

Influence of Saliva and Mucin on the Adhesion of *Candida* Oral Clinical Isolates

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Abstract

Objectives: This research work intends to clarify the role of artificial saliva, in particularly the role of mucin, a salivary protein, on the surface properties and adhesion ability of *Candida* spp. oral clinical isolates to abiotic surfaces. **Methods:** Four oral clinical isolates of *Candida* spp. were used: two *Candida albicans* strains (AC; AM) and two *Candida parapsilosis* strains (AD; AM2). The strains were isolated from patients using oral prosthesis. The microorganisms were cultured in the absence or presence of mucin and artificial saliva, and their adhesion to an abiotic surface (coated with mucin and artificial saliva) was evaluated. **Results:** The presence of mucin *per se* onto the abiotic surface decreased the adhesion of all strains, although the combination of mucin with artificial saliva had reduced this effect. No direct correlation between adhesion and the surface free energies of adhesion of the microorganisms was found. **Significance:** *Candida* spp. were human commensal microorganisms that became pathogenic when the host immune defenses were compromised. Medical devices were colonized by *Candida* spp. particularly, oral prostheses, which might lead to the degradation of the prostheses and systemic infections. The salivary secretions that constantly cover the oral cavity influenced *Candida* spp. adhesion process. Therefore, it was important to understand the interactions between *Candida* spp., salivary proteins and the characteristic of oral prosthesis when developing materials for oral prostheses.

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Keywords

Candida, Artificial Saliva, Mucin, Oral Adhesion, Surface Properties

1. Introduction

Candida spp. are opportunistic microorganisms present in the normal microbiota. On the right environment, these microorganisms are able to colonize, invade and multiply in tissues and organs, causing fungal infections that can go from superficial lesions to systemic infections [1] [2]. The ability of *Candida* spp. to adhere to host cells and inert substrates is one of the main driving forces for its pathogenicity, often leading to colonization, infection, and formation of biofilms [3].

Candida spp. are found in the oral cavity of more than 50% of the human population, and 80% of the clinical isolates are identified as *Candida albicans*. *Candida parapsilosis* is an emerging human pathogen that has dramatically increased in significance and prevalence over the past 2 decades, such that *C. parapsilosis* is now one of the leading causes of invasive candidal disease [4].

The human organism has many defence mechanisms in order to avoid colonization by microorganisms. Mucosal epithelial cells continuously secrete a mucosal fluid that acts as a barrier to maintain a healthy mucosa. In addition, saliva is formed by many defensive compounds, including mucins, antibodies, lysozyme or histatins that regulate the microorganism populations in the oral cavity [5]. Mucins are a major component of saliva. These large glycoproteins, with a high degree of glycosylation and potential for hydration, present antimicrobial activity, and opsonization ability, and are important components of the acquired pellicle [5] [6]. Nevertheless, saliva is also a source of water, nutrients and adherence factors. Salivary proteins can adsorb onto oral surfaces like tooth enamel and dentures, forming the acquired enamel pellicle [7]-[9], to which microorganisms are then able to adhere. Therefore, the precise role of saliva on *Candida* spp. adhesion to dentures is controversial. Several studies have shown that saliva reduces the adherence of *C. albicans* to dentures and epithelial cells [9], but other authors describe that saliva enhances the adherence of *Candida* to polystyrene [10] and polymethyl-methacrylate [11].

Salivary secretions constantly cover the oral cavity, so, it is important that, during the investigation of oral colonization, the interactions between *Candida* spp. and salivary proteins are considered. This research work intends to clarify the role of the salivary protein mucin, as well as artificial saliva, on the surface properties and the adhesion ability of *Candida* spp. oral clinical isolates.

2. Materials and Methods

2.1. Microbial Strains

Four oral clinical isolates of *Candida* spp. were used in this study: two isolates of *Candida albicans* (strains AC and AM) and two isolates of *Candida parapsilosis* (strains AD and AM2). The strains were isolated from patients using an oral prosthesis. *Candida albicans* AC and *Candida parapsilosis* AD were obtained from different individuals and *Candida albicans* AM and *Candida parapsilosis* AM2 from the same individual. The oral isolates were obtained from a Dentistry Clinic and belong to the Biofilm Group of the Centre of Biological Engineering, Minho University, where they were identified by molecular methods.

2.2. Culture Conditions and Conditioning Mediums

Candida isolates were subcultured on Sabouraud dextrose agar for 24 h at 37°C, after which each strain was inoculated in Sabouraud dextrose broth for 16 hrs at 37°C in an orbital shaker at 120 rpm. Cells were then harvested by centrifugation at 8000 rpm and washed twice with phosphate saline buffer (PBS, pH 7, 0.1 M). The cell pellets were resuspended in the conditioning mediums, and the cell concentration adjusted to 1×10^7 cells ml^{-1} .

Three different media were used in this study, namely artificial saliva without mucin (AS-Mu) and with mucin (AS + Mu) and mucin in PBS (Mu). The artificial saliva was prepared according to Lamfon *et al.* [12]: 2 g l^{-1} yeast extract (Liofilchem, Italy), 5 g l^{-1} peptone (Liofilchem, Italy), 2 g l^{-1} glucose (Applichem, Germany), 1 g l^{-1} mucin from bovine submaxillary glands—Type I-S (Sigma-Aldrich, USA), 0.35 g l^{-1} NaCl (Applichem,

Germany), 0.2 gL⁻¹ CaCl₂ (Riedel-de-Haën, Germany), and 0.2 gL⁻¹ KCl (Pronalab, Portugal) (pH 6.8 - 7).

2.3. Surface Coating with the Conditioning Media

A 6-well polystyrene plate was used as an abiotic substrate to study the influence of the presence of mucin on the adhesion properties of *Candida* spp. The three different conditioning media were used for coating. The polystyrene surfaces were incubated with each conditioning medium for 4 hrs at 37°C in an orbital shaker at 120 rpm, as described by Guggenheim *et al.* [13]. After the 4h incubation period, the wells were washed and stored in PBS at 4°C until needed (for a maximum of one week).

2.4. Contact Angle Measurement and Determination of Surface Free Energies

Contact angles were measured by the sessile drop technique, using a contact angle measurement apparatus model OCA 15 PLUS, DATAPHYSICS. Measurements were performed at room temperature using three different standard liquids (ultrapure water, formamide, and α -bromonaphthalene). Every assay was performed in triplicate and at least 10 measurements were performed for each sample.

Contact angles were measured on yeast lawns. Briefly, microorganisms were grown for 2 h in artificial saliva without mucin (AS – Mu) or in artificial saliva with mucin (AS + Mu). Subsequently, the cell suspensions were layered onto 0.22 μ m pore sized filters and dried for 4 h at 37°C, to standardize the humidity level [14].

The hydrophobicity and surface tension was determined using the Van Oss approach [15]-[17]. The acid-base nature of the surfaces was directly determined in terms of the surface free energy components γ_{sv}^{LW} (LW, Lifshitz-Van der Waals) and γ_{sv}^{AB} (AB, acid-base), according to Equation (1).

$$\gamma_{sv} = \gamma_{sv}^{LW} + \gamma_{sv}^{AB} \quad (1)$$

in which the AB component equals

$$\gamma_{sv}^{AB} = 2\sqrt{\gamma_{sv}^- \times \gamma_{sv}^+} \quad (2)$$

where γ_{sv}^- and γ_{sv}^+ are the electron-donating and electron-accepting surface free energy parameters, respectively. s stands for solid, v for vapor and l for liquid. Proper diagnostic liquids with known surface free energy components (γ_{lv}^{LW} , γ_{lv}^{AB} , γ_{lv}^+ and γ_{lv}^-) were selected.

Since α -bromonaphthalene is apolar ($\gamma_{lv}^{AB} = 0$), its contact angle on a surface can be used to calculate the γ_{sv}^{LW} component of the surface free energy

$$\gamma_{sv}^{LW} = \left(\left(\sqrt{\gamma_{lv}^-} \times (\cos \theta + 1) \right) / 2 \right)^2 \quad (3)$$

Water and formamide are both polar liquids and their contact angles can be used to calculate γ_{sv}^- and γ_{sv}^+ from the Young equation Equation (4).

$$\gamma_{lv} \times (\cos \theta + 1) - 2\sqrt{\gamma_{sv}^{LW} \gamma_{lv}^{LW}} = 2\sqrt{\gamma_{sv}^- \gamma_{lv}^+} + 2\sqrt{\gamma_{sv}^+ \gamma_{lv}^-} \quad (4)$$

The free energy of adhesion ΔG_{adh} can be separated into two components: ΔG_{adh}^{LW} is the free energy of adhesion due to Lifshitz-van der Waals interactions, and ΔG_{adh}^{AB} the free energy of adhesion due to electrostatic interactions, according to Equation (5).

$$\Delta G_{adh} = \Delta G_{adh}^{LW} + \Delta G_{adh}^{AB} \quad (5)$$

where

$$\Delta G_{adh}^{LW} = -2 \times \left(\sqrt{\gamma_{mv}^{LW}} - \sqrt{\gamma_{sv}^{LW}} \right) \times \left(\sqrt{\gamma_{sv}^{LW}} - \sqrt{\gamma_{lv}^{LW}} \right) \quad (6)$$

and

$$\begin{aligned} \Delta G_{adh}^{AB} = & 2 \left(\sqrt{\gamma_{mv}^+} - \sqrt{\gamma_{sv}^+} \right) \left(\sqrt{\gamma_{mv}^-} - \sqrt{\gamma_{sv}^-} \right) - 2 \left(\sqrt{\gamma_{mv}^+} - \sqrt{\gamma_{lv}^+} \right) \left(\sqrt{\gamma_{mv}^-} - \sqrt{\gamma_{lv}^-} \right) \\ & - 2 \left(\sqrt{\gamma_{sv}^+} - \sqrt{\gamma_{lv}^+} \right) \left(\sqrt{\gamma_{sv}^-} - \sqrt{\gamma_{lv}^-} \right) \end{aligned} \quad (7)$$

2.5. Yeast Adhesion Assays

The adhesion assays were performed on non-treated polystyrene surfaces, and polystyrene surfaces treated with the three different conditioning mediums: AS – Mu, AS + Mu and Mu. Yeast suspensions grown in AS – Mu or AS + Mu were used. Briefly, 500 μl of the standardized cell suspensions (1×10^7 cells ml^{-1} in AS – Mu or AS + Mu) were placed on the coated or uncoated well-plates for 2 hrs at 37°C in an orbital shaker at 120 rpm. Following this incubation period, the medium was removed and the wells washed with PBS to remove unattached cells. The wells were scraped to resuspend the adhered cells in PBS. The yeast cells were then sonicated for 45 sec, 30 W, in an Ultrasonic Processor. Viable counts for each *Candida* spp. were obtained by serial decimal dilutions in PBS, and plated on Sabouraud dextrose agar medium, followed by an incubation period of 24 hrs at 37°C .

2.6. Statistical Analysis

Statistical analysis was performed using the SPSS software. The one-way ANOVA test followed by a Bonferroni as a Post Hoc test (confidence level of 95%) was applied.

3. Results

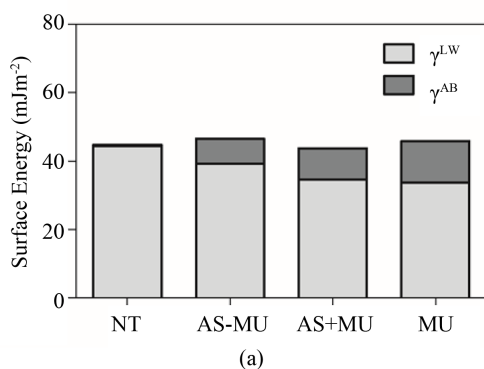
3.1. Effect of Conditioning Medium on Polystyrene Surface Characteristics

The treatments with AS – Mu, AS + Mu and Mu strongly affected the characteristics of the surface. The contact angles measured with the polar liquids (water and formamide) on the treated surfaces were lower than on the control surfaces (untreated), while the apolar liquid (α -bromonaftalene) formed higher contact angles (**Table 1**).

The surface free energy (total surface tension, composed by the Lifshitz-van der Waals γ^{LW} and acid-base γ^{AB} components) and hydrophobicity of treated and non-treated polystyrene surfaces was determined (**Figure 1**).

Table 1. Averaged contact angle values measured on the non-treated and treated polystyrene surfaces. Three standard liquids were used: water, formamide, and α -bromonaftalene. Average \pm standard error (SE) are represented. NT-non-treated polystyrene surface; AS – Mu-polystyrene surface treated with artificial saliva without mucin; AS + Mu-polystyrene surface treated with artificial saliva with mucin; Mu-polystyrene surface treated with mucin in PBS.

	$\theta_{\text{water}} (^\circ) \pm \text{SE}$	$\theta_{\text{formamide}} (^\circ) \pm \text{SE}$	$\theta_{\alpha\text{-bromonaftalene}} (^\circ) \pm \text{SE}$
NT	66.72 ± 2.08	49.84 ± 1.95	<0
AS – Mu	<0	29.27 ± 1.46	28.17 ± 2.25
AS + Mu	<0	34.66 ± 1.71	39.99 ± 1.34
Mu	28.76 ± 1.52	35.65 ± 1.37	41.75 ± 3.32



	NT	AS-Mu	AS+Mu	Mu
$\gamma^+ (\text{mJm}^{-2})$	0	0.2	0.28	0.70
$\gamma^- (\text{mJm}^{-2})$	14.84	66.82	71.27	53.7

(b)

	NT	AS-Mu	AS+Mu	Mu
$\Delta G_{\text{sws}} (\text{mJm}^{-2})$	-40.1	47.3	55.4	33.2

(c)

Figure 1. Free energy components and degree of hydrophobicity (ΔG_{sws}) determined for the non-treated and treated polystyrene surfaces. The total surface tension, composed by the Lifshitz-van der Waals component γ^{LW} and acid-base component γ^{AB} (a), the electron-accepting γ^+ and electron-donating γ^- parameters (b), as well as the degree of hydrophobicity (ΔG_{sws}) (c) are shown. NT-non-treated polystyrene surface; AS – Mu-polystyrene surface treated with artificial saliva without mucin; AS + Mu-polystyrene surface treated with artificial saliva with mucin; Mu-polystyrene surface treated with mucin in PBS.

Figure 1(a) shows a decrease on the Lifshitz-van der Walls component (γ^{LW}) after treating the polystyrene surface with AS – Mu, AS + Mu and Mu. The presence of mucin on the conditioning medium (AS + Mu and Mu) induced a higher reduction on the γ^{LW} component. Oppositely, treatments with AS – Mu, AS + Mu and Mu increased the acid-base (γ^{AB}) parameter of the free energy. Additionally, the electron-donating (γ^-) and electron-accepting (γ^+) parameter values increased significantly in the presence of AS or mucin (**Figure 1(b)**). For all the conditions, the γ^+ component was smaller than the γ^- component (inferior to 1 mJm^{-2}).

Combining the contact angles and surface free energies (**Table 1** and **Figure 1**), the hydrophobicity of the surfaces was determined as an expression of the free energy (ΔG_{sws}) interacting between two identical surfaces (s) immersed in water (w) (**Figure 1(c)**).

The ΔG_{sws} of the polystyrene surface was found to be -40.1 mJm^{-2} (**Figure 1(c)**), reflecting its hydrophobic character, since a hydrophobic surface is a surface for which $\Delta G_{sws} < 0$ [17]. Hydrophobic surfaces are usually surfaces with a low γ^- as well as a low γ^+ parameter, which was observed for polystyrene (**Figure 1(b)**). The treatments with AS – Mu, AS + Mu and Mu significantly increased the ΔG_{sws} values, changing the surface characteristic from hydrophobic to hydrophilic.

3.2. Effect of the Growing Media on *C. albicans* and *C. parapsilosis* Surface Characteristics

Interestingly, the four oral clinical isolates have different contact angle values, indicating different surface characteristics (**Table 2**). In fact, in the absence of mucin, *C. parapsilosis* AD had the highest water contact angle ($> 50^\circ$) and *C. albicans* AM had the lowest water contact angle (approximately 30°).

Moreover, the presence of mucin in the medium strongly influenced the water contact angles of all *Candida* spp. a decrease on the water contact angle value was observed for *C. albicans* AC and *C. parapsilosis* AM2, while the opposite effect occurred for *C. albicans* AM and *C. parapsilosis* AD. All the formamide contact angles increased, except for *C. parapsilosis* AD. The α -bromonaftalene contact angles also increased in the presence of mucin, except for *C. parapsilosis* AM2.

On the absence of mucin, *C. albicans* strains and *C. parapsilosis* AM2 had a γ^{AB} between 9 and 13 mJm^{-2} , while *C. parapsilosis* AD presented a very small γ^{AB} (around 2 mJm^{-2}) (**Figure 2**). When mucin was present in the medium, the γ^{AB} of *C. albicans* AM and *C. parapsilosis* AM2 was near 0 mJm^{-2} , the γ^{AB} of *C. albicans* AC decreased and γ^{AB} of *C. parapsilosis* AD increased. The γ^{LW} was stable when the medium was supplemented with mucin, which combined with γ^{AB} , resulted on a decrease of surface free energy for *C. albicans* AC, *C. albicans* AM and *C. parapsilosis* AM2, and in an increase for *C. parapsilosis* AD.

The electron-donating (γ^-) and electron-accepting (γ^+) parameters calculated for the *Candida* spp. are shown in **Table 3(a)**. In the absence or presence of mucin, all the *Candida* spp. have a γ^+ component smaller than the γ^- component. *C. albicans* AM presented the highest (47.6 mJm^{-2}) and *C. albicans* AC the lowest (21.33 mJm^{-2}) γ^- values when compared to the other microorganisms. *C. albicans* AC had the higher γ^+ value (1.93 mJm^{-2}) of all the *Candida* spp. When mucin is present, the surface characteristics of all *Candida* spp. are altered, with an increase in the γ^- parameter and a decrease on the γ^+ value, except *C. parapsilosis* AD.

Table 2. Averaged contact angle values measured on *Candida albicans* AC, *Candida albicans* AM, *Candida parapsilosis* AD and *Candida parapsilosis* AM2, grown in artificial saliva without mucin or artificial saliva with mucin. Three standard liquids were used: water, formamide, and α -bromonaftalene. Average \pm standard error (SE) are represented.

	Cells	Strains	$\theta_w (^\circ) \pm \text{SE}$	$\theta_f (^\circ) \pm \text{SE}$	$\theta_b (^\circ) \pm \text{SE}$
AS without mucin	<i>C. albicans</i>	AC	48.03 ± 2.38	19.20 ± 1.98	17.24 ± 1.21
		AM	29.28 ± 1.97	27.75 ± 1.97	22.02 ± 2.02
	<i>C. parapsilosis</i>	AD	54.47 ± 2.15	47.16 ± 2.49	26.41 ± 2.02
		AM2	44.18 ± 2.82	28.04 ± 1.41	26.97 ± 3.24
AS with mucin	<i>C. albicans</i>	AC	26.08 ± 1.80	39.33 ± 3.23	32.47 ± 1.54
		AM	38.14 ± 1.02	43.55 ± 2.48	23.34 ± 1.34
	<i>C. parapsilosis</i>	AD	57.62 ± 1.55	35.75 ± 1.44	33.59 ± 1.86
		AM2	28.97 ± 2.05	54.06 ± 3.54	23.34 ± 1.34

Table 3. Free energy components and degree of hydrophobicity (ΔG_{sws}) determined for *Candida* spp. Electron-accepting γ^+ and electron-donating γ^- parameters of the acid-base component γ^{AB} (a) and degree of hydrophobicity (ΔG_{sws}) (b) determined for the four *Candida* spp. in artificial saliva without mucin and artificial saliva with mucin.

		(a)			
		<i>C. albicans</i> AC	<i>C. albicans</i> AM	<i>C. parapsilosis</i> AD	<i>C. parapsilosis</i> AM2
AS without mucin	γ^-	21.32	47.60	28.86	29.50
	γ^+	1.93	0.44	0.03	1.30
AS with mucin	γ^-	60.79	49.78	17.00	76.20
	γ^+	0.04	0.00	1.69	0.00

		(b)			
		<i>C. albicans</i> AC	<i>C. albicans</i> AM	<i>C. parapsilosis</i> AD	<i>C. parapsilosis</i> AM2
AS without mucin	ΔG_{sws}	-19.9	20.2	-4.6	-4.7
AS with mucin	ΔG_{sws}	44.6	28.8	-22.2	62.5

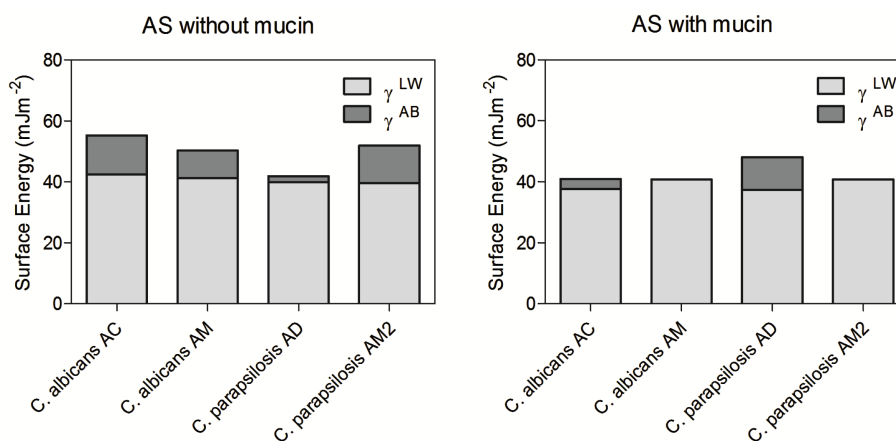


Figure 2. Free energy components determined for the *Candida* spp. in artificial saliva without mucin (a) and artificial saliva with mucin (b). The total surface tension, Lifshitz-van der Waals component γ^{LW} , acid-base component γ^{AB} , are shown.

On the absence of mucin, *C. albicans* AC and *C. parapsilosis* strains were found to be hydrophobic ($\Delta G_{sws} < 0$) (Table 3(b)), while *C. albicans* AM was found to be hydrophilic ($\Delta G_{sws} > 0$). Hydrophobic surfaces are usually surfaces with a low γ^- as well as a low γ^+ parameter, which is in accordance with these results. The presence of mucin changed the surface characteristic of *C. albicans* AC and *C. parapsilosis* AM2 from hydrophobic to hydrophilic. The ΔG_{sws} calculated for *C. parapsilosis* AD become even more negative, while for *C. albicans* AM it remained similar.

3.3. Effect of Saliva and Mucin on the Adhesion of *C. albicans* and *C. parapsilosis*

When mucin is not present in the growth medium, the adhesion of all *Candida* spp. is reduced only on the polystyrene surface treated with mucin (Mu). The number of viable of *C. parapsilosis* AD cells increased when attached to the surface with AS-Mu treatment (Figure 3).

The two *C. parapsilosis* strains adhered at a higher extend to AS – Mu polystyrene than the two *C. albicans* strains. For the treatment of the polystyrene surface with Mu the adhesion followed the order: *C. albicans* AM > *C. albicans* AC > *C. parapsilosis* AD = *C. parapsilosis* AM2.

It is also important to observe that the number of *C. albicans* AC cells adhered to the polystyrene surfaces increased when mucin was added. The same adhesion trend was observed for *C. albicans* AM, although there were no significant differences between the treatments with AS – Mu and AS + Mu. For the two *C. parapsilosis*

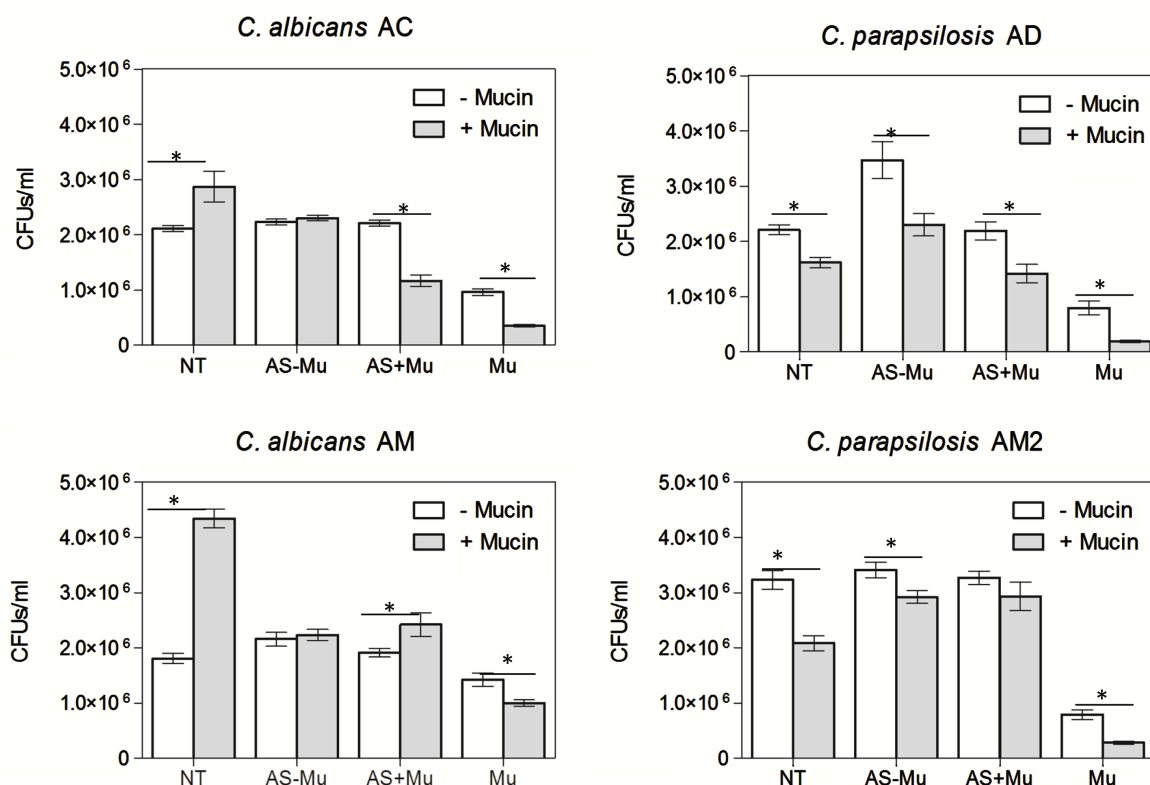


Figure 3. Number of oral clinical isolates of *Candida* spp. Adhered onto non-treated (NT) polystyrene or treated with artificial saliva without mucin (AS - Mu), artificial saliva with mucin (AS + Mu) and mucin in PBS (Mu).

strains only the treatment with Mu was able to significantly reduce the adhesion. Actually, the adhesion of *C. parapsilosis* AD and *C. parapsilosis* AM2 was increased for AS - Mu and AS - Mu/AS + Mu, respectively.

In the presence of mucin, the adhesion of *C. albicans* strains to the non-treated polystyrene surface was increased; while to the polystyrene treated with Mu was reduced. The adhesion to AS - Mu was not affected. The presence of mucin in the growth medium also reduced the adhesion of *C. parapsilosis* AD to all the surfaces, while the adhesion of *C. parapsilosis* AM2 was only reduced for the NT and Mu polystyrene surfaces.

3.4. Energies of Adhesion of *Candida* spp. to the Polystyrene Surfaces

In the absence and in the presence of mucin on the growth medium, the interaction energies between all the *Candida* spp. strains and the polystyrene surfaces treated with AS - Mu, AS + Mu and Mu were always unfavorable ($\Delta G_{adh} > 0$) (Table 4). ΔG_{adh} was higher for the AS + Mu, followed by AS - Mu and finally Mu. For the non-treated polystyrene surface, when grown in the absence of mucin, only *C. albicans* AM had an unfavorable ΔG_{adh} . When *Candida* spp. were in the presence of mucin, had $\Delta G_{adh} > 0$, except *C. parapsilosis* AD.

4. Discussion

The poor growth conditions on the oral environment oblige microbial cells to adhere in order to survive and colonize the oral cavity [7] [18]. Adhesion is a complex process, where surface free energies, van der Waals and electrostatic forces, hydrophobic interactions, cation bridging, receptor ligand binding, and the presence of nutrients are determinant for the adhesion of a microorganism [6]. In the particular case of the oral cavity colonization, the presence of saliva strongly influences adhesion. Saliva is a complex fluid secreted by the salivary glands, which forms a protective mechanical barrier against microbial colonization. It has antimicrobial properties given by different components such as lactoferrin, immunoglobulins, histatin or lysozymes [5]. Nevertheless, saliva is composed by other molecules, such as mucins and proline-rich-proteins that have been reported to

Table 4. Interfacial free energies of adhesion between *Candida* spp. and polystyrene surfaces non-treated and treated with AS – Mu, AS + Mu, and Mu. The total free energy (ΔG_{adh}) is presented.

		<i>C. albicans</i> AC	<i>C. albicans</i> AM	<i>C. parapsilosis</i> AD	<i>C. parapsilosis</i> AM2
	NT	-20.5	1.2	-15	-12
AS without mucin	AS – Mu	13	38.8	28.2	22.7
	AS + Mu	16.4	42.2	32	26
	Mu	8.8	31.6	21.2	17.3
	NT	10.3	1.3	-24.1	18.2
AS with mucin	AS – Mu	50.9	44.6	10.3	59.9
	AS + Mu	54.2	48.2	13.6	63.4
	Mu	41.9	36	6	50.1

facilitate adherence to surfaces [5]. There are contradicting reports on the literature regarding the influence of saliva on *Candida* adherence, with studies showing that the presence of saliva reduces the adherence of *C. albicans* acrylic resin based materials while others demonstrate an increased adherence [19].

This study gives a new insight on the effect of saliva on *Candida* spp. adhesion, and the precise role of a single component-mucin. The effect of mucin on the microorganism surface characteristics as well as its influence on the surface properties of polystyrene was assessed.

As expected, a high contact angle value ($\sim 67^\circ$) and a low free energy ($<10^\circ$) were obtained for the non-treated polystyrene surface, demonstrating its hydrophobic nature. All the treatments performed (AS – Mu, AS + Mu, Mu) changed the nature of the polystyrene surface from hydrophobic to hydrophilic (Figure 1 and Table 1). Nevertheless, there were no statistically significant differences in the total surface free energies calculated before and after treatments. Still, the treatments with AS – Mu, AS + Mu and Mu increased the γ^{AB} free energy parameter, which means a higher hydration of the surface. The increase in the γ^- values observed on Figure 1(b) is another indication of decreased hydrophobicity while the increase on γ^+ indicates an increase of the electron acceptor sites at the surface [17]. Even so, for all the conditions tested, the γ^+ component was always smaller than the γ^- component, indicating that the surfaces are electron donating in nature. The results show that the presence of the artificial saliva components mask the hydrophobic nature of the polystyrene surface, due to the adsorption of ions such as sodium, calcium, potassium, present in the artificial saliva that will influence the interactions between the electron-acceptor and electron-donor groups [20] [21]. The changes observed in the presence of mucin (in PBS) may be explained by the conformational adaptation and exposure of its hydrophilic terminals [14] [22] when bound to the surface. It is known that, although most proteins adsorb preferentially onto low-energy surfaces, some proteins, such as albumin, preferentially adsorb on substrates with surface energies that have a high polar (electron donor parameter (γ^-)) components [23]. This is a very important result, as it demonstrates the impact on the surface free energy parameters and on the hydrophobicity of the surface (Figure 1(c)) materials in the presence of different substances. The change on the physical-chemical characteristic influences the formation of the acquired enamel pellicle onto the oral surfaces and, therefore, microbial adhesion.

The contact angles measured on the yeast lawns showed important variations in the surface characteristics of the oral clinical isolates (Figure 2 and Table 2). In the absence of mucin, *C. albicans* AC and *C. parapsilosis* strains were found to be hydrophobic, while *C. albicans* AM was found to be hydrophilic (Table 3(b)). Also, both in the absence or the presence of mucin, all the *Candida* spp. presented a γ^+ component smaller than the γ^- component, indicating that the microorganisms are electron donors in nature (Table 3(a)).

When *Candida* spp. were grown in artificial saliva with mucin, *C. albicans* AC and *C. parapsilosis* AM2 changed from hydrophobic to hydrophilic (Table 3(a)). The presence of free mucin molecules in the medium, with the ability to bind to *Candida* cells surface [24] [25], lead to an adaptation of the *Candida* spp. Physico-chemical characteristics.

The energies of adhesion calculated based on the surface free energies of the *Candida* spp. and the polystyrene surfaces could not explain the adhesion profile on its own. Unfavorable energies of adhesion between the cells and the surfaces (Table 4) did not result in decreased adhesions (Figure 3). When *Candida* spp. strains

were cultured in artificial saliva without mucin, a similar adhesion profile was observed onto all surfaces except for the treatment with mucin in PBS (Mu). This result points for the fact the presence of mucin, by itself, on the polystyrene surface, reduces adsorption, while the presence of artificial saliva on the polystyrene surfaces also has a role on the adhesion of *Candida* to oral surfaces, neutralizing the effect of mucin. In this sense, the adhesion of *Candida* cells was not only influenced by the adsorption of mucin to the surface, but also by the ionic strength and other components from the artificial saliva present in the polystyrene surface, which masks the inhibitory effect of mucin.

In the presence of artificial saliva with mucin, an increased number of *C. albicans* adhered to the non-treated polystyrene surface was observed, while for *C. parapsilosis* strains the adhesion was reduced (**Figure 3**). This suggests that mucin or/and ions, present in the artificial saliva medium, interact with the cells' surface favoring *C. albicans* adhesion by increasing the interactions of electron-acceptor and electron-donor between polystyrene and the cell surface [20]. For *C. parapsilosis* strains, the effect of the presence of mucin in the medium seems to result in cells less apt to adhere. The effect of the mucin on the polystyrene surface (treatment Mu) further reduced adhesion of all the *Candida* strains while the treatments with As – Mu and AS + Mu were not always efficient on the reduction of adhesion (**Figure 3**). Interestingly, the presence of mucin in the fluid phase has a higher influence on the adhesion of *Candida* cells than when mucin is adsorbed onto the surface.

As mentioned before, adhesion is a very complex process, mediated by several factors such as surface free energies and hydrophobic interactions, but the interaction between adhesins and surface binding sites play a determinant role on the adhesion process of yeast [26]. *Candida* adhesion to oral surfaces is also mediated by cells surface receptors, mainly agglutinin-like sequence (ALS) that is hydrophobic and bound preferentially to the abiotic surfaces [27]. In fact, it is well known that *C. parapsilosis* has five ALS genes and six genes predicted for glycoposphatidylinositol-anchored protein 30 (Pga 30), but little is known about their role in adhesion [3] [28]. *C. albicans* has nine ALS genes involved on the adhesion mechanism [29]. The differences in *C. parapsilosis* and *C. albicans* ALS will probably explain the differences found on the adhesion of the both types of *Candida* strains.

5. Conclusions

This study shows that the role of mucin on *Candida* spp. adhesion is complex and must be carefully examined. The four *Candida* strains used in this study behave differently in the presence of mucin, showing either increased or decreased adhesion depending on the presence of mucin on the growing medium or on the polystyrene surface. Actually, while the presence of adhered mucin onto the surface decreases the adhesion of all the strains, the combination of mucin with artificial saliva diminishes this effect.

Although there is not a direct correlation between adhesion and the surface free energies of adhesion of these particular *Candida* strains, the presence of artificial saliva affects the physicochemical characteristics of the adherent surface, as well the hydrophobicity behaviour of the strains.

This study clearly demonstrates that it is important to evaluate the surface characteristics, as they will enhance or decrease the microbial attachment.

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References

- [1] Kabir, M.A. and Ahmad, Z. (2013) *Candida* Infections and Their Prevention. *ISRN Preventive Medicine*, **2013**, 13. <http://dx.doi.org/10.5402/2013/763628>
- [2] Kojic, E.M. and Darouiche, R.O. (2004) *Candida* Infections of Medical Devices. *Clinical Microbiology Reviews*, **17**,

- 255-267. <http://dx.doi.org/10.1128/CMR.17.2.255-267.2004>
- [3] Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W. and Azeredo, J. (2011) Adherence and Biofilm Formation of Non-*Candida albicans* *Candida* Species. *Trends Microbiology*, **19**, 241-247. <http://dx.doi.org/10.1016/j.tim.2011.02.003>
- [4] Coronado-Castellote, L. and Jiménez-Soriano, Y. (2013) Clinical and Microbiological Diagnosis of Oral Candidiasis, *Journal of Clinical and Experimental Dentistry*, **5**, e279-e286. <http://dx.doi.org/10.4317/jced.51242>
- [5] Dodds, M.W., Johnson, D.A. and Yeh, C.K. (2005) Health Benefits of Saliva: A Review. *Journal of Dentistry*, **33**, 223-233. <http://dx.doi.org/10.1016/j.jdent.2004.10.009>
- [6] Linden, S.K., Sutton, P., Karlsson, N.G., Korolik, V. and McGuckin, M.A. (2008) Mucins in the Mucosal Barrier to Infection. *Mucosal Immunology*, **1**, 183-197. <http://dx.doi.org/10.1038/mi.2008.5>
- [7] Cannon, R.D. and Chaffin, W.L. (1999) Oral Colonization by *Candida albicans*. *Critical Reviews in Oral Biology & Medicine*, **10**, 359-383. <http://dx.doi.org/10.1177/10454411990100030701>
- [8] Cannon, R.D., Holmes, A.R., Mason, A.B. and Monk, B.C. (1995) Oral *Candida*: Clearance, Colonization, or Candidiasis? *Journal of Dental Research*, **74**, 1152-1161. <http://dx.doi.org/10.1177/00220345950740050301>
- [9] Elguezabal, N., Maza, J.L., Dorransoro, S. and Ponton, J. (2008) Whole Saliva Has a Dual Role on the Adherence of *Candida albicans* to Polymethylmetacrylate, *The Open Dentistry Journal*, **2**, 1-4.
- [10] Vasilas, A., Molina, L., Hoffman, M. and Haidaris, C.G. (1992) The Influence of Morphological Variation on *Candida albicans* Adhesion to Denture Acrylic *In Vitro*. *Archives of Oral Biology*, **37**, 613-622. [http://dx.doi.org/10.1016/0003-9969\(92\)90123-P](http://dx.doi.org/10.1016/0003-9969(92)90123-P)
- [11] Edgerton, M., Scannapieco, F.A., Reddy, M.S. and Levine, M.J. (1993) Human Submandibular-Sublingual Saliva Promotes Adhesion of *Candida albicans* to Polymethylmethacrylate. *Infect Immunology*, **61**, 2644-2652.
- [12] Lamfon, H., Porter, S.R., McCullough, M. and Pratten, J. (2003) Formation of *Candida albicans* Biofilms on Non-Shedding Oral Surfaces. *European Journal of Oral Sciences*, **111**, 465-471. <http://dx.doi.org/10.1111/j.0909-8836.2003.00084.x>
- [13] Guggenheim, B., Giertsen, E., Schupbach, P. and Shapiro, S. (2001) Validation of an *in Vitro* Biofilm Model of Supragingival Plaque. *Journal of Dental Research*, **80**, 363-370. <http://dx.doi.org/10.1177/00220345010800011201>
- [14] Busscher, H.J., Bos, R. and Van der Mei, H.C. (1995) Initial Microbial Adhesion Is a Determinant for the Strength of Biofilm Adhesion. *FEMS Microbiology Letters*, **128**, 229-234. <http://dx.doi.org/10.1111/j.1574-6968.1995.tb07529.x>
- [15] Van Oss, C.J., Chaudhury, M.K. and Good, R.J. (1987) Monopolar Surfaces. *Advances in Colloid Interface Science*, **28**, 35-64. [http://dx.doi.org/10.1016/0001-8686\(87\)80008-8](http://dx.doi.org/10.1016/0001-8686(87)80008-8)
- [16] Van Oss, C.J., Ju, L., Chaudhury, M.K. and Good, R.J. (1989) Estimation of the Polar Parameters of the Surface Tension of Liquids by Contact Angle Measurements on Gels. *Journal of Colloid and Interface Science*, **128**, 313-319. [http://dx.doi.org/10.1016/0021-9797\(89\)90345-7](http://dx.doi.org/10.1016/0021-9797(89)90345-7)
- [17] Van Oss, C.J. (1995) Hydrophobic, Hydrophilic and Other Interactions in Epitope-Paratope Binding. *Molecular Immunology*, **32**, 199-211. [http://dx.doi.org/10.1016/0161-5890\(94\)00124-J](http://dx.doi.org/10.1016/0161-5890(94)00124-J)
- [18] Henriques, M., Gasparetto, K., Azeredo, J. and Oliveira, R. (2002) Experimental Methodology to Quantify *Candida albicans* Cell Surface Hydrophobicity. *Biotechnology Letters*, **24**, 1111-1115. <http://dx.doi.org/10.1023/A:1016083021525>
- [19] Pereira-Cenci, T., Del Bel Cury, A.A., Crielaard, W. and Ten Cate, J.M. (2008) Development of *Candida*-Associated Denture Stomatitis: New Insights. *Journal of Applied Oral Science*, **16**, 86-94. <http://dx.doi.org/10.1590/S1678-77572008000200002>
- [20] Henriques, M., Azeredo, J. and Oliveira, R. (2004) Adhesion of *Candida albicans* and *Candida dubliniensis* to Acrylic and Hydroxyapatite. *Colloids and Surfaces B: Biointerfaces*, **33**, 235-241. <http://dx.doi.org/10.1016/j.colsurfb.2003.10.012>
- [21] Oliveira, R. (1997) Understanding Adhesion: A Means for Preventing Fouling. *Experimental Thermal and Fluid Science*, **14**, 316-322. [http://dx.doi.org/10.1016/S0894-1777\(96\)00134-3](http://dx.doi.org/10.1016/S0894-1777(96)00134-3)
- [22] Busscher, H.J., Geertsema-Doornbusch, G.I. and Van der Mei, H.C. (1997) Adhesion to Silicone Rubber of Yeasts and Bacteria Isolated from Voice Prostheses: Influence of Salivary Conditioning Films. *Journal of Biomedical Materials Research*, **34**, 201-209. [http://dx.doi.org/10.1002/\(SICI\)1097-4636\(199702\)34:2<201::AID-JBM9>3.0.CO;2-U](http://dx.doi.org/10.1002/(SICI)1097-4636(199702)34:2<201::AID-JBM9>3.0.CO;2-U)
- [23] Comelles, J., Estévez, M., Martínez, E. and Samitier, J. (2010) The Role of Surface Energy of Technical Polymers in Serum Protein Adsorption and MG-63 Cells Adhesion. *Nanomedicine: Nanotechnology, Biology and Medicine*, **6**, 44-51. <http://dx.doi.org/10.1016/j.nano.2009.05.006>
- [24] Hoffman, M.P. and Haidaris, C.G. (1993) Analysis of *Candida albicans* Adhesion to Salivary Mucin. *Infection and Immunity*, **61**, 1940-1949.

- [25] Schenkels, L.C., Veerman, E.C. and Nieuw-Amerongen, A.V. (1995) Biochemical Composition of Human Saliva in Relation to Other Mucosal Fluids. *Critical Reviews in Oral Biology & Medicine*, **6**, 161-175. <http://dx.doi.org/10.1177/10454411950060020501>
- [26] Desai, C., Mavrianos, J. and Chauhan, N. (2011) *Candida glabrata* Pwp7p and Aed1p Are Required for Adherence to Human Endothelial Cells. *FEMS Yeast Research*, **11**, 595-601. <http://dx.doi.org/10.1111/j.1567-1364.2011.00743.x>
- [27] Aoki, W., Kitahara, N., Miura, N., Morisaka, H., Kuroda, K. and Ueda, M. (2012) Profiling of Adhesive Properties of the Agglutinin-Like Sequence (ALS) Protein Family, a Virulent Attribute of *Candida albicans*. *FEMS Immunology & Medical Microbiology*, **65**, 121-124. <http://dx.doi.org/10.1111/j.1574-695X.2012.00941.x>
- [28] Butler, G., Rasmussen, M.D., Lin, M.F., Santos, M.A., Sakthikumar, S., Munro, C.A., Rheinbay, E., Grabherr, M., Forche, A., Reedy, J.L., Agrafioti, I., Arnaud, M.B., Bates, S., Brown, A.J., Brunke, S., Costanzo, M.C., Fitzpatrick, D.A., Groot, P.W., Harris, D., Hoyer, L.L., Hube, B., Klis, F.M., Kodira, C., Lennard, N., Logue, M.E., Martin, R., Neiman, A.M., Nikolaou, E., Quail, M.A., Quinn, J.M., Santos, C., Schmitzberger, F.F., Sherlock, G., Shah, P., Silverstein, K.A., Skrzypek, M.S., Soll, D., Staggs, R., Stansfield, I., Stumpf, M.P., Sudbery, P.E., Srikantha, T., Zeng, Q., Berman, J., Berriman, M., Heitman, J., Gow, N.A., Lorenz, M.C., Birren, B.W., Kellis, M. and Cuomo, C.A. (2009) Evolution of Pathogenicity and Sexual Reproduction in Eight *Candida* Genomes. *Nature*, **459**, 657-662. <http://dx.doi.org/10.1038/nature08064>
- [29] Hoyer, L.L. (2001) The ALS Gene Family of *Candida albicans*. *Trends in Microbiology*, **9**, 176-180. [http://dx.doi.org/10.1016/S0966-842X\(01\)01984-9](http://dx.doi.org/10.1016/S0966-842X(01)01984-9)