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Effect of Gallic and Ferulic Acids on Oxidative Phosphorylation on *Candida albicans* (A72 and SC5314) During the Yeast-to-Hyphae Transition

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By

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Candida albicans is a dormant commensal in the mucosa of healthy individuals but can become an opportunistic pathogen when the host microflora is compromised. It has been reported as the most common cause of fungal infection among hospitalized U.S. patients, with elevated mortality rate. Candida species have been reported as resistant to antifungal drugs that increase the application of novel strategies to treat this infection. Among the natural compounds that have most gained attention as potential agents against fungi are phenolic compounds against fungi. C. albicans has the ability to switch phenotype from yeast-to-hyphae cells, with hyphae being the most invasive form; therefore, therapies aiming to impair this morphologic transition can be promising against this fungus. The main objective of this work was to evaluate the ability of isolated phenolics to prevent the yeast-to-hyphae transition of C. albicans by modulating the activity of the enzyme complexes involved in oxidative phosphorylation. Also, C. albicans strains (SC5314 and A72) responded significantly differently to the OXPHOS respiration even though they showed similar morphologies. Cells were treated with certain concentrations of gallic and ferulic acid in isolation determined to prevent hyphal growth by 25-50%. Results showed that gallic and ferulic acids inhibited the enzyme activity involved in oxidative phosphorylation of C. albicans (SC5314 and A72).

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1. LITERATURE REVIEW

1. A. *Candida albicans* Morphology and Pathogenicity:

Candida albicans inhabits the gastrointestinal tract as a typical commensal member of the microbiota but can become an opportunistic pathogen when the host microflora is compromised. *Candida* infections (candidiasis) can be life-threatening, particularly in individuals who are critically ill; (e.g., those with immunodeficiency syndrome, or hematological malignancy), causing mortality rates of over 30-40% (Morgan et al. 2005). The Centers for Disease Control has estimated that 46,000 cases of invasive candidiasis occur every year in the US (Centers for Disease Control and Prevention and US Department of Health and Human Services, 2013).

The most common form of invasive candidiasis is candidemia, which is now the most frequent bloodstream infection in the US (Magill et al. 2014). *Candida* species are the fourth main cause of hematogenous infections, such as candidiasis, in individuals with compromised immune systems (Horn et al. 2009; Wisplinghoff et al. 2004). Candidiasis has also been loosely associated with other non-emergent albeit debilitating conditions, such as denture stomatitis, allergic reactions, depression, diabetes and more recently obesity (Guggenheimer et al. 2000; Edward 1985; Srebrnik 1990; Salerno et al. 2011).

C. albicans is able to evade the immune system defenses of the host in order to survive due to its ability to switch phenotypes (Sudbury et al. 2004). Transcription of hyphal-specific genes is initiated by a network of environmental sensing and signaling transduction pathways (Biswas et al. 2007). Therefore, when *C. albicans* yeast is challenged by certain environmental cues, the hyphae switch is triggered (Schulze 2009). From the tip, a single germ tube evaginates and elongates. *Candida* then

synthesizes and maintains a continuous supply of materials as well as enzymes for elongating the new cell wall (Tournu 2011). Interestingly, *C. albicans* can even elongate without mitochondrial function when a fermentable carbon source is available, such as glucose (Gyurko et al. 2000; Geraghty and Kavanagh 2003). The hyphal form can survive in conditions of natural pH, temperatures above 37 ◦C, low O_2, serum and high CO_2 levels (Howard et al. 2007). This unique yeast can also survive under conditions of oxidative stress, which can regulate the expression of individual complexes present in the electron transfer chain and thereby control electron flow between the enzymes proton production and ultimately energy levels (Cavalheiro et al. 2004). Moreover, in the event that immune phagocytes are triggered by the presence of *C. albicans* yeast, either in the bloodstream or inoculating internal organs, *C. albicans* switches to the hyphae form during the early engulfing stage. As nutrients are limited in phagocytes, *C. albicans* hyphae are able to slow down glycolysis, switch to oxidative stress response and then resume rapid glycolytic growth upon escape (Lorenz et al. 2004) as the phagocytes break down.

Although both the yeast and hyphal form have been reported to infect internal organs (Felk et al. 2002), the hyphae are more adhesive than other *C. albicans* cells and more invasive (Jacobsen et al., 2012). When the bloodstream is infected by *C. albicans*, the yeast form is the typical phenotype present (Jacobsen et al., 2012). However, Dalle et al. (2010) showed via *in vitro* studies that the hyphae specifically invade both reconstituted epithelial and endothelial cells and cause damage. The study provides valuable information because *C. albicans* has to penetrate the mucosal barrier and endothelial cells to establish candidaemia and infect internal organs. There are two mechanisms *C. albicans* uses to invade epithelial cells: induced endocytosis by cell invasin Als3, which bind to host E-cadherin, and active penetration (Dalle et al. 2010;

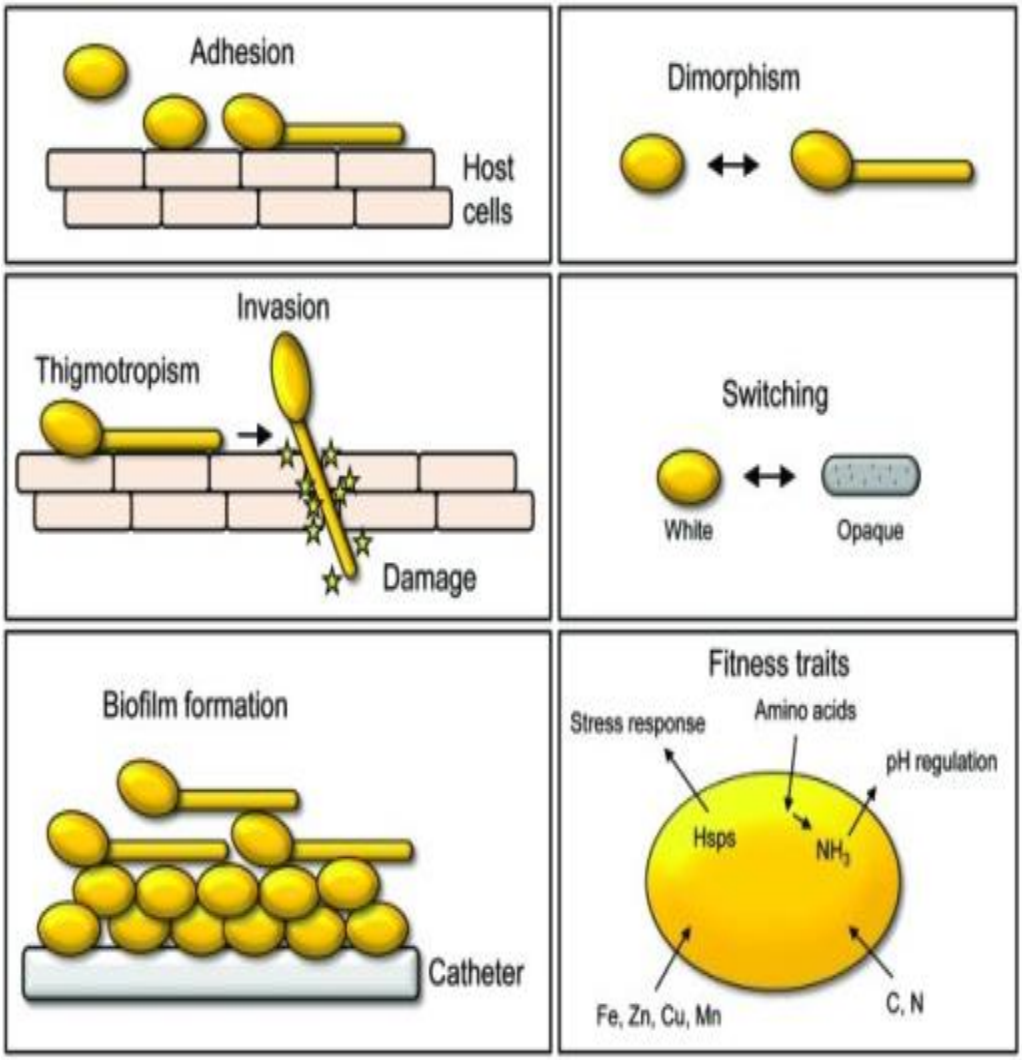


Figure 1. Virulent responses of *C. albicans.*

Phan et al. 2007). Indeed, active penetration is also responsible for an invasion of enterocytes (Dalle et al. 2010). (Figure 1. illustrates the different morphology and pathogenicity of *C. albicans*, some of which have not been discussed here as it beyond the scope of this project).

However, *C. albicans* yeast is able to thrive in the form of gastrointestinal induced transition (GUT) cells within a healthy host without imminent damage. Indeed, the phenotype of GUT cells maintains the fungus in a harmless state in which virulence-associated genes are downregulated (Pande et al. 3013). Considering that *C. albicans* benign yeast has the ability to adjust to a variety of available nutrients, virulent infections are dependent on the strength of the natural immune system (Brown et al., 2014). Oral candidiasis is the most common fungal infection of the oral mucosa, as it is usually associated with disequilibrium of the oral microbiota by prolonged use of broad-spectrum antibiotics (Soysa et al., 2008) and an immunocompromised defense system (Farah et al.; 2010). For example, oral candidiasis occurs in up to 90% of HIV patients before the introduction of active antiretroviral therapy (Thompson et al., 2010). This infection can be classified as pseudomembranous (also known as thrush), acute erythematous, denture-associated erythematous or chronic hyperplastic candidiasis, based on location, depth of epithelial invasion and host response [reviewed in Hebecker et al., (2014)]. Later stages of oral infection are characterized by destruction and loss of the superficial oral epithelium due to the invasion of long *C. albicans* hyphae cells (Farah et al., 2000), which appear to play a critical role in oral *Candida* infections because of their ability to penetrate the epithelial layer. Also, cells that are not able to switch morphology have been associated with the reduced capacity to invade epithelial cells *in vitro* and usually present reduced virulence in animal models of mucosal candidiasis (Park et al., 2005). Therefore, strategies that specifically target this virulence factor by inhibiting the yeast-

to-hyphae transition [reviewed in Jacobsen et al. (2012)] could be used to alleviate clinical symptoms of oral candidiasis and other types of *C. albicans* infections.

The majority of antifungal drugs commonly used today can be toxic to other cells, primarily eukaryotes, due to the similarity in host and fungal cells of mechanisms involving DNA, RNA and protein synthesis (Shoham and Levitz, 2005), which is one of the main difficulties in treating fungal infections. Also, the limited diversity of the available antifungal drugs used to treat these infections have caused the emergence of resistant organisms (Pfaller et al., 2008; Smith and Andes, 2008; Spanakis et al., 2006; Holmes et al., 2008). Only a few classes of agents are currently on the market to treat mucosal or systemic *Candida* infections (Matthew and Nath, 2009; Kathiravan, 2012; Denning and Hope, 2010). These main drug families consist of Azoles (e.g., fluconazole), polyenes (e.g., amphotericin B), echinocandins (e.g., caspofungin) and nucleoside analogues (e.g., flucytosine) with the first two cited drugs being the most commonly used (Denning and Hope, 2010; Dodds et al., 2010; Bekersky et al. 2002; Alves et al., 2014; Heimark et al., 2002). Azole-resistant *C. albicans* is frequent in HIV-infected patients with oropharyngeal candidiasis (Skiest et al., 2007; Sanglard and Odds, 2002), while minimal resistance to the polyene amphotericin B has been reported, although side effects and toxicity caused by this class are well-known (Laniado-Laborín and Cabrales-Vargas, 2002; Ellis, 2002). The major mechanisms leading to antifungal drug resistance in *Candida* is provided in an excellent review by Spampinato and Leonardi (2013). It must be emphasized that treatment with conventional antifungals can also eliminate microbiota members that are *Candida*'s natural competitors, which in turn can increase the severity of the infection (Candiracci et al. 2011; Kathiravan et al. 2012; Denning and Hope 2010).

The yeast to hyphae morphology can be induced in the laboratory depending on the growth medium and incubation conditions (Biwas et al., 2007; Veses and Gow,

2009) allowing for controlled experiments to develop novel anti-fungal agents that target the phenotypic switch. Moreover, due to the relationship of pathogenicity of *C. albicans* to its ability to switch from unicellular budding yeasts to a hyphae phenotype (Gow, 2013), this phenotypic switch presents a unique target for preventing or remediating the virulent state by compounds, such as natural products, without killing a commensal organism of our microbiome.

1. B. <u>Anti-hyphal Properties of Phenols</u>

Considering the emergence of *C. albicans* strains resistant to commonly used antifungal agents, the development of innovative and multiple targeted anti-fungal agents is critically needed. Phenolic compounds present in plant systems have shown such promise due to their ability to complex proteins, disrupt microbial membranes, or act as cell signaling agents (Papadopoulou et al. 2005; Kanwala et al. 2010; Candiracci et al. 2012; Bravo and Lazo 1996; Hirasawa and Takada 2004). The compounds, phenols, are uniquely positioned to fill this need because as secondary metabolites in plants, it is their function to protect their host against multiple stress conditions, including microbial invasions and abiotic (e.g., UV light) stresses (Tomás et al. 2000; Treutter, 2005).

Over 8000 polyphenolic structures have been identified (Baxter et al. 1998), and these are categorized by the presence of one or more aromatic rings bonded to one or more hydroxyl groups (Ignat et al. 2011). Phenols are further divided into several additional groups, such as flavonoids, phenolic acids, tannins, and lignans, based on their chemical structures (D'Archivio et al. 2007) (Figure 2), and then separated by the position of hydroxyl groups or acid groups (such as in the case of phenolic acids) (Figure 3 and 4). Also, the basic structure of each of these compounds can be derivatized to different positions on the main structure by various compounds, but typically sugar groups serve this purpose (Bravo 1998). Despite their different structures, many of these

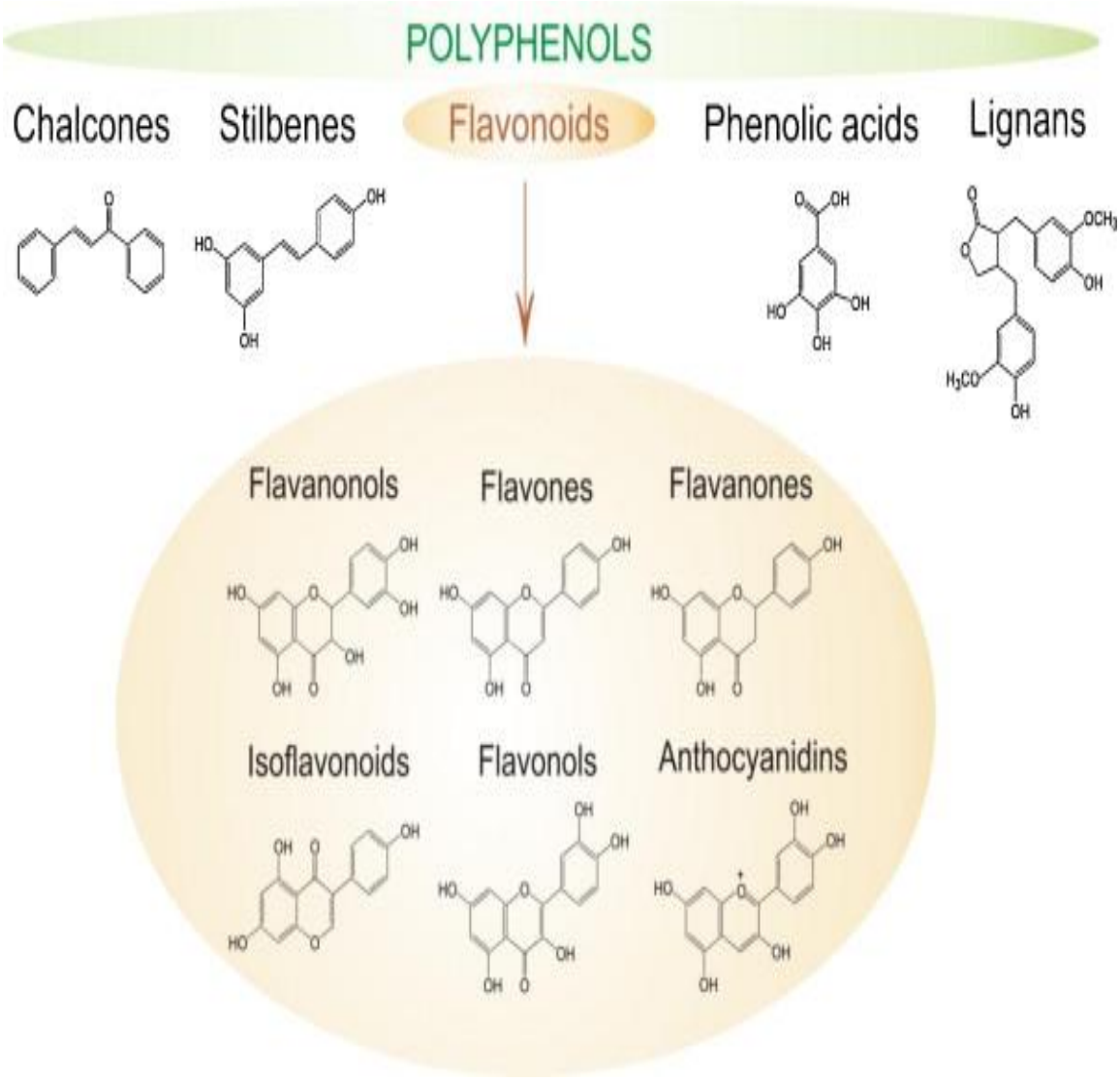
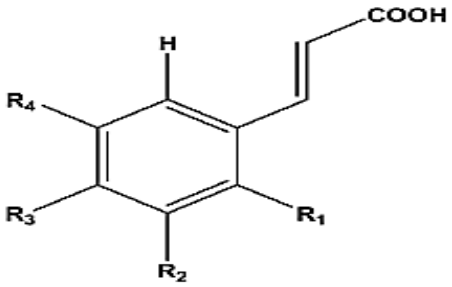


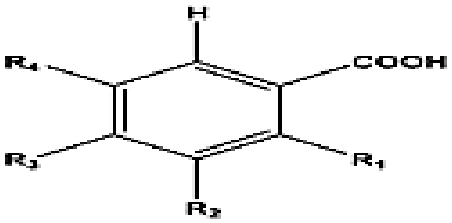
Figure 2: Categories of phenols based on structures.



Hydroxycinnamic Acids

Name	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	H	H
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

Figure 3: Structure of different types of phenolic acids classified as hydroxycinnamic acids due to the conjugated bond between the phenols and the acid groups.



Hydroxybenzoic Acids

Name	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Veratric acid	H	OCH_3	OCH_3	H
Salicylic acid	OH	H	H	H

Figure 4: Structure of different types of phenolic acids classified as hydroxybenzoic acids due to the covalent bond between the phenol and the acid group.

phenolic components share ordinary bioactive activities, albeit to varying degrees, which include the ability to scavenge free radicals and to modulate specific cellular enzyme activities (Cai et al., 2006; Chen and Blumberg, 2008; Masella et al. , 2005). As such, they have been shown to protect the human body against several chronic diseases, such as cancer, heart disease, Alzheimer's disease, and obesity (Hertog et al., 1993; Huang and Ferraro, 1992; Teixeira et al., 2013). Phenolic compounds are also able to enhance the healthy state of diseased cells by acting upon multiple carbohydrate metabolic pathways (Arathi and Sachdanandam, 2003; Kueck et al., 2007; Suolinna et al., 1975), including within the mitochondria. For example, Jagatha et al. (2008) determined that curcumin protected mitochondrial complex I activity from stress in dopaminergic neuronal cells. In addition, epigallocatechin gallate was able to increase enzyme activity of select tricarboxylic acid (TCA) cycle proteins and thus protect electron transfer complexes' function from mitochondrial oxidative damage in rat models (Srividhya et al. 2009).

Indeed, the antifungal activity of phenolic compounds against several fungal genera is well documented in the following review (Guzman 2014). As an example, epicatechin and quercetin isolated from the leaves of mango showed an increase of antifungal activity with an increase in their concentration against five different fungal species (Kanwal et al. 2010). Yet, the phenolic protection against yeast has primarily been studied based on the ability of phenolics to inhibit cellular growth (Gallucci et al. 2014; Faria et al. 2011; Candiacci et al. 2011; Guzman 2014; Kanwal et al. 2010). As an example, gallic acid extracted from hydrolyzable tannin was able to kill or inhibit the growth of *C. albicans* cells (Hong et al. 2011), but its effect on virulence factors was not determined. Gallic acid and quercetin identified from flowers of North Eastern Portugal showed antifungal activity against all *Candida* species biofilms (Alves el al. 2014). Also,

ferulic acid provided antimicrobial protection by inhibiting the growth of *Candida* species (Ergün et al. 2011).

Nonetheless, other, albeit limited, studies have targeted the virulence factors of *C. albicans*. Phenol-rich curcumin was able to inhibit reactive oxygen species produced by the *Candida albicans* hyphal phenotype, and thereby mitigate damage to engulfing phagocytes or epithelial layers for penetration into the bloodstream (Sharma et al. 2010). Kazuko et al. (2010) demonstrated that yeast-to-hyphae transition and the extent of hyphal growth was inhibited by resveratrol. Saito et al. (2013) showed the ability of the flavonoid, catechin, to inhibit the transition to *C. albicans* hyphal phenotype. The phenolic acid, gallic acid, has also been reported to prevent the *in vitro* formation of *C. albicans* biofilms (Wang et al. 2009). Also, benzyl was able to reduce hyphae formation and affect biofilm formation of *C. albicans* (Zhang et al. 2011).

Although phenolic compounds are consumed daily, they suffer from low bioavailability (5%) and spend little time in the body (< 24 h), which predicates the hypothesis that this diverse group of agents most likely are working together to provide a health benefit. For example, phenol-rich extracts obtained from honey and grapefruit were able to inhibit the hyphal transition in a dose-dependent matter that resulted in much lower inhibitory concentrations compared to the phenols in isolation (Candiracci et al. 2012; Cvetnic and Vladimir 2004). However, these studies remain limited and are beyond the scope of the current study, but warranted mention for future studies.

1. C. Respiratory chain and oxidative phosphorylation

Energy conversion by *Candida* species is the fourth main cause of hematogenous infections in individuals with compromised immune systems (Horn et al. 2009). This mechanism is achieved via functional mitochondria by two interconnected pathways that include the tricarboxylic acid (TCA) cycle and the electron transport chain / oxidative phosphorylation system, both of which occur in the mitochondria, or “the

power house" of the cell. The TCA cycle directly or indirectly supplies carbon, via carbohydrate, protein or lipids (Figure 5), which are then oxidized in the inner membrane of the mitochondria to produce reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide ($FADH_2$) and adenine trinucleotide (ATP) (Dröse and Brandt 2012).

However, the majority of energy in the form of ATP would not be possible without the four enzyme complexes forming the produced by the electron transport system (ETS) and another that catalyzes AMP to ATP, i.e., the oxidative phosphorylation (OXPHOS) enzyme, thus the most potent respiratory chain consists of five enzyme complexes. Electrons are transported by these enzymes, which are embedded in the inner mitochondrial membrane (Mandalari et al. 2010; Strauss et al. 2008) (Figure 6). The electrons from NADH and $FADH_2$ produced in the TCA are transferred to the electron transport chain at complexes I and II, respectively (Figure 6). This step regenerates NAD+ and FAD (the oxidized carriers) for use in the TCA pathway (Siedow and Umbach 1995). The electrons flow through the electron transport chain, causing protons to be pumped from the matrix to the intermembrane space. Eventually, the electrons are passed to oxygen, which combines with protons to form water (Moore et al. 1995). The proton gradient generated by proton pumping during the electron transport is a stored form of energy. When protons flow back down the concentration gradient (from the intermembrane space to the matrix), their only route is through ATP synthase, another enzyme embedded in the inner mitochondrial membrane (Figure 6). When protons flow through ATP synthase, water turns a water wheel and this motion catalyzes the conversion of ADP and P_i to ATP (Figure 6) (Nicholls and Ferguson 2002).

Complex I ubiquinone oxidoreductase oxidizes NADH to NAD+, passing the electrons to ubiquinone to form ubiquinol (Sarasota 1999; Pester and Minter 2016). Complex I has a unique particle L-shaped structure with two-dimensional arrays

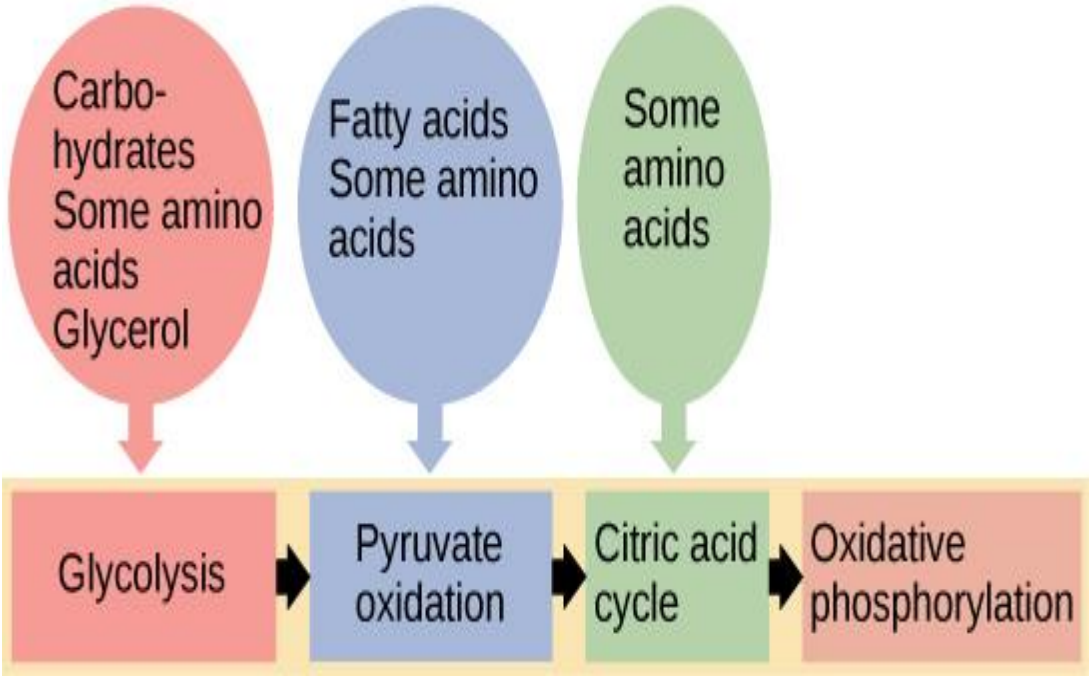


Figure 5: Sites of carbon entry by different compounds into the mitochondria via the TCA cycle.

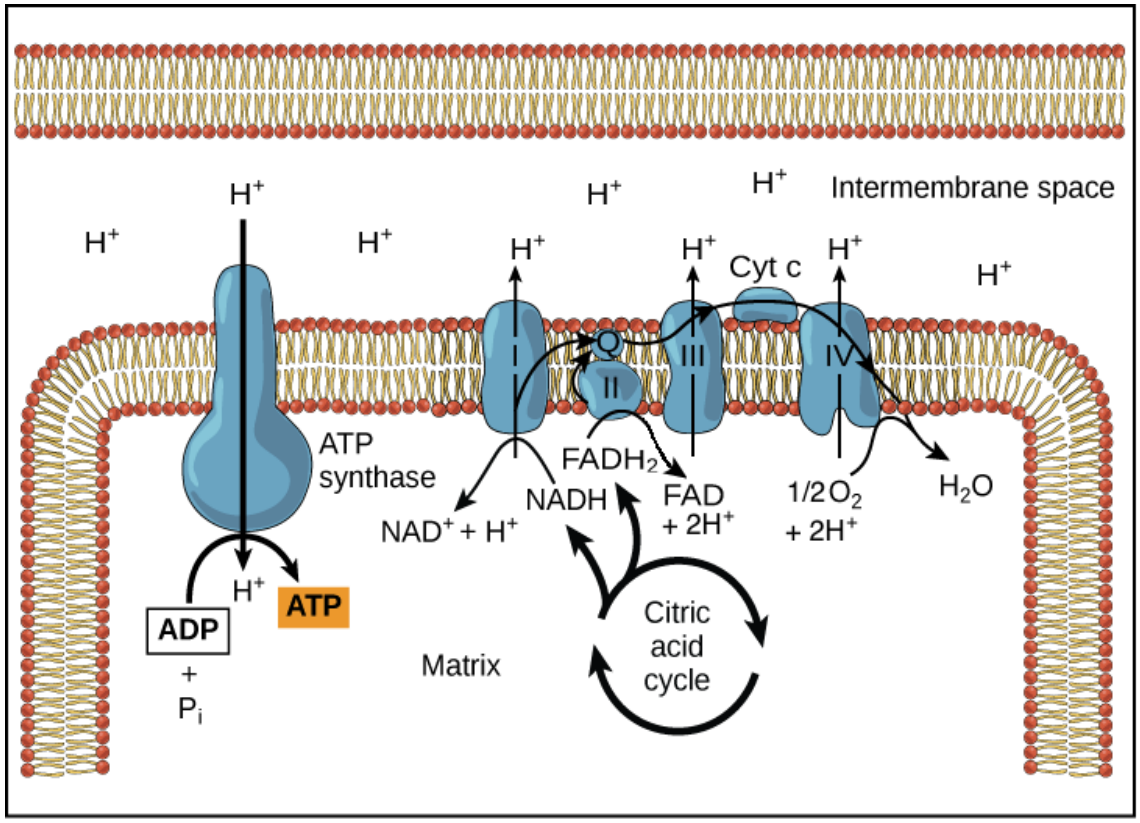


Figure 6: Electron transport chains, electron flow, and gradient pump and ATP synthase.

(Rich 2003). Complex II uses ubiquinone to oxidize succinate to fumarate. The reduction of ubiquinone to ubiquinol facilitates succinate oxidation resulting in additional electrons. Electrons are transported from Complex I or II as ubiquinone to complex III (Sun et al. 2005). Cytochrome bc1, or Complex III, then delivers electrons from oxidized ubiquinol to reduced cytochrome c. The Q-cycle is responsible for proton and electron transfer via two pathways, starting when ubiquinone attaches to Complex III upon binding two electrons that flow from the two different pathways.

One electron moves to the iron–sulphur cluster and then transfers to the heme of cytochrome c1 and the substrate cytochrome c. Another electron moves into the heme group of cytochrome b before being picked up by ubiquinone and this, in turn, reduces ubiquinone to ubiquinol. Cytochrome c oxidase or Complex IV is the enzyme responsible for transferring electrons from cytochrome c to molecular oxygen to deliver two electrons from reduced cytochrome c to further transfer to CuA and the heme a iron, followed by the heme a3 iron and lastly to CuB. A peroxide bridge then forms between the reduced form of CuB and heme a3. The proton breaks down the bridge and forms molecular water (Arnold 2012). Lastly, F_1F_oATPase or Complex V is responsible for the synthesis of ATP by using the proton motive force. The energy transfers between F_o membrane sectors to the F_1 catalytic sector. F_1 contains β (and α) subunits that each are composed of three-domain structures, which form the hexamer that catalyzes the synthesis of ATP (Saraste 1999; Belogrudov et al. 1995).

As mentioned previously, different types of phenols can positively impact a stressed cell; this includes acting upon multiple carbohydrate metabolic pathways, including oxidative phosphorylation (Arathi and Sachdanandam 2003). As an example, quercetin was able to protect against mitochondrial oxidative stress in isoproterenol-induced myocardial infarcted rats. More specifically, this study showed that by consuming (10 mg/kg body weight of) quercetin daily for a period of 7 days, the treated

rats presented with significantly decreased activity levels of enzymes involved in regulating the tricarboxylic acid cycle (TCA) and respiratory chain enzymes, which were comparable to the negative control group (Prince and Sathya, 2012). Makovec and Šindelář (1984) also determined that phenolic compounds affected the activity of respiratory chain enzymes of potato tuber mitochondria. This study shows that several types of phenolics were able to inhibit respiration and phosphorylation. Indeed, succinate dehydrogenase activity (complex II) was the most inhibited enzyme among the respiratory chain enzymes, with quercetin being the most effective inhibitory phenol. Xie and Chen (1999) determined that salicylic acid (SA) encourages rapid inhibition of ATP synthesis and respiratory O_2 uptake on mitochondrial electron transport and oxidative phosphorylation in tobacco cells. Because mitochondria play significant roles in plant diseases, the effect of the SA on electron transport was a higher chance in SA-mediated biological processes in mitochondria function.

Still, the effects on the activities of the respiratory proteins present during the *C. albicans* transition state have not been reported to our knowledge, yet alone when treated with phenols. Nevertheless, it is expected that this pathway is affected during the yeast-to-hyphae transition as reactive oxygen species (ROS) levels have been shown to change during this process. In a study conducted by Candiracci et al. (2012) the results showed that flavonoids extracted from honey were able to inhibit the yeast-to-hyphae transition of *C. albicans*. This response was attributed to the suppression both ROS generation and γ-glutamyl transpeptidase activity, which is responsible for GSH degradation during hypha elongation. Schröter et al. (2000) also reported that the levels of ROS production differ with *C. albicans* phenotypic transitions and is linked to its pathogenicity. More specifically, the highest ROS levels occurred during hyphae formation when compared to blastoconidia at the same protein level. Moreover, the

researchers proposed that the increased ROS levels were a consequence of disrupted complex I (CI) (Li et al. 2011).

2. OBJECTIVE AND SPECIFIC AIMS

The objective of this project was to determine the ability of the phenolic acids, ferulic and gallic, to modulate the activity of the enzyme complexes involved in oxidative phosphorylation as a means to maintain the benign yeast state. This research is linked to oxidative phosphorylation as changes in this highly interconnected energy pathway are expected in such a crucial event as a phenotypic switch. The activity of the individual complexes II, III, IV, V, the mitochondrial matrix marker enzyme citrate synthase, and the combined activity of complexes II and III will be monitored at each of the following time points: 0, 1, 3, and 6 hours of *C.albican* yeast –to hyphal transition . The cited times were selected because previous studies completed in our laboratory have shown that *C. albicans* is biochemically active at these time points even if the morphology appears similar.

Specific Aim 1: To determine the effects on the activities of the oxidative phosphorylation enzymes for two strains of yeast *C. albicans* (SC5314 and A72) during their transition from yeast to hyphae phenotype. Specific Aim I is needed as a means to understand the relationship of oxidative phosphorylation to the virulent phenotypic switch for two different strains, and thus if significant changes occur between both. This specific aim will be accomplished by first establishing each of 5 tests (Complex II, II + III, IV, and V), albeit citrate synthase will be used in the final calculations of each. The effects will then be compared using yeast exposed to 37°C (the transitioning temperature) and 30°C (the non-transitioning temperature). This information provides the foundation for Specific Aim 2.

Specific Aim 2: To determine the ability of ferulic and gallic acids, as isolated compounds, to target the oxidative phosphorylation enzymes as a means to suppress 25-50% or more of the hyphal stage using the two pathogenic *Candida* strains (SC5314 and A72). The working hypothesis for this specific aim is that different phenols will affect the oxidative phosphorylation chain, which will be reflected in the hyphal phenotype. This specific aim will be accomplished by treating *C. albicans* with each of the cited phenols in isolation and then measuring the activity of each oxidative phosphorylation enzyme in response to *C. albicans* during its yeast-to-hyphae transition.

3. MATERIALS AND METHODS:

3. A. Specific Aim 1:

3. A.1 *Preparation of* C. albicans *culture*: *C albicans* strains SC5314 and A72 were obtained from Kenneth Nickerson, University of Nebraska – Lincoln. A stock culture was grown to the stationary phase in Yeast extract-Peptone-Dextrose medium at 30 °C, washed and stored in potassium phosphate buffer (pH 6.6) at refrigerated temperatures. To transition the yeast-to-hyphae, the culture, at a cell density of $1X10^7$ per mL, was transferred to an Erlenmeyer flask containing 0.65mL of 0.1 M N-acetylglucosamine (GlcNAc), 2.80 mL of 0.1 M imidazole buffer (pH 6.5), 0.75 mL of 0.1 M $MgSO_4$, 0.65 mL of 0.1 M GlcNAc and 20.80 mL Nano pure water. The culture was then incubated at 37 °C in a water bath with shaking at 300 rpm to achieve aeration for 6 hr. A negative control was also prepared as cited above; only the temperature was maintained at 30°C to prevent transition. Aliquots (100 µl) of the cells were collected during the course of the transition period at 0, 1, 3 and 6 hr., and the cells from a 100 µL aliquot were counted at each time point using a hemocytometer. As the hyphae form tends to clump together making counting difficult, the number of hyphae cells vs. yeast cells were estimated by subtracting the number of yeast cells counted at each time point

(YC) from the number of initial cells (IYC) (at 0 h). Percent inhibition was then calculated using the formula: (YC / IYC) * 100 (Camara 2015).

3. A.2 *Enriched Mitochondrial Preparation from* C. albicans*:* Mitochondria of *C. albicans* were obtained from each cited transition time point were isolated from the pellet as described by Frazier (2012). Briefly, cells were suspended twice in a solution of 2 mM HEPES, 0.1 mM EGTA, 250 mM sucrose, pH 7.4 (with KOH) and then disrupted with a Mini-Beadbeater-1 for 3 min. The homogenate was transferred to an Eppendorf tube and spun at 600 *x g* in a chilled centrifuge for 10 min. The supernatants were combined and spun in a refrigerated tabletop centrifuge at 14,400 RCF for 10 min. The pellet containing enriched mitochondria was re-suspended in 2 mM HEPES, 0.1 mM EGTA, 250 mM sucrose, pH 7.4 (with KOH). Approximately 60–75 µL of the suspension was retained for CII + III and CIII assays. The remainder was treated with 25 mM potassium phosphate buffer (KPI), pH 7.2, 5 mM $MgCl_2$ for the CI, CII, CIV, CV and CS assays as described below. Samples were treated to three freeze/thaw cycles in a dry ice/ethanol slurry, to disrupt the mitochondrial membranes in order to make the substrates available to enzymes.

3. A.3. *Citrate Synthase (CS) Assay:* Citrate synthase (CS) buffer was prepared by combining 22.5 mL of 50 mM KPi (pH 7.4), with 2.5 mL of 0.1 mM, 5, 5'-dithiobis-dithio-bis-(2-nitrobenzoic acid) equilibrated to 30°C. The CS buffer (475 µL) was delivered to two matching UV cuvettes. The sample (20 µL) was then added to both cuvettes followed by 5 µL of acetyl CoA. The reaction was started by adding 5 µL oxaloacetic acid in one cuvette, while the other cuvette served as a blank. The reactions were monitored for 3 min at an absorbance at 412 nm. The results were calculated using Beer's law with the extinction coefficient for thionitrobenzoate anion of 13.6/mM/cm.

3. A.4 *Complex I (CI) Assay*: The buffer for the CI assay was prepared with 23 mL of 50 mM KPi (pH 7.4), 0.626 mL of 50 mM nicotinamide adenine dinucleotide, 0.5

mL of 1 mM KCN, 0.5 mL of 10 mM antimycin A, 0.25 mL of 0.1% (w/v) bovine serum albumin, 0.125 mL of 50 mM coenzyme Q1 and equilibrated to 30 °C. An aliquot of CI buffer (475 µL) was delivered to two UV cuvettes, while rotenone (5 µL) was added to one cuvette and ethanol (5 µL) was transferred to the other cuvette to serve as a blank. The reaction was started by adding 20 µL of the sample to each cuvette. The absorbance was measured at 340 nm for 3 min, and the results were calculated with an extinction coefficient for NADH of 6.81/mM/cm.

3. A.5 *Complex II (CII) Assay*: The CII assay buffer was prepared with 21.5 mL of 50 mM KPi (pH 7.4), 2.5 mL of 10 mM sodium succinate, 0.5 mL of 1 mM KCN, 0.5 mL of 10 mM antimycin A, and 0.25 mL of 2.5 mM rotenone and equilibrated to 30 °C. The CII buffer (485 µL) was delivered to two UV cuvettes followed by 10 µL of a sample and incubated for 10 min at 30^{o}C. The reaction was started by adding 5 µL of 50 mM CoQ1 in one cuvette, while 5 µL of 50 mM ethanol was added to second to serve as a blank. The reaction was monitored for 3 min at an absorbance of 280 nm, and the results were calculated with an extinction coefficient for CoQ1 of 12/mM/cm.

3. A.6 *Complex II + III (CII+III) Assay*: The CII + III assay buffer was prepared with 21.6 mL of 50 mM KPi (pH 7.4), 2.5 mL of 10 mM succinate, 0.5 mL of 1 mM KCN, 2.5 mL of 0.25 µM rotenone, 0.25 mL of 0.1% (w/v) BSA, and 0.188 mL of 0.075% ethylene diamine tetraacetic acid (EDTA), pH 7.0 and equilibrated to 30°C. An aliquot of CII+III buffer (472 µL), 10 µL sample and 5 µL of 1 mM ATP were then added respectively to each of two cuvettes and incubated for 5 minutes at 30 °C. The reaction was started by adding 12.5 µL of 50 mM cytochrome c to one cuvette, while the solution in the other cuvette served as a blank. The reaction was measured at absorbance 550 nm for 3 min, and the results were calculated with an extinction coefficient of reduced cytochrome c of 18.7/mM/cm.

3. A.7 *Complex III (CIII) Assay*: The CIII assay buffer was prepared with 23 mL of 50 mM KPi (pH 7.4), 1 mL of 1 mM n-dodecylmaltoside, 0.5 mL of 1 mM KCN, 0.25 mL of 2.5 mM rotenone, and 0.25 mL of 0.1% (w/v) BSA and equilibrated to 30°C. The CIII buffer (480 µL), 5 µL of sample and 5 µL of 0.1 mM (decylbenzylquinol) (reduced DB) were added to one visible light cuvette in the cited order. The CIII buffer and reduced DB were combined in the second cuvette to serve as a blank. The reaction was started by adding 3.7 µL of 15 mM cytochrome c. After the 20-second incubation time, the reaction was monitored for 3 min at 550 nm, and then L-ascorbic acid was added to the stop the reaction. The reaction was measured at absorbance 550 nm for an additional 3 min, and the results were calculated with an extinction coefficient for reduced cytochrome c of 18.7/mM/cm.

3. A.8 *Complex IV Assay*: The buffers 50 mM KPi (pH 7.4) (435 µL) and 2.5 mM n-dodecylmaltoside (50 µL) were added to each of two visible cuvettes and equilibrated to 30°C. The sample (10 µL) was delivered to one of the cuvettes and reaction was started by adding 3.8 µL of 15 mM of reduced cytochrome c. At the end of 3 min of incubation, a few grains of $K_3Fe(CN)_6$ was added to the reaction, which was then monitored for an additional 3 min at absorbance 550 nm. The results were calculated with an extinction coefficient for reduced cytochrome c of 18.7/mM/cm.

3. A.9 *Complex V Assay*: The V assay buffer was prepared with 21.25 mL of 40 mM Tris–HCO_3, 1 mM EGTA (pH 8.0), 2.5 mL of 0.2 mM NADH, 0.625 µL of 2.5 mM phosphoenolpyruvate (PEP), 0.25 µL of 0.5 mM antimycin A, 0.125 µL of 5 mM $MgCl_2$, 0.125 µL of 50 mg/ml lactate dehydrogenase (LDH), and 0.125 µL of 50 mg/ml pyruvate kinase (PK) equilibrated to 30°C. The V buffer (477.5 µL) and 12.5 µL of 2.5 mM ATP were added and allowed to equilibrate for 2 min. The reaction was started by adding 10 µl sample in one UV cuvette, while the other cuvette served as blank containing 10 µl of

2 µM oligomycin. The reaction was measured at absorbance 340 nm for 3 min. The results were calculated with an extinction coefficient of 6.22/mM/cm for NADH.

3. A.10 *Protein Assay*: The Bradford protein assay was used to a measured protein concentration of 150 µL of *C. albicans,* which was collected at 0, 1, 3 and 6 hr during the yeast-to-hyphae transition. One hundred µl Coomassie Brilliant Blue G-250 was added to the samples along with 50 µL of 50 mM KPi (pH 7.4) and allowed to incubate for 10-30 min. The reaction was measured in the microplate reader at 595 nm, and the protein levels were measured using bovine serum albumin as the standard.

3. A.11: *Activity Calculations used for each Assay (3. A.3-9)*: The activity for a given enzyme was determined from the data obtained from the associated assay and the protein levels as defined by the following equation: activity = (Δ Abs * V) / (ε * L * v *[prot]) in nmol /min/ mg where ε is extinction coefficient, V is reaction volume, v is sample, and [prot] is protein concentration of *C. albicans.*

3. B. Specific Aim 2:

3. B.1 *Culture Transition with a Single Treatment***:** The yeast cells were prepared for transition as described in Section 3.A.1. For this Specific Aim, each phenolic acid (ferulic and gallic acid) were prepared fresh, but in isolation, in 2% ethanol (v/v), as screening studies also using various concentrations of methanol and dimethyl sulfoxide showed this organic solvent at the designated concentration effectively solubilized the phenols but did not affect *C. albicans* transition (data not shown). The treatment solution was added to N-acetylglucosamine differentiation media at 5-7 final concentrations with the upper level soluble in 2% ethanol. The cells were allowed to transition, counted and percent inhibition determine as described previously.

3. B.2 *Enriched Mitochondrial Preparation from* C. albicans *cultures***:** The samples collected from the B1 experiments were then handled and tested as described in 3A.2 to 3A.11.

3. C Statistical Analyses:

A factorial design was used for analysis of the individual compound experiments with replication, time and compound as factors. The % inhibition data were obtained from at least three repetitions of each experiment, which includes the yeast-to-hyphae transition, and every phenolic treatment. The enzyme activities and % inhibition were expressed as the mean +/- standard deviation. One way ANOVA was applied to the results by using the with Tukey's honestly significant difference test to evaluate the significance of the mean of the data concerning the transition period (0,1,3 and 6 hours) and the negative control for untreated and treated samples with different enzymes. The results were considered significant at $p < 0.05$. The Grub's test was used to determine data outliers at the 95% confidence interval.

4. Results and Discussion:

4. A. Specific Aim 1: Activities of the oxidative phosphorylation enzymes of *C. albicans* (SC5314 and A72) during the transition from yeast to hyphae:

The enzymes of oxidation phosphorylation (OXPHOS), which include the electron transport (ECT) enzyme complexes (I, II, III, IV,) and the OXPHOS enzyme (V), synchronize their activity in order to effectively produce the potent energy molecule, adenine triphosphate (ATP), during normal respiration. Such synchronization is critical considering that these enzymes play the major role in producing the majority of ATP for a cell and thus collectively for a whole organism. As is expected, dysfunction or inhibition of one or more of these complexes will affect their harmonization leading to stress and even damage to the mitochondria, their site of location (Frazier and Thorburn, 2012). Mitochondrial dysregulation can, in turn, potentially affect one or more tissues that can progress into multisystem disorders that disregard age group, gender or ethnicity (Munnich and Rustin, 2001; McFarland et al, 2010; Di Donato, 2009). Indeed, mutation

in 100 genes have been identified in mitochondrial related diseases, which predicates that 1 in 5000 people will be affected by the most common cause of inborn metabolism errors (Skladal, 2003, Wong et al., 2010, Tucker et al, 2010, Kirby and Throburn, 2008).

Among these conditions, disturbing the OXPHOS mechanism can result in the changes to the morphology of the affected tissue organs. Torii et al. (1992) determined that the enzymes CI and CIV were both decreased in aging rats in the mitochondria of muscle tissue, , but not in other organs, such as the heart and liver. Boffoli et al. (1994) further showed a significant decrease in the enzymatic activity of Complex CI, CII and CIV in the human skeletal muscle of orthopedic patients, which was attributed to a decline in mitochondrial respiration. The authors further hypothesized that decreased activity of CI, CII and CIV enzymes was also involved in the structural and functional decline of the muscles and tissues associated with aging. Moreover, Bowling et al. (1993) reported that the damage that occurred to mitochondrial DNA in the brain tissue of aging monkeys was directly caused by a decrease in the activity of CI and CIV whereas CII+III and CV did not show significant differences. Combined, these studies demonstrate that a change in activity of one or more of OXPHOS enzymes can critically impact the phenotype of a cell or tissue leading to organ damage even when the other OXPHOS enzymes are operating properly (Frazier and Thorburn, 2012).

Other more current studies have shown that OXPHOS related defects are most common in cells or tissues with high energy demands, such as skeletal, neuromuscular or central nervous systems (Rahman and Hanna, 2009, Finsterer, 2006). Considering that cancer cells are metabolically adapted for rapid growth and proliferation, Rossignol et al (2004) studied the effects of OXPHOS and glycolytic respiration in a cervical cancer lines (HeLa) versus their normal cell counterparts to determine if a specific substrate availability played an important role in the respiration of the cancer cells. This research was particularly relevant for cancer cells as energy is primarily generated by glycolysis

(the Warburg effect) rather than OXPHOS (Vander Heiden, 2009). In this study, the expression of the OXPHOS proteins were down-regulated in the presence of glucose as was expected by the researchers. However, when galactose served as the energy substrate, OXPHOS enzymes were up-regulated indicating that respiration could be modulated to a more healthy state, even in cancer cells, when subjected to a different substrate.

All cells must respond to changes in respiration due to the availability of various substrates to maintain adequate energy levels (Calderone and Fonzi, 2001). *C. albicans* is not exempt from this circumstance. But, when certain cues in the environment require that these cells switch from yeast to hyphae, the ability of *C. albicans* to readily adapt metabolically to transition that may be similar to that cited for the cancer cells (Rossignol et al., 2004) due to the rapid response required. Therefore, it is critical to study the OXPHOS energy demand on *C. albicans* in an effort to understand how energy modulates this switch. This information could thus lead to a potential target for alternative and viable treatment options.

4A.1 *Cell morphology during yeast to hyphae transition*.

Figure 7 shows the morphology of phenotypic switch of *C. albicans* (SC5314) during 0, 1, 3 and 6 hr. These time points were selected as studies in our laboratory determined that *C. albicans* is biochemically active even if the morphology appears similar during certain stages of the transition process (data not shown). At 0 and 1 h, the cells were round to ovoid shape, albeit the latter were starting to adhere to one another (yet another virulence factor) (Calderone and Fonzi, 2001). Most of the cells had transitioned to the germ tube form by 3 h, with ~75%+ reaching the hyphal form. At 6 hr, the cell were 95% hyphae with longer and wider filaments that included high cell adhesion. It must be noted that the transition images of only *C. albicans* (SC5314) are

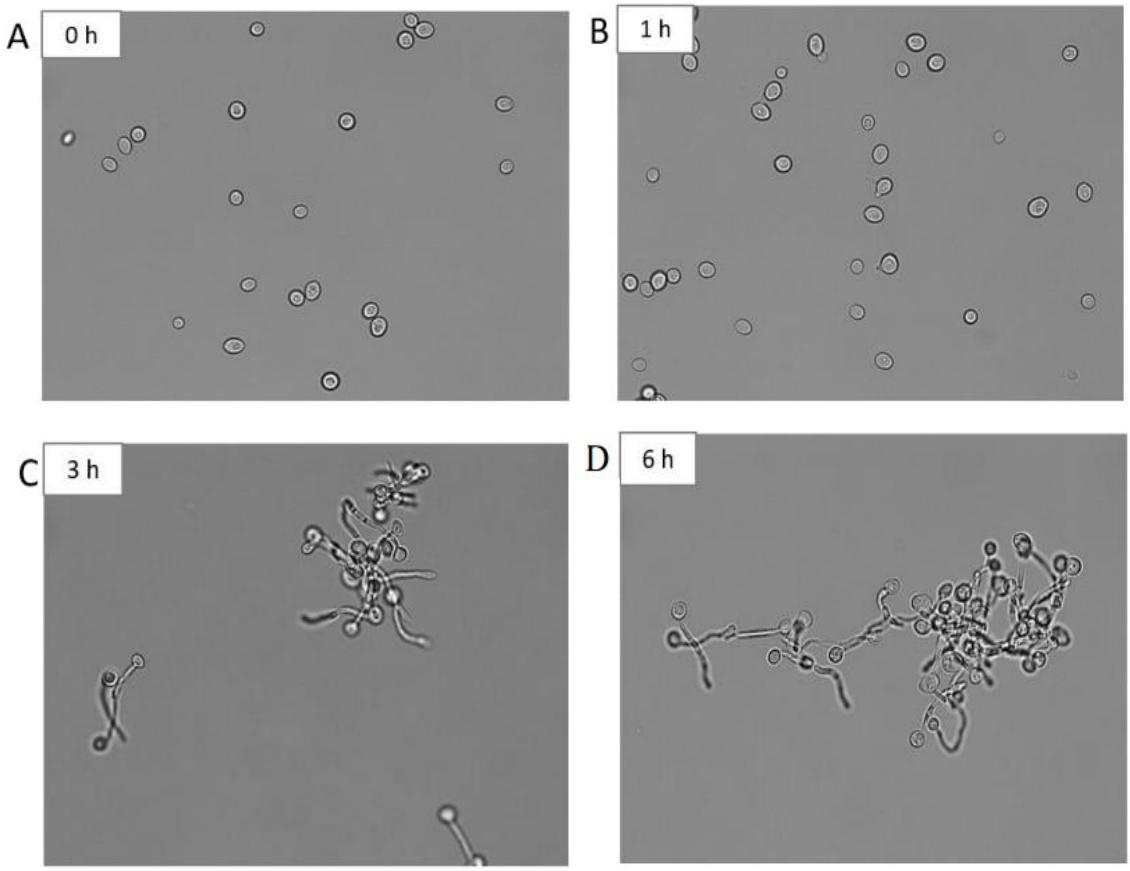


Figure 7: Morphology of *C. albicans* (SC5314) during the course of the phenotypic switch from a benign yeast to adhesive hyphae.

presented here in as *C. albicans* (A72) showed similar morphologies under similar conditions (shaking at 37^{o}C and using glucosamine media).

4A.2 *Comparison of* C. albicans *(strain A72 vs. SC53134) OXPHOS enzyme activity during yeast to hyphae transition at 0, 1, 3 and 6 hr*.

Because the morphologies appeared similar between *C. albicans* A72 vs SC53134 during the switch, the activities of each enzyme at transition time points (0, 1, 3 and 6 hr) were compared to each other to determine if differences occurred at the molecular level on a strain by strain basis. Such information is significant to understand whether different treatment options are necessary for different pathogenic *Candida* strains in terms of targeting OXPHOS enzyme activity to prevent the yeast to hyphae switch. To more accurately make such comparisons, the tricarboxylic acid (TCA) enzyme CS, which is also located in the mitochondria, was monitored to serve as a reference point for mitochondrial activity. By expressing the activity of each of the OXPHOS complexes as a ratio to CS activity, variability that may result from differing cell culture conditions (i.e. cell passage number or cell confluence) or even mitochondrial proliferation, which is typical in elongating *C. albicans* hyphae, can be prevented. Moreover, as different assays are displayed on the same graphic, each enzymatic value obtained at each hour was normalized to a given strain that was subjected to the non-transitioning condition, i.e., 30 ^{o}C (Figure 8 a-d), and was deemed the negative control. It also must be noted that the CI enzyme was not included in any of the analyses as no significant differences occurred in the activity of this enzyme regardless of strain type or time point throughout the transition. These results indicate that this sole OXPHOS enzyme, CI, does not differentially affect *C. albicans* respiration leading to the yeast-hyphae switch, but most likely continues to establish a potential across the inter-membrane and inner membrane space throughout the transition period.

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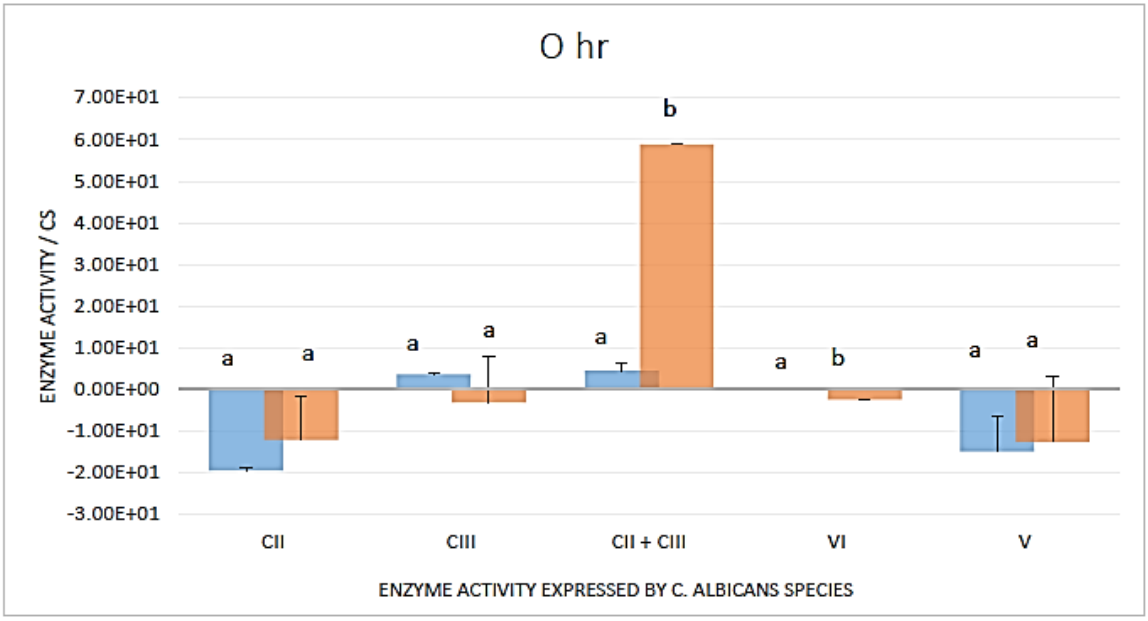

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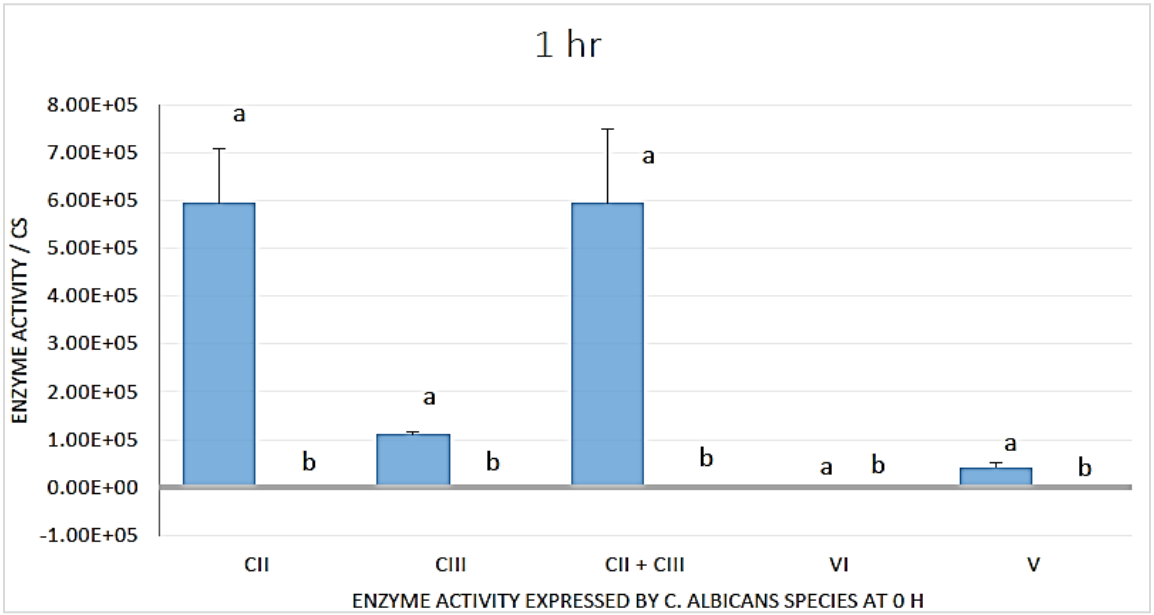
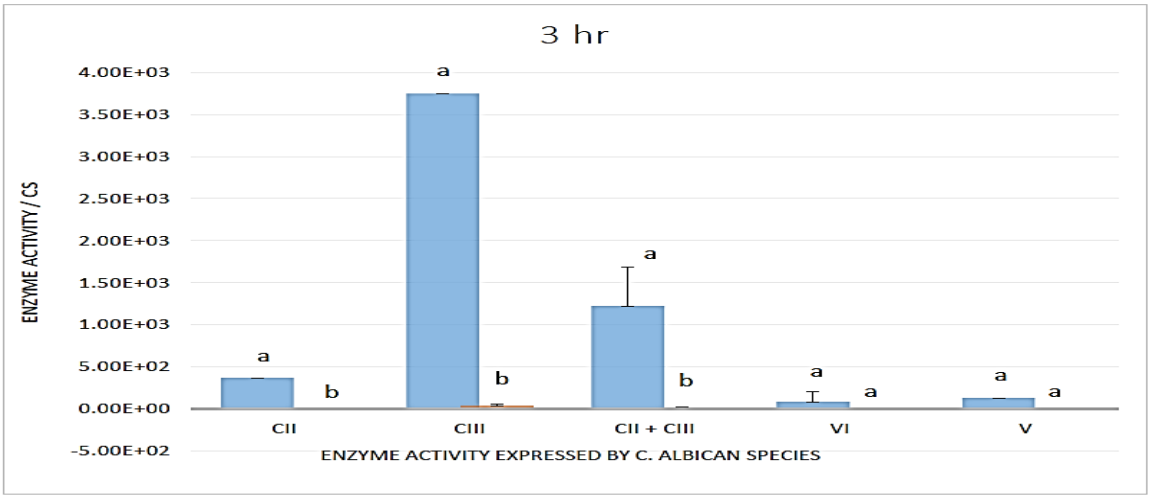


Figure 8. a and b. Statistical analysis of each enzyme for each strain, A72 (blue) and SC5314 (orange) at 0 hr (a) and 1 hr (b). Bars with different letters show significance for two strains based on a given enzymatic values only $P < 0.05$. All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line.

c.



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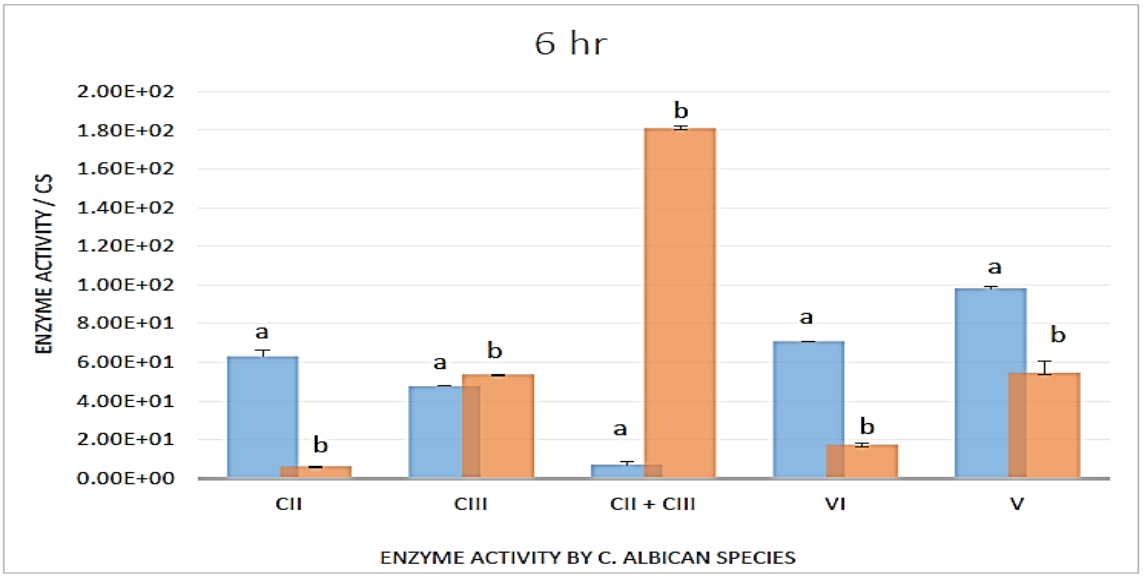

Figure 8. c and d: Statistical analysis of each enzyme for each strain, A72 (blue) and SC5314 (orange) at 3 hr (c) and 6 hr (d). Bars with different letters show significance for two strains based on a given enzymatic values only $P < 0.05$. All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line.

In terms of both enzyme activity and transition hr, it is clear the OXPHOS respiration of the two strains differ despite similar morphologies over the course of the transition (Figure 8). (It must be emphasized that bars with associated letter indicate significance of the two strains relative to only a given complex rather than all the complexes). Although the respiration of two species of *C. albicans* yeast, i.e., *C. albicans* and *C. parilosis*, have been monitored (Calderone and Clancy 2011), strains to strains differences have not been discussed in the literature to our knowledge, nor has OXPHOS enzyme activities been analyzed throughout the phenotypic switch of *C. albicans*. In this study, the results show that significant differences between most of the OXPHOS enzymes exhibited by the two strains (SC5314; SC) and (A72) regardless of transition time point (Figures 8 a-d) with the notable exception of the 0 hour time point (Figure 8 a). For the latter time points, the activity of CII + CIII expressed by SC was ~10 fold higher relative to that exhibited by A72. These results from the combined enzymes represents the transfer of electrons from CII to CIII where the reduction of cytochrome c occurs. The activity of the terminal enzyme of ETC (CIV), i.e., the site where cytochrome c is oxidized while generating water, was also statistically different between strains A72 and SC5314. The enzyme activity exhibited by SC5314 is lower than its A72 counterpart most likely as a means to regain homeostasis due to the increased electron transfer from CII and CIII as CV is statistically similar between the two organisms again (Figure 8a). Activation of CII+CIII may by may be an initial response of SC5314 to the new transition media considering that the cells were still in the yeast state and had just been transferred to a new environment (Calderone and Fonzii, 2001). Still, the values for all the enzymes are relatively low and trend near that of the baseline of the negative control for the 0 hr time point (Figure 8a).

However, by the 1 hr time point (Figure 8b), the values for all of the complexes were significantly different from each other, indicating that respiration readily changes

between the strains as they transition between the two phenotypes early in the process. More specifically, the activity for most of the complexes relative to *C. albicans* (SC5314) increased dramatically, i.e., from trending along the negative control to increasing to a high of 10^5, particularly for CII, CIII and CII+CIII (Figure 8b). These results indicate that electrons are being moved quickly through the ETC. However, at the 1 hour transition, the morphology of the yeast has not changed dramatically (Figure 7), but adhesion has initiated. Although the values for strain A72 do not even register on the bar graph (figure 8b) due to the elevated activities of strain SC5314, most of the associated enzymes increased by 10 fold with the notable exception of CIV, which remained close to baseline. For strain SC5314, CIV increased by a 2-fold factor. As such, even though substantially higher activities were exhibited by strain A72, a similar trend occurred across the enzymes for both strains.

When the yeast had transitioned by 3 hr mostly to the hyphae from (Figure 8c) the activities for both A72 and SC5314 began to reduce for all of the enzymes, which again show similar trends, but yet the two pathogenic *C. albicans* strains are statistically different relative to each enzyme activity due mainly to the higher activities produced by strain A72. Although additional virulence factors were expressed, as evidenced by the clumping effect (Figure 7) that is most likely due to the formation of biofilms and adhesion of cells and both strains exhibited elongated hyphae, no trend existed between the activities of the complexes (Figure 8d) across species. Indeed, the activity levels of enzymes for strain A72 were substantially higher than those expressed by strain SC5314, with the notable exception of CII + CIII, and CIII, where the activities of A72 were slightly but significantly lower than those expressed by its counterpart. Lastly, the activities decreased by 2-3 fold for most but not all of the A72 enzymes, except CIV remained at the same level as that expressed at 3 hr (Figure 8c). These results again show that respiration is acting much differently between the two strains at time point 6 hr

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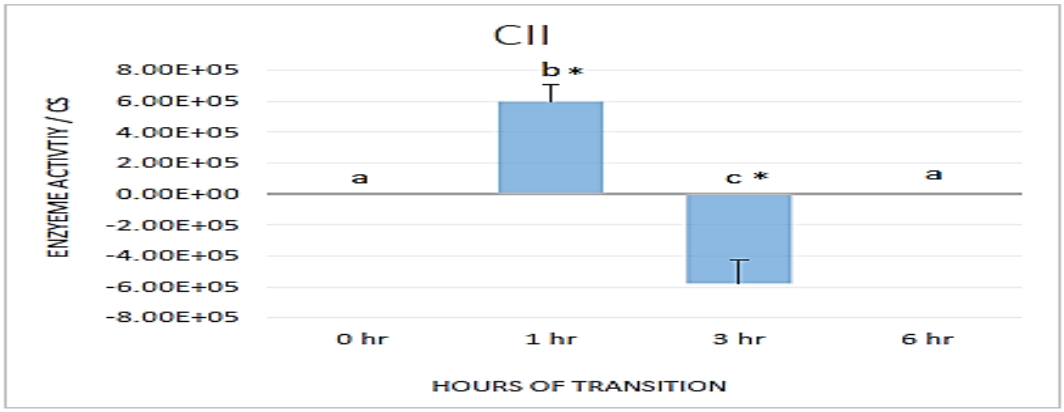

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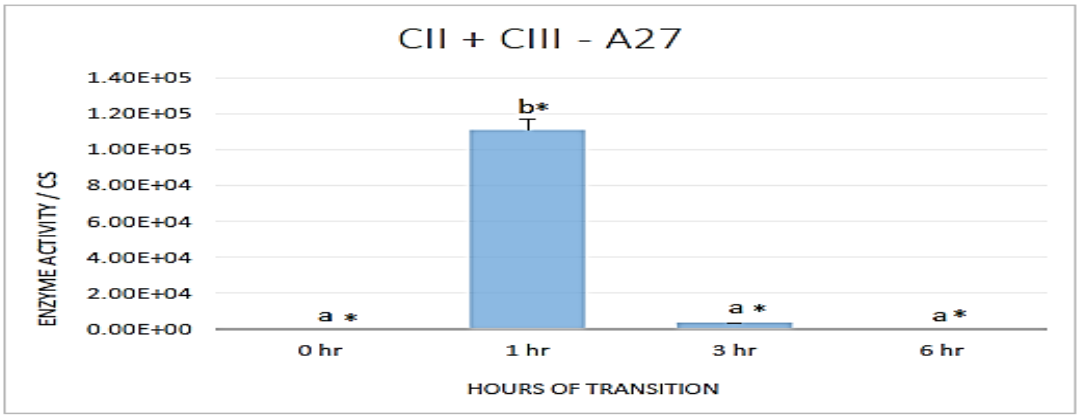

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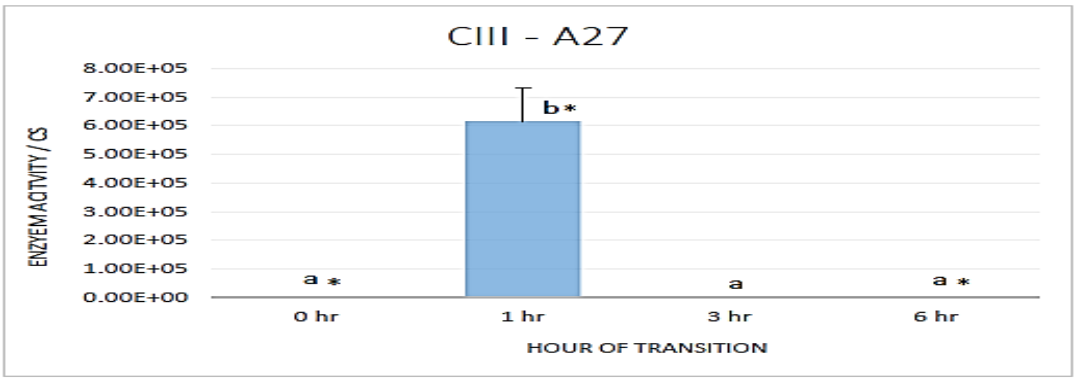

Figure 9, a, b, c. Activity of Complex II (a.), Complex II + III (b.) and CIII (c.) for *C. abicans* (A72) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

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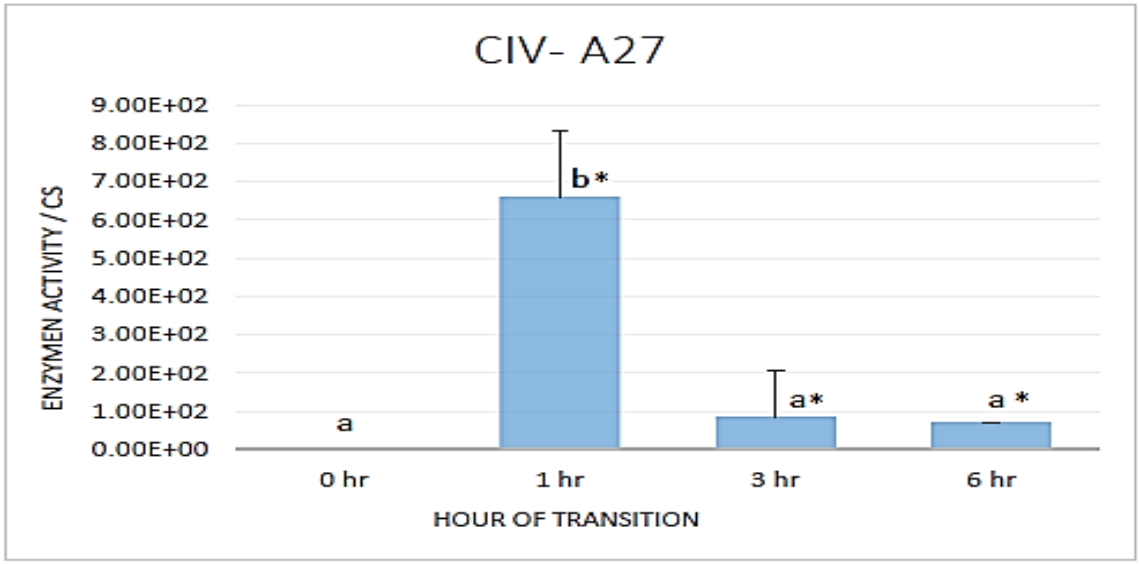

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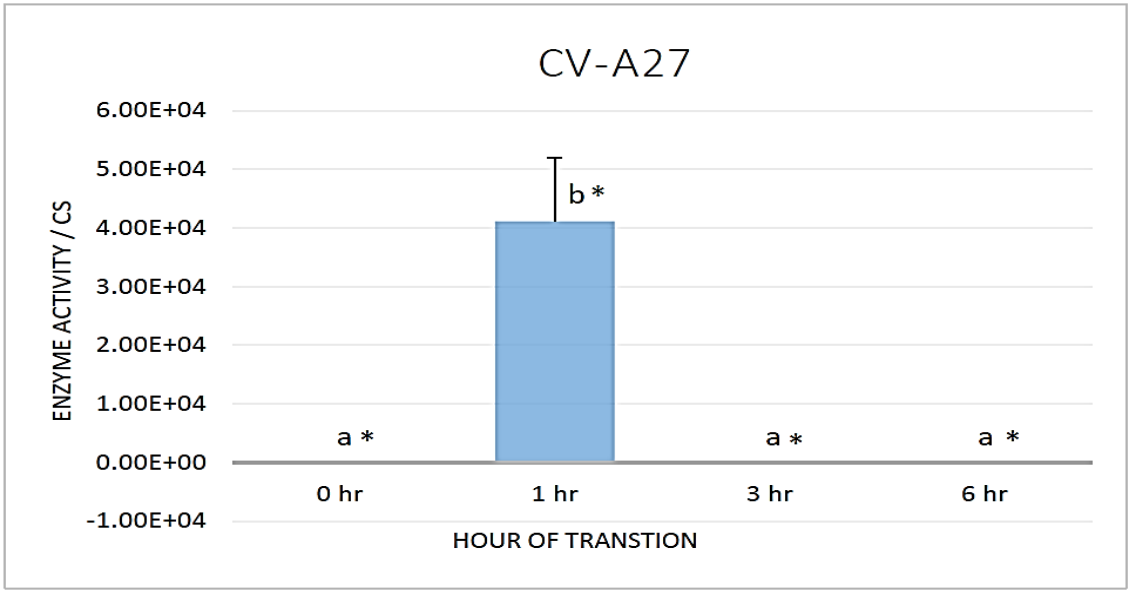

Figure 9 d, e. Activity of Complex IV (d.), and CV (e.) for *C. abicans* (A72) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

as the most of the enzyme activity now differs not only in intensity but in the overall trend that was exhibited during the 1 and 3 hr transition period. Thus, if OXPHOS was to be used a potential target for inhibiting the yeast to hyphae transition, different treatment options for different strains most likely are warranted.

4. A.3 *Comparison of* C. albicans *(strain A72) OXPHOS enzyme activity during yeast to hyphae transition at 0, 1, 3 and 6 hr*.

Based on the data discussed above, the activities of each enzyme for a given strain were analyzed across time to determine certain points (complexes) that could serve as potential targets (Figure 9.a-e). In these experiments, the activities were again statistically normalized to CS and then normalized to the control. This data was also evaluated to determine whether it was statistically different.

During time 0 of the transition (Figure 9 a-e), most of the complexes were statistically different than the negative control, with the notable exception of CII and CIV (Figure 9 a, d). The former complex obtains electrons from CI via the oxidation of the Coenzyme Q hub located in *C albicans*, which then enables CII to catalyze the oxidation of succinate to fumarate and the reduction of Coenzyme Q (Deepu, et al. 2012). This complex does not participate in electron transfer but has been linked to reducing powerful reactive oxygen species (ROS) (Ward, 2015). Therefore, CII may not yet have been actively subjected to the presence of ROS considering the time point was only 0 hr. However, as the time increased to 1 hr, the activity of this enzyme increased by 5-fold indicating that the transition is taking place and that the CII is suitably activated to protect *C. albicans* against ROS during the transition (Figure 9a). Indeed, by 3 hr, the activity decreased below the control when most of the yeast switched to hyphae. These results suggest that the ROS generation was complete, or that the ROS overpowered

the complex. Still, by 6 hr, CII was again significantly similar to the negative control thereby supporting the former hypothesis.

Referred to as cytochrome c oxidase, CIV (Figure 9d) is the final protein complex in the electron transport chain. It is therefore not surprising that this complex was statistically similar to the negative control at 0 hour. And, the substantial increase in activity by 1 hr is expected as the organism is in a state where it will need the energy to transition fully to the hyphae state and then continue to elongate and produce other virulent factors by 3 and 6 hr. Complex V (Figure 9e) showed a similar trend at 1, 3 and 6 hr, as would be also be expected considering that this complex catalyzes AMP to ATP. Interesting, CV was statistically different from the control at time point 0 even though CIV was not (Figure 9d and e). A possible reason could be that CV was able to sense the establishment of the proton gradient across a membrane to drive the synthesis of ATP from ADP and phosphate (P_i).

Complexes III and II +III (Figure 9 b and c) showed a similar pattern as CIV and CV, but their activities were much higher, reaching intensities comparable to CII at the 1 hr time point. Complex III is also known as cytochrome c reductase (Ward, 2015). Because only one electron can be transferred to the ubiquinol (QH2) donor from CII, this step is more elaborate as the enzyme binds three substrates, QH2, cytochrome C, and ubiquinone (Ward, 2015). After elaborate cross talk between these sites in the exchange of electrons, two electrons are eventually passed to CIV, while gaining two protons from the mitochondrial matrix, thereby adding to the proton gradient. Due to the number of substrates involved as well as both electrons and protons, the activity most likely elevated in order to maintain the flow of incoming electrons as well as the proton gradient. By 3 hr, the activity for this CIV is not significant, but it is well after transition of the hyphae had appeared (Figure 8). It does become significant again by 6 hr, probably to aid in providing the energy needed for elongating the hyphae and producing the other

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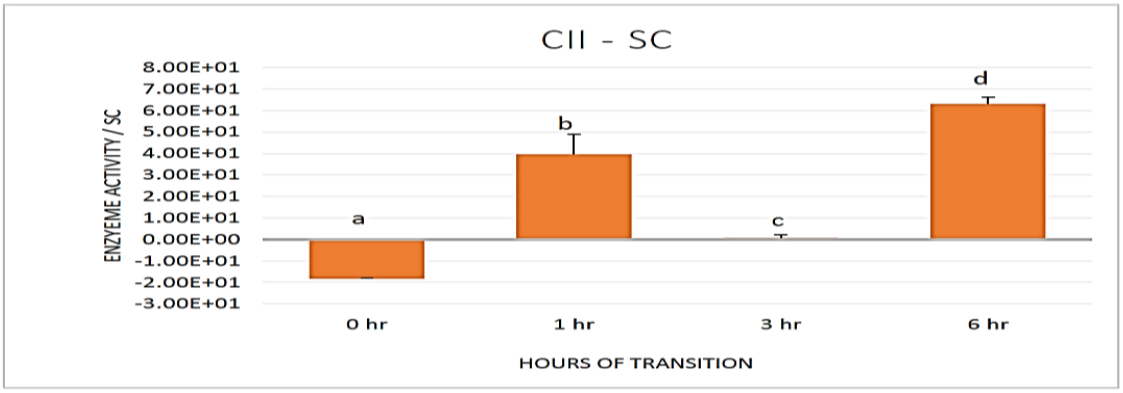

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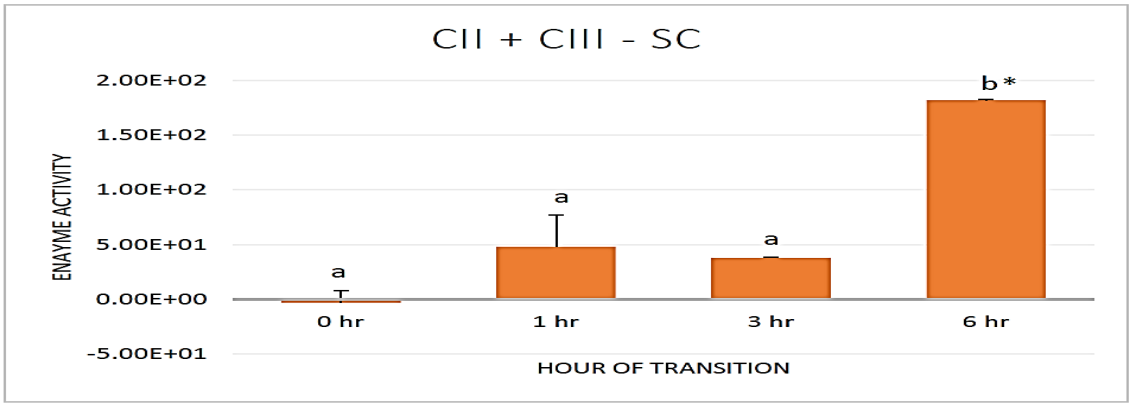

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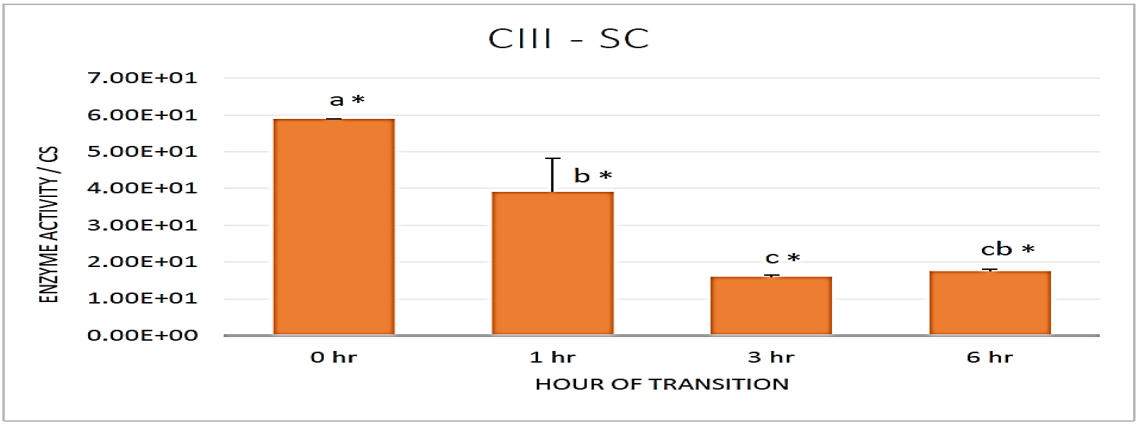
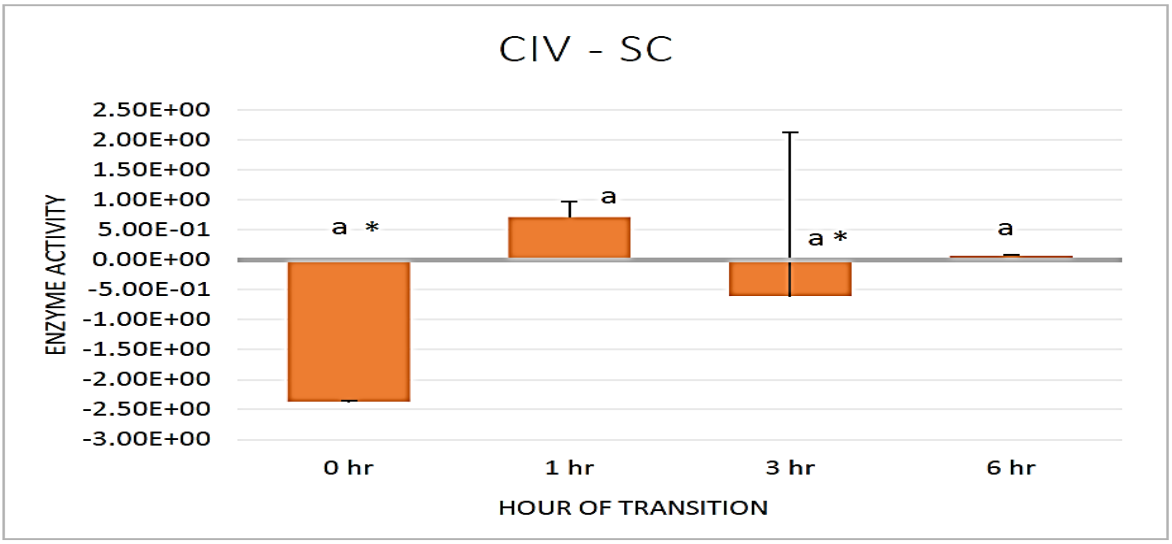


Figure 10 a.b.c. Activity of Complex II (a.), and CII + III (b.) amd CIII (c.) for *C. abicans* (strain SC5314) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

d.



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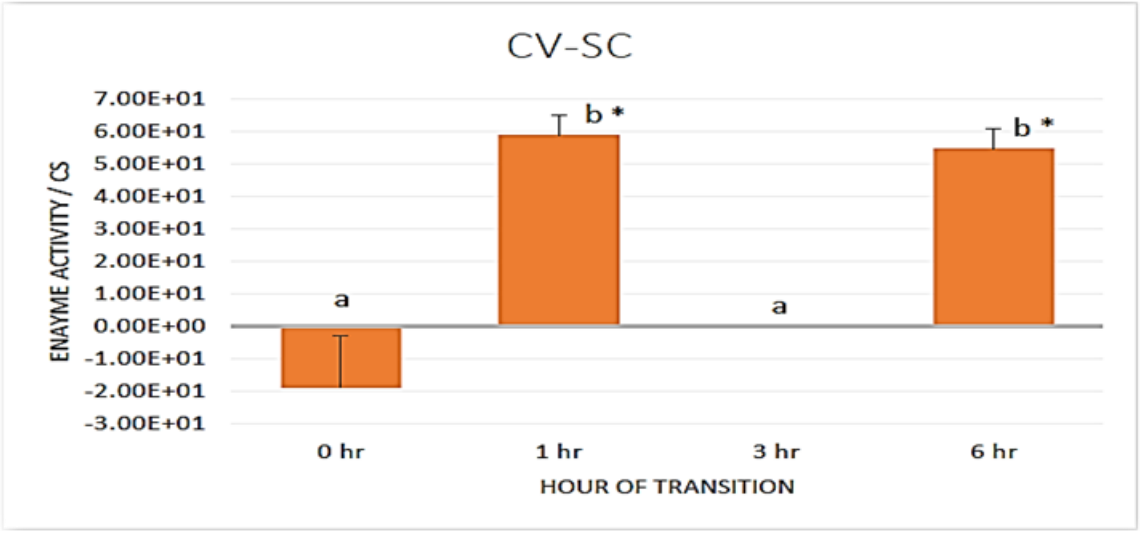


Figure 10 d.e. Activity of Complex IV (D.), and CV (E.) for *C. abicans* (strain SC5314) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

virulence factors. As stated previously, the assay associated with Complex II + III assesses the coupled transfer of electrons from CII to CIII through the CoQ hub (Deepu et al. 2012). By the 6 hr time period, all the enzymes are at much lower activity levels indicating that a high amount of energy is needed for A72 to transition to its yeast form. *As such, this may very well be the first target for developing a novel treatment approach for preventing the transition, namely, to stop CII+CIII as it is the first point of critical electron transfer to CIV, the enzyme for the final product, as well as depleting the proton pump.*

4A.4 *Comparison of* C. albicans *(SC5314) OXPHOS enzyme activity during yeast to hyphae transition at 0, 1, 3 and 6 hr*

Figure 10 a-e shows the OXPHOS effect for *Candida albicans* (SC5314) for each enzyme and time point. Again the transition time points, 0, 1, 3 and 6 hr were compared with each of the enzyme activities expressed by the OXPHOS enzymes. Moreover, the enzymatic activities of the transitioning cells were normalized of those that did not transition but were subjected to the same media.

At the 0 hr time point, three enzymes were statistically similar to the negative control, and included CII, CII+CIII and CV (Figure 10 a, b, e). With the exception the CV, the 0 hr time point did not vary in terms of activities between the control and the cited enzymes. Although CV was not significantly different than the control for strain SC5314 (Figure 10e), the activity was slightly but statistically higher compared to strain A72 (Figure 9e). We attributed this phenomenon to sensing of the proton gradient (refer to Section 4.A.3). However, by 1 hr, CV was significantly different from the control (Figure 10e). Moreover, CII was not significantly different at any of the transitioning time points, 0-3 h (Figure 10a). As this reaction is important for controlling ROS, these unchecked free radicals may have been in abundance, although their levels require measurement to test this hypothesis. In addition, CII + III (Figure 10b) was not statistically different at

either the 1 or 3 hr time point, probably due to the lower activity of CII (Figure 10a). Yet, electron transfer did occur as CIII was active at 0, 1, 3 and 6 hr indicating that this strain may have bypassed CII (Deepu 2002) and shuttled the electrons from CI directly through the CoQ hub into CIII, which also would explain the reason that CIV activity was statistically low at 0 hr as the electrons may have been overwhelming this enzyme even by bypassing CII and CII + III even at 0 h. But by 1 hr the final product was most likely being produced, as the activity of CIV and CV increased (Figure 9d and e). Yet, by 3 hr, the activities of all the enzymes are similar to the control, i.e., after the transition has occurred, but are statistically different at 6 hr when the hyphae elongate, and other virulence factors are formed. Based on the data, the other virulence factors may require more energy than the transformation from this strain. As such, in this strain it may not be as difficult to prevent the phenotypic switch, as the energy may be conserved for producing virulence factors that occur later in transition. It must be noted that although the enzyme activities are much lower for strain SC5314 compared to its counterpart, the event itself may occur more quickly if indeed CII is not involved.

4. B. Specific aim 2: To determine the ability of gallic and ferullic acid, as isolated compounds, to target the oxidative phosphorylation enzymes as a means to suppress 25-50% or more of the hyphal stage transition using the two *C. albicans* strains (SC5314 and A72).

Research is currently in progress for developing new drugs that are effective against *C. albicans* virulence as a means to reduce or prevent the effect of invasive candidiasis. However, most antifungal agents are either toxic or drug resistance exists (Ghannoum and Rice, 1999). Moreover, drug studies that target the virulence factors of *C. albicans* are essential considering that this is a benign commensal yeast when not virulent. Destroying the non-virulent microbe just opens a niche for potentially a more

dangerous organism. These types of studies remain limited but do include phenolic compounds, which have been shown to have antimicrobial activity, including preventing the hyphal form. For example, Saito et al., 2013 showed that 2.8 mM of catechin was able to inhibit the hyphal formation of *C. albicans* (NUD-202) isolated from the human mouth by reducing mRNA expression levels of hypha-specific genes and disrupting the MAP kinase cascade. In this study, the effect of phenolic acids, ferulic and gallic acid as isolated compounds on inhibiting the yeast-to-hypha transition were monitored for the two strains of *C. albicans* (SC5314 and A72). These phenolic acids were selected for this study as both were determined to be the most effective phenols in inhibiting the phenotypic switch of the phenols tested to date in our laboratory.

4. B.1. ***Dosage Selection of each Phenol***

The time selected to monitor the ability of the cited phenols to act upon OXPHOS complexes were correlated with the yeast to hyphae transition (Figure 11), which was 0, 1, 3 and 6 h. However, the concentration which was able to inhibit the yeast the longest and / or had the greatest effect was determined before proceeding any with the OXPHOS enzyme experiments.

As such, different concentrations of the phenolic acids were prepared that included 4, 2, and 0.4 mM. Aliquots of each solution (Table 1 and Table 2) was added to the transition media and the inhibition was monitored as described in the Materials and Methods section. Each of these concentrations were able inhibit the yeast-to-hyphal transition up to 92% within 3 hr for *C. albicans* (SC5314), and up to 86% within 3 hr for *C. albicans* (A72), which agreed with other published papers. For example, a previous study completed in our laboratory demonstrated that 95% of *C. albicans* (SC5314) remained in the yeast form for 3 hr in the presence of 3.5 mM catechin, with GlcNAc serving as inducing agent (Camara, 2015). Also, 50% of *C. albicans* (SC5314 and A72) were clearly in the yeast form 4 hr post-inoculation into in N-acetyl glucosamine using

Table 1

Comparing hyphae inhibition (%) by different concentrations of Gallic acid in different time points in the two *C. albicans* (SC5314 and A72).

	Gallic Acid (mM)					
	A 27			SC5314		
Time	0.4 mM	2 mM	4 mM	0.4 mM	2 mM	4 mM
1 hr	81.5% ± 4.9	35.9% ± 1.3	87.4% ± 7.2	39.0% ± 1.3	21.0 %± 0.9	16.8 %± 0.5
3 hr	4.9 %± 0.4	7.3% ± 0.7	14.0% ± 2.5	2.5 %± 0.4	3.1 %± 0.9	4.4 %± 0.7
6 hr	2.0% ± 0.4	4.5% ± 0.8	9.4% ± 3.3	0.7 %± 0.3	1.9 %± 0.5	2.9 %± 0.4

Table 2

Comparing hyphae inhibition (%) of different concentrations of Ferulc acid in different time points in the two *C. albicans (*SC5314 and A72).

	Ferulic Acid (mM)					
	A 27			SC5314		
Time	0.4 mM	2 mM	4 mM	0.4 mM	2 mM	4 mM
1 hr	86.9% ± 2.5	42.9 %± 3.6	89.4 %± 3.3	50.6% ± 3.3	49.9 %± 2.1	23.9 %± 0.6
3 hr	5.2% ± 0.9	8.7% ± 0.8	12.2 %± 0.9	4.8% ± 0.4	7.7% ± 0.7	2.6% ± 0.3
6 hr	1.1% ± 0.6	3.3% ± 0.4	5.0% ± 0.9	1.5% ± 0.2	3.6% ± 1.0	1.9% ± 0.2

different concentrations of farnesol (1-250 µM farnesol) (Mosel et al. 2005). Another study showed that *C. albicans* (SC5314) reduced the yeast-to-hyphae transition when exposed to (200-900 µM) resveratrol for 60-300 min using different serum, pH, and nutrient-induced conditions (Okamoto-Shibayama et al. 2010). These results indicate that *C. albicans* has the flexibility to respond to different strains due to their ability to metabolize available nutrients from other natural habitats present in inhabiting the host (Brown and Gow 1999). Another point of note from these studies is that strain A72 was the most responsive to both the ferulic and gallic acid treatments early into the transition period by delaying the onset of the hyphae formation.

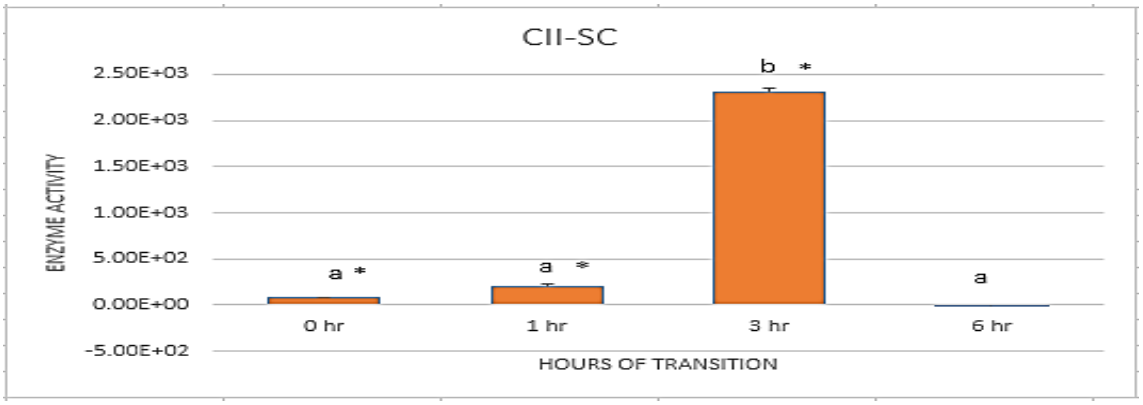
The lowest gallic / ferulic acid dosages used in this experiment produced the highest inhibition effects while decreasing and then increasing with increasing concentration for the strain A72. For strain SC5314, inhibition indirectly correlated with concentration, whether it be gallic acid or ferulic acid serving as the treatment. This is not an uncommon phenomenon, otherwise known as the low treatment effect (Casagrande and Darbon, 2001). Other researchers have reported similar but atypical responses for cells exposed to low doses of pure natural agents. For example, a cancer study resulted in cell viability that initially increased and then decreased; increased and decreased again with higher treatment doses. Casagrande and Darbon (2001) attributed such results to the ability of biological systems to be overcome by the low level toxicity of a bioactive agent while they are able to compensate at higher doses. Other studies completed in our lab have shown similar effects of a virulent factors, i.e., adhesion and biofilm formation, in that the lower concentrations tend to be the more potent the treatment (data not shown). More studies are needed to understand this low-dose response as these results may have important clinical implications.

4. B. 2 *Effect of phenols on the oxidative phosphorylation enzymes.*

As stated previously, phenolic compounds can act upon multiple carbohydrate metabolic pathways (Arathi and Sachdanandam, 2003; Kueck et al., 2007; Suolinna et al., 1975), including the electron transport chain. Indeed, phenolic compounds have shown potent effects on the generation of ATP. To this end, Morelnd and Novitzky (1987) showed that the phenols (coumarins, cinnamic acids, and vanillin) were active in in facilitating the ATP generating pathway by using 1-20 mM concentrations. The results also showed that higher concentrations lowered enzyme activity. Makovec and Šindelář (1984) determined that phenolic compounds (20 mM final concentration) affected the activity of respiratory chain enzymes of potato tuber mitochondria. Indeed, the researchers reported that gallic acid and quercetin significantly inhibited cytochrome c oxidase (Complex IV) by 72% of the control, while succinate dehydrogenase (complex II) was significantly inhibited by cinnamic and caffeic acid. On the other hand, reports have shown that phenolic compounds can have a negative impact on ATP generation by inhibiting the electron transport chain and OXPHOS (Moreland and Novitzky, 1987). The study showed that polyphenolic rich extracts obtained from chloroplasts (1 and 20 mM) significantly inhibited NADH oxidation and succinate dehydrogenase activity, or CI and CII respectively. Although the respiratory system of yeast, such as *C. albicans,* and plants differ, with the later missing the alternative oxidase (AOX) and parallel respiratory (PAR) pathways, both share the classical respiratory complex (CRC) pathway (Odds, 1988). To this end, Konishi et al. (1987) determined that 200 µg/ml tannic acid was able to inhibit the NADH dehydrogenase (CI) and the terminal oxidase (cytochrome b560-d complex) of the respiratory chain of *Photobacterium phosphoreum.*

In this study, the effect of isolated phenolic compounds on OXPHOS complexes (CII, CII+III, CIII, IV, V) present in *C. albicans* (SC5314 and A72) on the yeast to hyphal transition were investigated and correlated to the yeast morphology at a given time point (0, 1, 3 and 6 hr). As stated previously, gallic and ferulic acid were the most efficacious

a.



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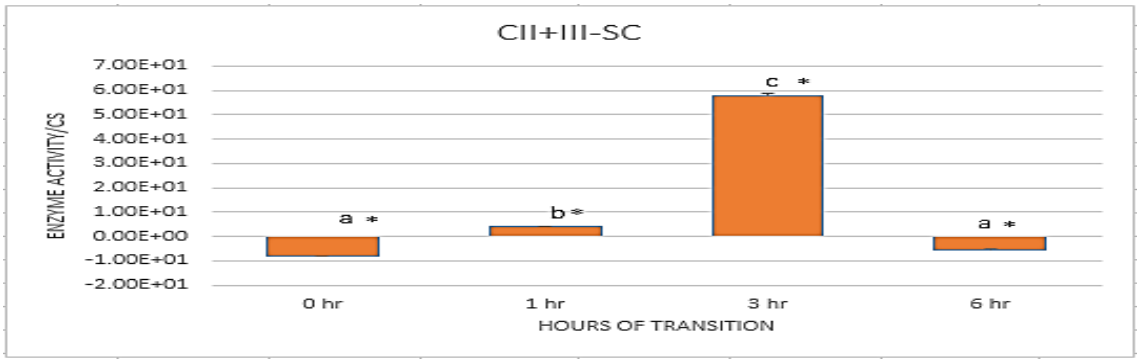


c.

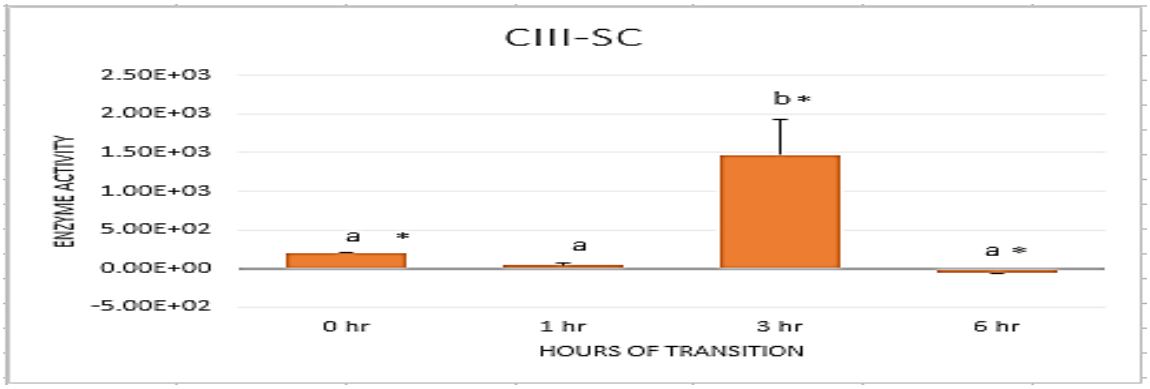
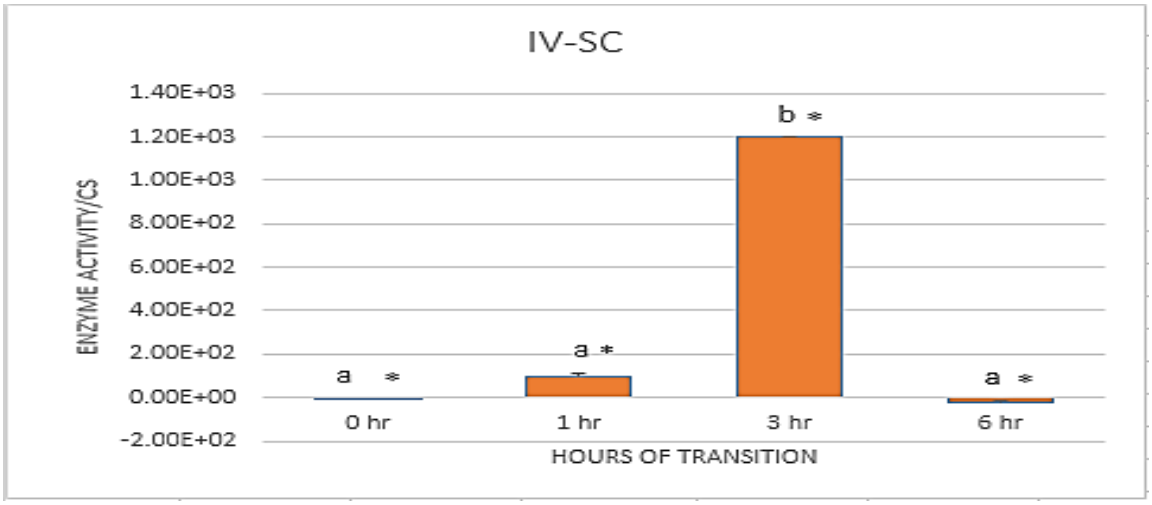


Figure 11 a,b,c. Effects of Gallic Acid on the activity of Complex II(a.), III (b.) CII + III) (c.) *C. abicans* (strain SC5314) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

d.



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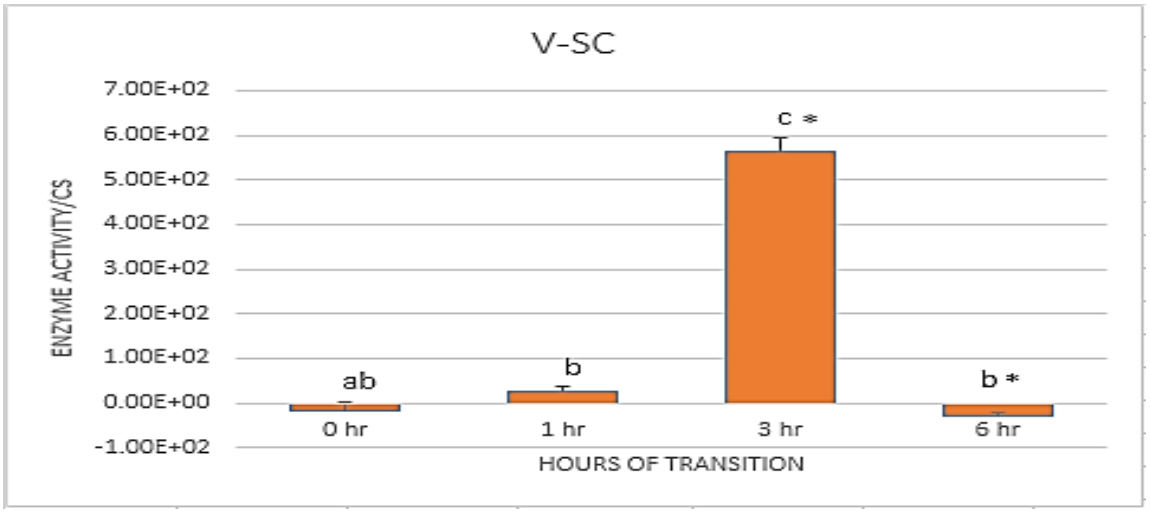


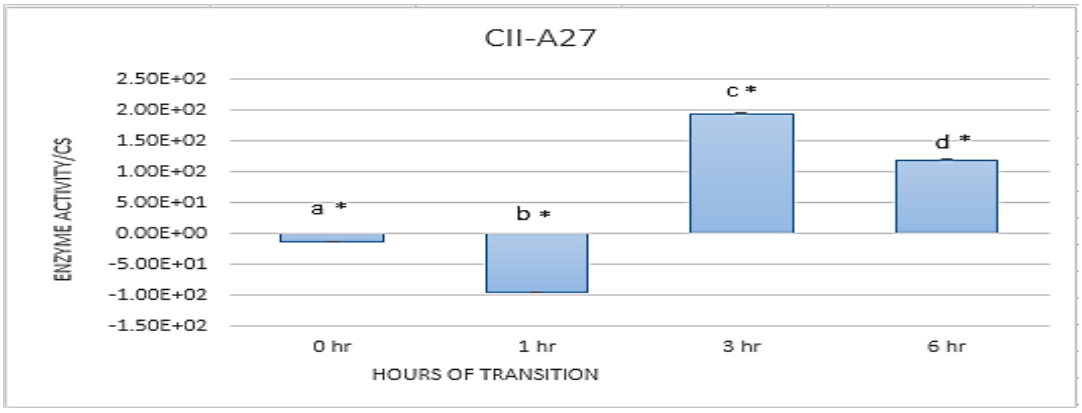
Figure 11 d.e. Effects of Gallic Acid on the activity of Complex VI (d.), and CV (d.) *C. abicans* (strain SC5314) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

compounds among the individual phenols tested in our lab to inhibit the yeast to hyphae transition. Therefore, these compounds were used for further studies to determine their ability to act upon the OXPHOS enzymes as possible target to mitigate the phenolic switch.

4. B. 3 *Effects of gallic acid during* C. albicans *(SC5314 and A72) yeast-to-hyphae transition on the oxidative phosphorylation enzymes.*

Figures 11 and 12 show the effect of gallic acid on *C. albicans* (SC5314 and A72) during the yeast-to-hyphae transition on OXPHOS, respectively. First, for strain SC5314, respiration shifted to increased activity for all the enzymes at the 3 hr transition point rather than what was typically the 1 hr point for the (untreated) positive control (Figure 11). Additionally, at 0 hr and 1 hr, CV enzyme was comparable to the negative control while the other enzymes, at time points 0, 1, and 6 hr, were low but statistically significantly different from the negative control. These results suggest that gallic acid may lead to reprogramming respiration. Although hyphae were fully present at 3 hr, other virulence factors could have been affected, as it proposed that high activity of the CV enzyme postive control (Figure 10e, 6 hr time point) continued to produce presumably for developing biofilms and other virulence factors. It must be emphasized again that by 1 hr, the activity of CV of cells treated with gallic acid was statisically comparable to the negative control indicating that no energy was being produced for the phenotypic switch at this time. Based on these data, the following hypothesis can be made. In the prescence of gallic acid, the duration of the switch from yeast to hyphae lengthend or shifted from 1-3 hr to 3+ hr with other viurlence factors most likely negatively impacted. However, Table 1 nullifies this hypothesis, as the inhibitory rate is only 2.5% at the 3 hr time point, thereby leaving the other potential negative effects on biofilm formation, hyphae elongation and cell adhesion.

a.



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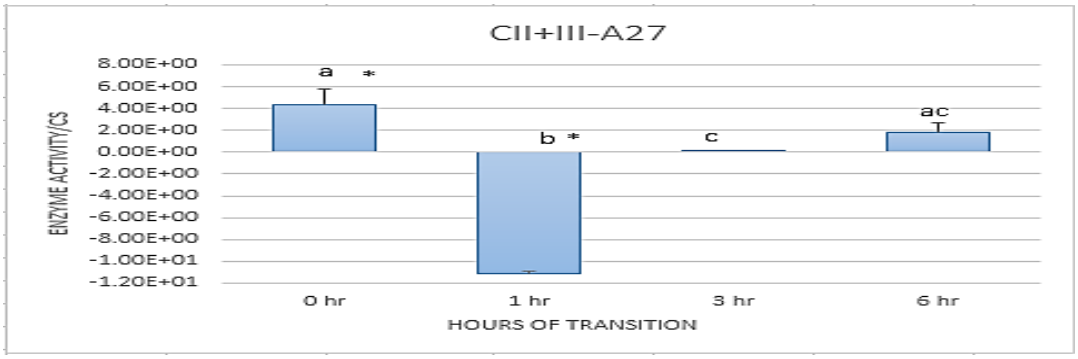

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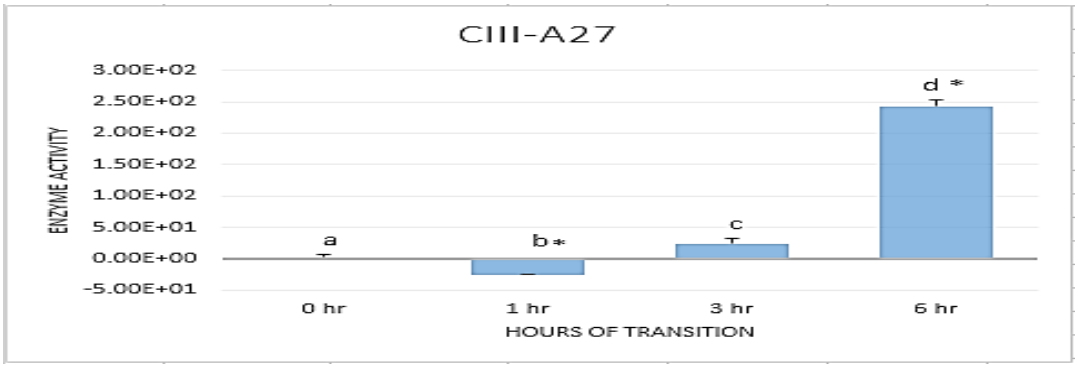
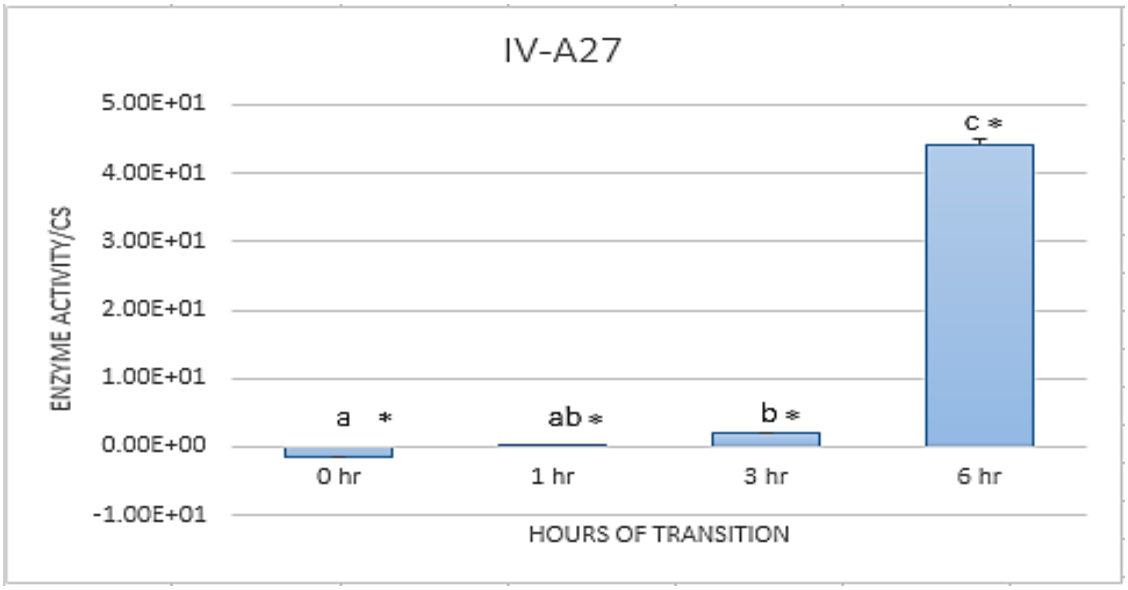


Figure 12 a,b,c. Effects of Gallic Acid on the activity of Complex II (a.), III (b.) CII + III (c.) *C. abicans* (strain A72) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters differ significantly across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

d



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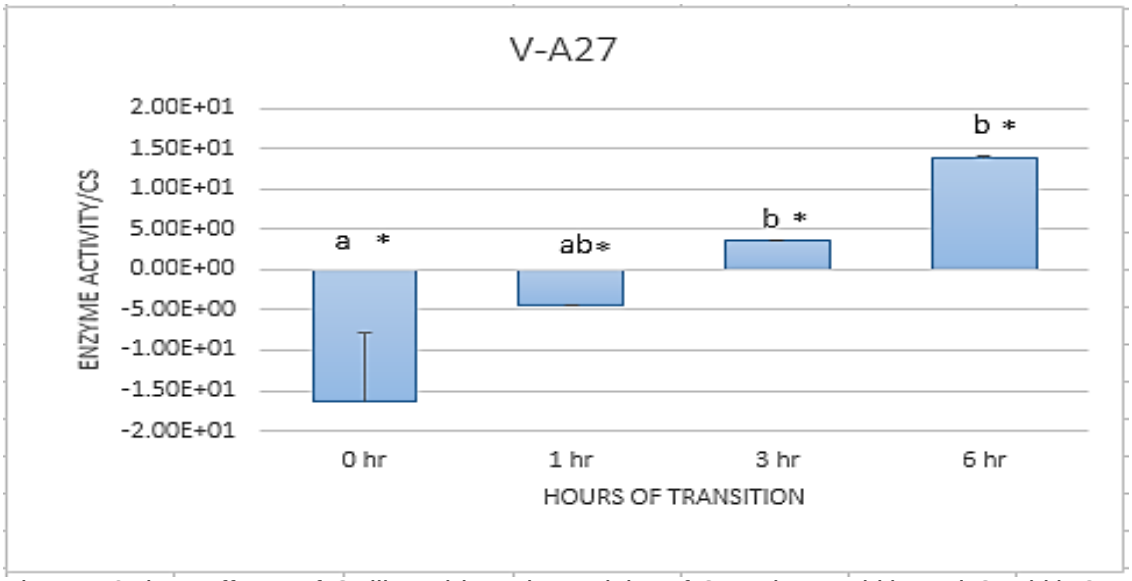


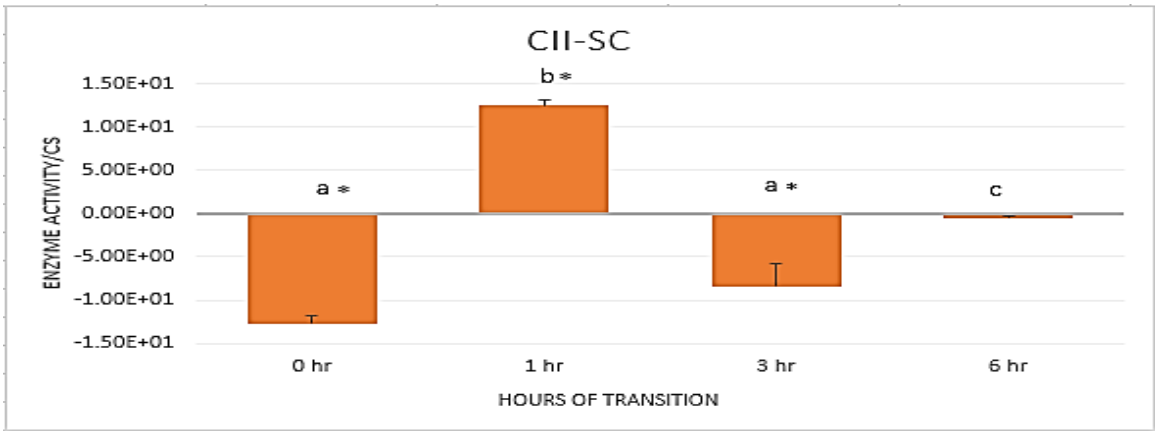
Figure 12 d.e. Effects of Gallic Acid on the activity of Complex VI (d.), and CV (d.) *C. abicans* (strain A72) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters differ significantly across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

Alternatively, the OXPHOS complexes of *C. albicans* (A72) showed significant differences between 0, 1, 3, and 6 hr, for all the enzymes (Figure 12 a-c) compared to the positive control (Figure 9a-B). The most notable difference in activity was that the overall intesity of each enzyme was substantially lower than that of either the positive control (Figure 9) or the negative control (grey bar on each figure), indicating a longer time period for a compete shift from yeast to hypae. Again, CV (Figure 12 e) produced a low activity, but lower than the negative control. Although the complex could be active, it probably could not catalyze enough ATP to sustain a speedy phenotypic switch, coupled with the decrease in overall enzyme activity by the complexes. According to Table 2, it took 3 hours for 95% of the yeast to form into the hyphal phenotype. These results indicate the gallic acid is partially protecting against *C. albicans* virulence by lengthening the time switch and possibly other virulence factors.

4. B. 4 *Effects of Ferulic acid during* C. albicans *(SC5314 and A72) yeast-to-hyphae transition on the oxidative phosphorylation enzymes.*

Ferulic acid was able to temporarily inhibit the yeast- to hyphal transition of *C. albicans* (SC5314 and A72) (Table 2), but was least effective against the SC5314 strain. Again, these results could be due to ferulic acid acting on the OXPHOS enzymes as most of the activities of the complexes at all the time points (Figure 13 a-e) varied substantially from the postive control (Figure 9a-e) while multiple enzyme activies were not significantly different than the negative control with the notable exceptions of CII (0,1,2 hr), (Figure 13a); CIV (0,1,3 and 6 hr) (Figure 13 d); and CV (0 and 1 hr) (Figure 13e). Lastly, (Figure 14 a-d) shows the effects of ferulic acid on transtion *C. albicans* (A72). Similar to *C. albicans* (SC5314), many of the enzymes at different time points are statistically similar to the negative control, with the exception of CII+ CIII (3 hr), (Figure 14b); CIII (0,1 and 3 hr); and CIV (0, and 3 hr); (Figure 14d). The data from both ferulic acid treaments show that when subjectied to ferulic acid, respiration of *C. albicans*

a.



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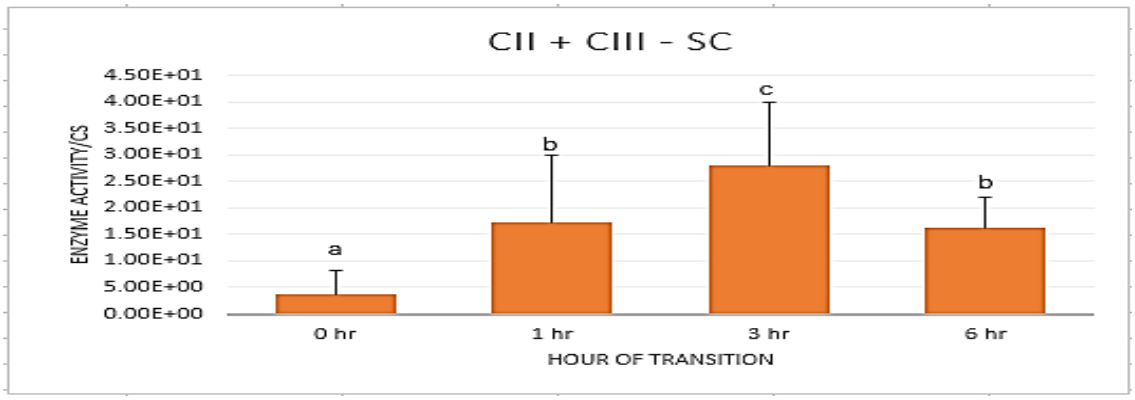

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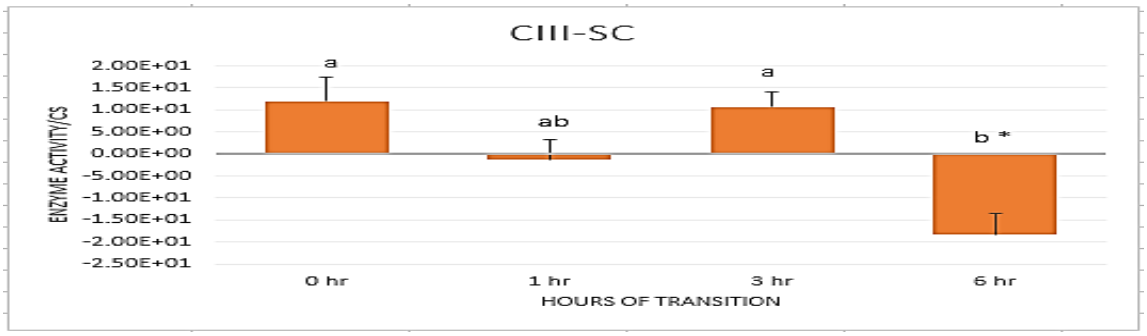
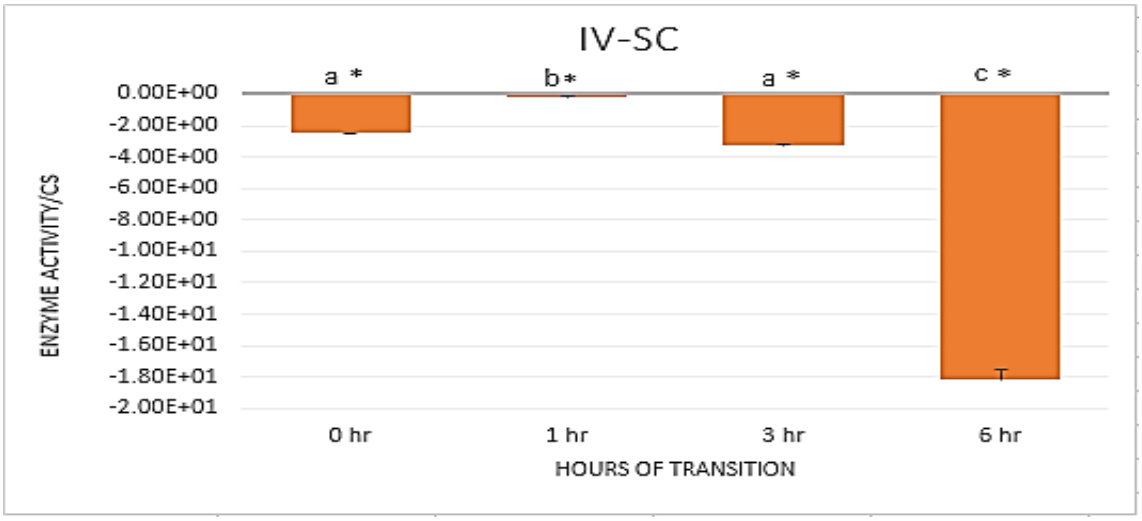


Figure 13 a,b,c. Effects of Ferulic Acid on the activity of Complex II (a.), III (b.) CII + III (c.) *C. abicans* (strain SC5314) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

d



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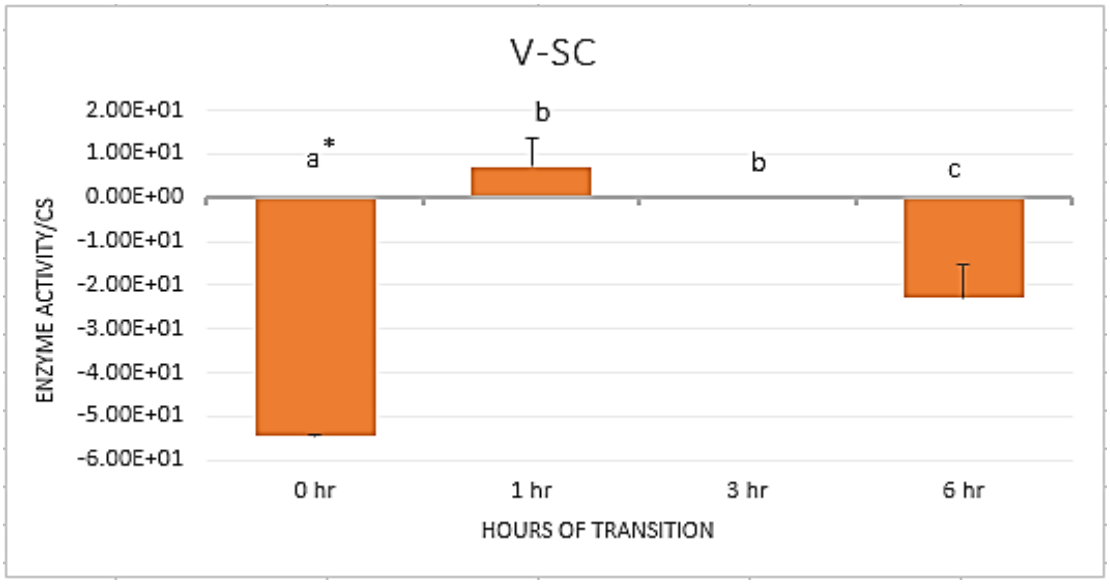
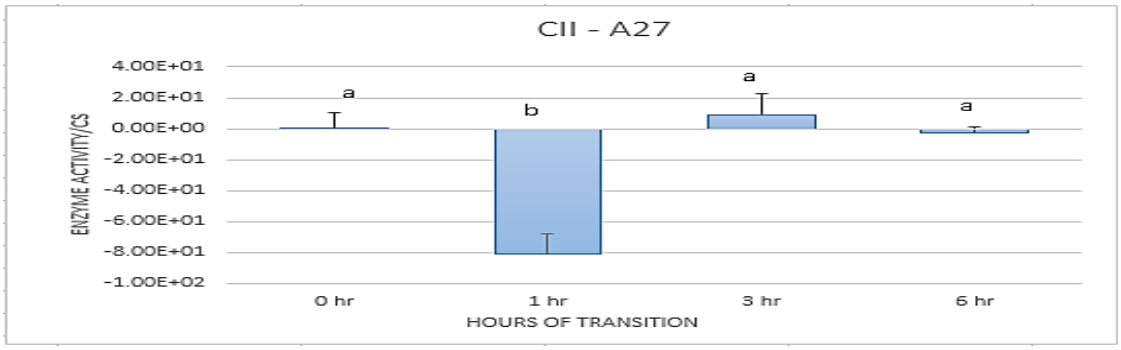


Figure 13 d.e. Effects of Ferulic Acid on the activity of Complex VI (d.), and CV (d.) *C. abicans* (strain SC5314) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

a



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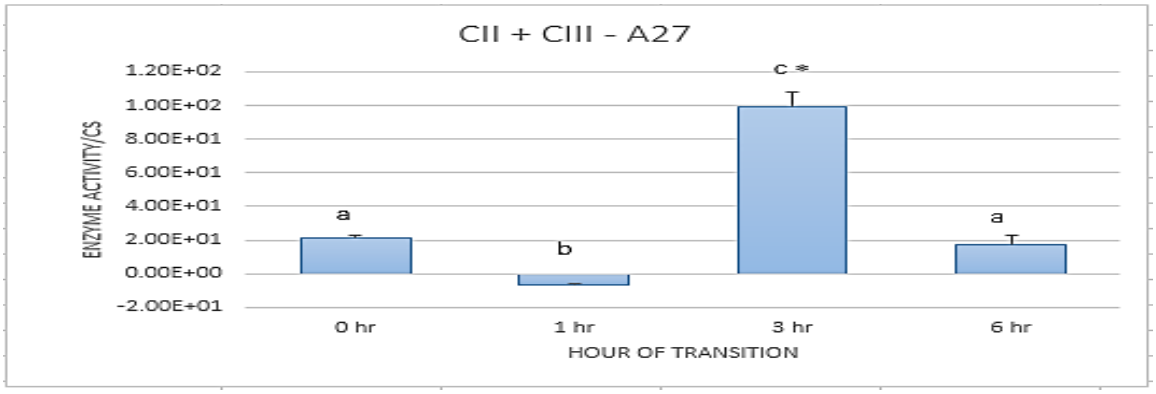

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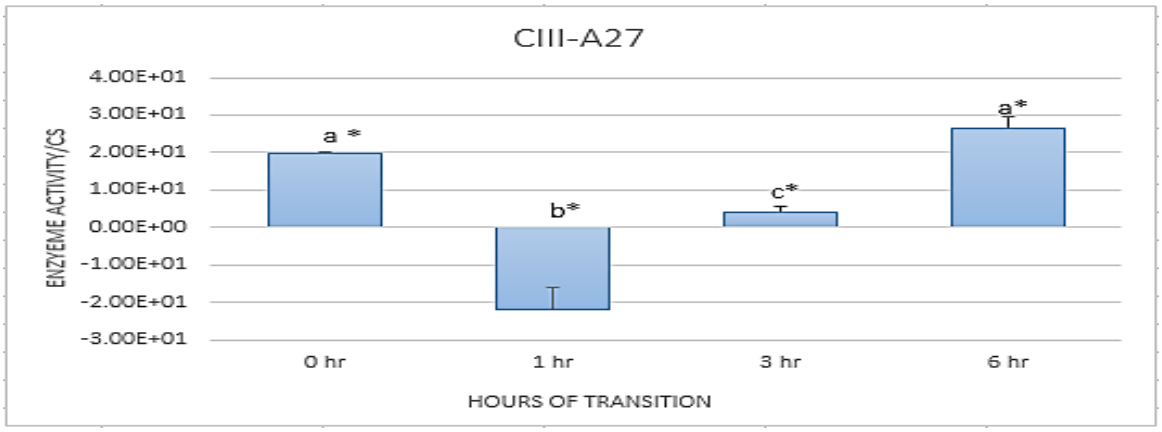
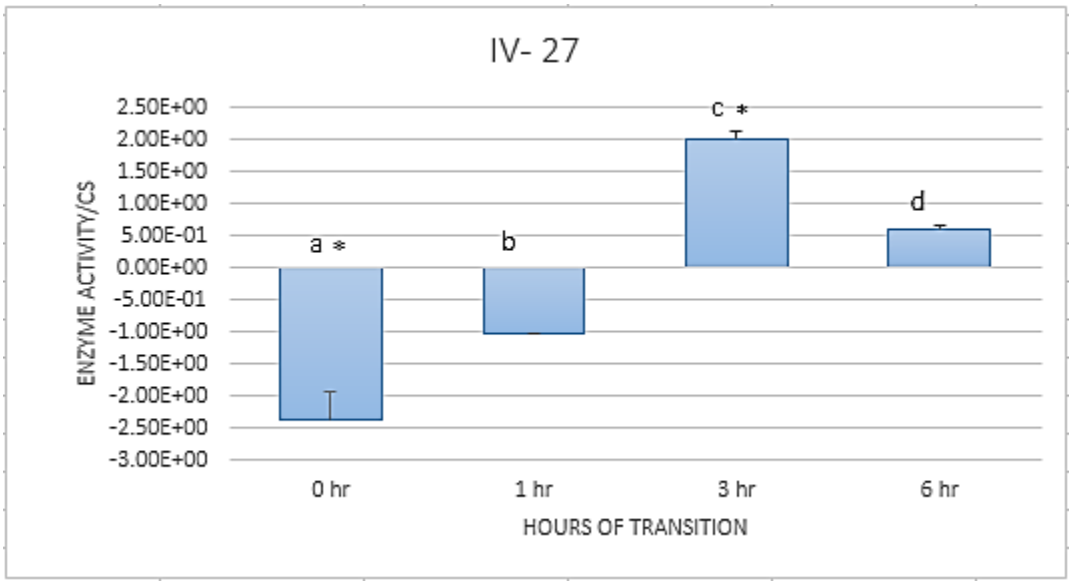


Figure 14 a,b,c. Effects of Ferulic Acid on the activity of Complex II (a.), III (b.) CII + III (c.) *C. abicans* (strain A72) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control

d.



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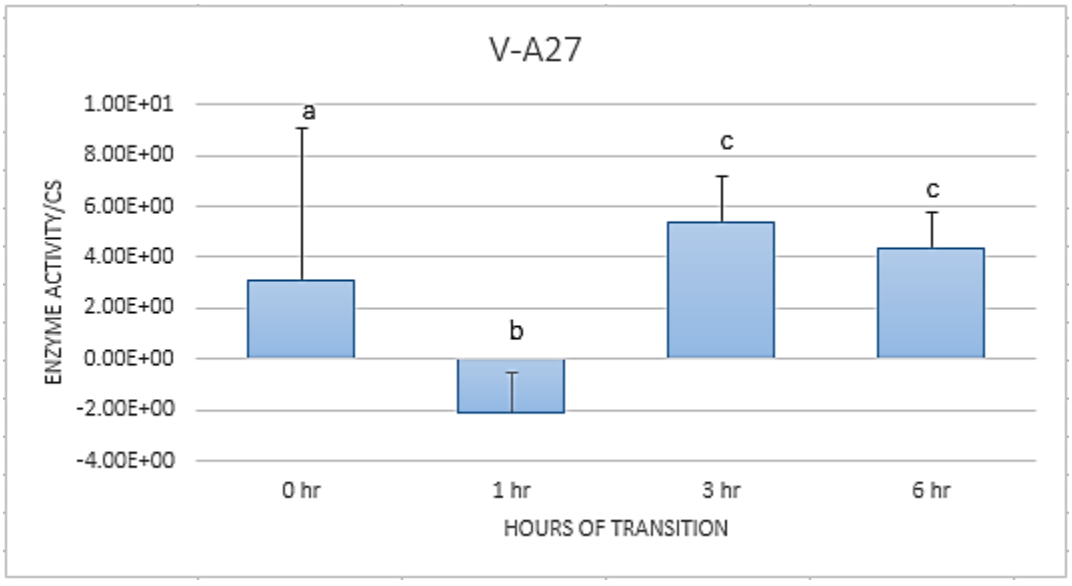

Figure 14 d.e. Effects of Ferulic Acid on the activity of Complex VI (d.), and CV (d.) *C. abicans* (strain A72) at 0, 1, 3 and 6 hr post-innoculation. Bars with different letters are significant across time period ($p < 0.05$). All values were normalized to the negative control, which was set at 0.00, as represented by the bold grey line. * represents an enzyme at a given time that is statistically different than the negative control.

(SC5314) appears to slow down at least compared to the negative control, as many values fall below the grey line (Figure 13a-d), and decrease compared to the postivie control (Figure 9-10). Although these results indicate that yeast to hyphae shift should be reduced or inhibited completely, 95% of the yeast cells have turned hyphae by the 6 h point (Table 2) for both strains.

These results indicate that *C. albicans* is obtaining energy form other sources to ensure that the yeast to hyphae phenotype is not altered. For example, at least three mitochontrial respiratory pathways have been identified in this yeast (Deepu 2012) which could be sustaining the energy needs for the phenotypic shift. These pathways must must be evaluated to fully understand the potential of targeting respriation using for a potential anti-fungal agent. Nonetheless, this research is significant as it the first to monitor the classic OXPHOS enzymes throughout transition and when treated with a potenial anti-fungal agent using repiration as the target.

5. CONCLUSION:

In this study we determined the ability of the phenolic acids, ferulic and gallic acid, to modulate the activity of the enzyme complexes (II, III, IV, V), the mitochondrial matrix marker enzyme citrate synthase, and the combined activity of complexes II and III involved in oxidative phosphorylation, of *C. albicans* (SC5314 and A72) during transitions from yeast to hyphae at each of the following time points: 0, 1, 3, and 6 hours. These results indicate that:

- OXPHOS enzyme, CI, does not affect *C. albicans* respiration leading to the yeast-hyphae switch, but most likely continues to establish a potential across the inter-membrane and inner membrane throughout the transition period.
- OXPHOS respiration of the two *C. albicans* strains (SC5314 and A72) differ despite similar morphologies over the course of the transition at 0, 1, 3, and 6 hr.

- For *C. albicans* strain A72 at the 6 hr period, all the enzymes are much lower which indicates that a high amount of energy is needed for the transition to its yeast form.
- The enzyme activities of *C. albicans* SC5314 are much lower compared to A72 which suggests it may not be as difficult to prevent the phenotypic switch in SC5314, as the energy may be conserved for producing virulence factors that occur later in transition.
- Different treatment options most likely are warranted when OXPHOS is used as a potential target for inhibiting the yeast to hyphae transition, since in the two strains of *C. albicans* respiration is acting much differently.
- Lower concentrations of isolated phenolic acids, ferulic and gallic acid, prevent hyphal growth by 25-50%. These concentrations also, inhibit the enzyme activity involved in oxidative phosphorylation of *C. albicans* (SC5314 and A72).
- Gallic and ferulic acids reduced or inhibited the yeast to hyphae shift should be complete, 95% of the yeast cells have turned hyphae by the 6 hr for both species. These results indicate that *C. albicans* is obtaining energy from other sources to ensure that the yeast to hyphae phenotype is not altered.
- Gallic acid could lead to reprogramming respiration at the 3 hr transition point in contrast to what was typically the 1 hr point for the positive control on *C. albicans* (SC5314). This may partially protect against *C. albicans* virulence by lengthening the time switch and possibly other virulent factors.
- *C. albicans* is obtaining energy from other sources to ensure that the yeast to hyphae phenotype is not altered when it is treated with ferulic acid.

In future work, further tests are needed to establish the mechanism of inhibition of OXPHOS on *C. albicans* by ferulic and gallic acid, such as by studying enzyme

kinetics. Also, it is worth studying the effect of phenols in combination and whether they act synergistically to positively modulate the activity of the oxidative phosphorylation enzymes, which would be reflected in the hyphal phenotype.

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