UNIVERSIDADE D
COIMBRA

Ana Patrícia Silva Abrantes

INTERKINGDOM MICROBIAL BIOFILM AND THE QUEST FOR NOVEL THERAPIES

Dissertação no âmbito do Mestrado em Farmacologia Aplicada orientada pela Professora Doutora Gabriela Conceição Duarte Jorge da Silva e coorientada pela Professora Doutora Sara Margarida dos Santos Domingues e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Setembro de 2022

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List of abbreviations

CLSI – Clinical and Laboratory and Standards Institute

HIV – Human Immunodeficiency Virus

ICU – Intensive care unit

MH – Mueller Hinton

MIC – Minimal Inhibitory Concentration

PCR – Polymerase Chain Reaction

qPCR – Real-time polymerase chain reaction

QS – Quorum Sensing

Rpm – Rotations per minute

RT-PCR – Reverse transcription-polymerase chain reaction

TSA – Tryptic Soy Agar

YPD – Yeats Peptone Dextrose

Abstract

Bacterial-fungal interactions are common in nature and clinical environments. These interactions usually occur in a form of biofilm, which is a community of microorganisms attached to an abiotic or biotic surface. Polymicrobial biofilms are more tolerant to antimicrobials than single species biofilm, limiting conventional drug therapy. The biological relevance of microbial interactions remains largely unknown. The main objective of this study was to evaluate the microbial interaction in a biofilm formation and the impact in the treatment of infection. The specific objectives were: to investigate the synergetic or antagonist interaction between bacteria and fungi, using *Acinetobacter* spp. and *Candida* spp. in a biofilm model, and to evaluate the effect of antibiotics in the biofilm formation.

Biofilms were formed in diverse culture media with different strains of *Acinetobacter* spp. and *Candida* spp. The methodology used was: evaluation of biomass in single species and mixed biofilms by using the crystal violet method after 24h of incubation; measurement of biomass in the absence and presence of antimicrobials; assessment of *C. albicans* virulence by microscopic observation of the formation of hyphal in the absence and presence of *Acinetobacter* spp.; and evaluation of expression of *Acinetobacter* spp. biofilm-associated genes (*ompA* and *csu*) by RT-qPCR.

We observed that the medium and the relative quantity of cells used can influence the biofilm production. Microscopic observation showed that *C. albicans* YP0037 formed pseudohyphae when in contact with *A. baumannii* 319 and *A. bereziniae* 118, while *C. tropicalis* M152540979 developed pseudohyphae in the presence and absence of the bacteria. Addition of sub-MIC of ciprofloxacin before biofilm incubation showed that mixed biofilms of *A. baumannii* 319 with *C. albicans* YP0037 and *C. glabrata* M14331 produced more biomass than single species biofilm; however, with *C. tropicalis* M152540979, biofilm formation was reduced. Addition to mixed biofilms of *A. bereziniae* 118 with *C. albicans* YP0037, *C. glabrata* M14331 and *C. tropicalis* M152540979, lead to a reduction in the biofilm formation. Sub-MICs of gentamicin added to an 8 hour-biofilm lead to an increase of biomass of the mixed biofilm. There was no possibility to conclude if the expression of *ompA* and *csu* was significant in *Acinetobacter* spp. Some studies showed that the interaction between *A. baumannii* and *C. albicans* are antagonistic, leading to the inhibition of biofilm, which is in contrast with our results for *Acinetobacter* spp.- when in contact with *Candida* spp.. Different methodologies can explain this observation or different strains/species do not behave identically, which accounts for the difficulty of treatment of polymicrobial infections. Moreover, virulence of fungi can be

enhanced in mixed biofilms. Antibiotics can interfere with biofilm production, even when already formed, increasing the biomass and challenging the treatment of polymicrobial infections. Overall, this study demonstrates the complex interactions of polymicrobial biofilms and how they can affect conventional therapy, urging for the study of mechanistic interactions to find new therapeutic targets.
Keywords: *Candida* spp., *Acinetobacter* spp., antimicrobial resistance, fungi, Gram-negative bacteria

Resumo

Interações entre bactérias e fungos são comuns na natureza, assim como em ambientes clínicos, e por vezes estas interações ocorrem em forma de biofilmes, que consistem em comunidades de microorganismos que se encontram ligados superfícies abióticas e bióticas. Os biofilmes polimicrobianos toleram mais os antibióticos do que biofilmes de apenas de uma espécie, o que leva a limitação no uso de terapêuticas convencionais. A relevância biológica destas interações, na maioria, ainda é desconhecida. O objetivo principal desde estudo era a avaliação das interações microbianas no decorre da formação de biofilme, e o impacto que podem ter no tratamento da infeção. Os objetivos específicos eram: investigar as interações de sinergismo ou antagonistas entre bactérias e fungos *Acinetobacter* spp. and *Candida* spp como modelo de estudo, e avaliar o efeito de antibióticos na formação de biofilmes.

Os biofilmes foram desenvolvidos em diversos meios de cultura, assim como foram usadas diferentes estirpes de *Acinetobacter* spp. and *Candida* spp., e a metodologia usada consistiu em avaliar a biomassa formada em biofilmes simples e polimicrobianos, usando o método de cristal violeta depois de 24h de incubação; medir a biomassa na presença e na ausência de antimicrobianos; avaliar a virulência de *C. albicans* através da observação ao microscópio da formação de hifas quando em contacto com *Acinetobacter* spp.; e avaliar a expressão de genes (*ompA* and *csu*) que se encontram associados à formação de biofilmes, usando a técnica de RT-qPCR.

Observou-se que o meio de cultura e a quantidade relativa de células (bactéria-fungo) que é usada pode influenciar a produção de biofilme. A observação microscópica demostrou que *C. albicans* YP0037 formou pseudo-hifas quando em contacto com *A. baumannii* 319 e *A. bereziniae* 118, no entanto *C. tropicalis* M152540979 formou pseudo-hifas estando sozinha ou em contacto com as *Acinetobacter* spp. A adição de concentrações sub-inibitórias (sub-MICs) de ciprofloxacina antes da incubação do biofilme demonstrou que os biofilmes polimicrobianos de *A. baumannii* 319 e *C. albicans* YP0037, e *C. glabrata* M14331produziram mais biomassa do que os biofilmes de cada espécies sozinhas. No entanto, *C. tropicalis* M152540979 demostra uma redução na produção de biomassa. A adição de concentrações sub-inibitórias (sub-MICs) de ciprofloxacina antes da incubação do biofilme demonstrou que os biofilmes polimicrobianos de *A. bereziniae* 118 e *C. albicans* YP0037, e *C. glabrata* M14331e *C. tropicalis* M152540979 levam a uma redução na formação de biofilme. A adição de sub-MICs depois do biofilme ter 8 horas de formação levou a um aumento na produção de biomassa nos biofilmes. Não foi possível concluir se a expressão dos genes *ompA* e *csu* são ou não significantes em *Acinetobacter* spp.

Alguns estudos afirmam que existe uma interação antagonista entre *A. baumannii* e *C. albicans* e que isso levaria a inibição do biofilme, no entanto os nossos resultados para *Acinetobacter* spp quando em contacto com *Candida* spp. demostram o oposto. Diversas metodologias podem explicar esta diferença nos resultados, como o facto de serem diferente espécies e estirpes e isso levar a que não se comportem de maneira igual, e isso realça um problema que é o tratamento destes biofilmes polimicrobianos. A virulência dos fungos pode aumentar quando se encontram em biofilmes, assim como a adição de antibióticos pode interferir com a produção de biomassa, aumentando-a, mesmo quando o biofilme já se encontra formado. Em conclusão, este estudo serve para demostrar a complexidade que são as interações nos biofilmes polimicrobianos, e como isso pode afetar a terapêutica convencional, assim como realçar a urgência para o estudo destas interações de forma a descobrir novos alvos terapêuticos.
Palavras-chave: *Candida* spp., *Acinetobacter* spp., resistência antimicrobiana, fungo, bactéria Gram-negativa

1. Introduction

Bacterial-fungal interactions are common in nature and in clinical environment, yet the molecular mechanisms underneath this interaction are still unclear and how they can affect the human health. Microorganisms have complex mechanisms so they can survive, defend themselves against advert environmental and nutritional conditions, as well as competing organisms, but also can impact one another's virulence. Interactions can be physical, chemical exchanges, changes in the environment, alteration of the host immune response or use of metabolic by-products (Fourie, R. and Pohl, C.H., 2019, Frey-Klett, P. [et al.], 2011, Stanley, C.E. [et al.], 2014). For example, the identification of penicillin was a result of a bacterial-fungal interaction (Fleming, A., 1929, Peleg, A.Y. [et al.], 2010).

Usually, polymicrobial interactions are commensal, however they can evolve to a disease, when occurs an imbalance in the normal microbial flora as a result of antimicrobial therapy or deficiencies in the host immunity. In situations of health, bacteria and fungi coexist on cutaneous and mucosal surfaces, such as the skin, the oral cavity, the gastrointestinal tract, and the vagina. Despite this, external factors can cause injuries to the skin, the poor oral hygiene can cause imbalance in the microorganisms, or as a consequence of chemotherapy, and that's when these interactions became pathogenic (Gupta, N. [et al.], 2005, Hermann, C. [et al.], 1999, Peleg, A.Y. [et al.], 2010, Stanley, C.E. [et al.], 2014). These injuries can damage the skin barrier and enter in the bloodstream, which should be sterile. For example, colonization by bacteria and fungi in the respiratory tract are very recurrent in patients with chronic lung diseases (Bauernfeind, A. [et al.], 1987, McAlester, G. [et al.], 2008) and in patients breathing thought the ventilator in intensive care units (Azoulay, E. [et al.], 2006, Ibrahim, S. [et al.], 2021, Raad, II [et al.], 2011, Rosenthal, V.D. [et al.], 2006), where bacterial-fungal biofilms are normally found (Adair, C.G. [et al.], 1999, Kojic, E.M. and Darouiche, R.O., 2004). Medical devices that go through the skin can be also affected by polymicrobial biofilms (MARRIE, T.J. and COSTERTON, J.W., 1984, Tchekmedyian, N.S. [et al.], 1986). Nevertheless, polymicrobial interactions can increase the morbidity and mortality when compared with single species interactions, duo to the complexity of the interactions (Carlson, E., 1982, Fourie, R. and Pohl, C.H., 2019, Stanley, C.E. [et al.], 2014, Thi, M.T.T. [et al.], 2020, Wambaugh, M.A. [et al.], 2020).

1.1. Biofilms

A biofilm is a structured 3D community of microorganisms attached to a surface, which can be an abiotic or biotic surface (Cavalheiro, M. and Teixeira, M.C., 2018, Harriott, M.M. and Noverr, M.C., 2009, Tsui, C. [et al.], 2016). They also offers a physical and metabolic barriers, which make microorganisms in biofilms much more resistant to antimicrobials when compared with microorganisms that grow independently or as planktonic bacteria (Ortega-Pena, S. [et al.], 2017, Rodrigues, C.F. and Cernáková, L., 2020), that is why polymicrobial infections are harder to treat clinically (Dixon, E.F. and Hall, R.A., 2015). The microorganism in this communities exhibit a lower growth rate; however, it exhibits a higher resistance to antimicrobial treatment (Cavalheiro, M. and Teixeira, M.C., 2018, Harriott, M.M. and Noverr, M.C., 2009, Sun, X. and Xiang, J., 2021), which is very different when compared with planktonic cells. They have the ability to adhere to different types of surfaces, which allows microorganisms to form biofilms on medical devices, such as intravascular catheters, prosthetic heart valves and joint replacements, or other tissues of the host, which can lead to permanent colonization and infections (Cavalheiro, M. and Teixeira, M.C., 2018, Harriott, M.M. and Noverr, M.C., 2009, Nett, J. and Andes, D., 2006). It is estimated that about 65% of bacterial infections are associated with bacterial biofilm (Lewis, K., 2001), which can be device or non-device-associated infections (Darouiche, R.O., 2004).

Even though, single microbial species can form biofilm*, in vivo* a mixture of bacterial and fungal species are the usual, given that 80% of the microorganisms live in this form (Cavalheiro, M. and Teixeira, M.C., 2018).

The mechanisms underlying biofilm resistance include: (1) an incomplete penetration by antibiotics and host immune cells through the matrix, (2) physiological changes in the biofilm microenvironment due to low growth and starvation responses, (3) phenotypic changes in biofilm cells, (4) quorum sensing (QS) between biofilm microorganisms, (5) expression of efflux pumps which can transport antimicrobial agents out of cells, and (6) the presence of a small fraction of microorganisms that are able to survive antibiotics (Rodriguez-Cerdeira, C. [et al.], 2019).

1.2. *Candida* spp.

Candida is one of the most common human fungal pathogens and represents the most important cause of opportunistic mycoses worldwide (Soliman, S.S.M. [et al.], 2017), as well as bloodstream infections with mortality rates of up to 60% (Glockner, A. and Cornely, O.A., 2015). However, *Candida* species are commensal fungi that are part of the human microbiota

(Atriwal, T. [et al.], 2021, Nobile, C.J. and Johnson, A.D., 2015, Shirtliff, M.E. [et al.], 2009). *Candida* species, namely *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida dubliniensis*, and *Candida tropicalis*, are responsible for a variety of infections linked to biofilm development (d'Enfert, C. and Janbon, G., 2016).

1.2.1. Candida albicans

Candida albicans is the most prevalent species, and can be found in a normal biota of the vagina, mouth, bowel, and skin (Matare, T. [et al.], 2017). *C. albicans* is an opportunistic pathogen, and in conditions of immune dysfunction, stress and prolonged use of antibiotics can cause a variety of infections, usually associated with a biofilm-related infection (Ponde, N.O. [et al.], 2021, Shirtliff, M.E. [et al.], 2009, Tsui, C. [et al.], 2016, Van Dyck, K. [et al.], 2021). For example, these infections can go from superficial mucosal and dermal infections, such as thrust/candidiasis, vaginal yeast infection, and diaper rash, to hematogenous disseminated infections with significant mortality rates that can go up to 40% in some cases (Nobile, C.J. and Johnson, A.D., 2015).

As a dimorphic fungus, *C. albicans* can transit from commensal to pathogen. Due to this ability, it can shift between yeast, pseudohyphae, and hyphal. The yeast form are single cells which are oval and can present both axial and bipolar budding forms. The pseudohyphae and hyphae are usually titled as filamentous morphologies because the cells normally grow in a differentiated manner, are elongated in form, and are attached end to end. Pseudohyphae are normally ellipsoid and have constrictions at the septal junctions, however hyphal cells generally have parallel sides, have a uniform size and own a true septal lacking constrictions (Chen, H. [et al.], 2020, Thompson, D.S. [et al.], 2011, Tsui, C. [et al.], 2016). This transition between yeast and hyphal is associated with pathogenesis and the ability to form biofilm (Soliman, S.S.M. [et al.], 2017, Tsui, C. [et al.], 2016).

Dispersion of biofilm-associated cells carries an enormous clinical significance, as released cells can form new biofilms or spread into host tissues. Therefore, they are associated with candidemia and disseminated invasive disease (Cavalheiro, M. and Teixeira, M.C., 2018, Ponde, N.O. [et al.], 2021, Tsui, C. [et al.], 2016). *Candida* biofilm-associated infections are one of the main challenges in clinical settings due to their resistance to antifungals and the host immune response (Matare, T. [et al.], 2017, Ponde, N.O. [et al.], 2021, Shirtliff, M.E. [et al.], 2009, Tsui, C. [et al.], 2016, Van Dyck, K. [et al.], 2021). The reduced susceptibility to antimicrobial agents of a biofilm associated infection is due to the incapability of the drug to

pass through the extracellular polysaccharide matrix and exposure of the cells to sub-minimum inhibitory concentrations (MICs) of the antimicrobial (Mba, I.E. and Nweze, E.I., 2020).

The cell wall of *Candida albicans* is composed by 4 main components: mannoproteins (which represents about 40% of the cell-wall biomass), β-1,3-glucan (the main stress-barrier polysaccharide of the wall), β -1,6-glucan (water-soluble component that interconnects mannoproteins to β-1,3-glucan and chitin chains) and a small amount of chitin (linear stress-bearing polysaccharide) (ten Cate, J.M. [et al.], 2009).

Farnesol is a quorum sensing molecule that inhibits filamentation and biofilm formation of *Candida albicans*. Farnesol is secreted continuously during *C. albicans* growth and it might prevent yeast-to-filament conversation. It's been showed that farnesol can also affect the viability and virulence of some bacterial species (Costa, A.F. [et al.], 2021, Kostoulias, X. [et al.], 2016, Rodrigues, C.F. and Cernáková, L., 2020). Quorum sensing is a cell-cell communication phenomenon in microorganisms, which can be mediated by secretion of small metabolites and plays an important role in biofilm formation. As well as it might have a bigger role in polymicrobial biofilms. (Kostoulias, X. [et al.], 2016, Ponde, N.O. [et al.], 2021, Tuttobene, M.R. [et al.], 2021).

1.2.1.1. *Candida albicans* biofilm

C. albicans produces an extremely structured biofilms composed of multiple cell types (i.e., round, budding yeast-form cells; oval pseudohyphal cells; and elongated, cylindrical hyphal cells) coated in an extracellular matrix (Chandra, J. [et al.], 2001, Nobile, C.J. and Johnson, A.D., 2015, Ramage, G. [et al.], 2009). The transition from planktonic cells to biofilm is followed by a complex alteration of phenotypic behavior underpinned by myriad changes in gene expression (Ponde, N.O. [et al.], 2021). The biofilm development happens in sequential phases: adherence, initiation, maturation, and dispersal, which occurs throughout 24–48 h (figure 1). The initial phase consists of the adhesion of single yeast cell to the substrate, establishing a foundation of a basal yeast cell layer. It is followed by cell proliferation across the surface and filamentation, where cells form elongated projections. The production of hyphae is fundamental for the initiation of biofilm formation, followed by the accumulation of an extracellular polysaccharide matrix as the biofilm matures. Finally, in the last phase, non-adherent yeast cells are released from the biofilm to the surroundings and colonize other surfaces (Cavalheiro, M. and Teixeira, M.C., 2018, Nobile, C.J. and Johnson, A.D., 2015, Ponde, N.O. [et al.], 2021, Tsui, C. [et al.], 2016).

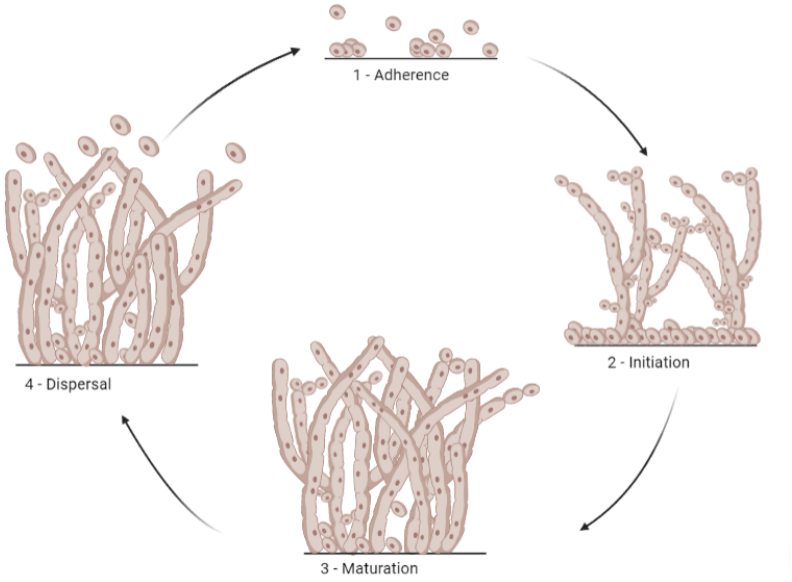


Figure 1 - Stages of *Candida albicans* biofilm formation.

1.2.2. Candida glabrata

C. glabrata is commensal fungi of the intestine tract (Glockner, A. and Cornely, O.A., 2015), and they also can colonize commensally the mouth, esophagus, and vaginal mucosal surfaces (Rodrigues, C.F. [et al.], 2014). As an infectious pathogen, *C. glabrata* is the second most common cause of Candida vulvovaginitis. It has an important role as an agent of candidemia and other invasive *Candida* infections, especially in immunocompromised patients and ICU patients. These *Candida* spp. is unable to form pseudohyphae, which leads to a less strong neutrophil response. *C. glabrata* expresses the adhesin *Epa1* which grants the ability of attachment to epithelial and endothelial surfaces in colonized and infected hosts (Glockner, A. and Cornely, O.A., 2015).

1.2.3. Candida tropicalis

Candida tropicalis is an emerging species that causes candidemia, among other things (Galan-Ladero, M.A. [et al.], 2019). *C. tropicalis* is a virulent *Candida* species with a greater capacity to form biofilms than *C. albicans*, as well as a variety of virulence characteristics such as antifungal resistance and osmo-tolerance (the ability to live at high salt concentrations). *C. tropicalis* is frequent in candidemia patients, particularly in Latin America and Asia. Antibiotic diffusion through *C. tropicalis* biofilms is the slowest of all *Candida* species, indicating that the biofilm structures may be unique (Phuengmaung, P. [et al.], 2021).

The process of yeast attachment to an available surface and the subsequent development of a biofilm can be divided into two stages: initially occurs adhesion between yeast and surfaces, which is generally mediated by nonspecific (e.g., hydrophobic) interactions, and then there's a second stage with a specific adhesion, or the anchoring phase, which is mediated by molecularly mediated binding between specific adhesins and the biotic surface and either host cells or other microorganisms (Galan-Ladero, M.A. [et al.], 2019).

1.3. *Acinetobacter spp.*

1.3.1. *Acinetobacter baumannii*

Acinetobacter baumannii is the member of the genus *Acinetobacter* and the family Moraxellaceae of the Eubacteria class Proteobacteria. *A. baumannii* is a non-motile, non-fastidious, non-fermentative, catalase-positive, oxidative-negative Gram-negative coccobacilli (Gaddy, J.A. [et al.], 2009, Ibrahim, S. [et al.], 2021, Sun, X. and Xiang, J., 2021). They are commonly found in intensive care units (ICUs) or surgery rooms, where antibiotics are frequently used which leads to appearance of resistance against most of the existing antibiotics (Bogdan, M. [et al.], 2018, Da Cunda, P. [et al.], 2020). *A. baumannii* is known for its environmental durability, surviving for several days on abiotic/inanimate objects and surfaces in medical surroundings, even in dry conditions (Da Cunda, P. [et al.], 2020, Tomaras, A.P. [et al.], 2003). These survival properties may have a significant role in the outbreaks caused by this pathogen (Tomaras, A.P. [et al.], 2003).

A. baumannii it is a multi-drug resistance bacteria (MDR), which means that she is resistant to more than tree antibiotic classes (Magiorakos, A.P. [et al.], 2012, Oliveira, D.M.P.D. [et al.], 2020). The World Health Organization (WHO) recognized *A. baumannii* as one of the most threatening bacterial pathogens (Lopes, S.P. [et al.], 2021, Tacconelli, E. [et al.], 2018, Tuttobene, M.R. [et al.], 2021, Walsh, B.J.C. [et al.], 2020). *A. baumannii* belongs to the ESKAPE group bacteria, which is the acronym for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* e *Enterobacter spp*. (Oliveira, D.M.P.D. [et al.], 2020), which are associated with hospital-acquired antibiotic-resistance (Da Cunda, P. [et al.], 2020, Gedefie, A. [et al.], 2021, Ibrahim, S. [et al.], 2021). *A. baumannii* causes several inflections, such as in skin and soft tissues, wound infections, bacteremia, endocarditis, urinary tract infections (UTIs), meningitis and pneumonia (Aliramezani, A. [et al.], 2019, Gedefie, A. [et al.], 2021, Ibrahim, S. [et al.], 2021, Tomaras, A.P. [et al.], 2003, Walsh, B.J.C. [et al.], 2020). The most common nosocomial infection associated with *A. baumannii* is pneumonia, normally associated with patients in ICU and

breathing thought the ventilator, which can cause ventilator-associated pneumonia (VAP), it is associated with mortality rate fluctuates between 40 and 70% (Ibrahim, S. [et al.], 2021, Raad, II [et al.], 2011). *A. baumannii* infections tend to appear in immunosuppressed patients, in patients with serious underlying diseases and in those subjected to invasive procedures and treated with broad-spectrum antibiotics (Perez, F. [et al.], 2007).

1.3.2. *Acinetobacter bereziniae*

A. bereziniae, also known as genomospecies 10, and is a strict aerobic, non-fermentative, nonmotile Gram-negative coccobacillus and member of the Gammaproteobacteria. It is considered an emerging nosocomial pathogen and has been reported to be responsible for health care infections including sepsis (Bonnin, R.A. [et al.], 2012, Domingues, S. [et al.], 2019, Reyes, S.M. [et al.], 2020).

1.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative rod-shaped bacterium which is a member of the Pseudomonadaceae family. Infections caused by this bacterium are widespread in nature and live in plants, animals (including humans), soil, and water. It is an opportunistic infection that causes nosocomial infections as well as possible fatal infections in immunocompromised individuals, such as patients with cancer, burn wounds, post-surgery, HIV and cystic fibrosis (Holcombe, L.J. [et al.], 2010, Thi, M.T.T. [et al.], 2020). *P. aeruginosa* was recognized as one of the most life-threatening bacteria and listed as priority pathogen for Research and Development of new antibiotics by the World Health Organization (Thi, M.T.T. [et al.], 2020)

Due to *P. aeruginosa* flexibility and strong intrinsic drug resistance, common antimicrobial treatments such antibiotics typically demonstrate low efficiency, increasing mortality. *P. aeruginosa* has the potential to form biofilms, which protect them from environmental stress and prevents phagocytosis, offer them the capacity for colonization and long-term survival, which results in complications in the treatment of these infections. These biofilms are highly structured and they are frequently found in patients with chronic infections, such as chronic bronchitis, chronic wound infections and chronic rhinosinusitis (Thi, M.T.T. [et al.], 2020).

1.5. Bacterial biofilm

Generally, biofilms can be developed on abiotic surfaces, such as medical implants or industrial equipment. The biofilm development is divided into five distinct stages. Stage I: where

the bacterial cells adhere to a surface via support of cell appendages such as flagella and type IV pili. Stage II: the bacterial cells undergo the switch from reversible to irreversible attachment. Stage III: bacteria attachment turns into a more structured architecture, known as microcolonies. Stage IV: these microcolonies develop into extensive three-dimensional mushroom-like structures, a hallmark of biofilm maturation. Stage V: cell autolysis disrupts the matrix cavity in the center of the microcolony, releasing scattered cells, which is followed by a change from sessile to planktonic growth mode, which allows the colonization of other areas (Colquhoun, J.M. and Rather, P.N., 2020, Da Cunda, P. [et al.], 2020, Thi, M.T.T. [et al.], 2020). In the figure 2 is an illustration of the biofilm formation.

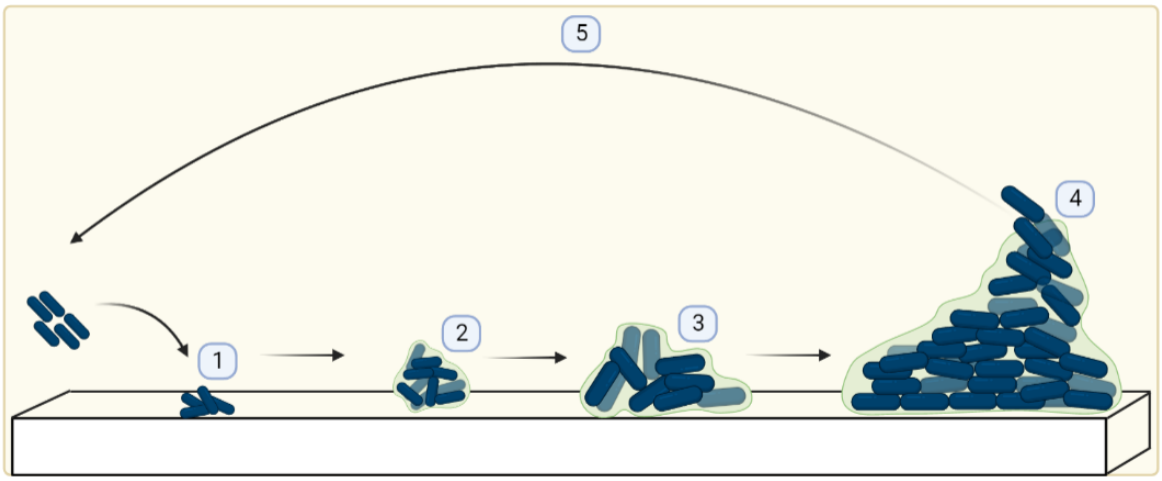


Figure 2 - Illustration of bacterial biofilm formation. 1 – Adhesion; 2 – Early attachment; 3 – Immature biofilm; 4 – Mature biofilm; 5 – Dispersal.

1.6. Bacteria-fungi interaction

1.6.1. Synergistic interaction

Polymicrobial synergy is a cooperative interaction between two or more species, which can produce an effect that an individual species alone could not. In biofilms or biofilms-related infections, these effects can lead to an increase in growth, antimicrobial tolerance, virulence and can enhance production of exopolysaccharide. Another possible interaction can be by metabolic cross-feeding or syntrophy, where one of the species produces a metabolic byproduct that enhances the growth of the other species (Gabrilska, R.A. and Rumbaugh, K.P., 2015, Shirtliff, M.E. [et al.], 2009).

1.6.1.1. Examples of synergetic interactions

Some examples of this type of interactions are: the interaction of ***C. albicans*** **and** ***Streptococcus mutans***, usually they are found together in the mouth. *S. mutans* glucosyltransferases can bind directly to the mannans presents on the surface of *C. albicans* yeast and hyphal cell walls, leading to the increase the formation and development of mixed biofilms (Ponde, N.O. [et al.], 2021). As well as the bacterial EPS can bind and retain fluconazole, reducing the uptake and intracellular transport of the drug, which enhances *C. albicans* tolerance to azole drug spectrum (Van Dyck, K. [et al.], 2021). There is QS molecules that are secreted, *C. albicans* secretes farnesol that when in dual species biofilm can enhance cell growth and microcolony development of *S. mutans* (Kim, D. [et al.], 2017, Ponde, N.O. [et al.], 2021) and *S. mutans* secretes mutanobactin A, trans-2-decenoic acid and competence-stimulating peptide which can inhibit *C. albicans* germ tube formation and the transition yeast to hypha (Jarosz, L.M. [et al.], 2009, Ponde, N.O. [et al.], 2021, Vilchez, R. [et al.], 2010).

The interactions of ***C. albicans*** **and** ***Staphylococcus epidermidis***. *Staphylococcus epidermidis,* is often associated with implant-associated infections. This interaction is often observed in *C. albicans* bloodstream infections and has been showed that the co-infection has an increased mortality when compared with mono-microbial infections (Carolus, H. [et al.], 2019, Van Dyck, K. [et al.], 2021). *S. epidermidis* produces extracellular polymers which protects *C. albicans* against fluconazole via inhibiting penetration of the drug in the polymicrobial biofilm (Adam, B. [et al.], 2002, Van Dyck, K. [et al.], 2021).

The interaction of ***C. albicans*** **and** ***Staphylococcus aureus*** are often found in a variety of biofilm-associated diseases, including periodontitis, cystic fibrosis, and denture stomatitis (Van Dyck, K. [et al.], 2021). *S. aureus* by himself is a poor biofilm producer, however, when in the presence of *C. albicans*, they can form a considerable amount of biofilm since the fungus creates a scaffold for the bacteria (Kong, E.F. [et al.], 2016). This interaction can leads to an increase of pathogenicity and enhanced drug tolerance (Van Dyck, K. [et al.], 2021).

1.6.2. Antagonist interaction

Polymicrobial antagonism can also be called antibiosis and can be defined as the suppression of one microbial species by another. Antagonistic mechanism consists of production of factors that kill or inhibit the growth of neighbors, production of chemical signals that can interfere or disrupt the behavior or physiology of neighbors or stealing the nutrients, letting the neighbors starve. One of the species can occupy all the attachment sites on a surface

so they can prevent the other to attach (Gabrilska, R.A. and Rumbaugh, K.P., 2015, Shirtliff, M.E. [et al.], 2009).

1.6.2.1. Examples of antagonist interactions

The interaction between ***C. albicans* and *P. aeruginosa*** can occur in cystic fibrosis patients, where the coexistence of the Gram-negative bacterium *P. aeruginosa* and *C. albicans* is frequent (Fourie, R. and Pohl, C.H., 2019, Van Dyck, K. [et al.], 2021). *P. aeruginosa* is known to attach to *C. albicans* filaments, forming biofilm over the hyphae instead of the surface, which leads to the death of *C. albicans* filamentous cells, although yeast cells remain viable (Cavalheiro, M. and Teixeira, M.C., 2018, Hogan, D.A. and Kolter, R., 2002). The presence of secreted factors by *P. aeruginosa* causes downregulation of genes involved in adhesion and biofilm formation and increases the expression of genes encoding drug exporters, such as *CDR1* and *SNQ2*, and the *YWP1* gene encoding a protein involved in biofilm dispersal, in *C. albicans* biofilms. The factors secreted by *P. aeruginosa* seem to have a specific effect in the maturation phase of biofilm formation by *Candida spp*. (Cavalheiro, M. and Teixeira, M.C., 2018, Holcombe, L.J. [et al.], 2010). *In vitro* studies, suggested that *P. aeruginosa* inhibits the growth of *C. albicans*, acting as an antagonistic (Fourie, R. and Pohl, C.H., 2019, Van Dyck, K. [et al.], 2021).

The interaction of ***Lactobacillus* spp. and *C. albicans*** is common in a normal vaginal microbiota, *Lactobacillus* spp. compete with *C. albicans* for receptors present on the surface of genitourinary epithelium for adhesion. As well as secrete lactic acid and hydrogen peroxide, which lowers the pH and it inhibits fungal attachment to the vaginal epithelium. It's been showed that both hyphal formation and biofilm development is affected by the bacteria (Boris, S. and Barbés, C., 2000, BORIS, S. [et al.], 1998, Ponde, N.O. [et al.], 2021).

The interaction of ***C. albicans* and *A. baumannii*** appears to inhibit *C. albicans* filamentation, which decreased the virulence of *C. albicans*. However, *C. albicans* also is able to inhibit *A. baumannii* growth via farnesol production which is a quorum sense molecule (Shirtliff, M.E. [et al.], 2009). Quorum sensing is a cell-cell communication phenomenon in microorganisms, which can be mediated by secretion of small metabolites and plays an important role in biofilm formation. As well as it might have a bigger role in polymicrobial biofilms. (Kostoulias, X. [et al.], 2016, Ponde, N.O. [et al.], 2021, Tuttobene, M.R. [et al.], 2021). Farnesol was first identified as an extracellular sesquiterpene that was responsible for mediating quorum sensing in *C. albicans* (Costa, A.F. [et al.], 2021, Kostoulias, X. [et al.], 2016). It's been showed that inhibits filamentation and biofilm formation of *Candida albicans*. The

molecule is secreted continuously during *C. albicans* growth, and it might prevent yeast-to-filament. Also, it's been showed that farnesol can also affect the viability and virulence of some bacterial species (Costa, A.F. [et al.], 2021, Kostoulias, X. [et al.], 2016, Rodrigues, C.F. and Cernáková, L., 2020). It's also been showed by using *Caenorhabditis elegans* polymicrobial infection assay, that *A. baumannii* can inhibit various virulence determinants of *C. albicans*, including hyphae and biofilm formation (Peleg, A.Y. [et al.], 2010, Peleg, A.Y. [et al.], 2008). Another study has showed that the outer membrane protein A (*ompA*) of *A. baumannii* is crucial for the attachment of the bacterium to *C. albicans* filaments, as well as to attach to mammalian epithelial cells and is followed by apoptotic cell death. However, *C. albicans* is capable of "attacking" the bacterium by secreting farnesol, a quorum sensing molecule that reduces the viability of *A. baumannii* growth (Gaddy, J.A. [et al.], 2009, Peleg, A.Y. [et al.], 2010).

2. Objectives

The main objective of this study is to better understand the microbial interaction in an interkingdom mixed biofilm and to unravel the challenges to treat these kinds of infections. Moreover, the majority of the studies involve the well-known pathogen *C. albicans*, and we will extend it to other clinical *Candida* species. The interaction of *Candida* spp. and *A. baumannii* is rarely reported and we will use different *A. baumannii* strains and other *Acinetobacter* species as a model.

Thus, to achieve the main goal, the specific objectives are: i, to investigate the synergetic or antagonist interaction of *Candida spp.* and Gram-negative bacteria in a biofilm; ii, to evaluate the activity of antibiotics in the biofilm formation (in *C. albicans* biofilm and mixed biofilm).

We expect changes in the virulence of *Candida albicans* when in the presence of Gram-negative bacteria. Also, it is expected that some antibiotics may inhibit the formation of biofilm and/or change the virulence of the fungi.

Overall, we expect to improve our scientific knowledge on the interkingdom microbial interaction in a biofilm and the impact in the virulence of *Candida* spp. and in the treatment of infection.

3. Materials e Methods

3.1. Microorganism Strains

Candida albicans YP0037 strain was kindly provided by Prof. Doctor Teresa Gonçalves from the Faculty of Medicine Microbiology Pathogenic Yeast Collection, University of Coimbra. *Candida glabrata* M14331 and *Candida tropicalis* M152540979 were clinical isolates from urine and blood samples, respectively, kindly provided by Dra. Cristiana Canha from Centro Hospitalar Universitário de Coimbra. *Acinetobacter baumannii* ATCC 19606, *Acinetobacter baumannii* 319, *Acinetobacter baumannii* 65JFFUC, *Acinetobacter baumannii* 189 HUC, *Acinetobacter bereziniae* 118, *Acinetobacter baumannii* 2798Pb, *Acinetobacter baumannii* 2813Pb mucosa, *Acinetobacter baumannii* 409231 (colónias pequenas) were obtained from the *Acinetobacter* sp. collection of the Laboratory of Microbiology of the Faculty of Pharmacy, University of Coimbra. Yeast and bacteria were stored at -80°C until use.

3.2. Biofilm formation assay

The biofilm assay was based on the method described by Stepanovic et al. (2000) and Peeters E, et al. (2008), with some modifications (Peeters, E. [et al.], 2008).

Yeast cells were grown at 37°C/18-24h in Sabouraud Dextrose Agar. Bacterial cells were cultivated at 37°C/18-24h in Trypticase-Soy agar (TSA) (Sigma-aldrich, USA). After the incubation they were transferred to 5mL of Yeast Peptone Dextrose (YPD) (Yeats extract (Quilaban, Portugal); Peptone (Scharlab, Spain); Dextrose (Scharlab, Spain)), and posteriorly were incubated at 37°C for 24h at 150 rpm (rotations per minute) in an orbital shaker (New Brunswick Scientific CO., USA). The inoculum with a turbidity identical to 0,5 McFarland standard was obtained by adding a few drops of medium to 5mL of YPD and measuring in a McFarland Densitometer (DEN-1 B, Biosan, EU). Then, 200 µL of each suspension were added to a 96 wells plates (Biosigma, Italy), in triplicate, as well as 200 µL of YPD for a sterility control. The plates were incubated at 37°C for 24h 150 rpm in an orbital shaker (New Brunswick Scientific CO., USA). After 24h of incubation, the supernatant was removed by inverting the plate. The wells were washed with milliQ water, then the plate was inverted and let it airdry for 5 min. Following, 200 µL of 99% (v/v) ethanol (VWR Chemicals, France) were added to each well for 15min for fixation, then the supernatant was removed by inverting the plate and let it airdry for 5min. Two hundred µL of crystal violet (Applichem, Germany) for 20min, afterward the excess of crystal violet was removed by inverting the plate. The wells were washed with milliQ water until the water came out clean, then the plate was inverted and let it airdry for 5 min. To each well were added 200 µL of 33% (v/v) acetic acid (Pronalab,

Portugal), in order to solubilize dye and biofilm biomass, followed by 1min homogenization of the plate in an orbital shaker (New Brunswick Scientific CO., USA). Subsequently, each well was transferred to a 96 wells plate and was read at 590nm in the plate reader (Synergy HT, BioTek, USA). The assay was performed in triplicate. The figure 1 illustrates the biofilm formation assay.

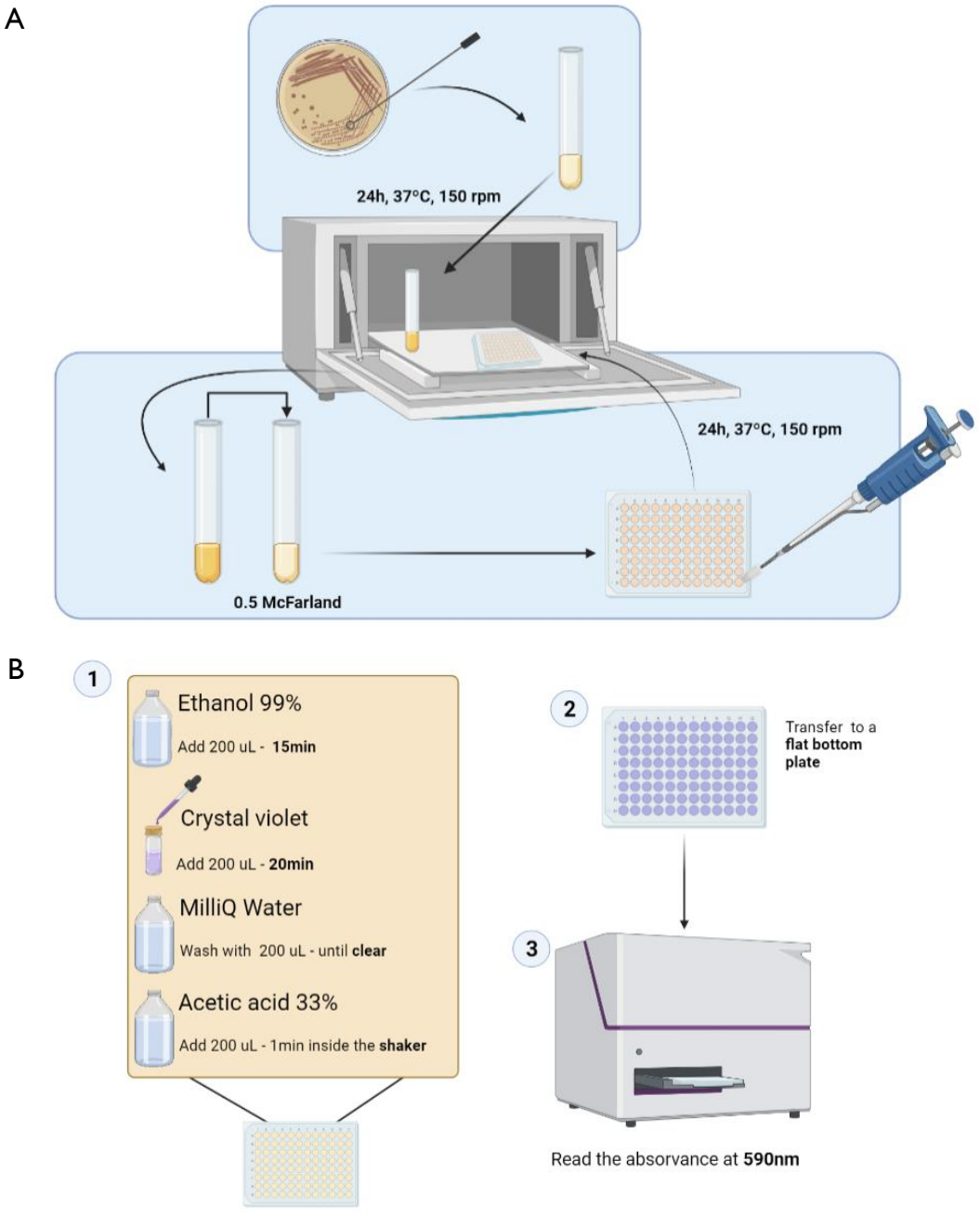


Figure 3 - Biofilm formation by using the crystal violet method. A – General procedure: preparation of the inoculum, inoculation of the 96 plate wells, incubation and reading; B – Protocol to read the biomass of the biofilm: staining and reading the 96 well plate.

3.3. Biofilm formation assay with different relative quantity of cells

The mixed biofilms were formed with one *A. baumannii* strain (ATCC 19606 and 319) and *C. albicans* YP0037. The inoculum of each microorganism and the formation of biofilm was prepared as previously described. The variations were the relative volumes of the inoculum of bacteria and yeast, where volumes of 100 µL -100 µL, 125 µL – 75 µL and 150 µL - 50 µL were tested. The 200 µL inoculum that was added to each well was divide in the previous volume, where 150 µL would be bacteria and 50 µL yeast and vice versa, as well as the other volume. The assay was performed in triplicate.

3.4. Determination of minimal inhibitory concentrations

In this assay, gentamicin (PanReac AppliChem, US) and ciprofloxacin (AppliChem, US) were used. Gentamicin (PanReac AppliChem, US) is an aminoglycoside, which has a bactericidal activity against aerobic gram-negative bacteria. After the Gram-negative membrane is passed through, once in the cytoplasm it binds to the 16s rRNA at the 30s ribosomal subunit. It will disrupt mRNA translation, which leads to the formation of truncated or non-functional proteins. Ciprofloxacin (AppliChem, US) is a 2nd generation fluoroquinolone, which inhibits DNA replication by inhibiting bacterial DNA topoisomerase IV and DNA-gyrase.

The minimal inhibitory concentrations (MICs) were determined by the microbroth dilution method according with extenso (CLSI) guidelines, using 96 wells plates.

Stock solutions of gentamicin and ciprofloxacin were prepared in 15mg/mL and 10 mg/mL concentrations, respectively. The antibiotic was added to a 15 mL Falcon tube, and it was added 10 mL of sterilized milli-Q water. After, a work solution was prepared in a 2 mL tube with a concentration of 1024 µg/µL. To each well of the plate 100 µL of Mueller-Hinton broth (MH) (Sigma-Aldrich, Spain) was added, and then it was added 100 µL of the work solution to the first well of each row, which resulted in a 512 µg/µL concentration in the first well. Then it was mixed by pipetting up and down movement and consecutive dilutions 1:2 were made, passing consecutively 100 µL to the next well and mixed. The process was repeated until the penultima well, where after mixed, it was discarded 100 µL and the tip. The last well of each row was a growth control, medium and microorganism were present. The assay was performed in triplicate.

3.6. Biofilm formation assay with addition of antibiotic

The inoculum of each microorganism, the formation of biofilm was prepared as previously described. The mixed biofilms were formed with one bacterial strain and a *Candida* spp. The variations were the addition of the antibiotic before adjusting the turbidity to 0,5 McFarland standard and to the well after 8 hours of incubation. The addition of antibiotic to the suspension was made according to the MIC of each antibiotic and species, and according to the volume of the suspension. The assay was performed in triplicate.

3.7. RT-qPCR

The RT-qPCR was based on the method described by (Amin, M. [et al.], 2019, Navidifar, T. [et al.], 2019), with some modifications.

In order to measure the genetic expression of biofilm-associated genes in *Acinetobacter* spp.: *ompA* and *csuE*. The *ompA* gene (major outer membrane protein) has an important role in the attachment of bacterial cells in abiotic surfaces and human alveolar epithelial cells and the *csuE* gene encodes part of the assembling system Csu-pilis and acts as an adhesin that binds to the surface in the beginning of the biofilm formation process (Amin, M. [et al.], 2019, Gaddy, J.A. [et al.], 2009).

3.7.1. RNA extraction

The RNA extraction was performed by using the Thermo Scientific GeneJET RNA Purification Kit (Thermo Scientific, EU) and following the kit protocol with a few alterations.

The RNA was extracted from mixed biofilms of a bacterial strain with *Candida* spp., as well as from single bacterial strain biofilm, and planktonic cells of bacterial strains.

The mixed biofilms were formed with one bacterial strain and a *Candida* spp. The inoculum of each microorganism, the formation of biofilm was prepared as previously described. The variations were the relative volumes of the inoculum of bacteria and yeast duo to the use of 24 wells plate instead of 96 wells plate, and the volumes varied from 200 µL to 2 mL.

The RNA was stored at -20°C until further use. The concentration and the purity of the RNA were assessed by using NanoDrop (ND-1000, Thermo Scientific, EU).

3.7.2. Reverse transcription

After the RNA extraction, it was reverse transcribed to cDNA and was used the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, EU),

following the manufacture protocol. The cDNA was kept at -20°C. The table 1 shows the components and volumes used, as well as the thermocycler (T-1 Thermoblock, Biometra, Germany) definitions.

Table 1 - Program and reaction components for reverse transcriptase PCR.

Program		Reaction (final volume – 20 µL)	
94°C, 3 min	1x		
94°C, 30 s 58°C, 30 s 72°C, 30 s	35x	5X Reaction Buffer	4 µL
		RiboLock RNase Inhibitor (20 U/µL)	1 µL
		10mM dNTP Mix	2 µL
		RevertAid M-MiLV RT (200 U/µL)	1 µL
		Primer	1 µL
		Water, nuclease free	1 µL
		Sample	10 L

3.7.3. Real time PCR

Real-time PCR amplification reaction was prepared in a final volume 20 µL, with 400 ng cDNA. To each well was added 10µL of SYBR Green (BIO-RAD, US), 0,8µL of the reverse and forward primer (Stabvida, Portugal), 6,4µL of RNase-free water and 2µL of sample (cDNA), as is shown in table 2. The primer sequences used for the genes involved in biofilm formation (*ompA and csuE*) are shown in table 3. The 16rRNA gene was used as an internal control for the normalization of the mRNA expression.

Real-time PCR was performed in a thermocycler as follow: on cycle of initial denaturation at 95°C for 15 s, 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, as table 2 shows.

Table 2 - Program and reaction components for real time PCR.

Program		Reaction (final volume – 20 µL)	
95°C, 15s	1x	MM SYBR Green	10 µL
95°C, 30s 55°C, 30s 72°C, 30s	40x	Primers (F)	0,8 µL
		Primers (R)	0,8 µL
		RNase-free water	6,4 µL
		Sample (cDNA)	2 µL

Table 3 - Primers used in real time PCR.

Gene	Primer seq.	Product	Reference
CsuE	F: TCAGACCGGAGAAAAACTTAACG		Amin, M. [et al.], 2019, Gaddy, J.A. [et al.], 2009
	R: GCCGGAAGCCTGTATGTAGAA		
AbOmpA	F: ATGAAAAAGACAGCTATCGCGATTGCA		
	R: CACCAAAAGCACCAGCGCCCAGTTG		

4. Results/Discussion

4.1. Microorganisms

Throughout the experiment *Candida albicans* YP0037 showed that it was possible to produce biofilm when alone and in the presence of Gram-negative bacteria. However, at some point *C. albicans* YP0037 stopped producing biofilm (alone or the presence of Gram-negative bacteria. Eventually, after some weeks, the biofilm formation was successful again. *C. albicans* YP0037 was able to produce biofilm when alone and in the presence of Gram-negative bacteria. The biofilm formation is a defense mechanism used by microorganisms to protect themselves against outside stress (Falanga, A. [et al.], 2022). When the rotations per minute were increased it was possible to observe this change. This strain may need extra stress to produce more biofilm (Simoes, L.C. [et al.], 2022).

This type of obstacle was not reported in the literature analyzed, yet it was personally communicated by Professor Teresa Gonçalves, and it is an important detail to be shared.

4.2. Hyphae and pseudohyphae formation

Hyphae formation is one of many virulence factors of *C. albicans*, and it's associated with the enhancement of biofilm formation (Henriques, M. and Silva, S., 2021, Talapko, J. [et al.], 2021). The switch between yeast and pseudohyphae represents an important role in biofilm production in *C. tropicalis*. In order to confirm hyphal formation a microscopic observation was made. Microscopic observation showed that *C. albicans* YP0037 did not developed hyphae in a single biofilm, however developed pseudohyphae when in contact with *A. baumannii* 319 and *A. bereziniae* 118, while *C. tropicalis* M152540979 developed pseudohyphae in the presence and absence of the bacteria. The figure 4 shows the microscopic images where it is possible to observe these modifications of phases.

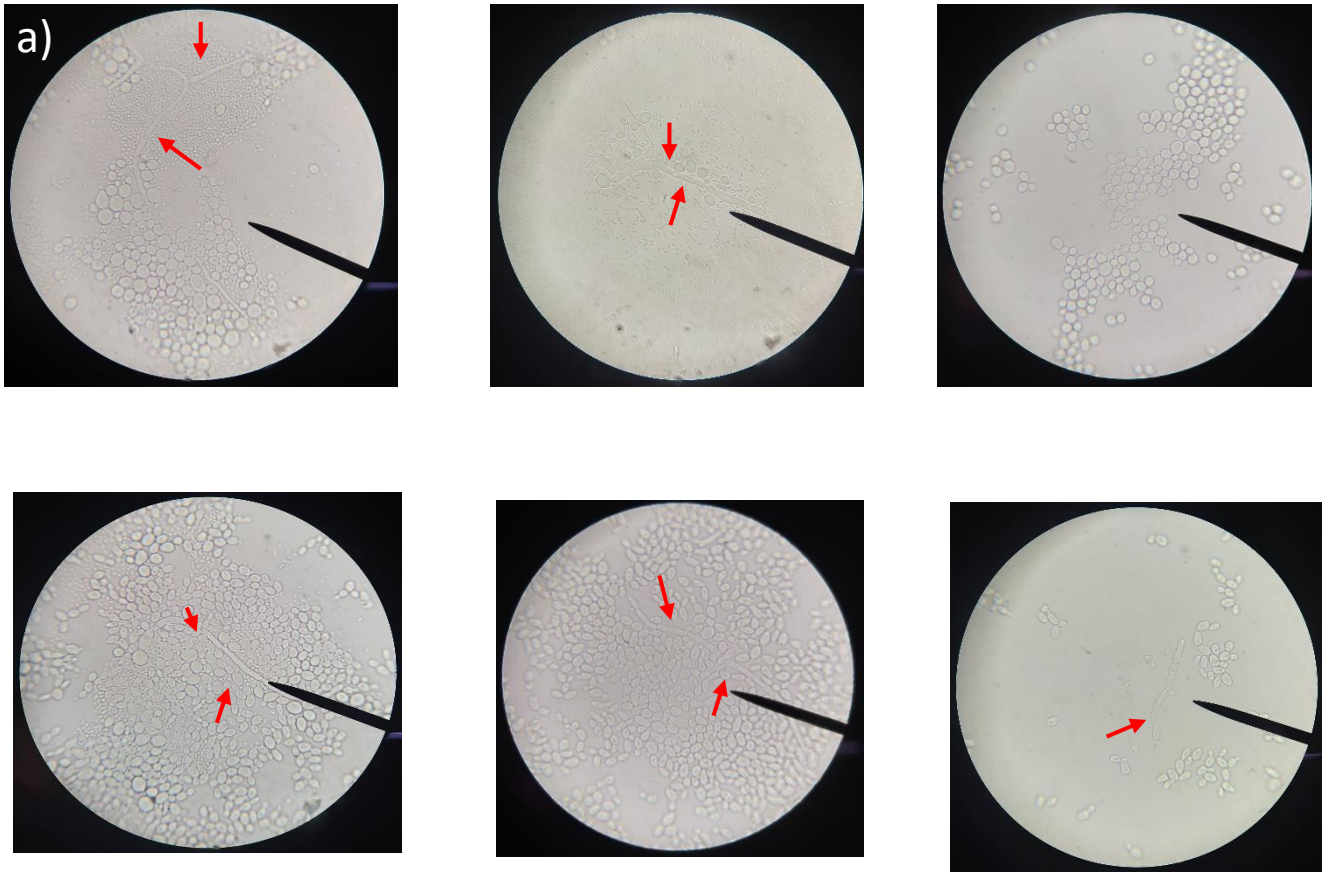


Figure 4 - Microscopic observation of pseudohyphae formation. The red arrows indicate pseudohyphae formations. a) *C. albicans* YP0037 + *A. baumannii* 319; b) *C. albicans* YP0037 + *A. bereziniae* 118; c) *C. albicans* YP0037; d) *C. tropicalis* M152540979 + *A. baumannii* 319; e) *C. tropicalis* M152540979 + *A. bereziniae* 118; f) *C. tropicalis* M152540979.

4.3. Effect of different mediums in biofilm formation

Duo to *C. albicans* YP0037 biofilm production problem, it was necessary to test different mediums: Sabouraud Dextrose, Tryptic Soy, and Mueller-Hinton. Biofilm formation depends on many factors including nutrient availability (Wijesinghe, G. [et al.], 2019). Each media has a different composition, which will influence the nutrient available. Sabouraud Dextrose has dextrose and peptone, Tryptic Soy has pancreatic digest of casein, peptic digest of soybean meal and sodium chloride, and Mueller-Hinton beef extract, acid hydrolysate of casein and starch.

In general, the microorganisms produced more biofilm in Tryptic Soy medium (figure 6).

It was possible to observe that *C. albicans* YP0037 biofilm production did not alter with these three types of medium. However, it was possible to observe that the medium used can influence the biofilm production. (Wijesinghe, G. [et al.], 2019)

There are many different mediums used in mixed biofilms. The most reported are Tryptic Soy Broth (TSB) (Adam, B. [et al.], 2002, Hacioglu, M. [et al.], 2020), RPMI (Falanga, A. [et al.], 2022, Fernandes, L. [et al.], 2020) and Yeast-peptone-dextrose (YPD).

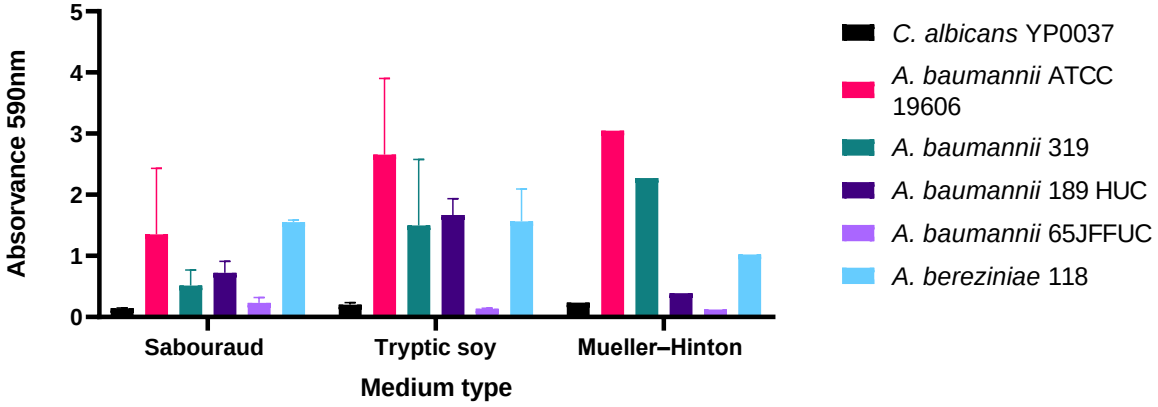


Figure 5 - Comparison of biomass production using different mediums after 24h of incubation.

4.4. Effect of different relative quantity of cells (yeast-bacteria) in biofilm formation

To address the influence of the cell concentration of one species versus the other, we used diverse quantities of yeast and bacteria cell: Volumes of 100 µL -100 µL, 125 µL – 75 µL and 150 µL - 50 µL. The figure 6 show that biofilm biomass is influenced by the relative quantity of cells. The volume 125 µL – 75 µL had the most biofilm production in general, so this was the volume used in the following assays.

Usually, the relative volume used in the studies is 100 µL of bacteria and 100 µL of yeast. (Hacioglu, M. [et al.], 2020, Holcombe, L.J. [et al.], 2010).

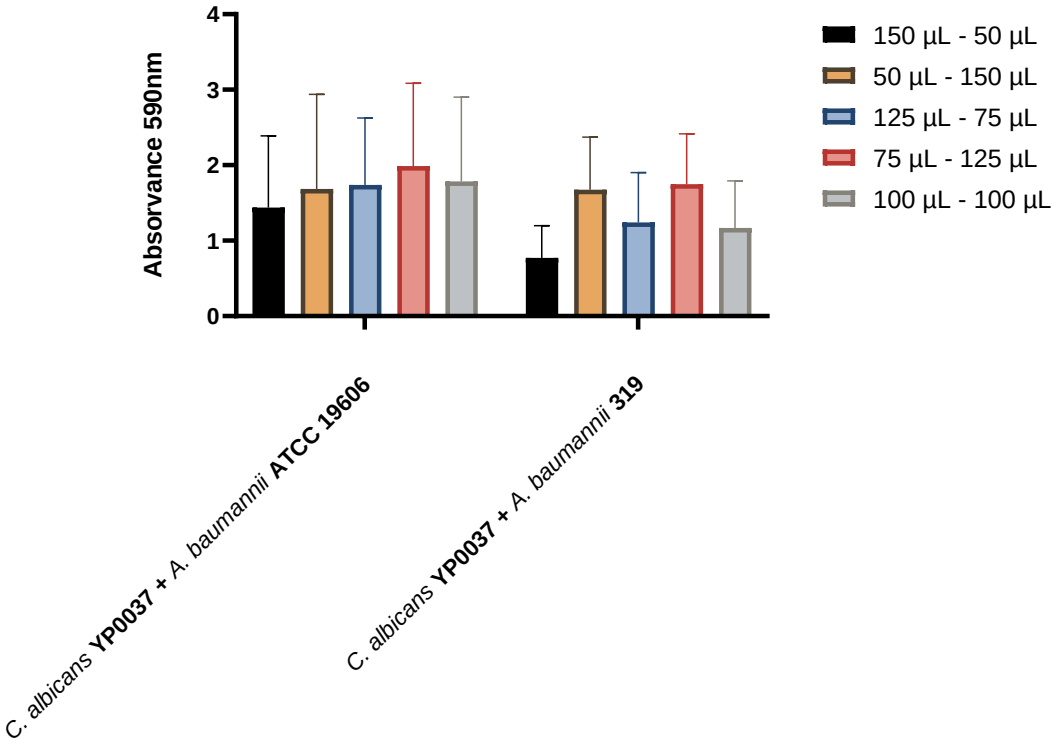


Figure 6 - Comparison of different relative quantity of cells in *C. albicans* YP0037 with *A. baumannii* ATCC 19606 and *A. baumannii* 319 biofilms.

4.5. Minimal Inhibitory Concentration determination

The table 4 shows the MICs for ciprofloxacin and gentamicin. This value is necessary for the next assays, where *A. baumannii* 319 and *A. bereziniae* 118 were selected (Domingues, S. [et al.], 2019). *A. baumannii* 319 is highly resistant to ciprofloxacin, while *A. bereziniae* 118 is susceptible to ciprofloxacin. As expected, *Candida* spp, were not inhibited by the antibiotics.

Table 4 - Minimal inhibitory concentration of two antibiotics in *Acinetobacter* spp. and *Candida* spp.

Species	*Ciprofloxacin*	*Gentamicin*
	MIC (µg/µL)	MIC (µg/µL)
A. baumannii 319	> 512	2
A. bereziniae 118	<0,5	16
C. albicans YP0037	ns	ns
C. glabrata M14331	ns	ns
C. tropicalis M152540979	ns	ns

* ns (not susceptible)

4.6. Effect of antibiotics in biofilms

An antibiotic must be administered in a series of doses at a concentration that gives the minimum inhibitory concentration (MIC) in order to kill/inhibit microorganisms at the site of infection. Concentrations below the MIC values are known as sub-minimum inhibitory concentrations (sub-MICs) (Davies, J. [et al.], 2006, Narimisa, N. [et al.], 2020). Have been demonstrated that antibiotics with sub-MICs can operate as signal molecules, changing the physicochemical properties and bacterial pathogenicity expression. The alterations brought on by sub-MICs of antibiotics reveal a state that bacteria experience in the wild and how they respond to it (Narimisa, N. [et al.], 2020).

Some studies show that once the biofilm is formed, antibiotic efficacy dramatically decreases and some antibiotics, stimulate biofilm growth at sub-inhibitory concentrations (Ferrer, M.D. [et al.], 2017).

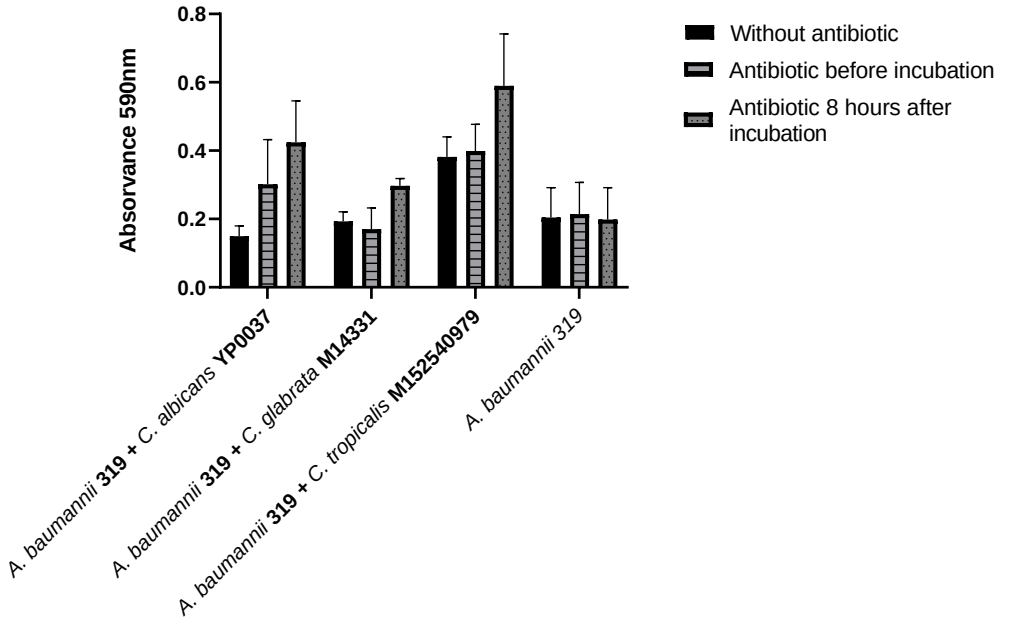
4.7. Effect of gentamicin in biofilms

The biofilms were performed in YPD media and with volumes of 125 µL – 75 µL and the assay was performed in triplicate and in two independent moments. Acinetobacter spp. and *Candida* spp. biofilms will be tested in different scenarios.

The different volumes of yeast and bacteria to form a biofilm were evaluated also with gentamicin, in order to see if it would alter the biofilm production when one of them was in a higher volume then the other. The concentration of antibiotic was in sub-MICs.

The figure 7 shows the effect of gentamicin in biofilms of *A. baumannii* 319 with different species of *Candida* spp. versus single biofilm and the impact of the antibiotic before and after the formation of the mixed biofilm. It is observed an increase of biomass of the mixed biofilm when the sub-MICs of gentamicin are added after the biofilm is already formed (8 hours). When the sub-MICs of gentamicin are added before the formation of the biofilm, it is observed an increase in biofilm production when compared with the biofilm formation without the antibiotic, except with the mixed biofilm of *A. baumannii* 319 with *C. glabrata* M14331 that decreases. The single biofilm of *A. baumannii* 319 is similar in the three conditions.

A.



B.

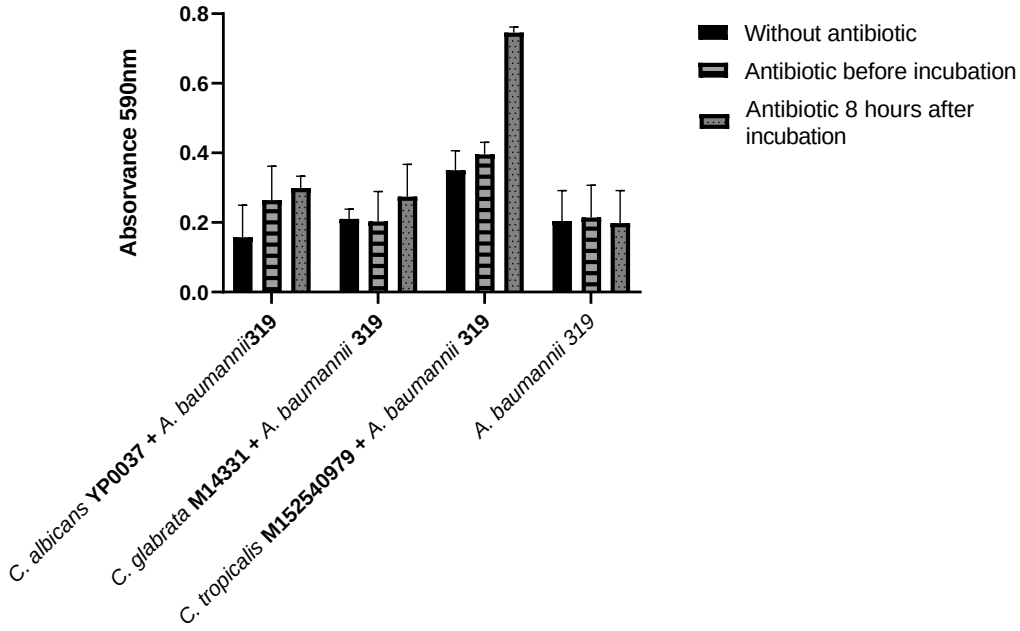
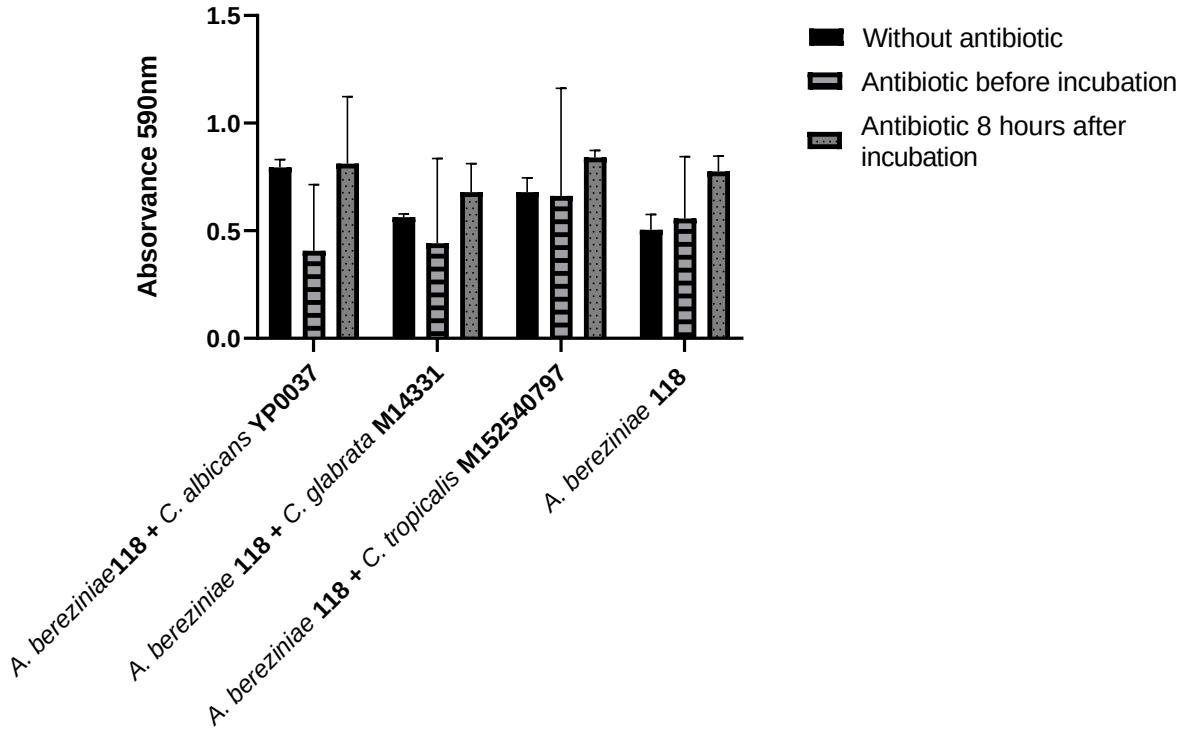


Figure 7 - Comparison of *A. baumannii* 319 and *Candida* spp. biofilm production with (before incubation and after 8 hours of incubation) and without addition of gentamicin in a sub-MICs concentration (1 µg/µL). A – volume of 125 µL of *A. baumannii* 319 and 75 µL of *Candida* spp. B – volume of 75 µL of *A. baumannii* 319 and 125µL of *Candida* spp.

The figure 8 shows the effect of gentamicin in biofilms of *A. bereziniae* 118 with different species of *Candida* versus single biofilm and the impact of the antibiotic before and after the formation of the mixed biofilm. It can be noted that the biomass of the mixed biofilm increases when the sub-MICs of gentamicin is added after the biofilm is already formed (8 hours). However, when the sub-MICs of gentamicin are added before the formation of the biofilm, it will decrease biofilm production when compared with non-addition of the antibiotic, except the mixed biofilm of *A. bereziniae* 118 with *C. tropicalis* M152540979 where they are very similar.

A.



B.

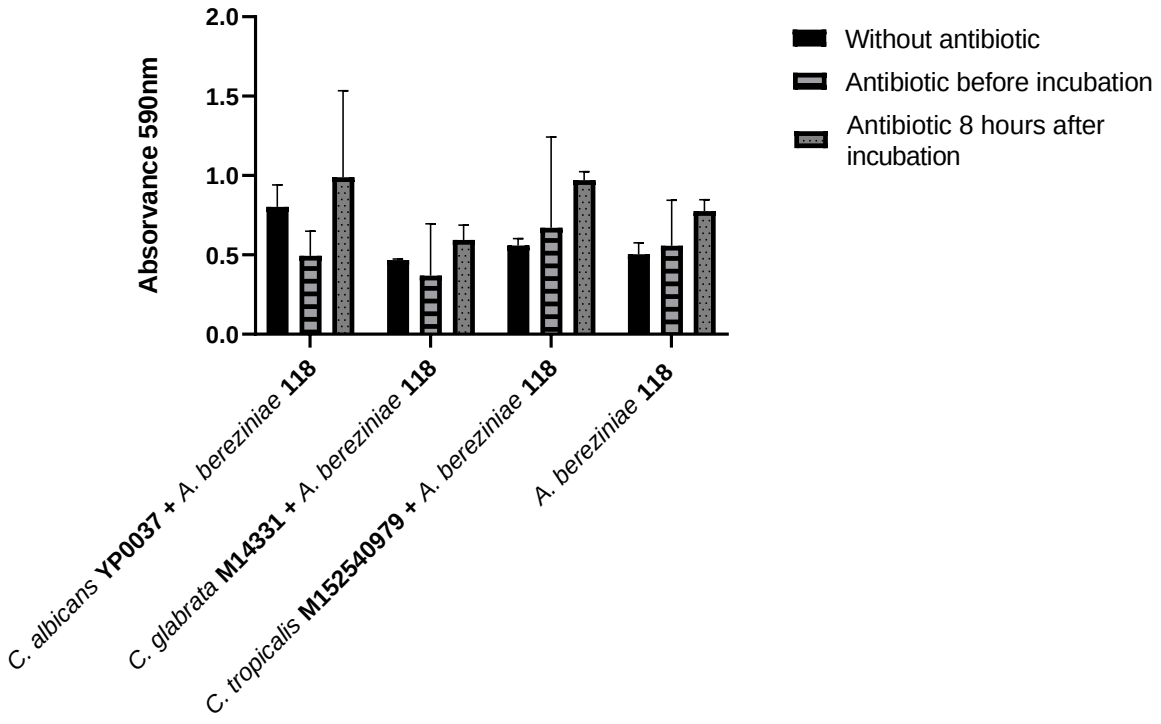


Figure 8 - Comparison of *A. bereziniae* 118 and *Candida* spp. biofilm production with (before incubation and after 8 hours of incubation) and without addition of gentamicin in a sub-MICs concentration (8 µg/µL). A – volume of 125 µL of *A. bereziniae* 11118 and 75 µL of *Candida* spp. B – volume of 75 µL of *A. bereziniae* 118 and 125 µL of *Candida* spp.

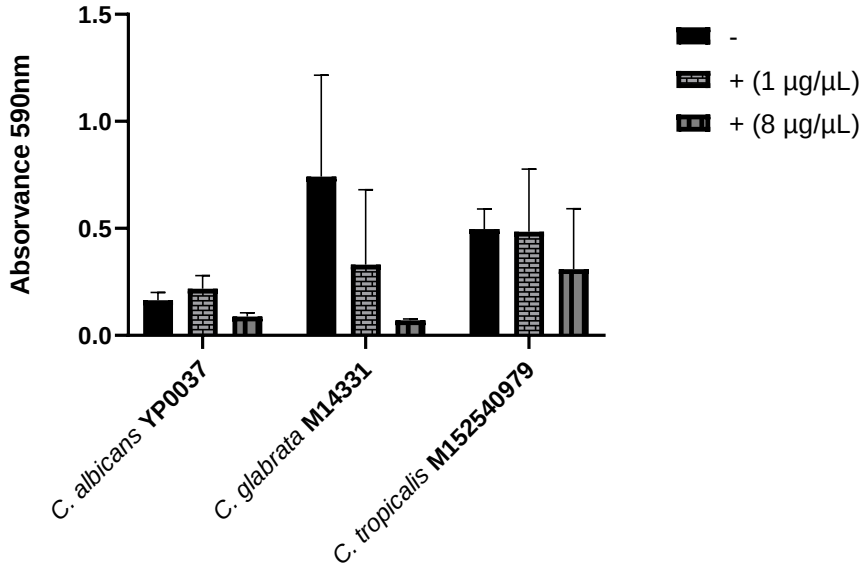
The figure 9 shows the effect of gentamicin in biofilms of different species of *Candida* biofilm with different concentrations of gentamicin and the impact of the antibiotic before and after the formation of the mixed biofilm. It can be observed that addition of gentamicin in a sub-MICs concentration to *C. albicans* YP0037 will result in increase in biofilm with a concentration of 1 µg/µL of gentamicin is added after the biofilm is already formed (8 hours) and when it´s added before the biofilm is formed. *C. glabrata* M14331 biofilm will decrease with the addition of gentamicin, independently if before or after the biofilm is formed. *C. tropicalis* M152540979 biofilm is very similar when the antibiotic is added before and after the biofilm is formed.

We cannot explain how *C. albicans* YP0037 increases biofilm formation when in contact with gentamicin and to our knowledge there are no other studies discussing this.

Some studies r that strains of *C. albicans* did not shown any susceptibility to gentamicin even at a concentration ≥512 µg/mL (Sushmasri, K. [et al.], 2022). However, if used in combination with azoles can have a synergetic effect and enhances their efficacy by inhibiting

the activity of extracellular phospholipase and suppressing the activity of efflux pump (Liu, Y. [et al.], 2019, Lu, M. [et al.], 2018). Non-*Candida albicans* species don't have information about studies with antibiotics.

A.



B.

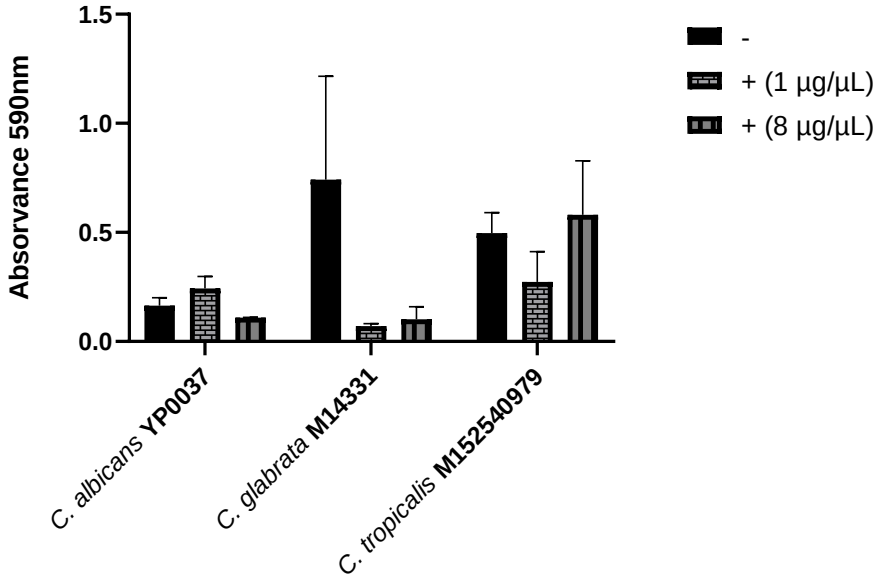


Figure 9 – Comparison of *Candida* spp. of biofilm production with (before incubation and after 8 hours of incubation) and without addition of gentamicin in a sub-MICs concentration (1 µg/µL and 8 µg/µL) A – Addition of gentamicin after 8 hours of incubation. B – Addition of gentamicin before of incubation.

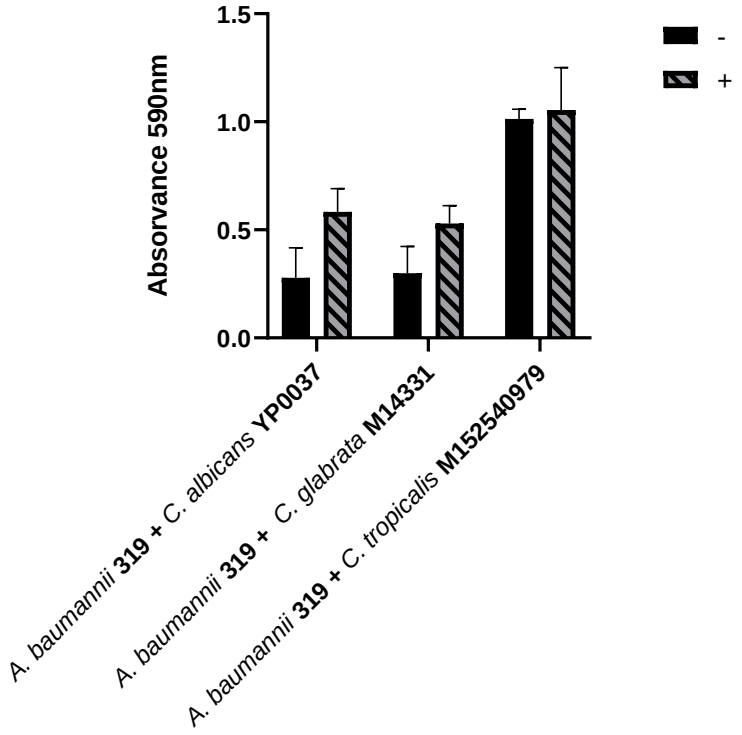
4.8. Effect of Ciprofloxacin

The biofilms were performed in YPD media and with volumes of 125 µL – 75 µL and the assay was performed in triplicate and in two independent moments. Where *Acinetobacter* spp. and *Candida* spp. biofilms will be tested in different scenarios,

The different volumes of yeast and bacteria to form a biofilm were evaluated also with ciprofloxacin, in order to see if it would alter the biofilm production when one of them was in a higher volume then the other. The concentration of antibiotic was in sub-MICs.

The addition of sub-MIC of ciprofloxacin before biofilm formation showed that mixed biofilms of *A. baumannii* 319 with *C. albicans* YP0037 and *C. glabrata* M14331 produced more biomass than single species biofilm, however, with *C. tropicalis* M152540979, the biofilm formation when compared with the biofilm without antibiotic was reduced when *A. baumannii* 319 is in a lower volume than *C. albicans* YP0037 (Fig. 10, B) and is similar when *A. baumannii* 319 is in a higher volume. The figure 10 shows effect in *A. baumannii* 319 and *Candida* spp. biofilm production.

A.



B.

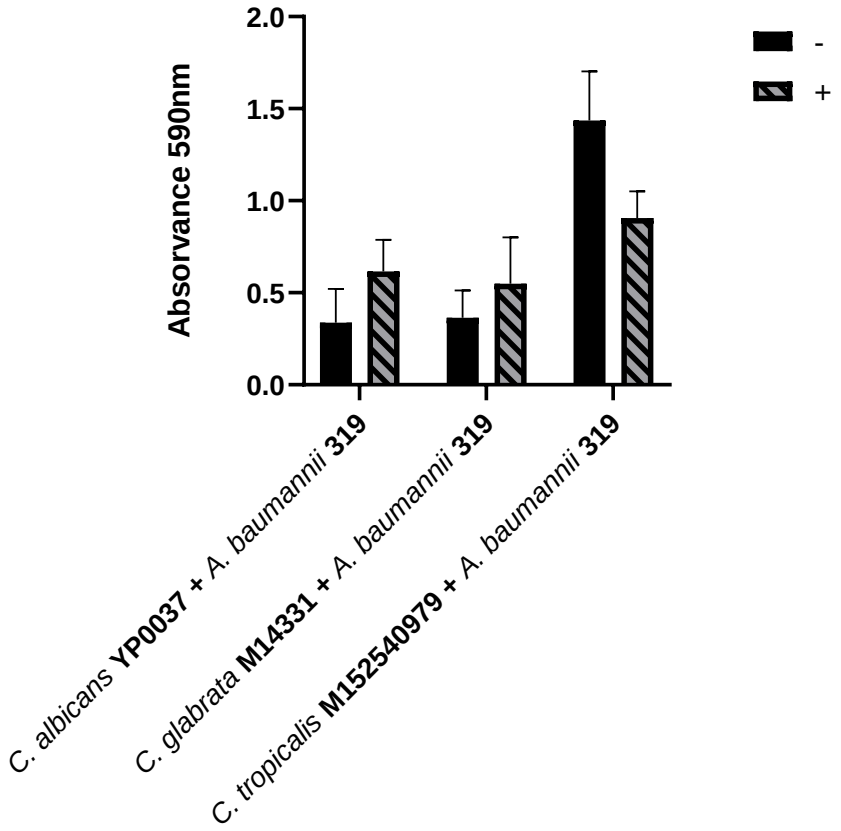
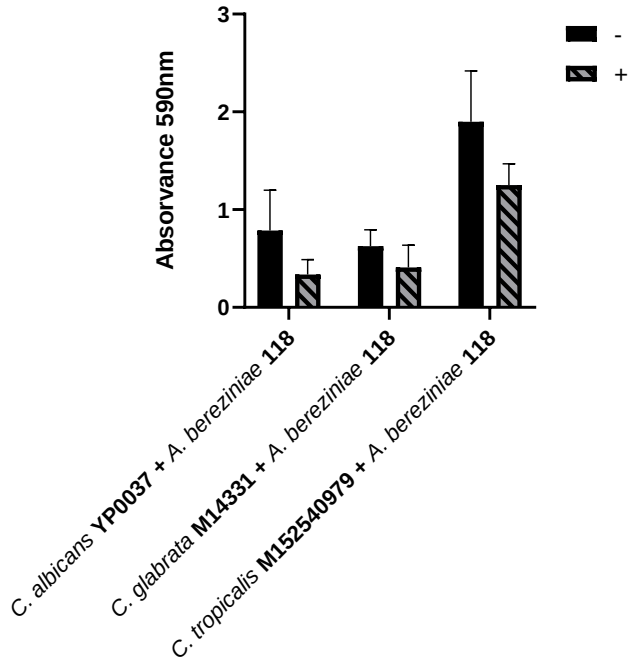


Figure 10 - Comparison of *A. baumannii* 319 and *Candida* spp. biofilm production with and without addition of ciprofloxacin in a sub-MICs concentration (512 µg/µL). A – volume of 125 µL of *A. baumannii* 319 and 75 µL of *Candida* spp. B – volume of 75 µL of *A. baumannii* 319 and 125 µL of *Candida* spp.

The addition of ciprofloxacin to mixed biofilms of *A. bereziniae* 118 with *C. albicans* YP0037, *C. glabrata* M14331 and *C. tropicalis* M152540979, lead to a reduction in the biofilm formation, as figure 11 shows.

A.



B.

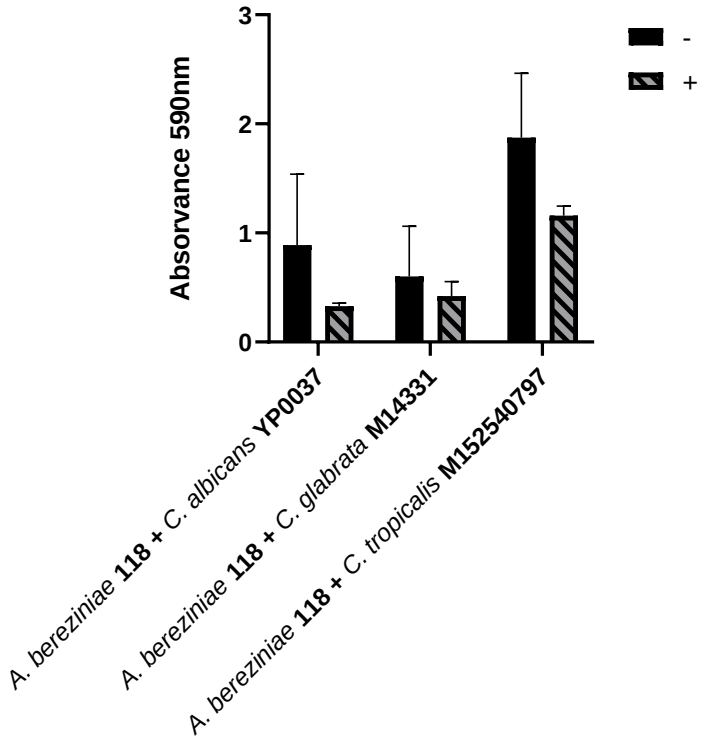


Figure 11 - Comparison *A. bereziniae* 118 and *Candida* spp. of biofilm production with and without addition of ciprofloxacin in a sub-MICs concentration (o,125 µg/µL). A – volume of 125 µL of *A. bereziniae* 118 and 75 µL of *Candida* spp. B – volume of 75 µL of *A. bereziniae* 118 and 125 µL of *Candida* spp.

The effect of the addiction of sub-MICs to *Candida* spp. biofilms is shown in Figure 12. It is possible to observe that *C. albicans* YP0037 biofilm will increase as higher is the concentration of ciprofloxacin. *C. glabrata* M14331 biofilm will decrease as the concentration gets higher. *C. tropicalis* M152540979 biofilm only decreases with the higher concentration (512 µg/µL).

We cannot explain how *C. albicans* YP0037 increases biofilm formation when in contact with ciprofloxacin and to our knowledge there's no other studies reporting this.

Regarding the effect of ciprofloxacin by themselves in *Candida* spp. it was no information, as well as information about antibiotic effects in non-*Candida albicans* species.

Some studies show that ciprofloxacin when combined with: amphotericin B can have a synergetic effect in some *C. albicans* strains; fluconazole had a synergetic effect but it was strain-dependent; and caspofungin did not show a significant synergetic or antagonist effect (Liu, Y. [et al.], 2019, Stergiopoulou, T. [et al.], 2008).

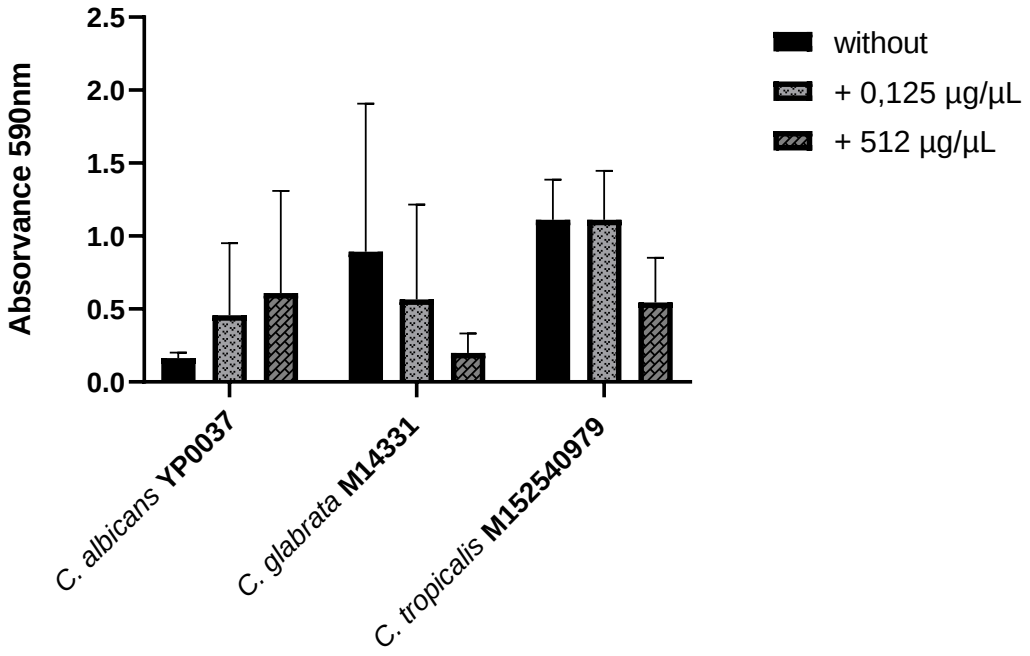


Figure 12 - Comparison of *Candida* spp. of biofilm production with and without addition of ciprofloxacin in a sub-MICs concentration (512 µg/µL and o,125 µg/µL).

4.9. Expression of *CsuE*. and *OmpA* genes

OmpA gene is involved in the attachment of bacterial cells in abiotic surfaces and human alveolar epithelial cells and the *csuE* gene acts as an adhesin that binds to the biotic surfaces in

the beginning of the biofilm formation process (Amin, M. [et al.], 2019, Gaddy, J.A. [et al.], 2009).

The table 5 shows the concentration of RNA extracted from a biofilm of *A. bereziniae* 118 that was in contact with *C. tropicalis* M152540979 and the assessment of its purity in multiple experiments.

Table 5 - Concentration of RNA extraction from polymicrobial and single biofilms, as well as from planktonic cells. 1 – *C. tropicalis* M152540979 and *A. bereziniae* 118 mixed biofilm, 2 – *A. bereziniae* 118 and *C. tropicalis* M152540979 mixed biofilm, 3 – *A. bereziniae* 118 biofilm, and 4 - *A. bereziniae 118* planktonic cells.

		ng/ul	**A260**	**A280**	**260/280**	**260/230**
1st assay	1	30,82	0,7705	0,3275	2,36	0,14
	2	26,085	0,652	0,306	2,135	0,19
	3	47,85	1,1965	0,592	2,02	1,76
	4	21,965	0,5495	0,261	2,11	1,755
2nd assay	1	163,9	4,0975	1,836	2,235	1,505
	2	116,68	2,917	1,3415	2,175	1,335
	3	118,795	2,9695	1,415	2,1	1,91
	4	27,51	0,688	0,336	2,055	1,04

The results of the RT-qPCR of the mixed biofilms of *A. bereziniae* 118 and *C. tropicalis* M152540979, single biofilm of *A. bereziniae* 118, and planktonic cells of *A. bereziniae* 118 are shown in table 6 where the Cq value represents the PCR cycle numbers ate which the sample's reaction curve intersects the threshold line.

It was not possible to conclude if there was an alteration of the expression of these genes. The assay must be repeated because there was amplification in samples that should not had it.

Moreover, other *Acinetobacter* genes involved in the formation of biofilms (Amin, M. [et al.], 2019, Gaddy, J.A. [et al.], 2009, Navidifar, T. [et al.], 2019) might be up- or downregulated. Also, *Candida* biofilm regulator genes were not evaluated. The influence of the yeast in the expression of biofilm regulator genes, and vice-versa, is an interesting study that deserves further attention, and this interaction must be studied at genetic level.

Table 6 - RT-qPCR results. a) 1st assay and b) 2nd assay. 1 – *C. tropicalis* M152540979 and *A. bereziniae* 118 mixed biofilms; 2 – *A. bereziniae* 118 and *C. tropicalis* M152540979 mixed biofilm; 3 – *A. bereziniae* 118 biofilm; and 4 - *A. bereziniae* 118 planktonic cells.

a)

Sample	*Cq*
1 csuE	28,33
2 CsuE	34,01
3 CsuE	28,80
4 CsuE	35,15
1 AbOmpA	32,55
2 AbOmpA	33,55
3 AbOmpA	33,43
4 AbOmpA	34,01
1 RT- AbOmpA	32,03
2 RT- AbOmpA	33,36
3 RT- AbOmpA	31,90
4 RT- AbOmpA	34,63
1 NTC- AbOmpA	34,56
2 NTC- AbOmpA	33,96
3 NTC- AbOmpA	34,75
4 NTC- AbOmpA	34,24
Blank	30,13
Blank	30,80
Blank	31,57
Blank	31,62

b)

Sample	*Cq*
1 csuE	0
2 CsuE	36,96
3 CsuE	0
4 CsuE	0
1 AbOmpA	29,90
2 AbOmpA	29,04
3 AbOmpA	33,29
4 AbOmpA	30,79
1 RT- CsuE	0
2 RT - CsuE	0
3 RT - CsuE	0
4 RT - CsuE	0
1 RT - AbOmpA	0
2 RT - AbOmpA	27,55
3 RT - AbOmpA	33,16
4 RT - AbOmpA	30,69
NTC - CsuE	0
NTC - AbOmpA	35,03

4. Conclusions

The main goal of this study was to assess the behavior of bacteria and yeast when in contact evaluating the formation of biomass and hyphae/pseudohyphae and under the exposure of antibiotics. Different strains/species of *Acinetobacter* sp. were used as model in contact with diverse *Candida* species.

The present study showed different results of biofilm formation and it was not possible to observe a uniform response of synergism or antagonism when yeast and bacteria are involved. In fact, different species and strains, both from *Acinetobacter* spp. and *Candida* spp., do not behave identically, which may explain our results. Moreover, our results also were not completely identical to other studies where it was showed that the interaction between *A. baumannii* and *C. albicans* is antagonistic (leading to the inhibition of biofilm). Different methodologies can explain this difference (strains and species used, relative cell concentration, media, time of incubation, biofilm formation method) or different strains behavior. This demonstrated the lake of uniformity of biofilm formation of bacteria and fungi, highlighting the difficulty of treatment of polymicrobial infections. Accounting for this difficulty, we observed that virulence of fungi can be enhanced in mixed biofilms.

It was also demonstrated that the exposure to antibiotics can interfere with biofilm production, even when already formed. Indeed, a biofilm formed will increase its biomass after contact with gentamicin.

Overall, this study demonstrates the complex interactions of polymicrobial biofilms and how they can affect conventional therapy, urging for the study of mechanistic interactions to find new therapeutic targets.

This topic deserves further investigation at genetic level to better comprehend the interaction at molecular level that are of key importance to the development of new therapies to inhibit /eradicate interkingdom biofilms.

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Annex I – Poster

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The 3rd edition of the International Virtual Conference on *Acinetobacter*

Interkingdom Biofilms: Interactions of *Acinetobacter* spp. and *Candida* spp. in Mixed Biofilms

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INTRODUCTION

Bacterial-fungal interactions are common in nature and clinical environments (1-3). These interactions usually occur in a form of biofilm, which is a community of microorganisms attached to an abiotic or biotic surface (4-6). Polymicrobial biofilms are more tolerant to antimicrobials than single species biofilm, limiting conventional drug therapy (7-9). The biological relevance of microbial interactions remains largely unknown.

AIM

- The main objective of this study was to evaluate the microbial interaction in a biofilm formation and the impact in the treatment of infection.
- The specific objectives are: to investigate the synergetic or antagonist interaction between bacteria and fungi, using *Acinetobacter* spp. and *Candida* spp. in a biofilm model; to evaluate the effect of antibiotics in the biofilm formation.

METHODS

- Biofilms were formed in diverse culture media with different strains of *Acinetobacter* spp. and *Candida* spp.
- The methodology used was:
  - evaluation of biomass in single species and mixed biofilms by the crystal violet method after 24h of incubation;
  - measurement of biomass in the absence and presence of antimicrobials;
  - assessment of *C. albicans* virulence by microscopic observation of the formation of hyphal in the absence and presence of *Acinetobacter* spp.;
  - evaluation of expression of *Acinetobacter* spp. biofilm-associated genes (*ompA* and *csu*) by RT-qPCR.

RESULTS

- We observed that the **medium** and the **relative quantity** (not shown) of cells used can influence the biofilm production (Fig. 1)

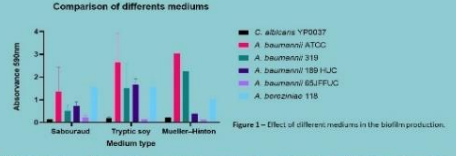

Figure 1 – Effect of different mediums in the biofilm production.

- Microscopic observation showed that ***C. albicans*** YP0037 **formed pseudohyphae** when **in contact** with *A. baumannii* 319 and *A. bereziniae* 118, while ***C. tropicalis*** M152540979 **developed pseudohyphae** in the **presence** and **absence** of the bacteria (Fig. 2)

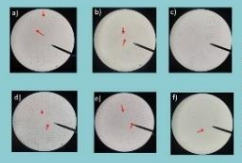

Figure 2 – Microscopic observation of pseudohyphae formation.
a) *C. albicans* YP0037 + *A. baumannii* 319;
b) *C. albicans* YP0037 + *A. bereziniae* 118;
c) *C. albicans* YP0037;
d) *C. tropicalis* M152540979 + *A. baumannii* 319;
e) *C. tropicalis* M152540979 + *A. bereziniae* 118
f) *C. tropicalis* M152540979

- Addition of **sub-MIC** of **ciprofloxacin before biofilm incubation** showed that **mixed biofilms** of ***A. baumannii*** 319 with *C. albicans* YP0037 and *C. glabrata* M14331 produced **more biomass** than single species biofilm (not showed); however, with ***C. tropicalis*** M152540979, biofilm formation was **reduced** (Fig. 3)

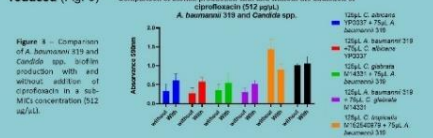

Figure 3 – Comparison of *A. baumannii* 319 and *Candida* spp. biofilm production with and without addition of ciprofloxacin in a sub-MICs concentration (512 µg/µL).

- Addition of **ciprofloxacin** to **mixed biofilms** of ***A. bereziniae*** 118 with *C. albicans* YP0037, *C. glabrata* M14331 and *C. tropicalis* M152540979, lead to a **reduction** in the biofilm formation (Fig.4)

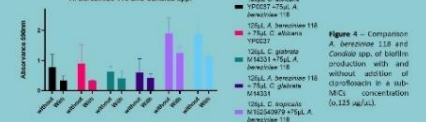

Figure 4 – Comparison *A. bereziniae* 118 and *Candida* spp. of biofilm production with and without addition of ciprofloxacin in a sub-MICs concentration (0,125 µg/µL).

- **Sub-MICs** of **gentamicin** added to an **8 hour-biofilm** lead to an **increase** of **biomass** of the mixed biofilm (Fig.5A and 5B).

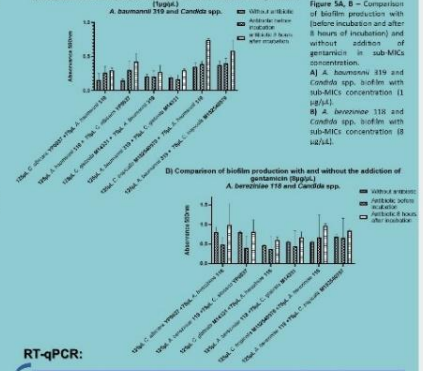

Figure 5A, B – Comparison of biofilm production with (before incubation and after 8 hours of incubation) and without addition of gentamicin in sub-MICs concentration.
A) *A. baumannii* 319 and *Candida* spp. biofilm with sub-MICs concentration (1 µg/µL).
B) *A. bereziniae* 118 and *Candida* spp. biofilm with sub-MICs concentration (8 µg/µL).

RT-qPCR:
There was **no significant expression** of *ompA* and *csu* in *Acinetobacter* spp.

CONCLUSIONS

- Some studies showed that the interaction between *A. baumannii* and *C. albicans* are antagonistic (10-12), leading to the inhibition of biofilm, which is in contrast with our results. Different methodologies can explain this observation or different strains do not behave identically, which accounts for the difficulty of treatment of polymicrobial infections. Moreover, virulence of fungi can be enhanced in mixed biofilms.
- Antibiotics can interfere with biofilm production, even when already formed, increasing the biomass and challenging the treatment of polymicrobial infections.
- Overall, this study demonstrates the complex interactions of polymicrobial biofilms and how they can affect conventional therapy, urging for the study of mechanistic interactions to find new therapeutic targets.

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