

Influence of the deletion of *FTH2* on lipids and fluconazole susceptibility of *Candida albicans*

By

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Dissertation summary

The opportunistic yeast *Candida albicans* resides harmlessly in the mucosal surfaces throughout the body but can become pathogenic under certain environmental conditions. Some of these mucosal surfaces that the yeast inhabits include the gastrointestinal tract, respiratory tract and even the oral cavity. With fungal infections becoming increasingly resistant to routinely used antifungals such as fluconazole, recent research has made efforts in exploring the role of iron availability on drug resistance. This arises from the fact that, the uptake of iron during infections is considered a virulence factor, and that colonisation and proliferation within the host are only possible when the pathogen has access to sufficient iron concentrations. As a result, the pathogen's heightened demand for iron necessitates the host to restrict its availability. Moreover, in the host's infected environment *C. albicans* can form biofilms fostering complex interactions that may also compete for micronutrients such as iron. This interspecies interaction is clearly demonstrated by the coexistence of *C. albicans* with the bacterium *Pseudomonas aeruginosa* which can persistently colonise airways or lungs of individuals with cystic fibrosis. In the realm of iron limitations, *C. albicans* resorts to its vacuolar stores where iron mobilisation into the cytoplasm is facilitated by vacuolar iron permeases such as *fth2p*. Thus, this study sought to explore the effect of *FTH2* deletion on fluconazole susceptibility and lipids of *C. albicans*. The initial focus was to determine the effect of *FTH2* deletion on *C. albicans* planktonic cells under varying fluconazole concentrations. Results indicated that both mutants ($\Delta/\Delta fth2_II$ and $\Delta/\Delta fth2$) exhibited less susceptibility towards all fluconazole concentrations when compared to their *C. albicans* parental strains (SC5314 and BWP17) respectively. This was followed by evaluating the effect of *FTH2* deletion on

fluconazole susceptibility, in both mono and polymicrobial biofilms. Similar results were observed in monomicrobial biofilms. However, $\Delta/\Delta fth2_{II}$ showed less metabolic activity when compared to its parental strain, *C. albicans* SC5314. As a result, these findings require further investigation. When evaluating the effect of fluconazole on polymicrobial biofilms, it became apparent that the combination of fluconazole and *P. aeruginosa* has a synergistic effect against $\Delta/\Delta fth2$. In contrast, the parental strain (*C. albicans* BWP17) remained less susceptible to fluconazole. We also evaluated the effect of *FTH2* on iron localisation in both mono- and polymicrobial biofilms. Although we were able to visualise differences in iron localisation within the cells, it was rather difficult to pinpoint exactly, which structures of the cells contained more iron. Considerably, more work still needs to be done, to determine the exact localisation of both forms of iron (Fe^{2+} and Fe^{3+}).

Moving forward, we evaluated the influence of *FTH2* mediated iron transport on the lipidome of *C. albicans*. This was done both in the presence and absence of *P. aeruginosa*. The lipid content was compared between the *C. albicans* parental strain (BWP17) and its mutant ($\Delta/\Delta fth2$). It was found that lipid droplet size and lipid content differed between *C. albicans* BWP17 and the mutant strain, $\Delta/\Delta fth2$. Competition for iron in polymicrobial biofilms also negatively influenced LD formation as well as the lipid content. However, the role and influence of *P. aeruginosa* in lipid droplet and lipid content production of *C. albicans* remains unclear and needs to be further explored. We have also demonstrated that there is a significant difference in the distribution of lipid species between *C. albicans* BWP17 and $\Delta/\Delta fth2$, and that these results may further point to the role of iron availability in lipid homeostasis and fluconazole susceptibility. Additionally, the results also showed that the presence of pyoverdine leads to a further decrease in lipid species in both *C. albicans* BWP17 and $\Delta/\Delta fth2$.

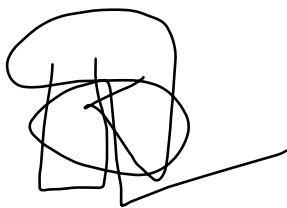
This study demonstrated the effect of *FTH2* deletion on fluconazole activity against *C. albicans* both in planktonic cells as well as mono-and polymicrobial biofilms. Furthermore, these findings also suggest that changes in lipid metabolism may influence membrane composition as well as fluconazole susceptibility. Although these initial findings provide insights on the influence of iron availability on fluconazole susceptibility and lipid homeostasis, more work is needed to establish a greater degree of accuracy in this matter.

Key words: *C. albicans*, Iron availability, Drug resistance, Fluconazole susceptibility, Lipid homeostasis, Pyoverdine

Declarations

It is hereby declared that this dissertation submitted for the Master's degree at the University of the Free State has been composed solely by myself and that it has not previously been submitted, in whole or in part at another University or Faculty. The copyright of this dissertation is hereby ceded in favour of the University of the Free state.

I, Setsiba Lekgoana Karabo hereby also declare that all royalties regarding intellectual property that was developed during the course of, and/or in connection with the study at the University of the Free State, will accrue to the University.



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Dedication

This work stands as a tribute to my family and friends for their unwavering support and love. Their constant believe in my abilities and words of encouragement have been the cornerstone of my academic journey.

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The Almighty, nothing held me down and kept me going as much as prayer.

Ethical clearance

I, Setsiba Lekgoana Karabo, who is the principal investigator for this research project declare that the project was approved after a thorough review of the aim, procedures and potential ethical concerns that may arise by the Biosafety & Environmental Research Ethics Committee of the University of the Free State with ethical clearance number: **UFS-ESD2022/0031/22**.

Environment & Biosafety Research Ethics Committee

07-May-2022

Dear **Ms Lekgoana Setsiba**

Project Title: **Investigation of the influence of vacuolar iron permease, Fth2p, on lipids of *Candida albicans* during competition for iron**

Department: **Microbiology and Biochemistry Department (Bloemfontein Campus)**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Environment & Biosafety Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is **UFS-ESD2022/0031/22**

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Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely



Prof. RR (Robert) Bragg

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Chapter 1

Literature review

Parts of this chapter have been submitted to Fungal Genetics and Biology for publication and is currently under review.

1.1.Motivation

While most fungi are non-pathogenic, there exists a large number of fungal species that are pathogenic to humans. Fungal infections are among the top causes of hospital-acquired infections and are an increasing public health concern globally (Farooque et al. 2019; Guliyeva et al. 2022). The world health organisation (WHO) has recently published its first ever fungal pathogen priority list with the aim of improving the overall response to these fungal pathogens to treatments (WHO 2022). A significant number of global fungal infections are caused by *Candida* species such as *Candida albicans*. The yeast *C. albicans* is a globally distributed opportunistic pathogenic microorganism that can be part of the normal human microbiome. However, this yeast has the potential to transition into a pathogenic lifestyle thus causing life-threatening infections (Auger and Joly 1977; Mayer et al. 2013). Like many other microorganisms, *C. albicans* possesses a remarkable ability to form biofilms which serve as mechanical barriers to protect the underlying microbial community from external factors (Lohse et al. 2018). These microbial communities are formed through a series of coordinated processes including the initial reversible attachment of planktonic cells to a surface of choice, proliferation, maturation and lastly the dispersion of cells from the complex (Lohse et al. 2018). Microorganisms within these communities also exhibit a complex architecture when compared to their planktonic counterparts thus making them more resistant to routinely used antifungals.

Resistance to antifungal treatment remains uncommon and thus, it is not surprising that over 60% of human fungal infections reported are associated with biofilm forming pathogens (Sadekuzzaman et al. 2015; Iñigo and Del Pozo 2018). Moreover, the advancement in preventative treatments (i.e., immune suppression during organ transplants) has further exacerbated the burden of drug resistance, thus posing a

challenge to patient care in intensive care units (Friedman and Schwartz 2019). The first line drug against fungal infections, particularly those caused by *C. albicans*, is fluconazole (Charlier et al. 2006; Leroy et al. 2016). However, resistance to this routinely used azole has been established and poses a substantial challenge in effectively treating and managing infections associated to *C. albicans* (Sanguinetti et al. 2005). Resistance to fluconazole primarily arises from changes in the affinity of the drug target, cell wall or plasma membrane composition which may in turn lead to impaired azole uptake (Venkateswarlu et al. 1996; Sanglard et al. 1998). Mutation or overexpression of the target enzyme lanosterol 14 α -demethylase and the increased activity of efflux pumps particularly those associated with membrane transport proteins *CDR1* (a major drug efflux ABC protein commonly encountered in azole resistance in *C. albicans*) and *CDR2* belonging to the ATP-binding cassette (ABC) transporter family have also been associated with drug resistance (Vanden Bossche et al. 1990; Venkateswarlu et al. 1996; Haque et al. 2007). This also includes members of the superfamily *MDR1* and *FLU1* (Wirsching et al. 2001). Moreover, it was also reported that the synergistic interplay between these resistance mechanisms further makes it difficult to treat these infections (Sanglard and Odds 2002).

Availability of iron also serves as a common adaptive factor for *C. albicans* to express virulence and pathogenesis within the host (Ramanan and Wang 2000). However, the availability of iron is highly restricted within the host, and this serves as a defence mechanism against the fungal pathogen (Hood and Skaar 2012; Frey and Reed 2012). As a result, *C. albicans* has evolved sophisticated strategies to acquire iron under iron limiting environments to maintain its normal cellular growth. The initial response to these iron limitations involves the use of the three central iron uptake systems namely, the haemoglobin iron-uptake system, the use of siderophores (xenosiderophores) and

the high-affinity reductive system (Ramanan and Wang 2000; Santos et al. 2003; Almeida et al. 2009; Bairwa et al. 2017). Moreover, the constant competition for micronutrients such as iron between *C. albicans* and *Pseudomonas aeruginosa* can influence their interactions and lead to extensive microbial antibiosis (Purschke et al. 2012).

During iron limiting conditions, *C. albicans* relies on its vacuolar iron deposits and thus the deletion of vacuolar iron permease genes such as *FTH2* may limit the mobilisation of iron from the vacuole into the cytoplasm (Mamouei et al. 2017; Mochochoko et al. 2021). Deletion of the iron permease gene has also been shown to result in lower iron levels within the cytoplasm, thus leading to reduced metabolic activity (Mochochoko et al. 2021). Therefore, a close relationship between iron availability and antifungal drug susceptibility in *C. albicans* has since been discovered and is yet seen as another novel determinant of the acquired multidrug resistance displayed by *C. albicans*. The effect of iron availability on drug susceptibility is especially linked to its overall impact on membrane lipid composition by targeting lipid metabolic pathways, particularly those of sphingolipids and ergosterol (Rella et al. 2016). This is because iron limitations have proven to lead to the downregulation of ergosterol biosynthetic genes such as *ERG11* which is responsible for encoding lanosterol 14 α -demethylase, an enzyme that plays a crucial role in the biosynthesis of ergosterol in fungal cells (Prasad et al. 2006). This also shows that iron deprivation results in the overall global remodelling of cellular lipids and that the possible link between iron and lipid homeostasis is yet to be explored.

1.2. Introduction

While the impact of invasive fungal infections on human health is often underestimated, invasive fungal infections are associated with alarmingly high mortality rates (Martínez-Pastor and Puig 2020). Statistics reveal that approximately a quarter of the global population has encountered non-fatal superficial mycosis at least once in their lifetime (Martínez-Pastor and Puig 2020). However, it is the invasive fungal infections that raise more concern, contributing to an estimated 1.5 million deaths annually (Rayens and Norris 2022; Fang et al. 2023). These infections are estimated to affect around 250,000 individuals worldwide, with the mortality rate exceeding 40%, particularly impacting those with compromised immune systems (Rayens and Norris 2022; Fang et al. 2023).

The elevated fatality rate associated with these infections can be attributed to several factors. For example, challenges in timely diagnosis, inadequate disease management, the occurrence of septic shock, selective pressures following antimicrobial treatments, and the overall critical condition of patients may collectively contribute to the severity of the situation (Antinori et al. 2018).

Recognising the severity of this global health concern, the World Health Organisation (WHO) released its inaugural fungal priority pathogens list on 25 October 2022 (Table 1), with the aim to extend and drive research efforts toward systemic invasive infections that threaten public health. The process of prioritisation was more centred on fungal pathogens that may cause invasive acute and subacute systemic fungal infections and for which there are currently treatment and management issues related to drug resistance or other factors. Following a ranking, the pathogens were further characterised into three groups based on priority (critical, high, and medium) (WHO 2022).

Candida albicans which is commonly found residing harmlessly on mucosal surfaces of healthy individuals, such as the oropharynx, gastrointestinal or genital tracts as well as the skin, stands as one of the primary sources of fungal infections in humans. This is because under certain conditions, the microorganism can transition into a pathogenic lifestyle and breach the epithelial barrier, thus causing bloodstream infections in immunocompromised individuals (Auger and Joly 1977; Mayer et al. 2013). Moreover, *C. albicans* possesses a vast spectrum of virulence factors that significantly enhance its ability to invade and colonise various sites within the host (Duval et al. 2020; d'Enfert et al. 2021). These virulence factors include the ability of the pathogenic microorganism to form highly organised microbial communities also known as biofilms. Microorganisms growing within these communities often exhibit resistance to frequently used antifungals, such as fluconazole and can withstand the host's innate defence mechanism (Almeida et al. 2009). Thus, treating biofilm-associated infections becomes very challenging to the global health, often leading to infections that are persistent or prove to be difficult to eradicate. The accelerated emergence of resistance to routinely used antifungals and the slowdown development of novel therapies is especially exacerbated in developing countries (Kuhn and Ghannoum 2004; Perumal et al. 2007).

Table 1: WHO fungal priority pathogens list (WHO 2022)

Critical group	High group	Medium group
 <i>Cryptococcus neoformans</i>	 <i>Nakaseomyces glabrata</i> (<i>Candida glabrata</i>)	 <i>Scedosporium</i> spp.
 <i>Candida auris</i>	 <i>Histoplasma</i> spp.	 <i>Lomentospora prolificans</i>
 <i>Aspergillus fumigatus</i>	 Eumycetoma causative agents	 <i>Coccidioides</i> spp.
 <i>Candida albicans</i>	 Mucorales	 <i>Pichia kudriavzevii</i> (<i>Candida krusei</i>)
	 <i>Fusarium</i> spp.	 <i>Cryptococcus gattii</i>
	 <i>Candida tropicalis</i>	 <i>Talaromyces marneffei</i>
	 <i>Candida parapsilosis</i>	 <i>Pneumocystis jirovecii</i>
		 <i>Paracoccidioides</i> spp.

Multiple lines of research have also pointed to treatment challenges posed by polymicrobial-associated infections. Within polymicrobial communities, there exists several complex interactions ranging from antagonistic to synergistic associations. One example of such an association is seen between *C. albicans* and the bacterium, *P. aeruginosa*, which are frequently isolated from patients suffering from cystic fibrosis (Hogan et al. 2004; Gupta et al. 2005; Trejo-Hernández et al. 2014; Fourie and Pohl 2019). The interaction between these two species can vary depending on environmental conditions or even the context of infection (Fourie et al. 2016).

In the quest of survival, all life forms, including bacteria and fungi, require trace metals such as for Iron, zinc, manganese, and copper, for growth and survival. The

aforementioned micronutrients are among the most essential metals, required for the proper functioning of a vast number of proteins within an organism (Porcheron et al. 2013). These proteins include iron-sulphur clusters or haem groups and are mainly involved in biological processes such as iron transport and storage, DNA synthesis, and mitochondrial respiration. The crucial aspect of microbial pathogenesis involves overcoming "nutritional immunity," where pathogenic microorganisms must surpass the host's mechanisms that restrict or locally enhance metals to impede microbial growth and minimise their potential harm (Luo et al. 2021). Despite the widely varying levels of micronutrients available within the host, *Candida* cells, have developed a variety of sophisticated strategies to acquire limiting micronutrients such as iron from the host. (Duval et al. 2020; Martínez-Pastor and Puig 2020). Furthermore, distinct and shared strategies for maintaining metal homeostasis are evident even among pathogenic fungi. For instance, *Candida* species exploits the presence of iron as a shared signal to induce the development of virulence traits (Duval et al. 2020). These mechanisms often differ significantly from those observed in bacteria.

Iron is a critical element essential for normal cellular functions in both the pathogen *C. albicans* and the host. In the context of infection, the absorption of iron is regarded as a virulence factor. Adequate iron availability is imperative for the successful colonisation and proliferation of the pathogen (Prasad et al. 2006). Consequently, the host's iron concentrations significantly influence susceptibility to *C. albicans* infection. Upon encountering a yeast infection, the host intentionally reduces iron availability in the blood, deep tissues, and the gastrointestinal tract to restrain the pathogen's growth and virulence. This limitation necessitates the pathogen to employ strategies to acquire iron from external sources when encountering environments deficient in iron (Fourie et al. 2018). *C. albicans* has evolved diverse iron uptake mechanisms to

procure iron from the host, including high-affinity reductive systems, siderophore uptake systems, and haemoglobin iron uptake systems (Ramanan and Wang 2000; Santos et al. 2003; Almeida et al. 2009; Bairwa et al. 2017).

Recent research has made efforts in exploring the effect of iron homeostasis on drug susceptibility within the host. This is mainly due to the limited availability of antifungal drugs and the escalating reports of azole resistance in hospital settings. Interestingly, these studies also suggest there may be a potential link between intracellular iron levels and the multidrug resistance in the host, hinting that lower concentrations of iron within the pathogen may lead to increased drug susceptibility (Nairz et al. 2010; Hameed et al. 2011; Hattab et al. 2022). Earlier investigations also propose a connection between altered lipid composition and changes in membrane integrity, which could result in increased drug diffusion and sensitivity (Wenk 2010). Notably, iron deficiency has been identified as a mechanism contributing to heightened drug susceptibility in *C. albicans* and other *Candida* species. These findings indicate that when iron is depleted, it prompts greater membrane fluidity, facilitating increased passive diffusion of drugs and consequently elevating drug susceptibility (Prasad et al. 2006).

The following sections highlight the significance of iron homeostasis and its availability to both the host and the pathogen, elucidating the diverse strategies of *C. albicans* exploiting and storing iron, particularly in iron-deficient environments. Furthermore, an in-depth exploration into the impact of iron availability on lipid homeostasis and its effects on drug susceptibility are discussed in more detail.

1.3. The opportunistic pathogenic yeast, *C. albicans*

The polymorphic yeast, *C. albicans*, is a usual member of the human microbiome and often resides harmlessly within the gut (Mayer et al. 2013). Yet under specific conditions (i.e., weakened immune systems), the yeast has the potential to cause infections, ranging from superficial infections of the skin to life-threatening systemic infections. Throughout both these types of infections, *C. albicans* relies on a vast number of virulence factors and fitness traits (Kuhn and Ghannoum 2004; Mayer et al. 2013). The ability of *C. albicans* to survive and thrive in diverse environments is attributed to its ability to transition between distinct morphological states, such as switching between yeast or filamentous hyphae. For example, switching between yeast and filamentous hyphae can be in response to certain stimuli, including pH, temperature, signalling molecules, and host factors (Duval et al. 2020). Moreover, this adaptive trait enables the yeast to modulate pH levels and induce hyphal growth in nutrient -poor settings, a characteristic essential for its virulence (Almeida et al. 2008; Almeida et al. 2009).

The yeast-to-hyphal transition plays an important role in the formation of *C. albicans* biofilms. Moreover, the ability of this microorganism to form biofilms on both biological and abiotic surfaces has also been recognised as an important virulence factor (Sudbery et al. 2004; Kumamoto and Vines 2005). The process of biofilm formation occurs in a sequential manner (**Figure 1.1.**) where yeast cells first adhere to favourable surfaces followed by the proliferation of the cells that results in the formation of hyphal cells in the upper part of the biofilm. This is then accompanied by the accumulation of an extracellular matrix and finally the dispersion of the cells from the biofilm complex. Compared to planktonic cells, mature biofilms exhibit significantly heightened resistance to antimicrobial agents and host immunological factors. These

mature biofilms comprise complex microbial communities embedded within a dense extracellular matrix. The intricate architecture of biofilms, the composition of the biofilm matrix, elevated expression of drug efflux pumps, and metabolic adaptability, collectively contribute to this amplified resistance (Mayer et al. 2013; Taff et al. 2013). (Mayer et al. 2013; Taff et al. 2013). Beyond biofilm formation, several other virulence factors contribute to the pathogenicity of *C. albicans*. These factors include the expression of adhesins, responsiveness to physical cues (thigmotropism), and the secretion of tissue-damaging hydrolytic enzymes (Mayer et al. 2013). Moreover, these attributes underlie the high virulence of the yeast, establishing it as one of the most invasive fungal pathogens. In healthcare settings, *C. albicans* ranks as the fourth leading cause of nosocomial bloodstream infections, further emphasising its clinical significance (Badiee and Hashemizadeh 2014).

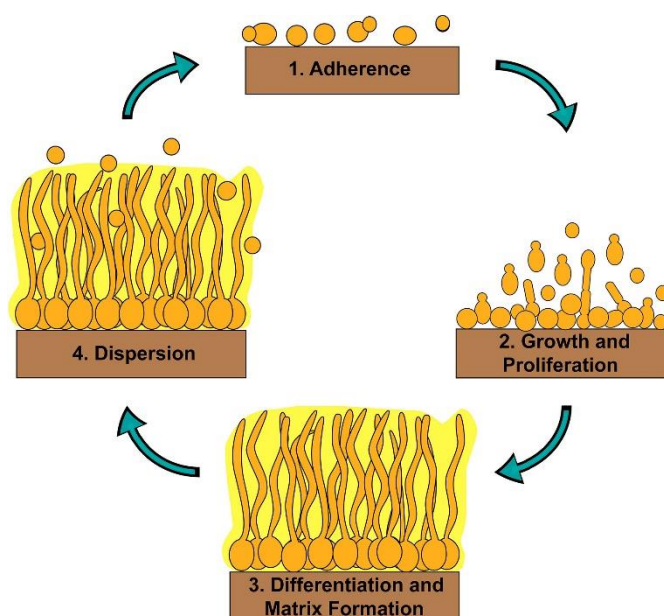


Figure 1.1. A schematic diagram of biofilm formation. Biofilm formation begins with the adherence of planktonic cells to a suitable surface (1). Once adherence occurs, the yeast cells begin to aggregate, thus forming microcolonies on the surface. This phase is also termed as growth and proliferation (2). Following this, yeast cells start to form a monolayer and irreversibly attach by producing an extracellular matrix containing proteins, polysaccharides and other molecules (3). The last step of biofilm formation involves detachment and dispersal of some cells into the surrounding environment. This allows microorganisms to colonise new surfaces and form new biofilms (4) (Rodriguez et al. 2020).

Micronutrients such as iron serve as a necessary cofactor of enzymes in a variety of biological processes, including DNA replication and electron transport for *C. albicans* to colonise and cause infections within the host (Zhang 2014). Colonisation by the pathogen can only take place if there is sufficient iron (Duval et al. 2020; Martínez-Pastor and Puig 2020). Thus, the demand for iron by the pathogenic microorganism necessitates the host to restrict iron availability as a defence mechanism termed "nutritional immunity" (Almeida et al. 2009; Luo et al. 2021). Additionally, the availability of iron within the human host is highly restricted due to the ability of the metal ion to catalyse the formation of free oxygen radical species with lipids or hydrogen peroxide, which have been highlighted to have damaging effects on cellular components (Smets et al. 2010; Martínez-Pastor and Puig 2020).

1.4. Iron acquisition and transport by *C. albicans*

Microorganisms lacking conventional iron excretory mechanisms, have evolved distinctive regulatory factors to sense and precisely modulate iron acquisition, storage, and utilisation (Haas, 2003). Extensive investigations into the mechanisms governing iron uptake and homeostasis in the model yeast, *Saccharomyces cerevisiae*, have laid a strong groundwork that has significantly influenced investigations into fungal pathogens, such as *C. albicans* (Kosman, 2003; Philpott and Protchenko, 2008). Confronted with limited iron within the human host, *C. albicans* has developed intricate and vital iron acquisition strategies (illustrated in **Figure 1.2.**) for its survival during infection. These strategic mechanisms include the utilisation of the high-affinity reductive iron uptake system (that resembles *S. cerevisiae*), acquisition of iron from haemoglobin, which is mediated by a family of extracellular membrane proteins and the utilisation of the siderophore uptake system (Bairwa et al. 2017).

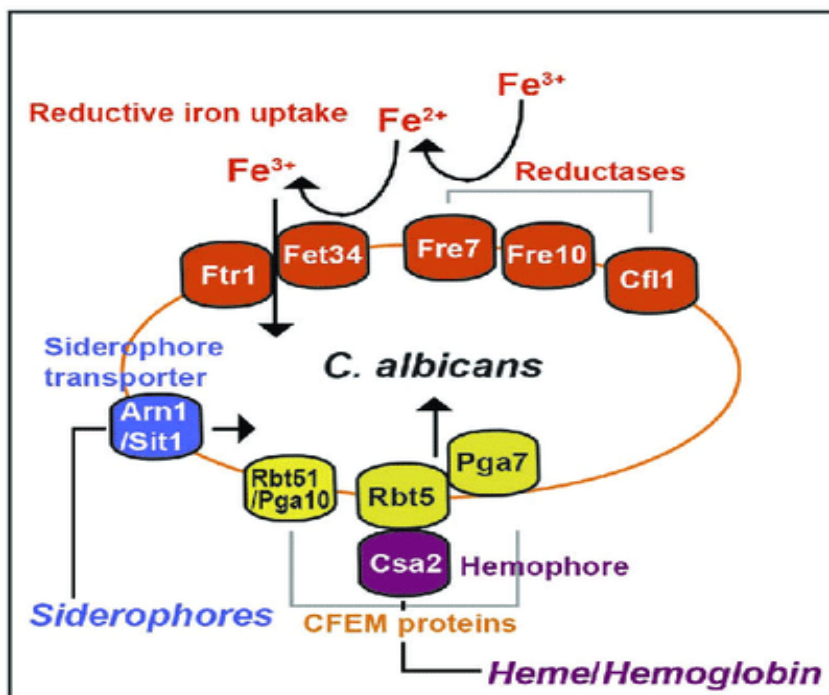


Figure 1.2. Iron acquisition mechanisms utilised by *C. albicans* under an iron-limiting environment. Three of the iron uptake systems include the Haem/haemoglobin uptake system which relies on a conserved family of proteins. The use of siderophores, that can retrieve iron from the surrounding environment and make it available for the pathogen. This system makes use of xenosiderophores that are mainly utilised using the Arn1/Sit1 transporter. Lastly, *C. albicans* can make use of the high-affinity reductive iron uptake system which consists of both ferroxidase enzymes coupled with iron permeases (Bairwa et al. 2017).

1.4.1. High-affinity reductive iron uptake system

The high-affinity reductive iron uptake system is responsible for the absorption of iron from the environment as well as two iron-containing host proteins transferrin and ferritin (Knight et al. 2005; Almeida et al. 2009). In *S. cerevisiae*, the high-affinity reductive iron uptake system is a two-step mechanism in which ferric iron is firstly reduced to ferrous iron (a more soluble form of iron) by ferric reductase (*FRE1* and *FRE2*). This soluble form of iron is later re-oxidised back to the ferric form by a multicopper ferroxidase (*FET3*), followed by transport into the cell by a permease (*FTR1*). The subsequent re-oxidation of ferrous iron back to ferric iron before intracellular absorption serves as a key mechanism for regulating and controlling the uptake of iron into the cell (Castells-Roca et al. 2011).

Candida albicans utilises a reductive iron absorption system with conserved orthologs of *S. cerevisiae* cell-surface ferric reductases, ferroxidases, and iron permease genes. Unlike *S. cerevisiae*, *C. albicans* possesses four distinct permease genes, either plasma membrane (*FTR1* and *FTR2*) or vacuole associated genes (*FTH1* and *FTH2*).

At least 17 genes encoding putative ferric reductases have been discovered in the genome of *C. albicans*. Among these are the genes *FRE7* and *FRE10* which encode highly active cell-surface ferric reductases, and whose deletion causes the pathogenic yeast to absorb less ferric iron (Knight et al. 2005; Jeeves et al. 2011). Moreover, recent research on the *CFL1* ferric reductase from *C. albicans* has increased our understanding of the involvement of these enzymes in fungal infections (Cain and Smith 2021). *CFL1* transcription is specifically regulated in response to iron and copper, and deletion of the gene results in a surprising number of abnormalities (Hammacott et al. 2000). For example, deletion of this gene resulted in several abnormalities including, sensitivity to oxidative stress, increased cellular iron levels, mitochondrial dysfunction, decreased filamentous development and alteration in the cell wall architecture, as well as reduced virulence in a mouse model of systemic candidiasis (Hammacott et al. 2000; Xu et al. 2014b). In *S. cerevisiae* the reduced ferrous iron is transported by a high-affinity heterodimeric transport complex consisting of the multicopper ferroxidase *FET3* and the permease *FTR1*, as discussed above, in the second step of reductive iron absorption. *FET3* oxidises ferrous iron to ferric iron, which is then delivered into the cell via *FTR1*. The transport of ferric iron within the cell employs a conventional metabolite channelling mechanism, where specific amino acid residues in *FET3* and *FTR1* interact to facilitate the passage of iron into the cell. In *C. albicans*, and other species such as *C. glabrata* and *Cryptococcus neoformans*, the corresponding components of the multicopper ferroxidase-iron permease system have been found and characterised (Ramanan and Wang 2000; Kosman 2003). In other species such as *C. glabrata*, *C. neoformans*, and notably in *C. albicans*, analogous components of the multicopper ferroxidase-iron permease system have been identified and characterised (Ramanan and Wang 2000; Kosman 2003). *C. albicans*

possesses five orthologs of the *S. cerevisiae* ferroxidase genes: *FET3*, *FET31*, *FET33*, *FET34*, and *FET99*. Transcriptomic analyses have also revealed the regulation of both *FET3* and *FET4* in environments deficient in iron (Jensen and Culotta 2002; Sanvisens et al. 2011). In a mouse model infected with systemic candidiasis, research revealed that *FET34* encodes a functional ferroxidase. This enzyme localises to the plasma membrane and significantly contributes to iron accumulation, hyphal growth, and pathogenicity (Heymann et al. 2002a). A recent study conducted by Mamouei and co-workers (2017) *in vivo*, on mutants lacking one or more ferroxidase or permease genes, demonstrated functional differentiation and redundancy among these genes during blood stream infection and colonisation of the gastrointestinal tract. Their hypothesis suggested that the flexible ferroxidase -permease partnership results in a more dynamic iron transport system, allowing *C. albicans* to adapt to various host niches consisting of varying iron concentrations.

Moreover, the high-affinity reductive iron uptake was reported to be intricately linked to the process of morphogenesis. The significance of this system is evident in fungal pathogens particularly those exhibiting dimorphic or pleiomorphic characteristic, that can switch between yeast-like growth and hyphal or pseudohyphal growth. For instance, in *C. albicans*, coordination between the high-affinity reductive iron uptake system and the adhesin-like protein Als33, facilitate iron acquisition from ferritin during hyphal development (Almeida et al. 2008). Studies conducted by Mochochoko and co-workers (2021) demonstrated that the deletion of the multicopper oxidase gene, *FET99*, or iron permease genes, *FTH1* and *FTH2*, influenced biofilm metabolic activity in *C. albicans*. This study further established that constructed mutants namely, $\Delta/\Delta fth2$, $\Delta/\Delta ccc1$ and $\Delta/\Delta ccc2$ (all part of the reductive iron acquisition pathway) also affected biofilm morphology and that strains deficient in iron uptake genes, particularly

FET99 and *FTH2* may play a role in virulence, biofilm formation as well as production of the immunomodulatory lipid mediators such as prostaglandin E₂ (PGE₂), which is responsible for the host immune response and invasion (Mochochoko et al. 2021). Thus, suggesting that the absence of these genes especially in iron-limiting conditions may disrupt iron transport from the vacuolar reservoirs leading to lower iron concentrations in the cytoplasm (Mochochoko et al. 2021).

1.4.2. Iron uptake from haemoglobin

The acquisition of iron by human fungal pathogens from various iron-binding host proteins has since been reported to significantly influence infection outcomes (Ene et al. 2014; Bruhn and Spellberg 2015). Hence, some pathogenic fungi target the haem as significant iron sources. Haem containing proteins such as haemoglobin, are known to be associated with over 80% of the iron in a mammalian host (Bairwa et al. 2017). Other haem-containing proteins include lactoferrin (Lf), transferrin (Tf), haptoglobin, haemopexin, lipocalin-1, and lipocalin-2 (Lcn1/Lcn2) (Nairz et al. 2010). These proteins often sequester iron, limiting its availability to microorganisms and serving as a defence mechanism against infection. However, some pathogenic fungi such as *C. albicans* have developed mechanisms to target haem and haemoglobin as critical sources of iron. In addition, the sequestration of iron in transferrin and ferritin also limits the availability of iron to microorganisms (Weissman et al. 2002; Santos et al. 2003; Nairz et al. 2010; Cassat and Skaar 2013). As such, some pathogenic fungi target haem and haemoglobin as significant iron sources. *C. albicans*, has sophisticated machinery for extracting iron from haem/haemoglobin. This system relies on a conserved family of proteins including, Rbt5, Rbt51/Pga10, Pga7, and Csa2 which also possesses the cysteine-rich Common in Fungal Extracellular Membrane (CFEM) domain. Interestingly, this system also functions independently of the

reductive and siderophore uptake mechanisms that the pathogenic yeast typically uses to acquire iron (Weissman et al. 2002; Santos et al. 2003; Weissman and Kornitzer 2004). Among these proteins, Csa2 plays a significant role in binding haemoglobin, potentially functioning as a haemophore, to transport the protein to other CFEM proteins such as Pga7 and Rbt5 (Kuznets et al. 2014). Recent structural studies conducted by Nasser and co-workers (2016) shed some light in the basis of the haem-iron acquisition mediated by Csa2. The CFEM domain in Csa2 exhibits a novel six- α -helical basket configuration with a flat hydrophobic platform on top that allows attachment of a planar haem molecule (Nasser et al. 2016). The specificity for Fe³⁺ haem-binding is facilitated by a conserved aspartic residue in the CFEM helical basket that acts as an axial ligand for the haem molecule (Nasser et al. 2016). This structural insight further provides a better understanding of how *C. albicans* effectively acquires iron from haemoglobin, a strategy crucial for its survival and virulence within the host.

1.4.3. Siderophore uptake

The third form of iron exploitation mechanism employed by *C. albicans* to acquire iron, is the siderophore uptake system. Siderophores are low-molecular-weight (<1 kDa) iron-binding modified peptides predominantly synthesised by specific bacterial and fungal species within an iron-poor environment. These proteins act as high-affinity iron chelators that can sequester or scavenge iron from the surrounding environment and make it accessible to pathogens (Holzberg and Artis 1983; Ismail et al. 1985; Heymann et al. 2002a). While *C. albicans* does not have the proper machinery to generate its own siderophores, the siderophore ion uptake system enables it to obtain iron from a variety of siderophores produced by other organisms (xenosiderophores). These exogenous siderophores include ferricrocin, ferrichrysin, ferrirubin, coprogen,

and triacetylfusarine (Holzberg and Artis 1983; Ismail et al. 1985; Heymann et al. 2002a). As a human commensal, *C. albicans* is likely exposed to xenosiderophores from bacteria that they share mucosal or gastrointestinal niches with (Heymann et al. 2002a). To utilise these xenosiderophores, *C. albicans* relies on the Sit1/Arn1 transporter, which recognises and transports these iron-binding compounds across the cell membrane, thus allowing microorganisms such as *C. albicans* to access the iron bound to these molecules. This transporter has also demonstrated a vital role epithelial invasion (Heymann et al. 2002a). The use of such a transporter was observed in a systemic candidiasis mouse model, where the Sit1/Arn1 transporter was required for effective invasion of reconstructed epithelium, simulating the human oral mucosa. However, interestingly, it did not significantly contribute to the pathogenicity of the yeast. This observation led to the logical conclusion that siderophore-mediated iron uptake might not exert significant influence during *C. albicans* bloodstream infections (Heymann et al. 2002a).

1.5. Iron transport and storage in *C. albicans*

Iron transportation across the plasma membrane in yeast appears to follow a unidirectional path, where the uptake and sequestration of iron remain largely irreversible, even in the presence of iron chelators (Urbanowski and Piper, 1999). At physiological pH, the chemistry of iron predominantly involves the hydrolysis and polymerisation of ferrous (Fe^{2+}) into insoluble ferric (Fe^{3+}) ions. Once inside the cell, organisms require a soluble, non-toxic, and bioavailable form of intracellular iron storage, a role commonly fulfilled by ferritin in many organisms, yet notably absent in yeast (Arosio et al., 2009).

S. cerevisiae adopts a strategy to store iron as low-molecular-mass Fe^{3+} polyphosphate complexes, and to a lesser extent, in the mitochondria where it

contributes to Fe-S cluster and haeme biosynthesis (Holmes-Hampton et al., 2013). The vacuole, a pivotal site for iron sequestration in yeast, serves as a storage compartment for various metals, functioning either to detoxify the cell or as a reserve for growth in low iron environments (Urbanowski and Piper, 1999). These vacuoles, acting as both lysosomes for protein breakdown and storage centres for ions and metabolites, play a critical role in maintaining cellular homeostasis (Li and Kane, 2009).

This cellular compartmentalisation within vacuoles allows for the binding and accumulation of numerous divalent cations, ensuring the regulation of essential ions such as Ca^{2+} , Mg^{2+} , Zn^{2+} , and Fe^{2+} as well as potentially harmful ones such as Sr^{2+} , Pb^{2+} and Cr^{3+} (Li and Kane, 2009). Iron transport into the vacuole occurs primarily through the Fe^{2+} and Mn^{2+} transporter, Ccc1p (**Figure 1.3**), with dynamic movement of iron into and out of the vacuole under varying conditions. The pH-dependent dynamics influence the protonated (at low pH) or deprotonated (at high pH) forms of iron species (Cockrell et al. 2011; Martínez-Pastor and Puig 2020).

In iron-replete conditions, iron can enter vacuoles through endocytosis or via the Ccc1p transporter on the vacuolar membrane. However, the specific form of iron species transported into vacuoles remains elusive but assumed to be favouring the Fe^{2+} state (Fu et al., 1994; Lapinskas et al., 1996; Li et al., 2001). *S. cerevisiae* also employs two mechanisms, Fth1p/Fet5p and Smf3p, on the vacuolar membrane to export Fe^{3+} from the vacuole into the cytosol, converting it to the Fe^{2+} state, particularly in iron-deficient environments (Cockrell et al., 2011).

In contrast, *C. albicans* utilises the Fet33p/Fth2p complex, which assembles on the vacuolar membrane and possesses functions similar to those of Fth1p and Fet5p in

S. cerevisiae (Martínez-Pastor and Puig, 2020). Fre7p facilitates the reduction of Fe^{3+} to Fe^{2+} by NADPH, subsequently transporting it to the Fet34p/Ftr1p complex (Martínez-Pastor and Puig, 2020). Furthermore, Smf3p, a vacuolar membrane protein, aids in transferring Fe ions and other divalent metal ions from vacuolar reserves to the cytosol (Stearman et al., 1996; Cohen et al., 2000; Xu et al., 2014a). However, the specific contribution of the vacuole to the overall iron metabolism within the cell remains to be fully elucidated (Martínez-Pastor and Puig, 2020).

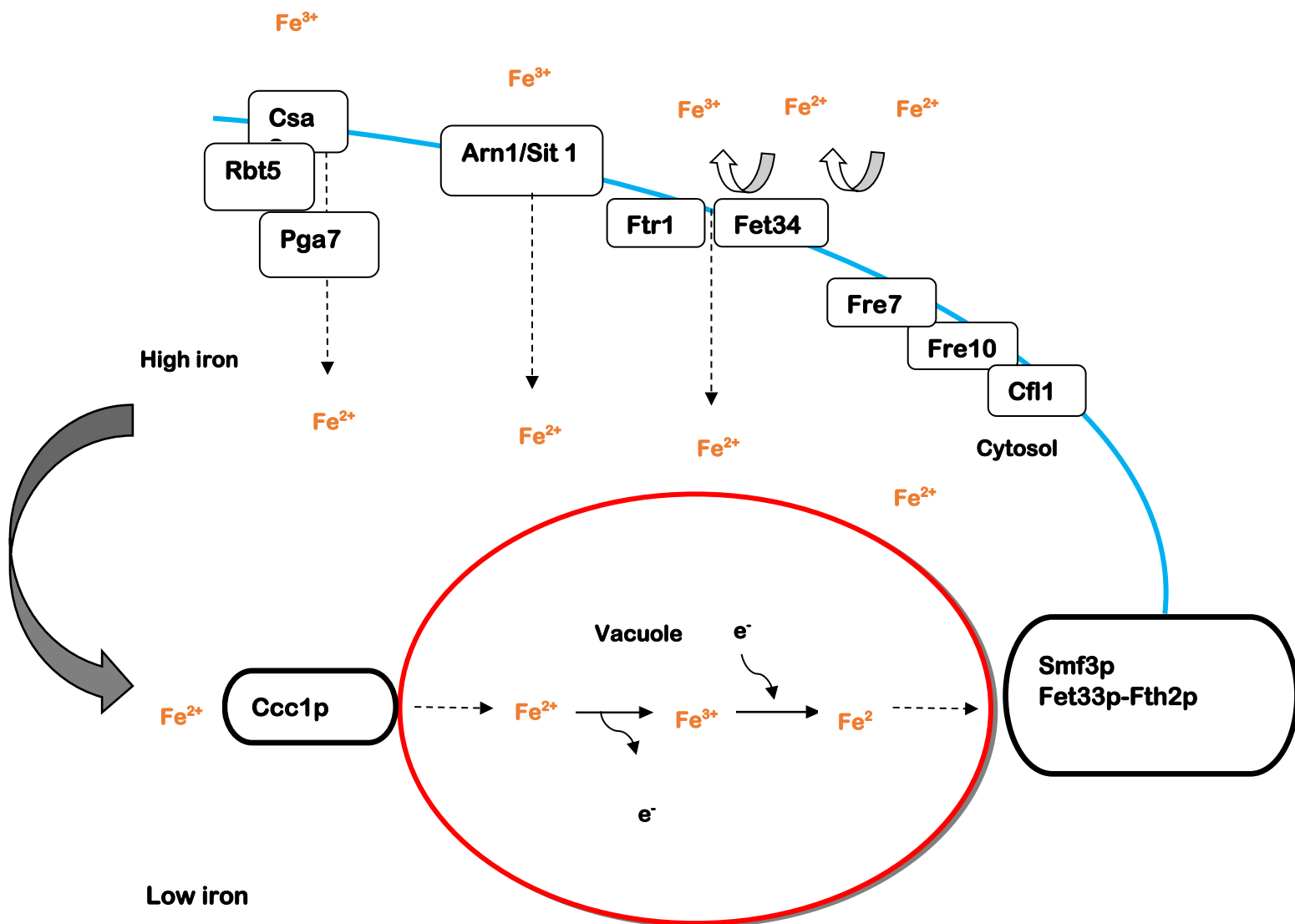


Figure 1.3. Intracellular trafficking and storage of iron in *C. albicans*. The vacuole serves as a storage compartment for micronutrients such as iron. In the vacuole, iron is specifically, stored in its oxidised, less soluble form Fe^{3+} (ferric iron); thus, making it more suitable for long-term storage in a less reactive and toxic state. This iron can later be reduced to Fe^{2+} (ferrous iron) and be released into the cytoplasm when needed. Several transporters such as Ccc1p, Smf3p and the Fet33p-Fth2p complex facilitate the movement of this micronutrient into the vacuole and out into the cytoplasm to maintain iron homeostasis (Cockrell et al. 2011; Martínez-Pastor and Puig 2020).

1.6. Metal handling and reprioritisation

The significance of mechanisms coordinating regulated transport, compartmentalisation, and utilisation of biometals for survival under host-imposed stress led to a proposal. This proposal suggested that a pathogen's access to and management of critical metals could influence its capacity to resist drug-induced stress (Hunsaker and Franz 2019a). As previously discussed, azoles bind to and inhibit the haem centre of the enzyme cytochrome P450 lanosterol 14 α -demethylase (Cyp51), which inhibits the formation of ergosterol, a crucial component of fungal cell membranes (Hitchcock 1991). Thus, the disruption of the ergosterol biosynthetic pathway by fluconazole necessitates extensive remodelling of the metal homeostasis networks by *C. albicans* in response to antifungal drug treatment (Hunsaker and Franz 2019b). This includes adaptation as a result of increased import and storage of Cu metal ions, altered utilisation of Cu- and Mn-dependent enzymes, increased production of the haemprosthetic group utilised by the enzyme target of fluconazole and increased retention of Fe, Mn and Zn (Hunsaker and Franz 2019b). Treatment

with fluconazole was found to specifically increase the cell's demand for transition metals. In response, *C. albicans* meets these increased needs by modifying metal transport, elevating the cellular metallome (Hunsaker and Franz 2019b). For example, cells treated with fluconazole maintained double the amount of Cu, Zn, Mn and Fe respectively, compared to untreated cells, suggesting that antifungal stress caused by fluconazole drives metal accumulation (Hunsaker and Franz 2019b). However, it is also important to note that despite the overall increase in metal content after treatment, fluconazole treatment led to a decrease in endoplasmic reticulum (ERP)-detectable Fe which may be as a result of Fe³⁺ stored in the vacuole (Hunsaker and Franz 2019b). This is to say that, during iron deficiency, vacuolar iron is reduced to Fe²⁺ and exported into the cytosol for cell use. Altogether, these data also suggest that antifungal stress caused by fluconazole within the cell influences biological and not necessarily chemical Fe or Cu deficiency.

Upon iron deficit, yeast uses the transcription factors Aft1 and Aft2 to activate the expression of the iron regulon, an ensemble of genes involved in iron absorption, recycling, and mobilisation (Chen et al. 2004; Rutherford et al. 2005; Gonçalves et al. 2014). Additionally, Aft1 and Aft2 stimulate the expression of Cth2, an mRNA-binding protein that suppresses the expression of genes, that code for iron-containing proteins or are involved in activities that use iron (Chen et al. 2004; Rutherford et al. 2005). Iron use in specific pathways is prioritised by Cth2 over other heavily iron-reliant processes, such as mitochondrial respiration, which are deemed to be non-essential (Sanvisens et al. 2014; Sanvisens et al. 2016).

The above findings are consistent with the study by Hameed and co-workers (Hameed et al. 2011), through which genome microarray analyses also showed that upon iron deprivation, *C. albicans* is able to redirect metals such as iron, that would otherwise

be incorporated into iron-independent pathways to other crucial pathways in order to sustain metabolic homeostasis. This is because not only do *Candida* cells increase the uptake of iron by regulating iron acquisition through its genes when iron is depleted, but also prioritised its utilisation in a manner that iron-independent enzymes or metabolic pathways are preferably utilised over dependent enzymes (Hameed et al. 2011). A research study by Ihrig and co-workers (2010) also showed that iron deficiency leads to a decrease in the activity of some of the iron-dependent enzymes which ultimately limits the levels of metabolic intermediates that are used as co-activators of specific transcription factors. For example, the authors found that there is a decrease in the overall expression of genes from branched-chain amino acid biosynthesis pathways that are iron-dependent and this is due to the sudden drop in the levels of the metabolic intermediate α -isopropylmalate, which serves as a co-activator of the Leu3 transcription factor (Ihrig et al. 2010).

1.7. Fungal-Bacterial interactions and the competition for iron

1.7.1. Interaction between *Candida albicans* and *Pseudomonas aeruginosa*

The constant competition for iron between microorganisms and their hosts is a constant battle with implications for both commensal colonisation and infections. Thus, it has been made evident that studying these interactions could offer insights into understanding the complex interplay between cross-kingdom and, co-infection of these agents. Over the past decade, the interplay between *C. albicans* and *P. aeruginosa* has become a focal point in clinical research, revealing their cross-kingdom interactions and communication patterns (Ader et al. 2011).

While initially non-pathogenic commensals in healthy individuals, both *C. albicans* and *P. aeruginosa* can transition to invasive pathogenic lifestyles, causing severe diseases or fatalities when infections occur at shared sites such as the gut, burn wounds, or lungs (Gupta et al., 2005; Ader et al., 2011; Mason et al., 2012). Their interaction, particularly evident in lungs of cystic fibrosis patients, often manifests as antagonism. Studies have also shown that *P. aeruginosa* has the ability to kill *C. albicans*. Historical observations from the 1970s detailed *P. aeruginosa*'s ability to inhibit *C. albicans* growth. In 2002, studies demonstrated *P. aeruginosa*'s preference for targeting and killing *C. albicans* hyphal cells, exploiting different morphotypes for lethal effects (Hogan and Kolter, 2002). Moreover, inhibition of biofilm formation and metabolic activity of *C. albicans* through bacterial components and secreted factors like phenazines and quorum sensing molecules has been well documented in their interaction (Grainha et al., 2020). *P. aeruginosa*'s pathogenesis is shaped by biofilm formation, virulence factor production, and antimicrobial resistance. These factors collectively contribute to the development of chronic infections and treatment resistance. Moreover, the ability of *P. aeruginosa* to form biofilms has been considered a defining trait of chronic infections and serves as a predictor of disease progression (Moradali et al., 2017). Within the biofilm environment, there is a promotion of the growth of small colony variants and persister cells, both of which exhibit heightened resistance against antibiotic treatments, enabling them to withstand higher doses of antimicrobial agents. This phenomenon further highlights the significance of biofilm formation in contributing to the overall resilience and persistence of *P. aeruginosa* in various clinical contexts. Interestingly, chemical substances released by *C. albicans* such as farnesol, impact not only its own resistance mechanisms against oxidative

stress but also influence *P. aeruginosa*'s growth and biofilm formation (Deveau et al., 2010).

Despite commonly being described as antagonistic, the interaction between these two microorganisms is remarkably complex, exhibiting both synergistic and antagonistic effects. These outcomes stem from their physical interactions as well as secretion of molecules mediated by quorum sensing. For example, interspecies competition between the two microorganisms leads to increased mutability and heightened production of virulence factors. This is evident in the differential expression of various proteins, including proteases, stress response elements, multidrug resistance-associated proteins, and iron-regulated proteins (Trejo-Hernández et al., 2014).

Moreover, *P. aeruginosa* has demonstrated the ability of employing diverse siderophores, including those from other microbes through siderophore piracy, and absorb haem from haemoproteins using distinct pathways for acquiring iron (Cornelis and Dingemans 2013). These mechanisms play a crucial role in sequestering iron and inhibiting *C. albicans* growth *in vitro* through siderophore production (Purschke et al., 2012; Trejo-Hernández et al., 2014). *P. aeruginosa* exhibits a versatile iron acquisition strategy through its Feo-system and redox-cycling phenazines, enabling the uptake of ferrous iron in microaerobic or anaerobic conditions. This adaptability allows the bacterium to switch between different iron absorption mechanisms depending on the nature of the infection. In addition, *P. aeruginosa* produces two siderophores, pyoverdine and pyochelin, each with distinct affinities for iron. Pyoverdine and pyochelin have been identified as inhibitors of *C. albicans* growth, exerting their effects by sequestering iron *in vitro* (Purschke et al., 2012; Trejo-Hernández et al., 2014). The interaction between *P. aeruginosa* and *C. albicans* extends beyond growth inhibition. The killing of *C. albicans* cells by *P. aeruginosa* may serve as a source of iron for co-

inhabiting organisms, including *P. aeruginosa*, providing a potential growth advantage in iron-limited environments (Morales et al., 2010). Thus, this dual siderophore system underscores the versatility of *P. aeruginosa* in efficiently competing for and acquiring iron resources. Moreover, this multifaceted strategy highlights the intricate dynamics of interspecies competition and cooperation in microbial communities.

1.8. The link between iron availability, lipid homeostasis, and drug susceptibility

Lipids play diverse roles in the context of pathogenic fungi, and their synthesis could serve as potential targets for drug development. Their significance arises from their integral role in membrane structure, creating a hydrophobic environment within the lipid bilayer that accommodates embedded proteins (Shevchenko and Simons, 2010).

Fungal lipids hold importance for two primary reasons. Firstly, they encompass lipids unique to fungi, absent in mammalian hosts, making them promising targets for innovative therapeutic strategies. Furthermore, the optimal functioning of several multidrug resistance (MDR) export proteins is contingent upon an ideal lipid environment, indicating the involvement of lipids in clinical drug resistance. Existing evidence establishes that alterations in membrane lipid composition, particularly in phospholipids and ergosterol, as well as changes in order (fluidity) and asymmetry, significantly influence the susceptibility of fungi to drugs (Kohli et al., 2002).

Microorganisms employ various mechanisms to counteract the effects of drugs like azoles, enabling them to endure drug exposure. These mechanisms include point mutations, overexpression of drug targets, reduced drug import, and enhanced drug efflux (Cannon et al., 1998; Maesaki et al., 1999). Drug tolerance, often arising from

single or combined processes, frequently involves the efflux protein-mediated expulsion of harmful substances from the cell in *Candida* species.

Clinical drug resistance, notably observed with widely used azoles like fluconazole, primarily targets ergosterol biosynthesis by inhibiting lanosterol 14 α -demethylase (Cyp51) at the heme center (Dunkel et al., 2008). This disruption in ergosterol biosynthesis, a vital component of the fungal cell membrane, is crucial for drug susceptibility in *C. albicans* cells. Interactions between membrane ergosterol and sphingolipids have been identified as significant, and reducing the concentration of either component heightens drug susceptibility in *C. albicans* (Prasad et al., 2006).

In iron-deficient *Candida* cells, the relationship between membrane fluidity and ergosterol becomes apparent. Low iron levels not only lead to a significant decrease in ergosterol levels but also prompt mechanisms that mitigate the effects of antifungal agents on the cell. These mechanisms include alterations in drug targets hindering drug binding and the overexpression of drug efflux pumps (**Figure 1.4**). During iron deficiency, the reciprocal regulation of *ERG11* and *ERG3* genes in the ergosterol production pathway occurs. Reduced *ERG11* levels result in decreased ergosterol levels, increased membrane mobility, and heightened drug passive diffusion. Additionally, the upregulation of *ERG3* contributes to the accumulation of detrimental sterols.

Moreover, iron deficiency in *C. albicans* has been associated with a highly conserved calcium-dependent serine/threonine-specific protein phosphatase known as calcineurin. This protein is linked to resistance against azoles and other drugs targeting ergosterol synthesis while mediating various stress responses within the cell (Srivastav et al., 2017). Iron deficiency mimics the phenotype of calcineurin signalling

mutants, increasing susceptibility to alkaline pH, salt, and membrane stress. Additionally, iron deficiency down-regulates calcineurin pathway genes *CRZ1*, *HSP90*, and *CMP1* (Cowen et al., 2014).

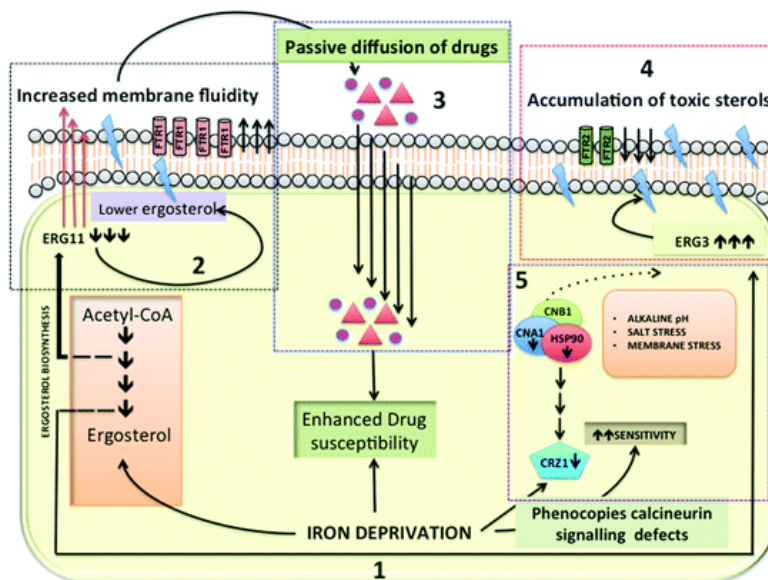


Figure 1.4. A schematic diagram depicting the events associated with lipid homeostasis in a MDR *C. albicans* upon iron deprivation. In the event of iron deficiency, *C. albicans* becomes susceptible to antifungal drugs such as fluconazole. This is because when the cell experiences iron deficiency, several cellular processes such as lipid homeostasis are affected. For example, when the yeast experiences nutrient stress, the *ERG11* becomes downregulated, thus leading to lower ergosterol production and increased membrane fluidity which in turn lead to a greater passive diffusion of drugs. Additionally, the calcineurin pathway genes encoding for transcription factors and genes that are responsible for membrane and salt stress as well as pH become downregulated (Srivastav et al. 2017).

Recently, Hattab and co-workers (Hattab et al. 2022) conducted inter-kingdom studies and observed a striking feature, where the combination of the bacterium *P. aeruginosa* and fluconazole produced a synergistic effect against *C. albicans* both *in vitro* and *in vivo*, thus making the drug fungicidal and improving its potency over 3-fold. This synergistic effect between the azole and *P. aeruginosa* was reported to be partly as a result of iron piracy and was reduced upon iron supplementation and the knockout of bacterial siderophores. These results demonstrated that the presence of co-colonising pathogens can to some extent influence drug susceptibility within the host. However, a thorough understanding of the effects of co-infection should also consider a number of factors ranging from nutrient availability within the host to the host's immune response.

1.9. Conclusions

Candida albicans exhibits an exceptional capacity to harness host metal resources across various infection scenarios, whether in commensal, superficial, or systemic conditions. This adeptness underscores its remarkable adaptability in effectively accessing and utilising essential metals, notably iron during its interaction with the host. This ability extends beyond standalone iron uptake systems, thus revealing a complex network intricately connected with other pathogenic mechanisms. Moreover, the high-affinity reductive pathway of *C. albicans* plays a significant role in iron acquisition within iron-limited environments. Genes within this pathway, such as *FTH2*, appear to be influencing biofilm formation, PGE₂ production and virulence, (Mochochoko et al. 2021), thus confirming the requirement for sufficient iron acquisition. However, despite recognising the profound impact of membrane lipids on drug susceptibility, our understanding of lipid homeostasis in *C. albicans* remains

relatively limited. As such, delving deeper into the mechanism of iron storage holds the potential to provide insights into the regulation of intracellular iron and its significant role in the pathogen's virulence.

1.10. Proposed Research Aims and Objectives

It is unknown what the influence between iron transport from the vacuole and lipid homeostasis in *C. albicans* is, therefore, the aim of this study was to investigate the role of the vacuolar iron permease Fth2p (which has been shown to play a role in prostaglandin production and virulence) in the lipid homeostasis and fluconazole susceptibility of *C. albicans*. This was done by pursuing the following objectives:

Chapter 2

1. Investigating the effect of *FTH2* deletion on *C. albicans* fluconazole sensitivity and iron localisation, both in the absence and presence of *P. aeruginosa*.

Chapter 3

1. Investigating the influence of deletion of *FTH2* on lipid droplet formation and lipid content. This was performed both in the presence and absence of *P. aeruginosa*.
2. Investigating the effect of deletion of *FTH2* on *C. albicans* lipidome, both in the absence and presence of pyoverdine (an extracellular iron chelator).

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Chapter 2

Determination of the effect of *FTH2* deletion on fluconazole susceptibility

2.1. Abstract

The past decades have witnessed a rapid increase in the development of resistance to widely used antifungal drugs. Interestingly, interference with iron homeostasis of *Candida albicans* has been reported to lead to improved antifungal response by the pathogen. With iron being an essential nutrient for almost all life forms, its uptake by pathogens is considered a vital virulence-associated characteristic. However, iron can also be toxic to microbial cells and thus its content in cells is tightly regulated. In yeasts, excess iron is stored in the vacuole, from where it can later be released into the cytoplasm during time of scarcity. *Candida albicans* genes *FTH1* and *FTH2* encode for vacuolar iron permeases, essential for iron transport from vacuolar stores into the cytoplasm. Thus, this study sought to explore the effect of *FTH2* deletion and iron availability on fluconazole susceptibility, on mono- and polymicrobial developing biofilms. To determine the effect of *FTH2* deletion on fluconazole susceptibility, two mutant ($\Delta/\Delta fth2$ and $\Delta/\Delta fth2_II$) and parental (BWP17 and SC5314) *C. albicans* strains were grown in both the absence and presence of fluconazole. Antifungal activity was firstly evaluated on planktonic cells, where both mutants showed resistance towards fluconazole when compared to their parental strains. The effect of fluconazole on developing biofilms was also determined using crystal violet assay (biomass assay) and XTT for metabolic activity. Our results showed a significant decrease in susceptibility and greater biomass production for both mutants upon drug exposure when compared to their parental strains. Even so, $\Delta/\Delta fth2$ showed greater metabolic activity. This was however not the case for $\Delta/\Delta fth2_II$. Co-culturing of *C. albicans* with *Pseudomonas aeruginosa* resulted in a synergistic effect against the $\Delta/\Delta fth2$ mutant in both assays with the parental strain however, showing less biomass reduction upon fluconazole exposure. Additionally, the localisation of Fe^{2+} was visualised using

microscopy. The results presented here reveal that deletion of *FTH2* influences fluconazole susceptibility and iron localisation. This may be due to the influence of iron on lipids, including membrane sterols.

Key words: *C. albicans*, Iron availability, Fluconazole susceptibility, Iron localisation

2.2. Introduction

A rapid increase in the number of biofilm-associated fungal infections and their resistance to routinely used antifungals has led to the need to develop new strategies to prevent and control these fungal infections. Biofilms consist of highly organised microbial communities that are either surface-associated or attached to one another and are encased within a self-produced extracellular matrix (ECM) that serves as a protective barrier (Ramage et al. 2009). Biofilms provide an organism with several benefits, including environmental protection, resistance to physical and chemical stress, metabolic cooperation, and community-based gene expression control (Ramage et al. 2012). Microorganisms growing within biofilms also exhibit distinctive phenotypic traits in comparison to their planktonic counterpart cells and from a clinical standpoint, the most significant characteristic of *Candida* biofilms is their high level of resistance to routinely used traditional antifungal treatment (Ramage et al. 2009). One such antifungal drug is fluconazole, that actively targets the lanosterol 14 α -demethylase cytochrome P450-dependent enzyme expressed by *ERG11* (Odds et al. 2003). The activity of fluconazole ultimately suppresses the formation of ergosterol and thus leads to its depletion within the cell membrane (White et al. 2002; Odds et al. 2003). Additionally, there is an accumulation of toxic membrane sterol pathway intermediates generated as a result of mutations of the *ERG3* gene, which tend to exert membrane stress when integrated into the fungal membrane and ultimately prevent proliferation (Prasad et al. 2006). Both intravenous and oral forms of fluconazole are available and appear to have no negative effects on the generation of steroid hormones (Casalinuovo et al. 2004). However, a challenge to successful patient outcomes is the specific fungistatic properties of azoles which cause a strong

directional selection on the surviving population to develop drug resistance (Anderson 2005; Cowen 2008; Arastehfar et al. 2020).

When compared to planktonic free-floating cells, *Candida albicans* biofilms have been found to be up to >1000 times more resistant to fluconazole, *in vitro* (Ramage et al. 2001; Lamfon et al. 2004; Hacıoglu et al. 2018). Yet, the current view is that biofilm resistance is a complex phenomenon that cannot be explained by a single mechanism alone. Rather, it is multifactorial and may entail various molecular mechanisms of resistance as compared to those demonstrated by planktonic cells (Hacıoglu et al. 2018). Some of these mechanisms include the expression of resistance genes, the existence of persister cells, the development of extracellular matrix with delayed antimicrobial drug penetration and altered growth, and metabolic rates of cells within the biofilms (Niimi et al. 2010) (**Figure 2.1.**). Thus, developing antifungals with novel targets is a crucial tactic for the successful control of fungal infections.

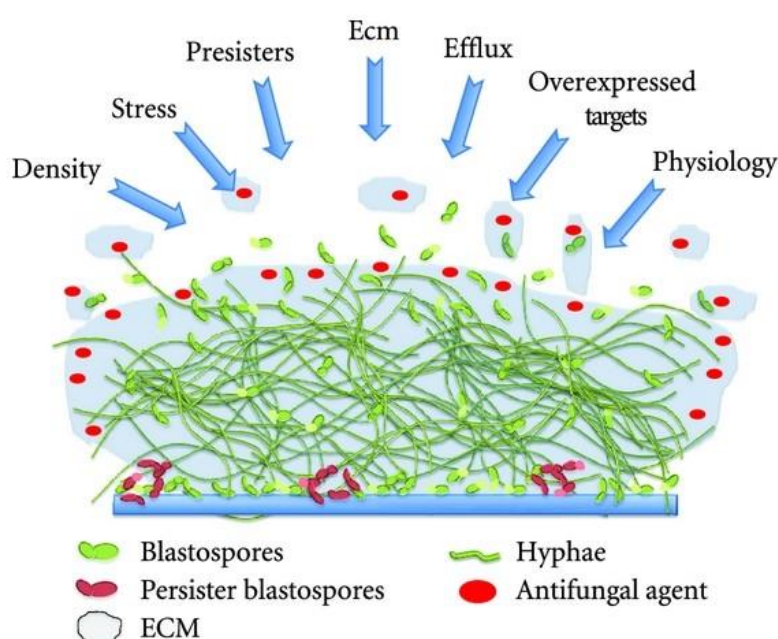


Figure 2.1. The adaptive fungal biofilm resistance mechanisms mediated by *C. albicans*. The biofilm's density, stress, persisters, extracellular matrix (ECM), efflux, overexpressed targets, and general physiology are represented by arrows as driving forces for antifungal resistance inside the biofilm (Ramage et al. 2012).

Similarly, the interaction of *C. albicans* with other opportunistic pathogenic microorganisms such as *Pseudomonas aeruginosa* within polymicrobial biofilms can influence the efficacy of antimicrobials (Alam et al. 2020; Hattab et al. 2022). It is also not clear if co-infections associated with these two pathogens should be treated with the same antimicrobials, as those used for treating mono-infections (Chotirmall et al. 2010; Horr   et al. 2010; Leclair and Hogan 2010; Gileles-Hillel et al. 2015). This is because both microorganisms can colonise similar niches and the interaction between the two species can be complex (F  rster et al. 2016; Fourie and Pohl 2019). Competition for available nutrients in the shared environment (i.e., iron) between *C. albicans* and *P. aeruginosa* has been well documented (Trejo-Hern  ndez et al. 2014). Both microorganisms require iron for their normal growth and consequently the production of iron chelators such as siderophore by *P. aeruginosa* may potentially limit iron availability for *C. albicans* (Purschke et al. 2012).

One of the most significant current discussions in fungal infections, is the role of ion homeostasis in antifungal drug resistance. Ion homeostasis is commonly acknowledged to be essential for all living cells and could potentially have a role in maintenance of the membrane potential, control of cell volume, cofactor synthesis for several enzymes, proliferation, and death (Yu et al. 2008). Ion-signalling and transduction networks are crucial for fungi to survive because they regulate gene

expression, morphological change, host invasion, stress response, and drug resistance (Juvvadi et al. 2017). A rising number of drugs that cause antifungal activity are mechanistically based on the dysfunction of metal ion homeostasis, which rapidly triggers cell death (Yu et al. 2008; Jiang et al. 2023).

One such important ion is iron ($\text{Fe}^{2+}/\text{Fe}^{3+}$), as it is an essential nutrient and serves as the most abundant trace metal in all eukaryotic cells (Lieu et al. 2001; Pantopoulos et al. 2012; Sherman et al. 2018). The fate of infection is dependent on iron competition between the host and the pathogen (Ramanan and Wang 2000). Even so, there is seldom any free iron in circulation since most of the iron in host cells is bound to iron-binding proteins including haemoglobin, ferritin, and transferrin (Weissman and Kornitzer 2004; Almeida et al. 2009; Lambert 2012). Thus, fungi such as *C. albicans* have developed a complex iron absorption regulatory network in the host environment, to carry out vital cellular functions. Three iron uptake systems, which include the haemoglobin uptake system, the utilisation of siderophores secreted by other species and the high-affinity reductive iron uptake system, have been described in *C. albicans* (Ramanan and Wang 2000; Santos et al. 2003; Almeida et al. 2009). In non-siderophore-producing *C. albicans*, the high-affinity reductive iron uptake system is particularly important for the continued growth and virulence of the organism (Heymann et al. 2002b). The high-affinity reductive uptake system makes use of iron transporters consisting of ferroxidase enzymes coupled to iron permeases, which are either localised within the plasma membrane or are vacuolar associated (Mamouei et al. 2017). Thus, during iron limitations, *C. albicans* makes use of the multicopper oxidase-permease complex to facilitate iron transport from its stores.

Apart from the processes associated with iron absorption, maintaining intracellular iron homeostasis necessitates the control of other internal factors such as oxidative stress,

morphogenesis and drug tolerance. In *C. albicans* the regulation of intracellular iron homeostasis is influenced by the Mrs4-Ccc1-Smf3 (MCS) pathway, which is made up of three iron transporters, Mrs4, Ccc1 and Smf3 situated on intracellular organelles, i.e., mitochondria and vacuole (**Figure 2.2.**). According to Xu et al. (2014a) dysregulation of the MCS pathway has a significant impact on several physiological processes that are closely related to cell survival, including response to oxidative stress, cell-wall stability, colony formation and filamentous growth. This pathway works in conjunction with the *Candida albicans* iron transporters Smf3, Fet33 and Fth2, to maintain iron homeostasis and facilitate the transport of iron to or from the vacuole into the cytosol (Weissman et al. 2008). Iron availability is also known to have an influence on the overall lipid homeostasis (Hameed et al. 2011; Rockfield et al. 2018; Ma et al. 2021). For example, iron deprivation leads to the global remodelling of cellular lipids. The deficiency of iron also targets the lipid metabolic pathways, particularly those involved in ergosterol and sphingolipid metabolism (Hameed et al. 2011). *C. albicans* relies on its vacuolar iron stores to maintain its normal growth and pathogenesis during iron limitations. The shuttling of iron from the vacuole into the cytosol is maintained by two iron permeases (complexed to multicopper-oxidases) Fth1 and Fth2, localised within the vacuole that are consistently induced by either iron limitations or high levels of iron respectively (Mamouei et al. 2017). As a result, the knowledge pertaining to regulation of iron homeostasis provides the foundation for exploring novel antifungal strategies. Thus, the aim of this chapter was to determine the effect of *FTH2* gene deletion on fluconazole susceptibility towards developing biofilms and see if this may result in changes in membrane fluidity and, eventually susceptibility to fluconazole.

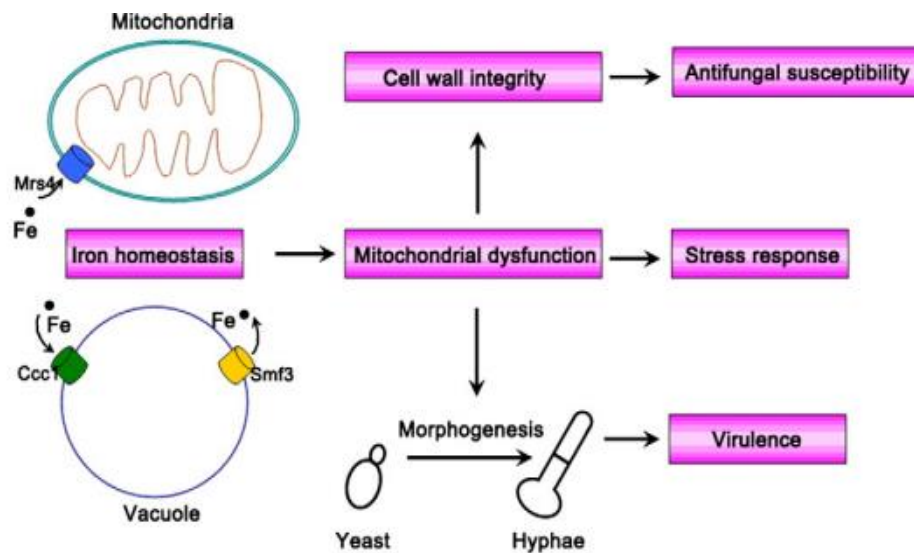


Figure 2.2. A schematic depiction of the influence of cellular iron homeostasis on fungal survival, drug tolerance and virulence through the Mrs4-Ccc1-Smf3 (MCS) pathway. Mrs4 is a mitochondrial carrier protein which transports iron across the mitochondrial inner membrane and maintains mitochondrial morphology (Froschauer et al. 2009). The Ccc1 transporter protein facilitates iron sequestration from the cytosol to the vacuole when iron concentrations are high for storage (Li et al. 2001). During iron-limitations, the Smf3 homolog protein belonging to the Nramp family assists in iron mobilisation from vacuolar stores through the Fth2 and Fet33 complex. This diagram further demonstrates how each of the cellular processes are related to one another and contribute to fungal pathogenesis as a result of iron homeostasis (Xu et al. 2014a).

2.3. Materials and methods

2.3.1. Strains used in this study

Two independent, previously constructed *C. albicans* *FTH2* deletion mutants and their respective parental strains (**Table 1**) were obtained from the yeast culture collection at the University of the Free State, Bloemfontein, South Africa. The yeasts were revived and maintained on Yeast Malt extract (YM) agar plates (3g/L malt extract, 3g/L yeast extract, 5g/L peptone, 10g/L glucose, 16g/L agar) at 30°C for 24 h and preserved at 4°C for all studies conducted. The bacterial strain, *P. aeruginosa* PAO1, which was a kind gift from Professor Robert Hancock (Department of Microbiology and Immunology at the University of British Columbia) was maintained on nutrient agar (1g/L malt extract, 2g/L yeast extract, 5g/L peptone, 8g/L sodium chloride and 20g/L agar) at 37°C. A 50% glycerol stock for each strain was also prepared and stored at -80°C for future use.

Table 1. *C. albicans* and *P. aeruginosa* strains used in this study

Name	Genotype	References
<i>C. albicans</i> SC5314	Wild type (WT)	(Gillum et al. 1984)
<i>C. albicans</i> BWP17	<i>ura3::λimm434/ura3::λimm434iro1::λimm434/iro1::λimm434his1::hisG/his1::hisGarg4::hisG/arg4::hisGRPS1/rps1::(URA3-HIS1-ARG4)</i>	(Wilson et al. 1999)

<i>C. albicans</i> $\Delta/\Delta fth2^*$	<i>fth2Δ::FRT/fth2Δ::ARG4/CIP10 URA3</i>	(Mamouei et al. 2017)
<i>C. albicans</i> $\Delta/\Delta fth2_{II}^{**}$	<i>fth2Δ::FRT/fth2Δ</i>	(Mochochoko et al. 2021)
<i>Pseudomonas aeruginosa</i> - PAO1	Wild type clinical strain	(Professor Hancock from the Department of Microbiology and Immunology at the University of British Columbia)

* Parental strain is BWP17

** Parental strain is SC5314

2.3.2. Planktonic cell growth studies

The effect of *FTH2* on fluconazole sensitivity on planktonic cells was evaluated by exposing all the revived *C. albicans* strains to different drug concentrations ranging from 0.25 µg/mL to 512 µg/mL. To do this, yeast strains represented were revived on YM agar plates for 24 h at 30°C, inoculated into 10 mL YPD broth (20 g/L, glucose, 20 g/L peptone, 10 g/L yeast extract) and incubated at 30°C for 24 h. Following incubation, the cells were then washed three times with 10 mL sterile Phosphate Buffered Saline [PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride (pH 7.4) (Sigma-Aldrich, St. Louis, MO, USA)] by centrifugation at 5000 rpm for 5 min and subsequently re-suspended in 10 mL sterile PBS. The cells were standardised to a final concentration of 1.0×10^6 cells/mL in 10 mL filter sterilised

(using a 0.22 µm nitrocellulose filter, Merck, Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA). The standardised cells were subsequently exposed to the different fluconazole concentrations ranging from 0.25 µg/mL to 512 µg/mL in 96-well polystyrene microtiter plates (Corning Incorporated, Costar, USA) containing RPMI-1640 media and incubated at 37°C. Readings to evaluate antifungal susceptibility on planktonic cells were taken in 2-h intervals, up to 8-h at an optical density (OD) of 595 nm. This was conducted with over three biological and technical replicates.

2.3.3. Monomicrobial *C. albicans* biofilm formation

To evaluate the effect of fluconazole on developing biofilms, a loopful of cells was inoculated into YPD (20 g/L, glucose, 20 g/L peptone, 10 g/L yeast extract) broth and incubated at 30°C for 24 h. Following incubation cells were harvested, washed three times with 10 mL sterile PBS by centrifugation at 5000 rpm for 5 min and subsequently re-suspended in 10 mL sterile PBS. These cells were standardised to a final concentration of 1.0×10^6 cells/mL in filter sterilised (using a 0.22 µm nitrocellulose filter, Merck, Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA) as previously described. The standardised cells were dispensed into flat-bottom 96-well polystyrene microtiter plates (Corning Incorporated, Costar, USA), exposed to 1 µg/mL fluconazole and incubated for 48 h at 37°C to allow biofilm formation (Kuloyo, 2020). Cell-free wells (containing sterile RPMI-1640 medium and a drug solvent) and drug-free wells (containing sterile RPMI-1640 medium, a drug solvent and cells) were included as blanks and negative controls respectively.

2.3.4. Polymicrobial biofilm formation in the presence of *P. aeruginosa*

All *C. albicans* yeast cells were prepared and cultivated as previously described in section 2.3.3. To cultivate the bacterium, an aliquot of *P. aeruginosa* PAO1 was inoculated in 5 mL nutrient broth (1g/L malt extract, 2g/L yeast extract, 5g/L peptone and 8g/L sodium chloride) and incubated in a shaking incubator for 24 h at 37°C. Importantly to note, the effect of *FTH2* on fluconazole susceptibility was only evaluated for *C. albicans* BWP17 and its mutant $\Delta/\Delta fth2$ (strain showing most resistance). The bacterial cells were washed three times with 10 mL sterile PBS by centrifugation at 5000 rpm (4°C) for 5 min. Following this, the cell solution was standardised to 0.05 at an optical density (OD) reading of 600 nm and inoculated in the presence of *C. albicans* standardised to a final concentration of 1.0×10^6 cells/mL in filter sterilised RPMI-1640. These cells were also exposed to 1 µg/mL of fluconazole in flat-bottom 96-well polystyrene microtiter plates (Corning Incorporated, Costar®, USA) and incubated for 48 h at 37°C to allow biofilm formation. Again, cell-free wells and drug-free wells were included.

2.3.5. XTT assay

Following both mono- and polymicrobial biofilm formation, the supernatant from each well was removed and biofilms were washed twice with 200 µL sterile PBS to remove non-adherent cells. A volume of 50 µL of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) (Sigma-Aldrich, USA) (1g XTT in 1L PBS, filter sterilised, aliquoted and stored at -20°C) containing 0.08 mM menadione (Fluka, USA) (stock solution of 10 mM menadione in acetone) was added to each well and incubated for 3 h in the dark at 37°C (Kuhn et al. 2003). The absorbance of each well was measured at 492 nm on an EZ Read 800 Microplate

Reader (Biochrom, UK). Cell-free control wells (RPMI-1640) were included as blanks to minimise the effects of variables. This experiment was performed in biological triplicates with three technical replicates per biological replicate.

2.3.6. Crystal violet assay

To determine the biomass of cells adhering to the 96-well plates in a quantitative manner, a crystal violet (CV) assay modified from Jin et al. (2003) was also performed following biofilm formation. Briefly, 0.4 % v/v of crystal violet solution (Merck Chemicals Pty. Ltd, South Africa) was prepared in double-distilled water (ddH₂O). Biofilms were washed twice with 200 µL of sterile PBS to remove non-adherent cells and left to air dry at room temperature for 45 min. The biofilms were then stained with 110 µL of CV and left for 45 min on the bench (away from light), to stain the formed biofilms attached to the cell culture plates. To remove the unbound CV, the biofilms were washed with 350 µL of sterile ddH₂O until the water was clear. To destain the biofilms, 200 µL of 95 % ethanol was added to the biofilms. 100 µL of the solubilised CV from each well was aliquoted into new 96-well plates and absorbance was measured at 595 nm on an EZ Read 800 Microplate Reader (Biochrom, UK). The average of blank absorbance values was deducted from experimental absorbance values to eliminate background interference and normalise values. This experiment was performed in biological triplicates with three technical replicates per biological replicate.

2.3.7. Confocal laser scanning microscopy

To monitor the localisation of Fe²⁺ iron, in both mono- and polymicrobial biofilms, yeast cells were cultivated for 24 h, at 30°C in YPD (20 g/L, glucose, 20 g/L peptone, 10 g/L

yeast extract). As previously described, bacterial cells were inoculated in 5 mL of nutrient broth and incubated for 24 h at 37°C. Both bacterial and yeast cells were subsequently washed three times with 10 mL sterile PBS by centrifugation, at 5000 rpm (4°C) for 5 min. The cells were then standardised to a final concentration of 1.0×10^6 cells/mL and 0.05 at an OD of 600 nm respectively in 10 mL filter sterilised (using a 0.22 µm nitrocellulose filter, Merck, Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA) respectively. Following this, cells were further cultivated as biofilms in 6-well plates (Corning Incorporated, Costar®, USA) containing coverslips, for 6 and 48 h respectively at 37°C using RPMI-1640 medium. After incubation, biofilms were subsequently washed twice with 200 µL sterile PBS to remove any non-adherent cells. These biofilms were then stained with an Fe^{2+} specific stain [(1µM Biotracker FerroOrange Live Cell Dye); Merck Chemicals Pty. Ltd, South Africa] prepared in 35 µL of Dimethyl sulfoxide [(DMSO), Sigma Aldrich]) which was further diluted in PBS to a ratio of 1: 2500. These biofilms were incubated for 30 min at 37°C in the dark to allow binding of the stain to iron within biofilms. After incubation, biofilms were subsequently washed once more, twice with 200 µL of sterile PBS to remove any unbound dye, transferred to glass slides and sent to the centre for microscopy, for confocal laser scanning microscopy (CLSM) imaging. The confocal images were acquired using a Zeiss LSM 900 airyscan laser scanning confocal microscope. The prepared samples were exposed to an excitation (ex) of 542 nm and an emission (em) of 572 nm, to visualise the stained biofilms. Image acquisition and subsequent analysis was done on the Zeiss ZEN 3.2 blue edition system (ZEISS, Germany).

2.4. Statistical analyses

All quantitative experiments were conducted in biological and technical triplicates. The averages and standard deviations were calculated using Microsoft excel 365 and GraphPad prism 10.0.3. The data of different groups were compared and evaluated for significance using the student's t-test. p -values ≤ 0.05 were considered statistically significant and indicated by an asterisk (*) on the graphs.

2.5. Results and discussion

2.5.1. The effect of *FTH2* on fluconazole susceptibility on planktonic cells

To begin with, we evaluated the effect of different fluconazole concentrations on planktonic cells. As previously described, yeast cells were exposed to a range of fluconazole concentrations and antifungal susceptibility was assessed in 2-h intervals from 2 to 8 h. The heatmaps illustrated in **Figure 3.1.** represent the response of *C. albicans* (SC5314, $\Delta/\Delta fth2_{II}$, BWP17 and $\Delta/\Delta fth2$) to different concentrations of fluconazole as percentage inhibition. As can be seen, in **Figure 3a and c**, there was an increase in fluconazole susceptibility with increased concentrations and incubation time across all parental strains (*C. albicans* SC5314 and BWP17) strains. Both parental strains displayed significant percentage inhibition towards fluconazole at concentrations between 8 $\mu\text{g/mL}$ to 256 $\mu\text{g/mL}$. On the contrary, the mutants displayed decreased susceptibility towards all tested concentrations overtime. Overall, all the isolates surprisingly showed less sensitivity to fluconazole at a concentration of 512 $\mu\text{g/mL}$.

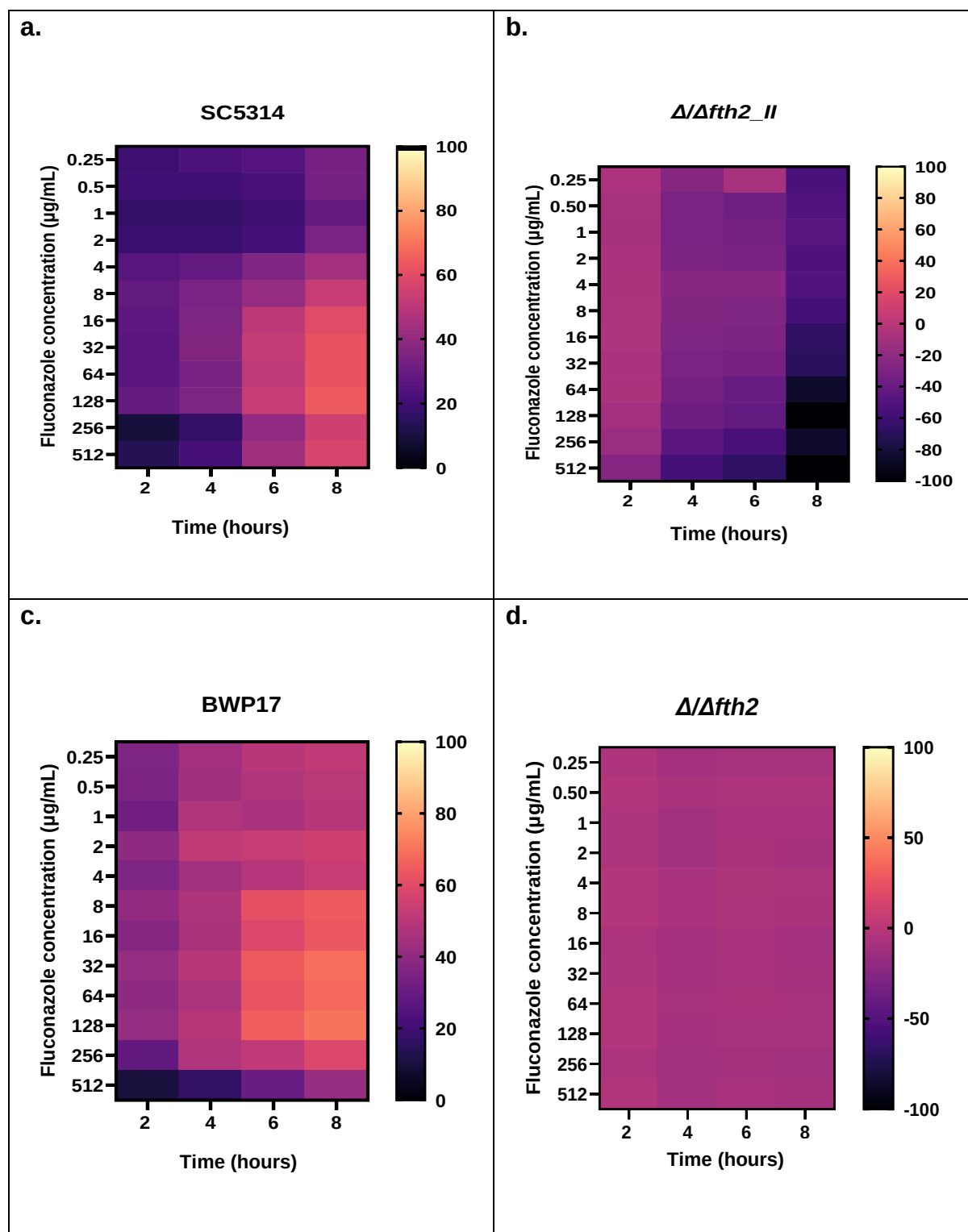


Figure 3. Susceptibility of the *C. albicans* to fluconazole on planktonic cells. To evaluate the influence of *FTH2* on fluconazole susceptibility, both parental (**a** and **c**) and their constructed mutant strains (**b** and **d**) were exposed to different

concentrations of fluconazole ranging from 0.25 µg/mL to 512 µg/mL. The heatmaps represent the percentage inhibition of different fluconazole concentrations at different time points. The different intensities represented by the scale bar show the susceptibility (percentage inhibition) of the different strains towards fluconazole.

Although the parental strains (*C. albicans* BWP17 and SC5314) displayed susceptibility to lower fluconazole concentrations, it was noted that there was an unusual trend at a concentration of 512 µg/mL in these strains, including mutants that displayed resistance. A complete decline in antifungal activity with prolonged times of exposure by susceptible strains has been shown in *C. albicans* planktonic cells by Shi and co-workers (Shi et al. 2019). Moreover, moderately resistant strains were shown to develop resistance more rapidly at MICs of 256 mg/L after 8 h. Within the same study, an important trend was seen in highly resistant strains, which displayed resistant throughout their growth and exposure to varying fluconazole concentrations. The mutants in this study both also showed resistance throughout, with increased incubation times as well as drug exposure.

Additionally, the effect of fluconazole against *C. albicans* has previously been evaluated by many researchers including Kaya and Ozbilge (2012) who established the minimum inhibitory concentration (MIC) of fluconazole against *C. albicans* biofilms to be 0.5 µg/mL. However, using concentrations higher than those formulated may be beneficial especially if resistance to fluconazole has been observed or suspected. Moreover, higher MICs may aid in reviewing the susceptibility threshold and establishing a broader range of susceptibility against the antifungal (Riesselman et al. 2000; Berkow et al. 2020; Wang 2020). As such, to

evaluate the effect of *FTH2* on fluconazole susceptibility, on both mono- and polymicrobial biofilms, it was decided to use a concentration of 1µg/mL to assess antifungal activity of fluconazole. The choice of this concentration was based on prior research findings by several scientists (Riesselman et al. 2000; Berkow et al. 2020; Wang 2020), as well as the findings from the assessment of fluconazole susceptibility on planktonic cells conducted in this study.

2.5.2. The effect of *FTH2* on fluconazole susceptibility on monomicrobial biofilms

The next objective was to investigate the influence *FTH2* deletion and fluconazole on developing biofilms which were incubated for 48 h. Some of the most commonly used colorimetric methods for evaluating biofilm formation phenotypically in *Candida* species include the use of XTT and CV assays, in which the cell viability and total mass are measured, respectively (Kuhn et al. 2003; Jin et al. 2003; Melo et al. 2011). The determination and standardisation of antifungal susceptibility tests in *C. albicans* biofilms have been made possible by the microtiter-plate-based biofilm model, which is compatible with the 96-well platform technology (Pierce et al. 2010). In this approach, biofilms are formed in 96-well microtiter plates, and are exposed to antifungal drugs for a predetermined amount of time. Since XTT and CV assays yield distinct types of information, it is advised to utilise them in conjunction (Muthamil et al. 2020).

Figure 2.4.a indicated that both *C. albicans* mutants ($\Delta/\Delta fth2$ and $\Delta/\Delta fth2_1I$) exhibited a slight but significant ($p \leq 0.05$) reduction in fluconazole susceptibility and thus less

percentage inhibition when compared to their *C. albicans* parental strains (SC5314 and BWP17) respectively. A decrease in fluconazole susceptibility displayed by both mutants also represents greater biomass production when compared to their parental strains. It should however be noted that CV non-specifically stains both dead and live cells together with the extracellular matrix which could lead to an erroneous assessment of biofilm biomass increase and sometimes high standard deviations (Li et al. 2003; Pitts et al. 2003; Shukla and Rao 2017). Using the XTT assay to determine metabolic activity, the decrease in fluconazole susceptibility was also seen for $\Delta/\Delta fth2$ when compared to *C. albicans* BWP17 (**Figure 2.4.b**). However, the $\Delta/\Delta fth2_{II}$ mutant displayed increased susceptibility (greater percentage inhibition) to fluconazole compared to its parental strain, *C. albicans* SC5314. Chen and co-workers (2018) screened *C. albicans* mutant strains including $\Delta/\Delta fth2$ against antifungals. This was done to identify gene mutations that either conferred resistance or heightened sensitivity against antifungals such as fluconazole. One key observation was that mutants, inclusive of the $\Delta/\Delta fth2$ mutant, displayed resistance to fluconazole at concentrations as high as 20 $\mu\text{g/mL}$. Thus, from the results obtained in this study, it is evident that the $\Delta/\Delta fth2$ mutant used in this study also showed resistance against fluconazole. It was also previously reported in section 2.5.1. on planktonic cell studies that both mutants displayed resistance to increased fluconazole concentrations ranging from 0.25 $\mu\text{g/mL}$ to 512 $\mu\text{g/mL}$. Thus, these findings further suggest that the deletion of *FTH2* causes a decrease in susceptibility to fluconazole, both in planktonic cells and biofilm formation. As a result, this may potentially provide escape pathways for cells facing challenges posed by fluconazole just as Chen and co-workers (2018) have described.

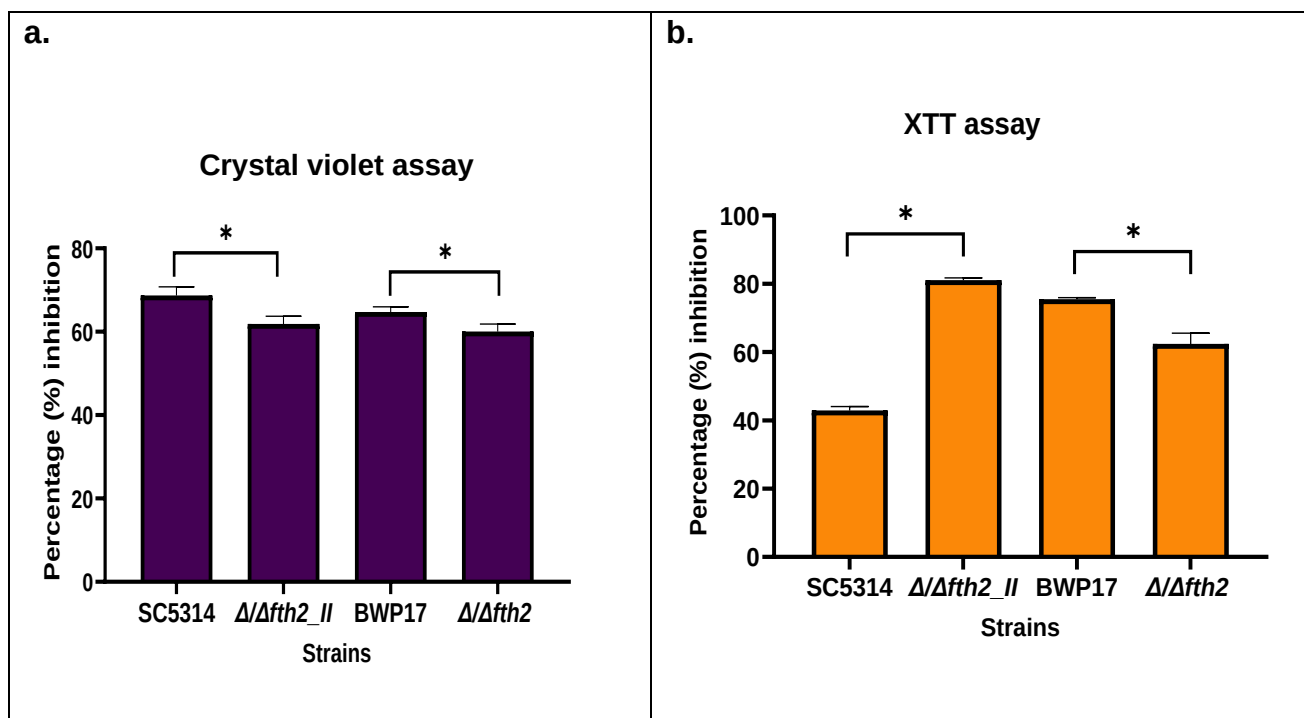


Figure 2.4. The effect of fluconazole on cell density (biomass), **(a.)** and metabolic activity **(b.)** of developing biofilms. Absorbance measured at 595 nm and 492 nm respectively for both the parental strains (SC5314 and BWP17) and mutant strains ($\Delta/\Delta fth2$ and $\Delta/\Delta fth2_II$) in the presence of 1 μ g/mL fluconazole are represented. The percentage inhibition values are the average of three biological and technical repeats. Error bars represent the standard deviations. p -value ≤ 0.05 was considered significantly different and is indicated by asterisks (*).

The resistance on $\Delta/\Delta fth2$ could be explained or further be supported by the metal handling and reprioritisation that the fungal pathogen (*C. albicans*) undergoes upon nutrient deprivation and fluconazole treatment reported by Hunsaker and Franz (2019b). The significance of *C. albicans*' capacity to modify and regulate metal homeostasis processes during fluconazole treatment is supported by multiple lines of evidence. Specifically, fluconazole treatment increases the cell's demand for transition

metals, and *C. albicans* meets this demand by adjusting metal transport to enhance to enhance cellular metal quotas. Fluconazole stress also alters how *C. albicans* utilises metals in the cell, as seen by the altered expression, activity, and synthesis of metalloenzymes and their cofactors (Hunsaker and Franz 2019b). Together, the relative increase in total Fe and corresponding decrease in labile Fe post fluconazole treatment reveal that the excess Fe is likely utilised by proteins, including cytochrome P450 enzyme, lanosterol 14 α -demethylase which is responsible for the biosynthesis of ergosterol (Henry et al. 2000; Whaley et al. 2016). Haem is a cofactor for the 14 α -demethylase (Cyp51) protein, the target of fluconazole, and is one of the most typical metalloporphyrins in nature (Correia et al. 2011). Correia and co-workers (2011) also showed that within 2 h of fluconazole treatment, *C. albicans* demonstrates the ability to upregulate the transcription of *ERG11*, an enzyme responsible for the expression of the Cyp51 protein (Correia et al. 2011). This would necessitate increased iron for haem production for this enzyme. Thus, these findings demonstrate a causal relationship between clinically significant drug resistance and this yeast's ability to maintain metal homeostasis.

Based on the findings of this study, it is clear that the susceptibility to fluconazole differs from that of its parental strain when *FTH2* is deleted, and this difference may be linked to the effect of iron on lipids, including membrane sterols. It is also worthy to note that the apparent sensitivity of *C. albicans* strains to fluconazole as a result of iron deficiency was observed in other tested drugs (Prasad et al. 2006), proving that this outcome is not only limited to fluconazole or *C. albicans* and was indeed as a result of iron availability, even in non-*albicans* strains such as *C. tropicalis*. Thus, suggesting that the influence of iron could be yet another novel determinant of multidrug resistance. It is however important to note that, although the disruption of the iron uptake systems

within *C. albicans* may have an impact on its overall fitness, fluconazole resistance displayed by $\Delta/\Delta fth2$ that is reported in this study may be a more complex phenomenon that does not entirely rely on iron availability.

2.5.3. *Pseudomonas aeruginosa* synergises with fluconazole against *C. albicans* mutant, $\Delta/\Delta fth2$

With the simultaneous colonisation of the two pathogens being linked to severe clinical outcomes, investigating antifungal efficacy during the co-infection and coculturing, (which may induce competition for iron) has become more recognised. As previously described, fluconazole susceptibility in polymicrobial biofilms was only evaluated for *C. albicans* BWP17 as well as its mutant, $\Delta/\Delta fth2$ which were co-cultured with *P. aeruginosa* PAO1 in the presence and absence of 1 $\mu\text{m}/\text{mL}$ fluconazole. As illustrated in **Figure 2.5.a** and **b** the bacterium had an influence on the fluconazole susceptibility of both strains. With the mutant strain exhibiting a higher percentage inhibition in comparison to its parental strain. A similar trend was also evident in the metabolic activity assay, where the $\Delta/\Delta fth2$ mutant showed 87% inhibition. While $\Delta/\Delta fth2$ demonstrated increased susceptibility to fluconazole in both biomass and metabolic assays, *C. albicans* BWP17 represented a rather different outcome with its biomass activity, showing a percentage inhibition lower than 40% and its metabolic activity percentage inhibition being lower than of the mutant, at 83 %.

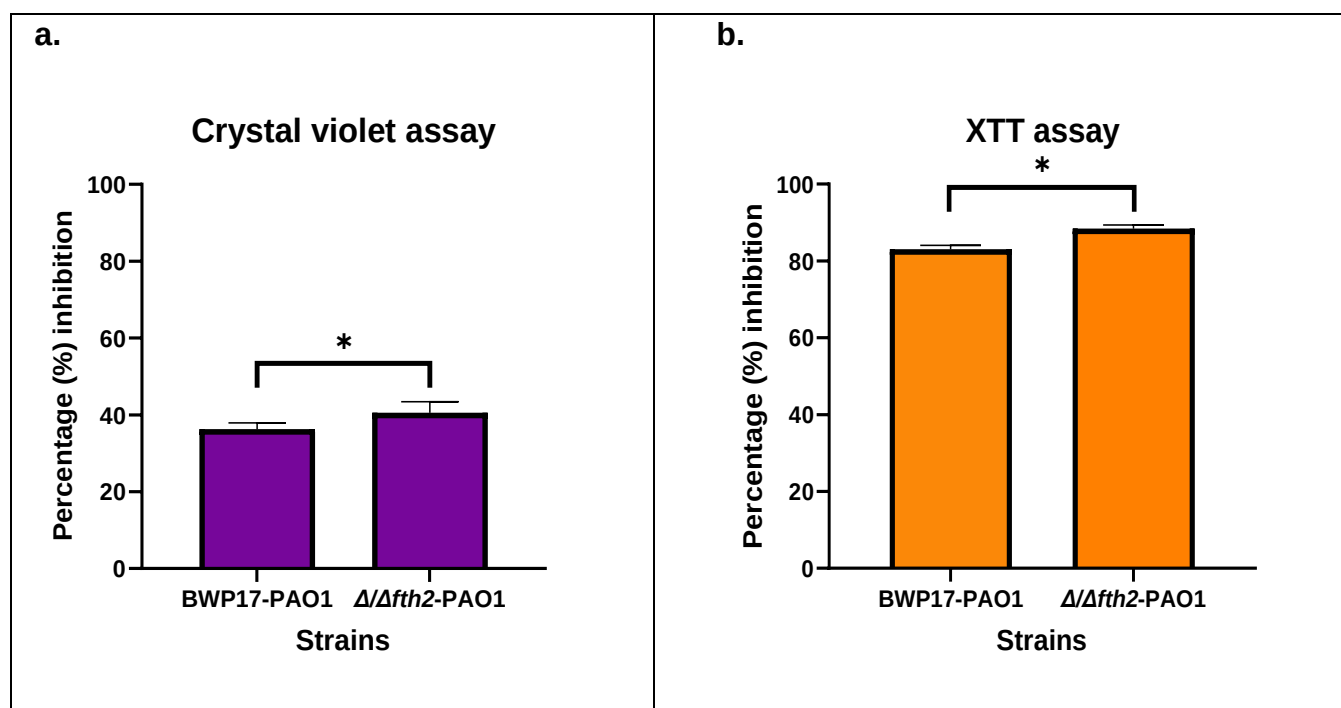


Figure 2.5. The effect of fluconazole susceptibility on polymicrobial biofilms.

Crystal violet and XTT assay were performed to determine biofilm biomass (a) and metabolic activity (b) following drug exposure. Absorbance measured at 595 nm and 492 nm respectively for both the parental strains (BWP17-PAO1) and mutant strain ($\Delta/\Delta fth2$ -PAO1) in the presence of 1 μ g/mL fluconazole are represented. The percentage inhibition values are the average of three biological and technical repeats. Error bars represent the standard deviations. p -value ≤ 0.05 and ≤ 0.005 were considered significantly different and are indicated by asterisks (*) and (**) respectively.

Previous work done by Hattab et al. (2022) established that the combination of *P. aeruginosa* and fluconazole was synergistic against *C. albicans*, thus making the drug fungicidal and resulting in the killing of *C. albicans*. Furthermore, the synergistic effect of fluconazole and *P. aeruginosa* against *C. albicans* elucidated to be partly as a result of iron competition between the two species or other previously identified mediators such as quorum sensing, production of toxins and filamentous growth.

In contrast to prior studies, our results present a different outcome in the parental strain of *C. albicans*, whose interaction with *P. aeruginosa* makes it less susceptible to fluconazole. It is however the iron-deficient mutant, $\Delta/\Delta fth2$ that shows a higher percentage inhibition of biomass production and metabolic activity. Based on these findings, it is clear that the interaction between *C. albicans* and *P. aeruginosa* needs to be further investigated. Thus, in light with these findings, it becomes apparent that fluconazole treatment is more effective in treating polymicrobial associated infections than mono-infections in both *in vivo* and *in vitro* studies.

2.5.4. Fe²⁺ iron uptake and mobilisation by *C. albicans*

In nature, free iron exists in its stable redox states, namely ferrous ion (Fe²⁺) and ferric ion (Fe³⁺) (Freeland-Graves and Proffitt 2002; Sánchez et al. 2017). Even so, understanding the behaviour of Fe²⁺ in living cells is considered more important than understanding that of Fe³⁺ because of the intracellular reductive environment, metal transporters, and the water solubility of Fe²⁺ (Freeland-Graves and Proffitt 2002; Sánchez et al. 2017). As such we investigated the localisation of Fe²⁺ using Biotracker Ferro Orange live cell dye. The Biotracker Ferro Orange is a fluorescent probe, that only detects labile Fe²⁺ ions (Tomita et al. 2019). The dye has no chelating ability and its interaction with Fe²⁺ reacts irreversibly, which is different from the detection principle of calcium-iron probes such as Fluo-3. As can be seen from the micrographs in **Figure 2.6.**, Fe²⁺ was detected within cells at both the early (6 h) and mature (48 h) phase of biofilm formation for both strains. Moreover, Fe²⁺ localisation in different parts of the cells can be seen and this was especially evident in *C. albicans* BWP17 biofilms (monomicrobial and polymicrobial).

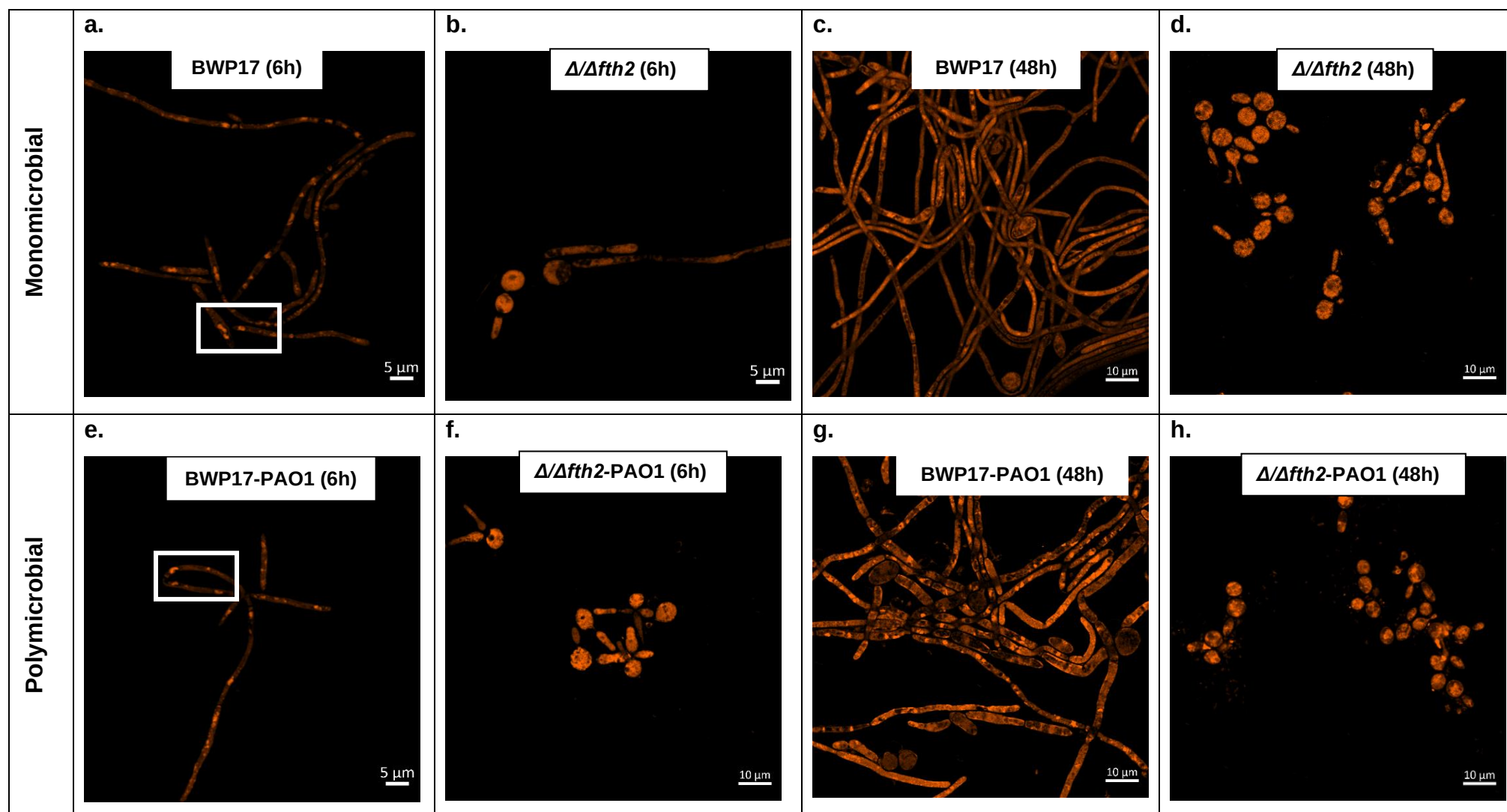


Figure 2.6. Representative confocal laser scanning micrographs of *C. albicans* mono- and polymicrobial biofilms. Biofilms were cultivated for 6 and 48 h respectively at 37°C. These biofilms were then stained with Biotracker Ferro Orange live cell dye, an Fe²⁺ specific staining solution to visualise iron localisation. **a)** BWP17 6-h monomicrobial biofilm; **b)** $\Delta/\Delta fth2$ 6-h monomicrobial biofilm; **c)** BWP17 48-h monomicrobial biofilm; **d)** $\Delta/\Delta fth2$ 48-h monomicrobial biofilm; **e)** BWP17 6-h polymicrobial biofilm; **f)** $\Delta/\Delta fth2$ 6-h polymicrobial biofilms; **g)** BWP17 48-h polymicrobial biofilm; **h)** $\Delta/\Delta fth2$ 48-h polymicrobial biofilm.

The presence of *P. aeruginosa* in polymicrobial biofilms however, does not show any influence on iron localisation of both strains. Although the localisation may be less evident in the mutant strain, this should be interpreted with care as this may be due to different microscopy settings. No significant movement or changes of Fe^{2+} from these localised areas could be detected during this experiment. Thus, this work is inconclusive in terms of the localisation of iron, thus further analyses may be necessary. In order to further investigate if iron is differentially released ($\text{Fe}^{2+}/\text{Fe}^{3+}$) from the vacuole stores in the case of the mutant or in the presence of *P. aeruginosa*, it will be well worth staining the cells with FM 4-64 [N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl)-hexatrienyl] pyridinium dibromide)], a vacuolar membrane staining dye (Vida and Emr 1995) and rhodamine dithenoyl hydrazide [(n-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)-n-(thiophene-2-carbonyl)thiophene-2-carboxamide], used for staining Fe^{3+} (Liu et al. 2020). This may also indicate a better understanding of the role of *FTH2* in iron mobilisation from the vacuole into the cytoplasm during iron limitations.

2.6. Conclusions

Owing to their wide range of activity, adequate safety profile and absorption, azoles have been the most often used class of antifungal drugs for treating both superficial and systemic fungal infections, for more than 20 years (Roemer and Krysan 2014; Robbins et al. 2016). Invasive mycoses are managed in large part, by the use of antifungal drugs, and their epidemiological characteristics are constantly changing. Despite efforts to standardise antifungal susceptibility testing through current guidelines, there are still limitations in establishing a clear relationship between *in vitro* susceptibility and a clinical response to treatment. It was demonstrated that the deletion of *FTH2* influenced fluconazole susceptibility both in planktonic cells and during biofilm formation. Contrary to what was expected, *C. albicans* BWP17 showed less fluconazole susceptibility in polymicrobial biofilms. This study also clearly demonstrated, that *C. albicans*, $\Delta/\Delta fth2$ polymicrobial biofilms become relatively more sensitive to fluconazole, possibly as a result of synergism exhibited by the Fluconazole-*P. aeruginosa* combination. These findings prompt a re-evaluation of the effect of iron availability on fluconazole susceptibility and further research into interspecies interactions are needed. This also paves a new way in understanding how antifungals are utilised during co-infections. Although it was demonstrated localisation of Fe^{2+} within the yeast cells, it would be imperative to also look at iron (Fe^{3+}) localisation with the vacuole. This will lead to a better understanding of where both forms of iron are localised within the cell. In addition to the possible link between iron availability and fluconazole susceptibility, the role of iron in lipid homeostasis is yet unexplored. As a result, the next chapter's focus shifts to the effect of iron availability on lipids of *C. albicans*.

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Chapter 3

The effect of *FTH2* deletion on lipids of *Candida albicans*

3.1. Abstract

The most encountered mechanisms of antifungal resistance include alterations in ergosterol biosynthesis pathways. This is as a result of the overexpression of *ERG11* gene as well as point mutation in target genes, which lead to a reduced affinity to azoles. With the increased occurrence of antifungal resistance being reported, the involvement of lipid homeostasis has since gained attention. This is because, the overall status of the membrane lipids serves as important determinant of drug susceptibility. Even more, the relationship between lipid metabolism and iron homeostasis may influence the pathogen's cellular response and susceptibility to certain drugs. To explore this interaction, lipid droplets as well as the lipidome of *Candida albicans* BWP17 (parental strain) and $\Delta/\Delta fth2$ (deletion mutant strain) with decreased fluconazole susceptibility were studied. Lipid droplets (LDs) were stained with BODIPY 493/503 and visualised using confocal laser scanning microscopy. The lipid content of the two strains was also quantified. This was performed both in the absence as well as presence of *Pseudomonas aeruginosa* PAO1 in order to assess the effect of possible competition for iron on LDs. In addition, untargeted lipidomic analyses in the absence and presence of pyoverdine (an iron chelator secreted by *P. aeruginosa* PAO1) was performed. The LD size and the lipid content differed between the parental strain and its mutant strain. Interestingly, the presence of *P. aeruginosa* PAO1 negatively influenced LD formation for both strains. Phosphoglycerides, sphingolipids, diacylglycerols and triacylglycerols were identified and quantified along with their molecular lipid species. Our analysis highlighted several marked changes in lipid compositions between the parental and mutant strain of *C. albicans*. Addition of pyoverdine led to a decrease in differentially produced lipid species. As a result, this

provides additional information regarding the interplay between lipid metabolism, iron homeostasis and drug resistance in *C. albicans*.

Key words: *Candida albicans*, Antifungal resistance, Lipid metabolism, Lipid droplets, Iron homeostasis

3.2. Introduction

Among the most commonly described mechanisms, those associated with lipids have emerged as one of the factors contributing to the acquired multidrug resistance (MDR) in *Candida albicans* (Mukhopadhyay et al. 2002). For example, studies have shown how an imbalance in ergosterol and sphingolipid (SL) levels may result in a reduced sensitivity of the yeast cells to antifungals (Rella et al. 2016). This is because both ergosterol and sphingolipids serve as important components of the fungal cell membrane (Rella et al. 2016). However, the interaction between ergosterol and sphingolipids and their reduced concentrations have also been linked to enhanced drug susceptibility (Mukhopadhyay et al. 2004). The increased interest in studying lipids is also linked to the role they play in regulating fungal pathogenicity and their contribution to other virulence mechanisms, such as biofilm formation and the release of extracellular vesicles (Rella et al. 2016). The ability of *C. albicans* to undergo morphological switching from yeast to hyphae or to form biofilms has been associated with its ability to evade the host immune response and cause pathogenesis (Almeida et al. 2008; Almeida et al. 2009). As such, expression profiling of the yeast's biofilms has revealed differential regulation of lipid biosynthesis genes. Moreover, lipidomic studies of planktonic cells and biofilms confirmed a significant remodelling of glycerophospholipids (GPLs) and SL composition within the two forms of the yeast (Lattif et al. 2011). Thus, suggesting the crucial role that lipids play in the development of hyphae and the modulation of biofilms in *C. albicans*.

Apart from the challenge of antifungal drug resistance mediated by single-species biofilms, the interaction between *C. albicans* and other species in polymicrobial biofilms serves as another conundrum to treating biofilm associated infections. The multispecies interactions (synergistic or antagonistic) have been reported to be

causing many hospital-acquired bloodstream infections and oral cavities, with nosocomial bloodstream infections contributing to approximately 27-56% of these infections (Pulimood et al. 2002; Klotz et al. 2007; Khan et al. 2017). Secretion of signalling lipid molecules, such as prostaglandins, by *C. albicans* plays a role in biofilm development and most importantly, in promoting mixed-biofilm formation with *Staphylococcus aureus* (Kim et al. 2013).

Similar to iron, lipid homeostasis is highly regulated within the host and the yeast *C. albicans*, in order to ensure the normal functioning of the cell. The regulation of lipid homeostasis involves maintaining a balance between the synthesis, storage and utilisation of lipids. All eukaryotic cells have the ability to form and store their lipids within lipid droplets (LDs). LDs are intracellular organelles that serve as storage compartments for neutral lipids (Radulovic et al. 2013). These neutral lipids include triacylglycerols (TAGs) and sterol esters (SEs) which make up the majority of the small, spherical yeast LDs, that may also contain a trace quantity of phospholipids and proteins (Leber et al. 1994). **Figure 3.1.** summarises LD biogenesis and illustrates how LDs are derived from the endoplasmic reticulum (ER). LDs do not only store and supply energy for the cell but can also function in a diverse number of cellular processes including RNA storage and protein-protein complex, degradation of protein molecules as well as microbial pathogenesis (McLauchlan 2009). However, these organelles do not function independently. As conditions change, they react to impulses started either on the cell surface or in the cytoplasm (McLauchlan 2009). The roles that LDs play in *C. albicans* pathogenesis have gained a significant amount of attention over the past few years. This is because during infection of the host cells, LDs accumulate to serve as storage depots for nutrients and survival within the host environment (Brink et al. 2021). In addition, LDs may aid in immune evasion of the

host as well as drug resistance by sequestering antifungal compounds away from the target (Brink et al. 2021).

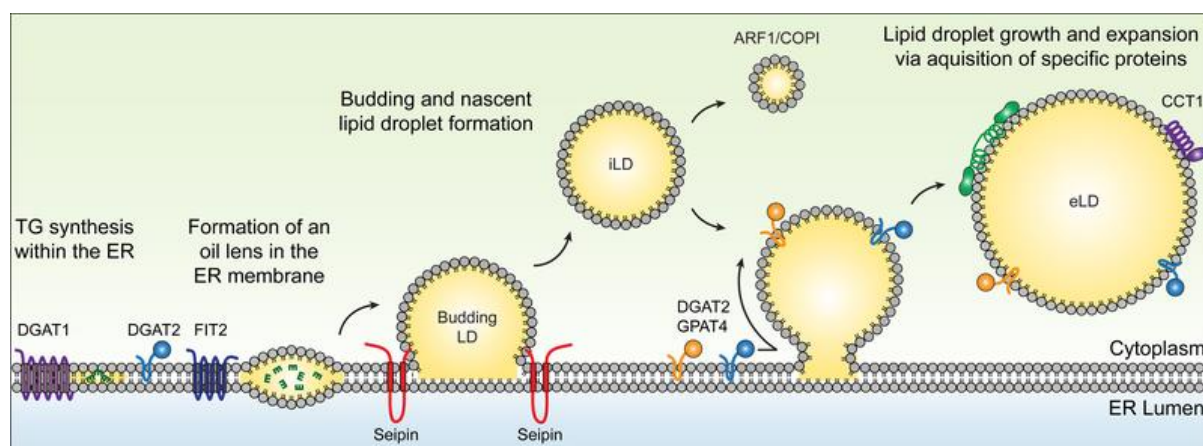


Figure 3.1. Lipid droplet biosynthesis. In yeast, LDs are derived from the endoplasmic reticulum (ER), where they can either remain attached or bud off and separate entirely from the ER upon maturation. A typical cycle of lipid droplet formation begins with the synthesis of TGs (also known as TAG) within the ER by the two central DGAT (diacylglycerol acyltransferase) enzymes: DGAT1 and DGAT2. This is followed by the formation of an oil lens in the ER membrane as a result of the accumulation of TG within the ER bilayer. Sufficient accumulation of the TG results in the budding of the LDs into the cytosol where in yeast, a substantial amount of the LDs remains attached to the ER, with FIT2 regulating the directionality of the budding reaction. Succeeding to the initial LDs, LDs grow and expand by acquiring specific proteins. Where a fraction of these newly formed LDs is converted to a subpopulation of LDs namely, expanding LDs (eLDs) which later give rise to two populations: initial LDs (iLDs) and eLDs in cultured cells (Walther et al. 2017).

Although the role of *FTH2* in lipid homeostasis is not well studied and fully understood, its influence on iron availability could indirectly have an impact on lipid metabolism and homeostasis. A large number of biochemical processes including lipid metabolism within the cell require iron-dependent enzymes for their synthesis, storage and utilisation (Jordá et al. 2020). To date, studies pertaining to how lipids contribute to biofilm formation in yeast is limited, when compared to bacterial biofilm development and cell-surface attachment. Thus, the aim of this chapter is to study the role of the deletion of *FTH2* in lipid metabolism in *C. albicans* in order to gain an understanding regarding the link between lipid homeostasis, iron transport and fluconazole susceptibility.

3.3. Materials and Methods

3.3.1. Strains used in this study

A previously constructed *C. albicans* *FTH2* deletion mutant ($\Delta/\Delta fth2$) and its parental strain *C. albicans* BWP17 (**Table 1**) were obtained from the yeast culture collection at the University of the Free State, Bloemfontein, South Africa. The yeasts were revived and maintained on Yeast Malt extract (YM) agar plates (3g/L malt extract, 3g/L yeast extract, 5g/L peptone, 10g/L glucose, 16g/L agar) at 30°C for 24 h and preserved at 4°C for all studies conducted. To cultivate the bacterium, an aliquot of *P. aeruginosa* PAO1 was inoculated in 5 mL nutrient broth (1g/L malt extract, 2g/L yeast extract, 5g/L peptone and 8g/L sodium chloride) and incubated in a shake-incubator for 24 h at 37°C. The bacterial strain, *P. aeruginosa* PAO1, was provided by Professor Robert Hancock (Department of Microbiology and Immunology at the University of British

Columbia was maintained on nutrient agar (1g/L malt extract, 2g/L yeast extract, 5g/L peptone, 8g/L sodium chloride and 20g/L agar) at 37°C. A 50% glycerol stock for each strain was also prepared and stored at -80°C for future use.

Table 1. Strains used in this study

Name	Genotype	References
<i>C. albicans</i> BWP17	<i>ura3::λimm434/ura3::λimm434iro1::λimm434/iro1::λimm434his1::hisG/his1::hisGarg4::hisG/arg4::hisGRPS1/rps1::(URA3-HIS1-ARG4)</i>	(Wilson et al. 1999)
<i>C. albicans</i> $\Delta/\Delta fth2$	<i>fth2Δ::FRT/fth2Δ::ARG4/CIP10 URA3</i>	(Mamouei et al. 2017)
<i>P. aeruginosa</i> - PAO1	Wild type clinical strain	Professor Hancock from the Department of Microbiology and Immunology at the University of British Columbia

3.3.2. Microscopic visualisation of lipid droplets

In order to visualise lipid droplets within mono- and polymicrobial biofilms, yeast cells were inoculated in 10 mL YPD broth (10 g/L yeast extract, 20 g/L, peptone, 20 g/L glucose) for 24 h at 30°C. The bacterium, *P. aeruginosa* PAO1 was inoculated in 5 mL nutrient broth (1g/L malt extract, 2g/L yeast extract, 5g/L peptone and 8g/L sodium

chloride) and incubated at 37°C for 24 h. Following incubation, the cells were washed three times with Phosphate Buffered Saline [PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride (pH 7.4) (Sigma-Aldrich, St. Louis, MO, USA)] at 5000 rpm (4°C) for 5 min and standardised to a final concentration of 1.0×10^6 cells/mL and 0.05 at an OD reading of 600 nm respectively, in RPMI-1640 medium (Sigma-Aldrich, USA). The cells were then cultivated in 6-well plates containing cover slips, for 6 h and 48 h at 37°C respectively, in RPMI-1640 medium (Sigma-Aldrich, USA) to allow mono- and polymicrobial biofilm formation. At the time-point of interest, the biofilms were subsequently washed with 200 μ L of sterile Phosphate Buffered Saline PBS to remove any non-adherent cells. Biofilms were then stained with 2 μ M BODIPY 493/503 staining solution which stains LDs green (Qiu and Simon 2016) and incubated for 20 min at 37°C in the dark to maintain the stability and reliability of the stain. Following incubation, the biofilms were washed twice with 200 μ L of PBS to remove any unbound excess dye. Following this, one drop of calcofluor white (Sigma-Aldrich) stain (a chitin and cellulose blue staining dye) (Hageage and Harrington 1984; Monheit et al. 1984) and one drop of 10% potassium hydroxide solution were added to the biofilms and incubated for 1 min on the bench away from the light. The excess stain was again washed with PBS and the coverslips were transferred to glass slides to visualise the stained biofilms; the samples were sent to the centre for microscopy. The samples were examined using confocal laser scanning microscopy (Zeiss LSM 900 airyscan 2), with the BODIPY 493/503 fluorescing at an excitation of 493 nm and an emission of 503 nm and calcofluor white at an excitation of 355 nm and an emission of 433 nm. The confocal images were visualised using Zeiss ZEN 3.2 (blue edition) system.

3.3.3. Quantification of lipid content

To facilitate the quantification of neutral lipid content within mono- and polymicrobial biofilms, yeast and bacterial cells were inoculated and standardised as previously described in section 3.3.2. These cells were subsequently washed with 10 mL sterile PBS at 5000 rpm (4°C) for 5 min and standardised to a final concentration of 1.0×10^6 cells/mL and 0.05 at an OD reading of 600 nm respectively, in RPMI-1640 medium (Sigma-Aldrich, USA). The cells were then cultivated in 96-well black plates (Costar® 96-Well Black Polystyrene Plate) at 37°C for 6 and 48 h respectively, in RPMI-1640 medium. The formed biofilms were washed twice with 200 µL PBS, stained with 50 µL of the 2 µM BODIPY 493/503 and incubated for 20 min at 37°C in the dark to maintain stain stability. The unbound dye was removed by washing biofilms twice with 200 µL sterile PBS. The fluorescence was measured at an excitation of 493 nm and an emission of 503 nm using a microplate fluorometer and luminometer [Fluoroskan Ascent® FL & Fluoroskan Ascent® (Thermo Fischer Scientific, Microplate Instrumentation)]. To normalise the fluorescence data against biomass, a crystal violet assay was performed as previously described (chapter 2, section 2.3.6.), on the same biofilms and absorbance was measured at 595 nm using a 96-well EZ Read 800 Microplate Reader (Biochrom, UK). The lipid content was denoted as the ratio of the **Relative Fluorescence Unit (RFU) / crystal violet assay**.

3.3.4. Untargeted lipidomic analysis of *C. albicans*

To evaluate the influence of *FTH2* mediated iron transport on the lipidome of *C. albicans* both the parental strain (*C. albicans* BWP17) and the mutant ($\Delta/\Delta fth2$) strain were inoculated in 10 mL YPD broth (10 g/L yeast extract, 20 g/L, peptone, 20 g/L

glucose) for 24 h at 30°C. These were subsequently washed three times with sterile PBS (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride (pH 7.4) (Sigma-Aldrich, St. Louis, MO, USA)] at 5000 rpm (4°C) for 5min. The cells were then cultivated for 48 h at 37°C in petri dishes containing RPM-1640 medium, in the absence and presence of 1 mg/mL pyoverdine, an extracellular iron chelator to allow biofilm formation. The biomass of each sample was then harvested by scrapping off the biofilms and centrifuging at 5000 rpm (4°C) to obtain a pellet. The pellet was then collected into 3.6/4 mL cryogenic vials and stored at -80°C until further analysis. The collected biomass of each sample was subjected to untargeted lipidomic analyses using a commercial platform (Creative Proteomics, <https://www.creative-proteomics.com>) to qualify and quantify all the various (up to 3100) lipids in them. This experiment was performed in four biological replicates per strain.

To perform the analysis, the samples were thawed on ice and 100 mg of the sample was transferred into a new tube. The samples were then homogenized with 600 µL of 70% ethanol, vortexed for 1 min and kept for 30 min at 4°C. 700 µL of chloroform was subsequently added to the samples and shaken for 5 min and kept for 30 min at 4°C. To collect the chloroform layers, samples were centrifuged at 12000 rpm for 10 min at a temperature of 4°C. The collected layers were then transferred to a new tube, where they were further dried under nitrogen. Following this, the dried extract was resuspended in 400 µL of isopropyl alcohol : methanol (1:1, v/v). Lastly, the samples were centrifuged for 15 min at 12000 rpm, at 4°C to collect the supernatant for LC/MS analyses. Samples are injected into a chromatographic column and the lipids are separated according to their chemical properties. The eluted samples are then subjected to electron spray ionisation (ESI). Both the negative and positive mode ESI's separation of compounds was by AQUITY Ultra-Performance Liquid Chromatography

(UPLC) in conjunction with AB SCIEX 5500, which is a high-performance instrument used to precisely analyse sensitive compounds in complex mixtures. This LC system also comprised of AQUITY UPLC BEH Amine (100 x 2.1 mm x 1.7 μ m). ESI in the negative mode consists of a mobile phase that is composed of solvent A and B with a gradient elution (0-3 min, 0%B; 3-7 min, 0%-50%B; 7-9 min, 50%-70%B; 9-11 min, 70%-100%B; 11- 14 min, 100%B; 14-15 min, 100%-0%B; 15-17 min, 0%B). The flow rate of the mobile phase in the negative mode is 0.1mL/min and the column are maintained at a temperature of 45°C with the sample manager at a low temperature of 4°C. In positive mode ESI, the flow rate of the mobile phase is 0.3 mL/min and is maintained at the same temperature as that of the negative mode with its sample manage temperature also set at 4°C. This mobile phase is composed of solvent A containing Water: Methanol: Acetonitrile=1:1:1, V/V/V, 5 mM CH₃COONH₄ and solvent B (5 mM CH₃COONH₄ isopropanol solution) with a gradient elution (0-0.5 min, 0%-20%B; 0.5-1.5 min, 20%-40%B; 1.5-3 min, 40%-60%B; 3-13 min, 60%-98%B; 13-13.1 min, 98%-20%B; 13.1-17 min, 20%B) (Creative Proteomics, <https://www.creative-proteomics.com>).

Table 2. The MS parameters ESI of both the negative and the positive mode are as follows:

	Negative mode ESI	Positive mode ESI
Curtain Gas	35 arb	35 arb
Collision Gas	9 arb	7 arb
Ion Spray voltage	4.5 KV	5.2 KV
Temperature	450 °C	450 °C
Ion Source Gas 1	50 arb	55 arb
Ion Source Gas 2	50 arb	55 arb

Units:

arb = arbitrary

KV = kilovolts

Following ionisation, the selected precursor ions undergo fragmentation, thus generating a mass spectrum for each of the lipid species collected from the samples. The raw data (four biological repeats per sample group) acquired for lipid analyses was aligned using the Lipid Search software (Thermo), which identifies all measurable lipids using the molecular fragmentation query language (MFQL). This also allows unconstrained data analysis that may be introduced by the traditional dependence on predefined spectral databases.

3.4. Statistical analyses

These experiments were conducted in at least biological and technical triplicates. The averages and standard deviations were calculated. The data of different groups were compared and evaluated for significance using the student's t-test. p -values < 0.05 , 0.01 and 0.005 were considered statistically significant and indicated by an asterisk (*, ** and ***) respectively on the graphs.

Ions from the ESI- or ESI+ were merged and imported into the SIMCA-P program (version 14. 1) for a multivariate analysis. In order to visualise data and identify outliers, a Principal Component Analysis (PCA) is firstly used as an unsupervised method. This is then followed by supervised regression modelling on the data set using Partial Least Squares Discriminant Analysis (PLS-DA) or Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) to identify the potential biomarkers. These biomarkers were then filtered and confirmed by combining the results of the VIP values (1,5), t-test ($p < 0,05$) and fold change (FC) > 2 .

3.5. Results and Discussions

3.5.1. Deletion of *FTH2* influences lipid droplet size and number

The influence of iron availability on LD formation was first evaluated using confocal laser scanning microscopy. Biofilms were stained as previously described in section 3.3.2. As depicted in **Figure 3.2**, LDs were successfully stained and changes in the size and number of LDs formed were evident upon deletion of *FTH2* as well as during interspecies competition. When comparing lipid droplet production in monomicrobial biofilms between the two strains, it was apparent that *C. albicans* BWP17 (parental

strain) formed many small lipid droplets (especially at 48h, **Figure 3.2.c**), while the $\Delta/\Delta fth2$ mutant strain contained fewer larger, LDs per cell (**Figure 3.2.b and d**). In terms of polymicrobial biofilms, both strains displayed similar patterns i.e., the production of small lipid droplets that are clustered (fused) together, thus resulting in irregular morphologies at 6 h (**Figure 3.2.e and f**). In 48-h polymicrobial biofilms, these small LDs were only seen in *C. albicans* BWP17, while the mutant still produced large LDs at 48 h (**Figure 3.2.h**).

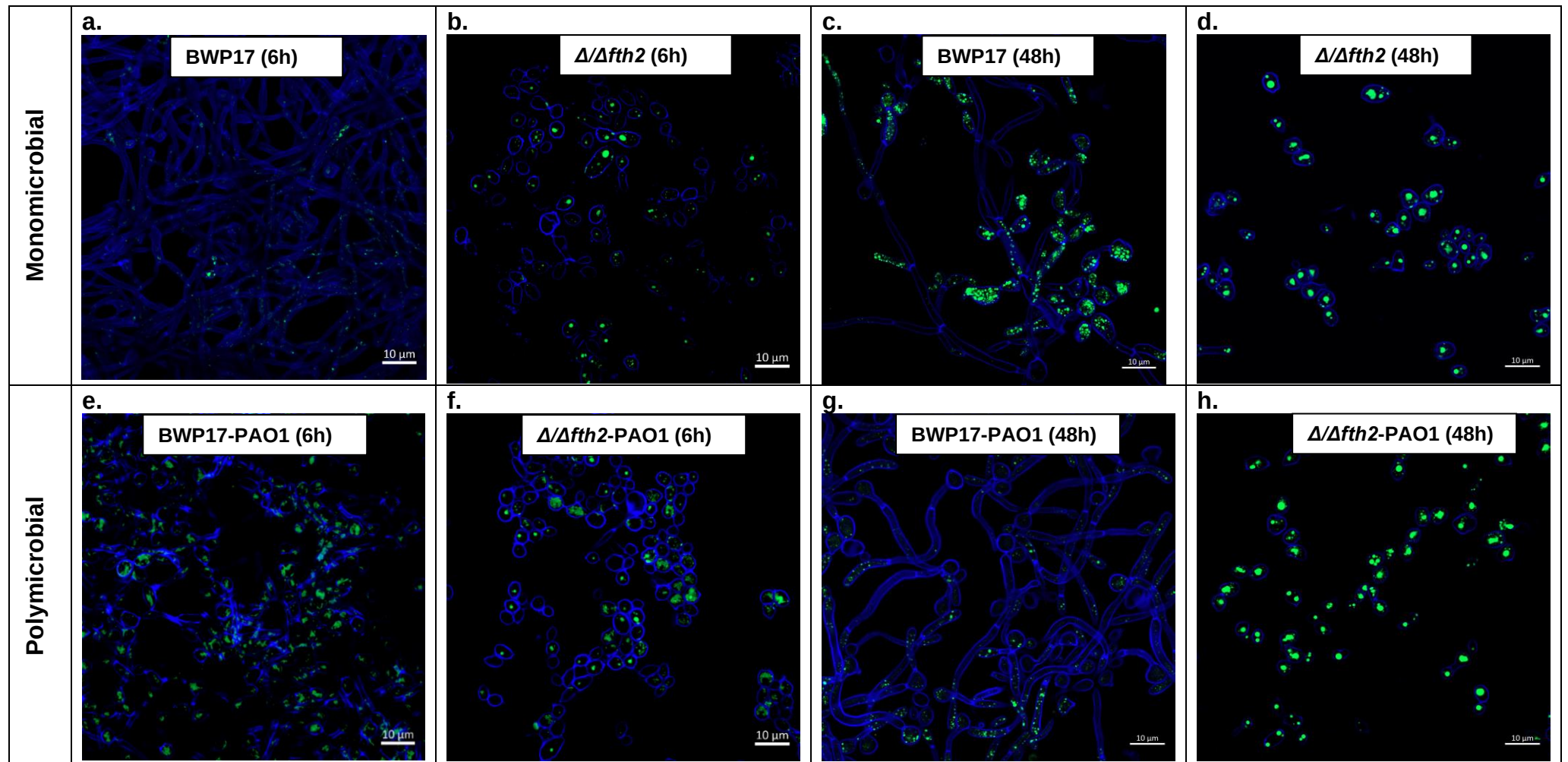


Figure 3.2. Representative confocal laser scanning micrographs of *C. albicans* mono- and polymicrobial biofilms. Biofilms were cultivated for 6 and 48 h respectively at 37°C. Biofilms were stained with calcofluor white (a blue chitin or cellulose staining dye) and BODIPY 493/503 (a green stain) to visualise the lipid droplet formation. **a)** BWP17 6-h monomicrobial biofilm; **b)** $\Delta/\Delta fth2$ 6-h monomicrobial biofilm; **c)** BWP17 48-h monomicrobial biofilm; **d)** $\Delta/\Delta fth2$ 48-h monomicrobial biofilm; **e)** BWP17 6-h polymicrobial biofilm; **f)** $\Delta/\Delta fth2$ 6-h polymicrobial biofilms; **g)** BWP17 48-h polymicrobial biofilm; **h)** $\Delta/\Delta fth2$ 48-h polymicrobial biofilm

It must be noted that this microscopic analysis was not quantitative, so in order to obtain quantitative data regarding the effect of *FTH2* deletion on lipid content a quantitative assay based on the fluorescence of LDs normalised against biomass was performed. A similar approach was also followed by Bozaquel-Morais and co-workers (2010), where the relative fluorescence measurements were normalised against the number of cells. As such, we measured fluorescence and quantified the biofilm biomass using crystal violet.

As depicted in **Figure 3.3**, the lipid content of monomicrobial biofilms for both strains increased over time, with *C. albicans* BWP17 displaying the highest content at both time points. Interestingly, deletion of *FTH2* significantly impacted the initial lipid content of the cells, showing significantly lower production in lipid content at 6 h. However, the most striking result was the reduction in lipid content of polymicrobial biofilms for both *C. albicans* strains, which were incapable of accumulating lipids over the 48 h period. In the polymicrobial scenario, deletion of *FTH2* did not have a further effect.

This decrease in lipid content for both strains in polymicrobial biofilms was unexpected owing to the fact that one of the contributing factors to lipid accumulation is nutrient stress (van Zutphen et al. 2014). Thus, we expected to see an increase in lipid content of the $\Delta/\Delta fth2$ mutant, either in monomicrobial or polymicrobial biofilms. These results indicate the complex nature of the effect of iron homeostasis as well as the presence of *P. aeruginosa* on lipid metabolism in *C. albicans* and requires further investigation.

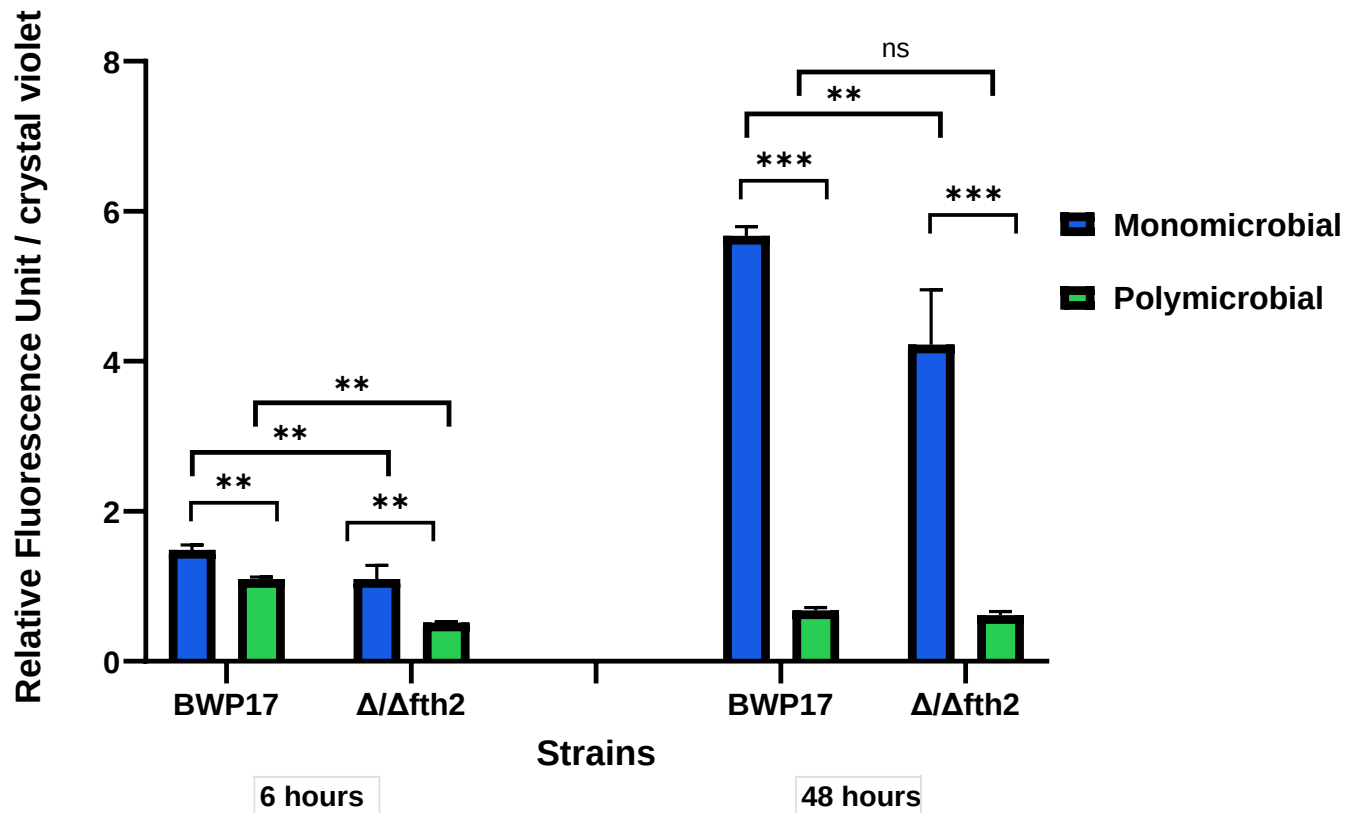


Figure 3.3. Quantification of lipid content. Lipid content of *C. albicans* strains, BWP17 and $\Delta/\Delta fth2$ mono- and polymicrobial biofilms at 6 and 48 h quantified using BODIPY 493/503 fluorescence normalised with crystal violet staining. Values represent the average of at least 6 values and the standard deviation is indicated by the error bars. *p*-values were determined by an unpaired student's t-test with ** representing $p \leq 0,01$, *** representing $p \leq 0,001$ and **ns** representing non-significant.

One of the most universally observed adaptive responses of yeast cells as a result of nutrient stress is metabolic remodelling and increased LD production (Philpott et al. 2012; van Zutphen et al. 2014). Because *C. albicans* may also store excess neutral lipids in LDs as a means to maintain lipid homeostasis (Rahman et al. 2021). Thus, this may explain the formation of bigger LDs evident in the mutant. Moreover, the delayed hyphal formation displayed by the $\Delta/\Delta fth2$ mutant could possibly be as a result of the slow growth caused by iron limitations, which may have an influence of the overall lipid metabolism.

The number, size and distribution of LDs, however, is highly dependent on the physiochemical state of a cell. Some of the key factors that drive LD production include scarcity of essential nutrients that may be associated with energy metabolism or an imbalance in the cellular energy homeostasis (Rahman et al. 2021). For instance, under normal growth conditions where the cells experience little to no nutrient stress, yeast cells may possess very few LDs that are smaller in size. However, upon nutrient deprivation, there is a sudden burst in LD production, that appear to be bigger in size (Wang et al. 2014).

De Bortoli and co-workers (2018) published a paper highlighting the relationship between iron homeostasis, LD formation and how an imbalance in iron availability influenced lipid pathways, mainly the accumulation of LDs. These authors showed that there was an accumulation of LDs in the cytoplasm and an expansion of the ER of breast cancer cells as a result of iron depletion. These findings led to other authors such as Tirinato et al. (2021) further investigating the role of LD formation and its potential connection to iron metabolism on radioresistant cancer cells isolated from different organs. Their research showed upregulation of *FTH1* (encoding ferritin heavy chain – the main intracellular iron storage protein) in all radioresistant cancer cell lines.

Silencing of *FTH1* caused a significant reduction in LD production, which led to an increase in radiosensitivity relative to the controls. Furthermore, overexpression of *FTH1* or treatment with a high-affinity Fe³⁺ iron chelator, Deferoxamine, was able to restore the LD pool as well as radio resistance.

All this suggests that not only starvation but disruption in cellular homeostasis or metabolism may influence LD formation. Moreover, this also proposes that there may be a common point where all the stress related pathways converge and connect with pathways associated with LD production. This may also explain the observed differences in LD size and lipid content in our experiments as both deletion of *FTH2* as well as the presence of *P. aeruginosa* may influence how *C. albicans* stores iron, which could change the expression to genes associated with iron storage to efficiently manage the available iron. This alteration could lead to changes in the intracellular iron levels available within the cell thus affecting LD production. In order to further investigate this, the effect of *FTH2* deletion as well as competition for iron on the lipidome of *C. albicans* was studied.

3.5.2. Overview of lipidome results

Our shotgun lipidome analysis data revealed the presence of at least 2080 differentially produced lipid species, from the following lipid classes: cholesterol esters (CE)¹, ceramides (CER), diacylglycerol, dihydroceramide (DCER), free fatty acids (FFA), hexosylceramides (HexCER), lactosylceramide (LacCER), lysophosphatidylcholine (LPCs), lysophosphatidylethanolamine (LPE), phosphatidic acid (PA) (bulk species), phosphatidylcholine (PC), phosphatidylethanolamine (PE),

¹ Lipid species designated as CE may not be correctly identified and require further investigation.

phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM) and triacylglyceride (TAG).

When conducting untargeted lipidomic analysis, the goal is to quantify all the detectable features from mass spectrometry (MS) and lipids are identified using LC-MS data. In doing so, this helps us to understand the lipid metabolism and analyse alterations in lipid metabolism. The analytical workflow encompasses annotation of lipids and interpreting their names, statistical analyses, preprocessing of the data as well as evaluating the structural and functional aspects of the lipids. To get the full spectrum of the lipids, two sets of data were generated, with one belonging in the negative mode while the other is in the positive mode and the lipids were profiled based on ionisation. In mass spectrophotometry, different ionisation modes are used to analyse the different sample groups. The different modes of ionisation influence how ions are generated and detected in MS (Singh et al. 2010; Prasad and Singh 2013). The positive ion mode is generally used to analyse small molecules with a single functional group which give singly charged ions as a result of an addition of a proton. Samples are ionised to produce positively charged ions (cations). In the negative mode, this can result in the loss of a proton which leads to the sample producing negatively charged ions (anions) after ionisation (Singh et al. 2010; Prasad and Singh 2013).

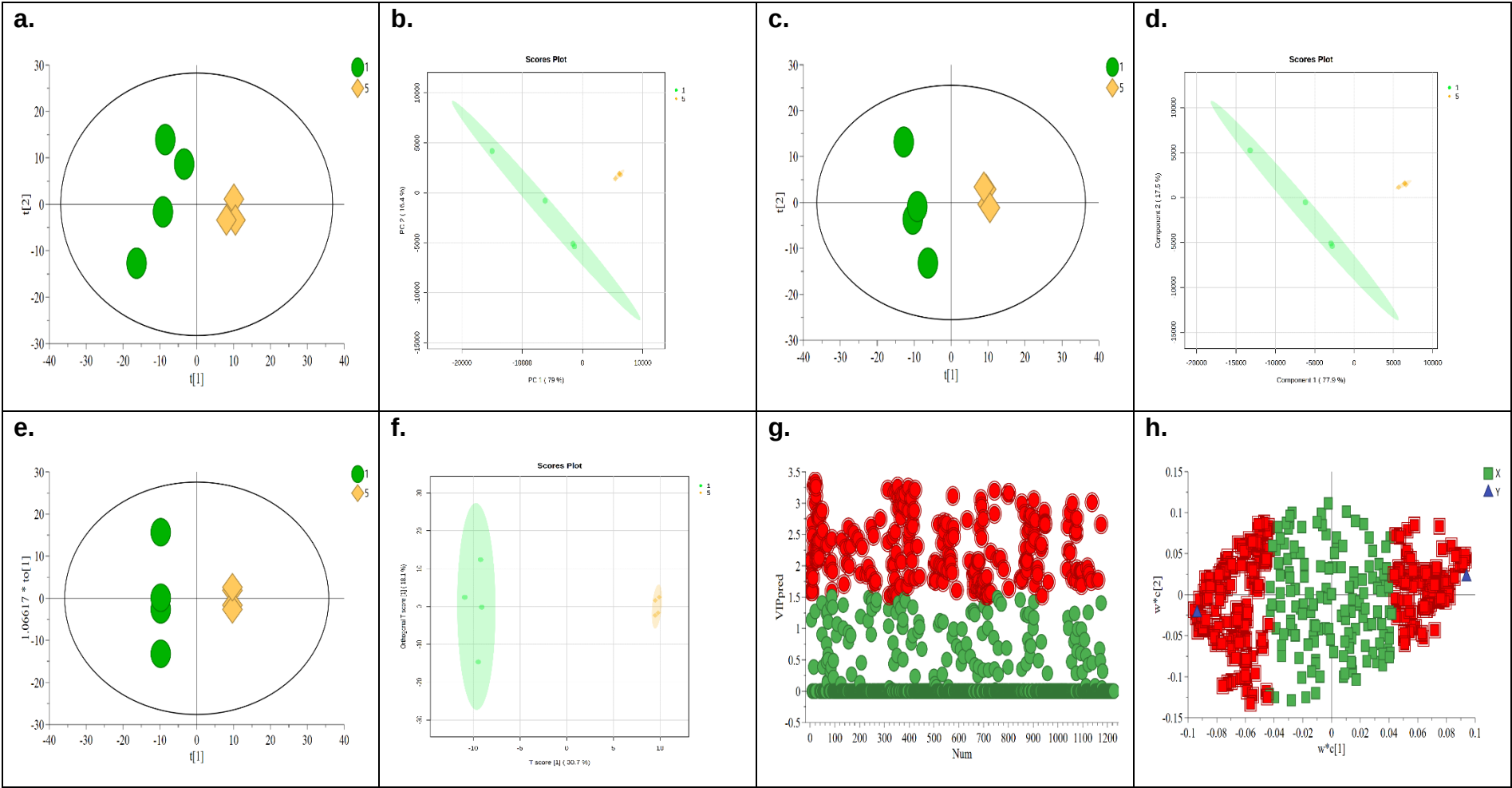
In order to perform lipid profiling, the principal component analysis (PCA) was used to investigate all observations. This helps to emphasise variations and bring about strong patterns between the different data sets analysed (Singh et al. 2010; Singh and Prasad 2011; Kokoszka and Kulik 2023). PCA plots allow a visual assessment on how the samples can be grouped based on the differences and similarities between them. This is then followed by a partial least squares-discriminant analysis (PLS-DA) or its

extension orthogonal projections to latent structures-discriminant analysis (OPLS-DA) which compare lipid changes in the two different groups analysed and helps eliminate any non-specific effects as well as confirm biomarkers (Sampaio and Calado 2021; Su and Li 2023). The significantly changed lipids are filtered based on their variable importance in projection (VIP) values greater than 1.5 ($VIP > 1.5$). VIP is a mathematical or statistical component used to identify the significance of each lipid variable in differentiating between the sample groups (Mahieu et al. 2023). Thus, a high VIP value indicates that a specific lipid variable plays a more important role in separating the groups while a low score proposes that the variable has a smaller role in explaining the differences between the sample groups. Examples of the analyses described above, comparing the parental strain with the mutant, are provided in **Figure 3.4.** As shown in the PCA plots (**Figure 3.4.a and b**) there is an unclear grouping trend exhibited between the two sample groups. PLS-DA (**Figure 3.4.d**) or OPLS-DA (**Figure 3.4.e**) showed a clear separation of the two groups.

Following this, lipid ions which showed significant changes between the two groups are filtered out based on their VIP values greater than 1.5, fold change (FC) >2.0 and p -values < 0.05 , and visualised on a volcano plot as shown in **Figure 3.5.** This highlights the lipid species that were significantly increased or decreased. Finally, these lipids are indicated on the heat maps generated using hierarchical clustering analysis (HCA) (**Figure 3.6.**). A hierarchical cluster analysis was performed using the complete algorithm of the program Cluster 3.0 (Stanford university) and visualised using Treeview (Stanford University). The mean values of the lipid contents from all the four biological replicates are used to calculate the lipid ratio and are normalised after log transformations of the data. These analyses were done for all the conditions

and the rest are provided in the supplementary data section together with the tables containing the quantitative data.

Negative mode analysis



Positive mode analysis

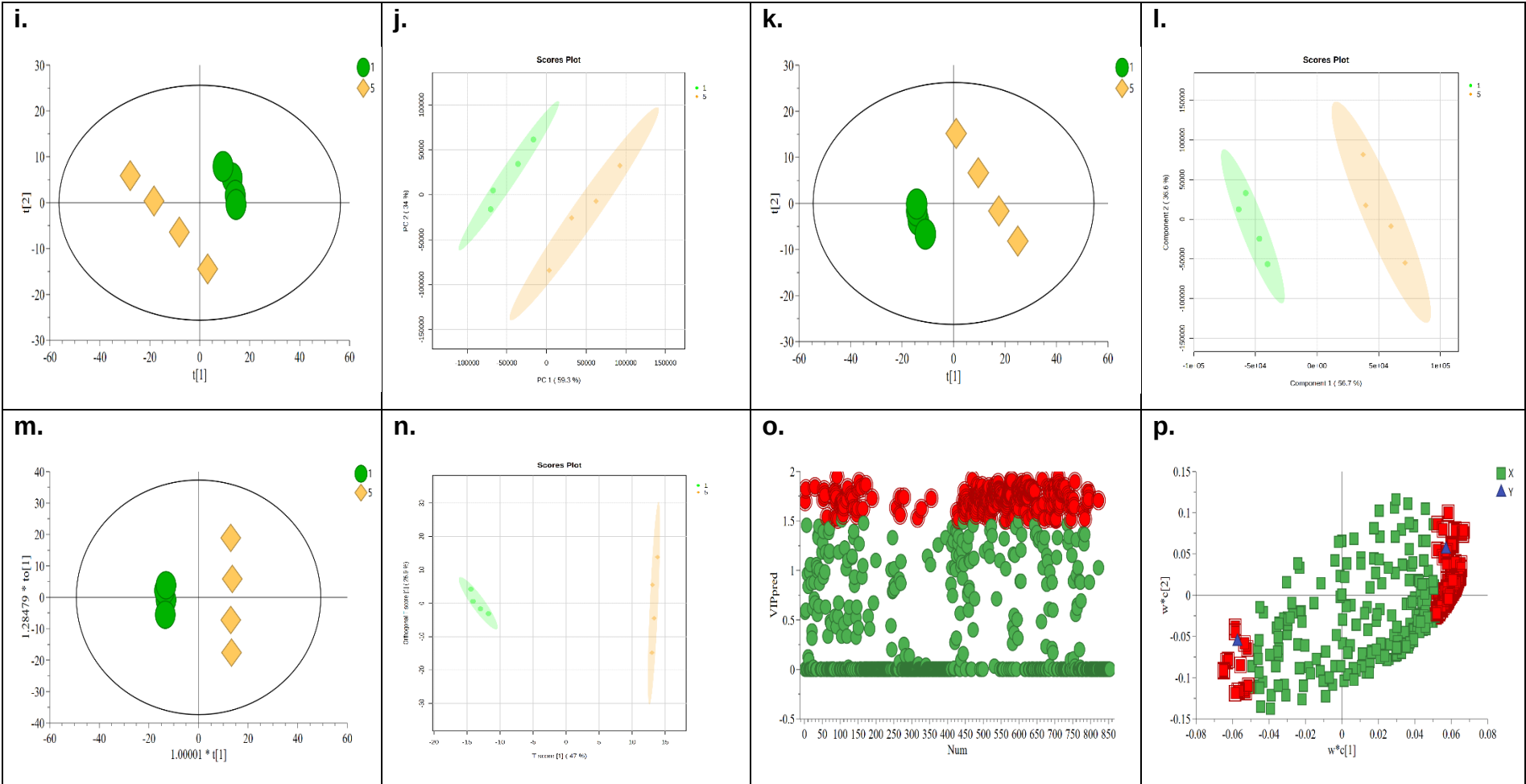


Figure 3.4. Negative and positive mode lipid profiling comparing the lipids of the $\Delta/\Delta fth2$ mutant (designated group 5) with *C. albicans* BWP17 (designated group 1). The first set of data (a-h) represent the negative mode of lipid analysis while the second set (h-p) represents the positive mode. The **PCA** plots in **Figure 3.4.a, b, i** and **j** show the grouping trend between the two sample groups. In the **PLS-DA** score plot (**c, d, k** and **l**) or OPLS-DA score plot (**e, f, m** and **n**), a clear separation of two groups is observed. **Figure 3.4.g** and **o** show the distribution of VIP values (**VIP > 1.5**). Lipids with a red box are labelled as significant compounds with a **VIP > 1.5** on the **PLS-DA** model loading plot (**h** and **p**).

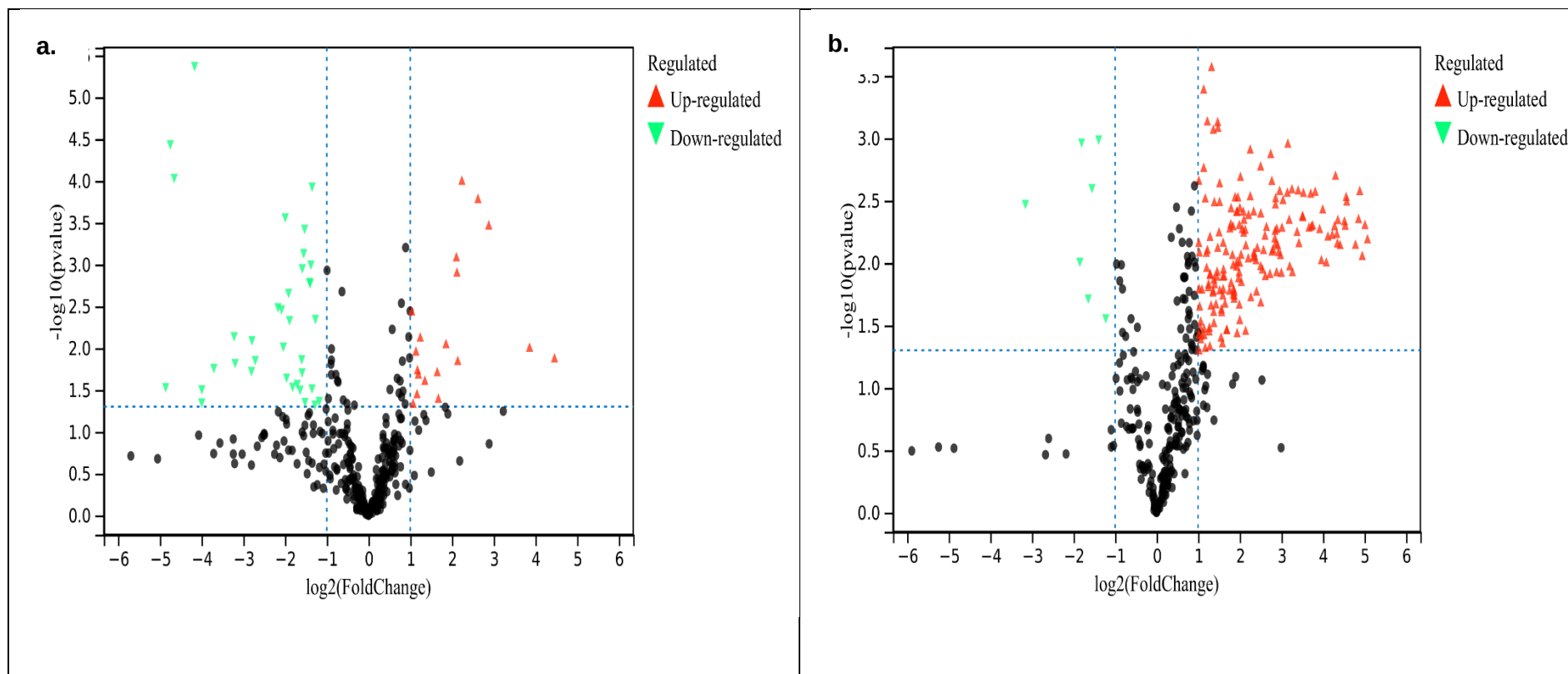


Figure 3.5. Comparative analysis of BWP17 vs $\Delta\Delta fth2$ lipid species. The volcano plot in (a) represent differentially produced lipids in the negative mode that are either up-regulated or down-regulated while (b) represents the differentially produced lipids in the positive mode. The range of $Y > 1.30$ and $X > 1$ is used to show a significant increase while the range of $Y > 1.30$ and $X < -1$ show a significant decrease on the volcano plot.

3.5.2.1. Effect of deletion of *FTH2* on *C. albicans* lipidome

The positive mode data set in **Figure 3.6.** showed a significant amount of differentially produced lipid species being increased in the mutant $\Delta/\Delta fth2$ compared to *C. albicans* BWP17, with most of the lipid species representing neutral and polar lipids such as TAGs and DAGs respectively.

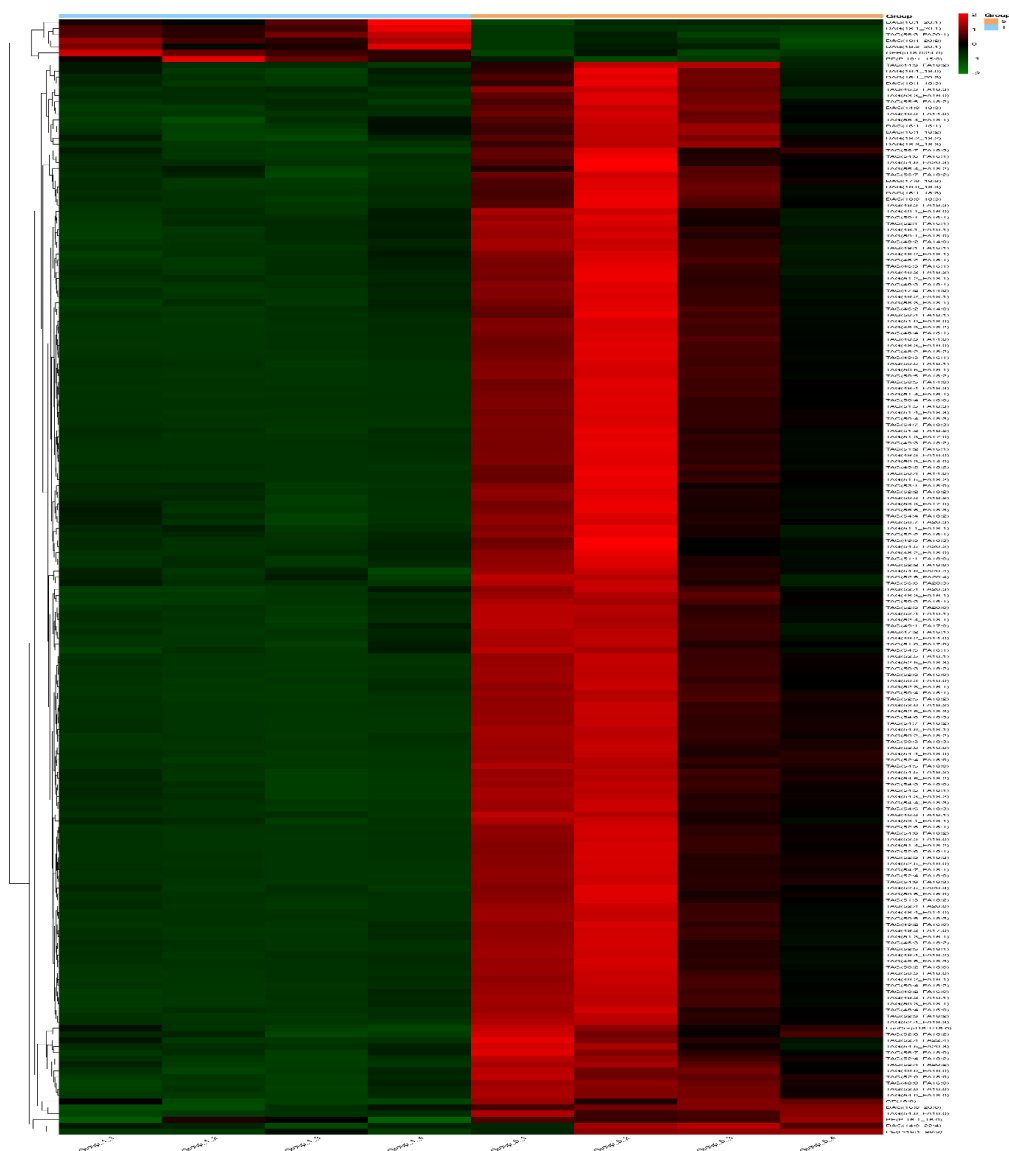


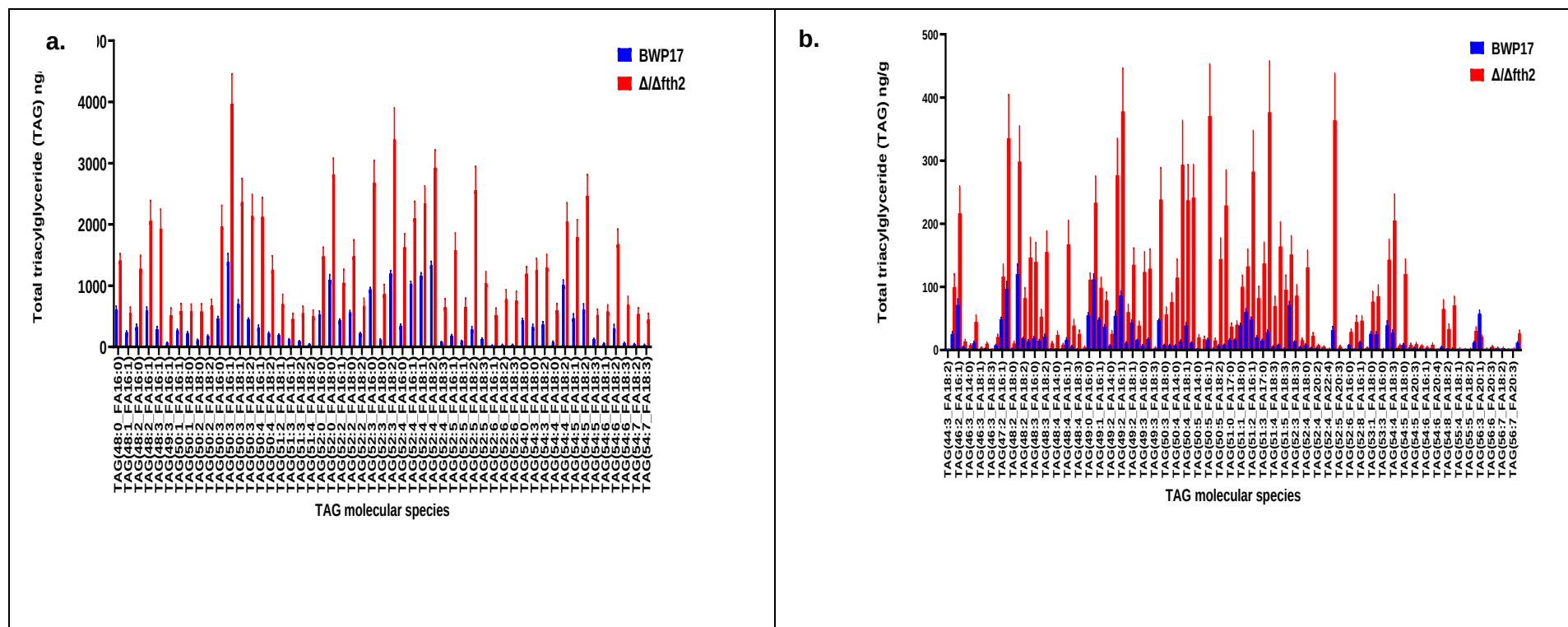
Figure 3.6. Positive mode lipid species analysis of differentially produced lipid species in *C. albicans* BWP17 (denoted as group 1) compared to $\Delta/\Delta fth2$ (denoted as group 5). Lipid species represented are mainly neutral (TAGs) and

polar (DAGs) lipids. The significantly upregulated lipid species are represented in red while the significantly downregulated lipids are represented in green.²

Since neutral lipids such as TAGs do not ionise, the TAG lipids were profiled using a product ion related to one of the fatty acyl chains. The targeted fatty acyl chain is indicated after the TAG abbreviation by **_FA carbon number: unsaturation** an example of this is **TAG (52:3_FA 16:0)**.

The significantly produced neutral lipid species of *Δ/Δfth2* are further illustrated in **Figure 7.c** using the log₂ FC values, with *Δ/Δfth2* displaying an overall log₂ FC above 0 with majority of the lipid species. The FC values indicate the relative difference in the abundance of lipid species between sample groups. Thus, a negative (-) log₂ FC value in this case would indicate a decrease in the abundance of specific lipid species while increase in the log₂ FC entails the opposite. Only one of the TAG molecular lipid species, TAG (56:3 _FA18:2), was differentially produced in lower amounts showing a log₂ FC below 0. (**Figure 3.7.c**).

² Figures referred to in this section, that may not be clearly visible are provided in the supplementary data section.



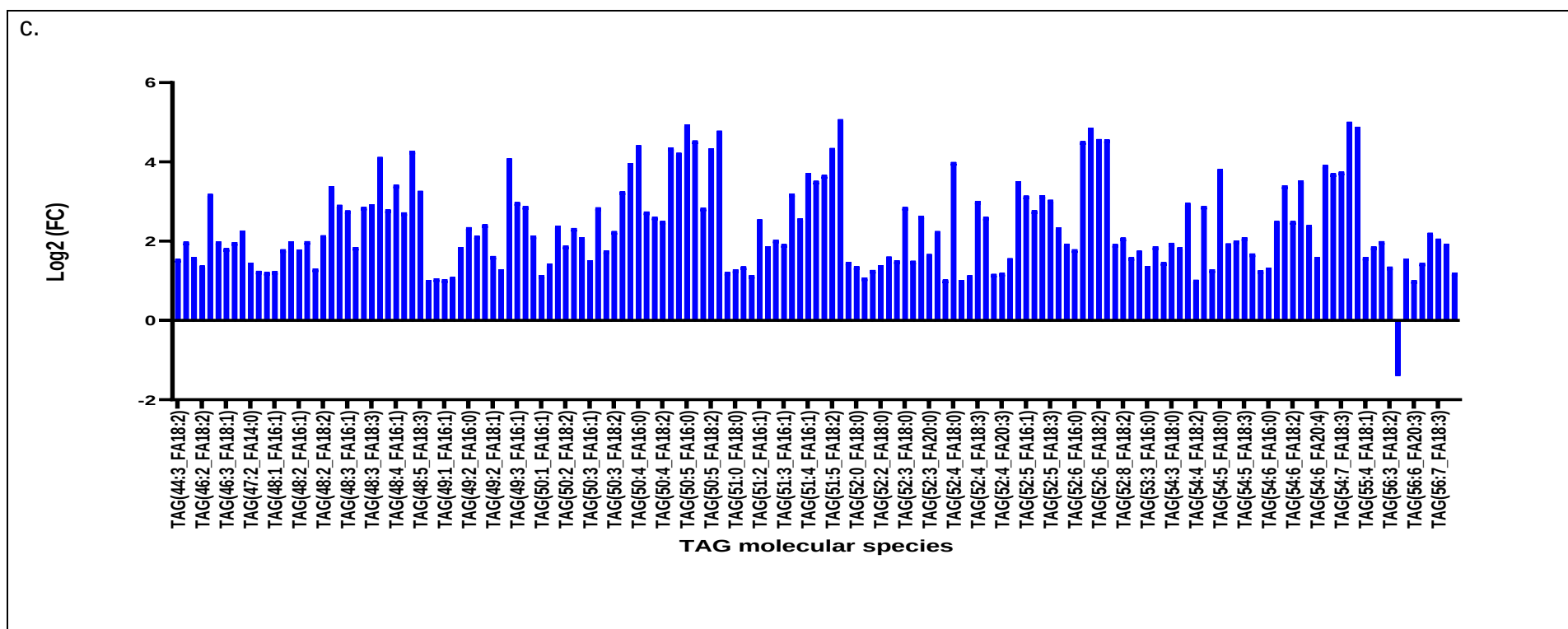
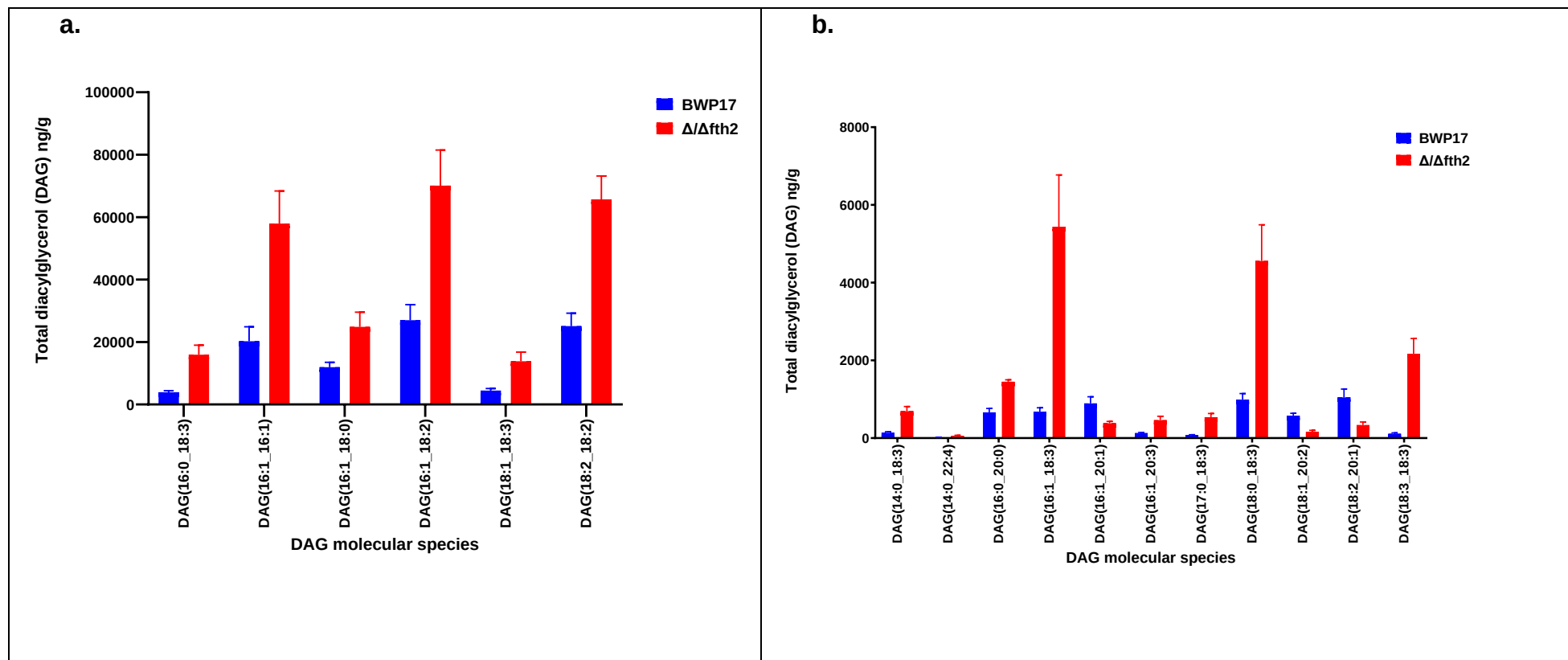


Figure 3.7. Differentially produced neutral lipids of BWP17 wild-type and $\Delta/\Delta fth2$ mutant strain. (a.) major TAGs and (b.) minor lipid species of BWP17 and $\Delta/\Delta fth2$. The TAGs in (c) annotated as log₂FC values, represent only to the $\Delta/\Delta fth2$ mutant. The molecular lipid species presented here are arranged according to their chain length and degree of unsaturation.

A similar trend was observed in DAGs (**Figure 3.8.**), with most of the differentially produced molecular species being increased in $\Delta/\Delta fth2$. Three of the DAGs molecular lipid species were differentially produced in lower amounts and this is made evident by the decrease in the $\log_2$ FC (**Figure 3.8.c**). Three of these lipid species are annotated as DAG (16:1 _20:1), DAG (18:1 _20:2) and DAG (18:2 _20:1).



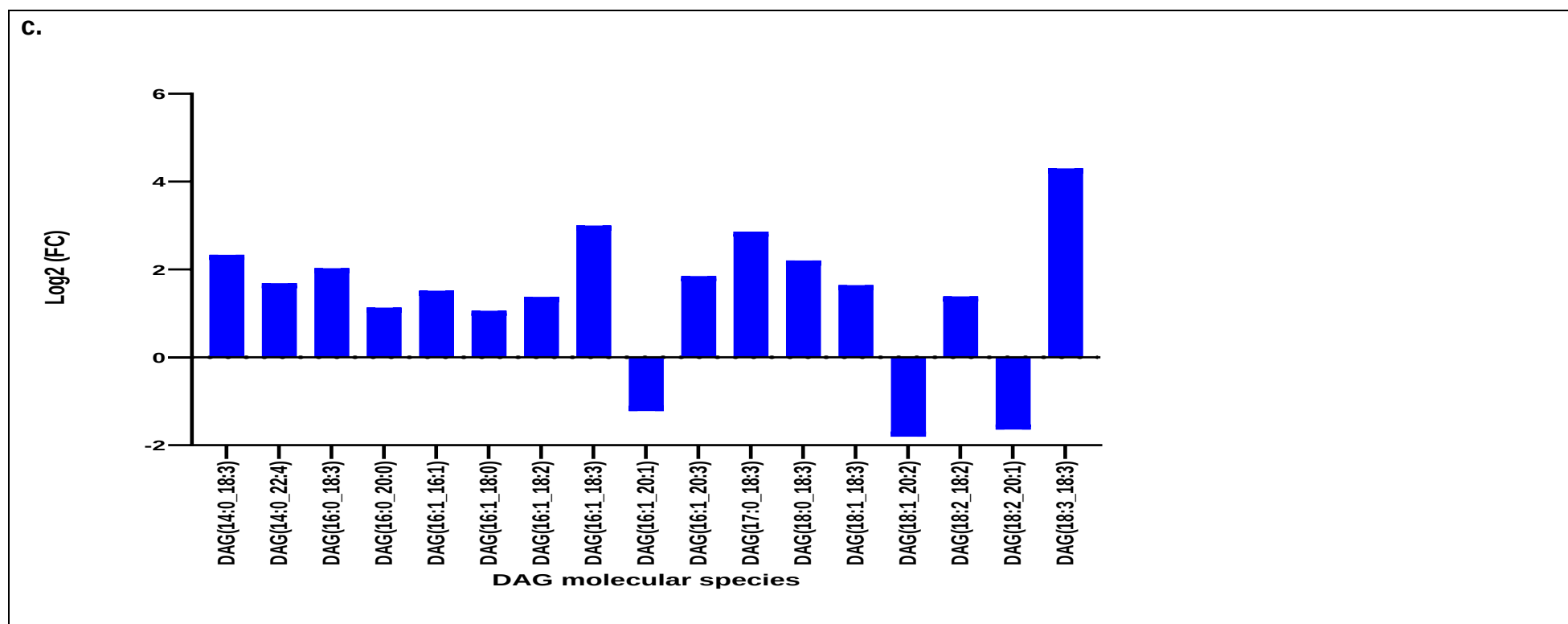


Figure 3.8. Differentially produced polar lipids of *C. albicans* BWP17 and $\Delta/\Delta fth2$ mutant strains. (a.) Major DAGs, (b.) Minor DAGs lipid species of BWP17 and $\Delta/\Delta fth2$ (c.) $\log_2$ FC values of the DAG lipid species ($\Delta/\Delta fth2$ compared to BWP17) arranged according to their chain length and degree of unsaturation.

Looking at the data generated in the negative mode, most of the differentially produced lipid species that were upregulated belong to the phospholipid class (**Figure 3.9.**). Since phospholipids are considered to be important for fluconazole resistance (Mukhopadhyay et al. 2004; Kopecka et al. 2020), it was important to look at this in more detail.

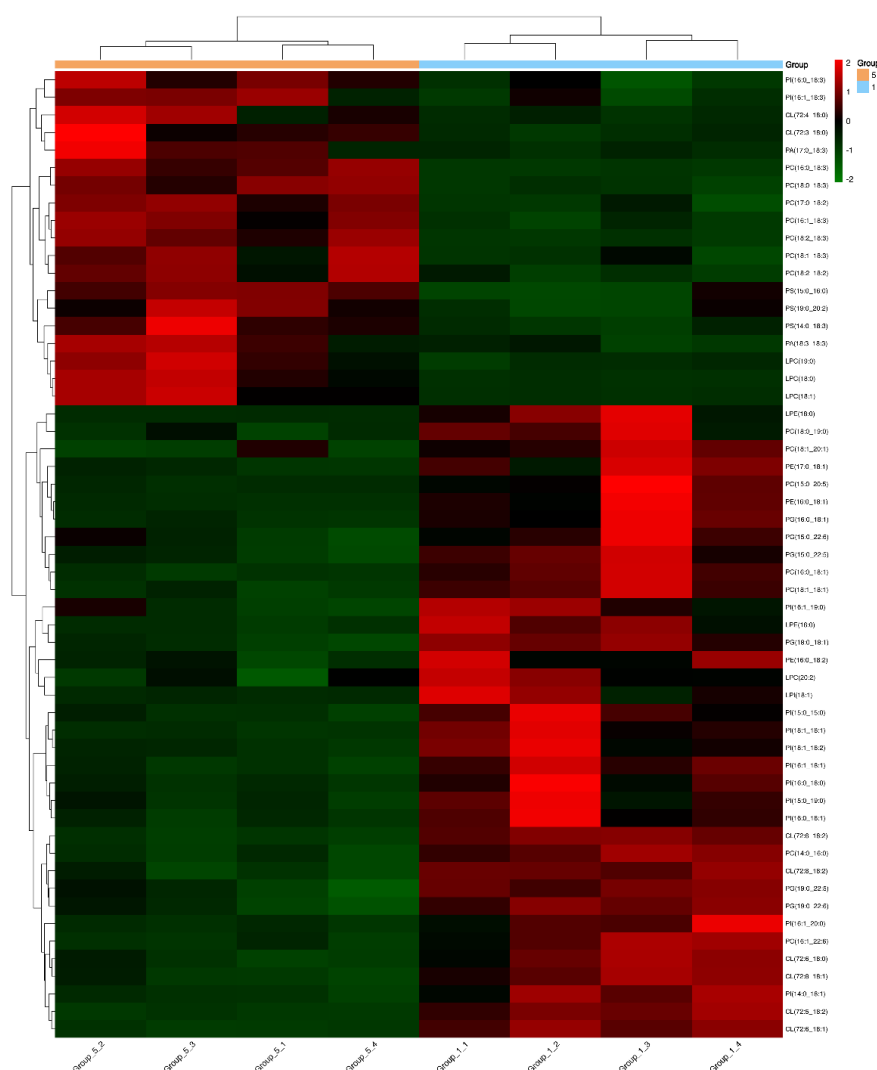


Figure 3.9. Negative mode analysis of differentially produced lipid species in *C. albicans* BWP17 (denoted as group 1) compared to $\Delta\Delta fth2$ (denoted as group 5). Lipid species represented mainly phospholipids including PA, PC, PE, PG and PS. The significantly upregulated lipid species are represented in red while the significantly downregulated lipids are represented in green.

Figure 3.10 shows the total concentration (ng/g lipid) of differentially produced phospholipids in both *C. albicans* BWP17 and $\Delta/\Delta fth2$. Overall, there were varying levels in phospholipid classes between the two strains with $\Delta/\Delta fth2$ showing lower levels of total PC, PE, PG and PI compared to BWP17. However, the levels of PA and PS were significantly higher in the $\Delta/\Delta fth2$ mutant. When the average PC to PE ratio of the two strains was calculated, it was evident that this ratio was higher in the mutant than in the parental strain (**Table 3**). The ratio PC to PE has been shown to be the key regulator of membrane integrity (Li et al. 2006). Thus, the increase in ratio could suggest the increased resistance demonstrated by the mutant upon fluconazole exposure.

Table 3. Average PC/PE ratio of *C. albicans* BWP17 and $\Delta/\Delta fth2$

Strain	Average PC (ng/g lipid)	Stdev	Average PE (ng/g lipid)	Stdev	PC/PE
BWP17	2955.76	627.32	9645.66	5005.33	0.31
$\Delta/\Delta fth2$	1687.63	215.47	977.46	366.87	1.73

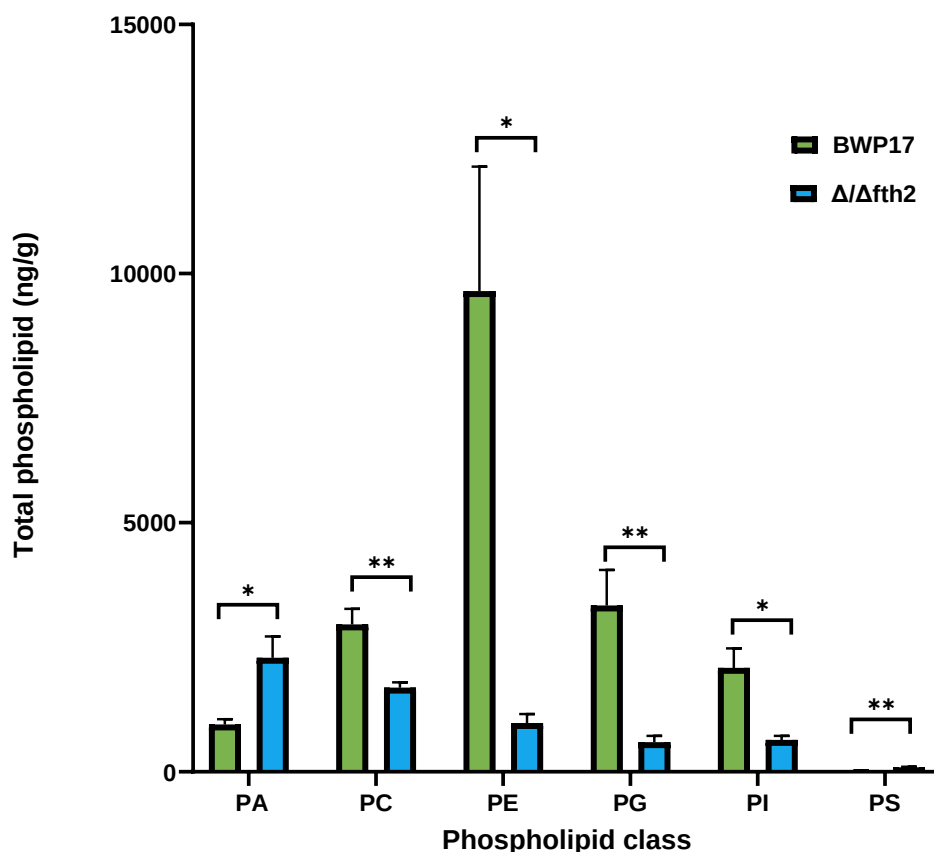


Figure 3.10. Total differentially produced phospholipids in *C. albicans* BWP17 and $\Delta/\Delta fth2$. The values presented are average of the four biological repeats and the standard deviation is indicated by the error bars. Statistical significance is indicated by the asterisk. * $p < 0.05$, **....***.

Studies have reported that several cellular stressors such as nutrient deprivation or high levels of reactive oxygen species (ROS) can induce *de novo* lipogenesis (Görlach et al. 2015). Thus, the change in the neutral lipid composition of TAGs with less LD production in iron- starved cells ($\Delta/\Delta fth2$ mutant) can further be explained by the cellular response to nutrient scarcity including iron deprivation. Moreover, it can also be seen that the TAGs molecular species displayed high levels of unsaturation. This also shows that iron deprivation can lead to lipolysis and TAGs mobilisation in an

attempt to generate energy in the absence of iron, resulting in increased levels of lipid intermediates. Even more, changes in lipid metabolism have indicated an active and a protective response to stress mediated by drugs, thus promoting cell survival even in the presence of the drug in cancer cells. Thus, we propose that, the increased levels of the observed TAGs and increased resistance displayed by the mutant (reported in chapter 2) could also be as a result of the high expression of fatty acid synthase (FAS), increasing TAG synthesis. The significantly produced TAGs that are upregulated in $\Delta/\Delta fth2$ could also explain the production of bigger lipid droplets seen in our confocal laser scanning micrographs (**Figure 3.2.**). LDs are composed mainly of neutral lipids including TAGs and sterol esters (SE). The sudden decrease in LD production in both the mutant biofilms and polymicrobial biofilms could also be explained by the metabolic shifts that the cells undergo upon nutrient deprivation, for example, changes in LD composition (i.e., TAG saturation). However, further studies are needed to better understand the effect of lipid metabolism of LD size and number.

3.5.2.2. Influence of pyoverdine on the lipidome of *C. albicans*

The influence of iron availability during competition on the *C. albicans* lipidome was further investigated by cultivating both strains (*C. albicans* BWP17 and $\Delta/\Delta fth2$) in the presence of 1 $\mu\text{g/mL}$ of pyoverdine for 48 h to allow mature biofilm formation. The siderophore, pyoverdine specifically binds and forms a stable complex with ferrous ion (Fe^{2+}) (Lopez-Medina et al. 2015; Bonneau et al. 2020). Since co-culturing *C. albicans* (BWP17 and $\Delta/\Delta fth2$) with *P. aeruginosa* PAO1 would complicate the lipidome study because *P. aeruginosa* produces its own lipids, one aspect of the presence of *P. aeruginosa* PAO1 was mimicked by the presence of this iron-chelator produced by *P. aeruginosa*.

A. Influence of pyoverdine of the lipidome of *C. albicans* BWP17

In order to assess the influence of pyoverdine on the lipidome of the parental strain (*C. albicans* BWP17), the first comparison was between the parental strain without pyoverdine and *C. albicans* BWP17 with pyoverdine. As shown in **Figure 3.11.** and **Table 4**, the presence of pyoverdine had a limited influence on the lipidome of BWP17 with only cardiolipin (CL) and phosphatidylethanolamine (PE) showing significant differences between the two sample groups (**Figure 3.12.**). This could be as a result of the iron chelator concentration being too low.

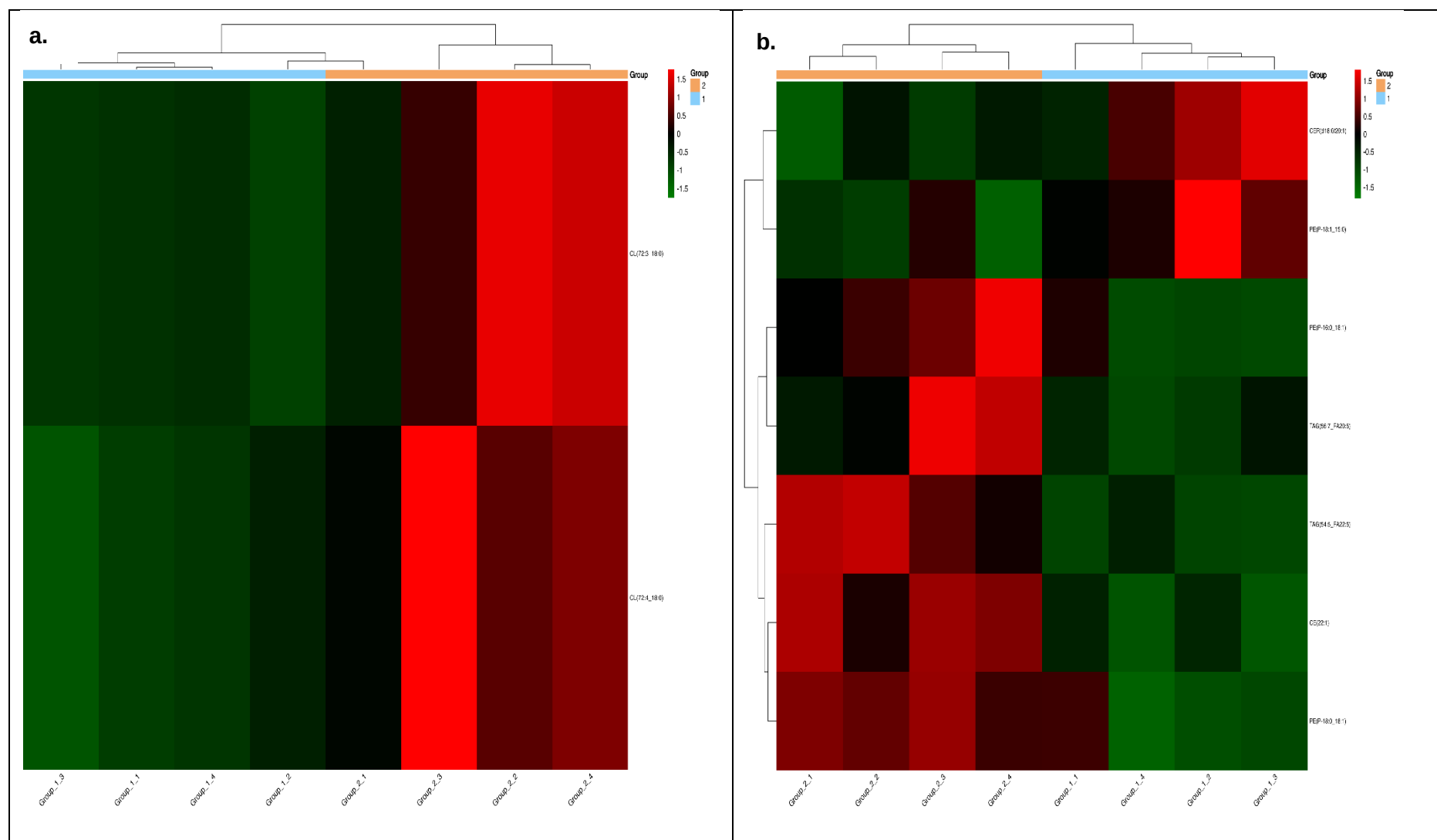


Figure 3.11. Negative and positive mode lipid profiling comparing the lipids of BWP17 without pyoverdine (designated group 1) to *C. albicans* BWP17 (designated group 2) with pyoverdine. The differentially produced lipid species are represented to show significantly upregulated (red) or significantly downregulated (green) lipid species.

Table 4. Effect of pyoverdine on the log₂ fold change (FC) of lipid species of *C. albicans* BWP17 biofilm

Lipid species	Log ₂ (FC)
CL (72:4_18:0)	1.09
PE(P-18:1_15:0)	-1.25
PE(P-18:0_18:1)	1.69
PE(P-16:0_18:1)	2.24
CER (d18:0/20:1)	-1.27
CE (22:1)	1.57
TAG (54:5_FA22:5)	1.18
TAG (56:7_FA20:5)	1.54
CL (72:3_18:0)	1.77

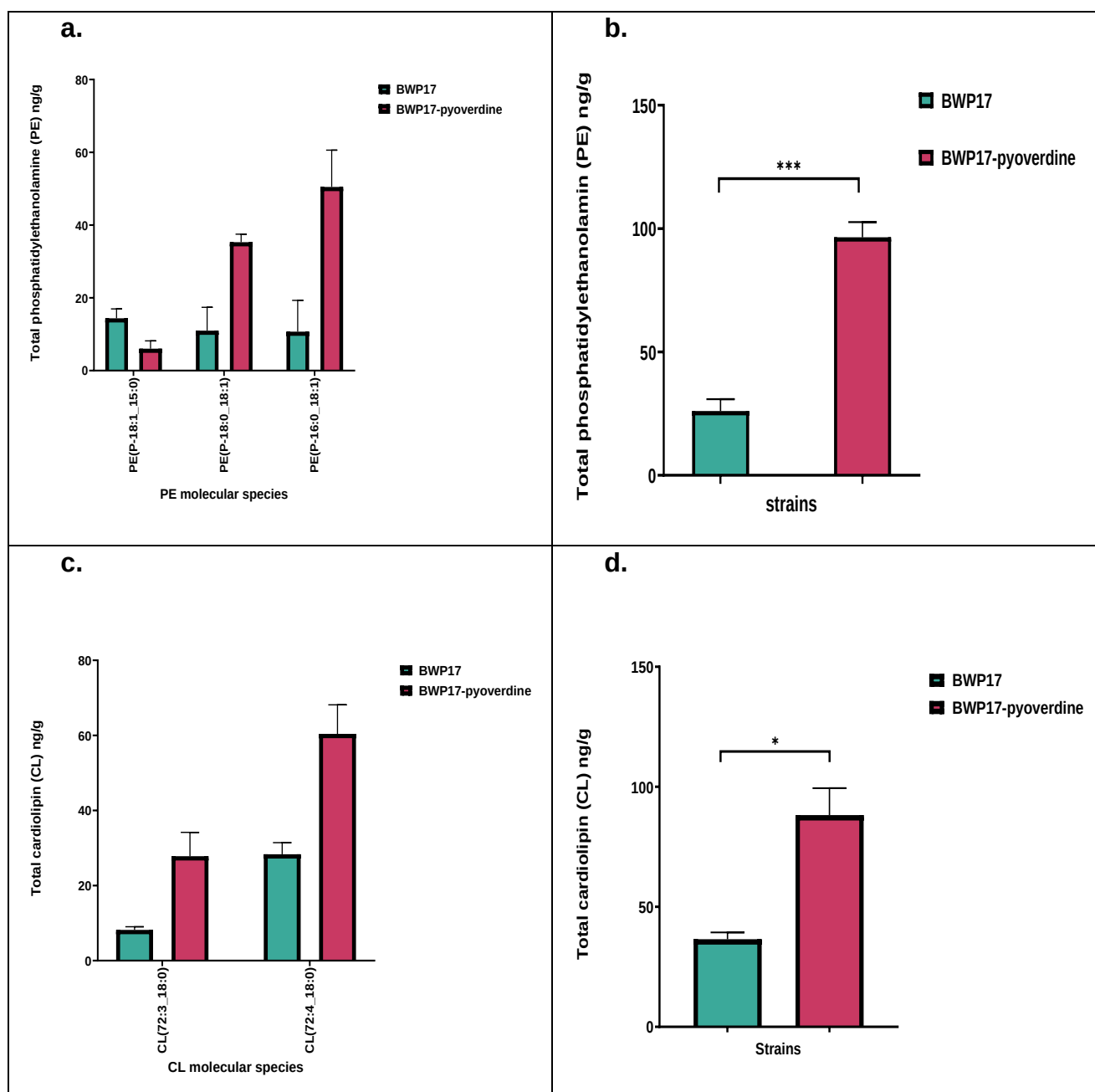


Figure 3.12. The graphs above illustrate the differentially produced lipid species of *C. albicans* in the parental strain, BWP17. The mean values of the individual PE species (a.), the total PE (b.), the mean values of CL species (c.) and the total CL in BWP17 versus BWP17 with pyoverdine (d.).

The ability of *C. albicans* to transition between yeast to hyphal growth is important for its pathogenesis. Luo and co-workers (2021) have recently established that strains

cultivated in iron limiting environments or those lacking *FTR1*, a plasma membrane associated gene whose expression is dependent on low iron concentrations failed to sustain hyphal formation. Moreover, lipid species such as CLs are known to be associated with the maintenance and functioning of mitochondrial membrane and could thus play a crucial role in hyphal development (Fry and Green 1981; Ke et al. 2021). The transition from yeast to hyphal growth requires energy and thus the mitochondria serves as a primary powerhouse for supplying energy through the respiratory chain (McBride et al. 2006; Shingu-Vazquez and Traven 2011; Calderone et al. 2015). The significantly produced CLs and PE lipid species that are mostly upregulated in the parental strain may contribute substantially to our understanding of why the mutant showed a delay in hyphal formation in comparison to the parental strain. Although it has been shown that other lipids such as PE and PG can compensate for the lack of CL, studies further showed that the growth rate of cells lacking CL was reduced, thus suggesting defects in mitochondrial energy (Pfeiffer et al. 2003; Zhong et al. 2004). However, it is important to acknowledge that many other factors besides lipids may play a role in the morphological switching in *C. albicans*. These include changes in environmental conditions, such as temperature and pH, cell signalling pathways, quorum sensing mechanisms or even various transcriptional regulators such as Efg1 and Cph1 (Stoldt et al. 1997; Braun and Johnson 2000; Davis et al. 2002; Pathak and Sahu 2018).

B. Influence of pyoverdine of *C. albicans* $\Delta/\Delta fth2$

The presence of pyoverdine only significantly influenced the production of a limited number of lipid species, without a specific pattern observed (**Figure 3.13.**).

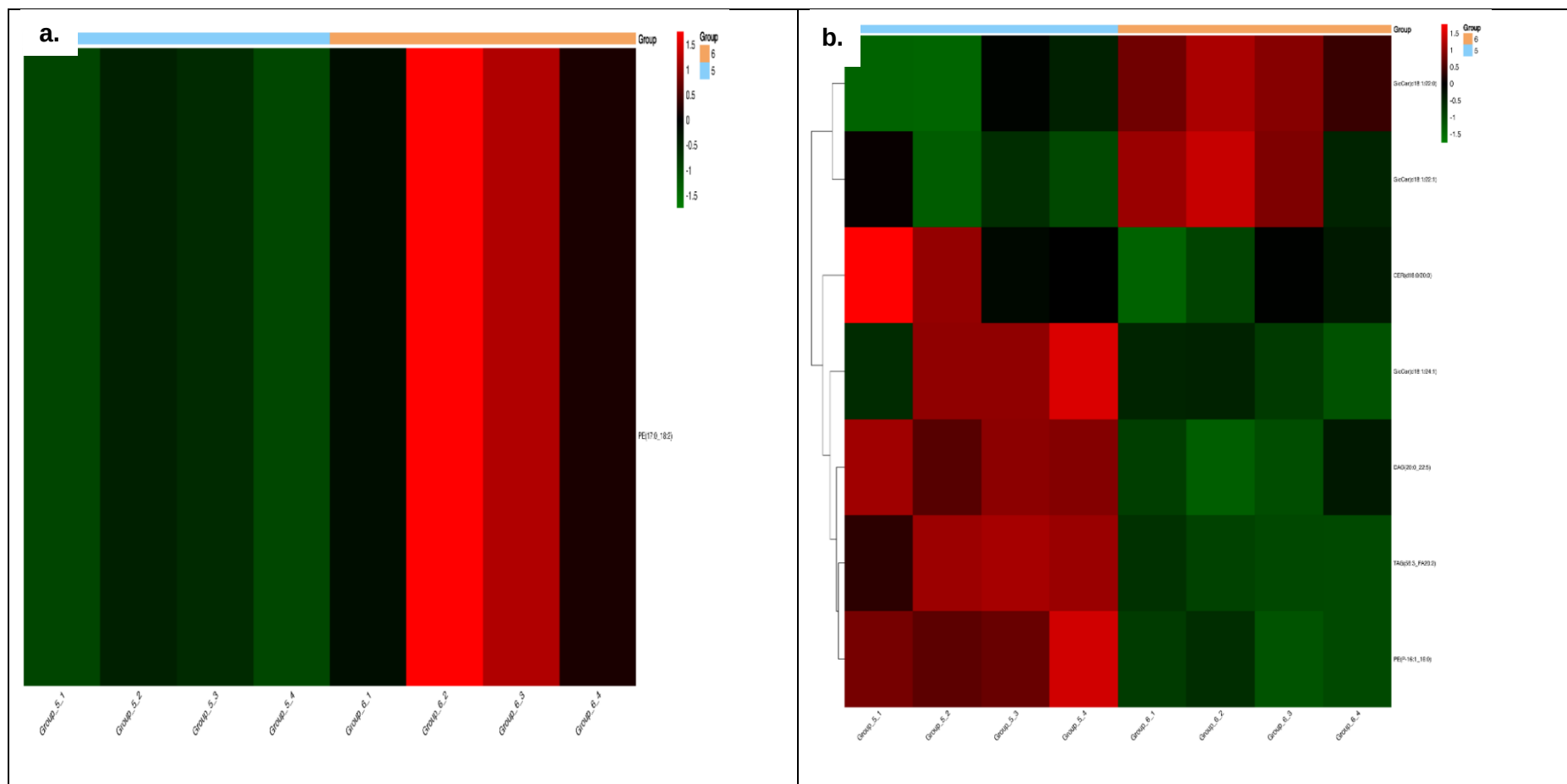


Figure 3.13. Lipid species of $\Delta/\Delta fth2$ without pyoverdine (annotated as group 5) compared to $\Delta/\Delta fth2$ with pyoverdine (annotated as group 6). Lipids profiled in the negative mode are represented in (a), while (b) represents lipids profiled in the positive mode. The significantly upregulated lipid species are represented in red while those that are regulated are represented in green in the different strains.

3.5.2.3. Influence of *FTH2* deletion on the lipidome of *C. albicans* in the presence of pyoverdine

The next condition compared *C. albicans* BWP17 to $\Delta/\Delta fth2$, both in the presence of pyoverdine. Here CLs, phospholipids (in the negative mode) as well as TAGS (in the positive mode) were differentially produced (**Figure 3.13.a and b**). Interestingly, when comparing the overall differentially produced lipids between BWP17 and $\Delta/\Delta fth2$ in the absence and presence of pyoverdine, it was noticed that pyoverdine caused a decrease in the log₂ FC observed for most of these lipid species (**Table 5**).

While pyoverdine primarily functions as an iron chelating molecule, its presence may indirectly affect the metabolic pathways of *C. albicans* including lipid metabolism. Pyoverdine concentrations as low as 3.125 μ M have shown to significantly interfere with the metabolism of *Aspergillus fumigatus* (*A. fumigatus*) biofilms (Sass et al. 2018). Although, only a few of the lipid species were differentially produced in the presence of pyoverdine, it is clear that addition of pyoverdine causes a further decrease in the abundance of lipid species. Given that only one concentration (**1 μ g/mL**) was tested, future studies would include different concentrations of the iron chelator to get a better view of its influence on *C. albicans*.

Table 5. Comparison of the log₂ FC of lipid species in the absence and presence of pyoverdine. The delta values indicate the differences in the log₂ fold change values of the two comparisons.

	$\Delta/\Delta fth2$ vs BWP17 with pyoverdine	$\Delta/\Delta fth2$ vs BWP17 without pyoverdine	
Lipid species	Log₂ (FC)	Log₂ (FC)	Delta
DAG(18:1_20:1)	-3.41	-3.16	0.25
DAG(18:1_20:2)	-2.04	-1.81	0.24
DAG(18:2_20:1)	-1.97	-1.64	0.32
CER(d18:0/24:0)	-1.38	-1.55	-0.17
TAG(56:3_FA20:1)	-2.13	-1.39	0.74
DAG(16:1_20:1)	-2.00	-1.22	0.78
LacCer(d18:1/18:0)	1.15	1.47	0.32
TAG(52:4_FA22:4)	1.24	1.56	0.33
TAG(53:1_FA18:0)	1.05	1.59	0.54
TAG(54:6_FA20:4)	1.55	1.59	0.04
TAG(53:1_FA18:1)	1.14	1.75	0.61
TAG(52:6_FA16:0)	1.97	1.79	-0.18
TAG(54:3_FA18:2)	1.18	1.84	0.65
TAG(50:2_FA18:2)	1.07	1.89	0.82
TAG(52:8_FA16:1)	1.10	1.92	0.82
TAG(56:7_FA20:3)	1.36	1.94	0.58
TAG(54:3_FA18:0)	1.22	1.95	0.73

TAG(46:3_FA18:2)	1.32	1.97	0.65
DAG(16:0_18:3)	1.60	2.03	0.43
TAG(51:2_FA18:2)	1.06	2.04	0.97
TAG(56:7_FA18:3)	1.83	2.06	0.23
TAG(54:5_FA18:3)	1.38	2.09	0.72
TAG(50:3_FA16:0)	1.18	2.10	0.92
TAG(49:2_FA16:1)	1.12	2.13	1.01
TAG(49:3_FA18:3)	1.09	2.14	1.05
TAG(48:2_FA18:2)	1.22	2.15	0.92
DAG(18:0_18:3)	1.61	2.20	0.60
TAG(52:4_FA16:0)	1.17	2.25	1.08
TAG(50:3_FA18:2)	1.31	2.25	0.95
TAG(50:3_FA14:0)	1.42	2.33	0.91
DAG(14:0_18:3)	1.80	2.33	0.53
TAG(49:2_FA16:0)	1.30	2.35	1.05
TAG(50:2_FA18:0)	1.55	2.39	0.84
TAG(49:2_FA17:0)	1.25	2.42	1.17
TAG(54:6_FA18:2)	1.38	2.50	1.12
TAG(50:4_FA18:2)	1.40	2.50	1.11
TAG(51:2_FA16:1)	1.38	2.55	1.17
TAG(51:3_FA18:2)	1.37	2.57	1.21
TAG(52:4_FA20:0)	1.35	2.61	1.26
TAG(50:4_FA18:1)	1.08	2.61	1.53
TAG(52:3_FA18:3)	1.77	2.64	0.87

TAG(48:4_FA18:2)	1.54	2.72	1.19
TAG(50:4_FA16:1)	1.48	2.74	1.26
TAG(48:3_FA16:1)	1.37	2.77	1.40
TAG(52:5_FA18:1)	1.72	2.78	1.05
TAG(48:4_FA16:0)	1.59	2.80	1.21
TAG(50:5_FA18:1)	1.29	2.84	1.55
TAG(50:3_FA18:0)	1.65	2.85	1.20
TAG(52:3_FA18:0)	1.87	2.86	0.99
DAG(17:0_18:3)	1.41	2.86	1.45
TAG(48:3_FA18:2)	1.71	2.86	1.15
TAG(49:3_FA18:2)	1.53	2.88	1.36
TAG(54:4_FA18:3)	2.14	2.89	0.75
TAG(48:3_FA16:0)	1.27	2.91	1.64
TAG(48:3_FA18:3)	2.01	2.93	0.92
TAG(54:4_FA18:0)	2.16	2.96	0.80
TAG(49:3_FA16:1)	1.48	2.98	1.50
DAG(16:1_18:3)	1.97	3.00	1.03
TAG(52:4_FA18:3)	1.88	3.02	1.13
TAG(52:5_FA18:3)	2.10	3.04	0.94
TAG(52:5_FA16:1)	1.89	3.14	1.25
TAG(52:5_FA18:2)	1.83	3.16	1.32
TAG(46:3_FA14:0)	1.81	3.19	1.38
TAG(51:3_FA17:0)	1.47	3.20	1.73
TAG(50:3_FA18:3)	2.36	3.25	0.89

TAG(48:5_FA18:3)	1.99	3.27	1.27
TAG(48:3_FA14:0)	2.16	3.38	1.22
TAG(54:6_FA18:1)	2.21	3.40	1.20
TAG(48:4_FA16:1)	1.68	3.42	1.74
TAG(52:5_FA16:0)	2.54	3.51	0.97
TAG(51:4_FA18:2)	2.19	3.52	1.33
TAG(54:6_FA18:3)	2.34	3.53	1.19
TAG(51:4_FA18:3)	2.78	3.67	0.88
TAG(54:7_FA18:2)	2.55	3.71	1.15
TAG(51:4_FA16:1)	1.93	3.72	1.80
TAG(54:7_FA18:3)	2.75	3.75	1.00
TAG(54:5_FA18:0)	2.82	3.81	0.99
TAG(54:7_FA18:1)	3.00	3.92	0.92
TAG(50:4_FA14:0)	2.50	3.96	1.47
TAG(52:4_FA18:0)	2.98	4.00	1.01
TAG(49:3_FA16:0)	2.18	4.08	1.90
TAG(48:4_FA14:0)	3.18	4.12	0.94
TAG(50:5_FA14:0)	3.22	4.22	1.00
TAG(48:4_FA18:3)	2.87	4.28	1.41
DAG(18:3_18:3)	3.49	4.30	0.81
TAG(50:5_FA18:2)	2.61	4.33	1.72
TAG(51:5_FA18:2)	2.58	4.35	1.76
TAG(50:4_FA18:3)	3.29	4.36	1.07
TAG(50:4_FA16:0)	2.82	4.41	1.60

TAG(52:6_FA16:1)	3.07	4.52	1.45
TAG(50:5_FA16:1)	2.91	4.54	1.63
TAG(52:6_FA18:3)	3.28	4.56	1.28
TAG(52:6_FA18:2)	3.05	4.57	1.52
TAG(50:5_FA18:3)	3.34	4.78	1.44
TAG(52:6_FA18:1)	2.79	4.86	2.07
TAG(54:8_FA18:3)	4.04	4.89	0.85
TAG(50:5_FA16:0)	2.95	4.95	2.00
TAG(54:8_FA18:2)	3.71	5.01	1.30
TAG(51:5_FA18:3)	3.52	5.07	1.55

3.5.2.4. Overlap of differentially produced lipid species

In **Figure 3.14**, it can be seen that there were unique differential production lipid profiles for each comparison. The deletion of *FTH2* had the biggest influence with most of the differentially produced lipid species (149) influenced, of which 137 were only influenced by the mutation. Ten (10) of these 149 lipid species were also influenced by pyoverdine in the mutant, while two were also influenced by pyoverdine alone. Thus, we can conclude that although there are unique effects of the deletion of *FTH2*, the presence of pyoverdine or the combination of these influences, there are some overlapping effects as well.

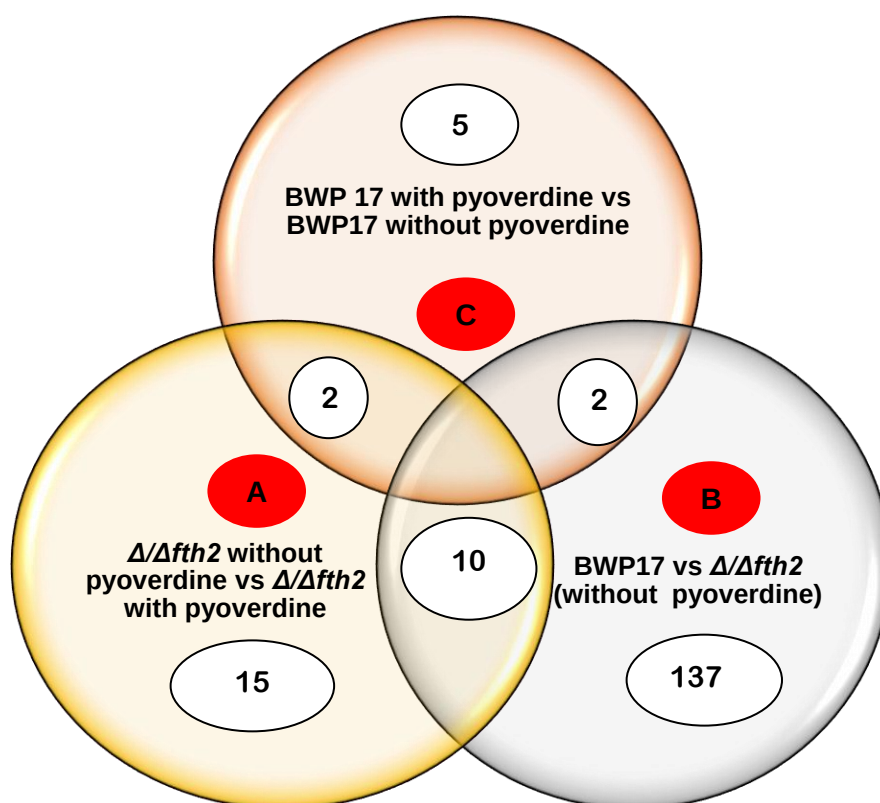


Figure 3.14. Venn diagram illustrating the distribution of differentially produced lipids for the *C. albicans* parental and mutant strain in the absence and presence of pyoverdine. **A.** $\Delta/\Delta fth2$ without pyoverdine vs $\Delta/\Delta fth2$ with pyoverdine, **B.** BWP17 vs $\Delta/\Delta fth2$ (without pyoverdine) and **C.** BWP17 with pyoverdine vs BWP17 without pyoverdine.

3.5.2.5. The effect of pyoverdine on BWP17 and $\Delta/\Delta fth2$ lipids

In the last condition, we compared the effect of pyoverdine on *C. albicans* BWP17 and $\Delta/\Delta fth2$ lipid species (**Fig. 15**). It is clear that the upregulated lipid species in the negative data mode contain mostly of phospholipids and CLs that we have previously described to be associated with membrane integrity and thus fluconazole

susceptibility. The upregulation of these lipids in *C. albicans* BWP17 could also explain why we see a decline in its percentage inhibition when compared to $\Delta/\Delta fth2$, whose viability was decreased in both the crystal violet assay and XTT assay (Chapter 2, section 2.5.3). However, this only forms a small part of the influence of lipid homeostasis on fluconazole susceptibility and further investigations must be done.

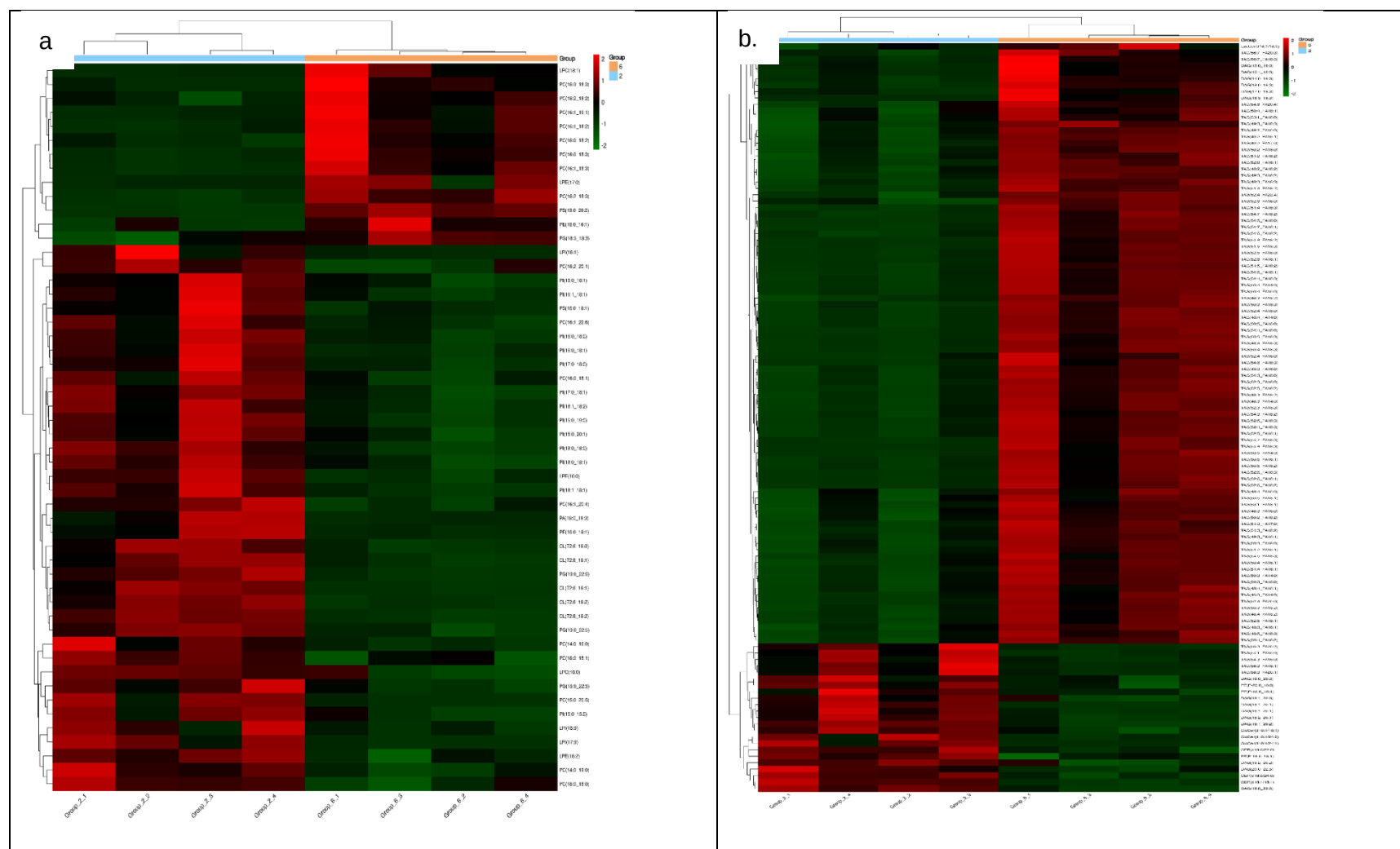


Figure 15. Lipid species analysis of *C. albicans* BWP17 with pyoverdine (denoted group 2) compared to $\Delta/\Delta fth2$ with pyoverdine (denoted group 6). Lipids profiled in the negative mode are represented in (a), while (b) represents lipids profiled in the positive mode containing mainly of TAGs molecular species.

Moreover, considering that the intracellular levels of antifungal drugs are dependent not only on the active drug efflux mechanisms but also the rate at which the drugs are imported (White et al. 1998), it is important to also consider the membrane lipid status. Studies were conducted to look at the lipid composition of azole susceptible (AS) and -resistant (AR) *C. albicans* strains (Singh and Prasad 2011). Some of these studies also revealed consistent differences in several lipid classes among the different AR isolates. These differences were observed in conjunction with the characteristic lipid composition that each isolate possessed. Importantly, however, it was concluded that the lipidome of each isolate was unique, which reflects the interplay of several host and genetic factors (Singh and Prasad 2011). One other mechanism which drives azole resistance is the alterations in the target enzyme lanosterol 14 α -demethylase (Marichal and Vanden Bossche 1995). Although we were not able to analyse the ergosterol levels, a study on clinically resistant *C. albicans* strains showed that changes in the status of membrane lipid phase and asymmetry could contribute to the acquired azole resistance (Kohli et al. 2002). The most highly resistance strain of *C. albicans* showed enhanced membrane fluidity which was postulated to be mainly associated with a decrease in ergosterol levels. The decrease in ergosterol levels were also proposed to be linked to the suppression of lanosterol 14 α -demethylase fluconazole target enzyme which is an iron-dependent cytochrome P450 monooxygenase enzyme belonging to the CYP51 family. Taken together, this could also account for the mutant ($\Delta/\Delta fth2$), whose ergosterol levels may be influence by iron limitations.

3.6. Conclusions

The importance of lipids in MDR is well established and the influence of iron availability was observed. It was demonstrated that each condition possessed a unique molecular species profile, and that the distribution of lipid species differs significantly between *C. albicans* BWP17 and $\Delta/\Delta fth2$. Moreover, the presence of pyoverdine may indirectly affect lipid metabolism. The decrease in lipid species caused by pyoverdine in both *C. albicans* BWP17 and its mutant may also account for the decrease in the production of lipid content in both *C. albicans* BWP17 and $\Delta/\Delta fth2$. The decrease in lipid species caused by pyoverdine may also be as a result of iron release from the fungal proteins, especially if the iron-dependent proteins affected play an important role in lipid metabolism and LD production. Overall, these results also support the notion that lipids play a crucial role in membrane integrity and may in turn influence fluconazole susceptibility. However, it is also important to note that the analysis was not fully comprehensive as, for instance, the ergosterol levels were not analysed. Thus further research on the influence of *FTH2* deletion on ergosterol levels still needs to be done.

3.7. References

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Chapter 4

General discussion, conclusions and future prospects

4.1. The effect of *FTH2* on fluconazole susceptibility

Fluconazole remains the first line drug against fungal infections such as those caused by *C. albicans* and this is due to its high bioavailability, low toxicity, its wide antifungal spectrum and low cost (Charlier et al. 2006; Vasicek et al. 2014; Lamothe et al. 2020). However, its fungistatic nature provides the opportunity for the development of antifungal resistance (Whaley et al. 2016). With the growing evidence of limited antifungals and the growing incidence of azole resistance, it is apparent that the widespread use of antifungals has led to an increased resistance to routinely used antifungals and that there is a need for new therapeutic strategies to counteract this issue (Rosenberg et al. 2017; Lamothe et al. 2020). The ability of *C. albicans* to grow and survive in diverse environments include its adaptive response to iron availability (Prasad et al. 2006). In our study, previously constructed *C. albicans FTH2* mutants ($\Delta/\Delta fth2_II$ and $\Delta/\Delta fth2$) associated with the high-affinity reductive iron uptake system, were used to evaluate the role of iron homeostasis on fluconazole susceptibility in planktonic cells and monomicrobial biofilms. The effect of *FTH2* deletion on fluconazole susceptibility within polymicrobial biofilms (in the presence of *P. aeruginosa*) was only tested for $\Delta/\Delta fth2$ and its parental strain, considering that the mutant displayed high resistance to fluconazole in prior studies. Both mutants displayed less susceptibility and decreased inhibition of biomass within planktonic wells and monomicrobial biofilms, upon fluconazole exposure in comparison to their parental strains. However, the mutant ($\Delta/\Delta fth2_II$) displayed a decrease in metabolic activity. This unexpected outcome may need to be further explored. A study by Chen and co-workers (Chen et al. 2018) demonstrated that fluconazole concentrations as high as 20 $\mu\text{g/mL}$ could not inhibit the growth of $\Delta/\Delta fth2$. The effect of fluconazole in

monomicrobial biofilms of $\Delta/\Delta fth2$ in this study conclusively demonstrate this previously reported role of *FTH2* in fluconazole susceptibility.

Interestingly, co-culturing of *C. albicans* with *P. aeruginosa* increases the activity of fluconazole towards $\Delta/\Delta fth2$. Hattab and co-workers (2022) recently demonstrated how *P. aeruginosa* enhances the activity of fluconazole against *C. albicans* both *in vivo* and *in vitro* studies. On the contrary, the parental strain (BWP17) did not show increased susceptibility in the presence of *P. aeruginosa*. Thus, the divergence from the expected pattern further highlights complexity of these interactions associated with these mixed microbial communities. For example, increase in pyoverdine production by *P. aeruginosa* in mixed biofilms has shown to increase the virulence of *P. aeruginosa* (Lopez-Medina et al. 2015; Bonneau et al. 2020). The intricate interplay between the two species emphasises the need to further explore mechanisms pertaining to these outcomes.

Like many other microorganisms, *C. albicans* possesses mechanisms that maintain intracellular iron levels including its mobilisation from storage compartments such as the mitochondria and vacuoles (Xu et al. 2014a). In this study, the role of *FTH2* on iron mobilisation from *C. albicans* stores. This was done with the aim of understanding if *FTH2* is needed for the release of iron from its vacuolar stores. Although differences in iron localisation within the cells, were noted, it could not be determined which structures may contain more iron. Thus, this study faced limitations in determining the exact cellular compartments where iron was localised. To address these limitations, future studies could look into incorporating a vacuole staining dye (FM4-64) in conjunction with rhodamine dithenoyl hydrazide, an Fe^{3+} staining dye. This may in turn elucidate the contribution of *FTH2* in iron transport with the fungal cell (Tong 2011; Liu et al. 2020).

4.2. The effect of *FTH2* deletion on lipids of *C. albicans*

The ability of *C. albicans* to tolerate antifungal treatment is associated with the specific global remodelling of lipid homeostasis. An imbalance in lipid species, such as ergosterol and sphingolipids, may affect drug susceptibility (Mukhopadhyay et al. 2004). In addition, the ability of *C. albicans* to store lipids in lipid droplets (LDs) for later use, may have an influence on the pathogenicity as well as the survival of yeast within the host (Chang et al. 2015). Lipid accumulation and lipid droplet formation are highly dependent on the physiological state of the cell (Zechner et al. 2009; Zechner et al. 2012). For instance, an imbalance in lipid metabolism or nutrient availability in yeast may lead to increased lipid synthesis and storage within the cells and thus induce lipid droplet biogenesis (van Zutphen et al. 2014).

Iron availability can have profound effects on the overall lipidome of the yeast (Hameed et al. 2011). For example, iron limitations can compromise the activity of enzymes responsible for processes such as fatty acid synthesis, sterol biosynthesis and most importantly, phospholipid synthesis, which is a major class of lipids that make up the cell membranes including plasma and intracellular membranes (Hameed et al. 2011).

As a result, the yeast's lipid production may be affected in terms of both quantity and composition. In our study, we observed differences in lipid droplet size and quantified lipid content between *C. albicans* BWP17 and $\Delta/\Delta fth2$. Additionally, comparison between *C. albicans* BWP17 and $\Delta/\Delta fth2$ lipid composition reveals a significant difference in the distribution of lipid species between the two strains. These results further suggest a correlation between iron availability and lipid homeostasis.

An important trend that emerged was the significantly upregulated triacylglycerols (TAGs) in the $\Delta/\Delta fth2$ mutant. Neutral lipids, mainly TAGs are stored within lipid droplets for later use during cellular starvation in membrane synthesis and energy production (Martin and Parton 2006; Zechner et al. 2012). It is suggested that significantly upregulated TAGs in $\Delta/\Delta fth2$ may account for the lipid droplet size displayed by the mutant (Singh et al. 2009; Vevea et al. 2015; Zechner et al. 2017).

In addition to ergosterol and sphingolipids, membrane lipids such as phospholipids are known to be closely linked to multidrug resistance in *Candida* (Mukhopadhyay et al. 2004).

Thus, we further evaluated the effect of *FTH2* gene on phospholipid production in *C. albicans* and have shown that although *C. albicans* BWP17 exhibited a high number in the total amount of differentially produced phospholipids, it was rather evident that $\Delta/\Delta fth2$ maintained its membrane integrity. This was made evident by an increase in the PC to PE ratio when compared to its parental strain, *C. albicans* BWP17. Moreover, these results may be indicative of the mutant's resistance to fluconazole. While these results are significant, it is important to note that this is one part of the overall lipid composition and as such, the role of ergosterol in drug susceptibility cannot be overlooked. Thus, it would be beneficial to further explore the effects of *FTH2* deletion on ergosterol levels. Ergosterol serves as a key component of the fungal cell membrane and is a primary target for many antifungal drugs including fluconazole in *C. albicans* (Mysyakina and Funtikova 2007). Studies pertaining to the effect of iron availability on drug resistance suggest that membrane ergosterol production is downregulated under iron-limiting conditions, thus leading to more fluid membranes and greater passive diffusion of drugs (Prasad et al. 2006; Hameed et al. 2011).

In this study the effect of pyoverdine on the production of lipid species was also demonstrated. Most of the total lipid species levels if not all decreased in response to pyoverdine in both strains. Although the interaction between *C. albicans* and *P. aeruginosa* has been well documented and the role of iron, in both microbial species, it is still unclear as to what extent their interactions may change as a result of evolutionary strategies. Studies also suggest that competition for nutrients such as iron may cause a shift in their normal interaction and this may include both species exploiting different resources to maintain their normal cellular processes and pathogenesis (Craig MacLean et al. 2005; Lawrence et al. 2012). In this study on the effect of pyoverdine may only constitute a small portion of the overall outcome of its interactions.

The overall findings suggest that changes in lipid metabolism may influence membrane composition and fluconazole susceptibility. It is however important to acknowledge the limitations of this study, such as the use of only one concentration of pyoverdine to evaluate the effect of iron availability on *C. albicans* lipidome and the exclusion of sterols such as ergosterol in the lipid profiling analyses. While phospholipids undoubtedly contribute to antifungal drug resistance, the structural and functional role of ergosterol makes it the prime target of antifungal drugs such as azoles and polyenes. Thus, to address these limitations, future studies could be to investigate the potential effect of *FTH2* deletion on ergosterol production.

In conclusion, although there may be other factors pertaining to the acquired multidrug resistance in *C. albicans*, this study adds valuable insights into the influence of iron availability on lipid homeostasis and fluconazole susceptibility.

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Supplementary data

Untargeted lipidomic analyses

The following supplementary data provides an in-depth look into our findings.

1. Negative data mode set:



CPLZ04212301-4_neg
ative data.xlsx

https://docs.google.com/spreadsheets/d/1Yzt6ku1RX-Igus7mDjZhxd-SYU_eGiA_/edit?usp=drive_link&oid=117176053009554672681&rtpof=true&sd=true

2. Positive data mode set



CPLZ04212301-4_posi
tive data.xlsx

https://docs.google.com/spreadsheets/d/1PH1ZR3JAOSMrKAOC8Vr0LHhmeMqrT7D-/edit?usp=drive_link&oid=117176053009554672681&rtpof=true&sd=true

3. The provided data (figures) is presented using the following annotations, for both the negative and the positive mode:

A1 = Analysis 1 (BWP 17 without vs BWP17 with pyoverdine)

A3 = Analysis 2 (BWP17 without pyoverdine vs $\Delta/\Delta fth2$ without pyoverdine)

A5 = Analysis 5 (BWP17 with pyoverdine vs $\Delta/\Delta fth2$ with pyoverdine)

A7 = Analysis 4 ($\Delta/\Delta fth2$ without vs $\Delta/\Delta fth2$ with pyoverdine)



CPLZ04212301-4_figures.zip

<https://drive.google.com/file/d/1nu8NBEIpr->

[i3z30AlUnVTcOrmdWglsmf/view?usp=drive_link](https://drive.google.com/file/d/1nu8NBEIpr-i3z30AlUnVTcOrmdWglsmf/view?usp=drive_link)

