Effect of cigarette smoke on *Candida albicans* growth and its interaction with human gingival fibroblasts

Mémoire

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Résumé

L'objectif principal de cette étude est d'étudier l'effet de la fumée de cigarette (FC) sur *C. albicans* et sur son interaction avec les fibroblastes gingivaux humains. Nous avons démontré que Candida se multiplie présence FC, en adoptant la forme hyphe. La FC rende *C. albicans* sensible au H_2O_2, mais résistant aux la chaleur et au NaCl. La FC augmente la production de chitine par *C. albicans*. Préincubé avec le FC, *C. albicans* adhère beaucoup plus aux cellules gingivales en monocouche, prolifère plus et adopte la forme hyphe plus facilement. Les fibroblastes quant à eux ils montrent une réduction de leur croissance, mais produisent plus de l'IL-1b. En conclusion: le CFC module d'un côté *C. albicans* et de l'autre côté les cellules de l'hôte. Ceci suggère un négatif important de la fumée de cigarette favorisant les candidoses orales chez les fumeurs.

Abstract

The main objective of this study was to investigate the effect of cigarette smoke (CS) on *C. albicans* and its interaction with human gingival fibroblasts. We have shown that Candida multiplies presence CS, by adopting the hyphae form. CS makes *C. albicans* sensitive H_2O_2, but resistant to heat and NaCl. CS increases chitin production by *C. albicans*. Preincubated with CS, *C. albicans* adheres more to gingival cells in monolayer, proliferating more and adopts the hyphae form more easily. Fibroblasts show a reduction of their growth, but produce more IL-1b. CSC modulates *C. albicans* and on the other side also CS modulates the host cells. This suggests a significant negative cigarette smoke promotes oral candidiasis in smokers.

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Als3	Agglutinin-Like Sequence 3
ANUG	Acute Necrotizing Ulcerative Gingivitis
AP-1	Activatory Protein-1
BAL	Broncho Alveolar Lavage
cAMP	Cyclic Adenosine MonoPhosphate
cdk	Cyclin-Dependent Kinase
C. albicans	*Candida albicans*
CHD	Coronary Heart Disease
Chs	Chitins
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette Smoke
CSC	Cigarette Smoke Condensate
CVD	Cardio Vascular Disease
CWPs	Cell Wall Proteins
Eap	Enhanced Adherence to Polystyrene
ECM	ExtraCellular Matrix
GPI	GlycosylPhosphatidylinositol
GPM1	PhosphoGlycerate Mutase 1
Hwp1	Hyphal Wall Protein 1
Hyr1	Hypha Regulated Protein 1
IL-1β	Interleukin-1β
MAPK	Mitogen-activated protein kinases
MLST	Multi-Locus Sequence Typing
NF-κB	Nuclear Factor Kappa-B
NNK	Nicotine-derived Nitrosamine Ketone
PAF	Platelet-Activating Factor
PAMPs	Pathogen Associated Molecular Patterns
PKA	Protein Kinase A
PRRs	Pattern Recognition Receptors
PVC	PolyVinyl Chloride
Saps	Secreted Aspartic Proteases
STAT	Signal Transducer and Activator of Transcription
TLR	Toll Like Receptor

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Thanks for my husband Shadaid Alanezi, who always does not let me disappointment and give me the energy to keep going.

Finally, thanks for my sister Sara, the second mother, she is my inspiration to be better in the thinking and the life.
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Foreword

Mahmoud Rouabhia, Witold Chmielewski, and Andrew Zakrzewski conceived the study. Humidah Alanazi, Abdelhabib Semlali, and Laura Perraud conducted the experiments. Humidah Alanazi, Abdelhabib Semlali, Laura Perraud, and Mahmoud Rouabhia analyzed and interpreted the data. Humidah Alanazi, Laura Perraud, and Abdelhabib Semlali drafted the Materials and Methods section. Mahmoud Rouabhia completed the paper with the help of Witold Chmielewski and Andrew Zakrzewski. All of the authors read and approved the final paper.

CHAPTER I

1.1 INTRODUCTION

1.1.1 The oral cavity and microbial niches

The human mouth is an appropriate environment for numerous microbes [1]. Microbial diversity in the human oral cavity was discovered long ago, thanks to culture methods and molecular-based approaches. The oral microbiome is heterogeneous showing different physicochemical properties [2]. The presence of shedding and solid surfaces in the oral cavity provides an opportunity for numerous microbial species to adhere and to grow, compared to other microbial habitats in the human body [3]. Multiple other factors such as oxygen content, saliva, gingival crevicular fluid, and diet play major roles in the microbial composition of these oral ecological niches [4]. Humans are able to modify the microbial load through daily hygiene habits such as cleaning the teeth and tongue [5].

The major constituents of the oral microbiome are bacteria and fungi. Many oral microbes, such as *Firmicutes* (genus *Streptococcus*, family *Veillonellaceae*, genus *Granulicatella*), *Proteobacteria* (genus *Neisseria, Haemophilus*), Actinobacteria (genus *Corynebacterium*, *Rothia, Actinomyces*), Bacteroidetes (genus *Prevotella, Capnocytophaga, Porphyromonas*), and Fusobacteria (genus *Fusobacterium*) are normal microbial complexes residing in the oral cavity in various intraoral niches (dental surfaces, cheeks, hard palate, tongue, and saliva) [3]. In contrast, the presence of fungi is associated with some diseases and infections, although this does not eliminate the role of fungi and fungi-bacteria interactions in such infections [6]. One of the most studied and most common fungi in the oral cavity is *Candida albicans (C. albicans*).

1.1.2 *C. albicans*

The yeast *C. albicans* is frequently found on the skin and in mucous membranes such as the mouth, rectum, etc. [7]. *C. albicans* is a mucosal commensal yeast capable of colonizing and proliferating within every organ of the body, sometimes causing serious infections. The immune system, can reduce *C. albicans* virulence and pathogenesis [8,9]. This highlights the importance of *Candida* both medically and biologically [10]. Of the 17 known *Candida* species, five (*C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, and C. krusei*) are invasive and are the cause of candidiasis. Interestingly, *C. albicans* is frequently identified in humans (42.1%) compared to *C. glabrata* (26.7%), *C. parapsilosis* (15.9%), *C. tropicalis* (8.7%), and *C. krusei* (3.4%) [11].

C. albicans is associated with clinical materials in 90 to 100% of mucosal infections, while 40 to 70% of candidemia is caused by *C. albicans* [11]. *C. albicans* has the capacity to modulate its growth according to environmental changes, leading to the expression of various genes in an appropriate manner. These genes involve specific signaling pathways (MAP kinases and RLM1) which play an important role in the virulence and morphological transitions of *C. albicans* [12].

C. albicans takes advantage of inappropriate physiological changes (such as impaired saliva or immune defense dysregulation) to proliferate and to invade, causing pathogenesis [9]. For example, *C. albicans* adheres better to denture materials, and forms a biofilm to maintain chronic infection [9]. Furthermore, the elderly are more prone to *C. albicans* infections because of an immune system dysregulation [13].
It is important to note that *Candida* species have some differences:

- Phylogenetically, *C. glubrata* is more similar to baker's yeast (*Saccharomyces cerevisiae*) than to other *Candida* species.

- Among all *Candida* infections, *C.albicans* is the most frequently involved in human infections;
- *C. dubiliniesis* evades the azole's effects faster than *C. albicans* does; this explains the association of *C. dubiliniesis* in HIV patients. Candidiasis has been found to be mainly caused by *C. albicans* [9].

C. albicans includes four major and eight minor clades typed among more than 400 *C. albicans* isolates identified by multi-locus sequence typing (MLST) techniques. This enabled the identification of flucytosine resistance associated with the major clades [14]. Pathogenesis of *C. albicans* involves the early adhesion steps involving cell wall proteins.

1.1.2.1 *C. albicans* cell wall

The *C. albicans* cell wall consists of 90% carbohydrate and 10% protein, each with an active role in the virulence of the yeast. Antigens and pathogen-associated molecular patterns (PAMPs) contribute to the conversion of *C. albicans* from commensalism to virulence. These PAMPs form two layers:

- The inner layer consists of polysaccharides which contribute to the immunological signature of *C. albicans* [15];
- The outer layer consists of glycoproteins which are formed by covalent links between the *O*-and *N*-linked mannose polymers (mannans) and proteins. These *O*-linked and *N*-linked mannans attract different Toll-like receptors (TLR) and C-type lectin pattern recognition receptors (PRRs) during *C. albicans* pathogenesis [16].

The outer layer mannoprotein contains β-glucan/chitin which give a strong shape to the cell wall; most of these glucans/chitins are glycosylphosphatidylinositol (GPI)-proteins contributing to the attachment of both the inner and outer layers of the yeast membrane (Figure 1-1). Moreover, many proteins expressed during the morphological changes from blastospore to hyphal form can contribute to *C. albicans* infection. Cell wall proteins (CWPs) are major players at various levels, from fungus adhesion and growth to invasion of the host. These CWPs are related to specific genes expressed during hyphal switching, such as hyphal wall protein 1 (Hwp1), hyphally regulated protein 1 (Hyr1), and agglutinin-like sequence 3 (Als3). *C. albicans* most often proliferates as a budding yeast under neutral or alkaline conditions; increasing the temperature triggers the morphogenesis [8,9,17,18].

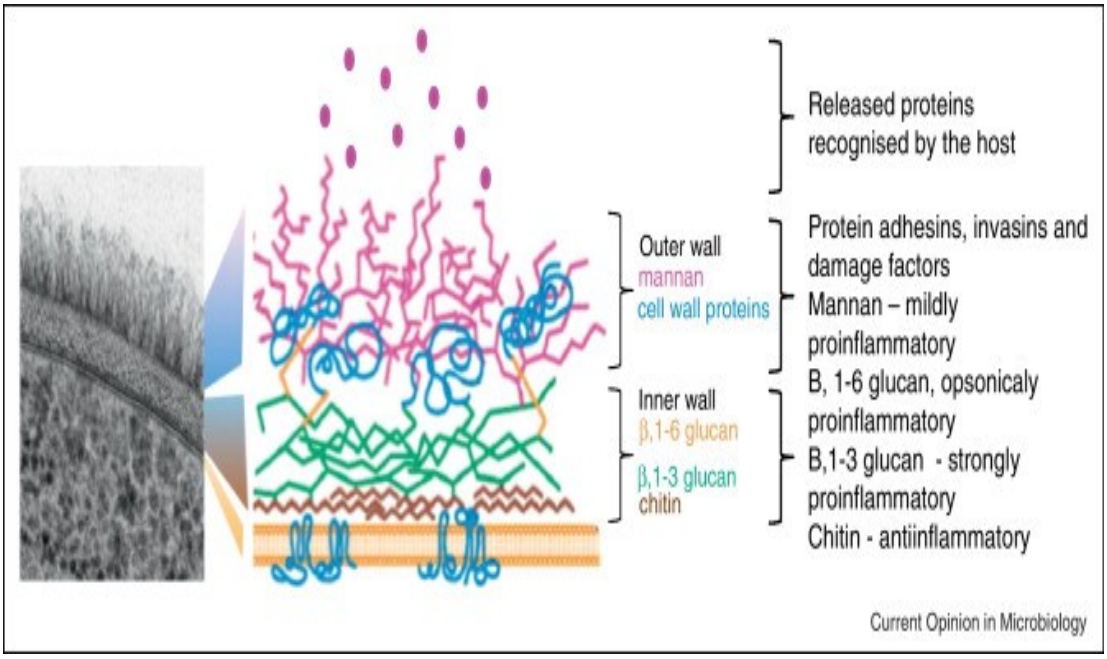

Figure 1-1: *C. albican* cell wall components. This figure is adapted from Gow and Hube. Curr Opin Microbiol. 2012 Aug;15(4):406-12. The figure shows transmission electron micrograph of the *C. albicans* cell wall. This was supported by showing key components this cell well along with their role interacting with the host [7].

1.1.2.2 *C. albicans* life cycle

C. albicans is capable of changing its morphogenesis under certain circumstances and morphing into many different forms (yeast, pseudohyphae, hyphae) which is considered the main virulence factor and pathogenic feature of this fungus [19], (figure1-2). These morphological changes lead to different life cycles (figure 1-3).

1. *C. albicans* proliferation occurs when the cells go from blastospore form, which is spherical (figure 1-3), to a septum ring formation, after which time the DNA separation takes place across the mother-bud neck. When the cell cycle progresses from G_1 to S at the same time as the DNA is replicated, the spindle pole body is duplicated and the cell separation takes place following cytokinesis. The new cells can subsequently enter their own cell cycle when they reach the appropriate size/maturation.

2. In pseudohyphal cells, similar to the budding yeast, the septum ring development also occurs prior to DNA separation which takes place across the mother-bud neck; however, following cytokinesis, the new cells remain linked to the mother cell to form elongated branched colonies. It has been reported that pseudohyphal cells require more time to polarize and remain more in G_2, compared to the yeast. The daughter cells then enter the life cycle along with the mother cells until the daughter cells reach the same size as the mother.
3. In hyphal cells, spetins are major proteins of the cell wall. Septins are filament-forming GTP-binding proteins and were first identified for their roles in cell-cycle progression in *S. cerevisiae*. With *C. albicans*, septins localize at the sites of germ tube emergence at the early stage of cell division, and later a fraction of the molecules appear to persist at the growing tips over extended. Interestingly, other cell cycles begin during hyphal development. The DNA moves and separates in the germ tubes (hyphal form is tighter than that of pseudohyphal cells) [19].

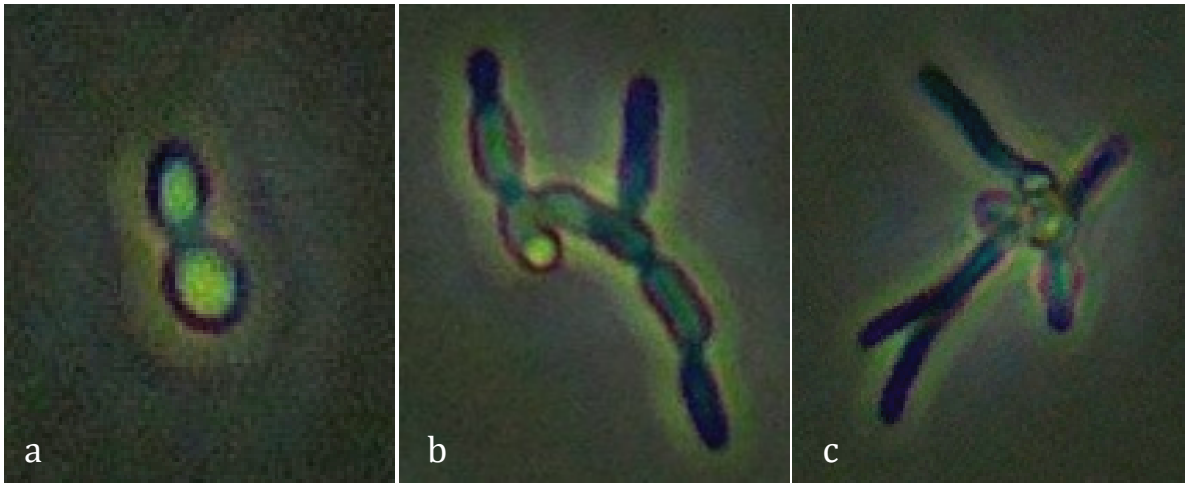

Figure1-2: *C. albicans* morphogenesis: a) Yeast cell, b) pseudohyphal, c) hyphe. This figures is adapted from our laboratory work.

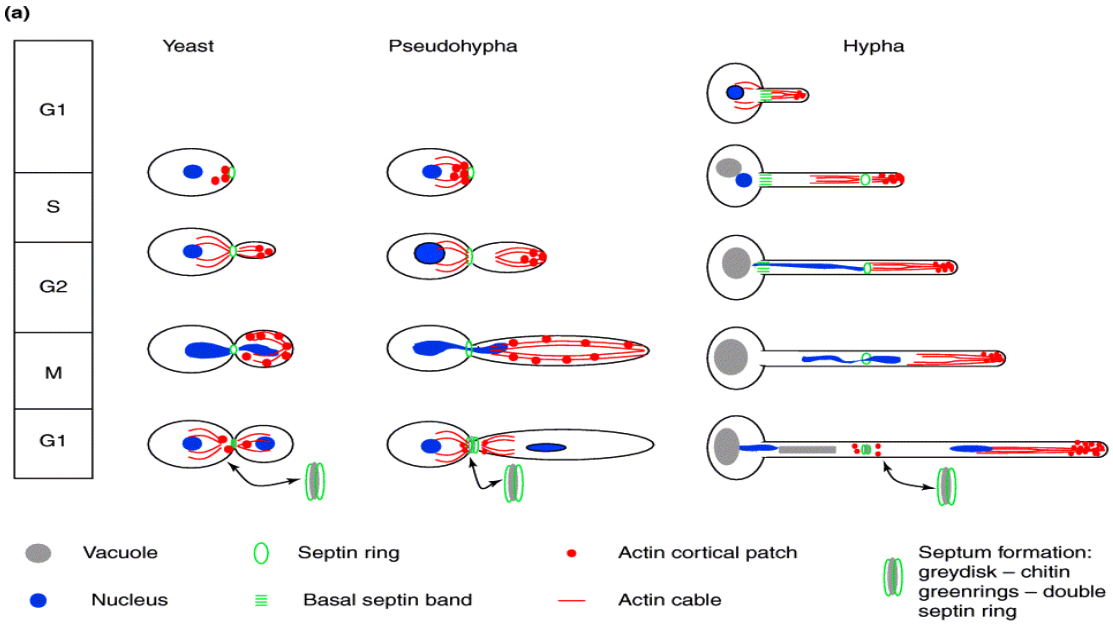

Figure 1-3: *C. albicans* cell cycle. Cell cycle of yeast and the first cell cycle of hyphae and pseudohyphae induced from unbudded yeast cells. Hyphal germ tubes emerge before the G1/S transition. This figure is adapted from Sudbery P and Gow N, Berman J. (Trends in microbiology. 2004;12:317-324.) [104].

1.1.3 *C. albicans* biofilms

Typical biofilm formation occurs when the microorganism attaches to a surface and/or to another microorganism and produces a protective extracellular matrix against the oral immune defense. This biofilm organization consists of survival mechanisms [20]. The ability to adhere to different abiotic and biotic structures is the first step of biofilm formation. This is supported by *C. albicans* morphological transition from blastospore to hyphal form, along with extra-polymeric materials [21]. It has been reported that microorganisms growing into biofilms are well protected against antimicrobial agents and that they are different than their planktonic (floating) counterparts [21]. Generally, infection by *C. albicans* biofilms on different surfaces (medical devices, human tissues) is modulated by several factors including different yeast species [22]. *C. albicans* has a tendency to grow more into biofilms than do other *Candida* species (*C. parapsilosis, C. pseudotropicalis, and Candida glabrata*). Furthermore, *C. albicans* forms biofilms more on silicone elastomer than on polyvinylchloride (PVC) [23]. In addition, the presence of conditioning film consisting of host saliva proteins was shown to facilitate biofilm formation on denture acrylic [24].

C. albicans biofilms are a mixture of extracellular matrix engulfing *C. albicans* cells in the form of blastospores and hyphae. Biofilm formation takes place through different yet sequentially organized stages (Figure 1-4). Microorganisms initially attach to available surfaces and after a certain time, the planktonic microorganisms undergo the morphogenesis process with significant surface adsorption through a thin extracellular polymeric material (Figure 1-1). The microorganisms will then proliferate and interact with each other to ensure their sustainability in the imposed environment. Through this proliferation, a thick extracellular matrix (mature biofilm) is formed to provide chemical as well as physical protection to the microorganisms. The formed biofilm then undergoes focal dispersion through specific signals, resulting in microbial release (Fig.1-1). The released microorganisms are then free to spread to other locations and form new biofilm [4-7].

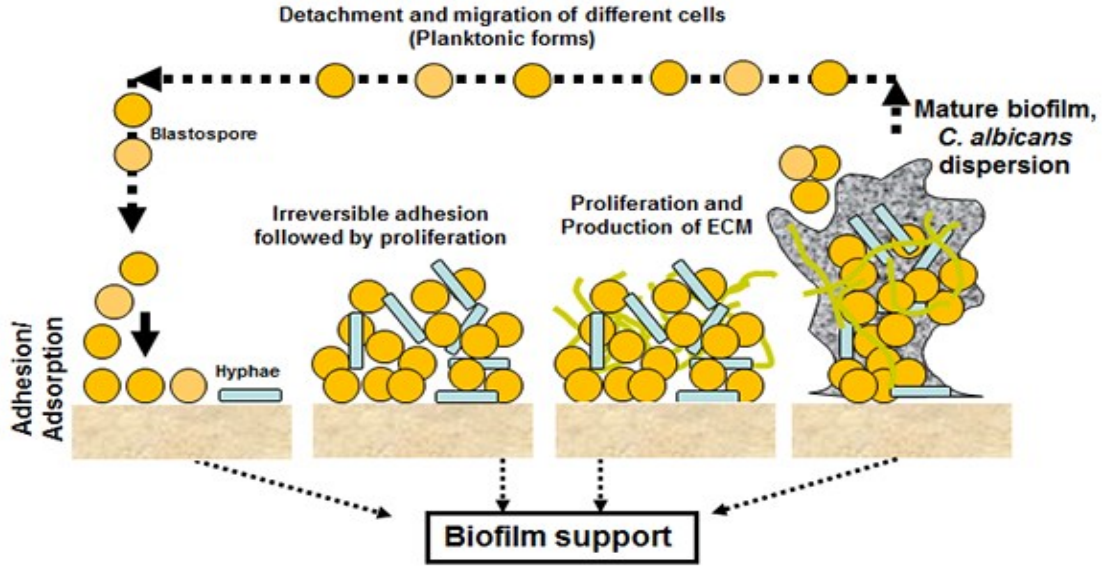

Figure 1-4: The different steps leading to biofilm formation. This figure is adapted from Rouabhia and Chmielewski (The Open Mycology Journal, 2012, 6, 27-32); showing the sequential formation of *C. albicans* biofilm.

1.1.3.1 *C. albicans* biofilm formation (phenotype switching)

The survival mechanism of *C. albicans* is the morphogenesis from blastospore to hyphal form and vice versa. This mechanism, known as 'phenotypic switching' from round to elongated form, confers to *C. albicans* the capacity to invade the host. In hyphal form, *C. albicans* is more virulent [9,25,26], adheres better to human tissue, and penetrates up to the basal layer. This is regulated by specific factors including fibrinogen-like and fibronectin-like receptors and non-specific factors such as hydrophobic or electrostatic interactions [15]. Phenotypic switching of *C. albicans* also involves different proteolytic enzymes (Saps) and specific signaling molecules [27].

1.1.3.1.1 Adhesion factors

Biochemical examination of the adhesion stage of *C. albicans* reveals the involvement of two key proteins, namely, enhanced adherence to polystyrene (Eap) and hyphal wall protein (Hwp) [9].

a) The Eap1 protein

Eap1, an adhesion protein, was first discovered in *S. cerevisiae* then in *Candida* [28]. Both Eap1 and Als proteins have a structure similar to that of the substrate-binding domain located in the N-terminus. In addition, there are tandem repeats in the central region and the C-terminus contains a GPI anchorage sequence. Studies on *eap1*Δ/Δ null mutants of *C. albicans* and the expression of *C. albicans EAP1* in *S. cerevisiae* revealed that Eap1 mediates adherence to epithelial cells and polystyrene [28,29]. Eap1 is also involved in biofilm formation both in vitro and in vivo. However, Eap1p is not known to be involved in *C. albicans* virulence during candidiasis.

b) Hwp1

Hwp1 is a GPI protein expressed on the surface of hyphae. This 9dhesion mediates the interaction of *C. albicans* with human oral epithelial cells through a specific adhesion mechanism [30,31]. Indeed, the N-terminal region of Hwp1 acts as a specific substrate for epithelial cells through small proline-rich proteins. Hwp1 reportedly mediates epithelial cell adherence by functionally mimicking host cell proteins [30,31]. The knockout of Hwp1 was shown to lead to reduced *C. albicans* virulence in vivo, suggesting the involvement of Hwp1 in *C. albicans* pathogenesis [32]. Hwp1 can interact with other adhesions, such as Als1 and Als3, and can mediate the adherence of one *C. albicans* hypha to another. This interaction contributes to biofilm formation [33].

c) Chitin

Adhesion of *C. albicans* to different biotic and abiotic structures is also mediated by chitin [34]. It has four synthase iso-enzymes (Chs1, Chs2, Chs3, and Chs8) controlling chitin synthesis through protein phosphorylation. For instance, the phosphorylation/de-phosphorylation of Chs3 at the S139 site (on the tips of growing buds and hyphae) regulates the position of Chs3 in *C. albicans* cell walls during cell cycle. On the other hand, the inactivation of Chs1and Chs2 can trigger *C. albicans* cell death [35, 36]. Chitin synthase can be affected by the BNI4 gene, which promotes *C. albicans* cell wall shape to hyphal form by increasing chitin expression. The deletion or mutation in the BNI4 gene leads to a significant change in *C. albicans* cell shape and reduces the hyphal form [36]. Chitins have been found to play a role in *C. albicans* adhesion to corneocytes. This adhesion involves chitin receptors known as lectin-recognizing chitins [34,38].

1.1.3.1.2 Transition factors and regulatory pathways

Multiple factors such as serum, high temperature, pH, N-acetylglucosamine, and CO_2 contribute to *C. albicans* phenotypic changes through various signaling pathways, including cAMP/PKA [38,39] [table 1-1]. Indeed, transition from blastospore to hyphal form is regulated by two major temporal pathways: the MAPK signal transduction pathway involving CPH1; and the Efg1 pathway which requires cAMP modulation [41].

These two pathways are also inter-regulated via positive and/or negative interactions that modulate hyphal development. For example, tup1 was found to repress hyphal formation through Rbf1p and Int1p (41). Changes in cell polarity, cell cycle, and cell wall architecture are virulence factors that appear throughout hyphal development [41]. Many studies confirmed that pseudohyphals were linked to an extension of the G2 phase. They may also be linked to a delay related to the synthesis of active cyclin-dependent kinase (cdk) complex coupled with Cdc28p phosphorylation [41]. Ste20-like protein and Cla4p are involved in merging

transduction signals which are needed for hyphal development and polarity modifications [41]. Morphological changes from blastospore to hyphal form are also regulated by Efg1 and Efh1 [39]. Hyphae facilitate *C. albicans* adhesion to tissue through $\alpha 5\beta 1$-like fibronectin receptors [40, 42].

Table 1-1: The environmental condition and pathways involved in filamentous growth regulation in *C. albicans* (adapted from Huang et al., [105])

Environmental Condition		**Pathways and Regulators**
Positive regulators	Serum, GlcNAc, starvation, poor nutrition (spider medium), glucose,	Ras1 → Cyr1, Bcy1 → cAMP → PKA (Tpk1 and Tpk2) → Efg1, Flo8 → hyphal specific genes (Hwp1, Ece1, etc.)
	CO2	Cyr1, Nce103
	Serum, spider medium	Ras1 → Cst20 → Cst11 → Hst7 → Cek1, Cek2 → Cph1, Tec1; Efh1; Hgc1; Ssn6; Cph2 → Tec1
	pH	Rim101, Phr1, Phr2
	GlcNAc	Ngt1 → Hxk1 → Dac1 → Nag
	Osmotic stress	Cst20 → Cst11 → Hst7 → Cek1, Hog1; Ssk1
	Physical interaction	Czf1, Mkc1
	Hypoxic conditions	Czf1, Efg1, Sch9
	Rapamycin	Tor1
Negative Regulators	Farnesol	Ras1 → Cyr1, Bcy1 → cAMP → PKA (Tpk1 and Tpk2) → Efg1
	Hypoxic conditions	Cyr1, Efg1, Flo8 Tup1, Nrg1, Rfg1, Rbf1

1.1.3.2 Gene expression of biofilm formation

C. albicans growth and biofilm formation are controlled by various genes [43], such as HSP90, TEF3, ADH1, PYK1, RP10, and GFA, along with other genes that are expressed as a response to morphological changes. One of these, HYR1, is expressed when *C. albicans* changes from yeast to hyphal form through a modulation of the hyphal cell wall protein [43]. Moreover, UME6 is found to be specifically related to the hyphal form, contributing to biofilm formation through two signaling partners, namely, Hgc1 and Sun41. UME6 is responsible for the expression of 5 filament-specific transcripts (HYR1, HWP1, RBT4, ECE1, and ALS3). UME6 and these transcripts are affected by Efg1 and Sun4 (figure 1-5) [44].

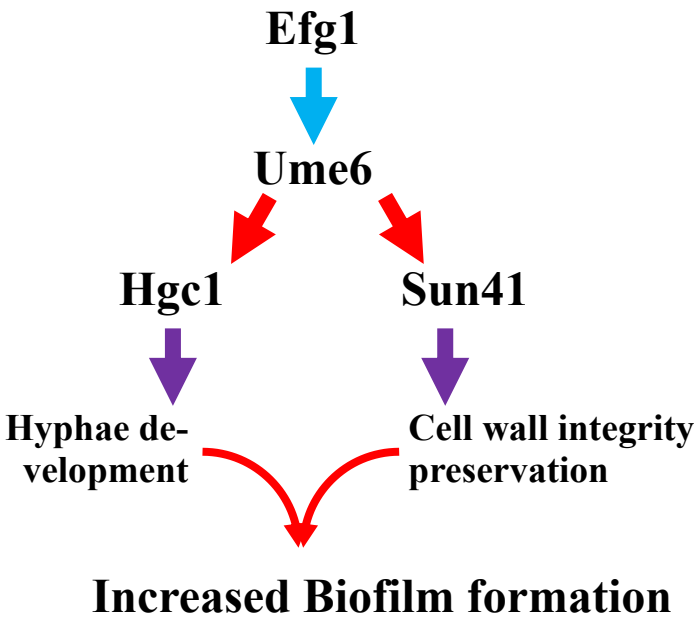

Figure 1-5: Roles of Efg1, Hgc1 and Sun41 in UME6-enhanced *C. albicans* biofilms formation. This figure is adapted from Banerjee et al.; [44].

1.1.3.3. Factors contributing to biofilm dispersion

Multiple factors contribute to biofilm dispersion. Among these is nutrition. Nutrition limitation mediates mature biofilm dispersion and reduces the capacity of detached *candida* cells to adhere and to form biofilm elsewhere [45]. Biofilm dispersion is the opposite of biofilm formation and means the detachment of *C. albicans* cells from the biofilms. Medium pH is another factor contributing to biofilm dispersion. It was reported that *C. albicans* biofilms tend to grow at acidic rather than alkaline pH which triggers the lateral yeast proliferation out of the upper-most hyphal layers of the *C. albicans* biofilms [45]. This leads to the overexpression of CaPES1 which supports the dispersion of biofilms [45]. Of interest is that the presence of bacteria as a co-culture with *C. albicans* reduces *C. albicans* biofilm formation [46]. A decreased *C. albicans* biofilm formation refers to the inhibition of the activities of certain genes involved in *C. albicans* adherence and morphology transition. These genes include Sap5, Als3, Ece1, and Hwp1. However, co-culture of two *C. albicans* strains (such as *C. albicans* 53 and 163) leads to increased biofilm formation [46,47]. Heat shock proteins play a critical role in biofilm formation. Exposure of *C. albicans* to high temperatures leads to an upregulation of HSP104 expression. This was suggested as a therapeutic target contributing to *C. albicans* biofilm inhibition [48].

RLM1 transcription factor also plays also a key role in *C. albicans* adhesion and biofilm formation because it manages and deals with different environmental stresses. Indeed, RLM1 has been found to participate in the recognition of *C. albicans* cell wall damage and to maintain cell integrity through the MAP kinase pathway by

- balancing the *C. albicans* polysaccharide cell wall;
- modifying the link between β-glucan, mannoproteins, and chitin; and
- producing β-,3-glucan synthase complex [49].

1.1.3.4. Biofilm resistance

Compared to growth as individual cells (planktonic form), growth as a biofilm is always more responsible for microbial resistance against antimicrobial agents [50,51]. *Candida* grown in biofilms escapes/resists anti-fungal drugs better compared to single *Candida* cells. This is linked to the capacity of *C. albicans* to adapt and to acquire many anti-fungal resistance mechanisms to maintain or increase its virulence. The presence of a thick extracellular matrix contributes to preventing antifungal agents from reaching the yeast cell. For example, β-1,3-glucans work as a barrier against anti-fungi drugs and thus impede attacks on biofilms [52]. This anti-fungi resistance of *C. albicans* biofilm is also demonstrated by the activation of certain genes. For example, FKS1 is essential for drug resistance, as it produces matrix β-1,3-glucans, thereby increasing bio-film formation and protecting *C. albicans* cells in this biofilm [53].

Biofilm may contain one or more species. It has been confirmed that mixed-species biofilm (such as fungus plus bacteria) is more resistant to antimicrobial activity compared to single-species biofilm. This may be linked to the various extracellular matrix compounds secreted by the fungi and the bacteria [54].

1.1.4 *C. albicans* infection

C. albicans infection begins with the yeast's adhesion, growth, and invasion of the host immune system. Once it overcomes this immune system, *C. albicans* adopts hyphal forms to facilitate the invasion [55]. In this way, *C. albicans* penetrates the host cells and reaches the blood to ultimately disseminate throughout the patient's body (figure 1-6) [17,56]. There are several causes leading to *C. albicans* infections. These causes include antibiotic treatment (which disrupts the normal flora population), immunosuppression, etc. [57,58]. The most prominent diseases promoting *C. albicans* infection are AIDS and diabetes. With AIDS, the disorder

is related to the immune system, causing flaws in immune cells, while in the case of diabetes, the carbon source is increased, contributing to *C. albicans* nutrition and survival [57,58].

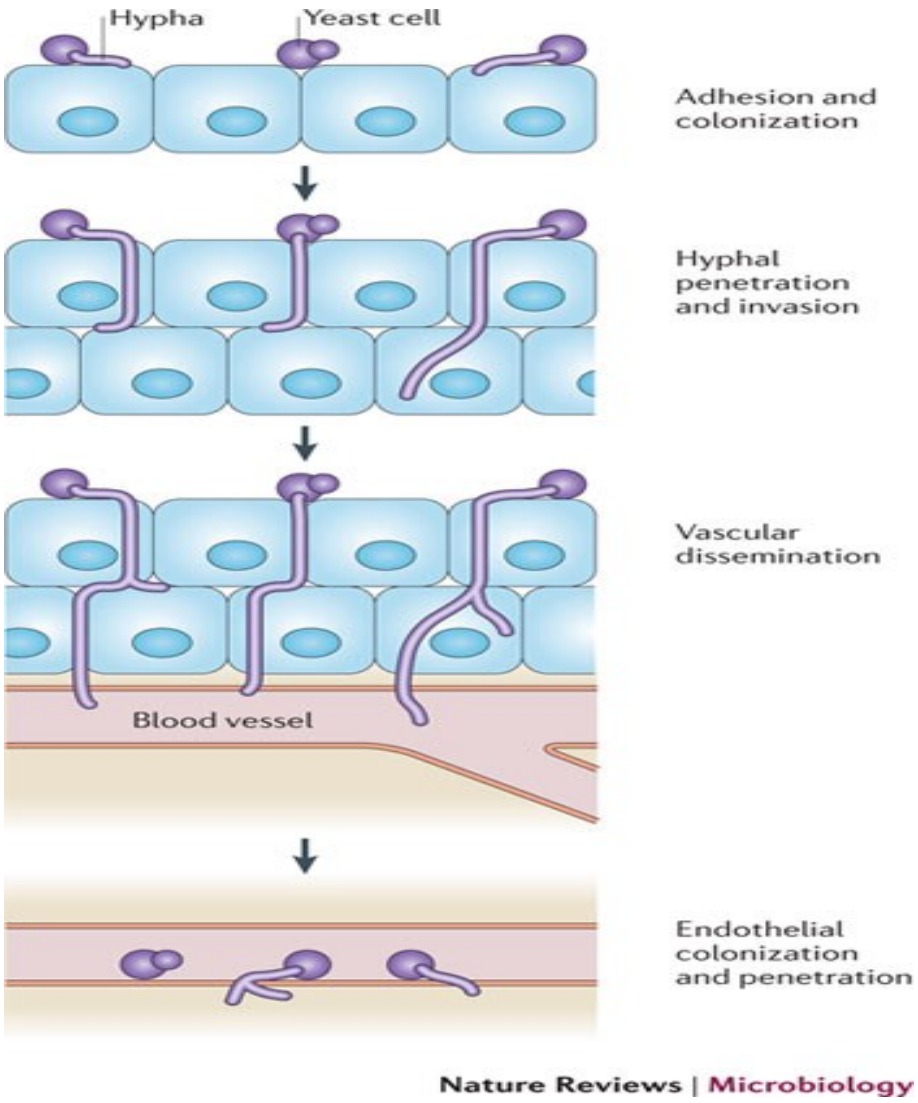

Figure 1-6: *C. albicans* invasion through epithelial cells. This figure is adapted from Gow and van de Veerdonk . Nature reviews. Microbiology, 2012; 10: 112-122. [106].

1.1.4.1 *C. albicans* and the host

In certain situations, *C. albicans* has a strong capacity to target the host innate immune system and to take advantage of it, converting from a commensal condition to an infection with an adaptation system that enables it to render the host suitable for its virulence purposes and ultimately reaching subendothelial matrix, endothelial cells, and keratinocytes [59,57].

1.1.4.2 *C. albicans* and different host tissues

In pathological conditions, *C. albicans* penetrates the tissue through its hyphae or can be endocytotic by epithelial cells. *C. albicans* can also secrete proteolytic enzymes, leading to cell/tissue destruction and yeast invasion [60]. It has been shown that *C. albicans* endocytosis by epithelial cells takes place through an endocytotic process. In blood, *C. albicans* can be phagocytized by leukocytes then with blood circulation which lead to the infection of various body sites [60]. To penetrate cells, *C. albicans* first interacts with them through different molecules, including N-acetylglycosamine glycoside, which is expressed by human cells such as human buccal or vaginal epithelial cells (protein-sugar interaction) [61]. Integrin is also a key protein interacting with *C. albicans* proteins CR2 transmembrane protein (CR2) or heterodimeric transmembrane protein (CR3) [61]. *C. albicans* also interacts with arginine-glycine-aspartic acid (RGD) peptides that present at the surface of epithelial and endothelial cells [Table1-2] [61].

Table 1-2: Candida-host recognition system. RGD is the amino acid sequence arginine glycine-aspartic acid. EMP is an extracellular matrix protein. This table is adapted from Calderone R. Trends in microbiology. 1993;1:55-58. [61].

Recognition system	*Candida*	Host cell	Type of host cell
I	Lectin	Fucose or GlycNAC glycosides	Epithelial
II	CR2 or CR3	RGD of EMP	Endothelial
III	Factor6	?	Epithelial
IV	Chitin	?	Epithelial

1.1.4.3 *C. albicans* adaptation

C. albicans displays unique properties in its adaptation process. It is able to take advantage of its interaction with the host to use all available host niches [57]. This adaptation system provides key factors to assist *C. albicans* during the transition (from commensal to pathogenic) through fungus cell wall modification, metabolism, and shape conversion [57]. When infectious conditions are available, *C. albicans* uses the host nutrition to produce the most virulent behaviours. For example, changing the sugar (carbon) source has been found to modulate *C. albicans* resistance by modifying the cell wall architecture through specific remodeling enzymes (figure 1-7) [62]. Moreover, *C. albicans* acquires many virulence factors during the metabolic adaptation in the host. Indeed, the change from yeast to hyphal form is affected by glucose. The production of aspartic proteinase (SAP) is also affected by glucose/nitrogen sources [63,64]. *C. albicans* can also bypass the host immune system surveillance. This may involve cell wall alteration, which can influence host immune sensing of PAMPs (figure 1-8) [57,65].

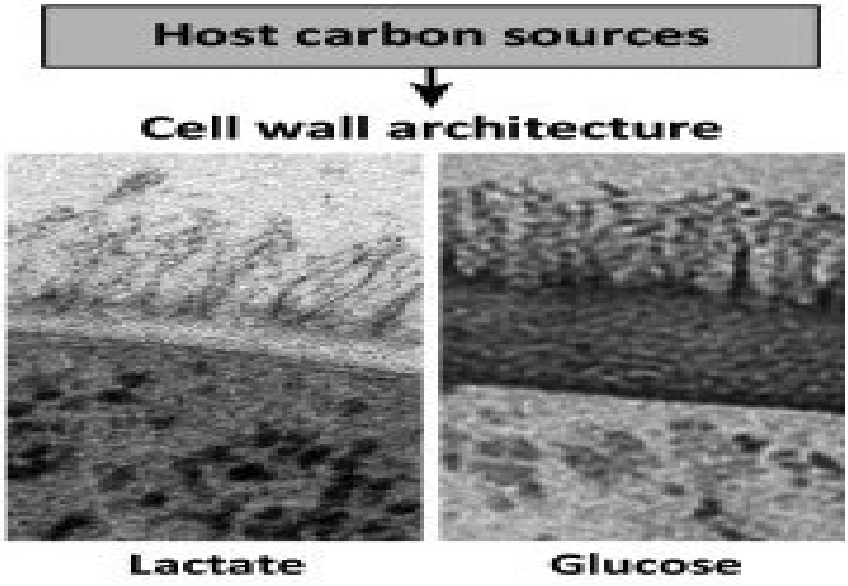

Figure 1-7: Changes in carbon source lead to major changes in cell wall architecture. This figure is adapted from Brown A and Brown G. Trends in microbiology. 2014 pii: S0966-842X(14)00139-5.[57]

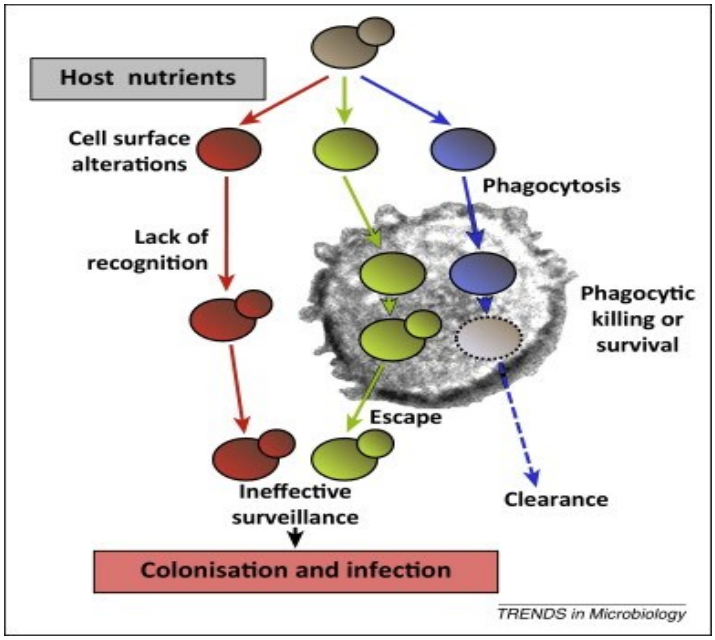

Figure1- 8: Changes in carbon source impact on immune surveillance by altering the recognition of *C. albicans* cells. This figure is adapted from Brown A and Brown G. Trends in microbiology. 2014pii:S0966-842X(14)00139-5.[57]

1.1.4.4 *C. albicans* and host immune response

C. albicans can dominate the different host tissues to reinforce its presence as an infection [59]. *C. albicans* escapes from the innate immune system by modulating and binding to different regulators such as H and H-like protein 1 factors [59]. C4BP and host extracellular matrix (ECM) proteins undergo lysis via plasmin stimulation through the binding of Gpm1 to plasminogen which later binds to *C. albicans* surface protein GPM1 (phosphoglycerate mutase1, which has glycolysis and plays a glyconeogenic role), factor H, and FHL-1 [59]. Furthermore, *C. albicans* also takes advantage of vitronectin (a part of ECM) to adhere and to grow [59].

Neutrophils are the most active cells against *C. albicans*. This involves different recognition receptors such as TLR2, TLR4, and mannose receptor (N-mannan) [66]. The binding of PRRs by their PAMPs leads to fungus recognition through various pathways that trigger protein profile changing (table 1-3). Interestingly, a discriminatory recognition pathway of *C. albicans* was discovered during infection and linked to the presence of a hyphal cell threshold promoting epithelial MAPKinase pathway activation (figure 9) [66].
Following *C. albicans* infection, epithelial cells secrete high levels of cytokines and chemokines, including IL-1α/β, IL-6, G-CSF, GM-CSF, TNF, RANTES, IL-8, and CCL20, and produce IL-12, IL-1α/β and TNF-α [67]. Of course, many other cells (neutrophils, dendritic cells, and T cells) contribute to the immune defense against *C. albicans* infection by increasing their anti-*Candida* properties [67].

Table 1-3: pattern recognition receptor that sense fungal –associated PAMPs. This figure is adapted from Moyes D and Naglik J. Clinical & developmental immunology. 2011;2011:1-9. [66]

Family	Receptor	PAMP
TLRs	TRL2	Phosoholipomannan
	TRL3	Double-stranded RNA Mannan
	TRL4	O-linked MAnnan residues
	TRL9	CPG DNA
CLRs	Dectin-1	B-1,3-glucan
	Dectin-2	High-mannose structures A-mannan
	Mannose receptor	Mannan
	MINCLE	Unknown
	Galectin-3	B-1,2-Mannosides
	DC-SIGN	High-mannose structures
NLRs	NLRP3	Unknown
Others	Cdw17	Unknown

1.1.5 *C. albicans* and oral cavity

In healthy conditions, the presence of *C. albicans* in the oral cavity is part of normal flora alongside many bacteria to maintain a microbial balance [68]. However, in certain conditions, this balance can be deregulated, resulting in infection in the oral cavity [69]. Many oral infections are caused by *C. albicans*. For example, one oral disorder caused by *C. albicans* is denture-related stomatitis which is an adhesion of *C. albicans* to dentures and the formation of biofilms leading to chronic inflammation [69]. Denture-related stomatitis is affected by patient age, smoking, oral hygiene, and the presence of yeast [69,70]. It is identified as an oral disorder known as oral candidiasis.

1.1.5.1 Oral candidiasis

Oral candidiasis is an oral infection most often caused by *C. albicans* which forms white patches and inflammation on the oral mucosa and can cover the tongue and other oral regions. This is due to the presence of large quantities of *C. albicans* in the biofilm. *C. albicans* overgrowth and biofilm formation is also the consequence of a reduced immune response (Table 1-4) [20]. For example; the compromised immune system in HIV patients is the primary reason for frequent *C. albicans* colonization causing oral candidiasis through the combination of fungi and bacteria [71]. Oral candidiasis groups pseudomembranous candidiasis or thrush, erythematous candidiasis, characterized as a red patch on the upper posterior part of the tongue, and hyperplastic candidiasis, identified as small, smooth, white areas changing to larger, dense, rough, and opaque plaques [71].

Table 1-4: Risk factors leading to oral candidiasis. This table is adapted from Rautemaa R and Ramage G. Critical reviews in microbiology. 2011;37:328-336. [20]

Decreased saliva production
Smoking
Atrophic oral mucosa
Mucosal diseases (Oral lichen planus)
Topical corticoids
Decreased blood supply (radiotherapy)
Impaired systemic defense mechanisms
Diabetes
Primary or secondary immunodeficiency
Immunosuppressive medication
Malnutrition
Infants (immature immunity)
Altered or immature oral flora
Yeast favoring
antibiotic treatment
narrow spectrum mouthwashes
high alcohol consumption
reflux, low pH
high carbohydrate diet
artificial materials (dentures)
diabetes
Infants
Poor oral hygiene
Mixed oral biofilms on non-renewing surfaces: teeth, dentures, intubation tube, tongue jewellery

1.1.6 *C. albicans* and risk factors

Oral candidiasis can be caused by a disruption in the oral microflora that affects oral *C. albicans* carriage and impairs local defense mechanisms. This disruption occurs following a decrease in saliva production, as well as by systematic diseases, antibiotic treatment, and tobacco smoke [21].

1.1.6.1 Saliva and oral candidiasis

Saliva has a role in fighting *C. albicans* infection. Because saliva contains many proteins, it can fight the presence of *C. albicans* infection with an anti-fungi composition [72]. The presence of *C. albicans* in the oral cavity for a long time due to decreased saliva flow can make oral candidiasis infection easier [73]. It has been demonstrated that placing epithelial cells with saliva inhibits *C. albicans* adhesion to these cells [74]. Thanks to specific adhesion proteins, *C. albicans* cannot adhere to free saliva [34]. Saliva thus has at least two major roles:

1- Continuous saliva flow contributes to eliminating non-adherent fungus and promotes the clearance activity [42]. Saliva composition also has an effect on *C. albicans* colonization; for example, the quantity of glucose present can affect the tendency by *C. albicans* to grow better, which explains the high probability of oral candidiasis in diabetic patients.

2- Saliva contains several non-specific defense factors capable of modulating the presence of *C. albicans* [75]. Both low salivation and low saliva quality contribute to *C. albicans* binding between cells and denture acrylic surfaces and increase the proliferation of the fungus [42]. Thus any decrease in saliva flow or volume will promote *C. albicans* adhesion and colonization in the oral cavity. Furthermore, a decrease in saliva quantity and quality alters the oral microflora which play a role in *Candida*

resistance to antifungal treatments [72,74]

1.1.6.2 Systematic diseases and oral candidiasis

Another factor contributing to *C. albicans* overgrowth is systematic disease. It has been demonstrated by many studies that 90% of HIV patients suffer from oral candidiasis [76]. Connections were found between oral candidiasis and immunologic mechanisms (such as T cells) in AIDS patients [77]. Another systematic disease that promotes oral candidiasis is diabetes mellitus due to the reduced capacity of neutrophils to phagocytose and kill *C. albicans* [78,79]. Moreover, oral candidiasis in persons with diabetes mellitus is the result of an increased glucose level in the blood and saliva which renders the oral cavity more attractive to *C. albicans* colonization, in addition to the host's suppressed immune system [80]. Children who have leukemia reportedly suffer from oral *Candida* infections, which may be due to the leukemia itself or to the chemotherapy and/or radiotherapy treatments which have an impact on immune dysfunction leading to *C. albicans* colonization and infection [81].

1.1.6.3 Antibiotic treatment and oral candidiasis

Another cause of oral candidiasis is the inadequate use of antibiotic treatments. It has been found that antibiotic treatment (wide-spectrum antibiotics) leads to *C. albicans* infection which takes advantage of a disrupted oral microflora [82,73]. This may be due to an inhibition of the anti-fungal immunity, including decreased phagocytosis which disrupts the equilibrium between *C. albicans* carriage and normal flora [83,84]. Dysregulation leading to oral candidiasis can also be promoted by external agents such as smoking.

1.1.6.4 Tobacco smoke and oral candidiasis

Cigarette smoke (CS) has been found to have a strong impact on oral cavity microflora leading to microbiome deregulation which may promote the growth of pathogenic microorganisms [85]. For example, CS has been shown to increase biofilm formation [86]. Several studies have suggested a link between oral *Candida* carriage and CS, as a strong percentage of smokers carry *Candida*, in contrast to non-smokers. It was suggested that CS provides an appropriate environment for *Candida* to grow and colonize the host. Furthermore, CS constituents such as aromatic hydrocarbon contents may serve to feed *C. albicans* [87]. CS-induced oral candidiasis may thus occur through a decrease in gingival cell immune activities. Indeed, CS reduces the level of oral antimicrobial peptide activity [87,88] which in turn leads to *C. albicans* growth and biofilm formation [87,86]. CS also appears to promote the production of lysis enzymes (such as phospholipase) by *C. albicans,* thereby contributing to tissue degradation as well as *C. albicans* invasion [89]. *C. albicans* virulence following exposure to CS can occur through gene activation. Indeed, HWP1, EAP1, and SAP2 gene expression was shown to increase in the presence of CS [90]. Thus CS can trigger oral candidiasis by activating various pathways that not only induce *C. albicans* virulence but alter the host's defense response. CS may also affect the interaction between *C. albicans* and host/cells, resulting in *C. albicans* overgrowth and infection [90,87].

1.1.7 Cigarette smoke (CS)

CS contains approximately 4000 chemicals (including chemical toxicants) which are found in the cigarette itself and include cigarette burning. Moreover, two types of smoke are achieved through the burning phase: the main stream (filtered) that the smoker produces by inhaling the cigarette and the side stream (unfiltered) which is the smoke emanating from the end of the cigarette bar (second hand smoke). Nicotine (another cigarette component) is responsible for the addiction. Other chemicals (N-nitrosamines, carbon monoxide, aldehydes hydrogen cyanide, polyaromatic hydrocarbons, and nitrogen oxides) are also present in cigarette smoke and are proven harmful [91,92].

1.1.7.1 Effect of cigarette smoke on human health

The different chemicals present in cigarettes are reported to be cytotoxic, mutagenic, carcinogenic, or anti-genic (innate and adaptive immunity). Entering the human body in different ways, including the oral cavity, nasal passages, and airway mucosa, these chemicals are reported to be responsible for genetic alterations in cell cycle, DNA damage, and tumor suppressor gene dysregulation (figure 1-9) [93,88]. Smoking is re-sponsible for 5 million human deaths each year [94].

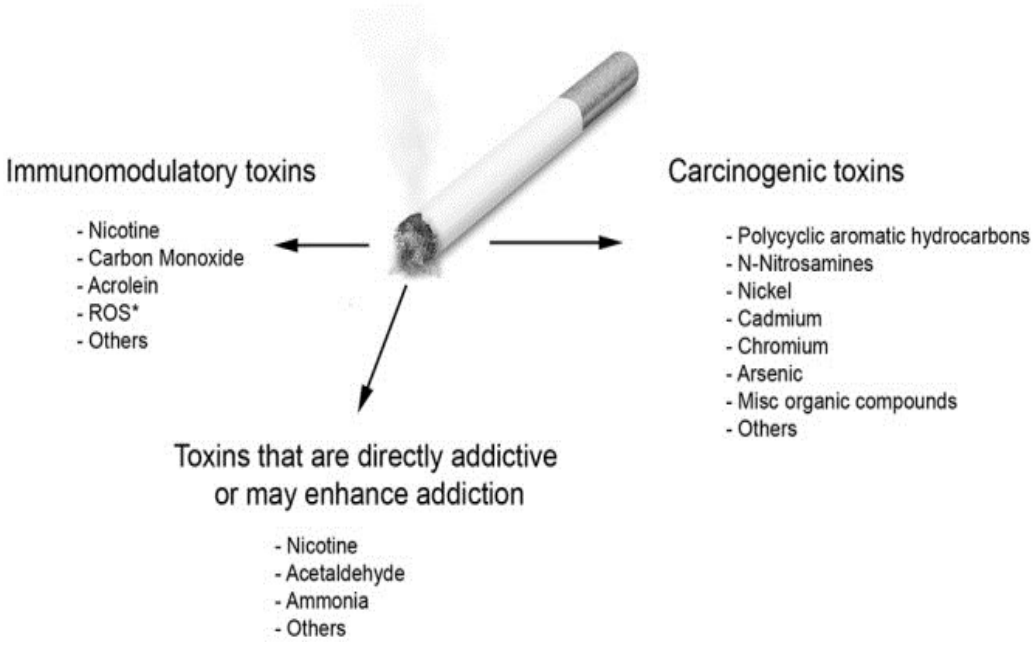

Figure 1-9: The harmful components of the cigarette. This figure is adapted from Journal of dental research. 2012;91:142-149. [93]

Burning one cigarette produces thousands of reactive oxygen species (ROS) which can destroy airway epithelial cell linings through the activation of intracellular signaling pathways (figure 1-10), induce the inflammatory response by increasing IL-8 and TNFα secretion, and finally, deregulate neutrophil function and fibroblast activity. CS also contributes to the activation of the inflammatory response through Toll-like receptors (TLRs), mitogen-activated protein kinases (MAPK), nuclear factor kappa-B (NF-κB), signal transducer and activator of transcription (STAT), and activator protein-1 (AP-1) [93,88]. Immune system dysregulation may thus explain cancer development in smokers [88].

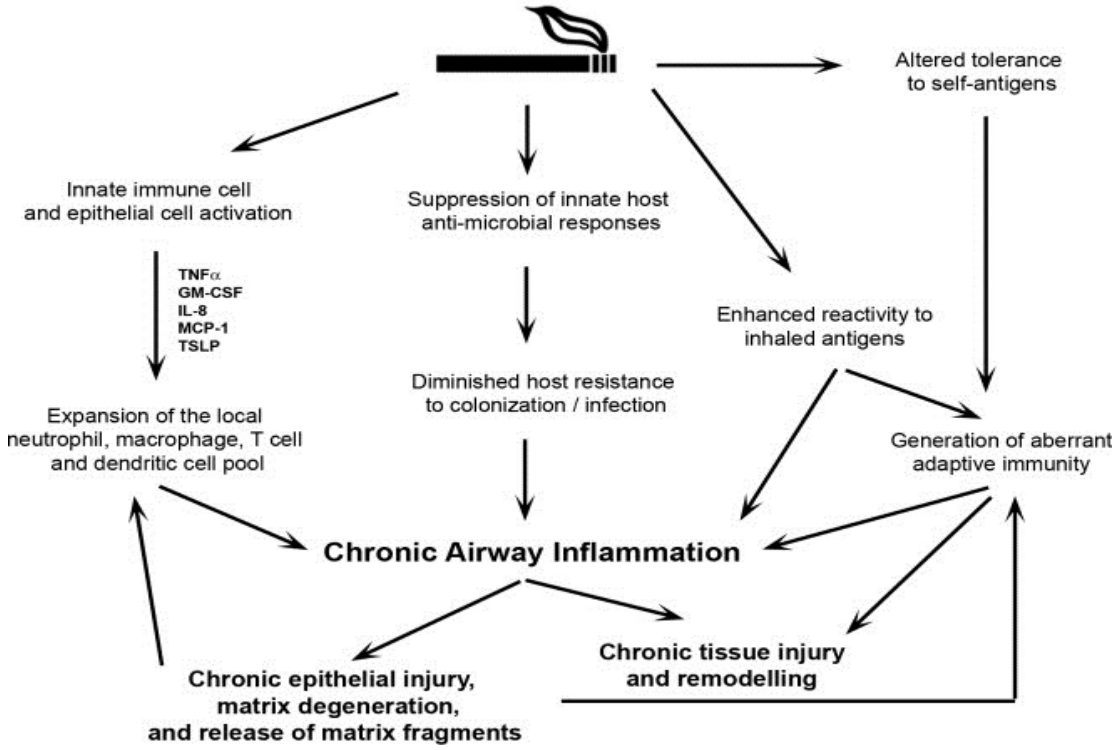

Figure1-10: Cigarette smoke modulates inflammation and promotes chronic inflammation in the conducting airways by a variety of mechanisms. This figure is adapted from Journal of dental research. 2012;91:142-149. [93]

1.1.7.1.1 Cigarette smoke and cancer

Research has shown that smoking is responsible for 25% of deaths in men and 4% of deaths in women by cancer. Tobacco-related cancers can be found in the kidneys, liver, stomach, uterine cervix, and breasts [95]. For example, several studies showed that breast cancer develops more in long-term female smokers than in non-smokers. Indeed, tobacco smoke can contribute to metastasis in the lung, as breast cancer cells are capable of adhering to the lung endothelium via endothelial PAF (platelet-activating factor) [96]. Multiple studies also report a link between tobacco and other cancers, such as esophageal cancer, laryngeal cancer and oral cancer [98].

1.1.7.1.2 Cigarette smoke and heart diseases

In the United States, cigarette smoke is responsible for 30% of deaths by coronary heart disease (CHD) [99] and incriminated in cardiovascular disease (CVD). Exposure to cigarette smoke in the form of second hand smoke also raises the incidence of cardiovascular disease [100]. Heart surgery failures, myocardial re-infarctions, and death are also exacerbated by smoking [100]. CS also causes coronary thrombosis, as it alters the role of endothelial cells, platelets, fibrinogen, and coagulation factors and leads to an altered equilibrium of the antithrombotic/prothrombotic and profibrinolytic/antifibrinolytic factors involved in heart attacks [101].

1.1.7.1.3 Cigarette smoke and respiratory diseases

Smoking is highly involved in respiratory diseases and causes significantly more health problems than does cancer [95]. The entire lung organ is affected by cigarette smoke, which promotes chronic obstructive pulmonary disease (COPD). Smokers run a greater risk of infection and lung dysfunction. Indeed, smokers have been shown to be more sensitive to respiratory tuberculosis, influenza, and pneumonia, with a greater percentage of deaths compared to non-smokers [95]. This is probably due to a reduced immune defense caused by smoking [98].

1.1.7.1.4 Cigarette smoke and oral diseases

The oral cavity is the first organ in contact with CS which is responsible for many oral diseases, including cancer. Oral cancers were found to increase 3 to 5 times more among smokers than in non-smokers and also in a dose-depending manner (number of smoked cigarettes/day and smoking years) [94]. CS promotes

periodontal disease (half caused by smoking). Furthermore, CS decreases tissue healing following oral surgery [102]. Moreover, much evidence suggests a link between smoking and tooth loss, inadequate dental restorations, and dentures changing color. Smoking also affects taste, promotes tooth decay [94], and promotes chronic gingivitis (inflammation and gingival bleeding) and acute necrotizing ulcerative gingivitis [94,103]. CS also alters the oral microflora, converting non-pathogenic *C. albicans* to pathogenic, resulting in oral candidiasis [94].

1.1.8 Hypotheses and objectives

Hypotheses

1. *C. albicans* may take advantage of the various products present in cigarette smoke to adhere, grow, and increase its infective capacity.
2. Cigarette smoke can reduce gingival cell defense reponses against *C. albicans* infection.

Objectives

Although some groups have reported a link between cigarette smoke and oral candidiasis, multiple questions remain as to the direct effect of cigarette smoke on *C. albicans* and human cells, and human cell interactions with *C. albicans* when present in the oral cavity. Our specific objectives were thus to investigate

1. The effect of cigarette smoke condensate (CSC) on *C. albicans* adhesion/growth and transition from blastospore to hyphal form;
2. The effect of CSC on *C. albicans* chitin production;
3. The effect of cigarette smoke-pretreated *C. albicans* on adhesion and growth following contact with gingival fibroblasts; and
4. The effect of cigarette smoke-pretreated *C. albicans* on gingival fibroblast proliferation and cytokine secretion.

Chapter II

Publication:

Cigarette smoke-exposed *Candida albicans* increased chitin production and modulated human fibroblast cell responses

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Abstract

The predisposition of cigarette smokers for development of respiratory and oral bacterial infections is well documented. Cigarette smoke can also contribute to yeast infection. The aim of this study was to investigate the effect of cigarette smoke condensate (CSC) on *C. albicans* transition, chitin content, and response to environmental stress, and to examine the interaction between CSC-pretreated *C. albicans* and normal human gingival fibroblasts. Following exposure to CSC, *C. albicans* transition from blastospore to hyphal form increased. CSC-pretreated yeast cells became significantly ($p<0.01$) sensitive to oxidation, but significantly ($p<0.01$) resistant to both osmotic and heat stress. CSC-pretreated *C. albicans* expressed high levels of chitin, with 2 to 8 folds recorded under hyphal conditions. CSC-pretreated *C. albicans* adhered better to the gingival fibroblasts, proliferated almost three times more, and adapted into hyphae, while the gingival fibroblasts recorded a significantly ($p<0.01$) slow growth rate but a significantly higher level of IL-1β when in contact with CSC-pretreated *C. albicans*. CSC was thus able to modulate both *C. albicans* transition through the cell wall chitin content as well as the interaction between *C. albicans* and normal human gingival fibroblasts. These findings may be relevant to fungal infections in the oral cavity in smokers.

2.1 Introduction

Mucosal candidiasis, especially the oropharingeal type (OPC), is a common opportunistic infection in both immunocompromised and immunocompetent persons [1]. The leading cause of candidiasis is *Candida albicans*, a commensal dimorphic yeast that colonizes up to 60% of normally healthy individuals [2]. Symptomatic OPC appears under a number of predisposing conditions [3, 4]. From these, tobacco smoking was considered predisposing to oral candidiasis [5]. Indeed, epidemiological studies in immunocompromised patients have identified tobacco as a major risk factor for symptomatic infection [6, 7]. Furthermore, the rate of oral *candida* carriage in tobacco smokers was reported to be higher in smokers than in non-smokers [8, 9]. This may explain why 98% of Indian villager's smokers suffer *candidal* leukoplakia that can be resolved after cessation of tobacco [10, 11].

The exact mechanism by which *candida*l carriage may be affected by tobacco is still to be discovered. Previous studies have demonstrated that smoking lead to innate immune reduction facilitating *candida* colonization and host infection [12]. This may suggest the use of tobacco compounds by *C. albicans* as nutritional factors, since aromatic hydrocarbons contained in cigarette smoke can be converted by *Candida* species to carcinogen end products [13]. It has also been reported that *C. albicans* can catalyze the formation of *N*-nitrosobenzylmethylamine supporting the high *candida*l leukoplakia level in smokers [14].

During the development of candidiasis, *C. albicans* adheres to and invades the tissue [15, 16]. Tis adhesion is promoted by yeast cell wall proteins [17]. *Candida* cell walls contain glucans, mannans, glycoproteins and chitins [18-20]. In *C. albicans*, three genes encoding different chitin synthases (*CHS1, 2 and 3)* were identified. Chitin production is dependent on the experimental culture condition. Indeed, the *CHS2* and *CHS3* are preferentially expressed under hyphal culture condition [21, 22], *whereas, CHS1* remained at low levels in

both yeast and hyphae [23]. Interestingly, Chs1p was found to be essential for cell integrity and virulence [23]. Mutants defective in chitin proteins are less virulent than the parental strain in a mouse model. Thus, with high level of chitin, *C. albicans* can overcome cigarette smoke effects, and escape the host immune defense [22].

Gingival fibroblasts are major actor in the host immune defense against *Candida* infection [24, 25]. Gingival fibroblasts play role in tissue structure and function [26]. They are active in the inflammatory response by secreting inflammatory cytokines, such as IL-6 and IL-8, as well as IL-1 in response to stimuli that include periodontopathic bacteria [27, 28]. The secretion of these cytokines may, thus be modulated when fibroblasts are exposed to cigarette smoke.

Exposure to smoke-derived toxicants has been shown to lead to immune dysfunction [29]. Smokers thus run a greater risk of contracting invasive diseases caused by various bacterial pathogens [30-32]. Exposure to cigarette smoke has also been shown to induce the formation of biofilm by various oral/respiratory pathogens *in vitro*, including *Porphyromonas gingivalis, Staphylococcus aureus, Streptococcus. pneumoniae, Klebsiella pneumonia* and *P. aeruginosa*, as well as *Streptococcus mutans* [30-32]. Cigarette smoke was also reported to increase *C. albicans* adhesion and growth, as well as biofilm formation [33, 34]. These effects were supported by the overexpression of *EAP1*, *HWP1*, and *Sap2* genes known to be active players in *C. albicans* virulence [34]. By modulating *C. albicans* adhesion, proliferation and biofilm formation in vitro, cigarette smoke may also modulate the yeast interaction with human cells such as fibroblasts. The aim of this study was thus to investigate the effect of cigarette smoke condensate on *C. albicans* transition from blastospore

to hyphal form, its response to environmental stress, its production of cell wall chitin, and its interaction with human gingival fibroblasts.

2.2 Experimental protocol

2.2.1 *Candida albicans*: *C. albicans* SC5314, known to be strongly invasive [35] was cultured for 24 h on Sabouraud dextrose agar plates (Becton Dickinson, Oakville, ON, Canada) at 30°C. For the *C. albicans* suspensions, one colony was used to inoculate 10 ml of Sabouraud liquid medium supplemented with 0.1% glucose, pH 5.6. The cultures were then grown in a shaking water bath for 18 h at 30°C, after which time the yeast cells were collected, washed with phosphate-buffered saline (PBS), counted by means of a haemocytometer, and adjusted to 10^6/ml prior to use.

2.2.2 Gingival fibroblast isolation and culture: Small biopsies of lamina propria tissue (gingival connective tissue) were collected from healthy non-smokers (18–25 years old) following their informed consent in accordance with Laval University Ethics Committee guidelines. To isolate the gingival fibroblasts, the connective tissue was placed in a collagenase P solution (0.125 U/ml; Boehringer Mannheim, Laval, QC, Canada) for 45 min at 37°C under gentle agitation. The isolated cells (2×10^5) were seeded in 75-cm^2 flasks (Falcon, Becton-Dickinson, Cockeysville, MD, USA) and grown in Dulbecco's modified Eagle's (DME) medium (Invitrogen) containing 10% fetal calf serum (Invitrogen Canada Inc., Burlington, ON, Canada). Upon 90% confluence, the gingival fibroblasts were used between passage two and four for the different experiments.

2.2.3 Preparation of the cigarette smoke condensate: 1R3F cigarettes were purchased from the Kentucky Tobacco Research & Development Center (Orlando, FL, USA) and used to prepare the cigarette smoke condensate solution. Each cigarette was placed into one end of a silicone tube linked to an Erlenmeyer flask

containing 200 ml of 0.09% sodium chloride. On the other end, a second silicone tube linked to the Erlenmeyer was connected to a standard vacuum. The cigarette was attached to the cigarette holder and lit and the smoke was extracted by applying vacuum, pulling the smoke directly into the 0.09% sodium chloride solution. The procedure was repeated with a total of ten whole cigarettes. The resulting cigarette smoke condensate (CSC) solution was then sterilized by filtration through a 0.22-µm filter and subsequently stored at 4°C until use.

2.2.4 Effect of CSC on *C. albicans* transition from blastospore to hyphal form: To determine the effect of CSC on the yeast-to-hyphae transition we used qualitative and quantitative assays. *C. albicans* (10^5 cells) was grown in 3 ml of Sabouraud dextrose broth supplemented with 0.1% glucose and 10% fetal bovine serum (FBS) with or without CSC at various concentrations (10, 30, and 50%). The negative controls refer to the *C. albicans* cultures without CSC. Different conditions were tested: (1) the hyphae-inducing conditions were previously reported [30], consisting of culture medium supplemented with 10% fetal calf serum followed by incubation at 37°C; (2) culture at 37°C, without serum; (3) culture in the presence of 10% serum at 30°C; and (4) culture at 30°C without serum. Following incubation for 3 or 6 h, the cultures were observed microscopically and photographed to record *C. albicans* morphology (n = 5). The density of the *C. albicans* transition was also measured. Furthermore, the length of the hyphal forms in each condition was measured by means of NIH-ImageJ software.

2.2.5 Effect of CSC on *C. albicans* response to stressful agents: To investigate the effect of CSC on *C. albicans* sensitivity/resistivity, we exposed the yeast cells to oxidative, osmotic or heat stress. To do so, *C. albicans* cells were cultured in Sabouraud with or without CSC at various concentrations (10, 30, or 50%). The culture medium was adjusted to have the same level of nutriments as did the control (absence of CSC).

Cultures were incubated in a water bath at 30°C for 24 h under agitation. Following the incubation, the cells were counted with a haemocytometer, after which time 10^6 cells/ml were treated with hydrogen peroxide (5, 10 or 50 mM) or NaCl (1.2 M) or heat treated at 45°C. Incubation under oxidative, osmotic or heat stress took place for 60 min under agitation. The cells were then washed twice with sterile PBS and suspended thereafter in Sabouraud medium at 10^6cells/ml. The sample volume referring to 10^3 or 10^4 of each condition was spotted on Sabouraud agar and incubated at 30°C and colony growth was monitored at 24 and 48 h. Culture on a PDA plate lacking the disrupting agents was also performed and used as a growth control. Cell viability following contact with oxidative, osmotic or heat stress was evaluated by means of the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) which measures viable cells as a function of mitochondrial activity as we previously reported [34]. Absorbance (optical density, OD) was subsequently measured at 550 nm by means of an xMark microplate spectrophotometer (Bio-Rad, Mississauga, ON, Canada).

2.2.6 Effect of CSC on *C. albicans* cell wall chitin content.

2.2.6.1 Cell wall isolation and purification: *C. albicans* in Sabouraud medium was cultured overnight in the presence or absence of CSC at various concentrations (0, 10, 30, or 50%). Sabouraud medium was supplemented or not with 10% fetal calf serum for hyphae culture conditions. Cells were collected by centrifugation at 1200 rpm for 10 min and were then used to extract cell wall proteins. The *C. albicans* pellets were suspended in 500 µl of sterile PBS and supplemented with 200 µl of glass beads (0.425-0.6 mm in diameter; Sigma-Aldrich, G9268). The samples were cooled at -80°C for 60 sec before being subjected to disruption by means of a MiniBead-beater (Biospec Products, Bartlesville, OK, USA) for 2 min at 5,000 rpm. The samples were then cooled for 2 min at -80°C and the disruption cycle was repeated 15 times. The cells were then washed twice with 1 M NaCl and extracted thereafter in an SDS-MerOH extraction buffer (50 mM Tris, 2%

sodium dodecyl sulphate (SDS), 0.3 M β-mercaptoethanol, and 1 mM EDTA; pH 8.0) at 100°C for 15 min. The resulting pellets were washed three times with 1 mM of PMSF in distilled water and the walls were freeze-dried, weighed, and used to determine the chitin level in each condition.

2.2.6.2 Chitin quantification: The amount of chitin present in the cell wall of each sample was determined as described previously [36]. Briefly, 50 mg of each cell wall were hydrolyzed in 6N HCl for 16 h at 100°C. Following evaporation at 65°C, the samples were dissolved in sterile water (1 ml). From this solution, 0.1 ml was supplemented with 0.1 ml of 1.5N Na_2CO_3 in 4% acetylacetone, and boiled (100°C) for 30 min, followed by the addition of 0.7 ml of 96% of ethanol to each sample. A volume of 0.1 ml of a solution containing 1.6 g of dimethylaminobenzaldehyde in 30 ml of HCl and 30 ml ethanol was added to each sample and the mixture was incubated for 1 h at 37°C in a dark atmosphere. Samples (3 x 200 ml) were aliquated into a 96-well plate at 200 µl/well and three wells per condition. Absorbance was measured at 520 nm by means of an xMark microplate spectrophotometer (Bio-Rad, Mississauga, ON, Canada) and compared with a standard curve of 0-200 mg of glucosamine. The glucosamine level in each sample was presented as the percentage of cell wall dried weight (n = 4).

2.2.7 Evaluation of CSC-pretreated *C. albicans* adherence to a human gingival fibroblast monolayer: To determine whether CSC promoted *C. albicans* adherence to gingival fibroblasts, cells were seeded into 6-well plates and grown at 37°C in a 5% CO_2 atmosphere until confluence, which was usually reached after four to five days. *C. albicans* was first treated with CSC at various concentrations for 24 h then washed twice with CSC-free culture medium. From this, 10^5 yeast cells were put in contact with a gingival fibroblast monolayer for 3 and 6 h at 30°C. Following each culture period, the infected and non-infected fibroblast monolayers were washed three times with PBS and subjected thereafter to crystal violet staining. One milliliter of 1% w/v

crystal violet solution in demineralized water was then added and the cultures were further incubated at room temperature for 15 min. Following incubation, the non-bound dye was removed from the wells by thorough washing with demineralized water, followed by drying at 37°C. Stained cells were then observed under an inverted microscope and the adherent *C. albicans* cells/clusters were counted and plotted as the number per mm^2 of fibroblast monolayer culture.

2.2.8 Effect of CSC on the interaction of *C. albicans* with gingival fibroblasts

2.2.8.1 Evaluation of CSC-pretreated *C. albicans* transition following contact with human gingival fibroblasts: To determine whether the CSC pre-treatment enhanced *C. albicans* transition when cultured with human gingival fibroblasts, *C. albicans* was grown in the presence of CSC at various concentrations for 24 h; 10^5 CSC-pretreated yeast cells were layered onto a confluent fibroblast monolayer under hyphae-promoting conditions. This refers to the presence of 10% serum in the medium and incubation at 37°C [37] for 6 h. At the end of this incubation period, the cultures were washed twice with warm PBS and subsequently stained with crystal violet dye, as described above. The stained cells were then observed and photographed (n = 4).

2.2.8.2 Evaluation of CSC-pretreated *C. albicans* and gingival fibroblasts growth: To determine the effect of CSC-pretreated *C. albicans* on gingival fibroblast growth and *vice versa*, a transwell (contact-independent) culture system was used. The lower chamber of this culture system was seeded with gingival fibroblasts. When the cultures reached confluence, CSC-pretreated *C. albicans* was seeded inside the upper chamber and integrated to the culture system. The interaction of the gingival fibroblasts with the *C. albicans* thus took place through the culture medium. The cultures were maintained at 37°C in a 5% CO2 humid atmosphere for either 24 or 48 h. Following each culture period, the *C. albicans* in the upper chamber was

collected separately, and cell density was determined by MTT staining, as described above. As for the gingival fibroblasts, they were collected from the lower chamber and their growth was determined using a haemocytometer (n = 5).

2.2.9 Effect of CSC-pretreated *C. albicans* on IL-1β secretion by gingival fibroblasts: To evaluate the level of IL-1 secreted by gingival fibroblasts stimulated or not with CSC-pretreated *C. albicans*, supernatants were collected from each condition after 24 and 48 h and were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA). The supernatants were collected in tubes containing one µl of a protease inhibitor cocktail for specific use with mammalian cell and tissue extracts (Sigma-Aldrich). Immediately after, the supernatants were centrifuged and used to measure IL-1β levels. ELISA plates were read at 450 nm and analyzed by means of a Microplate Reader Model 680 (Bio-Rad, USA). The minimum detectable concentration was under 1 pg/ml, as reported by the manufacturer. Each experiment was repeated four times and the means ± SD were calculated and presented.

2.2.10 Statistical analyses: Each experiment was performed at least four times, with experimental values expressed as means ± SD. The statistical significance of the differences between the control (absence of CSC, and or absence of *C. albicans*) and the test (presence of CSC alone or CSC-pretreated *C. albicans*) values was determined by performing a one-way ANOVA. Posteriori comparisons were performed using Tukey's method. Normality and variance assumptions were verified using the Shapiro-Wilk test and the Brown and Forsythe test, respectively. All of the assumptions were fulfilled. P values were declared significant at ≤ 0.05. The data were analyzed by means of the SAS version 8.2 statistical package (SAS Institute Inc., Cary, NC, USA).

2.3 Results

2.3.1 Cigarette smoke condensate promoted *C. albicans* transition from blastospore to hyphae forms:

Previously we reported t*hat CSC promoted C. albicans* growth [34]. *Candida* growth was supported by the transition from blastospore to hyphal form. As shown in Fig. 2-1A, form change was basically observable at 6 h of culture under specific hyphal conditions (presence of serum at 37°C). Indeed, hyphae numbers appeared to be greater with CSC than with the control. The transition was obtained with all CSC concentrations with the incubation at 37°C. Incubation time was also a factor, as hyphal forms were well-defined and were more frequent at 6 h than at 3 h of incubation. Hyphae size also differed in the presence of CSC when *C. albicans* was cultured in the presence of serum at 37°C. As shown in Fig. 2-1B, longer hyphal tubes were found at 3 and 6 h. It is important to note that CSC induced longer hyphae at as early as 3 h of incubation, with a significantly ($P < 0.05$) longer size observed compared to the control (absence of CSC). The hyphal tubes were longer at 6 h of incubation than at 3 h. Overall data thus demonstrate that cigarette smoke favored *C. albicans* growth and transition.

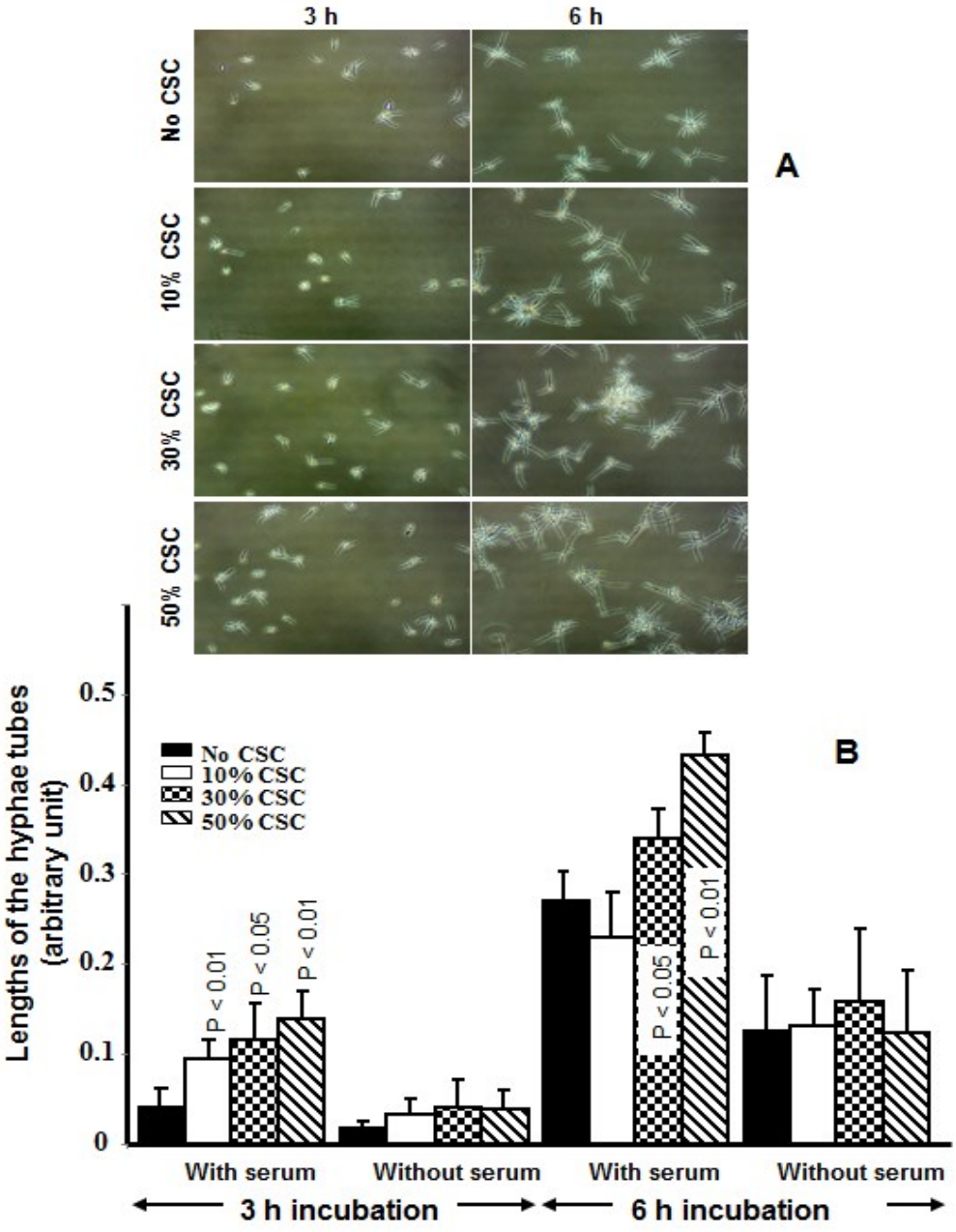

Fig. 2-1: *C. albicans* transition from blastospore to hyphal form following contact with CSC. *C. albicans* was cultured with or without CSC at various concentrations for 3 or 6 h at 37°C in a culture medium supplemented with 10% serum. Following each time point, the cultures were observed under an inverted microscope and photographed (Panel A). Hyphal tube length was measured by means

of NIH-ImageJ software (n = 5), and presented (Panel B). Significance was obtained by comparing the CSC-pretreated and untreated *C. albicans*.

2.3.2 CSC decreased *C. albicans* resistance to oxidative stress and increased its resistance to osmotic and heat stress: As shown in Fig. 2-2, following one hour of contact with 5 or 10 mM of hydrogen peroxide, *C. albicans* showed a marked decrease in resistance. Without CSC, a reduced colony size was observed following treatment, compared to non-treated samples. The decrease in colony size was greater when *C. albicans* was pre-cultured for 24 h with CSC. The effect was dependent on the hydrogen peroxide concentrations. It should be noted that at 50 mM of hydrogen peroxide, no colony was observed with either 5 or 10 μl of seeding volume (data not shown). Interestingly, when CSC-treated *C. albicans* was subjected to osmotic stress, an increased resistance was observed. As shown in Fig. 2-2, colony size was greater with 1.2 M NaCl treated *Candida* as compared to non-treated cells. The same observation can be made with heat stress (Fig. 2-2). These data were supported by cell viability as ascertained by MTT assay. As shown in Fig. 2-3, hydrogen peroxide significantly ($P < 0.05$) decreased the cell viability of non-primed or CSC-primed *C. albicans*. However, the decrease in cell viability was greater following hydrogen peroxide stress. Furthermore, this reduced cell viability was dependent on the hydrogen peroxide concentration used (Fig 3). Contrary to oxidative stress, osmotic and heat stress had no negative effect on cell viability. As shown in Fig.2-4A, osmotic stress led to a significant ($P < 0.05$) increase of *C. albicans* density, particularly with high levels of CSC (30 and 50%). The same effect was obtained under heat stress (Fig. 2-4B).

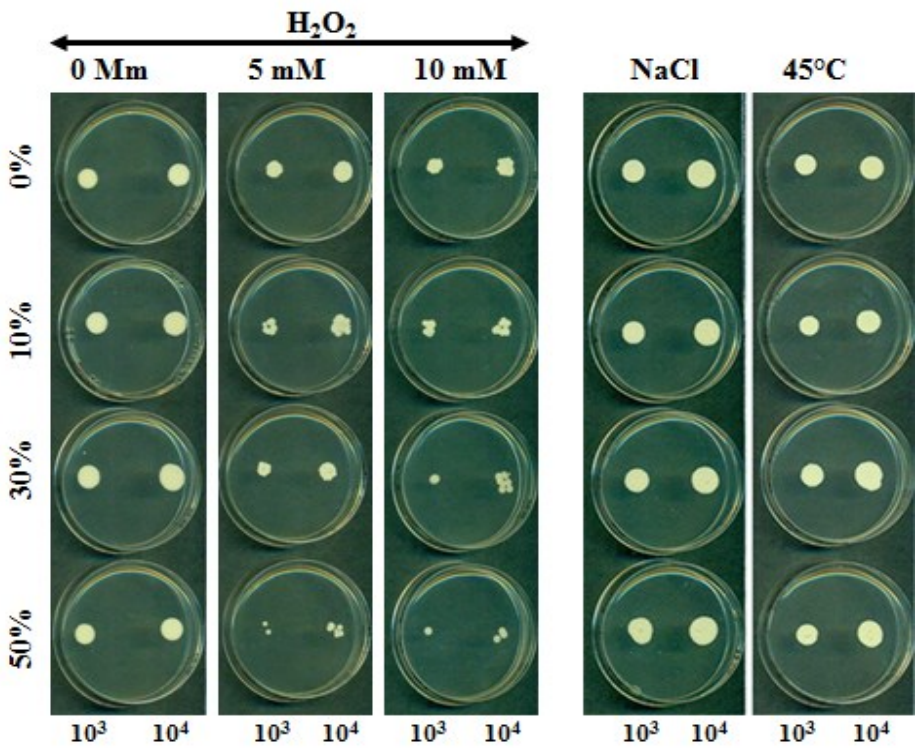

Fig. 2-2: Susceptibility of CSC-treated *C. albicans* to a cell surface-disrupting oxidative, osmotic and thermal stress agents. Cells were treated or not with CSC then washed and suspended in H_2O_2, NaCl or heat (45°C) for 60 min, then washed and spotted onto Sabouraud agar. Note the increased sensitivity of the CSC-treated *C. albicans* to 5 and 10 mM of H_2O_2, but the increased resistance to osmotic and thermal stresses.

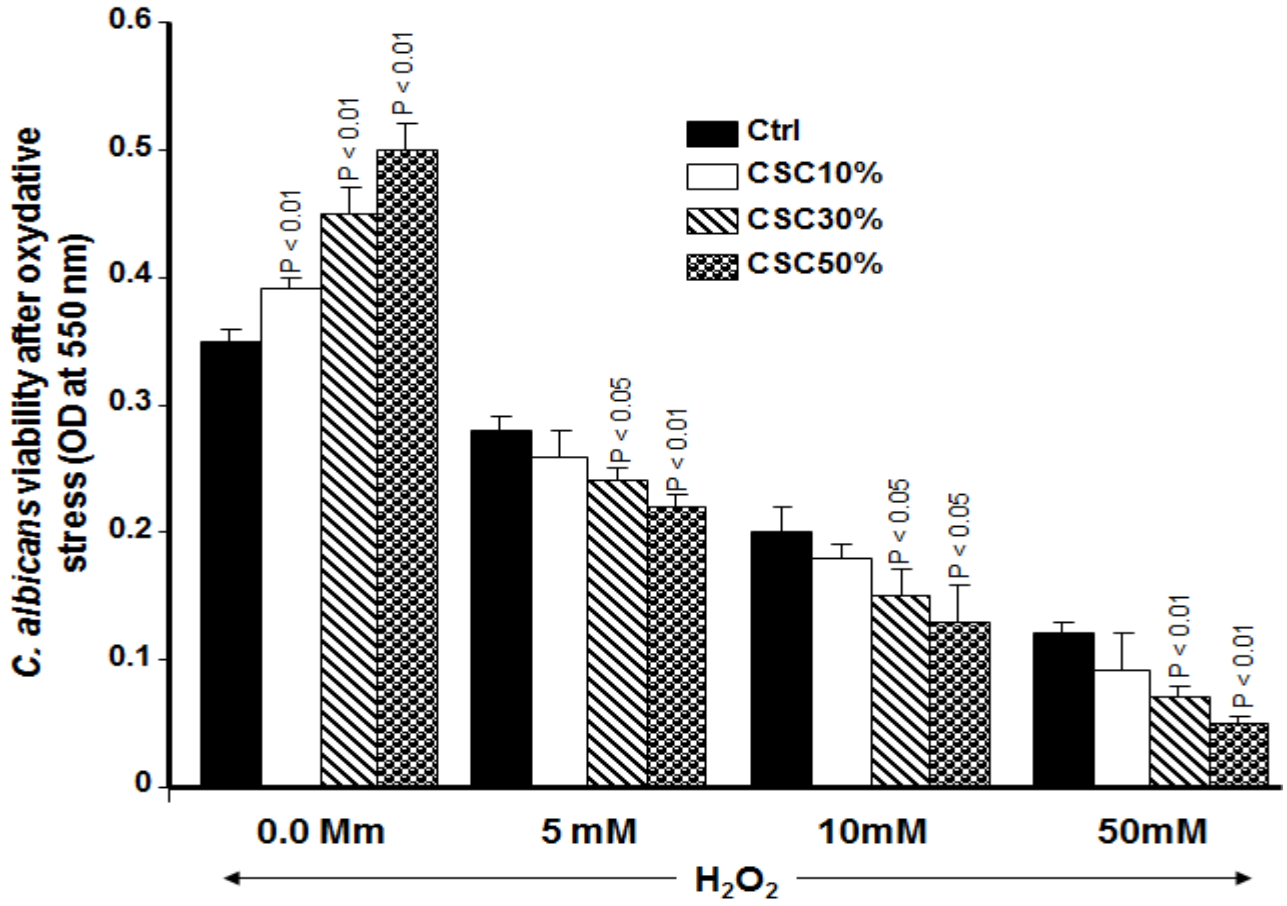

Fig. 2-3: Oxidative stress reduced the growth of the CSC-pretreated *C. albicans*. CSC-pretreated *C. albicans* was washed and 10^6 cells/ml were incubated with various concentrations of H_2O_2 for 1 h. The cells were then washed twice with warm medium and used to ascertain cell viability by MTT assay (n = 5). Significance was determined by comparing the CSC-pretreated and untreated *C. albicans*.

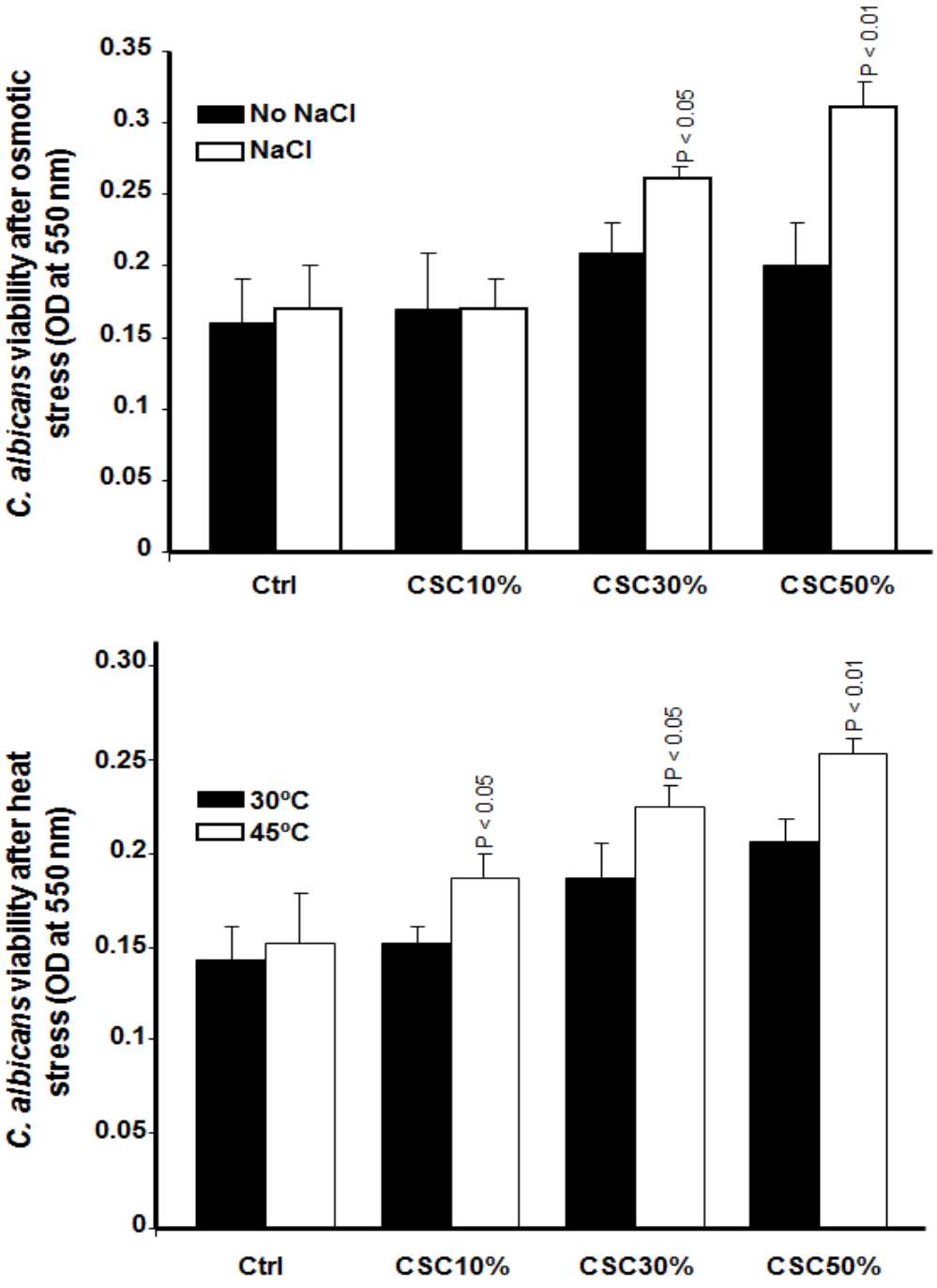

Fig. 2-4: Osmotic and heat stresses increased the growth of CSC-pretreated *C. albicans*. CSC-pretreated *C. albicans* was washed and 10^6 cells/ml were incubated with NaCl (1.2 M) or at 45°C for 1 h. The cells were then washed twice with warm medium and used to ascertain cell viability by

MTT assay (n = 5). Significance was determined by comparing the CSC-pretreated and untreated *C. albicans*.

2.3.3 Exposure of *C. albicans* to CSC increased chitin content: Fig. 2-5 reveals that the growth of *C. albicans* yeast cells in the presence of CSC resulted in elevated chitin content. Exposure of *C. albicans* to CSC under a non-hyphae-inducing culture condition (absence of serum and culture at 30°C) showed a slight increase and significant chitin content in CSC-exposed *C. albicans,* compared to that observed with the non-exposed cells. Chitin production thus increased under normal hyphae culture conditions compared to non-hyphae culture conditions, suggesting that hyphal conditions promoted chitin synthesis. When exposed to CSC, *C. albicans* cells showed significantly ($p < 0.01$) greater levels of chitin than did the control. This increase was more important with higher concentrations of CSC (Fig. 2-5).

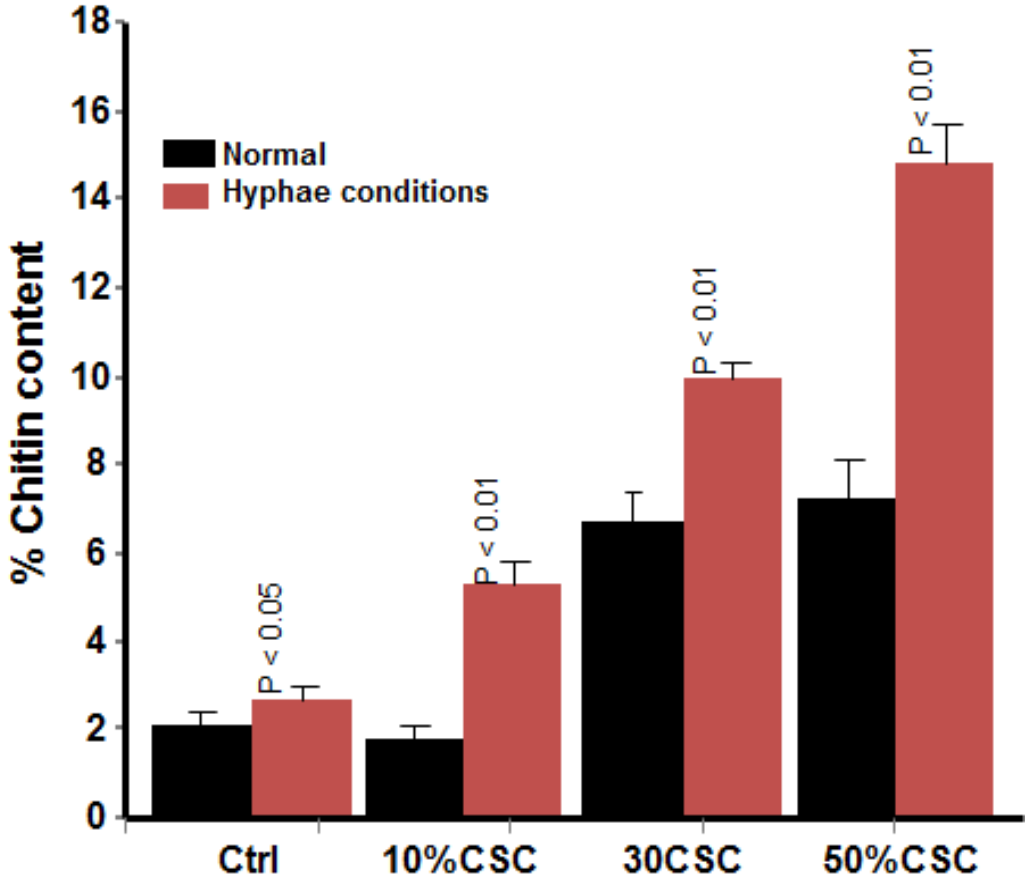

Fig. 2-5: Chitin content in the non-exposed and CSC-exposed *C. albicans*. The glucosamine released from the purified cell walls following acid hydrolysis was quantified and expressed as a percentage of cell wall dried weight. Data presented are from four independent assays performed in triplicate. Significance at $P < 0.05$ was determined by comparing the non-exposed and CSC-exposed yeast cells.

2.3.4 CSC-pretreated *C. albicans* adhered better and changed form when in contact with the gingival fibroblast monolayer: To investigate whether or not CSC modulated the interaction of *C. albicans* with human gingival fibroblasts, *C. albicans* was pretreated with CSC and its adhesion to a gingival fibroblast monolayer culture at 30°C was analyzed. As shown in Fig. 2-6A, an elevated number of adherent *C. albicans* colonies were observed in the gingival fibroblast monolayers placed in contact with CSC-pretreated *C. albicans*. These colonies contained more than four yeast cells with a mixture of blastospore and hyphal forms. Quantitative measurement of the colonies per mm^2 of fibroblast monolayer culture revealed (2-6B) a significantly high number of colonies in the CSC-pretreated conditions, compared to the CSC-untreated conditions. These high levels of adherent *C. albicans* on the gingival fibroblast monolayers were apparent with each tested CSC concentration. Furthermore, under the hyphae culture conditions, all of the *C. albicans* showed an elongated tube adhering to each culture surface, with a higher hyphal form density observed in the CSC-culture conditions compared to the non-CSC ones (Fig. 2-7). Overall data suggest that CSC promoted *C. albicans* adhesion and transition when interacting with human gingival fibroblast monolayers.

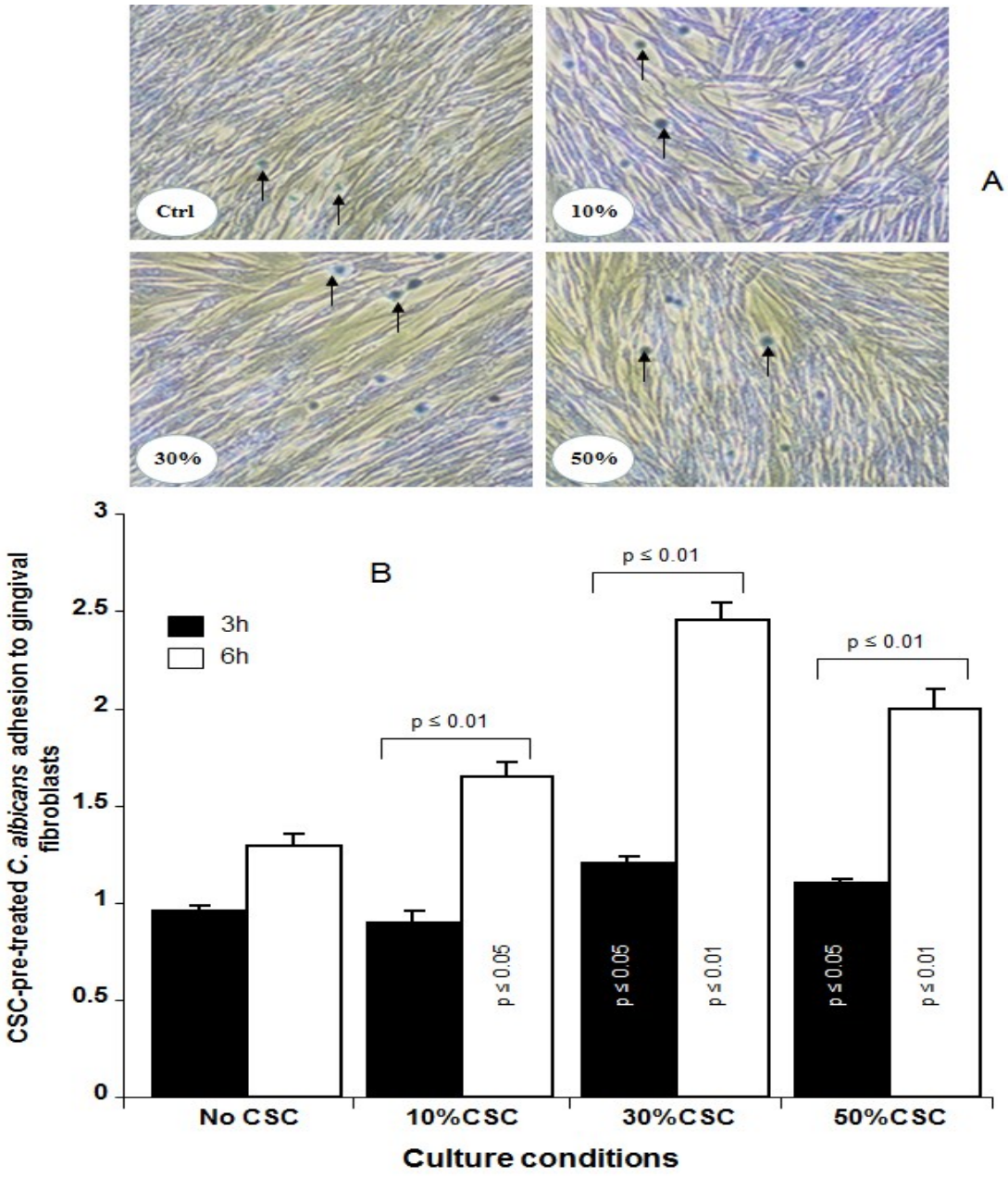

Fig. 2-6: CSC-pretreated *C. albicans* adhered more to gingival fibroblasts. *C. albicans* was incubated 24 h in the presence of CSC and then used to infect gingival fibroblast cultures for 3 or 6 h. Each culture condition was photographed following crystal violet staining (panel A, photos after 6 h). Adherent *C. albicans* colonies were counted under an inverted microscope and plotted (panel B, n = 4). Significance was obtained by comparing the CSC-pretreated and untreated *C. albicans*.

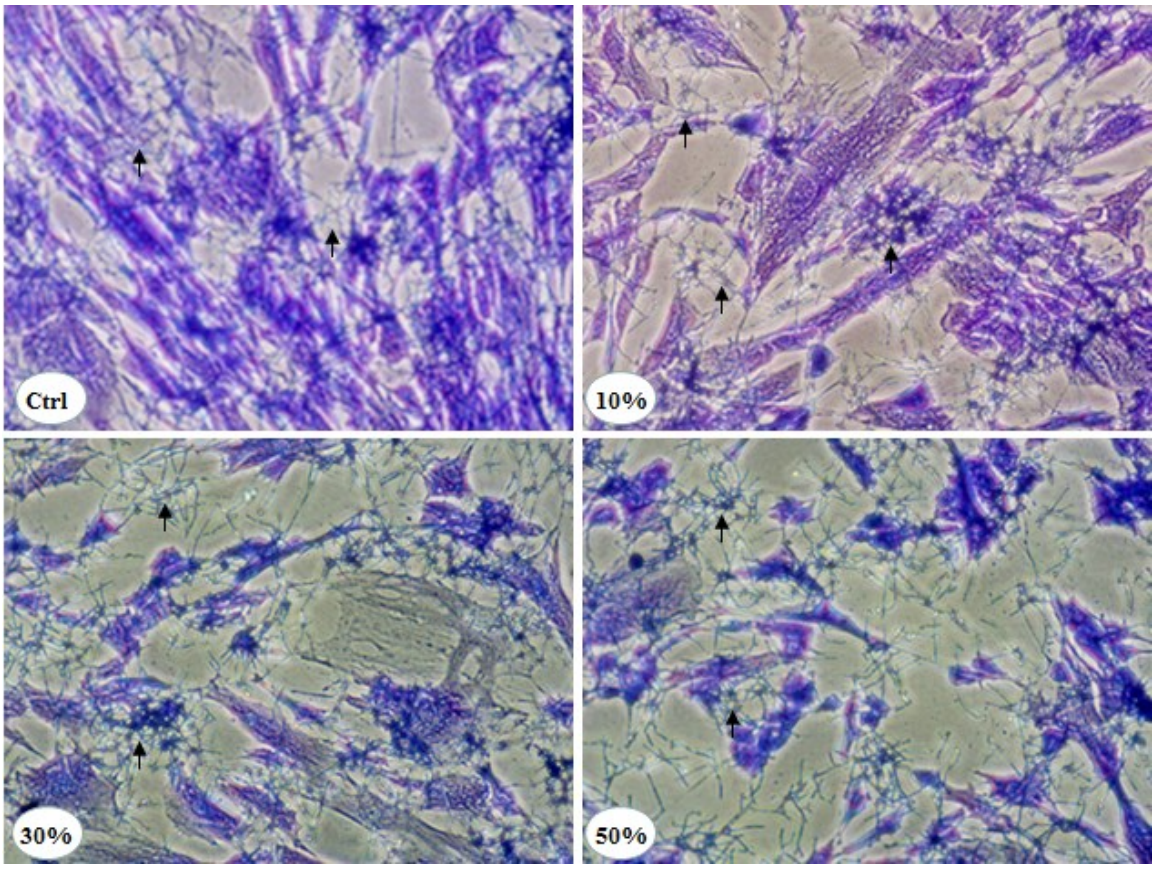

Fig. 2-7: Transition of CSC-pretreated *C. albicans* following contact with gingival fibroblasts. *C. albicans* was incubated 24 h in the presence of CSC and then used to infect gingival fibroblast cultures for 6 h under hyphal transformation conditions (presence of 10% serum at 37°C). Each culture condition was photographed following crystal violet staining. Note the high level of *C. albicans* hyphae. Representative photos are from 4 different independent experiments.

2.3.5 CSC-pretreated *C. albicans* proliferated better when interacting with the gingival fibroblasts: As shown in Fig. 2-8, the proliferation of CSC-pretreated *C. albicans* increased significantly after 24 h of interaction with the gingival fibroblasts. However, this growth was greater at 48 h than at 24 h. 2-8 shows that a

significant ($P < 0.05$) growth increase of *C. albicans* occurred with as low as 10% CSC, compared to the control, at 48 h. The highest growth rates were obtained with 30 and 50% CSC.

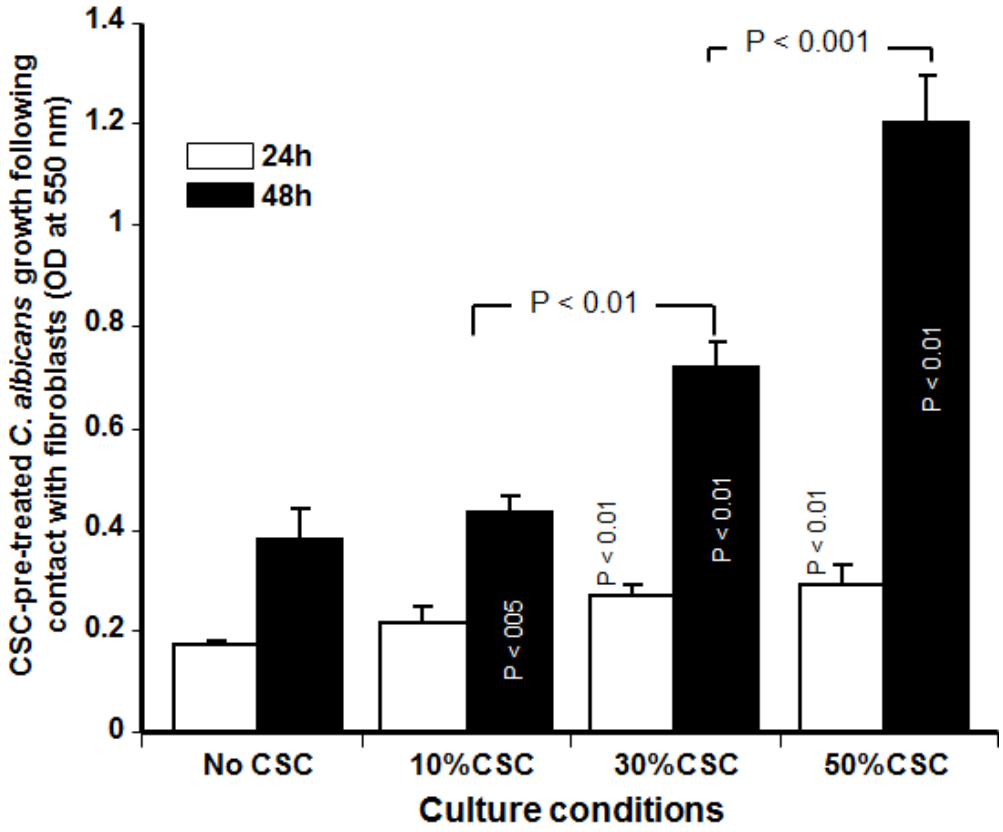

Fig. 2-8: Growth of CSC-pretreated *C. albicans* following contact with gingival fibroblasts. *C. albicans* was incubated 24 h in the presence of CSC and then used to infect gingival fibroblast cultures for 24 or 48 h at 37°C. The cultures were performed in a transwell culture system allowing for indirect contact between *C. albicans* and the fibroblasts. *C. albicans* cells were then collected and subjected to an MTT colorimetric assay (n = 5).

2.3.6 CSC-pretreated *C. albicans* decreased gingival fibroblast growth and increased IL-1β secretion: The indirect effect of CSC-pretreated *C. albicans* on gingival human fibroblasts was investigated using a transwell culture system. As shown in Fig. 2-9A, the number of viable fibroblasts at 24 h was significantly

($P < 0.01$) low with the 30 and 50% CSC concentrations, compared to the control. However, at 48 h, even the 10% CSC concentration led to a significant ($P < 0.01$) decrease in cell numbers. Of interest is that no detaching cells were observed in the cultures with and without CSC-pretreated *C. albicans*. The reduced number was therefore not the consequence of apoptotic/necrotic detaching cells but rather the result of a reduction in growth rate. This decreased fibroblast number (due to the interaction with CSC-pretreated *C. albicans*) may lead to the reduction of anti-inflammatory activity of gingival fibroblasts. To test this, we measured the level of IL-1β secreted by fibroblasts interacting with CSC-pretreated *C. albicans*. As shown in Fig.2-9B, after 24 h of contact, the approximate concentration of IL-1β secreted by the gingival fibroblasts infected with CSC-untreated *C. albicans* was 30 pg/10^4 fibroblasts. This increased to almost 40 pg/10^4 fibroblasts following contact with 30% CSC-pretreated *C. albicans* and to over 40 pg/10^4 fibroblasts with 50% CSC-pretreated *C. albicans*. IL-1β secretion was greater after 48 h of contact between the fibroblasts and the CSC-pretreated *C. albicans*, reaching close to 100 pg/10^4 fibroblasts with 50% CSC-pretreated *C. albicans*. The number of viable cells decreased, however, in the presence of CSC-pretreated *C. albicans* (Fig. 2-9A) and their secretion of IL-1β was elevated (Fig. 2-9B).

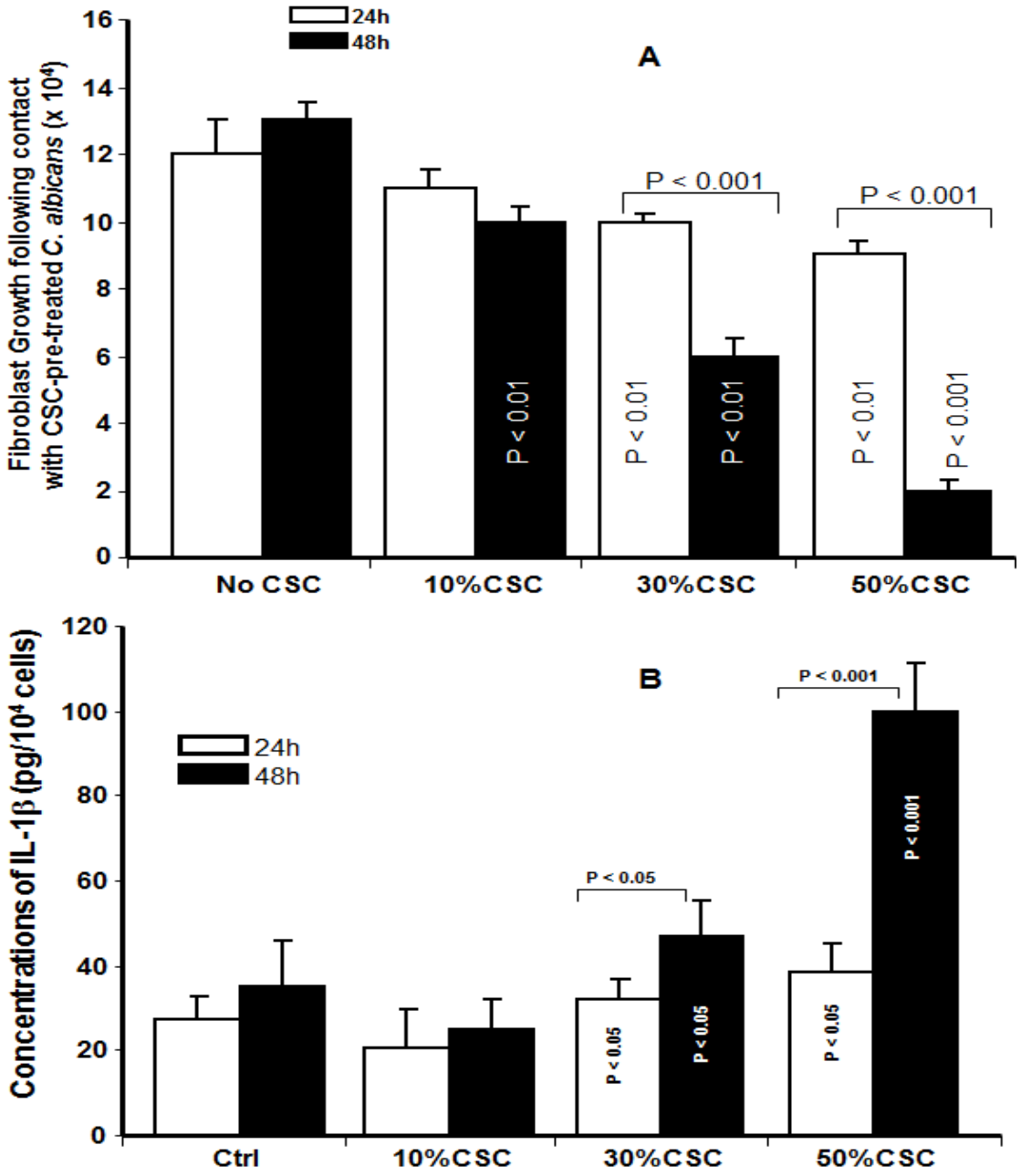

Fig. 2-9: CSC-pretreated *C. albicans* decreased gingival fibroblast growth. Cell cultures were pulsed with CSC-pretreated or untreated *C. albicans* for 24 or 48 h in a transwell culture system. (Panel A) = Fibroblasts were then trypsinized and viable/cell numbers were determined by trypan blue exclusion assay (n = 4). (Panel B) = Culture medium in each condition was used to measure the level of IL-1β by ELISA (n = 4).

2.4 Discussion

In a previous study, we demonstrated that the growth rate of *C. albicans* increased when it was cultured in the presence of CSC [34]. This is supported by the present study showing greater *C. albicans* morphological changes in the presence of CSC than in non-exposed controls.

One of the reasons for *Candida* pathogenesis is its ability to interchange between blastospore and hyphal forms. Hyphae are considered necessary for invasive growth, while blastospores are considered a clonal expansion form [38]. By promoting *C. albicans* growth and transition, CSC may contribute to *C. albicans* resistance against different stress agents such as oxidative, osmotic, and heat stress. In this study, we demonstrated that CSC-pretreated *C. albicans* was sensitive to H_2O_2, but was resistant to osmotic and thermal stress agents. This suggests that treatment with CSC selectively confers resistance of *C. albicans* to some stressful agents but not to others.

It has been reported that *C. albicans* is relatively resistant to reactive oxygen species (ROS), tolerating up to 20 mM of H_2O_2 under specific conditions [39]. This oxidative resistance involves AP-1-like transcription factor Cap1 through the Skn7 response regulator [40]; however, these data were generated under adaptive condition of *C. albicans*, which differs from our CSC-exposure experiment. We thus hypothesize that under our conditions, *C. albicans* sensed several stressful components in the CSC and adapted to them, but when in contact with a new and strong stress agent such as H_2O_2, it was no longer capable of preventing the damaging effect of this stress agent. Further mechanistic studies will confirm this and shed light on the signaling molecules and death pathway involved. Of interest is that the CSC-pretreated *C. albicans* displayed resistance to osmotic and heat stress. The mechanisms underlying such resistance are still to be investigated. These may include mitogen-activated protein (MAP) kinase pathway involved in osmo-adaptation in other

yeasts [41, 42]. Further studies are required to demonstrate the signaling mechanisms involved in CSC-pretreated *C. albicans* responses against stresses.

Cell wall proteins are involved in the sensing of stressful agents [43]. These are known to play a key role in yeast cell pathogenesis [43]. Since we demonstrated that CSC-pretreated *C. albicans* produced more chitin, this can be considered as a protective pathway used by *Candida* to overcome the damaging effect of CSC by modulating the cell walls. Indeed, it has been reported that fungi can overcome damaged cell walls by increasing chitin content to maintain cell wall integrity [44]. *In vitro* exposure of *C. albicans or A. fumigates* to echinocandins was shown to lead to a compensatory increase in chitin content [45, 46]. Thus the elevated chitin biosynthesis demonstrated in the present study may be considered as a potential mechanism of resistance or tolerance against the effects of CSC. This may translate to a significant clinical impact for smokers, as *C. albicans* cells with a high level of chitin reportedly reduced susceptibility to anti-fungi such as caspofungin and echinocandins [47].

Cell wall proteins are known to play key role in the interaction between the yeast cells and the host through adhesion processes [48]. *C. albicans* adhesion to host cells/tissue is a key factor promoting oral yeast carriage and possible infections [49]. This adhesion may be increased in the presence of cigarette smoke, as demonstrated in the present study, in which CSC-pretreated *C. albicans* adhered better to gingival fibroblast monolayers than did CSC-untreated *C. albicans*. CSC has been shown to favor the adhesion of *S mutans* to dental materials [50] and of *C albicans* on various materials [50]. We thus demonstrate, for the first time, the significant adhesion of *C. albicans* to gingival fibroblasts following contact with CSC. This supports previously reported research with cigarette smoke chemicals, showing the influence of nicotine on bacterial adhesion to soft tissues [51]. By adhering to gingival fibroblasts, *C. albicans* may have the appropriate conditions to proliferate. Indeed, the present study shows the increased growth of CSC-pretreated *C. albicans* when in contact

with human gingival fibroblasts. This may be due to the susceptibility of fibroblasts to CSC-pretreated *C. albicans* as previously reported with non-CSC-pretreated yeast cells [52].

Contact with host cells can be mediated by the fungal cell wall, an essential structure that provides physical strength and protects the fungus from hostile environments [53], including *C. albicans* protection against host cell inflammatory response [54]. The mechanisms underlying this process must be further examined.

The indirect interaction of gingival fibroblasts with CSC-pretreated *C. albicans* led to a decrease in the number of viable gingival fibroblasts. It is suggested that this decrease was not due to cell death through the detachment from the culture plate but was rather, most likely, due to a decrease in the cell growth rate. Further research will undoubtedly shed light on this process.

Interestingly, after being in contact with CSC-pretreated *C. albicans*, viable fibroblasts actively secreted IL-1β. Indeed, when comparing Fig. 2-9A related to the cell growth and Fig. 2-9B related to IL-1β secretion, a reverse image can be seen, where the lower number of viable fibroblasts corresponds to a high level of IL-1β secretion. The importance of IL-1β in protecting the mammalian host from invasive *C. albicans* infection has been clearly demonstrated, as mice deficient in IL-1β experienced decreased survival levels and increased fungal burdens relative to wild-type mice [55, 56]. The presence of CSC may thus contribute to the interaction of *C. albicans* with human gingival fibroblasts, leading to fibroblast stimulation and IL-1β secretion to overcome the damaging effect of CSC and *C. albicans*, as the *C. albicans* untreated with CSC led to low level of IL-1β secretion by the fibroblasts. This high level of IL-1β may support fibroblast protection against CSC-pretreated *C. albicans* virulence.

2.5 Conclusion

Cigarette smoke promoted *C. albicans* transition from blastospore to hyphal form. CSC-pretreated *C. albicans* was sensitive to oxidative stress, but resistant to osmotic and heat stresses. The CSC-pretreated *C. albicans* also displayed high levels of chitin, particularly under hyphae culture conditions. In addition, CSC modulated *C. albicans* interaction with the host by increasing the adhesion of this yeast to and proliferation on gingival fibroblasts. Contact with CSC-pretreated *C. albicans* affected the fibroblasts which exhibited a reduced growth rate and increased IL-1β secretion. Overall, this study suggests that cigarette smoke may promote *C. albicans* pathogenesis, supporting both the increased persistence of this pathogen in smokers and the severity of candidiasis.

2.6 References

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Chapter III

3.1 General discussion

The most important phase leading to *C. albicans* colonization in the oral cavity is its adhesion [34]. Many factors can positively influence this adhesion to oral tissues [42]. In our study, we investigated one environmental factor, namely, cigarette smoke, and found that CSC-pretreated *C. albicans* attached more to gingival fibroblast monolayer cells than did the controls (absence of CSC). This is in agreement with a previous study showing that exposure to cigarette smoke increased the binding of *C. albicans* to the host tissue [90]. Our data is also supported by bacterial studies showing the increased adhesion, growth, and biofilm formation of *P. gingivalis* and/or *Streptococcus gordonii* under the effect of tobacco smoke [86].

We also established a correlation between the adhesion and growth of *C. albicans* with CS. These data are in agreement with clinical observations showing that smokers are more prone to oral candidiasis compared to non-smokers [87]. Hypothetically, this may be due to the use of different CSC chemicals by *C. albicans* as fuel [87]. Furthermore, cigarette smoke may modulate cell wall proteins, thereby contributing to yeast adhesion followed by yeast growth and infection. Through this study, we were able to demonstrate a link between the presence of cigarette smoke and the increase in chitin production by *C. albicans*. Chitin was shown to play a key role in *C. albicans* growth and adhesion. Chitins are important to maintain *C. albicans* cell wall integrity during morphogenesis [37].

Although adhesion is a key factor for *C. albicans* to initiate pathogenesis [17], proliferation is another important factor to consider. Interestingly, we demonstrated that *C. albicans* continues to proliferate in the presence of CSC, even in contact with human gingival fibroblast cells. This suggests the ability of this yeast to adapt to a hostile environment, such as the host defense, and to take advantage of the CSC components to not only

survive but thrive. On the other hand, the presence of CS-pretreated *C. albicans* reduced gingival fibroblast growth but increased IL-1ß secretion. This suggests that the host cells sensed the damage occurring via *C. albicans*, and rather than proliferate, fibroblasts produced inflammatory mediators such as IL-1ß to counter the *C. albicans* infection. Further studies are warranted to confirm this response by investigating other inflammatory mediators and the presence of CSC with and without *C. albicans* and gingival fibroblast cultures.

Overcoming the host defense can take place through the yeast to hyphae transition. Of great interest is that we demonstrated that CSC promoted this transition. This may contribute to the yeast invasion leading to oral candidiasis by taking advantage of the CS products, thus supporting the high level of oral candidiasis in smokers compared to non-smokers [52]. Further studies will support this hypothesis. Investigating the link between smoking and *C. albicans* infection through biofilm formation will certainly shed light on the signaling mechanisms promoting candidiasis in smokers.

In the oral cavity, *C. albicans* can be accompanied by other bacteria. In general biofilms in the oral cavity is a mixture of microbial species. It will thus be very interesting to perform further investigations that include yeast and bacteria in the presence of CS. This will certainly provide further knowledge on the nature of mixed biofilms, the crosstalk between *C. albicans* and bacteria under smoke-induced stress, and the signaling pathways involved under these CS conditions [22].

Another future study which may increase our understanding of CS-modulated diseases will be to include both gingival epithelial cells and fibroblasts in the presence of CS and *C. albicans* with or without oral bacteria, such as *S. mutans* and or *P. ginvivalis*. Using this model, we will be able to investigate virulent genes at microbiological levels, as these genes can overexpress during *C. albicans* adhesion and transition from blastopore to hyphal form as well as when *C. albicans* invades the host cells. This model is already being used in our laboratory to investigate this hypothesis.

3.2 Conclusion

In the present study, we demonstrated that CS promoted *C. albicans* growth and transition from blastospore to hyphal form. CS-pretreated *C. albicans* was sensitive to H_2O_2 stress but was resistant to NaCl and heat stresses. Treating *C. albicans* with CSC led to an increased level of chitin, particularly under hyphal culture conditions. In addition, CSC modulated the interaction of *C. albicans* with the host by increasing the adhesion of *C. albicans* to gingival fibroblasts. Contact with CSC-pretreated *C. albicans* affected the fibroblasts, which exhibited a reduced growth rate but an increased IL-1β secretion. Overall results suggest that cigarette smoke may promote *C. albicans* infections, supporting both the increased persistence of this pathogen in smokers and the severity of candidiasis.

3.3 References

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Appendix:

List of publications and abstracts published and presented in scientific meetings

Publications:

Alanazi H, Semlali A, Perraud L, Chmielewski W, Zakrzewski A, Rouabhia M. Cigarette smoke-exposed *Candida albicans* increased chitin production and modulated human fibroblast cell responses. Biomed Res Int. 2014;2014:963156. doi: 10.1155/2014/963156. Epub 2014 Sep 11.

Semlali A, Killer K, **Alanazi H**, Chmielewski W, Rouabhia M. Cigarette smoke condensate increases *C. albicans* adhesion, growth, biofilm formation, and EAP1, HWP1 and SAP2 gene expression. BMC Microbiol. 2014 Mar 12;14:61. doi: 10.1186/1471-2180-14-61.

Abstracts:

Alanazi H, Semlali A, Alanazi M, Rouabhia M. poster titled: Cigarette smoke condensate promotes *C. albicans* growth, transition and interaction with human gingival fibroblasts. The International Union of Microbiological Societies (IUMS 2014) – XIVth International Congress of Bacteriology and Applied Microbiology, XIVth International Congress of Mycology and Eukaryotic Microbiology, XVIth International Congress of Virology (July 27-1 August) Montreal, Canada.

Alanazi H, Rouabhia M. *Candida albicans* growth, transition and interaction with human gingival fibroblasts were modulated by cigarette smoke. Oral presentation, 8th Search Day of the GREB/FMD, Faculty of dentistry, Laval University, May 9th 2013, Quebec city, Canada.

Alanazi H, Semlali A, Alanazi M, Rouabhia M. poster titled: Cigarette smoke condensate promotes *C. albicans* growth, transition and interaction with human gingival fibroblasts. In BiSP meeting (Bactériologie intégrative: Symbiose & Pathogenèse) held on 28 - 29 of November 2013 at the University Laval, Quebec city , Canada.