



Salivary pellicles equalise surfaces' charges and modulate the virulence of *Candida albicans* biofilm



Yuri Wanderley Cavalcanti^{a,c,*}, Melanie Wilson^b, Michael Lewis^b, David Williams^b, Plínio Mendes Senna^a, Altair Antoninha Del-Bel-Cury^a, Wander José da Silva^{a,**}

^a Periodontology and Prosthodontics Department, Piracicaba Dental School, University of Campinas, Piracicaba, SP, Brazil

^b Tissue Engineering and Reparative Dentistry, School of Dentistry, Cardiff University, Cardiff, Wales, UK

^c Department of Dentistry, School of Dentistry, State University of Paraíba (UEPB), Campina Grande, PB, Brazil

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ABSTRACT

Introduction: Numerous environmental factors influence the pathogenesis of *Candida* biofilms and an understanding of these is necessary for appropriate clinical management.

Aims: To investigate the role of material type, pellicle and stage of biofilm development on the viability, bioactivity, virulence and structure of *C. albicans* biofilms.

Methods: The surface roughness (SR) and surface free energy (SFE) of acrylic and titanium discs was measured. Pellicles of saliva, or saliva supplemented with plasma, were formed on acrylic and titanium discs. *Candida albicans* biofilms were then generated for 1.5 h, 24 h, 48 h and 72 h. The cell viability in biofilms was analysed by culture, whilst DNA concentration and the expression of *Candida* virulence genes (*ALS1*, *ALS3* and *HWP1*) were evaluated using qPCR. Biofilm metabolic activity was determined using XTT reduction assay, and biofilm structure analysed by Scanning Electron Microscopy (SEM).

Results: Whilst the SR of acrylic and titanium did not significantly differ, the saliva with plasma pellicle increased significantly the total SFE of both surface. The number of viable microorganisms and DNA concentration increased with biofilm development, not differing within materials and pellicles. Biofilms developed on saliva with plasma pellicle surfaces had significantly higher activity after 24 h and this was accompanied with higher expression of virulence genes at all periods.

Conclusion: Induction of *C. albicans* virulence occurs with the presence of plasma proteins in pellicles, throughout biofilm growth. To mitigate such effects, reduction of increased plasmatic exudate, related to chronic inflammatory response, could aid the management of candidal biofilm-related infections.

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1. Introduction

Candidiasis is an infection caused by fungi of the genus *Candida*, being also the most prevalent fungal infections in humans. Candidiasis can manifest both topically and systemically and may involve many different sites in the human body (Gow, van de Veerdonk, Brown, & Netea, 2011; Peleg, Hogan, & Mylonakis, 2010). As *Candida* species normally exist as a harmless commensals, and the onset of infection is usually associated with particular factors, such immunological debilitation, receipt of a broadspectrum

antibiotic, or the loss of homeostasis within microbial communities infections (Gow et al., 2011; Peleg et al., 2010; Williams et al., 2013).

Within the oral environment, *Candida*-associated denture stomatitis is the most prevalent form of oral candidosis. This condition affects a wide range of denture wearers, with prevalence varying between 35% to 75%, depending on the investigated population (Barbeau et al., 2003; Coco et al., 2008; Zomorodian et al., 2011). The infection is usually seen as chronic inflammation of the palatal tissue in contact with a poorly cleansed denture surface upon which candidal biofilm has developed (Barbeau et al., 2003; Coco et al., 2008; Williams et al., 2013). In these cases, *C. albicans* is the most frequently encountered *Candida* species, and whilst bacteria may also be present, their role in the infection is unclear (Barbeau et al., 2003; Coco et al., 2008; Zomorodian et al., 2011).

Candida albicans is regarded as the most pathogenic *Candida* species in all forms of human candidosis (Gow et al., 2011; Peleg et al., 2010; Williams et al., 2013). Virulence of *C. albicans* relates to

* Corresponding author at: Department of Dentistry, School of Dentistry, State University of Paraíba (UEPB), Rua Baraúnas, 351, Campina Grande 58429-500, PB, Brazil.

** Corresponding author at: Periodontology and Prosthodontics Department, Piracicaba Dental School, University of Campinas, Av. Limeira, 901, Piracicaba 13414-903, SP, Brazil.

E-mail addresses: yuri.wanderley@yahoo.com.br, dryuricavalcanti@gmail.com (Y.W. Cavalcanti), wanderjose@fop.unicamp.br (W.J.d. Silva).

its ability to form biofilms on a wide variety of surfaces (Nailis et al., 2010; Pereira-Cenci, Cury, Cenci, & Rodrigues-Garcia, 2007; Vukosavljevic, Custodio, Del Bel Cury, & Siqueira, 2012); to grow and proliferate as hyphae (Dongari-Bagtzoglou, Kashleva, Dwivedi, Diaz, & Vasilakos, 2009; Nobile, Nett, Andes, & Mitchell, 2006; Williams et al., 2013); to colonise and invade epithelial tissues (Alves et al., 2014; Dongari-Bagtzoglou et al., 2009; Malic et al., 2007); to produce and secrete proteolytic enzymes (Custodio, Silva, Paes Leme, Cury, & Del Bel Cury, 2014; Naglik et al., 2008; Naglik et al., 2003); and to evade host immune responses (Wei, Rogers, Lewis, & Williams, 2011). Importantly, *Candida* virulence factors can be triggered by local environmental stimuli (Ramachandra et al., 2014), including pH, nutrient availability and gaseous composition. Although the properties of the colonised surfaces (e.g. surface roughness and surface charge) and the composition of the salivary pellicle have been shown to increase biofilm development (Custodio et al., 2014; Zamperini et al., 2010; da Silva et al., 2014), the virulence of such biofilms has not been extensively studied.

In the case of denture stomatitis, at the interface between the denture and the epithelium, reduced salivary flow is evident and the acrylic surface is also non-shedding (Barbeau et al., 2003). Both of these factors can contribute to the accumulation of biofilm (Williams et al., 2013). Biofilm development over other intra-oral biomaterials, such as dental implants, has also been reported as a risk factor to oral infections including *peri-implantitis* (Kilic et al., 2014; Lehmann et al., 2013). Consequently, classical signs of chronic inflammatory responses (characterised by soft tissues swelling and increased blood plasma exudate) can be generated at the interface between the biomaterial and the mucosal surface.

Given the importance of the inflammatory exudate on the progression and pathogenesis of infection, greater understanding of the role that material surfaces and associated coating pellicles play in the development of *Candida* biofilm is required. At sites of inflammation, plasma components may also contribute to the pellicle formed or may alter surface free energy (SFE) (Custodio et al., 2014; Dodo, Senna, Custodio, Paes Leme, & Del Bel Cury, 2013; da Silva et al., 2014), the number of attached microorganisms (da Silva et al., 2014) and *C. albicans* phospholipase and proteinase activity (Custodio et al., 2014). However, the role of plasma containing pellicles and the surface material itself on *C. albicans* biofilm virulence has not been widely examined. Therefore, the aim of this study was to investigate the role of material type, pellicle composition and stage of development on the viability, bioactivity, virulence and structure of *C. albicans* biofilm.

2. Materials & methods

2.1. Study design

This *in vitro* study had a randomized, controlled and double-blinded design. Discs of titanium and acrylic resin were used for initial characterization and subsequent salivary pellicle formation and biofilm development. Factors under study consisted of material type, pellicle content, and stage of biofilm development. Response variables included quantification of the total number of bacteria, expression of putative virulence genes and analysis under Scanning Electron Microscopy (SEM). A total of 336 discs were used to performing this experiment, being half for acrylic and another half for titanium.

2.2. Experimental design

In vitro assessment of the effect of surface material, pellicle composition, and stage of biofilm development on *C. albicans* virulence was undertaken. Materials studied were acrylic resin and titanium, as used in the construction of dental prosthesis. The

surface roughness (SR) and SFE of these materials was initially characterised. Biofilms (n = 144, being n = 9 for each tested group) were then developed on acrylic and titanium discs, which had been coated with human whole saliva pellicles with, or without human blood plasma (20:1 v/v) (n = 36 for each material-pellicle group). After 1.5 h, 24 h, 48 h and 72 h incubation at 37 °C, biofilms were recovered by sonication (Custodio et al., 2014; da Silva et al., 2014) and the number of viable *C. albicans*, DNA content (Custodio et al., 2014; da Silva et al., 2014), and expression of *C. albicans* virulence genes (Alves et al., 2014; Nailis et al., 2010) (*ALS1*, *ALS3* and *HWPI*) was measured. Additional biofilms of both material types, pellicle and time of development were generated to analyse metabolic activity (n = 144, being n = 9 for each group; XTT assay) and biofilm structure (n = 48, being n = 3 for each group; Scanning Electron Microscopy SEM).

2.3. Sample preparation

Heat-polymerised (poly-methyl methacrylate; PMMA) acrylic resin (QC-20; Dentsply Int Inc., Weybridge, UK) was prepared according to the manufacturer's instructions and fabricated into disc-shaped (10 mm diameter, 2 mm thickness) samples. Acrylic discs were finished in a horizontal polisher (model APL-4; Arotec, São Paulo, Brazil) using progressively finer aluminium oxide papers (320-, 400-, and 600-grit).

Commercially pure grade IV titanium discs (12.5 mm diameter, 2 mm thickness) were obtained by cutting of titanium bars (Sandinox; Sorocaba, São Paulo, Brazil) using cold electron-erosion. The surfaces of the discs were finished and polished by barrelling. Barrelling involved mixing titanium discs in a barrel with ceramic particles and an abrasive paste for 8 h. The discs were then transferred to another barrel with porcelain particles and abrasive paste for an additional 3 h.

Prior to use, all discs were ultrasonically cleaned with 70% (v/v) alcohol and with sterile ultra-purified water (20 min) to remove surface debris. Acrylic discs were decontaminated by immersion in 1% sodium hypochlorite solution and then thoroughly rinsed with sterile water. Titanium discs were sterilised by autoclaving at 121 °C for 15 min.

2.4. Surface roughness (SR) characterisation

The SR of discs was measured using an optical tri-dimensional surface profilometer (NewView 7300, Zygo Middlefield, CT, USA), at a resolution of 0.3 µm (horizontally) and 0.05 µm (vertically). Five random areas (212 × 283 µm) of each disc (n = 5) were analysed under Gaussian filter to remove interferences. SR was expressed as average roughness (Sa and Sz), surface texture (Sdr), rate of peaks per area (Sds) and ratio between peaks and valleys (Ssk).

2.5. Salivary pellicle formation

Human whole saliva and blood plasma were used to generate pellicles on discs' surfaces. The ethical committee in research of the Piracicaba Dental School, University of Campinas, Brazil, had previously approved the collection and management of saliva and blood plasma, according to protocol number 021/2012. Stimulated human whole saliva was obtained from three healthy individuals after receipt of informed written consent, whilst pooled human blood plasma was obtained from a blood bank (Varginha – MG, Brazil).

After stimulation with paraffin film (Parafilm M; American Can Co, Neenah, WI, USA), saliva was collected in iced-chilled polypropylene tubes (Custodio et al., 2014; Dodo et al., 2013). This collection was performed at the same time in the morning,

over a 30 min period. Saliva was pooled, mixed with a serine protease inhibitor (0.1 mM phenylmethylsulfonyl-fluoride) and clarified by centrifugation (3800 × g, 5 min, 4 °C). The saliva supernatant was then sterilised by filtration, using a 0.22-µm-membrane filter (Corning, Horseheads, NY, USA) and maintained on ice until use (Custodio et al., 2014; Dodo et al., 2013). Aliquots (20 mL) of blood plasma in polypropylene tubes were stored at –20 °C until use (Custodio et al., 2014; Dodo et al., 2013).

Immediately prior to experiments, cleaned acrylic and titanium discs were horizontally placed in pre-sterilised 24-well plates (Custodio et al., 2014; Dodo et al., 2013). Prepared discs were incubated (35 °C), and gently rotated (75 rpm) on an orbital shaker (NT 150 Kline Shaker, Novatecnica, Piracicaba – SP, Brazil), for 2 h, with 2 mL of human whole saliva, or a mixture of whole saliva and blood plasma (20:1 v/v). The discs were then rinsed with phosphate buffered saline (PBS, pH 7.4) and immediately used in experiments.

2.6. Surface free energy (SFE) measurements

SFE was measured with a goniometer (Ramé-Hart 500; Ramé-Hart Instrument Co, Succasunna, NJ) using an acid-base method (Combe, Owen, & Hodges, 2004). Discs devoid of pellicle and those coated with saliva, or coated with saliva with plasma (20:1 v/v) were analysed (Custodio et al., 2014). Coated discs were initially dried at 35 °C for 4 h, prior to SFE analysis (Custodio et al., 2014). The contact angle between the material surface and the sessile drop (15 µL) was analysed for three different solutions: ultrapure water, 99.5% formamide (Sigma-Aldrich Corp, St. Louis, Mo), and 99% bromonaphthalene (Sigma-Aldrich Corp, St. Louis, Mo). Contact angles, the polar and dispersive components, and the SFE were computed with Ramé-Hart DROPimage Standard software (Ramé-Hart Instrument Co).

2.7. Development of biofilms

Candida albicans ATCC 90028 was cultured on Sabouraud's Dextrose Agar (SDA, Difco, Detroit, MI, USA) and 2–3 colonies were then subcultured in Yeast Nitrogen Base broth (YNB, Difco, Detroit, MI, USA) supplemented with 50 mM glucose. After 18–20 h incubation (35 °C, 75 rpm), cells were harvested by centrifugation (3000 × g, 5 min), washed twice in PBS, and re-suspended in YNB enriched with 100 mM glucose (Custodio et al., 2014; da Silva et al., 2014). The optical density was adjusted to 0.25 at 520_{nm} (Custodio et al., 2014; da Silva et al., 2014; da Silva et al., 2008) using a spectrophotometer (DU 800 UV/Visible Spectrophotometer, Beckman Coulter, Inc., Brea, CA, USA) and this standardised suspension corresponded to ~1 × 10⁶ colony forming units (CFU)/mL of *C. albicans* (Custodio et al., 2014; Dodo et al., 2013).

Prepared discs with associated pellicles were inoculated with a 10-fold diluted preparation of standardised *C. albicans* suspension and incubated aerobically (35 °C, 75 rpm) for 1.5 h (initial adherence) (Custodio et al., 2014; Dodo et al., 2013). The culture medium

was then removed and the discs washed (×2 in PBS) to remove non-adherent cells. Fresh culture medium (YNB enriched with 100 mM glucose) was added to each well and incubation continued for 72 h at 35 °C, under agitation (75 rpm) with the culture medium renewed every 24 h (Custodio et al., 2014; Dodo et al., 2013).

2.8. Biofilm analysis

Biofilms (n = 9, for each group) generated for 1.5 h, 24 h, 48 h and 72 h were recovered from acrylic and titanium surfaces by ultrasonic disruption (7 W, 30 s; Branson, Sonifer 50, Danbury, CT, USA) in 1 mL of PBS and collected in ice-chilled tubes.

A 200-µL aliquot of the biofilm solution was serially diluted in PBS and portions (20-µL) of these dilutions were cultured on SDA plates using a drop-counting technique (Custodio et al., 2014; da Silva et al., 2014). SDA plates were incubated aerobically at 35 °C for 24 h and the resulting CFU/mL enumerated. The remaining 800-µL of biofilm solution was divided equally (400-µL) and transferred into 1.5 mL microtubes. After centrifugation (11,000 × g, for 5 min, at 4 °C), the supernatant was removed and cell pellets stored at –80 °C for later qPCR and RT-qPCR analyses.

2.9. DNA and RNA extraction

Frozen biofilm pellets were suspended in 450 µL of lysis buffer (Tris-EDTA buffer solution, pH 8.0 and 1% lysozyme; Sigma-Aldrich) (Collart & Oliviero, 2001; Hoffman, 2001). Biofilm suspensions were mixed with 50 µL of 10% SDS (Sodium Dodecyl Sulphate, Sigma-Aldrich) and 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich), before high-speed homogenisation with glass beads in a Mini-Bead-Beater-8 (Strattech Scientific, Soham, UK). Total nucleic acid was obtained in aqueous phase, after separation with phenol:chloroform:isoamyl-alcohol (25:24:1) (Collart & Oliviero, 2001; Hoffman, 2001). These procedures were performed for both DNA and RNA samples, prior to respective nucleic acid purification (Collart & Oliviero, 2001; Hoffman, 2001).

After total nucleic acid extraction, DNA was precipitated with 100% ethanol and treated with 5 µL of 10 × diluted RNase A (20 µg/µL) (Invitrogen), for 30 min, at 40 °C. Purified DNA was suspended in 50 µL of 10 mM Tris-HCl and subjected to gel electrophoresis (Collart and Oliviero, 2001). Total DNA concentration was calculated using the absorbance ratio at 260/280_{nm} (Nanodrop, Thermo-Scientific, Life Technologies). Extracted DNA was used as an alternative method to perform biofilm cell quantification using quantitative polymerase chain reaction (qPCR).

In the case of RNA purification, total nucleic acid was precipitated with 100% ethanol and purified using the RiboPure Yeast Kit (Ambion, Life Technologies). Total RNA was recovered and after filter column purification and DNase I (Invitrogen) treatment, suspended in ultra-pure water. The integrity and purity of total RNA was assessed by gel electrophoresis. RNA concentration was spectrophotometrically determined by measuring the absorbance

Table 1
Oligonucleotide sequences used for qPCR and RT-qPCR assays.

Genes	Oligonucleotide sequences (5' → 3')	Product size
<i>ACT1</i> – Housekeeping gene	F – TGCTGAACGTATGCAAAGG R – TGAACAATGGATGACCAGA	186 bp
<i>ALS1</i> – Agglutinin-like sequence	F – CCCAACTGGAATGCTGTTT R – TTTCAAAGCGTCGTTACAG	223 bp
<i>ALS3</i> – Agglutinin-like sequence	F – CGTCCATTGTGACGCTTA R – GCGGTTAGGATCGAATGGTA	234 bp
<i>HWPI</i> – Hyphal Wall Protein	F – TCTACTGCTCCAGCCACTGA R – GTGGAATGGAAGCTTCTGGA	226 bp

ratio at 260/280_{nm} (Nanodrop, Thermo-Scientific, Life Technologies). Standardised (200 ng/ μ L) total RNA suspensions were prepared and expression of *C. albicans* putative virulence genes tested using two-step RT-qPCR (reverse transcription followed by qPCR).

2.10. qPCR and RT-qPCR analyses

Primers for qPCR and RT-qPCR are presented in Table 1. Primers were designed from full-length gene sequences obtained from the nucleotide platform in PubMed, using Primer3 Plus software (Koressaar & Remm, 2007; Untergasser et al., 2012). The specificity of each primer was confirmed using primer-BLAST (Ye et al., 2012), which compared the respective sequences with databases of *C. albicans* genomes. Primer specificity for qPCR was also confirmed in preliminary studies involving conventional PCR assays with extracted genomic DNA. *ACT1* primers were used for both DNA quantification (qPCR) and gene expression (RT-qPCR), whilst *ALS1*, *ALS3* and *HWP1* primers were only used for gene expression.

DNA quantification was performed in a Mini Opticon Real Time PCR System (Bio Rad, Hercules, CA, USA), which was used for amplification and detection by qPCR. Real time qPCR 20 μ L mixes comprised of 2 μ L of purified DNA, 10 μ L ($\times 2$) of iQTM SYBR[®] Green Supermix (Bio-Rad), 1 μ L of each (forward and reverse) *ACT1* primer (10 mM/ μ L), and 6 μ L of molecular biology grade water. Reactions were performed in a 48-well plate, using the following cycling profile: initial denaturation at 95 °C for 5 min; 40 cycles of 95 °C for 30s, 60 °C for 1 min, and 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. A dissociation stage at 60 °C was performed to generate a melting curve for verification of amplified product. Standard curves of 6 different DNA concentrations (600, 60, 6, 0.6, 0.06 and 0.006 ng/ μ L) were plotted using the cycle number at which the threshold fluorescence (Ct) was reached³⁰ and results were expressed as DNA concentration (ng/ μ L).

Purified RNA was used as template for gene expression analysis in a two-step RT-qPCR. This involved independent reverse transcription (RT) and qPCR. Synthesis of cDNA was performed using 1 μ g of purified RNA and the iScriptTM cDNA Synthesis Kit (Bio-Rad). RT reactions contained 5 μ L of purified RNA (200 ng/ μ L), 4 μ L ($\times 5$) of iScript buffer (Bio-Rad), 1 μ L of reverse transcriptase enzyme and 10 μ L of molecular biology grade water. RT reactions were performed in a conventional thermo-cycler, with the following thermal protocol: 25 °C for 5 min; 42 °C for 30 min and 85 °C for 5 min. Generated cDNA was used for qPCR analysis. *ACT1* gene served as an endogenous control for *C. albicans* gene expression, as its production is constitutive (Nailis et al., 2010; Nailis, Coenye, Van Nieuwerburgh, Deforce, & Nelis, 2006). Agglutinin-like sequence (*ALS1* and *ALS3*) and hyphal wall protein (*HWP1*) genes were targeted as putative virulence genes of *C. albicans*.

The qPCR for gene expression analysis was performed as described previously. After qPCR, the threshold was adjusted according to the amplification curves of all evaluated genes. Standard curves of 6 different DNA concentrations (600, 60, 6, 0.6, 0.06 and 0.006 ng/ μ L) were plotted using the cycle number at which the threshold fluorescence was reached (Ct) (Suzuki,

Nakano, Yoshida, Yamashita, & Kiyoura, 2004). Relative comparisons between groups were made according to the ratio between the absolute quantification of target and reference genes, for each group, within each time point. Results were expressed as relative fold change in relation to the expression of the reference (*housekeeping*) gene.

2.11. Metabolic activity of biofilms

Acrylic and titanium discs coated with the different pellicles (n=9, in each group) were used to develop biofilms for 1.5 h, 24 h, 48 h and 72 h. Biofilms were analysed with regards to metabolic activity by XTT reduction assay (da Silva et al., 2008). Intact biofilms were transferred to new 24-well plates containing 1580 μ L of PBS enriched with 200 mM glucose, 400 μ L of XTT solution (1 mg/mL, in ultra-purified water), and 20 μ L of menadione (0.4 mM in acetone). The plates were incubated for 3 h in the dark, at 35 °C, under rotation (75 rpm). Discs were removed and the well contents collected and centrifuged (6000 \times g, 5 min). Absorbance of supernatants at 490_{nm} was measured using a spectrophotometer (DU 800 UV/Visible Spectrophotometer, Beckman Coulter, Inc., Brea, CA, USA). The colorimetric change was directly proportional to the metabolic activity of biofilms.

2.12. Structure of biofilms

The structure of *in situ* biofilms (n=48, being n=3 for each group) was determined by SEM. Biofilms were fixed with Karnovsky solution (4% paraformaldehyde and 2% glutaraldehyde in 0.1 M Sorensen buffer pH 7.3) for at least 24 h (Seneviratne, Silva, Jin, Samaranayake, & Samaranayake, 2009; da Silva, Seneviratne, Samaranayake, & Del Bel Cury, 2010). Biofilms were washed three times in PBS and then dehydrated in increasing ethanol concentration (50%, 60%, 70%, 80%, 90% and 100%) washes for 5 min each (Seneviratne et al., 2009; da Silva et al., 2010). The discs were dried in a desiccator and gold sputtered coated before imaging by SEM (JSM 5600LV, JEOL, Tokyo, Japan). Images were taken under high vacuum mode (15 Kv) and $\times 1000$ magnification.

2.13. Statistical analysis

Statistical analysis was performed using SigmaPlot v. 11.3 (Systat Software Inc., London, UK) at 5% significance. The assumptions of equality of variances and normal distribution of errors were evaluated for each variable. The number of viable cells data was not normally distributed and then logarithmically transformed.

Statistical comparison between the SR of acrylic and titanium surfaces were made by independent *t*-test ($\alpha < 0.05$). Comparison of SFE components (Polar Component, Polar(+), Polar(-), Dispersive and Total SFE) was made using two-way analysis of variance (ANOVA 2-way). The substratum type (acrylic or titanium) and the presence of pellicle (saliva, or saliva with plasma, or uncoated) were considered as study factors. Tukey Honestly Significant Difference (HSD) test was used to show differences within the presence of pellicle

Table 2

Surface roughness (SR) parameters of acrylic (PMMA) and titanium discs evaluated by 3D profilometry.

	Sa (μ m)	Sz (μ m)	Sdr (%)	Sds (1/ μ m ²)	Ssk
Acrylic	0.28 \pm 0.05 ^A	4.22 \pm 0.65 ^A	6.29 \pm 1.99 ^A	0.12 \pm 0.03 ^A	-0.61 \pm 0.12 ^A
Titanium	0.29 \pm 0.02 ^A	5.26 \pm 0.51 ^B	3.36 \pm 1.15 ^B	0.08 \pm 0.04 ^A	-2.21 \pm 0.03 ^B

Sa: Average SR among peaks; Sz: Average SR between peaks and valleys; Sdr: Percentage of additional surface area provided by the texture of surface; Sds: Number of peaks per area; Ssk: Ration between peaks and valleys.

Different letters within the same column indicate statistically significant differences ($p < 0.05$) between Acrylic and Titanium.

With regards to the response variables of biofilm analyses (viable cell number, DNA concentration, metabolic activity and gene expression), statistical comparisons were made using three-way analysis of variance (ANOVA 3-way). In such situations, the substratum type (acrylic or titanium), the pellicle type (saliva or saliva with plasma) and the time of evaluation (1.5 h, 24 h, 48 h, or 72 h) were considered as study factors. Tukey HSD test was used to show differences within the periods of evaluation.

3. Results

The average SR regarded as difference among peaks (Sa) did not differ significantly ($p > 0.05$) between acrylic and titanium (Table 2). However, analysis of average SR regarded as differences between peaks and valleys (Sz) has shown significant ($p < 0.05$) differences between the discs. The additional surface area provided by texture (Sdr) was significantly ($p < 0.05$) higher for acrylic. Furthermore, titanium had a significantly lower Ssk ($p < 0.05$), which corresponded with a higher number of valleys.

Representative images of the topography of acrylic and titanium revealed homogeneity for both surface types as defined by the polishing techniques used (Fig. 1). Higher number of scratches was observed on acrylic surfaces, whilst titanium exhibited minor grooves of different sizes.

The total SFE of acrylic and titanium only differed ($p < 0.05$) when the discs of such materials were devoid of pellicles (Table 3). Pre-coating acrylic and titanium with saliva, or saliva with plasma, produced equivalent SFE for both material types (acrylic and titanium) ($p > 0.05$). Compared to uncoated discs, saliva pre-coating decreased total SFE of acrylic, whilst increased total SFE of titanium, leading to no differences in these parameters for the materials. The same standardisation effect was also evident in discs with saliva containing plasma pellicle. Compared to other groups, the saliva with plasma pellicle had significantly ($p < 0.05$)

higher total SFE and greater polar component with negative charge.

The number of viable microorganisms (Fig. 2A) increased significantly ($p < 0.05$) with incubation time. Biofilm cell number was highest between 48 h and 72 h, when differences between the numbers of viable microorganisms were not significant. For each period of evaluation, biofilm growth was not affected by the substratum type, or by pellicle composition. A similar slope profile along the stages of development was observed for DNA quantification (Fig. 2B). The maximum concentration of DNA was reached after 48 h, when biofilm cells were highest, although the DNA concentration did not change significantly for 24 h, 48 h and 72 h samples nor in relation to the material or coating pellicle. Biofilm metabolic activity (Fig. 2C) increased with time, being maximal after 48 h incubation and this was significantly ($p < 0.05$) higher compared with the initial adhesion phase (1.5 h). Acrylic and titanium biofilms developed in the presence of saliva, or saliva with plasma, did not differ in metabolic activity at 1.5 h and 24 h. However, at 48 h and 72 h time points, biofilms developed over saliva with plasma-coated discs had significantly ($p < 0.05$) higher metabolic activity, compared to those grown on saliva-coated discs. Additionally, biofilms developed in the presence of saliva pellicle exhibited a decrease in their metabolic activity at 72 h.

Expression of *C. albicans* putative virulence genes varied with incubation period for all genes (Fig. 3). *ALS1* expression was up regulated with time for biofilms developed on both acrylic and titanium with saliva, or saliva with plasma pellicles (Fig. 3A). However, biofilms developed on saliva with plasma pellicles, exhibited significantly higher expression of *ALS1* at all periods of evaluation. Expression of *ALS3* was similar for all samples at 1.5 h (Fig. 3B). However, after 24 h, biofilms developed on saliva pellicles had significantly lower *ALS3* expression, whilst biofilms grown in the presence of saliva with plasma, maintained *ALS3* expression until 72 h *ALS3* expression on acrylic and titanium biofilms did not differ significantly for the same pellicle types. *HWP1* expression did

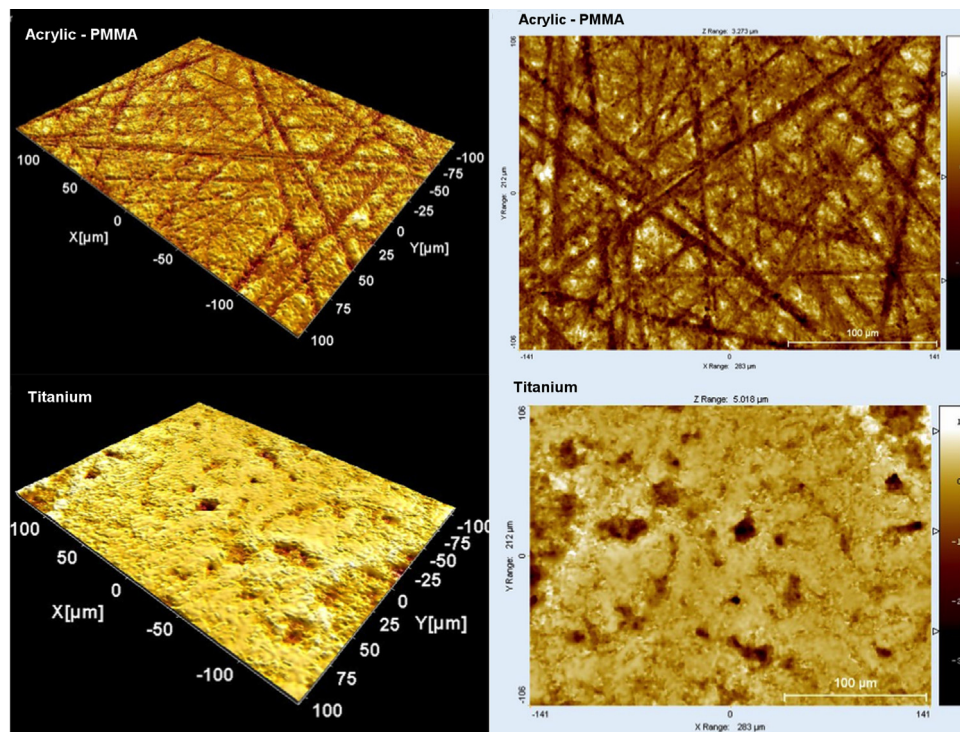


Fig 1. Representative microscopy of acrylic (PMMA) and titanium surface topography evaluated by white light 3D profilometry. Note the presence of polishing artifacts on acrylic surface, whilst titanium has numerous small grooves.

Table 3
Surface Free Energy (SFE) parameters of acrylic (PMMA) and titanium discs. Samples were evaluated without pellicle, or after pellicle formation in the presence of saliva, or saliva with plasma.

SFE Components	Without pellicle ^A		Saliva ^B		Saliva with Plasma ^C	
	Acrylic	Titanium	Acrylic	Titanium	Acrylic	Titanium
Polar Component	6.1 ± 0.9 ^a	2.9 ± 1.4 ^b	2.9 ± 1.3 ^a	1.3 ± 1.4 ^a	12.4 ± 2.3 ^a	13.6 ± 1.3 ^a
Polar(+)	0.8 ± 0.2 ^a	0.1 ± 0.1 ^b	1.9 ± 0.6 ^a	1.4 ± 0.3 ^a	2.1 ± 0.2 ^a	3.4 ± 0.3 ^a
Polar(–)	11.8 ± 2.3 ^a	21.3 ± 0.9 ^b	13.8 ± 1.4 ^a	21.3 ± 4.6 ^b	22.5 ± 2.7 ^a	33.9 ± 1.9 ^b
Dispersive	30.5 ± 2.3 ^a	25.1 ± 1.9 ^b	28.9 ± 2.1 ^a	32.3 ± 1.5 ^a	31.5 ± 3.5 ^a	32.3 ± 3.4 ^a
Total SFE	36.6 ± 2.5 ^a	28.0 ± 1.8 ^b	31.8 ± 1.4 ^a	33.6 ± 2.1 ^a	43.9 ± 2.2 ^a	45.9 ± 2.9 ^a

Different uppercase letters (top row) show differences among the SFE of discs with different coatings. Different lowercase letters show statistically significant differences ($p < 0.05$) among the SFE components of acrylic and titanium discs, within different coatings.

not change for discs with the same pellicle type (Fig. 3C). However, biofilms grown on saliva with plasma pellicles had statistically higher *HWP1* expression at 1.5 h and 24 h. Also, expression of *HWP1* was progressively down regulated for all periods of evaluation, and was similar for all evaluated groups at both 48 h and 72 h.

SEM of biofilms on acrylic (Figs. 4 and 5) and titanium (Figs. 6 and 7) showed that biofilm cell densities increased significantly with time, as seen by the few cells attached at 1.5 h and the dense biofilm structure observed at 72 h. A more condensed biofilm developed in the presence of saliva after 72 h. Biofilms developed over saliva-coated discs (Figs. 4 and 6) were comprised predominately of yeast forms of *C. albicans*, with few pseudohyphae evident at 1.5 h and 24 h. Biofilms developed over saliva with plasma pre-coated discs (Figs. 5 and 7) appeared less compact with numerous pseudohyphae and hyphae at all periods of evaluation.

Discussion

Previous studies have shown that surface properties of biomaterials have a significant role in development of biofilms (Dorkhan, Chávez de Paz, Skepö, Svensäter, & Davies, 2012; da Silva et al., 2014). Perhaps this is best exemplified by research that demonstrates increased microbial colonisation over rougher surfaces (Dorkhan et al., 2012; Pereira-Cenci et al., 2007; da Silva et al., 2014). In the present study, however, both evaluated materials (acrylic and titanium) had similar SR parameters. Acrylic did have more scratches and increased surface texture due to polishing procedures. However, these features did not significantly differentiate it from titanium with regards to biofilm accumulation.

Research of SFE effects on biofilm development has resulted in contradictory reports (Moura, da Silva, Pereira, Del Bel Cury, & Rodrigues Garcia, 2006; Zamperini et al., 2010; da Silva et al., 2014). A study has shown that SFE does not influence biofilm development, (Moura et al., 2006) whilst other research has reported that increased SFE can lead to both higher and lower biofilm accumulation (Dorkhan et al., 2012; Lima, Koo, Vacca Smith, Rosalen, & Del Bel Cury, 2008; Pereira-Cenci et al., 2007; Zamperini et al., 2010; da Silva et al., 2014). However, the SFE changes reported in these previous studies were most likely related to different pellicle types on the biomaterial surfaces. In the present study, pellicles comprised of saliva with plasma, increased the SFE and the negative charge of both acrylic and titanium discs, resulting in equivalency between these materials within the same parameters.

Generally, saliva pellicles on biomaterials increase the SFE with subsequent reduction in the number of adherent cells (Custodio et al., 2014; Lima et al., 2008; Pereira-Cenci et al., 2007; Zamperini et al., 2010; da Silva et al., 2014). Whilst the absence of saliva appears to increase the number of attached cells (Jin, Samaranyake, Samaranyake, & Yip, 2004; Lima et al., 2008; Pereira-Cenci et al., 2007), this scenario is not representative of the *in vivo*

situation. The composition of the salivary pellicle also influences the adherence of microorganisms (Dorkhan et al., 2012; Vukosavljevic et al., 2012). In this present study, however, pellicles of saliva-only or saliva with plasma did not affect *Candida* cell number or total DNA concentration. However, the presence of plasma increased the metabolic activity and expression of *C. albicans* virulence factors within biofilms on both acrylic and titanium.

In this study, the content of pellicles on the acrylic or titanium was not actually determined. However, previous studies performed by our research group have demonstrated that blood plasma-only pellicles formed on titanium are rich in fibronectin, serum albumin, apolipoprotein-AI and fibrinogen (Dodo et al., 2013). In turn, within the same material, saliva-only pellicles consisted primarily of alpha-amylase 1, cystatinS, cystatin-SA and mucin 5AC oligomeric mucus/gel-forming (Cavalcanti et al., 2014). With regards to acrylic surfaces, saliva-only pellicles were rich in α -amylase 1, cystatin-SN, cystatin-S and prolactin-inducible protein (Custodio et al., 2014). Saliva with plasma pellicles on the same substratum (acrylic) was however, composed of isoform 1 of albumin (most abundant) and fibrinogen (Custodio et al., 2014). Therefore, we consider that blood plasma makes a significant contribution to the composition of saliva with plasma pellicles, as seen by the incorporation of serum albumin and fibrinogen proteins (Custodio et al., 2014; Dodo et al., 2013; Samaranyake, Cheung, Yau, Yeung, & Samaranyake, 2013).

The results of this present study suggest that proteins present in blood plasma pellicle, as serum albumin and fibrinogen, may have a significant effect on stimulating both the metabolic activity and expression of *C. albicans* virulence genes. Previous studies have shown *C. albicans* has affinity to fibrinogen and other coagulative proteins, which may protect *Candida* from phagocytosis during blood-stream infection (Bertling et al., 2010; Fehrmann et al., 2013). Serum albumin proteins have been shown to increase *C. albicans* adherence to substrates and promote hyphal development (Samaranyake et al., 2013; Ovchinnikova, van der Mei, Krom, & Busscher, 2013; Ramachandra et al., 2014). The increase of negative surface charges may also have an effect on the accumulation and virulence of biofilms (Custodio et al., 2014; Lima et al., 2008; Zamperini et al., 2010).

Furthermore, serum albumin proteins increase sterol uptake through ATP-binding cassette (ABC) transporters (Marek, Silvestro, Fredslund, Andersen, & Pomorski, 2014). This phenomenon interferes with the respiratory chain, allowing *Candida* to utilise exogenous sterol under anaerobic conditions (Li and Prinz, 2004; Marek et al., 2014). Also, cholesterol uptake may serve as a protective measure against ergosterol-specific antifungals. (Marek et al., 2014; Nakayama, Tanabe, Bard, Hodgson, & Wu s Takemori, 2007). Therefore, incorporation of both fibrinogen and albumin into the salivary pellicle has the potential to significantly increase the virulence of *C. albicans* biofilm.

Total metabolic activity is generally directly proportional to the number of viable cells (Moura et al., 2006; Seneviratne et al., 2009;

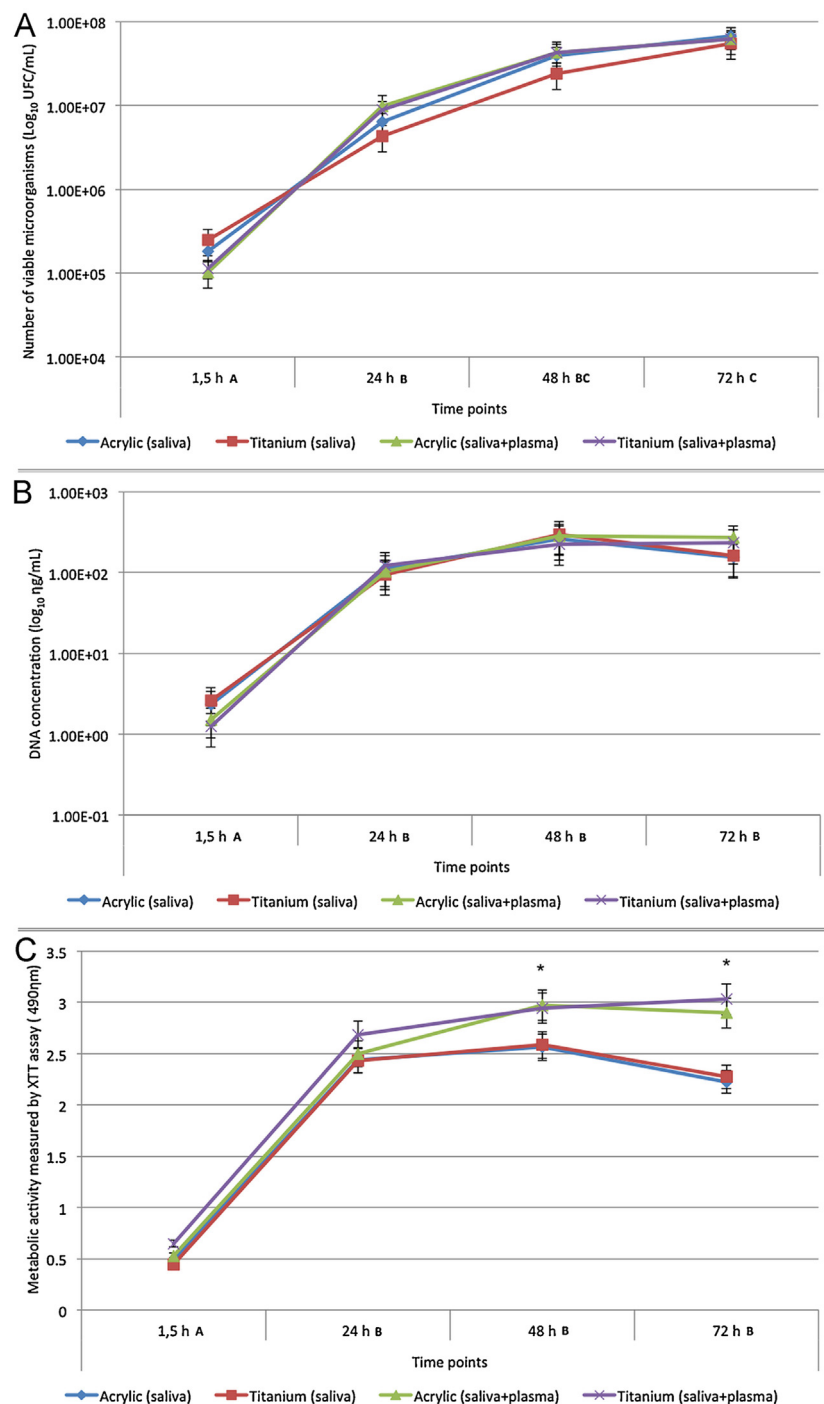


Fig. 2. Viability and bioactivity of *C. albicans* biofilm formed on Acrylic and titanium surfaces pre-coated with saliva or saliva with plasma. Biofilms (n=9, for each group) were formed for 1.5 h, 24 h, 48 h and 72 h. Panel A shows the number of viable microorganisms (log₁₀ UFC/mL); Panel B shows the DNA concentration (log₁₀ ng/mL) and Panel C presents the metabolic activity measured by XTT assay (490 nm). Different letters for the time points indicate statistically significant differences between periods of evaluation (p<0.05). *Indicate statistically significant differences between saliva and saliva with plasma-coated discs (p < 0,05).

da Silva et al., 2008; da Silva et al., 2010) and this was evident in the present study. However, when the number of microorganisms reached a constant (after 48 h and 72 h) for the groups, higher metabolic activity was evident with biofilms grown on pellicles of saliva with plasma. The effect may, again, be due to serum albumin within pellicle, which might induce higher respiratory activity (Li & Prinz, 2004; Marek et al., 2014). Biofilms developed on saliva-coated discs, maintained constant metabolic activity between 24 h and 48 h, which then reduced at 72 h. These results confirm those

of previous studies involving biofilms grown over similar periods *in vitro* (Seneviratne et al., 2009; da Silva et al., 2010).

The plasma-supplemented salivary pellicle not only promoted metabolic activity, but also overall biofilm maturation. *ALS1* gene expression is directly related to *C. albicans* biofilm maturation⁴⁷ and expression of this gene increased in biofilms grown in saliva with plasma. The increased expression was likely responsible for more rapid and enhanced biofilm development. As *ALS1* has been associated with different *C. albicans* adhesion strategies (Murciano

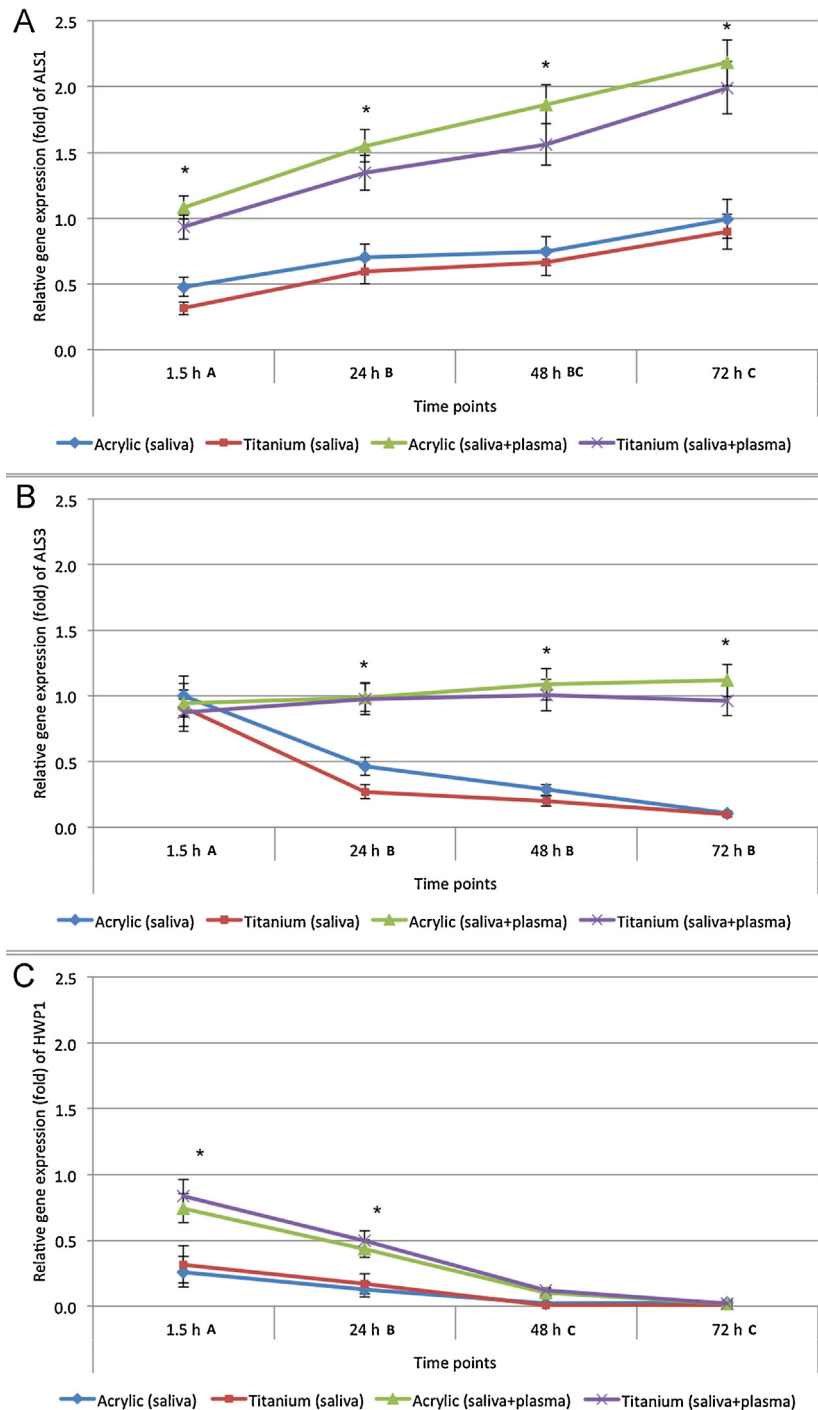


Fig. 3. Relative gene expression of *C. albicans* agglutinin-like sequence (*ALS1* and *ALS3*) and hyphal wall protein 1 (*HWP1*) genes, measured in biofilms formed on acrylic and titanium pre-coated with saliva or saliva with plasma. Panels A, B and C, respectively, illustrate the relative gene expression of *ALS1*, *ALS3* and *HWP1*, in acrylic and titanium biofilms developed in the presence of saliva, or saliva with plasma, for 1.5 h, 24 h, 48 h and 72 h. *C. albicans* reference gene (*ACT1*) was used for data normalisation. Results correspond to the fold change relative to the reference gene's (*ACT1*) constitutive expression. Different letters for the time points indicate statistically significant differences between periods of evaluation ($p < 0.05$). *Indicates statistically significant differences between saliva and saliva with plasma coated discs, within each gene expression evaluation ($p < 0.05$).

et al., 2012; Zhu & Filler, 2010), its up regulation may confer a more adherent biofilm, which in turn is an important virulence factor of *C. albicans*.

ALS3 and *HWP1* genes were also up regulated in biofilms developed on saliva with plasma coated discs. These genes translate hyphal-specific proteins, which are directly involved with *C. albicans* morphogenesis and hyphal adherence (Murciano et al., 2012; Nailis et al., 2009; Orsi et al., 2014; Ramachandra et al.,

2014; Zhu & Filler, 2010). Up regulation of *ALS3* and *HWP1* may be associated with the serum albumin and higher protein content present in saliva with plasma pellicles (Samaranayake et al., 2013; Orsi et al., 2014; Ovchinnikova et al., 2013; Ramachandra et al., 2014). These proteins can directly up regulate expression of putative virulence genes, or induce an associated environmental condition that promotes *C. albicans* virulence (Samaranayake et al., 2013; Gow et al., 2011; Ramachandra et al., 2014).

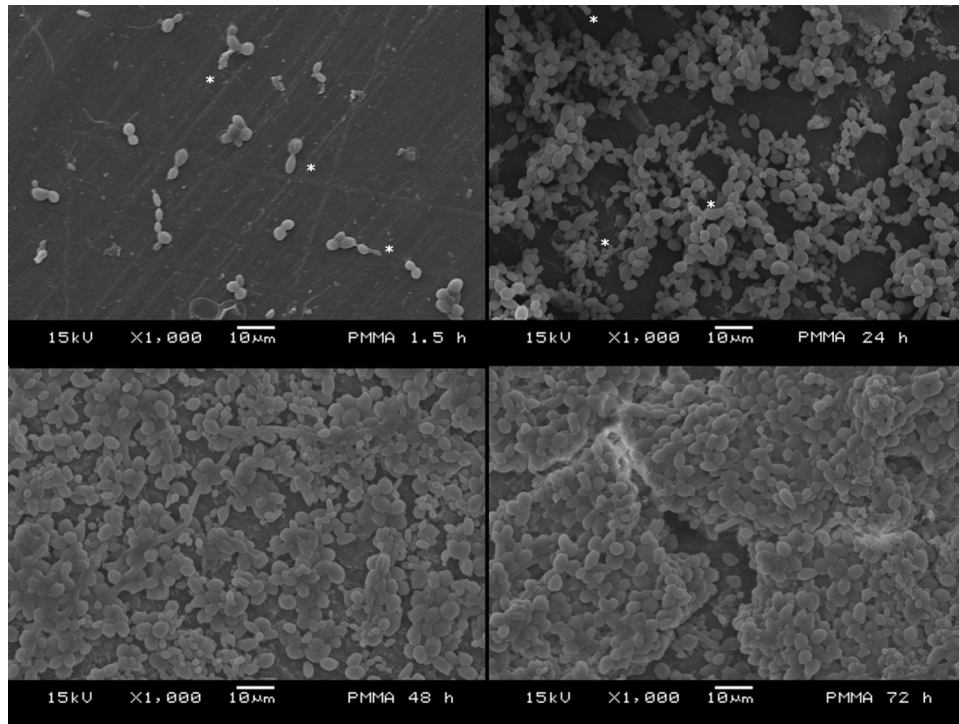


Fig. 4. SEM of *C. albicans* biofilm formed on acrylic surfaces pre-coated with saliva. Biofilms were imaged at 1.5 h, 24 h, 48 h and 72 h of development. Asterisks show structures similar to pseudo-hyphae at early stages of biofilm formation (1.5 h and 24 h).

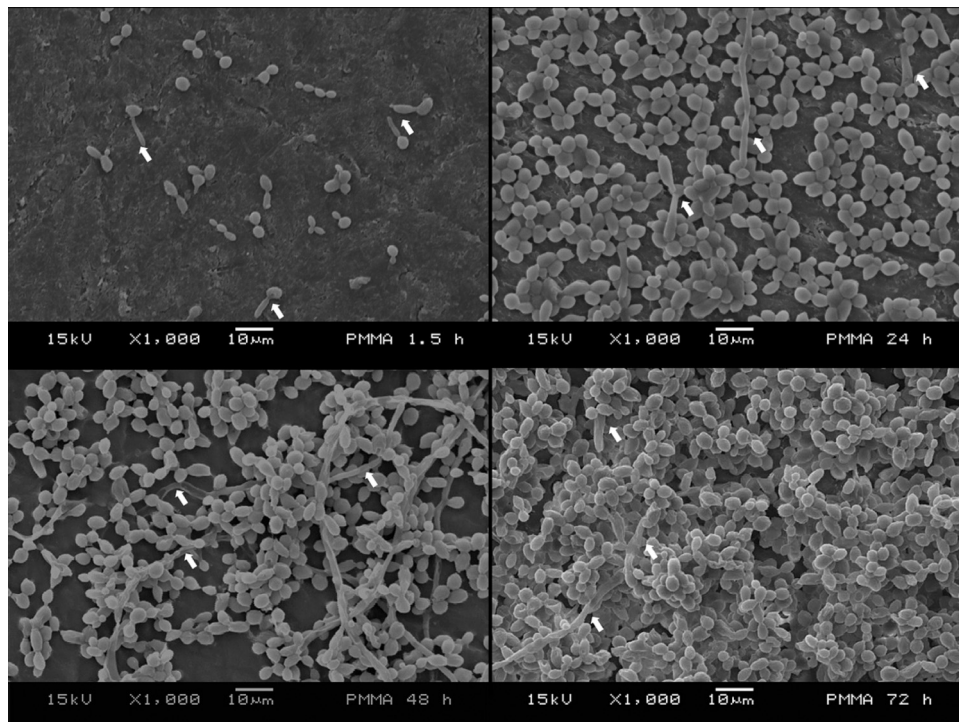


Fig. 5. SEM images of *C. albicans* biofilm formed on acrylic surfaces pre-coated with saliva with plasma. Biofilms were imaged at 1.5 h, 24 h, 48 h and 72 h of development. White arrows indicates the presence of hyphae, which were more prevalent in the presence of saliva with plasma pellicle.

Although biofilms grown on saliva-only pellicles exhibited decreased *ALS3* expression with time, biofilms on saliva with plasma coated discs maintained constant *ALS3* expression with up regulation of *HWP1* expression for a longer period. This notably contributed to a more extensive biofilm with increased hyphal

production, as confirmed by SEM. Deletion of the *C. albicans ALS3* gene has been found to reduce candidal morphogenesis in biofilms (Nailis et al., 2009) with subsequent lower epithelial cell adhesion, reduced tissue damage and cytokine production. (Murciano et al., 2012), Although *HWP1* deletion has been shown not to interfere

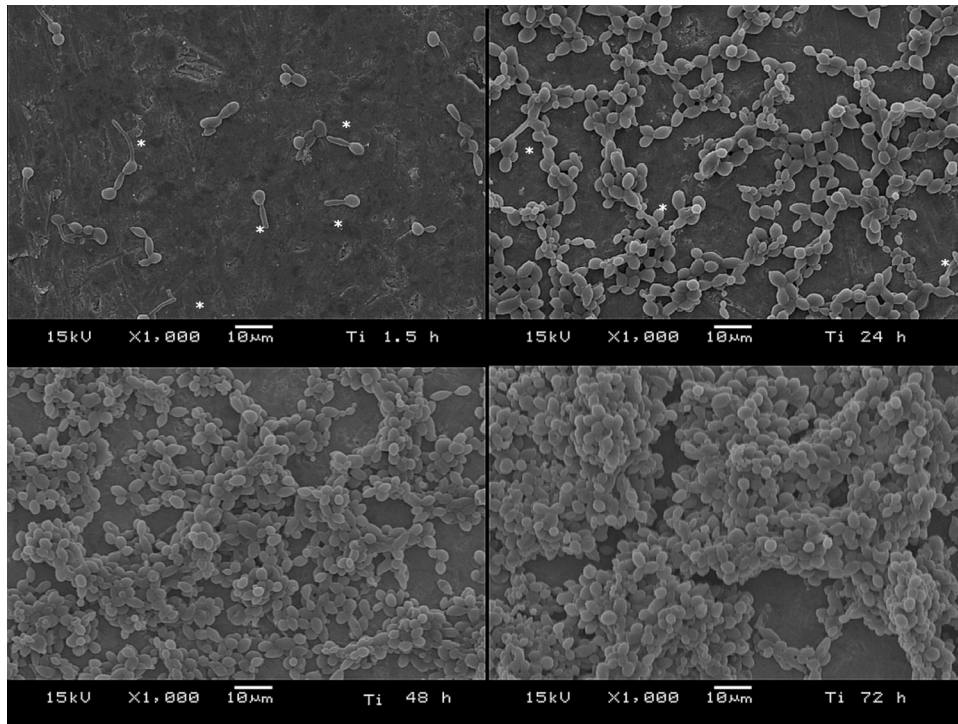


Fig. 6. SEM images of *C. albicans* biofilm formed on titanium surfaces pre-coated with saliva. Biofilms were imaged at 1.5 h, 24 h, 48 h and 72 h of development. Asterisks show structures similar to pseudo-hyphae at early stages of biofilm formation (1.5 h and 24 h).

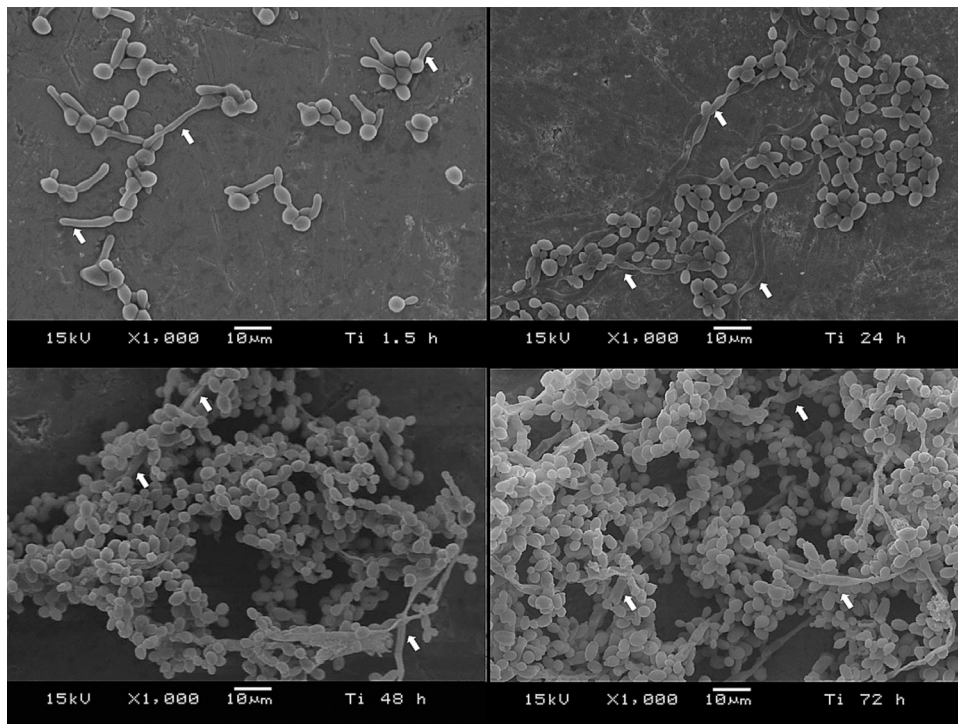


Fig. 7. SEM images of *C. albicans* biofilm formed on titanium surfaces pre-coated with saliva and plasma. Biofilms were imaged at 1.5 h, 24 h, 48 h and 72 h of development. White arrows indicate the presence of hyphae, which were more prevalent in the presence of saliva with plasma pellicle.

with biofilm accumulation, mutant samples exhibit reduced pathogenesis and increased fungal membrane defects. (Orsi et al., 2014)

SEM revealed hyphal development during initial stages of biofilm formation, for both materials (acrylic and titanium) and

pellicles types (saliva only and saliva+plasma). This coincided with higher expression of *ALS3* and *HWP1* at 1.5 h. Also, SEM confirmed increased hyphal production in biofilms developed in the presence of saliva with plasma, with occurrence of hyphae at all

time points. Expression of *ALS3* and *HWP1*, therefore, represent relevant indicators of *C. albicans* virulence.

The results of present study show that salivary pellicles contribute to the equalisation of surface charges of different materials. The results also confirm that pellicles of saliva containing plasma promote virulence of *C. albicans* biofilm, by inducing hyphal proliferation with associated up regulation of *ALS3* and *HWP1*. Collectively, these results are important towards our understanding of how saliva and blood plasma proteins influence biofilms on different biomaterials. The management of biofilm-associated infections in chronic inflammation should not only involve antimicrobial treatment, but could also aim to reduce tissue inflammation responses. This could diminish the virulence of biofilms and aid management of *Candida*-associated infections.

5. Conclusion

Saliva with plasma pellicles on acrylic and titanium surfaces contributed to the equalisation of surface charges, but increased SFE. This pellicle also promoted higher metabolic activity and up regulation of *C. albicans* putative virulence genes. Although more significant effects of saliva with plasma proteins were observed within 24h, activation of *C. albicans* virulence persisted for longer periods of biofilm development. Therefore, management of biofilm-associated chronic inflammation should consider the role of blood plasma proteins in the triggering more virulent infections.

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