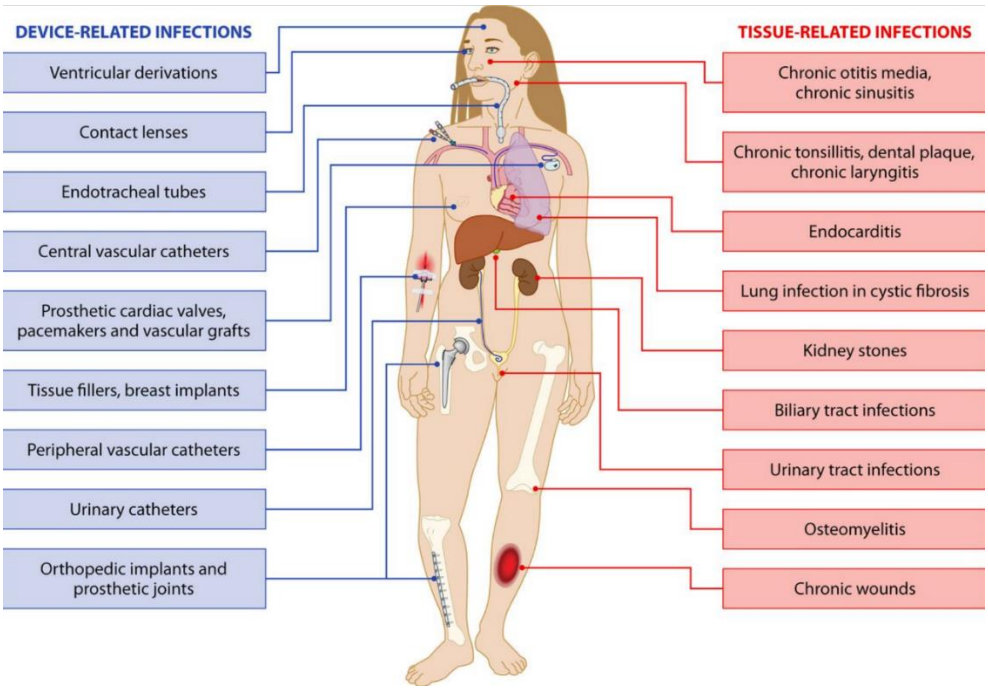

Figure 1.4 - Prevalent biofilm-related infections, including device (left) and non-device (right) related infections.(39)

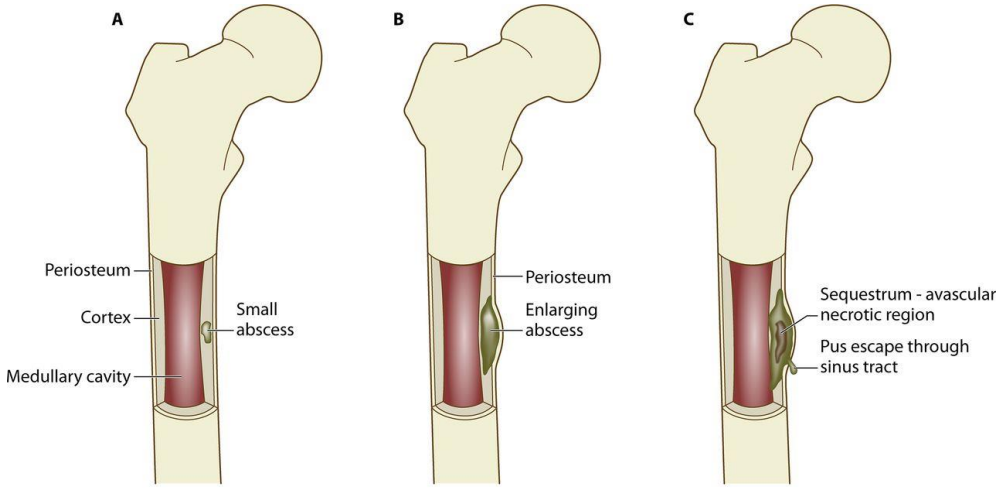

Figure 1.5 - Sequential progression of bone infections. (A) A small abscess localized in the cortical region is formed, which constricts the proper blood flow to the infected site. (B) As the abscess grows, it invades the sub-periosteal region, lifting the periosteum. (C) Blockage of blood supply to the bone contributes to bone necrosis.(30)

Concerning species that are typically involved in bone infections, several strains of microorganisms have been identified, as illustrated in Figure 1.6.

S. aureus is a typical causative agent found in bone infections, such as osteomyelitis, being present in 60 % of cases.(40) Over the past years, a drastic increase of *Candida albicans* (*C. albicans)* has been registered in osteomyelitis (e.g., vertebral osteomyelitis), and bone infections secondary to trauma or surgery.(41-44)

Several studies have been reporting the coexistence of *S. aureus* with *C. albicans* in complex polymicrobial biofilm-related bone infections, displaying increased drug tolerance and pathogenicity. It has been reported that the co-isolation of *S. aureus* with *C. albicans* increases the mortality in several mouse models.(45-47) Dyck *et al* (2021) described that the coexistence of *S. aureus* with *C. albicans* in a peritoneal infection was lethal, while the corresponding mono-microbial infections were not.(46) Similarly, in another study, they were injected intraperitoneal, separately and combined. The combination of the two microorganisms resulted in 100 % mortality, whereas the corresponding mono-microbial infections produced no animal deaths.(47)

Considering these facts, it is crucial to explore strategies for managing polymicrobial biofilms and, therefore controlling and treating bone infections related to polymicrobial biofilms.

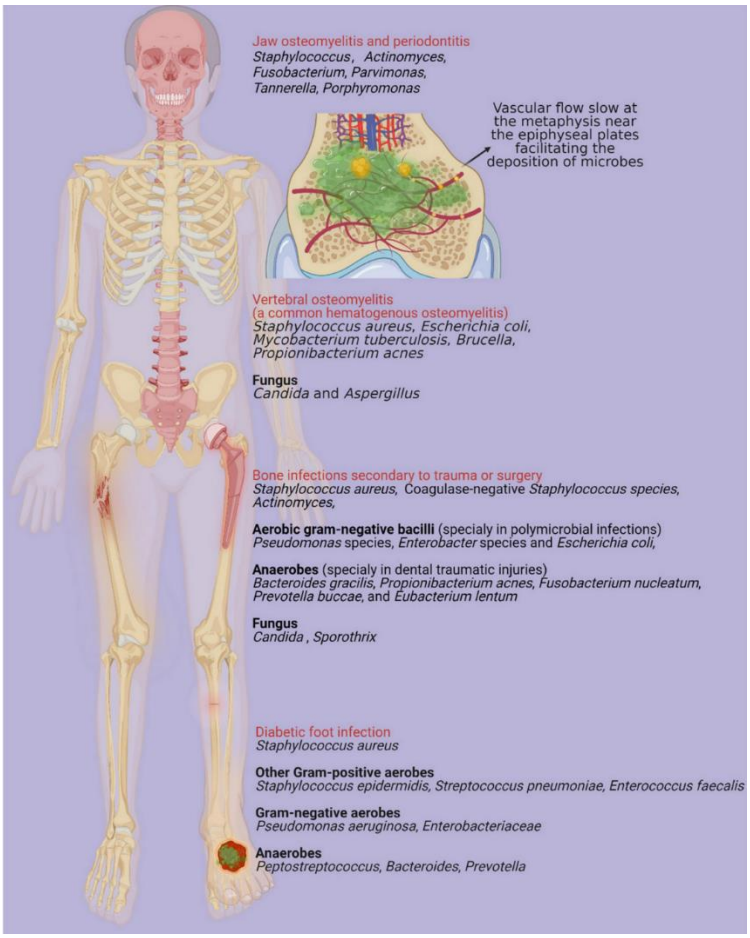

Figure 1.6 - Species of microorganisms involved in distinct bone infections. *S. aureus* is the most typical causative agent of bone infections. *C. albicans* has been identified in several types of bone infections, namely vertebral osteomyelitis and bone infections secondary to trauma or surgery.(33)

1.2 Anti-biofilm Therapies as Strategies for Biofilm Management

Managing biofilms are essential to avoid serious bone infections and to maintain the integrity of natural bone tissue. Current treatment options encompass physical methods and the administration of antibiotics. These methods have disadvantages (e.g., invasive procedures and antimicrobial resistance) and instigate collateral damage to surrounding tissues. In light of this, novel therapies are being studied and found promising. However, further research is mandatory to perceive the most effective treatment option.

1.2.1 Traditional Therapies

Currently, there are four standard available approaches to manage microbial biofilms (Figure 1.7) which are applied depending on the stage of the biofilm. These approaches include: (i) the use of antimicrobials (antibiotics or antifungals); (ii) physical removal through jets, sprays and brushes; (iii) medical device removal (in cases of device-related infections); and (iv) the use of dispersing agents. Concerning biofilm-related bone infections, the treatment is

complex, requiring the administration of specific antimicrobial and, in some cases, the removal of infected bone or soft tissue by the surgical procedure followed by the administration of a long-term specific antimicrobial regimen.(48, 49)

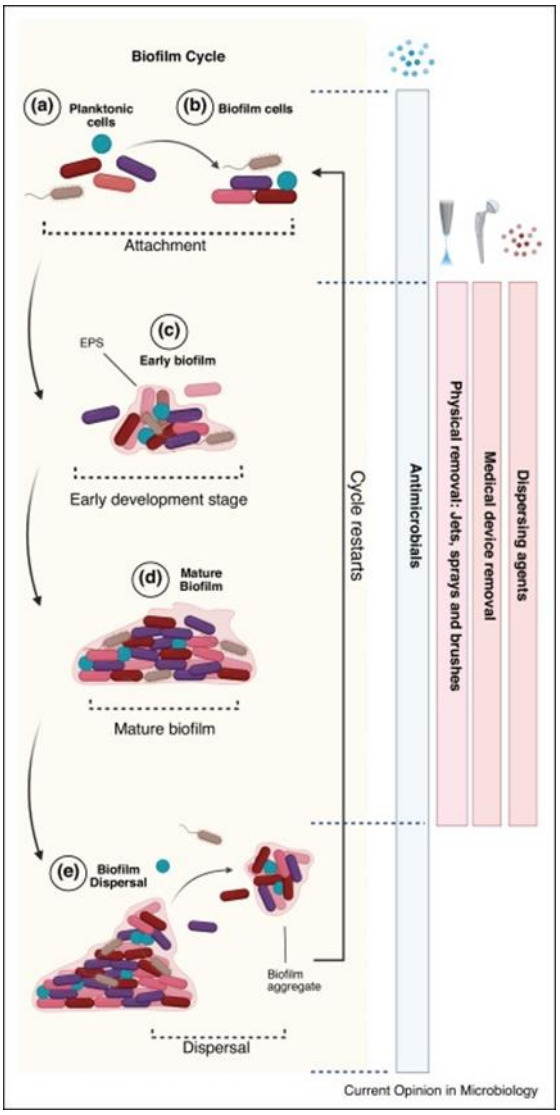

Figure 1.7 - Current strategies to manage biofilms. In the attachment phase ((a) and (b)), antimicrobials are the therapeutic use to manage biofilms. In the early development stage ((c)) and mature stage ((d)), the overall outcome of conventional treatments decreases as a result of enhanced antimicrobial resistance provided by ECM. Herein, clinical practice comprises physical removal by jets, sprays and brushes, medical device removal altogether or the use of dispersing agents followed by the administration of antimicrobials. In the dispersal phase ((e)), antimicrobials are typically administered alone.(48)

Antibiotics are able to kill, neutralize or even slow down the growth of a given disease-causing bacteria. To date, they have been widely used to treat human sickness and death-causing infectious diseases. Several factors must be considered when choosing an antibiotic for clinical use, such as proven antibacterial efficacy, low cytotoxicity, low microbial resistance development risk, capability to penetrate into the target tissue, inexpensive cost, high stability, and dosing convenience.(50)

Based on the type of action, antibiotics can be divided into two main groups: (i) bacteriostatic; (ii) bactericidal. Bacteriostatic antibiotics are capable of intervening in protein synthesis and blocking bacteria metabolic pathways which inhibit or slow down the growth of bacteria, whereas bactericidal can cause cell death by targeting the cell membrane or cell wall of bacteria.(51) The current standard of care for *Staphylococcal* infections consists of administrating antibiotics, namely nafcillin, oxacillin, cefazolin and vancomycin.(52, 53) Nevertheless, over the past decade, most of *Staphylococcus* strains have acquired resistance to potent antibiotics (e.g., penicillin, methicillin).(54, 55) Thus, there is an urge to beat this developing pattern of resistance.

Concerning antifungals, the five major classes of antifungals are azoles, polyenes, echinocandins, allylamines, and pyrimidine analogues. Overall, they target either the cell wall or fungal plasma membrane.(56-58) Azoles (e.g., ketoconazole, fluconazole, voriconazole, itraconazole and posaconazole) are the most commonly prescribed to treat harsh fungal infections.(56, 57, 59) Azoles interfere in the biosynthesis of ergosterol, a fungal-specific membrane sterol that has a key role in cellular functions, such as maintaining fungal membrane fluidity and integrity, as well as the proper functioning of the membrane-bound enzymes.(56, 59) Albeit azoles are the most common antifungal drugs in clinical use, many studies have shown and documented reduced azole antifungal susceptibility of *Candida* species.(59) Oxman *et al*. (2010) reported that *Candida* species generally considered fully prone to fluconazole became highly resilient to fluconazole, based on several cases of candidemia.(60)

1.2.1.1 Antibiotics and Antifungals

The combination of antibiotics and antifungals has been shown as a viable strategy in the control of osteomyelitis caused by *S. aureus* and *C. albicans* mixed biofilms. The choice of antibiotic and antifungal should be done according some fundamentals and guidelines, in order to potentiate the combined action. For example, Food and Drug Administration (FDA) approval and clinical data of a given antimicrobial agent are key requirements. In addition, the selection is made according to their costs, toxicities, site of infection and local susceptibility patterns.(48, 61-64) The combination of drugs must produce an increased spectrum of antimicrobial coverage and increased drug-efficacy with lower dosages. Furthermore, it must decrease drug toxicity and suppress antimicrobial resistance.(65)

In order to select effective doses of antimicrobials, it is demanded to know the minimal inhibitory concentration (MIC) and minimal biofilm inhibitory concentration (MBIC). MIC is defined as the lowest concentration of an antimicrobial agent (usually expressed in µg/ml), which prevents microbial growth.(66, 67) MBIC is described as the lowest concentration of a given antimicrobial at which there is biofilm eradication.(66)

Recently, studies have been conducted to deeply understand the impact of an antifungal – antibiotic combined therapy in fungal – bacteria mixed biofilms. A study performed by Sorribas *et al* (2022) documented an increased effect of caspofungin combined with meropenem against *C. albicans* – *S. aureus* and *C. albicans* – *Escherichia Coli* dual-species biofilms.(68) Similarly, Rogiers *et al* (2018) studied the activity of the antifungal drug anidulafungin and antibiotic tigecycline against polymicrobial biofilms composed of *S. aureus* and *C. albicans* associated with intraperitoneal infections, and observed an increased effect, suggesting a synergistic action between both antimicrobial agents.(69)

1.2.1.1.1 Ceftriaxone

Cephalosporins are a group of antibiotics generally used to manage serious infections from both Gram-positive and Gram-negative bacteria. These compounds have a ß-lactam ring bonded to a dihydrothiazine ring (or cepham) and side-chains (R1 and R2) in their core structure (Figure 1.8).(70) R1 site is related to cross-reactivity, since it can enhance the affinity to penicillin-binding proteins and enhance the resistance against bacterial ß-lactamases, whereas R2 is related to half-life and toxicity.(70, 71)

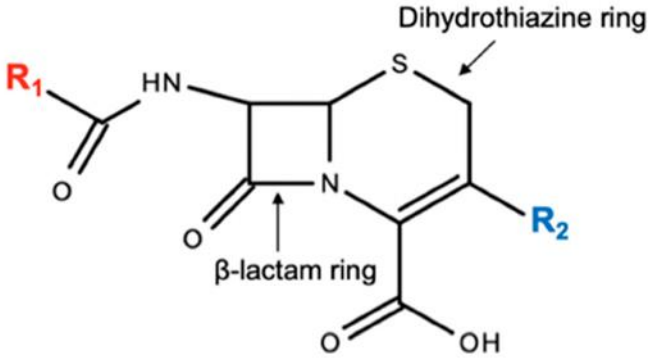

Figure 1.8 - Cephalosporin core structure. Side-chains are represented in red (R1 site) and blue (R2 site). R1 site is related to cross-reactivity, whereas R2 relates to half-life and toxicity.(70)

There are five generations of cephalosporins, based on their spectrum of activity and temporal discovery, with the third-generation currently being the recommended class for bone infections, since they easily penetrate in the bone tissue and they exhibit broader spectrum of activity than earlier generation, covering pathogens that are typically involved in such infections (e.g., *S. aureus*).(72-77)

First-generation cephalosporins (e.g., cefazolin, cephalothin, cephapirin, cepharadine, cefadroxil and cephalexin) are effective against most Gram-positive cocci (e.g., *Staphylococci spp.* and *Streptococci spp.*), but has minimal coverage against Gram-negative bacteria, such as *Escherichia Coli (E. coli)*, *Proteus mirabilis (P. mirabilis)* and *Klebsiella pneumoniae (K. pneumoniae)*.(70, 72, 78)

Second-generation cephalosporins (e.g., cefmetazole, cefotetan, cefoxitin, cefuroxime and cefprozil) display stronger activity against Gram-negative bacilli (e.g., *E. coli*, *P. mirabilis* and *K. pneumoniae*) despite having less activity against Gram-positive *cocci*. They also have coverage against *Haemophilus influenzae (H. influenzae)* and *Neisseria spp.*(70, 72, 79)

Third-generation (e.g., cefotaxime, ceftazidime, cefdinir, ceftriaxone, cefpodoxime and cefixime) has a wide-ranging spectrum of activity against Gram-positive and Gram-negative aerobic and some anaerobic bacteria, including *Streptococci spp.*, methicillin-susceptible *S. aureus* and *Pseudomonas aeruginosa (P. aeruginosa)*.(70, 72)

Fourth-generation cephalosporin (e.g., cefepime) has a similar spectrum of activity to third-generation. It easily penetrates the outer membrane of Gram-negative bacteria, as well as is capable of penetrating the cerebral spinal fluid. However, its use is reserved for patients with septicemia and multi-resistant microorganisms.(72)

The fifth-generation cephalosporins (e.g., ceftaroline, ceftibiprole) present a broad spectrum of activity, including methicillin-resistant *S. aureus*, which make them unique compared to other cephalopsorins. However, they do not have activity against *P. aeruginosa*.(70, 72, 80)

In Table 1.1, a brief description of every single generation of cephalosporins is presented relative to their spectrum of activity, as well as some examples included in a specific generation/family.

Table 1.1 – Cephalosporins at a glance.

Family	First-Generation	Second-Generation	Third-Generation	Fourth-Generation	Fifth-Generation
Spectrum of activity (Examples)	*Staphylococci spp., Streptococci spp., E. coli, P. mirabilis, K. pneumoniae*	*Staphylococci spp., Streptococci spp., E. coli, P. mirabilis, K. pneumoniae, H. influenzae, Neisseria spp.*	Second-generation's spectrum of activity, methicillin-susceptible *S. aureus* and *P. aeruginosa.*	Third-generation's spectrum of activity	Second-generation's spectrum of activity, methicillin-susceptible and resistant *S. aureus*, *Listeria monocytogenes*, *Enterococcus faecalis.*
Cephalosporins (Examples)	Cefazolin,Cephalothin, Cephapirin, Cepharadine, Cefadroxil, Cephalexin	Cefmatazole, Cefotetan, Cefoxitin,Cefuroxime, Cefprozil	Cefotaxime, Ceftazidime, Cefdinir, Ceftriaxone, Cefpodoxime, Cefixime	Cefepime	Ceftaroline, Ceftibiprole
References	(70, 72, 78)	(70, 72, 78, 79)	(70, 72, 81, 82)	(72, 78)	(70, 72, 78, 80)

Regarding their mechanism of action, cephalosporins inhibit the synthesis of peptidoglycan and, consequently cell wall synthesis. Their ß-lactam rings establish a connection with the penicillin-binding proteins (PBP), inactivating them. PBP are important during cell wall synthesis, since they strengthened cross-linking between different units of peptidoglycan. Meanwhile, the normal activity of the bacteria is affected and bacteria die.(72) In Figure 1.9 is illustrated an interaction between a ß-lactam antibiotic with both Gram-negative and Gram-positive bacteria, as representative of cephalosporins' mode-of-action.

Ceftriaxone is a third-generation semi-synthetic cephalosporin with an extensive half-life and, as represented in table 1.1, has a wide-ranging spectrum of activity against Gram-positive and Gram-negative bacteria, including methicillin-susceptible *S. aureus*.(70, 72, 78) It has shown effectiveness against several uncomplicated infections, such as urinary tract infections, lower respiratory tract infections, as well as soft tissue, skin, joint and bone infections.(72, 83) Moreover, ceftriaxone is relatively inexpensive and well tolerated.(83, 84)

Tests were performed to established interpretative breakpoints for ceftriaxone, which are determined as follows: (i) susceptible – MIC < 32 µg/ml; and (ii) resistant – MIC ≥ 64 µg/ml.(85)

Ceftriaxone shows active against some Gram-positive bacteria, including *S. aureus*, presenting MICs equal or greater than 25 µg/mL.(86) According to the established breakpoints, *S. aureus* show susceptibility against ceftriaxone.

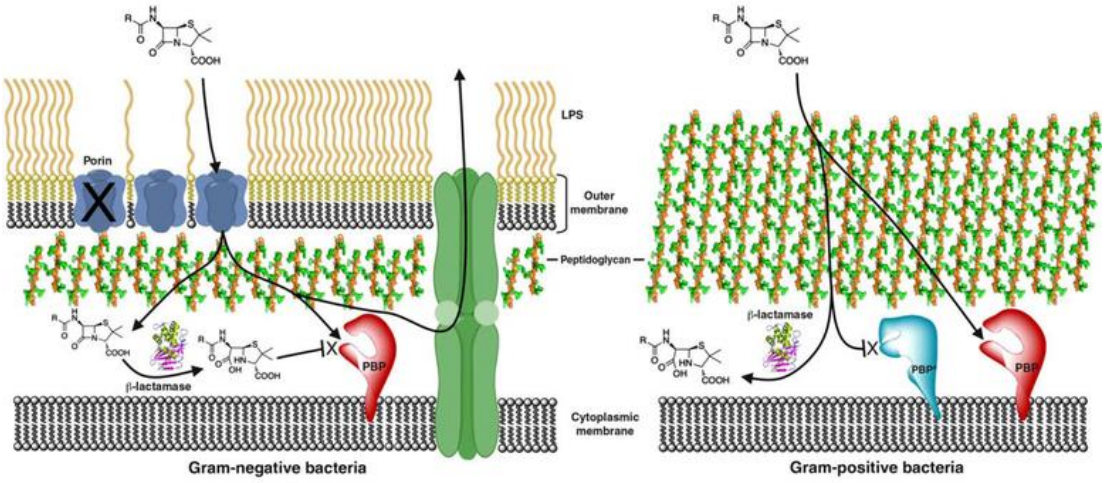

Figure 1.9 – Representation of the interaction between ß-lactams antibiotics with PBP, in a Gram-negative (left) and Gram-positive (right) bacteria. In a Gram-negative bacterium, ß-lactams pass through the layer by channels (porins). As they reach the periplasmatic space, they could be intercepted by ß-lactamases (ß-lactam resistant mechanism), or they could reach deeper sites towards the inner layer. Herein, ß-lactam rings bind to PBPs. In a Gram-positive bacterium, ß-lactams pass through outer membrane of peptidoglycan. They could be intercepted by ß-lactamases, or they could reach deeper sites towards the inner layer. Herein, ß-lactam rings bind to non-modified PBPs. Overall, the synthesis of peptidoglycan and cell wall could be inhibited, altering the normal activity of the bacteria.(87)

1.2.1.1.2 Amphotericin B

The polyene antifungals include nystatin, natamycin and amphotericin B, those are administrated to treat different fungal infections, such as: (i) nystatin – for mucosal infections (e.g., oral or vulvovaginal candidiasis); (ii) natamycin – for ophthalmic infections; and (iii) amphotericin B – for systemic mycoses, candidiasis and bone infections. Polyene molecules

consist of a macrolactone ring of polyunsaturated carbons and they have an amphipathic character due to the presence of a hydrophobic "tail" and a hydrophilic "head" (Figure 1.10).(88-90)

Polyenes are suitable surrogates for azoles to fight against fungal infections, due to their fungicidal properties, broadest spectrum and rare resistance development.(89) Regarding their mode-of-action, polyenes interact with ergosterol leading to disruption of the fungal cytoplasmatic membrane.(91, 92) Polyene-ergosterol binds generate pores across the fungal membrane, as well as extract part of the total ergosterol present in the plasma membrane, forming extra membranous aggregates.(57) It should be noticed that recently it was documented isolated of *Candida tropicalis*, *Candida auris* and *Candida lusitaniae* resistant to polyenes.(92)

Amphotericin B is a polyene macrolide antifungal extracted from the actinomycete *Streptomyces nodosus* and has an almost planar macrolactone ring with seven double bonds in trans configuration responsible for the hydrophobic part of the molecule.(88, 89, 93, 94)

Amphotericin B is the most common polyene used in antifungal therapy, concretely to treat invasive and life-threatening fungal infections, such as systemic mycoses, candidiasis and bone infections, due to low mycological resistance and its broad spectrum of activity (e.g., *C. albicans*, *Candida parapsilosis*, *Candida glabrata*).(90, 92, 93, 95) Developing a considerable resistance to the drug is rare and only occurs in certain strains where an alteration in their ergosterol biosynthesis pathway occurs. In those strains, instead of producing ergosterol, they produce ergosterol-like compounds which have a low binding affinity with amphotericin B.(93) In amphotericin B non-resistant strains, their ergosterol biosynthesis pathway is intact and, thereby they can produce ergosterol, which entices amphotericin B. The hydrophobic part of the molecule binds to ergosterol, generating pores and channels in the fungal plasma membrane used as transmembrane pathways for intracellular medium's electrolytes, such as potassium, phosphate, and ammonium, as well as carbohydrates and proteins. Also, amphotericin B induces oxidative damage in the fungal cell. The whole of this process ends up in cell death (Figure 1.11).(96)

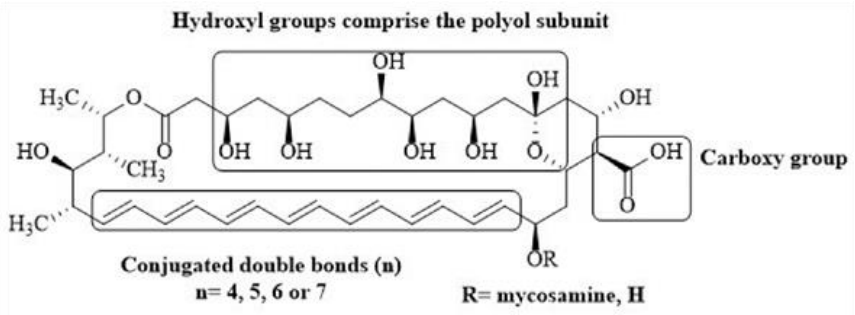

Figure 1.10 - General chemical structure of polyenes.(88)

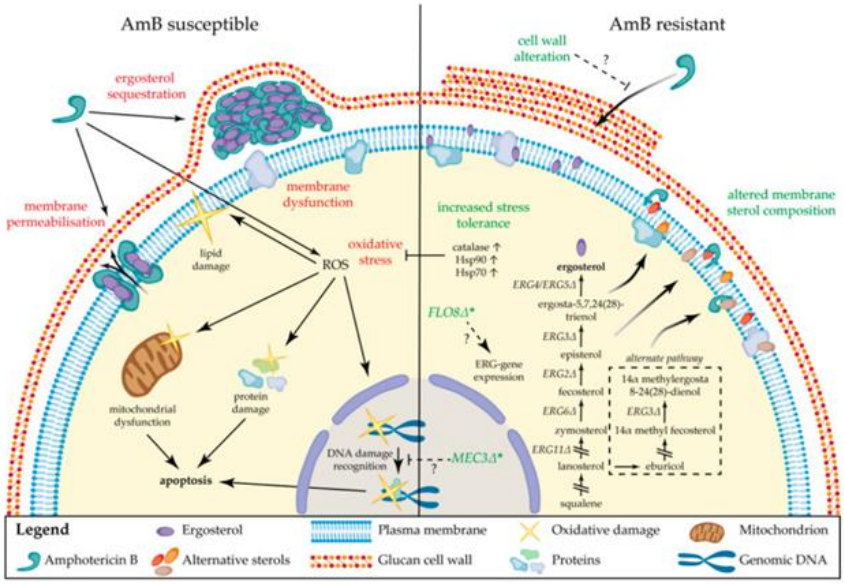

Figure 1.11 - Overview of the interaction between a susceptible (left) and resistant (right) fungal cell and amphotericin B (AmB). In a susceptible fungal cell, AmB binds to the ergosterol, generating pores and channels in the fungal membrane and inducing oxidative stress, leading to cell death. On the other way around, AmB resistant strains display an altered cell wall and produce alternative sterols with low affinity to AmB, hindering antifungal action.(89)

Breakpoints for amphotericin B were established by antifungal susceptibility testing to evaluate the spectrum of activity of the drug in a given clinically important yeast. Based on Ellis (2002), breakpoints for amphotericin B are determined as follows: (i) susceptible - MIC < 1µg/ml; (ii) resistant- MIC > 1 µg/ml. According to the same article, the amphotericin B spectrum of activity was determined based on *in vitro* susceptibility data from the literature in several different species by finding the MIC at which 90% of isolates are neutralized (MIC_{90}). *C. albicans* showed a range of MIC_{90} between 0.25 and 1 ug/ml, thus it is considered fully susceptible to amphotericin B.(93)

Mahmoudabadi *et al* (2014) tested amphotericin B MBIC for 120 isolated strains of *C. albicans,* isolated from different sources, such as vulvovaginal candidiasis, candiduria, bucal cavity and other environmental surfaces. Results showed that 65% of the total isolates displayed susceptibility to concentrations lower than 10 µg/ml. Furthermore, all strains isolated from mouth and other environmental surfaces were found to be fully susceptible to amphotericin B at concentrations lower than 10 µg/ml (MBIC < 10 µg/ml).(97)

1.2.2 Emerging Therapies

As stated, biofilms can withstand antimicrobial agents, due to several factors, such as an outer layer composed of EPS and high mutation rates. Moreover, the use of single agents against, for instance, polymicrobial biofilms could be ineffective, since they only target bacteria or fungi.(46) Hence, a demand to develop alternative approaches using new or a combination of antimicrobial agents to control and/or eradicate the biofilm formation and growth, thus preventing the development of bone infections.(98)

Several alternative antimicrobial agents are being studied for the management of biofilm-associated infections, including quorum-quenching compounds, bacteriophages, photodynamic

therapy, plant-derived components, nanoparticles and probiotics.(46, 61, 98) Quorum-quenching compounds, bacteriophage therapy and nanoparticles are still undergoing clinical trials.(46, 48, 61, 99, 100)

Quorum-quenching compounds are molecules that inhibit QS, preventing the expression of virulence and biofilm formation genes. They are biofilm specific and there is a low risk of acquired resistance.(61) However, as stated, quorum-quenching compounds are still in clinical trials.(61)

Bacteriophage therapy is another potential alternative therapeutic approach. Bacteriophages are viruses that specifically infect bacteria, controlling the host bacterium's cellular machinery for replication. Finally, they are capable of lyse the host's bacterium.(101) The efficacy of the therapy does not appear to be affected by the stage of the biofilm and it could perform well in conditions where nutrients and temperature are low. (48, 61) Phage production is fast and inexpensive. Yet, there is a risk of acquired resistance, as well as phages can be neutralized by the host's immune system.(48, 61)

Photodynamic therapy produces reactive oxygen species, capable of damaging cell components. This therapy is a minimally-invasive approach, with a wide-ranging spectrum of activity, and presents a low risk of acquired resistance and no toxicity. However, it has a limited effect against biofilms, due to the complexity of ECM.(46, 98)

Plant-derived components (e.g., essential oils) inhibit the QS and disrupt the cell membrane and matrix structures. They present low toxicity, wide pharmacological activity (e.g., curcumin has antifungal and antibacterial activity) and may display great activity against biofilms. In spite of this, the small scale production, low availability and stability, and high volatility are shortcomings that limited their application.(46)

Nanoparticles have been studied for drug delivery purposes, since they exhibit unique magnetic, chemical, electrical and mechanical properties. Due to their small sizes, nanoparticles penetrate the biofilm matrix, allowing the release of drugs *in situ*. They may exhibit intrinsic activity (e.g., gold and silver), inhibiting QS and producing reactive oxygen species capable of cell damage. Nanocarriers allow targeted delivery of drugs, reaching deep biofilm's regions, as well protecting drugs from the external environment. However, they generally present toxic for mammalian cells.(46, 99)

The use of probiotics has been studied to target polymicrobial biofilms. Probiotics are live microorganisms with health benefits when administrated in sufficient quantities. They trigger competitive adhesion to human bone tissue with microorganisms and produce bacteriocins and altered environmental conditions, blocking the production of QS molecules. Allow to maintain and restore the microbiota. Despite their advantages, they have limited survival. Besides, there is poor available research data on probiotics.

Table 1.2 summarizes modes-of-action, as well as advantages and shortcomings of the alternatives for antimicrobial agents to manage biofilm-associated infections.

Table 1.2 - Summary of some novel treatment approaches for biofilms

Novel therapy	Quorum quenching compounds	Bacteriophages	Photodynamic Therapy	Plant-derived components	Nanoparticles	Probiotics
Anti-biofilm effect	Inhibit QS.	Infect and lyse bacteria.	Produces reactive oxygen species.	Inhibition of QS. Disruption of the cell membrane and matrix structures.	Release of drugs *in situ*. Inhibition of QS and production of reactive oxygen species.	Competitive adhesion to human bone tissue with microorganisms. Inhibition of QS.
Strengths	Specificity. Low risk of acquired resistance.	Specificity. Inexpensive and fast phage production. May perform well in conditions where nutrients and temperature are low.	Wide-ranging spectrum of activity. Low risk of acquired resistance. No toxicity. Minimally-invasive approach.	Low toxicity. Wide pharmacological activities.	Targeted delivery of drugs. Protect drugs. Easier penetration inside a biofilm. Intrinsic antimicrobial properties	Maintain and restore the balance of microbiota.

Table 1.2 - Summary of some novel treatment approaches for biofilms

Novel therapy	Quorum quenching compounds	Bacteriophages	Photodynamic Therapy	Plant-derived components	Nanoparticles	Probiotics
Shortcomings	Still in clinical trials.	Risk of acquired phage resistance. Neutralized by the host's immune system. Still in clinical trials (phase I and II).	Limited effect against biofilms.	Small scale production. Low availability and stability. High volatility.	Possible toxicity for mammalian cells. Poor available research data.	Limited survival. Poor available research data.
References	(61)	(48, 61)	(46, 98)	(46)	(46, 99)	(46, 102)

Conventional antimicrobials are still the approach of choice due to their availability and well-documented side effects and efficacies. However, the discoveries of new antimicrobials are motionless and coincide with a time when there has been an increase in antimicrobial resistance. Thus, there is an urge in optimizing antimicrobial utilization.(103)

The combination of two or more antimicrobial agents, known as "combined therapy" (CT) seems to be a worthwhile strategy, as it has been reported to be effective against biofilms' resistance mechanisms. It can be used in particular cases where a causative agent is resistant to conventional monotherapy. The CT may produce innovative modes-of-action that could overcome the main survival mechanisms of biofilm cells against antimicrobials. In addition, it may bolster drug-efficacy by lower doses of antimicrobials and, consequently lower drug toxicity. In this regard, polymicrobial biofilm could be treated by using a CT, due to multi-target against different microorganisms within the biofilm.(61, 62, 64, 104) However, CT presents potential hurdles, such as increased side-effects, need for optimizing dosages and duration to bolster efficacy and minimize toxicity. Its outcome could be limited by biofilms' resistance mechanisms.(61, 64)

The combination of different drugs can trigger different kinds of activities: (i) synergistic – when the total effect of the drugs is greater than the sum of the individual effects of each drug; (ii) additive – the effect is equivalent to that produced by combining the individual antimicrobials; (iii) antagonistic – the produced effect is lower than that obtained by combining the individual antimicrobials.(103)

Sueke *et al* (2010) investigated several antibiotic (teicoplanin, moxifloxacin, ciprofloxacin, meropenem and linezolid) combinations and concentrations against *S. aureus* strains isolated from bacterial keratitis. They showed that, depending on concentrations, certain combinations of antimicrobials may display a synergistic action.(105) Tamma *et al* (2012) showed that the combination of penicillin with gentamicin displayed synergism against *S. aureus* isolated from the cardiac tissue of rabbits. The eradication of bacteria was verified in half the time required to obtain the same level of eradication by using penicillin monotherapy.(106) Moreover, Dall *et al* (2018) revealed that the combination of gentamicin with daptomycin, vancomycin or ciprofloxacin showed also synergistic action against *S. aureus*, besides they showed that the minimal biofilm eradication concentration (MBEC) decreased. In opposition, the combination of gentamicin with rifampicin, clindamycin or linezolid produces an antagonistic effect, increasing MBEC.(107) It should be noticed that the antimicrobial CT using ceftriaxone is seen as a standard approach for the treatment of some pathologies, especially infective endocarditis (IE). IE instigated by *Enterococcus faecalis* are frequently handled by administrating ampicillin combined with ceftriaxone. Moreover, benzylpenicillin/ceftriaxone CT is seen as an alternative to forswore *Enterococcal* endocarditis.(108)

Cui et al (2015) showed synergistic combinations of antifungals with anti-virulence agents and antibiotics against *in vitro* and *in vivo C. albicans*. The authors combined for instance, amphotericin B with caspofungin, fluconazole and doxycycline and observed a synergic effect between combinations, i.e., the combined treatment showed greater antifungal activity than the sum of the individual drug effects.(104) Some combinations between amphotericin B and other antimicrobial agents against several microorganisms' strains have been tested in clinical trials. For example, Rex *et al* (2003) tested the combination of fluconazole with amphotericin B against *Candida spp* isolated from candidemia.(109) Additionally, Day *et al* (2013) used an amphotericin B-flucytosine CT against *Cryptococcus spp* isolated from meningitis. The results

indicate that mortality was lower when the combination therapy was used, compared to amphotericin B administered alone.(110)

Li *et al* (2015) described the impact of the fluconazole – minocycline CT polymicrobial biofilms composed by *S. aureus* and *C. albicans*. This treatment showcased a strong anti-polymicrobial biofilm, as the antifungal and minocycline displayed a synergistic effect.(111)

Given these facts, the combination of antimicrobials may result in a synergistic CT that might yield promising results in the treatment of bone infections related to mixed-species biofilms. However, further research in animal models and clinical trials are required to test and prove safety and efficacy.(104)

1.3 Objectives

Primarily, this research work has as its main purpose to investigate the activity of ceftriaxone and the antifungal drug amphotericin B, alone and in combination, against mono- and dual-species *S. aureus*/*C. albicans* biofilms. Given this, this work seeks to accomplish the following objectives:

- Study the possible interactions and relationship between *S. aureus* and *C. albicans* when both coexist within a biofilm;
- Evaluate the efficacy of mono- and combined-therapy against mono- and dual-species biofilms.

Thereby, mono- and dual-species biofilms of *S. aureus* and *C. albicans* were developed and their growth kinetics were evaluated in order to perceive at which time-point they reach a mature stage. Furthermore, ceftriaxone and amphotericin B were tested alone and in combination against either mono- or dual-species biofilms to assess the efficacy of both mono- and combined-treatment.

Chapter 2

Materials and Methods

2.1 Microorganisms, Antimicrobial Agents and Growth Conditions

S. aureus ATCC (American Type Culture Collection) 49230 and *C. albicans* ATCC 10231 were selected as reference strains for the study. Tryptic Soy Broth (TSB, Merck Millipore), 0.9% NaCl (Merck Millipore), Mannitol Salt Agar (MSA, Liofilchem), Tryptic Soy Agar (TSA, Merck Millipore), Sabourad 2% Dextrose Broth (SDB, Merck Millipore) and Sabourad 2% Dextrose Agar (SDA, Merck Millipore) were prepared according to producer's guidance. TSA plates were used to quantify the total cultivable cells of both *C. albicans* and *S. aureus*, while SDA and MSA were used as selective plates to quantify the total cultivable cells of *C. albicans* and *S. aureus*, respectively. Regarding antimicrobial agents, ceftriaxone (Cayman Chemical) and amphotericin B (Biowest) were used to assess their effect on mono- and dual-species biofilms. They were prepared according to manufacturer's datasheet, at a concentration equivalent to 10 times the MIC.

Before each experiment, a suspension at 1×10^6 CFU/mL of *C. albicans* ATCC 10231 and *S. aureus* 49230 in exponential phase was prepared and used in the following sections.

2.2 Formation of Mono- and Polymicrobial Biofilms

To form single-species biofilms, one mL of previously prepared suspension of *C. albicans* ATCC 10231 and *S. aureus* 49230 was added to polystyrene 24-well tissue culture plates (TCPs) whereas, for dual-species biofilms, 500 µL of each culture at 2×10^6 CFU/mL were used. The TCPs underwent incubation for two hours at 37 ºC in an orbital shaker at 120 rpm to promote the adherence of microorganisms. Afterwards, the wells were gently washed twice with 0.9 % NaCl to remove weakly attached cells and then one mL of fresh medium was pipetted to the wells containing adhered biofilms. The TCPs containing single-species biofilms were incubated for 24, 48 and 72 h, at 37 ºC in an orbital shaker at 120 rpm to determine the time required to reach a mature biofilm, while those containing dual-species biofilms underwent incubation for 48 h, at the same temperature and shaking. Following each incubation time, the wells containing formed biofilms were gently washed twice with 0.9 % NaCl to remove loosely

adherent cells. For controls, microorganism-free wells were used. All experiments were performed in triplicate as independent experiments.

2.3 Growth Kinetics of *S. aureus* and *C. albicans* Biofilms

Colony-forming unit (CFU) counting was the quantitative method chosen to evaluate the growth kinetics of mono-species biofilms.

To perform CFU counting, the sessile microorganisms were dislodged during a 10-min sonication using an ultrasonic water bath (BactoSonic Ultrasonic Bath BS14, 200 W, 40 kHz, Bandelin) and serial dilutions were performed and seeded onto TSA or SDA plates for CFU counting.

2.4 Anti-biofilm Activity of Antimicrobial Agents

Susceptibility trials were performed to assess the effect of the antimicrobials on mono- and dual-species biofilms. In this sense, mono- and dual-species biofilms were developed in the wells of polystyrene 24-well TCPs, following the previously described protocol. After 48 h of incubation, the wells were slowly rinsed twice with 0.9 % NaCl to remove loosely adherent cells. Fresh medium containing ceftriaxone (0.25 mg/mL) and amphotericin B (10 µg/mL) were added to wells and, then plates underwent incubation for 24 h, at 37 ºC in an orbital shaker at 120 rpm. Afterwards, the wells were slowly rinsed twice with NaCl 0.9% and sessile population were detached during a 10-min sonication using an ultrasonic water bath (BactoSonic Ultrasonic Bath BS14, 200 W, 40 kHz, Bandelin). CFU assay was performed according to the previously described protocol. Untreated biofilms were used as control.

2.5 Data Analysis

All experiments were performed in triplicate as independent experiments. The results were represented as mean ± standard deviations (SD). Statistical analysis was performed by using two-tailed Student's *t*-test, assuming unequal variances. Differences between multiple experiments were considered statistically significant at a $\rho<0.05$, corresponding to a confidence level of 95 %.

Chapter 3

Results and Discussion

A large proportion of bacterial and fungal bone infections are related to biofilm formation.(4, 36-38) The conjunction of *S. aureus* with *C. albicans* is typical in these infections, altering their sensitivities to antimicrobials.(46) In this sense, efforts have been put into the discovery for novel therapies and strategies to control these infections. The combination of different antimicrobial agents is an emerging therapy currently used in several infections, including candidemia, meningitis and endocarditis, as it may produce positive effects over conventional monotherapy.(104, 108-110, 112)

In the present study, mono- and dual-species biofilms composed of *S. aureus* and/or *C. albicans* were developed and characterized in terms of biofilm formation, as well as antimicrobial susceptibility, particularly ceftriaxone and amphotericin B, used individually and in combination.

3.1 Growth Kinetics of Mono-species *S. aureus* and *C. albicans* Biofilms

The ability to form biofilm density was tested for both strains of reference and confirmed using CFUs assay.

The results (Figure 3.1) showed that *S. aureus* biofilm displayed a higher density compared to *C. albicans*, as observed by other authors. (41, 113, 114) Zegre *et al* (2022) showed that *S. aureus* presented a higher ability to form mono-species biofilm than *C. albicans*.(41) This fact can be explained by the differed size of the microorganisms, as *C. albicans* is larger than *S. aureus*.(115) Hyphae of *C. albicans* tend to elongate when growing, increasing its surface area for attachment, thus leading to lower cell densities.(24, 116)

Significant differences in biofilm densities between 48 and 72 h were observed for both mono-species biofilms studied, indicating that the biofilms stabilized at 48 h, reaching maximum values of 7.44 Log CFU/mL for *S. aureus* and 6.27 Log CFU/mL for *C. albicans* (Figure 3.1). After 72 h of incubation, both biofilms seem to enter in a declining phase, since biofilm density suffered significant decrease. The phase of declining growth is typically concomitant with nutrient exhaustion and production of detrimental metabolic end products.(117) As

referred, mono-species biofilms reached the mature phase at 48 h, which is in accordance with previous studies.(118-121) For instance, Zago *et al* (2015) investigated the growth kinetics of mono- *S. aureus* and *C. albicans* biofilms and observed that both biofilms reached the peak values at 48 h.(121) This data is crucial to determine the moment at which a stabilized infection is established, corresponding to a mature biofilm. (113) According to Li (2020), mature biofilms promote cell-to-cell interactions, leading to the production of virulence factors.(113)

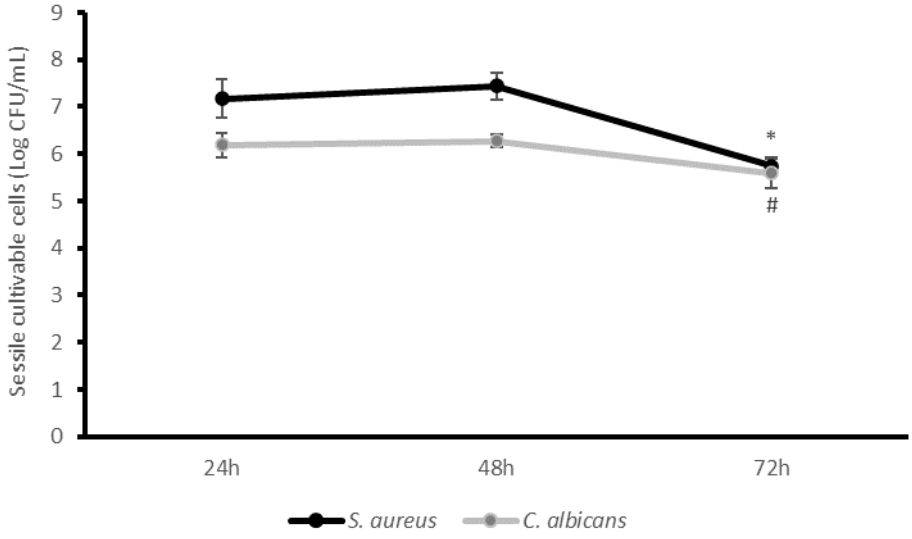

Figure 3.1 - Quantification of sessile population over 72 h of mono- *S. aureus* (represented by black) and *C. albicans* (represented by grey) biofilms. Biofilm densities were expressed in Log CFU/mL. The symbols * and # mean significant differences ($\rho<0.05$) between time-points, for mono-*S.aureus* and *C.albicans* biofilms, respectively.

3.2 Sensitivity to Antimicrobials of Mono-*S. aureus* and *C. albicans* Biofilms

The effects of ceftriaxone and amphotericin B alone or in combination were further studied on mature mono-species biofilms and assessed by performing the CFU assay in order to perceive their sensitivities to the antimicrobials.

The antibiofilm activity of mono- and CT after 24 h in contact with mono-species biofilms is depicted in Figure 3.2.

Concerning *S. aureus* biofilm, the results show the bacterial density of the untreated biofilm (7.09 ± 0.36 Log CFU/mL) was significantly higher than ceftriaxone-treated biofilm (4.11 ± 0.52 Log CFU/mL), similar than amphotericin B-treated biofilm (6.93 ± 0.36 Log CFU/mL) and significantly higher than CT-treated biofilm (4.19 ± 0.37 Log CFU/mL). In this sense, the ceftriaxone (0.25 mg/mL) displayed a strong antibiofilm activity, reducing 3 Logs of biofilm density (99.9 % of reduction), proving its activity against *S. aureus*, as a result of its mode-of-action. Based on Bui (2022), ceftriaxone inhibits the synthesis of peptidoglycan and cell wall, leading to bacteria death.(72) Amphotericin B did not affect the *S. aureus* growth and proliferation, as expected. Similarly, Rogiers *et al* (2018) evaluated the activity of

anidulafungin in mono- *S. aureus* biofilm and observed that it lacked any activity.(69) In what concerns the CT, it showed a similar antibiofilm activity as ceftriaxone, since it reduced 3 Logs of biofilm density (99.9 % of reduction). These results suggest that the combination of the two antimicrobials did not interfere in the ceftriaxone's activity. In a study conducted by Li *et al* (2015), the effect of minocycline and fluconazole, alone and in combination, was tested in mono- *S. aureus* biofilm. The authors observed that fluconazole did not impact the bactericidal activity of minocycline.(111)

In what concerns *C. albicans*, the results show the fungal density of the untreated biofilm (6.51 ± 0.08 Log CFU/mL) was similar to ceftriaxone-treated biofilm (6.59 ± 0.17 Log CFU/mL), significantly higher than amphotericin B-treated biofilm (5.53 ± 0.36 Log CFU/mL), and significantly higher than the CT-treated biofilm (4.19 ± 0.37 Log CFU/mL). In this sense, the amphotericin B (10 µg/mL) reduced 1 Log *C. albicans* density (90 % antifungal activity), proving its activity against *C. albicans*, as a result of its mechanism of action. As reported by Carolus *et al* (2020), amphotericin B acts in the ergosterol biosynthesis pathway and induces oxidative damage in the fungal cell, leading to cell death.(89) As expected, the ceftriaxone did not affect the *C. albicans* biofilm. Similarly, Ku (2010) tested the activity of vancomycin in a mono-*C. albicans* biofilm. It was noticed that it had no activity against *C. albicans*.(122) Concerning the CT, it showed a similar antibiofilm activity as amphotericin B, since it reduced 1.26 Logs of fungal density (91.6 % of reduction). These results suggest that the combination of the two antimicrobials did not interfere in the amphotericin B's activity. Ku (2010) also tested the activity of amphotericin B, individually and in combination with tigecycline, in mono-*C. albicans* biofilm. It was observed that the combination of the two antimicrobial agents did not alter the antifungal activity of amphotericin B.(122)

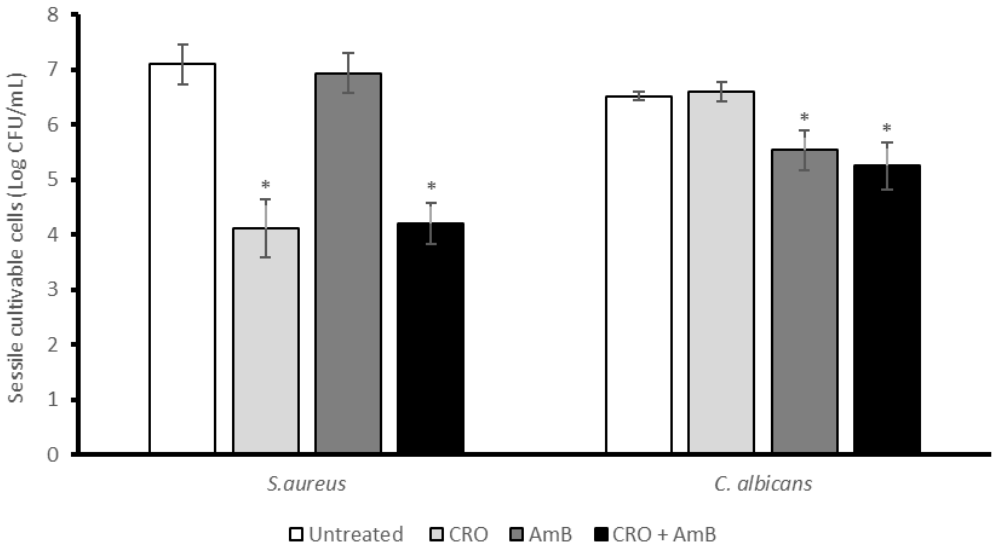

Figure 3.2 – Antibiofilm activity of ceftriaxone (CRO), amphotericin B (AmB) and ceftriaxone–amphotericin B (CRO + AmB) against mono-*S. aureus* and *C. albicans* biofilms. Biofilms densities were expressed in Log CFU/mL. The symbol * means significant differences ($\rho<0,05$) between the treatments (mono- and combined) and untreated *S. aureus* and *C. albicans* biofilm groups (controls).

3.3 Sensitivity to Antimicrobials of Dual-*S. aureus* and *C. albicans* Biofilms

The effects of ceftriaxone and amphotericin B alone or in combination were further studied on mature dual-species biofilms and assessed by performing the CFU assay.

In table 3.1 are represented the cell densities obtained for mono- and dual-species, for each condition (untreated, CRO, AmB and CRO + AmB).

The results show that the total and viable cell density of untreated dual-species biofilm (6.97 ± 0.11 Log CFU/mL) was similar to untreated mono-*S. aureus* biofilm (7.09 ± 0.36 Log CFU/mL), but relatively higher than the density obtained for untreated mono-*C. albicans* biofilm (6.51 ± 0.08 Log CFU/mL). This suggests that the cell density of the dual-species biofilm is mainly due to *S. aureus*. However, further discrimination of the sessile population within dual-species biofilm is needed.

When administered ceftriaxone, dual-species biofilm registered a cell density of 6.90 ± 0.10 Log CFU/mL, being expressively higher than mono-*S. aureus* biofilm (4.11 ± 0.52 Log CFU/mL) and similar than mono-*C. albicans* biofilm (6.59 ± 0.17 Log CFU/mL). These results allow to infer that the cell density of the dual-species biofilm is mainly due to *C. albicans*. In addition, they suggest that ceftriaxone might produce an effect in *S. aureus* within dual-species biofilm, decreasing its cell density. However, further discrimination of the sessile population within dual-species biofilm is needed.

Amphotericin B presented a similar impact in dual-specie biofilm (6.91 ± 0.09 Log CFU/mL) compared to mono-*S. aureus* biofilm (6.93 ± 0.36 Log CFU/mL). In contrast, the effect of the antifungal drug was dissimilar comparing with mono-*C. albicans* biofilm (5.53 ± 0.36 Log CFU/mL). These results allow to infer that the cell density of the dual-species biofilm is mainly due to *S. aureus*. Additionally, they suggest that amphotericin B might produce an effect in *C. albicans* within dual-species biofilm, decreasing its cell density. However, further discrimination of the sessile population within dual-species biofilm is needed.

Regarding CT, the cell density of dual-species biofilm (5.34 ± 0.34 Log CFU/mL) was higher than mono-*S. aureus* biofilm (4.19 ± 0.37 Log CFU/mL), and similar to mono-*C. albicans* biofilm (5.25 ± 0.43 Log CFU/mL). These results allow to speculate that the cell density of the dual-species biofilm is most likely due to *C. albicans*. They suggest that the CT might had impact on *S. aureus*. Nevertheless, further discrimination of the sessile population within dual-species biofilm is needed.

Table 3.1 – Cell densities expressed by mean ± SD Log CFU/mL, obtained in mono- and dual-species biofilm, for each condition. The symbol * means significant differences (ρ<0,05) between mono- and dual-species biofilms (control), for each condition.

Condition	Mono-species biofilm		Dual-species biofilm
	S. aureus	*C. albicans*	
Untreated	7.09 ± 0.36	6.51 ± 0.08 (*)	6.97 ± 0.11
CRO	4.11 ± 0.52 (*)	6.59 ± 0.17	6.90 ± 0.10
AmB	6.93 ± 0.36	5.53 ± 0.36 (*)	6.91 ± 0.09
CRO + AmB	4.19 ± 0.37 (*)	5.25 ± 0.43	5.34 ± 0.34

The antibiofilm activity of ceftriaxone and amphotericin B, alone and in combination, after 24 h in contact with dual-species biofilms is depicted in Figure 3.3. Non-selective growth media, namely TSA, were used to assess the total density of dual-species biofilms. Selective growth media, namely MSA and SDA, were used to discriminate the sessile population within dual-species biofilms. MSA was used to quantify *S. aureus* and SDA to quantify *C. albicans*.

In Figure 3.3 it is observed that untreated dual-species biofilm has a similar total cell density as ceftriaxone-treated dual-species biofilm, being 6.97 ± 0.11 and 6.90 ± 0.10 Log CFU/mL, respectively. Also, amphotericin B-treated dual species presents a similar cell density of 6.91 ± 0.09 Log CFU/mL. Conversely, CT-treated dual-species biofilm presents a lower cell density, being 5.34 ± 0.34 Log CFU/mL. In this sense, the CT was able to reduce the total cell density by 1.63 Logs (96.9 % of reduction).

Looking for within dual-species biofilm (Figure 3.3), the untreated dual-species biofilm reached densities of 6.97 ± 0.11 Log CFU/mL, presenting higher amount of *S. aureus* (6.88 ± 0.11 Log CFU/mL) than *C. albicans* (5.43 ± 0.31 Log CFU/mL), which as in accordance with single-species biofilms, supporting that these differences are most likely due to distinct microorganisms' sizes.(68)

Regarding ceftriaxone, the total cell density of the treated dual-species biofilm was 6.90 ± 0.10 Log CFU/mL, presenting, in its composition, a higher amount of *C. albicans* (6.83 ± 0.18 Log CFU/mL) than *S. aureus* (4.09 ± 0.20 Log CFU/mL), which is in line with the results obtained in single-species biofilms. In this sense, ceftriaxone reduced bacterial density within dual-species biofilm by 2.81 Logs (99.8 %). This reduction was noteworthy, demonstrating the effectiveness of ceftriaxone in targeting and reducing *S. aureus* within dual-species biofilm. Besides, these results suggests that the presence of *C. albicans* did not affect the activity of ceftriaxone against *S. aureus*. Shivaji (2023) evaluated the activity of ceftriaxone in mono- *S. aureus* and dual-*S. aureus* and *Fusarium solani* (*F. solani*) biofilms. The results demonstrated that the bactericidal activity of ceftriaxone was not altered by the presence of *F. solani*.(123) In another study, the activity of minocycline against mono- and dual- *S. aureus* and *C. albicans* was evaluated. Minocycline was able to target and reduce *S. aureus* within dual-species biofilm. It was also observed that the presence of *C. albicans* did not alter the bactericidal activity of minocycline.(41)

Concerning amphotericin B, the total density of the treated biofilm was 6.91 ± 0.09 Log CFU/mL, presenting, in its composition, higher amount of *S. aureus* (6.86 ± 0.11 Log CFU/mL)

than *C. albicans* (5.25 ± 0.17 Log CFU/mL), which as in accordance with single-species biofilm. In this regard, amphotericin B reduced fungal density within dual-species biofilm by 1.66 Logs (97.7 % of reduction). This reduction demonstrates the effectiveness of amphotericin B in targeting and reducing *C. albicans* within dual-species biofilm. In addition, the provided results imply that the presence of *S. aureus* did not affect amphotericin B's activity against *C. albicans*. Sorribas (2022) tested the effect of caspofungin in dual-*S. aureus* and *C. albicans* and observed that it reduced *C. albicans* within dual-species biofilm.(68)

In what concerns CT, the total density of the treated biofilm was 5.34 ± 0.34 Log CFU/mL, presenting, in its composition, a higher amount of *C. albicans* (5.16 ± 0.16 Log CFU/mL) than *S. aureus* (3.44 ± 0.34 Log CFU/mL), which is in accordance with the results obtained for mono-species biofilm. In this sense, the CT significantly reduced bacterial density by 1.9 Logs (98.9 %), whereas it had no significant impact in fungal density, within dual-species biofilm. These results suggest that the presence of *C. albicans* did not affect the activity of the CT against *S. aureus*. Conversely, the presence of *S. aureus* seemed to affect the activity of CT against *C. albicans*. This implies that the *S. aureus* might somehow shield *C. albicans*. Hu *et al* (2021) observed an enhanced resistance of *C. albicans* against amphotericin B within dual-*S. aureus* and *C. albicans* biofilms, most likely due to an alteration in the antifungal resistant gene expression of *C. albicans* triggered by *S. aureus*. (124) Zegre *et al* (2022) reported that *S. aureus* tend to be located mainly in the upper layers of the biofilm, being attached to the morphological forms of *C. albicans*.(41) In this sense, due to this stratified growth, *S. aureus* is exposed to the action of the ceftriaxone, whereas *C. albicans* is protected from amphotericin B's activity. In light of this, the remaining *S. aureus* within dual-species biofilm might be sufficient to protect *C. albicans* from the fungicidal activity of amphotericin B.

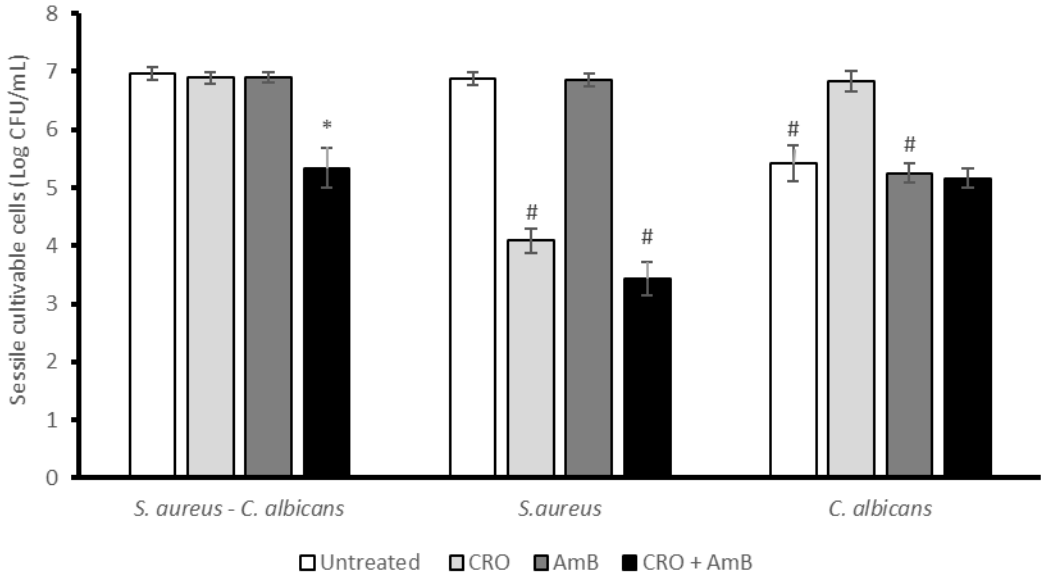

Figure 3.3 – Antibiofilm activity of ceftriaxone (CRO), amphotericin B (AmB) and ceftriaxone-amphotericin B (CRO + AmB) within dual-species biofilms. Biofilms densities were expressed in Log CFU/mL. TSA plates were used to quantify the total cell density of dual-species biofilm, whereas MSA and SDA were unsed as selective growth media for *S. aureus* and *C. albicans*, respectively. The symbol * means significant differences (ρ<0,05) between the mono- and combined treatment and untreated biofilm, in *S. aureus* – *C. albicans* group. The symbol # means significant differences (ρ<0,05) between the mono- and combined treatment and respective condition in *S. aureus* – *C. albicans*.

Chapter 4

Conclusions

Biofilm-driven bone infections, namely caused by *S. aureus* – *C. albicans* polymicrobial biofilms, are considered a relevant and dramatic health problem, since they may exhibit enhanced pathogenicity and chronicity, leading to bone loss. A CT that uses at least two antimicrobial agents is seen as a potential strategy to address and control such infections.

In this work, a CT using two different antimicrobial drugs (ceftriaxone and amphotericin B) was tested against mono- and dual-species biofilms of *S. aureus* and *C. albicans*. For those purposes, growth kinetics of mono-species biofilms were studied to assess at which moment they reach maturation, corresponding to an infection. Additionally, mature mono- and dual-species biofilm were developed to evaluate the activity of the two antimicrobial drugs.

Growth kinetics of mono-species biofilms were characterized by CFUs counting and showed that both mono-*S. aureus* and *C. albicans* biofilms reached a mature stage at 48 h, corresponding to a stabilized infection.

Regarding the sensitivity of mono-*S. aureus* biofilms to the antimicrobial agents, ceftriaxone displayed high antibiofilm activity against *S. aureus* and its bactericidal activity was not altered by the presence of amphotericin B. The antifungal had no impact in *S. aureus*.

Mono-*C. albicans* biofilms showed susceptibility to amphotericin B and its fungicidal activity was not altered by the presence of ceftriaxone. The antibiotic had no impact in *C. albicans*.

Concerning dual-species biofilms, they show a higher amount of *S. aureus* than *C. albicans*, presumably due to difference in size of the microorganisms. Within dual-species biofilm, ceftriaxone was able to target and reduce *S. aureus*, whereas amphotericin B targeted and reduced *C. albicans*. The CT significantly reduced *S. aureus* density, but it had no impact in *C. albicans* density, within dual-species biofilm, speculating that *S. aureus* might have a protective role on *C. albicans*.

In summary, the CT was able to reduce the density of mono-species biofilms, as well as the overall density of a dual-*S. aureus* and *C. albicans* biofilm. Moreover, the results proved that the combination of two different kind of antimicrobials did not affect their antimicrobial activity.

Nevertheless, more studies should be done to verify the activity of both mono- and CT in other time-points and to bolster their effectiveness. As they were able to reduce mono- and dual-species biofilms, increasing their concentrations might ensure biofilms eradication.

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