

**IRON-RESPONSIVE CARBON SOURCE UTILIZATION IN  
*CANDIDA ALBICANS***

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A Thesis  
Submitted to  
the Temple University Graduate Board

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of the Requirements for the Degree  
MASTER OF SCIENCE

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by  
Khushi Gandhi  
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Thesis Approval(s)

Sumant Puri, Thesis Advisor, Oral Health Sciences  
Nezar Al-Hebshi, Oral Health Sciences  
Mehmet Hakan Ozdener, Oral Health Sciences  
Rishabh Sharma, Oral Health Sciences

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## ABSTRACT

**Background:** *Candida albicans* colonization in the oral cavity, particularly in immunocompromised individuals, can progress from oral candidiasis to invasive, life-threatening candidiasis if it enters the bloodstream. The virulence of *C. albicans* is linked to its ability to form biofilms, transition between yeast and hyphal forms, and alter its cell wall structure in response to environmental changes. The individual effect of changes in iron and carbohydrates (carbon sources) on virulence has been previously studied. However, the contribution of different carbon sources in the presence of low or high iron has not been examined. **Objective:** Thus, the objective of this study was to investigate the synergistic effects of iron availability and different carbon sources on *C. albicans* growth and virulence mechanisms. **Methods:** The *C. albicans* strains used in this study were cultured in yeast nitrogen base (YNB) minimal media supplemented with respective carbon sources in the presence of 1  $\mu\text{M}$  iron (low-iron condition) or 100  $\mu\text{M}$  iron (high-iron condition). Growth of *C. albicans* was monitored in 96-well plates over 24 hours ( $\text{OD}_{600}$ ) using a spectrophotometer. Evos light microscope was used to visualize yeast-to-hyphae ratios. Biofilms were grown in 6-well plates for 24 hours and quantified using 0.1% crystal violet. Cell wall mannan assessment was done by staining with ConcanavalinA dye and microscopic imaging. **Results:** *C. albicans* exhibited significantly enhanced hyphae formation under high iron condition with all the carbon sources. Sucrose in the presence of high iron yielded the highest yeast-to-hyphae ratio. Similarly, biofilms were also significantly more robust under this condition. **Conclusion:** Iron levels can alter the effect of various carbon sources on *C. albicans* virulence.

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# CHAPTER 1

## INTRODUCTION

### 1.1 Oral Microbiota: A Living Ecosystem

The oral cavity represents an intricate ecosystem, hosting one of the most complex microbial communities, second only to the human gut microbiome (Caselli et al., 2020). It consists of diverse microorganisms occupying oral habitats such as the teeth, gingiva, tongue, mucosa, and hard and soft palates, which further connect to the tonsils, esophagus, middle ear, and upper respiratory tract. The oral cavity is the port of entry for the organism from where it is transported to different visceral areas through saliva and food (Dewhirst et al., 2010). Even though the fundamental functions of these microbial communities remain consistent across populations (Gillings et al., 2015), the composition of the oral microbiome varies, and individuals have their own microbial identity (Franzosa et al., 2015).

The symbiotic microbiome plays a key role in supporting body functions, nutrition, and the immune system, and helps in maintaining oral and systemic health (Caselli et al., 2020). However, when the microbial balance is disrupted, it can lead to oral health issues like caries, root infections, periodontal disease, bone inflammation, and tonsil infections (Kerr, 2015) (Abusleme et al., 2013) (Fan et al., 2016). These can progress to systemic conditions like cardiovascular disease (Beck & Offenbacher, 2005), stroke (Joshi-pura et al., 2003), preterm birth (Offenbacher et al., 1998), diabetes (Qin et al., 2012), pneumonia

(Awano et al., 2008), obesity (Ley et al., 2006), colon cancer (Ahn et al., 2013), and psychiatric disorders (Foster & McVey Neufeld, 2013).

Within this complex network, over 700 species of microorganisms coexist, including bacteria, fungi, viruses, archaea, and protozoa (Bäckhed et al., 2012). The oral cavity also hosts a diverse fungal community, with at least 85 different fungal species identified. Prominent genera include *Candida*, *Cladosporium*, *Alternaria*, *Aspergillus*, *Fusarium*, *Cryptococcus*, and *Aureobasidium* (Ghannoum et al., 2010), as well as *Malassezia* (Dupuy et al., 2014). Although fungi constitute a mere 0.004% of the total microbiome population (Caselli et al., 2020), their potential impact on biofilm formation is significant due to their cellular size and ability to form hyphae. *Candida* represents the predominant genus in this oral mycobiome, with *Candida albicans* constituting approximately 70% of the fungal population (Ghannoum et al., 2010).

## **1.2 *Candida albicans* and its Pathogenic Shift**

*Candida spp.* resides as a part of normal human flora of the oral cavity, gastrointestinal tract, and vaginal tracts (Kim & Sudbery, 2011). 30-50% of people carry this organism, and the rate of carriage increases with the age of the patient (Agrawal et al., 2014). *C. albicans* has distinctive characteristics to grow into three different morphological forms, i.e., yeast, pseudohyphae, and hyphae (Sudbery et al., 2004). The latter two are considered invasive as they promote tissue penetration in the early stages of infection, while the yeast form is more involved in dissemination through the bloodstream (Sudbery et al., 2004). *Candida* species demonstrate significant morphological adaptability, shifting between spherical yeast cells and filamentous structures.

Intermediate forms, termed pseudohyphae, arise when elongated daughter cells remain connected after incomplete separation at septation sites, forming chains of linked cells (Sudbery et al., 2004). This phenotypic plasticity extends to colony morphology, where reversible transitions occur between distinct colony types. For instance, colonies may alternate between smooth, opaque appearances and irregular, textured forms. These colonies often harbor heterogeneous populations, blending yeast cells with pseudohyphal and true hyphal growth forms. Hyphal growth is considered one of the important virulence factors and is promoted by a higher temperature of 37°C that is present in their potential host (Sudbery et al., 2004).

*Candida albicans* typically maintains a symbiotic relation within the oral microbiome by establishing cooperative interactions with resident microorganisms. However, when environmental equilibrium becomes compromised, this fungus demonstrates opportunistic behaviour, rapidly multiplying and colonizing the oral tissues (Díez et al., 2021). This cross- kingdom interactions leads to its involvement in pathogenesis of oral diseases such as pulp and periapical inflammation, periodontal diseases, peri- implantitis, oral cancer, and dental caries like root surface caries and most importantly early childhood caries (ECC) (Du et al., 2022). The prevalence of *Candida* species in both saliva and dental plaque samples correlates directly with Early Childhood Caries (ECC) severity (Du et al., 2022). Although innocuous in immunocompetent hosts, when infecting individuals with compromised immunity due to malignancies, HIV infection, or diabetes mellitus, *C. albicans* proliferation can induce oral discomfort, gustatory dysfunction, swallowing difficulties, and subsequent nutritional compromise; furthermore, disseminated infection via hematogenous or digestive routes presents life-

threatening complications in vulnerable populations, with associated mortality approaching 79% in cases of systemic candidiasis (Pemmaraju et al., 2016; Viudes et al., 2002).

The inter-conversion between commensal and pathogen is governed by several factors, including host immunocompetence, resistance to antifungal agents, and various virulence determinants, particularly cellular adhesion mechanisms, structured biofilm development, and the environmentally responsive morphological shift between yeast and hyphal forms (Fourie et al., 2018; Nantel et al., 2002).

### **1.3 Biofilm Formation as a Virulence Factor**

Biofilms form when organisms attach to and grow on surfaces. The surface characteristics and cell wall structure of microorganisms significantly influence biofilm formation (Donlan, 2001).

Researchers study *C. albicans* biofilms through lab experiments, animal models, and by examining medical devices (Lohse et al., 2017). It showed that biofilm formation happens in four stages: attachment, growth, maturation, and spreading. First, yeast-shaped cells attach to a living or non-living surface, creating a foundation layer. These cells then multiply and begin forming different cell shapes, including pseudohyphae and hyphae, which help build the biofilm structure. Within about 24 hours, the biofilm matures, containing a mixture of cell types surrounded by a protective matrix made of proteins, fats, sugars, and genetic material. This matrix shields the biofilm and helps it resist disruption. The mature biofilm has a structured arrangement with yeast cells at the bottom and hyphae extending outward (Lohse et al., 2017). When mature, the biofilm releases

yeast-form cells that can spread infection to new areas. Similar biofilm structures have been observed in animal testing (using catheters, dentures, and vaginal models) and in clinical settings on medical devices like catheters and dentures. This ability to form biofilms allows *C. albicans* to persist on artificial surfaces and resist removal, creating significant problems in healthcare settings (Lohse et al., 2017). Moreover cell wall remodelling also affects biofilm formation and nutrients such as zinc, iron, and sugars, along with environmental factors like pH and temperature, substantially modify the fungal cell wall structure which is other virulence factor (Tripathi et al., 2020). Among these, iron appears to be a particularly important micronutrient.

#### **1.4 Iron Availability on Microbial Growth and Pathogenesis**

Iron serves as an essential micronutrient vital for numerous cellular processes, including the biosynthesis of critical biomolecules such as hemoglobin, amino acids, DNA, lipids, and sterols (Misslinger et al., 2018; Schaible & Kaufmann, 2004). Research has demonstrated that elevated iron levels can significantly enhance *Candida albicans* growth by modifying its cell wall structure, which has been associated with increased resistance to antifungal agents (Tripathi et al., 2020). In microbial ecosystems, organisms continuously compete for iron stores – both microorganisms within communities and pathogens with their host during commensal relationships and active infections. Since iron is indispensable for both host and *C. albicans*, efficient iron acquisition during infection represents a significant virulence factor, and successful colonization and proliferation depend largely on the fungus's ability to access sufficient iron (Sutak et al., 2008).

The relationship between host iron status and susceptibility to *C. albicans* infections has been well-documented. Studies have shown that pretreating endothelial cells with the iron chelator phenanthroline reduces damage caused by *C. albicans* and diminishes fungal invasion compared to untreated cells (Fratti et al., 1998). Similar protective effects were observed when oral epithelial cells were pretreated with bathophenanthroline disulphonate (BPS) (Almeida et al., 2008). The impact of iron availability on *C. albicans* virulence has been further validated in vivo in study done by (Abe et al., 1985). In a mouse model of systemic candidiasis, animals receiving intravenous colloidal iron (60 mg/kg body weight) for three consecutive days prior to *C. albicans* inoculation exhibited significantly higher mortality rates. Within 28 days post-infection, 80% of iron-supplemented mice succumbed to infection, compared to only 40% mortality in the control group (Abe et al., 1985).

### **1.5 Carbon Source Utilization and Influence on Virulence Mechanisms**

In addition to iron, the availability and type of carbon sources profoundly influence *Candida albicans* virulence and infection dynamics (Lok et al., 2020). This fungal pathogen has evolved remarkable metabolic flexibility, allowing it to thrive in diverse host microenvironments where glucose may be scarce but alternative carbon compounds are abundant (Lok et al., 2020). This nutritional adaptability represents a critical virulence attribute that directly impacts colonization efficiency and pathogenic potential. Carbon utilization significantly shapes several key aspects of *C. albicans* biology (Lok et al., 2020). First, different carbon substrates drive distinct growth patterns and reproductive strategies, directly affecting the pathogen's ability to proliferate within host tissues (Lok et al., 2020).

Second, carbon source variation triggers specific metabolic pathways and signaling cascades that regulate virulence factor expression (Lok et al., 2020). Carbon sources are particularly crucial for *C. albicans* cell wall structure and function. Assimilation of different carbon compounds modulates glucan structure and cross-linkages within the cell wall, directly influencing adhesion capabilities, surface hydrophobicity, colonization efficiency, cellular signaling, and immune recognition (Kulkarni et al., 1980).

Beyond cell wall effects, carbon availability serves as a critical environmental cue governing *C. albicans* morphological transitions between yeast and hyphal forms (Lok et al., 2020). This dimorphic switching capability is essential for pathogenesis, as hyphal forms enable tissue invasion and biofilm development while yeast cells facilitate dissemination (Lok et al., 2020). A notable attribute of *C. albicans* is its metabolic versatility regarding carbon utilization. The pathogen can metabolize glucose under both aerobic and anaerobic conditions (Agrawal et al., 2014), enabling it to adapt to oxygen-variable environments within the host, and enhancing its ability to thrive across diverse host niches. Understanding the intricate relationship between carbon metabolism and *C. albicans* virulence offers promising avenues for therapeutic intervention. By targeting specific carbon utilization pathways or disrupting carbon-dependent morphological transitions, novel antifungal strategies could potentially overcome current treatment limitations and improve clinical outcomes for patients suffering from candidiasis.

## **1.6 Gap in Knowledge and Study Objectives**

While previous research has explored the isolated effects of iron availability and carbohydrates on *C. albicans*, there remains a critical knowledge gap regarding their

combined influence. This intersection presents a promising opportunity for developing novel therapeutic strategies that combine dietary sugar restriction with iron level management to enhance antifungal drug efficacy, attenuate fungal virulence mechanisms, and strengthen host immune responses against *C. albicans* infections.

This study aimed to address this knowledge gap by analyzing the combined effects of iron availability and carbon sources on various aspects of *C. albicans* growth and virulence. We examined how iron levels impact *C. albicans* growth kinetics when utilizing different carbon sources. Additionally, we sought to characterize potential morphological transitions in *C. albicans* in response to iron supplementation while growing on various carbon sources. We also assessed whether iron availability and carbon sources influence *C. albicans* biofilm formation. Moreover, to examine cell wall structural changes under the influence of iron, we analyzed cell wall components like mannan.

## CHAPTER 2

### MATERIALS AND METHODS

This research was carried out in the Oral Microbiome Laboratory at the Kornberg School of Dentistry, Temple University, Philadelphia, PA. Before initiating the study, the protocol was submitted for institutional review and approval. All required training, including laboratory safety training, certifications in Biosafety Levels, Chemical and Environmental Hazards, and Bloodborne and Airborne Pathogens, were completed by the researchers directly involved in conducting the experimental work. The compliance measures were secured to align with institutional and regulatory standards.

#### **2.1 Fungal Strain, Media, and Culture Conditions**

For all the experiments, 1.7 g/L of minimal Yeast nitrogen base (YNB) media without copper, ammonium salt, and iron ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ), supplemented with 2.5  $\mu\text{M}$  copper sulfate ( $\text{CuSO}_4$ ), 5 g/L ammonium sulfate ( $\text{NH}_4\text{SO}_4$ ) was used as a base media. Twenty-five  $\mu\text{L}$  of 50 mM iron chelator named Bathophenanthrolinedisulfonic acid (BPS) was added to 5mL of YNB media. Basal media supplemented with 5  $\mu\text{L}$  of 1mM concentration of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  was used as low iron media to commence the experiment. Basal media supplemented with 5  $\mu\text{L}$  of 100mM concentration of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  was used as high iron media for the experiment (Tripathi et al., 2020). 2% glucose, sucrose, and fructose were added as carbon sources in low and high iron media respectively to make six different combinations. Microbiological loops were used to inoculate colonies of *C. albicans* into the tubes with 5mL of media. These tubes were placed in 30°C for a period of 24 hours for

incubation on the first day (D<sub>1</sub>). On the second day (D<sub>2</sub>) cells from the overnight culture were reinoculated into 5ml respective fresh media for second overnight incubation at 30°C.

## **2.2 Growth Assessment**

For growth assessment after two overnight incubations on day 3, optical densities (OD) of the cultures were measured and calibrated to a final OD<sub>600</sub> = 0.1. 200 µL all of media containing cultures were deposited into each well of a 96 well polystyrene plate. The plate was further incubated at 30°C, and growth curve was assessed by measuring absorbance at 600 nm over 24 hours, every 30 minutes with gentle shaking, using a spectrophotometer (BioTek Synergy Multi Mode Reader) (Sharma et al., 2023).

Using standard mean deviation growth was assessed under high and low iron conditions with different carbon sources using GraphPad Prism (version 9) software.

## **2.3 Morphological Analysis**

### ***2.3.1 Yeast to Hyphae Ratios***

For this experiment, the culture and media conditions were the same as mentioned above. After two days of overnight incubation in different carbon sources and iron medium at 30°C, the culture was washed and diluted to OD<sub>600</sub> = 0.3 in each respective medium and incubated under shaking conditions at 37°C for 24 hours. For hyphal analysis, 7 µL of cell suspension was extracted using a micropipette, placed on a glass slide, covered with a coverslip, and visualized using an EVOS Axio Fluorescence Microscope at 40× magnification.

For quantification, minimum 200 cells/condition/field were counted and recorded as either yeast cells or hyphae from the recorded data across three independent biological replicates. Statistical analysis was performed using GraphPad Prism (version 9) software, with significance assessed via Student's t-test. Representative fields were randomly selected using a systematic sampling approach to minimize observer bias. For morphological classification, standardized criteria were applied to distinguish between yeast and hyphal forms based on length-to-width ratio and presence of constrictions at septal junctions. P-values < 0.05 were considered statistically significant.

### **2.3.2 Solid Media Hyphae Formation**

In the YNB agar medium with three different carbon sources under high and low iron conditions, 5  $\mu$ L of *C. albicans* culture was added using a micropipette and allowed to solidify for one hour. The plates were then incubated at 37°C for 72 hours. Spots on agar were photographed using ChemiDoc imaging system.

## **2.4 Biofilm Experiment**

### **2.4.1 Biofilm Formation by *C. albicans***

Biofilm formation in different concentrations of iron with different carbon sources was carried out using YNB medium in a 96 well plate. Two-day overnight culture was used and calibrated to OD600 = 1.0. A total volume of 200  $\mu$ L per well was deposited and the plate was incubated at 37°C for 2 hours. Non adherent cells were removed by gentle washing and 200  $\mu$ L of respective media will be added to each well. The methods are followed as described previously (Puri et al., 2012).

### ***2.4.2 Biofilm Quantification***

For biofilm quantification, after thoroughly washing the wells, 200 $\mu$ L of 0.1% crystal violet was added to each well and kept aside for 15 minutes. The dye was pipetted out and the wells were thoroughly washed using 1x PBS. For the measurement of Optical Density, the well plate was air dried for 30 minutes and 30% acetic acid was added to dissolve the biofilm (Parolin et al., 2021). Absorbance was measured using a spectrophotometer (BioTek Synergy Multi-Mode Reader) at 37°C for 24 hours every 30 minutes with gentle shaking. For assessment a One-way ANOVA test was conducted to assess the statistical significance using GraphPad Prism (version 9) software.

### **2.5 Cell Wall Mannan Assessment**

For this experiment, the culture conditions were similar as mentioned above. On the third day, after two overnight incubation, the OD of the cultures was measured and an OD<sub>600</sub> of 0.3 was established in YNB media with respective carbon sources and iron conditions. The cultures were then incubated at 37°C for 4 hours to achieve log phase of the growth that was required for experimentation. The cells were centrifuged at 5000 revolutions per minutes (rpm) for 5 minutes, the supernatant was discarded and washed with 1x PBS after the incubation period. After washing, 1ml of 4% Paraformaldehyde was added to fix the cells and incubated at 37°C for 20 minutes. Post incubation, these cells were centrifuged, supernatant was removed and 200 $\mu$ L of 1x PBS was added to preserve the cells at 4°C for subsequent uses.

We prepared cell samples for microscopic analysis using specific fluorescent stains to visualize cell wall components. From each culture, 20 $\mu$ L of cellular sediment was

suspended in 1mL of 1X PBS. Samples were then stained with ConcanavalinA (ConA, 50µg/mL) to detect mannan. Due to the photosensitivity of these dyes, all staining procedures were conducted in a darkened environment. The ConA-treated samples were incubated for 45 minutes. Following incubation, cells were pelleted by centrifugation at 12,000 rpm, and the supernatant was discarded. The cell pellets were washed twice with 1X PBS to remove excess dye. The resulting stained cell preparations were mounted on microscope slides for subsequent image analysis. Fluorescence intensities were measured using a EVOS fluorescence microscope at 40x magnifications in GFP light cube filters used for ConA (Tripathi et al., 2020).

## CHAPTER 3

### RESULTS

#### 3.1 Growth Kinetics of *C. albicans* Under Various Iron and Carbon Source

##### Conditions

To obtain the results for this experiment, we evaluated *C. albicans* growth in 96-well polystyrene plates over a 24-hour period, starting with a standardized baseline optical density (OD<sub>600</sub>) of 0.1. Figure 1 illustrates the growth patterns of *C. albicans* under various conditions. The data points represent the aggregate means calculated from three independent replicates for each experimental variable. The growth kinetics of *C. albicans* were monitored under different iron conditions (high and low) with three distinct carbon sources (glucose, sucrose, and fructose) at 2% concentration (Figure A and B). Under high iron conditions (Figure A), all three carbon sources supported similar growth patterns with minimal differences between glucose, sucrose, and fructose. The growth curves exhibited a typical sigmoidal pattern with a lag phase until approximately 3 hours, followed by an exponential growth phase between 3-12 hours, and a subsequent stationary phase. The final optical density (OD<sub>600</sub>) values after 24 hours of incubation reached approximately 1.1 for all three carbon sources, with fructose showing a marginally higher final OD<sub>600</sub> (1.12) compared to glucose (1.09) and sucrose (1.07), although these differences were not statistically significant ( $p > 0.05$ ).

Under low iron conditions (Figure B), more pronounced differences in growth patterns were observed among the three carbon sources. While all carbon sources still exhibited the characteristic sigmoidal growth curve, the exponential growth phase

appeared to begin slightly earlier in sucrose-supplemented media compared to glucose and fructose. Interestingly, by the end of the 24-hour period, glucose and fructose supported higher growth (OD<sub>600</sub> of 1.12 and 1.14, respectively) compared to sucrose (OD<sub>600</sub> of 1.03). The difference between sucrose and the other two carbon sources was not statistically significant ( $p > 0.05$ ). All the experiments were performed in at least three biological repeats with triplicates, and the results were expressed as mean  $\pm$  standard error (SEM)

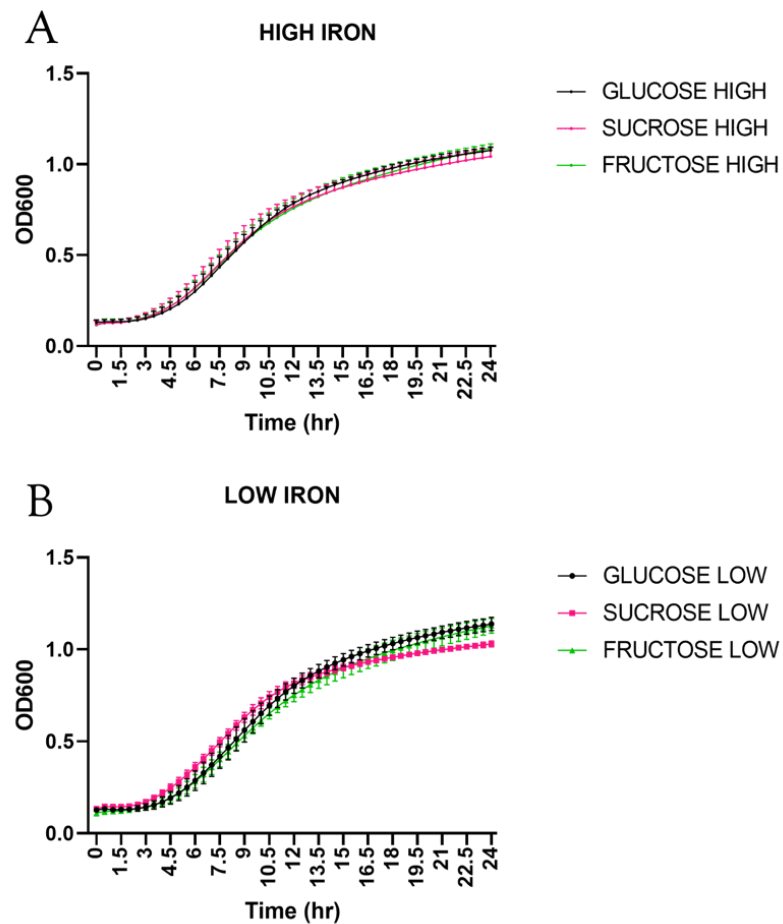


Fig 1. Growth Curves. *C. albicans* growth in presence of three different carbon sources. (A) High iron 100  $\mu$ M, (B) Low iron 1  $\mu$ M concentrations. The results of three independent biological repeats are represented as means  $\pm$  SEM.

## 3.2 Hyphal Changes

### 3.2.1 Yeast vs Hyphae Ratio

The morphological transition from yeast to hyphal form represents a critical virulence factor in *C. albicans*. To investigate how the interaction between iron availability and carbon source affects this transition, we quantified the hyphal-to-yeast ratio under various experimental conditions (Figure 2).

Our results demonstrate that both iron availability and carbon source significantly impact the hyphal morphogenesis of *C. albicans*. Under high iron conditions, sucrose elicited the highest proportion of hyphal formation (42.40%), which was higher, but not statistically significant ( $p = 0.1189$ ) than that observed with glucose (21.3%). Fructose + high iron conditions supported intermediate hyphal formation (10.5%), which was significantly lower than sucrose + high iron ( $p = 0.032$ ).

Strikingly, iron limitation substantially reduced hyphal formation across all carbon sources. Glucose + low iron conditions nearly abolished hyphal formation (0.5%), representing a significant reduction compared to glucose + high iron ( $p = 0.0436$ ). Similarly, sucrose + low iron resulted in markedly reduced hyphal formation (5.02%) compared to sucrose + high iron ( $p = 0.0207$ ). Fructose + low iron also showed minimal hyphal development (0.6%) significantly lower than fructose + high iron conditions ( $p = 0.0232$ ). Statistical comparisons between different carbon sources under low iron conditions revealed no significant differences ( $p > 0.05$ ), suggesting that iron limitation overrides the differential effects of carbon sources on hyphal formation.

These findings highlight the critical role of iron in promoting hyphal formation in *C. albicans*, particularly in the presence of sucrose as a carbon source. The data suggest

that iron limitation represents a stronger determinant of morphological behavior than carbon source variation, effectively suppressing the yeast-to-hyphae transition regardless of the available sugar. Data were analyzed using the student's t- test and p values lower than 0.05 were considered significant.

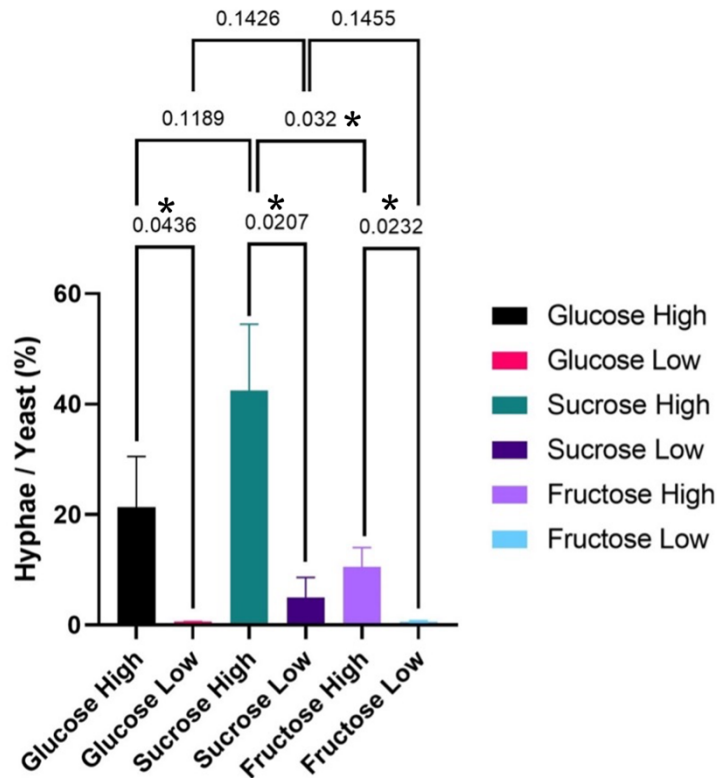


Fig 2. Hyphae vs Yeast Ratio. Quantification of hyphae vs yeast ratio of *C. albicans* in glucose, sucrose and fructose with high (100  $\mu$ M) and low (1  $\mu$ M) iron conditions plotted using GraphPad prism software. The results of three independent biological repeats are represented as means  $\pm$  SEM. Statistical significance analysis was assessed by Student's t- test

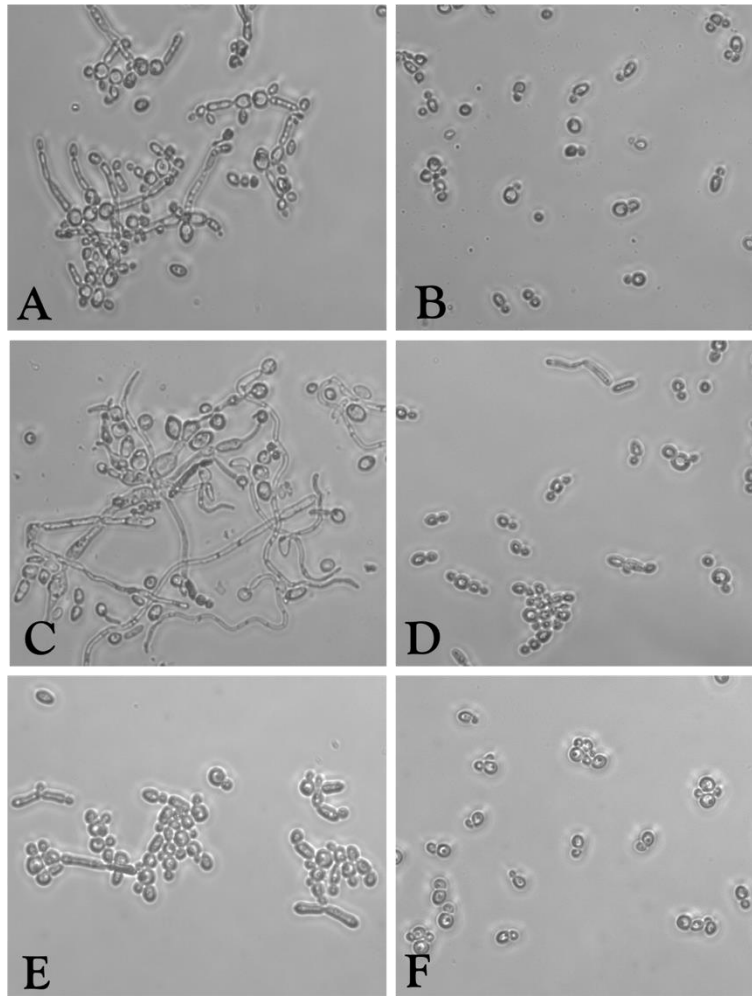


Fig 3. Microscopic Images of *C. albicans* Morphological Changes Using Axio Fluorescence Microscope. (A) Glucose high iron condition, (B) Glucose low iron. condition, (C) Sucrose high iron condition, (D) Sucrose low iron condition, (E) Fructose high iron condition, (F) Fructose low iron condition. This experiment was conducted in triplicate with independent biological replicates, and the figure presented is a representative image from one of these experimental iterations.

Microscopic pictures of *C. albicans* morphological changes with different carbon sources under high and low iron conditions is seen in figure 3. There is a stark difference between hyphal formation based on iron availability. Under iron-sufficient conditions, robust hyphal development was observed, while iron-limited environments predominantly maintained the yeast form regardless of carbon source. However, among the three carbon sources tested, sucrose (Figure 3, panel C) demonstrated the most pronounced effect on hyphal elongation in iron-replete conditions.

In the microscopic images of *C. albicans* hyphal growth in solid media with same combinations of the media, we found that the hyphal growth and surface roughness is significantly seen in sucrose + high iron conditions as compared to others (Figure 4, panel C). This represents sucrose utilization under high-iron conditions, demonstrating the potent morphological transition-inducing properties of this specific nutrient combination. The smooth, dome-shaped colonies in panels B and F exhibit characteristic glossy surfaces indicative of minimal hyphal formation, while panels A, D, and E show subtle variations in colony topography that may reflect intermediate morphological states. This notable difference suggests that the interaction between sucrose metabolism and iron availability may play a critical role in regulating *C. albicans* morphological transitions.

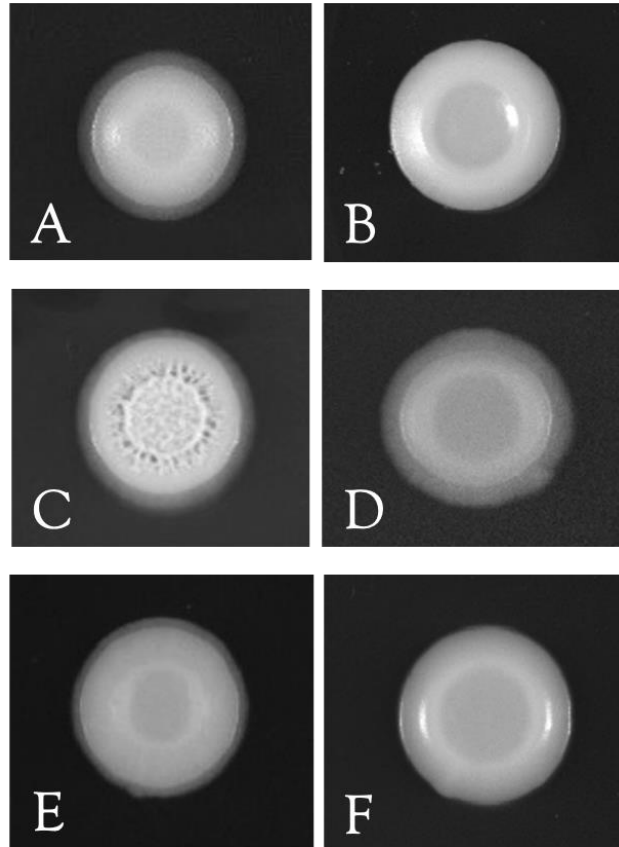


Fig 4. Microscopic Images of *C. albicans* Morphological Changes in Solid Agar. (A) Glucose high iron condition, (B) Glucose low iron condition, (C) Sucrose high iron condition, (D) Sucrose low iron condition, (E) Fructose high iron condition, (F) Fructose low iron condition. This experiment was conducted in triplicate with independent biological replicates, and the figure presented is a representative image.

### 3.3 Biofilm Analysis

The biofilm formation capacity of *C. albicans* was assessed under different iron availability conditions (high and low) with three carbon sources (glucose, sucrose, and fructose) (Figure 4). Biofilm density was quantified by measuring optical density at 600nm (OD600) after the established incubation period.

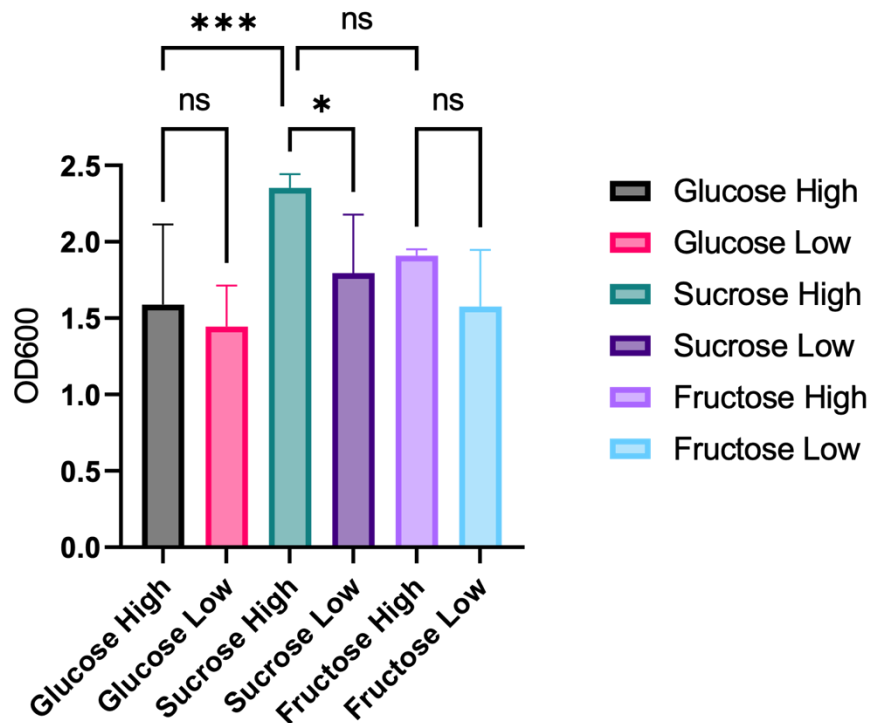


Fig 4. Biofilm Quantification. Biofilm formation of *C. albicans* in different media quantified by measuring optical density at 600nm wavelength. The results of two independent biological repeats are represented as means  $\pm$  SEM. Statistical significance analysis was assessed by One-way ANOVA test.

Biofilms formed in sucrose-rich, high-iron media demonstrated the most robust growth, achieving the highest optical density measurements (OD600 = 2.35). This represented a statistically significant increase compared to biofilms formed in low-iron sucrose media ( $p < 0.05$ ). The enhanced biofilm formation observed under high-iron sucrose conditions suggests a synergistic effect between sucrose metabolism and iron availability. The values were also significant for comparison between high iron + sucrose and high iron + glucose media ( $p < 0.001$ ).

Fructose-supplemented media also supported substantial biofilm formation under high-iron conditions (OD600 = 1.91), though this was not statistically different from its low-iron counterpart (OD600 = 1.57). Glucose media yielded comparable biofilm measurements regardless of iron concentration (high: OD600 = 1.58; low: OD600 = 1.44), with neither condition showing statistical significance between high and low iron states.

These findings demonstrate that while carbon source significantly impacts biofilm development, the interaction between carbon metabolism and iron availability appears particularly critical, with the combination of sucrose + high iron creating optimal conditions for robust biofilm formation. This relationship may represent an important factor in understanding the organism's virulence and colonization capabilities in environments where these nutrients are abundant.



Fig 5. Biofilm Formation. Visual representation of biofilm of *C. albicans* in different media starting from left is glucose, sucrose, fructose + high iron condition.

### 3.4 Cell Wall Studies

The cell wall architecture of *Candida albicans* was assessed through component-specific fluorescence microscopy to determine the impact of iron availability on cell wall composition. Fluorescent micrographs (Figure 6) revealed distinct patterns of mannan distribution visualized by Concanavalin A (ConA) staining under varying iron conditions. Qualitative analysis of these images demonstrated a notable decrease in mannan content within the cell walls of *C. albicans* cultured under high-iron conditions compared to those grown in iron-limited environments.

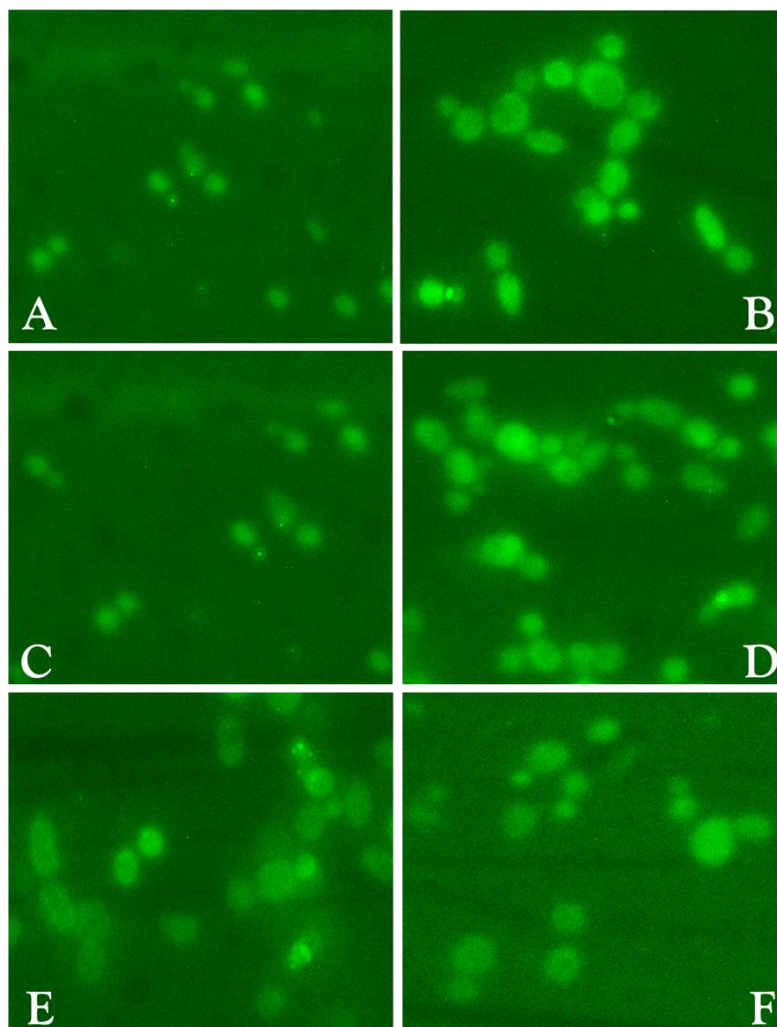


Fig 6. Qualitative Analysis of Cell Wall Component Mannan. Fluorescence microscopic images of *C. albicans* in (A) Glucose high iron condition, (B) Glucose low iron condition, (C) Sucrose high iron condition, (D) Sucrose low iron condition, (E) Fructose high iron condition, (F) Fructose low iron condition.

## CHAPTER 4

### DISCUSSION

The interplay between iron availability and carbon source utilization represents a critical determinant of *C. albicans* virulence potential. Our findings reveal that iron levels modulate the response of this opportunistic pathogen to different carbon sources. This affects the key virulence traits of the microorganism such as morphological changes like converting from yeast to hyphal form which also enables it to invade the tissues, biofilm formation which increases the resilience of the organism and cell wall remodelling, which increases its antifungal resistance are its key virulence traits (Fourie et al., 2018) (Nantel et al., 2002) (Tripathi et al., 2020). ). This study highlighted the role of nutrient-dependent regulation of the virulence factors to govern *C. albicans* pathogenicity.

Previous research has established iron as a critical modulator of virulence traits in *C. albicans*, demonstrating its profound influence on pathogenicity (Sutak et al., 2008; Almeida et al., 2008). Similarly, multiple studies have documented how different sugars independently affect the organism's morphology, adhesion properties, and overall virulence potential. However, a significant gap existed in our understanding of how these two essential environmental factors—iron availability and carbon source—might interact to collectively shape *C. albicans* pathogenicity. The current study is the first study that addresses this knowledge gap by systematically investigating the combined effects of iron concentration (high versus low) across three distinct carbon sources (glucose, sucrose, and fructose) on multiple virulence parameters through multiple measurement techniques.

Previous studies have shown robust growth of *C. albicans* under high iron condition which signifies its iron adaptability (Sharma et al., 2023). However, our findings reveal no

significant changes in the growth pattern of the organism under high and low iron conditions with all three carbon sources.

The morphological transition assessment suggested that the organism shows significant morphological changes under high iron conditions as compared to low iron conditions for all the carbon sources. Amongst all three carbon sources the finding that sucrose as a carbon source in high-iron environments produced the highest yeast-to-hyphae ratio is particularly significant. In the context of solid media cultivation, *C. albicans* grown on sucrose under high-iron conditions exhibited remarkable hyphal development, characterized by a distinctive wrinkled colony morphology indicative of extensive filamentous growth (Figure 4-C). Notably, sucrose demonstrated a unique capacity to induce hyphal formation even under iron-limited conditions, a phenotype not observed with the other carbon sources tested (Figure 4-D). This differential response highlights the specialized relationship between sucrose metabolism and morphological switching in *C. albicans*, suggesting that sucrose may activate hyphal-inducing pathways that can partially overcome the morphological constraints typically imposed by iron limitation. The enhanced hyphal formation observed specifically with sucrose under iron-replete conditions indicates that this dietary sugar might play a more substantial role in promoting pathogenicity than previously recognized, especially in iron-rich host niches. This might be related to sucrose's rapid metabolism providing abundant energy for the energetically demanding process of hyphal elongation, with iron serving as a critical cofactor for enzymes involved in this morphological transition. However, a notable limitation of this study lies in the fundamental structural differences between the carbon sources examined. Sucrose, as a disaccharide composed of glucose and fructose, possesses distinct

physicochemical properties compared to its monosaccharide constituents tested individually. The enhanced biofilm formation observed under high-iron sucrose conditions may therefore reflect not only metabolic preferences, but also structural advantages conferred by disaccharide molecules that facilitate adhesion or extracellular matrix formation. Future investigations should systematically compare multiple disaccharides (e.g., maltose, lactose) with monosaccharides to determine whether the observed effect is specific to sucrose or represents a broader phenomenon related to disaccharide structure. Additionally, examining the role of bonding in cell-surface interactions could provide mechanistic insights into the carbon source dependency of biofilm development under varying iron conditions.

Another limitation of this study concerns the potential influence of significant hyphal changes observed under high iron conditions on the growth measurements. Hyphal morphology can substantially interfere with spectrophotometric readings. When *C. albicans* transitions from yeast to hyphal forms, several factors affect OD600 measurements: hyphal cells tend to aggregate and form clumps, creating uneven suspension in culture media, while the elongated, filamentous structure of hyphae scatters light differently than spherical yeast cells. This methodological consideration suggests that the apparent similarity in growth curves across conditions may be due to the pronounced morphological differences we observed between high and low iron treatments.

Similarly, the observation of significantly more robust biofilm formation under high-iron and sucrose conditions aligns with the hyphal findings, as hyphal elements constitute a structural framework for biofilm architecture (Nobile & Johnson, 2015). A methodological limitation of this study relates to our use of the crystal violet assay for

biofilm quantification. Despite its widespread application, we observed considerable variability in absorbance measurements between experimental replicates. This inconsistency may be attributed to the inherent fragility of *Candida* biofilms, which can be disrupted during washing steps, combined with potential variations in dye intensity and retention. For future investigations, we recommend implementing complementary or alternative quantification methods, particularly dry weight assessment, which would provide a more direct measure of biomass independent of dye binding properties.

Furthermore, fluorescence microscopy analysis revealed a decreased mannan exposure across all carbon sources tested under high-iron conditions (Figure 6). The cell wall of *C. albicans* exhibits a distinct two-layer architecture. The outer layer comprises mannans covalently linked to various glycosylated proteins (mannoproteins), constituting approximately 35-40% of the total cell wall mass. This outer mannan layer serves as the primary interface with the host environment. The inner layer consists of  $\beta$ -glucans networked with chitin, forming the structural foundation of the cell wall (Tripathi et al., 2020). Our observations are similar to (Tripathi et al., 2020), who found that high iron decreased mannan in the outer cell wall. Pradhan et al. (2019) demonstrated that high iron reduces the thickness of the mannan layer in the outer cell wall supporting our observations. These previous findings may help explain our results, suggesting that the changes we observed could reflect both actual compositional differences and alterations in how cell wall components are exposed or arranged. While these observations provide compelling evidence for iron-dependent modulation of cell wall architecture, it should be noted that this analysis was limited to qualitative assessment. Future studies employing quantitative

fluorescence intensity measurements would further elucidate the magnitude of these compositional changes.

These results collectively point toward a potential therapeutic strategy involving dietary management of both iron and specific carbon sources, particularly sucrose, in patients susceptible to candidiasis. Based on our data it becomes crucial to assess the susceptibility to candidiasis in diabetic patients with iron overload.

The data reveal that sucrose appears to be a particularly potent carbon source for promoting hyphal formation in *C. albicans*, but only when sufficient iron is available. The findings indicate that while sucrose may be the most effective "culprit sugar" for triggering hyphal growth, iron availability ultimately serves as the master regulator that can override sucrose's effects. This relationship warrants further investigation to understand how nutritional factors might influence *C. albicans* pathogenicity in clinical settings.

## CHAPTER 5

### CONCLUSION

It can be very well concluded from this study that iron plays a significant role in altering the influence of carbon sources on *C. albicans*' mechanisms. Our findings demonstrate that the combination of sucrose and high iron conditions yields the most pronounced yeast-to-hyphae transition ratio, a critical virulence determinant. Furthermore, biofilm formation, another key pathogenic attribute was substantially enhanced under these specific nutrient conditions. Cell wall analysis confirms that iron plays a role in cell wall remodelling of its components with all the carbon sources.

This intersection is particularly relevant in the context of human physiology, where both nutrients coexist in various forms across different anatomical niches. Our results establish a foundation for further investigation into nutrient-dependent virulence regulation in *C. albicans* and highlight the importance of considering nutritional complexity in developing novel antifungal strategies that target the pathogen's metabolic adaptability rather than conventional drug targets.

**Clinical Implication:** Our results motivate us towards a clinical paradigm shift in managing oral diseases associated with *Candida*, particularly early childhood caries (ECC), as iron and sucrose may synergistically influence *Candida* virulence. Thus, sucrose should be seen as a function of iron for a complete clinical picture

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