UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

Dissertação

Candida albicans e cárie radicular: análise do transcriptoma

Candida albicans and root caries: a transcriptomic analysis

Laís Daniela Ev

Orientação: Profª Drª Marisa Maltz

Co-orientação: Profª Drª Clarissa Cavalcanti Fatturi Parolo

Porto Alegre, 2016.

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Dissertação apresentada como parte dos requisitos obrigatórios para a obtenção do Título de Mestre em Clínica Odontológica

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“Ninguém é tão sábio que não tenha o que aprender, nem tão ignorante que não tenha nada para ensinar”

J.J.Camargo

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Resumo

Os microrganismos associados à cárie são, em sua maioria, microrganismos acidogênicos e acidúricos, anaeróbios estritos e facultativos. A presença de fungos é associada à microbiota de cárie radicular, sendo a espécie fúngica mais relacionada a *Candida albicans*. Embora estudos de cultivo e de análise de DNA comprovem a presença de fungos na microbiota associada a lesões de cárie radicular, demonstrando um gradiente crescente de colonização com a progressão da doença, pouco se sabe sobre o papel que estes microrganismos desempenham no processo de doença. O objetivo deste trabalho foi estudar o papel de *Candida albicans* na cárie radicular, através da análise de transcriptoma de biofilmes naturais de superfícies radiculares hígidas (n=10, SRS) e de lesão de cárie radicular ativa (n=9, RC). Foi avaliada a expressão diferencial de genes de *Candida albicans*, as funções específicas e vias metabólicas associadas a este microrganismo. O RNA total microbiano foi extraído e o mRNA isolado e sequenciado na plataforma Illumina Hi-Seq2500. Foram formados *pool* (grupamentos) das amostras com valores inferiores a 30ng/RNA para a construção de bibliotecas genômicas. Os dados gerados pelo sequenciamento de RNA-Seq foram compilados em uma tabela de contagem (*reads*) e mapeados com o genoma de referência (*C. albicans* SC5314) utilizando o software CLC Genomics Workbench 7.5.1. Para o cálculo do nível de expressão gênica os dados foram normalizados com o algoritmo DESeq. A expressão diferencial foi calculada com binomial negativa e *False Discovery Rate* (FDR<0,05). Os genes com maior expressão em RC e em SRS foram analisados pela mediana relativa de expressão (RME; *Relative Median Expression*) e expressão diferencial, assim como as vias metabólicas associadas a genes de virulência e metabolismo de açúcares. Dois genes (CaO19.610, FDR=0.009; CaO19.2506, FDR=0.018) apresentaram expressão diferencial significativa em superfície radicular hígida (SRS) e tem suas funções relacionadas a formação de biofilme. Enquanto que em superfície radicular cariada (RC) sete genes (UTP20, FDR=0.018; ITR1, FDR=0.036; DHN6, FDR=0.046; CaO19.7197, FDR=0.046; CaO19.7838, FDR=0.046; STT4, FDR=0.046; GUT1, FDR=0.046) apresentaram expressão diferencial significativa e tem suas funções relacionadas a atividade metabólica, transporte de açúcares, tolerância ao estresse,

invasão e regulação de pH. *Candida albicans* é um microrganismo importante no desenvolvimento da doença cárie radicular.

Palavras-chave: RNA-seq; *Candida albicans*; Cárie Radicular; Transcriptoma.

Abstract

The microbiota associated with root caries must be acidogenic and aciduric. *S. mutans, Lactobacillus, Actinomyces, Veilonella, Bifidobacterium,* and other bacteria play important roles in root caries biofilm. Yeasts are also present on carious and non-carious root biofilms, being the specie *Candida albicans* the most prevalent yeast found in root biofilms. Although the presence of *Candida albicans* is stablished in the literature, and there are an increasing gradient of *Candida* species. colonization with caries progression, the role of this microorganism in root caries has not being totally elucidated. The aim of this study is to analyse the role of *Candida albicans* in root caries thought a transcriptomic analysis of biofilms of sound root surfaces (n=10, SRS) and root caries lesions (n=9, RC) using a high-throughput sequencing of cDNA (RNA-Seq). The differential expression of genes of *Candida albicans* SC5314, the specific functions and pathways associated with the gene expression of the present study were investigated. The total bacterial RNA was extracted and the mRNA was isolated (Illumina Hi-Seq2500). Samples with low RNA concentration (less than 30ng/RNA) were pooled, yielding a final sample size of SRS=10 and RC=9. Sequence reads were compiled in a count table and mapped to *C. albicans* SC5314 genome of reference, using the software CLC Genomics Workbench 7.5.1. Gene expression was calculated in the algorithm DESeq, and the differential expression was calculated with binomial negative (Log2FoldChange) and *False Discovery Rate* (FDR<0,05). The genes with higher expression in RC and SRS were analysed by the Relative Median Expression (RME), and the virulence factors and pathways and sugar metabolization related with *Candida albicans* pathogenicity in root caries were analysed. Two genes (CaO19.610, FDR=0.009; CaO19.2506, FDR=0.018) are up-regulated in Sound Root Surface (SRS) have their functions related to biofilm formation and seven (UTP20, FDR=0.018; ITR1, FDR=0.036; DHN6, FDR=0.046; CaO19.7197, FDR=0.046; CaO19.7838, FDR=0.046; STT4, FDR=0.046; GUT1, FDR=0.046) are up-regulated in biofilm of carious dentin (RC) have functions related to metabolic activity, sugar transport, stress tolerance, invasion and pH regulation. *Candida albicans* is, therefore, important to root caries development.

Key-words: RNA-seq; *Candida albicans*; Root Caries; Transcriptome.

Equipe executora

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Orientadoras:

Profª. Drº. Marisa Maltz, Doutora em Odontologia, Professora Titular do Departamento de Odontologia Preventiva e Social da UFRGS.

Profª. Drª. Clarissa Cavalcanti Fatturi Parolo, Doutora em Odontologia, Professora Adjunta do Departamento de Odontologia Preventiva e Social da UFRGS.

Colaboradores:

Nailê Damé-Teixeira: Doutora em Odontologia. Professora substituta do Departamento de Odontologia Preventiva e Social da UFRGS.

David Beighton: Doutor em Microbiologia Oral. Professor de Microbiologia Oral do King's College London Dental Institute.

Thuy Do: Doutora em Microbiologia. Professora do Dental Institute, University of Leeds.

Instituições envolvidas

Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

Faculdade de Odontologia, Universidade de Leeds, Leeds, Reino Unido.

Área de pesquisa

Diagnóstico das afecções buco-faciais.

Sumário

Lista de abreviaturas

ACC1 =acetyl-CoA carboxylase

ACT1 =hypothetical protein CaO19.12474

ACT2 = hypothetical protein CaO19.5293

ADH1 =alcohol dehydrogenase

ALS =agglutinin-like sequence (ALS1-7 and ALS9)

BCR1 =Biofilm and Cell wall Regulator1 gene

BH =Benjamini-Hochberg

Brg1 =Biofilm ReGulator gene

°C =Celsius degree

C. albicans =*Candida albicans*

cAMP/PKA =cyclic adenosine monophosphate

CaO19.610 =potential DNA binding regulator of filamentous growth

CaO19.2506 =hypothetical protein CaO19.2506

CaO19.2877 =pyruvate decarboxylase

CaO19.7197 =hypothetical protein CaO19.7197

CaO19.10395 =pyruvate decarboxylase

CaO19.12474 =hypothetical protein CaO19.12474

CaO19.5007 =hypothetical protein CaO19.5007

CAP1 =hypothetical protein CaO19.1623

cDNA =Complementary DNA

CdC19 =pyruvate kinase

Cdc24 =hypothetical protein CaO19.10684

Cdc28 =cyclin-dependent protein kinase Cdc28

Cdc42 =likely rho family Ras-like GTPase

Cek1 =ERK-like Kinase

CFO1 =Ferroxidase

CO_2 =carbon dioxide

Cph1 =STE-like transcription factor fragment

Cph1p = STE-like transcription factor fragment protein

Cph2 =hypothetical protein CaO19.8778

Cst20 =likely signal transduction kinase

Cyr1 =adenylate cyclase fragment

Czf1 =potential fungal zinc cluster transcription factor

DHN6 =hypothetical protein CaO19.2344

DNA =desoxyribonucelic acid

EC number =Enzyme Commission Numbers

Ece1 =hypothetical protein CaO19.10882

EFG1 =positive regulator of filamentous growth

ENA1 =ATPase transporter gene

ENO1 =enolase

FBA1 =fructose-bisphosphate aldolase

FDR =False Discovery Rate

Flo8 =Transcription factor

GlcNAc =N-acetylglucosamine

GP1 =glycophosphatidylinositol

GPM1 =probable phosphoglycerate mutase

Gtfβ =glucosyltransferase beta

GUT1 = potential glycerol kinase Gut1p

HAP =hydroxyapatite

HGC1 = Hypha-specific expression and relatedness to G1 Cyclins

HOG1 = likely protein kinase

HOMD = Human Oral Microbiome Database

HSF1 = Essential transcription factor, mediates heat shock transcriptional induction

Hsp =heat shock protein

Hsp90 =heat shock protein 90

HXK2 =hexokinase

H^+ATPase =proton ATPase

Hst7 =likely pheromone pathway MAP kinase kinase

HWP1 =hyphal-specific cell wall protein aka ECE2

Hyr1 =hyphally regulated cell wall protein

ITR1 =potential myo-inositol transporter

KEGG =Kyoto Encyclopedia of Genes and Genomes

LEA =Late Embryogenesis abundant proteins

Lip9 =lipase

MAP kinase =mitogen-activated protein kinase

mRNA = Messenger RNA

MUC1 =cell surface glycoprotein involved in filamentous growth

n =number of samples

NCBI =National Center of Biotechnology Information

Ndt80 = meiosis-specific transcription factor

Nrg1 =potential zinc finger protein

RBT5 =likely cell surface antigen

Rob1 =Regulator Of Biofilm

ORF =Open reading frame

PCK1 =phosphoenolpyruvate carboxykinase

PGI1 =glucose-6-phosphate isomerase

PGK1 =phosphaglycerate kinase

pH =potential of hydrogen

PHR1 = hypothetical protein CaO19.11310

PHR2 = *C. albicans* pH regulated cell wall protein Phr2p

PKA =protein kinase A

Plb2 =phospholipase

PspC = polymorphic protein

QS =quorum sensing

QSMs =quorum sensing molecules

Ras1 = Ras family GTPase involved in hyphal growth

RC =Root Caries

RME =Relative Median Expression

Rim13 =hypothetical protein CaO19.11478

Rim20 =positive regulator of alkaline-induced genes

Rim101 =proteolytically activated regulator of alkaline-induced filamentation

RNA =Ribonucleic acid

RNA-Seq =RNA sequencing; whole transcriptome shotgun sequencing

Rox1 =domain transcriptional repressor of filamentous growth and hyphal genes

Saps =secreted aspartic proteases (SAP1, SAP2, SAP3, SAP4, SAP5, SAP6, SAP7, SAP9, SAP10, SAP98, SAP99)

S. cerevisiae =*Saccharomyces cerevisiae*

SCFAs =short chain fatty acids

S. mutans =*Streptococcus mutans*

snoRNA =small nucleolar RNAs

spp = species

SRS =Sound Root Surface

SSA1 =HSP70 family chaperone; cell wall fractions

SspA =polypeptide adhesins A

SspB =polypeptide adhesins B

Ste11 =potential pheromone pathway MAPKKK

Ste12p =Putative transcription factor protein

STT4 = hypothetical protein CaO19.1814

TDH1 =glyceraldehyde-3-phosphate dehydrogenase

TEC1 =potential TEA/ATTS type DNA binding protein

Tpk1 =cAMP-dependent protein kinase catalytic subunit

Tpk2 =cAMP-dependent protein kinase catalytic subunit

Tup1 =transcriptional repressor of filamentous growth

UME6 = transcription factor

UTP20 =potential U3 snoRNP protein Utp20p fragment

18s rRNA =18S ribosomal RNA

Revisão de literatura

Background

The increase in life expectancy around the world is related to improvements of general health conditions and medical and technological advances. These improvements also reflected in oral health, with the retention of teeth in the mouth over the years, and consequently more exposed root surfaces because of gingival recession [Shen *et. al*., 2002]. Root caries is therefore emerging as a significant problem in the middle aged and elderly people due to the vulnerability of the exposed root surfaces against the acidic attack by oral microbiota. The exposed tissue, cement or dentin, is much less resistant than enamel, due to the mineral compositions, in enamel approximately 98% and in cement and dentin 50%. Consequently, the critical pH for demineralization is higher for root caries than for enamel (6.8 *versus* 5.5) [Schüpbach *et al.,* 1990].

Root caries and the related microbiota

Root caries lesions are most often originated at the gingival margin, as it is a biofilm retention area. The biofilm in this area must harbor microorganisms that are able to produce acid from carbohydrates (acidogenic) and must be able to growth in a low-pH environment (aciduric) [Brailsford *et al.,* 2001]. Until now, it is understood that diverse microorganisms are involved in the etiology of root caries. Microbiota of sound root surface is more complex than the one associated with root caries, that could be different from the one associated with enamel caries [Syed *et al.,* 1975; Shen *et al.,* 2002; Preza *et al.,* 2008]. Previous researches using culture and DNA analysis demonstrated that *Streptococcus mutans, Lactobacillus* species (spp.), *Candida albicans* and *Veillonella* spp. are present in major proportion in root caries than in sound root surface [van Houte *et al.,* 1994; Marchant *et al.,* 2001]. *Actinomyces* spp., *Veillonella* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Rothia, Candida* spp., *Entetococcus, Staphylococcus* spp., *Capnocytophaga* spp. and *Prevotella* spp. also were related with root caries [Syed *et al.,* 1975; Shen *et al.,* 2002; Mantzourani *et al.,* 2009; Brailsford *et al*., 2001].

Yeast in root caries

Candida albicans asymptomatically colonizes the skin, mucosal surfaces and oral cavity of most healthy individuals [Klinke *et al.,* 2011; Lu *et. al.,* 2014]. It is a common opportunistic fungal pathogen of humans and may be present in several locations on the human body due to its opportunistic nature, being able to invade tissues causing candidiasis. Oral candidiasis is one of the most common opportunistic oral fungal infections [Raja *et al.,* 2010]. *C. albicans* has the capacity to invade mouth tissues and artificial materials such as acrylic, which is used in several types of dental prostheses [Barbieri *et al.,* 2007]. The levels of yeast in saliva increased in the presence of dentures [Loesch *et al.,* 1999], a common feature in elderly patients.

Candida species has been associated with dental caries, especially with early childhood caries and root caries [Aamdal-Scheie *et al.,* 1996; Loesche *et al.,* 1999; de Carvalho *et al.,* 2006]. A strong association was found between the prevalence of *C. albicans* and dental caries [Beighton *et al.,* 2004]. Several authors showed that the proportion of *Candida* species was higher in individuals with caries than in individuals without caries. Beighton and Lynch [1995] reported the relatively high prevalence of *Candida* spp. (58.5%) from 82 root caries lesions. Brailsford *et al.* [2001], showed that the proportion of yeasts was significantly greater in root caries samples compared to sound root surface. Arslan *et al*. [2016] shown that *Candida* spp. was isolated at a rate of 40% of individuals with caries and at a rate of 27.8% of individuals without caries. Some authors also suggested that *Candida albicans* might be a risk factor for root or dentin caries of abutment teeth, particularly in the elderly with low salivary flow [Nikawa *et al.,* 2003], and may serve as a marker organism for active root caries [Beighton and Lynch, 1993]. According to Mantzourani *et al.* [2009] the proportion of yeasts was significantly greater in the dentin samples from soft lesions than from biofilm of sound root surfaces, in adults and elderly individuals. Previous reports support the evidence that the proportion of *C. albicans* was significantly greater in the carious dentin than in the dental biofilm sample of children with caries [Marchant *et al.,* 2001]. Therefore, there are an increasing gradient of *Candida* spp. colonization with caries progression, soft lesions present a major proportion of *Candida* spp. than leathery lesions and sound surfaces.

Although there are reports about the presence of *Candida* spp. in dental caries, little is known about their functions and importance in root carious biofilm.

Candida species

Approximately 200 species comprising the genus *Candida*, only 20 have been associated with human diseases [Williams *et al.,* 2013]. Most of the studies has shown yeast presence in oral biofilms but they did not define the species involved [Syed *et al.,* 1975; Loesche *et al.,* 1999; Beighton and Lynch, 1993; Brailsford *et al.,* 2001; Mantzourani *et al.,* 2009]. Different species can be related to different environment conditions, different cariogenic potential, hence performing different functions. *C. albicans* is the most commonly found species in the oral cavity [Beighton and Lynch, 1995; Shen *et al.,* 2002] followed by *C*. *tropicalis*, *C. krusei* and *C. dubliniensis* [Shen *et al*., 2002; de Carvalho *et al.*, 2006; Raja *et al.,* 2010; Marchant *et al.,* 2001] and *C. glabrata* [Shen *et al*., 2002]. *In vitro* studies analyzing calcium release in root caries lesions demonstrated that *C. albicans* has a more aggressive calcium releasing activity related to other *Candida* species [Szabó *et al.,* 2014]. In spite of these evidences, little is known (or almost nothing) about these fungi functions in carious biofilms.

Cariogenicity and virulence factors of Candida albicans

There are several characteristics of *C. albicans* that are related to its cariogenicity [Klinke *et al.,* 2011]. Several *in vitro* studies demonstrated the high acidogenic potential [Samaranayake *et al.,* 1983; Samaranayake *et al.,* 1986; Nikawa *et al.,* 1994]. *Candida* species are hetero-fermentative microorganisms and have a high potential to metabolize dietary sugars to acid [Thein *et al.,* 2006] which leads to the dissolution of hydroxyapatite crystals in enamel and dentin [Nishimura *et al.,* 2002; Charone *et al.,* 2013]. An i*n vitro* study showed that *C. albicans* has 20 times higher potential to dissolve hydroxyapatite than *S. mutans* [Nikawa *et al.,* 2003]. Yeast growth and adhesion may be enhanced by high glucose concentration in saliva, this glucose can serve as nutrients for *Candida* organisms [Manfredi *et al.,* 2004; Arslan *et al.,* 2016]. They are able to produce high concentrations of acids

(lactic acid in higher proportion), extracellular polysaccharides and proteins, and also to lower the pH of glucose-supplemented saliva down to a value of 3.2 by secreting organics acids [Saramanayake, 1986; Brighenti *et al.,* 2014], contributing to the cariogenicity of oral biofilms. Furthermore, it is able to reduce the salivary pH at a lower value than acids produced by *Streptococcus mutans* [Klinke *et al.,* 2009; Charone *et al.,* 2013; Saramanayake, 1986; Szabó *et al.,* 2014; Brighenti *et al.,* 2014], suggesting that fungi can have a crucial role in the biofilm disbiosys. Besides acid production, another function of microorganism that should be important in the root caries progression is the collagen degradation [Takahashi and Nyvad*,* 2016], although little is known about any involvement of *Candida* in this process.

Candida spp. are capable of colonizing the hard surface of the teeth, invading the dentinal tubules, participating in the formation of microbial biofilm, and producing large amounts of acids that are responsible for demineralization of tooth enamel and dissolution of hydroxyapatite [Nikawa *et al.,* 2003; Samarayake *et al.,* 1986]. Dentin comprises not only hydroxyapatite (HAP) but also organic material, such as type I collagen, which are likely to be partly degraded or denatured with acids or enzymes, particularly in caries lesions exposed to the oral environment [Makihira *et al.,* 2002]. In the pathogenesis of root caries the ability to degrade collagen it's a very important feature in the second phase of caries progression [Takahashi and Nyvad*,* 2016]. The cervical region, the periodontal fibers and dentin from carious lesions contempt collagen, which could be degraded to various extents and then serve as colonization sites for other oral microorganisms [Makihira *et al.,* 2002]. *C. albicans* is capable to adhere to saliva-coated hydroxyapatite with a relatively high affinity [Cannon *et al*., 1995] and shows strong adherence to collagen [Makihira *et al.,* 2002]. *C. albicans* possesses collagenolytic activity [Nishimura *et al.,* 2002] and adheres to both denatured and intact collagen through different mechanisms [Makihira *et al.,* 2002]. This process may contribute to the persistence of *C. albicans* on the root surface. Exposed collagen from dentin creates an adherence site for *C. albicans* [Makihira *et al.,* 2002; Nikawa *et al.,* 2003]. The cervical regions of teeth surfaces and root surfaces are usually covered not only by saliva but also by an exudate or serum components derived from subgingival fluids [Nikawa *et al.,* 1998]. *In vitro* studies observed that a serum pellicle was significantly more effective in promoting the colonization of *C. albicans,* than saliva pellicle and uncoated

specimens, it is possible to say that the adherence of *C. albicans* to saliva-coated hydroxyapatite with type I collagen is enhanced when compared with uncoated HAP [Nikawa *et al.,* 1998; Nikawa *et al.,* 2006].

Cariogenicity depends not only on the terminal pH and collagen degradation, but also on several other factors, including the ability of organisms to colonize the tooth surfaces, the length of time the pH remains below the critical pH for demineralization of root surfaces, and the quantity and quality of the acids produced during fermentation [Clarkson *et al.,* 1987].

Pathogenicity of *Candida* spp. are related with several survival strategies: 1) morphological flexibility; 2) white-to-opaque switching and mating; 3) contact-induced filamentation; 4) hypha-associated expression of adhesins; 5) invasion into host cells by induced endocytosis; 6) active penetration; 7) release of hydrolytic enzymes (e.g., secreted aspartic proteases (Saps)) that support penetration and the breakdown of tissue material; 8) acquisition of nutrients and micronutrients from host cells, e.g., zinc and iron uptake systems; 9) stress response pathways facilitating resistance to adverse environmental conditions, e.g., reactive oxygen species, reactive nitrogen species, low pH, and starvation; 10) active modification of the phagosome to promote hyphal growth, facilitating macrophage damage and escape [Polke *et al.*, 2015].

Many of this virulence factors might be associated with root caries pathogenicity, including [Arslan *et al.,* 2016; Klinke *et al.*, 2009; Calderoni and Fonzi, 2001]:

a) Invasion
b) Biofilm formation and co-aggregation
c) Adherence and damage
d) Morphogenesis (Phenotypic diversity, switching form)
e) Acid production
f) Acid tolerance
g) Stress response

In order to understand the role of *C. albicans* in the root caries, we will discuss the literature findings about possible genes, genes interactions and pathways associated with *C. albicans* virulence.

Candida albicans colonization

Figure 1 shows a hypothetical model indicating different microenvironments that *C. albicans* could be exposed in host. This microenvironments exposed *C. albicans* to a variety of signals leading to an activation of several distinct signalling pathways. Activation of these pathways must trigger specific responses that allow the fungal cells to adapt, and grow in the particular microenvironment. Some signals will be specific to some microenvironments (A and C), whereas others will be common to several microenvironments (B), leading to the activation of different pathways (1, 2, 3). There is probably some overlap in the specificity of the signal transduction pathways with respect to their inputs as well as some redundancy in their outputs (multiple arrows). Nevertheless, specific signalling pathways must respond to specific subsets of signals and must generate specific responses. Their outputs include (a) yeast-to-hyphal morphogenesis, (b) adherence to host tissue, (c) production of hydrolytic enzymes, including secreted aspartyl proteinases (sspa) and (d) trigmotropism (the ability to penetrate and orientate growth in response to contact with a solid surface).

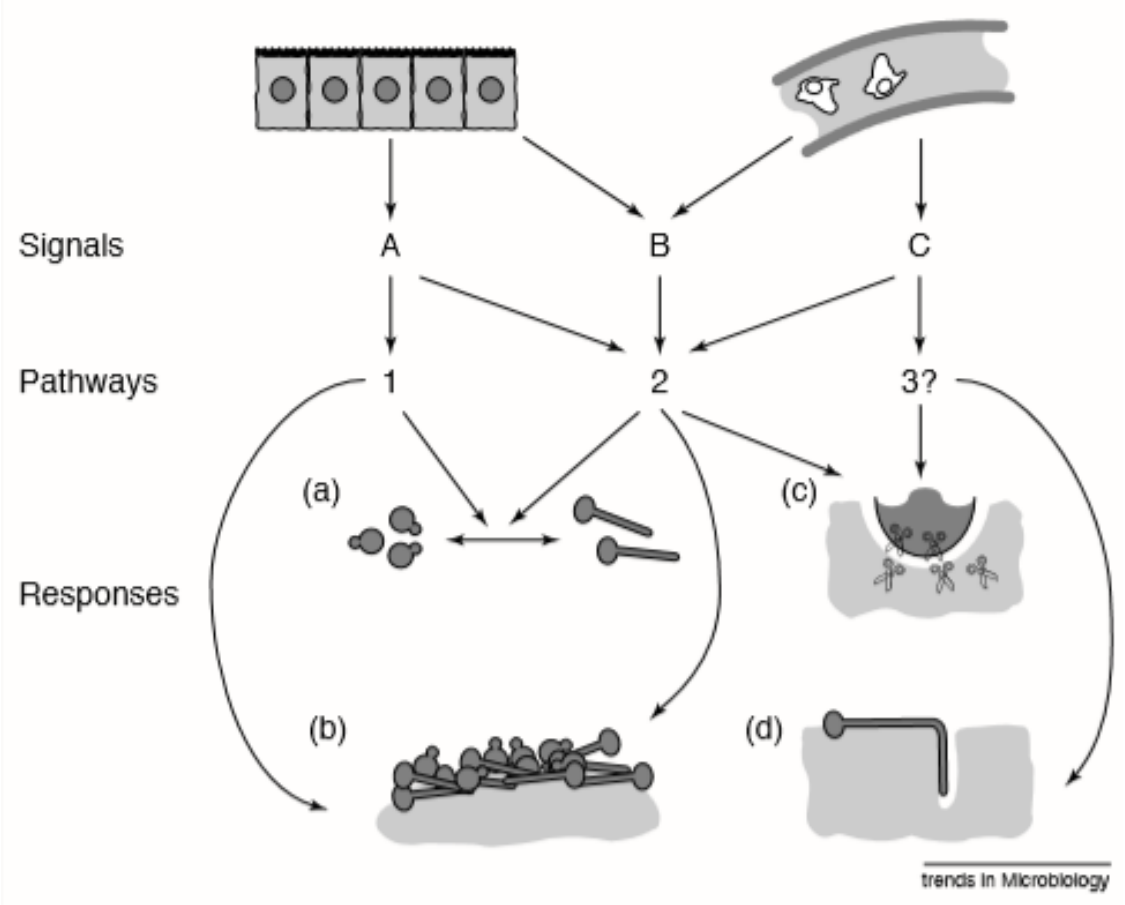


Figure 1: Hypothetical model indicating different microenvironments that *Candida*

albicans could be exposed in host. (a) yeast-to-hyphal morphogenesis, (b) adherence to host tissue, (c) production of hydrolytic enzymes, including secreted aspartyl proteinases (sspa) and (d) trigmotropism (the ability to penetrate and orientate growth in response to contact with a solid surface)[Brown and Gow, 1999].

a) Invasion

Like other pathogens, virulence in *C. albicans* includes host recognition and invasion. Adhesion to host cells is essential for colonization and survival in the host [Höfs *et al.,* 2016]. *C. albicans* utilizes two distinct and complementary mechanisms for host cell invasion: induced endocytosis (passive) and active penetration [Wächtler *et al.,* 2012]. The mechanisms used by *C. albicans* are dependent on many different factors, e.g. the invasion stage, fungal morphology and the epithelial lineage to be invaded [Yang *et al.,* 2014; Wätchler *et al.,* 2012].

The importance of Als3, Ssa1 and Act genes in *C. albicans* invasion process is well stablished in the literature [Höfs *et al.,* 2016]. The invasion process is initiated via the interaction of *C. albicans* invasins Als3 and Ssa1 on the hyphal surface with target host cell receptors [Mayer *et al.,* 2013]. Induced endocytosis, cytokinesis, determination of cell polarity and morphogenesis in yeasts are all actin-dependent process [Chen *et al.,* 2005; Moseley and Goode, 2006].

Microorganisms have constitutive hydrolytic enzymes to facilitate active penetration of host tissues [Arslan *et al.,* 2016]. These cells secrete large amounts of hydrolases, which facilitate penetration into the host cell and promote efficient nutrient acquisition by means of host tissue degradation, facilitating fungal invasion [Naglik *et al.,* 2008]. Lipolytic enzymes, such as phospholipases and esterases, are associated in *Candida* infections with host cell penetration, adhesion to epithelial cells, invasion of reconstituted human oral epithelium, and even host signal transduction pathways [Trevino-Rangel *et al.,* 2013]. Phospholipase could play an important role in the invasion of host tissues in lesions of candidiasis by destroying the epithelial cell membranes (that are made up of lipids and proteins) and allowing the hyphal tip to enter the cytoplasm [Banno *et al.*, 1985]. Proteinase production is considered to increase colonization and penetration to host tissues of microorganisms, and to avoid the host's immune system by degrading a number of proteins important in host defences, such as immunoglobulins, complements, and

cytokines [Hube, 1996]. The hydrolytic enzymes aspartyl proteinase (Sap5), lipase (Lip9), and phospholipase (Plb2) are important virulence factors of *C. albicans* [Freire *et al*., 2015]. The enzyme Sap5 is able to degrade human proteins such as salivary antibodies, mucin, and collagen and to activate the production of other virulence factors by *Candida* [Naglik *et al.*, 2008], in addition to acting directly on the innate immune system, degrading components of the complement system [Gropp *et al.*, 2009]. Lipase (Lip9) and phospholipase (Plb2) contribute to colonization and infection by degrading components of the host cell membrane [Barros *et al*., 2008]. Enzymatic activity may play an essential role in the capacity of *Candida* to establish itself as a colonizing and/or infectious microorganism [Arslan *et al.,* 2016].

b) Biofilm formation and co-aggregation

Biofilm consist of structured microbial communities that are attached to a surface and surrounded by a self-produced extracellular matrix and often contribute to and/or cause disease in humans [Costerton *et al.,* 1995; Hall-Stoodley and Stoodley, 2009] such as dental caries. Dental biofilm forms naturally on teeth and is of benefit to the host by helping to prevent colonization by exogenous species [Herrero *et al.,* 2016]. Microbial homeostasis is part to a dynamic balance of both synergistic and antagonistic microbial interactions. However, homeostasis can break down, leading to shifts in the balance of the microbiota, thereby predisposing sites to disease, according to the current knowledge [Ecological Plaque Hypothesis - Marsh, 1994]. *Candida albicans* can form biofilms on abiotic and biotic surfaces [Höfs *et al*, 2016]. Besides the diverse virulence mechanisms of *C. albicans*, the capacity to form biofilm is one of the most important factors. It facilitates the adhesion, proliferation and spread of this microorganism to other infection sites and it could reflect in its caries potential [Finkel and Mitchell, 2011]. Binding of the organism to host cells, host cell proteins or microbial competitors (co-agregation) more than likely prevents or at least reduces the clearance by the host [Calderoni and Fonzi, 2001].

The extracellular matrix is the most critical component of biofilms, because it is responsible for maintaining cells adhered [Cury *et al.,* 2000]. The biofilm matrix

of *C. albicans* is mainly composed by β-1,3-glucan that builds up its structure, provides antifungal resistance and also provides sites for glucosyltransferase beta (Gtfβ) activity in dual species biofilms with *S. mutans* [Falsetta *et al.,* 2014].

Quorum sensing (QS) system are also correlated to biofilm formation [Avbebelj *et al.*, 2016]. *Candida albicans* was the first fungus reported to have a QS system [Hogan, 2006]. QS is the process of cell-to-cell communication in biofilms in which individual cells can regulate their phenotype in response to the extracellular concentration of quorum sensing molecules (QSMs). Production of a QSMs occurs in response to certain environmental changes, coordinating gene expression in many types of yeast. In *C. albicans* the major regulation of QS is the QSM alcohol farnesol [Lu *et al.,* 2014]. *C. albicans* produces high amounts of farnesol in comparison to other *Candida* species. Farnesol has several physiological effects, being able to affect genes involved in drug resistance, cell wall maintenance, phagocytic response, surface hydrophobicity, iron transport, and a range of heat shock proteins [Hornby *et al.,* 2001; Polke *et al.,* 2016]. Furthermore, farnesol affects several signalling pathways, and the most prominent action of this QSM is the inhibition of the yeast-to-hypha transition, one of the most important virulence factors of *C. albicans*. Although farnesol are related to biofilm formation, there are no information about its role in caries pathogenicity.

Diverse genes are related with biofilm formation of *Candida albicans.* Nobile *et al.* [2012] showed that the transcription factors BCR1, TEC1, EFG1, Ndt80, Rob1, and Brg1 play a role in controlling biofilm formation. Barros *et al.* [2016] confirmed that SAP5 and HWP1 were the genes more expressed in the formation of biofilm *in vitro*. Genes TEC1, BCR1 and EFG1 are essential for the formation of a mature and stable biofilm resistant to antifungal therapy and to the protection of the immune system, allowing the spread of infections caused by this microorganism [Finkel and Mitchell, 2011; Nobile *et al.*, 2012].

As part of a biofilm, *C. albicans* are closely related with other yeast and bacteria. Fungal-bacterial interactions can be antagonist or mutually beneficial. The proportion of certain bacterial classes such as *Fusobacteriia, Bacteroidia* and *Flavobacteriia* correlated negatively with the *Candida* load [Kraneveld *et al.,* 2012]. There are also antagonistic fungal-bacterial interactions between Lactobacillus and *Candida*. *Lactobacilli* are particularly crucial to antagonizing an excessive *Candida*

growth and thereby conferring a health benefit to the host [Höfs *et al.,* 2016]. Most lactobacilli are recognized as probiotics regarding *Candida* control interfering in the yeast to hypha transition [Boris and Barbes, 2000; Höfs *et al.,* 2016]. Latic acid as well as short chain fatty acids (SCFAs) like acetate, propionate, butyrate and isobutyrate, produced by Lactobacilli contribute to a low environmental pH, thereby inhibiting the yeast-to-hypha transition of *C. albicans* [Noverr and Huffnagle, 2004]. On the other hand, *Lactobacillus casei* was shown to stimulate germ tube growth [Boris and Barbes, 2000; Nair *et al.*, 2001].

The interactions between *C. albicans* and streptococci and *Actinomyces naeslundii* are mutually beneficial and strongly contribute to the formation of early dental plaque [Holmes *et al.,* 1996; Jenkinson *et al.,* 1990]. Streptococci regularly found in association with *C. albicans* include *Streptococcus gordonii, S. oralis,* and *S. sanguinis* [Sullivan *et al.,* 2000]. Interaction of *C. albicans* with *S. gordonii* enhances the formation of hyphae to which *S. gordonii* can bind via the cell wall proteins SspA and SspB [Bamford *et al.,* 2009]. It is already known that *C. albicans* adheres to *Staphylococcus aureus* and *S. gordonii* via the hyphal adhesin Als3p, the mechanism regarding *S. mutans* is yet unknown [Peters *et al.*, 2012; Silverman *et al.,* 2010; Ricker *et al.,* 2014]. Nikawa *et al* [2001] showed that when C*. albicans* grown *in vitro* in media supplemented with high concentration of galactose, they lost the ability to adhere to *S. sanguis*, and acquired the ability to adhere to *S. subrinus*, *S. salivarius* and *Actinomyces* [Nikawa *et al.,* 2001; Samaranayake and MacFarlane, 1980].

The hypothesis of the association between *S. mutans* and *C. albicans* is based on their mechanisms of virulence and biochemical characteristics as well as host factors that provide a buccal environment favoring the action of both microorganisms [Thein *et al.*, 2006; Raja *et al*., 2010; Barbieri *et al.,* 2007]. The coexistence of *S. mutans* and *C. albicans* induces the expression of important virulence factors from both species [Brighenti *et al.,* 2014]. With *S. mutans* and *C. albicans* interactions, the fungus does not have to compete for adhesion sites, being able to adhere to preattached organisms [Barbieri *et al.,* 2007]. In addition to providing adhesion sites, the streptococci excrete lactate that can act as a carbon source for *C. albicans* growth [Holmes *et al.,* 2006; Metwalli *et al.*, 2013]. In return, *C. albicans* reduces the oxygen tension to levels preferred by streptococci and provide growth stimulatory factors for the bacteria [Bamford *et al.,* 2009]. Some

evidence has demonstrated that the presence of *C. albicans* has been shown to enhance the adherence of *Streptococcus mutans* to the oral biofilm and carious lesion *in vitro* [Barbieri *et al*., 2007]. When *S. mutans* was cultivated separately, the adherence to the dental surface was characterized by a thick compact biofilm, with tightly grouped cells of uniform size and morphology. When *C. albicans* was cultivated separately, the biofilm showed the presence of cellular morphology with a predominance of filaments, being characterized by a thinner aspect, with a greater uniformity in the biofilm, and all regions of the tooth being equally colonized [Barbieri *et al.,* 2007]. Therefore, this synergism could be important to the formation of a cariogenic biofilm.

c) Adherence and damage

Cell wall adhesins are the major proteins responsible by the *C. albicans* adhesion. Als (agglutinin-like sequence) of *C. albicans* is a member of a family of eight glycosylated proteins (ALS1-7 and ALS9), all of them are cell surface proteins and are glycosylphosphatidylinositol (GPI)-linked to the β-1,6-glucans of the fungal cell wall[Höfs *et al.,*2016; Gaur and Klotz, 1997; Hoyer, 2001; Sheppard *et al.,* 2004]. Als1-5 appear to provide an adhesion function, this abilities can be related with the infection of *Candida* spp. in root caries [Calderoni and Fonzi, 2001; Höfs *et al.,* 2016]. HWP1 and RBT5 encode cell wall proteins that are important for adhesion to host cells and iron acquisition from the host [Lu *et al.,* 2014]. Hyphal wall protein1 (Hwp1) is a hyphal-and germ-tube-specific gene [Calderoni and Fonzi, 2001], and the N-terminal region of Hwp1 serves as a substrate for epithelial cell-associated transglutaminases, resulting in covalent attachment of *Candida* to host epithelial cells via cross-linking of glutamine residues of the N-terminal region of Hwp1 to as yet unidentified host proteins [Moyes *et al.,*2015].

Regarding to damage, it has been reported that the *Candida* spp. produce secreted aspartyl proteinases (SAPs) in the presence of high nitrogen sources, such as serum albumin, presented in the crevicular fluid. These proteinases have been proposed as one of the important virulence factors in oral infections because they are capable of degrading dentinal collagen at different pH (detected in a range between 7.5 to 2.2 being optimal between 3.5 – 2.2), and have suggested that this

proteinase might be related to the progression of dentinal caries [Nishimura *et al*., 2002; Klinke *et al*., 2009; Naglik *et al*., 2008]. SAP gene family may have distinct roles at the colonization and invasion of the host, morphogenesis and degradation of collagen [Hube *et al.,* 1994; Nishimura *et al.,* 2002]. Secretory aspartyl proteinase (Sap) activity has been implicated in the attachment and penetration stages of *Candida* infections [Hube *et al.,* 1994]. Sap5 and Sap6 are biofilm-specific proteolysis in *Candida albicans,* these proteases are required for proper adhesion by *C. albicans*, and are essential for proper initiation of biofilm formation both *in vitro* and *in vivo* [Winter *et al.,* 2016].

d) Morphogenesis (Phenotypic diversity, Switching form)

Morphogenesis refers to the transition between unicellular yeast cells and a filamentous growth form and is considered to represent the most important virulence factor of *C. albicans* [Calderoni and Fonzi, 2001; Höfs *et al.,* 2016]. The morphological transition, allow *C. albicans* to switch reversibly between yeast, pseudohyphae, and hyphal growth forms (Figure 2). Pseudohyphae are morphologically distinguishable from hyphae because pseudohyphase have constrictions at the sites off septation and are wider than hyphae. By contrast, hyphae form long tube-like filaments with completely parallel sides and no constrictions at the site of septation [Sudbery, 2011].

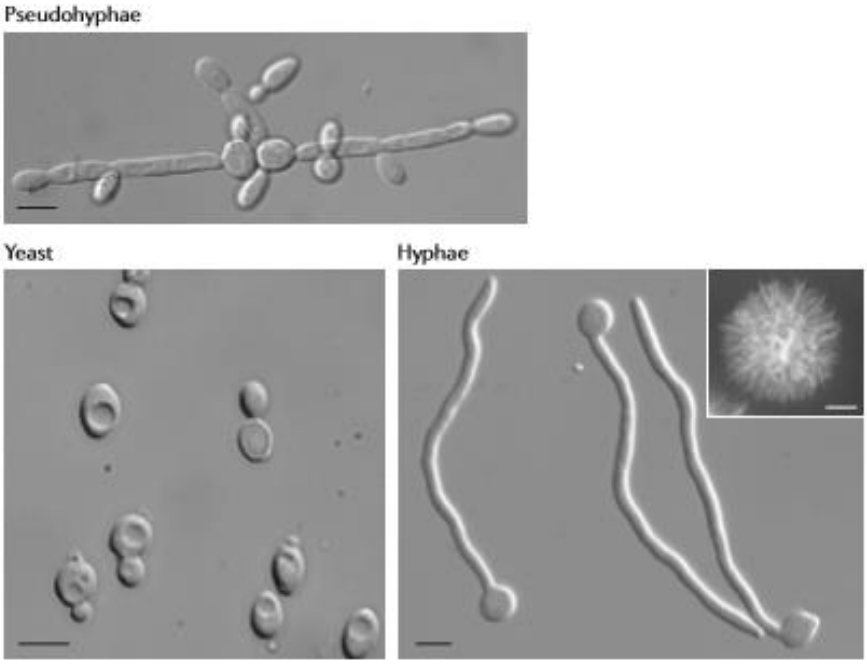


Figure 2: *Candida albicans* morphogenetic forms [Sudbery, 2011].

Both morphologic forms have been suggested to be important for pathogenicity, although the hyphal form is invariably connected with a pathological condition, being more invasive and contributing to host tissue damage [Calderoni and Fonzi, 2001; Höfs *et al.*, 2016]. Morphogenesis in *C. albicans* requires hyphal initiation and maintenance [Polke *et al.,* 2016]. Environmental stimuli leading to morphogenetic transition of *C. albicans* i*n vitro.* The hyphal growth is stimulated by the presence of serum, growth at a temperature of 37°C, N-acetylglucosamine (GlcNAc), neutral pH, high concentration of CO_2. Yeast growth is favored at 30 °C, acid pH (pH 4,0) and high glucose concentrations, although pseudo-hyphae growth is favored at 35 °C at a 5,5 pH [Sudbery, 2011]. This environmental stimuli modulate two signaling pathways, the cAMP (cyclic adenosine monophosphate) and MAP Kinase (mitogen-activated protein kinase) (figure 3), who modulated the activation of several genes.

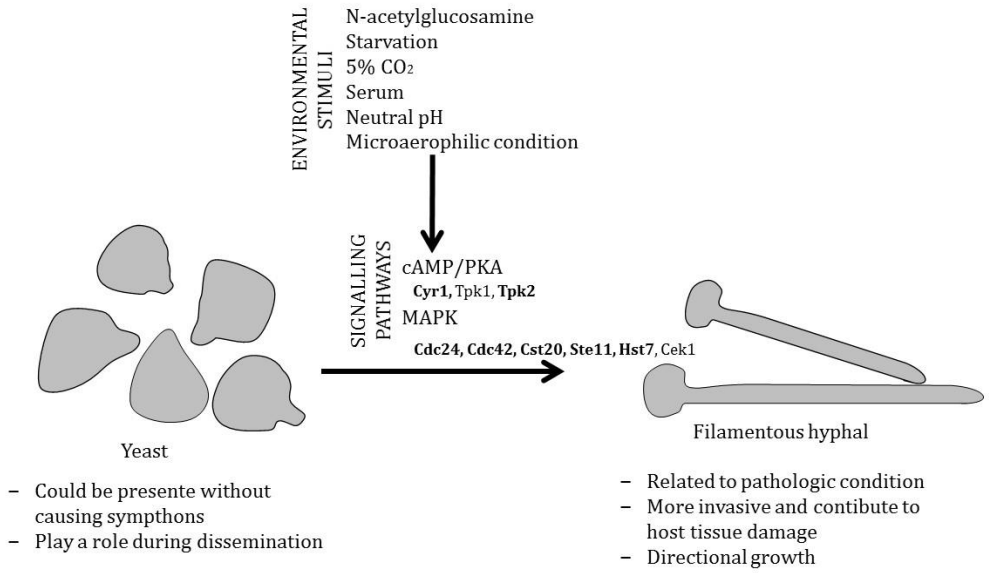


Figure 3: Schematic model of yeast-to-hypha transition induced by environmental stimuli and activation of signaling pathways. The genes involved in cAMP/PKA and MAPK pathways are described above this pathways.

The MAPK pathway involves the activation of Cdc24, Cdc42, Ste11, Cst20, Hst7 and Cek1 genes. In *C. albicans*, Cdc24 correspond to a hypothetical protein similar to Cdc42, being both involved in establishment of cell polarity. Ste11, Cst20, Hst7 and Cek1 are signals transduction kinase involved in pheromone response pathway. Regarding cAMP pathway, it is involved in the activation of Cyr1, Tpk1 and Tpk2 genes. Cyr1 stimulates cAMP production, which then activates protein kinase A (PKA), which has two catalytic subunits: Tpk1 and Tpk2 [Lu *et al.,* 2014]. This two pathways acts in combination with some key transcription factors to modulated the mechanisms of *C. albicans* morphological transition [Lu et al., 2014]. This transcription factors involve Efg1, Czf1 and Cph1. Efg1 is a key transcriptional regulator in *C. albicans* and control various aspects of morphogenesis and metabolism of *C. albicans* [Noffz *et al.*, 2008]. Ste12p homolog (Cph1p) recently has been shown to be involved in hyphal growth of *C. albicans* during some conditions [Liu *et al.,* 1994]. Czf1 is a potential fungal zinc cluster transcription factor. Morphogenesis is modulated by Nrg1 protein (promoter of hypha-specific genes), also UME6 and HGC1 are hypha-specific genes regulators of hyphal transcription and morphogenesis [Lu *et al.,* 2014]. UME6 expression is reduced on hyphal induction in the absence of any one of the transcription factors that mediate the

expression of hypha-specific genes, such as Efg1, Cph1, Cph2, Czf1 and Flo8 [Zeidler *et al.* 2009].

Hypha formation is associated with the expression of characteristic hypha-associated genes, these include the hyphal wall protein Hwp1, the agglutinin-like sequence protein Als3, the secreted aspartic proteases Sap4, Sap5, and Sap6 and the hypha-associated proteins Ece1 and Hyr1 [Sudbery, 2011; Mayer *et al.,* 2013]. These proteins contribute to the special properties of hyphae that are important for virulence, e.g. by aiding in adhesion to and invasion into host cells [Brown and Gow, 1999]. Hwp1 is important for the formation of hyphal-and germ-tube [Calderoni and Fonzi, 2001]. Hypha-specific gene expression is negatively regulated by a complex consisting of the general transcriptional corepressor Tup1 in association with Nrg1 or Rox1p-like regulator of filamentous growth (Rfg1), TUP1 transcription is induced by farnesol in wild-type cells [Kebaara *et al.,* 2008]. Positive regulation of hypha-specific gene expression is carried out by a panel of transcription factors, including Efg1, Cph1, Cph2, Tec1, Flo8, Czf1, Rim101 and Ndt80 [Sudbery, 2011]. Efg1 is required for hyphal formation in response to serum, CO_2, neutral pH and GlcNAc [Stoldt *et al.,* 1997; Lo *et al.,* 1997], Efg1 is thought to be the major regulator of hyphal formation under most conditions [Braun and Johnson, 2000]. Rim13, Rim20, Rim101 are associated with pH sensing pathway.

Other factors involved in signal transduction: Hsp90 and Ras1. Ras1 stimulates both the cAMP and MAPK pathways [Feng *et al.,* 1999; Leberer *et al.,* 2001]. Elevated temperature is a requirement of all hypha inducing conditions except growth in an embedded matrix. Temperature seems to be sensed by heat shock protein 90 (Hsp90), a protein sensible to stress [Shapiro *et al.,* 2009]. Furthermore, strains that are engineered to have a moderate reduction in Hsp90 levels form hyphae in response to serum at 30 °C rather than 37 °C. Hsp90 signalling requires an intact cAMP pathway as a mutation in any of the components upstream of Efg1 blocked the hypha inducing effects of Hsp90 inhibition. Interestingly, an EFG1−/− mutant still formed hyphae when Hsp90 was inhibited, suggesting that an alternative downstream target is activated by Hsp90 signalling.

Hgc1-Cdc28, a hyphal-specific-G1-cyclin 1, is expressed during hyphal growth and is a key regulator of *C. albicans* morphogenesis pathway that is active through the cAMP-PKA or MAPK pathway [Zeng *et al.,* 2004; Liu *et al.,* 1994]. This

gene is associated with diverse cellular machines responsible for different aspects of hyphal growth, such as cell polarity, membrane trafficking, and cell separation, being responsible for polarized growth at the hyphal tips and cell chain formation [Lu *et al.,* 2014; Kim, 2016].

e) Acid production

Dietary sugars, like glucose and galactose, play an important role in biofilm formation of *C. albicans* in oral cavity [Jin *et al.,* 2004]. Klinke *et al.* [2009] analysed the velocity of acid production of *C. albicans* at different pH and concentrations of glucose *in vitro*. The authors conclude that there are at least three different processes that participate in the acidification of surrounding environment by yeast cells. Firstly, the extrusion of several organic acids by *C. albicans* like pyruvic acid and acetate. Secondly, the plasma membrane of yeasts is abundantly equipped with an H^+-ATPase which, in presence of glucose, actively pumps out protons from the cell to generate an electrochemical gradient which is used in the co-transport of nutrients and makes a major contribution to the net acidification. A third source of acidification is based on the excretion of carbon dioxide resulting from oxidative or fermentative glucose metabolism. In an aqueous medium, CO_2 is partially dissolved forming carbonic acid, which in neutral and alkaline conditions dissociates to produce bicarbonate and hydrogen ions. Presumably this process is responsible for the massive rise in at velocity of acid production in *C. albicans* [Klinke *et al.,* 2009; Barbieri et al., 2007].

Oral *Candida* isolates have been shown to be highly acid tolerant and acidogenic [Klinke *et al.,* 2009], and to be able to reduce the pH of a culture during growth from pH 7.5 to pH 3.2 [Samaranayake *et al.,* 1986]. The proportion of organic acids to overall acid production by the yeast wad below 10% at neutral conditions, in contrast to 42-66% at pH 4.0 [Klinke *et al.,* 2009].

C. albicans is dependent on high concentrations of dietary sugars to produce acid. *In vitro* straved cells of *C. albicans* required a 50-fold higher concentration of extracellular glucose than lactobacilli to enable half-maximum acid formation [Klinke *et al.,* 2009]. *C. albicans* excretes organic acids, mainly pyruvic and acetic acid

[Klinke *et al.,* 2009], the former being even more potent than lactic acid in decreasing the pH of an already intensely acidified environment [Klinke *et al.,* 2011].

f) Acid tolerance

The acidification of the environment coincides with the high load of *Candida* and it could disturb the oral commensal microbiome leading to an increase of aciduric microbiota and reduction of natural diversity of the bacterial microbiome [Kraneveld *et al.,* 2012].

C. albicans must be able to adapt its growth to a range of physiological extremes such pH. One example of how *C. albicans* adapts to physiological extremes is that at the neutral pH the organism expresses PHR1, a gene whose function is associated with cell wall synthesis and whose expression is optimum around neutrality. PHR1 expression is switched off and a second pH-regulated gene PHR2 provides a similar function but at an acid pH [Calderoni and Fonzi, 2001].

g) Stress response

Some genes are known as stress response functions, such as RIM101 (a pH-dependent regulator), ENA1 (an ATPase transporter gene) and CFO1 (a ferroxidase), Hsp90 (chaperone temperature dependent regulator) [Chen et al., 2014; O'Meara *et al.,* 2016]. Several functions were enriched among these genes, including some typically associated with virulence, such as adhesion, iron homeostasis, stress response, response to starvation, and biofilm formation [Amorim-Vaz and Sanglard, 2016]. *C. albicans* responds to host stress by activating stress pathways that include the mitogen-actived protein kinase (MAPK) Hog1, the AP1-like transcription factor Cap1, and the heat shock transcription factor Hsf1 [Erwig and Gow, 2016].

Final Remarks and aim of the present study

C. albicans is a very adapted microorganism able to colonize and survive in the oral cavity. Although many studies provided indirect evidence to support the hypothesis that *Candida* species may play an important role in the pathogenesis of

dental caries, related to poor oral hygiene, dentinal carious lesions and cariogenic diet [de Carvalho *et al.,* 2006], a strong relationship between these microorganisms and caries has not been completely elucidated [Szabó *et al.,* 2014]. The study of *Candida* species and caries may be important for a better understanding of the colonization processes and induction of this disease [de Carvalho *et al.,* 2006]. The proportion of *C. albicans* is merely 0.2% of the total cultivable flora [Calderone and Fonzi, 2001]. However, when carious lesions achieve cavitation, a new ecological niche is developed where *Candida* can increase in proportion due to a new retentive site with collagen exposed [Metwalli *et al.,* 2013; Marchant *et al.,* 2001; Mantzourani *et al.*, 2009].

The real role of this microorganism in root caries is uncertain. Although culture and molecular (based on DNA sequence) techniques increased the knowledge about oral biofilms, it has been shown that each site has a different microbial composition [Beighton, 2005; Belda-Ferre *et al.,* 2012]. So, recently the focus of oral biofilms studies is the microbial functions, to better understand the microbial profile in oral health and disease [Do *et al.,* 2015; Damé-Teixeira *et al.*, 2016]. To unveil its pathogenicity in root caries, a complex gene expression (RNA-Seq) study can be advantageous. This highly sensitive technique uses next generation sequencing platforms, enabling transcriptomic analysis of individual species without the need for species-specific protocols, as necessary for the micro-array approach [Do *et al.,* 2015; Amorim-Vaz and Sanglard, 2016]. This technique may be helpful to exploit *Candida* and other fungi role and will allow us to answer the question: is *Candida albicans* a “partner in the crime” regarding root caries or it is just opportunist?

Hence, the aim of this study was to:

1. Observe the gene expression of *Candida albicans* in biofilm of root surfaces.
2. Evaluate the overview of the most prevalent genes by sample in sound root surface and root caries.
3. Identify genes related to *Candida albicans* virulence traits.
4. Evaluate the differential gene expression in SRS and RC.

Artigo 1

Title: *Candida albicans and root caries: a transcriptomic analysis*

Abstract

Background: Candida albicans is the most prevalent yeast found in root caries lesions. Despite that, little is known about its role in root caries pathogenesis. *Objective:* The aim of this study was to observe the gene expression of *Candida albicans* in the microbiota of root surfaces with and without caries. The differential gene expression of *Candida albicans* SC5314 and the specific genes related to cariogenic traits were studied in association with sound and carious root surfaces. *Design*: The oral biofilms from exposed sound root surface (SRS; n=10) and active root caries (RC; n=30) samples were collected. The total bacterial RNA was extracted and the mRNA was isolated. Samples with low RNA concentration were pooled, yielding a final sample size of SRS=10 and RC=9. cDNA libraries were prepared and sequenced on the Illumina Hi-Seq2500. Sequence reads were mapped to *C. albicans* SC5314 genome. Count data were normalized using DESeq2 to analyse differential gene expression applying the Benjamini-Hochberg correction (FDR<0.005). *Results*: Transcription of 14,217 genes in root biofilms without caries (SRS) and with caries lesions (RC) were performed for *Candida albicans* SC5314. Two genes (CaO19.610, FDR=0.009; CaO19.2506, FDR=0.018) are up-regulated in SRS, and their functions are related to biofilm formation. Seven genes (UTP20, FDR=0.018; ITR1, FDR=0.036; DHN6, FDR=0.046; CaO19.7197, FDR=0.046; CaO19.7838, FDR=0.046; STT4, FDR=0.046; GUT1, FDR=0.046) are up-regulated in RC groups and have their functions related to metabolic activity, sugar transport, stress tolerance, invasion and pH regulation. *Conclusion*: *C. albicans* is a microorganism metabolically active in biofilm of sound root surface as well in carious dentin, playing different roles in health and disease, therefore it is, important in root caries development.

Keywords:

RNA-seq; *Candida albicans*; Root Caries; Transcriptome.

Introduction

Root caries lesions are most often originated at the gingival margin, as it is a biofilm retention area. The biofilm in this area must harbor microorganisms that are able to produce acid from carbohydrates (acidogenic) and that must be able of growth in a low-pH environment (aciduric) [Brailsford *et al.,* 2001]. Until now, it is understood that diverse microorganisms are involved in the etiology of root caries. Previous researches using culture and DNA analysis demonstrated that *Streptococcus mutans, Lactobacillus* species (spp.)*, C. albicans and Veillonella* spp. are present in major proportion in root caries than in sound root surface [van Houte *et al.,* 1994; Marchant *et al.,* 2001]. *Actinomyces* spp., *Veillonella* spp., *Streptococcus* spp*., Bifidobacterium* spp*., Rothia, Candida* spp., *Entetococcus, Staphylococcus* spp*., Capnocytophaga* spp. and *Prevotella* spp. also were related with root caries [Syed *et al.,* 1975; Shen *et al.,* 2002; Mantzourani *et al.,* 2009; Brailsford *et al*., 2001]. Microbiota of sound root surface is more complex than the one associated with root caries [Syed *et al.,* 1975; Shen *et al.,* 2002; Preza *et al.,* 2008].

Candida albicans asymptomatically colonizes the skin, mucosal surfaces and oral cavity of most healthy individuals [Klinke *et al.,* 2011; Lu *et. al.,* 2014]. *C. albicans* has the capacity to invade mouth tissues and artificial materials such as acrylic, which is used in several types of dental prostheses [Barbieri *et al.,* 2007]. Furthermore, *C. albicans* is an important colonizer of carious lesions and it has been found frequently in dentin caries lesions rather than in biofilm or saliva [Mantzourani *et al.,* 2009]. However it is still not known whether the yeast acts as caries pathogen or plays a role as a commensal microbe. *C. albicans* possess some important properties that can characterized an important root caries pathogen. It is capable of adhering to saliva-coated hydroxyapatite and possess strong adherence to collagen. Although it is extremely acid tolerant and acidogenic microorganism such as *S. mutans* and *Lactobacilli,* two well stablished cariogenic pathogens [Aas, 2008]. In order to discover the role of *C. albicans* in root caries a high-throughput sequencing of cDNA (RNA-Seq) transcriptome under two distinct conditions, sound root surface versus root carious lesions was performed. This technique may be helpful to exploit *Candida* and other fungi role and will allow us to answer the

question: is *Candida albicans* a “partner in the crime” regarding root caries or it is just an opportunist colonizer?

Materials and Methods

This study is part of the project “metatranscriptome of root caries” performed in collaboration between Federal University of Rio Grande do Sul and the University of Leeds. The complete methodology of this study has been previously described [Dame-Teixeira *et al.*, 2016]. Briefly, volunteers to this research were selected in two groups: sound exposed root surface group (SRS; n=10) and root caries group (RC; n=30; one primary active cavitated root lesion in need of restauration treatment per person). The diagnosis of RC was done according to the activity criteria, using visual-tactile examination [Nyvad and Fejerskov, 1982]. Biofilm and dentin were collected and immediately placed in 1mL of RNAprotect reagent (Qiagen inc.). The total RNA was extracted using the UltraClean® Microbial RNA Isolation (Mo-bio, San Diego, USA) with on-column DNAse digestion (Qiagen, Inc). The extracted RNA samples were quantified using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen) and samples with total RNA concentration <30ng/RNA were pooled, leading to a final sample size of 10 SRS and 9 RC. The Ribo-Zero™ Meta-Bacteria Kit (Epicentre, Illumina) was used for mRNA enrichment and Illumina®TruSeq™ library prep protocols (Illumina, San Diego, CA) were used to library preparation and sequencing was performed with Illumina HiSeq2500. RNA-seq sequencing data are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive, under the accession numbers SRS779973 and SRS796739. FASTQ files were obtained for each sample, and imported into the CLC Genomics Workbench 7.5.1 software (CLC Bio, Qiagen) for mapping against 163 oral microbial genomes. The number of sequence reads that have been assigned to each gene is considered as the read count data.

Candida albicans genome and analysis

The *C. albicans* SC5314 was the genome selected for this study. This strain was chosen because its genome has been fully sequenced, and because of it large information in the literature. The associated information from the 163 oral microbial genomes was downloaded from the DNA Data Bank of Japan, NCBI, the Broad Institute and HOMD (Human Oral Microbiome Database) and mapped against the short reads sequences. After mapping, a count table was generated containing the read count for 14,217 oral *C. albicans* SC5314 genes.

To consider the putative presence of the organism in the sample, we calculated the sum of reads divided by the total number of genes for each sample and considered sample with ≥14,217 reads as valid. Doing that, samples with less than 30% of gene with at least one read were excluded from the analysis.

Statistical analysis for inferring differential gene expression between sample groups was also carried out using the R package DESeq2 [Love *et al.,* 2014]. The cut-off for designating a gene as being differentially expressed was a change in transcript levels of at least 1-log fold change (negative values= up-regulated in SRS and down-regulated in RC, positive values=down-regulated in SRS and up-regulated in RC) and Benjamini-Hochberg (BH) adjusted P-value (False Discovery Rate; FDR) of less than 0.05. The functions and putative pathogenicity in root caries of genes up regulated in SRS and RC were analyzed.

The comparison of the total number of reads were analyzed by Mann Whitney test ($p<0.005$). The number of reads and the relative median expression (RME) (25th-75th) level for genes were calculated for each of the sample groups, as described previously [Do *et al.,* 2015]. Then, the RME was ranked, to observe the most highly expressed transcripts in RC and SRS samples. In order to draw a profile of gene expression, the median of RME of transcripts in SRS and RC conditions were considered low expression RME between 0-10, medium 11-100, and high above 100. This cut off points were decided according to data distribution were 10% higher than 10 RME. Genes related to *C. albicans* virulence factors (invasion, biofilm formation and co-aggregation, adherence and damage, morphogenesis, acid production, acid tolerance and stress response) and putative cariogenicity were analysed. The platform Kyoto Encyclopaedia of Genes and Genomes (KEGG), were

utilized for pathways and Enzyme Commission Numbers (EC number) identification.

For the overview of the most prevalent genes we ranked all RME median for SRS and RC and analysed all genes with median RME values ≥100 per group. Doing that, 37 genes were analysed.

Ethic considerations

This study was approved by the ethic committee of Federal University of Rio Grande do Sul (process n° 427.168) and by the ethic committee of the National Research Ethics Service Committee Yorkshire &The Humber - Leeds West (protocol n° 2012002DD). Volunteers to the study were patients who attended the dental clinics for dental treatment in two centres: Faculty of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Brazil; and School of Dentistry, University of Leeds, Leeds, United Kingdom. All volunteers signed a written consent.

Results

According to the cut-off point chosen to determine the putative presence of a mapped organism in each sample, *C. albicans* is present in n=4 biofilms from root surfaces without caries (4 out of 10) and in n=6 biofilm from root surfaces with caries (6 out of 9) (Figure1). Table 1 shows that the number reads distribution in sound and disease samples are not different (p=0.522).

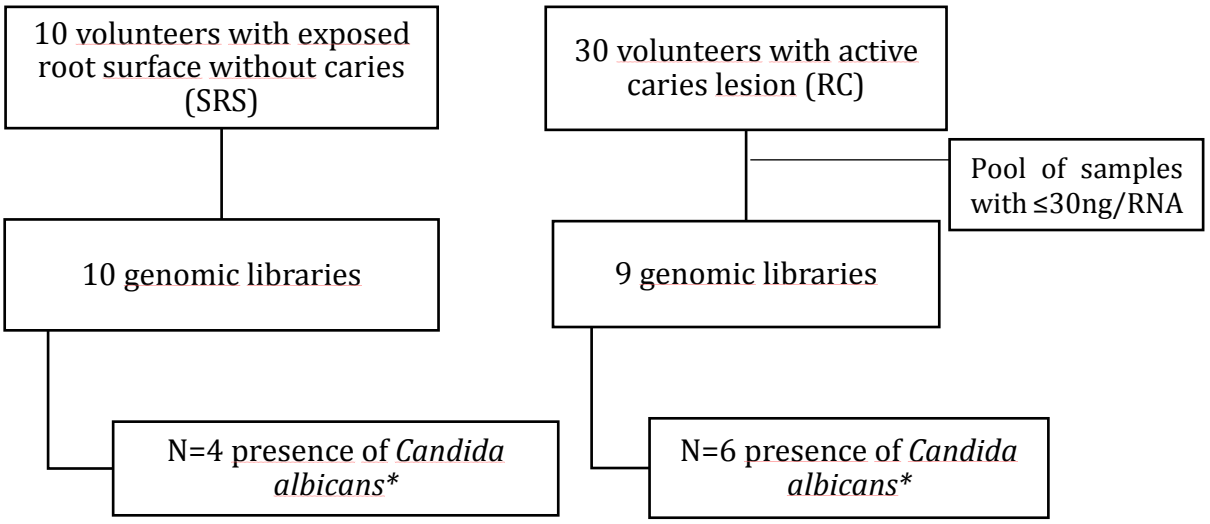


Figure 1. Fluxogram of the sampling. *presence of *C. albicans* based on the presence of at least 14,217 genes per sample.

Table 1. Total reads numbers (median/percentile/range) by group.

	Median	25th-75th	Range
SRS	157,175	48,329 – 197,776	22,721 – 223,511
RC	209,495	79,770 – 571,876	48,759 – 738,400

SRS =sound root surface. RC=root caries. Mann-Whitney p=0.522.

Gene expression per sample

Figure 2 shows an overview of the mostly prevalent genes in *C. albicans* biofilm with and without caries. A total of thirty seven genes with median RME>100 were analysed being thirty four in SRS and twenty in RC.

Although we can observe some similarities in some samples (RC_H, RC_D, RC_A) and (SRS_12, SRS_8, SRS_16, RC_F). It is evident that a pattern to RC and SRS is not observed.

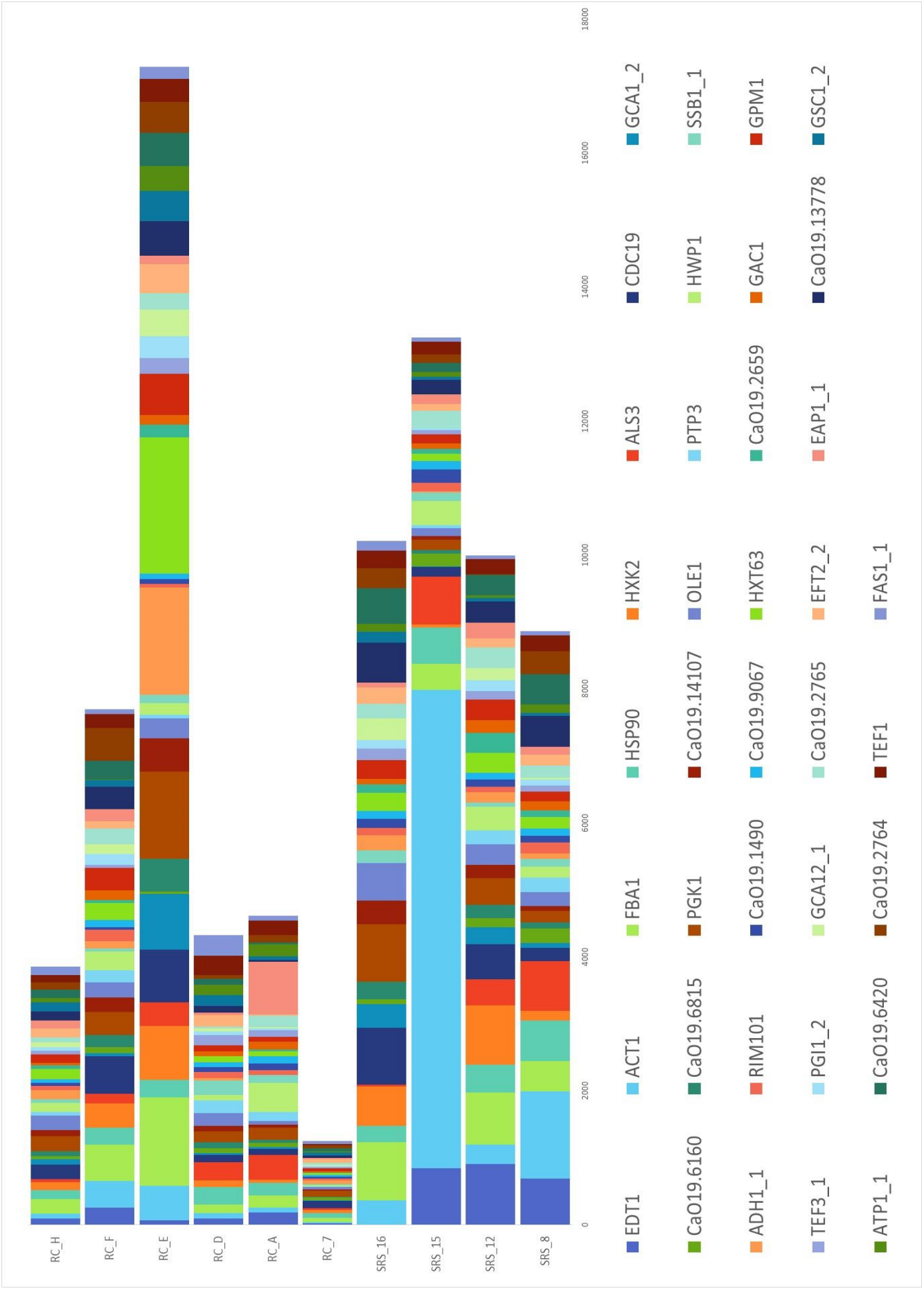


Figure 2. Relative median expression (RME; log10) of genes in the Sound Root Surfaces (SRS; n=4) and Root Caries (RC; n=6) samples. RME was calculated from the median values of normalized read counts. The top highest median of RME values for SRS and RC were selected and sorted, indicate the genes that are most highly expressed by *C. albicans* SC5413.

Expression of genes related to possible cariogenic traits

Table 2 shows the *C. albicans* genes associated with possible virulence factors (RME and percentiles of these genes). In both conditions, we found transcript for 51 out of 67 genes related to virulence traits that are presented in the literature as important factors.

In the invasion factor, genes LIP9, PLB2 and SAP5 presented low expression. While ACT and ALS3 genes seems to have high expression in SRS and medium expression in RC.

In the biofilm formation factor, the genes SAP5 and SAP6 had low expression. While TEC1, EFG1 genes presented medium gene expression in both conditions. The gene HWP1 seems to have a high expression in SRS group them in RC.

Related to adherence, 6 genes (ALS2, ALS4, ALS5, ALS6 and ALS9) had low expression in both conditions. ALS7 presented low expression in SRS group, while in RC ALS7 presented a medium expression. The HWP1 and the ALS3 genes presented high expression in SRS.

Related to collagen degradation genes SAP1, SAP2, SAP3, SAP4, SAP6, SAP8, SAP10, SAP98 and SAP99 presented low expression, while SAP7 and SAP9 presented a medium expression level in both conditions.

Related to acid tolerance, the genes PHR1 and PHR2 presented medium expression values. Regarding stress response, genes with high expression were Hsp90 in both conditions and RIM101 in SRS. Gene RIM101 presented medium expression in only RC, while genes HOG1 and CAP1 presented low expression in both conditions.

The genes SSA1 (invasion), BCR1, NDT80, ROB1, BRG1 (biofilm formation), CEK1, TPK1, STE12, UME6, HGC1, FLO8, ROX1 (morphogenesis), ENA1, CFO1, HSF1 (stress response), that were described in the literature as related to virulence factors in *C. albicans* were not detected in our study.

Table 2. Relative median expression (RME) and percentiles (25^{th}-75^{th}) of genes related to virulence factors in *Candida albicans* in the Sound Root Surfaces (SRS; n=4) and Root Caries (RC; n=6) samples. RME was calculated from the median values of normalized read counts using DE-Seq algorithm.

Accession ID	Median SRS (25^{th}-75^{th})	Median RC (25^{th}-75^{th})	Virulence Trait
ACT			
ACT1_1	416.13(156.10-2871.60)	38.92(2.07-221.80)	
ACT1_2	415.74(147.50-2811.20)	37.65(2.06-209.30)	Invasion
ACT2_1	3.75(0.93-3.85)	8.21(2.82-12.75)	
ACT2_2	5.02(4.02-9.45)	6.81(6.69-10.22)	
LIP9			
LIP9_2	0(0-0)	0.47(6.36-1.85)	Invasion
LIP9_1	0(0-0)	0(6.10-1.22)	
PLB2			
PLB2_1	0(0-0.47)	0.95(0-7.49)	Invasion
PLB2_2	0(0-0)	0.18(0-4.16)	
TEC1			
TEC1_1	21.98(18.5-26.30)	14.46(2.65-26.40)	Biofilm Formation
TEC1_2	18.23(6.75-21.38)	12.08(6.96-37.16)	
EFG1	70.10(38.95-86.02)	22.14(0-62.93)	Biofilm Formation/ Morphogenesis
HWP1			
HWP1_1	127.29(19.59-176.94)	80.53(0-167.24)	Biofilm Formation/
HWP1_2	126.31(20.02-188.10)	72.18(0-148.94)	Adherence
ALS1			
ALS1_1	9.26(0.78-36.07)	26.58(0-84.96)	Adherence
ALS1_2	39.03(13.44-64.22)	22.85(0-147.17)	
ALS2			
ALS2_1	4.61(0.93-13.47)	4.64(0.78-6.23)	Adherence
ALS2_2	2.79(0.62-9.47)	3.73(0-7.46)	
ALS3			
ALS3_1	199.32(45.47-274.46)	88.26(0-122.72)	Invasion/
ALS3_2	50.15(3.16-98.69)	33.79(0-77.51)	Adherence
ALS3_3	171.47(34.94-255.39)	91.19(2.51-124.35)	
ALS3_4	159.55(34.17-225.69)	24.39(1.47-89.82)	
ALS4	5.93(1.04-13.48)	10.63(0-23.22)	Adherence
ALS5			
ALS5_1	3.47(2.07-4.99)	5.04(0-11.62)	Adherence
ALS5_2	1.35(0.16-3.42)	5.65(0-12.03)	

Continuation:

Accession ID	Median SRS (25th-75th)	Median RC (25th-75th)	Virulence Trait
ALS6	2.76(0-6.47)	9.35(0-17.69)	Adherence
ALS7	9.38(6.41-37.42)	18.73(0-36.87)	Adherence
ALS9			
ALS9_1	3.80(0-11.14)	4.03(0-13.27)	
ALS9_2	6.48(1.35-9.75)	2.30(0-8.02)	Adherence
ALS9_3	0(0-0)	1.80(0-4.62)	
RBT5			
RBT5_1	51.14(18.72-76.96)	38.22(0-70.20)	Adherence
RBT5_2	43.62(15.26-72.99)	33.44(0-74.34)	
SAP1			
SAP1_1	0(0-0.27)	1.30(0-2.84)	Collagen
SAP1_2	0(0-0.52)	0(4.06-2.01)	degradation
SAP2			
SAP2_1	0.31(0-1.19)	0.93(55.86-4.55)	Collagen
SAP2_2	0.35(0-4.2)	1.18(115.19-4.03)	degradation
SAP3			
SAP3_1	0(0-0)	0(26.17-1.93)	Collagen
SAP3_2	0(0-0.27)	0.36(29.39-2.27)	degradation
SAP4			
SAP4_1	0(0-0)	0(0-0.61)	Collagen degradation
SAP4_2	0(0-0)	1.65(0-4.38)	
SAP5			
SAP5_1	0(0-0.27)	0.41(1.16-3.76)	Collagen degradation/ Biofilm formation/ Invasion
SAP5_2	0(0-0)	1.85(0-3.56)	
SAP6			
SAP6_1	0(0-0)	0.59(0.52-3.61)	Collagen
SAP6_2	0(0-0)	1(0-6.06)	degradation
SAP7			
SAP7_1	16.52(14.19-21.6)	11.59(6.80-28.20)	Collagen
SAP7_2	18.89(6.9-25.58)	15.71(0-25.99)	degradation
SAP8			
SAP8_1	0(0-4.03)	1.295(0-4.85)	Collagen
SAP8_2	0(0-0)	0.06(0-3.67)	degradation
SAP9			
SAP9_1	13.75(5.16-18.51)	16.32(0.54-25.66)	Collagen
SAP9_2	16.27(5.08-25.63)	17.15(1.07-30.05)	degradation

Continuation:

Accession ID	Median SRS (25th-75th)	Median RC (25th-75th)	Virulence Trait
SAP10			
SAP10_1	0.31(0-0.94)	1.84(4.84-6.65)	Collagen
SAP10_2	0(0-0.27)	1.06(13.5-3.99)	degradation
SAP98			
SAP98_1	0.31(0-0.94)	0.52(0.35-2.27)	Collagen
SAP98_2	0(0-0)	0.53(1.92-1.64)	degradation
SAP99			
SAP99_1	0(0-0)	1.24(0-4.56)	Collagen
SAP99_2	0.18(0-1.01)	0.12(0-1.93)	degradation
CDC24			
CDC24_1	6.25(1.13-13.59)	9.45(1.21-13.71)	Morphogenesis
CDC24_2	6.66(4.24-11.23)	5.8(0-12.67)	
CDC42			
CDC42_1	6.58(1.99-11.37)	3.45(0-4.37)	Morphogenesis
CDC42_2	1.38(0-3)	2.95(0-3.9)	
STE11			
STE11_1	9.07(5.47-17.16)	7.33(0.54-14.34)	Morphogenesis
STE11_2	14.44(4.44-28.53)	7.30(2.34-18.04)	
CST20			
CST20_1	16.77(8.48-24.6)	12.58(0-23.91)	Morphogenesis
CST20_2	16.99(12.72-21.04)	15.66(0-22.24)	
HST7			
HST7_1	6.02(5.50-7.33)	4.55(0-11.04)	Morphogenesis
HST7_2	5.06(1.24-5.33)	3.73(0-9.39)	
CYR1			
CYR1_1	5.06(1.24-9.98)	4.47(0-13.31)	Morphogenesis
CYR1_2	8.35(5.96-10.10)	7.98(0-17.23)	
TPK2			
TPK2_1	6.71(5.57-7.61)	5.79(0-9.88)	Morphogenesis
TPK2_2	5.64(1.13-7.49)	7.98(0-10.83)	
PKA1			
PKA1_1	1.85(0-3.82)	2.04(24.88-3.86)	Morphogenesis
PKA1_2	15.85(8.48-21.81)	6.53(15.73-9.34)	
CZF1			
CZF1_1	0.52(0-2.11)	1.27(5.05-5.85)	Morphogenesis
CZF1_2	1.75(0.26-4.65)	1.35(6.26-3.44)	
NRG1	2.28(0.64-4.99)	6.05(6.01-13.69)	Morphogenesis

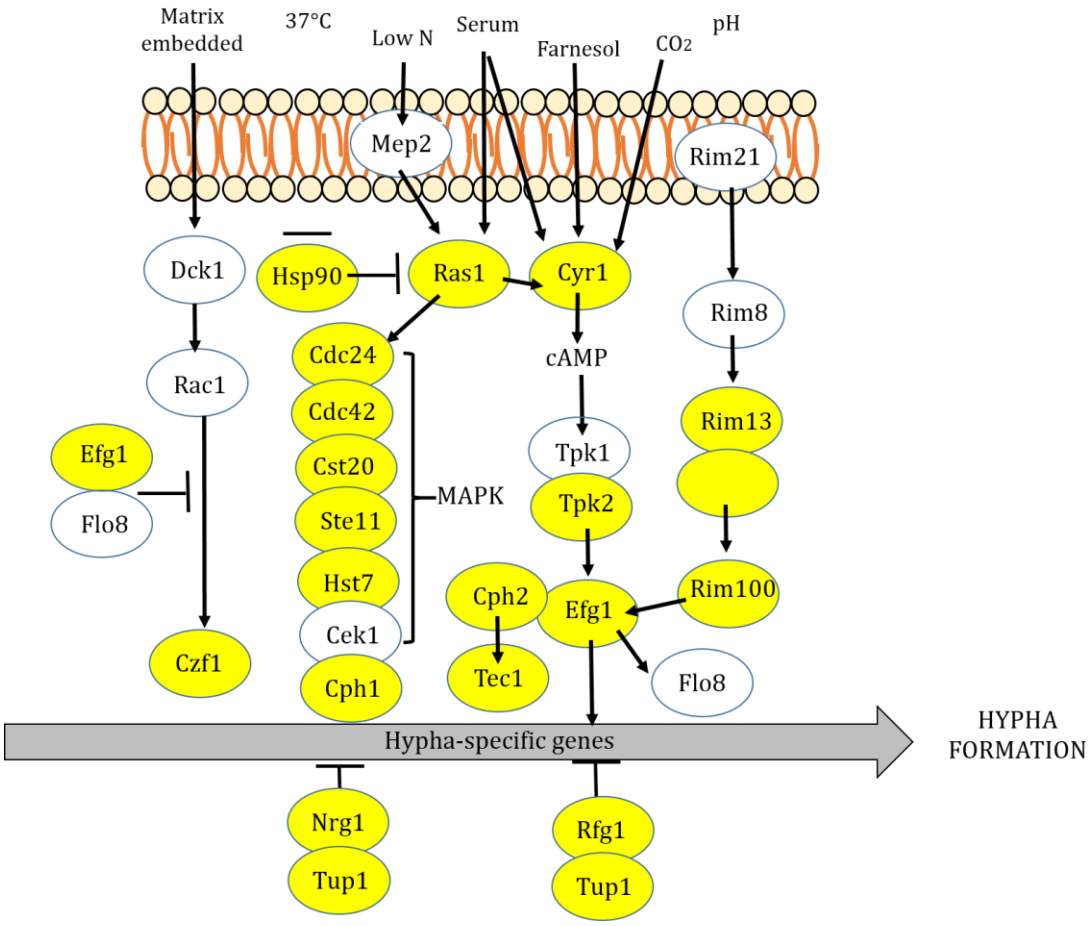
Continuation:

Accession ID	Median SRS (25th-75th)	Median RC (25th-75th)	Virulence Trait
CPH1			
CPH1_1	9.58(1.56-35.08)	8.13(0-17.53)	Morphogenesis
CPH1_2	0(0-0.47)	0.59(0-1.48)	
CDC28			
CDC28_1	0.35(0-1.56)	1.69(0-3.16)	Morphogenesis
CDC28_2	1.04(0-8.58)	2.83(0-6.96)	
CPH2			
CPH2_1	52.42(21.47-56.66)	23.81(0-46.74)	Morphogenesis
CPH2_2	26.53(14.35-43.96)	13.48(0-20.62)	
HSP90			
HSP90_1	212.35(129.29-284.69)	89.73(19.53-129.49)	Morphogenesis
HSP90_2	254.89(158.85-313.28)	111.99(14.61-143.75)	Stress Response
RAS1			
RAS1_1	2.59(0-5.46)	5.45(0-6.90)	Morphogenesis
RAS1_2	3.30(0.62-5.07)	4.68(0-5.76)	
TUP1			
TUP1_1	24.91(19.85-32.21)	10.47(0-14.82)	Morphogenesis
TUP1_2	20.69(9.82-28.32)	8.03(0-10.93)	
RFG1			
RFG1_1	0(0-1.04)	2.28(0-6.62)	Morphogenesis
RFG1_2	7(1.35-8.9)	5.59(0-11.98)	
HYR1			
HYR1_1	0(0-0.47)	0.47(0-1.67)	Morphogenesis
HYR1_2	0(0-0.47)	1.42(0-7.26)	
ECE1			
ECE1_1	0.18(0-2.40)	0.42(0-1.93)	Morphogenesis
ECE1_2	1.23(0-4.65)	1.03(0-4.44)	
PHR1			
PHR1_1	33.62(25.20-94.04)	38.18(0-57.07)	Acid tolerance
PHR1_2	36.96(27.23-104.02)	48.26(0-57.07)	
PHR2			
PHR2_1	4.71(2.27-29.58)	7.77(0.78-32.06)	Acid tolerance
PHR2_2	51.11(2.69-118.06)	58.10 (0.18-137.34)	
RIM101	120.39(89.45-151.32)	65.67(2.72-111.54)	Stress response/ Morphogenesis
HOG1			
HOG1_1	4(1.89-5.07)	4.36(1.30-5.09)	Stress response
HOG1_2	3.96(0.77-6.99)	4.2(1.065-7.63)	

Continuation:

Accession ID	Median SRS (25th-75th)	Median RC (25th-75th)	Virulence Trait
CAP1			
CAP1_1	7.15(1.73-7.64)	5.21(3.12-8.725)	Stress response
CAP1_2	11.01(6.34-14.78)	9.25(1.65-11.7)	

Regarding morphogenesis, we look up at all the genes associated with cAMP/PKA (Cyr1, Tpk1, Tpk2) and MAPK (Cdc24, Cdc42, Cst20, Ste11, Hst7, Cek1) pathways and other related genes (Figure 3). Most of the morphogenesis genes presented low (CDC24, CDC42, STE11, HST7, CYB1, TPK2, PKA1 in RC, CZF1, CPH1, NRG1, CDC28, RAS1, RFG1, HYR1, ECE1) or medium expression (EFG1, CST20, PKA1 in SRS STE11 TUP1 in SRS, CPH2). The only gene with high expression is Hsp90 that was already mentioned for being related to stress response too (Table 2).



[Adapted from Sudbery, 2011].

Figure 3. MAPK/cAMP pathways and genes related to morphogenesis in *Candida albicans*. Highligthed genes (yellow) were transcripts in SRS and RC.

Figure 4 shows the pathway related with the metabolism of Glycolysis and Gluconeogenesis. Regarding to both SRS and RC samples, 7 genes are transcripts with RME>100 for both conditions. This genes are TDH1 (glyceraldehyde 3-phosphate dehydrogenase), PGK1 (phosphoglycerate kinase), ENO1 (enolase), PCK1 (phosphoenolpyruvate carboxykinase), CaO19.10395 (pyruvate decarboxylase), CaO19.2877 (pyruvate decarboxylase) and ADH1 (alcohol dehydrogenase). Others 5 genes presented transcripts with RME>100 only in SRS group, HXK2 (hexokinase), PGI1 (glucose-6-phosphate isomerase), FBA1 (fructose-bisphosphate aldolase), GPM1 (probable phosphoglycerate mutase) and CDC19 (piruvate kinase).

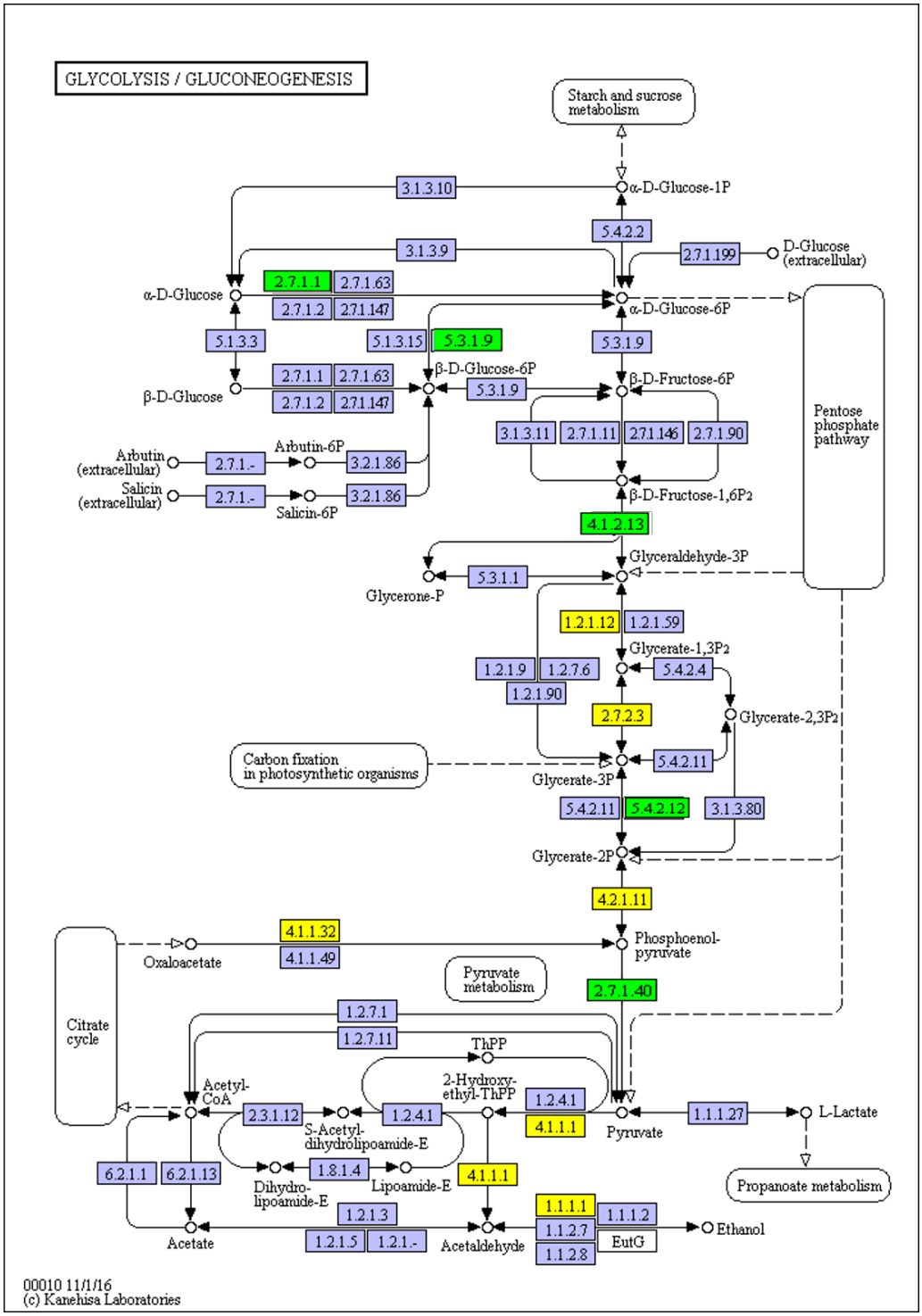


Figure 4. Glycolysis/Gluconeogenesis pathway obtained from Kyoto Encyclopaedia of Genes and Genomes (KEGG). Highlighted genes in yellow are highly expressed in SRS and RC (RME>100). TDH1 glyceraldehyde 3-phosphate dehydrogenase [EC: 1.2.1.12]; PGK1 phosphoglycerate kinase [EC: 2.7.2.3]; ENO1 enolase [EC: 4.2.1.11]; PCK1 phosphoenolpyruvate carboxykinase (GTP) [EC: 4.1.1.32]; CaO19.10395 pyruvate decarboxylase [EC: 4.1.1.1]; CaO19.2877 pyruvate decarboxylase [EC: 4.1.1.1]; ADH1 alcohol dehydrogenase [EC: 1.1.1.1]. Highlighted genes in green are highly expressed only in SRS (RME>100). HXK2 hexokinase [EC: 2.7.1.1]; PGI1 glucose-6-phosphate isomerase, archaeal [EC: 5.3.1.9]; FBA1 fructose-bisphosphate aldolase, class I [EC: 4.1.2.13]; GPM1 probable phosphoglycerate mutase [EC: 5.4.2.12]; CDC19 encodes Piruvate Kinase, related with conversion of phosphoenol-pyruvate in pyruvate [EC: 2.7.1.40];

Looking up at pyruvate metabolism (Figure 5), 3 genes seems to be relevant in *C. albicans* metabolism related to root surfaces biofilms. PCK1 (phosphoenolpyruvate carboxykinase) are highly expressed (RME>100) for both conditions. The gene CDC19 (piruvate kinase) are highly expressed only in SRS, meanwhile ACC1 (acetyl-CoA carboxylase) are highly expressed only in RC.

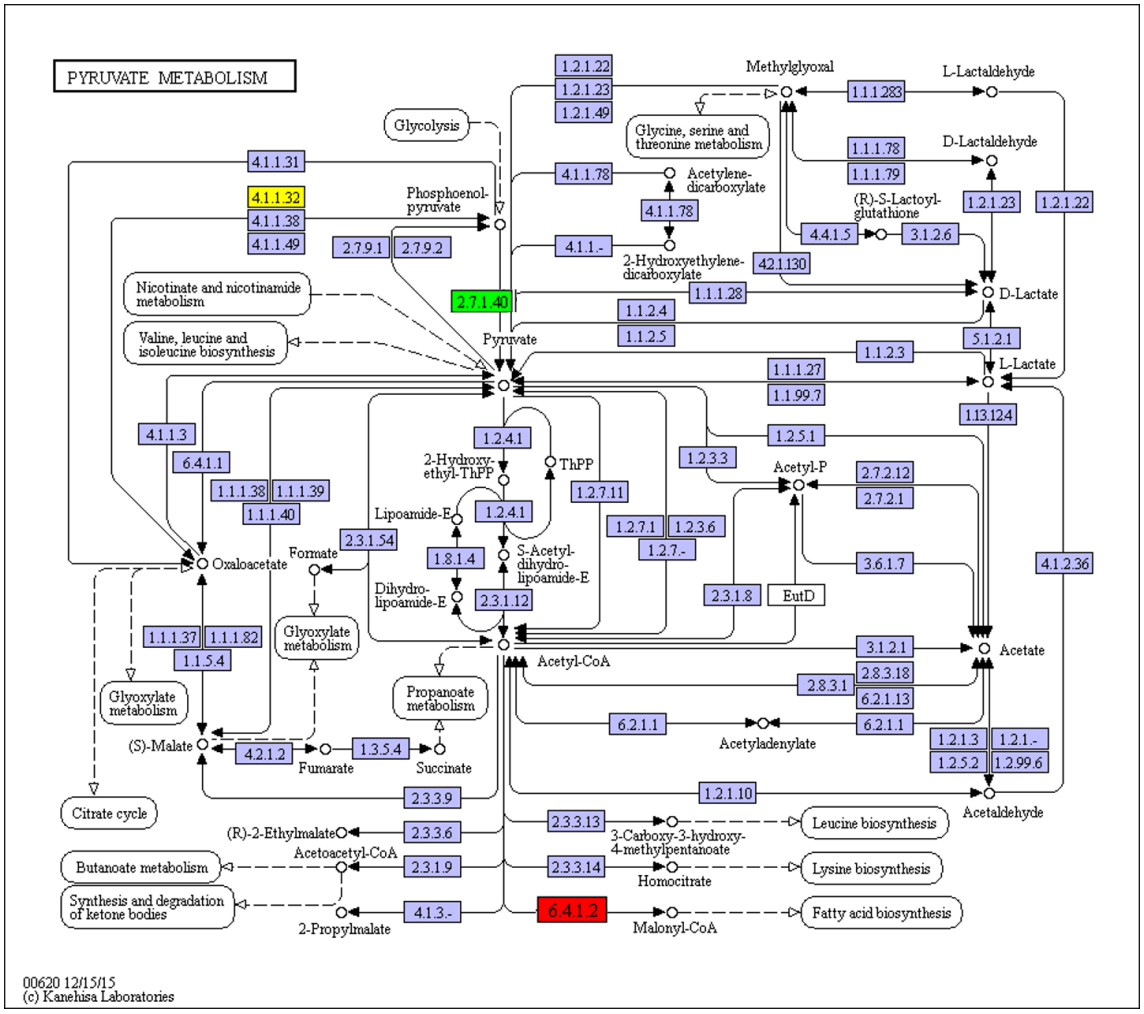


Figure 5. Pyruvate pathway obtained from Kyoto Encyclopaedia of Genes and Genomes (KEGG). Highlighted gene in yellow are highly expressed (RME>100) in SRS and in RC (PCK1 phosphoenolpyruvate carboxykinase (GTP) [EC: 4.1.1.32]). Highlighted gene in green are highly expressed (RME>100) in SRS (CDC19 encodes Piruvate Kinase, related with conversion of phosphoenol-pyruvate in pyruvate [EC: 2.7.1.40]). Highlighted gene in red are highly expressed (RME>100) in RC (ACC1 acetyl-CoA carboxylase / biotin carboxylase 1 [EC: 6.4.1.2]).

Differential expression analysis (DE)

The DE analysis has shown the overexpressed genes in root biofilms with and without caries (Figure 6). The up-regulated genes in health group were CaO19.610 and CaO19.2506. The CaO19.610 (FDR= 0.009) codes for a potential DNA binding regulator of filamentous growth (NCBI). This gene is a version of *C. albicans* EFG1 with altered C terminus. The CaO19.2506 (FDR= 0.018) codes for a hypothetical protein with a very weak similarity to *Streptococcal* proline-rich surface protein PspC.

The up-regulated genes in root caries group were UTP20, ITR1, DHN6, CaO19.7197, CaO19.7838, STT4, and GUT1. The UTP20 (FDR= 0.018) codes for a potential U3 small nucleolar RNAs (snoRNA) protein. The ITR1 (FDR= 0.036) codes for a potential active sugar transporter, potential myo-inositol transporter, similar to *S. cerevisiae* ITR1 (YDR497C). The DHN6 (FDR= 0.046) codes for a dehydrin hypothetical protein. The CaO19.7197 (FDR= 0.046) codes for a hypothetical protein similar to *S. cerevisiae* YLR002C, with unknown function. The CaO19.7838 (FDR= 0.046) codes for a flocculin-like protein serine-rich, repetitive ORF similar to *S. cerevisiae* MUC1 (YIR019C) cell surface flocculin. The STT4 (FDR= 0.046) codes for a hypothetical protein phosphatidylinositol-4-kinase. The GUT1 (FDR= 0.046) codes for a potential glycerol kinase Gut1p, likely carbohydrate kinase similar to *S. cerevisiae* GUT1 (YHL032C) glycerol kinase (NCBI).

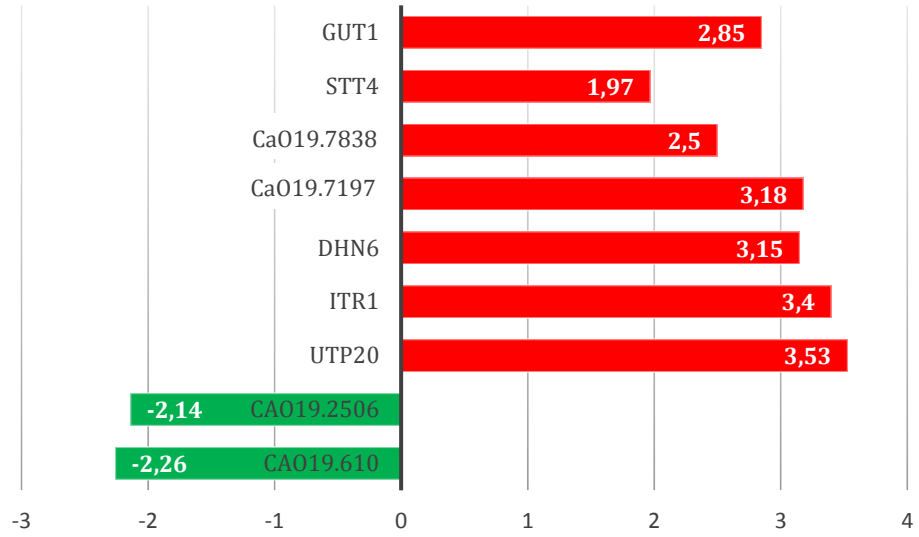


Figure 6. Differential expression (Log2FoldChange) of genes up-regulated in sound root surface (negative values - green) and up-regulated in root caries lesions (positive values - red) calculated using DESeq2 algorithm. FDR<0.05. GUT1= potential glycerol kinase; STT4= hypothetical protein phosphatidylinositol-4-kinase; CaO19.7838= flocullin-like protein serine-rich; CaO19.7197= hypothetical protein; DHN6= dehydrin hypothetical protein; ITR1= potential myo-inositol transporter; UTP20= potential U3 small nucleolar RNAs protein; CaO19.2506= hypothetical protein with a very weak similarity to S*treptoccal* proline-rich surface protein PspC; CaO19.610= potential DNA binding regulator of filamentous growth.

Discussion

The distribution of the most prevalent genes in SRS and RC are similar. Assuming that the gene expression not seem to be different in SRS and RC in a first view, we investigate in the literature genes related with virulence traits of this microorganisms (Sudbery, 2011; Arslan *et al.,* 2016; Höfs *et al.,* 2016). The expression of 67 genes associated to *C. albicans* virulence were investigated in the literature and none of this genes presented differential expression in our samples. Pathogenicity of *Candida* spp. are related with several survival strategies like the capacity to exploit and invade the host tissues, the capacity to form biofilms and co-aggregate to various microorganisms, the capacity to switch form to adapt to environmental, the capacity to produce acids and react to stress. Some virulence trait are expressed in both conditions which seems to be relevant to *Candida albicans* survival in root surface biofilms. The most expressed genes related to virulence traits are discussed below.

ACT1 and ALS3 are genes related to invasion factor. ACT1 is a potential housekeeping gene associated to *C. albicans* SC5314 related in the literature as actin [Freire *et al.,* 2015]. ACT1 codes for 2 hypothetical proteins, CaO19.12474 and CaO19.5007 [NCBI]. ALS3 is a cell surface agglutinin-like partial protein, also related with biofilm formation, adherence and morphogenesis [Sudbery, 2011; Mayer *et al.,* 2013; de Barros *et al.,* 2016; Höfs *et al.,* 2016]. ALS3 expression is regulated by growth conditions [Bruno *et al.,* 2010].

Regarding to biofilm formation, the gene HWP1 seems to have an important expression level. Hyphal wall protein1 (Hwp1) is a hyphal-and germ-tube-specific cell wall protein, and their expression is regulated by growth conditions [Bruno *et al.,* 2010; Calderoni and Fonzi, 2001]. De Barros *et al.* [2016] confirmed that HWP1 was one of the genes more expressed in the formation of biofilm *in vitro*. The N-terminal region of Hwp1 serves as a substrate for epithelial cell-associated transglutaminases. This cell-association results in covalent attachment of *Candida* to host epithelial cells via cross-linking of glutamine residues of the N-terminal region of Hwp1 to as yet unidentified host proteins [Moyes *et al.,* 2015], having an important role in adhesion too. Cell wall adhesins are the major proteins responsible by the *C. albicans* adhesion. Als (agglutinin-like sequence) of *C. albicans* is a member

of a family of eight glycosylated proteins (ALS1-7 and ALS9), all of them are cell surface proteins [Höfs *et al.,* 2016; Gaur and Klotz, 1997; Hoyer, 2001; Sheppard *et al.,* 2004].

The *C. albicans* ability to degrade collagens was observed in the literature [Nishimura *et al.,* 2002]. The genes of *C. albicans* that are related to this ability belong to SAPs family. This family are composed by a conjunct of secretory aspartyl proteinase that are described as proteins related with collagen degradation [Hube *et al.,* 1994; Hube, 1996]. Most of genes related to SAPs family presented low/medium expression in the present study. So, the collagens degradation by these genes does not seems to be of great relevance for root caries progression as expected.

The pH in carious lesion can be very acid in some periods. Therefore, in order to colonize such environment *C. albicans* must be able to adapt to a range of extremes pH. One example of how *C. albicans* adapts to physiological extremes is that at the neutral pH the organism expresses PHR1, a gene whose function is associated with cell wall synthesis and whose expression is optimum around neutrality. PHR1 expression is switched off and a second pH-regulated gene PHR2 provides a similar function but at an acid pH [Calderoni and Fonzi, 2001]. In the present study both genes are transcripts at low level. Other genes related to acid tolerance are Hsp90 (chaperone temperature dependent regulator) and RIM101 (a pH-dependent regulator) [Chen et al., 2014; O'Meara *et al.,* 2016]. In the present study both genes are highly expressed. Several functions were enriched among these genes, including some typically associated with virulence, such as adhesion, iron homeostasis, stress response, response to starvation, and biofilm formation [Amorim-Vaz and Sanglard, 2016]. Hsp genes are overexpressed in the initial stage of *C. albicans* biofilm formation [Becherelli *et al.,* 2013].

Changes in pH, nutrients availability, temperature, which could be viewed as stresses, active hyphal development [Brown and Gow, 1999]. In carious lesions several of this environmental stresses could be related to induce hyphal growth. In both oral and vaginal candidosis the hyphal form is related to tissue invasion and consequently its pathogenicity [Naglik *et al.,* 2008; Moyes *et al.,* 2015]. In root carious dentin, the degradation of dentin provide an environment that could be compared to a necrotic niche, a favourable environment for *C. albicans* growth. Some

genes are related as specific hyphae genes, such as ASL1, ALS3 and HWP1 [Nobile *et al.,* 2012], being ALS1 with medium expression in our samples while ALS3 and HWP1 has high expression. The MAPK and cAMP pathways are associated in the literature with morphogenesis process [Sudbery, 2011]. Although, major part of genes related with this pathways were identified in our samples, most of them present low or medium expression. A possible explanation lay down in the fact that hyphal growth is stimulated in neutral pH [Sudbery, 2011] that is not found in an active carious lesion site, usually acid.

Looking up to glycolysis gluconeogenesis and pyruvate metabolism it is possible to see that *C. albicans* from root biofilms with and without caries are involved in the acid production of pyruvate, ethanol and acetaldehyde and indirectly in lactate formation. In the present study, many genes related to glycolysis/gluconeogenesis and pyruvate pathways were identified, concluding that *C. albicans* generate energy mainly from glycolysis in both healthy and carious root sites. This suggests the importance of sugar availability for the genus prevalence in the plaque. The genes that presented RME>100 in glycolysis and gluconeogenesis metabolism in these study were TDH1 glyceraldehyde 3-phosphate dehydrogenase, PGK1 phosphoglycerate kinase, ENO1 enolase, PCK1 phosphoenolpyruvate carboxykinase (GTP), CaO19.10395 pyruvate decarboxylase, CaO19.2877 pyruvate decarboxylase, ADH1 alcohol dehydrogenase. Enolase (2-phospho-D-glycerate hydrolyase) is responsible to catalyses the dehydration of 2-phosphoglycerate to phosphoenolpyruvate. It also catalyses the reverse reaction during gluconeogenesis. It is among the most abundant proteins in *C. albicans* and *S. cerevisiae* [Chaffin, 1998]. Phosphoglycerate kinase (PGK) catalyses the hydrolysis of 1,3-bisphosphoglycerate to 3-phosphoglycerate with the production of ATP [Chaffin, 1998]. Other genes with RME>100, are HXK2 hexokinase, PGI1 glucose-6-phosphate isomerase, FBA1 fructose-bisphosphate aldolase, GPM1 probable phosphoglycerate mutase and CDC19 encodes pyruvate kinase, related with conversion of phosphoenol-pyruvate in pyruvate. Looking up to pyruvate metabolism genes that have RME>100 are PCK1 (phosphoenolpyruvate carboxykinase), CDC19 (encodes pyruvate kinase, related with conversion of phosphoenol-pyruvate in pyruvate) and ACC1 (acetyl-CoA carboxylase / biotin carboxylase 1). These genes that belong to pyruvate pathway with RME>100 are

majorly related with the formation of pyruvate as carbon source and with the conversion of Acetyl-CoA in Malonyl-CoA to form fatty acids.

The differential expression of genes in our study shows that up-regulated genes in health are related to biofilm formation while in disease the genes are related to stress tolerance, metabolism and invasion.

The genes up-regulated in biofilm of sound root surface are CaO19.610 (FDR= 0.009) and CaO19.2506 (FDR= 0.018). The CaO19.610 (FDR= 0.009) codes for a potential DNA binding regulator of filamentous growth [NCBI]. This gene is a version of *C. albicans* EFG1 with altered C terminus. Efg1 protein is a key transcriptional regulator in *C. albicans* and controls various aspects of morphogenesis and metabolism [Noffz et al., 2008], being required for the true hyphae growth, biofilm formation, cell adhesion and filamentous growth in *C. albicans* [Gancedo 2001]. EFG1 gene, confers to *C. albicans* the capacity of transition from commensal microorganism to opportunistic pathogen status [Pierce *et al.,* 2012]. In an *in vitro* experiment the gene expression of EFG1 had significantly higher expression levels at 12 h of observation, and decreased gradually their expression at 24 h and 48 h, suggesting that these genes are important in the initial biofilm formation stage (12 h) but not in the mature biofilms (24 and 48 h) [de Barros *et al.,* 2016]. However, others studies shown that EFG1 are essential for the formation of a mature and stable biofilm resistance to antifungal therapy and to the protection of the immune system, allowing the spread of infections caused by this microorganism [Finkel and Mitchell, 2011; Nobile *et al.*, 2012]. The CaO19.2506 (FDR= 0.018) codes for a hypothetical protein with a very weak similarity to Streptococcal proline-rich surface protein PspC. In *Streptococcus pneumoniae*, PspC has a well-established importance in adherence and colonization [Rosenow et al., 1997]. Its possible function could be related to adhesion and coding for a membrane adhesin. Both filamentous growth and cell wall adhesin are important for biofilm formation and that is why they were found up-regulated in sound root surface condition in the DE analysis. It is well stablished that *C. albicans* is able to form biofilm and co-aggregate with other microorganisms from the oral cavity [Charone *et al.,* 2013; Falsetta *et al.,* 2014].

The up-regulated genes in root caries group were UTP20, ITR1, DHN6, CaO19.7197, CaO19.7838, STT4, and GUT1.

UTP20, ITR1 and GUT1 are genes related to *C. albicans* metabolism. The up-regulation of these genes shows that *C. albicans* is active in disease. The UTP20 (FDR= 0.018) codes for a potential U3 small nucleolar RNAs (snoRNA) protein. UTP20 has been reported as a component of U3 snoRNA protein complex and has been implicated in 18S rRNA processing, being essential for 18 rRNA function [Wang 2007; Dez *et al.,* 2007]. The ITR1 (FDR= 0.036) codes for a potential active sugar transporter, potential myo-inositol transporter, similar to *S. cerevisiae* ITR1 (YDR497C). Myo-inositol is an essential substrate for *C. albicans*, and it can be utilized as carbon source. For its survival, *C. albicans* must be able to synthesize the essential metabolite inositol or acquire it from the host. In the absence of ITR1, *C. albicans* could not transport inositol and was nonviable [Chen *et al.,* 2008]. The GUT1 (FDR= 0.046) codes for a potential glycerol kinase Gut1p, likely carbohydrate kinase similar to *S. cerevisiae* GUT1 (YHL032C) glycerol kinase (NCBI). In *Saccharomyces cerevisiae* glycerol utilization is mediated by two enzymes, glycerol kinase (Gut1p) and mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p). The carbon source regulation of GUT1 depends on carbon source availability. The promoter activity of GUT1 was lower during growth on glucose and highest on the non-fermentable carbon sources, glycerol, ethanol, lactate, acetate and oleic acid [Grauslund *et al.,* 1999]. The lactate utilization by *C. albicans* could be related to the pH regulation in biofilm which is important for the microbiome survival. This ability may explain the importance of *C. albicans* as a partner in crime in the maintenance of a viable biofilm preventing excessive acidification of the carious habitat. Besides that, this ability could explain the synergism between *mutans streptococci* and *C. albicans* already related in the literature [Falsetta *et al.,* 2014]. Althougt *C. albicans* is important for the biofilm dysbiosis. Looking at the *C. albicans* gene expression in root caries it does not seems to be a risk factor as suggested by Nikawa [2003].

The up-regulated genes DHN6 and STT4 could be related to the ability to survive in an extreme environment such as the root cavitated caries lesions. The DHN6 (FDR= 0.046) codes for a dehydrin hypothetical protein. Dehydrin is a stress tolerance gene in plants. Dehydrins (DHNs) constitute a distinct biochemical group of LEA (Late Embryogenesis Abundant) proteins. These proteins can be induced in vegetative tissues by various stress factors that cause cell dehydration (i.e., drought,

salinity, cold, heat, low temperature, etc) [Hanin *et al.*, 201; Drira *et al.,* 2015]. The STT4 (FDR= 0.046) codes for a hypothetical protein phosphatidylinositol-4-kinase. The gene STT4, is essential for viability and plays an important role in the phosphatidylinositol-mediated signal transduction pathway required for cell wall integrity [Pramanik *et al.,* 1997].

The CaO19.7838 (FDR= 0.046) codes for a flocculin-like protein serine-rich, repetitive ORF similar to S*. cerevisiae* MUC1 (YIR019C) cell surface flocculin. MUC1 encodes for cell-surface flocculin and is required for pseudohyphal and invasive growth of *C. albicans* [Gancedo, 2001]. The up-regulation of these invasive growth gene shows the importance of this virulence trait for the colonization/penetration of *C. albicans* in the carious dentin.

The CaO19.7197 (FDR= 0.046) codes for a hypothetical protein similar to *S. cerevisiae* YLR002C, with unknown function. In the present study we found several hypothetical proteins and genes with uncharacterized function, highlighting the importance of more studies related with *C. albicans* transcriptome.

Conclusion

Our data shows that *Candida albicans* has an active metabolism in biofilm of sound root surface and biofilm of carious dentin of root caries as well. The differential expression analysis shows that in health the up-regulated genes are related to metabolic activity, sugar transport, stress tolerance, invasion and pH regulation. *C. albicans* is, therefore, important to root caries progression.

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Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

References

Aamdal-Scheie A, Luan WM, Dahlén G, Ferjerskov O: Plaque pH and Microflora of Dental Plaque on Sound and Carious Root Surfaces. J Dent Res 1996;75(11):1901-1908.

Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, Leys EJ, Paster BJ. Bacteria of Dental Caries in Primary and Permanent Teeth in Children and Young Adults. J Clin Microbiol 2008;46(4):1407-1417.

Amorim-Vaz, S.; Sanglard, D; Novel Approches for Fungal Transcriptomics from Host Samples. Frontiers in Microbiology 2016;6:1571.

Arslan S, Koç AN, Şekerci AE, Tanriverdi F, Sav H, Aydemir G, Diri H: Genotypes and virulence factors of *Candida* species isolated from oral cavities of patients with type 2 diabetes mellitus. Turk J Med Sci 2016;46:18-27.

Avbelj M, Zupan J, Raspor P; Quorum-sensing in yeast and its potential in wine making. Appl Microbiol Biotechnol 2016;100(18):7841-52.

Bamford CV, d'Mello A, Nobbs AH, Dutton LC, Vickerman MM, Jenkinson HF; *Streptococcus gordonii* modulates *Candida albicans* biofilm formation through intergeneric communication. Infect Immun 2009;77:3696–3704.

Banno Y, Yamada T, Nozawa Y: Secreted phospholipases of the dimorphic fungus, *Candida albicans*; separation of three enzymes and some biological properties. Sabouraudia 1985;23:47-54.

Barbieri DSAV, Vicente VA, Fraiz FC, Lavoranti OJ, Svidzinski TIE, Pinheiro RL: Analysis of the *in vitro* adherence of *Streptococcus mutans* and *Candida albicans*. Braz Journ Microbiol 2007;38:624-631.

Barros LM, Boriollo MF, Alves AC, Klein MI, Gonçalves RB, Höfling JF: Genetic diversity and exoenzyme activities of *Candida albicans* and *Candida dubliniensis* isolated from the oral cavity of Brazilian periodontal patients. Arch Oral Biol 2008;12:1172– 1178.

De Barros PP, Rossoni RD, De Camargo Ribeiro F, Junqueira JC, Jorge AO.: Temporal Profile of Biofilm Formation, Gene Expression and Virulence Analysis in *Candida albicans* Strains. Mycopathologia 2016;9.

Becherelli M, Tao J, Ryder NS: Involvement of Heat Shock Proteins in *Candida albicans* biofilm formation. Mol Microbiol Biotechnol 2013;23:396–400.

Beighton D, Lynch E: Relationships between yeasts and primary root-caries lesions. Gerodontology 1993;10:105-108.

Beighton D, Lynch E: Comparison of selected microflora of plaque and underlying carious dentin associated with primary root caries lesions. Caries Res 1995;29(2):154-8.

Beighton D, Brailsford S, Samaranayake LP, Brown JP, Ping FX, Grant-Mills D, Harris R, Lo EC, Naidoo S, Ramos-Gomez F, Soo TC, Burnside G, Pine CM:A multi-country comparison of caries-associated microflora in demographically diverse children. Community Dent Health 2004;21(1):96-101.

Beighton D: The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol 2005;33:248–55.

Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, Romero H, Simón-Soro A, Pignatelli M, Mira A: The oral metagenome in health and disease. ISME J 2012;6:46-56.

Boris S, Barbes C: Role played by *lactobacilli* in controlling the population of vaginal pathogens. Microbes Infect 2000;2:543-546.

Brailsford SR, Shah B, Simons D, Gilbert S, Clark D, Ines I, Adams SE, Allison C, Beighton: The Predominant Aciduric Microflora of Root-caries Lesions. J Dent Res 2001;80(9):1828-1833.

Braun, B. R. & Johnson, A. D: TUP1, CPH1 and EFG1 make independent contributions to filamentation in *Candida albicans*. Genetics 2000;155:57–67.

Brighenti FL, Medeiros AC, Matos BM, Ribeiro ZEA, Koga-Ito CY: Evaluation of caries-associated virulence of *Candida albicans* isolated from saliva of pediatric patients with sickle-cell anemia. J Appl Oral Sci 2014; 22 (6): 484-9.

Brown AJP, Gow NAR: Regulatory networks controlling *Candida albicans* morphogenesis. Trends Microbiol 1999;7(8): 333-338.

Bruno VM, Wang Z, Marjani SL, Euskirchen GM, Martin J, Sherlock G, Snyder M: Comprehensive annotation of the transcriptome of the human fungal pathogen *Candida albicans* using RNA-seq. Genome Res 2010;20(10):1451-8.

Calderone RA, Fonzi WA: Virulence factors of *Candida albicans*. Trends Microbiol 2001;9(7):327-335.

Cannon RD, Holmes AR, Mason AB, Monk BC. Oral *Candida*: clearance, colonization, or candidiasis? J Dent Res 1995;74:1152.

De Carvalho FG, Silva DS, Hebling J, Spolidorio LC, Spolidorio DMP: Presence of *mutans streptococci* and *Candida* spp. in dental plaque/dentin of carious teeth and early childhood caries. Arc Oral Biol 2006;51:1024-1028.

Chaffin WL, López-Ribot JL, Casanova M, Gozalbo D, Martínez JP: Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol Mol Biol Rev 1998 Mar;62(1):130-80.

Charone S, Portela MB, das Chagas MS, Soares RMA, Castro GFBA: Biofilm of *Candida albicans* from oral cavity of an HIV-infected child: challenge on enamel microhardness. Oral Surg Oral Med Oral Pathol Oral Radio 2013;115(4):500-504.

Chen CP, Posy S, Shaul B, Shapiro L, Honing BH: Specificity of cell-cell adhesion by classical cadherins: Critical role for low-affinity dimerization through β-strand swapping. Proc Natl Acd Sci USA 2005;102:8531-8536.

Chen YL, Kauffman S, Reynolds TB: *Candida albicans* uses multiple mechanisms to acquire the essential metabolite inositol during infection. Infection and Immunity 2008;76(6):2793–2801.

Chen Y, Toffaletti DL, Tenor JL, Litvintseva AP, Fang C, Mitchell TG, *et al:* The *Cryptococcus neoformans* transcriptome at the site of human meningitis. *mBio* 2014; 5, e1087-e1093. doi: 10.1128/mBio.01087-13.

Clarkson BH, Krell D, Wefel JS, Crall J, Feagin FF: *In vitro* Caries-like lesions production by *Streptococcus mutans* and *Actinomyces viscosus* using sucrose and starch. J Dent Res 1987;66:795–8.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scoth HM: Microbial biofilms. Annu Rev Microbiol 1995;49:711-745.

Cury JA, Rebelo MAB, Del Bel Cury AA, Derbyshire MT, Tabchoury CP: Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. Caries Res 2000;34:491-7.

Dame-Teixeira N, Parolo CC, Maltz M, Tugnait A, Devine D, Do T: *Actinomyces* spp. gene expression in root caries lesions. J Oral Microbiol 2016;6(8):32383.

Dez C, Dlakic M, Tollervey D: Roles of the HEAT repeat proteins Utp10 and Utp20 in 40S ribosome maturation. RNA 2007;13:1516–1527.

Do T, Sheehy EC, Mulli T, Hughes F, Beighton D: Transcriptomic analysis of three *Veillonella* spp. present in carious dentin and in the saliva of caries-free individuals. Front Cell Infect Microbiol 2015;5:1-8.

Drira M, Saibi W, Amara I, Masmoudi K, Hanin M, Brini F: Wheat dehydrin K-segments ensure bacterial stress tolerance, antiaggregation and antimicrobial effects. Appl Biochem Biotechnol 2015;175(7):3310-21.

Erwig LP, Gow NA: Interactions of fungal pathogens with phagocytes. Nat Rev Microbiol 2016;14(3):163-76.

Falsetta ML, Klein MI, Colonne PM, Scott-Anne K, Gregoire S, Pai CH, Gonzalez-Begne M, Watson G, Krysan DJ, Bowen WH, Koo H: Symbiotic relationship between *Streptococcus mutants* and *Candida albicans* synergizes virulence of plaque biofilms *in vivo*. Infect Immun 2014;82(5):1968-1981.

Feng Q H, Summers E, Guo B, Fink G: Ras signaling is required for serum-induced hyphal differentiation in *Candida albicans*. J Bacteriol 1999;181:6339–6346.

Finkel JS, Mitchell AP: Genetic control of *Candida albicans* biofilm development. Nat Rev Microbiol 2011;9:109–18.

Freire F, de Barros PP, da Silva Ávila D, Brito GN, Junqueira JC**,** Jorge AO: Evaluation of gene expression SAP5, LIP9, and PLB2 of *Candida albicans* biofilms after photodynamic inactivation. Lasers Med Sci 2015;30(5):1511-8.

Gancedo JM: Control of pseudohyphae formation in *Saccharomyces cerevisiae*. FEMS Microbiology Reviews 2001;25:107-123.

Gaur NK, Klotz SA: Expression, cloning, and characterization of a *Candida albicans* gene, ALA1, that confers adherence properties upon Saccharomyces cerevisiae for extracellular matrix proteins. Infect. Immun. 1997;65:5289–5294.

Grauslund M, Lopes JM, Rønnow B: Expression of GUT1, which encodes glycerol kinase in *Saccharomyces cerevisiae*, is controlled by the positive regulators Adr1p, Ino2p and Ino4p and the negative regulator Opi1p in a carbon source-dependent fashion. Nucleic Acids Res 1999;27(22):4391–4398.

Gropp K, Schild L, Schindler S, Hube B, Zipfel PF, Skerka C: The *yeast Candida albicans* evades human complement attack by secretion of aspartic proteases. Mol Immunol 2009;47(2-3):465-75.

Hall-Stoodley L, Stoodley P: Evolving concepts in biofilm infections. Cell Microbiol 2009;11:1034-1043.

Hanin M, Brini F, Ebel C, Toda Y, Takeda S, Masmoudi K: Plant dehydrins and stress tolerance. Plant Signaling & Behavior 2011;6(10):1503-1509.

Herrero ER, Slomka V, Boon N, Bernaerts K, Hernandez-Sanabria E, Quirynen M, Teughels W. Dysbiosis by neutralizing commensal mediated inhibition of pathobionts. Sci Rep 2016;29(6):38179.

Höfs S, Mogavero S, Hube B: Interaction of *Candida albicans* with host cells: virulence factors, host defense, escape strategies, and the microbiota. J Microbiol 2016;54(3):149-169.

Hogan DA: Talking to themselves: autoregulation and quorum sensingin fungi. Eukaryot Cell 2006;5:613–619.

Holmes, A.R., McNab, R., and Jenkinson, H.F: *Candida albicans* binding to the oral bacterium *Streptococcus gordonii* involves multiple adhesin-receptor interactions. Infect. Immun. 1996;64:4680–4685.

Holmes AR, van der Wielen P, Cannon RD, Ruske D, Dawes P: *Candida albicans* binds to saliva proteins selectively adsorved to silicone. Oral Surg Oral Medic Oral Radiol Endodontol 2006;102:488-494.

Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, et al: Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl Environ Microbiol 2001;67:2982–2992.

Hoyer, L.L: The ALS gene family of *Candida albicans*. Trends Microbiol 2001;9:176–180.

Hube B, Monod M, Schofield DA, Brown AJ, Gow NA: Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans.* Mol Microbiol 1994;14(1):87-99.

Hube B. *Candida albicans* secreted aspartyl proteinases. Curr Top Med Mycal 1996;7:55-56.

Jekinson HF, Lala HC, Sherpherd MG: Coaggregation of *Streptococcus sanguis* and other streptococcis with *Candida albicans*. Infect Immun 1990;58:1429-1436.

Jin Y, Samaranayake LP, Samaranayake Y, Yip H:. Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. Arch Oral Biol 2004;49:789-98.

Kebaara, B. W. *et al*: *Candida albicans* Tup1 is involved in farnesol-mediated inhibition of filamentous growth induction. Eukaryot Cell 2008;7:980–987.

Kim JY: Human fungal pathogens: Why should we learn? Journal of Microbiology 2016;54(3):145–148.

Klinke T, Kneist S, de Soet JJ, Kuhlisch E, Mauersberger S, Förster A, Klimm W: Acid Production by Oral Strains of *Candida albicans* and Lactobacilli. Caries Res 2009;43:83–91.

Klinke T, Guggenheim B, Klimm W, Thurnheer T: Dental caries in rats associated with *Candida albicans*. Caries Res 2011;45:100-106.

Kraneveld EA, Buijs MJ, Bonder MJ, Visser M, Keijser BJ, Crielaard W, Zaura E: The relation between oral *Candida* load and bacterial microbiome profiles in Dutch older adults. PLoS One. 2012;7(8):e42770.

Leberer E et al: Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*. Mol. Microbiol 2001;42:673–687.

Liu H, Kohler J, Fink GR: Suppression of hyphal formation in *Candida albicans* mutation of a STE112 homolog. Science 1994;266:1723-1726.

Lo H J et al: Non filamentous *C. albicans* mutants are avirulent. Cell 1997;90:939–949.

Loesch WJ, Taylor GW, Dominguez LD, Grossman NS, Stoll J: Factors which are associated with dental decay in the older individual. Gerodontology 1999;16(1):37-46.

Love M, Anders S, Huber W: Differential analysis of count data – the DESeq2 package: bioRxiv. 2014.

Lu Y, Su C, Liu H: *Candida albicans* hyphal initiation and elongation. Trends Microbiol 2014;22(12):707-714.

Makihira S, Nikawa H, Tamagami M, Hamada T, Samaranayake LP: Differences in *Candida albicans* adhesion to intact and denatured type I collagen in vitro. Oral Microbiol Immunol 2002;17:129-31.

Manfredi M, McCullough MJ, Vescoci P, Al-Kaarawi ZM, Poster SR: Update on diabetes mellitus and related oral diseases. Oral Dis 2004;10:187-200.

Mantzourani M, Fenlon M, Beighton D: Association between *Bifidobacteriaceae* and the clinical severity of root caries lesions. Oral Microbiol Immunol 2009;24:32-37.

Marchant S, Brailsford SR, Twomey AC, Roberts GJ, Beighton D: The predominant microflora of nursing caries lesions. Caries Res 2001;35:397-406.

Marsh PD: Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 1994;8(2):263-271.

Mayer FL, Wilson D, Jacobsen ID, Miramón P, Groβe K, Hube B: The novel *Candida albicans* transporter Dur31 is a multi-stage pathogenicity factor. PLoS Pathog 2012;8:e1002592.

Metwalli KH, Khan SA, Krom BP, Jabra-Rizk MA: *Streptococcus mutans, Candida albicans,* and the Human Mouth: A Sticky Situation. PLOS Pathogens 2013;9(10):1-5.

Moseley JB, Goode BL: The yeast actin cytoskeleton: from cellular function to biochemical mechanism. Microbiol Mol Biol Reviews 2006;70(3):605–45.

Moyes DL, Richardson JP, Naglik JR: *Candida albicans*-epithelial interactions and pathogenicity mechanisms: scrarching the surface. Virulence 2015;6:338-346.

Nair RG, Anil S, Samaranayake LP: The effect of oral bacteria on *Candida albicans* germ-tube formation. APMIS 2001;109:147-154.

Naglik JR, Moyes D, Makwana J, Kanzaria P, Tsichlaki E, Weindl G, Tappuni AR, Rodgers CA, Woodman AJ, Challacombe SJ, Schaller M, Hube B: Quantitative

expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. Microbiology 2008;154:3266–80.

Nikawa H, Yamamato T, Hayashi S, Nikawa Y, Hamada T: Growth and/or acid production of *Candida albicans* on soft lining materials *in vitro.* J Oral Rehabil 1994;21(5),585-594.

Nikawa H, Hamada T, Yamashiro H, Murata H, Subiwahjudi A: The effect of saliva or serum on *Streptococcus mutans* and *Candida albicans* colonization of hydroxylapatite beads. J Dent. 1998;26(1):31-7.

Nikawa H, Egusa H, Makihira S, Yamashiro H, Fukushima H, Jin C, Nishimura M, Pudji RR, Hamada T: Alteration of the coadherence of *Candida albicans* with oral bacteria by dietary sugars. Oral Microbiol Immunol 2001:16;279–283.

Nikawa H, Yamashiro H, Makihira S, Nishimura M, Egusa H, Furukawa M, Setijanto D, Hamada T: *In vitro* cariogenic potential of *Candida albicans*. Mycoses 2003;46:471-478.

Nishimura M, Nikawa H, Yamashiro H, Nishimura H, Hamada T, Embery G: Cell-associated collagenolytic activity by *Candida albicans*. Mycopathologia 2002;153:125-8.

Nobile CJ, Fox EP, Nett JE, TR Sorrells, QM Mitrovich, AD Hernday, BB Tuch, DR Andes, AD Johnson: A recently evolved transcriptional network controls biofilm development in *Candida albicans*. Cell. 2012;148:126–38.

Noffz CS, Liedschulte V, Lengeler K, Ernst JF. Functional Mapping of the *Candida albicans* Efg1 Regulator. Eukaryotic Cell 2008;7(5):881–893.

Noverr MC, Huffnagle GB: Regulation of *Candida albicans* morphogenesis by fatty acid metabolites. Infect. Immun. 2004;72:6206–6210.

Nyvad B, Fejerskov O: Root surface caries: clinical, histopathological and microbiological features and clinical implications. Int Dent J 1982;32:312-26.

O'Meara TR, Veri AO, Polvi EJ, Li X, Valaei SF, Diezmann S, Cowen LE: Mapping the Hsp90 Genetic Network Reveals Ergosterol Biosynthesis and Phosphatidylinositol-4-Kinase Signaling as Core Circuitry Governing Cellular Stress. PLoS Genet 2016;12(6):e1006142.

Peters B, Ovchinnikova E: Staphylococcus aureus adherence to *Candida albicans* hyphae is mediated by the hyphal adhesin Als3p. Microbiology. 2012;158:2975-86.

Pierce VJ, Kumamoto AC: Variation in *Candida albicans* EFG1 expression enables host-dependent changes in colonizing fungal populations. MBio 2012;3.

Polke M, Hube B, Jacobsen ID: *Candida* survival strategies. Adv Appl Microbiol 2015;91:139-235.

Polke M, Sprenger M, Scherlach K, Albán-Proano MC, Martin R, Hertweck C, Hube B, and Jacobsen ID: A functional link between hyphal maintenance and quorum sensing in *Candida albicans.* Mol Microbiol 2016.

Pramanik A, Garcia E, Ospina R, Powell M, Martinez M, Alejo W, McKoy J, Moore CW: Cloning, characterization and identification of the gene encoding phosphatidylinositol 4-kinase. Cell Mol Biol 1997;43(7):1007-18.

Preza D, Olsen I, Aas JA, Willumsen T, Grinde B, Paster BJ: Bacterial Profiles of Root Caries in Elderly Patients. J Clin Microb. 2008;46(6):2015–2021.

Raja M, Hannan A, Ali K: Association of oral candidal carriage with dental caries in children. Caries Res 2010;44:272-276.

Ricker A, Vickerman M, Dongari-Bagtzoglou A: *Streptococcus gordonii* glucosyltransferase promotes biofilm interactions with *Candida albicans*. J Oral Microbiol 2014;6.

Rosenow CP, Ryan JN, Weiser S, Johnson P, Fontan A, Ortqvist HR, Masure: Contribution of novel choline-binding proteins to adherence, colonization, and immunogenicity of *Streptococcus pneumoniae*. Mol Microbiol 1997;25:819.

Samaranayake LP, MacFarlane TW: An *in vitro* study of the adherence of *Candida albicans* to acrylic surfaces. Arch Oral Biol 1980;25:603-609.

Samaranayake LP, Geddes DA, Weetman DA, MacFarlane TW: Growth and acid production of *Candida albicans* in carbohydrate supplemented media. Microbios 1983:37(148),105-115.

Samaranayake LP, Hughes A, Weetman DA, MacFarlane TW: Growth and acid production of *Candida* species in human saliva supplemented with glucose. J Oral Pathol 1986:15(5),251-254.

Schüpbach P, Guggenheim B, Lutz F: Histopathology of root surface caries. J Dent Res 1990;69:1195-1204.

Shapiro R S *et al:* Hsp90 orchestrates temperature dependent *Candida albicans* morphogenesis via Ras1–PKA signaling. Curr Biol 2009;19:621–629.

Shen S, Samaranayake LP, Yip HK, Dyson JE: Bacterial and yeast flora of root surface caries in elderly, ethnic Chinese. Oral Microbiol 2002;8:207-217.

Sheppard DC, Yeaman MR, Welch WH, Phan QT, Fu Y, Ibrahim AS, Filler SG, Zhang M, Waring AJ, Edwards JE: Functional and structural diversity in the Als protein family of *Candida albicans*. J Biol Chem 2004;279:30480–30489.

Silverman RJ, Nobbs AH, Vickerman MM *et al*: Interaction of *Candida albicans* cell wall Als3 protein with *Streptococcus gordonii* SspB adhesin promotes development of mixed-species communities. Infect Immun 2010;78:4644-52.

Stoldt V R, Sonneborn A, Leuker CE, Ernst JF: Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J 1997;16:1982–1991.

Sudbery PE: Growth of *Candida albicans* hyphae. Nature Reviews 2011;9:737-748.

Sullivan JMO, Jenkinson HF, Cannon RD: Adhesion of *Candida albicans* to oral *streptococci* is promoted by selective adsorption of salivary proteins to the streptococcal cell surface. Microbiology 2000;146:41–48.

Syed SA, Loesche WJ, Pape HL, Grenier E: Predominant cultivable flora isolated from human root surface caries plaque. Infect Immun 1975;11:727-731.

Szabó B, László M, Papp-Falusi E, Szabó Z, Szabó J, Márton I, Kelentey B: Studies on the possible aetiological role of diferente *Candida* species in pathogenesis of dentin caries by monitoring the calcium release from tooth particles. Acta Microbiol Imm Hung 2014;61(1):11-17.

Takahashi N, Nyvad B: Ecological Hypothesis of Dentin and Root Caries. Car Res 2016;50:422–431.

Thein ZM, Samaranayake YH, Samaranayake LP: Effect of oral bacteria on growth and survival of *Candida albicans* biofilms. Arc of Oral Biol 2006;51:672-680.

Trevino-Rangel R de J, Gonzalez JG, Gonzalez GM: Aspartyl proteinase, phospholipase, esterase and hemolysin activities of clinical isolates of the *Candida parapsilosis* species complex. Med Mycol 2013;51:331-335.

van Houte J, Lopman J, Kent R: The predominant cultivable flora of sound and carious human root surface. J Dent Res 1994;73:1727-1734.

Wang Y, Liu J, Zhao H, Lu W, Zhao J, Yang L, Li N, Du X, Ke Y: Human 1A6/DRIM, the homolog of yeast Utp20, functions in the 18S rRNA processing. Biochim Biophys Acta 2007;1773(6):863-8.

Wätchler B, Citiulo F, Jablonowski N, Förster S, Dalle F, Schaller M, Wilson D, Hube B: *Candida albicans* epithelial interactions: dissecting the roles of active penetration, induced endocytosis and host factors on the infection process. PLoS One 2012;e36952.

Williams DW, Jordan RP, Wei XQ, Alves CT, Wise MP, Wilson MJ, Lewis MA: Interactions of *Candida albicans* with host epithelial surfaces. J Oral Microbiol 2013;5.

Winter MB, Salcedo EC, Lohse MB, Hartooni N, Gulati M, Sanchez H, Takagi J, Hube B, Andes DR, Johnson AD, Craik CS, Nobile CJ: Global Identification of Biofilm-Specific Proteolysis in *Candida albicans*. MBio. 2016;7(5):13.

Yang W, Yan L, Wu C, Zhao X, Tang J: Fungal invasion of epithelial cells. Microbiol Res 2014;169:803–810.

Zeidler, U. et al: UME6 is a crucial downstream target of other transcriptional regulators of true hyphal development in *Candida albicans*. FEMS Yeast Res 2009;9:126–142.

Zeng L, Choi SC, Danko CG, Siepel A, Stanhope MJ, Burne RA: Gene regulation by CcpA and catabolite repression explored by RNA-Seq in *Streptococcus mutans*. PLoS One. 2013;8(3):e60465.