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Vývoj směsných společenstev patogenních kvasinek *Candida albicans* a *Candida guilliermondii*

Evolution of mixed cultures of the pathogenic yeasts *Candida albicans* and *Candida guilliermondii*

BAKALÁŘSKÁ PRÁCE

Školitel: Doc. RNDr. Olga Heidingsfeld, CSc.

Praha 2022

I affirm this thesis was elaborated independently under the supervision of Doc. RNDr. Olga Heidingsfeld, CSc., that all sources used were cited properly and that any part of the thesis has not been submitted for obtaining another degree.

(Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

May 27, 2022, Prague ……..

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Abstract

The objective of this thesis was to investigate how cocultures of *Candida albicans* and *Candida guilliermondii* change overtime under control conditions and under the influence of fluconazole. These species are opportunistic fungal pathogens but widely differ in their susceptibility to antimycotic of interest – fluconazole.

After a brief introduction to special commonalities, the mechanism of pathogenesis, and the treatment of infection, this work explores each organism's growth curves under selected conditions and the process of artificial evolution using the model of passaging of cocultures. Afterwards, these populations of *C. albicans* and *C. guillieromondii* were investigated using qPCR and chromogenic media.

qPCR analysis revealed that under control conditions, *C. albicans* (CA) prevails; the possible reason behind this is a 20% shorter generation time, as revealed by the growth curve. In the presence of fluconazole, two trends occurred. One is related to the innate resistance of C. guilliermondii (CG), where CG was dominant by the end of passaging. The second trend led to CA being the dominant one, despite its susceptibility. This is a result of a heightened resistance, where minimal inhibitory concentration 50 (MIC_{50}) increased almost 10-fold, possibly due to mutations.

The change in populations investigated using chromogenic media (CHROMagar) follows and supports the trends found by qPCR; however, their interpretation proves to be rather tricky and more data are needed to conclude.

Key words: *Candida albicans*, *Candida guilliermondii*, fluconazole, passaging, drug resistance, MIC_{50}

Abstrakt

Cílem této práce bylo zjistit, jak se mění kokultury kvasinek *Candida albicans* a *Candida guilliermondii* v průběhu času, jednak za kontrolních podmínek a též pod vlivem flukonazolu. Tyto druhy jsou oportunní fungální patogeny, které se značně liší ve své citlivosti k nejčastěji používanému antimykotiku, flukonazolu.

Po krátkém úvodu do společných rysů studovaných druhů, jejich mechanizmu patogeneze a léčby infekcí, které způsobují, se tato práce věnuje růstovým křivkám obou mikroorganizmů za vybraných podmínek a taktéž procesu umělé evoluce pomocí modelu pasážování kokultur. Poté byly tyto populace *C. albicans* a *C. guillieromondii* zkoumány pomocí qPCR a chromogenních médií.

qPCR analýza odhalila, že za kontrolních podmínek v kulturách převládá *C. albicans* (CA). Možným důvodem je 20 % kratší generační doba, jak ukazuje růstová křivka. V přítomnosti flukonazolu bylo možno pozorovat dva trendy. Jeden souvisí s přirozenou rezistencí *C. guilliermondii* (CG) a vedl k tomu, že CG byla na konci pasážování dominantní. Druhý trend vedl k dominanci CA, přestože tato kvasinka je za normálních okolností k flukonazolu citlivá. Na základě tohoto zjištení byla testovaná minimalní inhibiční koncentrace (MIC_{50}) náhodně vybraných kolonií CA. V experimentu, kde ve 12. pasáži CA převládla, došlo až k 10-násobnému zvýšení její MIC_{50}, pravděpodobně v důsledku mutací.

Změny v populacích zkoumaných pomocí chromogenního média (CHROMagar) podporují trendy zjištěné qPCR, ale jejich interpretace je poměrne náročná a k plnohodnotnému závěru bude potřeba více dat.

Klíčová slova: *Candida albicans*, *Candida guilliermondii*, flukonazol, pasážovaní, léková rezistence, MIC_{50}

Table of Contents

List of abbreviation

CA	*Candida albicans*
CG	*Candida guilliermondii*
FLU	fluconazole
DNA	deoxyribonucleic acid
LOH	loss of heterozygosity
PAMP	pathogen-associated molecular pattern
spp.	species
MAPK	Mitogen-activated protein kinases
YNB-glu	yeast nitrogen base + glucose medium
YPD	yeast peptone dextrose medium
MIC	minimum inhibitory concentration
GIT	gastrointestinal tract
CFU	colony-forming units
BR	biological replica
NCBI	National Center for Biotechnology Information
qPCR	quantitative polymerase chain reaction
TRIS	trisaminomethane
ALS	agglutinin-like sequence
MKP1	MAPK phosphatase 1
TCA	tricarboxylic acid
CoA	coenzyme A
SDS	sodium dodecyl sulfate
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
IOCB	Institute of Organic Chemistry and Biochemistry
AIDS	Acquired immunodeficiency syndrome
PBS	phosphate-buffered saline
MDR	multi-drug resistance transporters
ABC	ATP-binding cassette
CDR	Candida drug resistance gene
OD_{nm}	optical density (lower index represents wavelength)

1. Introduction

Kingdom of fungi can be an umbrella term for a diverse group of eukaryotic organisms with widely disparate metabolisms, reproduction strategies, and morphologies. Specifically, our focus falls on the part of the Ascomycota phylum - yeast. Due to their variability, yeasts have not been classified as taxon and classifying these organisms tends to be complicated. Yeasts can generally be found in nature and as commensals in the human body. The most commonly discussed yeast is *Saccharomyces cerevisiae*, which has often been used as a model organism. It is also the first eukaryotic organism whose genome has been sequenced[1].

The yeast species associated with various diseases attracted a comparable degree of attention during the last two decades. *Candida albicans* plays a prominent role as the most frequent cause of mycotic diseases worldwide, with most of the research focused on it. The significance of other *Candida* species, often designated as non-albicans, increases as well, like *C. guilliermondii* in our case. Therefore it is important to discuss common characteristics of our focal genus *Candida* in more general terms.

As previously stated, the genus comprises a large number of different species. However, the motivation to study cocultures of *C. albicans* and *C. guilliermondii* stems, among others, from the disproportion of studies published. There are about 140,000 studies published about *S. cerevisiae,* about 45,000 studies published about *C. albicans*, but not even 1,000 research papers published about *C. guilliermondii* (data corresponds to NCBI database, as of March 2022).

2.1.Candida albicans

C. albicans is one of the most common human fungal pathogens across several continents, responsible for systematic and non-systematic candidiasis.[2] It is commensally present in the GIT or genitourinary tract, where dysbiosis, usually induced by antibiotics or immunosuppression, can over-turn this opportunistic pathogen into a threat. It is a polymorphic species, which means it can present itself as an oval yeast cell, thought to be non-invasive. But also present itself in its hyphal form, helping with infection and penetration of the host's immune system. *C. albicans* is often contracted nosocomially and is linked to high mortality in patients in intensive care units and hospitalised children[3].

2.2. Non-*albicans Candida* species

While *C. albicans* is the most frequently isolated fungal pathogen in clinical practice, the importance of other *Candida* species cannot be underestimated.

2.2.1. Candida guilliermondii

Previously known as *Pichia guilliermondii*, of the *Meyerozyma* genus, is a haploid, opportunistically pathogenic yeast abundantly found on various biotic (e.g. skin) and abiotic surfaces (e.g. catheters)[4].

Although only 3-5% of all candidemia cases are attributed to *C. guilliermondii*, such infections lead to "cluster infections" of an immunocompromised individual.[5] Collection of studies between June 1997 and December 2003 have identified 134,715 medical isolates of *Candida spp.,* among which *C. guilliermondi* has placed sixth out of 16 species focused on[6]. It has also revealed a significantly lower susceptibility to fluconazole (75.2%) in comparison to *C. albicans*. Change in susceptibility to fluconazole has also been observed in correlation to the location of the hospital specimen. Isolates collected from dermatology and surgery specimens were about 20% less susceptible than those isolated from other areas[6]. The study found that fluconazole-resistant strains were still susceptible to voriconazole[6].

A study investigating hematologic malignancies and accompanying diseases, carried out for over 20 years, has shown that in all fungemia cases caused by *C. guillermondii*, a central venous catheter was present, as well as patients were subjected to immunosuppressant therapy[4].

C. guilliemondii isolates, connected to underlying malignant conditions, are characterised by low virulence and medium formation of biofilms. Yet, with high innate resistance to azole antifungals and echinocandins, the prognosis of *C. guilliermondii* candidiasis is in most cases unfavorable[7].

This ability to withstand commonly used antimycotics, combined with the increasing number of fungal infections, exacerbates our interest in this multi-resistant species[8].

2.2.2. Candida glabrata

C. glabrata is the second most commonly isolated of the *Candida sp.* in clinical practice, it lacks the ability to form hyphae, and its cells are significantly smaller in size. Although some consider the change in morphology as a major virulence factor, *C. glabrata* relies on other strategies (e.g. adherence, antigenic modulation). In addition, it possesses innate azole antimycotic resistance. This pathogen causes systemic infection and usually infects the elderly[9,10].

2.2.3. Candida krusei

C. krusei cells have a "rice-like" shape compared to other *Candida spp.* and possess an innate fluconazole resistance. Although systemic infections caused by this species are not as ordinary, individuals with immunosuppressive treatments, neutropenia or long-term antibiotic users are at risk[11].

2.2.4. Candida parapsilosis

The commonality of this pathogen has been rapidly increasing over the past two decades. *C. parapsilosis* causes mainly nosocomial infections, primarily affecting neonates. The transmission of this pathogen is usually vertical – mother to child. One virulence factor this species highly relies on is the ability to form hydrophobic biofilms, allowing the colonisation of medical equipment (e.g., catheters)[11]. In contrast to *C. glabrata*, *C. parapsilosis* targets newborns[9].

3. Commonalities of *Candida* species

While each Candida species has its specific set of features and strategies for survival within the host, there are certain commonalities, including the cell wall, polymorphism, pathogenic mechanisms, and others.

3.1.Morphology

The cell wall of pathogenic *Candida spp.* provides similar physical advantages to that of other unicellular fungi but reflects the pathogen's niche. It consists of two layers, where the inner layer consists of polysaccharides – β-glucan and chitin. Chitin is an acetamidated cellulose-like polysaccharide with β-(1→4) glycosidic linkage. The addition of polar groups allows for further hydrogen bonding, thereby promoting insolubility[*]. The amount of this N-acetylglucosamine polymer increases in hyphal forms, suggesting its role in virulence. β-glucan is a glucose polymer vastly abundant in fungi – glucose units are bound via β-(1→3) glycosidic bond, attributing to about 50-60% of dry structural weight. The outer layer is comprised of mannoproteins associated with glycosylphosphatidylinositol. Although mannoproteins do not play a significant role in maintaining the cell shape like chitin and β-glucans, these glycoproteins are capable of cell wall remodelling and adhesion. They are coupled with pathogen-associated molecular pattern (PAMP)[12].

Yeasts potentially exist in one of three forms, producing oval yeast cells (blastospores) or filamentous forms of pseudohyphae and hyphae. Due to environment-induced morphological

switching, most but not all yeasts can be found in any of these morphologies and undergo a transition between them. For example, polymorphism plays a vital role in pathogenesis in some *Candida* sp., such as *C. albicans*. Other species, such as *C. glabrata,* are not capable of hyphal formation and rely on other mechanisms[13].

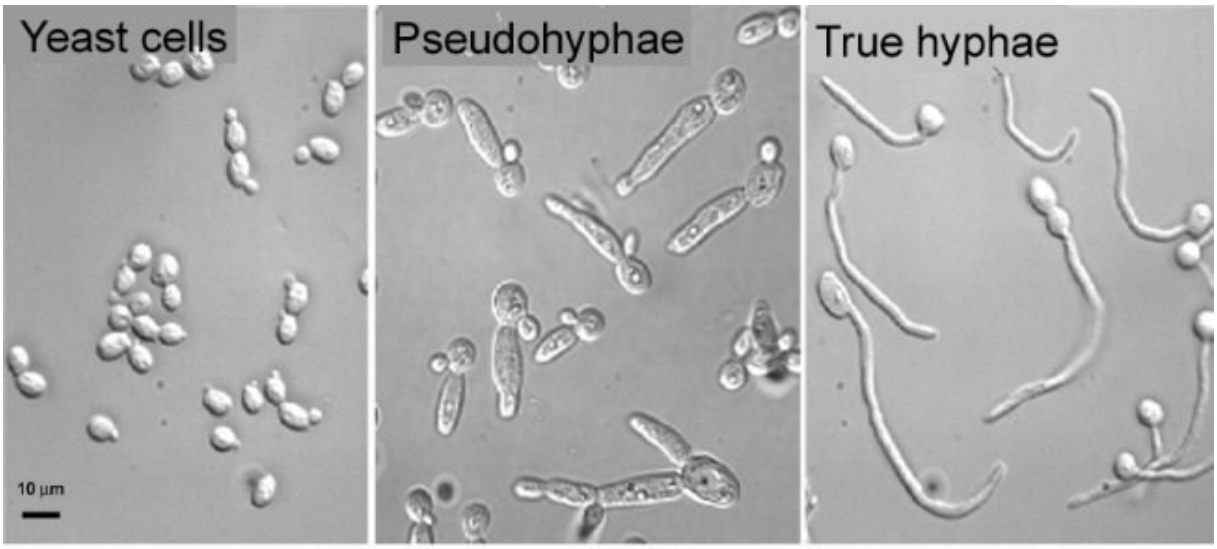

Figure 1: Microscopic images of the three different forms Candida albicans can present itself as. Starting from the left are oval yeast cells, then filamentous pseudohyphae and true hyphae[14]

3.2.Mechanism of pathogenesis

Considering the massive variety of fungi, only 0,001% are deemed pathogenic[15]. *Candida albicans,* with its abundant presence in our lives, is the primary cause of fungal infections[16]. Although this opportunistic pathogen shares many virulence-contributing traits with other yeasts, the virulence strategies of individual pathogenic yeasts vary. The factors related to the pathogenesis of *C. albicans* include polymorphism, the versatility of protein apparatus conferring adherence to various surfaces, the ability of tissue penetration, and metabolic adaptation enabling utilisation of a wide range of nutrients and multi-stress response. *Figure 2* shows the interaction between how various stresses imposed by the host can activate virulence factors of *C. albicans*. Although this section primarily discusses *C. albicans*, some of these mechanisms can be, to a certain extent, applied to other species, sometimes referred to as non-*albicans*.

3.3. Polymorphism

A critical factor in the change of the *C. albicans* morphology is the environment. Blastospores thrive under acidic pH; however, with increasing pH, the yeast undergoes bud-hyphae transition[17]. This transition can also be induced by increased CO_2 levels, lack of carbon and nitrogen, or reactive oxygen species, commonly present in host phagocytic cells. Research

indicates that *C. albicans* can generate subtoxic levels of hydrogen peroxide, self-inducing the change in morphology[18]. After phagocytosis by macrophages, this process can help rapture the host cell and escape immune response, increasing virulent potential and survival.

Another example leading to morphological change is higher CO_2 levels, *C. albicans* uses the enzyme carbonic anhydrase, converting carbon dioxide to bicarbonate anion, activating the second messenger system - crucial in yeast-to-hyphae switching[19].

Both filamentous and non-filamentous morphologies play an equal role in virulence. In a study of mutants that could not form hyphae, virulence was significantly reduced[20]. Non-filamentous cells play a role in dissemination, whereas filamentous help with invasion[21]. Although filamentation is common in *C. albicans,* non-*albicans* species such as *C. glabrata* can achieve pathogenesis without such morphological change, proposing that filamentation is only one of the ways *Candida* is capable of virulence[9].

Hyphae are able to penetrate mucosal membranes, stimulating transcription factor c-Fos and MAPK phosphatase MKP1, promoting pro-inflammatory cytokine response[22].

C. albicans employs thigmotropism, self-induced directional growth, where cells change to filamentous morphology after contact with a surface. Very similarly, the formation of biofilms is induced[23].

3.4. Genetic variability

Even though *C. albicans* is diploid, it is unable to divide meiotically into its haploid form, it can maintain its genetic variability via chromosomal polymorphism – changes in ploidy of chromosomes, mitotic recombination, and loss of heterozygosity (LOH)[24]. These factors enable micro adaptations in the host, contributing to the survival of the yeast cells. Studies show that LOH is proportionally increased with environmental stressors within the host[25].

3.5. Cell wall proteins

Since adhesion can be considered an initial stage in pathogenesis, mannoproteins present in the outer fibrillar structure facilitate the interaction of host and cell interaction and can be generalised as adhesin proteins[26]. Secondly, cell wall proteins also moderate the morphology of the cell, thereby massively influencing the pathogenic potential of yeast-to-hyphae transformation and the integrity of the cell wall[27]

3.5.1. Secreted proteins

The ability to invade the host can be attributed to hydrolytic enzymes – extracellular proteinase, phospholipase, hemolytic factor. Secretory aspartyl proteinases can degrade proteins present

in skin, immunoglobulins, and albumins, disrupting the first line of defence[28]. Murine model mutants deficient in phospholipase secretion had shown to be less virulent[29]. Hemolytic factors (hemolysins) play a role in the hyphal invasion of disseminated candidiasis[30].

3.5.2. Adhesins

Candida spp. are known for adhering to biotic (skin, mucosal tissue) and abiotic (catheter, contact lenses, …) surfaces. Adhesins are an outer-surface protein that facilitates the vital step of pathogenesis - adhesion.

ALS (agglutinin-like sequence) genes are widely present in *Candida* spp. and code for the GPI-anchored proteins (glycophosphatidylinositol). ALS proteins bind to the endothelium, epithelium, and extracellular matrix proteins[31,32]. Studies with homologous gene *ALA1* in *S. cerevisiae* show that *ALS1* gene acts as a fibronectin binder.

ALS3 protein can also act as an invasin, where a cell is able to bind to E-cadherin, initiating phagocytosis and penetrating the host.

Another adhesin HWP1 (hyphae wall protein) is associated with the covalent binding of yeast and the host[33].

3.5.3. Als3 mediated biofilm formation

In vitro studies show that mutants with Als3 gene mutation are able to produce biofilms; however, such mutants are very defective[34].

3.6. Adaptation to the host and virulent trait expression

A vital element of pathogenesis is the ability to adapt to the host's environment, suggesting a close correlation between the expression of virulence and adaptation to the host's environment in effective pathogenesis. These adaptations occur on multiple levels—the tropism switches from saprophytic to parasitic. Ambient gas concentrations change to that inside of the host – hypoxia.

3.6.1. pH adaptation

Due to the high variability of pH throughout the body, *C. albicans* has to be able to manipulate it in a brief period. To alkalinise*, C. albicans* can utilise amino acid metabolism, secreting ammonia extracellularly[35]. This action can also trigger yeast-to-hyphae switching. Such process has been observed under conditions present in phagocytic cells, where neutralisation of acidic conditions within phagolysosomes increases the chance of survival[17]. Extracellular acidification is connected to glucose metabolism, where the cell secretes small organic acids[36, 37].

When *C. albicans* colonises the host, usually via slightly alkaline mucosal membranes, the pH-dependent zinc finger transcription factor (Rim101p) activates a cascade mobilising proteins present in the plasma membrane, endosomal complex and proteasomes. *Rim101* upregulates the synthesis of ferric reductase, proteinase (Sap5p), helping in pathogenesis[38].

3.6.2. Thermal shock

During the change from ambient temperatures to the temperature of the host, the yeast suffer from heat shock. This action upregulates the expression of heat shock genes involved in yeast-to-hyphae switching, where mutants with distorted heat shock transcription factor were less virulent[39].

3.6.3. Carbon metabolism

The preferred carbon source is glucose; however, in the absence of saccharides (e.g., the presence of lactate), *C. albicans* can utilise "non-preferred carbon sources" such as glycerol, ethanol, acetate, fatty acids, and others. With the aim of energy production, and replenishment of TCA intermediates and CoA; fungi, effectively employ pathways of β-oxidation, glyoxylate cycle or gluconeogenesis[40]. In a study involving mutants with deletions related to enzymes crucial in utilising non-preferred carbon sources, the defectives' metabolism was negatively impacted, leading to either full or partial attenuation and suggesting multiauxic carbon source utilisation during infection[41].

Carbon metabolism is implicitly associated with stress resistance – when *C. albicans* is exposed to glucose, it starts to upregulate genes involved in oxidative stress adaptation, suggesting its evolutionary adaptation to possible phagocytosis after entering the bloodstream[42].

Utilisation of different carbon sources in *C. albicans* is usually accompanied by cell wall remodelling and changes in the cell wall proteome, contributing to heightened resistance to antifungals[37]

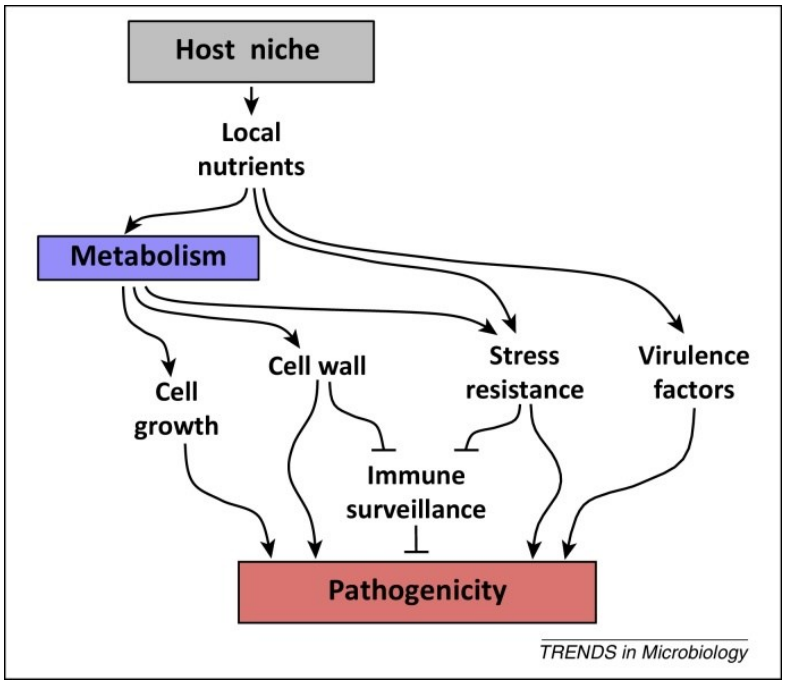

Figure 2: Map of possible adaptations of C. albicans to the host and its connection to pathogenesis[11].

3.6.4. Biofilm formation

It is known that biofilms are more resistant to stress than their planktonic counterparts[43]. Seemingly static biofilms display inner dynamics, including morphological changes of the cells, extracellular matrix formation, and alteration of gene expression relative to planktonic cultures. The genes regulated differentially in biofilms include hypoxic metabolism factors, β-glucan synthesis pathway and other advantageous proteins like multi-drug resistance transporters (MDR) and adhesins (ALS)[44].

In *C. albicans,* biofilms comprise various morphological forms – blastospores are attached to a basal surface, and filamentous cells inhabit the top layer. After the biofilm matures, planktonic cells can be released and are responsible for disseminating and spreading of infection[45,46].

3.6.5. Candidalysin

Candidialysin is a cytolytic toxin, instigating two effects – penetrating the host cell with the ability to stimulate the host's immune system. It is encoded by the *ECE1* gene and is up to 10,000-fold more expressed during the hyphae formation. This hyphae-associated peptide activates a pro-inflammatory response in epithelial cells and macrophages when engulfed. However, this mechanism is not applied when escaping the macrophage membrane[47].

4. Candidiasis

Some yeasts commensally present in the human mycobiome can be considered pathobionts – organisms that are a part of the human microflora but are capable of causing an infection. Due

to the comensallic nature of this relationship, conditions within the host influence the outcome and any imbalance can induce a pathogenic potential[48].

An example of this is *Candida* spp. – the cause of candidiasis. The commonality of yeast infections has only increased with the widespread use of antibiotics. There are several mechanisms proposed as to why this happens – change in the GIT microbiome and allowing colonisation of resistant species or the ability of specific antibacterial agents altering the host's response to *Candida sp.* and others[49,50]. It is also essential to know that these statistics highly vary geographically[51,52]. Candidiasis is associated with high crude mortality rates of 30-81% and a range of 5-71% of attributed mortality rates[53].

Candidiases can be separated according to their origin - endogenic or exogenic. Endogenic infections are preceded by the disruption of mucosal membranes or microbiome imbalance allowing for dissemination and triggering pathogenic potential, of yeasts already present in the host. Exogenic infections are highly associated with nosocomial conditions (e.g., catheterisation). Most susceptible to fungal infection are immunocompromised patients, for example, transplant recipients, AIDS patients, or those who have undergone surgery.

4.1.Types of candidiasis

4.1.1. Oral candidiasis

Thrush is an oral fungal infection characterised by opaque white patches of plaque forming on the inner side of the mouth and tongue. When removed manually, inflamed, red tissue is exposed, possibly leading to haemorrhage. These sections are painful and can cause difficulties ingesting food[54].

4.1.2. Cutaneous candidiasis

Segments of skin that are not appropriately ventilated trap moisture and, in combination with bodily temperature, serve as a matrix for fungal infections, forming wet red patches on the skin[55].

4.1.3. Genital candidiasis

Vulvovaginal candidiasis is a fungal infection of the female reproductive system. Presently it is one of the most common venereal diseases among women. 75% of women contract this infection at least once in their fertile period, with 50% reoccurring[56].

Balanitis is a venereal disease caused by the *Candida* spp., causing inflammation and discomfort in people with male reproductive organs. Red lesions form on the glans or under the foreskin[57].

4.1.4. Systemic candidiasis

Candidiasis affects both the organs and bloodstream. The most lethal form is sepsis, the dissemination of pathogens into the bloodstream – most found in immunosuppressed individuals (AIDS patients, transplantees). Symptoms include fever, hypertension, and myalgia. The mortality rate is 40%[58].

5. Antimycotics

Fungi are opportunistic pathogens, which means they are only capable of causing an infection under specific conditions. Risk group entails immunosuppressed individuals - e.g., HIV patients, patients with cytotoxic chemotherapy, catheterised individuals, transplantees, diabetes patients)[59] Fungi can also infect plants or animals, negatively affecting agriculture via crop destruction and biodiversity via mass extinction[60]. Antifungal research has sprouted in the mid-50's by finding two polyenes – nystatin and amphotericin B. The FDA divides antifungals into four groups – polyenes, echinocandins, flucytosine and azoles[61].

5.1.Flucytosine

Flucytosine is an antimetabolite, a molecule antagonistically inhibiting the utilisation of a metabolite[62]. This halopyrimidine analogue inhibits proteosynthesis by incorporating fluorouracil into the rRNA and tRNA. Due to rapidly built resistance, it is commonly used in combination with amphotericin B. It is usually used against *Cryptococcus* and *Candida* spp., with *C. parapsilosis* and *C. tropicalis* being less susceptible[63]. After the uptake via cytosine permease, rapid deamination to fluorouracil occurs within the cell. Fluorouracil cannot be used due to its high toxicity and low uptake by the fungal cells[64].

5.2.Polyenes

These ionophores form a complex with ergosterol in the cytoplasmatic membrane, causing punctures in the cell wall[65]. The most commonly known polyene is Amphotericin B. The drawback in use is their non-selective binding to mammalian cholesterol molecules, leading to nephrological toxicity[66].

5.3.Azoles

Antimycotics containing azole groups can be divided into imidazoles, triazoles, and thiazoles. Azoles compromise the ergosterol synthesis by inhibiting 14-α demethylase. This enzyme is a part of the cytochrome P450 group encoded by the *ERG11* gene. Nowadays, azoles are the most used antimycotics due to their low toxicity, limited interaction with mammalian P-450 demethylase, and various administrations[67]. The advantage of using these antimycotic is that

yeast use ergosterol for cell membrane fluidity, whereas humans use cholesterol. Fluconazole's structure can be seen in *Figure 3*.

5.3.1. Fungistatic mechanism of azole antimycotics

The nitrogen atom present in the azole ring binds as a ligand to the iron cation present within the heme group of lanosterol 14-α demethylase (Erg11p), impeding the biosynthesis of ergosterol. This atom binds as a sixth ligand, blocks the activation of oxygen, necessary for the conversion of lanosterol and its intermediates, leading to the accumulation of 14-α methyl sterols within the cell[68]. Ergosterol is the most abundant sterol present in the fungal cytoplasmic membrane and modulates its fluidity[69]. Azoles can also bind to the apoenzyme and, in high enough concentrations, can interact with lipids within the membrane[70].

5.3.2. Resistance

Candida resistance to antimycotics can be achieved via three main paths – the first being alterations in *ERG11* (mutation, overexpression), which either decrease the affinity of the targeted enzyme towards fluconazole or stimulate overproduction of Erg11p by the cell. This can cause the commonly used concentration of FLU is not sufficient anymore. Secondly, *C. albicans* can change synthesis pathways by using different enzymes (e.g. C5,6-desaturase) to synthesise various sterols, benefitting from resistance to other antimycotics. Another pathway is an overexpression of genes (*CDRs, MDR*) encoding for efflux pumps. This can subsequently decrease the concentration of antifungal agents within the cell. These pumps involve the ABC (ATP-binding cassette) family and major facilitator superfamily efflux proteins[71]. Aneuploidy is closely related to these mechanisms since the multiplication of chromosomes associated with resistance can lead to a similar outcome. It is also seven times more likely to occur in fluconazole-resistant isolates. Such change often leads to a multiplication of chromosome 5, from which *ERG11, UPC2* – macrophage cell death mediator, and *TAC1* – drug resistance protein stimulator, are transcribed from[72].

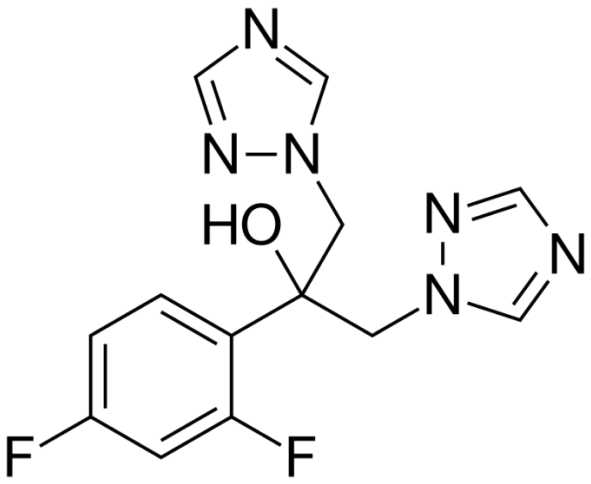

Figure 3: Chemical structure of fluconazole, an antifungal agent used in our experiment. This fungistatic possesses two triazole groups that bind to iron cation found in the lanosterol 14-α demethylase.

5.4.Echinocandins

Echinocandins are a group of large semisynthetic lipopeptides comprised of a hexapeptide nucleus and a variable N-linked fatty acid chain. It is deemed that the conformation and structure of the side chain are vital in its function[73]

This antifungal agent non-competitively inhibits an enzyme complex glucan synthetase. It disrupts the polymerisation of the 1,3-beta-D-glucan present in the cell wall, causing osmotic stress and lysing the cell. The advantage of using echinocandins is the lack of 1,3-beta-glucans in the human cells[74].

6. Aims

This project aimed to investigate how cocultures of *C. albicans* and *C. guilliermondii* change over time and how is this dynamic influenced by fluconazole.

In order to do so following steps were taken:

- construction of separate growth curves of individual species with and without fluconazole
- process of serial passaging and isolation of DNA from each passage
- qPCR analysis and assessment of microorganism ratio in each passage
- analysis of CFU in some of the passages using chromogenic medium
- assessment of MIC in *C. albicans* after passages

7. Material and methods

7.1. Materials used

Material	Composition
Agar (Sigma-Aldrich)	
Candida albicans	Obtained from the Czech Collection of Microorganisms
Candida guilliermondii	https://ccm.sci.muni.cz/en
CHROMagar™ (CHROMagar, France)	
Citric acid (Sigma-Aldrich)	
D-(+)-Glucose anhydrous (Sigma-Aldrich)	
Dimethyl sulfoxide (Sigma-Aldrich)	
EliZyme™ Green MIX AddROX (ELISABETH PHARMACON, spol. s r. o)	
Ethanol 96% p.a. (PENTA s.r.o.)	
Extraction solution A	0.2M NaCl (11.6g/l); 20mM EDTA (7.4g/l); 10% SDS (100g/l) (w/w)
Extraction solution B	100mM TRIS-HCl, pH 7.5
Falcon tubes	
Final extraction buffer	A+B (1:1)
Fluconazole (Sigma-Aldrich)	0.267 mg/ml (in DMSO)
Glass beads, acid-washed (Sigma-Aldrich)	
Glycerol (Sigma-Aldrich)	
PCI (VWR Chemicals, USA)	Phenol-chloroform-isoamyl alcohol (50:50:1)
Primers (GENERI BIOTECH s.r.o.)	
SDS (Sigma-Aldrich)	
Sodium acetate (Sigma-Aldrich)	
Sodium chloride (PENTA s.r.o.)	
YNB-glu medium (Sigma-Aldrich)	7g/l YNB medium + 20g/l glucose, 20mM citrate, pH 5
YPD agar medium (Sigma-Aldrich)	50g/l (20g/l dextrose, 20g/l peptone, 10g/l yeast extract) + 1% agar (w/w)
YPD medium (Sigma-Aldrich)	

7.2.Instruments used

Analytical scale	Adam®, UK
Autoclave	Panasonic, USA
Centrifuge HERMLE Z383 K	Hermle Labortechnik GmbH, Germany
Centrifuge 5424R	Eppendorf, USA
Shaking incubator ES-60	MIUlab, China
Flow box	Labox, Czechia
Magnetic stirrer	VELP Scientifica, Italy
Microtubes	Eppendorf, USA
pH meter pHenomenal®, pH 1100L	VWR® Avantor®, USA
Vortex	IKA, Germany
UV-VIS Spectrophotometer Helios Alpha	Thermo Electron Corporation, USA
Nanodrop spectrophotometer DS-11	Denovix, USA
CFX Connect Real-Time PCR Detection System	Bio-Rad Laboratories, USA

7.3.Cultivation of yeast cells

Before inoculation to liquid media (YNB-glu/YPD), yeasts have always been inoculated from stock cultures stored at -80°C onto separate YPD agar plates and incubated for 24h at 37°C in an incubator.

7.4.Growth curve

The colony of each microorganism was inoculated into separate Falcon tubes in YPD medium and incubated in an orbital shaker overnight at 37°C. Then OD_{600} was measured and standardised to OD_{600}=1.0, and 100 µl of culture were added into 20 ml of YNB-glu medium in a 50 ml Falcon tube.

OD_{600} was measured every hour until it reached a plateau. The whole experiment was performed in triplicate.

7.5. Cell passaging

C. albicans and *C. guilliermondii* (CA, CG) were inoculated from glycerol stock cultures (stored at -80 °C) onto two separate YPD agar plates and incubated for 24h at 37°C. A colony from each plate was then separately inoculated into a 5 ml YPD liquid medium and incubated for 24h at 37° in an orbital shaker incubator.

After incubation, OD_{600} was measured, and both yeasts were inoculated into 20 ml of YNB-glu medium, in proportion, 500 µl if OD_{600} = 1.5. Then 20 µl of fluconazole solution (c=0.267mg/ml) or pure DMSO were added and incubated for 24h at 37°C in an orbital shaker incubator. After incubation, OD_{600} was measured, and the second passage was inoculated with 500 µl of past passage into corresponding Falcon tubes. Then this process was repeated ten times (or prepare 12 passages), see *Figure 4*.

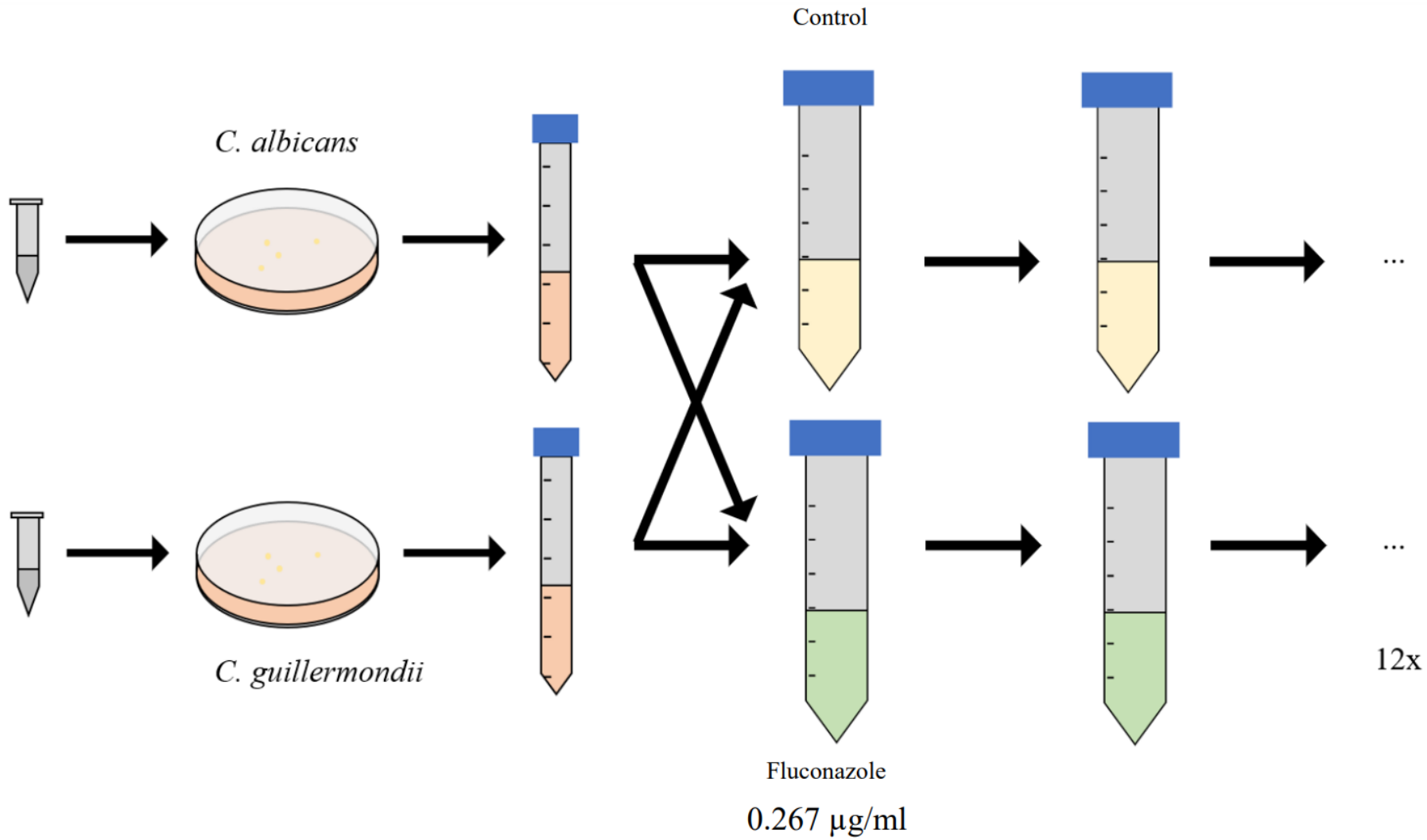

Figure 4: The process of passaging. Final tow steps have been repeated twelve times, resulting in twelve passages. This served as a model of artificial evolution of this coculture.

7.6.Preservation of mixed cultures passages and storing

An aliquot from each passage was always taken and stored at -80°C as a glycerol stock solution (final concentration of 20% (w/w) of glycerol). Extra aliquot was taken from passages that were not inoculated right away (e.g. over the weekend storage).

If the passage was inoculated again from -80°C stock solution (e.g. freezing cultures over the weekend), OD_{600} was measured at first (after thawing) and the passage was inoculated with 500 µl if the OD_{600} of the stock solution was 1.5.

7.7.DNA extraction

DNA extraction was performed according to the protocol of Hoffmann and Winston with slight modifications.[75] Briefly, pellets were resuspended in an extraction buffer, composed of 0.2 M NaCl, 20 mM EDTA, 10% SDS, 100 mM TRIS-HCl (pH 7.5), then the same amount of PCI was added and the suspension of cells was vortexed for 5 minutes in the extraction buffer and PCI (RNase was not applied). This PCI extraction and purification was repeated again. After this extraction, the water phase was then precipitated overnight in 96% ethanol and 3 M sodium acetate. After precipitation pellets were resuspended in 50 µl of dH_2O.

7.8.Concentration of isolated DNA

DNA concentrations for qPCR analysis were measured using a Denovix DS-11 spectrophotometer.

7.9.Measuring optical density (OD_{nm})

Each one of the experiments used a standardised cell suspension. This was achieved by measuring the optical density of inoculum or measurement of OD_{600} for the growth curve. To do this UV-VIS spectrophotometer was used. Measuring was always carried out in a polyethylene cuvette with an optical path of 10 mm. For chromogenic analysis OD_{530} was used.

7.10. CHROMagar analysis

Cultures stored at -80°C were taken out of the freezer, kept on ice and subsequently inoculated using a sterile pipette tip into 5 ml of YNB-glu medium under the conditions present during the passaging (FLU/ no FLU) and incubated overnight in an orbital shaker. After incubations, OD_{530} was measured, and cultures were diluted with respect to the lowest $OD_{530,}$ where OD = 0.284 should correspond to 10^6 cells/ml; according to Costa et al. (the size of CG is roughly similar to CA)[76]. After the first trial, if OD_{530} was 0.1, a dilution of 1:15000 with PBS was chosen.

CHROMagar plates were then inoculated with 100 µl of diluted cultures and incubated for at least 48h at 37°C, and CFUs were counted. Although the provided protocol suggests incubation of 48h, agar plates were incubated for 72h for a better colour distinction of the colonies..

7.11. qPCR analysis

The concentration of each DNA sample was first assessed using DeNovix DS-11 Series Spectrophotometer / Fluorometer and diluted to concentration in correspondence with the protocol, which was, in our case, ≤100 ng/20 µl. Then the protocol provided with the EliZyme^{TM} Green MIX AddROX (ELISABETH PHARMACON, spol. s r.o.) was used.

Table 1: Protocol used for qPCR

Step	Temperature [°C]	Time [s]	Cycles
Initial denaturation	95	180	1
Denaturation	95	5	40
Annealing	60	30	
Extension	60	30	

7.11.1. Primers used

Primers used to assess the ratio of each organism were designed using Primer3+ software[77]. Primers themselves have been designed by Dr. Leszek Pryszcz, who compared *C. albicans* and *C. guilliermondii* genomes and selected orthologous genes containing distinct segments of DNA suitable for qPCR primer design.

Table 2: Primers designed by Dr. Leszek Pryszcz (Centre for Genomic Regulation, Barcelona, Spain) for each species. In our case, primers for the genes ACC1 and GSC1 were used.

Strain	Genes	Name	Sequence (5′-3′)	Product size (bp)
C. albicans	FAS2†	CA1-F	CAGCACCCAAACCAAATCTT	177
		CA1-R	AACCGGAAGACACTCCATTG	
	GSC1‡	CA2-F	TTGGTAACGAAATGCAACCA	219
		CA2-R	CAAGCGTGAAAAAGACCACA	
	ACC1§	CA3-F	TGGCTTAGGGGTTGAATGTC	159
		CA3-R	CCATCGATTTGAATGGCTCT	
C. guillermondii	FAS2	CG1-F	TGGTCGATAGTGCTGCTTTG	230
		CG1-R	CTTCAAAGGCGAGAAAGTGG	
	GSC1	CG2-F	CGGCTTCCAAAGAGTAGTCG	234
		CG2-R	TACACGCGGATTACATTGGA	
	ACC1	CG3-F	AAATTGGGATTGGATGACCA	190
		CG3-R	GGTGCCCCTGCTATTAA	

† α subunit of fatty-acid synthase
‡ β-1,3-Glucan Synthase Catalytic subunit
§ acetyl-coenzyme-A carboxylases

7.12. MIC analysis

Colonies from the 12th passage from the CHROMagar plate were inoculated into the YNB-glu medium and incubated for 24h at 37°C. OD_{530} of all cultures was standardised to 0.1, and each well on a microtitration plate was inoculated with 99 µl of the culture and 1 µl of fluconazole solution at various concentrations – 10, 5, 2.5, 1, 0.5 µg/ml. Microtitration plates were then incubated in an orbital shaker for 24h. OD_{530} was measured at 6h and 24h after inoculation. Inhibition was identified if the OD_{530} of the well was lower than $0.5 \cdot OD_{530}$ of the positive control wells.

8. Results

8.1. Growth curve

The growth curves of *C. albicans* and *C. guilliermondii* monocultures were measured to compare each organism's growth under selected conditions – with and without the influence of fluconazole.

Figure 5 represents monocultures of both organisms under control conditions used in the passaging process. *C. albicans* has a shorter generation time in comparison to *C. guilliermondii*. However, both organisms reach very similar OD_{600}. According to these findings, *C. albicans* is more efficient at utilising nutrients.

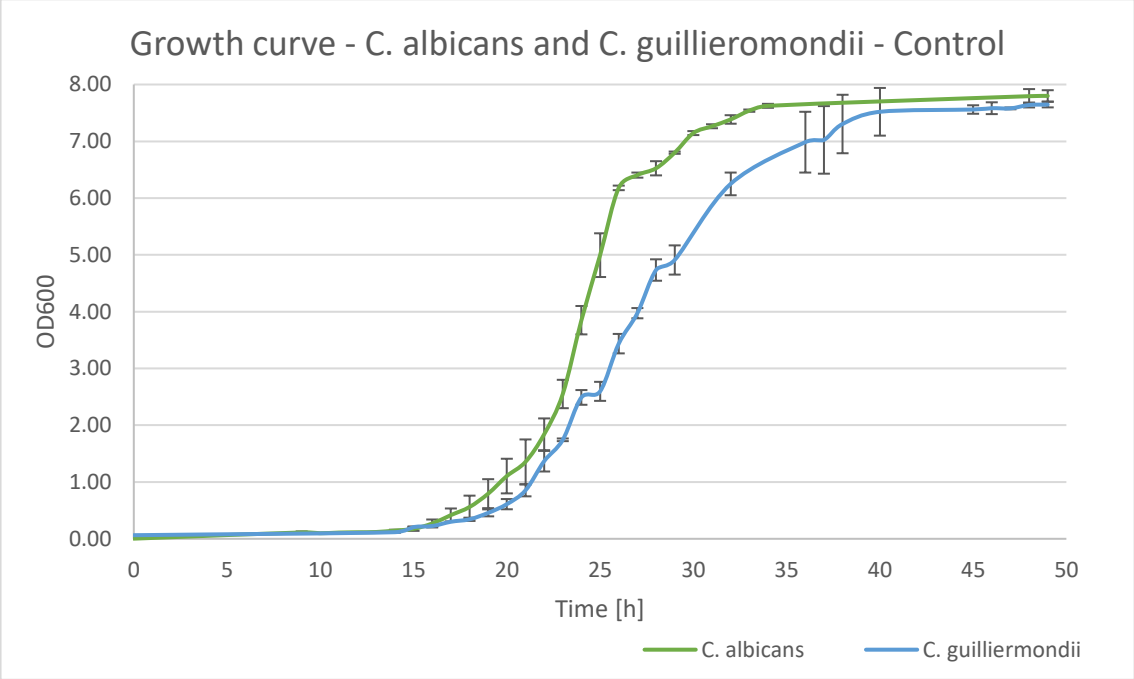

Figure 4: Growth curve of both monocultures in YNB-glu medium, without fluconazole. Both organism reach the same optical density, however C. albicans has a shorter generation time. Each point on the graph is represented as an average of a triplicate, where error bars represent the standard deviation.

Figure 6, shows the growth curve of each microorganism under the influence of fluconazole. *C. albicans* reaches only about half of the optical density, and its generation time is significantly extended. *C. guilliermondii* was not vastly impacted by the presence of fluconazole and has reached a similar optical density to that of control conditions. However, the exponential phase was slightly prolonged.

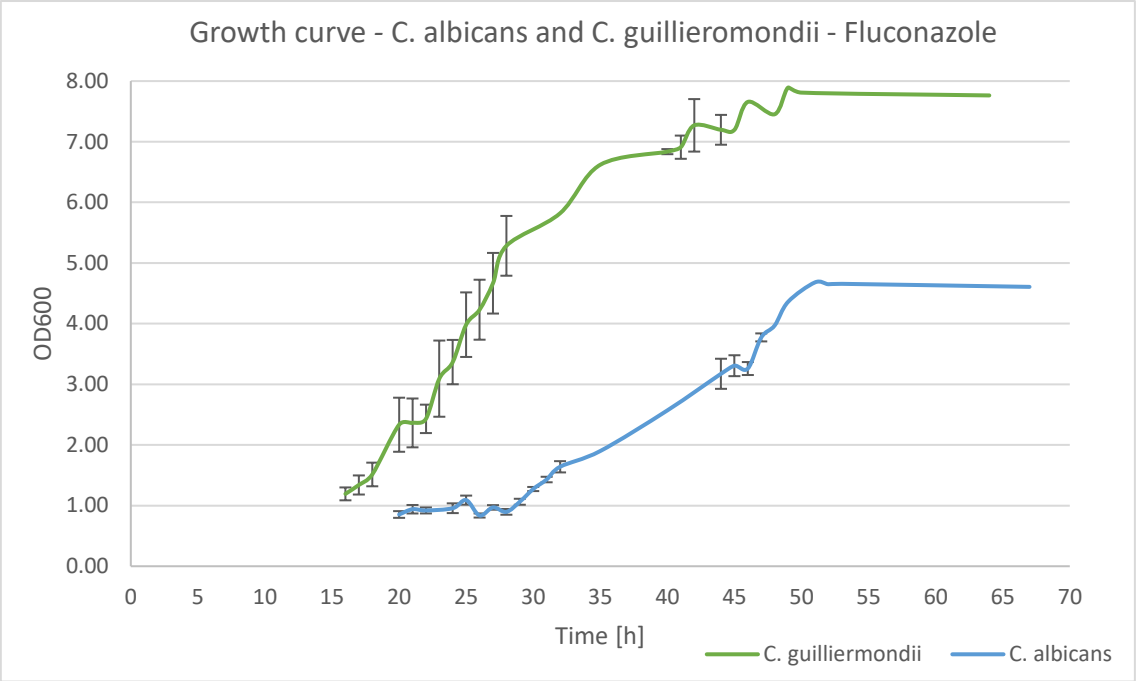

Figure 6: Growth curve of each organism under the influence of fluconazole (c=0.267mg/l). This concentration represent 53% of MIC of C. albicans. This significantly set back CA in both optical density and in the onset of exponential phase. Each point on the graph is represented as an average of a triplicate, where error bars represent the standard deviation.

8.2. Changes in CA to CG ratio during serial passaging

In order to quantify how cocultures change in the time horizon, qPCR was used. Firstly, DNA from each passage was isolated and then analysed using organism-specific primers (*Table 2*), which allowed for their distinction and comparison. Passages were also analyzed using a second method of chromogenic media.

Data set consist of two biological replicas A and B, where each replica was carried out in separate facilities (IOCB and PřF UK). Furthermore, each of the biological replicas consisted of two parallel experiments, represented by blue and orange lines.

8.2.1. qPCR

Figures 7 and *9,* represent separate biological replicas (BRs), but their outcome is quite similar and expected due to the same conditions and results shown in the growth curves (*Figure 5 and 6*). *C. albicans*, as one of the most common fungal pathogens, was able to prevail in the coculture. However, in the biological replica A (BR A) (*Figure 7*), this occurred between the first and fifth passage, whereas in the BR B *C. albicans* became dominant early on (*Figure 9*). Graphs use a logarithmic scale because the obtained data vary in order of magnitude. Therefore, logarithmic scale better represents the change in the culture.

Figure 8 and *10* represent cultures under the stress of fluconazole. In the biological replica A (*Figure 8*), *C. guilliermondii* was more successful at withstanding the stress and remained in the majority throughout. In the case of biological replica B (*Figure 10*), *C. albicans* could adapt to the stressor and hence take over *C. guilliermondii.*

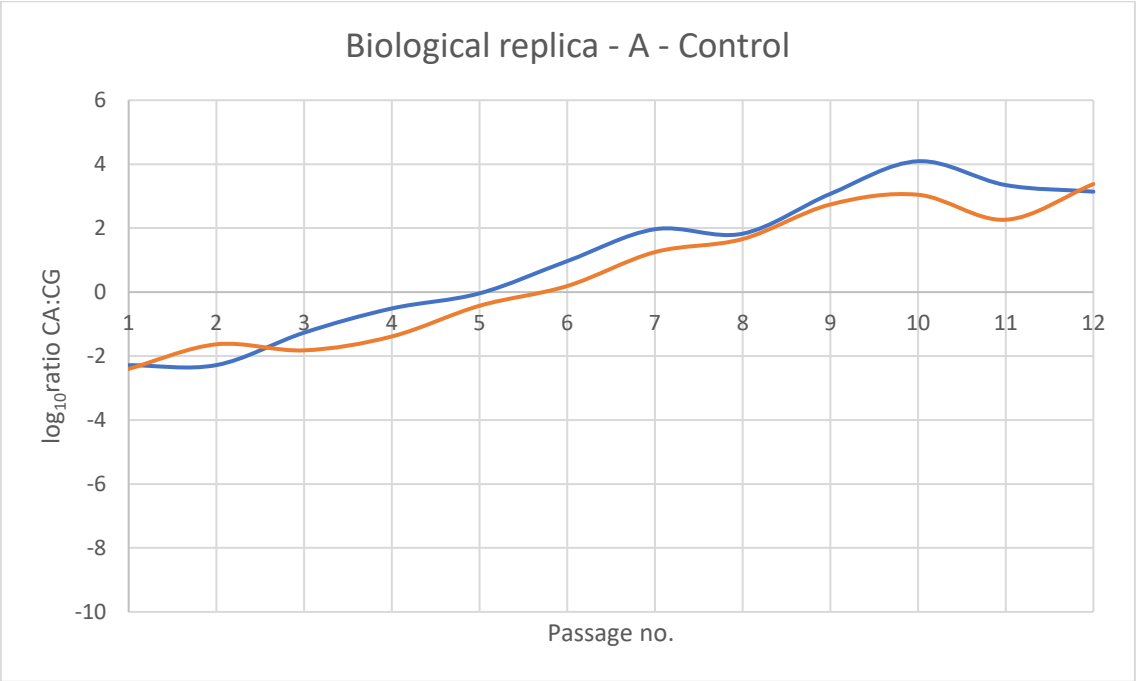

Figure 7: Ratio of organisms in each passage in biological replica A - Control. The two lines represent two parallel experiments. In this run, C. albicans prevailed over C. guilliermondii after around fifth passage.

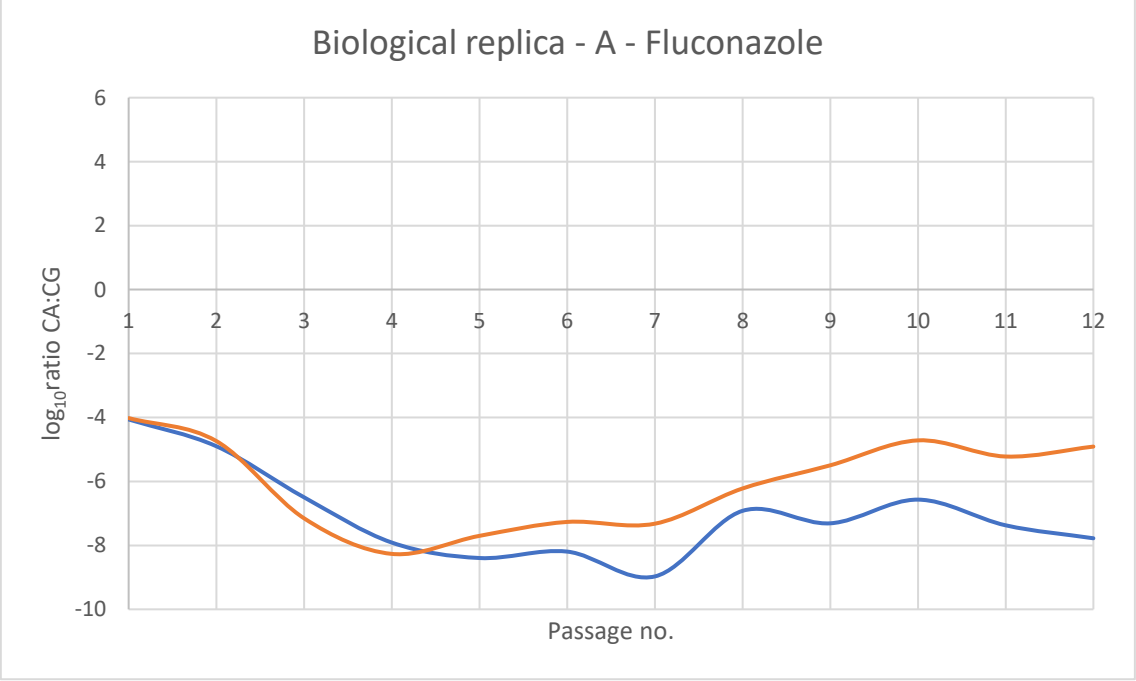

Figure 8: Ratio of organisms in each passage in biological replica A – Fluconazole (c=0.267mg/l). Two lines represent two parallel experiments. In this run, C. guilliermondii was in the majority of the coculture.

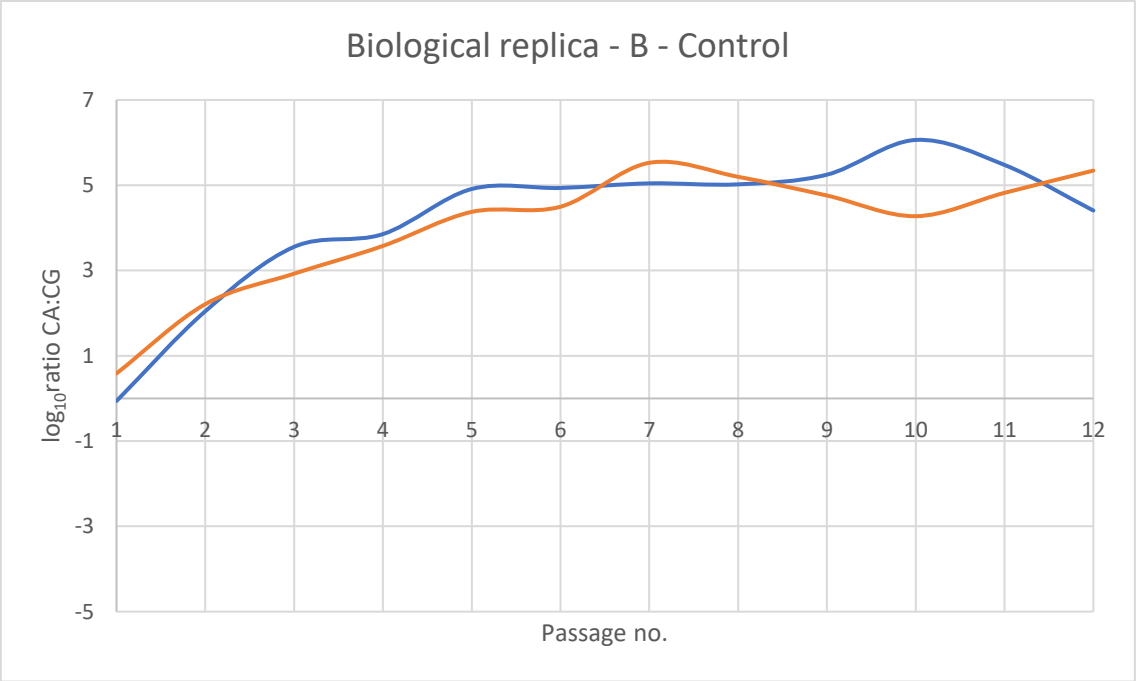

Figure 9: Ratio of organisms in each passage in biological replica B - Control. Lines represent the course of passaging, as observed in two parallel experiments. In this run C. albicans has quickly prevailed in the coculture.

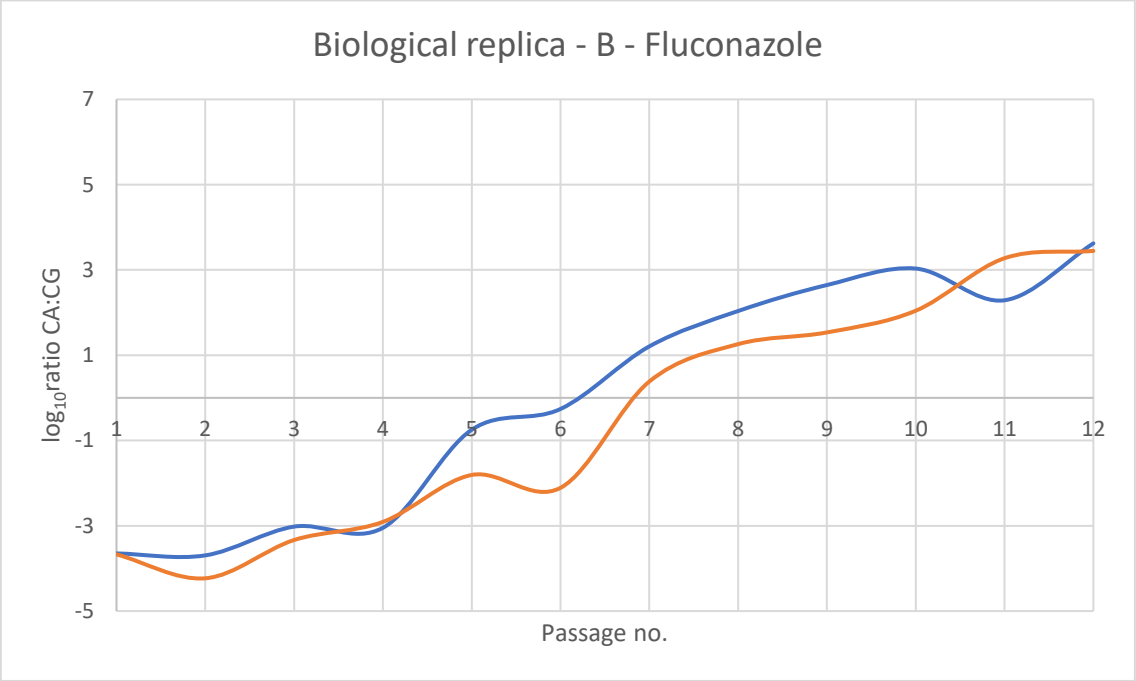

Figure 10: Ratio of organisms in each passage in biological replica B – Fluconazole (c=0.267mg/l). Lines represent the course of passaging, as observed in two parallel experiments. In this run C. albicans was able to take over C. guilliermondii, which led to MIC testing.

8.2.2. Chromogenic analysis of CFU

After the lengthy process of passaging, glycerol stock cultures of the 12. passage from each run were inoculated on a CHROMagar plate, a medium that allows for colony distinction based on the chromogenic change. Plates were cultivated for at least 48 hours to distinguish the colonies

present correctly. For better illustration and connection to qPCR analysis, the ratio of *C. albicans* to *C. guilliemondii* was chosen.

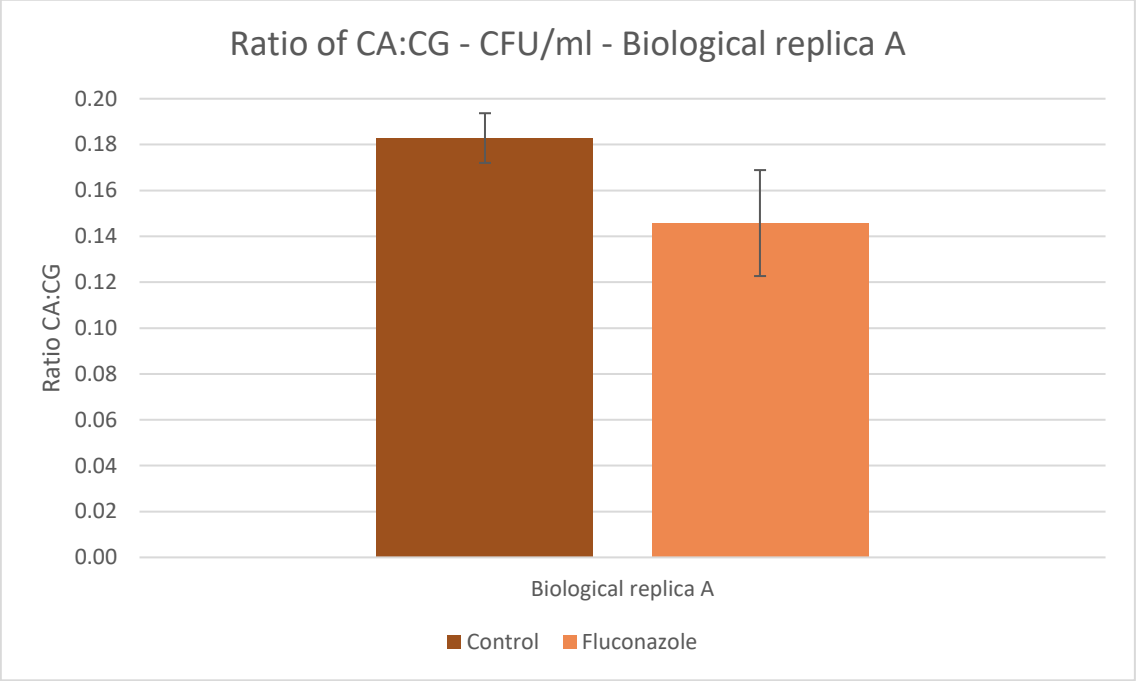

Figure 11: Bar graph showing the ratio of CA to CG and their CFU/ml of BR A. There is a visible difference in ratios between the control and fluconazole group, which correlates with data collected from the qPCR analysis in Figure 7.

Figure 11, portrays a similar trend found in *Figure 7* and *8*, where the control group has a higher ratio of CA CFU/ml, than that of the fluconazole group.

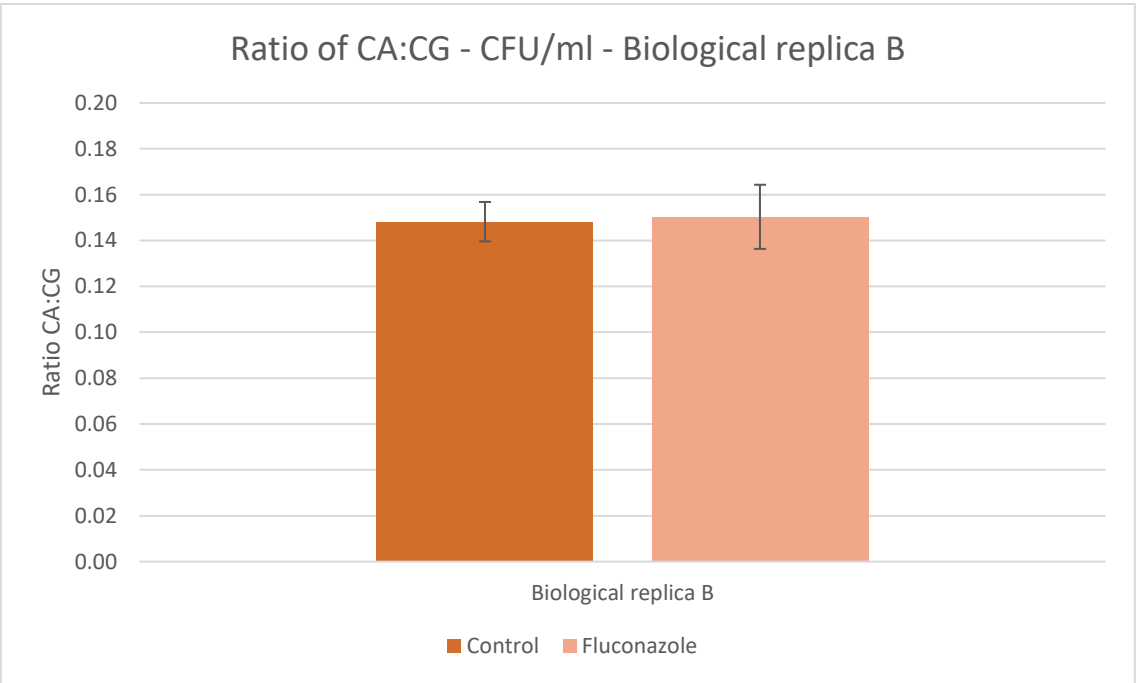

Figure 12: Bar graph showing the ratio of CA to CG and their CFU/ml of BR B. Graph is again using ratio of these organism for easier comparison to qPCR graphs.

Figure 12 shows ratios that are similar to each other. However, the fluconazole group corresponds with the change in trend (Figure 10), where there is an increase in CA population compared to the CFU/ml ratio of *Figure 11*.

These graphs represent the average values of two parallel experiments from each of the biological replicas. While the CFU counts roughly corresponded to the trends observed using the qPCR analysis (*Figure 7-10)*, the amount of data was insufficient to consider this experiment conclusive.

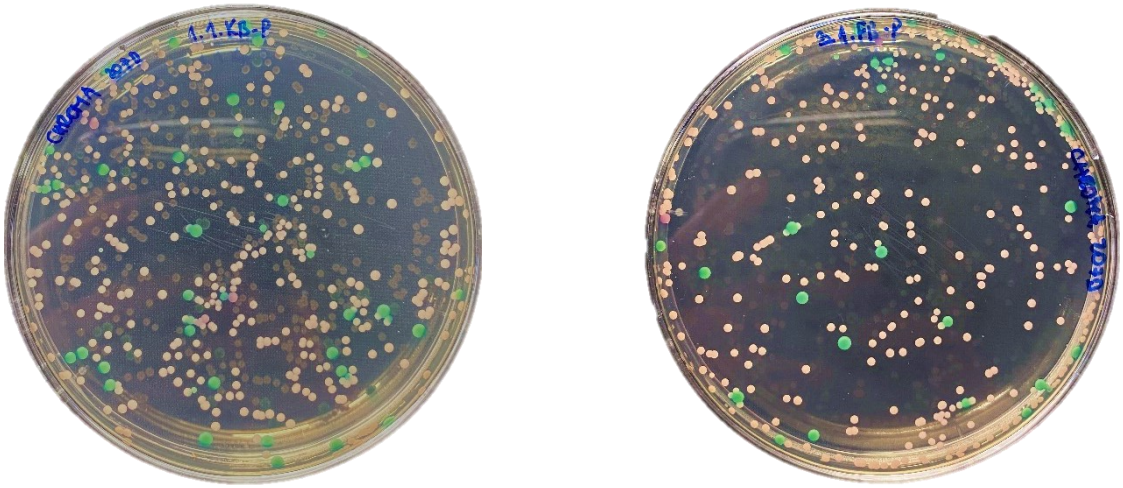

Figure 13: CHROMagar plates representing CFUs of the first passage of BR A. Green colonies represent C. albicans, and beige colonies represent C. guilliermondii. The plate on the left was of the control group, and after better inspection, it contains more CA colonies. The plate on the right was of the fluconazole group, and the decreased CFU of both organisms is visible. However, compared to the plate on the left, it contains fewer CA colonies, as expected.

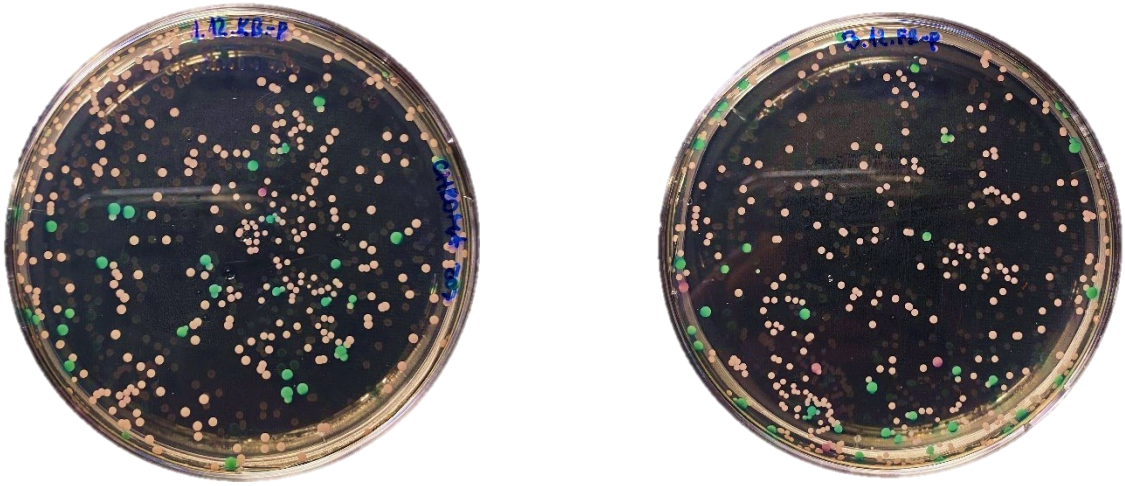

Figure 14: CHROMagar plates represent the control group's twelfth passage (left) and fluconazole group (right) of BR A. Green colonies represent CA, whereas beige colonies represent CG. After closer inspection, the control group still contains more CA than the fluconazole group, as expected. However, interesting is the still present mixed nature of both cocultures.

Figure 13 and *14* demonstrate the susceptibility of CA to fluconazole where the number of its colonies by the twelfth passage decreases. Also, worth mentioning is the mixed nature of these plates.

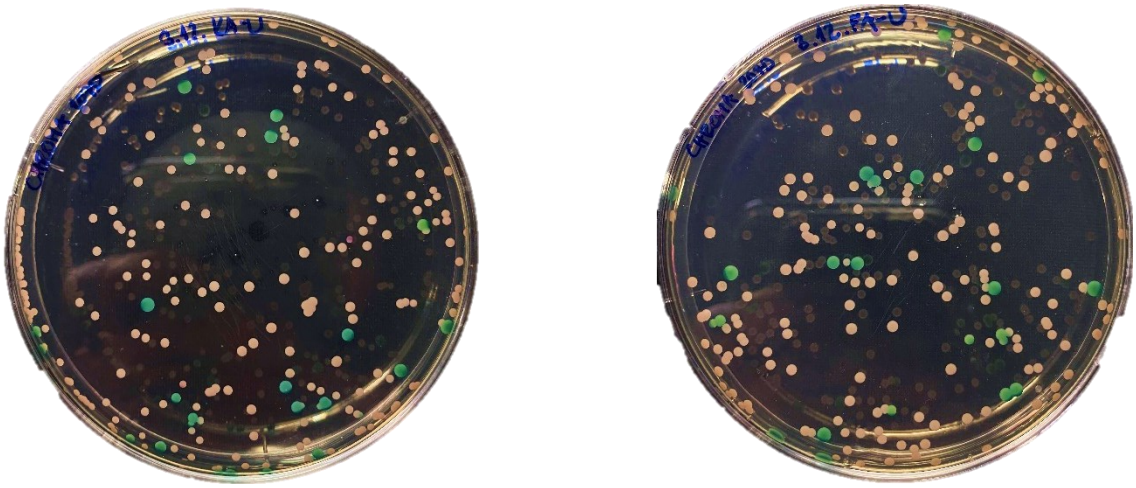

Figure 15: CHROMagar plates representing the twelfth passage of the control group (left) and fluconazole group (right) of the BR B. Green colonies represent CA and CG is represented by beige colonies. Overall, both plates look very similar which corresponds with the similar trend found during qPCR analysis, where CA was prevalent in both passages.

Comparison of *Figure 14* and *15* support the trend observed in qPCR analysis, however it is really difficult to quantify the data collected from this method.

8.3.MIC_{50} analysis

Figure 8 portrays that BR A culture, under the influence of fluconazole, mainly contains *C. guilliermondii*. In contrast, *Figure 10* of BR B, eventually comprises *C. albicans* primarily, despite being susceptible to fluconazole. Therefore several random colonies from the 12. passage were taken, and the MIC_{50} was tested. As illustrated in *Table 4*, isolates of *C. albicans* from the BR B after 12. passages were less susceptible to fluconazole than those of a BR A run under fluconazole or those of control groups. MIC of *C. albicans,* isolated from BR B after 12 passages with fluconazole, was higher and, in some cases, has reached 0.5-2.5 µg/ml.

Table 4: MIC_{50} of C. albicans colonies isolated after 12 passages, to see if MIC of this microorganism increased after this process. MIC_{50} of C. albicans is 0.25 µg /ml. The numbers 1-3 correspond to randomly chosen colonies.

Sample			Concentration of FLU [ug/ml]
Biological replica A	**Control**	1	0.5
		2	0.0-0.5
		3	0.0-0.5
	Fluconazole	1	0.0-0.5
		2	0.0-0.5
		3	0.0-0.5
Biological replica B	**Control**	1	0.0-0.5
		2	0.0-0.5
		3	0.0-0.5
	Fluconazole	1	0.5-1.0
		2	0.5
		3	1.0-2.5

9. Discussion

9.1. Growth curves

The growth curves of each monoculture were measured to assess and compare their behaviour in the YNB-glu medium. The reasons for the medium choice include the fact that it is chemically defined and may thus enable analyses of secreted molecules, such as quorum sensing factors, in future. It is a rich medium containing ideal carbon and nitrogen sources – glucose and ammonium ions. While both yeasts reach similar OD_{600} in their stationary phase, *C. albicans* seems to be more efficient in mobilising its enzyme apparatus and utilising the nutrients. Under control conditions, this observation is logical, and corresponds to the epidemiological prevalence of *C. albicans* candidiasis. However, with the rising trend of antimycotic resistance and overuse of azoles, *C. guilliermondii* could pose a future threat.

The growth curve has also helped assess the correct time for inoculation and sample selection. All samples have been harvested in their exponential phase. Therefore, it was only up for discussion when the samples should be collected, and 24h time point was agreed upon.

Furthermore, the growth curve was measured in the same medium (YNB-glu), including fluconazole with a 0.267 µg/ml concentration (close to MIC_{50}). There was a significant increase in the time it takes the monoculture to achieve the stationary phase. Under control conditions, cultures have reached the same OD_{600}, *C. albicans* after 35h and *C. guilliermondii* after 45h. Though under the influence of fluconazole, *C. guilliermondii* has reached a very similar $OD_{600,}$ and the plateau was achieved after 60h, which equates to a 67% increase. Nevertheless, the start of the exponential phase stayed merely similar.

C. albicans was hampered under the influence of fluconazole. The onset of the exponential phase was delayed by 54%, with the culture only achieving about half the OD_{600} of the control group.

According to these growth curves, *C. albicans,* under normal conditions, is better at utilising nutrients with its earlier exponential phase and can easily impede the growth of *C. guilliermondii* by consuming the nutrient resources. With the use of fluconazole, *C. guilliermondii* achieves an exponential phase earlier and, in turn, impedes *C. albicans*.

This model reflects the advantages *C. albicans* may have over other *Candida* species. While the experiments performed in the present study cover only C. *albicans* and *C. guilliermondii*, it appears logical to assume that the metabolic efficiency of *C. albicans* is one of the factors bringing this species to the top of the candidiasis chart.

9.2. Different concentrations of fluconazole

This project initially used very high concentrations of fluconazole of 3.2 μg/ml; nevertheless, these attempts were usually ended prematurely due limited growth in the fluconazole culture. Therefore, a concentration of FLU subinhibitory for CA was chosen. Although such change can lead to change in the dynamics of the cocultures, it is still beneficial to look at how it changes with subinhibitional concentration of fluconazole (c=0.267 μg/ml)

Passages with a concentration of 3.2 μg/ml of fluconazole were previously analysed by Ing. Lucie Michalcová (University of Pardubice). These passages show a strong trend of an immediate decrease of *C. albicans* population under fluconazole and a constant increase in the population of *C. guilliermondii*. This trend supports the known claim of innate fluconazole resistance of *C. guilliermondii*.

In order to investigate the dynamic of culture in a time horizon, a model of artificial evolution, passaging, was selected. This procedure imposes a challenge with each step of the experiment. Contamination and all kind of irregularities caused delays in this research. In fact, five replicas were set up, but only two were performed successfully up to the end and were used for subsequent analysis. These are the replicas presented in this work and denominated A and B.

9.3.qPCR

qPCR was used as the most precise and reliable way of quantifying the ratio of *C. albicans* and *C. guilliermondii* in the mixed populations. The design of primers suitable for this purpose was not the subject of this thesis. Nevertheless, since qPCR is being used increasingly as a diagnostic tool in hospitals, a hands-on experience with novel primer pairs, as presented here, may be helpful for microbiological laboratories involved in routine screening.

The sensitivity of this technique enabled the observation of how ambient condition of passaging can cause discrepancies in the trends in between biological replicas. Biological replica B was carried out in a different laboratory by Doc. RNDr. Olga Heidingsfeld, CSc at the IOCB, potentially influencing the outcome. This trial has shown a trend where over time, around 6./7. passage, *C. albicans* has prevailed over the *C. guilliermondii* (*Figure 10*).

Interestingly, separate duplicates of the same BR have ended up with similar outcomes (under control conditions, *Figure 7* and *9*). This might suggest that slight changes in the procedures used may lead to a very different result. Biological replica A has been carried out at the Department of Chemistry, Charles University. This implies that either slight ambient changes

can alter the mechanism of resistance or that the reduction of susceptibility is random/depends on unknown circumstances.

Figure 7, portrays a steady increase in the ratio of *C. albicans* over time and around sixth passage is in majority. Under the lack of fluconazole, *C. albicans* has a shorter generation time (as shown in *Figure 5*), therefore better at utilising nutrients and gaining its lead. This also makes sense when considering the commonality of infections by this particular yeast.

The results of *Figure 9* are not surprising a follow a similar trend to that found in *Figure 7*, where due to a similar reason *C. albicans* becomes dominant. Altogether, under control conditions, *C. albicans* will prevail.

In *Figure 8*, *C. guilliermondii* is the dominant species in this mixed culture, which makes sense, considering the observation in *Figure 6,* where *C. albicans,* under the influence of fluconazole, hardly reaches similar optical density and its generation time is considerably postponed. The dominance of CG is almost immediate; therefore lower concentration of fluconazole could be of interest.

At first glance, *Figure 10* might seem like an outlier, as it should have followed a similar trend to that of *Figure 8.* However, in this case *C. albicans* becomes the dominant species in the coculture. It seem that when these cocultures tend to follow two trends when exposed to FLU. Either CG takes advantage of its innate resistance to FLU and prevails, or CA is able to undergo selective evolution of less susceptible strains and prevail. However, the concentration chosen does not favour any of these trends and higher concentration do not provide time long enough for CA to evolve, leading to CG being dominant.

9.4.CFU analysis

The motivation to use this method was to assess the living organisms, not merely their DNA. This chromogenic medium allows for colony distinction based on the colour change. The control group of BR A (*Figure 11*) exhibits properties of those observed during the qPCR analysis, where the ratio of *C. albicans* increases (*Figure 7* and *8*).

The CA population of BR B shows an observable increase in CFU (Figure 15) compared to that of the BR A (*Figure 14*). This corresponds with the findings of qPCR when we compare the different outcomes under FLU in each BR. However, to draw any concrete conclusions, more data has to be collected due to the error rate of this method.

The important difference between these two methods is that CFU count represents living organisms and, in that sense, is selective. qPCR, although routinely used, can be influenced by means of contamination, moreover, in this case, by the presence of already dead cells. Therefore the validity of this comparison falls on the observer and the applicability of these results. When looking at these methods as diagnostic tools during infections, CFU count could better represent the ongoing candidiasis. Since qPCR is not alive-or-dead selective, with the primers used here, this method can be used to diagnose mixed infection. Another thing to consider is the time merit that differs between these methods.

9.5. Increase in MIC_{50}

Each of the colonies that have been tested was taken randomly but roughly copied the unexpected results of *Figure 10. C. albicans* is naturally susceptible to fluconazole which is usually the golden standard of candidiasis treatment in a clinical setting. This model shows how, after twelve passages, C. albicans could adapt to a certain degree to this stressor. In some cases, the MIC_{50} has increased 10-fold (*Table 4*).

To a certain extent, this case is very similar to how bacteria are able to build resistance within its host, if for example the antibiotic treatment is neglected, and via natural selection, such strain can prevail.

Overall, during our research, two trends have emerged – one shows how opportunistic pathogenic yeast *C. albicans* can easily mutate to a more resistant species over the course of 12. passages. This trend is alarming and similar to the trend where antibiotics and their overuse and underuse can negatively impact the epidemiology of this species. The second trend connected to *C. guilliermondii* shows the importance of research of non-*Candia* species, which exhibit innate resistance to the most commonly used antimycotic – fluconazole and can quickly prevail. Passaging under the subinhibitory concentration of FLU demonstrates a process of selection of *C. albicans* strains with decreased susceptibility to fluconazole and the potential of *C. guilliermondii* to thrive even under the influence of this particular stressor.

To our knowledge, the mechanism ensuring the reduced susceptibility of C. guilliermondii to fluconazole has been explored very little. A study focusing specifically on this consists of a very limited sample and investigated only one of the resistance mechanisms[78].

10.Conclusion

Mixed cultures of CA and CG were analyzed during the process of 12 rounds of passaging. Following observations were made:

- the ratio of each microorganism was assessed in every passage
    - under control conditions – *C. albicans* usually dominated, which is possibly linked to shorter generation time
    - presence of fluconazole either resulted in *C. guilliermondii* dominating or favoured less-susceptible strains of *C. albicans*
- constructed growth curves had shown the difference in generation time under control conditions, as well as shown how fluconazole negatively impacts both organisms
    - fluconazole had strongly negatively impacted the generation time of *C. albicans* and the optical density this monoculture achieves
    - fluconazole has only prolonged the generation time of *C. guilliermondii*, but did not affect the optical density of the monoculture
- analysis using chromogenic culture media, trend-wise support findings of qPCR analysis; however, the data are difficult to interpret
    - this analysis revealed the presence of both organisms even in the last passage
- analysis of MIC of *C. albicans* after the process of 12 passages had shown the evolution of more resistant strains of CA

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