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Summary

Polymicrobial infections, including bacterial-fungal interactions are often associated with increased resistance to antimicrobial treatments. Additionally, diagnostics of polymicrobial infections is not part of the scope of clinical diagnostics.

Bacteria and fungi can communicate via quorum sensing systems with the production of peptides, metabolites and toxins, capable of modulating growth of other microbial species thus, resulting in antagonistic, synergistic or neutral effects. The molecules produced during a bacterial-fungal interaction also affect the host immune response and can even alter the efficacy of a given treatment. The fungus *Candida albicans* and the bacterium *Klebsiella pneumoniae* are both human commensals but, once the epithelial barriers are broken, they become opportunistic pathogens. However, the mechanisms mediating the interaction between *C. albicans* and *K. pneumoniae* have not yet been elucidated. In this study, we aimed to characterize *C. albicans-K. pneumoniae* interaction both *in vitro* and *in vivo*. For this, we have assessed the capacity of biofilm formation of co-cultures compared to single cultures. Co-cultures have shown decreased biofilm formation, independently on the growth media used, suggesting an antagonistic effect. At the molecular level, we were able to identify key genes that play a role in this interaction. Upon exposure to *K. pneumoniae*, *C. albicans* has upregulated the gene *ECE1,* associated with the production of the toxin *Candida*lysin. In co- culture, *K. pneumoniae* has upregulated genes associated with cell wall degrading enzymes such as chitinase and endoglucanase. Interestingly, *C. albicans* seems to counteract the cell wall degradation by increasing chitin and β-glucan contents. Finally, an *in vivo* model of *Galleria mellonella* was used to assess the *C. albicans-K. pneumoniae* interaction. Expectedly, the survival of *G. mellonella* was significantly decreased upon co-infection compared to the respective single infections.

In conclusion, in this study we characterized the antagonistic interaction of *C. albicans* and *K. pneumoniae* and gained new insights on the molecular mechanisms and host responses. This will add knowledge for further exploitation of new diagnostic tests and treatment of polymicrobial infections.

Zusammenfassung

Polymikrobielle Infektionen, einschließlich Interaktionen von Bakterien und Pilzen, sind oft mit einer erhöhten Resistenz gegen antimikrobielle Therapien verbunden und deren Diagnostik gehört nicht zur Routine in der Klinik. Bakterien und Pilze können über sogennante Quorum Sensing Systeme mittels der Produktion von Peptiden, Metaboliten und Toxinen kommunizieren. Dies kann das Wachstum anderer mikrobieller Arten modulieren und so antagonistische, synergistische oder neutrale Effekte erzielen. Die Moleküle, die bei einer Interaktion von Bakterien und Pilzen entstehen, beeinflussen auch die Immunantwort des Wirtes und können sogar die Wirksamkeit einer bestimmten Therapie verändern. Der Pilz *Candida albicans* und das Bakterium *Klebsiella pneumoniae* sind beide humane Kommensalen. Sobald jedoch die schützenden epithelialen Barrieren des Körpers überwunden sind, werden sie zu opportunistischen Krankheitserregern. Die Mechanismen, die die Interaktion zwischen *C. albicans* und *K. pneumoniae* vermitteln, sind noch völlig unklar. In dieser Studie zielen wir darauf ab, *C. albicans-K. pneumoniae* Interaktionen sowohl *in vitro* als auch *in vivo* zu charakterisieren. Dazu haben wir die Kapazität der Biofilmbildung von Co-Kulturen im Vergleich zu Einzelkulturen bewertet. Co-Kulturen haben eine verminderte Biofilmbildung unabhängig von den verwendeten Wachstumsmedien. Dies deutet auf eine antagonistische Interaktion zwischen *C. albicans* und *K. pneumoniae* hin. Auf molekularer Ebene konnten wir Schlüsselgene identifizieren, die bei dieser Interaktion eine Rolle spielen. Nach der Interaktion mit *K. pneumoniae* hat *C. albicans* das Gen *ECE1* hochreguliert, welches für das Toxin *Candida*lysin kodiert. In der Co-Kultur reguliert *K. pneumoniae* Gene hoch, die die zellwandabbauenden Enzyme Chitinase und Endoglucanase kodieren. Interessanterweise scheint *C. albicans* dem Zellwandabbau entgegenzuwirken, indem es den Chitin- und β- Glucan-Gehalt in der Zellwand erhöht. Schließlich wurde ein *Galleria mellonella in vivo* Modell verwendet, um die *C. albicans-K. pneumoniae* Interaktion zu untersuchen. Erwartungsgemäß war das Überleben von *G. mellonella* bei einer Co-Infektion im Vergleich zu den jeweiligen Einzelinfektionen signifikant reduziert. Zusammenfassend lässt sich sagen, dass wir in dieser Studie die antagonistische Interaktion von *C. albicans* und *K. pneumoniae* charakterisiert haben und neue Erkenntnisse über die molekularen Mechanismen und Reaktionen des Wirtes gewonnen haben. Dieses Wissen könnte nützlich sein für die Entwicklung neuer diagnostischer Tests und die Behandlung von polymikrobiellen Infektionen.

List of abbreviations

AIDS: acquired immune deficiency syndrome

AMPs: antimicrobial peptides

AUC: area under the curve

BFIs: bacterial fungal interactions

CAS : caspofungin

CBP: clibical breakpoint

CDC: centers for disease control (and prevention)

CFW: Calcofluor White Stain

CHL: chloramphenicol

CLSI: clinical and laboratory standard institute

CNS: central nervous system

CRE: carbapenem-resistant Enterobacteriaceae

CST : colistin

DMEM: Dulbecco's modified eagle medium

DNA: deoxyribonucleic acid

ESBLs: extended-spectrum-β-lactamase

ESCs: extended-spectrum-cephalosporins

EUCAST: European committee on antimicrobial susceptibility

FITC: Fluorescein isothiocyanate

GlcNAc: N-Acetylglucosamine

GIs: gastrointestinal tracts

HiFCS: heat-inactivated fetal calf serum

KAN : kanamycin

KEGG: encyclopedia of genes and genomes

MAP: mitogen activated protein

MAPK: mitogen-activated protein kinase

MIC: minimum inhibitory concentraition

MR: mannose receptor

OD: optical density

PKA: protein kinase A

PAMP: pathogen associated molecular pattern

PRR: pattern recognition receptor

RNS: reactive nitrogen species

RNA: ribonucleic acid

ROS: reactive oxygen species

TLR: Toll-like receptor

YNBNP: yeast nitrogen base + N-acetylglucosamine + phosphate buffer

1. Introduction

1.1 *Candida albicans,* a human fungal pathogen

Candida albicans is part of the human microbiome and asymptomatically colonizes different parts of the body including, the gastrointestinal tract (GI), oral mucosal, skin and reproductive tract [1]. A fully functional immune system is able to control the growth of *C. albicans.* However, dysbiosis, alterations of the host immunity, antibiotic and Immunosuppressant therapies, stress conditions, environmental changes such as pH or nutrient shifts, can affect the homeostasis and fitness of *C. albicans* [1-3]. Transition from a commensal to a pathogenic state will lead to initiation of infection. The infections can vary from skin rashes and superficial mucosal infections to hematogenously disseminated bloodstream infection with striking mortality rate of up to 40 % [1,3]. In certain cases, the mortality rates can be even higher (71 %), depending on the host immunity and prognostics mostly in patients with candidemia [4]. *C. albicans* is especially important in hospital acquired infections often leading to 15 % of the total cases of sepsis [2,5-9]. *Candida* infections are often associated with several diseases, including cancer, cystic fibrosis and AIDS, it is not only considered to be the most important fungal infection in individuals with AIDS or patients going through anticancer therapies, but also very serious in patients having implanted medical devices [10].

In addition to single *C. albicans* infections, this fungal strain has been most frequently isolated from mixed bacterial-fungal infections [2].

1.2 *Candida albicans* virulence factors

Candida albicans has developed mechanisms that permit survival within the human host. Several virulence factors account for this adaptability. Here we describe each associated virulence factor in detail:

Morphogenesis

One of the most important biological features of *C. albicans* is the capability to switch from yeast to filamentous forms. *C. albicans* is a polymorphic fungus, as it can be presented

as unicellular budding yeast, pseudohyphae and true hyphae [11] Figure1.1 shows different morphologies of *C. albicans* [11]. The morphological switch between yeast and filamentous forms (hyphae) can be induced by different environmental conditions such as elevated temperature, elevated temperatures, serum, nutrient availability, carbon dioxide, serum, nutrient availability, carbon dioxide, certain amino acids or N-acetyl glucosamine (GlcNAc) activates signaling pathways leading to morphological changes. There are many known signaling pathways that transduce environmental signals into morphological switching [12]
e.g. the first known singling pathway is based on cAMP and protein kinase A (PKA) targets that targets Efg1 transcriptional factor. The other known signaling pathway for morphological changes is a mitogen activated protein (MAP) kinase-based that also transduces pheromone response. Other than that a pH response is mediated by Rim101 transcription factor and response to solid matrix is mediated by pathways which activate a transcription factor called Czfl [11]. The morphological plasticity has a direct link to pathogenicity of *C. albicans* in humans [11]. The hyphae and pseudohyphae forms of *C. albicans* are intrinsically invasive on solid media [13]. These filamentous forms have also been shown to facilitate the penetration to epithelial cell layers during invasive growth and escape the phagocytic cells [14,15].

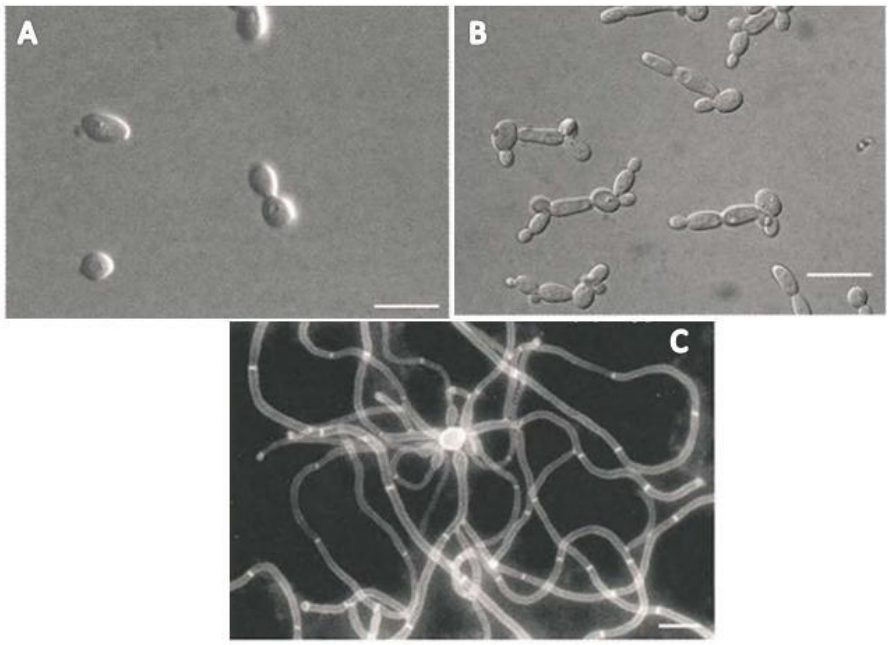


Figure 1.1: Yeast, pseudohyphal and hyphal morphologies . (A) Budding yeast cells, shortly after inoculation. (B): Pseudohyphal buds that exhibit morphologies like yeast that starts to be polarized. (C): mature hyphal mycelia. (*Adapted from* [11])

Recombinant DNA technologies have given the chance to researchers to create more than 1,000 gene knockouts from *C. albicans* strains. Out of 6,000 genes of *C. albicans* many are described as hyphal formation genes. Many proteins are also screened and identified with the help of approaches including genome-wide transcriptional profiling and proteomics techniques that are specific in hyphal structures [24,25].

Hyphal structures express several factors that can accelerate infection, for example:

HWP1 gene (hyphal wall protein) is a hyphal specific gene that also plays a role in adhesion. homozygous mutants of *hwp1Δ/Δ Candida* strains showed increased susceptibility to microglia cell-mediated damage and a difference in abundance in surface adhesin-like structures, emphasizing the importance of *HWP1* gene in pathogenicity of *Candida albicans*. [16,17].

One of the invasions of *C. albicans* is the *ALS3* gene. The Als3 hyphal specific protein binds to host cell receptors such as E-cadherin and N-cadherin to induce the endocytosis. In addition, Als3 protein binds to host ferritin and by this way allows *C. albicans* to utilize the protein as an iron source [18].

The *C. albicans ECE1* gene (extend of cell elongation) is highly expressed by hyphae when infecting epithelial barriers. In 2016 Moyes DL, *et al*. created a double mutant of *ece1Δ/Δ Candida albicans,* where they still observed epithelial invasion and penetration. Despite the invasion observed by the mutant *Candida* strain no damage to epithelial cells or induce in p-MKP1/c-Fos were observed. These observations uncovered the critical role of *ECE1* in epithelial damage and innate immune recognition during *C. albicans* infection and introduced the only known cytolytic peptide toxin in a human fungal pathogen (explained in 'toxin production' section separately) [19]. The other hyphal specific gene of *C. albicans* is *EFG1*, a member of a conserved class of bHLH (basic helix-loop-helix) proteins. *EFG1* is an essential dual regulator of morphogenesis of the human fungal pathogen *C. albicans* by promoting filamentation under normoxia condition, but also but repressing it under hypoxia conditions [20]. In *Candida albicans*, there are many genes controlling hyphal formation. *BRG1* which encodes a putative GATA family transcription factor is an important hyphal specific gene. Deletion of *BRG1* both copies prevents hyphal formation [21]. Another hyphal specific gene which plays an important role in the pathogenicity and virulence of *C. albicans* is the transcription factor *UME6*. A double mutant strain of *ume6Δ/ume6Δ* showed a clear defect in hyphal formation/extension both *in vivo* and *in vitro.* However hyphal extension is controlled by other factors such as *NRG1*, a transcriptional repressor. It has been proved that *BRG1* expression is being controlled by a feedback loop with *NRG1,* thus controlling hyphal

formation [21]. *UME6* transcription factor also functions with *NRG1* through a negative feedback loop to control the level and duration of hypahl-specific gene expressions [22].

Phenotypic switching

Phenotypic switching is an *in vitro* and *in vivo* phenomenon that is reversible and happens with spontaneous emergence of *Candida* colonies altering the morphology at higher rates than somatic mutations [23,24]. This strategy allows cells to rapidly adapte to environmental challenges, that is an important ability to change the life style from being commensal to the existence as a pathogen [25-27]. The "white-opaque" transition is a bistable phenotypic switching in *C. albicans* that can be observed at sites of infection and recurrence of infection, especially in immunodeficiency cases and diabetic individuals [24].

Differences between white and opaque cells start from shape of them. White cells are round with smooth and domed colonies, whereas opaque cells are flatter with translucent colonies [28]. The interaction of these two cell forms with host immune system is also different. White cells secrete chemoattractant for leukocytes, but opaque cells do not. This difference is suggested as a escape mechanism for *C. albicans* from the host immunity [29]. The other important difference between white and opaque cells is the expression of genes that are involved in glucose metabolism. Under both aerobic and anaerobic conditions, white cells express genes involved in fermentative metabolism, whereas opaque cells express genes enrolled in oxidative metabolism [29]. In addition to gene expression profile, white and opaque cells also differ in virulence characteristics and mating competency [30,31]. White cells are more virulent in systemic candidiasis, but opaque cells colonize at cutaneous infections and mate more efficiently [29].

In white and opaque cells of *C. albicans*, the gene *CPH1* is the master transcription factor mediating MAPK signaling for pheromone-stimulated biofilm formation. However, loss of *CPH1* had no effect on conventional biofilm formation [15]. *C. albicans* strain of *cph1/cph1 efg1/efg1* double mutant is unable in filamentous growth and hyphae or pseudohyphae formation in response to stimuli, including serum or macrophages [32].

Biofilm formation

In last decades, microbial studies were mainly based on studying them in free-floating (planktonic) cultures or as colonies that are grown on the surface of nutrient agar plates

[1,33]. Now, researcher have come to an agreement that biofilms are the preferred and natural state of growth for many microorganisms such as *C. albicans* [34].

Biofilms are community of microbial cells that are adhered to a surface or at air-liquid interface which are surrounded by a self-produced extracellular matrix [34]. Microbial biofilms are observed in a variety set of environments including biotic and abiotic surroundings like plant and mammalian tissues, catheters and medially implanted devices and biomaterials. Biofilm formation has been seen in many microbial species such as *Candida* spp., Staphylococcus spp., Streptococcus spp., *Escherichia coli* and etc. A universal feature of all biofilms is the increasing resistance to physical and chemical (drug) interruptions, making them very difficult to battle in clinical settings as a huge risk for human health. Biofilm-associated infections lead to over 5 million central venous catheter replacement each year [35]. Often, patients need to be treated with 1000 x higher dosage of drugs that will further bring more complications such as liver and kidney damages. Often these treatments are not enough and patients are not able to tolerate them [36-39].

Candida albicans biofilm formation consists of four stages. Figure 1.2 shows the four stages of biofilm formation in *C. albicans.* This process starts with adherence of yeast cells onto a solid surface called seeding stage (Figure 1.2.A). Typically, an *in vitro* culture of *C. albicans* is added onto a solid surface to start the adherence phase which takes approximately 60 to 90 minutes. Following this time frame, non-adherent or loosely adherent cells are washed away resulting in the formation of a basal layer of anchoring yeast cells [40,41]. The next step is proliferation and biofilm development of the attached yeast cells (Figure1.2.B). This stage is followed by biofilm maturation which consists of a complex network of several layers of polymorphic cells from round yeast forms to cylindrical and pseudohyphal cells which are encased in an extracellular matrix comprised of glycoproteins (55%), carbohydrates (25%), lipids (15%), and nucleic acids (5%) (Figure 1.2.C) [42]. This matrix gives a thick and structured appearance to the biofilms and also provides protection from physical injury and drugs [42].

In some cases, it is possible to visualize a mature biofilm by naked eye. Under a microscope, a mature biofilm appears as a structured complex of different cell types (yeast, hyphae and pseudohyphae). The final stage of biofilm formation comprises the dispersal step, where cells migrate to other locations to form new biofilms. In the human body, a biofilm seeded at a primery location can disperse its cells to other tissues and organs in the body (Figure 1.2.D)[42].

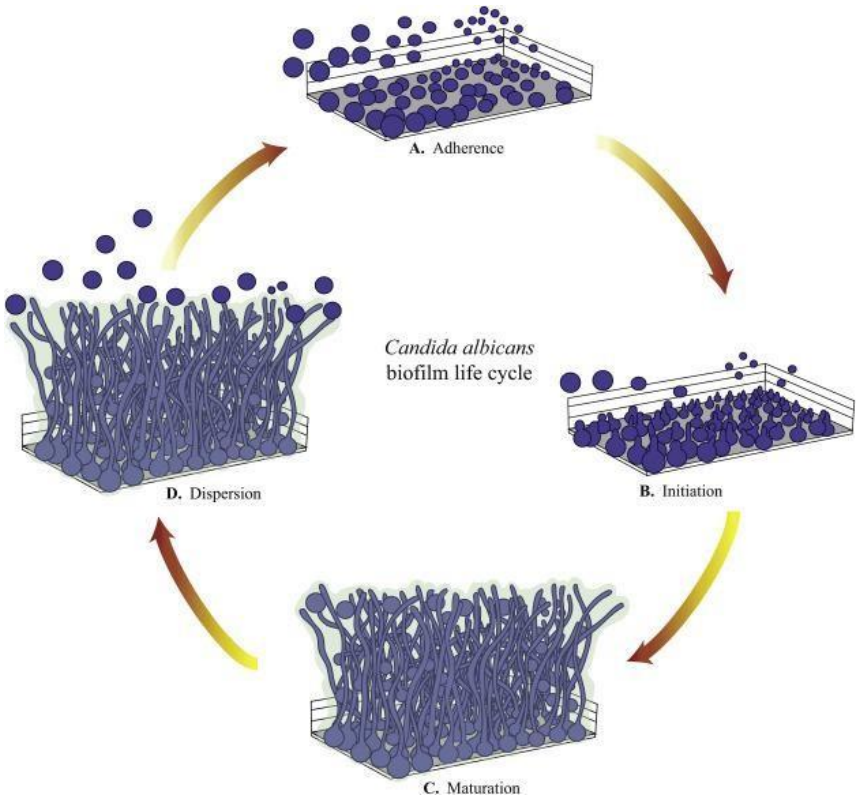


Figure 1.2: *C. albicans* biofilms. A. Adherence of round yeast cells to an abiotic surface. **B**. Initiation of biofilm formation. **C**. Maturation of the biofilm, where complex layers of polymorphic cells develop and become encased in an extracellular matrix which protects them from environmental stress. **D**. Dispersion, where the round yeast cells leave the matured biofilm to seed new sites (*adapted from* [42]).

In 2012, a transcriptional network that consists of eight master regulators controlling biofilm formation in *C. albicans* was described [43]. This network consists of Efg1, Tec1, Bcr1, Ndt80, Brg1, and Rob1 transcriptional regulators and each of this is required for biofilm development in rat *in vivo* and *in vitro* catheter and denture models [43]. These master regulators are controlling the expression of each indi idual regulator, therefore resul ng in a complex regulatory network. Together, these master regulators bind to di erent promoters and regulate the expression of 1,000 target genes. Studies both in planktonic and biofilm conditions revealed that all of these genes play key roles in hyphal formation, adhesion, drug resistance and matrix production [44]. Many newly identified target genes in biofilm network are not studied yet and therefore no sequences are available. But, the orthology mapping shows that entire newly identified target genes are highly enriched for genes that called "young" genes, suggesting that *C. albicans* has aquired the biofilm formation ability with

evolution. This shows us that why only few fungal organisms are able to form biofilms [1]. In addition to these six master regulators, 44 more transcriptional regulators have been linked to biofilm formation that if any is lacking within cells, *C. albicans* biofilm formation will be affected [1,45].

Adhesion and "adhesins"

Adhesion or aggregation is an important property of fungal colonization [46]. *Candida albicans* has a set of specific proteins, namely adhesins that mediate adhesion of the fungus to abiotic surfaces, host cells and other microorganisms [47,48]. The gene family of *ALS* (agglutinin-like sequence) is intensively described in *C. albicans*, reviewed in [49]. These genes encode cell-surface glycoproteins that play key roles in ligand binding, aggregative effects, and attachment [49]. From all the *ALS* gene members, *ALS3* is shown to play a key role in hyphae-associated adhesion [50,51]. *ALS3* is important for infections of oral epithelial cells and mucosal surfaces as increased exression levels of *ALS3* were found in *in vitro* and in *in vivo* models of mice [50]. Except from *ALS* gene family and *HWP1* (hyphal wall protein explained in morphogenesis section), there are other regulators that play important roles in adhesion reviewed in [52].

Toxin production of *C. albicans*

Until 2016 it was believed that only bacteria are able to produce cytolytic proteins and peptide toxins as typical virulence factors that disturb epithelial barriers, damage cells and activate or modulate host immune systems. Moyes DL, *et al*. has discovered the first peptide toxin in *Candida albicans*. This toxin permeabilizes membranes by positively charged C-terminus and thereby triggers a current concomitant with calcium influx. The *Candida*lysin directly damages epithelial membranes and activates epithelial immunity by triggering a danger response signaling pathway. *ECE1* gene is responsible gene for the toxin production [19].

1.3 *C. albicans* cell wall

C. albicans cell wall is presented within two main layer of outer layer and inner layer. The outer layer is mainly composed of glycoproteins and the inner layer contains skeletal polysaccharides [12]. Cell wall is composed of glucans including β-1,3-, mixed β- 1,3-/β- 1,4-,

β- 1,6, α -1,3- glucans (approximately 50-60 % of the wall by dry weight), mannans and/or galactomannans (The cell walls of *S. cerevisiaeandand* and *C. albicans* contain glycosylated mannoproteins with chains rich in mannose, known as mannans which are 20 -30 % of cell mass) and chitin (only 1-2 % of dry weight) [53-56]. Fungal cell wall is a complex of polysaccharide and lipid moieties that activates host immune responses [57]. Exterior to the plasma membrane, fungal cell wall innermost layer is composed of chitin, an N-acetylglucosamine polymer. The next layer is composed of immunoreacti e β-(1, 3) and β-(1, 6) glucans. The outer cell wall consists of glycoproteins that incorporate N- and O-linked mannans and causes inflammatory responses via mannose receptor and TLR-4 [58]. Figure 1.3 shows the structure of *C. albicans* cell wall [59].

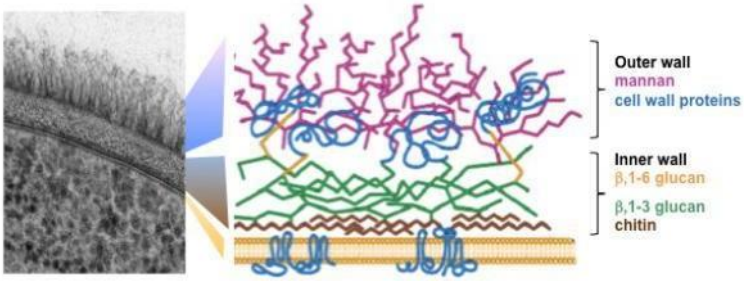


Figure 1.3: *Candida albicans* cell wall structure. Two layers can be distinguished in *C. albicans* cell wall. The outer layer is mainly composed glycoproteins and the inner layer contains chitin and β-1,3 glucan which helps to shape and strengthen the cell. The outer layer and the inner layer are linked together through more flexible β-1,6 glucan that are attached to the outer cell wall proteins.

1.4 Antifungal drug resistance mechanisms

As a major global health issue, nearly 1.35 million deaths annually, is caused by fungal infections [60]. A successful patient management suffering from fungal infections requires antifungal therapies. The current antifungal drugs are limited classes including azoles, echinocandins and flucytosine [61]. In these between echinocandins family is the best antifungal choice to treat severe infections such as candidiasis [62]. Echinocandin antifungals including caspofungin and micafungin are cell wall active agents that work by inhibiting the biosynthesis of the 1,3-D-glucanin in fungal cell wall [62]. Echinocandin drugs are active against most of *Candida* species, but have limited spectrum and are inactive against Mucormycetes, Cryptococcus spp. or Fusarium spp. [63,64]. Echinocandins are fully functional in some of *Candida* biofilms and azole-resistant yeasts such as *C. glabrata* and *C. krusei,*

because of the mechanism of action that is unrelated to azoles that inhibits the biosynthesis of fungal the plasma membrane [65-69]. The privilege of echinocandins is their specificity in the target which this target doesn't exist in humans, therefore echinocandins have lower toxicity. Echinocandins are prescribed intravenously, because they have a low oral bioactivity. From the concentration and dosage point of view, they have a notable C_{max}, but also largely AUC/MIC (Area under the curve/Minimum inhibitory concentration) drugs. They are distributed into central nervous system (CNS) and eyes, but well distributed through tissues [70]. The drugs comprise from two subunits: a GTP-binding protein, Rho, that plays a role in regulation of biosynthetic capacity of glucan synthase [71] and the second subunit is a catalytic, Fks. This subunit is encoded by *FKS1, FKS2* and *FKS3* genes. In *Candida albicans*, *FKS1* is an important and essential gene, while *FKS2* and *FKS1* are redundant in *C. glabrata* [72-74]. However, *FKS3* is not expressed in high le els and doesn't ha e a major impact on overall biosynthetic capacity.

The predominant *Candida* spp. are highly susceptible to echinocandins [75,76]. Echinocandins potent activity against most *Candida* species was confirmed by standardized microbroth dilution susceptibility tests which were established by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [75,76]. From over 100 centers worldwide, 8271 *Candida* species isolates were collected from 2003 to 2007, MIC values and epidemiological cutoff values (ECV) were tested with the CLSI M27-A3 broth microdilution method. All major *Candida* species were tested with the three echinocandins and the alues obtained as: MIC alues of ≤ 0.06 mg/ml and ECV alues ≤ 0.25 mg/ml.

Both organizations of EUCAST and CLSI have established species and drugs specific clinical breakpoints (CBP) for echinocandins [70,77], although no CBP has been established for caspofungin and it has not been recommended to use caspofungin MIC testing for clinical assessment [78]. *Candida* spp. are increasingly reported to gain resistant against echinocandins [79-86], however the prevalence of *C. albicans* echinocandins resistant is about 2-3 % , the other specis such as *C. glabrata* show higher resistance (8-13 %). Mostly the echinocandin resistance emerges after prolonged therapy [87].

1.5 Fungal recognition and immune system activation

Several pathogen-associated molecular patterns (PAMPs) expressed by invading microorganisms such as bacteria and fungi are recognized by pattern recognition receptors (PRRs) that results in signaling-mediated transcription and production of inflammatory mediators such as cytokines and chemokines [88]. These signals induce the recruitment of immune cells such as neutrophils to the site of infection [89]. Different PRRs are localized on the surface of macrophages and dendritic cells such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). An immune response initiated through multiple series of signaling cascades is activated upon ligand binding. This results in fungal internalization via phagocytosis and production of cytokines, reactive nitrogen species (RNS) and reactive oxygen species (ROS) which all contribute for activation of the adaptive immune system and killing of the invasive pathogen [90-93].

In more detailed way, β-mannan is recognized through galectin-3 [94] and α-linked N-mannans are recognized through the mannose receptor (MR), dectin-2, mincle, and DC-SIGN [95,96]. Chitin is recognized and taken up by MR and further induces TLR9- and NOD2-dependent IL-10 production [58,96]. In summary, many fungal cell wall components are recognized by TLR2, TLR4, TLR9 that causes activation of macrophages and dendritic cells and further production of proinflammatory cytokines such as IL-1β, IL-6 and IL-23 [97,98]. For further immune cell recruitments, produced proinflammatory cytokines activate Th17 cells by binding to their receptors.

Neutrophils are primary cellular defenses against infections. They are one of the key immune cells in invading bacterial and fungal infections through multiple mechanisms including antimicrobial peptide production, protease release and reactive oxygen species (ROS) production [98]. Neutrophils migrate to the site of infection in response to the chemotactics produced by pathogens [89,99], hence neutropenia can be detrimental in bacterial and fungal infections. In other hand, excessive accumulation of neutrophils can have an inverse effect on immune system by damaging tissues [100]. In addition to neutrophils, other immune cells such as monocytes, macrophages and dendritic cells are also counted as phagocytes during invasive fungal infections (candidiasis). Although immune system catches invading microorganism soon after infection, fungi have developed some strategies to overcome the defenses including PAMPs shielding by cell wall components and capsules, asteroid bodies, dimorphism from yeast to hyphae and biofilm formation . *C. albicans*

virulence factors are manly expressed when the fungi is in hyphae form and hyphae induces lower amount of cytokines in comparing to yeast cells [99].

Candida albicans has two strategies of evading the host immune system including endocytosis and active penetration [101,102]. For endocytosis, some proteins are expressed on the cell surface (invasins) that these proteins mediate the binding to host ligands [50,103], thereby engulfment of fungal cells into the host cells. Killed hyphae are even taken up, indicating that the process of induced endocytosis is a passive program and does not need activities of viable fungal cells [104,105]. In contract to endocytosis, penetration needs viable *C. albicans* hyphae. Although it is still not clear how exactly the penetration mechanism works and which factors are mediating it, but it is believed that physical force and fungal adhesion are important factors [106]. Another factor that is important in penetration is secreted aspartic proteases (Saps) [102].

2. *Klebsiella pneumoniae (quasipneumoniae)*

Klebsiella pneumoniae is a member of *Enterobacteriaceae* family which is part of the human microbiome. *Klebsiella* can be found as a commensal microorganism in the gastrointestinal tract and lungs [107]. However, depending on the microbiome equilibrium composition and the host immunity, this microorganism can turn into an opportunistic pathogen. Infections by *Klebsiella* species account for one third of all Gram-negative infections [107]. These infections include pneumonia, cystitis, urinary tract infections, surgical wound infections, pyogenic liver abscesses, endogenous endophathalmitis and even more life threatening infections such as endocartitis and septicemia [108]. In pediatric wards, premature neonates and infants as well as in immunocompromised individuals, this bacterium can cause sepsis and meningitis [107]. *Klebsiella pneumoniae* strains are assigned to different terms such as classical *K. pneumoniae* (cKp) which are persistent in hospital environments, hyper virulence *K. pneumoniae* (hvKp) that are distinct from cKp and were firstly described in Asian Pacific Rim causing invasive and metastatic infections. *Klebsiella* also exists as multi-drug resistant (MDR) clones that are spreading internationally, raising awareness for investigation of resistance mechanisms [109-112]. Navon-Venezia and colleagues have investigated the wide resistome of this pathogen [113]. Importantly, they report the ability of *K. pneumoniae* to accumulate antibiotic resistance genes (ARGs) under antibiotic selective pressure. This occurs by *de novo* mutations via acquisition of plasmids and

transferable genetic elements, which lead to development of drug-resistant (XDR) strains [113].

Klebsiella pneumoniae sub-species *similipneumoniae* strain ATCC 700603, known as *K. pneumoniae* K6, was previously classified in three phylogroups called KpI, KpII, KpIII [114]. Recent studies suggested that the second subgroup (KpII) should be reclassified as a new species called *Klebsiella quasipneumoniae* sub-species *quasipneumoniae* and subspecies *similipneumoniae* which correspond to two sub-species of KpII-A and KpII-B, respectively [115]. In this thesis, the *Klebsiella* strain used in all the experiments is designated as *Klebsiella quasipneumoniae* and the related data are elicited from Kyoto Encyclopedia of Genes and Genomes (KEGG) data base.

2.1 Virulence factors of *Klebsiella* species

Biofilm formation

K. pneumoniae is able to form biofilms that are aggregates of cells adhering to each other and also surfaces. Biofilms are embedded within a self-produced matrix of extracellular polymeric substance (EPS) [116]. The EPS is a complex mixture of polysaccharides, proteins and DNA. Biofilms contribute to invasive infections especially in immunocompromised patients and colonize in gastrointestinal, urinary and respiratory tracts [107]. The most clinically significant effect of biofilms can be seen in inner surfaces of catheters and medical devices. Biofilm development starts from cell adherence to formation of microcolonies. Furthermore, it maturates and finally dispersal of the cells happens. In *Klebsiella,* the most important surface structures in biofilm formation are three fimbriae and the capsular polysaccharide (CP) [117]. The function of fimbriae is to mediate stable adherence of *Klebsiella* cells, but CPs affect the cell-to-cell communication in biofilm structures. As the environmental conditions are constantly changing within biofilms, cells need to be dynamic and adoptable. Therefore, in biofilms the gene expression must be swift and capable of extensive changes. In different bacteria such as *Klebsiella pneumoniae*, transcription regulation is controlled by quorum sensing, a communication and signaling system that coordinates the gene expression within organism communities [117]. Known quorum sensing molecules are described in *Klebsiella pneumoniae* [117-120], but the present data on Kp quorum sensing molecules are not complete.

The matrix formed within *Klebsiella pneumoniae* biofilms has a key role to block the access of antibiotics, host antibodies and phagocytes to the cells [121]. Being highly

resistance to antibiotics is the most notorious feature of *Klebsiella* biofilms. Within biofilms cells are adapted to starvation and low oxygen levels which results in excessive growth of the bacteria. This feature diminishes the efficiency of antibiotics that target the metabolically active cells [107].

Antibacterial resistance

In 2013, a landmark report on ''Antibiotic Resistance Threats'' was released by Centers for Disease Control and Prevention (CDC) [122]. In this report, 3 microorganisms were introduced as posing a threat level of urgent. These microorganism were *Clostridium difficile*, carbapenem-resistant *Enterobacteriaceae* (CRE) and drug-resistant *Neisseria gonorrohoeae* [122]. Within these microorganisms, CRE that includes organisms like *Escherichia coli* and *K. pneumonaie* (Kp), are posing resistance to almost all currently available antibiotics. In health care settings, CRE which are typically mediated by the production of beta-lactamases [123] have increased over the past decade such a way that nearly 50 % of patients developing blood stream infections with these microorganisms die from infection [122,124]. The last-resort antibiotics to treat such infections is colistin [125], however CRE are developing colistin resistance strains.

Klebsiella pneumoniae naturally produces a chromosomal penicillinase (SHV-1), therefore it is resistant to ampicillin, carbenicillin and ticarcillin. However, it has a property that makes this strain quite virulent that is the propensity to collect resistance plasmids. Previously Kp was known for plasmids encoding extended-spectrum-β-lactamase (ESBLs) changing to expanded-spectrum-cephalosporins (ESCs). These resistant plasmids were TEM- AND SHV-type ESBLs and they coexisted on the same plasmids encoding the resistant genes to aminoglycosides (e.g. kanamycin, gentamicin; inhibiting protein synthesis of bacteria by binding to 30s ribosomal subunit), cotrimoxazole (blocks the making and usage of folates) and tetracycline (binds to 30s ribosomal subunit and block the tRNA binding). In 90s, the new ESBLs family in Kp (CTX-M group) emerged which had resistance mark to penicillin and ESCs, but not effective to carbapenems [107,126]. Studies in the United States showed that the prevalence of exhibiting resistance to third generation cephalosporins in *Enterobacteriaceae* has increased from 1.39 % to 3 % since 1999 till 2001 [127]. Interestingly, 7.7 % of total isolates were *Klebsiella pneumoniae*; they posed 33.1 % of ESBL-producing *Enterobacteriaceae.*

Carbapenems which kill bacteria by binding to penicillin-binding proteins and inhibits bacterial cell wall synthesis were used as the first line of treatment when a meaningful

proportion of bacterial isolates developed fluorinated quinolones resistant. However, the extensive use of carbapens resulted in appearance of multi-drug resistant (MDR) carbapenemase *Klebsiella pneumoniae* strains. The carbapenemase encoding genes are on MDR transferable plasmids that causes resistant to multiple antibiotics (CP-Kps) [128]. These strains are a major cause of neonatal and children infections especially in south-east Asia and western pacific regions [129-131]. Several reports have shown the carbapenemase-producing *Enterobacteriaceae* distribution in pediatric and adult patients in Europe, North and South America, too [132-134]. Since CP-Kp exhibit a wide range of drug-resistance phenotype, the treatments are more complicated [135,136]. Polymyxins including colistin and polymyxin B (inhibitors of bacterial membrane functions) are listed as the most effective antibiotics against CP-resistant-Kp (CPR-Kp), however the minimum inhibitory concentration (MIC) of these antibiotics are quite high. It was reported that *K. pneumoniae* outbreak, was belonging to the ST512 clone [137,138]. It was proved in 22 of 24 isolates that Kp is resistant to colistin by broth microdilution following the CLSI-EUCAST recommendations [139] (MIC of colistin were: 1 and 2 mg/ml and 0.25 and 1 mg/ml in duplicate assays for the 2 other isolates) [140].

The polymyxin resistance in Kp is mediated by some modifications in lipid A with 4 -amino-4deoxy-L-arabinose (L-Ara4N, *arnBCADTEF-ugd* loci) and/or phosphoethanolamine (pEtN, *pmrC* or plasmid-borne *mcr-1*) [141-143]. The modification of lipid A which is encoded chromosomally, is inducible by several two component systems (TCSs) such as PhoPQ and PmrAB [144,145]. There is a negative feedback regulation on *phoPQ* by a small transmembrane protein called MgrB [146]. Mutations (loss-of-function) of *mgrB* constitutive inductions of *phoPQ* which leads to upregulation of the lipid A conferred this strain to polymyxin resistance strain [146-151]. In a recent study that 8 patients were treated with colistin and Kp samples were taken before and after drug treatments, 449 genes were predicted to play roles conferring multi-drug resistance. In 15 of 16 Kp isolates, an *ISKpn26*-like element disrupted *mgrB.* 7 colistin-susceptible Kp isolates were observed where *phoQ* was mutated with disturbed *mgrB* [152]. This report was the first to show any *phoQ* mutations within *mgrB*-disturbed colistin–susceptible Kp isolates [152]. These studies indicate the complex and multifaceted mechanisms of colistin resistance in *K. pneumoniae* strains highlights the need of more extensive research on this topic.

KEGG database is a resource for high-level functions in biological functions, especially large-scale molecular databases which are generated by genome sequencing and other high-throughput technologies. Some of the genes related to *Klebsiella pneumoniae* (*quasipneumonaie* in this study) are listed as code or numbers within this database. However

some of the genes have not been studied extensively, but one can find an orthologue of that gene in other similar/related species. In this study we had used this database to find more of interesting genes related to our study.

2.2 Immune recognition and evasion strategies of *K. pneumoniae*

Innate immunity

Klebsiella pneumoniae, like any other microorganism, has to overcome the chemical and mechanical barriers to escape the host immunity to be able to cause infection. After accessing the host, the organisms are recognized by pattern recognition receptors (PRRs) of host immune cells and thereby triggering the immune mediator's production. The key player of host innate immunity is monocytes and macrophages as phagocytes which further orchestrate the immune response via cytokine and chemokine production. Neutrophils are among the first immune cells that are recruited to the site of infection. In order to perform this process some mediators such as interleukin (IL)-8 and IL-23 which further induces IL-23 production, are required [153,154]. Also IL-12 is involved in this process by production of interferon-gamma and further expanding the expression of IL-17. Upon pathogen recognition, NOD-like receptor pyrin domain which contains NLRP3 inflammasome pathway is activated that causes IL-1β production. In addition, other pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-6 are also induced upon bacterial recognition [155]. The recruitment and maturation of dentric cells (DCs) happenes in *Klebsiella pneumoniae* infections via TLR-9 dependent manner which lead to intracellular killing of bacteria with these innate immune cells [156].

Adaptive immunity

In some studies, the link between the innate and the adaptive immunity has been described and provided by PRRs, especially TLR-mediated maturation of DCs which further on activated the pathogen-specific T lymphocytes [157]. After antigen uptake, DCs become active and migrate to lymph nodes to present the foreign peptides in the context of relevant MHC molecules. After maturation, DCs endowed with the ability to stimulate naïve $CD4^+$ T lymphocytes into T helper (Th) subset [158]. It has been discovered that Th1 and Th 17

responses are important for protection against intracellular bacteria and bacterial infections with possible roles in the development of autoimmunity, respectively [158,159].

Evasion strategies

The circumvent of the host immune system by *Klebsiella pneumoniae* is still not fully understood, but there are four well-characterized virulence factors that helps the bacteria to evade from immune system. These factors include fimbriae, the capsule, siderophores and the lipopolysaccharide (LPS) [153,160]. *Klebsiella pneumoniae* is adhering to epithelial and immune cells as well as abiotic surfaces by using the type 1 and type 3 fimbriae to facilitate the adherence. Capsular polysaccharides (CPs) are composed of 78 (K1 -K78) distinct serotypes. In these between K1 and K2 are the most virulent *Klebsiella pneumoniae* strains by producing excessive capsular materials (hypermucoviscous phenotype). Other *Klebsiella pneumoniae* strategy that helps the bacteria to dampen the host immune response is the LPS variation. Some of the strains modify the LPS till a degree that it is not recognized by host immune cells and therefore escaping the immune recognition. Other than LPS amount variation, the capsule masks LPS from detection by the TLR-4 [161,162]. All of these are strategies that help the bacteria to dampen the immune system by preventing inflammatory responses. Also, Kp steals iron from host to accelerate the growth and replication. In Kp four iron acquisition molecules (siderophores) have been described including enterobactin, yersiniabactin, salmochelin, and aerobactin. In these between, enterobactin, the primary iron system, has been described to have the highest affinity for iron which is present in both classical and hypervirulent strains. More recent studies have been suggesting that hvKp produces larger amount of enterobactin siderophores than the cKp strains which causes more virulence and pathogenicity of these strains [163]. Although there are many biological factors described for the hvKp to be distinguished from other strains, but there is no single marker known that specifies these strains from other *Klebsiella* strains.

2.3 Polymicrobial infections

Why should we study polymicrobial infections?

Simultaneous isolation of bacteria and fungi has been done from oral cavities infections associated with periodontitis, wound infections, chronic pulmonary diseases such as cystic fibrosis, urinary tract infections, cancer and patients with immune deficiency

problems such as AIDS [42,164,165]. Current diagnostics of bacterial and fungal infections are solely aimed to identification of individual species or genera but do not consider the occurrence of a polymicrobial infection [2](Nogueira, MF et al. 2019, *in press*). Only recently more attention has been devised to the potential interaction between microorganisms which can lead to poorer outcomes for the patient [42]. For example, it was shown that mice infected with a sub-lethal inoculum of *P. aeruginosa* and *C. albicans* together showed higher mortality rate than single infections of either species alone [166]. The mortality rate of patients having double infection of *C. albicans* with different bacterial strains is 50 % higher in comparison to individuals having only *C. albicans* infections [167-169]. Fungal infections are the cause of death in more than 45 % in patients with immunodeficiency or cancer. These infections are mostly nonbacterial and are probably due to treatments with inefficient drugs [170]. Other study showed that synergistic interaction between *C. albicans* and one of the oral bacteria *Streptococcus oralis*, *Streptococcus sanguinis*, or *Streptococcus gordonii* in an *in vitro* mucosal model, made both organisms stronger to invade and cause pathogenic biofilms [171]. Importantly, antimicrobial drug treatment leads to alterations of the normal flora of the human body, therefore disturbing the microbiome equilibrium. Other factors including diet, stress, age, chronic inflammation, all contribute to dysbiosis and affect the progression of a variety of diseases [172-175]. Ineffective diagnostics can lead to extensive inflammatory responses, organ damage or failure and in the end higher mortality rates for patients [42,176]. Early and targeted diagnostics should therefore consider the potential microbial interactions.

C. albicans in polymicrobial infections

Up to date, *Candida albicans* has been reported as the most frequently isolated fungal species from bacterial-fungal infections [2]. [172]. Figure 1.2 shows the multi-species biofilms formed by the fungi *C. albicans* and different bacteria through human body. *C. albicans* shares some specific niches of the human body with different bacteria. These niches include oral cavities, lungs, gastrointestinal tracts (GIs), vulvovaginal region and skin. In addition the bacterial-fungal interactions (BFIs) can be found on implanted medical devices and burn wounds, too (Figure 1.2, a). The most common ways by which *C. albicans* interact with bacteria are physical interactions, synergistic or antagonistic relationships, produced signaling molecules such as quorum sensing molecules and finally via nutrient exchange [177-179]. Bacterial and fungal communications are mediated through produced proteins and molecules called quorum sensing molecules. Finding these molecules and determining their relations

with host immunity makes them a great target for efficient diagnostics and treatments. For example, studies have shown that produced signaling molecules via human intestinal microbiota could be used for disease diagnostics [180]. *C. albicans* can have a close physical interaction with both gram positive and gram negative bacteria. For example studies have shown that *C. albicans* adhesion proteins like Als3, Als2 and Hwp1 and also transcriptional regulators such as Bcr1 and Tec1 play important roles in physical interaction of this fungus with different bacterial species such *as S. epidermidis, S. gordonii* and *S. aureus* [181-183]. Other type of BFI relationship described within *C. albicans* is synergistic, providing protection for one or even both species present in biofilms. For example when *C. albicans* and methicillin-resistant *S. aureus* (MRSA) were grown together in biofilm mode, *C. albicans* could protect the MRSA strain from vancomycin that normally have negative effect of the resistant strain (Figure 1.2. c)[184,185]. A second example for the synergistic BFIs is the dual-species biofilm between *C. albicans* and either *Bacteroides fragilis* or *Clostridium perfringens*, both as anaerobic bacteria existing in human gut. The biofilm formed by *C. albicans* provide a protective hypoxic microenvironment for these bacterial species [186]. For antagonistic BFIs we could name *C. albicans* and *P. aeruginosa* relationship, that in this case bacterial cells attach to the hyphal structure of the fungi and kill it. Although the exact underlying mechanism is not understood, we should consider the complexity of these interactions and multiple factors affecting the outcome [187,188].

The BFIs are not limited to direct physical interactions. Some of BFIs rely on the secretion and diffusion of produced signaling molecules and others on changes in local environment such as pH variations, oxygen or carbon dioxide levels for altering the behavior of the other species present in interaction (Figure 1.2. d,e) [42,189]. One example for inhibition via altering the environmental pH is *Lactobacillus* species present in vaginal microbiota. This bacterial species lower the pH in this part of body by producing lactic acid and therefore inhibiting fungal (*C. albicans*) initial adhesion to the vaginal mucosal surfaces (Figure 1.2. e) [190]. Studies on the interaction between *C. albicans* and *P. aeruginosa* revealed that each of these strains is producing quorum sensing molecules that are detectable by the other species. For instant, *P. aeruginosa* secrets 12-carbon acyl homoserine lactone that inbibits hyphal growth of *C. albicans* (Figure1.2. d)[191,192]. *C. albicans* hyphal and biofilm formation is also disturbed in a similar situation with *Enterococcus faecalis* which produces bacteriocin EntV [193]. One of the interaction modes that has been less studied, is the nutrients and electrons exchange between different microbial species forming biofilms [194,195]. This interaction provides the gain of energy from multiple series of reactions to

microorganisms that a single species in that particular biofilm might lack. The known interaction of *C. albicans* and *P. aeruginosa* is relevant for human health, in which a positive feedback loop affect the biofilm formation by exchanging the phenazine produced from bacteria and ethanol secreted from the fungi [196,197]. As explained previously, there are multiple bacterial strains interacting with *C. albicans* in biofilms. One of the less studied BFIs is the interaction between *Klebsiella pneumoniae* and *Candida albicans.* These microorganisms are both present in human microbiota and are co-isolated from patients such as cystic fibrosis [198-202]. In previous studies, it was shown that *C. albicans* biofilm thickness was reduced when it was co-cultured with *K. pneumoniae.* While this BFI, gene expression profiles were highly changed in both of the microorganisms, such as particular genes like *WOR1* (master regulator of white-opaque switching). Figure 1.2 indicates different niches in human body that are shared between fungal and bacterial species and the type of interactions that could be modulating the BFIs.

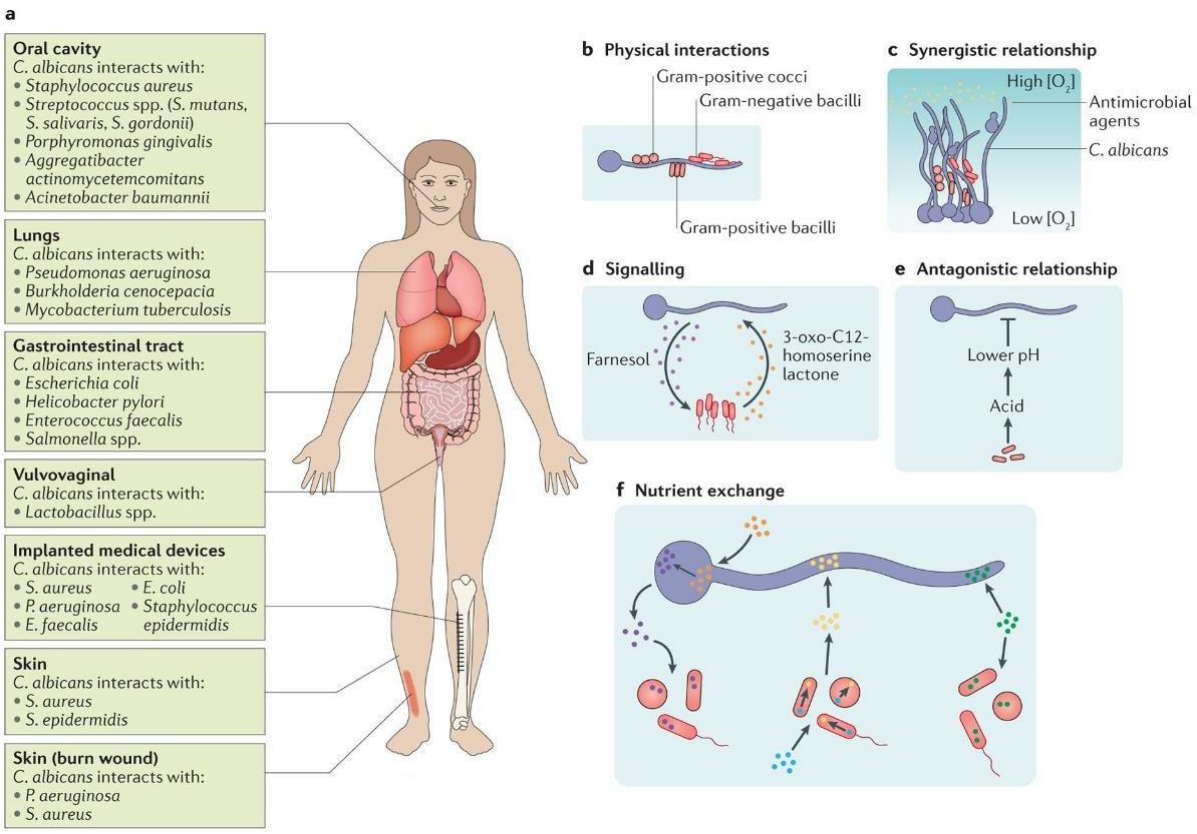


Figure 1.3: Multi-species biofilm formation in human body a) Different niches through human body that are shared between bacteria and fungi. Through infections of these parts of the body, bacterial strains are co-isolated with *C. albicans*. **b-f)** Common ways in which *C. albicans* is forming biofilm in combination with different bacterial strains. **b)** Bacterial cell, gram positive and gram negative, are

able to bind to *C. albicans* hyphal structures while forming biofilms. **c)** BFIs can be synergistic. For example, *C. albicans* hyphae can protect the bacteria from antibiotics (in yellow) or the fungal hyphae can make a low oxygen concentration in depth of the biofilms and therefore protecting the anaerobic bacteria. **d)** Inter-kingdom communication is possible by signaling molecules produced by microorganisms within mixed-species biofilms. In this example, *P. aeruginosa* produces the signaling molecule 3-oxo-C12-homoserine lactone (in orange) and *C. albicans* secretes farnesol (in purple) that influence the behavior of the front strain. **e)** BFIs can be antagonistic, too. For instance, *Lactobacillus* strains are lowering the pH in vaginal mucosal surfaces, therefore inhibiting the hyphal formation of *C. albicans*. **f)** Microorganisms within mixed biofilms can exchange nutrients. They can either share nutrients, or one species can make use of a nutrient and in turn produce other nutrients that can be used by the second species (Figure *adapted from* [2]).

Hence, understanding the complexity of bacterial-fungal interactions and the interplay within the human host could provide us the knowledge to discover novel biomarkers that facilitate diagnostics and treatment of polmicrobial infections.

1.4 Galleria mellonella, an *in vivo* model to study bacterial and fungal infections

Galleria mellonella larvae (greater wax moth or honeycomb moth) are insects that belong to the *Lepidoptera* order. *G. mellonella* has been extensively used as an *in vivo* model to study virulence of human pathogens and the host-pathogen interactions [203-208] One of the reasons which make *Galleria* model a useful tool for researchers, is because of the similarities of this insect innate immunity to the innate immunity of mammals. The life cycle of this insect can vary from larval to pupal/moth form. During each step of their life cycle, the size differs from 1-3 cm in larva and 3-4 cm in pupae and finally maturing into moth [204,207]. The small size of this model makes the manipulations fast and easy without any need of special equipments. In addition, this model has a very low cost comparing to mouse models, easy to handle and they tolerate a wide range of temperatures such as 37 °C. Over 1000 publications on PubMed have used this model to study the virulence factor of microbial pathogens and also the efficiency of antimicrobial compounds [209] and to aim so, mostly adult larvae (before they get into the pupa stage in their life cycle) are used. The body structure of the larvae is quite simple and it contains a neural tube that works from frontal part through the dorsal section and a digestive tube. The whole body includes a large amount of fat. All of the organs in *Galleria mellonella* are surrounded by hemolymph. This model was used to study fungal infections such as *C. albicans* [203,210,211], *A. fumigatus* [212] and *Cryptococcus neoformans* [213].

Although this model has been widely used in research and has numbers of advantages, but there are also some limitations for using *Galleria* model. The genome of this model has been sequenced recently [214], but there are no ways for genetic manipulation of this model [215].

Innate immune system of *Galleria mellonella*

The immune system of *Galleria* consists of components such as fatty body, lymph nodes and hemocytes. The general cavity of the *Galleria* body is occupied by the open circulation system called hemolymph. These insects have only the innate immunity which is composed of three parts: physical and chemical barriers that help to protect the insect from any external environment, humoral response and the cellular response which is mediated by hemocytes as *Galleria*'s blood cells [216]. Hemocytes have various functions such as storage of different type of substances, providing defense for the organism, phagocytosis, nodulations and melanization. The humoral system is based on different molecules that are secreted while infection caused by pathogens, such as lytic enzymes (lysoyome), antimicrobial peptides (AMPs) and melanins, opsonins and other molecules that are directly attaching the pathogens, like phenoloxidase (that protects the insect by catalyzing the melanin) [217]. Hemocyte function is the main defense of these insects against pathogens. There are 6 different types of hemocytes (prohemocytes, plasmatocytes, granular cells, coagulocytes, spherulocytes, and enocytoids) reviewed in [209]. Most importantly, the process of noduluation happens when the invading microorganisms are recognized and killed by hemocytes trapping aggregation system (nodules) and it is the time that melanization happens inside this aggregates [218]. And also, the process of phagocytosis that happens in two steps and starts by the contact of granular cells contacting the pathogen and releasing he granules that follows by the second step which is completed by the adhesion of plasmatocytes to the pathogen [219].

Response of *Galleria mellonella* to *C. albicans* infection

Studies have shown that both cellular and humoral responses are recruited in *Candida* infections. Also, some antifungal such as caspofungin have immunomodulatory effects and increased the immune response of *Galleria* during fungal infection [220]. The susceptibility of larvae has increased during fungal infections, when the hemocytes were inhibited by cytochalasin and nocodazole [221]. During fungal infections, hemocytes induce nodulation at

the site of infection and that avoids fungal replication. The process is carried out by the fast and early accumulation of melanin at the side of infection [222]. GmCD8 receptor in *Galleria* binds to apolipophorin III in *C. albicans* and recognizes the pathogen and induces phagocytosis [223]. β-glucans in fungal cell wall induces the production of AMP genes and therefore protecting the larvae from extensive *C. albicans* doses [224].

Other than fungal infections, bacterial infections, either Gram-positive or Gram-negative such as *Klebsiella pneumoniae* have been studied in this model (reviewed in [209].) Polymicrobial infections have also been explored in this model, where *C. albicans* was co-infected with *S. aureus* in *Galleria* model and the viability analysis showed a higher pathogenicity in the presence of both bacterial and fungal strains. In this co-infection, *Candida* supported the bacterial colonization and antifungal resistance has been enhanced [208]. In another study, it was proven that *A. fumigatus* pre-exposure enhanced the mortality of the infection caused by *P. aeruginosa* infection [225].

Hypothesis and aims of the study

Hypothesis

We hypothesize that, upon *Candida-Klebsiella* interaction, *Klebsiella* might produce enzymes such as chitinase and endoglucanase that could cause the cell wall degradation of the fungus. In this between, the gene expression pattern of *C. albicans* could be changed and may also produce some defense molecules or toxins such as *Candida*lysin upon interaction. The sensitivity of antimicrobial drugs in the bacteria and/or the fungus might be changed upon interaction. Gene expression pattern might be changed in both of the species and therefore affecting the virulence factors such as biofilm formation.

Aims

In this study, we aimed to investigate and characterize the interaction between the human pathogens *C. albicans* and *K. quasipneumoniae*. We hypothesize that particular proteins and molecules produced by these pathogens mediate the bacterial-fungal interaction and may be exploited as microbial biomarkers for diagnostic and treatment. In more detail, we aim to:

- Determine the biofilm biomass of *Candida-Klebsiella* co-cultures and compare them with single cultures to find out about the type of BFI interaction.
- Investigate MIC values of antifungal and antibacterial drugs against *Candida* and *Klebsiella,* respectively, to check for the possible gain of drug resistance in co-cultured strains.
- Analyze the expression of key gene regulators, important in this BFI, from both bacterial and fungal strains.
- Quantify cell wall components of *C. albicans* upon exposure to *K. quasipneumoniae.*
- Examine the host viability of *G. mellonella* after BFI infection and compare them with single infected individuals.

2. Materials and Methods

2.1 Microbiological methods

2.1.1 Strains used in this study

Table 2.1: Fungal and bacterial strains used in this study

Strain	**Reference**
Candida albicans SC5314	[226]
Candida auris CBS10913	[227]
Klebsiella quasipneumoniae ATCC 700603	[228,229]
Candida albicans SC5314 pENO_dTom_NATr	[230]
Klebsiella quasipneumoniae pUA66_PrpsM_gfp_KANr	[231-233]

2.1.2 Fungal and bacterial cultivation media

- YPD Liquid (Yeast extract Peptone Dextrose) media (1 x)

10 g/l yeast extract (Formedium, Norfolk, UK), 20 g/l Bacto-Peptone (Formedium, Norfolk, UK), 2 % (w/v) Glucose (ITW Reagents, Germany)

Yeast extract and peptone were suspended in ddH_2O. A 20 % Glucose (ITW Reagents, Germany) stock solution was prepared and diluted in the YP-medium to the appropriate final concentration and autoclaved for 20min at 121°C

- YPD plates

10 g/l yeast extract (Formedium, Norfolk, UK), 20 g/l Bacto-Peptone, 2 % (w/v) Glucose (ITW Reagents, Germany)

200 ml of 2 x liquid YPD-media were prepared, supplemented with 50 ml 20 % Glucose (ITW Reagents, Germany) and added to 250 ml of 4 % (w/v) agar.

- LB Liquid (Lysogeny Broth) media (1 x)

10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract (Becton Dickinson Austria Ges.m.b.H) were mixed in 950 ml of ddH_2O and autoclaved for 20min at 121°C

- LB plates

10g/l Tryptone

5g/l Yeast extract

10g/l NaCl

For LB-plates, add bacteriological agar (BD) at a final concentration of 2% (w/v).

- Media used for bacterial and fungal cultures

DMEM (Dulbecco's Modified Eagle Medium-high Glucose) (ITW Reagents, Germany) + glutamine (Gibco, Thermofisher) + 10 % heat Inactivated Fetal Calf Serum (hiFCS)

- M9 (Minimal Medium), low osmolarity:

M9 salt (64 g Na_2HPO_4, 15 g KH_2PO_4 (VWR International GmbH, Austria), 2,5 g NaCl (ITW Reagents, Germany), 5 g NH_4Cl (VWR International GmbH, Austria)

Glucose 0,4 % (ITW Reagents, Germany)

$MgSO_4$ 0,002 M (Merk KGaA, Germany)

$CaCl_2$ 0,0001 M (Sigma-Aldrich Handels GmbH, Austria)

- YNBNP media

YNB (Difco Yeast Nitrogen Base w/o AAs and Ammonium Sulfate) (BD Biosciences, Austria)	1,7 g/l
N-acetyl-glucosamine (Sigma-Aldrich Handels GmbH, Austria)	2,5 mM
Phosphate buffer (KH_2PO_4 + K_2HPO_4) (VWR International GmbH, Austria)	25 mM
Glucose (ITW Reagents, Germany)	0,20 %
Maltose (Sigma-Aldrich Handels GmbH, Austria)	0,10 %
Ammonium Sulfate (Sigma-Aldrich Handels GmbH, Austria)	5 g/l

2.1.3 Bacterial and fungal cell counts

- For cultivation of bacteria and fungi in either biofilm or planktonic growth mode, cells were counted and adjusted to the same value. For this, bacteria and fungi were grown overnight at 37 °C and 30 °C, respectively.

- From overnight cultures, 1 ml was taken in a 1.5 ml Eppendorf tube and spun down at 16662 rcf for 2 min.

- Cells were washed once with 1 x PBS. After the washing step, cells were resuspended into 1 ml PBS.

- To count fungal cells, a 1:100 dilution was prepared in PBS. Cell suspension was vortexed and 10 µl was loaded on the chamber. Cell counts were performed according to the following formula:

Number of cells per ml: number of cell counts x 5 squares x 10^4 to estimate the number of cells per ml x dilution factor.

- To count bacterial cells, a 1:100 dilution in PBS was prepared and the OD_{600} nm was measured with a spectrophotometer (U-2900, Metrohm Gmbh, Austria)

Growth curve of *Klebsiella quasipneumoniae* 700603 strain was previously performed in our laboratory. An OD_{600} nm = 1.0 corresponds 1 x 10^9 cells/ml. To convert the OD_{600} value into cell numbers the following formula was used:

Cells/ml: OD_{600} x dilution factor x 1.2 x10^9 cells

Phosphate-Buffered Saline (PBS)

NaCl 140 mM

KCl 27 mM

Na2HPO4 90 mM

KH2PO4 15 mM

Reagent	Amount to add (for 1× solution)
NaCl (ITW Reagents, Germany)	8 g
KCl (ITW Reagents, Germany)	0.2 g
Na_2HPO_4 (VWR International GmbH, Austria),	1.44 g
KH_2PO_4 (VWR International GmbH, Austria),	0.24 g

2.1.4 Growth of *C. albicans* and *K. quasipneumoniae* in co-culture in biofilm mode

- On a weekly basis, *Candida* and *Klebsiella* species were streaked out from a frozen stock (-80 °C) on YPD and LB agar plates and grown at 30 °C and 37 °C, respectively.
- For overnight cultures, a single colony of the *Candida* or *Klebsiella* strain was transferred from a streaked plate into a snap cap tubes containing 5 ml liquid YPD or LB media and incubated at 30°C/37 °C and 200/180 rpm for overnight cultures.

- Next day, 1 ml of the overnight cultures was spun down at 16662 rcf for 2 min. Cell pellets were washed once with 1 ml 1 x PBS and resuspended in 1 ml 1x PBS.

- Upon measurements of OD_{600} nm, cells were adjusted to a certain concentration. Each replicate containing *C. albicans* single, *K. quasipneumoniae* single and co-cultures 1 x 10^6 cells/ml of each fungal and bacterial species were cultured in 3 ml DMEM + 10 % hiFCS in 35 mm (CytoOne, Starlab GmbH, Ahrensburg, Germany) dishes (STARLAB, Germany).

- For biofilm formation, cells were incubated at 37°C. Upon 90 min incubation, non-adherent cells were removed followed by a washing step with 1 x PBS, incubated for 24 hours.
As controls, *C. albicans* and *K. quasipneumoniae* single cultures were present in each experiment to compare the results with the co-culture (depending on the experiment goal).

2.1.5 Growth of *C. albicans* and *K. quasipneumoniae* in co-culture in planktonic mode

- For planktonic growth, *C. albicans* and *K. quasipneumoniae* cells were prepared from overnight cultures as explained in section 2.1.4.

- *C. albicans* and *K. quasipneumoniae* cells (1 x 10^6 cells/ml) were added to a final volume of 30 ml DMEM medium (+ 10 % hiFCS) into 200 ml Erlenmeyer flasks. As controls, *C. albicans* single and *K. quasipneumoniae* single cultures were used for comparison with co-cultures.

- Cells were incubated at 37 °C, 180 rpm for 24 hours. Upon 24 hours incubation, cells were transferred into 50 ml falcon tubes and spun down at 16662 rcf at 4 °C for 15 min. Cells were washed with 1 x PBS, and resuspended in PBS in the volume of the initial medium.

2.2 Crystal Violet assay

Biofilm biomass of single and co-cultures of *C. albicans* and *K. quasipneumoniae* was assessed by crystal violet assay.

For this, cells were inoculated for overnight cultures as explained in section 2.1.4

Next day, each strain was cultured in 200 ml of different media (DMEM + 10% hiFCS, YNBNP, M9),to a final concentration of 1 x 10^6 cells/ml. At 90 min washed once with 1 x PBS and new media was added. The plates were removed after 24 hours and crystal violet assay was performed. The OD_{600} nm was obtained with VICTOR Nivo machine (Perkin Elmer, Germany).

Crystal violet assay

- After 24 h incubation, the medium was removed
- Plate was washed 2 x with PBS
- 200 µl methanol was added and incubated for 15 min at room temperature. Methanol was removed and plate was dried completely overnight.
- The next morning, 200 µl of 0,1 % (w/v) crystal violet (Thermofisher) was added and incubated for 10 min and then crystal violet was removed
- Plate was washed 2 times with 1 x PBS and wells dried completely, afterwards.
- Biofilms were destained by adding 200 µl of 33 % acetic acid (v/v), mixed by gentle pippeting
- Suspension was transferred into a clean 96 well plate
- The optical density 600 nm was measured.

2.3 Determination of MIC changes of *C. albicans* and *K. pneumoniae* upon co-interaction

2.3.1 MICs determination by E-test

- In order to test the changes in the MICs of antifungal and antibacterial drugs before and after interaction, *C. albicans* and *K. quasipneumoniae* were cultivated in biofilm and planktonic modes (as explained in the sections 2.1.4- 2.1.5).

Media supplemented with antifungal or antibacterial drugs

- YPD plates supplemented with drugs

- YPD + 100 µg/ml Streptomycin (Sigma-Aldrich Handels GmbH, Austria)
- YPD + 500 µg/ml Kanamycin (Life Technologies, Austria)
- YPD + 0,0156 µg/ml Caspofungin (Merck & Co., Whitehouse Station, NJ)
- YPD + 100 µg/ml Nourseothricin (NAT) (Werner Bioagents, Germany)

Biofilm growth for BFI

- After 24 hours of interaction, 3 technical replicates each containing 3 ml of cell suspensions were scraped and pooled together in a 15 ml falcon tube. Cells were spun down at 16662 rcf at 4 °C. Supernatant was removed and cells were washed with 9 ml 1 x PBS.
- The cell pellet was resuspended in 9 ml PBS and a cotton swab was used to take the cell suspension and plate onto YPD plates supplemented with drugs (explained in the 2.1 section) for selection against the opposite strain.

- After plating the cells, E-test strips were immediately placed on the plate.

E-test strips used were the following:

Colistin (CS) 0,016 – 256 mg/ml (*In vitro* diagnostika Werner Uhl-Michelin, bestbion^{dx} , Germany)

Caspofungin (CAS) 0,002-32 mg/ml (*In vitro* diagnostika Werner Uhl-Michelin, bestbion^{dx} , Germany)

Plates were incubated for 48 hours at 37 °C. After 48 hours, pictures were taken (Canon EF-S 60mm) to determine the MICs.

Planktonic growth for BFI

- Cells from planktonic growth were pooled in a 50 ml falcon tubes and spun down for 15 min at 4 °C. Following the centrifugation step, cells were washed with 1 x PBS.
- Cell pellets were resuspended into 30 ml 1 x PBS.

A cotton swab was used to plate the cells on YPD agar plates. The E-test drug strips were placed immediately onto the agar plate.

- Incubation was performed at 37 °C for 48 hours. Pictures were taken to determine the MICs.

2.3.2 MICs (in liquid media) based on EUCAST guidelines

- MICs of antibacterial and antifungal drugs were tested in liquid media from single and co - cultures of *C. albicans* and *K. quasipneumoniae* fluorescently labeled. Cells were grown in biofilm mode for 24 hours at 37 °C as explained in the 2.1.4 section.
- Upon 24 hours incubation, cells were transferred into FACS tubes with filter –caps and then cells were sorted. A total number of 1 x 10^6 cells/ml were sorted into 5 mg/ml Caspofungin to prevent possible cross contamination with *Candida albicans*. Meanwhile, a 96-well plate ((CytoOne, Starlab GmbH, Ahrensburg, Germany)) was prepared with different antibacterial drugs to determine the MICs difference of the *K. quasipneumoniae* strain before and after co-interaction with *C. albicans*.

Drugs used for Minimum Inhibitory Concentration (MIC) test (EUCAST guidelines)

Drug	Stock concentration
Caspofungin (sigma-Aldrich Handels GmbH, Austria)	5 mg/ml
Colistin (sigma-Aldrich Handels GmbH, Austria)	50 mg/ml
Chloramphenicol (Sigma-Aldrich Handels GmbH, Austria)	10 mg/ml
Kanamycin (Life Technologies, Germany)	50 mg/ml

Drugs were dissolved in H_2O. Initially, stock plates with antibacterial drugs were prepared.

Table 2.2 Final drug concentration range used in MICs determination

Colistin µg/ml	Kanamycin µg/ml	Chloramphenicol µg/ml
0,0976	0,976	0,488
0,1953	1,953	0,976
0,390625	3,90625	1,953
0,78125	7,8125	3,90625
1,5625	15,625	7,8125
3,125	31,25	15,625
6,25	62,5	31,25
12,5	125	62,5
25	250	125
50	500	250

100	1000	500
200	2000	1000

- Following cell sorting, cells were spun down at 16662 rcf at 4 °C for 5 min and washed once with PBS. Cell pellets were resuspended in 3 ml 2 x YPD liquid medium and 100 µl of the cell suspension was added to the 96-well plate which was already containing the antibacterial drugs.
- Incubation was performed at 37 °C for 24 hours. At 24 hours, the optical density (OD_{600} nm) was measured by VICTOR Nivo machine (Perkin Elmer, Germany).

2.4 Cell preparation for confocal microscopy visualization of single and co-cultures of *K. quasipneumoniae* and *C. albicans* in biofilm and planktonic growth mode

- Single and co-cultured *C. albicans* and *K. quasipneumoniae* (fluorescently labelled strains) in biofilm and planktonic growth mode were prepared as explained in the section 2.1.4 – 2.1.5. The growth modes are briefly explained bellow:

- Biofilm mode**:** To be able to take confocal microscopy pictures for the biofilm growth mode, 1 x 10^6 cells/ml (3 ml in total) were grown on IBIDI plates (µ-dish, 35 mm high, ibiTreat, 35 mm) for 24 hours at 37 °C. At 24 hours, media was remo ed and the dish surfaces were gently washed with 1 x PBS.

-Planktonic mode: In the case of planktonic growth, 1 x 10^6 cells/ml (15 ml in total) cells were grown in 50 ml Erlenmeyer flasks for 24 hours at 37 °C, 180 rpm on shaker. At 24 hours, cell suspension was transferred to a 15 ml falcon tube and spun down at 16662 rcf speed, 4°C for 15 minutes. After a washing step, the cell pellet was resuspended into 1 ml PBS and 5 µl were taken on a glass slide for microscopy pictures.
- Microscopy pictures were taken with CLSM, Zeiss LSM 700 confocal microscope.

2.3 DNA and RNA methods

2.3.1 Genomic DNA extraction from Bacteria and Fungi and PCR

1. Bacterial and Fungal growth

- Cells were grown in biofilm mode (section 2.1.4), in 35mm (CytoOne, Starlab GmbH, Ahrensburg, Germany) dishes in 3 different media (M9, DMEM, YNBNP. The total growth was 24 hours at 37 °C (triplicates were used for each condition)
- Cells were scraped and collected in 15 ml falcon tubes and spun down for 10 min at 16662 rcf at 4 °C.
- Cells were washed in 1 x PBS and supernatant was removed
- Cells were resuspended in 500 µl H_2O.
- Cells were transferred into 1.5 ml Fast Prep tubes.

2. The suspensions were spun down at 18188 rcf at 4 °C for 5 min and the supernatants were removed.
3. The pellets were resuspended in 200 µl of Yeast lysis buffer.
4. 200 µl of phenol-chlorophorm isoamyl alchohol (Sigma, Austria) and one scoop of acid washed glass beads (Sigma) were added_ these steps have all been performed under the hood!
5. FAST prep was done -> 3 cycles of 45 sec 6 m. After each cycle 5 min incubation on ice was performed.
6. 200 µl of TE (pH 8) was added – Spun for 5 min at 4 °C and transferred the aqueous phase into new 1.5 ml eppendorf tubes (approx.200 µl). 500 µl 100 % EtOH was added; mixed by inversion and incubated at -20 °C for 20 min.
7. Spun 10 min at 18188 rcf at 4 °C and the supernatant was removed. 400 µl TE and 4 µl RNaseA (10 mg/ml) were added to the cell pellets, vortexed and incubated for 20 minutes at 37 °C.
8. 10 µl 4 M ammonium acetate (NH_4AC) and 1 ml 100 % EtOH were added and samples were incubated 20 min at -20 °C.
9. Samples were spun for 10 min at 4 °C and the supernatants were discarded. Samples were washed with 1 ml 70 % EtOH, the pellets were air dried for 5 min and ressuspended into 50 µl H_2O for 5 min at 37 °C.
10. Samples were kept at -20 °C.
11. DNA quality was checked on a 1 % agarose gel.
12. Quality of the primers for bacteria and fungi were checked before performing any tests with them (performed PCR check)

Buffer	Composition	
Yeast Lysis Buffer (for 200 ml)	Triton X- 100	4 ml

10 % SDS	20 ml
5M NaCl	4 ml
0,5 M EDTA	400 µl
1M Tris (pH 8.0)	2 ml
dH_2O up to 200 ml	

TE (Tris- EDTA buffer) for 500 ml

1 M Tris (pH 8)	5 ml
0.5 M EDTA (pH 8)	1 ml
dH2O	496 ml

2.3.2 RNA extraction & cDNA synthesis

Sample collection for RNA extraction

- Bacterial-Fungal Interaction (BFI) in biofilm growth mode (see section 2.1.4) for 24 hours at 37 °C in triplicates per condition in 35 mm (CytoOne, Starlab GmbH, Ahrensburg, Germany) Petri dishes. Used media for all the conditions is DMEM + 10 % hiFCS.

Harvesting the cells

- All the cells were collected into falcon tubes (cell scraper was used; all on ice, 4ºC centrifuge) – pooled together 3 x Petri dishes
- Spun down 15 min at 4.4 rpm x 1000
- Cells were washed in cold PBS (2 x); spun down 15 min
- Cells were collected into 2 ml size fast prep tubes (4ºC) with cold water
- Supernatant was removed
- Froze in 100 % EtOH and stored at -80ºC until use or use directly for RNA isolation.

RNA isolation

- Samples were thawed on ice
- Centrifuge was cooled down to 4ºC; all steps were performed on ice; exclusively filter tips and clean hood were used + pipettes with RNAse and DNAse-free disinfectant were cleaned

- Ressuspend the pellet in 1 ml Trizol (Invitrogen GmbH, Austria), incubated 5 min at room temperature
- A scoop of 0.3 g RNAse-free glass beads (450-600 microns, acid-washed, Sigma-Aldrich Handels GmbH , Austria) were added to each tube
- Samples were mixed and incubated 5 min at RTºC
- Fast prep was perfomed: 2 x cycles 6.0 m/s, 45 seconds
- Samples were spun down for 15 min, 13500 rpm, 4ºC
- Upper phase was transferred (~ 400 µl) into new Eppendorf tubes
- 200 µl chloroform was added
- The tubes were shaked vigorously by hand for 15 seconds
- Samples were incubated at RTºC for 3 min
- Samples were spun at 13500 rpm for 15 min, 4ºC
- The aqueous phase (~ 200 µl) was transferred into a fresh tube
- 500 µl of cold isopropanol was added
- Samples were incubated on ice for 20 min
- Spun down for 30 min at 13500 rpm, 4ºC
- Supernatant was removed
- The pellet was washed with 1 ml of cold 70 % EtOH
- Samples were spun at 13500 rpm for 5 min at 4 °C
- EtOH was removed; spun down for a couple of seconds; removed remaining EtOH
- Pellet was air dried for ~1 min
- RNA was dissolved in 20 µl RNAse-free water and vortexed several times; spun down for a couple of seconds
- RNA was stored at -80 ºC (froze with cold 100 % EtOH or liquid nitrogen, if available) or proceeded for RNA quality check

RNA quality check

- Nanodrop was used (1 µl of sample)

2.3.3 DNAse treatment and cDNA synthesis for qPCR

Digested 15 µg of RNA sample: (All the buffers and enzymes were from ThermoFischer, Austria)

- RNA: 5 µg
- 10x buffer DNAse I: 10 µl

- DNAse I : 10 µl
- Ribolock RNase inhibitor 1.25 µl
- (RNAse-free water: up to 100 µl)
- Incubate at 37ºC for 30 min with water (no shaking!)
- Only after heating, add RNAse-free water: up to 100 µl

RNeasy kit (Qiagen) protocol was followed to clean up the RNA.

PCR (as a control, check for presence of DNA in the samples)

1 µl of a 1:15 dilution of RNA (1 µl RNA + 14 µl H_2O) was used for control PCR to confirm the absence of DNA

Genomic DNA was used as positive control sample

H_2O was used as negative control sample

Reaction

2.5 µl	10 x DreamTaq Buffer Green
2 µl	dNTPs [2.5 mM]
1.25 µl	Primers for fungi (ITS2) - 25 µM (Fw + Rv)
1.25 µl	Primers for bacteria (16S) - 25 µM (Fw + Rv)
1 µl	Template RNA (from 1:15 dilution)
0.125 µl	DreamTaq Polymerase
16.9 µl	H_2O
25 µl	Total volume

PCR program

2min	95°C	
30sec	95°C	
30sec	62°C	30 cycles
30sec	72°C	
3min	72°C	
∞	10°C	

5 µl of sample was loaded onto a ~2 % agarose gel + Midori green (1:30000) (NIPPON Genetics EUROPE, Germany)

Run: 90V, 30 min

DNAse treatment was repeated if DNA was present in the samples

If there was no DNA in the samples, proceeded for determination of RNA concentration and cDNA synthesis.

A master-mix of 1:1 mix of oligo dTs (10 µl) and random primers (0,2 µg/µl) 10µl was prepared

2.3.4 cDNA synthesis from the RNA samples

(All buffer and enzymes from ThermoScientific, Austria)

Reaction

0.4 µg RNA (400ng/ml)	x µl (max 10,5 µl)
5 x reaction buffer	4 µl
1:1 Oligo dTs & random primers	2 µl
dNTP mix (10 mM)	2 µl
Ribolock RNase inhibitor	0.5 µl
RevertAid Reverse Transcriptase	1 µl
H2O	up to 20 µl

PCR for reverse transcriptase (RT)

PCR program for "RT"	Minutes
25 °C	10
42 °C	60
70 °C	10
4 °C	∞

- ~ 1h and 30 min
- PCR product was kept at -20°C
- cDNA was diluted 1:10 (use the total cDNA from the reaction 20 µl + 180 µl H_2O)
- cDNA was stored at -20°C

Testing the cDNA using house-keeping genes (HKG)

Reaction

2.5 µl	10x DreamTaq Buffer Green

2 µl	dNTPs [2.5mM]
1.25 µl	Primers for fungi (HKG: PAT1 - 25 µM (Fw + Rv)
1.25 µl	Primers for bacteria (HKG: GapA) - 25 µM (Fw + Rv)
3 µl	Template cDNA (from 1:10 dilution)
0.125 µl	DreamTaq Polymerase
16.9 µl	H_2O
25 µl	Total volume

PCR program

2min	95°C	
30sec	95°C	
30sec	62°C	30 cycles
30sec	72°C	
3min	72°C	
∞	10°C	

10 µl of sample was loaded onto a ~ 3 % agarose gels + 1:30000 Midori green

Run: 90V, 30 min

100 bp DNA marker New England Biolabs (NEB)

Note: if the bands corresponded to the expected product size, the cDNA was used for further qPCR test with genes of interest.

2.3.5 Real-time reverse transcription PCR (real-time RT-PCR)

For this method, the following RT-PCR mix was prepared

Stock solution	final conc.	Total vol. 10 µl
water		1,85
Primer Mix [25µM]	375nM	0,15
Mastergreen Mix 2x (LUNA Universal - New England Biolabs)	1x	5
distribute to wells		7
Template		3

- Next, the RT-PCR mix with the respective cDNA samples were pipetted in triplicates (3 x technical replicates) in real-time PCR plates (Eppendorf) and the qPCR program was run. By using the Realplex4 Master cycler (Eppendorf), the following PCR program was chosen:

	denaturating	95 °C	5 min
	denaturating	95 °C	10 sec
40 cycles	annealing	60 °C	15 sec
	extension	72 °C	15 sec
	denaturating	95 °C	20 sec
	annealing	60 °C	20 sec
	melting curve		30 min
	program end	95 °C	15 sec

- For relative quantification of gene expression, analysis was carried out according to the ΔΔCt method with the following equations:

ΔCt: 1/((2^Ct mixed culture)/(2^Ct single culture))

Each Ct value was normalized to House Keeping Genes (HKG), in case of *C. albicans* to *PAT1* and in *K. quasipnaumoniae* case to *GAPA.*

The normalized values of the mixed samples were then divided to the single ones.

2.3.6 Primer used to test the bacterial and fungal genes involved in the co-interaction

Primers used for RT-qPCR for *C. albicans*

Gene name	Forward primer (5´ to 3´)	Re erse primer (5‘ to 3‘)
CPH1	TTGGAACCTCCTCGTTCAGAG	TCTCGTTCCAAGGCATCTGCC
EFG1	CATCACAACCAGGTTCTACAACCAAT	CTACTATTAGCAGCACCACCC
HWP1	GCTGGTTCAGAATCATCCATGC	AAGGTTCAGTGGCAGGAGCTG
ECE1	TGCCATTTGTTGTCAGAGCTG	TAGCTTGTTGAACAGTTTCCAGG
RIP1	TGCTGACAGAGTCAAGAAACC	GAACCAACCACCGAAATCAC
PAT1	TTATCGGAATGGTCCTCGTG	CCAGAAGAACCATCATCAAC
ALS3	CTCGTCCTCATTACACCAACC	GAAACAGAAACCCAAGAACAAC
FKS1	GGAGCGGAAGTGGTTTATTC	CTGTATACATATAGATAGATAGATAGATAG
UME6	TCATTCAATCCTACTCGTCCACC	CCAGATCCAGTAGCAGTGCTG
NRG1	GGTTGCACGTTGTCGAAACC	TGTTGCTGCTGCTGCTTGG

BRG1	ACGATCAACCATTAGTGGAGG	GAAGAAGTAGGTGTAGATGATCCAC
ITS2	GAATCATCGAATCTTTGAACGCAC	TACCACTACCGTCTTTCAAGCA

Primers used for RT-qPCR for *K. pneumoniae*

Gene name	Forward primer (5´ to 3´)	Re erse primer (5‘ to 3‘)
AVR78_03180	TGATTGCAGTAGACCTGTCC	GGTCGGAGTAATTGACATCCA
AVR78_04690	CTGGTTTATTATCTGGCGTGG	TGTAGAACCTTCACCTGCTC
AVR78_09300	TGTTCAAAGCACTGCAACTC	GGTTTACCTACCAGTTCTGAC
AVR78_07185	CCCGCTATCTCGATATCCAG	TTAAGGATCACCGTTTGCTC
CHIT	ATCCGCAGAAACGCTTTACC	CCCTTCGCCTTGATGTAGTC
ENDOGLUC	AAATTTCCACTTCCGAAGGACAG	AGGTTGTTCTGTGTCCAGGT
SDIA	GGATGTCTCATTACCAGGCAG	GAAACAGCCCATCTTCCCAC
16S region	CCAGCAGCCGCGGTAA	TTACGCCCAGTAATTCCGATTAA
GAPA	AGAACTGAACGGCAAACTGAC	TGGCTTTCTTGATTTCTTCGTAGG

2.4 Triple staining and FACS quantification of *C. albicans* cell wall components upon exposure to *K. quasipneumoniae*

Growth of cells

- Overnight cultures of wild type strains of *C. albicans* and *K. quasipneumoniae* were prepared as explained in the section 2.1.3.
- Two technical replicates of each condition (consisting of *C. albicans* alone, *K. quasipneumoniae* alone and a co-culture from both strains) were prepared in 35 mm (CytoOne, Starlab GmbH, Ahrensburg, Germany) Petri dishes (3 ml per dish) in DMEM + 10 % hiFCS media in biofilm mode (see section 2.1.4).
- After biofilm formation for 24 hours at 37 °C:
    - Cells were collected by scraping into falcon tubes
    - spun down at 16662 rcf for 15 min at 4 °C
    - Cells were washed in 1 x PBS
    - Pellets were resuspended into 1000 µl PBS
    - 200 µl (depends on cell density!) of cells were taken into new eppendorfs
    - Spun down at 1.5 rcf for 2 min and remove the supernatant

- Heat-killed cells were used for single staining controls: 10 min at 99 °C on a thermoblock (Eppendorf, Germany)

2.4.1 Fc: Dectin1 protein staining of β-glucan

- Samples were blocked with cold FACS block (1 ml) (0.5 % BSA, 5 % HI-rabbit serum (Sigma-Aldrich Handels GmbH), 5 mM EDTA, 2 mM NaAzide in PBS) for 30 min at room temperature in a Hula mixer; spun down and removed the supernatant
- Washed 3 x with 1 ml cold FACS wash (0.5 % BSA, 5mM EDTA, 2 mM NaAzide in at 4°C in 1 x PBS); spun down and the supernatant was removed
- Diluted Fc-Dectin1 protein ([stock] = 50 µg/ml) to 1 µg/ml in FACS block and used 100 µl/sample
- Incubated for 1 hour on ice
- Washed 3 x with 1 ml FACS wash; spun down and removed the supernatant
- The pellet was reuspended in 200 µl FACS block (45 min on ice) + 1/200 anti-human Fc + 488 (Invitroge) (prepare the Fc + 488 in FACS block solution) or anti-human Fc + Cy3 or APC (OR incubate with 1/100 anti-human Fc+Biotin (Jackson Immuno # 709-066-149) (Use 1 µl of Fc+488) (1990 µl FACS block + 10 µl of Fc+488; kept 45 min on ice); Scaled up for the sample; spun down and removed the supernatant
- Washed 3 x with 1 ml cold FACS wash

2.4.2 ConcanavalinA-Texas Red (ConA-TR) staining of mannans

- From the cells stained with Alexa Fluor 488 (BioLegend, US) for β-glucans, resuspended cells in 1000 µl PBS, added ConA-TR and only then transferred to dark brown eppendorfs (25 µl ConA-TR into 9975 µl PBS; scaled up and added 1000 µl to each sample
- Incubated 45 min in dark at 30 °C for yeasts with ConA-TR (LifeTech Austria) to a final concentration of 25 µg/ml; [ConA-TR stock] = 10 mg/ml; spun down and removed the supernatant
- Washed once with PBS; spun down and removed the supernatant
- Resuspended in 500 µl 1x PBS (depending on the cell density)

- Used 50 µl (depending on the cell density) of the resuspension into 500 µl FACS buffer or PBS into FACS tubes

2.4.3 CFW (fluorescent brightener 28; Sigma) staining - chitin

- CFW (Sigma-Aldrich Handels GmbH) was immediately added before running the samples in a FACS Fortessa
- A dilution of 1:400 from stock 10 mg/ml was prepared in H_2O; scaled up for all the samples
- 500 µl of CFW ([CFW stock] = 10 mg/ml; [CFW f] = 25 µg/ml was added to the FACS tubes from the glucans staining
- The samples were incubated at least for 10 min in the dark.
- Samples were ready for FACS.

All the centrifugation steps were performed with bench centrifuge (Eppendorf-5417 R) at 1.5 x g for 2 minutes at 4 °C (1.5 ml Eppendorf tubes were used).

2.4.4 Solutions for triple staining

FACS block (Vf= 100 ml)
0.5% BSA
5% HI-rabbit serum (5 ml) (Sigma-Aldrich Handels GmbH) 5
mM EDTA
2 mM NaAzide (NaN_3) in PBS
Kept it at 4ºC

FACS wash
0.5% BSA
5 mM EDTA
2 mM NaAzide in PBS
Kept it at 4ºC

Fc-Dectin1
Dilute in 1 ml H_2O
Stock solution = 50 µg/ml

Working solution: dilute the stock solution 10 x and use 100 µl/sample; or use directly 10 µl into 490 µl FACS block

ConA-TR (LifeTech Austria)

Prepared sodium bicarbonate (=sodium hydrogen carbonate = NaHCO3)

- Stock solution 1 M
- Working solution 0.1 M

Prepare ConA-TR stock solution

- Stock solution 5 mg/ml in 0.1 M $NaHCO_3$
- Added 2 mM of NaN_3 for longer storage

2.5 Establishment of an *in vivo* model of *Galleria mellonella* for BFI

Galleria mellonella larvae (XXL) were ordered from Evergreen, Germany).

Upon arrival, larvae were kept at 12-15 °C at least for 24 h prior inoculation. This permits stability of the larvae from any possible reactions to stress over transportation.

- To test the bacterial-fungal interaction *in vivo,* bacterial and fungal cell suspensions were prepared directly from overnight cultures (as explained in section 2.1.3). *Galleria mellonella* model was injected by total cells number of 1 x 10^7 cells/ml of *C. albicans* and 1 x 10^2 cells/ml of *K. quasipneumoniae* to the very last left prolegs (10 µl per injection).

- After injection, larvae were incubated at 37 °C for several days. The viability of each single larva was checked every 24 hours.

- Dead larvae were considered as having increased levels of melanization (black or dark brown color of the larvae and no movement should be observed upon gentle touch.

3. Results

3.1 Biofilm biomass of *C. albicans-K. quasipneumoniae* co-culture is decreased compared to single cultures

Biofilm biomass of bacteria and fungi is dependent on both, species and growth conditions [234,235]. In previous studies, it has been shown that *C. albicans* biofilms are decreased when co-cultured with bacterial strains [234]. In these interactions, quorum sensing molecules are produced from both bacterial and fungal strains. The entire secreted proteins from biofilms were higher in a co-culture, but each individual strain has decreased the protein secretion [235]. In previous studies it has been shown that low-complex medium such as YNBNP, could support the growth of mixed biofilms [235].

In this study, we tested three different media that could support the growth of both *C. albicans* and *K. quasipneumoniae*. Therefore, M9 (contains high phosphate that make the iron requirements less easily shown [236]) and YNBNP (since it supports the biofilm formation of both microorganisms in single and co-cultures and also doesn't contain complex protein mixtures that would further make the results analysis complicated) [235] were selected as representative of starvation media and DMEM supplemented with 10 % hiFCS, representative of a complete medium, commonly used in *ex vivo* assays. Formation of biomass of single- and co-culture biofilms was quantified upon crystal violet assay for the three different media. This assay is based on detachment of death cells from cell culture plates. During this assay, the death and detached cells are washed away and the attached cells are stained with crystal violet. After a washing step, the dye is solubilized and the absorbance is measured at ~600 $_{nm}$. A significant decrease in biomass formation of the co-culture was only observed in M9 medium (Figure 3.1). Although not significant, the same trend was observed for YNBNP and DMEM/FCS media, corresponding to a decrease in biomass formation of *Candida-Klebsiella* co-cultures in comparison to single cultures. Since the phenotype of the co-culture *Candida-Klebsiella* is similar for the three media, we have selected DMEM/FCS medium to perform further assays. DMEM/FCS medium is commonly used in cell culture assays to study the immune response of e.g. macrophages and neutrophils to pathogens. All the data generated with this medium will be the basis for future *ex vivo* assays.

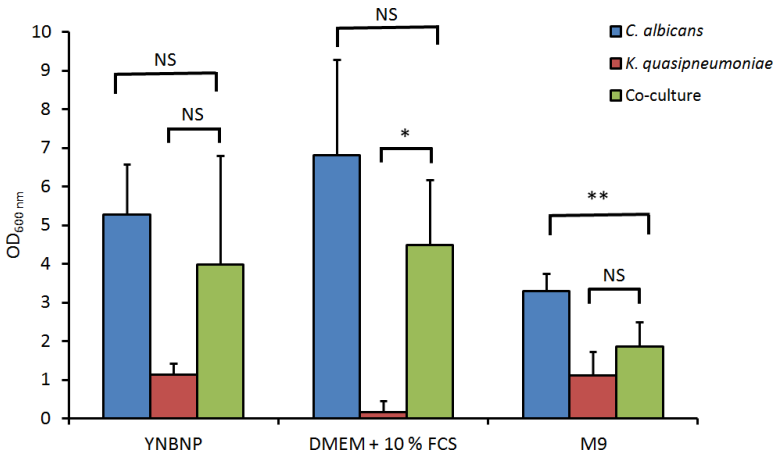


Figure 3.1: Crystal violet assay of single- and co-cultures of *C. albicans* and *K. quasipneumoniae*. Crystal violet staining after 24 h of interaction in YNBNP, DMEM+10 % hiFCS and M9 media. Error bars indicate standard deviation (SD): p> 0.5: non-significant (NS), p<0.05(*), p<0.01(**). n=3.

3.2 MICs determination of antibacterial and antifungal drugs in polymicrobial infections

Echinocandins including caspofungin and micafungin are the major antifungal drugs used against *Candida* spp. and are fully functional against some *Candida* biofilms. However, in recent years there are reports on gained resistant against these drugs by *Candida* species [62,79,84].

Multidrug resistant *Klebsiella pneumoniae* that has the ability to gain more resistant plasmids are spreading over the globe and are a major concern of hospital-acquired infections. An important concern of health care communities is Carbapenem-resistant *Klebsiella pneumoniae* (CRKp) infections. There are few antimicrobials agents that possess activity against CRKp. These include aminoglycosides (such as kanamycin), tigecycline, and also recently approved ceftazidime/avibactam. In addition, polymyxins including colistin are an important treatment option [237]. In case of severe CRKp infections, colistin is often used with other antimicrobial agents such as tigecycline, meropenem, gentamicin, or fosfomycin [238]. However increasing consumption of colistin has caused colistin-resistant *Klebsiella pneumoniae* carbapenemase (KPC)–producing strains are reported globally [239].

In general microbial biofilms which are the communities of adherent cells to solid surfaces or at liquid-air interfaces are more resistant than the other growth form, planktonic (free floating) cells. Biofilms are harder to treat or be removed such a way that they need ~1000 x more drug to be removed. Existing antifungal drugs at a concentration effective

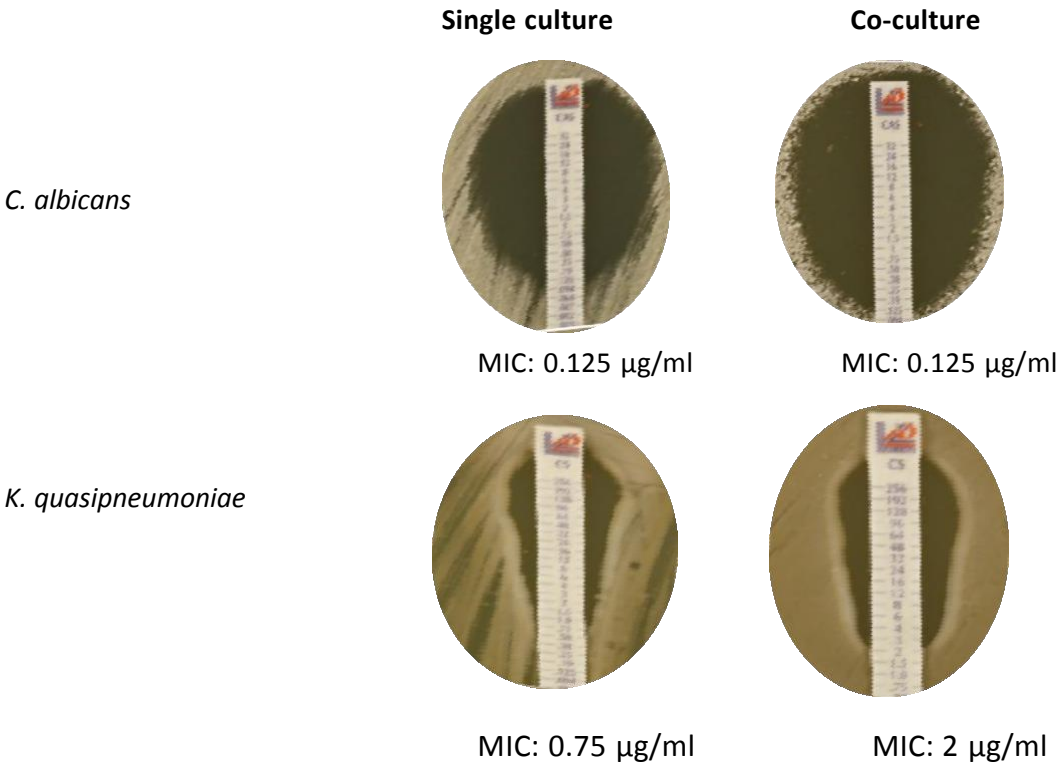
against the planktonic *C. albicans*, have a large difference and mostly ineffective against *C. albicans* in biofilm mode [2,240]. Other differences between biofilm and planktonic growth modes are the differences between metabolisms and the gene regulations [2].

The polymicrobial infections need almost 100-1000 x more drug concentration to be treated [198,241]. And also the biofilm mode of growth exhibits higher MIC values in comparing to non-adhering (planktonic) grown cells [69]. Mostly the dual species biofilm/planktonic growth has decreased drug efficiency and increased MIC values [208].

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) have established guidelines on examining the susceptibilities of antifungal and antibacterial drugs on the Minimum Inhibitory Concentration (MIC) against *C. albicans* and *K. quasipneumoniae* [242,243].

In this study, we have performed MICs of an antifungal drug - caspofungin and, an antibiotic – colistin. The aim is to determine if *Candida* and *Klebsiella* have increased tolerance to drug treatment upon interaction. For this, MICs were performed according to two different methodologies, including E-tests and liquid-based assays following the EUCAST guidelines. Using E-test strips of caspofungin and colistin, the MIC values were interpreted upon 24 h growth in agar plates. Single- and co-cultures of *Candida-Klebsiella* grown in planktonic and biofilm mode were compared. MICs results within *K. quasipneumoniae* showed a 2 fold sensitivity reduction to colistin when exposed to *C. albicans* in both planktonic and biofilm growth modes, whereas in case of *C. albicans*, there was no caspofungin MICs difference observed before and after BFI. Figure 3.2.A indicates the biofilmic grown *Candida-Klebsiella* cells that have been tested for MICs against caspofungin and colistin, respectively. The MICs of caspofungin have not been changed within single culture and co-culture of *C. albicans* and the MIC values were 0.125 µg/ml in both *Candida* cultures. However*, K. quasipneumoniae* has decreased the sensitivity to colistin upon exposure to *C. albicans* and the MIC values increases from 0.75 µg/ml in single culture to 2 µg/ml in co-culture. The part B of figure 3.2 shows the MIC variation from single cultures to co-cultures of *C. albicans* and *K. quasipneumoniae.* MIC values of caspofungin against *C. albicans* were stable before and after interaction with *K. quasipneumoniae*, although *K. pneumoniae* has decreased sensitivity to colistin upon exposure to the fungi and the MIC values change from 2 µg/ml in single cultures to 4 µg/ml in co-cultures.

A) Biofilm growth mode



B) Planktonic growth mode

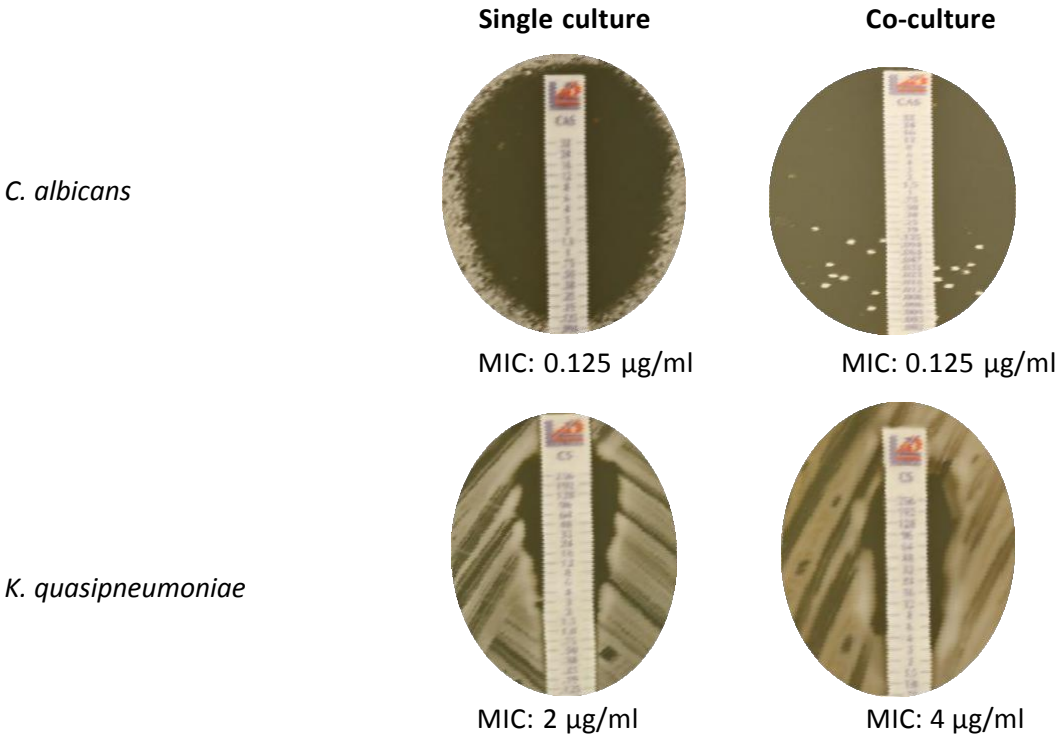


Figure 3.2: Determination of MICs with caspofungin and colistin E-test strips. Single- and co-cultures of *Candida-Klebsiella* were grown in A) biofilm or B) planktonic mode. A sample of these cultures was streaked onto YPD agar plates containing antibiotic or antifungal drug for selection of fungal or bacterial growth, respectively. E-test strips of caspofungin and colistin were placed onto the agar

plates. Upon incubation at 37°C, MIC readings were performed at 48 h and the MICs of *Candida* and *Klebsiella* previously grown in co-culture were compared with MICs of single cultures. The pictures shown are representative of 4 biological replicates.

Since *Klebsiella* has shown differences in the MIC values upon co-culture with *Candida*, we aimed to investigate how the MIC values for *Klebsiella* would vary in a liquid-based assay. This assay is based on EUCAST guidelines. This technique works by microdilution of antibacterial drugs against the sorted *Klebsiella* that was exposed to *C. albicans*. *Klebsiella* was grown as single culture and also in co-culture with *Candida* in biofilm mode. At 24 h, a cell sorter was used to separate *Klebsiella* from *Candida* cells. *Klebsiella* was then distributed into 96-well plates containing serial dilutions of antibiotic drugs. Upon 24 h incubation, the $OD6_{00nm}$ was assessed and MIC values determined accordingly. Figure 3.3 shows the MIC determination performed in liquid media based on EUCAST guidelines. As indicated in Figure 3.3.A, colistin, the same antibiotic used in MIC determination by E-test to which *Klebsiella* has shown 2-fold decreased in sensitivity, was microdiluted in YPD liquid media and the sorted *Klebesiella* was plated in 96-well plates for 24 h. The optical density comparison showed 2-fold increased in sensitivity to colistin within MICs of *Klebsiella* from co-cultures to *Klebsiella* from single cultures. However, in part 2 and 3, there were no MIC differences observed in 2 other antibiotics of kanamycin and chloramphenicol.

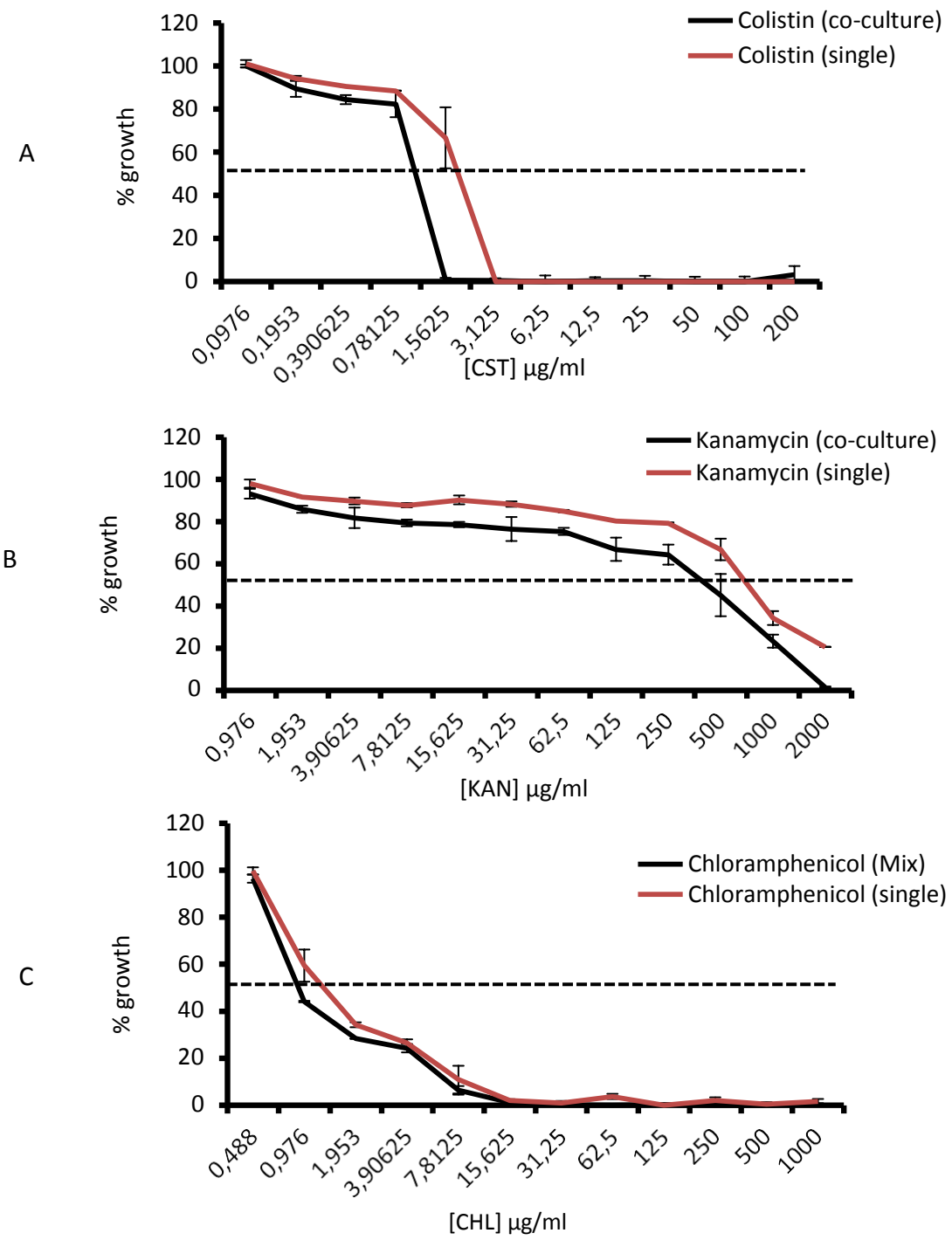


Figure 3.3: Determination of MICs of colistin, chloramphenicol and kanamycin. Single- and co-cultures of *Candida-Klebsiella* were grown in biofilms. *Klebsiella* cells were sorted and plated in liquid YPD medium in 96-well plates supplemented with microdilutions of antibacterial agents A: colistin, B: kanamycin and C: chloramphenicol. Cells were incubated for 24 h at 37°C, and then OD_{600nm} was measured. The dashed lines show the half maximal inhibitory concentration (IC50). Error bars indicate the standard deviations of two biological replicates each with two technical replicates. (n=2).

3.3 Confocal microscopy shows adhesion of *Klebsiella* to hyphal structures and yeast forms of *C. albicans*

Bacteria and fungi survive in nature by forming resistant structures of biofilms. Biofilms are structured consortium of bacterial or fungal cells that are embedded in a self - produced extracellular matrix containing proteins, DNA and polysaccharides that protects the biofilm from environmental stress and chemicals. Cells appear different in biofilm mode, because they are closely located together [244,245]. In addition to that, microorganisms have lower metabolic activities in biofilm mode, but instead they expose higher expression of genes needed for anaerobic growth. Bacterial and fungal cells communicate by means of molecules that further activate and regulate the gene expression of virulence factors of these microorganisms. All of these changes might be under the control of quorum sensing instructions [246]

K. pneumoniae has been shown to be able to adhere to both yeast and hyphal structures of *C. albicans* while forming biofilms *in vitro* [2,186]. Confocal scanning laser microscopy (CSLM) images have shown that when having polymicrobial infections with *C. albicans* and *K. pneumoniae*, both fungal and bacterial species incorporated into biofilm formation [2,186].

In this experiment, we aimed to investigate the physical interaction between *C. albicans and K. quasipneumoniae* in different growth modes, including biofilm and planktonic. Upon 24 h incubation of single- and co-cultures of *Candida*-dTom and *Klebsiella*-GFP tagged, cells were visualized under a confocal microscope. Figure 3.4 shows the confocal microscopy of *Candida*-dTom and *Klebsiella*-GFP in biofilm and planktonic growth modes when single and co-cultured. Biofilms of *Candida-Klebsiella* co-cultures have shown an apparent decrease of *Candida* hyphal structures. Additionally, *Candida* hyphal structures were hardly found in planktonic growth in co-cultures. The confocal images also show adhesion of *Klebsiella* to both hyphal structures and yeast cells when grown in biofilm and planktonic, suggesting that adhesion is independent of *Candida* morphology. Interestingly, *K. quasipneumoniae* has shown different morphologies depending on the growth mode. In biofilm mode, *Klebsiella* was elongated whereas in planktonic mode, *Klebsiella* was shown in spherical shape. Also, the morphology of *Candida* seems to vary according to the growth mode. In biofilm mode, *Candida* hyphal structures were thinner in comparison to hyphae grown in planktonic mode. However, co-cultures have not affected those morphologies. These observations suggest possible alterations of *Candida* cell wall components and hyphal structures by *Klebsiella*-

produced molecules and/or alterations in *Candida* hyphal gene regulations in the presence of *Klebsiella*. Therefore, the key gene regulators in biofilm formation of both microorganisms and hyphal specific genes of *Candida* were examined in the next steps.

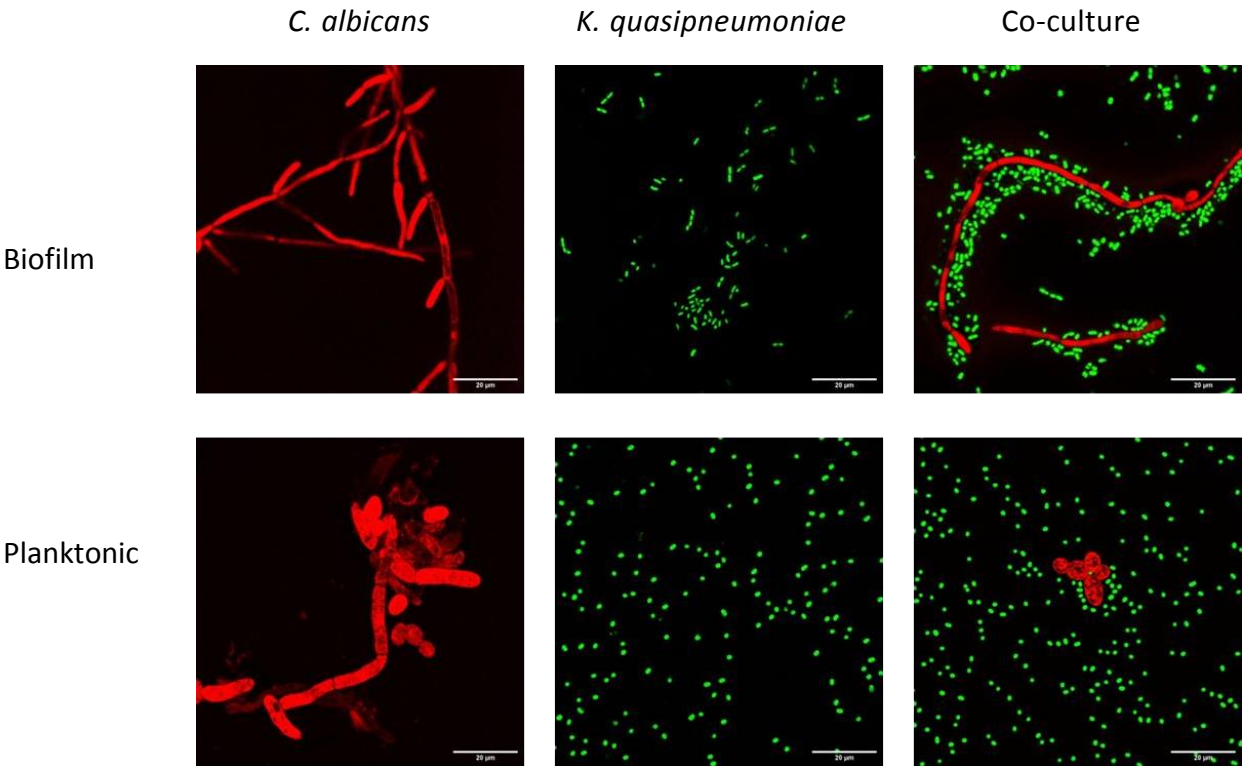


Figure 3.4: Confocal microscopy of single- and co-cultures of *C. albicans*-dTom and *K. quasipneumoniae*-GFP-tagged in biofilm and planktonic growth mode. Cells were grown in DMEM medium supplemented with 10 % hiFCS. For biofilm growth, cells were incubated at 37°C incubators for 24 h. For planktonic growth, cells were shaking at 180 rpm, at 37°C for 24 h **.** Confocal microscopy were taken. Scale bars represent 20 µm. n=3

3.4 Gene expression analysis of *Candida albicans* and *K. quasipneumoniae* single and co-cultures when grown in biofilm mode

Previous studies have shown that expression of certain genes is changed in bacterial-fungal interactions [2]. For example, growth of *C. albicans* has been shown to be inhibited by innumerous bacterial genera including, *Aeromonas, Bacillus, Clostridium, Enterobacter, Klebsiella, Kluyvera* and *Serratia marcescens* [247]. In aerobic conditions, bacterial-produced enzymes such as chitinases and mannan-derading enzymes inhibited *Candida* yeast growth by

hydrolyzing the cell wall components [247]. Bandara, HM, *et al*. (2013) showed that *Pseudomonas aeruginosa* lipopolysaccharide inhibits *Candida albicans* hyphae formation and alters gene expression during biofilm development by upregulation of *Candida* hyphal specific genes (HSGs) including *RBT1, RBT4, HWP1, HYR1, ALS3, ALS8* and *ECE1* and the transcription factor *EFG1* [248]. Another study on the inhibitory effect of *Lactobacillus* strains, that the closely related taxa *L. rhamnosus, L. casei* and *L. paracasei* showed a strong activity against *Candida* hyphae formation. Examining the activity of purified compounds and mutants of the model strain *L. rhamnosus* GG releaved the major peptidoglycan hydrolase Msp1, which is conserved in the three closely related taxa, was identified as a key effector molecule for this inhibition. This enzyme was able to break-down the chitin, as the most important component of *Candida* cell wall and therefore inhibit *Candida* hyphal formation in mixed culture [249]. In this study, we planned to analyze the expression of genes involved in *Candida-Klebsiella* interaction. This included genes associated with biofilm formation and hyphal specific genes such as *ECE1, HWP1, CPH1, UME6, BRG1*, *FKS1* and *ALS3* in *Candida* and biofilm formation, stress response and secreted enzymes such as chitinase and endoglucanase from *Klebsiella* that may play a key role in the interaction. To investigate the gene expression, RNA isolation was performed from single- and co-cultures of *Candida* and *Klebsiella* upon growth in biofilm mode for 24 h. RNA isolation was based on Trizol method (see details in the methods section). Following DNase treatment, the RNA was cleaned up with a RNeasy kit. To test for the presence of genomic DNA, a PCR test was performed and samples were run on 2 % agarose gels. Figure 3.5 shows samples corresponding to three different biological replicates. The positive control for *C. albicans* shows a band at ~140 bp corresponding to the expected product size from the amplification of the reference gene *ITS2*. The positive control for *Klebsiella* shows a band at ~50 bp, corresponding to the expected product size from the amplification of the reference gene *16S* region. The negative control for *C. albicans* is clear whereas the negative control for *Klebsiella* shows a weak band which may indicate primer dimerization. For all the samples, with the exception *Klebsiella* single culture in replicate number 1, we observed clearance of DNA bands compared to the positive controls, which indicates that the DNase I treatment has worked properly. Only the samples shown to be clear of DNA were considered for further cDNA synthesis.

Figure 3.5: A 2 % agarose gel for testing the presence of genomic DNA in RNA samples. Samples: *C. a1: C. albicans* biological replicate 1 - *K. p1*: *K. quasipneumoniae* biological replicate 1 - *C. a* M1: *C. albicans* in co-culture (mix) biological replicate 1 - *K. p* M1: *K. quasipneumoniae* in co-culture (mix) biological replicate 1. *C. a2: C. albicans* biological replicate 2 - *K. p2*: *K. quasipneumoniae* biological replicate 2 - *C. a* M2: *C. albicans* in co-culture (mix) biological replicate 2 - *K. p* M2: *K. quasipneumoniae* in co-culture (mix) biological replicate 2. *C. a3: C. albicans* biological replicate 3 - *K. p3*: *K. quasipneumoniae* biological replicate 3 - *C. a* M3: *C. albicans* in co-culture (mix) biological replicate 3 - *K. p* M3: *K. quasipneumoniae* in co-culture (mix) biological replicate 3. *C. a +:* positive control from *C. albicans* genomic DNA. *C. a* -: negative control (H_2O). *K. p* +: positive control from *K. quasipneumoniae* genomic DNA. K. p -: negative control (H_2O). Marker: 100 bp DNA ladder from New Englan Biolabs (NEB).

Following cDNA synthesis, a PCR was performed to amplify the cDNA and the PCR products were visualized on a 3 % agarose gel. Figure 3.6 shows samples corresponding to three biological replicate. The positive control for *C. albicans* shows a band at ~43 bp corresponding to the expected product size from the amplification of the reference gene *PAT1.* The positive control for *Klebsiella* shows a band at 118 bp, corresponding to the expected product size from the amplification of the reference gene *GAPA.* The negative control for *Klebsiella* is clear whereas the negative control for *Candida* shows a week band which may indicate primer dimerization. For all the samples, we observed a clear cDNA band compared to the positive controls, which indicates the successful cDNA synthesis.

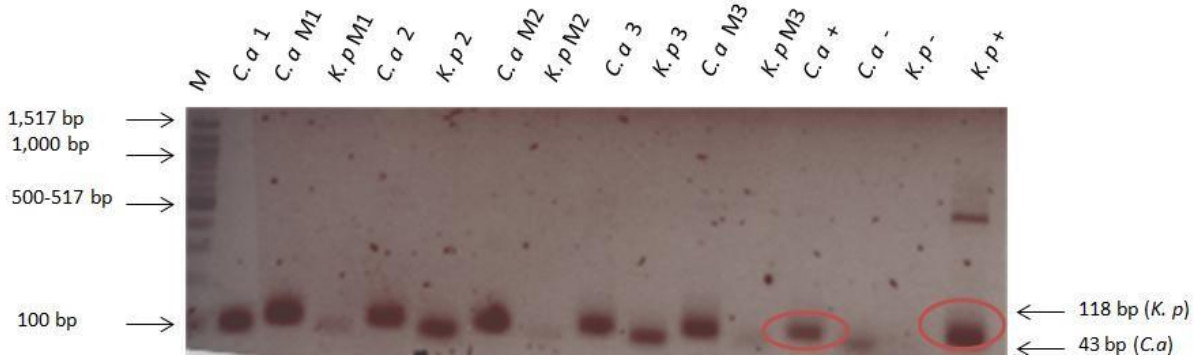


Figure 3.6: A 3 % agarose gel for testing the quality check of cDNA synthesis from RNA samples. Samples: *C. a1: C. albicans* biological replicate 1 - *K. p1*: *K. quasipneumoniae* biological replicate 1 - *C. a* M1: *C. albicans* in co-culture (mix) biological replicate 1 - *K. p* M1: *K. quasipneumoniae* in co-culture (mix) biological replicate 1. *C. a2: C. albicans* biological replicate 2 - *K. p2*: *K. quasipneumoniae* biological replicate 2 - *C. a* M2: *C. albicans* in co-culture (mix) biological replicate 2 - *K. p* M2: *K. quasipneumoniae* in co-culture (mix) biological replicate 2. *C. a3: C. albicans* biological replicate 3 - *K. p3*: *K. quasipneumoniae* biological replicate 3 - *C. a* M3: *C. albicans* in co-culture (mix) biological replicate 3 - *K. p* M3: *K. quasipneumoniae* in co-culture (mix) biological replicate 3. *C. a +:* positive control from *C. albicans* genomic DNA. *C. a* -: negative control (H_2O). *K. p* +: positive control from *K. quasipneumoniae* genomic DNA. K. p -: negative control (H_2O). Marker: 100 bp DNA ladder from New England Biolabs (NEB).

Primers that did not already exist in our database were designed by PerlPrimer software and ordered from Eurofins. All the used primers for testing *Candida* genes by qPCR already existed in our database. Only primers of the genes *7185* (450 bp), *9300* (354 bp), *3180* (50 bp) and *4690* (55 bp) for *Klebsiella* were newly designed and tested by PCR. PCR products were visualized on a 1 % agarose gel. Figure 3.7 shows the PCR products and the negative control (H_2O) indicated by (-). Only the labeled and named primers were used for qPCR analysis. For all the samples, we observed a clear DNA band corresponding to the expected size, which indicates the successful DNA synthesis; therefore primers were considered for qPCR tests.

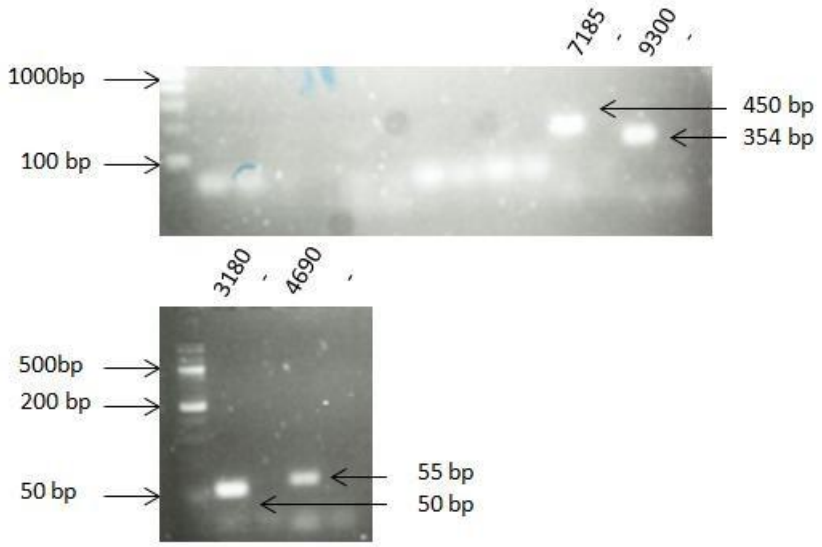


Figure 3.7: A 1 % agarose gel for testing the newly designed primers for qPCR analysis . The primers designed for genes *7185, 9300, 3180* and *4690* in *Klebsiella* were tested by PCR. Negative controls (H_2O) are indicated by (-). Marker: 100 bp and 50 bp DNA ladder from New England Biolabs (NEB).

3.4.1 Gene expression analysis of *C. albicans* upon exposure to *K. quasipneumoniae*

A more in-depth analysis of the *Candida-Klebsiella* interaction was performed at the molecular level. A pre-selection of key genes potentially involved in the regulation of this interaction was analyzed by qPCR. According to the literature, genes involved in adhesion such as *ALS3*, biofilm formation and hyphal specific genes such as *ECE1*, *UME6*, *BRG1*, and genes involve in cell wall synthesis and component such as *FKS1* and *HWP1* have been shown to play a key role in bacterial-fungal interactions [165,186,247,248]. Therefore, in our studies, we have analyzed the gene expression of hyphal specific genes (HSC) *ECE1, CPH1, UME6, BRG1*, (*NRG1* as an inhibitor) and *EFG1*, adhesion genes *ALS3*, and genes responsible for *Candida* cell wall synthesis *HWP1* and *FKS1*. Gene expression analysis was carried out from cDNA of single- and co-cultures of *Candida-Klebsiella*. For the gene amplification, design of primers was performed for each gene, except for those available in-house. Here we explain the qPCR results for each individual gene within a background related to our study:

ECE1: Studies on gene *ECE1* showed that this gene is only expressed in *Candida* hyphal population and not in yeast form, suggesting that *ECE1* expression is linked with the extent of cell elongation. [250]. Also, recently, *ECE1* was identified as the responsible gene of the only

known fungal cytolytic peptide toxin in *C. albicans* as essential for damaging epithelial membranes, triggering a danger response signaling pathway and activating epithelial immunity [19]. We tested the expression of this gene for its correlation with hyphal extend and also for being responsible for *Candida*lysin production which could have been affected in the presence of *Klebsiella*. As shown in figure 3.7, expression of *ECE1* is significantly upregulated when *Candida* was exposed to *Klebsiella* in co-cultures. This observation shows that the main target of *Klebsiella* is *Candida* cell wall within this interaction and the fungus may activate defense mechanisms through the production of the cytolytic enzyme *Candida*lysin.

HWP1*:* The hyphal wall protein 1 gene (*HWP1*) seizes significant antigen and hyphal specificity. It encodes a cell wall mannose protein, which is essential for normal growth of the mycelium[16]. In this study, we observed a significant decrease in expression of *HWP1* gene of *Candida albicans* in the co-cultures. *Klebsiella* may be responsible for the negative effects on the *Candida* cell wall degradation.

CPH1*: Candida albicans* can stochastically switch between white and opaque phenotypes. Not only *CPH1* is the master transcriptional regulator of pheromone-stimulated biofilms in *C. albicans*, but also *CPH1* is essential for pheromone-stimulated biofilm formation in white cells [15]. In this study, we examined *CHP1* expression levels of *C. albicans* in single cultures and compared the levels with co-cultures. The qPCR results showed a significant increase of the expression of this gene in the co-cultures. The effect of *Klebsiella* stress and cell wall degradation cause an increased struggle of *Candida* to overcome the pressure, therefore *Candida* might induce upregulation of *CPH1* to tolerate the bacterial stress factors.

EFG1: Deletion mutants of *EFG1* showed inability to filament and did not form biofilms, but rather formed sparse monolayers of loosely attached elongated, rod-like cells [251]. Therefore, the importance of *EFG1* gene in *Candida* biofilm formation ability encouraged us to test its expression levels when it was cultured with *Klebsiella.* Since the data from confocal microscopy showed reduced hyphal structures of *Candida*, we tested the expression of *EFG1* in co-cultures and compared the expression levels with the single cultures. The qPCR data showed no difference in the expression levels of *EFG1* in single and co-cultures of *Candida* and *Klebsiella*, showing that *EFG1* expression stays intact in this interaction.

UME6, BRG1*: UME6* gene has been reported as a transcriptional regulator in *C. albicans*, which is expressed in response to multiple host environmental triggers and is very important in hyphal extension. The double mutant *ume6*Δ/Δ showed a clear defect in hyphal extension both *in* an *in vitro* and *in vivo* model of systemic candidiasis. In the same study,

NRG1 was also suggested to work with *UME6* by a negative feedback loop to control the levels and duration of hyphal-specific gene expression [22,43]. *NRG1* is not only controlling the *UME6* expression, but also the GATA-family transcription factor *BRG1* [21]. Therefore, we tested *UME6*, *NRG1* and *NRG1* expression levels in *C. albicans* when it was exposed to *K. quasipneumoniae* when grown in biofilm mode. However, there was a significant increase in expression of *UME6* and *BRG1* genes, but no significant difference was observed in the expression of *NRG1* in *C. albicans* co-culture in comparison to single culture. These data suggest that fungal cells try to overcome cell wall degradation by expressing more of hyphal specific genes.

FKS1: It has been pro en that synthesis of β-(1,3)-glucans in biofilms is essential for tolerance to antifungal agents and, without it, cells exhibit increased susceptibility to drugs. In previous studies, it was shown that *FKS1* is important for biosynthesis of *C. albicans* cell wall and matrix β-(1,3)-glucan during biofilm growth [252]. For this reason, we assessed *Candida albicans FKS1* expression levels in single and co-cultures with *Klebsiella.*. A significant decrease in the expression of *FKS1* gene of *C. albicans* in the presence of *K. quasipneumoniae* was observed.

ALS3: The double mutant strain *als3*Δ/Δ showed that *C. albicans* is unable to bind to multiple host cell surface proteins such as N-cadherin on endothelial cells and E-cadherin on oral epithelial cells. To examine this *Candida* virulence factor, we compared the expression levels of *ALS3* in single and co-cultures. The qPCR results showed no significant difference of *Candida albicans ALS3.* Although we have observed adhesion of bacterial cells to the hyphal structures of *C. albicans* in the microscopy pictures *ALS3* seems to have no direct effect in this interaction. Figure 3.7 shows the ratio of gene expression in co-cultures and single cultures of *C. albicans* upon 24 hours of biofilm growth.

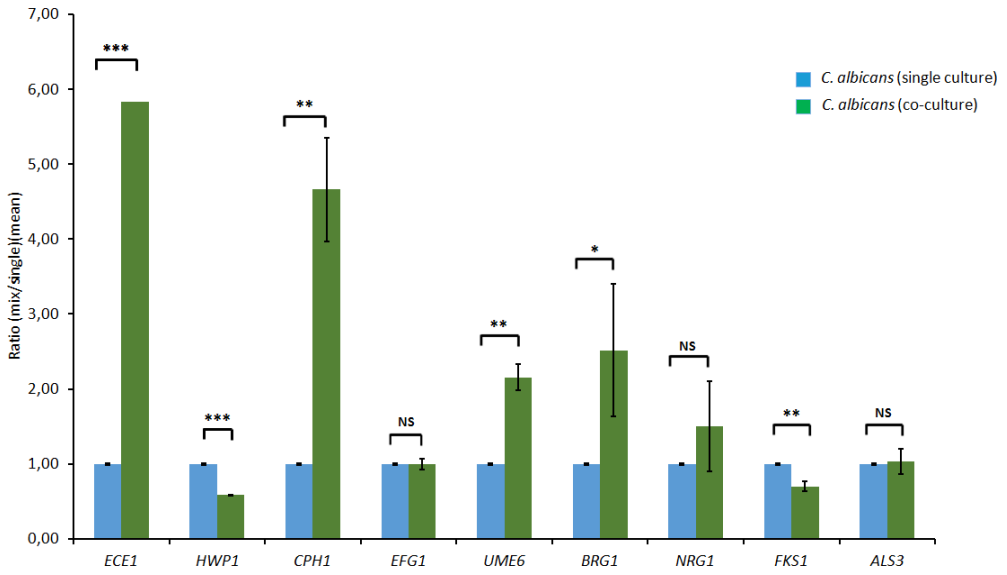


Figure 3.7: Gene expressions of *C. albicans* upon interaction to *K. quasipneumoniae*. qPCR from cDNA corresponding to single- and co-cultures of *Candida* and *Klebsiella* grown in biofilm mode for 24 hours at 37°C. Transcript levels were calculated by CT values division of co-cultures to the single cultures and normalized to expression levels of *PAT1*. Data are shown as mean of three independent biological replicates. Error bars indicate standard deviation (SD): p> 0.5: non-significant (NS), p<0.05(*), p<0.01(**), p<0.0001(***) .

3.4.2 Gene expression analysis of *K. quasipneumoniae* upon exposure to *C. albicans*

All the genomic information of *Klebsiella quasipneumoniae* ATCC 700603 and genes tested in this study were collected from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Some of the genes in this database are solely designated in numbers. Genes contributing to biofilm formation (#*7185*), stress responses (#*9300, #3180* and *#4690*), and cell wall degrading enzymes (*CHIT*, *ENDOGLUC*) and quorum sensing gene *SDIA* were tested in single and co-cultures *Candida-Klebsiella* interaction in biofilms.

#7185: The role toxin-antitoxin (TA) has been described and found on the chromosome of many bacterial species. There are several hypothetical descriptions that could be explained by TA systems [253]. The toxin-antitoxin system TabA was previously described in *Escherichia coli* known to influence biofilm formation through YjgK (TabA) and fimbriae [254]. Although not described yet in any studies, we found an ortholog of this gene in KEGG database in *Klebsiella quasipneumoniae* (Entry AVR78_07185). We tested expression of this gene in the BFI. The qPCR data showed a significant increased expression of # *7185* gene,

when *Klebsiella* was co-cultured with *C. albicans*. This indicates upregulation of biofilm formation in this BFI influenced by the presence of the bacteria in co-cultures.

#4690, #3180* and *#4690: Three stress response genes were tested in this study. The stress response system protein RcsF (#*9300*), universal stress global response regulator UspA (#*3180*) which is an ortholog in *E. coli* as a universal stress protein that is co-ordinately regulated and co-operate in the defense against DNA damage [255] and also stress response serine/threonine protein kinase YihE (#*4690*) were tested in this study. From all these three genes, only #*4690* was significantly upregulated when *K. quasipneumoniae* was co-cultured with *C. albicans.*

CHIT: Recently, it has been shown that the *Candida albicans* cell wall component chitin can be degraded by an enzyme -chitinase, produced by *Lactobacillus rhamnosus* GG and thereby inhibiting *C. albicans* morphogenesis [249]. Although not yet described in *K. quasipneumoniae,* we found the associated gene in KEGG database and assessed the expression of *CHIT* in the presence of *C. albicans*. Interestingly, the expression levels were highly upregulated in the co-cultures compared to *Klebsiella* single cultures. This indicates that cell wall degrading enzymes produced by the bacterial may negatively affect *Candida* cells.

ENDOGLUC: The crystal structure of the endoglucanase Cel10 was isolated from *Klebsiella pneumoniae* [256]. There are other orthologs of this gene in KEGG database for *K. quasipneumoniae*. In this study, we tested the expression of *ENDOGLU* in *Klebsiella* when co-cultured with *C. albicans* and compared the values with single cultures. The qPCR data indicated a significant increase in expression of this gene, when *Klebsiella* was exposed to *Candida*. This suggests that *Klebsiella* has the potential to degrade glucans on the fungal cell wall.

SDIA: Expression levels of a quorum sensing molecule named SdiA were also analyzed. *SDIA* gene is found in *Salmonella typhimurium* that encodes a SdiA homolog, a putative quorum sensor of the LuxR family, that regulates virulence genes genes [257]. In KEGG database, the same gene was found in *K. quasipneumoniae*. Therefore, expression levels of this quorum sensing molecule were tested in single and co-cultures of *Candida* and *Klebsiella*. The qPCR data indicates that *SDIA* is significantly upregulated in the presence of *Candida*. In Gram-negative bacteria, quorum sensing is sometimes programmed by the LuxR family of transcriptional regulators, which affect the gene expression patterns such as virulence gene expression [257]. Therefore, having a high *SDIA* gene expression in the presence of *Candida* would be explained by an increase in virulence of the bacteria.

Figure 3.8 shows the ratio of gene expression in co-cultures and single cultures of *C. albicans* upon 24 hours of biofilm growth.

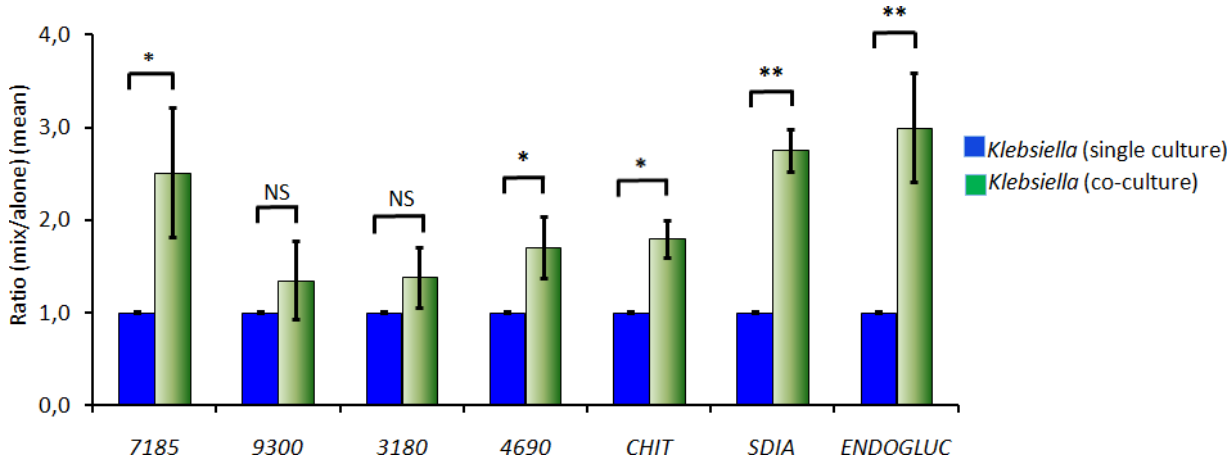


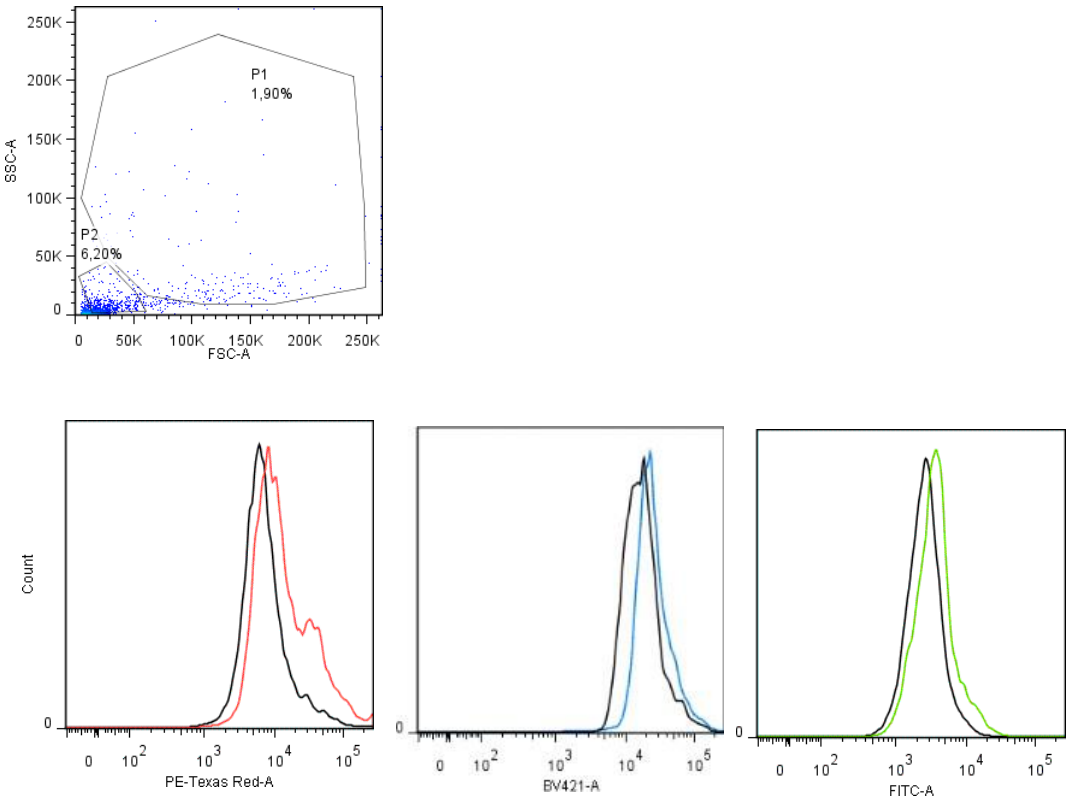
Figure 3.8: Gene expression of *K. quasipneumoniae* upon exposure to *C. albicans*. qPCR of cDNA corresponding to single- and co-cultures of *Klebsiella* and *Candida* grown in biofilm mode for 24 hours at 37°C. Transcript levels were calculated by CT values division of co-cultures to the single cultures and normalized to expression levels of *GAPA1*. Data are shown as mean of three independent replicates (n=3). Error bars indicate standard deviation (SD): p> 0.5: non-significant (NS), p<0.05(*), p<0.01(**).

3.5 Triple staining of *C. albicans* cell wall components showed variation in cell wall content upon exposure to *Klebsiella*

The fungal cell wall is a protective barrier against several environmental stresses including temperature shifts, oxidative and osmotic stress, and also other microbes [53]. Modulation of the cell wall can occur upon stress exposure [258,259]. This triggers the activation of particular signaling pathways that result in the synthesis of cell wall components such as fungal cell wall modifying enzymes, adhesions and iron acquisition proteins located in the cell wall [260,261]. In addition to environmental stress, the presence of other microorganisms can alter fungal cell wall composition. Recently it has been shown that Msp1 enzyme of *Lactobacillus rhamnosus* GG has chitinase activity when exposed to *C. albicans* and inhibits fungal biofilm formation and hyphal growth [249]. The fungal cell wall is composed of glucans including β-1,3-, mixed β- 1,3-/β- 1,4-, β- 1,6, α -1,3- glucans (approximately 50-60 % of the wall by dry weight), mannans and/or galactomannans (The cell walls of *S. cerevisiaeandand* and *C. albicans* contain glycosylated mannoproteins with chains rich in

mannose, known as mannans which are 20-30 % of cell mass) and chitin (only 1-2 % of dry weight) [53-56].

In this study, we have observed that *Klebsiella* has upregulated chitinase and endoglucanase genes upon exposure to *Candida*. We therefore have hypothesized that these enzymes may contribute to degradation of the *Candida* cell wall components chitin and β-glucans. Differences in the cell wall composition of *Candida* were investigated by flow cytometry with triple-fluorescence staining [262]. Single- and co-cultures of *Candida* and *Klebsiella* were grown in biofilm mode for 12 h at 37°C. The three cell wall components were stained as explained in the methods section. Briefly, Fc: Dectin1 was used to stain the β-glucans, concanavalinA-Texas Red stained mannans and CFW was used to stain chitin. Quantitative analysis of each cell wall component was based on the direct correlation with the fluorescence intensity. In a triple-staining approach, an increase in mannans (increased fluorescence of PE-Texas Red) and chitin (increased fluorescence of BV421) of *Candida* in co-culture was observed but, these changes were not significant (Figure 3.9). However, after two biological replicates of triple staining after 12 h of BFI, there were no significant differences in fluorescence intensity corresponding to mannans, β-glucans and chitin. Figure 3.9 shows the gating strategy, histogram and fluorescence intensity of each cell wall staining.



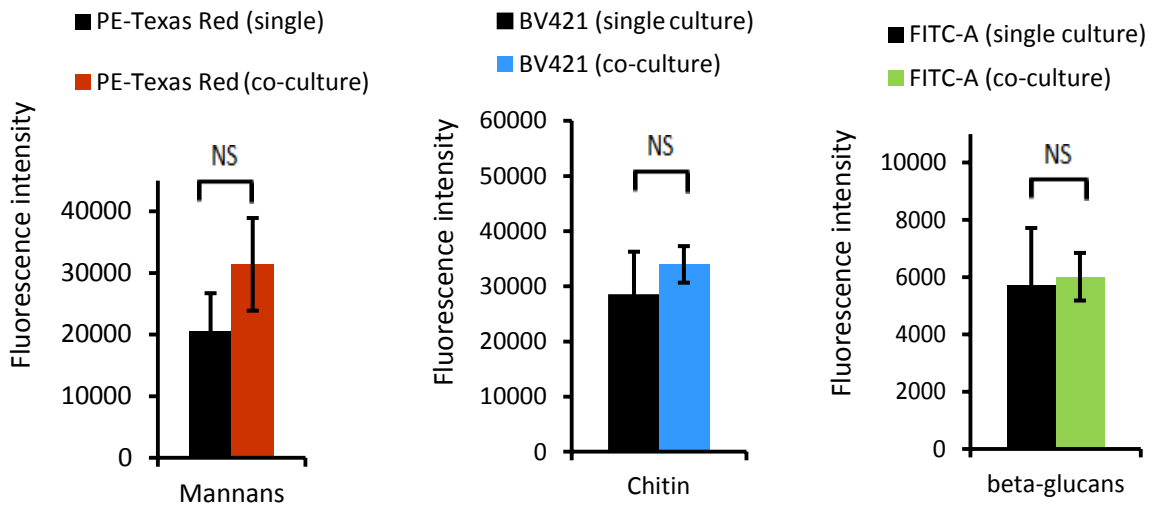


Figure 3.9: Gating strategy, Histogram and fluorescence intensity of FITC-A (β-glucan staining), PE-Texas Red-A (mannan staining) and BV421 (chitin staining) after 12 hours of interaction (in histograms the black line is *C. albicans* in single culture and the colored line is *C. albicans* in co-culture). Data are shown as mean of two independent replicates (n=2). Error bars indicate standard deviation (SD) p> 0.5: non-significant (NS).

Since the cell wall components chitin and β-glucans might be degraded by *Klebsiella* enzymes, as observed by the upregulation of chitinase and endoglucanase in the qPCR data, we then focused on investigating the differences on chitin and β-glucans by flow cytometry with a double-staining approach. In single and co-cultures, while gating for stained *Candida* cells, two different populations were observed (Figure xxx). Cells positive for BV421 (chitin stain) and FITC-A (β-glucan stain) were selected for fluorescence intensity (P1). Figure 3.9 shows the gating strategy to select the positively-stained cells in co-cultures (P2 population analysis is explained in the next two pages).

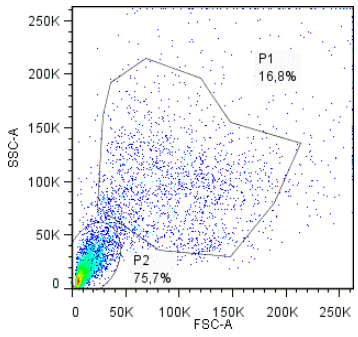


Figure 3.9: Gating strategy of stained *Candida* cells in co-culture with *Klebsiella*. *Candida* cells were double stained with BV421 (chitin stain) and FITC-A (β-glucans stain). P1 and P2 populations were considered for fluorescence intensity separately.

Analysis was based on two different time points of 12 and 24 hours to have a better understanding into this interaction and assess the effect of *K. quasipneumoniae* produced enzymes on the cell wall of *C. albicans*. Figure 3.10 shows the fluorescence intensity of the fluorophores used to identify chitin and β-glucans and the respective histograms. After 12 hours of interaction, a significant increase in fluorescence intensity correspondent to β-glucans was observed in co-cultures compared to *Candida* single cultures. However, no significant changes were observed for chitin. This suggests that *Candida* cells are under the effect of bacterial-derived chitinase and try to overcome this stress by increasing the synthesis of β-glucans. After 24 hours of BFI interaction, the stress coming from produced enzymes from *Klebsiella* is higher against *Candida*, therefore the effort of *Candida* to produce more β-glucans and chitin rises, therefore a significant increase of fluorescence intensity in BV421 (chitin staining) and FITC (β-glucans staining) was observed. Figure 3.10 shows the fluorescence intensity histograms and graphs after 12 and 24 hours of interaction.

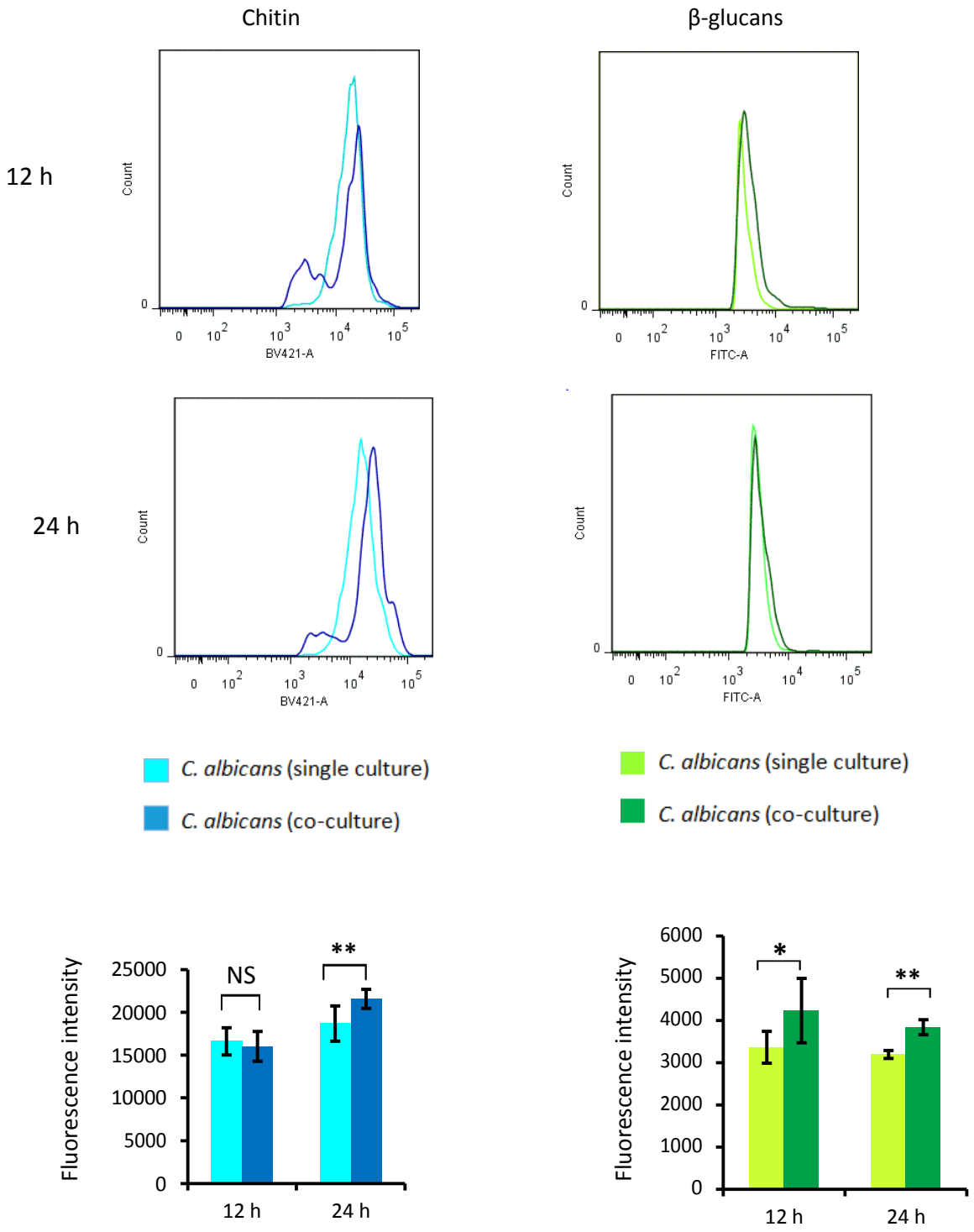
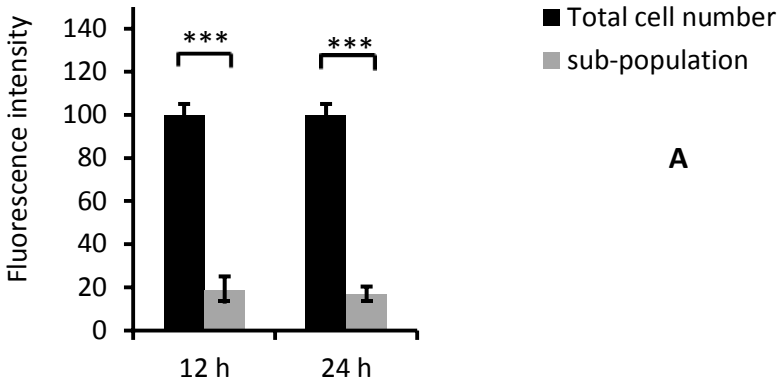
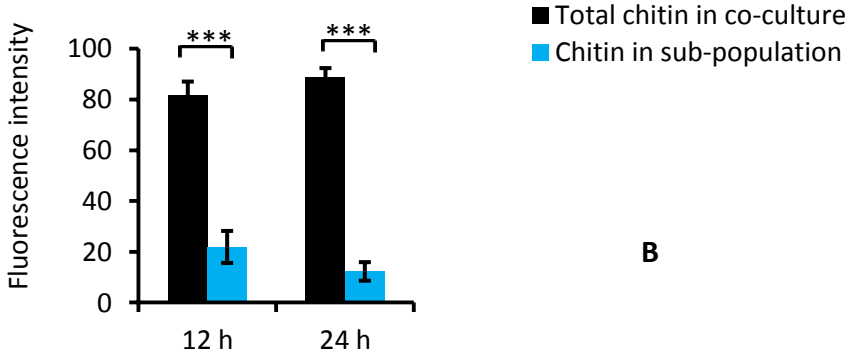


Figure 3.10: Double staining of *C. albicans* cell wall components upon exposure to *Klebsiella*: Graphs are representative histograms of the counts for BV421 (chitin staining) and FITC-A (β-glucan staining) after 12 and 24 hours of co-interaction with *K. quasipneumoniae*. (Light color represents *C. albicans* single cultures and dark color represents *Candida* in the co-cultures). Error bars indicate standard deviation (SD): p> 0.5: non-significant (NS), p<0.05(*), p<0.01(**). n=3.

The sub-population (P2) that appeared in *Candida* staining was separately considered for assessment of fluorescence intensity. The gating strategy and fluorescent intensity are shown in Figure 3.9. The cell number of this sub-population was compared with the total cell number of the co-culture (Figure 3.11.A). Not only the fluorescence intensity of BV421 (chitin stain), but also FITC-A (β-glucans stain) of P2 population was assessed (Figure3.11.B). The created trend populations (P2) which are smaller in size are only 20 % of the total cell number after 12 h of *Candida-Klebsiella* interaction, whereas the sub-population has decreased to ~15 % after 24 h. P2 sub-population shows significant decrease in fluorescence intensity of both BV421 (chitin stain) and FITC-A (β-glucans stain) after 12 and 24 hours on *Candida-Klebsiella* interaction. However, the fluorescence intensity of FITC-A staining β-glucans was significantly decreased in P2 population after 24 h interaction (Figure 3.11.C). Figure 3.11 shows the cell numbers of sub-population compared to the total cell number in co-culture; BV421 fluorescence intensity of P2 sub-population compared to the total BV421 in co-culture and the FITC-A fluorescence intensity in P2 sub-population compared to total FITC-A in co-culture in both 12 and 24 hours of *Candida-Klebsiella* interactions.





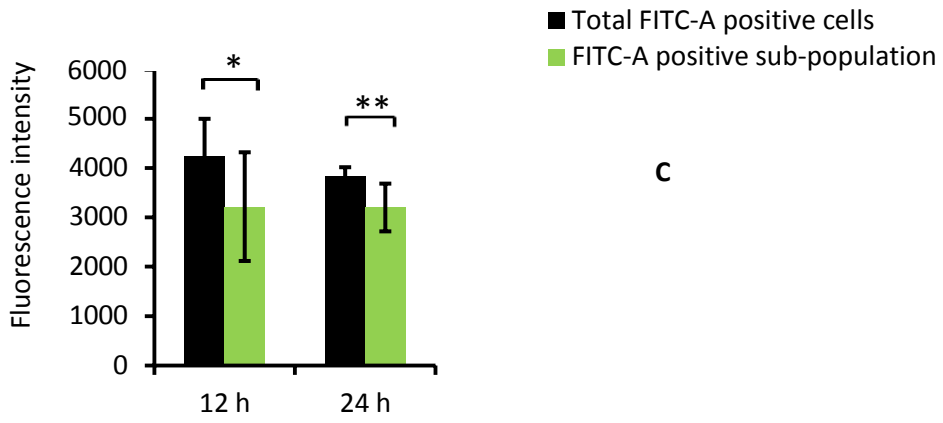


Figure 3.11: P2 sub-population of chitin stained *Candida* cells. Cell numbers in P2 sub-population was compared with total cell numbers of *Candida* in co-cultures (A) - Fluorescence intensity of BV421 for chitin staining in P2 was compared with total chitin intensity in co-cultures (B) - Fluorescence intensity of FITC-A in P2 sub-population was compared with total FITC-A positive cells after 12 and 24 h of *Candida-Klebsiella* interaction. (C). Error bars indicate standard deviation (SD): $p<0.05$(*), $p<0.01$(**) and $p<0.0001$(***). n=3.

3.6 *Candida-Klebsiella* co-infection decreases the viability of the *in vivo* model *Galleria mellonella*

Galleria mellonella, commonly referred as greater wax moth or honeycomb moth, has been used as an *in vivo* model in several studies, including microbial infections [204,207,213,215,225,263]. This model offers some advantages compared to mouse models, as they are cheap, easy to handle and free of ethics approval [206]. Therefore, they can be useful for wide screening studies thus, avoiding the unnecessary use of large numbers of mouse models. The immune system of *Galleria mellonella* has remarkable similarities to human and mice innate immunity. This makes *G. mellonella* a suitable model to study bacterial and fungal infections. [264]. Some studies reported that the immune system of *Galleria mellonella* was able to distinguish between virulent and avirulent strains of *Klebsiella pneumoniae* in a mono-infection assay [205,206]. However, only the avirulent strains were completely phagocytosed [205]. Infection of *G. mellonella* larvae with a single culture of *C. albicans* has shown increased fitness of the fungal cells [210,211]. Changes in protein synthesis, switch to cellular respiration and invasion strategies were some of the features used by *C. albicans* to overcome the immune response of the larvae [265]. Polymicrobial infections have been also studied in this model, where *C. albicans* was co-infected with *S.*

aureus in *G. mellonella* model. The viability analysis showed a higher pathogenicity in the presence of both fungal and bacterial strains, where *Candida* supported the bacterial colonization and has enhanced antimicrobial resistance [208].

In this study, we aimed to infect *G. mellonella* larvae with single- and co-cultures of *Candida* and *Klebsiella* to address the viability of the larvae over time. A total number of 20 larvae were infected per each condition. As controls, a group of larvae were not infected and another group was injected solely with PBS. Following incubation at 37°C, each group was checked for signs of morbidity every 24 h for 5 consecutive days. The viability of the larvae was assessed by counting the yellow-healthy ones and discarding those which were turning into dark-brown or black color and simultaneously had no movement upon gentle touch.

Our data have shown that *Candida-Klebsiella* co-infection resulted in the highest mortality of *G. mellonella*. The survival rate at 5 days was around 10 %, compared to 50 % of each single culture (Figure 3.12).

Interestingly, single infection with *Klebsiella* has resulted in a fast drop in *G. mellonella* viability, compared to *Candida* single infection. This suggests that *Klebsiella* might be hypervirulent for the larvae and, as previous studies suggest, the innate immune system is not able to clear out *Klebsiella* virulent strains. However, *G. mellonella* has been shown to be capable of conceptually approximate *K. pnuemoniae*-caused pneumonia and mimics the *Klebsiella* infection by cell death associated with bacterial fast replicates, escaping phagocytosis and attenuation of the host immunity [205].

The untreated and PBS-injected larvae have shown no variance in viability after 5 days, which discards any potential associated risks of the injection process. *Galleria* infected with a single culture of *C. albicans* tolerated the infection for two days and after the second day a negative effect was visible. A co-infection with both bacterial and fungal strains resulted in the lowest viability of the larvae.

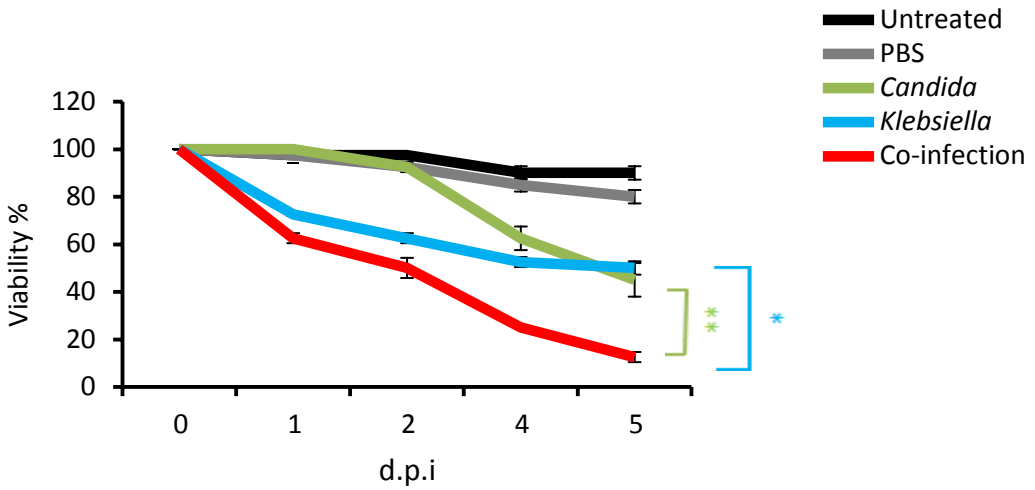


Figure 3.13: Viability of *Galleria mellonella* upon infection with single- and co-cultures of *Candida* and *Klebsiella*. Groups of 20 larvae were injected with *C. albicans, K. quasipneumoniae* and *Candida-Klebsiella* co-cultures. As controls, one of the groups was left uninjected (untreated) and another one injected solely with PBS. d.p.i. – days post infection. Error bars indicate standard deviation (SD). $p<0.05$(*) and $p<0.01$(**). n=4.

5. Discussion

5.1 A medium supporting biofilm formation of polymicrobial communities

Biofilms are considered as a strategy used by fungi and bacteria for protection against harsh environmental conditions [266]. Biofilms reduce the efficiency of antimicrobial therapies and prolong recovery of patients carrying bacterial or fungal infections [267]. Therefore, *in vitro* and *in vivo* studies are required to investigate the mechanisms underlying biofilm formation and drug resistance development.

Both bacterial and fungal strains are able to form biofilms [234,235] however, the amount of biofilm biomass formed in a microbial community is dependent on the interaction, either synergistic or antagonistic. Previous studies have shown that *Candida* biofilms decrease upon interaction with different bacteria [235], and in this between used media has an important impact on the experiment outcome.

In this study, three media including YNBNP, DMEM+ 10 % hiFCS and M9, were tested to measure the biofilm biomass formed during *Candida-Klebsiella* interaction to examine firstly the type of interaction, whether it is synergistic or antagonistic, and secondly to find a medium that supports the biofilm formation of both strains.

M9 as a minimal medium could not be used in further *ex vivo* studies such as analyzing the interaction within bone-marrow derived macrophages; therefore it wouldn't be a cle er choice for the respective BFI. Although not significant, but the same trend of decreased biofilm biomass was observed in YNBNP and DMEM + hiFCS media in co-cultures of *Candida-Klebsiella*. However, there was a significant decrease in biofilm formation of *Klebsiella* single cultures compared to co-cultures in DMEM medium. This could be a result of detachment of the bacterial cells during several washing steps associated with the crystal violet assay. *Klebsiella* might also need more incubation time to be appropriately attached to the plate surface. Here, the focus was to compare the biofilm biomass of single and co-cultures; however, because of too much variance in the measurement represented by the error bars, more replicates with modifications and other assays such as dry mass, XTT (tetrazolium dye) and also genomic quantification of biofilms could be used for biofilm biomass measurements to further explore the *Candida-Klebsiella* interaction

5.2 Determined MIC value varies by different experimental settings

Drug resistance is a major health issue which is increasing globally. Drug resistant *Candida* and *Klebsiella* isolates have been reported including echinocandins as the major antifungal [240,243,268] and colistin as one of the last resources to treat carbapenem-resistant *Klebsiella* (CRKp) [237]. In biofilms, the minimum inhibitory concentra on (MIC) of an microbial drugs rises 1000 x in comparison to other growth stages like planktonic or free floating cells [238]. Although in polymicrobial infections are a life threatening issue, MICs of each individual strain contributing in infection have not been studied intensively. Up to now, the contribution of each single species in a polymicrobial biofilm with an effect on MICs has not yet been fully addressed. Here, we determined the MICs of biofilms and planktonic *C. albicans and K. pneumoniae* upon interaction either in liquid YPD medium (based on EUCAST guidelines) or on solid YPD (E-test). While MIC values of caspofungin for*C. albicans* have not changed upon interaction in biofilms and planktonic cells, colistin MIC values for *K. quasipneumoniae* upon interaction varies. According to the gene expression results, there was a significant upregulation of the "*7185*" gene which is in ol ed in biofilm formation. Often biofilms are associated with increased resistance to drug treatment. Our results suggest that, upon interaction with *Candida*, *Klebsiella* increases biofilm formation and therefore, is less sensitive to antibiotics treatment. However, in an MIC assay conducted according to EUCAST guidelines, *Klebsiella* has shown increased sensitivity to colistin. This contradiction may be due to differences in growth mode, as cultures were grown in solid media for the E - test assay and liquid media for the MIC based on EUCAST guidelines. These observations could be explained by a more homogeneous distribution of antimicrobial drugs in liquid medium compared to solid medium. Therefore, the choice of the antimicrobial test is extremely important for diagnostics and more studies should be devised to assess antimicrobial susceptibility.

Of note, studying pharmacokinetic and pharmacodynamics of colistin is difficult to carry out, since it binds to many different laboratory materials [269], which might directly affect the MIC values.

The negatively-charged colistin binds to bacterial LPS and displaces calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions from the phosphate groups and disturbs the membrane [269]. kanamycin and chloramphenicol, which both affect protein synthesis, did not show a difference in MIC values for *Klebsiella* upon exposure to *Candida*. These observations could suggest an effect of this BFI on the cell wall of *Klebsiella*, because if a drug targets *Klebsiella*

cell wall and *Klebsiella* was sensitive to the drug in BFI, this could suggest that there were alterations in the cell wall during interaction.

5.3 Microscopic visualization of *Candida-Klebsiella* co-cultures

Previous studies have confirmed the adhesion and close physical interaction of *Candida* with different bacteria such as *Klebsiella* in biofilm growth mode [245,246]. Confocal scanning laser microscopy data have confirmed that both microorganisms are contributing in biofilms [2,244-246,270-273]. In this study, *Candida* and *Klebsiella* were grown in both biofilmic and planktonic growth modes and visualized using a confocal microscope.

Confocal microscopy revealed adherence of *Klebsiella* cells to *Candida* hyphal structures. Different morphologies of *Candida* and *Klebsiella* cells in biofilms and planktonic stage could be explained by the differences in the growth condition and also shaking might affect cells in planktonic stage. Reduced hyphal structures shown in co-cultures may come from the effect of this interaction on biofilm formation, but also the washing steps could lead to a loss of less strongly adhered biofilm structures.

5.4 *C. albicans* cell wall is affected by cell wall degrading enzymes produced by *K. quasipneumoniae*

C. albicans genes involved in biofilm formation, adhesion and hyphal development have been shown to play key roles in polymicrobial infections [247,248,250,270]. Our data from crystal violet assays showed a reduction in biofilm biomass in co-cultures of *Candida-Klebsiella* and also microscopy pictures showed a reduction in *Candida* hyphal structures in co-infection. Gene expression analysis of *Candida* genes showed that *ECE1*, which is an important gene that is only expressed in hyphal forms and is also responsible for production of *Candida*lysin, has been significantly increased in the co-cultures. We therefore hypothesize that *Candida* may counteract *Klebsiella*-induced stress by producing this toxin. Although qPCR data showed a significant increase of *ECE1* gene in co-cultures, it would be necessary to test the levels of the Ece1 protein by other methods such as proteomic analysis of the supernatant or western blotting by using antibodies against *Candida*lysin.

Other hyphal genes such as *HWP1* (Hyphal Wall Protein), which is important for adhesion to host cells, and *FKS1*, which is responsible for synthesis of β- (1,3)- glucans in the fungal cell wall, are significantly decreased in the presence of *Klebsiella* during biofilm formation. These observations could be explained by the cell wall degrading enzymes

produced by *Klebsiella*. Hyphal specific genes such as *UME6* and *BRG1* and the transcriptional factor *CPH1* required for mating and biofilm formation have been significantly increased when *Candida* was exposed to *Klebsiella*. These observations could be explained by a neutralizing mechanism of fungal cells to cope with cell wall degradation effects from the *Klebsiella* side. Gene expression analysis from planktonic-grown cells and comparison with the biofilm-grown cells could give more information about *Candida-Klebsiella* interactions.

5.5 *K. quasipneumoniae* produces cell wall degrading enzymes against *C. albicans* upon interaction

A recent study on bacterial-fungal interactions has shown that *Lactobacillus rhamnosus* GG produces a chitinase enzyme and thereby inhibits *C. albicans* morphogenesis [249]. From the KEGG database we found out that *K. quasipneumoniae* not only encodes a *CHIT* gene, but it also possesses *ENDOGLUC* as fungal cell wall degrading enzymes. Gene expression analysis of *K. quasipneumoniae* not only showed a significant increase of *CHIT* and *ENDOGLUC* as cell wall degrading enzymes upon exposure to *C. albicans*, but also revealed high expression of the quorum sensing molecule coded by SdiA, that might play a role in coordinating these enzymes. For further analysis, western blotting with antibodies against chitinase and endoglucanase enzymes and/or proteomic analysis from co-culture supernatants could be performed to further prove the presence of these enzymes in the co-cultures. Although the biofilm formation gene 7185 was upregulated in co-cultures, we observed a reduction in biofilm biomass in crystal violet assays. More in-depth analysis of biofilm related genes on both the bacterial and fungal side could give more information about biofilm-related genes involved in this interaction. Only the stress response gene *4690* (protein kinase) was upregulated upon exposure to *C. albicans*. In order to study this interaction in depth, we need to test more gene regulators that might be important in *Candida-Klebsiella* interaction.

5.6 *Klebsiella*-produced cell wall degrading enzymes affect *Candida* β-glucans and chitin significantly upon longer incubation times

Candida cell wall is an important barrier to protect cells from external and environmental stresses. Presence of other microbes can also be counted as another threat for fungal cells and can therefore trigger the pathways leading to cell wall modifications [205,231,274,275]. For example *C. albicans* biofilm formation is inhibited by chitinase activity

of *L. rhamnosus* GG. As *K. quasipneumoniae* is also able to express *CHIT* [249], we hypothesized that the produced enzyme could be able to degrade chitin of the *Candida* cell wall. Other than chitinase, *Klebsiella* also expresses endoglucanases. About 50-60 % of the *Candida* cell wall is composed of β-glucans, therefore we suggest that enzymes such as endoglucanase could have a great effect on cell wall composition. Cell wall staining showed a significant increase in chitin and β-glucan in the *Candida* cell wall in co-cultures. Therefore we suggest that *Candida* cells counteract the negative effect of enzymes produced by *Klebsiella* by inreasing cell wall components. More replicates with longer and different incubation times of *Candida-Klebsiella* co-cultures could give a more complete picture of this effect. However, fluorescence intensity of mannans as the most outer layer of *Candida* cell wall has not changed significantly. Knock out models of *Klebsiella* lacking *CHIT* and *ENDOGLUC* genes could prove cell wall degrading activities.

5.7 *Galleria mellonella* as an *in vivo* model to study bacterial-fungal interactions

G. mellonella has been extensively used to study microbial infections over the recent years [204,207,215,225,263]. Not only in bacterial or fungal infections, but also in polymicrobial infections it has been shown that *G. mellonella* is a model that can conceptually mimic infections and the mammalian innate immunity [205,208]. A co-infection of *Candida-Klebsiella* resulted in high mortality in the *G. mellonella* model compared to single species controls. We hypothesize that an infection caused by two pathogens may exacerbate the inflammatory process and cause the immune system to collapse. Increased inflammation, and in addition, the potential secretion of toxins and molecules derived from the pathogens may altogether contribute to a less effective immune response, resulting in the host death. More assays are needed to assess the immune response against a polymicrobial infection. For example, gene expression of both the pathogen and the host would help to uncover the mechanisms behind this complex bacterial-fungal-host interaction. These results also suggest that *Candida* and *Klebsiella* may have a synergistic interaction *in vivo*.

Additionally, *ex vivo* experiments could be performed for interaction of these pathogens with murine-derived immune cells such as macrophages or neutrophils. ELISA, western blotting and also gene expression analysis of immune cells would give more insights into immune modulations of this polymicrobial infection.

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