Molecular characterization of Candida in the oral cavity and factors involved in biofilm formation and virulence
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Chapter 1

Introduction:

Molecular Characterization of *Candida* in the Oral Cavity and Factors Involved in Biofilm Formation and Virulence
Growing population of older adults

Improvements in social living conditions and medical care have resulted in extension of the average human life span. As a consequence, the number of older adults and people needing (intensive) healthcare services is expected to increase significantly in the next decades. In the European Union, the proportion of persons of 65 years and older is predicted to reach 53% of the total population by the year 2025 (Muenz, 2007). At the same time, a decline is seen in the absence of natural teeth in this population (Müller et al., 2007). With increasing number of older adults retaining their natural teeth, risk is enhanced for diseases in the oral cavity. Aging is a complex multidimensional process of physical, physiological, and social changes in a person over time, which may lead to various health-related problems. For example, senescence of tissues can cause mucosal fragility, and senescence of functions can cause a lowered immune response (Reynolds, 2014). Polypathologies (e.g., diabetes and malignancies), polymedications, and malnutrition are also factors that contribute to changes during aging, and may disturb the microbial homeostasis in the oral cavity of older adults (Bodineau et al., 2009; Reynolds, 2014).

The oral cavity and its resident microbiota

The human oral cavity consists of several different components, including the teeth, gingiva (gums), tongue, palate, cheeks, lips, and floor of the mouth. Saliva flows over the oral surfaces. Glycoproteins, such as the salivary mucins MUC5B, MUC7, and secretory immunoglobulin A (IgA) (Gibbins et al., 2013), form a thin layer on oral surfaces, named a pellicle. The surfaces in the mouth are lined by mucosal- and epithelial cells (shedding surfaces), except for the teeth, prostheses or implants (non-shedding surfaces). Shedding and non-shedding surfaces in the mouth are heavily colonized by microorganisms, the so-called oral microbiome.

Of the about $10^{14}$ cells that make up human beings only ten percent are mammalian cells (Bianconi et al., 2013; MacDougall, 2012). The majority are residing microorganisms, the resident microbiota, which consists of hundreds of different viral, archaeal, bacterial, fungal, and protozoan species (Dewhirst et al., 2010; Wade, 2013).

A variety of viruses, mostly disease-associated, can be found in the mouth. Herpes viruses are among the most prevalent in saliva, and may cause
diseases of the oral mucosa or the periodontium (Slots and Slots, 2011). Herpes simplex for instance causes inflammation of the gingiva and possibly oral mucositis in vulnerable patients (van der Beek et al., 2012). Human papilloma virus can be detected as a resident, in oral mucosa and saliva of healthy adults, but DNA is also detected in gingival biopsies from periodontitis lesions (Slots and Slots, 2011). Papillomavirus type 16 is associated with a subset of oropharyngeal squamous cell carcinoma (Marur et al., 2010). Human immunodeficiency virus (HIV) and hepatitis viruses are blood borne pathogens, which, via gingival crevicular fluid, can be detected in saliva. Some viruses such as mumps and measles can be transmitted by inhalation of aerosols. Rabies virus resides in dogs and other animals, and can be transmitted through a bite. Mumps and rabies cause infection of the salivary gland, and thus can be found in saliva of affected persons (Slots and Slots, 2011).

The Archaea species Methanobrevibacter oralis, Methanobacterium curvum/congolense and Methanosarcina mazeii are detected in the oral cavity of healthy subjects and patients suffering from periodontitis (Matarazzo et al., 2011).

The oral bacterial community is dominated by the phyla Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Spirochaetes (Dewhirst et al., 2010). We have applied barcoded pyrosequencing of the bacterial hypervariable regions V5-V7 of the 16S rRNA gene, and found that these bacterial phyla were also present in salivary samples of Dutch older adults (Kraneveld et al., 2012). The genera predominantly found in our study were Streptococcus, Rothia, Veillonella, Prevotella, Gemella, Actinomyces, Neisseria, Porphyromonas, Haemophilus, Lactobacillus, and Lepotrichia (Kraneveld et al., 2012).

In an oral mycobiome study, 85 fungal genera were found in the oral cavity of healthy individuals. Candida, Cladosporium, Aureobasidium, Saccharomycetales, Aspergillus, Fusarium, and Cryptococcus species were among the predominant genera (Ghannoum et al., 2010). Candida species are a group of opportunistic pathogenic yeasts. In most individuals they reside as harmless microorganisms in the oral cavity (Kraneveld et al., 2012). However, if given the opportunity, Candida species can cause a local oral infection, called candidiasis (Pankhurst, 2013), see Figure 1. Candida albicans is the most prevalent fungal pathogen in oral mucosal and systemic infections (Pfaller and
Diekema, 2007). \textit{C. albicans} prevalence is also reported to be associated with dental caries (Gábris et al., 1999; Moalic et al., 2001; Yang et al., 2012). Besides \textit{C. albicans}, non-\textit{albicans} species such as \textit{C. glabrata}, \textit{C. parapsilosis}, \textit{C. tropicalis}, \textit{C. krusei}, and \textit{C. kefyr} have been detected in healthy and diseased individuals, and can also cause mucosal and systemic infections (Bagg et al., 2003; Furlaneto-Maia et al., 2008; Laheij et al., 2012; Yang et al., 2011). Recently, \textit{C. glabrata} has emerged as an important opportunistic pathogen due to widespread use of immunosuppressive therapy, indwelling medical devices, and broad-spectrum antibiotics (Rodriques et al., 2014; Silva et al., 2012). Treatment of \textit{C. glabrata} infections poses a medical problem due to its ability to form resilient biofilms and its high intrinsic resistance to commonly used azole antifungals (Rodriques et al., 2014).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Images.png}
\caption{Images of Candida infections in the oral cavity. (A) Candidiasis in the mouth of a baby, also known as oral thrush. (B) Candidiasis on the tongue of a child who had taken antibiotics. (C) Median rhomboid glossitis, a type of chronic atrophic candidiasis, on the tongue of a 72-year-old man. (D) Candidiasis in the mouth and oropharynx in a person with human immunodeficiency virus (HIV). (E) An elderly woman with angular cheilitis, a result of a \textit{Candida} infection associated with the wearing of a denture. Images A, B, and C are taken from Wikimedia Commons, and are from James Heilman (A and B) and Klaus D. Peter (C). Images D and E are from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification numbers #6053 (D) and #2925(E).}
\end{figure}
Two protozoan species have been found among oral resident microbiota: an amoeba, *Entamoeba gingivalis*, and *Trichomonas tenax* (Wade, 2013; Wantland et al., 1958).

All the above described oral resident microorganisms together have evolved to co-exist with the host; there is a mutually beneficial relationship. For example, the presence of microbiota on all surfaces of the mouth limits colonization by exogenous, possibly pathogenic microorganisms, a process termed colonization resistance (He et al., 2013). The resident oral microorganisms have continual nutrients available for growth, and they produce inhibitory factors, which create an unfavorable environment for potential microbial invaders so that their adhesion and/or colonization is restricted (Wilks, 2007; Wilson, 2005). Another example is that certain oral bacteria contribute to maintenance of cardiovascular health by reducing dietary nitrate into nitrite (Hezel and Weitzberg, 2013). Digested nitrate is absorbed in the intestine but up to 25% is actively taken up by the salivary glands and secreted by saliva (Govoni et al., 2008; Kapil et al., 2010). Resident oral bacteria can use that nitrate as an alternative to oxygen during respiration, reducing it to nitrite (Govoni et al., 2008). The latter is then swallowed and via the stomach taken up into the bloodstream where it is converted into nitric-oxide, which is essential for vascular health and helps to keep blood vessels supple (Govoni et al., 2008; Hobbs et al., 2013; Kapil et al., 2013; Petersson et al., 2009). There is also evidence for interaction between the microorganisms and the host, a process termed cross-talk. For instance, cross-talk between some of the resident bacteria and host mucosal cells resulted in down-regulation of potentially damaging pro-inflammatory host responses to the normal oral microbiota, while the host retained the ability to respond to microbial threats (Cosseau et al., 2008; Srinivasan, 2010).

The oral cavity harbors a diverse microbiome, and each surface in the mouth may display a different microbial community (Dewhirst et al., 2010; Huttenhower and Consortium, 2012). The wide range in pH, redox potential, nutrient availability, shedding and non-shedding surfaces, and salivary and crevicular fluids all select for specific microbial communities. Each of these may alter in composition and metabolic activity but tend to reach a kind of homeostasis with the host (Marsh, 1994). Changes in the oral environment, whether induced by aging, illness, diet, or medications (e.g. long term broad-
spectrum antibiotic treatment), can disrupt this delicate homeostasis and may lead to oral infectious disease (Abaci et al., 2010; Kleinegger et al., 1996; Schumann et al., 2005; Tylenda et al., 1989).

**Biofilm formation**

The resident oral microbiota forms spatially organized, interactive, multi-species communities embedded in an extracellular polysaccharide (EPS) matrix, called biofilms, on mucosal and dental surfaces (Do et al., 2013; ten Cate, 2006; Wilson, 2005). Formation of biofilms begins with a crucial step: surface attachment of free-floating (planktonic) microbial cells, see Figure 2. Some species may not be able to attach to a surface on their own but anchor themselves to earlier attached microorganisms or to the EPS matrix (Kolenbrander, 2000). They require this adhesion to a surface or to other microorganisms for their sustainance in the oral cavity, or else they will be swallowed by the constant flow of host secretions (Kolenbrander, 2000). This adhesion may come about through cellular recognition of specific or non-specific attachment sites on particular cells (coaggregation) or immobilized cells on surfaces (coadhesion) (Kolenbrander et al., 2010). After attachment, multiplication follows and the biofilm grows and matures (Figure 2). The microbial cells growing in a biofilm change physiologically and start communicating via signalling molecules, transfer DNA, have higher adhesion forces, and become more resistant to mechanical stress or antibiotic/antifungal treatment than planktonic cells (Douglas, 2003; Kolenbrander et al., 2010; Ramage et al., 2010; Wessel et al., 2014). Sets of genes have shown to be differentially regulated in biofilms when compared to planktonic cells in a variety of species (Dötsch et al., 2012; Nett et al., 2009; Wang et al., 2012; Yeater et al., 2007). For instance, upregulation of adhesin genes involved in attachment (Kranerveld et al., 2011) and downregulation of adhesin genes involved in dispersal (Barraud et al., 2009) has been reported. The final stage of biofilm formation is known as dispersion (Figure 2), it enables biofilm cells to detach and, subsequently, spread and colonize new surfaces (Kaplan, 2010).
Figure 2. Biofilm formation. *Candida* infection starts with biofilm formation and this requires attachment of *Candida* cells to 'a surface'. After attachment, multiplication follows and the biofilm grows, matures, and is embedded in an extracellular polysaccharide (EPS) matrix. The final stage of biofilm formation is dispersion, biofilm cells detach and, subsequently, spread and colonize new surfaces.

Of clinical relevance is the fact that microorganisms in a biofilm are less susceptible to antimicrobials. This may be due to different (combinations of) mechanisms: (1) a slow or failing penetration of antibiotics through the EPS matrix, (2) an altered (phenotypic) protected state of some bacteria/fungi, (3) the antibiotic action may be antagonized by nutrient depletion or waste product accumulation in the deeper layers of the biofilm (Stewart and Costerton, 2001).

The many different conditions and different environments in the health and biology of a host will influence oral biofilm development, species composition, and host niche (location). pH, oxygen, nutritional cues (Karatan and Watnick, 2009), an individual’s diet or oral hygiene (Marsh, 2003) and, in some cases, exposure to antibiotics (Hoffman et al., 2005) are all factors that may affect biofilm development.

Thus, an oral biofilm is a very dynamic ecosystem, and in healthy humans there is an ecological homeostasis between the host and the resident microorganisms (Kolenbrander et al., 2010; Marsh, 2005). Minor disturbances of the homeostasis within a biofilm may lead to proliferation of pathogenic species and, consequently, may cause oral infectious disease.
Older adults may at some point be affected by one or more of the following factors: the use of antibiotics and/or corticosteroids, (prolonged) hospitalization, radiation, chemotherapy, immunosuppression therapies, malnutrition, hyposalivation (dry mouth), indwelling medical devices, and the wear of a (partial) denture (Bodineau et al., 2009; Pfaller and Diekema, 2010). Most likely, combinations of factors will contribute to changes in the oral biofilm homeostasis. Imbalance of this homeostasis may result in an oral community that contains excessive pathogenic fungi, e.g. *Candida* spp. (De Resende et al., 2006; Kleinegger et al., 1996; Percival et al., 1991). In an aging population, preventing fungal infections and their costly pharmacological treatment is a growing challenge in human medicine (Bodineau et al., 2009; Pfaller and Diekema, 2010).

*C. albicans* is the most frequent cause of oral candidiasis with a total estimated prevalence between 15 and 75% (Ghannoum et al., 2010; Yang et al., 2011), and up to 90% in elderly (De Resende et al., 2006; Kleinegger et al., 1996), especially in denture wearers (Gusmão et al., 2010; Vanden Abbeele et al., 2008). Reported prevalence varies between epidemiological studies. This is most likely due to differences in the studied populations and the sampling- and identification methods that are used.

In recent years, either as co-infecting agent with *C. albicans* or as sole detectable pathogen, *C. glabrata* emerged in oral mucosal lesions and in systemic infections in humans (Hajjeh et al., 2004; Kaur et al., 2005; Krcmery, 1999; Krcmery and Barnes, 2002; Pfaller and Diekema, 2004; Vazquez et al., 1998). Oropharyngeal candidiasis in HIV and cancer patients caused by both *C. albicans* and *C. glabrata* seemed to be more difficult to treat than infections that are solely due to *C. albicans* (Redding, 2001). Moreover, *in vitro* work by Luo and Samaranayake showed significant intraspecies variability in both cell surface hydrophobicity (CSH) and adhesive ability to denture acrylic surface for *C. glabrata* strains as compared to *C. albicans* strains (Luo and Samaranayake, 2002). In addition, systemic infections caused by *C. glabrata* are associated with a higher mortality rate than those caused by *C. albicans* (Li et al., 2007). Resistance against human salivary histatins (Helmerhorst et al., 2005) and commonly used antifungals such as azoles, echinocandins, and Amphotericin B (Hitchcock et al., 1993; Rodrigues et al., 2014) in strains of *C. glabrata*
complicates therapeutic treatment. Therefore, understanding the mechanisms underlying virulence of *C. glabrata* is critical for developing new methods to prevent infection, diagnose, or cure disease.

**Candida glabrata virulence factors**

One of the main differences between *C. albicans* and *C. glabrata* is that *C. albicans* is a diploid whereas *C. glabrata* has a haploid genome. Morphologically and phylogenetically, *C. glabrata* is much more related to the non-pathogenic yeast *Saccharomyces cerevisiae*. In comparison with *S. cerevisiae*, *C. glabrata* has lost several genes, e.g. genes involved in galactose, phosphate, nitrogen, and sulphur metabolism (Dujon et al., 2004; Kaur et al., 2005; Roetzer et al., 2011). Moreover, *C. glabrata* is auxotroph (unable to synthesize a compound required for its growth) for nicotinic acid, pyridoxine, and thiamine, and these compounds thus have to be retrieved from the host environment (Kaur et al., 2005; Rodrigues et al., 2014). Lacking the ability of *C. albicans* to form hyphae, which helps the latter to evade host immune responses and penetrate and damage host tissues, *C. glabrata* must possess other virulence factors that contribute to its high mortality rate and rapid dissemination in the immunocompromised host. Thus far, there is limited knowledge on the pathogenic mechanisms of *C. glabrata*. One of the major virulence factors is probably its ability to adhere to and form biofilms on host tissues and/or medical devices. Indeed, adherence to host tissue is essential for tissue invasion and infection. The relative cell surface hydrophobicity (CSH) of *C. glabrata* cells is also likely to play a role in this process (Luo and Samaranayake, 2002). However, other factors, for instance the production of tissue-damaging hydrolytic enzymes such as aspartyl proteases (Kaur et al., 2007), phospholipases, lipases (Kumari et al., 2013), and hemolysins (Luo et al., 2004; Sachin et al., 2012) also contribute to its pathogenesis. Upon release into the local environment, hydrolytic enzymes contribute to survival and replication of the yeast within macrophages as well as to destruction of host epithelial cells (Rodrigues et al., 2014).

*Candida*-microbial (Hogan and Kolter, 2002; Holmes et al., 1996), *Candida-Candida* (Verstrepen and Klis, 2006) or *Candida*-host (Busscher et al., 2010; Luo and Samaranayake, 2002; Ramage et al., 2006) interactions all take place at the *Candida* cell wall. *Candida* cell walls consist of 1,3-β-glucan, 1,6-β-
glucan, chitin, and mannoproteins, see Figure 3 (De Groot et al., 2008). The majority of the cell wall mannoproteins are glycosylphosphatidylinositol (GPI)-modified proteins that are covalently bound to 1,6-β-glucan via a remnant of their GPI anchor. GPI proteins occupy the outer layer of the cell wall in Candida and are, among others, involved in cell shaping, invasion, biofilm formation, and resistance to antifungal drugs (Figure 3) (De Groot et al., 2013). Among the Candida GPI cell wall proteins are glycoside hydrolases, aspartyl proteases, and adhesins. Adhesins enable the fungal cell to interact with and adhere to specific substrates. Most known fungal adhesins are GPI proteins. Their precursors contain signal peptides for entry into the endoplasmic reticulum (ER) and GPI anchoring, which are cleaved off in the secretory pathway after exerting their function.

**Figure 3.** *Candida glabrata* cell wall model. The cell wall consists of a 1,3-β-glucan matrix to which 1,6-β-glucan, chitin, and mannoproteins are covalently attached to form a strong but flexible macromolecular network. Adopted and adjusted form (De Groot et al., 2008).

Mature adhesins consist of an N-terminal functional/effector domain, responsible for the specific ligand interactions (protein-protein, protein-sugar, or other protein-ligand), followed by a low-complexity domain that is often rich in serine and threonine residues and spiked with tandem repeats (TRs) (de Groot et al., 2013). Thus far, a limited number of Candida adhesins have been shown to be involved in adhesion to human tissues and in other infection-related processes such as biofilm formation: Als (agglutinin-like sequence) proteins, Eap1 (epithelial adhesion protein), and Hwp1 (hyphal wall protein 1) in *C. albicans*. 
(Hoyer, 2001; Li and Palecek, 2003; Sundstrom, 2002), and Epa1 (epithelial adhesion protein), Epa6, and Epa7 in *C. glabrata*. Until recently, the only adhesins known in *C. glabrata* were the members of the EPA gene family. Epa1, Epa6, and Epa7 all mediate adherence to human epithelial and endothelial cells (Cormack et al., 1999; Domergue et al., 2005; Iraqui et al., 2005; Zupancic et al., 2008). Epa6 and Epa7 are involved in biofilm formation (Iraqui et al., 2005). The modular structure of Epa proteins is similar to that of the well-studied Als family of adhesins in *C. albicans*. Work on Als proteins showed that Als3 mediates adherence to epithelial cells, endothelial cells, EPS, and *Staphylococcus aureus* (Nobbs et al., 2010; Peters et al., 2012; Sheppard et al., 2004; Zhao et al., 2004). Als3 has been found to be involved in *C. albicans* biofilm formation and in binding to *Streptococcus gordonii* (Nobbs et al., 2010; Nobile et al., 2008). Furthermore, Als3 has been shown to act as an invasin by inducing endocytosis in the host cell, and mediates iron acquisition by binding to host cell ferritin, displaying Als3 as a multifunctional protein (Almeida et al., 2008; de Groot et al., 2013; Liu and Filler, 2011). Variations in TR regions of Als3 have been shown to modulate protein function and adhesion (Oh et al., 2005). TR regions in *C. albicans* Als proteins seem to interact with hydrophobic surfaces and to facilitate amyloid formation and aggregation (Frank et al., 2010). TR regions in *C. glabrata* adhesin-encoding genes are shown to have strain-dependent variations in length (decrease or increase of TR copy numbers) (de Groot et al., 2013). The ligand binding domains of Epa proteins have lectin properties and are called PA14 (anthrax protective antigen 14) domains (De Groot and Klis, 2008). Glycan array analysis has shown that Epa1, Epa6, and Epa7 bind to oligosaccharides containing terminal galactose residues, such as those occurring in host cell mucin-type O-glycans (Maestre-Reyna et al., 2012; Zupancic et al., 2008). A previous bioinformatics study by Weig and colleagues, using an early release of the sequenced genome of *C. glabrata* strain ATCC2001/CBS138, predicted 106 putative GPI proteins of which about 50% had features of adhesin-like cell wall proteins (Weig et al., 2004). For the majority of the putative cell wall adhesins in *C. glabrata*, the regulation, function, and substrate specificity is still unknown. Therefore, part of this thesis focuses on the regulation of adhesin-encoding genes in *C. glabrata*. Elucidating the relevance, structures, functions, and specificities of adhesin-like wall proteins may be of help in the search of possible new antifungal drug targets in *C. glabrata*. 

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Objectives of the thesis:
The aim of this thesis was to characterize interactions of Candida in the oral cavity and study factors involved in biofilm formation and virulence. Specifically, three studies were undertaken:

1. The relationship between Candida load and the composition of bacterial microbiome profiles in saliva was assessed.

2. Candida–bacterial interactions were studied in vitro. A model was used with different surfaces for oral bacteria and Candida spp. to adhere, interact and form a biofilm on.

3. Cell wall analyses were performed in Candida glabrata to identify potential novel virulence factors or targets for antifungal drug development.

Outline of the thesis:
1. The aim of the first study was to increase our knowledge on fungal-bacterial ecological interactions in the oral cavity of older adults in the Netherlands (Chapter 2).

2. In the second study, an in vitro model of the oral cavity was used to assess interactions between Candida albicans, Candida glabrata and Streptococcus mutans in biofilms grown on various surfaces either coated with saliva or not (Chapter 3).

3. The aim of the third study was to improve our understanding of the cell wall in C. glabrata in relation to pathogenesis:

   3.1 The genome and the cell wall of C. glabrata were studied in search for (genes encoding) novel adhesin-like proteins (Chapter 4).

   3.2 Subsequently, gene expression of known and newly identified adhesin genes, under biofilm and planktonic conditions, was studied using quantitative real-time polymerase chain reaction (qPCR) (Chapter 5).

   3.3 Finally, lineages of the C. glabrata CBS138 strain, obtained from independent research laboratories, were compared for karyotypic and phenotypic alterations including an analysis of adhesin genes (Chapter 6).
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