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Escuela de Ciencias Biológicas e Ingeniería

Analysis of vegetable juice-based medium components for the induction of Candida filamentation

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniera Biomédica

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“This is ground control to my Major Tom...

To David Nicolás, I may not understand much about life, but one thing is clear, as long as our love lasts, the world will not perish. My soul loves you.”

Jannys Valles

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Finally, like Nietzsche when he wrote: "Throw roses into the abyss and say: Here is my thanks to the monsters who didn't succeed in swallowing me alive."

Jannys Valles

Resumen

Candida es el género más común de hongos patógenos unicelulares y es responsable de una amplia variedad de infecciones humanas. La capacidad de este hongo para inducir un "cambio morfológico", transformando células en forma de levadura a células filamentosas, es un factor de virulencia ligado a su patogenicidad. En este estudio, se investigó la inducción de la filamentación en *Candida* utilizando medios de cultivo basados en jugos de verduras, inspirados en el jugo comercial V8. Además, se examinó el proceso de morfogénesis en cada vegetal por separado, y se analizaron otros factores moduladores, como el pH, la temperatura y la concentración. Los resultados sugieren que los vegetales individuales pueden inducir por sí solos la filamentación estándar en *Candida*, lo que respalda la hipótesis de que este hongo podría tener un origen como simbionte o patógeno de plantas. Se propone una futura investigación para explorar las vías metabólicas involucradas en la filamentación, utilizando mutantes de factores de transcripción conocidos y genes reporteros. Esto proporcionaría una base molecular para la regulación de la filamentación de *Candida* en medios vegetales. Además, esto podría tener implicaciones importantes para explicar los mecanismos de filamentación de hongos en vegetales y su transición a humanos. Este estudio no solo ofrece perspectivas sobre la biología de *Candida* en relación con los vegetales, sino que también sienta las base para el desarrollo de medios de cultivo más seguros y estables que reduzcan el riesgo de falsos positivos y transmisión de enfermedades, así como nuevos enfoques para tratamientos antifúngicos.

Palabras Clave:
Candida; Filamentación; Vegetales; V8; Morfología; Patogenicidad; Adaptación.

Abstract

Candida is the most common genus of unicellular pathogenic fungi and is responsible for a wide variety of human infections. The ability of this fungus to induce a "morphological change", transforming yeast-like cells to filamentous cells, is a virulence factor linked to its pathogenicity. In this study, the induction of filamentation in *Candida* was investigated using vegetable juice-based culture media inspired by commercial V8 juice. In addition, the morphogenesis process was examined for each vegetable separately, and other modulating factors, such as pH, temperature and concentration, were analyzed. The results suggest that individual vegetables alone can induce standard filamentation in *Candida*, supporting the hypothesis that this fungus could have an origin as a plant symbiont or pathogen. Future research is proposed to explore the metabolic pathways involved in filamentation, using mutants of known transcription factors and reporter genes. This would provide a molecular basis for the regulation of *Candida* filamentation in vegetable media. Furthermore, this could have important implications for explaining the mechanisms of fungal filamentation in vegetables and their transition to humans. This study not only offers insights into the biology of *Candida* in relation to vegetables but also lays the foundation for the development of safer and more stable culture media that reduce the risk of false positives and disease transmission, as well as new approaches for antifungal treatments.

Keywords:
Candida; Filamentation; Vegetables; V8; Morphology; Pathogenicity; Adaptation.

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Chapter 1

Introduction: Theoretical Framework

1.1 General Features of *Candida spp.*

Distinctively, '*Candida spp.*' constitutes a group of species belonging to the fungal kingdom. More specifically, its taxonomy falls within the phylum Ascomycota, which further includes the subdivision Saccharomycetes. Here, *Candida* stands as a notable member of this class. Among its main characteristics, *Candida* is a diploid fungus capable of both sexual and asexual reproduction, and is pathobiotic in nature; that is, it can act as both a commensal organism and an opportunistic pathogen [1]. Additionally, this fungus exhibits a remarkable dispersal capacity. In healthy individuals, *Candida* can coexist normally in the human body, assuming a commensal role [2]. However, as the most prevalent fungal pathogen in humans, it can transform into an opportunistic agent under certain circumstances, leading to mucosal and systemic infections in various body parts, including the skin, mouth, gastrointestinal tract, and genitourinary system [3]. Furthermore, *Candida* is the predominant fungus causing nosocomial infections. These infectious processes normally occur in hospital environments, presenting challenges at surgical sites or through contamination in critically ill patients with intravenous catheters [4].

1.1.1 Common *Candida* Species

There are approximately 150 species within the genus *Candida*, which coexist with the human host as opportunistic commensal fungi, as mentioned above. Despite this diversity, the predominant species is *Candida albicans*, accounting for 60% of infections [4]. However,

there is a noticeable shift towards the presence of non-albicans species (NACS). These include *C. glabrata, C. guilliermondii, C. krusei, C. lusitaniae, C. parapsilosis, and C. tropicalis*, which are considered emerging opportunistic pathogens. Notably, *C. glabrata, C. parapsilosis, and C. tropicalis* pose several medical challenges, including difficulties in diagnosis and resistance to antifungal drugs [1].

It is impossible not to talk about *C. albicans* when mentioning common *Candida* species. *C. albicans* is the most significant fungal pathogen and the primary responsible behind invasive infections with high mortality rates. Despite medical efforts, *C. albicans* has a mortality rate of 40%, even with clinical treatment [5]. Its predominance as a major infectious threat can be attributed to specific genetic traits, particularly those related to reproduction and plasticity. These aspects will be further explored in this research.

The distinguishing characteristics of other *Candida* species cannot be overlooked. There are differences in the evolution of these species that are reflected in changes in their morphology and pathogenicity. Although they do not possess the same virulence properties as *C. albicans*, they express genetic traits that allow them to cause dangerous systemic infections in humans [6]. Notably, *C. tropicalis* and *C. parapsilosis*, in addition to *C. albicans*, have the capacity for filamentation both in vitro and in vivo. These species can form true hyphae, thereby increasing their virulence. Hence, non-albicans species also play a significant role in infections, particularly due to their filamentation abilities [7].

1.1.2 *Candida* Morphology

The morphology of *Candida spp.* plays a crucial role in the development of its adaptive and pathogenic capabilities. The anatomy of *C. albicans* is particularly notable for its variability and virulence, distinguishing it from other species. This morphology includes yeast, hyphal, and pseudohyphal forms. Yeasts are characterized by their round or oval shape. In contrast, hyphae are thin, elongated cells, similar to the shape of a tube. These can form complex filamentous structures, resembling a bunch of tangled wires. Pseudohyphae, on the other hand, represents an intermediate structure between yeasts and hyphae, sharing characteristics of both forms. This results in a transitional morphology that shifts from yeasts to hyphae [3].

The differentiation among these three morphological structures of *Candida* can be complex. These differences can be seen in Figure 1.2. It is crucial to recognize that yeast forms are spherical and separate completely following cell division. Pseudohyphae usually appear as yeast chains wider than true hyphae and joined by contractions (septa). Hyphae, however, are distinguished by their narrow diameter, resulting in elongated, individual cells. When *Candida* assumes the yeast form, it typically functions as a commensal within the host. The transition to pseudohyphae and hyphae, however, signifies a shift towards a pathogenic role in the host organism [3, 8].

It is crucial to detail the reproductive cycle of *C. albicans* in order to discuss other morphotypes. As mentioned above in general terms, *C. albicans* is a diploid fungus that reproduces both sexually and asexually. Thus, it can exist in two distinct cell types: the 'white cells,' representing its 'sterile' form, and the well-known 'opaque cells,' which are competent for reproduction/mating [9]. Despite having the same genome, these two cell types express different genes, follow different metabolic pathways, and exhibit varied sensitivities to antifungals. It is also noteworthy that opaque cells are larger than white cells and feature pimple-like protrusions. Moreover, these differences enhance their virulence and alter their interactions with the host.

1.1.3 *Candida* Phylogenetics

The enigmatic 'Third Kingdom' comprises a fascinating array of organisms known as fungi; those organisms that oscillate between the bifurcation of animals vs. plants. Within this group, belongs the even more mysterious and chameleon-like '*Candida*'. This designation was initially given to fungi that were challenging to characterize and had an ambiguous evolutionary origin, leading to a somewhat chaotic and disorganized taxonomy. However, thanks to the advent of genomics and phylogenomics, these puzzles are slowly being solved [10]. In evolutionary biology, phylogenetics is a discipline that investigates the genealogical relationships of different species. In order to illustrate this set of relationships, phylogenetic trees are made to show the evolution and relatedness to common ancestors. These relationships are crucial in understanding pathogenic processes, virulence, and treatment resistance among *Candida albicans* and other non-albicans *Candida* species.

Within the *Ascomycota* phylum, the subdivision *Saccharomycotina* encompasses several classes, including *Saccharomycetes*, which further contains the order *Saccharomycetales*. This order includes notable species such as *Candida parapsilosis*, *Candida tropicalis*, and *Candida albicans*, the latter being the human pathogenic fungus par excellence. This classification is illustrated in Figure 1.1. These *Candida* species are part of the CTG (or CUG) clade, characterized by a unique codon usage in which the CTG codon codes for serine rather than leucine. This serine encoding allows these *Candida* species to effectively camouflage their β-glucan, thereby evading detection by the host's immune system. This adaptation is a hallmark of pathogenic species within this lineage [11, 12].

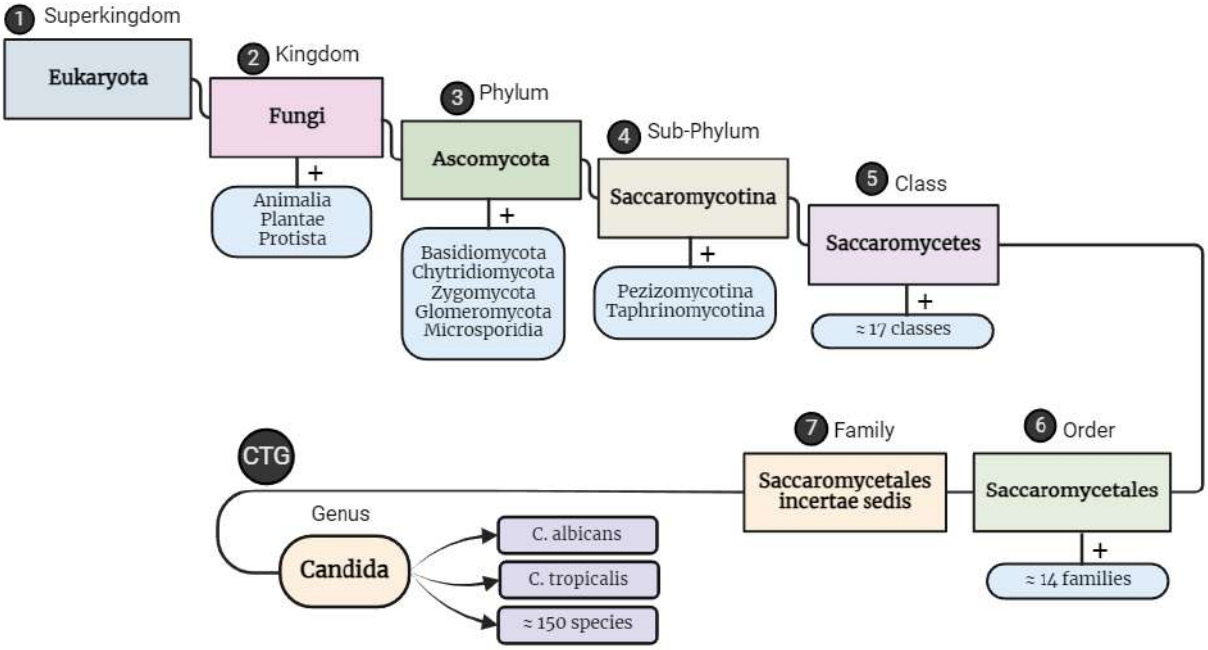


Figure 1.1: General taxonomic classification of *Candida*; *Candida* species diverge from a common ancestor (*Ascomycota*) within *Saccharomycetales*, highlighting the specific evolution of each species and their phylogenetic relationship with other species of the same order.

Phylogenetic analyses have revealed that the *Saccharomycotina* group, which includes *Candida* species, is polyphyletic [13]. This means that although these organisms are classified together based on common characteristics, they do not share a direct common ancestor. *Candida* has evolved along different evolutionary lines, which highlights their diversity, evolutionary complexity, adaptability and its plasticity. This adaptability enables them to thrive in adverse and hostile environments. The *C. albicans* genome is notably complex

and versatile, consisting of eight pairs of chromosomes totaling approximately 16 Mb in its haploid state. Predominantly a diploid organism, *C. albicans* displays considerable heterozygosity and chromosomal variations, potentially leading to aneuploidy, a condition marked by alterations in chromosome numbers. Although its reproduction is mostly clonal, *C. albicans* can also undergo a parasexual cycle, forming tetraploid progeny that eventually lose the extra chromosomes. In addition, the flexibility of its genome is central to its adaptation and survival in diverse environments [11, 14, 7].

Candida dubliniensis, shares certain similarities with *C. albicans*, but also possesses "improvements". Notably, *C. dubliniensis* exhibits a more variable karyotype (shape and size of chromosomes), suggesting a potential for greater genetic variability and adaptability to diverse environments. Surprisingly, this makes it less adaptable to hostile environments. This divergence could be due to the different evolutionary trajectories taken by these species [7].

Phylogenetic analyses utilize DNA microarrays of multiple *C. albicans* isolates from various clades, geographic locations, anatomical sources, and pathological states to deduce their evolutionary pathways, gene family expansion, and molecular epidemiology. Unfortunately, very few *C. albicans* strains have been isolated from non-animal sources for study. While *C. albicans* is typically recognized as a principal pathogen in humans; there are cases of its isolation from non-human environments like trees, shrubs, and grass. For instance, the genomic diversity of *S. cerevisiae* indicates that forests may be its ancestral habitat, and by extension, forests and plants could be the original habitat for the entire Saccharomyces class. Several yeast species live in trees, including other opportunistic pathogenic *Candida* species such as *Candida tropicalis* and *Candida parapsilosis* [15, 16].

The *C. albicans* genome, extracted from ancient oak trees, demonstrates a significant phylogenetic similarity to strains isolated from humans and other animals in clinical settings. This closeness suggests that *C. albicans* can endure in tree environments for extended periods, maintaining an ecological niche similar to that of clinical strains. Strains derived from oaks display a notable phenotypic variety and heterozygosity, which could reflect differences in mating and clades. Such genetic diversity in oak-sourced *C. albicans*, characterized by increased heterozygosity, is thought to represent an ancestral trait common to multiple *Candida* species [15]. Comparative studies between clinical and environmental

strains of *C. albicans*, as well as investigations into related species like *C. tropicalis* and *Cryptococcus*, are crucial to understand the evolutionary aspects of their commensalism and pathogenicity. Moreover, the discovery of species including *Candida carvajalis sp.* and *Candida ecuadorensis sp.* in Ecuador, isolated from decaying wood and other organic substrates, underscores the genus *Candida*'s adaptability to varied habitats [17, 18].

1.2 *Candida* Infections

Throughout the first sections of this work, the importance and dangerousness of *C. albicans* was emphasized as the most common pathogenic species in humans and, notably, the most extensively studied. Nevertheless, preventing infections caused by this fungus remains a challenge in healthcare. It is estimated that at least 30% of healthy individuals are colonized by *C. albicans* at some point [2]. This prevalence is higher in specific groups, including women, diabetics, pregnant women, the elderly, and intensive care unit (ICU) patients. Approximately 75% of women are expected to experience at least one *Candida* infection (vaginal candidiasis) in their lifetime, with 5% likely to suffer recurrent infections [3, 19, 20]. The risk of infection is particularly significant in patients with compromised immune systems, such as those in ICUs. Contributing factors to infections in these scenarios include the patient's immune response, the presence and number of medical devices susceptible to contamination (e.g., catheters), the duration of ICU stay, and the overall degree of morbidity [21, 4].

1.2.1 Common *Candida*-Associated Infections

Globally, *Candida spp.* is ranked as the fourth leading cause of healthcare-associated infections [22]. *C. albicans*, along with the four major non-albicans species (NACS) - *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* - are responsible for 90% of all cases of invasive candidiasis [23]. The incidence of these infections has progressively increased worldwide, attributed to the significant rise in the population at risk, including individuals infected with HIV, those with autoimmune diseases, patients undergoing cancer treatment, or those frequently using indwelling medical devices [6, 19]. Consequently, the immune status of the host makes it easier for *C. albicans* to establish an

infection. This pathogenic fungus finds optimal conditions to evade the immune system of the patient, allowing it to reproduce and disseminate within the host environment.

Even though the *Candida* fungus may colonize individuals, they may not experience any discomfort or symptoms. This asymptomatic nature is a notable characteristic of this pathogen, allowing it to proliferate unchecked until an internal imbalance leads to a severe infection. *Candida* infections can be classified in several ways. For example, depending on the origin or source of the infection, they can be endogenous, when the source is the organism itself (e.g., an overgrowth of the fungus in some anatomical site of the organism itself), or exogenous, arising from external sources (like hospital environments, contaminated medical devices, or hospital staff) [24, 1]). Furthermore, these infections are also classified based on the affected area, divided into superficial and systemic/invasive types. These fungal infections can be subdivided into cutaneous (skin-related), mucosal (mucous membranes: esophageal, oropharyngeal, and vulvovaginal) and the most dangerous and serious type, systemic infections (such as candidemia, affecting the bloodstream and other internal organs) [25, 13].

As is evident, *Candida* infections can evolve from superficial colonization to life-threatening systemic conditions. Among the most common *Candida*-associated infectious manifestations are vulvovaginal candidiasis (VVC), biofilm formation on medical devices (including prosthetic devices, intravenous and urinary catheters, and stents, among others), oral candidiasis, and candidemia. The latter, candidemia, is a frequent condition in hospitals and accounts for approximately 15% of bloodstream infections [26]. It is also a major contributor to morbidity and mortality among hospitalized patients or those with compromised immune systems [14, 27].

This section highlights the complex scenario of *Candida* infections. These phenomena require more effective treatments for their elimination, since resistance to antifungal therapies and drugs is increasing. Diagnosis, prevention and treatment of *Candida* infections represent a significant challenge in modern medicine. Therefore, understanding their pathogenic mechanisms, filamentation processes, and growth patterns is crucial to develop new intervention strategies for these diseases.

1.2.2 Clinical and Epidemiological Relevance

The ability of *Candida albicans* to cause infections represents a significant public health challenge [3, 28]. Global reports indicate that invasive candidiasis affects approximately 750,000 individuals annually, leading to around 50,000 deaths. This implies a mortality rate of up to 40% among adults [29]. *Candida* ranks as one of the top four pathogens causing hospital-acquired infections [13]. The mortality rate from these fungal infections is comparable to that of severe diseases like malaria, breast cancer, tuberculosis, or HIV [30]. Moreover, these infections also incur significant economic costs, which can reach up to $40,000 in the United States and Europe [29].

Although data on the mortality and global incidence of *Candida* infections are available, accurate estimation is difficult due to the lack of standardized criteria and guidelines. The incidence varies based on factors such as patient age, hospital admissions, population health data, and the presence of risk factors like transplantation, intensive care, or specific treatments. In Latin America, the information on the incidence, morbidity, and mortality of these infections is even more limited and varies according to geographic region, local epidemiology and economic factors, including poverty and limited access to public health services [31].

Candida spp. is a facultative pathogen that causes disease mainly in hosts with compromised immune systems [3]. The prevalence of *Candida* is higher in pregnant women, the elderly, AIDS patients, diabetics, those on antibiotics or corticosteroids, and hospitalized patients exposed to medical devices [24, 7]. Additionally, the ability of these fungi to infect and cause disease has led to the development of resistance to drugs such as azoles, and first-line treatments, increasing their ability to spread [7, 32]. The following sections will provide detailed information about the virulence processes in Candida infections and the importance in the development of improved isolation and treatment strategies.

1.3 Pathogenicity and Virulence Mechanisms

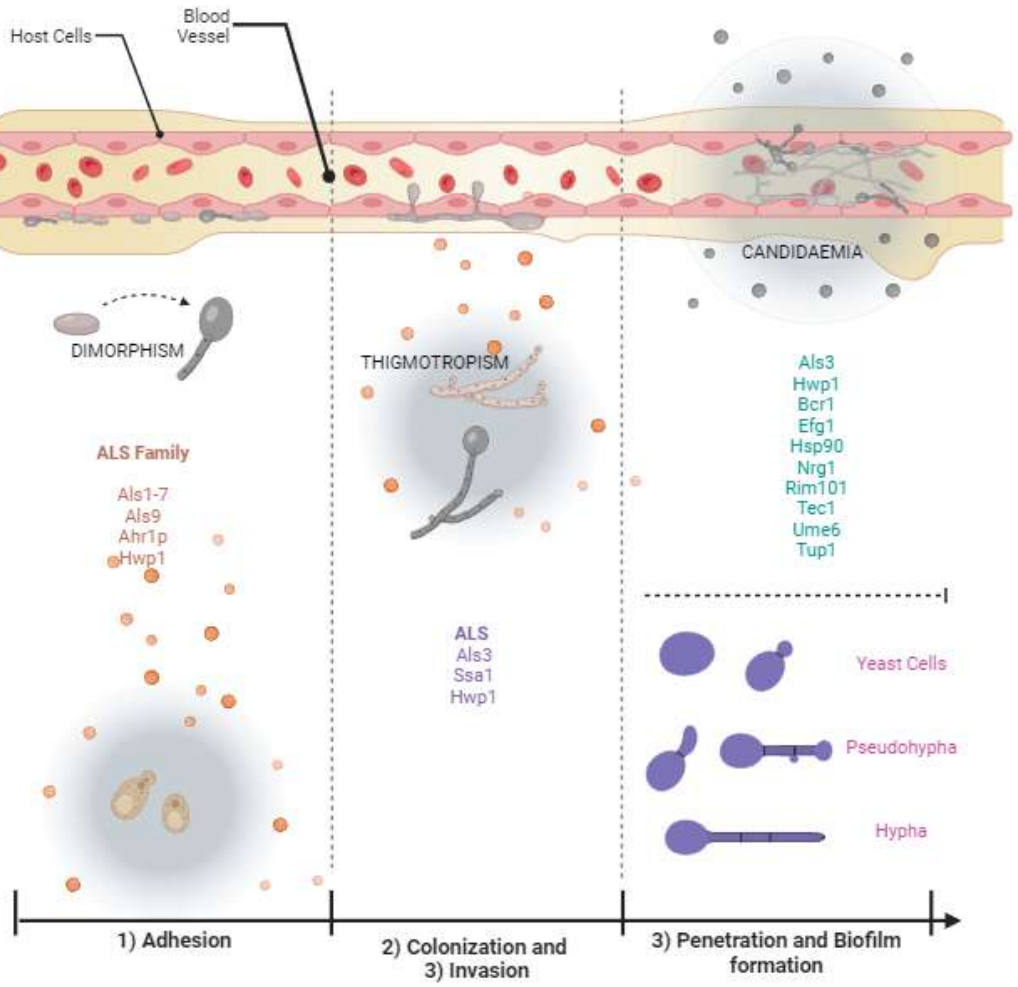


Figure 1.2: Comprehensive overview of *Candida* pathogenic processes, including the phases of invasion, colonization, dissemination and biofilm formation through which *Candida* establishes and maintains infection. The transcription factors essential for each pathogenic phase are detailed, as well as the morphological diversity of *Candida*, from its yeast form to pseudohyphae and hyphae.

Candida pathogenicity is characterized by its transition from a commensal yeast form to a pathogenic hyphal form, marked by a complex interplay of various activation pathways and virulence mechanisms [33]. As an organism adaptable to different biological conditions, *Candida* can respond to environmental changes, triggering a pathogenic response [34, 1, 12].

The general steps involved in *Candida* pathogenesis include:

1. Adhesion facilitated by adhesins.
2. Colonization of the host environment.
3. Invasion involving dimorphism (morphological change) and secretion of proteases.
4. Dissemination through enzyme secretion.
5. Penetration and biofilm formation.

These steps facilitate fungal growth and help the pathogen evade the immune system, allowing *Candida* to penetrate and invade endothelial tissue (see Figure 1.2). Deep invasion into human body tissues leads to the activation of further virulence processes [1].

Other virulence factors associated with *Candida* include:

a) Endocytosis: This allows *Candida* to evade immune recognition and survive for reproduction.
b) Morphological and Phenotypic Change: *Candida* growth from yeast to filamentous forms and phenotypic transition from 'white' to 'opaque' cells.
c) Thigmotropism: directed growth of hyphae toward epithelial cells for invasion.
d) Biofilm Formation: This facilitates the spread to sensitive tissues and establishes infection.
e) Enzyme Secretion: *Candida* secretes extracellular hydrolytic enzymes, adhesins, and cytolytic proteins.
f) Interaction with Heat Shock Proteins.
g) Metabolic flexibility: This allows *Candida* to adapt to various environments.
h) Escape from Phagocytosis.
i) Resistance to antifungals, among others.

This is a network of virulence factors of *Candida*, there are many connections between these mechanisms and they are regulated by suitable conditions for its development [1, 35].

1.3.1 Adhesion, Invasion, and Biofilm Formation

Adhesion

During the adhesion process, *Candida* species, particularly *C. albicans*, bind to various surfaces, both biological (such as tissues, mucous membranes, and organs) and abiotic (including medical devices and instruments, catheters, etc.) [36]. The success of this process depends on the effectiveness of adhesion factors and is mediated by proteins known as adhesins [37, 1]. Adhesins, essential in this process, belong to the Agglutinin-like sequence (ALS) family, composed of glycosylated agglutinin-like sequences. This group includes 8 members (Als1-7 and Als9), which share features such as 3' and 5' domains (amino acid sequences with adhesion functions) and a 108 bp core domain consisting of tandem repeats (proline and serine/threonine amino acid repeats, contributing to biofilm formation). The glycoprotein Als3 and the adhesin Hwp1 are particularly relevant in this family due to their prominent roles in the adhesion process. The other proteins involved can be observed in detail in Table 1.1 [37, 38].

Invasion

C. albicans can invade the host through two distinct mechanisms: active penetration and induced endocytosis. For active penetration, the formation of true hyphae is required to invade host cells. However, the activation pathways and molecular factors involved in this mechanism are not yet completely understood. On the other hand, the mechanism of induced endocytosis involves the fungus secreting proteins on the cell surface that function as invasins. These invasins bind to different host ligands, such as E-cadherin in epithelial cells and N-cadherin in endothelial cells. The main proteins that are essential for invasion in this process are listed in Table 1.1 [36, 37].

Biofilm Formation

The degree of virulence of *Candida albicans* is directly related to its ability to form biofilms [38]. In addition, morphological change is a crucial virulence factor, allowing yeast cells to transform into hyphae, which are essential to form the complex networks known as biofilms. Therefore, biofilms are composed of different morphological forms [14].

These structures develop in three stages:

1. Individual fungal cells enter an adherent phase (on substrates), which can be either abiotic or biotic, such as stents, catheters, prostheses, mucosal cell surfaces, and tissues, thus forming the basal layer of the biofilm.
2. The second phase involves growth or proliferation, where morphogenic changes, such as the transition from yeasts to hyphae and pseudohyphae, are essential for forming dynamic communities. Filamentation initiates the creation of the biofilm.
3. In the third phase, the extracellular matrix is established, and the remaining cells disperse through it, forming the biofilm.

Thus, the biofilm comprises two main layers: the first is a basal layer with blastospores (single cells produced by budding) encapsulated by hyphae within a matrix. The second is a discontinuous monolayer formed by various *Candida* morphologies, predominantly the pathogenic forms (hyphae and pseudohyphae). Additionally, biofilms can be composed of different *Candida* species and strains.

The clinical importance of biofilms is attributed to several factors:

a. Microorganisms in biofilms are less sensitive to drugs and tend to be resistant to antibiotics, so higher concentrations of drugs are needed for their eradication [38].
b. The biofilm matrix increases the metabolic plasticity of cells, conferring protection and favoring the expulsion of antifungal drugs [39].
c. Medical devices colonized by these biofilms, such as intravascular catheters and prostheses, can exacerbate infections in patients with complex medical conditions [37, 1].
d. The extracellular matrix of these networks contains β-glucans, which hide the cells making them invisible to the host immune system [39].
e. *C. albicans* biofilms are resistant to death by neutrophils and do not trigger reactive oxygen species (ROS) production [37, 39].

1.3.2 Secretion of Proteases and Cytolitic Peptides

Protease secretion is a virulence attribute that contributes significantly to the pathogenicity of *Candida*. To achieve colonization and invasion of the host, fungal cells secrete hydrolytic enzymes such as proteases, lipases, phospholipases, and peptidases, playing a

key role in facilitating invasion [33]. Secreted aspartyl proteinases (SAPs) exhibit significant proteolytic activity, degrading human proteins at injury sites, including albumin, hemoglobin, keratin, and immunoglobulins. These enzymes are produced and secreted by *C. albicans* and other *Candida* species. Nine different SAP genes (SAP1-9) have been studied in *C. albicans*. These aspartic proteases function well in acidic and neutral pH (pH 3-5-7) and are involved in colonization, pathogen cell spreading, extracellular adhesion, and tissue penetration [33, 40, 20].

The main families of aspartic proteases include candidapepsins and cathepsins. Candidapepsins play multiple virulent roles in the host organism, such as contributing to adherence on biological surfaces, enhancing the penetration of pathogenic cells, and degrading protective barriers. They can also engulf proteins from the invaded subject and affect the patient's defenses to evade the immune system [2].

On the other hand, the secretion of cytolytic peptides facilitates the invasion and dissemination of the fungal infection. In its pathogenic hyphal form, *C. albicans* secretes cytolytic peptides, most notably candidalysin. This cytolytic toxin is produced by *C. albicans*, *C. tropicalis*, and *C. dubliniensis*, the main pathogenic species. Candidalysin is secreted as a mechanism for invasion into the human body [1, 30]. Candidalysin, the main toxin identified in human pathogens, is produced by the ECE1 gene, which is expressed in the presence of hyphae. This gene is responsible for secreting the Ece1p protein, associated with the release of candidalysin. This is related to pathological processes since hyphae release candidalysin, damaging host epithelial cells. The characteristics of this peptide include its amphipathic properties, with a secondary structure composed mainly of alpha helices. These features confer plasticity and adaptability to diverse environments [41]).

Secretion of this peptide is considered a virulence factor for the following reasons:

a. Candidalysin triggers the production of lactate dehydrogenase, which damages cells and disrupts the cell membrane.
b. It is critical in mucosal and systemic infections, driving host cell activation and neutrophil recruitment, as well as type 17 immunity [30].

c. Causes epithelial damage and calcium influx, activating intracellular signaling pathways and cytokine secretion.

d. Destabilizes the plasma membrane of epithelial cells.

e. Causes cellular stress leading to necrotic cell death.

f. Facilitates fungal translocation through gastrointestinal epithelial cells (Richardson et al., 2022).

g. Triggers epithelial immunity via MAPK signaling molecules that activate immune cell recruitment (Richardson et al., 2022).

Table 1.1: Summary of key elements in the regulation of *Candida* pathogenicity and adaptability processes.

Element	**Type**	**Related Process**	**Function**	**References**
Ace2 (Angiotensin-Converting Enzyme 2)	TF	Filamentation	Regulates *Candida* filamentation in response to low oxygen concentrations (hypoxia).	[42]
Ahr1p	Adhesin / TF	Adhesion / Filamentation	-Increase in extracellular pH. -Regulates *Candida* adherence.	[3])
Als3	Adhesin / Invasin / TF	Adhesion / Invasion / Biofilm Formation	-Multifunctional protein. -Iron acquisition. -Binds to host cell receptors (E-cadherin and N-cadherin). -Induces cells to endocytose.	[43, 37, 38]
Bcr1	TF	Biofilm Formation	-Biofilm regulator on abiotic surfaces. -It is one of nine transcription factors (Brg1, Efg1, Flo8, Gal4, Ndt80, Rob1, Rfx2 and Tec1) critical for biofilm development.	[44, 39]

Efg1	TF	Filamentation	-Key factor in the regulation of morphogenesis. -Essential for biofilm formation. -A member of the APSES group of proteins (regulate morphogenesis). -Required for filamentation (in vitro and in vivo).	[45, 3, 46, 1, 39, 47]
Epidermal Growth Factor (EGFR)	Transmembrane Protein	Filamentation	Induction of endocytosis of filamentous cells.	[37, 38]
Hsp90	Heat Shock Protein	Biofilm Formation	-Main heat shock protein. -Key factor in the biofilm dispersion process. -Has chaperone function (protein folding).	[37]
Hwp1	TF	Adhesion / Invasion / Biofilm Formation	-Regulates filamentous growth. -Hwp1, together with Als3, contribute to biofilm formation by acting as complementary adhesins. -It is involved in adhesion to host cells. -Regulates the expression of genes associated with filamentation.	[37, 38]
Nrg1	TF	Filamentation	-Acts as a repressor in filamentation. -Mutants (deletion or alteration) of Nrg1 (and Tup1) exhibit filamentous growth. -Represses or activates genes associated with hyphal formation.	[1, 47]

Rim101	TF	Filamentation	-Regulates and promotes filamentation by alkaline pH. -Controls cell wall protein gene expression through alkalinity.	[48]
Ssa1	TF	Invasion / Filamentation	-It is a member of the heat shock protein 70 (Hsp70) family. -Als3 and Ssa1 interact with Egfr and Her2 to induce endocytosis of *Candida* hyphae.	[37, 38]
Tec1	TF	Filamentation	Transcription factor in yeast-to-hyphal transition and filamentous growth.	[44, 1]
Ume6	TF	Filamentation	Controls morphogenesis and filamentous growth.	[1]
Tup1	TF	Filamentation	Inhibits the induction of filamentous growth mediated by farnesol depletion.	[1]

1.3.3 Metabolic Plasticity

The *C. albicans* species exists as a commensal organism within the gastrointestinal regions, forming part of the microbiome. This nutrient-rich environment guarantees the survival of the fungus, but it is an environment of competition with other microorganisms that coexist in the microbial flora. Consequently, *Candida* must have skills to be able to compete for the available nutrients in this niche [37, 1]. The metabolic plasticity of this species is evident in its ability to assimilate nutrients from the environment even under adverse conditions. During infection, pathogenic fungi must rapidly adapt to host-induced changes, including responses to their virulence mechanisms, which may involve a decrease in nutrient availability. Their ability to overcome this dynamic niche corresponds to the success of their metabolic plasticity.

During an infection, *C. albicans* feed primarily on glucose, lipids, proteins, and amino acids, depending on availability and the colonized site [37]. Although glycogen is the main source of fungal nutrition, *Candida* often faces glucose shortages and needs to use

alternative metabolic pathways to access other nutrients.

The growth and colonization of fungi are limited by the nutrient availability in the anatomical niche where the fungus establishes itself, and it is here that its metabolic plasticity plays a crucial role. This limitation triggers a series of metabolic processes, such as glycolysis, glycogenesis, and, in response to starvation, the glyoxylate cycle, which promotes pathogenic processes. Alternative pathways include the utilization of carbon sources, lipids, lactate, amino acids and GlcNAc. GlcNAc, an amino sugar derived from glucose, stands out for being a component of fungal cell walls with a significant ability to induce filamentation in *C. albicans* [36]. Furthermore, *C. albicans* is capable of inducing supply processes in response to particular nutrient deficiencies. For example, in the face of zinc shortage, it generates C. albicans 'Goliath' cells to uptake this element. This is a giant phenotype of C. albicans that enhances the secretion of ferritin and transferrin to overcome this limitation and establish infection [36].

1.3.4 Morphological Switching and Filamentation

1.3.4.1 Morphological Switching

Morphological switching is a pathogenic process in which *Candida*, especially *C. albicans*, has the ability to transform from its commensal yeast form to its pathogenic hyphal form. This reversible transition, known as 'dimorphism', is crucial in establishing infection. At this point, it is important to distinguish between hyphae and 'germ tubes.' Germ tubes refer to elongated structures that appear during the transition from yeast to hyphal form, marking the initial phase of true hyphae development [49].

Candida morphogenesis allows it to invade tissues and mucous membranes, causing deep damage. This shape change confers the ability to defend itself against phagocytosis by macrophages and escape the host immune system by forming complex structures [5, 38]. For this process to occur there are different signaling pathways involved in this morphological change, and there is a close relationship between anatomical niches, morphotypes, and biochemical changes undergone by these cells [3, 27].

Another important aspect is the phenotypic transition of *Candida*. Although this process also involves other *Candida* morphotypes, it is more associated with their reproductive

capacity and involves changes in gene expression associated with different transcriptional factors. This phenotypic diversity enables adaptation to extreme conditions and survival in various niches. The transition occurs between white (sterile) and opaque (reproductively competent) cells, with differences in growth and virulence capabilities. These morphological and phenotypic changes are controlled and regulated transcriptionally and by different signaling pathways [39].

The morphological switch of *C. albicans* can be subject to external and internal stimuli. There are several signaling pathways, transcription factors (TFs), and environmental stimuli that control this transition, among the most studied of these:

a. Temperature; generally, an elevated temperature change around 37°C.
b. Serum exposure/incubation.
c. N-acetylglucosamine (GlcNAc): a monomer of chitin polymers present in fungal cell walls.
d. Low concentrations of nitrogen (N), oxygen (O_2), carbon (C); in the case of oxygen it is hypoxia.
e. High concentrations of carbon dioxide (CO_2); hypercapnia.
f. Peptidoglycan: a carbohydrate polymer found in bacterial cell walls that facilitates the formation of germ tubes and hyphae.
g. Amino acids
h. Alkaline or neutral pH; changes in pH activate MAP kinases that induce morphological transition.
i. Rim 101; transcription factor involved in transduction of pH changes.
j. Starvation or adequate nutrition; inhibits or promotes morphological transition.
k. Oxidative stress
l. Osmotic stress
m. Genetic pathways
n. Insertion matrix; substance that surrounds and supports the cells.
o. Environmental stimuli
p. Fungal signaling pathway, example: cAMP-PKA.

The integration of all these factors and conditions from external and internal signals and

stimuli can act independently or together to induce the morphological transition process and increase the virulence potential of *Candida* [5, 1, 50].

1.3.4.2 Filamentation Process

Filamentation, the process by which *Candida* yeasts transform into hyphae, significantly enhances their pathogenic capabilities and intensifies infection. This process begins after cell attachment to the host. Interestingly, filamentation is an anisotropic process, i.e. expansion of cell volume occurs along a specific axis, not randomly. The Golgi complex plays a crucial role in distributing necessary components to the hyphal end. Septins, plasma membrane proteins, form a double ring-like structure that transforms into the shape of an hourglass, followed by bud development and redistribution of septins during mitosis [3, 51].

Filamentation is essential for the survival of the fungus and thus for the success of an infection. The formation of hyphae ensures better cell adhesion and invasion, as well as nutrient acquisition and mobility through the biome. Virulent characteristics that benefit the fungus through hyphal formation include:

- The elongation and growth of the hyphae allow perforation of the macrophage membrane, as a defense mechanism against the cells of the immune system of the invaded host.
- Filamentation maintains the morphological plasticity of the fungus during infection and makes it adaptable to various factors.
- It allows the formation of biofilms, facilitates tissue invasion and phagocyte escape.
- It induces trigmotropism involving contact growth in different media and contributes to its pathogenicity.
- Contributes to endocytic uptake by damaging patients' cells and penetrating their tissues and epithelial cells.

The main actors in this process are transcription factors (TFs). TFs are proteins that regulate the expression of genes involved in filamentation and other pathogenic mechanisms. These TFs are activated through various signaling pathways [46]. Although the exact mechanisms that facilitate filamentation are not fully understood, several factors

are involved and control each other. TFs help *Candida* coordinate various virulent functions, such as morphological change, biofilm formation, and antibiotic resistance. Table 1.1 identifies several transcription factors and the function of each [52].

1.3.4.2.1 *Candida* Filamentation in Different Media Conditions After describing the pathogenicity processes of *Candida*, including its filamentation, it is crucial to note that several in vitro conditions induce morphological change. As mentioned in the 'metabolic plasticity' section, nutrients and other conditions are essential for *Candida* survival and transformation. Investigating the process of *Candida* filamentation in different culture media, both traditional and innovative, allows us to develop new strategies for its control and treatment.

Filamentation in Standard Culture Media

YNB + GlcNAc

This medium comprises two main elements: yeast nitrogen base (YNB) and N-acetylglucosamine (GlcNAc). YNB is used as a minimal medium, providing basic nutrients for cell growth. On the other hand, GlcNAc, an amino sugar derived from glucosamine, is used as a carbon source and promotes the biosynthesis of chitin, which is essential for fungal cells. This is a component of the fungal cell wall, and has been shown to chemically induce filamentation in vitro and to modify gene expression in various cells [53, 54, 55].

Through proteomic analysis in the plasma membrane of *Candida* albicans, the Ngt1 protein, the first eukaryotic GlcNAc transporter identified, was found to mediate the uptake of this amino sugar, facilitating the transformation of the yeast form to hyphae [56, 54]. *C. albicans* yeast cells can be cultured in YNB medium supplemented with GlcNAc as a carbon source that will induce morphogenesis and growth of pathogenic hyphae [56, 40].

YPD + serum

This rich medium consists of yeast extract-peptone-dextrose (YPD) which can be supplemented with serum. Serum types, such as fetal bovine serum (FBS) or human serum, are effective inducers of hyphal growth in vitro studies [53]. This medium facilitates the growth of *S. cerevisiae* and certain *Candida* species. Furthermore, adding serum and incubating at 37°C promotes *C. albicans* filamentation. Additionally, these media are suitable for germ tube production and differentiation between *Candida* species [57].

Lee's medium

Developed by Lee et al. (1975), this synthetic medium is chemically composed of six amino acids, biotin, inorganic salts, and glucose. It was developed for the growth of yeast cells and filamentous cells of *C. albicans*. This medium with synthetic amino acids and the absence of peptones promotes the growth of dimorphic pathogens by controlling factors such as pH and temperature. Although its use has declined, it is still relevant for pre-culture studies [34, 58, 57].

RPMI medium

This medium refers to the "Roswell Park Memorial Institute" medium (RPMI). There are several types of RPMI medium, its main components include: amino acids, salts, vitamins (B12, biotin, inositol, and choline), L-glutamine, and an interesting sodium bicarbonate buffer system that confers extra nutritional capabilities. As this medium does not contain proteins, lipids, or growth factors, it usually requires additional supplementation, the most common is to supplement with a percentage of fetal bovine serum. Among the formulations of these media, we have RPMI 1640 which is used for the culture of human lymphoid cell lines and malignant leukocytes [59, 60, 61]. Among the main ingredients of RPMI 1640 we have insulin, oxaloacetic acid, sodium pyruvate, and vitamin B12 in high concentrations [62]. The characteristics of this medium make it an interesting choice for studying the in vitro process of fungal cell filamentation.

Spider medium

Spider medium includes mannitol, peptone, and dextrose. It is mainly used to study filamentation and biofilm growth in *Candida* species. The presence of nutrients such as peptides and the control of factors such as temperature (37°C) favor hyphal formation. This medium is particularly useful for non-albicans species that cause filamentation, such as *C. parapsilosis* and *C. tropicalis* [54, 63]. The elements of Spider medium make it ideal for the investigation of filamentation and other virulent processes of *Candida* species in the laboratory [34, 64].

Filamentation in Tomato Juice Medium

Tomato juice, which functions as an enriched culture medium, contains nutrients such as beta-carotene, niacin, calcium, lycopene, hydroxycinnamic acid derivatives, flavonoids, and vitamins (A, C, and E) [65]. This medium can replace human serum in germ tube testing, offering a safe and effective alternative. Human serum can cause false negative results due to biological inhibitors present in serum and carries the risk of transmission of infections. Therefore, tomato juice, being a rich source of nutrients, has been shown to be effective for fungal cell growth and filamentation and can be used as a suitable substitute.

Shiyamalee et al. (2020) conducted a study using tomato juice ('Thilina' variety) to investigate germ tube formation and filamentation of *C. albicans* to facilitate differentiation between these species. In their comparative assays, they used human serum and tomato juice, incubating 66 *Candida* isolates in test tubes at 37°C to induce filamentation. The results demonstrate the potential of tomato juice as a new source for the study of *C. albicans* filamentation [65].

This coincides with the research of Ghosh, (2009) who demonstrated that fungi are more virulent than bacteria in decomposition processes of fruits such as tomato [66, 67]. Tomato juice being a mineral-enriched medium has encouraged experimentation with other vegetable juices, such as the 'V8 Original' juice marketed by Campbell Soup Company. This mixture of eight vegetables (celery, watercress, spinach, lettuce, parsley, tomato, beet, and carrot) has been used in mycology studies, as it is believed to contain factors that enhance the growth and allow the identification of *Candida* species [68].

Black, (2020) used a standard formulation of V8 medium with agar to compare various media types and techniques in the study of fungal transmission, highlighting some difficulties associated with solid medium. Alves, et al. (2006), also used V8 medium to analyze its potential in differentiating between *Candida* dubliniensis and *Candida* albicans, based on chlamydospore production. In addition, Kent, et al. (2008), studied the ability of V8 medium to induce sporulation and sexual development of *Cryptococcus neoformans* and some *C. albicans* species [69, 68, 70].

Factors favoring hyphal formation in plant juice-based culture media include pH, nutrients, and auxotrophic factors. Vegetable juices are usually acidic, with a pH between approximately 4.5 and 5. However, low pH typically inhibits filamentation, while alkaline pH promotes filamentation through the Rim101 pathway. The nutrient content of the media can also promote microbial growth of bacteria and fungi [66]. To conclude this section, tomato juice can be used as a culture medium, both liquid and solid with agar, for research in the area of mycology. Its ease of obtaining ingredients, its preparation, its low cost, and its organic nature make it a viable option that avoids false positives induces the differentiation of yeasts and hyphae, and potentiates this transformation process into *Candida* species.

Chapter 2

Problem Statement and Hypothesis

2.1 Problem Statement

The present study focuses on a critical research problem in microbiology: the analysis of the factors that stimulate filamentation of *Candida*, a single-celled pathogenic fungus causing severe infections. Despite advances in the understanding of its pathogenicity, there is a gap in the understanding of the mechanisms behind its morphological transition to filamentous forms, a key factor in its virulence. This research seeks to deepen the ability of the components/ingredients of an innovative culture medium inspired by the commercial vegetable juice 'V8' to induce *Candida* filamentation. The study also focuses on analyzing the impact of specific variables such as pH, temperature, and nutrient concentration on this process. The central hypothesis suggests that the ingredients of vegetable juice may simulate environmental conditions that induce the morphological 'switch' in *Candida*, which could indicate a possible plant origin in its evolution. It is hoped that the results of this research will provide a clearer insight into the regulation of filamentation, opening avenues towards the development of more stable and economical culture media, and offering an innovative approach to study the mechanisms of pathogenicity in *Candida* and the management of fungal infections.

2.2 Hypothesis

The hypothesis of this research focuses on the relationship between the ingredients of the vegetable juice-based culture medium (celery, watercress, spinach, lettuce, parsley, beet, tomato, and carrot) and the induction of filamentation in *Candida*. It is proposed that the combination of these ingredients, together with variations in pH, temperature, and nutrient concentration, may enhance the morphological transition of *Candida* from yeast to hyphae. It is suggested that some vegetables may have a more pronounced effect on this process due to their unique nutritional properties and a possible symbiotic relationship with *Candida*. Furthermore, it is presented that the molecular regulation of this process is mediated by specific transcription factors and metabolic pathways, opening the possibility for future investigations through gene deletion techniques and mutant analysis. This hypothesis implies that the components of the culture medium have a direct effect on *Candida* filamentation and suggests the possibility of symbiotic evolution with plants that could have facilitated the transition of *Candida* to human hosts.

Chapter 3

Justification and Objectives

3.1 Justification

Due to the global prevalence of *Candida* infections, particularly in medical settings, research on the virulence of this fungus is needed. Candidiasis ranks among the most common infections worldwide, and its treatment and diagnosis face multiple challenges. As noted by Mba & Nweze (2020), *Candida* pathogenesis involves a complex network of factors, including metabolic plasticity, filamentation, and biofilm formation, underscoring the need to investigate these mechanisms [22, 1]. Furthermore, according to Shiyamalee et al. (2020), there is a demand for inexpensive alternatives to commercial media for the identification of *C. albicans*, thus this research addresses the need to develop an economically viable culture medium [65]. The use of V8 vegetable juice-based media could offer an affordable and safe solution, overcoming the limitations of traditional media such as human serum, which carries risks of disease transmission and false negative results. The proposed research seeks to fill a gap in the understanding of *Candida* pathogenesis, offering new insights by investigating the impact of vegetables on filamentation. This work not only contributes to the field of microbiology but also presents an opportunity to develop safer and more affordable detection and cultivation methods.

3.2 General and Specific Objectives

3.2.1 General Objectives

The general objective of this research involves understanding the mechanisms and factors that induce filamentation in *Candida*, in order to determine how this pathogenic process influences the spread of infections and diseases. This involves a detailed study of the components of a culture medium based on V8 vegetable juice. In this way, by analyzing the individual ingredients as well as environmental conditions (pH, temperature, and nutrients) we seek to determine the influence on the morphological transition of *Candida* and its pathogenic behavior. In addition, this could lead to the development of innovative, safe, accessible, and inexpensive culture media.

3.2.2 Specific Objectives

- To analyze the influence and effect of eight vegetables on the morphogenesis process of *C. albicans* through the implementation of growth and filamentation assays, using optical microscopy techniques to document morphological changes under various temperature conditions.
- To assess and modulate the impact of pH, temperature, and stress on the pathogenic behavior of *C. albicans*, using precise temperature control and pH measurement techniques. This approach will help to evaluate both specific and generalized pathogenic responses under various environmental conditions through the implementation of both rapid and gradual temperature shifts from 30°C to 37°C, coupled with incubation periods of 2 hours or more.
- To establish an experimental foundation for future studies, including the use of gene deletion techniques and transcription factor mutant analysis, to better understand the molecular mechanisms that regulate the interaction between *C. albicans* and vegetable components.

Chapter 4

Methodology

4.1 *Candida* Strain Selection, Culture and Maintenance

In this study, we used strains of *C. albicans* (PMRCA18) [71], *C. tropicalis* (clinical isolate) and *C. glabrata* (clinical isolate) preserved at -80 °C in a glycerol solution (15% v/v) in the laboratories of Yachay Tech University, Ibarra, Ecuador. For culture, strains were transferred from frozen stock to YPD agar plates (For 1 liter, 20 g agar, 20 g bacto peptone, 20 g dextrose, and 10 g yeast extract), using sterile swabs. Plates were incubated at 30 °C for 2-3 days to promote fungal cell growth, which allowed their use in subsequent in vitro experiments. To ensure the viability of the cultures and to avoid contamination of the plates, fungal cells were subcultured onto new YPD agar petri plates monthly, using aseptic techniques.

Overnight cultures were incubated on a V8 control medium and individual vegetable-based media at 150 rpm and 30 °C. To minimize the risk of contamination by other fungi or bacteria, 200 µg/ml of ampicillin and 50 µg/ml of chloramphenicol were added to the media, as will be further detailed in section 4.3. Microscopic examinations were regularly performed as part of the experimental protocols to confirm the absence of contaminants. Culture conditions were regularly monitored to ensure the consistency and quality of the experiments. In addition, it is important to note that the *C. albicans* wild type was used for all filamentation assays in this research work and the *C. tropicalis* and *C. glabrata* strains were reserved only for the final assays with non-albicans species.

4.2 Media Preparation

4.2.1 Preparation of Standard Media

V8 Original + Supplements (Liquid)

After analyzing various enriched media and inspired by the work of Shiyamalee et al. (2020), [65] a positive control liquid medium was developed based on Campbell Soup Company's commercial "V8 Original" juice, which includes a mixture of eight vegetables (celery, watercress, spinach, lettuce, parsley, tomato, beet, and carrot). To 5 mL of V8 juice, we added 0.1 g of yeast extract, 0.1 g of dextrose, and 0.05 g of sodium carbonate ($NaCO_3$), making up to 40 mL with H_2O. The pH was adjusted with 3.2 mL of 0.2 M HCl to achieve a pH of 5.7. The medium was subjected to a sterilization process, as detailed in section 4.3.

V8 Original Agar

A mixture of 200 mL of V8 juice, 0.5 g of $CaCO_3$, 5 g of dextrose, 1 g of yeast extract, and 20 g of agar was distributed in Petri dishes. This medium was autoclaved (120 °C/60 min).

V8 Homemade

Not knowing the precise recipe for Campbell Soup Company's commercial V8 juice, a homemade mixture equivalent to the commercial V8 juice was prepared for this positive medium by mixing equal proportions of each homemade vegetable juice. Details of the preparation of each individual juice are described in section 4.2.2.

4.2.2 Preparation of Vegetable Juice Media

Individual culture media were prepared for each of the eight vegetables. The vegetables were purchased fresh in 2023 from a local supermarket (Supermaxi, Ibarra, Ecuador). Samples of the 8 vegetables were collected with gloves and placed in individual clean bags for aseptic transport. A total of 8 vegetables (celery, watercress, spinach, lettuce, parsley, tomato, beet and carrot) were analyzed. 50 g of each vegetable, after being washed with water and alcohol (and peeled in the case of beet and carrot), was mixed with 50 mL of H_2O, and cooked to boiling (about 5 min). Subsequently, another 50 mL of water was added, and each mixture was blended, strained, and stored in sterile recipients for each

vegetable. Each juice (50 mL) was stored in Falcon tubes (8 flasks in total) for subsequent sterilization, detailed in section 4.3.

4.3 Sterilization, Filtration, and Antibiotic Use

All media, both liquid and solid, were autoclaved at 120 °C for 60 min. Subsequently, liquid media were centrifuged at 4700 rpm for 10 min and filtered with a 0.40 μm filter. For dense vegetable media, (especially carrot, tomato, and spinach) an additional centrifugation was performed in 1 mL microfuge tubes retaining the liquid part and discarding the pellet. Antibiotics (ampicillin and chloramphenicol) were added to each medium at twice the standard concentration to prevent contamination. Sterilization was performed in the order described in this section.

We established sterilization and antibiotic use procedures, to ensure the purity of the media and to verify the absence of contamination of other fungi or bacteria in the samples. These observations will be described in more detail in section 5.1.2.

4.4 Assay Conditions for Candida Inoculation in Vegetable Juice-Based Media

4.4.1 Cell Concentration for Inoculation

C. albicans strains for inoculation were preserved on YPD plates as described in section 4.1. Inoculation was carried out using sterile cotton swabs, and the optical density of the medium was measured at 600 nm (OD600). Overnight cultures in V8 medium were incubated using a MaxQ™ 4450 Benchtop Orbital Shaker (SHKE4450, Thermo Scientific) at 30°C and 150 rpm. For filamentation assays, 34 µl of the overnight culture was taken to perform each inoculation, maintaining consistency and ensuring a constant cell concentration during all experiments. Using a calibration table specific for *C. albicans* that correlates OD600 with the number of viable cells, the 34 μL was estimated to represent approximately 6.8×10^4 cells (CFU/ml). This calculation is based on the relationship established in the calibration curve between the measured OD600 and the colony-forming unit count (CFU/ml), thus ensuring accuracy in the estimation of cell concentration.

4.4.2 Time and Temperature Protocols

The general description of the protocol is detailed as follows:

1. **Preparation of culture media;**

   a. Section 4.2 details instructions for the preparation of both control media and individual vegetable media. It is important at this step to ensure the homogeneity and sterility of each medium.

2. **Setup of the Culture Media Assays;**

   a. Two test tubes were used for each culture medium. Each test tube was labeled with the name of the medium and the temperature at which it would be incubated (30°C or 37°C).

   b. During the pre-culture phase, all tubes were incubated at 30°C, except those intended for heat-fast transition experiments, which were marked at 37°C and should be kept at that constant temperature during all phases.

   c. Each set of experiments would include a test tube for the positive control medium where the pre-culture was done and another tube with the same medium as a negative control, i.e., there would be no inoculation and it was intended to evaluate purity and absence of contamination.

   In this section, we clarify that the tubes were prepared a day before inoculation and incubated without inoculum to achieve the desired temperature, thereby eliminating the wait for temperature adjustment and ensuring they were contamination-free.

3. **Pre-culture of *Candida*;**

   a. The wild type *C. albicans* strain was used to inoculate the test tube containing the positive control, using an aseptic technique.

   b. This is done with the help of a sterile swab from the frozen stock of the standard *Candida* strain. For all filamentation assays and parameter variation assays, the *C. albicans* strain (PMRCA18) was used, and the other strains were reserved only for assays with non-albicans species.

4. **Cell Growth;**

   a. All tubes were placed in a shaker at 150 rpm at 30°C overnight, ensuring optimal conditions for *Candida* growth,

   b. All tubes should be checked for the absence of contamination during the culture process. Only the tube containing the positive control medium should show signs of cell growths.

5. **Post-Cultivation Screening;**

   a. After the overnight incubation of the wild-type cells (or positive control), the content of the test tubes was transferred to microfuge tubes and centrifuged at 4500 rpm for 2 minutes to sediment the cells.

   b. The pellet was carefully washed with 1 ml of distilled water and centrifuged again under the same conditions.

6. **Inoculation and Filamentation Observation;**

   a. The pellet was resuspended with 1 ml of distilled water and 34 µl were taken for inoculation of the different culture media.

   b. Details of the cell concentration are given in section 4.4.1.

   c. The inoculated media was incubated at the preset temperatures (30°C or 37°C) for 2 hours and 30 minutes, which is crucial to observe morphological changes in *Candida*.

7. **Microscopic Examination;**

   a. After incubation, samples were transferred from each culture to labeled microfuge tubes, corresponding to the medium and incubation temperature.

   b. They were centrifuged at 4500 rpm for 2 minutes and pellet samples were prepared for microscopic observation.

   c. Approximately 5 µl of each sample was placed on a slide, covered with a coverslip, and examined under an optical microscope at 40X magnification.

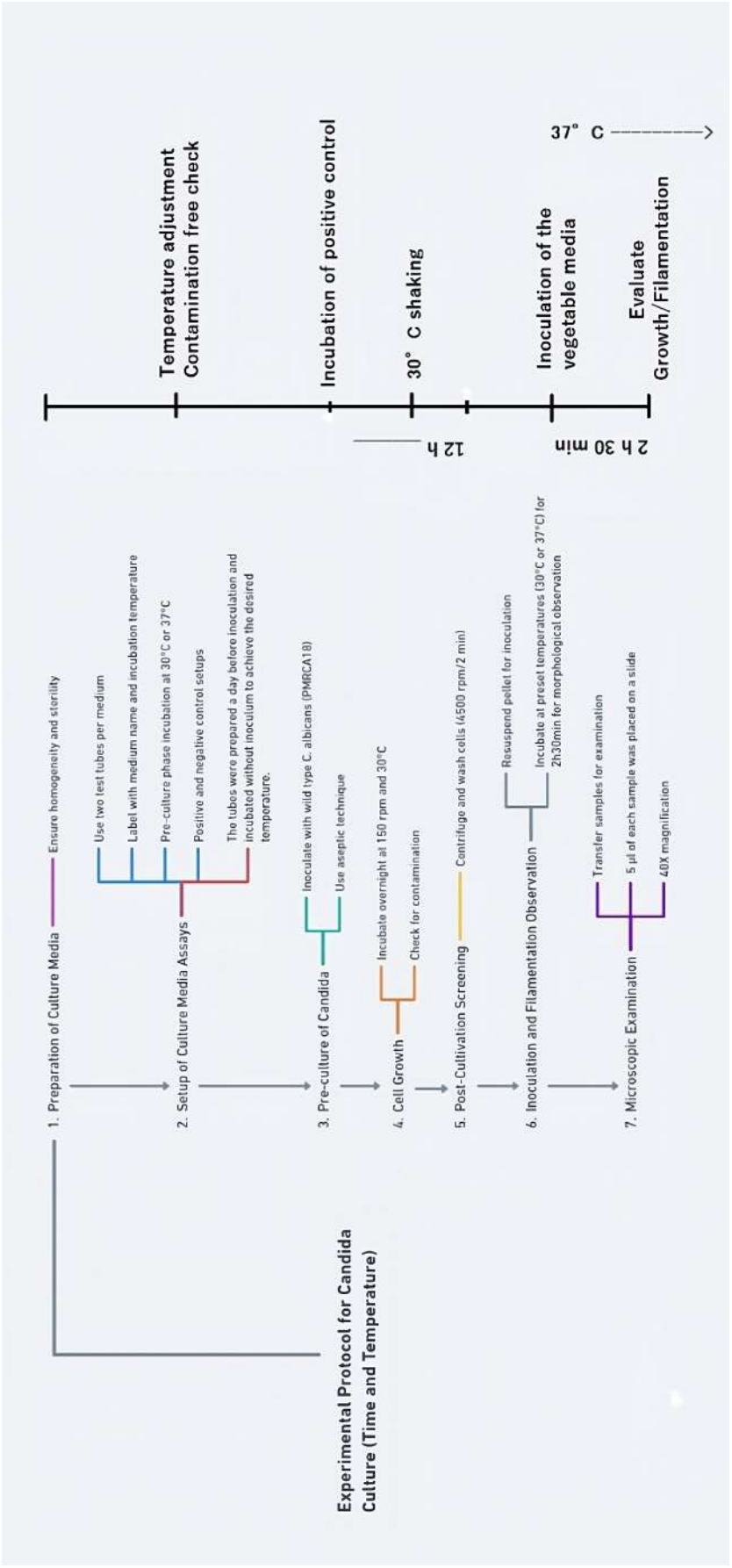


Figure 4.1: Experimental protocol summary: culturing and growing *Candida* in control and vegetable-based media

4.5 Data Registration

Light microscopy was used to capture detailed images, facilitating a comparative analysis between media. All observations and measurements were meticulously recorded. Microscopic images functioned as visual evidence of the observed morphological changes. The tests were performed by duplicate microscopic readings, observing the cells in the same medium at 30°C and 37°C to evaluate their transformation with 40X objectives. In this way, it was possible to easily sort yeast cells at 30°C and hyphae at 37°C, in almost all culture media.

Chapter 5

Results and Discussion

5.1 Evaluation of Growth Media Conditions for Filamentation

5.1.1 Preliminary Tests: Protocol Efficacy and Observations

The methodology of this research work adopts a qualitative approach to evaluate the morphological changes of *Candida* in different culture media based on vegetable juices. Using microscopy, the transformation of yeast cells to filamentous structures in these culture media was evaluated, allowing us to see the differences in their potential to induce filamentation. Evaluation criteria included cell shape, presence of true hyphae, mycelial density, presence of media contamination, and clarity of visualization on each. These observations were recorded using microscopic images and detailed descriptions of experimental variables, such as incubation time, temperature, pH, and media purity, to ensure consistency and reliability of the results and an accurate assessment of the impact of vegetable media on *Candida* morphogenesis.

Culture Media Comparison

It was observed that all of the vegetable juice-based media induced variations in *Candida* morphology, although some media performed better than others. We believe that these differences are due to the nutrient composition of each medium.

- **Control Media:** We used [V8 Original + Supplements] liquid medium to highlight its presumptive potential in the filamentation process of *C. albicans*. In addition,

we employed a V8 Homemade culture medium containing equal proportions of the 8 vegetable juices. It was observed that both media were capable of inducing a filamentation process in *C. albicans* when performing a switch to 37°C. The liquid medium [V8 Original + Supplements] was initially tested for its potential to induce germ tubes, which mark the onset of hyphal formation. This medium provided good results during filamentation assays. However, the [V8 Homemade] medium could be considered to have superior characteristics to [V8 Original + Supplements], as it does not contain supplements and forms hyphae of greater length and stronger mycelial density. In addition, the supplements in the Original V8 medium make it susceptible to contamination, compared to the V8 Homemade. This will be further detailed in section 5.1.2 contamination testing.

Filamentation Observation

Detailed evaluations of *Candida* growth on the different media were carried out. Significant differences in cell morphology were noted between the vegetable juice-based media and the positive control medium V8. These observations were directly related to the specific culture conditions, such as the temperatures and incubation times established in the time-temperature protocols (section 4.4.2), and the components of each medium, including its nutrients.

Morphological changes, especially the transition from the yeast form to filamentous structures, were documented under different culture conditions. These differences can be better visualized in section 5.3. The images corresponding to the different media show variations such as the shape of the yeast cells, some apparently budding, the length of the hyphae, their formation into filamentous structures, or their dispersion. In addition, in some denser media, sedimentation was observed causing hyphae to form compact circles or hindering the clear view of the cells, among other characteristics.

These results suggest that these media are reliable as an alternative tool to study the morphological change of *C. albicans*. These preliminary observations provide valuable information for adjusting and optimizing experimental protocols for subsequent phases of the research, thus ensuring the validity and reliability of the results obtained.

5.1.2 Media Contamination Testing

For our preliminary assays, we did not have a specific sterilization protocol. Therefore, we opted to use the control media and supplements without autoclaving them, which caused high bacterial contamination, as seen in Figure 5.1. In addition, we experimented with raw tomato and beet media, i.e., without autoclaving or cooking the vegetables, just washing and blending them with water. In these initial media, we only added antibiotics and performed centrifugation to remove possible sediments. The differences were significant: the media were completely contaminated by bacteria and other types of fungi, as can be seen in Figure 5.2.

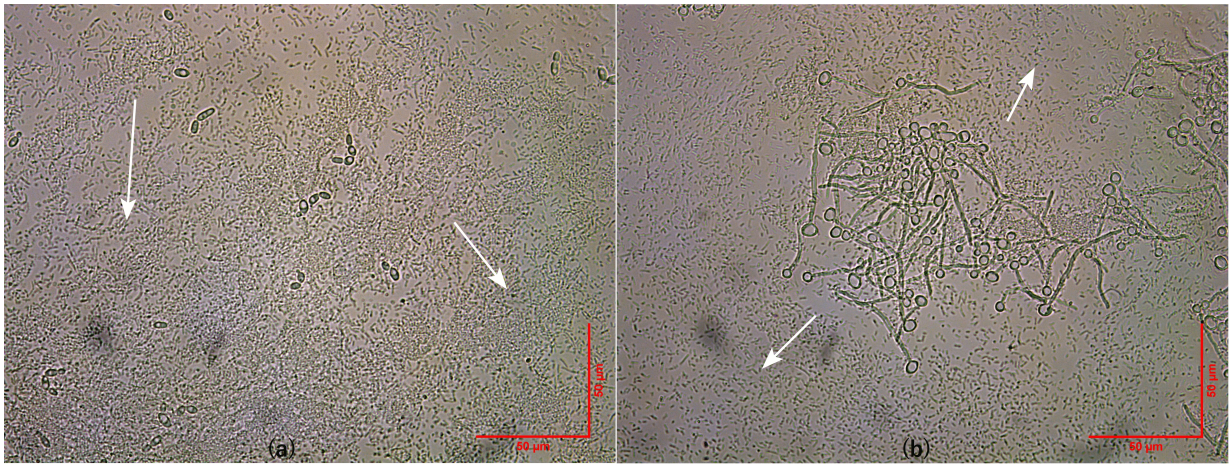


Figure 5.1: Contamination was observed in the [V8 Original + Supplements] culture medium, which had not been subjected to autoclaving or filtering; only antibiotics were added. Despite the presence of contamination, a 'morphological change' in *Candida* was noted. However, due to the potential interference of bacteria with the filamentation process, this medium was ultimately discarded. Figure (a) illustrates the bacteria observed in the culture medium incubated at 30°C, while figure (b) depicts the bacterial contamination surrounding the hyphae formed at 37°C.

For these reasons, it was essential to establish a sterilization protocol (section 4.3) to clarify these vegetable juice-based media and perform the filamentation assays. One of the techniques that presented the most inconvenient was the filtration of the juices. We attempted to sterilize the media with a 0.20 μm pore diameter filter, but due to the density of the media, we opted for a 0.40 μm one. Still, filtration proved almost impossible due to media thickness. Therefore, a double centrifugation process was necessary to remove precipitates that could contaminate the media. In separate experiments, we also performed

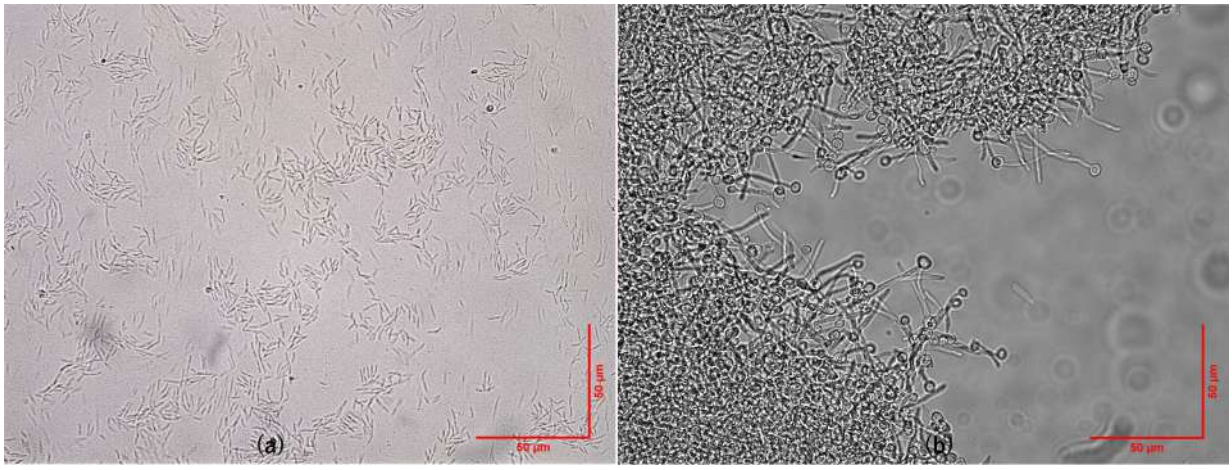


Figure 5.2: Two culture media based on beet juice: (a) The raw beet juice (not autoclaved or filtered), incubated at 37°C, showed the presence of bacteria, with an apparent absence of *Candida* cells. This outcome may be attributed to the contaminant presence that inhibits *Candida* growth. (b) Once autoclaved, filtered, and with added antibiotics, it facilitated a morphological switch to hyphae when incubated at 37°C.

autoclaving of the vegetable juices in sterile 50 ml plastic tubes with a slightly loose lid. The sterile tubes where the media were stored were only opened inside a laminar flow cabinet that had been previously disinfected under aseptic conditions. We observed that tomato, beet, and lettuce juices changed significantly in color upon autoclaving from a more vivid color to an opaquer, darker color, although this did not perceptibly affect colony morphology.

Moreover, it is important to mention that culture media based on vegetable juices must undergo a sterilization process that includes: autoclaving of the media, filtration, double centrifugation, and addition of antibiotic. However, these media should be kept refrigerated (2 - 8°C) and stored in sterile containers with lids, opening only inside a laminar flow cabinet. In any case, the media should not be kept for more than one and a half months, as they may become contaminated. It is advisable to discard them and prepare new ones under the most aseptic conditions possible. We believe that microbial contamination of vegetables may be due to poor handling during storage, transport, or preparation of the media. However, it is not easy to rule out environmental factors, such as opportunistic contaminations or cross-contamination by microorganisms (airborne fungi) on other products in transit.

5.2 Analysis of Filamentation Parameters

Several authors have already described the influence of different factors in the induction or inhibition of the *Candida* filamentation process. These factors not only depend on the nutrient system present in the culture media but are also related to pH, temperature changes, heat shock proteins, enzymes, and other stresses. This section discusses how modulation of temperature and pH factors may influence the ability of *Candida* to respond effectively, which in turn could affect their survival and pathogenicity.

5.2.1 Temperature Effects on Filamentation

It has already been demonstrated that temperature is a determining factor in the morphology of *Candida*. Therefore, in this research, we tested this factor. According to the existing literature, at low temperatures (24-30°C), *C. albicans* tend to grow mainly as blastopores (yeast form). However, by increasing the temperature to values equal to or higher than 37°C, a noticeable shift towards hyphal formation is observed.

An interesting finding arose when *C. albicans* cells were maintained at a temperature of 30°C. Under these conditions, and with an incubation time greater than two and a half hours, a tendency of *Candida albicans* to filament on spinach, lettuce, beet, and carrot media was evident (see Figure 5.3). This behavior suggests that, even at not extremely high temperatures, the ability of *Candida* to change its morphology is present and could be relevant to its pathogenicity.

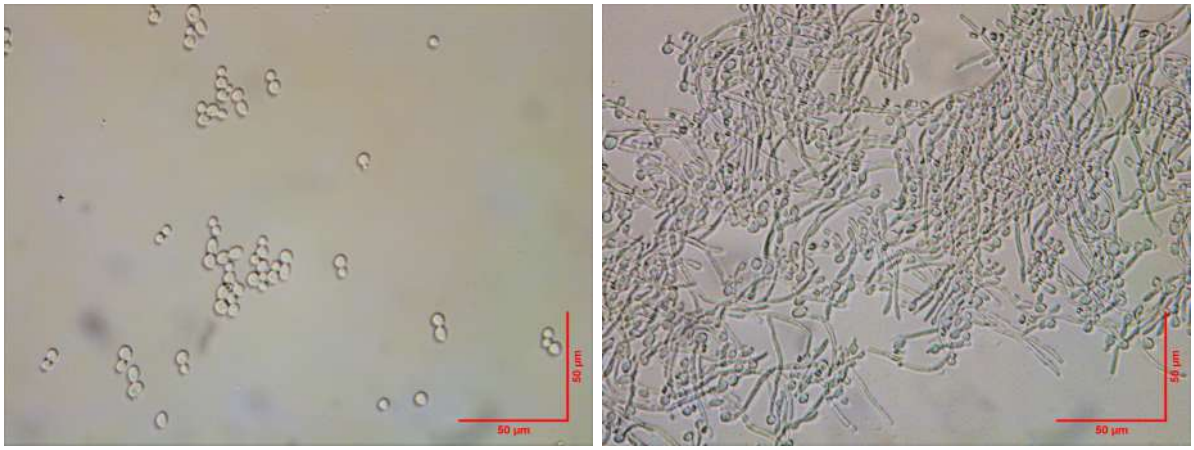
Figure 5.3: *C. albicans* cells exhibit filamentation at 30°C in the media of (a) spinach, (b) lettuce, (c) beet, and (d) carrot when incubated for more than two and a half hours.

These results underline the importance of temperature as a critical environmental factor in their morphology. Being enriched media, nutrients may play a key role in the induction of filamentation we can also consider that they are the media with the highest pH, as evidenced in Table 5.1. It is possible that these factors are more relevant in these media even above the temperature factor. This knowledge is vital to understand how *Candida albicans* can adapt and potentially cause infections in different environmental conditions.

5.2.2 Slow vs. Fast Temperature Transitions in Control Medium

The idea in this experiment was to subject *C. albicans* cells to a sudden exposure to high temperatures (see Figure 5.4b). In parallel, these cells were compared with other cells exposed to a slow temperature transition (see Figure 5.5b). This approach provided information on how this fungus responds to different heat stress conditions. In particular, we analyzed how *C. albicans* responds to an abrupt change in temperature, which is closely related to its ability to adapt to hostile environments.

Slow Transition



(a) *C. albicans* cells exposed to growth at a temperature of 30°C.

(b) *C. albicans* cells exposed to a slow transition from 30°C to 37°C.

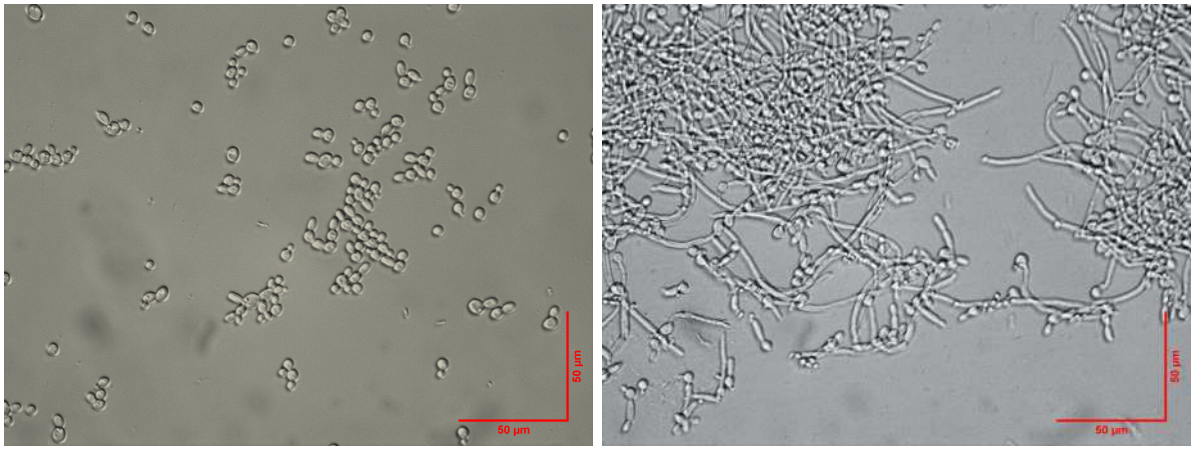
Figure 5.4: Control medium [V8 Original + Supplements] inoculated with *C. albicans* cells exposed to a slow temperature transition from 30°C to 37°C.

In this experiment, five test tubes were used, each containing 1 ml of the culture medium [V8 Original + Supplements]. Tube 1 was inoculated with *C. albicans* cells for pre-culture, following the protocol detailed in section 4.4.2. Tube 2 acted as a negative control, i.e. no inoculation was performed in it to ensure the absence of contamination. For Tube 3, *C.*

albicans was inoculated and incubated at 30°C. Tubes 4 and 5, labeled 'Fast Transition' and 'Slow Transition', respectively, were incubated at 37°C. The key difference was that the 'Fast Transition' Tube was pre-warmed overnight at 37°C before inoculation. The results of these assays are presented in Figure 5.4 and Figure 5.5

These studies have found that both a quick and a slow temperature transition can induce hyphal formation in *C. albicans*. It is possible that the gradual increase in temperature allows *C. albicans* to adapt to these changes more efficiently. However, abrupt fast transition forces *C. albicans* to a more violent adaptability. In any case, *C. albicans* is able to morphologically transform in the face of these changes and show plasticity in hostile environments, which indicates its ability to survive and is an important characteristic for colonization and establishment of infections.

Fast Transition



(a) Inoculation of the medium with *C. albicans* cells at a temperature of 30°C.

(b) *C. albicans* cells exposed to thermal transition from 30°C to 37°C.

Figure 5.5: Control medium [V8 Original + Supplements] inoculated with *C. albicans* cells exposed to a thermal fast transition from 30°C to 37°C.

When this enriched culture medium induces stress, for example, by this abrupt change in temperature, it could lead to increased expression of heat shock proteins (HSPs), which affect the morphology of *C. albicans*. However, this study does not encompass a monitoring of HSPs expression to correlate with stress induction and thus with changes in fungal morphology. Future work could overcome the limitations of this work and investigate the expression of HSPs and their role in the adaptation and survival of *C. albicans* at elevated temperatures.

5.2.3 pH Influence on *Candida* Filamentation

The pH plays a fundamental role in the filamentation of *Candida albicans*. Several studies indicate that alkaline environments can trigger morphological changes in this fungus. *C. albicans* keeps its internal pH balanced by producing ammonia (NH_3) outside the cell, which is converted to ammonium ion (NH_4^+) through the enzyme urease. This increase in NH_4^+ raises the pH, which promotes *Candida* morphogenesis [5, 1].

The transcription factor Rim101 plays a critical role in this process, being activated under alkaline conditions, triggering the morphological switch of *C. albicans*. Understandably, these findings suggest that both neutral and basic pH favor the yeast-to-hypha transition, whereas acidic pH inhibits this transformation.

Surprisingly, in our experiments, it was observed that the vegetable juice-based culture media presented acidic pH values, as detailed in Table 5.1. Among the media based on individual vegetables, the medium made with tomato juice recorded the lowest pH value (pH = 4.46), while the spinach culture medium had the highest value, with a pH of 6.61. This result raises the hypothesis that the ability of *C. albicans* to filament under these conditions could be attributed to other stimulating factors, such as the availability of nutrients and the presence of organic compounds specific to each medium.

Table 5.1: pH of positive culture media and individual vegetable-based culture media.

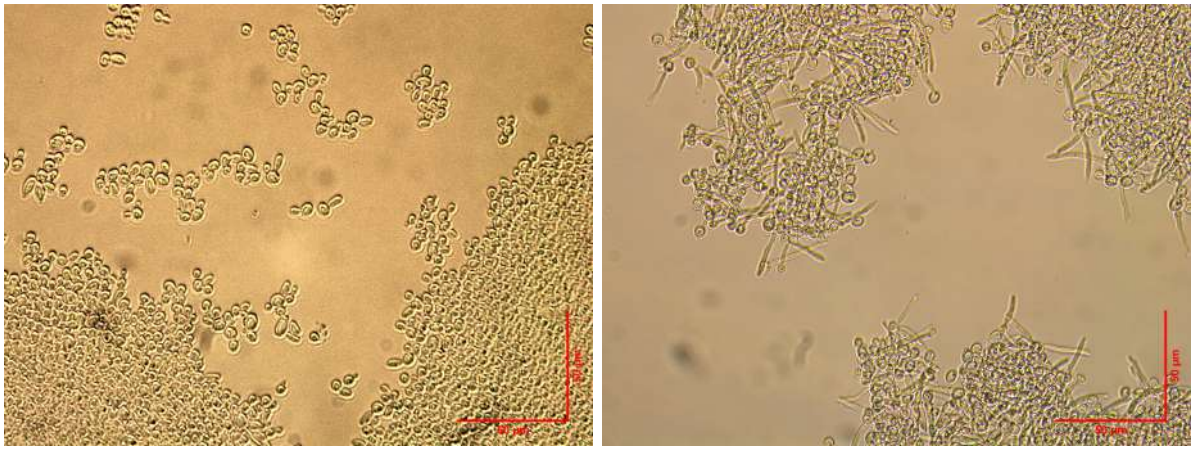
Culture Medium	**pH**	**Filamentation**
Positive control medium: V8 Original + Supplements	5.70	++
Positive control medium: V8 Homemade	5.55	++
Celery Culture Medium	5.82	+
Watercress Culture Medium	5.94	+
Spinach Culture Medium	6.61	+++
Lettuce Culture Medium	6.40	+
Parsley Culture Medium	5.95	++
Tomato Culture Medium	4.46	+
Beet Culture Medium	6.43	+++
Carrot Culture Medium	6.32	++

This research presented unexpected results. Although there is no ideal pH value for *Candida*, a pH close to neutral or slightly alkaline is usually more conducive to the induction of filamentous forms. The response of *C. albicans* to pH can be modulated by other environmental factors, such as temperature, nutrient availability, and the presence of certain vegetable-derived signaling molecules. Although it is considered that *C. albicans* does not usually filament in acidic media, we observed that it did. This challenges the belief that acidic environments do not favor filamentation. One possible explanation is that certain virulence processes, such as the secretion of aspartic proteases, are better activated or adapted in acidic conditions (pH 3-5-7) and are involved in colonization and pathogenicity. These findings provide an experimental platform for future research to confirm and expand these results.

5.3 Comparative Study of Filamentation in Vegetable Juice-Based Media

The literature reviewed in the introduction has corroborated the development of specific culture media to enhance the growth of yeasts or filamentous structures of *C. albicans*. In this section, the morphology of cells grown on these vegetable-based media was examined in detail, revealing growth patterns in response to the specific components of these media. Commercial V8 juice was used for the preparation of an enriched culture medium. Inspired by this concept, individual media were developed for each of the plant components of V8 juice. This provided an effective technique to differentiate between yeast and filamentous forms of *C. albicans*, similar to the germ tube test method.

Positive control medium: V8 Original + Supplements

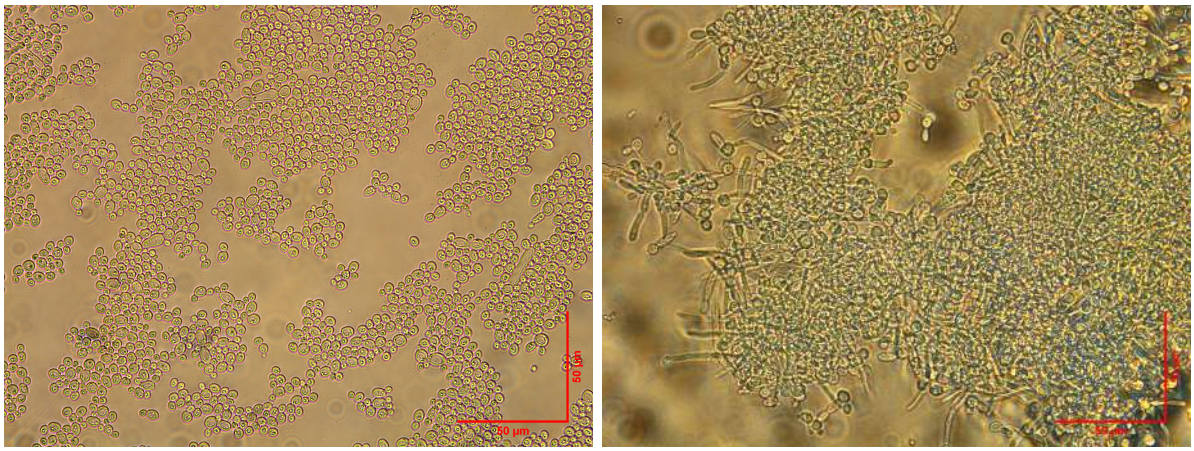


(a) Yeast cell growth observed in [V8 Original + Supplements] medium at 30°C.

(b) At a temperature of 37°C, a morphological change was induced in *C. albicans*.

Figure 5.6: Conducted filamentation assays to study *C. albicans* morphogenesis in control medium 'V8 Original + Supplements'

Positive control medium: V8 Homemade

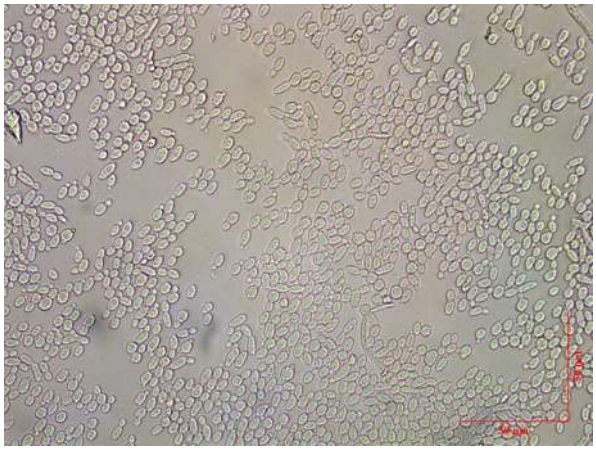


(a) Growth of yeast cells was noted in [V8 Homemade] medium at 30°C.

(b) Transformation of yeast to hyphae in [V8 Homemade] medium at 37°C.

Figure 5.7: Filamentation assays in 'V8 Homemade' medium.

Celery Culture Medium



(a) In [Celery Culture Medium] at 30°C, yeast cell proliferation was observed.

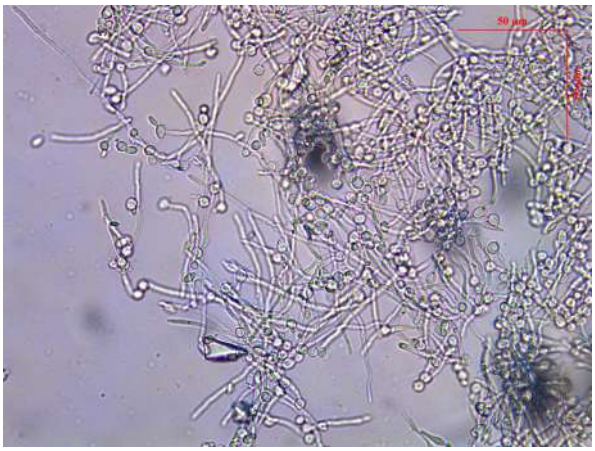
(b) The increase in temperature to 37°C triggered a morphological shift in *C. albicans*.

Figure 5.8: Morphogenesis of *C. albicans* was investigated through filamentation assays in [Celery Culture Medium]

Positive results were achieved with culture based on celery and watercress juices. Notably characterized by their green color, these juices exhibited low density, thus facilitating the filtration process. Both types of media effectively induced morphological changes in *C. albicans* and facilitated cellular growth. As can be seen in Figures 5.8 and 5.9

Watercress Culture Medium

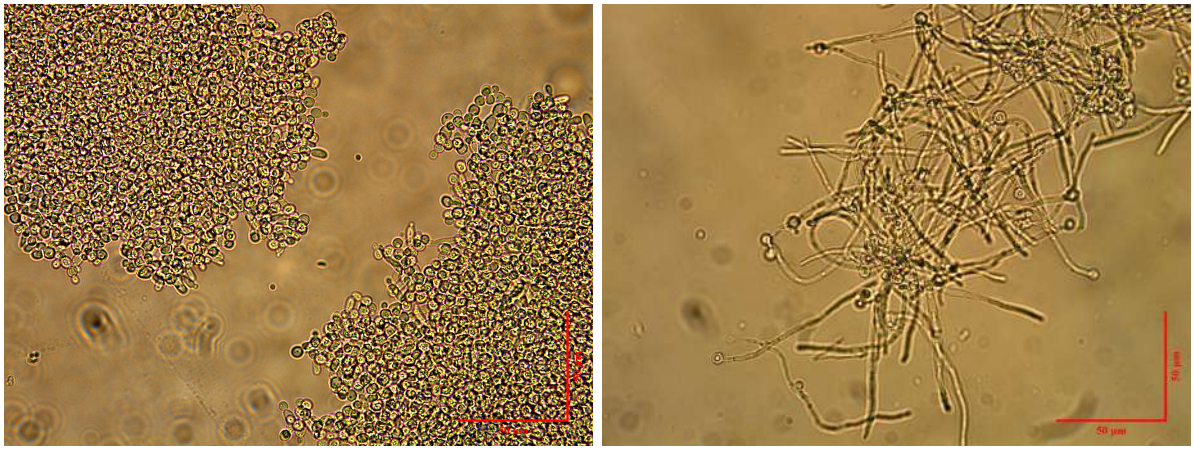
(a) Yeast cells showed growth in [Watercress Culture Medium] when incubated at 30°C.°



(b) Adjusting the temperature to 37°C induced a morphological change.

Figure 5.9: Filamentation assays in [Watercress Culture Medium] were conducted to explore *C. albicans* morphogenesis

Spinach Culture Medium



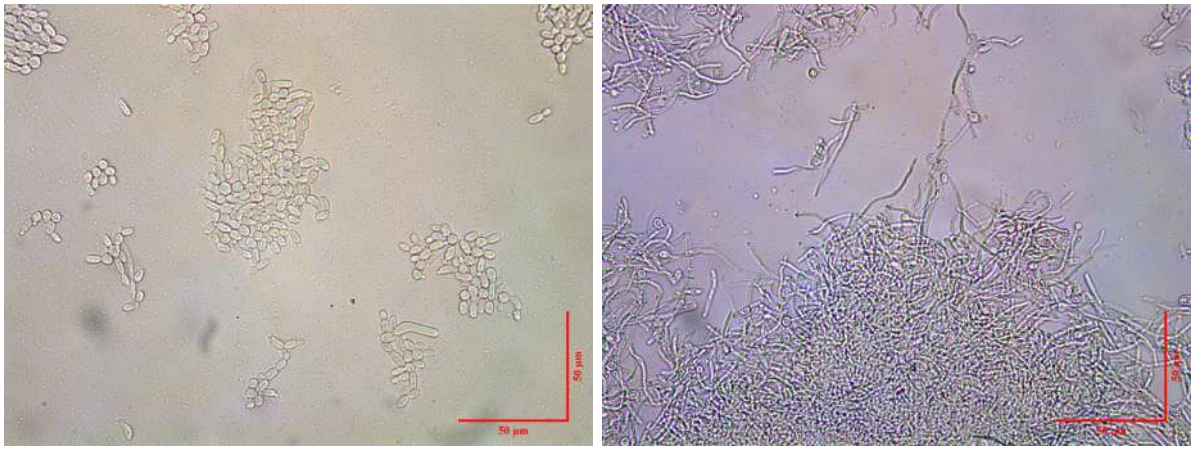
(a) [Spinach Culture Medium] at 30°C supported the growth of yeast cells.

(b) A temperature increment to 37°C stimulates a morphological switch

Figure 5.10: Filamentation assays of *C. albicans* in 'Spinach Medium'.

The spinach-based culture medium, with the highest pH (see Table 5.1), effectively induced true hyphae at 37°C and allowed a morphological switch at 30°C, provided the incubation time exceeded 2.5 hours. In contrast, the lettuce-based medium also promoted hyphae formation at 37°C, but these were shorter and formed aggregates.

Lettuce Culture Medium

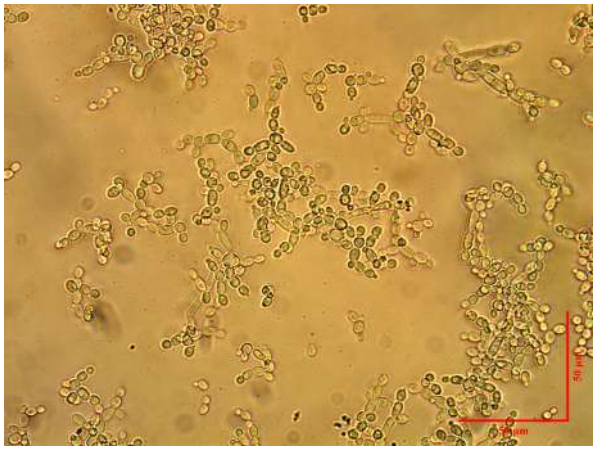


(a) Observation of yeast cell growth occurred in [Lettuce Culture Medium] at 30°C.

(b) Elevating the temperature to 37°C leading to the emergence of hyphae

Figure 5.11: Assays for filamentation were executed to investigate the morphogenesis of *C. albicans* in [Lettuce Culture Medium].

Parsley Culture Medium



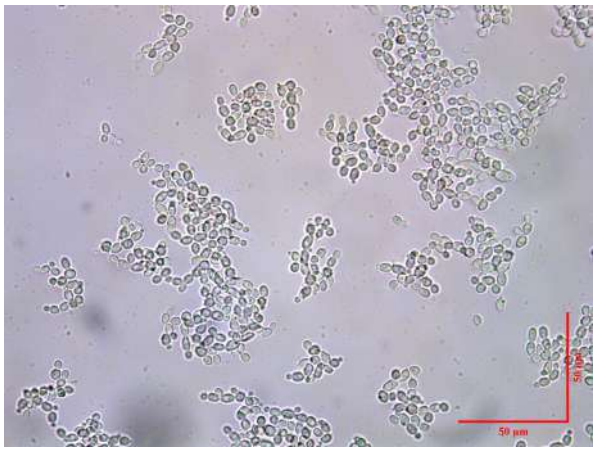
(a) Yeast cell growth was evident in [Parsley Culture Medium] maintained at 30°C.

(b) The temperature was increased to 37°C to trigger hyphal formation in *C. albicans*.

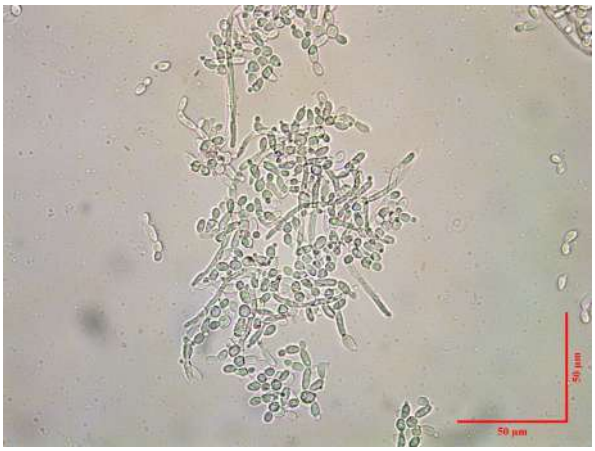
Figure 5.12: Filamentation assays to study the morphogenesis of *C. albicans* in [Parsley Culture Medium].

Successful filamentation is evident in the parsley medium, characterized by the significant length of its true hyphae (see Figure 5.12). Conversely, the tomato medium exhibited minimal filamentation, especially featuring pseudohyphae and germ tube formation (see Figure 5.13). For optimal hyphal formation (at 37°C), extend the incubation time beyond 2.5 hours.

Tomato Culture Medium



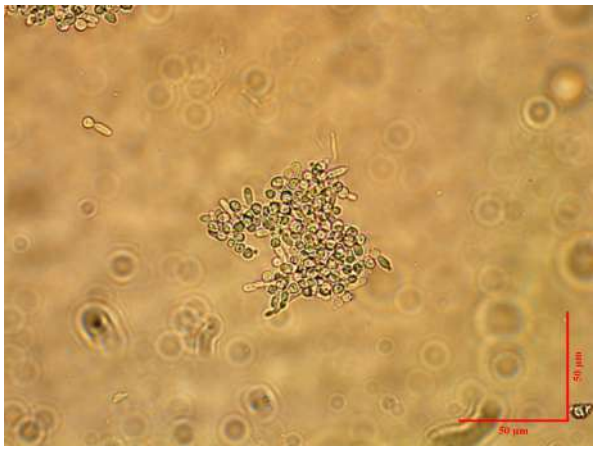
(a) Growth of yeast cells in [Tomato Culture Medium] at a temperature of 30°C.



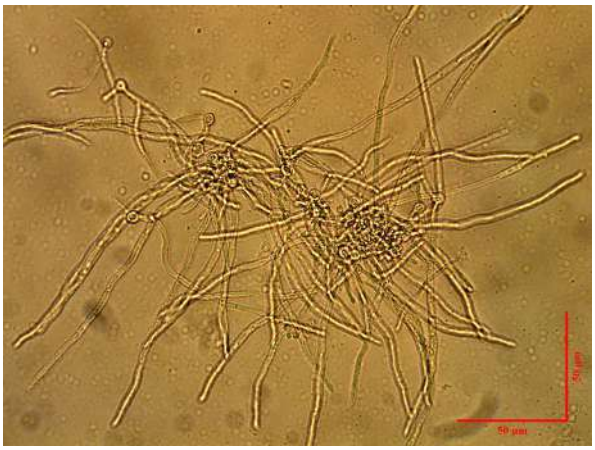
(b) At a temperature of 37°C a slight morphological change is induced.

Figure 5.13: Results of filamentation assays in tomato culture medium to further investigate the morphogenesis of *C. albicans*.

Beet Culture Medium



(a) In [Beet Culture Medium] at 30°C, there was notable yeast cell growth.

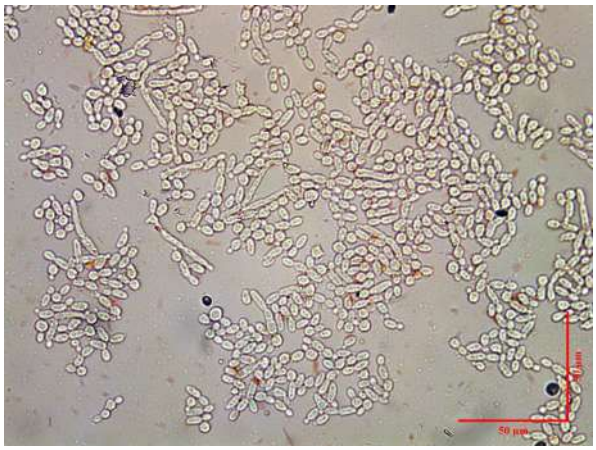


(b) Morphological transition was induced by setting the temperature to 37°C.

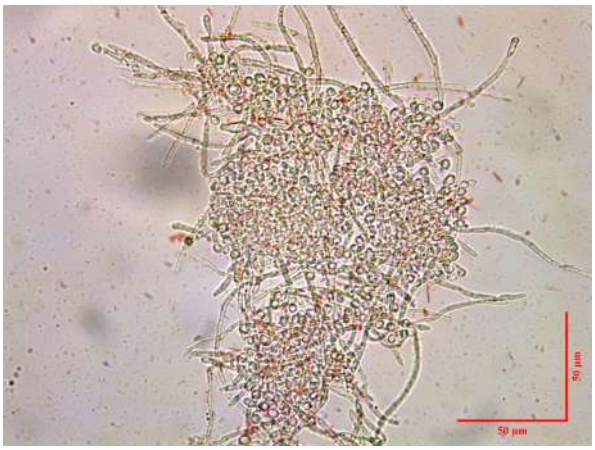
Figure 5.14: Morphogenesis assays for *C. albicans* were conducted in [Beet Culture Medium] focusing on filamentation.

The beet and carrot media were among the thickest, with small precipitates visible in the images even after filtration. Notably, the beet medium featured hyphae of considerable size, (see Figure 5.14) whereas in the carrot medium, hyphae tended to cluster together (see Figure 5.15).

Carrot Culture Medium



(a) [Carrot Culture Medium] facilitated yeast cell growth at 30°C.



(b) Temperature increase (37°) induces hyphae in Beet culture medium

Figure 5.15: The process of *C. albicans* morphogenesis was examined through filamentation assays in [Carrot Culture Medium].

These microscopic images highlight variations in the ability to induce filamentation in each of these media. These variations include significant differences in cell morphology, such as cell shape and size, hyphal length, presence of filamentous structures, and other distinctive features specific to each medium. The results obtained are promising and positive, demonstrating that in each of these media *C. albicans* is capable of undergoing significant morphological change.

In the case of the eight vegetable juice-based media, they all have the potential to induce the filamentation process in *C. albicans*. Regarding the density of their contents, carrot, tomato, and parsley media proved to be the most difficult to filter due to the thickening of their juices, which caused problems in cell visualization. In terms of incubation times, spinach, lettuce, beet, and carrot media seemed to induce the morphological change in *C. albicans* more easily. In particular, it was observed that with these four media, *C. albicans* cells could filament at a temperature of 30°C if the incubation time exceeded 2 hours and 30 minutes, which did not occur with the other vegetables. Specifically, in the parsley and tomato media, if the incubation time exceeded the established time, filamentation only occurred at 37°C.

Throughout this research, it has been highlighted how the composition of the culture media plays a key role in the growth and development of pathogenic processes in fungi. However, it is also evident that environmental factors, including pH, temperature, incubation time, and nutrient content of the medium, significantly impact fungal morphogenesis. In fact, some culture media have been developed specifically for the study of certain fungal capabilities. The culture medium based on the commercial juice 'V8 Original' made with a mixture of vegetables satisfies the right conditions to favor the growth and filamentation of *C. albicans*. This culture medium works properly because of the mixture of nutrients specific to each vegetable that constitutes it. 'V8 Original' juice contains salt, potassium chloride, citric acid, natural flavors, ascorbic acid, and vitamin A (Beta Carotene), which provide the necessary trace elements to stimulate the growth of fungi.

5.4 Analyzing V8 Original Agar

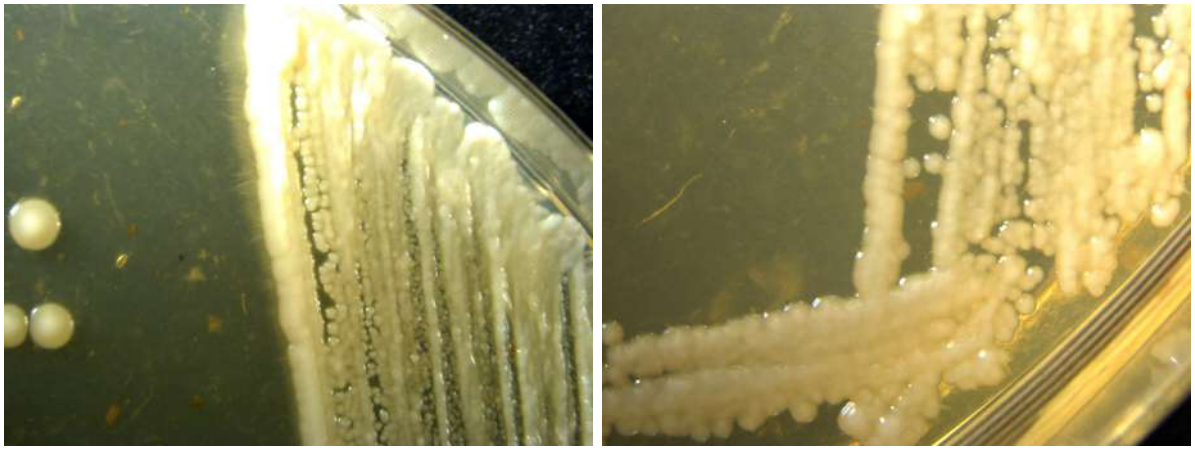
The preparation of the solid medium 'V8 Original Agar' was performed as described in section 4.2. For analysis, we inoculated approximately 10μl of a cell suspension of the standard *C. albicans* strain (PMRCA18) onto the surface of V8 Original Agar. Incubation was carried out at 37°C for 24 hours, with daily observations. After 72 hours, the first signs of filamentous cells were observed. These results allowed us to compare the ability of both liquid and solid positive control media. Specifically, commercial V8 juice, combined with agar, provides a unique set of nutrients and growth factors, thus influencing the morphological transformation of *C. albicans*. The rich and varied composition of V8 could facilitate different physiological responses, including filamentation.

(a) Colony formation of *C. albicans* observed in the [V8 Original Agar] culture medium.

(b) Presence of filamentation around the colonies on the culture medium plates.

Figure 5.16: Conducted filamentation assays with *C. albicans* on solid [V8 Original Agar] medium plates.

The results obtained on the solid medium 'V8 Original Agar' were analyzed microscopically through 40X objectives, revealing filamentation only in a few colonies or areas of high cell density in the plate. Figure 5.16a depicts well-formed colonies, while Figure 5.16b illustrates filaments surrounding the colonies, although not all colonies exhibited this morphological change. As shown in Figure 5.17, a higher concentration of hyphae, indicative of increased filamentation, is observed at points where the inoculation stick seeded the plate areas with a higher cell density. This pattern suggests that nutrient competition may trigger enhanced plasticity, facilitating morphological transformation.



(a) Notable filamentation of *C. albicans* at the contact point where the inoculation stick first meets the plate.

(b) Increased cell concentration in the contact zone, accompanied by a higher number of hyphae.

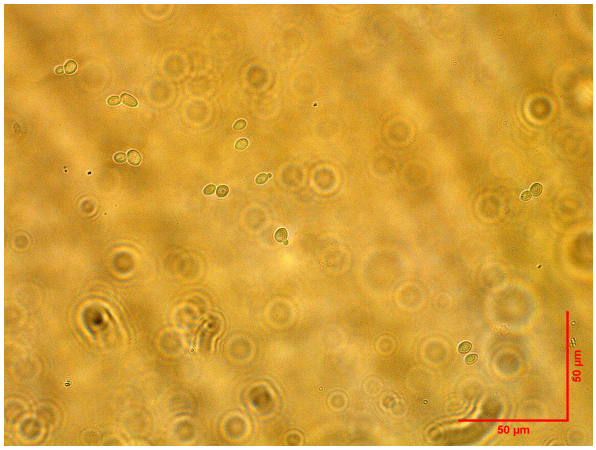
Figure 5.17: Observed growth patterns of *C. albicans* cells on 'V8 Original Agar' culture medium plates.

Although the results of this experiment on solid media are limited, it is important to note that liquid media offer superior advantages for the cultivation of *C. albicans*. The choice of a liquid culture medium for this investigation is based on its efficacy in promoting the growth and proliferation of *C. albicans*. Above all, liquid media offer a more favorable environment for growth, facilitating the exchange of gases and nutrients. Another reason is that they allow precise control of temperature and pH conditions. Adjustments of environmental factors in these vegetable-based culture media during filamentation assays could be used to study and manipulate pathogenic processes, which is relevant for biomedical research and the development of better culture media.

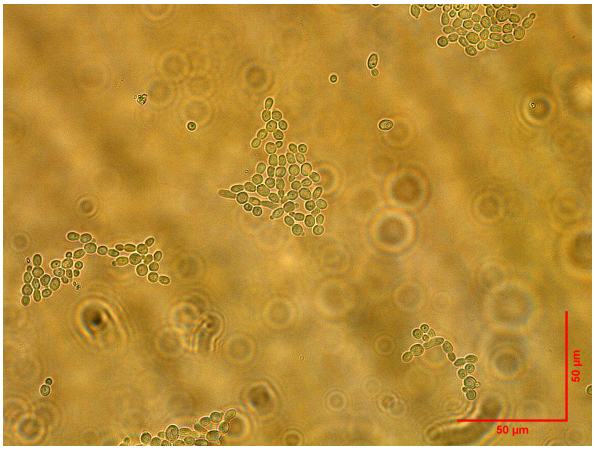
5.5 Filamentation Assays with Non-albicans Strains

The literature recognizes that, in addition to *C. albicans*, *C. tropicalis* and *C. parapsilosis* species have the ability to transition from their yeast form to hyphae. For this study, filamentation assays were performed with clinical isolates of *C. tropicalis* and using *C. glabrata* as a negative control. These assays were carried out using the control medium [V8 Original + Supplements], following the protocol described in section 4.4.2.

C. tropicalis



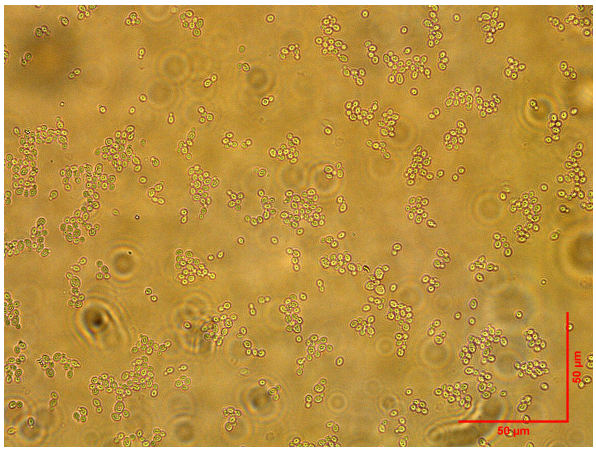
(a) *C. tropicalis* cells inoculated at 30°C in [V8 Original + Supplements] medium.



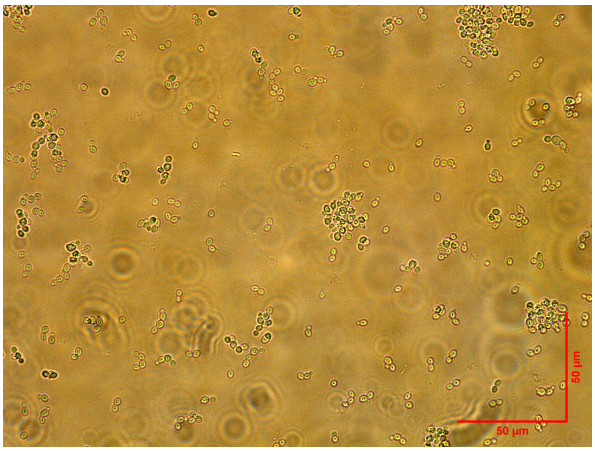
(b) *C. tropicalis* cells inoculated at 37°C in [V8 Original + Supplements] medium.

Figure 5.18: Filamentation assays conducted with *C. tropicalis* in [V8 Original + Supplements] medium.

C. glabrata



(a) *C. glabrata* cells inoculated at 30°C in [V8 Original + Supplements] medium.



(b) *C. glabrata* cells inoculated at 37°C in [V8 Original + Supplements] medium.

Figure 5.19: Filamentation assays conducted with *C. glabrata* in [V8 Original + Supplements] medium.

Clear differences were observed between the behaviors of albicans and non-albicans *Candida* species. *C. glabrata* cells maintained their yeast form under both temperature conditions (30°C and 37°C), while *C. tropicalis* showed signs of minimal transformation at 37°C, forming pseudohyphal-like cells. These findings can be visualized in Figure 5.18 and Figure 5.19. Although these results show that the V8 medium has potential for the growth

of non-albicans cells, its effectiveness appears to be reduced compared to that observed in *C. albicans* species. It will be important to validate these results in future research with a wider range of non-albicans *Candida* species on different plant culture media, a limitation present in this assay due to time.

Conducting these comparative assays with non-albicans *Candida* species is critical to understanding the variability in the response of different *Candida* strains to the same nutritional environment of a plant-based culture medium. This information is relevant to developing more effective strategies for understanding and treating fungal infections.

5.6 Summary of Culture Media Findings

Table 5.2: Overview of the morphological responses of *C. albicans* to temperature variations and incubation times in various vegetable-based culture media.

Culture Medium	**pH**	**Incubation time for inoculated medium 2 hours**		**Incubation time for inoculated medium 3 - 4 hours**	
		30°	**37°**	**30°**	**37°**
Positive control medium					
V8 Original + Supplements	5.70	Yeast	Hyphae	Yeast	Hyphae
V8 Homemade	5.55	Yeast	Hyphae	Yeast	Hyphae
Individual vegetable-based culture media					
Celery Culture Medium	5.82	Yeast	Hyphae	Yeast	Hyphae
Watercress Culture Medium	5.94	Yeast	Hyphae	Yeast	Hyphae
Spinach Culture Medium	**6.61**	Yeast	Hyphae	Hyphae	Hyphae
Lettuce Culture Medium	6.40	Yeast	Hyphae	Hyphae	Hyphae
Parsley Culture Medium	5.95	Yeast	Hyphae	Yeast	Hyphae
Tomato Culture Medium	4.46	Yeast	Pseudohyphae/ germ tube formation	Yeast	Hyphae
Beet Culture Medium	6.43	Yeast	Hyphae	Hyphae	Hyphae
Carrot Culture Medium	6.32	Yeast	Hyphae	Hyphae	Hyphae

Table 5.3: Experimental overview of the impact of thermal transitions and the utilization of non-albicans strains in positive culture media.

Positive control medium	**Strains**			
V8 Original + Supplements	***C. albicans***	**Slow Temperature Transitions**	**30°**	Yeast
			37°	Hyphae
		Fast Temperature Transitions	**30°**	Yeast
			37°	Hyphae
	C. tropicalis	**30°**	Yeast	
		37°	Pseudohyphae	
	C. glabrata	**30°**	Yeast	
		37°	Yeast	

Chapter 6

Conclusions

In conclusion, this research has demonstrated that *C. albicans* exhibits filamentation when cultured in vegetable juice-based media. This filamentation is not limited to a single type of vegetable source; our investigations included eight distinct vegetable juices, among them tomato juice which has already been studied as a serum replacement in culture media. This consistent induction of filamentation in diverse vegetable-based media implies that the morphological adaptability of *Candida spp.*, particularly its filamentation capacity, might have roots in an ancestral relationship with plants. The phenomenon, initially perceived as an interaction with animal hosts, may actually be an inherent feature rooted in the evolutionary relationship of *Candida spp.* with plant organisms. This finding opens new insights into understanding the evolutionary biology of *Candida spp.* and its mechanisms of interaction with different hosts.

Incubation times and nutritional richness of the media also played an important role in the ease of filamentation. We observed that spinach, lettuce, beet, and carrot media more effectively induced morphological change in *C. albicans*. Specifically, these cells could filament at 30°C if the incubation time exceeded 2 hours and 30 minutes. We controlled and analyzed the influence of environmental factors such as pH and temperature. It was observed that the acid pH of the media favored fungal growth and suppressed bacterial growth. In addition, we subjected *C. albicans* cells to a quick or slow transition to 37 degrees to test their reaction.

Despite the limitations of this study, it is important to consider the development of new vegetable-based culture media for pathogenic or antifungal studies and to understand

how these vegetables and stress factors affect the physiology and pathogenicity of the fungus. In addition, the relationship between heat shock proteins and *Candida spp.* adaptation under stress conditions deserves further attention. Regarding media contamination and sterilization protocols, it is essential to investigate how bacteria control fungal colonization, particularly in the interaction between *Candida spp.* and bacteria in vegetable decomposition processes. This area could be key to determining the importance of aseptic techniques. Genetics, transcriptomic, and proteomics approaches could complement this research, providing a deeper understanding of the underlying mechanisms and transcription factors associated with filamentation on vegetable media.

Finally, these investigations have also revealed the practical benefits of using vegetable juices as culture media. Derived from common vegetable materials, including those potentially considered waste, these vegetable-based media present a cost-effective and sustainable alternative to traditional culture media. Notably, these media do not require the addition of supplemental components such as human serum to induce filamentation. This characteristic not only simplifies the culture process but also reduces the risks of false positives. The application of these media can revolutionize fungal research and clinical diagnostics, especially in resource-limited settings. They provide an environmentally friendly and cost-effective solution, aligning with the principles of sustainability and accessibility. Finally, the absence of animal protein hydrolysates in this medium renders it a more ethical choice.

Chapter 7

Future Directions

Based on this research, a transcriptomic analysis can be performed in the future to identify which specific genes are activated during filamentation in vegetable juice-based media, and contrast these with the genes activated in traditional media and human tissues. This comparison could reveal certain molecular mechanisms associated with the adaptation and pathogenicity of *Candida albicans* in plant environments. Additionally, it is essential to search for specific components of vegetables juices that enhance *Candida* filamentation. So far, the complexity of these media has limited this type of study, but a focus on media fractionation and analysis can be addressed that could provide significant insights into nutritional and environmental factors related to *Candida* morphogenesis.

Additionally, this research justified the necessity to investigate the expression of heat shock proteins in the adaptation and survival of *C. albicans* within stressful environments, such as exposure to elevated temperatures. This exploration could expand our comprehension of the mechanisms contributing to the resistance and adaptability of *Candida*. Incorporating a broader range of non-albicans *Candida* species in these studies could validate and generalize the results obtained. However, it is understood that not all *Candida* species may undergo filamentation. Focusing efforts in this area could provide a more balanced understanding of the mechanisms of morphological transition in *Candida* species. Finally, the success of this study with traditional vegetables suggests potential extensions to other vegetables or fruits, particularly those for which countries like Ecuador are natural producers.

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