Combining nanofabrication with natural antimicrobials to control denture plaque

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Abstract

Management of fungal biofilms represents a significant challenge to oral healthcare. As a preventive approach, minimising adhesion between intra-oral devices and microorganisms would be an important step forward. Denture stomatitis (DS) is a multifactorial denture-associated inflammation of the oral mucosa where candidal biofilms are one of the contributing factors. Therefore, understanding candidal biofilms on dentures and finding novel strategies to control these biofilms are of significance. Interference with the adhesion step of biofilm formation is hypothetically effective strategy to control biofilms.

To understand the relationship between denture candidal load, denture material type and *C. albicans* biofilm forming heterogeneity in DS, quantitative polymerase chain reaction (qPCR) molecular method and crystal violet (CV) assay were used. This study investigated two novel strategies to control *C. albicans* biofilms through interfering with adhesion: natural polyphenol curcumin (CUR) and modifying the topography of the denture material surface. Based on the optimised effective CUR concentrations, CUR adsorption to PMMA denture material was spectrophotometrically analysed. Based on these data, the effect of adsorbed CUR to PMMA and CUR pre-exposure on adhesion of *C. albicans* were assessed. The effect of CUR on *Candida-Candida* adhesion was investigated and the expression profile of selected adhesion and aggregation-associated genes was assessed using qPCR method. Micro/nano-fabricated polycarbonate and PMMA materials were replicated using injection and compression moulding techniques, respectively and were characterised using scanning electron microscopy (SEM). Adhesion of *C. albicans* on the micro and nano-scaled patterns was assessed using microscopic and qPCR molecular methods, respectively. The physical characteristics of the materials were assessed using theta tensiometer and a white light profiler.

The data demonstrated that although *C. albicans* was detected in greater quantities in diseased individuals, it was not associated with increased biofilm biomass. Denture substrata were shown to influence biofilm biomass, with poly(methyl methacrylate) providing the most suitable environment for *C. albicans* to reside.

Subsequent studies showed that CUR concentrations of 50 μg/ml could prevent adhesion to PMMA. This effect was enhanced by the CUR pre-treatment of yeast cells (>90% inhibition, $p < 0.001$). Investigation of the biological impact of CUR showed that it preferentially affected immature morphological forms (yeast and germlings), and actively promoted aggregation of the cells. Transcriptional analyses showed that CUR temporally modulated adhesion and aggregation associated genes. Finally, PMMA denture material was replicated to show nano features. These topographies influenced adhesion of *C. albicans*, depending on the candidal morphological form and the shape. Nano-pit spatial arrangements variably affect the adhesion of *C. albicans*, where SQ arrangement demonstrated a significant anti-adhesive capacity. Differential adhesin expression was observed on these surfaces, which were affected by the wettability and roughness of surfaces tested.

In summary, *C. albicans* is an important determinant of denture disease, so preventing its adhesion and biofilm formation were worthwhile objectives. This thesis has shown that CUR molecules and SQ nano-pit topographies reduced *C. albicans* adhesion, demonstrating that chemical and physical inhibition strategies are useful. The data presented in this thesis showed the high potential of the novel strategies to be used against *C. albicans* biofilms, and encourages the further investigation of these approaches against polymicrobial denture biofilms.

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Author's Declaration

I declare that I have carried out the work described in this thesis unless otherwise acknowledged or cited, under the supervision of Professor Gordon Ramage, Dr Douglas Robertson, Dr Christopher Nile and Professor Nikolaj Gadegaard.

I further declare that this thesis has not in whole or in part, been submitted for any other degree.

Hasanain Alalwan

May 2018

Abbreviations

AAF1: Adhesion-aggregation factor 1

ALS: Agglutinin-like sequences

AMP: Antimicrobial peptide

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

AS: Artificial saliva

BSA: Bovine serum albumin

cDNA: Complementary deoxyribonucleic acid

CFU: Colony forming units

CHX: Chlorhexidine

CLSI: Clinical and laboratory standards institute

COPD: Chronic obstructive pulmonary disease

CSH: Cellular surface hydrophobicity

Ct: Cycle threshold

CUR: Curcumin

CV: Crystal violet

ddH2O: Double distilled water

DS: Denture stomatitis

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EAP1: Epithelial adhesion protein 1

EDTA: Ethylene diamine tetraacetic acid

ELISA: Enzyme linked immunosorbent assay

F: Flat

G: Germling

H: Hyphae

HBD: human beta defensin

HBF: High biofilm former

HEX: Hexagonal

HIS: Histatin

HMDS: Hexamethyldisilazane

HSP: Heat shock protein

IL-8: Interleukin-8

INF: Intermediate biofilm formation

LBF: Low biofilm former

MAP: Mitogen activated protein

MIC: Minimum inhibitory concentration

MOLLO: Molloplast

MWD: Microwave disinfection

NC: Negative control

NRT: No reverse transcriptase

NSQ: Near square

NTC: Non-template control

OD: Optical density

OMIG: Oral microbiology and immunology group

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PDT: Photodynamic therapy

PEM/THFM: Poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate

PMIC: Planktonic minimum inhibitory concentration

PMMA: Poly (methylmethacrylate)

qPCR: Quantitative polymerase chain reaction

rDNA: Ribosomal DNA

RNA: Ribonucleic acid

ROS: Reactive oxygen species

rpm: revolutions per minute

RPMI-1640: Roswell Park Memorial Institute - 1640 media

RT: Reverse transcription

RT-qPCR: Real time-qPCR

SAB: Sabouraud dextrose agar

SAP: Secreted aspartyl proteinases

SD: Standard deviation

SEM: Scanning electron microscopy

SEM: Standard error of mean

SMIC: Sessile minimum inhibitory concentration

SOD: Superoxide dismutase

SQ: Square

TBAEMA: Tert butylaminoethyl methacrylate

TUP1: Thymidine uptake 1

UFI: Ufi gel

WCA: Water contact angle

XTT: 2,3 bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide

Y: Yeast

YPD: Yeast peptone dextrose

1 Introduction

1.1 Introduction

As the elderly population expands to a predicted two billion by 2050, the number of denture wearers will concomitantly rise. Edentulousness is an irreversible clinical condition that can be described as an ultimate marker of oral disease burden (Cunha-Cruz *et al.*, 2007). It is also associated with socioeconomic factors, with higher prevalence reported in the poor and women (Bedos *et al.*, 2003). All types of dentures (complete, partial and overdenture) can be associated with oral mucosal lesions with predisposing factors that can be physical such as chronic irritation, immunological such as hypersensitivity or microbiological (Jainkittivong *et al.*, 2010).

Currently, around 20% of the UK population wear removable dentures of some form, with 70% of UK adults older than 75 years old wearing dentures (Hannah *et al.*, 2017), with many of these individuals suffering from denture stomatitis (DS), an inflammation of the palate (Gendreau & Loewy, 2011). Poor oral hygiene is frequently observed within this patient group and several factors can impact the onset of DS such as salivary flow, denture cleanliness, age of denture, smoking and diet (Martori *et al.*, 2014b). A large proportion (>two-thirds) of individuals who wear removable complete dentures may suffer from DS, though most individuals are asymptomatic (Gendreau & Loewy, 2011). DS represents the most frequent oral mucous lesion in elderly individuals (Rivera *et al.*, 2017). Denture-oral hygiene instruction and professional guidance is required, which is more significant in the elderly population who could suffer from cognitive and manual dexterity obstacles (Zenthofer *et al.*, 2013). Only a minority of sufferers experience pain, itching or burning sensation, discomfort or taste disturbance. Soft tissue inflammation below or above the denture, as a result of persistent exposure to microorganisms, is characteristic of DS (O'Donnell *et al.*, 2017)

A recent systematic review showed that there is no well-defined treatment strategy of DS because of the multi-causative nature of this inflammatory response (Yarborough *et al.*, 2016). This may rationalise why research was focused on prevention and inhibition of denture microbial biofilms (Park *et al.*, 2015;Tsutsumi *et al.*, 2016). Nevertheless, endeavours to create antimicrobial denture materials

often result in a collateral damage of the mechanical properties that may fail the prosthesis (Paleari *et al.*, 2011). This chapter will review the influences of removable prosthodontic appliances on health, focus on how various factors influence a move towards disease, and introduce existing and novel clinical management strategies.

1.2 Evolution and importance of removable prosthodontic appliances

Prosthodontic appliances have been used since ancient times right through to our own contemporary period. The ancient Egyptians in 2500 BC tried to replace missing teeth using natural teeth connected to one another by gold wires (Forshaw, 2009). The first endeavour to fabricate a realistic denture, including denture base, was in the fifteenth century in Europe using blocks of ivory or bone (Murray & Darvell, 1993). US president George Washington had ivory dentures (Ring, 2010). The use of ivory or bone as raw materials for denture fabrication continued until the year 1780 when it was replaced by porcelain, and latterly in 1820 gold had been introduced to prosthodontic field as a denture base (Murray & Darvell, 1993). Vulcanite then superseded all the previous denture base materials in 1850 when Goodyear was granted a patent for mixing sulphur with natural rubber, then celluloid and bakelite (a phenol-formaldehyde resin) were introduced, though were not typical substitutes for vulcanite. After around a century of vulcanite reigning, in 1935 the acrylic resins, specifically the poly methylmethacrylate (PMMA) were introduced. Having evolved after more than 3 decades of extensive research it had many advantages over vulcanite, including dimensional and colour stability, inertness, and the possibility of chemical bonding of artificial teeth made from the same material. Given these important properties, in 1946 approximately 95% of fabricated dentures were manufactured from PMMA, with cobalt-chromium base metal alloys playing an adjunct role in denture fabrication (Corrado, 1990;Murray & Darvell, 1993;Williams, 2015). Despite the general trend of declining tooth loss, the world-wide demographic change to an aging population has seen an overall net gain in the demand for dental prostheses (Douglass & Watson, 2002).

Dentures are removable prosthetic replacement of missing teeth and associated soft and hard tissues for complete or partially edentulous patients. Functional (mastication and phonetics) and aesthetic restoration, and therefore restoration of the somatic and psychological health, are the main advantages of denture wear, where oral health-related quality of life is usually enhanced after prosthodontic treatment (Montero *et al.*, 2013;Swelem *et al.*, 2014). Several studies have revealed a positive impact of prosthodontic treatment on the temporomandibular joint disorders (Goiato *et al.*, 2010;Abdelnabi & Swelem, 2015). Moreover, improvement of oral functions after head and neck cancer surgery, such as hemimaxillectomy, can be achieved by obturator dentures that aim to close the defect. The obturator denture separates the oral and nasal cavities and prevents nasal regurgitation of food and liquids and avoid hyper-nasal speech, besides the facial profile support and ordinary benefits of dentures (Chen *et al.*, 2016a). Thus, dentures are considered of significant importance for the general wellbeing of denture wearers, though are not without their disadvantages.

1.3 Mechanopathological responses to removable prosthodontic appliances

Through the last two decades, intensive research had been performed to confirm the link between the denture and oral, as well as systemic health (Nikawa *et al.*, 1998b). Poorly fitted dentures may reduce chewing and masticatory performance, which in turn negatively impact the general health and can deteriorate the nutritional status of the denture wearer (Garrett *et al.*, 1996;Sahyoun & Krall, 2003). Wearing a removable oral prosthesis alongside poor denture plaque management could influence systemic health. Although causality has not been well established, vigilant hygienic measures should be considered for patients with systemic diseases (Le Bars *et al.*, 2015). Indeed, there is even suggestions that treatment of DS is able to improve endothelial function and minimise risk of atherosclerosis and hypertension (Osmenda *et al.*, 2017). It has also been reported that denture plaque has a role in initiating unanticipated lung infections such as aspiration pneumonia in immunocompromised and medicated elderly population (Nikawa *et al.*, 1998b). Poor oral hygiene was significantly related to nosocomial and aspiration pneumonia, as several serious pathogenic microbes were isolated

from the oral cavity of institutionalized elderly persons, especially those in hospital ICUs and nursing home settings (Scannapieco, 2006;Kuyama *et al.*, 2010;Iinuma *et al.*, 2014). Furthermore, a systematic review indicates that teeth brushing after meals, a thorough cleaning of denture at least once a day, in addition to professional oral hygiene care once a week, are all necessary to reduce aspiration pneumonia and associated mortality in fragile elderly people individuals (van der Maarel-Wierink *et al.*, 2013). Further studies report that life-threatening pneumonia was doubled in elderly denture wearers, indicating a potential relationship between denture wear and respiratory infections (Iinuma *et al.*, 2014). This is endorsed by a recent report demonstrating the abundance of several potential respiratory pathogens on denture surface of healthy and diseased individuals using a molecular approach (O'Donnell *et al.*, 2016). Therefore, the denture has an inherent capacity to be a hazardous reservoir of infectious pathogens, that have the potential under certain circumstances to influence systemic health. An association between denture plaque and chronic obstructive pulmonary disease (COPD) has also been reported by Przybylowska *et al.* (2014), where 90% of the study patients had pathogenic pulmonary microorganisms in their denture plaque, with 75% of these having a yeast.

From another systemic perspective, and given the association between *Candida* species and denture wearing (Webb *et al.*, 1998b;Radford *et al.*, 1999;Ramage *et al.*, 2006), a positive relationship between oral *Candida* species and occurrence of oral cancer was uncovered by Alnuaimi *et al.* (2015). That study observed a highly significant oral *Candida* carriage rate in oral cancer subjects, which has been corroborated by Uittamo *et al.* (2009). It was reported that *C. albicans* is associated with the formation of acetaldehyde (a potent carcinogenic compound) via metabolism of glucose. Moreover, the carcinogenesis process and metastasis could be developed and progressed by the *C. albicans* through several mechanisms, such as the production of carcinogenic nitrosamines and activation of CD4 T-cells to produce specific interleukins that stimulate the angiogenesis of the tumors (Ramirez-Garcia *et al.*, 2016). Collectively, denture wearing has more profound systemic implications than we may generally acknowledge.

Locally, the potential oral pathological response to denture wearing is multifactorial. Studies have shown a significant role of medication on oral health of denture wearers (Carr *et al.*, 1993), a significant physical effect of denture surface and biomechanics on denture plaque accumulation and associated dental and periodontal status (Drake & Beck, 1993), the positive role of maintenance of a good denture hygiene (Shay, 2000), and the negative role of the inappropriate use of a product or a technique (Verran *et al.*, 2014). Besides, other factors may complicate the denture-oral cavity relationship, such as salivary flow, dietary effect and microbial colonisation (Turner *et al.*, 2008;Altarawneh *et al.*, 2013;Martori *et al.*, 2014a). These contributing factors are summarised in Figure 1.1.

Denture biomechanics can contribute to oral disease, where poorly fitted and fabricated complete or partial dentures could lead to deterioration of the hard tissues (teeth and bone), such as exposure of the abutment teeth to excessive and poorly distributed forces that produce bone resorption and increased teeth mobility (Aydin & Tekkaya, 1992). In some cases, poorly fitted partial dentures may lead to root fracture of the abutment tooth (Mizuno *et al.*, 2016). Another example on the local mechano-pathological response to dentures is combination syndrome, which is characterised by increased bone loss in the anterior region under the maxillary complete denture opposing a lower anterior natural teeth, causing a hyperplastic flabby ridge (Palmqvist *et al.*, 2003).

Dentures might also physically affect soft tissue (oral mucosa), and spots of traumatic ulcers could evolve due to high extension of the borders of denture flanges and occlusal instability. Additionally, hyperplastic fibrous tissue, or denture irritation hyperplasia, could evolve in response to chronic irritation from poorly fitted denture or overextended flanges (Carlsson, 1997). Burning mouth syndrome, which is characterised with painful burning sensation in the oral cavity without any obvious lesion, was positively associated with denture wearing, though it is of multifactorial aetiology (Svensson & Kaaber, 1995;Mukatash-Nimri *et al.*, 2017).

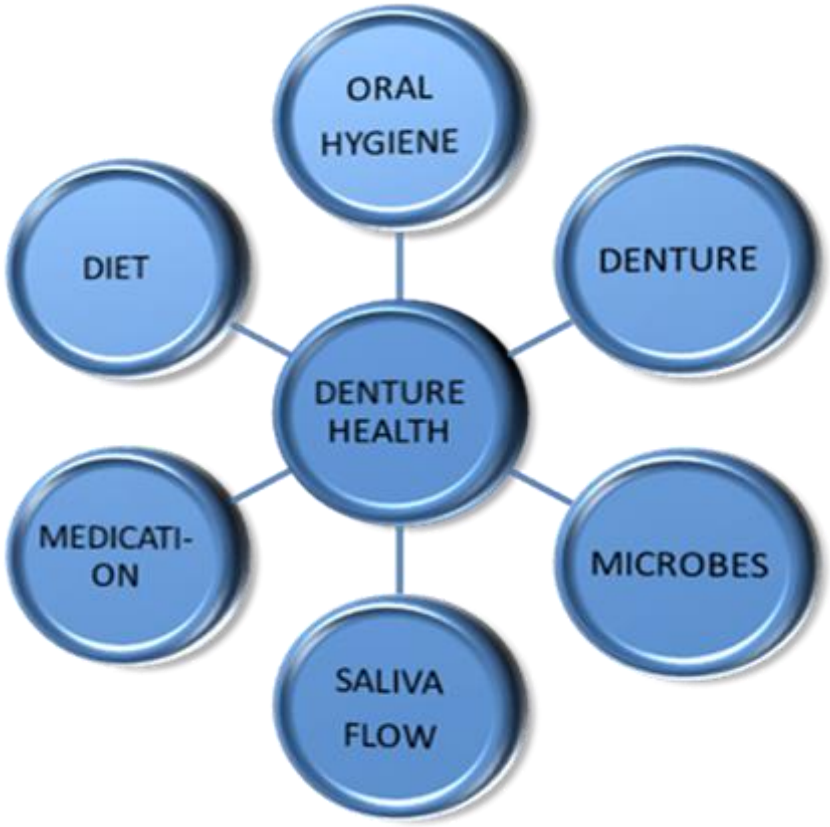

Figure 1.1 The multiple contributing factors in oral health of denture wearers.

From a microbiological point of view, denture-associated detriments might be more complicated and recalcitrant to therapy. This is attributed to the oral environment, host immunological response and associated microbiome, where the oral cavity represents a typical milieu for microbial colonisation (Preshaw *et al.*, 2011;Benso *et al.*, 2013). Furthermore, denture material surfaces especially the unpolished fitting surface that is in an intimate contact with the mucosa exaggerate such a problem because of the inherent roughness, voids and crevices that could provide a shelter for the microbes and increase their capacities to shear forces, which can clearly observed by scanning electron microscopy (Verran & Maryan, 1997;Ramage *et al.*, 2004), as shown in Figure 1.2.

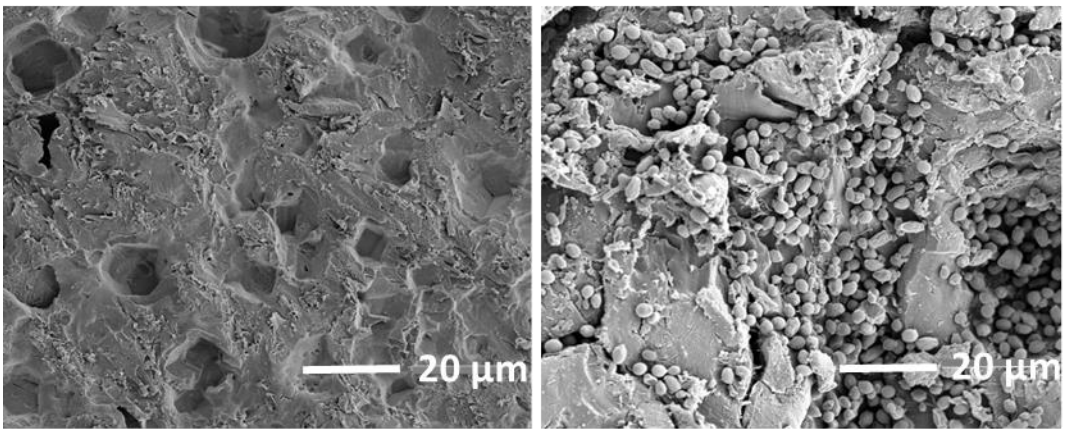

Figure 1.2 SEM image of non-polished acrylic denture base material with and without yeast cells. Scale bar is 20 um**.**

1.4 Denture stomatitis

1.4.1 Signs and symptoms and classification

DS is a chronic erythematous and oedematous inflammation of oral mucosa localized to areas in direct contact with the fitting surfaces of the removable prosthodontic appliances, although it could be detected in association with orthodontic appliances and obturators (Webb *et al.*, 1998a). The denture bearing area of the maxillary mucosa is the typical inflammation site. Usually, it is asymptomatic, although it could be accompanied by swelling and bleeding of the mucosa, dryness, burning sensation and unpleasant taste and halitosis of the oral cavity. Moreover, it was reported that one to two thirds of the denture stomatitis patients complain discomfort in their oral cavity (Arendorf & Walker, 1987;Webb *et al.*, 1998a). Newton's classification has divided its clinical manifestation into three types based on the grade of inflammation severity. Type I: localized inflammation or pinpoint hyperaemia, Type II: diffuse hyperaemia (erythematous), where more diffused erythema involving large part or entire denture covered mucosa; Type III: granular with papillary hyperplasia distributing over the centre of the hard palate and the alveolar ridge (Scully & Felix, 2005) (Figure 1.3).

(i) **(ii)** **(iii)**

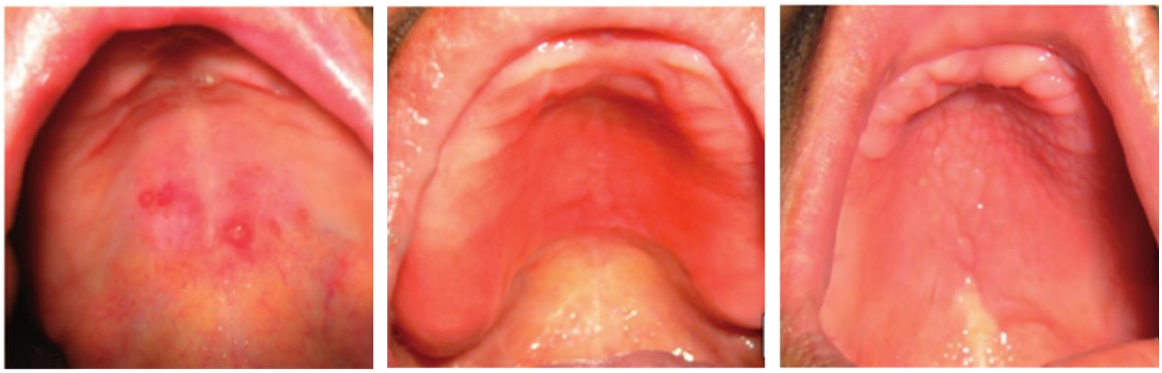

Figure 1.3 Denture stomatitis clinical presentations according to Newton's classification. (i) localised hypermedia: Grade I **(ii)**, widespread erythematous inflammation: Grade II, **(iii)**Granular and papillary hyperplasia: Grade III (da Silva *et al.*, 2011).

1.4.2 Epidemiology and aetiology

The epidemiological reports indicate a varied prevalence of DS among denture wearers ranging between 15->70% (Gendreau & Loewy, 2011). Age and gender are important factors in incidence of DS, where elderly people and women are more liable to this disease and showed higher incidence rate (Gendreau & Loewy, 2011). Moreover, DS is a frequent oral mucosal disorder correlated with institutionalized elderly people (Magalhães & Moreira, 2010), as well as those with a cognitive impairment due to dementia (Bramanti *et al.*, 2015). Socioeconomic status (Evren *et al.*, 2011) and levels of education (Baran & Nalcaci, 2009), also negatively impact prevalence rates for DS.

DS is a multifactorial disease, initially described with three main aetiological factors driving its initiation: trauma, infection and allergy (Budtz-JöRgensen, 1974). However, malnutrition, hormonal disturbances and antibiotics are also considered as predisposing factors (Jeganathan & Lin, 1992). *Candida* species, smoking and nocturnal wear are also correlated to DS (Barbeau *et al.* (2003). Salerno *et al.* (2011) attributed DS into systemic and local factors. These systemic factors included diabetes, deficiency of nutritional factors, kidney disease and xerostomia, while the local factors included trauma, saliva, pH of the oral cavity, the permeability of the acrylic resins, and the presence of microbial plaque. Given

that DS is proposed to be a type of oral candidiasis, it can therefore be influenced by systemic contributory factors that drive thrush, including cigarette/tobacco smoking, antibiotic treatment, cytotoxic/ radiotherapy, and diabetes mellitus (Soysa *et al.*, 2004;Soysa & Ellepola, 2005;Soysa *et al.*, 2006).

Trauma from ill-fitted denture can be considered one of the causes, which is endorsed by research showing the using of implant supported over-dentures. The over-denture reduces the direct forces on the mucosa and facilitates a good distribution of forces, which reduces the trauma of mucosa and subsequently the possibility of DS occurrence (Emami *et al.*, 2008). Moreover, poor denture fit, poor denture hygiene, and night-time wearing, in addition to denture surface imperfections and using silicon denture liners, facilitate the establishment of denture microbial biofilm and plaque formation (Gendreau & Loewy, 2011). Regular sugar consumption, low salivary pH and the presence of *Candida* in the oral cavity were also shown as risk factors and correlated with establishment of DS (Martori *et al.*, 2014a). Finally, the microbiome and level of dentition in the oral cavity can have a significant impact on DS development and exacerbation, where O'Donnell *et al.* (2015b) showed an associated with DS in partially edentate patients, suggesting bacterial-fungi inter-kingdom interactions play an important role. Overall, the factors that drive DS are multifactorial, which makes the clinical management problematic.

1.4.3 Diagnosis

The diagnosis of DS is fundamentally based on clinical features of the palatal mucosa, such as direct visual observation for the presence of pin-point or diffuse erythematous lesions, and papillary hyperplasia that correspond to the fitting surface of the denture (Puryer, 2016). Optical devices have also been advocated, such as an erythema meter that can be used to measure the degree of erythema of the palatal mucosa (Cross *et al.*, 1998;Cross *et al.*, 2004). Any suspicious immunocompromising condition should be excluded, such as diabetes and HIV infection (Scully, 2013). Laboratory tests are also useful for confirmation, including haematological evaluation (full blood count and iron, folate and vitamin B12 screening) and histological evaluation (via obtaining biopsy), where significant differences in palatal epithelial thickness and haematological abnormalities are

reported (Jennings & MacDonald, 1990). Microbiological and immunological diagnostic approaches that detect the presence of *C. albicans* and the antibody titre to its antigen are often used (Jeganathan & Lin, 1992).

Microbiologically, direct microscopy of smears of the palate and the denture, culturing of palatal swabs, and use of imprint culture are all useful (Webb *et al.*, 1998a). Historically, smears were examined by staining with Periodic acid-Schiff stain for fungal yeasts detection (Davenport, 1970;Budtz-Jorgensen, 1972), and for culture, swabs taken from the mucosa or denture are plated onto Sabouraud's agar to detect *Candida* (Cawson, 1965). Imprint culture can also be performed by using a foam pad that is pressed against the area of mucosal interest, then removed and pressed firmly on to the Sabouraud's plate and incubated (Arendorf & Walker, 1980). The oral rinse culture is a method that involves instructing the patients to rinse their mouth with 10 ml of phosphate buffer saline for 1 min, which is collected, centrifuged and resuspended into 1ml (concentrated method) prior to plating on Sabouraud's agar, is also regularly used for candida diagnostics (Samaranayake *et al.*, 1986). In today's microbiology laboratories, *Candida* chromogenic agars are used that enable quantitative and qualitative assessment of different species, such as *C. albicans* and *C. glabrata* (Coco *et al.* (2008b).

To expedite conventional microbial diagnostics beyond the concentrated oral rinse methodology then the successful use of real time quantitative polymerase chain reaction (RT-qPCR) with a high level of sensitivity has been reported (1-10 CFU/ml) (White *et al.*, 2004). The use of RT-qPCR has also been recently reported to detect the presence of pathogenic pulmonary bacteria in dentures using this advanced technique (O'Donnell *et al.*, 2016). Given the general move in clinical microbiology to become more molecular, then rapid and specific detection of key pathogenic culprits is likely, though we still need live organisms in order to perform sensitivity testing to inform the best chemotherapeutic intervention.

Immunological biomarkers for candidal detection could also be additionally helpful, though are not widely implemented. These include radioimmunoassay and enzyme-linked immunosorbent assay (ELISA), which have been reported to recognise *Candida* serologically (Byadarahally Raju & Rajappa, 2011). For

example, significant increases of salivary anti-*Candida* IgA antibodies have been detected in in DS patients (Jeganathan *et al.*, 1987). Other immunological biomarkers include elevated interleukin 2 (IL-2) (Rodriguez-Archilla *et al.*, 1996), the alteration in neutrophil morphology and activity (Gasparoto *et al.*, 2012b), and the modulation of other cytokines, such as IL-1ß, IL-4,IL-10, IL-6, IL-12, TNF-α, CXCL8, MCP-1(Gasparoto *et al.*, 2012a;Pinke *et al.*, 2016). However, at this stage these are largely used in a research capacity and their general utility in routine testing will always be limited.

1.5 Denture plaque and the role of *Candida* in DS

Microbial biofilms are associated with 65-80% of human infectious diseases (Joo & Otto, 2012). These are characterised by highly structured microbial communities that are attached to the colonised surfaces, in which the microbes communicate to each other through a system called quorum sensing. Furthermore, production of extra cellular matrix is a distinctive phenomenon of biofilms that give this microbial community the utmost protection against shear forces, penetration of antimicrobial agents and immune cells (Ramage *et al.*, 2002b;Ramage *et al.*, 2009). Moreover, persister cells, a small sub-population of resilient cells, ensures maintenance and repopulation following chemical insult (Lewis, 2005;LaFleur *et al.*, 2006). Biofilm development on dentures has been demonstrated by Ramage *et al.* (2004) and others (Sachdeo *et al.*, 2008;Susewind *et al.*, 2015).

The presence of an oral prosthesis is significantly correlated with deteriorating oral health, specifically mucosal integrity (O'Donnell *et al.*, 2015b), but also cariogenic and periodontal status (da Fonte Porto Carreiro *et al.*, 2016). Oral malodour, halitosis, is also a notable consequence of denture microflora (Verran, 2005;Nalcaci & Baran, 2008). Compositionally, the denture microflora is heterogeneous and dynamic given its close proximity to other oral microenvironments (O'Donnell *et al.*, 2015b), and can influence or be influenced by other surfaces. Indeed, Marsh *et al.* (1992) showed the capability of partial dentures to increase cariogenic bacteria within the oral cavity. Moreover, the prevalence of gingivitis and root caries was shown to increase with removable partial dentures wearing (Preshaw *et al.*, 2011), where fungal species also

contribute together with bacteria in such pathologic abnormalities (Coulthwaite & Verran, 2007). Indeed, it has been shown that there is a synergistic relation between *C. albicans* and *Streptococcus mutans* in developing virulent decay-inducing biofilms (Koo & Bowen, 2014). Angular cheilitis, another common disease in denture wearers, is characterized by erythematous fissures at the corners of the mouth that is highly associated with *C. albicans* and *Staphylococcus aureus* (Martori *et al.*, 2014a). However, biofilms formed on denture surfaces often include *Candida* species, which is thought and consistently reported to be a principal player in denture biofilm-associated disease (Ramage *et al.*, 2004;Taylor *et al.*, 2008;Øilo & Bakken, 2015). Figure 1.4 demonstrates the growth and development of a *C. albicans* biofilm on denture and silicone substrates.

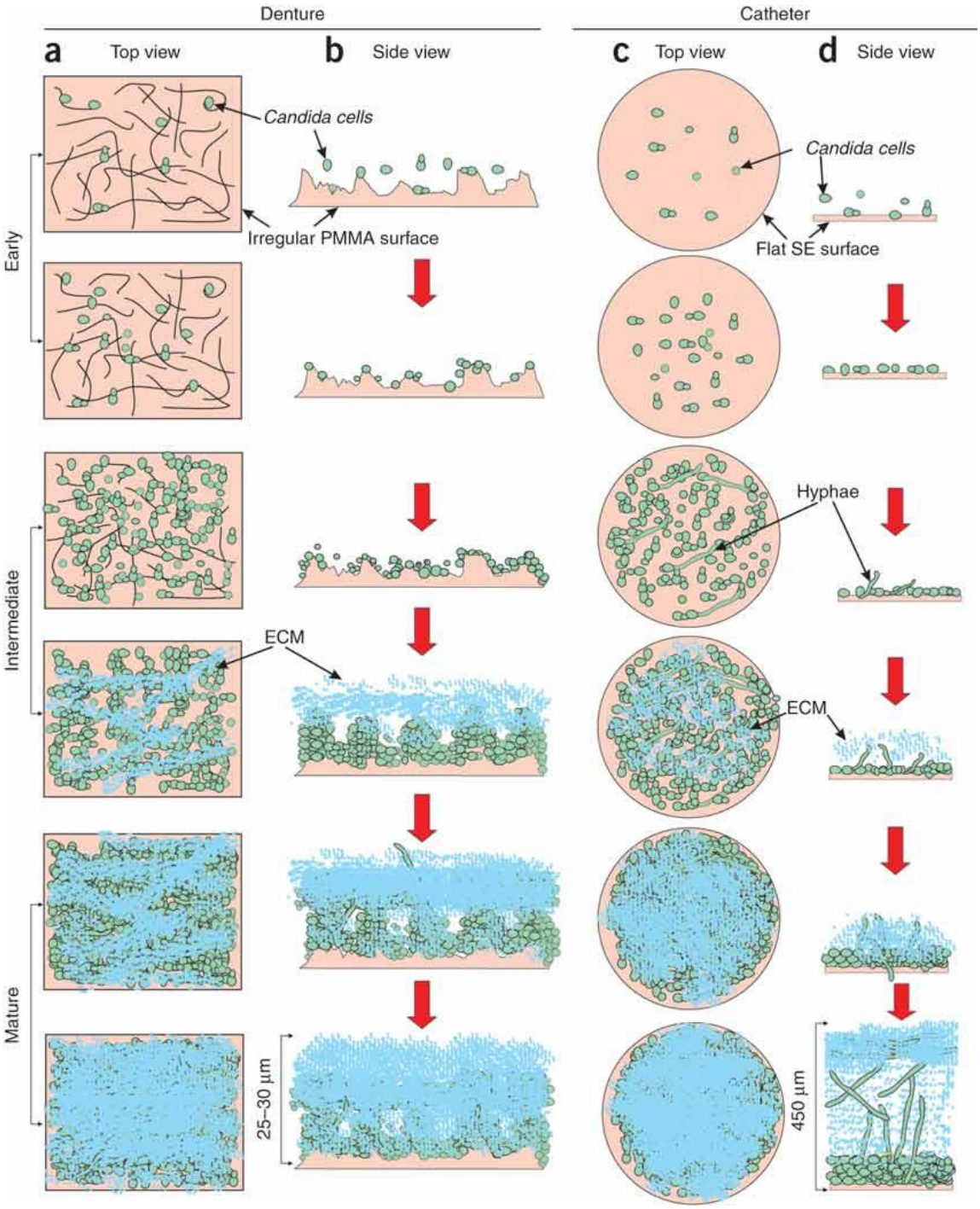

Figure 1.4: Schematic representation of *Candida albicans* biofilm formation on different device substrates. Schematics show biofilms formed on **(a,b)** PMMA denture strips or **(c,d)** silicone elastomer catheter disks. Panels a and c represent the substrate seen from the top, whereas panels b and d show the side view of biofilms formed on the PMMA strip and SE disk, respectively. ECM, extracellular material. This schematic was derived on the basis of data obtained from fluorescence and confocal microscopy analyses (Chandra *et al.*, 2001).

It is suggested that *Candida* and bacteria are correlated with DS, where higher numbers of yeasts and bacteria were cultured from DS patients (Budtz-Jorgensen *et al.*, 1983). Formation of the microbial biofilm is a successful survival strategy that different microbes can follow, and it is associated with different infectious diseases that may be related to indwelling medical devices (Donlan & Costerton, 2002). Denture material has the capability, by help of oral environment, to initiate an inter-kingdom microbial biofilm (Cavalcanti *et al.*, 2016). The diversity of bacterial and fungal species (*Candida* species specifically) within the oral cavity in health and disease is complex, and relies on the particular environment in which they coexist, and the physical or chemical nature of cohabitation (O'Donnell *et al.*, 2015a). Maintenance of homeostasis in the oral cavity between bacteria and fungi is important and influenced by the host environment (Xu & Dongari-Bagtzoglou, 2015). Therefore, dysbiosis could cause pathological consequences, such as that induced by antibiotics (McLean, 2014). The extensive microbiome of the denture fitting surface *in vivo* ranges from 10^4 to $10^6/cm^2$, thus maximising the significance of the denture biofilm ecosystem (Monsenego, 2000). Furthermore, it was demonstrated that 1 mg of the denture plaque could contain up to 10^{11} microbes (Nikawa *et al.*, 1998a).

Generally, accumulation of microbial denture plaque on the denture fitting surface is considered a significant factor in DS (Nikawa *et al.*, 1998b). Higher number of *Candida*, the initial positive effect of antimycotics, adhesion capabilities to both denture surfaces and palatal mucosa and the much greater mass of *Candida* in comparison to bacteria (> 50 times) make *Candida* an essential element in DS occurrence and development. Nevertheless, the role of bacteria should not be underestimated (Salerno *et al.*, 2011). It was shown that collaboration between the bacteria and *Candida* begins from the first stage of biofilm formation 'adhesion' (Busscher *et al.*, 2010). Adherence of the *Candida* cells to surfaces is a mainstay in their virulence and ability for colonization and reproduction and biofilm formation. Moreover, given the biofilm formation and dimorphism capacities of *C. albicans* that could act as a scaffolding for other microorganisms and instigate co-aggregative coexisting habitat onto dentures (O'Donnell *et al.*, 2015a) (Figure 1.5). Indeed, this is recently endorsed by Kean *et al.* (2017), where *Staphylococcus aureus* benefited from *C. albicans* in

formation of a biofilm via using it as a structural scaffold. Bacterial adherence to denture surfaces has also reported, where *Streptococcus mutans* and *Staphylococcus aureus* were the most isolated bacterial microorganisms from the upper complete denture with a rate of 53.3 and 34.4% respectively (Ribeiro *et al.*, 2012). Pereira-Cenci *et al.* (2008b) showed that *Streptococcus mutans* had a negative effect on the hyphae formation capacity in *Candida*, despite significant quantities of denture biofilm. Moreover, *Streptococcus oralis* adhesion to dentures was reported elsewhere (Charman *et al.*, 2009), which alongside *C. albicans* have the potential to produce complex denture biofilms (Cavalcanti *et al.*, 2016).

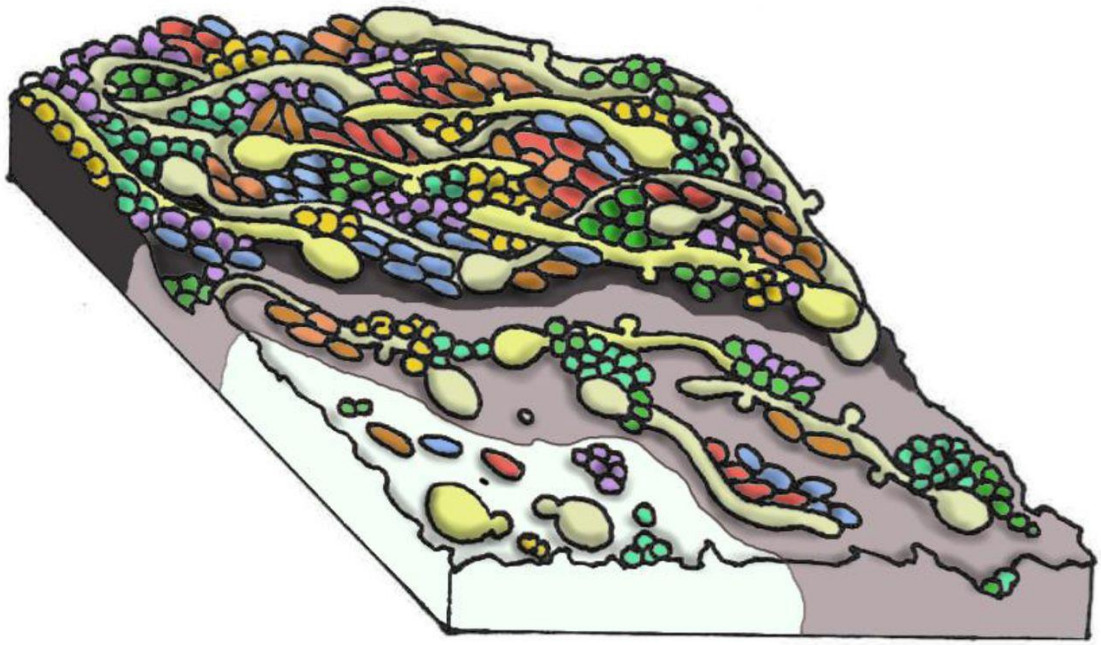

Figure 1.5 The *Candida*-bacteria colonisation of denture surface. A schematic drawing represents the Polymycrobial community with *C. albicans* in yeast and hyphal forms (O'Donnell *et al.*, 2015a).

Several studies have shown that the pathogenicity of *Candida* can be synergised by the presence of some types of bacteria within the oral cavity, such as *Streptococcus mutans* and *Staphylococcus aureus* (Harriott & Noverr, 2009;Diaz *et al.*, 2012;Falsetta *et al.*, 2014). This notion was supported by Cavalcanti *et al.* (2015), where up-regulation of ALS3, EAP1, HWP1 and SAP6 *Candida* virulence-significant genes was reported in presence of bacteria. Another report demonstrated that lactic acid bacteria such as lactobacilli could induce filamentation in opaque cell type *C. albicans* (Liang et al., 2016). This is relevant as the presence of hyphae in the saliva or on the mucosa had a positive effect on

raising the load of *C. albicans*. Likewise, there was a statistically significant relation between the hyphae incidence and number of lactobacilli, so this bacterium appears to play an important adjunctive role in DS (Bilhan *et al.*, 2009). However, Lamfon *et al.* (2005) did not find a significant difference in the quantities of the *Candida*, *Lactobacillus*, *Streptococcus*, *Veillonella* or *Actinomyces* species in mucosa and dentures swabs of DS diagnosed patients group and control group.

To demonstrate the importance of *Candida* spp in DS, Ramage *et al.* (2004) used SEM to inspect development of candidal biofilms directly from dentures from DS patients. Mesh of yeast and hyphal candidal forms in an entangled biofilm was observed, with denture cracks and imperfections representing niches for attachment, and biofilm thriving. The potential contribution of non-*albicans* types of *Candida* in DS pathogenesis was explored by Coco *et al.* (2008b). Traditional microbiological plating method with counting of colony forming units was followed and revealed a significant difference between the total *Candida* burden of the advanced grades of DS (grade II & III) and the control. In the same report, the percentages of presence of *C. albicans* and *C. glabrata* throughout the healthy and diseased groups were 75 and 30% of participating patients, while the rate of *Candida glabrata* was 80% among the highest grade of Newton's DS classification, with significant association with *C. albicans*, indicating an important co-association in DS.

Further studies have shown that *C. tropicalis* was the focus of DS pathogenesis deterioration. This was reported in a study evaluating the prevalence of *Candida* species among DS diabetic type 2 and non-diabetic people. *C. albicans* showed a prevalence of 81% with 15% for both *C. glabrata* and *tropicalis*. *C. albicans* and *tropicalis* were higher in prevalence of DS group. Moreover, *C. tropicalis* had a significant predilection for the highest degree of DS inflammation (Sanita *et al.*, 2011). Production of enzymes may be considered an explicit liaison between *Candida* and DS. The role of secreted aspartyl proteinases (SAP protease enzymes) in DS development and severity was explored by Ramage *et al.* (2012a). Transcriptional quantitative PCR results showed a highly significant difference in all SAP genes expressions between control and diseased group. Further studies have shown that phospholipase production by *Candida* species of clinical isolates

was significantly related to DS and was limited to *C. albicans* and *C. dubliniensis*, (Marcos-Arias *et al.*, 2011). Indeed, there are a number of studies that have shown that haemolytic activity, germ tube formation, induction of clot formation (coagulase activity) and biofilm formation of *Candida* spp. may all contribute to DS (Pereira *et al.*, 2016), highlighting the importance of candida related virulence.

1.6 *Candida albicans* virulence attributes

C. albicans has sufficient ammunition to exhibit pathogenicity when given an opportunity (Ramage *et al.*, 2009). The adaptation of *C. albicans* to environmental changes makes it a pleiotropic organism (Poulain, 2015), existing in two main morphological forms: round to oval yeasts, or long mycelial form hyphae (Figure 1.6). The pathogenicity of *Candida* can be facilitated through many determinants displayed in Figure 1.7, including adhesion, dimorphism, development of biofilms, switching (phenotypic plasticity), metabolic flexibility (glycolysis, gluconeogenesis), thigmotropism (directional hyphal growth), invasion, secretion of proteases and rapid adaptation to pH environmental fluctuations (Mayer *et al.*, 2013). Moreover, a thick, protective (0.5 μm) cell wall in addition to the ability to fight the free radicals released by immune cells through formation of superoxide dismutase enzyme (Poulain, 2015). Thigmotropism can be described as a representative of virulent contact sensing capacity of *C. albicans*. The contact sensing definitive mechanism is still elusive, although mechanosensitive ion channels and integrin-like signalling could be significant potential mechanisms that elucidate this fundamental physio-pathogenic fungal process (Kumamoto, 2008).

(i) **(ii)** **(iii)**

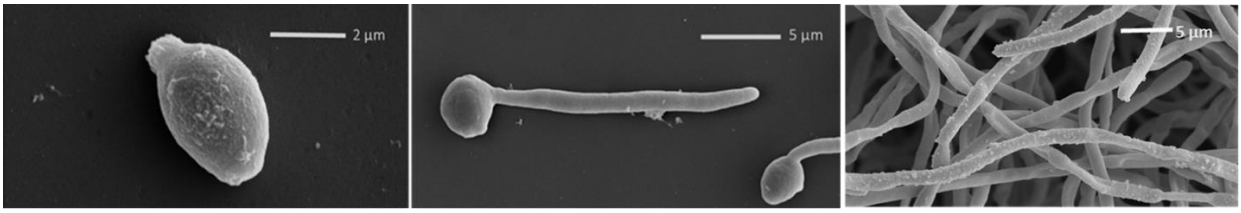

Figure 1.6 The main morphological forms of *C. albicans*. (i) Yeast morphological form. **(ii)** Germling morphological form **(iii)** Hyphae morphological form. Images were acquired at 10,000, 4,000 and 2500 x magnifications, respectively.

Highly structured biofilm formation could be the most important strategy, as it acts as a refuge, reservoir, scaffold and communication for microbes (Ramage *et al.*, 2009). In biofilm formation, the steps of biofilm formation include adherence of yeasts, their proliferation, hyphal cells on the top of yeasts basal layer, accumulation of extracellular matrix and dispersion of yeast cells away from the biofilm complex (Figure 1.4), (Finkel & Mitchell, 2011). These dispersed yeasts can then take part in the dissemination of candidiasis in different human body organs (Uppuluri *et al.*, 2010).

The first stage in biofilm formation is adhesion, which is principally driven by the ALS genes (Hoyer & Cota, 2016). The proteins they encode control cell surface hydrophobicity and the attachment to abiotic and biotic surfaces. The Als3 protein plays a multifunctional and potent role in *C. albicans* pathogenicity. Alongside Hwp1 and Als1, Als3 adhesins function together to form biofilms. It also has a role in *Candida* iron acquisition via binding to ferritin. *ALS3* gene is significantly upregulated in *C. albicans* hyphae and pseudohyphae, and is inhibited by an abundant nutrient environment. Moreover, Als3 could have a functional role in co-aggregation with bacteria (Liu & Filler, 2011).

Following adhesion, biofilm formation is driven by the Efg1 protein, a morphogenic regulator (Ramage *et al.*, 2002a). Other key proteins include Hwp1 (hyphal wall protein), Als3 (agglutinin-like sequence protein), and Sap4, Sap5, Sap6 (secreted aspartic proteases) (Mayer *et al.*, 2013). *C. albicans* can adapt to different pH, changing morphology depending on its environment - pH >7 encourages the *Candida* to grow with hyphal predominance, while pH <6 induce yeast form growth

(Mayer *et al.*, 2013). Related to this hyphal transition is another important *C. albicans* weapon recently discovered by Moyes *et al.* (2016). This hyphally regulated peptide toxin is called candidalysin, which has been shown to impact cell membranes of epithelial cells, damaging the permeability equilibrium. This represents a critical determinant in breaching the epithelial tissue barrier role. Regulation of these processes is in part driven by central regulators, that include *BCR1* and associated transcription factors, as well as adaptive protective mechanisms. Mayer *et al.* (2013) demonstrated that the heat shock protein (*HSP90*) gene has a role in dispersion of yeasts from the mature biofilm, increased biofilm antifungal resistance, and is co-regulated with a network of genes to drive biofilm formation. Moreover, *HSP90* has a role in regulation of candidal filamentation (Lu *et al.*, 2011), a pivotal aspect of *C. albicans* biofilms development. Many of these attributes are driven by the microenvironment that they exist within, and nutrient availability.

In nutrient starvation, *C. albicans* modulates the extracellular acidity by alkalinizing its surrounding to trigger an autoinducing hyphal form transformation (Vylkova *et al.*, 2011). This transformation could be associated with up taking of surrounding amines containing molecules instead of glucose, though this may be a strain dependant attribute. It has recently been reported that the metabolism of the amino acids, such as aspartate, arginine, proline and glutamate, has been upregulated in *C. albicans* strains capable of high biofilm formation, whereas sucrose, starch and purine metabolism have been upregulated in low biofilm formers (Rajendran *et al.*, 2016b). The absence of glucose as a main source of carbon may press the *Candida* to use an alternative source, which could increase their virulence. Lactate grown *C. albicans* has a more rigid cell wall than the glucose grown one, enabling it to deal more effectively with osmotic shock (Ene *et al.*, 2015). Moreover, lactate grown *C. albicans* is less detectable by the innate immune cells (Ene *et al.*, 2013). Overall, this shows that *Candida* has a genome that is well adapted to survive and persist within the oral cavity.

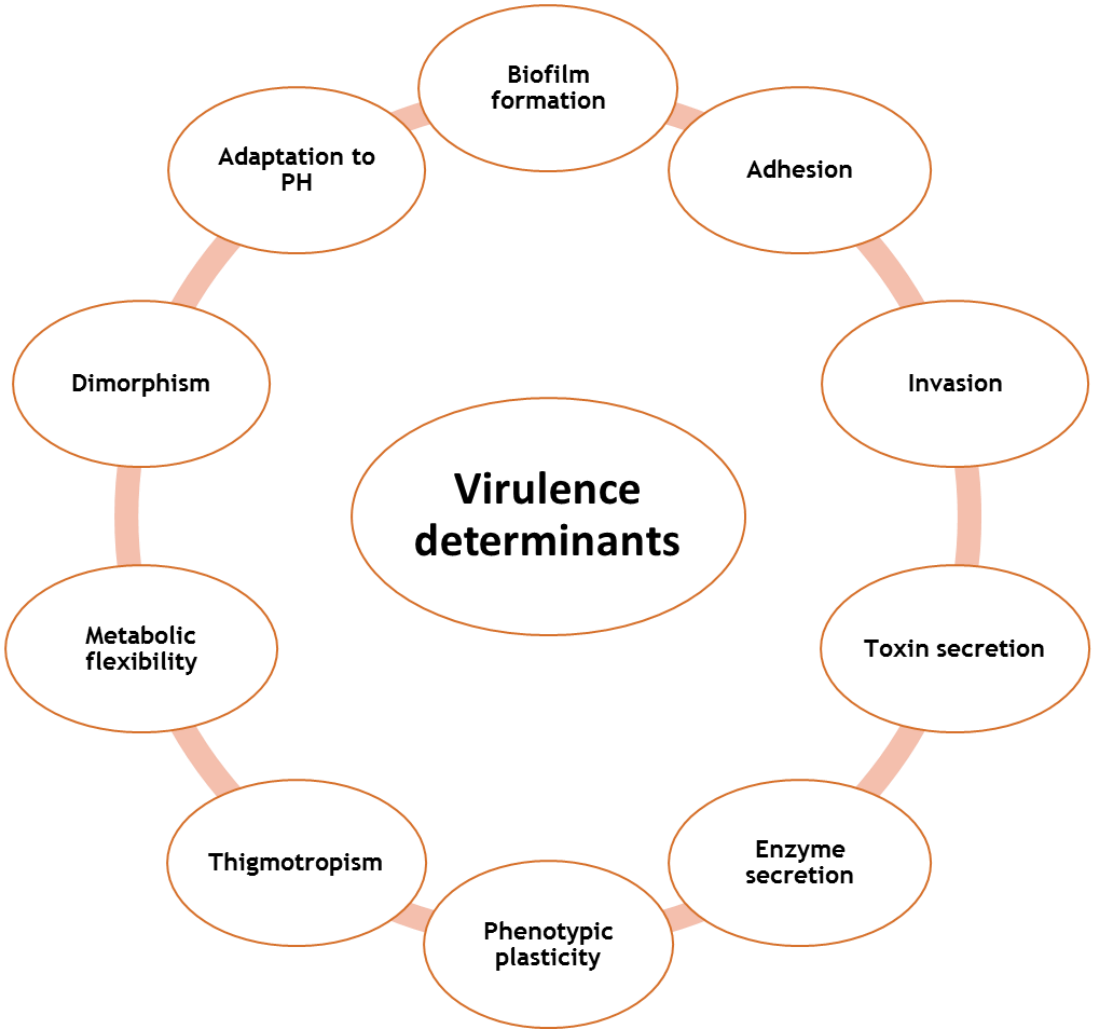

Figure 1.7 Virulence factors of *C. albicans:* A schematic plot.

1.7 Treatment strategies of DS

A diligent sanitizing regimen, regular dentist visits, and overnight denture removal are mandatory to reduce the incidence of DS. Nevertheless, development of anti-biofilm strategies to reduce the microbial adhesion could be a significant measure to ameliorate denture hygiene and reduce DS (Gendreau & Loewy, 2011). However, commitment to strict sanitizing measures and appropriate cleaning without compromising the denture surface can be considered as a difficult target to attain within denture wearers (Jagger & Harrison, 1995). In a clinical study, denture hygiene indices statistically deteriorated after long-term follow up in comparison to last recall check-up. The patient sample of this study were elderly people who were able to clean their dentures themselves, having passed manual dexterity and cognitive capacity tests. This indicates the importance of the need for periodical professional intervention with such a group, which gives an indication that the status will be worse with people who need extensive care and unable to perform denture hygiene care themselves.

Webb *et al*. (1998b) reviewed DS treatment modalities, which included antimicrobial, antiseptic and disinfectant measures. They concluded that there was not a comprehensively effective treatment *per se,* because of the multifactorial etiological nature of DS, and asserted on patient's compliance playing an imperative role in DS management. Therefore, DS management require multiple mutually supportive factors. Ramage (2015) reviewed the denture cleansing chemical and mechanical procedures and their influence on oral microbial response and associated denture stomatitis, clarifying some of their drawbacks. In the same report, general guidelines for denture management from a microbiological angle were presented, which could be summarised by the following: reducing denture plaque using daily non-abrasive brushing and denture cleansers, annual professional cleaning, and the potential beneficial role of denture adhesives in increasing denture stability.

A recent systematic review by Yarborough *et al*. (2016) for the DS treatment measures, reported the importance of antifungal treatment to the immunocompromised individual, while focusing on prosthesis disinfection as an effective strategy for healthy individuals. Multiple strategies were clinically

followed to reduce the pathogenic established biofilms. Furthermore, the recurrence of DS remains as a problem although its prevalence is unknown.

1.7.1 Physical strategies

Microwave irradiation could be considered as a practical strategy in DS management. Neppelenbroek *et al.* (2008) confirmed the significant impact of microwave disinfection in treatment of DS in contrast to miconazole, besides the recurrence in microwaved denture group was reduced. Microwave disinfection was investigated in another study, where rinsing with nystatin and microwave disinfection (MWD) were evaluated to treat DS. Three denture microwaving treatment (650w-3min) weekly was as effective as 4 times daily nystatin. Recurrence was reported after 3 months with approximately half of the participants in every treatment modality (Silva *et al.*, 2012). Three sequential exposures of 60 sec had effectively disinfected maxillary dentures and reduced the oral candidiasis of the denture wearers, although it could affect the dimensional stability of the denture and it is an inappropriate disinfecting technique to metal containing dentures (Banting & Hill, 2001). Furthermore, several reports proved the detrimental effects of microwave denture disinfection on the physico-mechanical properties (Seo *et al.*, 2007;Hamouda & Ahmed, 2010;Vasconcelos *et al.*, 2013).

Photodynamic therapy has emerged in multiple aspects of dentistry (Konopka & Goslinski, 2007). As a helpful weapon to fight oral candidiasis, photosensitizer erythrosine illustrated excellent impact on planktonic cells, though was less effective against biofilms (Costa *et al.*, 2011). Another report confirmed the efficacy of photodynamic approach in reducing the virulence traits of *Candida* species isolated from DS individuals (Pereira *et al.*, 2015). In a clinical report, harnessing of photodynamic therapy (photosensitizer: hematoporphyrin derivative) to manage DS *in vivo* showed a positive response and it required several sequential sessions, although recurrence was observed (Mima *et al.*, 2011). Further analysis has shown that *C. albicans* was less susceptible than *Escherichia coli* and *Staphylococcus aureus* (Demidova & Hamblin, 2005).

Other strategies include ultrasonics, where *in vitro* and *in vivo* evidence suggests positive benefits from ultrasonic cleaning of acrylic denture base to removing *C.*

albicans (>80%) (Kawasaki *et al.*, 2014). Nishi *et al.* (2014) showed that distilled water ultrasonic cleaning of dentures was not as effective as performing in the presence of denture cleanser. Use of ultrasonic or cleanser alone led to significant surviving of microorganisms in comparison to combining cleaning method.

1.7.2 Chemical strategies

It was demonstrated that patient responsibility for accurate denture cleanliness is more difficult to do than it appears. This was established by clinical and microbiological evidence, as treatment of DS without antifungals represented an unrealistic management option. Using systemic itraconazole for 2 weeks to manage DS, a significant myco-clinical enhancement was reported, while 50% of subjects have recurred with DS after 6 months (Cross *et al.*, 2000). Martin-Mazuelos *et al.* (1997) showed that fluconazole and itraconazole antifungals had improved the clinical status of DS, though fluconazole resistance was problematic.

Making new dentures as an alternative way to DS management has not show clinical or mycological success in comparison to combined antifungal-antiseptic methods that use systemic fluconazole and local application of chlorhexidine to the fitting surface of dentures (Kulak *et al.*, 1994). However, a meta-analysis of randomized clinical trials indicated the significant possibility of using alternative ways of managing DS rather than antifungal synthetic drugs. The alternative methods were enumerated as disinfectants, mouthwashes, photodynamic approaches, natural antimicrobials and microwave irradiation (Emami *et al.*, 2014). In a clinical study related to DS, the study participants were instructed to rinse their mouths with chlorhexidine gluconate 0.12% and to soak their dentures overnight in it for 24 days. The results showed a significant mycological and clinical improvement, but once treatment stopped, the disease recurred from both microbiological and clinical diagnostic angles (Kamalakshi *et al.*, 1992). However, evidence of significant dental staining drawback were reported with using chlorhexidine (Van Strydonck *et al.*, 2013).

To better understand how clinical management can be improved, we can draw on laboratory investigations. Ramage *et al.* (2011) reported a significant efficacy of some of the over-the-counter mouthwashes in comparison to some antifungal drugs against developed biofilms. Caspofungin (an echinocandin drug) was as

effective to biofilm as to planktonic *C. albicans*, but it is limited by its intravenous delivery method. Additionally, they showed that Corsodyl mouthwash (0.2% chlorhexidine and 7% ethanol) exhibited anti-biofilm activity to *Candida*. Caveats to these approaches can be that mouthwashes can exhibit undesirable side effects, such as taste problems and staining (Hong *et al.*, 2009;Barao *et al.*, 2015;Sadat Sajadi *et al.*, 2015). There are also links from oral mouthwashes to head and neck cancer, which still remain controversial and need further investigation (Conway, 2009;La Vecchia, 2009;Gandini *et al.*, 2012;Ahrens *et al.*, 2014).

In an *in vitro* study, 10 different disinfection procedures were used for denture soft liner cleaning. It was found that only sodium hypochlorite (1%), microwave irradiation at 850W (6min), and effervescent cleansing were effective (Buergers *et al.*, 2008). Denture cleansers are a good, but not perfect strategy, for anti-candidal biofilm approaches. Therefore, an adjunctive mechanical removal of the residual biofilm to prevent denture recolonization seems imperative (Jose *et al.*, 2010). In an *in vitro* study, Ramage et al (2012) have reported the anti-biofilm efficacy of two different sequential denture cleaning regimens. Logically, a 4 day sequential continuous denture cleanser chemical approach was more effective than a 4 day intermittent chemical/mechanical one. SEM of the day 2 showed regrowth of DS clinically isolated *C. albicans* and this was attributed to retention of *Candida* in denture material cracks or crevices and production of extracellular protecting matrix. This could necessitate the need for enhanced chemical and ultrasonic approaches to manage denture biofilms (Ramage *et al.*, 2012c). In another study, concerns were raised in respect to the influence of denture cleanser on multi-species biofilm, where the counts of *C. albicans* increased 10 times in comparison to *Streptococcus mutans* after 7 days sequential daily 3 min immersion in alkaline peroxide denture cleanser (Lucena-Ferreira *et al.*, 2014). Moreover, the alkaline peroxide effervescent denture cleansers were investigated from the physical and mechanical point of view, and negative effects were reported (Peracini *et al.*, 2010). From a disinfection point of view, 0.5% sodium hypochlorite has been suggested by Skupien *et al.* (2013) in their systematic review as an efficacious anticandidal disinfectant for denture liners (denture liners are used to reline the fitting surface of the denture). Furthermore,

disinfection of dentures with sodium hypochlorite soaking has promised a clinical anti-DS effect and reduced number of *Candida* CFU, but metal containing removable appliances represent a hurdle in respect to this method (Webb *et al.*, 2005).

Given the less toxic effect of phytomedicines, phytotherapeutics such as *propolis*, *Punica granatum*, *Melaleuca alternifolia* and *Vitis vinifera* increasingly become potential rival to conventional antifungals in treatment of DS, although further clinical studies are needed to confirm a standard preparation (Casaroto & Lara, 2010). *Zataria multiflora* 0.1% gel and miconazole 2% gel were investigated for their efficacy on DS subjects. The results were positive and comparable, while relapse was observed after 4 weeks of completing the treatment, they have suggested that dentures are the source of reinfection (Amanlou *et al.*, 2006). Likewise, *Artemisia sieberi* herbal mouthwash (1%) was equivalent to nystatin in treatment of DS (Sefidgar *et al.*, 2010).

Propolis extracts (ethanolic and non-ethanolic) with antifungal and anti-inflammatory capacities have also been suggested for DS treatment (Santos *et al.*, 2005;Santos *et al.*, 2008). The anti-planktonic and anti-biofilm effect of several types of natural polyphenolic compounds against *C. albicans* were investigated by Shahzad *et al.* (2014). They suggested that affinity of the oral surfaces to adsorb the polyphenols and their potential saliva bioavailability may potentiate their oral hygiene impact. It was concluded that curcumin and pyrogallol showed promise against the planktonic and biofilm form cells. Other studies have investigated the aqueous extract of garlic, *Melaleuca alternifolia* (tea tree oil) and *Punica granatum* efficacy in treating DS. These studies indicated significant treating action (Vasconcelos *et al.*, 2003;Catalan *et al.*, 2008;Bakhshi *et al.*, 2012).

1.8 The polyphenol curcumin

1.8.1 The structure and biomedical significance of curcumin

Curcumin is a polyphenolic natural compound. It was isolated from turmeric (Asian spice) two centuries ago. The turmeric was extracted from the rhizomes of *Curcuma longa* plant in the thirteen century (Esatbeyoglu *et al.*, 2012). The curcumin (Diferuloylmethane) chemical formula is $C_{21}H_{20}O_6$, International Union of Pure and Applied Chemistry (IUPAC) nomenclature is (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, and its molecular weight is 368.38. Curcumin consists of two phenolic O-methoxy containing groups that are connected by a seven carbon conjugated (alternating single and multiple bonds) linker consisting of an α,β-unsaturated β-diketone moiety. The B-diketone functionality contribute in the intramolecular H^+ atom transfer which is called tautomerism, therefore, curcumin can be existed in two different forms enol form and keto form (Figure 1.8) (Priyadarsini, 2009;Priyadarsini, 2014). Curcumin's physical-chemical properties can be summarised as solid, orange to yellow in colour, non-flammable, hydrophobic, poorly soluble at physiological pH, behaves as a proton donor at acidic pH where the keto form is dominated and as an electron donor at basic pH, where the enol form is dominated, melting temperature 170-175^{o}C, light sensitive, odourless, soluble in ethanol, dimethyl sulfoxide and acetic acid (Esatbeyoglu *et al.*, 2012).

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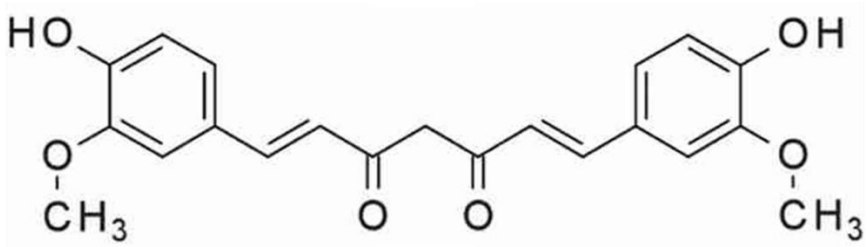

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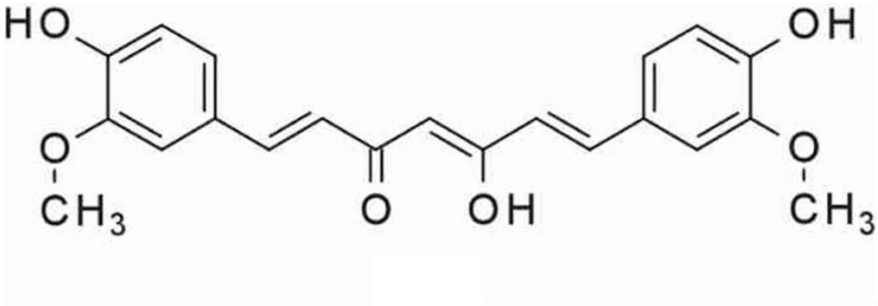

Figure 1.8 Chemical structure of curcumin. (i) Keto form, **(ii)** Enol form.

Several clinical trials showed the non-toxicity, good toleration, and beneficial preventive and therapeutic effects of curcumin on a plethora of human diseases such as cancer, arthritis, cardiovascular diseases, Crohn's disease, allergies, ulcerative colitis, neurodegenerative diseases, diabetes, obesity, cerebral edema and psoriasis among others (Gupta *et al.*, 2013;Shehzad *et al.*, 2013;Yang *et al.*, 2013;Furst & Zundorf, 2014;Panahi *et al.*, 2017).

Anti-oxidative and anti-inflammatory effects, besides inducing molecular targets such as transcription factors and enzymes could be considered as potential mechanisms of its multiple biological effects, although a comprehensive understanding is elusive so far (Mahmood *et al.*, 2015). China, United States, India, Korea, Thailand, Pakistan, South Africa, Nepal and Japan have used curcumin as a supplement, although it is not permitted yet as a treatment (Gupta *et al.*, 2012). The daily dietary consumption of curcumin was 2.7-14.8 mg in Korea while it has quadrupled in south Asia (Kwon, 2014).

In spite of its advantages, a disadvantage was frequently reported which is the low bioavailability, while significant scientific research has been conducted to reduce this drawback using nanonisation and conjugation strategies (Prasad *et al.*, 2014). Remarkably, it was demonstrated by Esatbeyoglu *et al.* (2015) that degradation of curcumin at 180ºC could not minimise its biological beneficial effects because of the significant biological effects of its degradation products, which are ferulic acid, 4-vinyl guaiacol and vanillin. In an *in vivo* study, it was demonstrated that the efficacy of curcumin mouth rinse was higher than chlorhexidine mouth wash in treating oral mucositis in cancer patients treated with radio-chemotherapy (Patil *et al.*, 2015). The safety of curcumin has been reported by several studies (Lao *et al.*, 2006;Chandran & Goel, 2012;Shehzad *et al.*, 2013;Sahebkar & Henrotin, 2016), all these studies share the support for the good-toleration of curcumin and absence of adverse effects even with daily high doses that may reach up to 6-8 g/day.

1.8.2 The antimicrobial effect and mechanism of curcumin

Epidemiological studies indicate the importance of the phenolic compounds in the prevention of many diseases that are common to western communities. Furthermore, the microbes could transfer the phenolic compounds into more efficacious antimicrobials (Cueva *et al.*, 2010). The activity of polyphenols against the microbial causative agents of dental caries and periodontal disease was recently reviewed (Slobodnikova *et al.*, 2016), the review authors thought the capability of these compounds might lead to their being introduced into practice soon. In an *in vitro* study by Rai *et al.* (2008), it was shown that the bacterial cytokinesis of *Escherichia coli* and *Bacillus subtilis* can be inhibited by curcumin through interfering with polymerization of FtsZ protein assembly that forms the Z ring constricting septum, which is very critical structure during the cytokinesis process.

Periodontitis is a degenerative chronic disease of the gingiva and is highly correlated with bacteria such as *Porphyromonas gingivalis* (Slots, 1986). In an *in vitro* study conducted by Shahzad *et al.* (2015), curcumin was reported as the most potent investigated polyphenol among 48 different types. Curcumin showed an effective inhibition of planktonic growth of *Porphyromonas gingivalis* and

Aggregatibacter actinomycetemcomitans bacteria, which are commonly associated with periodontitis. Besides, that study reported the high affinity of curcumin to hydroxyl apatite crystal coated surfaces, which might add an advantage for fighting periodontitis because of the dental biofilm-developing nature of the periodontitis-associate bacteria.

Given that the dental caries-bacteria relationship is notorious, perhaps biofilm formation and the associated cariogenicity of *Streptococcus mutans* could be reduced by curcumin (Hu *et al.*, 2013). Therefore, curcumin molecule could be a significant potential candidate in caries prevention. Hu *et al.* (2013) showed the inhibition of Sortase A enzyme that catalyses the tethering of some of adherence proteins to the cell wall by curcumin, accompanied by reduced biofilm biomass. This aforementioned report has confirmed a former *in vitro* study by Song *et al.* (2012), where a significant effect of curcumin was observed on the vitality and adherence of *Streptococcus mutans* to collagen and fibronectin human teeth surfaces. It is noteworthy to mention that the anti-adherence capacity of curcumin to the bacteria that is inferred by the Sortase A inhibiting capacity of curcumin was firstly reported by Park *et al.* (2005) in *Staphylococcus aureus*. Furthermore, a significant synergism between antibiotics and curcumin was concluded by an *in vitro* study that investigated the possibility of reducing the minimum inhibitory concentration (MIC) of several antibiotics on methicillin-resistant *Staphylococcus aureus* (MRSA) (Mun *et al.*, 2013). Moreover, Brown (2015) prioritised some of the compounds that could be repurposed for development of antibiotic resistance breaker strategies, where the curcumin was selected and preferred for its significant bi-functionality (direct antibiotic and anti-inflammatory effects) on both classes of bacteria (Gram positive and Gram negative). The mechanism of curcumin antibacterial efficacy could be explained by affecting bacterial cell membrane permeability, which was investigated by propidium iodide uptake and calcein leakage fluorescence assays (Tyagi *et al.*, 2015), where all the investigated Gram-positive and negative bacteria (*Enterococcus faecalis, Staphylococcus aureus, Pseudomonas aeruginosa Escherichia coli)* showed a significant concentration and time dependent influence by curcumin.

The prophylaxis of oral candidiasis could benefit from the potential antifungal activities of some natural bioactive compounds, which could be added to the oral health products (Rautemaa & Ramage, 2011). The antifungal capacity of nutraceuticals and natural phenolic extracts have been reviewed by Rodrigues *et al.* (2016), these types of extracts were considered as one of the growing novel armamentarium and attractive prototypes in fighting *Candida* infection. The antifungal capacity of crude *Curcuma longa* extracted oil was firstly reported against dermatophytes mold type species (Apisariyakul *et al.*, 1995). Another report showed the anti-*Candida* (*albicans* and *krusei*) effect of curcumin, especially when conjugated with glycine/alanine molecules that increased its bioavailability (Mishra *et al.*, 2005).

The proliferation of *C. albicans* can be inhibited by curcumin. This was confirmed by a study that relied on flow cytometry technology to determine the level of S-G2-M during the cell cycle, where a decline was demonstrated in these important events, but that study used high curcumin concentrations (>1000 µg/ml) (Jianhua & Hai, 2009). In an *in vitro* study conducted by Martins *et al.* (2009), results suggested the potential clinical antifungal efficacy of curcumin and a significant antifungal role of curcumin against *Candida* species (except *glabrata*) and *Paracoccidioides brasiliensis*, while *Aspergillus* different species were invulnerable to curcumin. Furthermore, Martins *et al.* (2009) microscopically measured the number of adhered *Candida* cells to the buccal epithelial cells using different species of *Candida*, and they noticed a remarkable reduction with curcumin treated *Candida* cells.

In a study that interrogated the mechanism of the curcumin anticandidal capacity against laboratory and clinical strains, Neelofar *et al.* (2011) reported the effective capacity of curcumin on both types of strains and identified three essential mechanisms which are: reduction of ATPase activity, inhibition of proteinase production and interference with ergosterol biosynthesis, although all the ergosterol targeted antifungals display upregulation of the proteinase activity. Notably, Sharma *et al.* (2010b) have shown the anti-hyphal capacity of curcumin to *C. albicans* and they related that to the up-regulation of global suppressor thymidine uptake 1 gene (TUP1) in response to curcumin, where the tup1 null mutant did not show an influence by curcumin. The antifungal mechanism of

curcumin was interrogated, where the level of the reactive oxygen species (ROS) in all the tested species of *Candida* was raised after curcumin treatment. Additionally, the oxidative stress associated genes were upregulated. Consequently, they correlated these perturbing cellular events to early apoptosis and cell growth inhibition of *Candida* species. Moreover, another antifungal mechanism was reported by the same research group, where *C. albicans* ergosterol depletion was associated with cell membrane disturbances, besides accumulation of the ergosterol biogenisis precursors and the generation of ROS (Sharma *et al.*, 2012). The curcumin function in compromising fungal cell membrane equilibrium was endorsed by a different study (Lee & Lee, 2014). In that study, depolarisation of the membrane potential of the curcumin treated *C. albicans* was shown which was potentially attributed to electrostatic and hydrophobic interactions. Further, the potassium efflux of the tested cells was increased after curcumin treatment, which compromising the cellular homeostasis and leading to cell death. Another research group attributed the anti-*Candida* mechanism of curcumin to the increased intracellular acidity that resulted from the inhibition of the H^+ efflux capability of the *Candida* cell suggesting the disturbance of functional role of the cell membrane in controlling such activities (Khan *et al.*, 2012). Nevertheless, the notion of breaching the cell wall integrity mechanism has emerged using candidacidal concentration. This notion was reported by Kumar *et al.* (2014), who demonstrated the down-regulation of many cell wall integrity genes and disruption of calcinurin and mitogen activated protein (MAP) kinase pathways that are associated with cell wall homeostasis. Recently, the dual anti-fungal and anti-carcinogenic mechanism of curcumin was demonstrated (Chen *et al.*, 2016b). Figure 1.9 summarises the aforementioned literature suggested antifungal mechanisms.

The invasive type of fungal infection in cancer patients provokes a logical interest in molecules of bi-pharmacological effects such as curcumin, specifically with its high dose safety, besides its capacity to inhibit the efflux pump strategies that are frequently used by cancer cells (Chearwae *et al.*, 2006). The light sensitivity feature of curcumin was used to fight the *Candida* species in a planktonic and biofilm growth modes, displaying the possibility of curcumin use in photodynamic therapy (Dovigo *et al.*, 2011). All the investigated light excited-curcumin treated

Candida species were significantly inhibited, where the colony forming units of the plated planktonic cells and the metabolic activity and the biofilm biomass were reduced of the biofilms. The former report was endorsed by murine *in vivo* study (Dovigo *et al.*, 2013). However, a study conducted by Carmello *et al*. (2015) indicated a temporary genotoxic effect after light excitement (without curcumin), while a significant slowing in DNA repair was noticed with curcumin-light excited *Candida albicans*. In a supporting context of the aforementioned potential capacity of curcumin to augment existing conventional treatments, significant synergism between nystatin, amphotericin B, ketoconazole, miconazole, fluconazole, itraconazole, voriconazole antifungal drugs was reported by Sharma *et al*. (2010a). Similarly, fluconazole and curcumin reduced the resistance of clinical isolates of *Candida albicans* to fluconazole (Garcia-Gomes *et al.*, 2012).

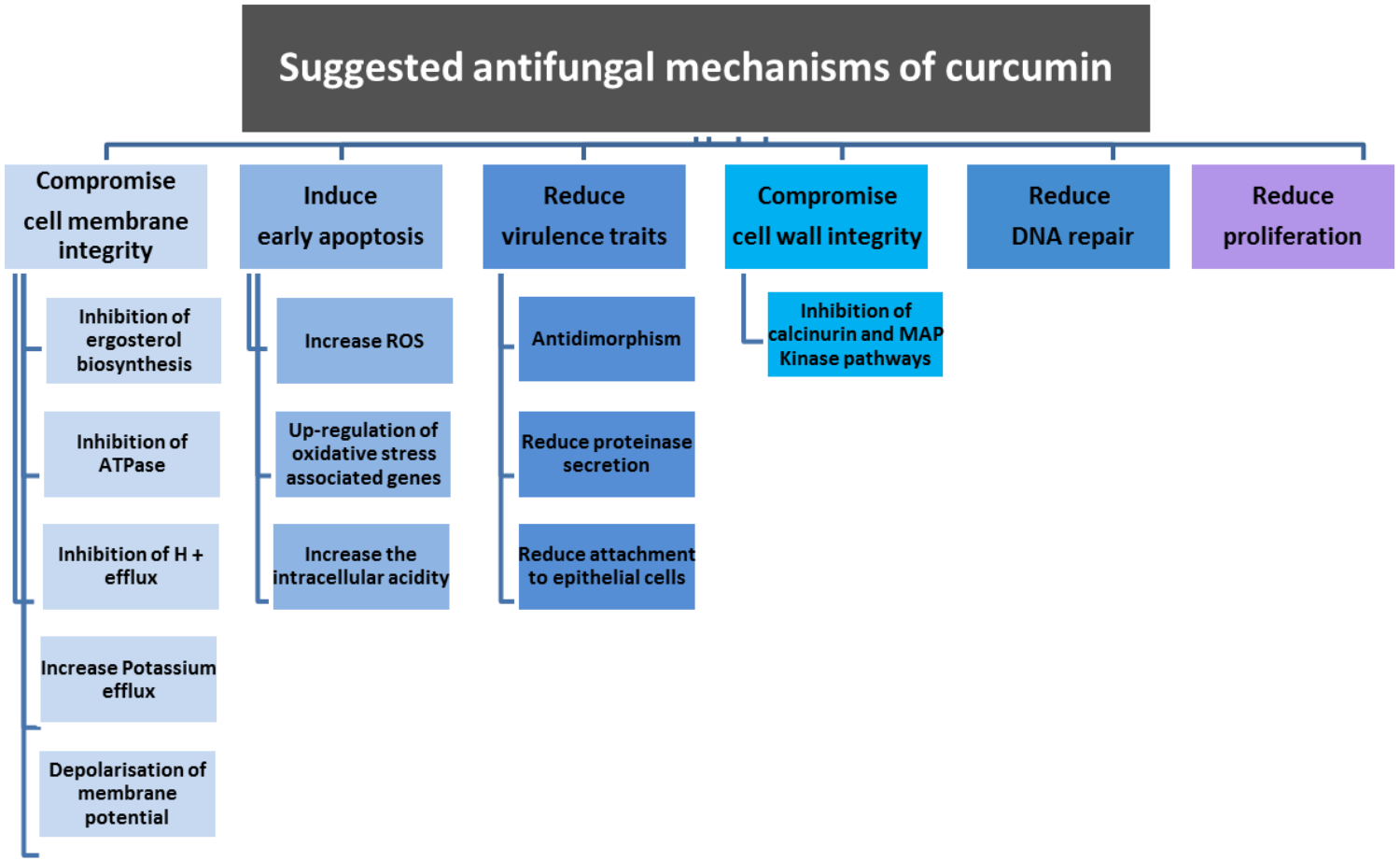

Figure 1.9 The multiple targeted antifungal mechanism of curcumin.

1.9 Preventive antimicrobial strategies in denture material

1.9.1 Chemical modification

Polymethyl methacrylate (PMMA) is the most utilized material for denture fabrication, while soft liners of different chemical constituents are used to reline fitting surface of the denture (Sakaguchi & Powers, 2012). There were many experimental endeavours to modify the bulk chemical structure of polymer denture base materials.

Microbial biofilm represents a major challenge for biomaterials. Incorporation of antimicrobials and a sustained release approach have been used to manage candidal biofilms. Examples are described below. A thin film polymer coating with high or low porosity that was incorporated with chlorhexidine (CHX), nystatin or amphotericin B was developed by Redding *et al.* (2009) to inhibit *C. albicans* biofilm. The incorporation of chlorhexidine in the coated samples proved its superiority in biofilm inhibition. Silver nano-particles were added to PMMA to investigate the adherence and biofilm formation of *C. albicans*, though no significant effect was observed in either case (Wady *et al.*, 2012). This was attributed to the non-release of Ag ion. However, another study reported a significant reducing capacity of modified denture base material with silver nano-particles although poor colour stability was reported as well (Nam *et al.*, 2012), which was recently confirmed by different group (Li *et al.*, 2016). In spite of the increased surface roughness, combined release of small amounts of F^-, $BO_3^{3-}$and Na^+ ions could be sufficient to reduce the adhesion of *C. albicans* to denture resin material incorporated with glass ionomer filler (Tsutsumi *et al.*, 2016). Salim *et al.* (2013) investigated the anti-*Candida* biofilm efficacy of impregnation of CHX or fluconazole to a poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) over 28 days. The CHX anti-biofilm efficacy was apparent, where the biofilm biomass and the metabolic activity reduction were 75% and 84% respectively, while in contrast the fluconazole impregnation was 8.8% and 12.6%, respectively. The researchers suggested that the CHX-impregnated PEM/THFM polymeric drug carrier system could potentially be used as a denture liner for the treatment of DS. Another study investigated the incorporation of chlorhexidine

different salts in prevention of denture *Candida* biofilm. The chlorhexidine diacetate seemed effective due to its release to the adjacent areas in comparison with chlorhexidine hydrochloride which had not anti-*Candida* impact (Bertolini *et al.*, 2014). Pusateri *et al.* (2009) evaluated the anti-candidal effect of pre-coated PMMA with 0.12% CHX gluconate, histatin-5 (His-5) or human ß-defensin-3 (HBD-3). The results showed that His-5 and CHX had an effect on biofilms, while HBD-3 had not shown any significant effect on biofilms. Moreover, immersion coating of nanoparticles of CHX digluconate on dental silicones reduced proliferation of *Candida* by releasing effective concentrations (Garner *et al.*, 2015). An anti-*C. albicans* effect of zinc oxide nano-particles incorporation as a composite or coating to acrylic denture base material was reported by Cierech *et al.* (2016). A successful rechargeable anti-*Candida* denture material was developed by (Cao *et al.*, 2010;Villar *et al.*, 2013), through copolymerization of methacrylic acid and diurethane dimethacrylate. The click-on click-off (recharging) miconazole controlled release was investigated for elongated period up to 2 months that could be washed off by EDTA to quench the antifungal drug. Likewise, a rechargeable denture material using poly(N-vinyl-2-pyrrolidinone)-grafting polymerisation was demonstrated by Sun *et al.* (2013). They reported a significant antifungal efficacy of controlled sustained release that persisted for weeks with chlorhexidine and months with miconazole. However, the antimicrobial release approach can be criticised for the initial burst stage that being followed by release of low ineffective concentrations (Huang & Brazel, 2001), besides the potential of short period of activity and discontinuity of release (Kitagawa *et al.*, 2014).

Contact killing surfaces could be more reliable and promising than antimicrobial releasing surfaces (van de Lagemaat *et al.*, 2017). Chemicals such as quaternary ammonium and 2-tert butylaminoethyl methacrylate (TBAEMA) have been used for that purpose. Incorporation of 2% quaternary ammonium polymer compounds in heat cured acrylic resin have showed successful bacteriostatic and fungistatic effects but it was stated that development of resistance ,toxicity and long-term stability need further studies (Pesci-Bardon *et al.*, 2006).

However, dangerous side effects were reported on quaternary ammonium, such as negative effect on the reproductive system (Melin *et al.*, 2014) and stimulation of respiratory and skin allergic reactions (Lipinska & Walusiak, 2014). Another

study investigated the impregnation of 10 and 25% of TBAEMA, which is a poly-cationic substance with pendant amino groups acting as a contact biocide, into PMMA (Marra *et al.*, 2012). An antibacterial efficacy against *Streptococcus mutans* and *Staphylococcus aureus* biofilms was shown. There was no significant effect on *C. albicans* biofilm formation, which was attributed to the rigid physical properties of the *Candida* cell wall that prevented the biocidal compound from replacing the $Mg^{+2}$and Ca^{+2} of the cell membrane.

Studies using modification with anionic charged surfaces were also of interest. The effect of copolymerization of methacrylic acid with PMMA on *C. albicans* adherence was investigated by Park *et al.* (2003). The resulted negatively charged surface showed candidal adherence inversely related to increasing the ratio of methacrylic acid, which support the advocates of the effect of electrostatic interaction on initial adherence of *C. albicans*. Similar results have been revealed with modification of the acrylic resin surface charge and characteristics (Park *et al.*, 2008). Further, the absence of phosphate anions in PMMA may reduce the adsorption of cationic salivary antimicrobials that could minimize and control microbial colonization. That notion was investigated in an *in vitro* study, where PMMA has been modified by incorporation of phosphate group using mixtures of methyl methacrylate and methallyl phosphate as monomers. The anti-adherence efficacy of the new polymer to *C. albicans* and their capability to adsorb histatin 5 was improved in comparison to the control group (Raj & Dentino, 2011).

A major problem with adding antimicrobials to biomaterials is the negative impact on the mechanical properties, where incorporation of antimicrobial polymers to denture base acrylic resins may compromise its flexural strength as in using of tert-butylaminoethyl methacrylate (Paleari *et al.*, 2011) or incorporating of apatite-coated TiO_2 photocatalyst (Shibata *et al.*, 2007). Also, phosphate containing monomer with an anti-*Candida* effect affected some of the PMMA physico-mechanical properties (Dhir *et al.*, 2007). Moreover, addition of methacryloyloxyundecyl pyridinium bromide (MUPB) as an antimicrobial monomer, showed a higher fibroblasts cytotoxicity than methyl methacrylate (MMA) by 20 times (Regis *et al.*, 2012).

These drawbacks could indicate that modification of the surface only would be advantageous. Bazaka *et al*. (2011) reported that coating and surface modification strategies for removable biomaterials seem to be more realistic in maintaining the polymer bulk properties, and might enhance the surface bio-functionality and biocompatibility. Graft polymerization of 2-methacryloyloxyethyl phosphoryl choline (MPC) on acrylic denture surfaces create a covalently bonded durable brushing-induced friction surface and anti-adhesive to *Streptococcus mutans* biofilm. The grafting method had confirmed its superiority to the coating with dipping method. Anti-adhesiveness was attributed to the lower liability for protein adsorption where proteins produce a scaffold for bacterial adhesion (Takahashi *et al.*, 2014).

Lazarin *et al*. (2013) investigated the effect of different experimental photo polymerized coatings containing hydrophilic monomers (2 hydroxyethyl methacrylate, 3-hydroxypropyl methacrylate and 2-trimethylammonium ethyl methacrylate chloride or zwitterionic monomer (sulfobetaine methacrylate) on acrylic denture material. They noticed that the increased hydrophilicity led to a decrease of the *Candida* adhesion and concluded that the physicochemical properties of the substrate surface dictate the formation and composition of the acquired pellicle that influences the biofilm development. In contrast to the former study, coating the PMMA with Silane-SiO2 nanocomposite film reduced the adherence of *C. albicans* though it decreased the surface energy and reduced its hydrophilicity (Yodmongkol *et al.*, 2014). Hydrophobic-fumed silica treated with hexamethyl disilane was added to silicone soft liner, the results revealed lower penetration percentage of *Candida* with increasing the filler percentage (Rodger *et al.*, 2010).

Improved bio-functionality of biomaterials may increase the opportunity to the adsorption and bonding of bioactive molecules (Dang *et al.*, 2014). Surface modification of PMMA beads by copolymerisation with methyl methacrylate acid had positively influenced on the adsorption and desorption of histatin 5 anti-microbial peptide. It was concluded that controlled release of this from PMMA had reduced the adherence of *C. albicans* while, adsorbed histatin had not shown any candidacidal effect (Edgerton *et al.*, 1995). Following on from this, Yoshinari *et al*. (2006) used oxygen cold plasma to improve the adsorption of histatin 5 to

PMMA. The results showed a six times increase in the rate of adsorption of histatin 5 with plasma treatment that reduced colonisation by *Candida*. Recently, the acrylic resin denture base was bio-functionalised by plasma initiated grafting polymerisation of several coating polymers (Wen *et al.*, 2016). The adsorption of miconazole antifungal drug was significantly higher to the bio-functionalised PMMA and the biofilm formation was inhibited.

1.9.2 Physical modification

To the best of my knowledge there is no research article that has investigated the modification of the PMMA denture material surface physically using micro/nano fabrication approaches. Therefore, given the relative novelty of the field as a whole, a general view will be presented on the biomedical impact of micro/nano patterned surfaces on mammalian cells and the microorganisms, then a brief paragraph about the fabrication.

Nano fabricated surfaces are well-studied in the electronic engineering and optical fields, while, in the biology and medicine fields they remain to some extent novel and poorly explored, although initial research of cell-substrate interaction displayed a response to the nano-structured materials (Anselme *et al.*, 2010). The surface energy of the micro-structured patterns is affected with Cassie-Baxter model (air trapped between the microstructures) or Wenzel model (water trapped between the microstructures). This could be more pronounced with regularly nanostructured patterns presenting potential ambivalent phenomena (the nano-patterned surface could increase the hydrophilicity or the hydrophobicity of the surface) (Martines *et al.*, 2005).

The Interactions between micro/nanotopographies and human cells have been investigated in several studies. In orthopaedic/dental implant associated surgeries, fibroblast and osteoblast cell adhesion, differentiation and survival are critical (Chatakun *et al.*, 2014). Downing *et al.* (2013) demonstrated the possibility of the biophysical microtopographies represented by highly ordered microgrooves replacing the biochemical molecules in reprogramming the somatic cells to pluripotent cells. This is a critical issue in cell-engineering and regenerative medicine. Furthermore, nano-patterned topographies of 100-200 nm diameter

nanodots effectively modulated the adherence capacity of the fibroblast cells and induced apoptosis like events (Pan *et al.*, 2009).

Biggs *et al.* (2007) reported a significant interaction between the nano-patterened substrate and the cells. They showed a reduced adherence capacity of the primary human osteoblast cells onto highly ordered arrays of nanopit topographies in comparison to the planar control substrates, but on the contrary, the controlled slightly disordered nano-patterned arrays displayed higher adhesion capacities. They suggested a compromisation of the protein adsorption in addition to the surface free energy changes as potential mechanisms that led to the reduction in the focal adhesion (cell-extra cellular matrix adhesion). While, completely random nanotopographies have led to an increase in the focal adhesion, which was evidenced by modulation of the genetic expression (Biggs *et al.*, 2009). Support for regenerative therapies by controlling stem cell differentiation and cell fate through nanotopographical systems was reviewed by Dalby *et al.* (2014). They expected an evolution of the next generation of regenerative medicine biomaterials using the knowledge base about nanotopographies-cells interaction.

Furthermore, micro/nanotopographies have been investigated for their potential interaction with microorganisms, although it is notable to mention the presence of only one (according to the best of our knowledge) research article that investigated the interaction between such surfaces and fungal species (Whitehead *et al.*, 2005). Whitehead *et al.* (2005) interrogated micro and sub-micro titanium engineered topographies to reveal the effect of different feature diameters on the retention of *C. albicans* in addition to *Staphylococcus aureus* and *Psuedomonas aeruginosa*. These titanium-engineered topographies did not show differences with *C. albicans*, in opposite to the other two investigated microorganisms due to the disparity of the sizes of theses microbes relative to the size of the features.

Generally, an increase in uncontrolled surface roughness could offer a shelter for the microorganism from cleaning shear forces and by maximising the surface area of attachment.

This was elucidated by Boyd & Verran (2002) as they demonstrated an increase of the adherence capacity of *Staphylococcus aureus* to unpolished and randomly abraded stainless steel surfaces. On the other hand, antifouling and self-cleaning

natural systems such as lotus leaf effect and shark skin that are characterised by characteristic hydrophobicity and distinctive topographies at micro/nano scale, are main drivers of interest in the interaction of micro-nano topographical features with microorganisms in an endeavour to control the bacterial adhesion and subsequent biofilm formation (Gu & Ren, 2014).

Inspiration by nature has frequently affected surface design engineering, therefore self-cleaning windows, submarine low drag and wall-climbing robots could be considered mimicking designs of the hydrophobic lotus leaf, dolphin shape and gecko feet respectively. This is termed biological mimicry, which describes the copying of natural designs to solve different challenges (Bixler & Bhushan, 2012). The finer Sharklet AF™ microtopographies that are inspired by the shark skin design reduced the adhesion of *Ulva linza* zoospores by 85%, and the authors suggested the wettability thermodynamics as a causative mechanism (Carman *et al.*, 2006). The same microtopography (Sharklet AF™) was further investigated by Chung *et al.* (2007) using polydimethyl siloxane elastomer substrate. The authors reported a significant reduction in 7-21 day biofilm of *Staphylococcus aureus* in comparison to the smooth surface control. In another report, bacterial adhesion could be modulated by ordered micro/nano topographical surfaces, where *Pseudomonas fluorescens* early stage biofilms were hindered by using submicron ordered grooves (Diaz *et al.*, 2009).

The traditional concept of the smoother surfaces can allow less adhered bacteria could be endangered by Mitik-Dineva *et al.* (2009) study. They have reported the increased adherence of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* on nano-scale smoothened surfaces in comparison to the control surfaces. This was attributed to the increased extra cellular polysaccharides formation by the bacteria. Ploux *et al.* (2009) showed the opposite behaviour of human osteoblast progenitor cells to the *Escherichia coli* bacterial cells in adhesion to ridge-groove microtopography, where more osteoblasts and fewer bacterial cells adhered at the initial stage of incubation. However, a review discussed the adhesion of the human cells and bacteria to micro-nanotopographies, the deformity of the cytoskeleton of the bacteria in response to micro-nanostructures is less likely to occur in comparison to the eukaryotic human cells, which could hinder the targeting of mechano-stimulus

mechanism driven by the contact with micro-nanostructures. Besides, the surface chemical composition play a critical role in bacterial adhesion that may mask the topography effect (Anselme *et al.*, 2010). The notion of possible interaction between the surface chemistry and topography was reported, when a mathematical model was developed by Decuzzi & Ferrari (2010) that demonstrated the possibility of tailoring the surface energy and the topography of the biomedical surfaces to reduce bacterial adhesion to the nanotopographical features. A significant coherent relation between surface energy, which might be dictated by the surface chemical composition, and the nanotopography was reported.

A silane based-fluorinated nanosilica superhydrophobic coating displayed antibioadhesive properties against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Privett et al., 2011). In contrast, a different study showed a massive role for the topographical shape, size and feature in reduction of bacterial adhesion and subsequent biofilm formation while the surface hydrophobicity/hydrophilicity did not demonstrate a significant impact (Perera-Costa *et al.*, 2014).

Different bacteria show different behaviour towards nanostructured surfaces that may complicate the evolution of universal conclusion (Hsu *et al.*, 2013). In an endeavour to mimic the superhydrophobic, antifouling, lotus leaf surface, a titanium surface was fabricated in dual scale micro-nano projecting features (Fadeeva *et al.*, 2011). In that report, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the testing models. The first type of bacteria was capable of attaching to the surface while the other type was not. It was concluded that different bacteria behave differently on superhydrophobic surfaces.

Cicada wings could represent a different nanofeatured antibacterial mechanism. As shown by Ivanova *et al.* (2012), *Pseudomonas aeruginosa* could be killed within minutes through a purely mechanical approach, where the cell membrane is ruptured due to the high pressure applied by the tapered nano pillars of the wings that cause stretching of the cell membrane although, the hydrophobic self-cleaning nature of these wings did not prevent the bacteria from adhering. Furthermore, the cicada wings model was a logical basis for investigating the

bactericidal effect of TiO_2 nanowire via another study. Diu *et al.* (2014) showed a significant killing of *Pseudomonas aeruginosa*, while *Staphylococcus aureus* was not affected. The bactericidal mechanical-stretching mechanism of cicada wing was confirmed, using a biophysical model and 3 different bacterial species (Pogodin *et al.*, 2013). This inherent physical antibacterial strategy has been recently endorsed with dragonfly wings that presented a specific nanofeatured surface (Bhadra *et al.*, 2015;Mainwaring *et al.*, 2016). However, in a different study, a variety of submicron topographical arranged features showed higher affinity to bacterial adherence, where the attached bacteria used different mechanisms to maximise their attachment (Hsu *et al.*, 2013).

The generation of micro/nano master patterns and the subsequent replication methods will now briefly described. The fabrication technology of master patterns encompasses a range of methodologies which are photolithography, electron beam lithography, colloidal lithography, polymer demixing among others (Kearns *et al.*, 2011). The first and second methods present the possibility of elaboration of highly ordered topographies. Photolithography technology depends on developing a film of photosensitive layer (photoresist) on the substrate and applying a light that transfer through a specific patterned mask to limit the illumination to the targeted areas, and then an etchant is applied for removal of the photoresist and preparation of the pattern in a synchronising manner. The electron beam lithography method does not use the mask role in developing the patterns but use the electron beam to directly develop the pattern on the photoresist. It elaborates more accurate patterns but takes longer time and more expensive (Kearns *et al.*, 2011).

Moreover, the 'soft lithography' which is an alternative and complement to the photo and electron beam lithography can be used as successful way to generate the replicas in inexpensive and more convenient approach that facilitates the introduction of the micro/nano topographies to the biomedical research field. Soft lithography uses non-photolithography techniques (to avoid the optical diffraction disadvantage and the subsequent low resolution) such as self-assembly monolayer, microcontact printing, microtransfer molding, replica molding and embossing for replicating features using mostly elastomeric 'soft' stamps or molds (Xia & Whitesides, 1998).

The replication of polymer patterns via imprinting of the master patterns can be divided into 2 main non-photolithographic approaches: Hot embossing technique, which is simple and requires heating the polymer slightly above its glass transition temperature then applying a given force. The second approach is the injection moulding technique that is characterised by its capacity to produce larger number of replicates in a shorter time but it requires a more complicated equipment (Xia & Whitesides, 1998;Gadegaard *et al.*, 2006b).

1.10 Hypothesis & Aims

The preceding introduction has demonstrated the importance of microorganisms on the denture surface. The hypothesis of this study is that *C. albicans* plays a critically important role in DS, and that new methods of controlling these microorganisms will lead to improved clinical management. Therefore, this PhD thesis aimed to undertake the following objectives:

- To screen and assess the ability of *Candida albicans* to form biofilms on clinically relevant surfaces

- To evaluate whether the polyphenol curcumin could be used to manage candidal adhesion

- To evaluate whether physical manipulation of denture relevant substrates could be used to manage candidal adhesion.

2 *Candida albicans* biofilms formation on denture materials

2.1 Introduction

Tooth loss and edentulousness is related to age, and with changing population demographics this problem is expected to continue to rise. Generally, microorganisms living within biofilms are more resistant to antimicrobial agents, therefore prevention of biofilm formation is a priority (Ramage *et al.*, 2004;Ramage *et al.*, 2009). Within the biofilm, *C. albicans* co-aggregates with a range of bacterial species forming the basis of denture plaque, which is often associated with other oral diseases, including root caries, gingivitis, halitosis and angular cheilitis (O'Donnell *et al.*, 2015a). In severe cases, more serious systemic effects are reported, such as aspiration pneumonia, which is a potentially life threatening lung infection (van der Maarel-Wierink *et al.*, 2013;Iinuma *et al.*, 2014;O'Donnell *et al.*, 2016). In light of the local and systemic impact of denture wearing, investigating the ability of microbes to colonize denture surfaces, in particular *C. albicans*, is a worthwhile aim for oral health research.

DS is a common inflammatory condition of the oral mucosa that sits adjacent and in direct contact with the fitting surface of a removable prosthesis. The prevalence of DS ranges between 15 to 70% in these patients (Gendreau & Loewy, 2011), and is associated with multiple factors, including night-time denture wearing, poor oral hygiene, denture surface imperfections, denture stability, medications, salivary flow, smoking, poor general health, and diseases causing compromised immunity (Jeganathan & Lin, 1992;Rodriguez-Archilla *et al.*, 1996;Soysa & Ellepola, 2005;Gendreau & Loewy, 2011). In addition to these contributory factors, microbial infection with *Candida* species and other oral bacterial pathogens is also key to the development of DS (Gendreau & Loewy, 2011;Salerno *et al.*, 2011). The microorganism most pertinent to DS is *C. albicans*, a commensal opportunistic yeast capable of colonizing different surfaces. Existing as a biofilm on denture related surfaces *C. albicans* displays a number of adaptive resistance mechanisms, making it difficult to manage solely through chemotherapeutic intervention (Rautemaa & Ramage, 2011). Its capability of expressing a range of key virulence determinants during this sessile lifestyle, including morphogenetic switching (yeast to hyphae transition), the release of hydrolytic enzymes, and the production of protective extracellular matrix

components, makes this an important denture related pathogen (O'Donnell *et al.*, 2015b). Moreover, clinical isolate heterogeneity means that we cannot conclude that all strains will be equally pathogenic (Tumbarello *et al.*, 2007;Sherry *et al.*, 2014;Rajendran *et al.*, 2016a;Kean *et al.*, 2018), thus complicating our ability to fully understand the basis of candidal induced DS.

Poly (methyl methacrylate) (PMMA) is one of the most commonly used denture materials because of the appropriate mechanical, physical and economical properties. Furthermore, the ease of manipulation, despite its varied topography that promotes the development of microbial communities (Raj & Dentino, 2013;Jackson *et al.*, 2014;Susewind *et al.*, 2015). The rigid mechanical feature of PMMA may cause discomfort to the underlying mucosa. Therefore, resilient soft liners such as silicone- and acrylic-based liners are becoming increasingly popular. These materials showed enhancement in the patient masticatory function and minimised appearance of pressure sore spots inside the oral cavity and the associated pain (Kimoto *et al.*, 2007;Palla *et al.*, 2015). However, these liners cannot resist microbial colonisation and could deteriorate denture hygiene even further (Nevzatoglu *et al.*, 2007;Susewind *et al.*, 2015). Attachment of *Candida* can be influenced by a number of factors, though the physicochemical nature of the denture substrate is important (Pereira-cenci *et al.*, 2008a), and given the variety of denture related substrates available then it is not surprising we observe so much clinical variation. Whether this variation is biologically driven or material driven remains to be determined.

2.2 Aims

It is hypothesised that candidal denture biofilm formation is multifactorial, influenced both by the surface and microorganisms, though which is the more dominant is unclear. Therefore, the overall aim of this chapter was to:

1. Investigate the quantitative impact of denture candidal burden in DS patients.

2. To assess the biofilm-forming capacity of *Candida albicans* clinical denture isolates.

3. To investigate the impact of different denture surface materials on the adherence capacity and biofilm formation of different clinically isolated *C. albicans*.

Part of the data presented in this chapter has been published in the Journal of Medical Microbiology:

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Findings from this chapter have been presented at the following academic meetings:

1. Postgraduate research prize seminar held in Glasgow Dental School on 27th of April, 2016.

2. Oral microbiology and immunology group (OMIG) postgraduate research prize symposium held in the College of Clinical Dentistry at the University of Sheffield on 8th of Feb, 2017.

2.3 Materials and Methods

2.3.1 Molecular quantification of *Candida* from dentures

Denture plaque samples were collected from denture wearers attending Glasgow Dental Hospital (NHS Greater Glasgow & Clyde). Removable prosthodontic appliances from 129 patients were submitted to this study, as previously described (O'Donnell *et al.*, 2015b). Written informed consents were obtained from all participants, with ethical approval granted by the West of Scotland Research Ethics Service (12/WS/0121). Every patient was examined by a dentist to determine the presence of DS. Patients with treatment history of antibacterial or antifungal drug subscription within 6 weeks before sampling were excluded. Eighty-one patients were categorized as healthy (no signs of oral inflammation in denture-foundation areas) and 48 patients were categorized as diseased. Newton's classification was utilised to subdivide the DS group (Newton, 1962). DS group was sub grouped into Grade 1 inflammation (localised hyperemia [n=24]), Grade 2 (diffuse erythematous hyperemia[n=14]) and Grade 3 (diffuse granular erythematous with papillary hyperplasia[n=10]). As a general instruction, every patient was asked to not clean his/her dentures on the day of sample collecting.

After removal of the denture from the patient's oral cavity, it was placed in a plastic bag (Fisher Scientific, Loughborough, UK) filled with 50 ml 1x phosphate-buffered saline (PBS [Sigma–Aldrich, Poole, UK]). Each appliance was sonicated in a water bath at 35 kHz (Ultrawave, Cardiff, UK) for 5 min to collect the adherent biofilm (Coco *et al.*, 2008a). The denture sonicate of each sample was centrifuged at 3700 × g for 10 min and the pellet resuspended in 2ml of RNAse free water® (Qiagen, Manchester, UK), which was then frozen at -80°C to be used in further experiments. Dr Lindsay O'Donnell undertook this component of the study during her PhD thesis (O'Donnell, 2016).

2.3.1.1 DNA extraction of clinical samples

DNA purification to investigate *Candida* denture burden was performed using the QIAGEN QIAamp mini kit for isolation of genomic DNA (QIAGEN, Germany). Frozen samples were thawed from the -80°C freezer; vortexed for 30 s and 1 ml pipetted

into a 1.5 ml Eppendorf tube. These were then centrifuged at 10000 rpm for 10 min. The supernatant was carefully pipetted, leaving the pellet intact. To disintegrate proteins and for enzymatic lysis purposes, 20 µl of proteinase K and 180 µl of ATL Buffer (a tissue lysis buffer) was added to the pellet and vortexed. The mixture was incubated in a heat block (Pierce Reacti-Therm® Heating Module) at 55°C for 20 min. Further steps were undertaken to guarantee cell lysis, using a bead beater [BeadBug™ microtube homogenizer (Sigma-Aldrich, Gillingham, UK)]. For this, 0.25 ml of 0.5mm diameter sterile glass beads (Thistle Scientific, Glasgow, UK) were transferred to the lysis suspension within defined O-ring screwcap microtubes and 3 × 30 s disruptions were performed at maximum velocity (400 rpm), incubating for 10 s on ice between disruptions. Supernatants were pipetted and transferred into a fresh Eppendorf tube after centrifuging at 7000 rpm for 10 min.

Thereafter, QIAmp mini DNA extraction kit steps were followed as per the manufacturers protocol, beginning with the addition of 200 µl of Buffer AL (lysis buffer) to the sample, pulse vortexing for 15 s, and incubating in the heat block at 70°C for 10 min. Next, 200 µl of 100% ethanol was added and pulse vortexed for 15 s. This was then pipetted in to a QIAmp mini spin column and centrifuged at 8000 rpm for 1 min. The mini spin column was removed from its collecting tube, the collecting tube containing the filtrate was disposed, and the mini spin column was placed in a new collecting tube. Next, 500 µl of Buffer AW1 was added and centrifuged at 8000 rpm for 1 min. Again, the mini spin column was removed from its collecting tube and placed in a new collecting tube after disposal of the collecting tube containing the filtrate. Five hundred microlitres of buffer AW2 was then added and centrifuged at 14000 rpm for 4 min. The mini spin column was transferred into a new eppendorf. Finally, 50 µL of AE buffer was added and incubated for 1 min at room temperature, and centrifuged at 8000 rpm for 2 min. Next, the mini spin column was discarded and the filtrate containing purified DNA was stored in -20 ºC after assessment of the purified DNA quality and quantity by a NanoDrop®-1000 spectrophotometer (ThermoScientific, Loughborough, UK) prior to moving to the subsequent step with quantitative polymerase chain reaction molecular method (qPCR).

2.3.1.2 Culture conditions

Laboratory stocks of the reference strain *C. albicans* SC5314 were maintained weekly on Sabouraud dextrose agar [Oxoid, Cambridge, UK] plates at 4°C. This strain was selected because it is genetically well characterised as it is the origin of strains conventionally used for molecular analysis (Fonzi & Irwin, 1993;Jones et al., 2004;Thewes et al., 2008). Yeast peptone dextrose (YPD) broth medium [1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose (Oxoid, UK)] was used for propagation of the yeast cells. This was performed by inoculating a loopful of *C. albicans* colonies in 10ml YPD in 50ml polyethylene tubes, and incubating overnight in a shaking incubator with 150 rpm at 30ºC. The overnight propagated cells were centrifuged for 5 min at 3000 rpm and washed twice with 1 x PBS, then resuspended in 10 ml of 1 x PBS.

2.3.1.3 Quantitative standard curve

Preparation of a standard curve is an essential step for qPCR analysis from clinical specimens. To standardise *C. albicans* SC5314 cells to 1×10^8 per/mL, the yeast cells were counted using a Neubauer haemocytometer. Ten ml of 1×10^8 CFU/ml was distributed to a series of eppendorf tubes with 1 ml in each; DNA was extracted as described in the previous section (2.3.1.1) for each one of the eppendorfs. After Purified DNA was assessed for quantity and quality using a NanoDrop-1000 spectrophotometer. It was ten-fold serially diluted with AE buffer to 10^{-5}, then stored at -20ºC.

2.3.1.4 Quantification of *Candida* CFEs in denture sonicates

A real time qPCR (RT-qPCR) approach was used to assess the dentures' candidal colony forming equivalents (CFEs) burden. Following extraction of DNA, 129 denture sonicate DNA samples were distributed into MicroAmp™ Optical 96-Well

Reaction PCR Plates (Applied biosystems, USA). Briefly, a master mixture was prepared according to the number of the targeted reactions by mixing 1µL of forward (F) and reverse (R) universal *Candida* species primers (10 µM), 7 µL Nuclease free water (Hyclone®, Utah, USA) and 10 µL Fast SYBR® Green PCR Master Mix (Applied Biosystems, USA). These quantities were equivalent to only

one targeted reaction. The 18S-rDNA primer sequences were as following: F - CTCGTAGTTGAACCTTGGGC and R - GGCCTGCTTTGAACACTCTA, as previously described (Rajendran et al., 2015). Nineteen microlitres of this mixture was dispensed in every target well of a 96-well reaction PCR plate. Then, 1 µL of the purified DNA per sample was added in duplicate. Negative control wells (no DNA template) were set as duplicate. A duplicate of reaction wells was allocated for each dilution of the ten-fold serially diluted standard curve-purified DNA, which was isolated as annotated in 2.3.1.3. The 96- well PCR plate containing aliquots was briefly centrifuged at 1000 rpm for 1 min to remove any trapped bubbles.

A thermal cycler [StepOnePlus™ Real-Time PCR System unit (Applied biosystems, USA)] was used to monitor the amplification of the 18S rDNA candida specific gene through monitoring the fluorescence of the DNA-intercalating fluorescent dye in all the targeted reactions. This was performed under the following thermo-cycling conditions: 50°C for 2 min, 95°C for 2 min followed by amplification procedure of 40 cycles of 95°C for 3s (denaturation) and 60°C for 30s (annealing/extension). The analysis of the cycle threshold (Ct) values was carried out using StepOne software v2.3. This was performed by generating a specific standard curve equation for each PCR plate and analysing the data (Ct values) accordingly. The number of the colony forming equivalents (CFEs) per denture was doubled to be meaningful for the whole sample, because the CFEs was quantified for only 1 ml of the finally centrifuged denture sonicate sample (2 ml), as previously annotated in 2.3.1 and 2.3.1.1.

2.3.2 Standardised *C. albicans* biofilm assessment of denture isolates

The independent detection and identification of *Candida* species of the denture sonicates was performed by Dr Lindsay O'Donnell during her PhD thesis (O'Donnell, 2016). Briefly, 100 µl of the denture sonicate was plated on Colorex Candida agar (E & O labs, Bonnybridge, UK) and incubated at 30ºC for 72 h. Species identification was performed as per the manufacturers protocol, with green colonies representing *C. albicans*, pink colonies representing *C. glabrata* and the blue colonies representing *C. tropicalis*. Isolates were then were further sub-cultured on sabouraud dextrose agar for purification purposes. These denture

isolates were stored in Microbank vials (Pro-Lab Diagnostics) at -80ºC, until further use.

2.3.2.1 Biofilm formation from denture clinical isolates

To perform this biofilm assessment experiment, 31 and 37 *C. albicans* clinical isolates were selected that were isolated from denture sonicates of healthy and DS patients, respectively. The *C. albicans* cells were propagated in YPD, washed twice with PBS, centrifuged and resuspended in PBS as annotated in section (2.3.1.2). To develop *C. albicans* mature biofilm, the cell count was standardised in RPMI-1640 medium to 1×10^6 CFU/ml using Neubauer haemocytometer as previously established for 96-well microtitre plates (Ramage et al., 2001;Pierce et al., 2008). Briefly, 100 µl of the standardised suspension were inoculated in the targeted wells of the 96-well flat-bottomed polystyrene plates (Corning Incorporated, NY, USA) and the microtitre plates were covered with their original lids and sealed with parafilm. Each isolate was tested in triplicate, and negative controls containing media without *C. albicans* were included. Microtitre plates were then statically incubated at 37ºC for 24 h. Following growth, media were carefully aspirated and the resultant biofilms were washed twice with PBS to remove non-adherent cells from the biofilm.

2.3.2.2 Crystal violet assay for biofilm biomass assessment

The biomass of these biofilms was assessed using the crystal violet (CV) assay, as described previously by our group (Mowat *et al.*, 2007;Sherry *et al.*, 2014). Briefly, following washing, biofilms were left overnight to dry at room temperature. One hundred microlitre of 0.05% weight/volume CV solution was then added to each dried biofilm and incubated at room temperature for 10 min to allow absorption of the dye. Following incubation, the CV solution was discarded, and biofilms were carefully washed with running tap water to remove any unbound dye, and then 100 µl of 100% ethanol was applied to destain each biofilm. The contents of every well were mixed thoroughly by pipetting, before 75 µl was transferred to a new 96-well plate for spectrophotometric measurement. Biomass was then spectrophotometrically measured by reading absorbance at 570 nm in a microtitre plate reader (FluoStar Omega, BMG Labtech). All absorbance values were blank

corrected based upon two negative controls wells, where no biofilms were formed. Grouping of the isolates were based on their level of biomass distribution. All isolates observed below the first quartile (Q1, OD570=0.382) were classified as low biofilm former (LBF), and isolates with a biomass higher than the third quartile (Q3, OD570=1.192) were considered high biofilm former (HBF), as described previously (Sherry *et al.*, 2014;Rajendran *et al.*, 2016a).

2.3.3 Investigation of biofilm formation upon denture materials

Development of candidal biofilms is usually assessed using RPMI-1640 medium and a polystyrene plate as a substrate. The artificial nature of testing environments of these biofilms can be modulated to be more clinically relevant using different denture materials as substrates and artificial saliva (AS) as a medium. Two clinical isolates [n=1 LBF (GSK106) and n=1 HBF (GSK090)] and one laboratory strain (SC5314) were selected for observation and measurement of the initial (4 h) and mature (24 h) biofilm formation upon different denture materials.

2.3.3.1 Fabrication of denture material samples

Discs of denture prosthetic materials (12 mm ±0.2 diameter × 1 mm thickness) were fabricated using the lost wax technique (McCord, 2009). Discs of sheet wax were invested in die stone molds in prosthodontic metal flasks. After boiling out the wax, different denture materials were applied and processed using a compression molding technique using a dental hydraulic press (Kavo, Germany). Briefly, a die stone investing medium (Super yellow, John & Winter co. Ltd, UK), was poured in the lower half of the metal flask, then the wax discs were placed and left to set. Afterwards, a layer of a separating medium (DENTSPLY, USA) was applied. Then, the upper half of the flask was fitted and the second layer of die stone investing medium was poured. After reaching the final setting, the wax was eliminated with 5 min boiling water immersion and washing. Three denture materials were utilized to prepare the samples: heat cured polymethylmethacrylate PMMA (C&J De-luxe denture base polymer, Surrey, UK), Molloplast B® (GmbH & Co. KG, Germany) heat cured silicone-based denture soft liner and Ufi Gel® SC (VOCO GmbH, Germany) cold cured acrylic based denture soft liner. The heat cured samples were fabricated in a hot water bath (QD, UK)

according to the manufacturer's instruction for each material. After completion of the specific curing regimen for each material, the discs were deflasked and finished by removing the border flashes with an acrylic bur.

In order to reflect the clinical situation where the fitting surface of the denture is not highly polished, no further finishing was carried out on the surface of discs. Each disc was visually examined and discs with visible internal or external imperfections were excluded. Discs were then immersed in distilled water for 7 days to remove any remnants of residual monomer. The discs were thoroughly disinfected by sonication in distilled water at 35KHz- for 15 min before immersion in 100% ethanol for one hour. Discs were then re-sonicated as above for a further 15 minutes before exposure to ultraviolet light (TripleRed®, NUAIRE cabinet, Plymouth, UK). Both sides of each disc side were exposed to 15 minutes UV light to complete the process (Ramage *et al.*, 2012c). The prepared discs were then stored in dry sterile tubes until use.

2.3.3.2 Preparation of artificial saliva

Artificial saliva (AS) was prepared to be used as an incubation medium as previously defined (Pratten *et al.*, 1998;Millhouse *et al.*, 2014), this is to relatively simulate the oral cavity environment. AS was prepared by mixing of porcine stomach mucins 2.5 g, sodium chloride 3.5 g, potassium chloride 0.2 g, calcium chloride dihydrate $CaCl_2.2H_2O$ 0.2 g (Sigma- Aldrich); and yeast extract 2.0 g, Lab Lemco powder 1.0 g, proteose peptone 5.0 g (Oxoid). Next, 1 litre of sterile distilled water was added to mix the ingredients thoroughly. The mixture was autoclaved at 121°C for 15 min. Following autoclaving, 1.25 mL of 40% sterile Urea v/v (Oxoid) was added to AS which was aliquoted into 50 mL volumes, kept out of the light and stored at 4°C for a maximum of two weeks. Prior to every use, it was vortexed to homogenise the precipitated contents.

2.3.3.3 Biofilm formation on different denture materials

The selected *C. albicans* isolates (GSK106 and GSK090) and the laboratory strain SC5314 were maintained, propagated and washed as explained in 2.3.1.2 and 2.3.1.3. Colony forming units (CFU) were standardized using a haemocytometer and light microscope to 1×10^7 CFU/ml. Discs of each denture material were distributed in triplicate for each *C. albicans* strain and denture material in polystyrene 24-wells plates (Corning Incorporated, NY, USA), with AS used as medium of incubation. One millilitre of the standardised AS-*C. albicans* suspension was dispensed in each well containing discs, then the plates were covered with their original lids and sealed with parafilm and incubated for 4 or 24 h at 37ºC in static condition. The experiment was repeated on 3 independent occasions.

2.3.3.4 Metabolic activity quantification assay

For *in vitro* candidal biofilm optimisation and characterisation purposes the XTT assay was used. Quantitative assessment of *C. albicans* adhesion and biofilm formation was assessed using an XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide]formazan salt-based metabolic reduction assay (Ramage *et al.*, 2001). Briefly, XTT (Sigma-Aldrich, Dorset, UK) was prepared as a solution of 0.25 g/L in distilled water and placed on a magnetic stirring platform for 30 min and wrapped in tin foil to ensure complete darkness. To ensure sterility, 0.22 µm filters (Cole-Parmer CA Syringe Filters, UK) were used to filter the solution, which was aliquoted and stored at -80ºC. Prior to use, XTT solution was thawed and menadione (Sigma-Aldrich, Dorset, UK), prepared in acetone to 10 mM, was added to a final concentration of 1 µM. After washing, developed biofilms with 1 ml of PBS, 1 mL of XTT/menadione mixed solution was aliquoted to all washed biofilms, including negative control wells. Blanks were included for background correction purposes. Plates were incubated in the dark for 3 h at 37°C. Then, 750 µl was pipetted and transferred to a new 24 wells plate. A colorimetric change in XTT reduction was measured at 492 nm in a microtitre plate reader (FluoStar Omega, BMG Labtech, Aylesbury, Buckinghamshire, UK).

2.3.3.5 Macroscopic visual inspection

The ability of *C. albicans* to form biofilms on denture materials was visually inspected by eye through staining with crystal violet in a way analogous to dental plaque disclosing agents (Wu *et al.*, 2013). Early (4h) and mature (24h) biofilms were developed at optimal densities of cells (10^7 CFU/ml) in AS at 37°C on all the three denture materials tested using three *C. albicans* strains, grown as described above in 2.3.3.3. Negative control (NC) discs (immersed in AS only) were also included. Following incubation, biofilms were washed with 1 ml of PBS and left to dry overnight. One ml of crystal violet stain 0.05% w/v was added to each well and incubated in room temperature for 10 min. Then, the discs were washed thoroughly by dipping five times in two Petri dishes each containing 40 ml of distilled water to eliminate unbound stain. Then, the discs were air-dried and photographic images were taken using a digital camera (Sony, Japan).

2.3.3.6 *In vitro* molecular quantification

For molecular analysis, *C. albicans* DNA from 4 and 24h biofilms was extracted. Biofilms were washed once with 1 ml of PBS gently to remove the non-adherent cells. Discs were picked with sterile tweezer and put in sterile bijous (Sterilin Ltd, Newport, UK) containing 1 ml of PBS. Bijous were sonicated in water bath (Ultrawave, Cardiff, UK) for 10 min to collect the adherent cells then the suspension was pipetted into Eppendorf ® microtubes, which were centrifuged at 10000 rpm for 10 min. The supernatant was pipetted carefully; leaving the pellet intact. Then, DNA extraction was performed as described in section 2.3.1.1.

Quantification of the adherent colony forming equivalents by Real Time-quantitative PCR assay was performed as described in 2.3.1.4.

2.3.4 Statistical analysis

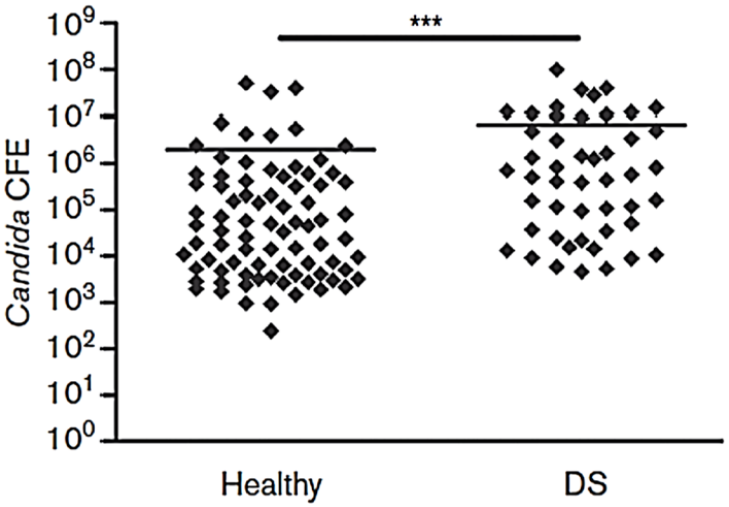
Data distribution, statistical analysis and graphics were performed using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA). All data were investigated for being normally distributed or not using D'Agostino-Pearson omnibus and Shapiro-Wilk tests. Level of significance with 0.05 was used as an accepted probability of 5% for incorrectly rejecting the null hypothesis. Transformation of the data, if necessary, and selection of the specific statistical test are described in the results sections of this chapter.

2.4 Results

2.4.1 Denture carriage of *Candida* in health and disease

To determine whether there was an association between denture carriage/colonisation of *Candida* spp. and DS, qPCR was employed to investigate levels of *Candida* in denture sonicates from 81 healthy and 48 diseased patients with DS. Data were not normally distributed and therefore $\log_{10}$ transformation was undertaken. Figure 2.1(i) demonstrates that *Candida* quantities were greater on dentures from DS patients compared to healthy denture wearers ($p<0.001$). Nevertheless, an observable overlap was shown between these two groups. Candidal carriage for healthy patients ranged from 2.45×10^2 to 5.03×10^7 CFEs and from 4.57×10^3 to 9.77×10^7 CFEs for DS patients, across the 129 dentures tested. When the DS patients were stratified based on Newton's grade of inflammation, no significant difference between *Candida* carriage of healthy denture wearers and patients classified with Newton's grade 1 was observed. However, significant differences were observed between healthy denture wearers and patients classified with Newton's grade 2 ($p<0.01$) and Newton's grade 3 ($p<0.01$) (Figure 2.1[ii]).

(i)



(ii)

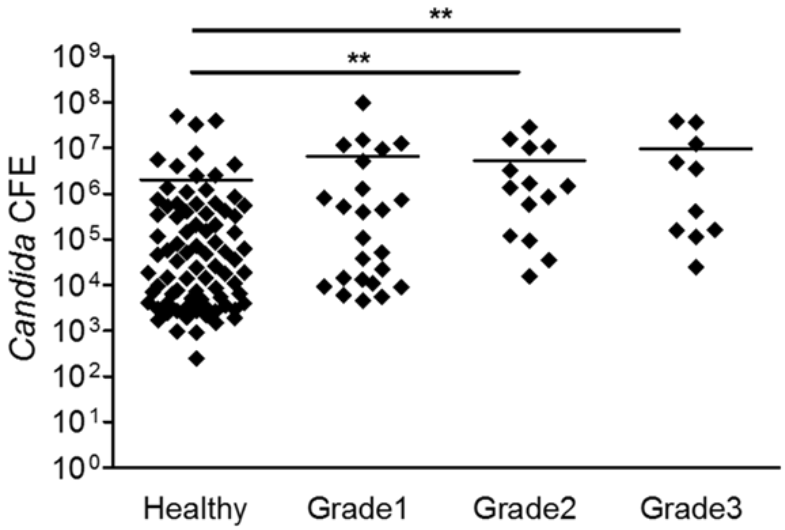


Figure 2.1: Candida carriage in dentures in respect to DS status. (i) *Candida* cells in colony forming equivalents (CFEs) in health and diseased groups (N=129). Higher number was observed in diseased group samples. An unpaired *t*-test was performed to compare the independent groups, and horizontal line refer to mean. **(ii)** *Candida* carriage in dentures in respect to the DS grades of inflammation. One-way ANOVA showed a high significant difference with *p* value of <0.0001. Bonferroni multiple comparison test analysed the statistical relation of healthy group samples with different sub groups of diseased group and between the different grades of inflammation themselves. Horizontal lines represent means.

2.4.2 Variability of denture isolates in biofilm formation

C. albicans clinical isolates have various capabilities to form biofilms. Evaluation of denture candidal biofilm forming heterogeneity with respect to DS was investigated through assessment of the capacity of the denture *C. albicans* isolates to form biofilms. These isolates were cultured from the dentures of 31 and 37 selected healthy and DS diseased study participants, respectively. CV assay was used to evaluate the biofilm-forming ability by measuring the biofilm biomass. Figure 2.2 shows the differential biofilm formation of these clinical isolates. The isolates were categorised into three classes: low, intermediate and high biofilm formers. Isolates with optical densities of the first quartile (Q1, OD_{570}=0.382) were deemed as low biofilm formers (LBF), isolates of the third quartile (Q3, OD_{570}=1.192) were deemed as high biofilm formers (HBF) and the in-between optical densities were considered as intermediate biofilm formers. The range of optical densities for healthy patients was from 0.09 to 2.81 and from 0.1 to 2.06 for DS patients, through the 68 isolates assessed. A high variability in the biomasses of healthy and DS patients was noticed, and a statistical significance was not observed between them (P>0.05). This confirmed the heterogeneous nature of *C. albicans* biofilm forming ability within both groups.

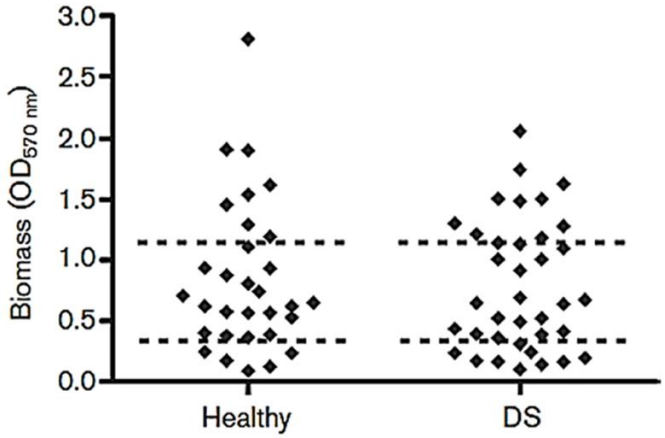


Figure 2.2: Quantification of biofilm biomass from denture isolates. *C. albicans* isolated from dentures of healthy (n=31) and DS (n=37) patients were grown as biofilms for 24 hr and their biofilm biomass assessed using the crystal violet assay. Unpaired *t*-test was used to analyse the data (p>0.05). The dotted lines represent demarcation of LBF and HBF isolates by the lower and upper quartiles, respectively.

2.4.3 Characterisation of *in-vitro C. albicans* biofilms

To observe the effect of variable conditions associated with the development of *C. albicans* biofilms, XTT and CV assays were used for quantifying the biofilm metabolism and biomass, respectively. Initially, early biofilms were developed on PMMA denture material and standard polystyrene plates using clinically relevant medium (AS) to determine any possible differences. Figure 2.3(i) showed that an inoculum of 1×10^7 CFU/ml resulted in a significantly greater metabolism of early AS-developed biofilms on PMMA denture material than 1×10^6 CFU/ml ($p<0.01$), 1×10^5 CFU/ml and 1×10^4 CFU/ml ($p<0.001$). Duplication of the experiment on the standard polystyrene 24 well culture plates displayed a similar trend, as shown in Figure 2.3(ii). Therefore, prospectively further optimisation and characterisation experiments were performed using polystyrene 24 well plates for feasibility purposes.

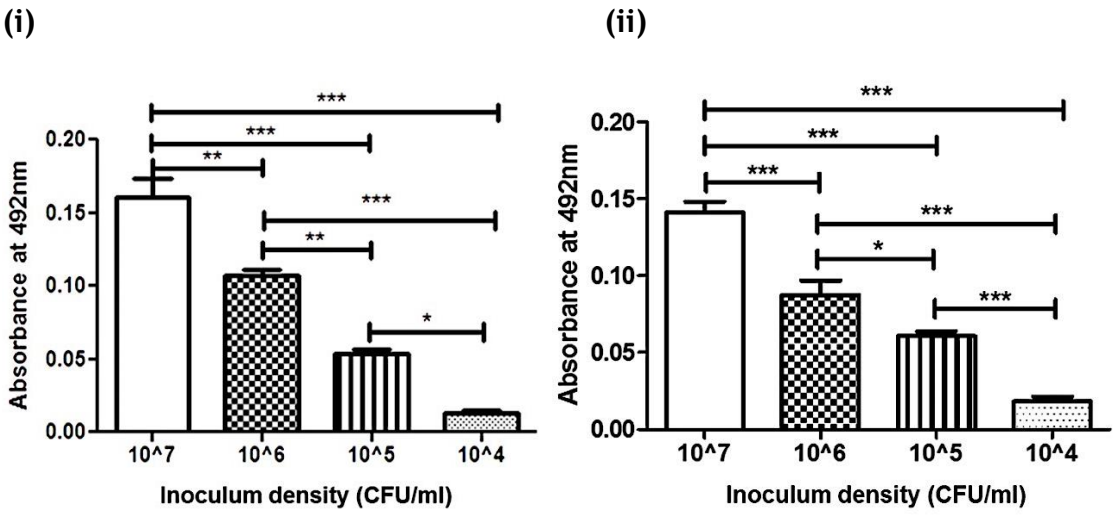


Figure 2.3: Optimisation of *C. albicans* early biofilm on PMMA and polystyrene. XTT metabolic assay was used to assess the biofilms tested, with the experiment repeated in 3 independent occasions in triplicate. **(i)** PMMA discs were colonised for 4 hr with different CFU/ml concentrations using AS medium. (ii) Replication of the same aforementioned experiment using polystyrene as substrate. One-way ANOVA test displayed P value < 0.0001. Bonferroni post hoc assay was used for further analysis. *= P< 0.05, **= P<0.01, ***= P< 0.001. Error bars represent standard error of mean.

2.4.3.1 The effect of the inoculum density on biofilm formation

For an initial screening purpose, first, several inoculum densities were investigated for any differences in mature biofilm biomass, which were developed on 24 well plates and using the standard RPMI-1640 medium. Data showed a significantly higher biomass for the inoculum density of 5×10^5 over 2.5×10^5 ($p<0.05$) but not for any of the other densities investigated (Figure 2.4). These data indicated that CV assay is not the most appropriate to detect any differences among biofilms developed from different inoculum densities. Based on these data, metabolic activity of early and mature biofilms were interrogated for two inoculum densities 5×10^5 or 1×10^7 CFU/ml using the XTT assay in two media (AS & RPMI). These densities were selected according to the previous data shown in Figures 2.3 and 2.4. Figure 2.5 (i) and (iii) show the non-significant difference between the early (4 hr) and mature (24hr) biofilms when the 1×10^7 CFU/ml cell density was used with all strains tested in both AS and RPMI media. In contrast, the 5×10^5 CFU/mL inoculum density showed a significantly greater metabolic activity for all of the strains tested and in both media used (AS and RPMI) as shown in Figure 2.5 (ii) and (iv), respectively. Therefore, 5×10^5 CFU/ml inoculum density significantly increased the metabolic activity of the mature biofilms (24 hr) in comparison to their early counterparts (4 hr).

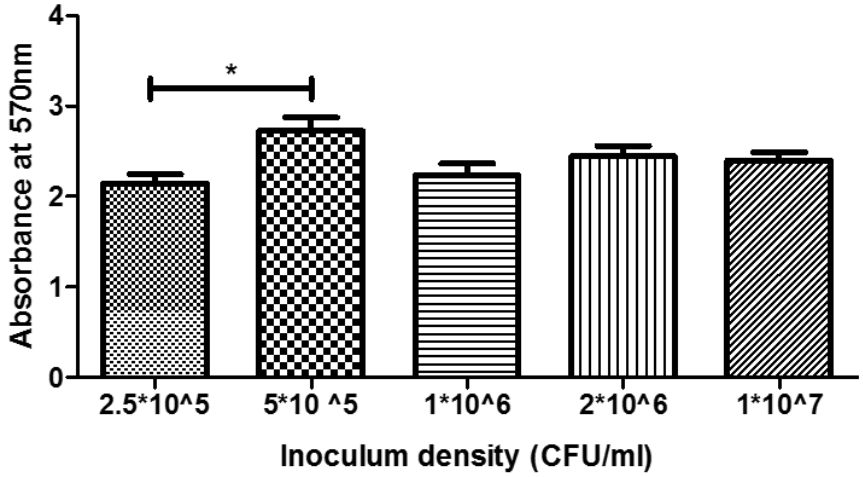


Figure 2.4: Effect of different inoculum densities on RPMI-developed mature biofilms. Biofilms of *C. albicans* SC5314 were grown for 24 h in RPMI media at varying inoculum densities. Following growth, biofilm biomass was assessed using the crystal violet assay. The experiment repeated in 3 independent occasions using triplicates. One-way ANOVA test displayed P value < 0.05. Bonferroni *post hoc* assay was used for further analysis. * = P< 0.05, Error bars represent standard error of mean.

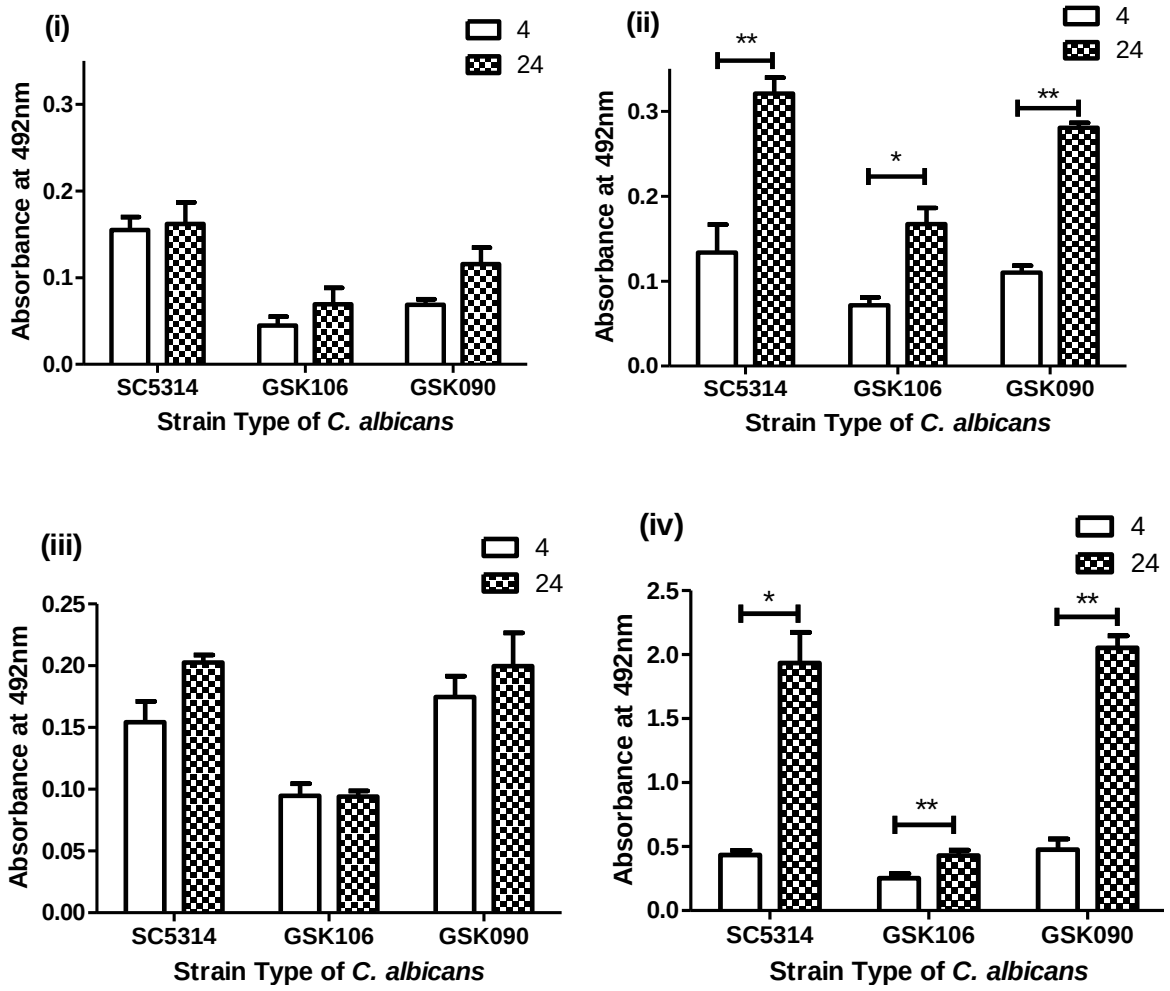


Figure 2.5: Optimisation of the CFU/ml concentration for development of candidal biofilm. XTT assay was used to assess the metabolism of the biofilms tested. The experiment repeated in 3 independent occasions using triplicates. **(i)** and **(ii)** represent the metabolic activity of AS-developed biofilms with 1×10^7 and 5×10^5 CFUs/ml, respectively. **(iii)** and **(iv)** represent the metabolic activity of RPMI-developed biofilms with 1×10^7 and 5×10^5 CFUs/ml, respectively. Student's *t* test was used for statistical analysis. * = P< 0.05, **= P< 0.01. Error bars represent standard error of mean.

2.4.3.2 The effect of incubation medium on biofilm formation

The comparison of AS medium to RPMI medium for biofilm development was statistically interrogated (student's *t* test) using the data sets derived from the 5×10^5 CFU/ml inoculum density. Figure 2.6 shows the higher capacity of RPMI medium to develop early and mature biofilms relative to AS, where significant differences were observed between the AS and the RPMI media for all tested strains at both time points. Thus, RPMI medium considerably promotes biofilm metabolism in comparison to the AS.

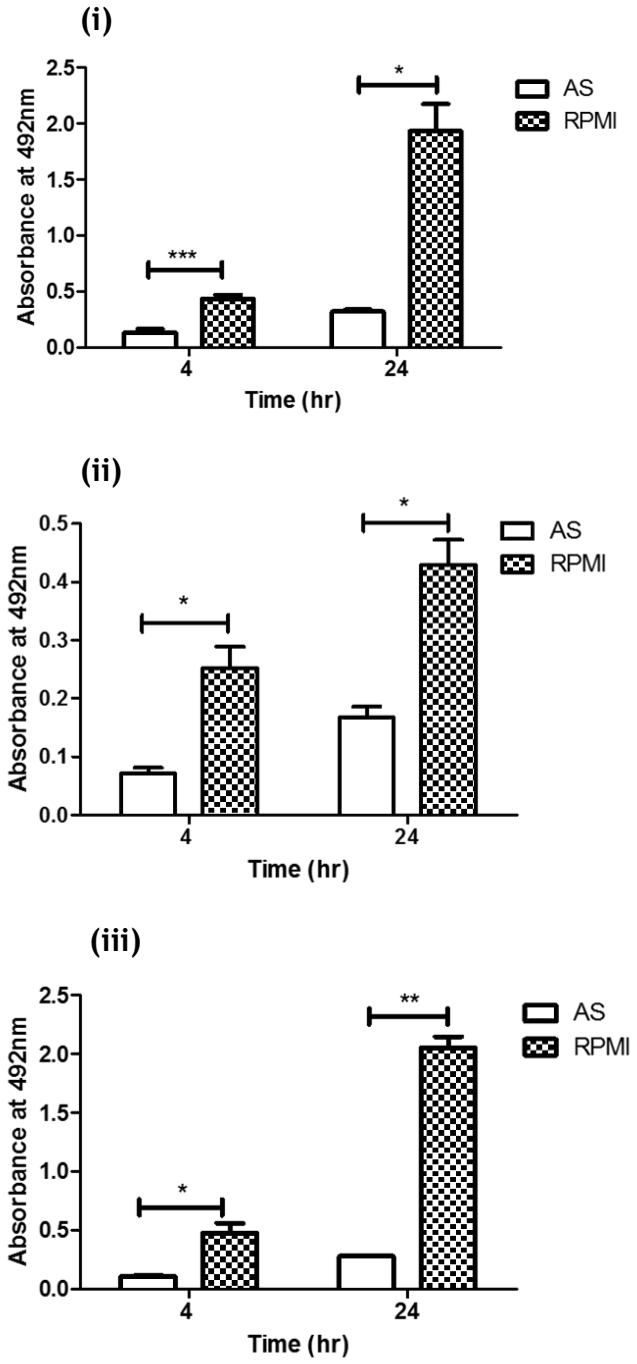
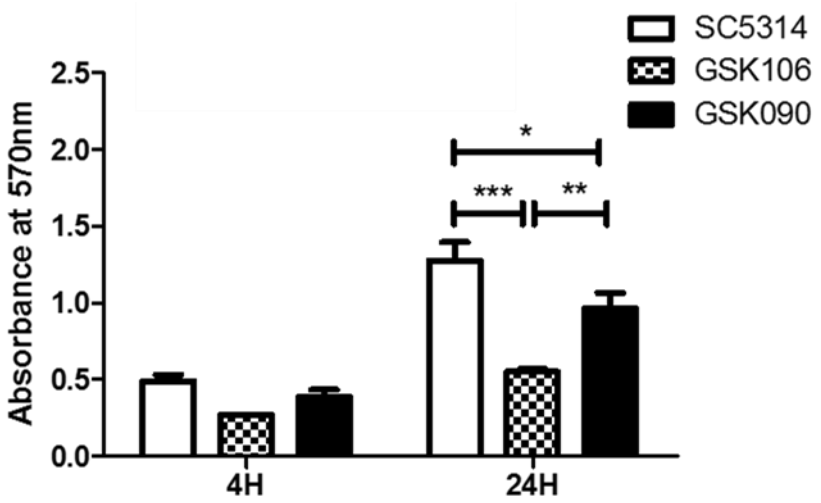


Figure 2.6: Optimisation of the incubation medium for development of candidal biofilm. XTT assay was used to analyse the metabolism of the biofilms tested. **(i)** represents the biofilm metabolism for the reference strain SC5314, while **(ii)** and **(iii)** represent the biofilm metabolisms for the clinical isolates GSK106 and GSK090 respectively. Student t test was used to analyse the data statistically. * = P< 0.05, **= P< 0.01, ***= P< 0.001. Error bars represent standard error of mean.

2.4.3.3 The effect of strain variability on biofilm formation

Interrogation of the effect of strain variability on biofilm development requires an alternative assay, as the XTT assay is not optimal for comparison of different strains due to the possible difference in the metabolic rate among different strains (Ramage, 2016;Azeredo *et al.*, 2017). Therefore, the CV assay was used for measurement of the biofilm biomass and comparing between different strains using two media (AS and RPMI) and incubation periods (4 and 24 hr). The statistical analysis by two-way ANOVA signified the important roles of strain variability and incubation period as significant sources of variation in candidal biofilms. Bonferroni's multiple comparison *post hoc* test clarified the non-significant effect of strain variability on 4hr early biofilms as opposed to 24 hr mature biofilms (Figure 2.7). Within the mature biofilms, the clinical isolate GSK106 showed a significant low biofilm forming capacity in comparison to the other investigated strains. Accordingly, strain variability was a vital source of variation in the development of mature biofilms but not early biofilms.

(i)



(ii)

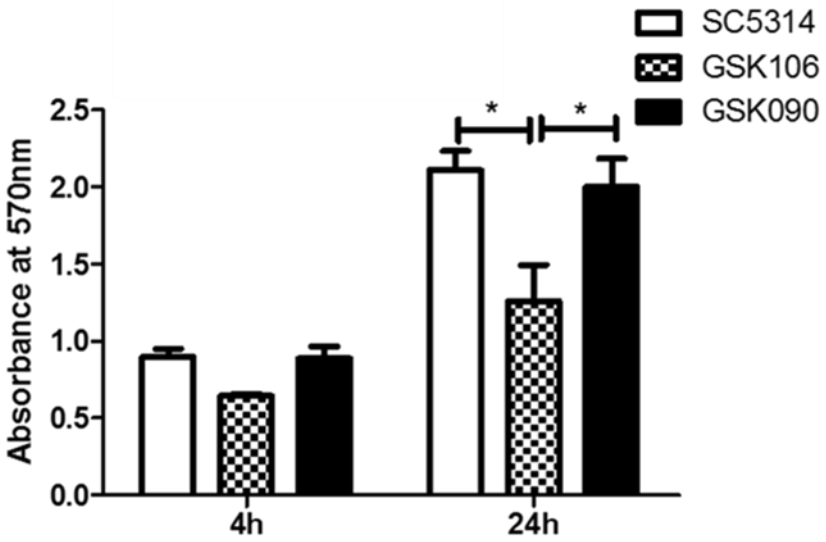


Figure 2.7: The effect of strain variability on development of candidal biofilm. CV assay was used to assess the biofilm biomass of the biofilms tested. Data collected from 3 replicates in 3 independent occasions. The biofilm biomass in AS-developed biofilm (i) and biofilm biomass in RPMI-developed biofilm (ii). Two-way Anova followed by Bonferroni multiple comparison test were used for statistical analysis. * = P< 0.05, **= P< 0.01, ***= P< 0.001. Error bars represent standard error of mean.

2.4.4 Impact of denture substratum type on candidal colonisation

The influence of the denture material on candidal attachment and colonisation has revealed conflicting data (Verran & Maryan, 1997;Pereira-cenci *et al.*, 2008a;Lazarin *et al.*, 2013). Therefore, this study aimed to evaluate colonisation of *C. albicans* on three different denture materials using a variety of methodologies. The effect of denture material upon the formation of *C. albicans* early and mature biofilms was investigated using three types of denture materials, two clinical isolates and one laboratory strain of *C. albicans*.

2.4.4.1 Visual investigation

Crystal violet staining of 4 h colonised *C. albicans* laboratory and clinical *C. albicans* strains did not show clear visual differences between the different denture materials. Although it revealed the capability of *C. albicans* to colonise the denture material surfaces irrespective of denture material (Figure 2.8).

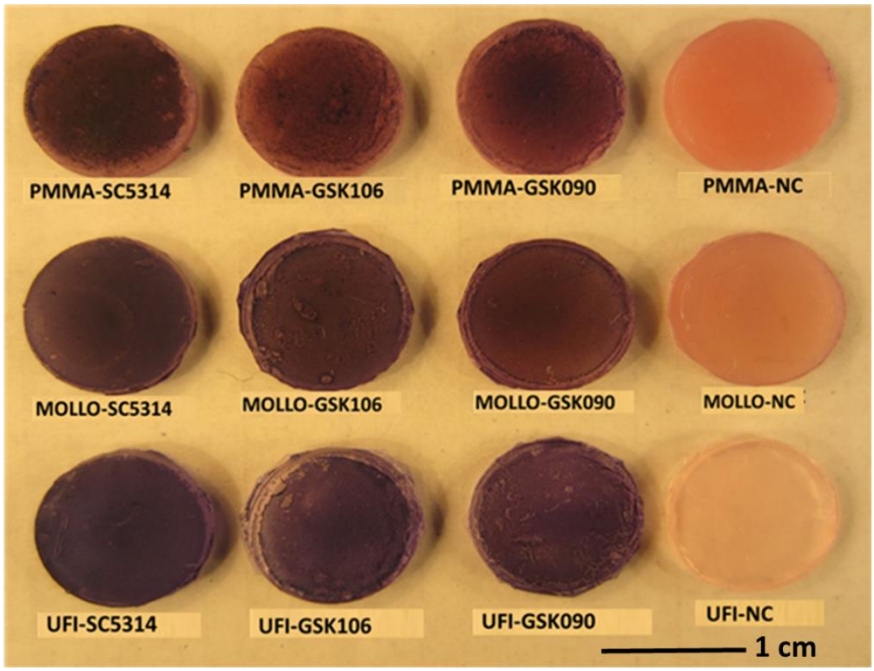
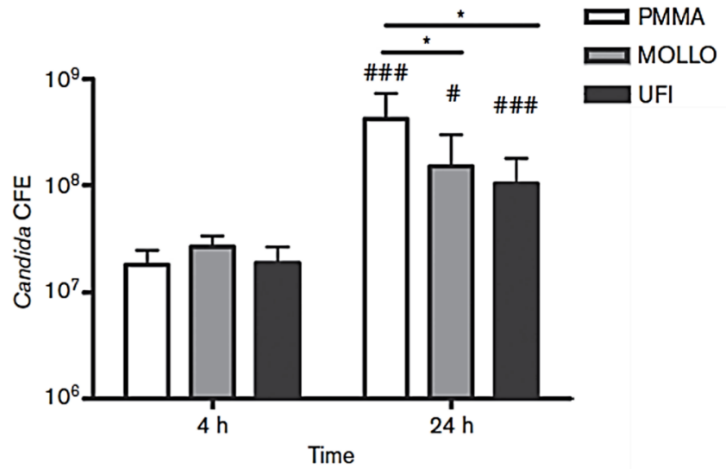


Figure 2.8: CV staining of early biofilms developed on different denture materials. SC5314, GSK106 and GSK090 *C. albicans* were colonised on the denture materials for 4 hr. PMMA, MOLLO and UFI stand for the denture substrata used polymethyl methacrylate, Molloplast® and Ufi gel®, respectively. NC represent negative control (no biofilms were developed). Scale bar is 1 cm.

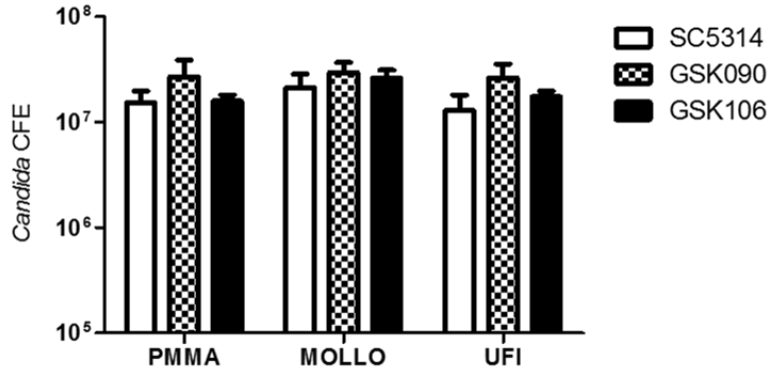
2.4.4.2 Molecular quantification

The number of the colonising *C. albicans* on denture materials tested were quantified by molecular qPCR methodology. For statistical analysis, one-way ANOVA and post hoc Bonferroni multiple comparison test were undertaken after data $\log_{10}$ transformation, with data is shown in figure 2.9(i). Comparison of early (4h) biofilms revealed no statistical significance observed among the three substrata tested ($P>0.05$). At the mature stage (24h) of biofilm formation, PMMA denture material revealed an increased affinity for *C. albicans* colonisation. A colonising average of 4.21×10^8 *C. albicans* CFEs for PMMA was 2.8 times and 4.1 times greater than those of Molloplast® ($P<0.05$) and Ufi gel® ($P<0.05$), respectively. Expectedly, *C. albicans* burden was significantly higher in the mature biofilms than in their early counterparts in all denture materials tested: PMMA ($P<0.001$), Molloplast® ($P<0.05$) and Ufi gel® ($P<0.001$). However, there was no statistical significance among the tested strains on early or mature biofilms of *C. albicans* that were colonised onto different denture materials, as shown in figure 2.9(ii) and (iii).

(i)



(ii)



(iii)

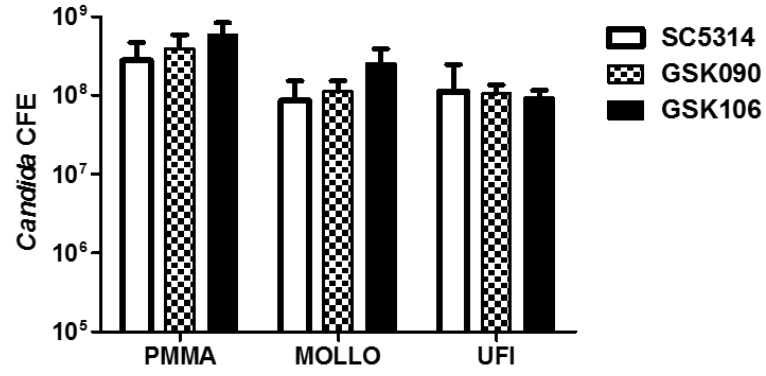


Figure 2.9: Effect of the investigated denture materials on the biofilm formation of *C. albicans*. (i) The early (4h) and mature (24h) biofilms were grown on PMMA, MOLLO and UFI denture substrata; and were assessed by molecular quantification. Data of three isolates were combined together and denoted by mean±SD. Data of 24h biofilms were compared to their 4h counterparts (# P<0.05, ###P<0.001). Statistical significances were detected between different denture substrata at 24h (*P<0.05). Non-significant effect of strain variability on early **(ii)** and mature **(iii)** biofilms that were developed on different denture materials.

2.5 Discussion

Over the last fifty years, there has been significant advances in our understanding of oral hygiene. Nevertheless, with an ageing population, prosthodontic treatment with partial or complete denture remains an inevitable option for a vast majority of adult society. Improvements in conventional therapy for edentulous people is important because the demand for full prosthodontic appliances is unlikely to decrease in the near future. The necessity of conventional dentures is still high because osseointegration-associated therapies are region-specific. Furthermore, these implant-associated therapies could involve a supplementary conventional prosthodontic treatment as an overdenture option (Carlsson & Omar, 2010). Moreover, dentures showed high esthetic, acceptable chewing capability, comfort and phonetics satisfaction in denture wearers that may exceed the patient expectations (Santos *et al.*, 2015;McCunniff *et al.*, 2017). Therefore, the necessity of studying biomedical associated-diseases of denture wearing is still of great importance. The high prevalence of DS (15-<70%) and its high recurrence makes its prevention a real need (Gendreau & Loewy, 2011). It has been reported that *Candida* species, specifically *C. albicans*, play a vital role in progression of DS and denture associated diseases (Webb *et al.*, 1998a;Dagistan *et al.*, 2009;Salerno *et al.*, 2011).

This chapter reports that the denture candidal load has a significant role in DS, in agreement with earlier investigations (Barbeau *et al.*, 2003;Dagistan *et al.*, 2009;Salerno *et al.*, 2011). Traditional microbiological techniques (culturing and staining techniques), have revealed higher quantities of *Candida* cells from dentures of the DS diseased group in comparison to those of the healthy group, especially dentures taken from patients diagnosed with moderate to severe grades of inflammation. While biofilm forming heterogeneity of *C. albicans* different strains (strain variability) did not show such a significant role in respect to DS, denture material appeared to have an impact on *C. albicans* mature biofilms.

The biofilm forming heterogeneity phenomenon is a clear and intriguing observation shown among the denture *C. albicans* clinical isolates investigated, displaying a yet, uncharacterised role in DS. From a clinical view, the hypothesis

that biofilm forming heterogeneity of *C. albicans* can contribute in deciding morbidity and mortality levels is deserving of examination concerning the oral cavity (Kean *et al.*, 2018). Our group revealed a relationship with this phenotype and mortality in a cohort of candidaemia patients in Scotland (Rajendran *et al.*, 2016a), therefore examining this in a cohort of denture patients was a reasonable hypothesis. Despite the fact that biofilm forming heterogeneity was observed utilizing *in-vitro* models, a relationship with patient clinical presentation was not observed. Failure to exhibit a connection might be clarified by the way that DS in immunocompetent people is milder than candidaemia from an extremely immunocompromised population. Nonetheless, a significant relationship between the denture candidal load and DS was observed, proposing a notion that the physical cooperation between extensive quantities of yeasts and hyphae on the denture surface was more vital than the phenotype *per se*, and this is possibly impacted by the various microbiota in the oral cavity as previously described (O'Donnell et al., 2015b). Undoubtedly, there is a growing body of evidence to suggest that *Candida* and bacteria compose polymicrobial communities, where various bacterial species that are communal to the oral environment can influence the pathogenicity of each other (Harriott & Noverr, 2009;Diaz et al., 2012;Falsetta et al., 2014;O'Donnell et al., 2015a), and particular oral microbes can stimulate the transition of *C. albicans* LBF into a HBF (Arzmi et al., 2016).

Real time qPCR quantification was previously reported for quantifying fungal cells (Lievens *et al.*, 2006;Willger *et al.*, 2016). Our research group has recently used qPCR methodology to quantify respiratory pathogens residing on dentures (O'Donnell *et al.*, 2016). Yet, according to the best of our knowledge, this is the first study to quantify denture-adherent *Candida* cells associated with DS using molecular methodologies. It has been observed that candidal denture biofilms are characterised by yeast-hyphae cellular aggregations (Ramage *et al.*, 2004;Coco *et al.*, 2008a;Bilhan *et al.*, 2009), thus, traditional CFU counting methods could underestimate the real number of the denture-colonising cells. For this reason, qPCR offers specific advantages over conventional microbiology techniques (Azeredo *et al.*, 2017).

Conflicting results were previously reported in the literature regarding the adhesion and colonisation capacity of *Candida* on denture liners (Pereira-cenci et al., 2008). Therefore, an experiment was designed to address this problem. AS medium was used to mimic the oral environment and prolonged incubation periods were not targeted because dentures are generally frequently cleaned (Soll & Daniels, 2016). A high inoculum density of 1×10^7 CFU/ml was used as previously defined in the literature (Verran & Maryan, 1997;Chandra' *et al.*, 2001;Raj & Dentino, 2011;Marra *et al.*, 2012;Lazarin *et al.*, 2013;Jackson *et al.*, 2014;Yodmongkol *et al.*, 2014). Moreover, this high cell density induces the development of a mature biofilm with less interlocking hyphae and more yeasts (Mayer *et al.*, 2013). Therefore, this high cell density may facilitate the process of collecting the biofilm as it was reported that it is difficult to collect the hyphae form of *C. albicans* (Hoyer & Cota, 2016). The qPCR methodology was undertaken to prevent the negative influence of the results by clumping of the cells, as we have used 4 and 24hr incubation periods that considered enough to develop an early and mature biofilm that could facilitate this clumping. The reason for selecting the denture materials investigated was based on a clinical standpoint. PMMA is the most routinely used denture material because of its low cost and easy handling, despite its varied surface, which clearly impacts on microbial establishment and biofilm growth (Jackson *et al.*, 2014;Susewind *et al.*, 2015). Discomfort could be promoted by the close contact of PMMA denture material to the mucosa because of its stiffness. As a result, resilient, less rigid denture liners are progressively become popular. Reports suggest that denture liners improve patient masticatory function and decrease discomfort (Kimoto *et al.*, 2007;Palla *et al.*, 2015), yet denture liners are liable for microbial colonisation (Bulad *et al.*, 2004;Vural *et al.*, 2010), and biofilm accumulation (Nevzatoglu *et al.*, 2007;Valentini *et al.*, 2013). In this chapter, samples were fabricated in stone molds, and were not polished. This was in order to simulate the ordinary clinical manipulation of the intaglio surface of the dentures, which is usually not polished to preserve the surface details and prevent compromising denture retention. However, investigating linear (polished) PMMA surfaces can be suggested for further research because of the possible capability of *C. albicans* to form biofilms on these surfaces.

Data shown in this chapter revealed no statistical difference in candidal colonization upon various denture materials among the early *in-vitro* biofilms, which is in agreement with previous studies (Bulada et al., 2004;Pereira-Cenci et al., 2007;Hahnel et al., 2012). However, at a later phase of biofilm development, PMMA was demonstrated to be the denture material surface that is most susceptible to biofilm development. A conceivable explanation as to why increased numbers were observed on PMMA is potentially because the porosity of soft liners are significantly more than that of hard acrylics (Wright *et al.*, 1998;Pereira-cenci *et al.*, 2008a;Øilo & Bakken, 2015). Hence, large quantities of microorganisms could be embedded in these porous surfaces (Taylor *et al.*, 1998). Consequently, even an forceful strategy for biofilm removal such as sonication, may not remove all microorganisms colonising soft liner cracks and crevices. Therefore, traditional quantification techniques used in previous studies could explain the differences observed in this study (Radford *et al.*, 1998;Bal *et al.*, 2008;Mutluay *et al.*, 2010).

The heterogeneity of clinical isolates of *C. albicans* has a potential role in complicating the clinical presentation of diseases (Sherry *et al.*, 2014;Pereira *et al.*, 2016;Rajendran *et al.*, 2016b), besides a potential role in their adhesion capacity (Cannon *et al.*, 1995). Therefore, it was important to investigate early and mature biofilms on denture materials using clinical isolates together with a commonly used reference strain. Contrary to Waters *et al.* (1997), the data displayed a non-significant impact of strain variability on early and mature biofilms formed on all materials investigated.

Investigation of *in vitro* biofilm models is critical and consideration of the variables that are related to the biofilm formation is of high importance. This is firstly because of the association of *Candida* biofilm formation with DS (Ramage *et al.*, 2004;Rautemaa & Ramage, 2011;Pereira *et al.*, 2016), and secondly the positive link between *in vitro* biofilm models and their *in vivo* counterparts obtained from DS patients (Nobile & Johnson, 2015). There is clear disparity in the literature regarding the starting cell density inoculum used in candidal biofilm formation models; ranging from ~ 1×10^7 through 1×10^6 to 1×10^4 cells/ml (Hawser & Douglas, 1994;Ramage *et al.*, 2004;Johnson *et al.*, 2012). Consequently,

investigation of 1×10^7 and 5×10^5 cell densities after a brief preliminary optimization was performed. The highest cell density did not show significant biofilm development in either of the considered media for all investigated strains. This can be interpreted by terms of the quorum-sensing systems that are used by *Candida* in response to the density of cell population. In high cell densities, quorum-sensing molecules such as farnesol and tyrosol inhibit biofilm formation and filamentation (Ramage *et al.*, 2002b;Albuquerque & Casadevall, 2012). The positive effects of RPMI-1640 medium on promoting *C. albicans* adhesion, biofilm development and antifungal susceptibility testing are well established in comparison to yeast nitrogen base and other media (Kucharikova *et al.*, 2011;Soll & Daniels, 2016;Weerasekera *et al.*, 2016). Therefore, RPMI-1640 medium was used as a standard medium for development of *C. albicans* biofilm. However, the effect of AS medium on biofilm development is still not fully established. Data shown in this chapter revealed RPMI as an appropriate medium to develop filamentous biofilms characterized with high metabolic activity. While, AS-grown biofilms demonstrated a less filamentous phenotype and lower metabolism, which could be resulted from the rich-nutrient nature of the AS. This is in line with Arzmi *et al.* (2016), although they used a low concentration of AS (25%). Moreover, mucins that are found in AS may suppress *Candida* cells during biofilm development (Kavanaugh *et al.*, 2014). However, this is a controversial issue, because some authors reported a supportive role for the mucin in *C. albicans* adhesion (Edgerton *et al.*, 1993;Burgers *et al.*, 2010). Irrespective of the type of medium of incubation, the characterisation data shows the importance of strain variability in outlining the biomass of the mature biofilms, while this is not the case in early biofilms. In conclusion, the biofilm characterisation data presented herein shows the significance of a rational approach to selecting the appropriate cell density, medium of incubation for the development of *in vitro* biofilms. Additionally, it highlights that strain variability should be considered as it has an impact on mature biofilms.

Chapter findings

- Molecular quantification revealed the importance of *Candida* denture burden in DS.

- Heterogeneity in biofilm formation of the denture isolated *C. albicans* strains was observed, although no significant association with DS was observed.

- Characterisation data of *C. albicans in vitro* biofilms showed the importance of a rationale in selection of the biofilm development variables.

- The XTT assay was more sensitive than CV assay in detecting differences among *C. albicans* biofilms developed from various inoculum densities. In contrast to the low inoculum density and RPMI medium, high inoculum density and AS medium did not show significant differences between early and mature biofilms. Strain variability showed significant effect on biofilm development in mature biofilms only.

- Early *C. albicans* biofilms were not influenced by denture material, however their mature counterparts were, with PMMA denture materials yielding the highest quantity of *C. albicans*.

- Strain variability (3 strains levels of biofilm forming capability) did not significantly affect early or mature phases of *C. albicans* biofilm development on denture materials using clinically relevant medium.

3 The anti-adhesive activity of curcumin on *Candida albicans* biofilms on PMMA denture material

3.1 Introduction

Increasingly, there are fewer effective antimicrobials available to treat clinical infections (Thabit *et al.*, 2015). This can be attributed to the increased prevalence of anti-microbial resistance, driven largely by poor antibiotic stewardship, combined with a gradual decrease in investment in antimicrobial development from the pharmaceutical industry (Renwick *et al.*, 2016). The failure of the development of effective antimicrobials to keep pace with emerging bacterial resistance has the potential to reverse the medical, surgical and social advancements of the last century and place us in a pre-penicillin era. Microbial diseases deemed unimportant now, may pose a greater threat in the future. We must therefore aim to be proactive and consider novel antimicrobial strategies (Crofts *et al.*, 2017).

Nature has an abundance of possibilities, where botanically isolated chemotherapeutic extracts can provide benefits over purely synthetic agents due to their co-evolution alongside harmful microbes. Polyphenolic natural compounds are intriguing potential sources in this context. The macromolecular composition of polyphenols and the characteristic phenolic functional group or "phenolic moiety" are found in many plants and can be synthetically prepared (Shahzad *et al.*, 2014). The polyphenol curcumin (diferuloylmethane), isolated from the rhizomes of the *Curcuma longa* plant (Mahmood *et al.*, 2015) is one such compound which merits consideration. Curcumin (CUR) is the vital element of turmeric used in Asiatic cuisine and as a dietary supplement or food additive in the food industry. Turmeric contains up to 5% CUR (Esatbeyoglu *et al.*, 2012;Kwon, 2014). Gupta *et al.* (2012) indicated the relative safety and active biological potentials of CUR, including inhibition of the damaging oxidation and inflammation reactions. Critically, CUR shows wide range antimicrobial activities (Moghadamtousi *et al.*, 2014), including antifungal (Martins *et al.*, 2009;Khan *et al.*, 2012), antibacterial activities (Shahzad *et al.*, 2015;Tyagi *et al.*, 2015), and additionally a potential capacity to impact biofilm and adhesion properties (Shahzad *et al.*, 2014;Shahzad *et al.*, 2015).

Research from the Ramage laboratory has demonstrated the capability of CUR to modify the attachment of periodontal microbes to the hydroxyapatite-coated substrates and influence biofilm development (Shahzad *et al.*, 2015). In parallel, investigations on the denture pathogen *C. albicans* showed that CUR was highly effective against sessile biofilm cells (Shahzad *et al.*, 2014). Kumar *et al.* (2014) further demonstrated that CUR could specifically affect the biosynthesis and permeability of the *C. albicans* cell wall through targeting of the calcineurin-mediated signalling and mitogen activated protein kinase pathway.

The globally important opportunistic pathogen *C. albicans* is known for its ability to form clinically important biofilms (Ramage *et al.*, 2006;Uppuluri *et al.*, 2009;Rajendran *et al.*, 2016c). Moreover, this property is complemented by a range of colonisation determinants, including adhesion and invasion proteins, such as agglutinin-like sequences (ALS) and secreted aspartyl proteases (SAPs), that encourage attachment and invasion into the host (O'Donnell *et al.*, 2015a). The yeasts capacity to alter morphology and exhibit dimorphism and thigmotropic tendencies makes it a very well-adapted and tenacious pathogen. *C. albicans* has the capacity to form biofilms on both biotic and abiotic surfaces making this microorganism particularly important in oral diseases. To survive and compete in the oral cavity the organism possesses an array of phenotypic characteristics that provide physical protection from internal and external antimicrobial mechanisms (Ramage *et al.*, 2014). Given that *C. albicans* plays a significant role in DS means we need to consider antimicrobial strategies that target this microorganism as a basic prerequisite for DS prevention. Hence, it seems plausible that CUR might promote oral health through counteracting or limiting the effect of *C. albicans* to denture wearers.

3.2 Aims

It is hypothesised that CUR could be used to actively inhibit *C. albicans* biofilm formation on denture materials through interfering with the initial adhesion phases. The aim of this chapter was to assess this hypothesis using the following key investigations:

1. To investigate the capacity of PMMA denture material to adsorb CUR.
2. To investigate the impact of adsorbed CUR on adhesion of *C. albicans* to PMMA denture material.
3. To investigate the capability of CUR pre-treated *C. albicans* to adhere to PMMA denture material and the possibility of synergistic combination with CUR adsorbed on to the surfaces.
4. To investigate the influence of CUR on different morphological forms of *C. albicans* and biofilm formation.
5. To investigate the effect of CUR on the inter-*C. albicans* adhesive interactions.
6. To assess the response of *C. albicans* to CUR at the molecular level through investigation of the expression of selected adhesion-aggregation associated genes.
7. To investigate the impact of CUR incorporation in the PMMA material on candidal adhesion.

Part of the data presented in this chapter has been published in *Frontiers in Microbiology* journal.

Alalwan H, Rajendran R, Lappin DF, Combet E, Shahzad M, Robertson D, Nile CJ, Williams C, Ramage G. Front Microbiol. 2017 Apr 20;8:659. doi: 10.3389/fmicb.2017.00659.

Findings from this chapter have been presented at the following academic meetings:

1. Postgraduate research prize seminar held in Glasgow Dental School on 27th of April 2016.
2. OMIG postgraduate research prize symposium held in College of Clinical Dentistry in University of Sheffield on 8th of Feb, 2017.

3.3 Materials and Methods

3.3.1 Culture conditions and standardisation

C. albicans SC5314 laboratory strain was used for these studies. Operational stocks of yeast cells were prepared on fresh Sabouraud agar (Sigma-Aldrich, UK), cultivated for 48 h at 30°C, and maintained at 4ºC as annotated in chapter 2 (section 2.3.1.2). Using one colony, the cells were cultured in yeast-peptone-dextrose (YPD) medium (Sigma-Aldrich) for 18 h at 30°C and 150 rpm in an orbital shaker. The cells were washed twice by centrifuging in sterile phosphate buffered saline (PBS) (Sigma-Aldrich, UK), then standardised to the required colony-forming unit per millilitre (CFU/ml) using a Neubauer haemocytometer.

3.3.2 Antifungal susceptibility testing

The two cellular modes of growth (planktonic free-floating cells and sessile biofilm cells) were first examined for their sensitivity to the polyphenol curcumin (CUR) (HPLC grade, Acros Organics, Belgium). Prior to each experiment, a standard stock of CUR was prepared, using a non-antimicrobial concentration of dimethyl sulfoxide (DMSO) as a solvent, and adjusted to <5% v/v in RPMI-1640 medium (Sigma-Aldrich, UK) for preparation of working concentrations (Shahzad *et al.*, 2015). At the commencement, standardised CLSI M-27A broth microdilution methodology was conducted for planktonic yeast cultures in 96 well round bottomed microtitre plates (Corning Incorporated, NY, USA) (CLSI-M27-A, 2008). Briefly, serial doubling dilutions were performed using RPMI-1640 medium after addition of 200 µl of CUR to the first column of the microtitre plate and 100 µl of medium in the next nine columns. Thereafter, 100 µl of 1×10^4 of standardised *C. albicans* cells were inoculated into appropriate wells. The treatment concentration range was (0.39 - 200µg/ml). Plates were incubated at 37ºC for 24 h. Negative (medium only) and positive controls (medium + cells only) were included. Clear wells with no visible growth were considered as the planktonic minimum inhibitory concentration (PMIC).

For sessile susceptibility testing, pre-formed mature biofilms were treated with CUR using standardised sessile antifungal testing (Ramage *et al.*, 2001;Pierce *et*

al., 2008). Briefly, 100 µl of 1×10^6 of standardised *C. albicans* cells were grown in 96 wells flat-bottomed microtitre plates (Corning Incorporated, NY, USA) at 37ºC for 24 h using RPMI-1640 medium. Then, biofilms were washed with PBS and treated with 100 µl of CUR for a further 24 h at 37ºC, prepared using serial doubling dilutions as described above. The same treatment regimen described above was used, where CUR was double diluted in a fresh 96 well microtitre plate immediately prior to the treatment of the biofilms. Subsequently, treated biofilms were washed with PBS and assessed for reduction of tetrazolium to formazan using the XTT assay (Ramage *et al.*, 2001) as described in chapter 2 section (2.3.3.4), and the optical densities measured at 492 nm using a microtitre plate reader (FluoStar Omega, BMG Labtech, UK). Negative (medium only) and positive (medium + cells only) controls were included. The anti-sessile impact was calculated via comparing the experimental wells data to the positive control data to reveal the $SMIC_{80}$, i.e. where the optical density is reduced more than 80% in comparison to the positive control optical density. These experiments were repeated in 3 independent occasions with triplicate wells for each condition.

3.3.3 Investigating the effect of CUR on *C. albicans* growth kinetics

The growth kinetics of CUR-treated *C. albicans* was assessed to determine if 0.5 x PMIC concentration could influence the rate of 24 h growing yeasts. The cells were standardised to 1×10^4 CFU/ml in YPD, with 200 µl inoculated to the appropriate well of a 96 well round-bottomed plate. Alongside, CUR-treated cells (50 µg/ml= 0.5 ×PMIC) were inoculated. The microtitre plate was incubated at 37°C for 24 h and every 1 h the absorbance was measured at 530 nm after automated shaking for 30 s at 100 rpm. This experiment was repeated on two independent occasions using six replicates. Negative controls (medium only) and (medium + CUR) were included for appropriate background correction of the untreated and treated cell, respectively.

3.3.4 Investigating the capacity for adsorption of CUR onto denture material

The possible affinity of CUR to fabricated PMMA denture material was explored by measuring the quantity of the adsorbed CUR spectrophotometrically. Heat cured PMMA denture base material (Chaperlin and Jacobs Ltd, Surrey, UK) was used to produce 12 mm diameter discs using the dental compression moulding technique as described in chapter 2 (2.3.3.1). PMMA sections were immersed in ddH_2O for one week to ensure removal of any residual toxic monomers. To prepare varying operational concentrations of CUR suspensions, stock CUR was diluted in RPMI-1640 medium to 200, 400 and 800 µg/ml. The PMMA sections were distributed in the appropriate wells of 24 well plates (Costar, Corning Incorporated, USA) and 1 ml of the CUR suspension was added. The plates were then incubated at room temperature for a series of time points (1, 5, 10, 30, 60, 120, 240 and 1440 min), covered with tin foil to prevent light exposure. After the incubation period the CUR suspension was removed. The PMMA sections were then transferred with tweezers to fresh wells and washed by gentle immersion in 1 ml of distilled water, then distributed to fresh well. Next, 1 ml of DMSO (100%) was added for 5 min to dissolve the adsorbed CUR. For quantification of the released CUR, a standard curve was developed by serially diluting CUR in DMSO to prepare concentrations ranging from 100 to 0.39 µg/ml. Based on that standard curve, the DMSO- dissolved CUR was measured at 436 nm using the spectrophotometer (FluoStar Omega, BMG Labtech) including appropriate blank correction wells.

3.3.5 Investigating the effect of CUR adsorption on adhesion of *C. albicans* to PMMA

Based on the previous experimental data, PMMA denture material sections were each immersed in 1 ml of 800 µg/ml of RPMI-CUR suspension for 10 min in order to obtain approximately 50 ug/ml of adsorbed CUR, then washed with ddH_2O to eliminate the unadsorbed molecules. The CUR-adsorbed PMMA sections were distributed in a 24 well plate and immediately inoculated with 1 ml of 5×10^5 CFU *C. albicans* SC5314 cells, then incubated for 30 min at 37°C. Positive controls were included, which were initially immersed in RPMI medium only for 10 min then inoculated with the cells. When the incubation period was completed, the sections

were gently washed twice in PBS and sonicated in bijou tubes contained 1 ml of PBS at 35 kHz for 10 min (Ultrasonic bath, Fisher scientific, UK) to collect the adherent cells. These collected cells were counted using the Miles and Misra plate counting method (Miles *et al.*, 1938). In brief, using PBS, ten times serial dilutions were performed of the collected cells (10^{-1} to 10^{-4}). A twenty microlitre drop of each diluted suspension was plated in triplicate on SAB agar plates. Plates were incubated for 24-30 h at 37°C. The most clearly separated colonies in the least dilution were considered in counting.

The following equation was applied to quantify the adhered cells:

Number of colonies × dilution factor × 50 = colony forming units per cm^2 PMMA

The final cell number was then compared to a CUR untreated control. All experiments were performed in triplicate on 3 independent occasions.

3.3.6 Microscopic imaging of adherent *C. albicans* to CUR-adsorbed PMMA

To examine the impact of adsorbed CUR on candidal adhesion visually, scanning electron microscopy (SEM) was also performed using the same experimental parameters that were described in the aforementioned section (3.3.5), then processed and imaged, as described previously (Erlandsen *et al.*, 2004;Sherry *et al.*, 2016). Briefly, *C. albicans* cells were grown on CUR adsorbed PMMA sections. These sections were washed twice with PBS, before being fixed for 20 h in 2% paraformaldehyde, 2% glutaraldehyde, 0.15M sodium cacodylate, and 0.15% w/v alcian blue, at pH 7.4, and prepared for SEM on the next day. In brief, sections were triple washed with 0.15M sodium cacodylate buffer, then incubated in 1% osmium tetroxide for 1 h. After triple washing with distilled water, 0.5% uranyl acetate was added and incubated for 1 h. After this, a sequential ethanol dehydration was followed with 30%, 50%, 70%, 90%, absolute alcohol and dried absolute alcohol. Later, HMDS (Hexamethyldisilazane) was added for 5 min. The samples were incubated in a desiccator at room temperature for 24 h. Next, the samples were sputter-coated with a layer of gold-palladium (15-20 nm) and inspected under a JEOL JSM-6400 scanning electron microscope.

3.3.7 Investigating the biological effect of CUR on *C. albicans* adhesion and biofilm formation

Following CUR adsorption to PMMA denture material, which was conducted using same experimental parameters as described above, the PMMA discs [untreated and treated (PMMA- and PMMA+, respectively)] were distributed in the appropriate wells of 24 well plates and inoculated with 1 ml of 5 x 10^5 CFU *C. albicans* SC5314 cells. The inoculated cells were either + 3 min CUR (50 µg/ml) pretreated or PBS (control) pretreated at 37°C. These discs then were incubated for 30 min at 37°C. Subsequently, the discs were gently washed twice with PBS. Candidal adhesion was then assessed and quantified using the Miles and Misra plate counting method as described in section (3.3.5). The levels of adhesion were expressed as a proportion of the control (PMMA-/*C. albicans*).

In parallel, an assessment was conducted to determine whether extended CUR pretreatment time negatively influenced adhesion via treating cells for 3, 30 and 90 min, then the levels of adhesion to PMMA were quantified as described above.

CUR treatment of *C. albicans* cells at different growth phases was assessed to determine whether or not can CUR played a role in biofilm development, with the hypothesis that there might be dissimilarities in how yeast (Y), germlings (G) or hyphae (H) reacted to this molecule. Briefly, yeast cells were grown overnight, standardised to 1 x 10^6 CFU/ml in RPMI and inoculated into a 96-well microtitre plate as described in chapter 2 (section 2.3.2.1). Cells were then exposed to 50, 100 or 200 ug/ml CUR at either 0 h (Y), 2 h (G) or 4 h (H) post inoculation and incubated for a further 24 h at 37ºC. Subsequently, the developed biofilms were washed with PBS and the resultant biofilm assessed using an XTT metabolic reduction and crystal violet assays as described in chapter 2 (sections 2.3.3.4 and 2.3.2.2, respectively). Moreover, the developed biofilms were microscopically evaluated using a light microscope (Model BX40F4; Olympus, Tokyo, Japan). All experiments were performed in duplicate on 3 independent occasions.

3.3.8 Investigating the aggregative effect of CUR

With the purpose of assessing whether CUR has further effects on *C. albicans* yeasts, its impact on aggregation was assessed. SC5314 laboratory strain cells (Y) were standardised (1 x 10^6 cells) in PBS and exposed ± to 50 µg/ml of CUR (sub-inhibitory concentration) for 90 min at 37°C in an orbital shaker (200 rpm). Following incubation, vortexing of the standardised inoculum was avoided in order to reduce any disaggregating forces. The cells were serially ten-fold diluted in PBS and plated onto Sabouraud agar using the Miles and Misra method (Miles *et al.*, 1938). Next, the plates were incubated for 24-30 h at 37°C and the colonies counted as annotated in section (3.3.5). In parallel, a light microscope was used to observe the cells and to evaluate aggregation visually. All experiments were performed in triplicate on 4 independent occasions.

3.3.9 Investigating the effect of CUR on cell surface hydrophobicity

Microbial adhesion to the hydrocarbon xylene was utilised to measure the cell surface hydrophobicity (CSH) of ± CUR exposed *C. albicans* (SC5314) using a modified version of the CSH assay (Yoshijima *et al.*, 2010;Sherry *et al.*, 2014). Cells were propagated overnight and standardised to 1.0 at OD 600 in PBS using a spectrophotometer and semi-micro cuvettes. 1.1 ml of the standardised cells were added to a 1.5 ml Eppendorf and spun for 10 min at 7000 r/min, the supernatant was discarded leaving the pellet intact. CUR (50 µg/ml) diluted in PBS was added to the pelleted samples. Control samples (cells not exposed to CUR) were included. Afterwards, the samples were vortexed and incubated at 37°C for 3, 30 and 90 minutes in an agitated state (200 rpm). Next, samples were washed twice with 20% DMSO to remove the CUR, and controls were similarly treated. Later, xylene (0.275 ml) was added to form 1/5 of the final volume, and vortexed for 2 min and left for 30 min phase separation. The lower aqueous layer was carefully pipetted and its optical density was measured at OD600. Negative controls (PBS only + xylene) were included for background correction. The optical densities were measured for the standardised treated and untreated samples that did not contain any xylene (incubated at 37°C and 150 r/m) at the specific time point and considered as OD before xylene overlay.

The following equation was applied to calculate the CSH percentage: ([OD before Xylene overlay- OD after Xylene overlay] / OD before Xylene overlay) × 100. The experiment was independently repeated six times using duplicates.

3.3.10 Assessing the molecular impact of CUR on adhesion and biofilm formation

Y and H morphological forms of *C. albicans* SC5314 cells were prepared via an initial inoculum of 1 x 10^8 and 5 x 10^5 CFU/ml in RPMI-1640 medium, respectively. For H cells, these were colonised on PMMA denture material sections within 24 wells plates for 4 h. Both Y and H cells were then exposed ± CUR (50ug/ml) in RPMI for 3, 30 and 90 min and incubated at 37°C. After incubation, cells were either centrifuged (for Y cells) or sonicated (for H cells) in a 35 kHz for 10 min (Ultrasonic bath, Fisher scientific, UK) to harvest the cells. The harvested cells were then washed by centrifugation and RNA was extracted from these cells.

RNA extraction was performed using a combined mechanical disruption (0.5mm glass beads) and chemical TRIzol™ method (Invitrogen, Paisley, UK). The extracted RNA was further treated to remove any residual DNA, and then were processed for production of complementary DNA, which are quantitatively analysed for the expression profile of targeted genes using qPCR. This multiple procedure is described in detail in the following sub-sections:

3.3.10.1 Extraction of total RNA

The first step in RNA extraction was the addition of 1 ml of TRIzol™ solution (Invitrogen, Paisley, UK), which is a monophasic solution of phenol and guanidine isothiocyanate, to the pelleted candidal cells (Y and H). The mixture was vortexed for 30 seconds, then transferred to screw cap microcentrifuge tubes containing 0.25 ml of 0.5mm diameter sterile glass beads (Thistle Scientific, Glasgow, UK) to facilitate disruption of the cell wall, using a Bead beater [BeadBug™ microtube homogenizer (Sigma-Aldrich, Gillingham, UK)]. Three cycles of bead beating were performed, each cycle lasted for 30 seconds at maximum velocity (400 rpm), the tubes were kept on ice in-between the cycles.

Next, 100 µl of 1-bromo-3-chloropropane was added and vortexed for 30 seconds, then left for 3 minutes. The next step was to centrifuge at 13,000 rpm for 15 min at 4°C (Heraeus centrifuge) to isolate an upper aqueous clear layer. This layer was transferred to a sterile RNase-free microtube, and 500 µl of absolute isopropanol was added in order to precipitate the RNA. The tubes were then inverted 30-40 times, and placed at -20°C overnight to increase RNA precipitation.

The next day, sedimentation of RNA was implemented by centrifuging for 10 min at 13,000 rpm at 4°C. The supernatant was discarded, and the remaining pellet washed with 800 µL of ice cold 70% ethanol to be centrifuged for 10 min at 6500 rpm at 4°C. Ethanol was carefully pipetted, and samples were air dried for 20-30 min. The final step was RNA resuspension in 20 µL of RNase free distilled water and incubation at 65°C in a heat block (Techne, Staffordshire, UK) for 5 min to facilitate RNA recovery.

3.3.10.2 DNA digestion by DNase

RNase-free DNase kit (QIAGEN GmbH, Germany) was used to eliminate residual genomic DNA. One and half µl of DNase was added to every RNA sample and incubated at room temperature for 30 minutes, then incubated at 75°C in a heat block (Techne, Staffordshire, UK) for 10 min to inactivate the DNase enzyme. Finally, the RNA samples were stored at -80°C.

3.3.10.3 Synthesis of complementary DNA (cDNA)

RNA concentration and purity was measured by NanoDrop™ ND-1000 spectrophotometer (Labtech International, East Sussex, UK) to standardise RNA quantity reverse transcripted to cDNA. cDNA synthesis was performed using High-Capacity RNA-to-cDNA™ reverse transcription (RT) (Applied Biosystems, UK). The mastermix was prepared by mixing 2 µL of RT Buffer, 2 µL of RT Random Primers, 0.8 µL of dNTP Mix, 4.2 µL RNase free distilled water and 1 µL of MultiScribe® Reverse Transcriptase enzyme. Ten microliters of mastermix was added to 10 µL of RNA-distilled water standardised solution to obtain a final volume of 20 µL in 200 µL dome-capped PCR microtubes (Abgene, ThermoFisher, Surrey, UK).

Solutions were prepared containing 500 and 50 ng of the purified RNA from the yeasts and hyphae samples, respectively. NRT (No Reverse transcriptase) samples were included to monitor the efficacy of the Reverse transcriptase enzyme. Next, the samples were placed in a Thermo-cycler (Bio-Rad, Hertfordshire, UK). The cycle program consisted of 10 min at 25°C, 120 min at 37°C, 5 min at 85°C and a final hold stage at 4°C. cDNA was then stored at -20°C until used in subsequent PCR assays.

3.3.10.4 Primers used

Oligonucleotide primer synthesis was conducted by (Invitrogen, Paisley, UK). The primers were designed against the *ALS1*, *ALS3*, *ALS5* (agglutanin-like sequence 1, 3 and 5), *EAP1* (epithelial adhesion protein 1) and *AAF1* (adhesion and aggregation factor 1) genes. The primers were synthesised to match the sequences taken from peer-reviewed papers. Except, *AAF1* which was designed by the Oral Sciences Research Group in Glasgow Dental Hospital and School. The primers are listed in Table 3.1.

Table 3.1: Primers used for real time qPCR transcriptional analysis of *Candida albicans*

Gene	Sequence (5' to 3')	Reference
ALS1	F - TTCTCATGAATCAGCATCCACAA	(Nailis *et al.*, 2009)
	R - CAGAATTTTCACCCATACTTGGTTTC	
ALS3	F - CAACTTGGGTTATTGAAACAAAAACA	(Nailis *et al.*, 2009)
	R - AGAAACAGAAACCCAAGAACAACCT	
ALS5	F - CTGCCGGTTATCGTCCATTTA	(Green *et al.*, 2005)
	R - ATTGATACTGGTTATTATCTGAGGGAGAAA	
EAP1	F - ACCACCACCGGGTATACAAA	(Sherry *et al.*, 2014)
	R - GCCATCACATTTGGTGACAG	
AAF1	F - CTGCCCTTGTTGGTACATCT	This study
	R - TGGGATAGTTGGTGGAGGAG	
ACT1	F - AAGAATTGATTTGGCTGGTAGAGA	(Ricardo *et al.*, 2009)
	R - TGGCAGAAGATTGAGAAGAAGTTT	

3.3.10.5 Gene expression assessment and analysis utilising real time PCR

Fast SYBR® Green PCR Master Mix (Applied biosystems, USA), intercalating, fluorescent dye was harnessed to perform RT-q PCR, using the same procedure and thermo-cycler program annotated in chapter 2 (2.3.1.4). Each parameter was analysed in duplicate using the StepOnePlus™ Real-Time PCR System unit (Applied biosystems, USA)] and StepOne software v2.3. The primers listed in Table 3.1 were used and 1 µl of synthesised cDNA was added instead of genomic DNA. No cDNA template and no reverse transcriptase controls were included.

ACT1 housekeeping gene was selected for normalisation; all the generated Ct values of the interrogated genes were relative to the Ct values of that standard housekeeping gene. $2^{-\Delta\Delta Ct}$ arithmetical method (Percentage of expression= $2^{-\Delta Ct}$, where ΔCt= Ct of targeted gene – Ct of *ACT1* housekeeping gene) was undertaken, to quantify the relative expression (Livak & Schmittgen, 2001). A heatmap was created for the differential expression of genes (log_2) over the period of 3 to 90

min from the untreated control compared to CUR exposed cells. Maps and clusters were generated in R with the use of heatmap.2 function from the gplots package. Dr Ranjith Rajendran supported these analysis.

3.3.11 Investigating the effect of CUR incorporation on adhesion

The capacity of CUR-incorporated PMMA denture material to reduce adhesion of *C. albicans* was explored. Prior to fabrication of PMMA discs, stock CUR was mixed with the monomer to obtain a standardised solution of 200 µg/ml. Then, the discs were fabricated as annotated in chapter2 (2.3.3.1). Control discs were included taking into consideration the CUR dissolvent (DMSO) concentration. All discs were appropriately distributed into a 24 well plate. *C. albicans* SC5314 was propagated and standardised in RPMI-1640 medium. One ml of 5×10^5 cells were inoculated and statically incubated for 30 min at 37°C. After the incubation, the sections were washed in PBS, sonicated to collect the adherent cells and the collected cells were counted using the Miles and Misra plate counting method (Miles *et al.*, 1938) as annotated in section (3.3.5). Three replicates were used and the experiments were repeated on five independent occasions. In parallel, the release of CUR from the fabricated discs was investigated via immersion of the targeted discs in DMSO solvent for 24 h and measuring released CUR spectrophotometrically as annotated in section (3.3.4).

3.3.12 Statistical Analysis

As we were unable to ascertain that the data conformed to a Gaussian distribution data analysis was performed on non-parametric data using either a Mann-Whitney test or a Kruskal-Wallis test with Dunn's multiple comparison post-test. All independent data points were presented, with error bars representing the median with interquartile range. Where proportional data were presented, analysis was performed on the original data sets. All statistics and figures were produced using GraphPad Prism v.5 (GraphPad Software Inc., La Jolla, CA).

3.4 Results

3.4.1 CUR can be adsorbed onto denture material up to effective concentrations

Initially, the potential inhibitive capacity of CUR to planktonic and biofilm *C. albicans* cells was tested to establish biologically active working concentrations appropriate for use in downstream analyses. The planktonic minimum inhibitory concentration (PMIC) was 100 µg/ml of CUR, while the sessile (biofilm) minimum inhibitory concentration that produced an ≥80% reduced metabolic activity ($SMIC_{80}$) was 200 µg/ml, representing a significant reduction in the biofilm's activity and/or viability. Furthermore, the growth kinetics of 0.5 x PMIC CUR-treated *C. albicans* was investigated. Figure 3.1 shows that the start of exponential phase of CUR-treated cells was delayed for a period of 4 h compared to the untreated control (6h v 10h). Both sets of cells reached the stationary phase at the same time (20 h), with approximately same cellular quantity. Statistical analysis did not show any difference ($p>0.05$), which could clarify that the selected concentration of CUR did not influence growth of *C. albicans* and subsequently did not interfere with the results of the next adhesion investigations.

According to these data, it was important to evaluate whether these effective concentrations of CUR could be adsorbed to PMMA denture material to interfere with *C. albicans* adhesion. Therefore, the PMMA samples were immersed in 200, 400 and 800 µg/ml CUR for different time periods and the adsorbed concentrations were measured using an elution method in conjunction with an optimised standard curve. Figure 3.2(i) displays the visible light absorption spectrum of the CUR in DMSO. This figure shows the absorbability of CUR at 436 nm at ≤100 µg/ml concentrations. The kinetics of adsorption for each concentration is presented in figure 3.2(ii). PMMA had the capacity to adsorb biologically effective CUR concentrations and immersion in 800 µg/ml CUR solution for 90 min was required to achieve adsorbed concentrations with anti-biofilm activity (200 µg/ml). Nonetheless, 50 µg/ml and 100 µg/ml concentrations were adsorbed from this initial concentration when immersed for 10 and 30 min. The lower concentration CUR solution (400 µg/ml) was able to attain 50 µg/ml and PMIC levels, though this approximately required 90 and 240 min immersion. Finally, the lowest

concentration CUR solution (200 µg/ml) did not show the capability to achieve any antimicrobial level concentrations, even after 24 h adsorption. Consequently, using of 800 µg/ml of CUR in adsorption experiment appeared biologically effective and efficient.

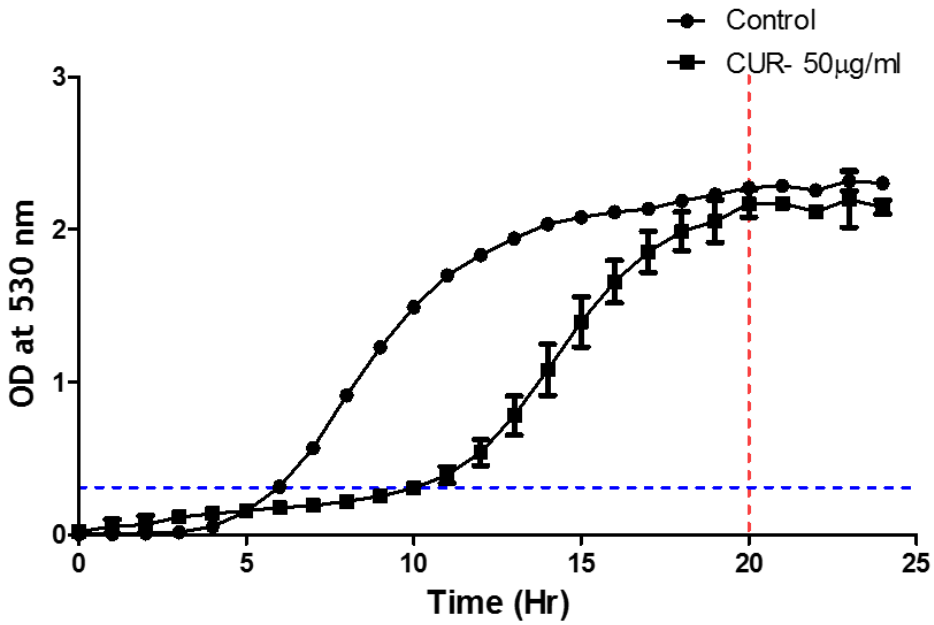
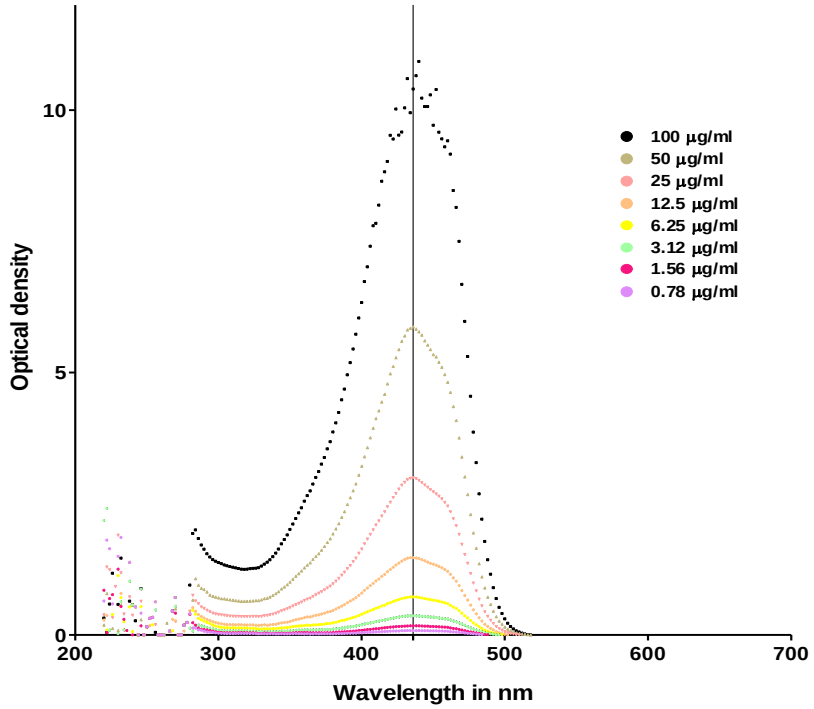


Figure 3.1: Growth kinetics of half PMIC CUR-treated *C. albicans*. The blue and the red dotted lines represent tangents to the start points of exponential and the stationary phases, respectively. Mann-Whitney statistical test was used to analyse the data.

(i)



(ii)

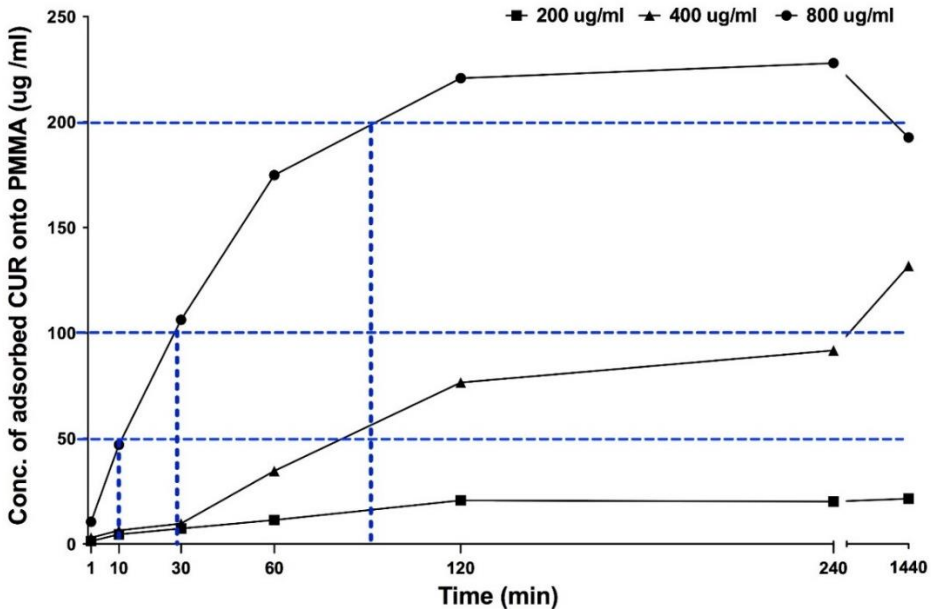


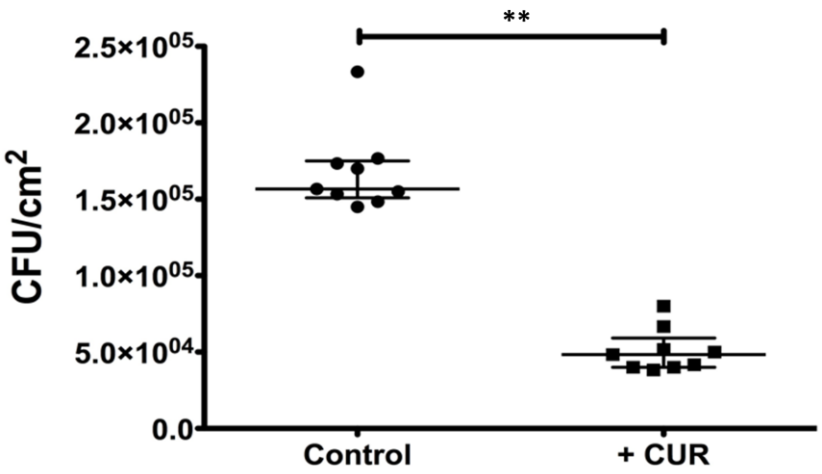
Figure 3.2: The adsorption capacity of CUR onto PMMA denture material. (i) Visible light spectrophotometric analysis of CUR in DMSO using a range of CUR concentrations, where the vertical line that contacts the peaks represents the absorption wavelength. **(ii)** Time and concentration dependent adsorption of CUR to PMMA (blue dotted line) at 0.5 x PMIC (50 µg/ml), PMIC (100 µg/ml) and $SMIC_{80}$ (200 µg/ml).

3.4.2 CUR adsorption reduces *Candida albicans* adhesion

Based on the data in the previous section, the focus was on immersion samples in 800 µg/ml of CUR for 10 min, which achieved 50 µg/ml on the surface of the PMMA for downstream analysis. Selection of a 0.5 x PMIC concentration was aimed to ensure exposure of the cells to a non-inhibitory concentration.

Next, the adherence capability of *C. albicans* SC5314 to PMMA denture material for 30 min was evaluated, where surfaces adsorbed with CUR (50 µg/ml) were compared to an unadsorbed control [Figure 3.3(i)]. A three-fold significant reduction in adhesion of *C. albicans* was observed ($p<0.004$), where the median number of adherent cells were reduced from approximately 1.56×10^5 to 4.8×10^4 cells/cm^2 with interquartile ranges of $1.5\text{-}1.75 \times 10^5$ and $4\text{-}5.9 \times 10^4$, respectively. Besides, SEM analysis showed a visible decline of adherent yeasts cells on the PMMA surfaces [Figure 3. 3(ii)].

(i)



(ii)

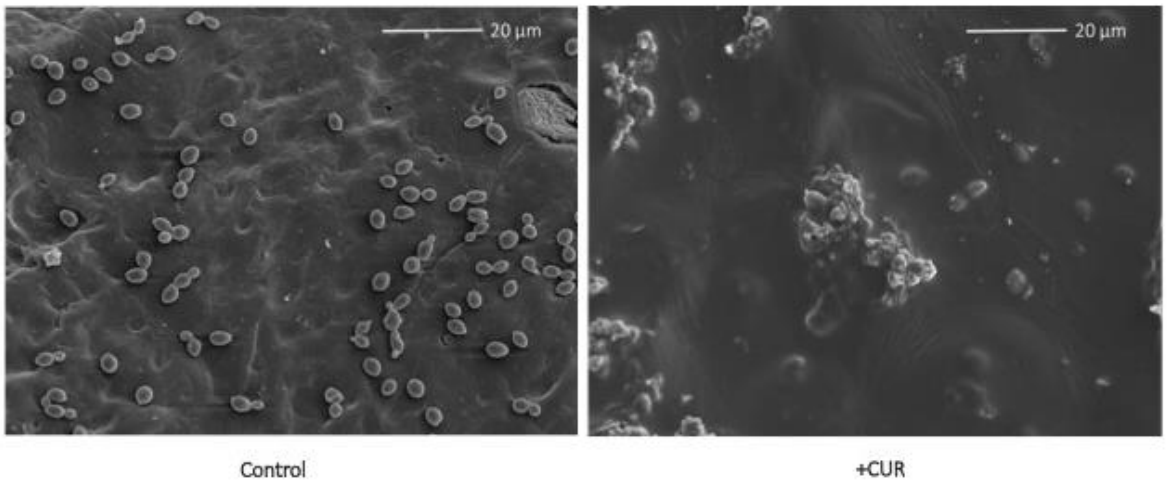


Figure 3.3: The impact of CUR adsorption to PMMA on *Candida albicans* adhesion. (i) *C. albicans* anti-adhesive capacity of approximately 50 µg/ml adsorbed CUR onto PMMA (+CUR) compared to non-adsorbed control surfaces, where a Mann-Whitney test was performed on data from nine independent experiments (n=9). All independent data points are presented, with error bars representing the median with interquartile range (**= $p < 0.01$). **(ii)** SEM micrographs of 30 min adherent *C. albicans* cells onto CUR adsorbed (+CUR) and non-adsorbed (control) PMMA surfaces. Scale bar is 20 µm.

Given that CUR adsorption showed capacity for a direct anti-adhesive effect, we explored the possibility that combining a short exposure to CUR with that of CUR adsorbed to PMMA would result in a synergistic increase in overall activity and prevent adhesion and colonization. *C. albicans* was exposed to CUR 50 µg/ml for 3 min (CA + CUR) then compared to both PMMA adsorbed with CUR (PMMA + CUR) or a combination of CUR exposed *C. albicans* and CUR adsorbed PMMA (PMMA CA

+ CUR). Figure 3.4 shows that the adhesion was reduced by 27% only with the short, direct exposure of *C. albicans* alone, which was lower than CUR adsorption alone (70% reduction), where a significant difference ($p<0.05$) was observed between these reductions. Moreover, when both the cell and surface were co-exposed to CUR a significant reduction of 93% ($p<0.001$) was observed.

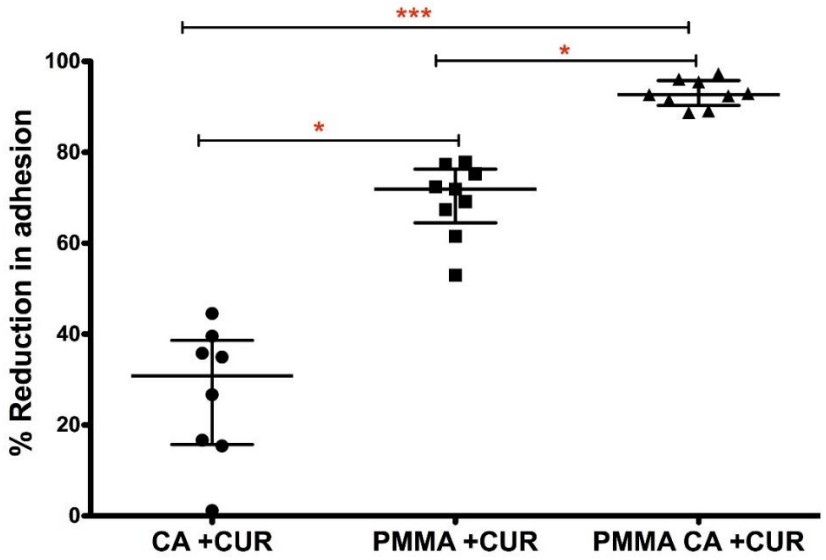


Figure 3.4: The impact of single and dual-treatment of CUR on *C. albicans* adhesion. A synergistic anti-adhesive capacity of (PMMA CA +CUR) was observed, where short pre-exposure of cells to CUR (CA +CUR) with PMMA adsorbed CUR (PMMA +CUR) were combined. The statistical analysis was performed using a Kruskal-Wallis test with Dunn's multiple comparison post-test performed on the data from nine independent experiments. The percentages shown relative to the control. All independent data points (n=9) are presented, with error bars representing the median with interquartile range (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4.3 CUR inhibits biofilm formation and enhances *Candida albicans* aggregation

The data above displayed a positive anti-adhesive impact to *C. albicans* with respect to the adsorption of CUR to PMMA surfaces, and displayed that a brief CUR pre-exposure (3 min) resulted in diminished adhesion of *C. albicans*, proposing that sub-inhibitory concentrations can provoke some biological activity. To further investigate this, the CUR pre-exposure time on *C. albicans* was extended. It was shown that increasing the pre-exposure time from 3 to 30 and 90 min,

respectively, significantly improved anti-adhesion ($p>0.01$). However, between 30 to 90 min there were no significant enhancements in anti-adhesive capabilities (Figure 3.5).

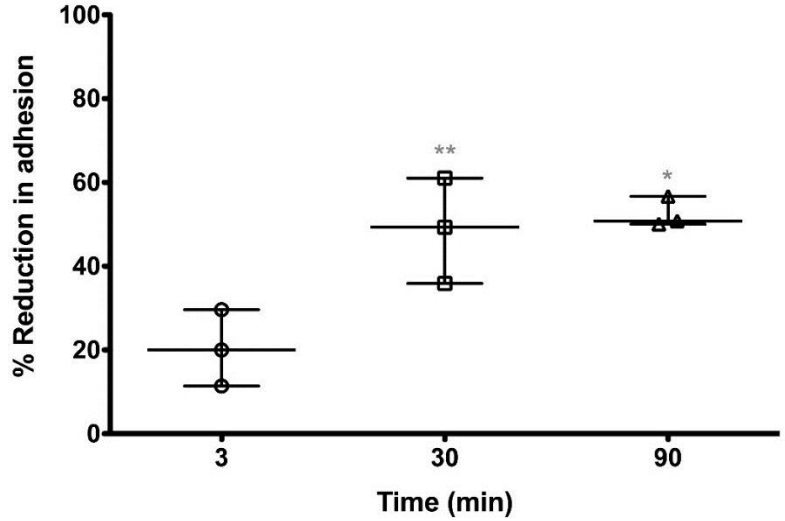
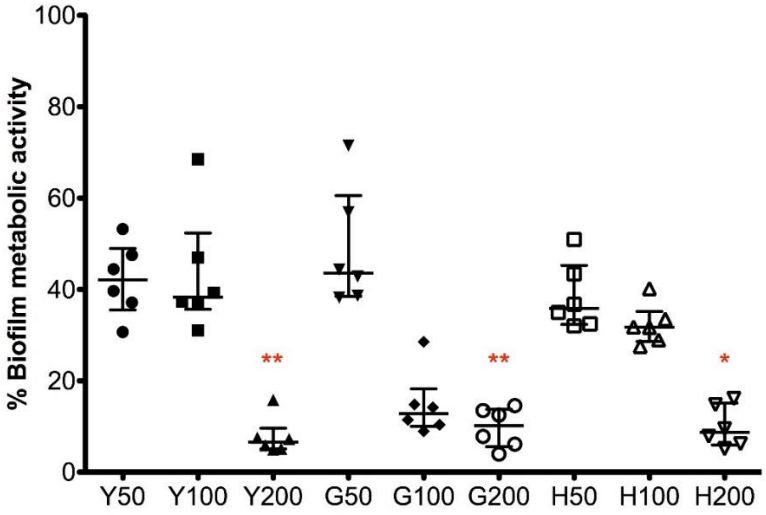


Figure 3.5: Impact of longer-term CUR pre-exposure on candidal adhesion. *C. albicans* SC5314 was pre-exposed to 0.5 x PMIC sub-inhibitory concentration of CUR (50 µg/ml) for 3, 30 and 90 min and adhesion to PMMA was evaluated in triplicate in 3 independent experiments. The percentages shown are relative to their respective controls. All independent data points are presented, with error bars representing the median with interquartile range (* $p < 0.05$, ** $p < 0.01$).

Next, the longer-term impact of CUR and response of the different morphological forms of *C. albicans* to CUR and how this could impact biofilm formation was investigated. To achieve this aim, yeast cells (Y = 0 min), germlings (G = 120 min) and hyphae (H = 240 min) were prepared prior to CUR exposure at 50, 100 and 200 µg/ml, which were then incubated for 24 h to develop biofilms. The developed biofilms were assessed using metabolic and biomass data [Figures 3.6(i) and (ii)]. The resultant metabolic and biomass data mostly showed similar patterns, where at lower sub-inhibitory concentrations (50 µg/ml) and PMIC levels (100 µg/ml) the anti-candidal properties moderately impacted the overall biofilm metabolism and biomass with a lesser effect on the latter. At SMIC levels (200 µg/ml) a significant drop in biofilm metabolism was observed for Y and G of approximately >90% of the control ($p<0.01$), though H cells were least impacted ($p<0.05$).

That considerable anti-biofilm effect of SMIC concentration on metabolism was less noticeable on biofilm biomass, with statistically significant 85% to 54% reduction in Y and G ($p<0.05$), respectively, dropping to an insignificant 11% reduction in H cells. The inhibited biofilms were microscopically examined, where the light microscope images showed an anti-biofilm impact on all biofilms investigated in a concentration and morphological form dependent manner (Figure 3.7).

(i)



(ii)

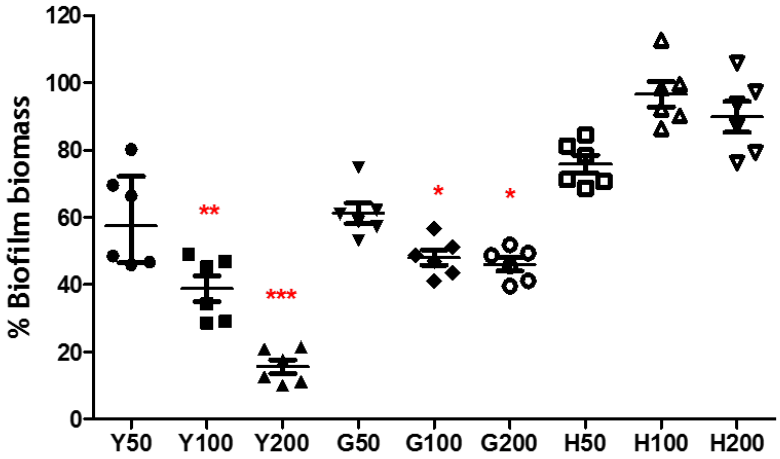


Figure 3.6: The impact of CUR on *C. albicans* biofilm formation. Three morphological forms of *C. albicans* SC5314 (Y = yeast; G = germlings; H = hyphae) were exposed to CUR at different concentrations 50,100 and 200 µg/ml, and after 24 h the resultant biofilm formation assessed through metabolic **(i)** and biomass **(ii)** analytical assays. Data represents six independent experiments, Kruskal-Wallis test with Dunn's multiple comparison post-test were used for statistical analysis.

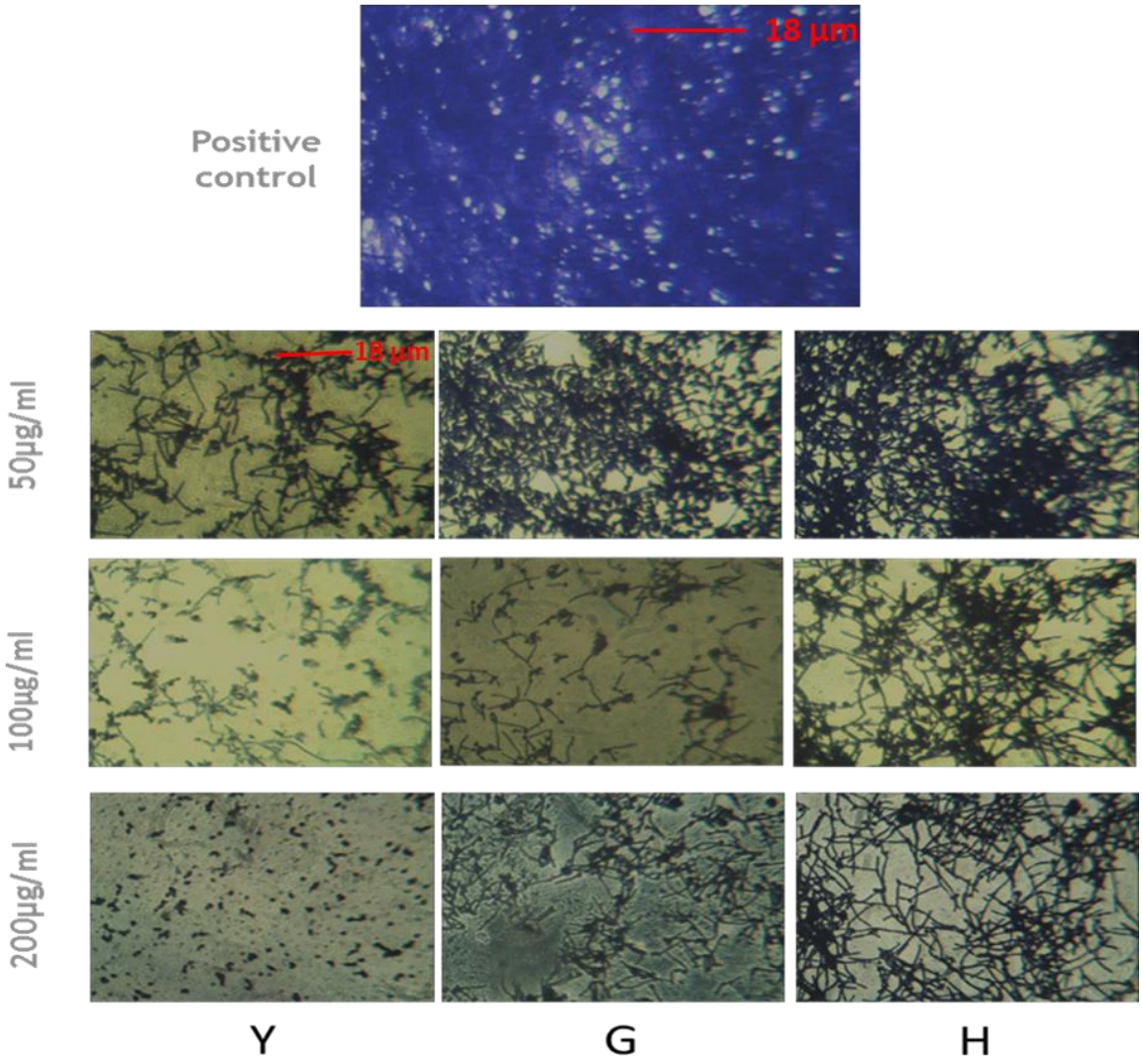
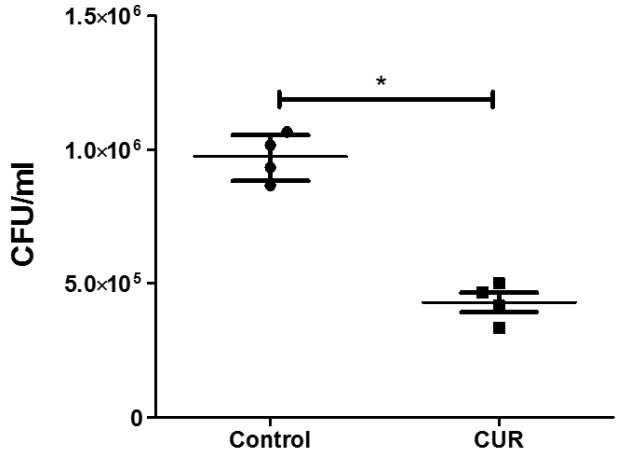


Figure 3.7: The impact of CUR on *C. albicans* biofilm architecture. Light microscope images of biofilms tested, which show a reduction in the biofilm mycelial quantity and density in a concentration and morphological form dependent manner, images acquired at 250 X magnification. Scale bar 18 µm. (Y = yeast; G = germlings; H = hyphae)

To assess whether CUR induces aggregation through alteration of the cell wall surface a plate counting based approach was performed. To quantify aggregation it was hypothesized that sub-MIC levels would lower the resultant CFU compared to its respective control, where each aggregate would produce only one CFU and reduce the overall homogeneity of the suspension. Indeed, a significant drop in CFU counts was demonstrated in the CUR group ($p<0.01$) [Figure 3.8(i)], which was additionally confirmed microscopically [Figure 3.8(ii)].

(i)



(ii)

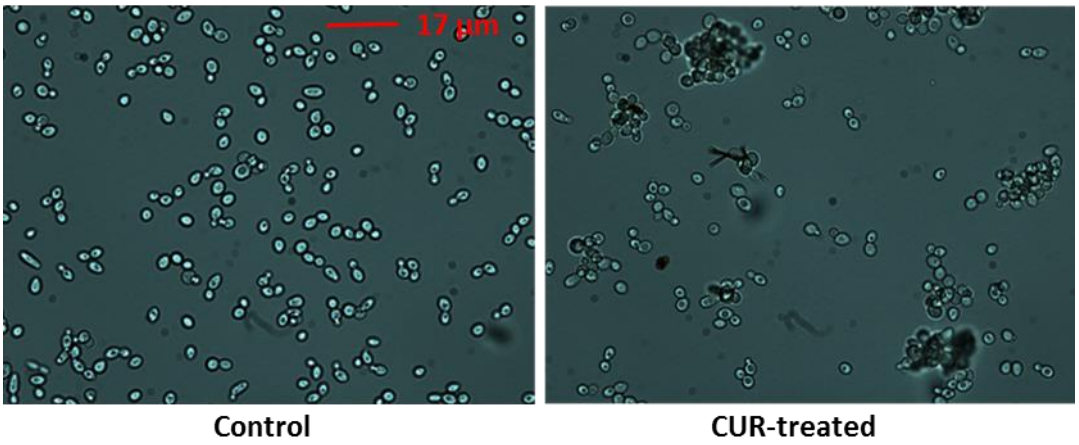


Figure 3.8: The impact of CUR on aggregation of *C. albicans*. (i) Aggregation of *C. albicans* SC5314 treated with (50 ug/ml) was evaluated by total viable cell counts, analysed using a Mann-Whitney test on triplicate data from 4 independent experiments, All independent data points are presented, with error bars representing the median with interquartile range (* $p < 0.05$). Additionally, validation of the phenotype by light microscopy (400 X magnification) was performed **(ii)**, where aggregation possibility was observed. Scale bar is 17 µm.

3.4.4 CUR reduces cell surface hydrophobicity of *Candida albicans*

Given the potential positive relationship between the microbial CSH and its adhesive capability (Wang *et al.*, 2015), this was tested for CUR with respect to *C. albicans* using the aqueous-hydrocarbon biphasic partitioning assay. The resultant data showed an overall significant reduction in the CSH of CUR exposed *C. albicans* at all time-points investigated, with 13, 51 and 37% reduction (Figure 3.9). Additionally, 30 min of CUR exposure showed a statistically significant difference compared to 3 min ($p < 0.05$). These data suggested reduction of *C. albicans* CSH as a potential biological mechanism of CUR antiadhesive capability.

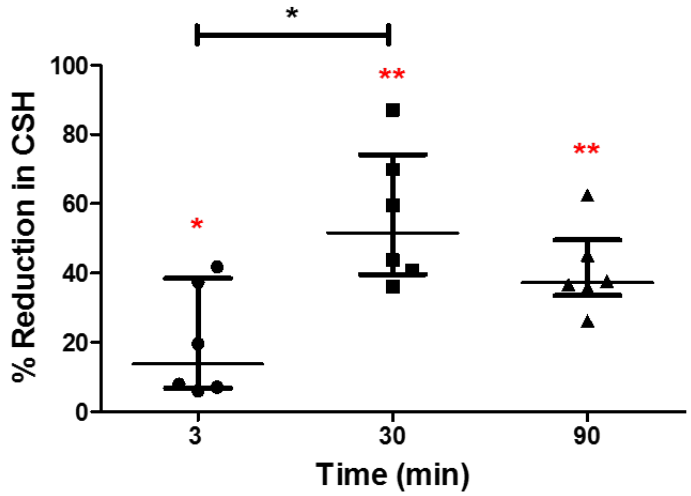


Figure 3.9: The impact of CUR on cell surface hydrophobicity of *C. albicans*. Kruskal-Wallis test with Dunn's multiple comparison post-test were used for statistical analysis. All independent data points are presented, with error bars representing the median with interquartile range (* $p < 0.05$, ** $p < 0.01$). Red asterisks represent the significant difference between each unexposed control and its Cur exposed at the relevant time point.

3.4.5 CUR affects the temporal expression of *Candida albicans* adhesins

The data above suggested that CUR induced biological effects on *C. albicans*, most considerably on preventing biofilm development, influencing aggregation and reducing CSH. Therefore, the aim was to investigate a panel of related genes through transcriptional analysis. To perform this, Y and H cells at 3, 30 and 90 min post CUR exposure were prepared. Graphical and visual representation of the data obtained were featured in bar charts (Figure 3.10) and heat map analysis with hierarchical clustering (Figure 3.11).

It was shown that that Y cells (Figures 3.10i, ii & iii) exposed to CUR showed temporal changes in gene expression, most notably the progressive down-regulation of the most potent adhesion-related gene *ALS3* that became statistically significant after 90 min of CUR exposure. Whereas *ALS1*, which was related to *ALS3* as the hierarchical clustering illustrated, showed a variable modulation in response to CUR, where it was significantly down-regulated at 3 min ($p< 0.01$) and then up-regulated at 30 and 90 min, though it was significant at 30 min only. However, the clustered aggregative and flocculation genes *AAF1*, *EAP1*, and *ALS5* transcripts were all significantly up-regulated ($p< 0.05$-0.01) in a time-dependent manner.

The clustering of expression for the H cells (Figures 3.10iv, v & vi) was similar to that of Y cells, and the patterns showed moderate similarity. More specifically, *ALS3* gene expression was significantly down-regulated ($p<0.05$) at 30 and 90 min time points though it was non-significantly up-regulated at 3 min. Whereas, *ALS1* showed non-significant up-regulation up to 30 min and down-regulation at 90 min. *ALS5*, *EAP1* and *AAF1* were considerably upregulated with *AAF1* showing the highest levels of expression at 30 min in comparison to the control, and reciprocally *ALS3* being the most down-regulated. The heat map clustering (figure 3.11) showed the relative contrast between the main two gene clusters in response to CUR exposure, where the expression of *ALS5* and *ALS3* genes were clear examples. Levels of differential expression were consistently higher in the Y than H cells.

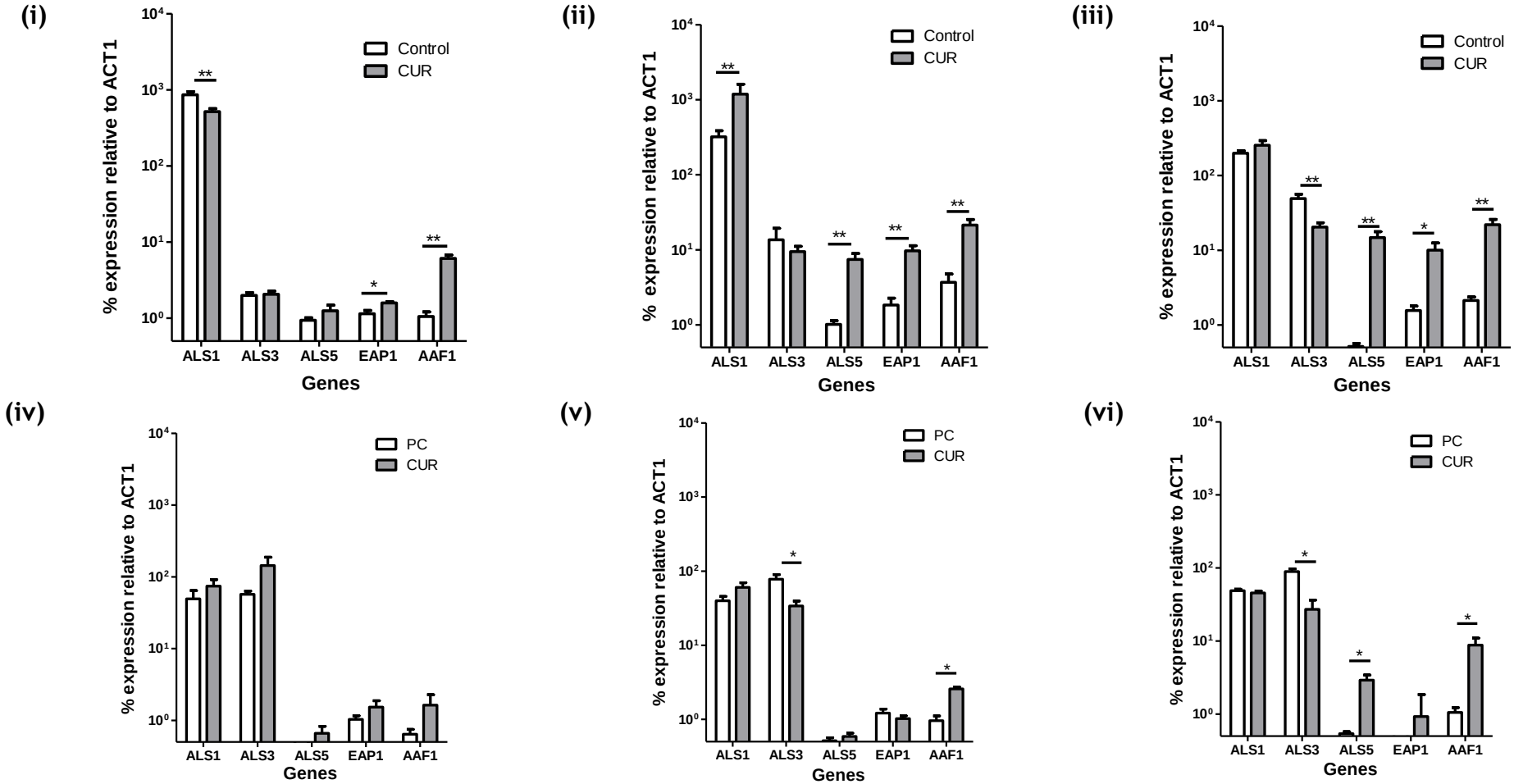


Figure 3.10: Transcriptional expression of CUR-treated *C. albicans*. Y cells **(i, ii & iii)** were prepared and exposed to CUR for 3, 30 and 90 min, respectively. The same was also performed to the H cells **(iv, v & vi).** Expression of *ALS1, ALS3, ALS5, EAP1* and *AAF1* were then assessed using qPCR and relative gene expression assessed the *ACT1* housekeeping gene. An unpaired *t* test was used (*p < 0.05, **p < 0.01), where a natural log transformation was performed on the data. Error bars represent standard error of mean.

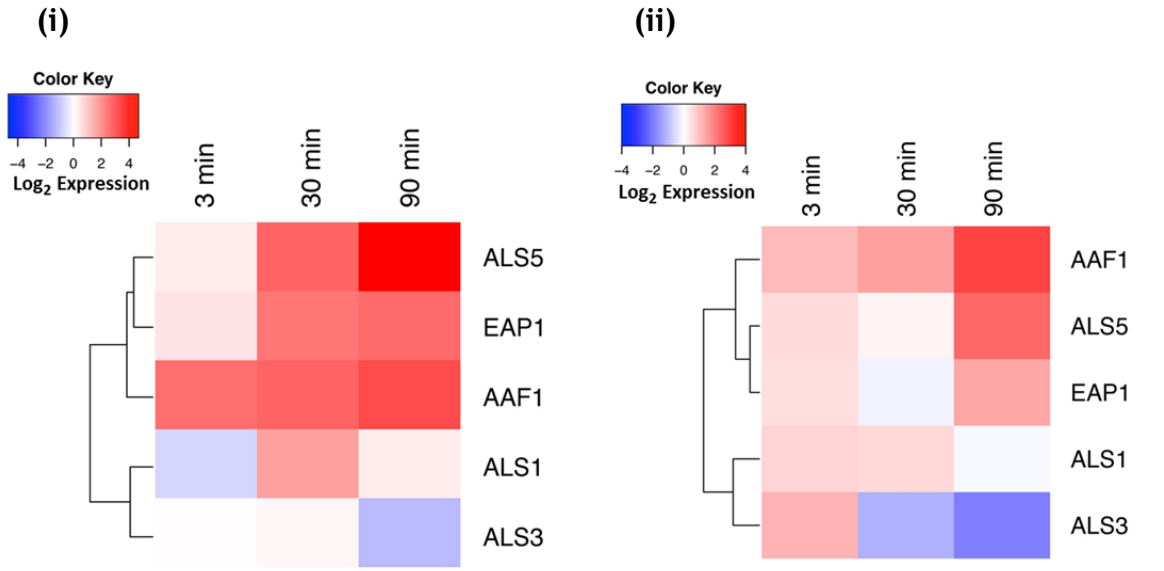


Figure 3.11 Visual transcriptional analysis of CUR treated *C. albicans*. (i) Y and (ii) H cells. Heatmap and clustering was performed for the differential expression of genes (log_2).

3.4.6 PMMA-incorporated CUR does not inhibit Candida albicans adhesion

Based on the previous data, adhesion of *C. albicans* was reduced when freely adsorbed CUR was permitted to physically adsorb to the already cured (completely polymerized) PMMA surface. Therefore, it was hypothesized that incorporation of CUR within PMMA may provide longer sustained release of bioactive concentrations. The effect of incorporation of CUR into the uncured PMMA was tested by adding CUR to the monomer (200 µg/ml) before mixing it with the powder, and candidal adhesion (30 min) tested using Miles and Mirsa plate counting methodology. Figure 3.12 shows the non-significant difference in the number of the counted CFU between the CUR-incorporated and control ($p>0.05$). Thus, incorporation of CUR during fabrication of PMMA denture material did not show the predicted impact on *C. albicans* adhesion. To investigate why this was not the case, CUR release from the processed CUR-incorporated PMMA was spectrophotometrically assessed. No CUR release was observed, suggesting covalent chemical interaction between CUR and PMMA monomers that could restrict CUR solubility and release.

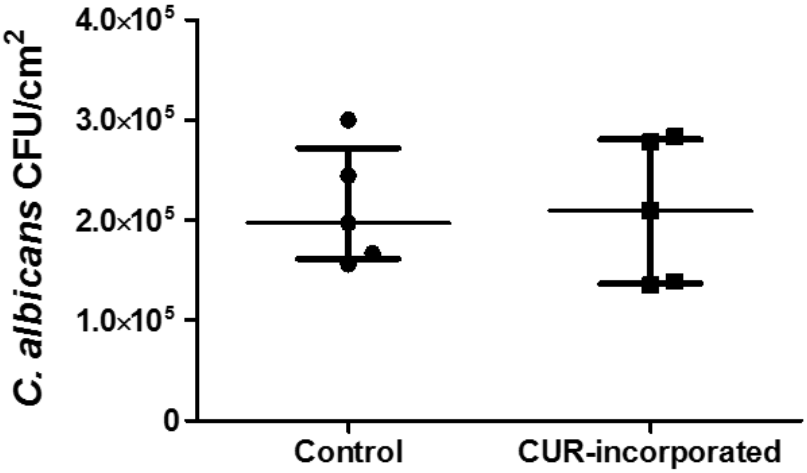


Figure 3.12: The non-effective role of CUR incorporated to PMMA on *Candida albicans* adhesion. *C. albicans* anti-adhesive capacity incorporated CUR to PMMA (CUR-incorporated) compared to non-incorporated control surfaces, and a Mann-Whitney test was performed on data from 5 independent occasions, where 3 surfaces were used as replicates and an average was considered ($p>0.05$). The averaged independent data are presented, with error bars representing the median with interquartile range.

3.5 Discussion

The potent biological activities of the polyphenol CUR has enabled some researchers to describe it as a modern biological regulator (Esatbeyoglu *et al.*, 2012). The data presented in this chapter display the possibility for use of CUR within the perspective of oral health for denture wearers. It has been shown that CUR can adsorb to denture substrates and reduce *C. albicans* adhesion, rather than actively kill or inhibit the microorganisms. Remarkably, exposure of *C. albicans* to CUR elicited cellular aggregation, an effect that also reduced its adhesion capacity, besides the decline in the cell surface hydrophobicity. Transcriptional analyses revealed that the key ALS3 adhesin [the most important 'king' of the ALS family as illustrated by Hoyer *et al.* (2008)] was negatively impacted, whereas genes associated with aggregation were positively impacted. Collectively, the data shown reveal that CUR has the potential to be used in denture care as a means of preventing *C. albicans* biofilms and subsequently denture-induced stomatitis (O'Donnell *et al.*, 2015a).

Initially, the aim was to evaluate and confirm CUR antimicrobial capabilities. These data are in general agreement with others (Martins *et al.*, 2009;Sharma *et al.*, 2010b;Khan *et al.*, 2012), displaying that concentrations around 100 µg/ml are necessary to inhibit cellular growth. Besides, 200 µg/ml demonstrated a considerable anti-biofilm activity (Shahzad *et al.*, 2014). For further confirmation, 0.5 x PMIC concentration (50 µg/ml) showed no impact on the cellular growth kinetics (Neelofar *et al.*, 2011). Any discrepancies noted might be attributed to the protocols used for the broth microdilution method, variability of strains used, variability of CUR source and purity and proportions of curcuminiods involved.

Considering the principal interest of this research was in preventing *C. albicans*, rather than actively inhibiting and killing *C. albicans*, then the focus was on using a lower sub-inhibitory concentration (half PMIC) of 50 ug/ml. This was motivated by the hypothesis that CUR could be clinically used as a supplement, or as part of a nutritional regimen, where, the concentration of CUR could be maintained through salivation along with adsorbing on to the oral tissues and the related prostheses.

The results presented herein indicate that approximately 6% of the CUR provided can be adsorbed to the prosthesis surface within 10 min. Theoretically, polyphenol CUR might be directly and indirectly delivered to the oral cavity. The direct method would be during ingestion, and indirectly when it is absorbed by the digestive system and becomes bioavailable to bodily fluids, i.e. blood and saliva. Indeed, literature showed that polyphenols have high affinity for the salivary proteins, especially the proline-rich proteins and histatins (Bennick, 2002;Soares *et al.*, 2011). The daily CUR consumption is very variable across cultures, for example, in Nepal and India daily consumption of CUR can reach up to 100 mg (Shahzad *et al.*, 2015), and in South Korea this may only reach 15 mg (Kwon, 2014), whereas, in the United Kingdom, according to the European Food Safety Authority, it may reach up to 0.9 to 3.3 mg/kg of the body weight daily, which is equivalent to 60-230 mg/day (EFSA, 2010). Therefore, providing an anti-candidal concentration just through nutritional approach is not without challenges, though the potential of achieving an anti-adhesive concentration is a more conceivable goal. Indeed, it was possible to show that 50 µg/ml could be adsorbed onto PMMA, the polymer most commonly used to construct denture prostheses, within a relatively short time. The resultant adsorption optimisation process showed that the optimised CUR-adsorbed PMMA reduced *C. albicans* adhesion by up to 70%, which was further improved to 93% with a short CUR pre-exposure of *C. albicans*. This combination revealed that CUR has dual functionality, through surface adsorption and directly against *C. albicans* producing a synergistic-like effect.

For further understanding of the biological function of the sub-inhibitory CUR effect, a series of studies were undertaken to define its impact on the kinetics of adhesion, whilst also evaluating how its influence on the dissimilar morphological forms of *C. albicans*. The Ramage laboratory have formerly shown that other natural molecules, such as tea tree oil (TTO) derivatives and carbohydrate-derived fulvic acid (CHD-FA) affect *C. albicans* colonisation and growth depending on the stage of biofilm development (Ramage *et al.*, 2012b;Sherry *et al.*, 2012). Therefore, it was reasoned that CUR may also act on *C. albicans* in a similar manner. It was shown that prolonged exposure (30 and 90 min) of *C. albicans* yeast cells considerably minimised its adhesive capability onto PMMA, signifying that CUR was capable of modifying its adhesive capability in some manner, which was

further investigated through observing cells grown to different phases of morphological development, namely yeast, germling and hyphae cells. It was hypothesised that the morphological phase of *C. albicans* at which the cells were treated with CUR could influence overall development and integrity of the biofilm. Indeed, it was shown that all treated morphological forms exhibited reduced general biofilm formation, though only the inhibitory concentrations, specifically 200 µg/ml, reduced the yeast and germling cells developed biofilms in a significant way in comparison to the sub-inhibitory concentration. However, hyphae cells were not influenced in a concentration dependent manner, suggesting that the efficacy of CUR was more apparent against immature morphological phases. Remarkably, though the biofilms were generally reduced compared to control levels, there were still significant biofilms remaining, indicating once more the advantage of early preventative intervention. A key limitation of this interpretation is the sample sizes used during these analyses (Vaux, 2012). Indeed, it raises questions whether the statistical analyses are valuable, which is why individual data points are presented. Nonetheless, when the data is examined in its entirety there are indicative trends that CUR demonstrates positive biological influences, though further research is required to confirm these data.

The anti-oxidative and hydrophobic capacities of the polyphenol CUR molecules (Priyadarsini, 2013;Mirzaei *et al.*, 2017) might give an explanation why it preferentially adsorbs to PMMA and the *C. albicans* cell wall. It was hypothesised that the hydrophobic nature of the CUR molecule might initiate an aggregation process of the coated *C. albicans* with one another. This notion could be of importance within the context of oral delivery with the saliva, specifically if the possibility of creating complexes of cells that reduce their interaction with the denture surface was considered, which also may minimise the opportunity for individual cells to adhere and be shielded inside denture surface crevices and imperfections. Indeed, this was verified both quantitatively and visually, which could also explain why a synergistic inhibition of adhesion at sub-inhibitory concentrations was observed.

Further mechanistic causes of microbial aggregation might result from failure of daughter cells separation, as in *Candida auris* (Borman *et al.*, 2016). The possibility of this to occur in response to CUR in this study is low because PBS was used as a non-nourishing medium although the phenomenon might occur when CUR- exposed *C. albicans* tested in nourishing media. The other potential cause is an increase of the cell surface hydrophobicity (CSH). Therefore, the CSH of CUR-exposed (50 µg/ml) *C. albicans* was tested over different periods of exposure.

In parallel, the effect of sub-inhibitory concentration of CUR on reducing the CSH of *C. albicans* was demonstrated. Several expressed proteins participate in CSH modulation (Bujdáková *et al.*, 2013). The CSH experiment helped to achieve two objectives in a single action. It suggested a potential mechanism for the anti-adhesive effect of sub inhibitory concentration of CUR, and also these experiments supported the notion of coating of the cells with CUR, because the CSH experiment was modified by washing the cells with diluted DMSO to remove the proposed coating CUR. This data comes in line with several studies that confirmed the positive association between CSH and adhesion to surfaces (Krasowska & Sigler, 2014;Ellepola *et al.*, 2016;Wang *et al.*, 2017b), though some reports did not confirm this relationship (Bujdáková *et al.*, 2013;Silva-Dias *et al.*, 2015) and others presented a complicated role for CSH in adhesion (Hoyer & Cota, 2016). We can suggest that the decrease of *C. albicans* CSH in response to CUR exposure is an evasion endeavor from these cells to distance themselves from the organic hydrophobic molecules, which resembles a mechanism observed in hydrophilic microorganisms that protect themselves from attachment of organic molecules (Krasowska & Sigler, 2014).

An inherent range of genetic and morphological properties of *C. albicans* provide it with enhanced capacity for colonisation and biofilm formation (Blankenship & Mitchell, 2006). Finding strategic approaches to controlling these properties offers potential for innovative anti-candidal treatments. Mechanistically, it was intriguing to understand how CUR elicited *C. albicans* specific effects. A previous study has demonstrated that CUR has the capability to modulate the global repressor of filamentation *TUP1* (Sharma *et al.*, 2010b). Indeed, Ramage's research group showed that *HWP1*, which is a key hyphal wall associated element,

was down-regulated when a focused study on mature biofilms was conducted (Shahzad *et al.*, 2014). To this end, a transcriptional approach was employed to assess genes involved in adhesion and aggregation. CUR downregulated the *ALS3* gene in both yeast and hyphae cells; *ALS3* has a well-established and key role in *C. albicans* adhesion, and plays a pivotal role in biofilm formation and invasion (Zhao *et al.*, 2004;Nobile & Mitchell, 2005;Hoyer *et al.*, 2008;Liu & Filler, 2011). The down- regulation of such an important gene results in a reduction in cellular adhesive capacity. However, the *ALS1* gene was variably modulated by CUR and its transcriptional data did not steadily come in a line with the adhesion data. Indeed, it was reported that *ALS1* has less impact on adhesion than *ALS3* and complicated interrelationships among *ALS* family members have been observed (Zhao *et al.*, 2004). Nevertheless, both genes have been shown to have significant functions in early biofilm events (Nailis *et al.*, 2009;Fox *et al.*, 2015).

There is controversy about the role of the ALS5 gene in initial biofilm formation and adhesion of *C. albicans* (Hoyer et al., 2008). *ALS5* has a less well-defined function within the ALS family, and even though defined as an adhesin, functionally it appears to have amyloid characteristics and the capability to enhance aggregation (Rauceo *et al.*, 2004;Garcia *et al.*, 2011). This aggregation-driving role might explain why it is up-regulated following exposure of the *C. albicans* cells to CUR and this corresponds to the observed phenotype in this study. Furthermore, the expression of *AAF1* was also up-regulated by CUR in both morphological forms tested, which is a gene highly associated with flocculation and aggregation (Fu *et al.*, 1998). It was interesting that *AAF1* appears to have an insignificant adhesive function (Rieg *et al.*, 1999), subsequently further supports the developing concept of the phenotypes and anti-adhesive capacities detected next to exposure to CUR.

Surprisingly, *EAP1* gene expression manifested comparable trends to *ALS5*, although this gene expresses a protein recognised to improve adhesive properties and biofilm formation (Li *et al.*, 2007;Fox *et al.*, 2015). It was expected that we would see a similar level of down-regulation to that of *ALS3*. This unexpected data suggests that *EAP1*, whereas displaying these adhesion driving capacities, might have supplementary functions in cellular aggregation, nevertheless this

necessitates further research. Collectively, these molecular data reveal the capacity of sub-inhibitory concentrations of CUR to elicit the expression of biological properties that could be beneficial in reducing colonisation of *C. albicans*.

Finally, it was intriguing to investigate the possibility of CUR-incorporated denture material to induce effective anti-adhesive capability. CUR-incorporated polymer conjugates have previously shown beneficial biological activity (Yoncheva *et al.*, 2015;Requejo-Aguilar *et al.*, 2016), where water-soluble polymers were considered to facilitate the delivery of the drugs. Hence, it was not surprising that incorporation of CUR inside the water-insoluble PMMA denture material during fabrication did not demonstrate the potential anti-adhesive capacity. Furthermore, this also could be attributed to the very low solubility of CUR in water (Esatbeyoglu *et al.*, 2012), and the potential chemistry between the CUR molecules and PMMA polymer, where CUR may be bound within the PMMA. Indeed, submersion of the incorporated PMMA in DMSO solvents for 24 h showed absolutely no CUR release, which further supports our explanation.

While it is true that existing advice on denture cleaning will largely allow patients to maintain oral health these do not appear to be being followed consistently leading to a high prevalence in disease. This study explores the opportunities to circumvent to a certain extent the poor compliance with existing denture hygiene measures enhancing the effectiveness of existing approaches through dietary intake of biologically significant polyphenols such as CUR. Not only is CUR active against *C. albicans*, but against other pathogens of the oral cavity such as periodontopathic bacteria (Shahzad *et al.*, 2015). Taking into consideration the high diversity of the microbiome and mycobiome of denture wearers (O'Donnell *et al.*, 2015b), then the wide-spectrum of activity shown means that CUR has the potential to be beneficial in areas of oral health beyond denture health. The identified biological activity of CUR would support further investigation and development of a CUR based solution as a denture soak. Such a solution could have the potential to effectively manage adhesion of these pathogenic biofilm related microorganisms. It is also possible that CUR could be used in conjunction with other effective strategies for example photoactivation. There is a potential

to promote the CUR antimicrobial activity induced via photoactivation (Cieplik *et al.*, 2015), which may yield both a dual preventative and decontaminative strategy. Indeed, clinical research has previously demonstrated an additive advantage to photodynamic therapy (PDT) alone (Pereira *et al.*, 2015), as well as in conjunction with CUR in the context of oral healthcare measures (Leite *et al.*, 2014). The mechanistic philosophy of PDT works through locally acting light-activated photo-antimicrobial particles that generate excessive quantities of highly reactive oxygen species (ROS). The dramatic increase of ROS has a damaging effect on the targeted site of action (Wainwright *et al.*, 2017). For this reason, PDT could provide an additional advantage in improving the low concentrations of orally supplied natural antimicrobials, where it might be light-activated bi-daily or more frequently.

Nevertheless, establishment and maintenance of activatable concentrations that exert an anti-adhesive influence might represent a hurdle. Consideration must be given to the route of delivery of these active particles. Another potential way to use this molecule would be to partner with nanotechnological strategies, such as the formation of nanosized CUR, which has previously been demonstrated to enhance the biological activity and cellular response (Gopal *et al.*, 2016), and would optimise our capability to deliver biologically active concentrations. Another hurdle is in the potential discoloration of the dental prosthesis and related oral tissues due to the yellowish colour of CUR, though some CUR analogous or derivatives such as the tetrahydrocurcumin have overcome this chemical property (Aggarwal *et al.*, 2014).

Chapter findings

1. CUR can be adsorbed to PMMA denture material up to effective concentrations in a time and concentration dependant manner.

2. Adsorption of CUR to PMMA reduces *C. albicans* adhesion.

3. CUR has the biological activity to reduce the adhesion of the pre-exposed *C. albicans*.

4. Combination of adsorption of CUR to PMMA with pre-exposure of *C. albicans* to CUR provides a synergistic antiadhesive-like effect.

5. CUR inhibits the metabolic and biomass of *C. albicans* biofilms in a morphological form, concentration and time point dependent manner.

6. CUR induces *C. albicans* aggregation.

7. CUR reduces the CSH of *C. albicans*.

8. CUR modulates adhesion and aggregation associated genes within a relatively short time.

9. Incorporation of CUR inside PMMA denture material does not induce biological activity.

4 The effect of micro and nano-patterned surfaces on *Candida albicans* colonisation

4.1 Introduction

Current anti-biofilm strategies for dental biomaterials, such as coating and sustained release of antimicrobial molecules, remain under-developed and unexploited (Øilo & Bakken, 2015). Scientists have recognised that surface modification is an effective strategy to fight microbial biofilms, though chemical alteration using polyethylene glycol brush-like surfaces, and incorporation of antimicrobial compounds such as antibiotics, silver ions, cationic peptides and quaternary ammonium, whilst transiently effective, eventually lose efficacy (Perera-Costa *et al.*, 2014). Moreover, the functional chemical moieties of the surface can be masked by the microbial secretions and adsorbed host proteins (Perera-Costa *et al.*, 2014), and there is also the potential for associated cytotoxicity (Wang *et al.*, 2017a). Therefore, developing anti-fouling mechanisms through a physical alteration appears promising, as this would negate some of these negative consequences of chemical modification.

The commensal *C. albicans* has the capacity to switch morphology and become a versatile opportunistic pathogen through its capacity to produce hyphae and a range of adapted virulence factors (Nobile & Johnson, 2015). The adhesion capacity of *C. albicans* to biotic and abiotic surfaces represents a key step in a key virulent attribute, i.e. biofilm formation (Fox *et al.*, 2015). Interference with *C. albicans* adhesion and biofilm formation through a natural chemotherapeutic approach was presented in the previous chapter (Chapter 3). Chemical modification of the denture material surface has been reported elsewhere (Park *et al.*, 2008), which involve changing the chemical properties of the polymer surface. Physico-chemical modification of plastic polymers is also reported with plasma modification through bombarding the surface with a beam of gas in a plasma status to modify the surface roughness, wettability and adhesion properties (Ozdemir *et al.*, 1999). More specifically, plasma physico-chemical treatment for denture polymers was also reported, which was designed to increase the surface free energy and minimise the microbial adhesion (Zamperini *et al.*, 2013;Qian *et al.*, 2016). However, there is lack of information within the literature regarding purely physical modification approaches of denture material surface

that is devoid of any chemical processing. Thus, focussing on this area of research seems appropriate and timely.

The problem that exists with denture substrates is that the increase of uncontrolled surface roughness offers shelter and protection for microorganisms from the host salivary dynamics and mastication, as well as adjunct cleaning shear forces. Moreover, uncontrolled surface topography enhances the surface area of attachment to support further microbial retention (Charman *et al.*, 2009). This was elucidated by Boyd and Verran, as they demonstrated the increase of the adherence capacity of *Staphylococcus aureus* to unpolished and randomly abraded stainless steel surfaces (Boyd & Verran, 2002). On the other hand, antifouling and self-cleaning natural systems such as lotus leaf effect and shark skin, which are characterised by characteristic hydrophobicity and distinctive well organised topographies at micro/nano scale are main drivers in the interest of the interaction of micro-nano-topographical features with microorganisms and subsequent biofilm formation (Gu & Ren, 2014). Therefore, controlled surface topographies may provide a positive benefit for these clinical problems.

Nano-fabricated surfaces are firmly established in the electronic engineering and optical studies, while in the biology and clinical fields this remains a poorly explored area of research (Anselme *et al.*, 2010). Pioneer research endeavours on cell- nano-structured substrate (Dalby *et al.*, 2002;Teixeira *et al.*, 2003) and microorganism- nano-structured substrate (Diaz *et al.*, 2007;Ploux *et al.*, 2009) interactions reported the induction of a biological response to the nano-structured materials, suggesting that similar approaches could have potential antimicrobial effects on nano-structured denture surfaces. During the last decade, several studies have investigated the biomedical interaction of the biomimetic and bioinspired micro-nano patterned topographies with the bacteria that showed promising antifouling results (Ploux *et al.*, 2009;Crawford *et al.*, 2012;Diu *et al.*, 2014;Gu & Ren, 2014;Perera-Costa *et al.*, 2014). However, conflicting results have also been observed with microbial attachment to engineered surfaces (Hsu *et al.*, 2013). To date, at the time of writing this thesis, only one study investigated adhesion of a fungal species with micrometre and sub-micrometre dimensionally patterned surfaces (Whitehead *et al.*, 2005). Here, titanium-coated surfaces were

used that did not show any significant differences between the regularly distributed micrometre and non-regularly distributed sub-micrometre featured surfaces on the retention of *C. albicans*. This chapter aimed to take this one step further and investigate the interaction between micro/nano topographies and *C. albicans*.

4.2 Aims

The general objective of this chapter is to explore the possibility of development of an engineered patterned denture material surface that could enhance the anti-adhesive capacity of *C. albicans* colonisation. Therefore, it was important to explore the possibility of the most widely used denture material (PMMA polymer) to replicate topographies of micro-, sub-micro and nano-scale features.

The specific objectives are as follows:

1. To investigate the capacity of PMMA denture material to replicate micro-, submicro- and nano-scale features.

2. To investigate the adhesion of *C. albicans* on engineered surfaces of micro and sub-micro features of different forms.

3. To investigate the adhesion of *C. albicans* on nano-scale featured surfaces.

4. To evaluate the molecular response of *C. albicans* to the engineered surfaces.

5. To evaluate the wettability of the engineered surfaces and the surface roughness of the non-patterned polymers.

6. To assess the efficacy of combining the engineered-patterned surface with CUR adsorption on the adhesion and biofilm formation of *C. albicans*.

Some of the shown data in this chapter has been published in "Nanomedicine: Nanotechnology, Biology and Medicine" journal.

Hasanain Alalwan, Christopher J. Nile, Ranjith Rajendran, Robert McKerlie, Paul Reynolds, Nikolaj Gadegaard, Gordon Ramage. Volume 14, Issue 3, April 2018, Pages 1045-1049.

Findings from this chapter have been presented at the following academic meetings:

1. Oral presentation for OMIG Postgraduate Research Prize Symposium in College of Clinical Dentistry in University of Sheffield on 8th of Feb, 2017.

2. Poster presentation in the annual conference of the British Society of Prosthodontics (BSSPD) in London on 6th April, 2017.

4.3 Materials and methods

4.3.1 Replication of micro/nano-featured materials

The polymer surfaces that were used and colonised with *C. albicans* in this study were engineered topographies of thermoplastic polycarbonate and PMMA denture materials. Production of the master patterns and micro- and the nano-fabricated polycarbonate topographies was performed by Dr Paul Reynolds in collaboration with Professor Nikolaj Gadegaard lab, Biomedical Engineering, School of Engineering, University of Glasgow.

4.3.1.1 Replication of micro/nano-featured polycarbonate material

Generally, the process consists of three sequential steps: Patterning (pattern generation), die fabrication and replication. The injection molding technique was the replication method of choice for polycarbonate polymers, because of the high temperature required for melting the polycarbonate, the capacity of introducing of very small sub 10 nm features and the successful high throughput production. For pattern generation, electron beam lithography was used to prepare the metal patterned master substrates (Gadegaard *et al.*, 2003b). In brief, silicon substrates were coated with PMMA and exposed in an electron beam lithography tool (Vistec VB6 UHRWF). After development, the substrates were electroplated to form nickel shims that were made of a nickel vanadium alloy (7% vanadium) (Gadegaard *et al.*, 2003a). These shims were used as die for replication with injection moulding (Victory 28, Engel GmbH) of polycarbonate (Makrolon OD2015) substrates after melting it at 280°C (Reynolds *et al.*, 2012).

All the materials tested for *C. albicans* colonisation were sterilised by immersion in 70% ethanol for 5 min, left over night to dry in the hood in an oblique position (Johansson *et al.*, 2002), then exposed to UV light for 5 min per aspect (ULTRA-LUM UVC-508).

4.3.1.2 Investigating the replicability of micro/sub-micro-featured PMMA denture material

Initially the feasibility of using PMMA denture material in the replication of micro-scale features was evaluated. A photo lithographically fabricated master silicon wafer (Figure 4.1) was supplied by the Professor Nikolaj Gadegaard lab (Biomedical Engineering, School of Engineering, University of Glasgow) to enable an initial screening of the possibility of generating micro features in a heat cure-high viscosity dough of PMMA denture material. The silicon master wafer had 0.04 cm^2 micro-patterned surface area of micro-patterned pits, therefore it will produce micro pillars on replication. Powder/liquid ratio of heat cure PMMA denture material (C&J De-luxe denture base polymer, Surrey, UK) was mixed and left at room temperature to reach the dough stage as annotated in manufacturer instructions. The silicon wafer was placed on a glass slide and a small dough of the PMMA was applied over it, sandwiched with another glass slide, and pressed with thumb pressure until the engraved micro- imprinted pattern of the master was clearly shown through the material. Then the material was cured in a water bath following to the manufacturer instructions.

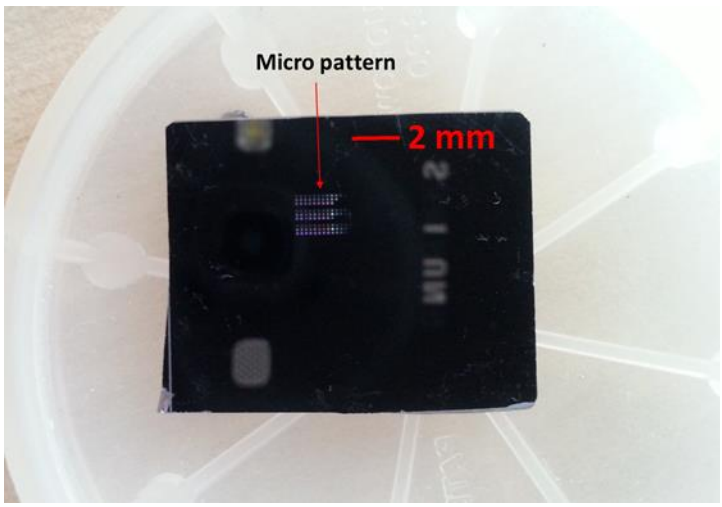


Figure 4.1: Silicon master wafer including micro-fabricated patterns. Scale bar is 2 mm.

4.3.1.3 Replication of micro/nano-featured PMMA denture material

Alternatively, the traditional dental compression moulding technique using dental flasks and dental hydraulic press was used. A polydimethyl siloxane resilient pad was fabricated in customised manner for the fragile silicon wafer and was placed underneath the master silicon wafer to avoid the risk of its fracture because the resilient pad will absorb and evenly distribute the applied forces. Subsequently, because of the fragility of the silicon master pattern, metal master patterns were used. The inherent nature of the metal for non-breakability cancelled the need for a resilient pad underneath the master pattern. Therefore, a nickel shim was moulded directly in die stone material. The micro-patterned nickel shim has a micro-patterned surface area of 0.25 cm^2 (0.5×0.5 cm) of micro-pillars, therefore, it will produce micro-pits when replicated. After moulding the nickel shim in the lower half of the dental flask, a polythene sheet was placed over the lower half of the flask covering the nickel shim so as to separate it from the moulding wax. Then, to provide a universal thickness for the replicated PMMA, 2 mm thickness of the moulding wax was applied upon the pattern and the upper half was articulated with the lower half for completing the mould. The die stone was poured into the upper half and left to set at room temperature for 1 h. Then, the two halves of the flask were separated and the wax was removed using boiling water. An alginate separating medium was applied to the set die stone moulds and left to dry for 30 min. At that moment, the die stone mould containing the nickel shim was ready for the replication process.

The PMMA denture material dough was prepared as described above and directly placed upon the nickel shim and pressed using a dental hydraulic press with 1250 psi for 5 min (Consani *et al.*, 2002). Then, the flask was immersed in a water bath for curing according to the manufacturer instructions. After completing the curing cycle, the flask was bench cooled for 3 h, and then the cured replicated specimen was deflasked and edges finished using acrylic burs. For PMMA denture material replication of nano-patterns, the same aforementioned procedure was followed as illustrated in Figure 4.2. The surface area of the nano-patterned area was 1 cm^2 (1×1 cm) of nano-pillars.

After completing the replication process, the nickel shim was directly placed in absolute isopropanol and cleaned using overnight 50°C hot acetone, then sonicated at 35 kHz for 5 min (Ultrasonic bath, Fisher scientific, UK), transferred to absolute isopropanol and sonicated for further 5 min. Then, the nickel shim was kept in fresh isopropanol until next use. Before every use, it was removed from the isopropanol and left at the bench to dry for 30 min. All the work was performed in the laboratory hood.

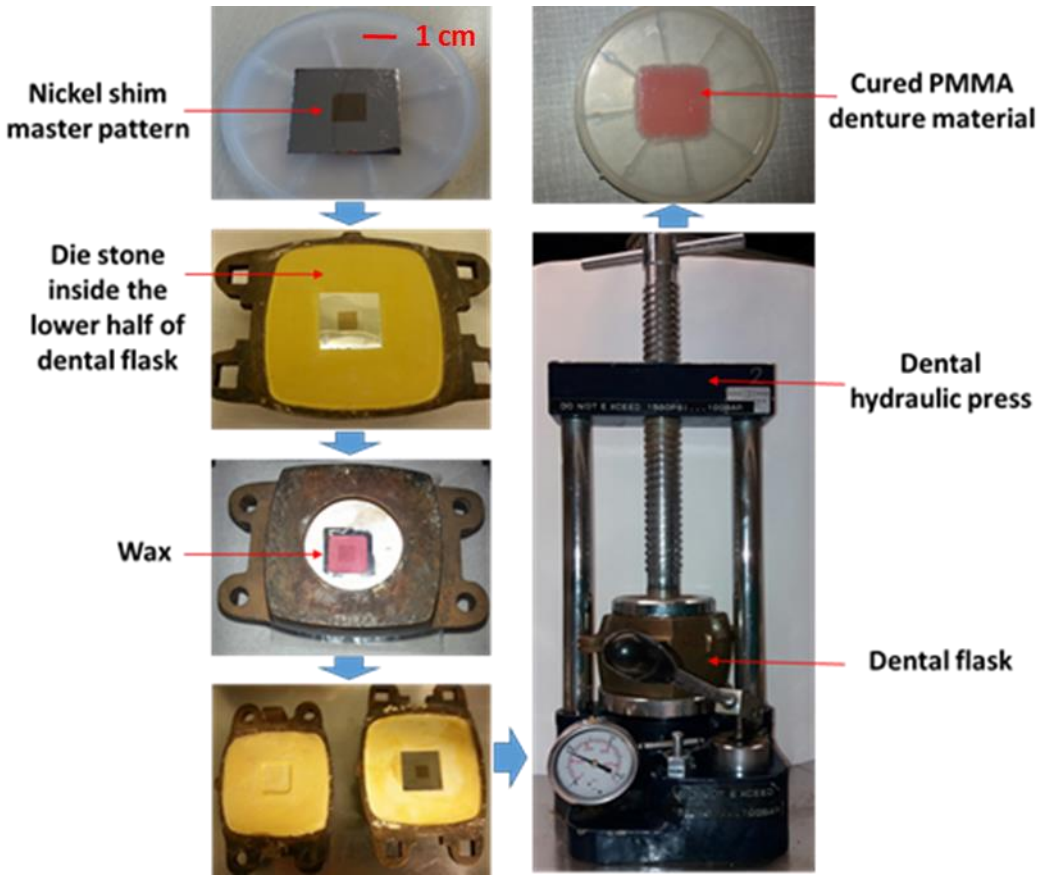


Figure 4.2: Replication of micro/nano-patterns on PMMA denture material. The process was performed using dental flask compression molding technique. Scale bar is 1 cm.

4.3.2 *Candida albicans* adhesion on the micro-pillar patterns

Polycarbonate micro-patterned pillars of 0.04 cm^2 of three different dimensions (2, 1 and 0.5 µm in diameter) and two different arrangement forms (square form (SQ) and near square (NSQ) form of arrangements) were challenged for adhesion of *C. albicans* (Figure 4.3). Before the adhesion experiment, these polycarbonate sections were characterised by scanning electron microscopy and the sections were prepared and coated with a layer of gold-palladium (15 nm). *C. albicans* SC5314 was propagated in yeast-peptone-dextrose (YPD) medium (Sigma-Aldrich) for 18 h at 30°C in an orbital shaker at 150 rpm. The cells were washed by centrifugation in sterile phosphate buffered saline (PBS, Sigma-Aldrich, UK) and standardised to an inoculum density of 1×10^4 CFU/ml in RPMI-1640 medium (Sigma-Aldrich, UK). A duplicate of sections of every form of arrangement were distributed in petri dishes and 1.5 ml of the *C. albicans*-medium suspension was dispensed upon the section. The inoculated cells were allowed to adhere to the targeted sections and incubated for 30 and 90 min at 37ºC statically. This incubation period was sufficient to obtain yeast (Y) and germling (G) *C. albicans* morphological forms

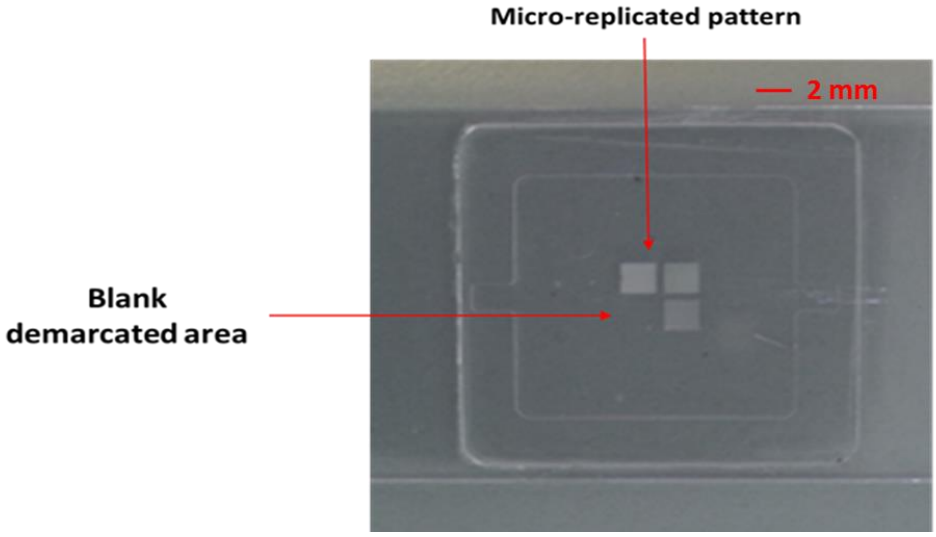


Figure 4.3: Polycarbonate replicated micro-patterns. Every square represents different dimention. The blank area is demarcated at a microsopic scale to represent the control flat area. Scale bar is 2 mm.

After completing the incubation period, the medium-cells suspensions were aspirated and the sections were washed twice with 1.5 ml of PBS to remove any planktonic non-adherent cells. Then, the sections were left to dry at room temperature for 6 h and stained with 1.5 ml of crystal violet dye (0.05% v/v) for 10 min at room temperature. Afterwards, the dye was removed, and to remove any unbound stain the sections were comprehensively washed ten times through five consecutive immersions in two Petri dishes each containing 40 ml of distilled water. Then, the sections were air-dried for 30 min and examined under a light microscope at 400 x magnification for counting the total number of adherent cells in every targeted area. Counting was performed in 16 fields of view and repeated twice where an average was considered. The experiment was repeated on four independent occasions.

4.3.3 *Candida albicans* adhesion on the micro-pit patterns

PMMA denture material with micro-pit patterns were replicated using the dental compression moulding technique as described in section 4.3.1.3. The replicated sample dimensions were 5×5×2 mm. Two forms of micro-pit inter-distance were used (short and long micro-pits inter-distance). The replicated samples were characterised by scanning electron microscopy (SEM) and the pit diameter was 0.63 µm±0.02, the inter-pit distances were 0.3 and 0.8 µm±0.02 and the depth was 1.3 µm±0.02. *C. albicans* were propagated as annotated in the previous section and standardised to 1×10^6 CFU/ml in RPMI-1640 medium. Control (flat surface) PMMA denture material were involved. The sections were distributed in 24 well plate and 1 ml of the cells-medium suspension was dispensed and statistically incubated for 30 and 90 min to allow adherence of the *C. albicans* and to obtain yeast (Y) and germling (G) morphological forms. Then, the incubated suspensions were pipetted and the sections were washed twice by gentle dipping in fresh 1 ml of PBS. Subsequently, the *C. albicans* containing samples were prepared for scanning electron microscopy imaging as annotated in chapter 3 (section 3.3.6). After completing the gold-palladium coating procedure, every sample was imaged at 500 x magnification in four random fields of view.

The experiment was duplicated on two independent occasions. For counting the number of the adhered cells, the acquired images were processed and analysed by ImageJ 1.51h software (National Institutes of Health, USA).

4.3.4 *Candida albicans* adhesion on the nano-pit patterns

At the beginning, polycarbonate nano-pit patterned samples of 22×22×2 mm dimensions were challenged for *C. albicans* adhesion (Figure 4.4). Three different arrangement forms of nano-pit arrays were tested. These arrays were square (SQ), near square (NSQ50) and hexagonal (HEX). The pits were of 120 nm diameter, 100 nm depth and 300 nm pitch (pit centre to pit centre) with an offset of ±50 nm for the NSQ50 topography. The nano-pit topographies were characterised with SEM. The cells were propagated and standardised in RPMI-1640 medium as annotated in the previous section. The sections were distributed in 35 mm diameter petri dishes (Thermo Fisher scientific, USA). Four ml of the standardised cell-medium suspension was aliquoted. Control (flat surface) specimens were involved.

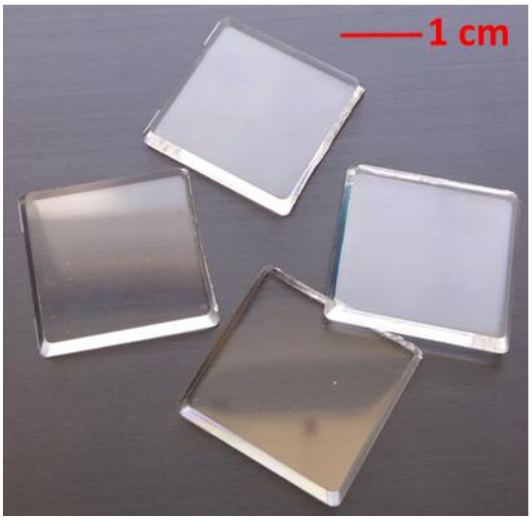


Figure 4.4: Polycarbonate nano-pit patterned sections. The sections were manufactured using injection moulding machine. The bevel up surface was the surface of interest (nano-patterned and tested). The nano patterned sections are completely identical to the non-patterned (flat) sections when they were macroscopically examined (vision with unaided eye). Scale bar is 1 cm.

The inoculated cells were allowed to adhere to the targeted sections and incubated for 30 and 90 min at 37°C in static position. These sections were then washed with PBS and retained cells removed through sonication at 35 kHz for 10 min (Ultrasonic bath, Fisher scientific, UK), followed by 15 sec vortex.

DNA and RNA were extracted using a combination of mechanical (disruption with 0.5mm glass beads) and chemical methods [(QIAGEN QIAamp mini kit for isolation of genomic DNA (QIAGEN, Germany)] and (TRIzol™, Invitrogen, Paisley, UK for isolation of RNA) as described in chapter 2 (2.3.1.1) and chapter 3 (3.3.10.1), respectively. The adherent cells were quantified using qPCR through amplification of the Candida-specific 18S DNA as described in chapter 2 (2.3.1.4). The expression of specific *C. albicans* adhesion genes (*ALS1*, *ALS3* and *EAP1*) was also investigated at each experimental parameter using real-time qPCR as described in chapter 3 (3.3.10.5). The gene expression data were analysed and processed via $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Generation of maps and clusters was through R programming language with the use of heatmap 0.2 function from the gplots package. The experiment was repeated at least in four independent occasions using two sample replicates.

After assessment of the results, the SQ form of arrangement was selected for replication into PMMA denture material. Therefore, nano-pit replicated PMMA specimens were fabricated as described in section (4.3.1.3). The dimensions of the replicated nano-imprinted PMMA were 10×10×2 mm. Flat surfaces PMMA were fabricated as described in chapter 2 (2.3.3.1) and used as controls. The samples were distributed into 24 well plate and 1 ml of the standardised cell-medium suspension was aliquoted. The same incubation parameters were used; 30 and 90 min. Then, DNA and RNA of the *C. albicans* were extracted to evaluate the adhesion capacity and related molecular response of the adherent cells. Two replicates were used in this experiment and it was repeated, at least, in three independent occasions.

4.3.5 Development of biofilms on the SQ nano-pit pattern

SQ nano-pit polycarbonate were challenged for development of *C. albicans* biofilms using RPMI-1640 medium. Measurement of biofilm metabolism and biomass were considered for evaluation of the biofilms developed. The cells were propagated and standardised as described in chapter 2 (2.3.2.1). The substrates were distributed in 35 mm petri dishes and 5 ml of the cell-medium suspensions were inoculated. Flat surfaces were involved as control. These petri dishes were incubated for 4 and 24 h at 37^{o}C in static position. Then, the substrates were washed twice with 5 ml of PBS and placed in fresh 35mm petri dishes for adding 5 ml of the XTT solution. And the XTT assay was performed as described in chapter 2 (2.3.3.4). The same biofilm formation procedure was repeated for CV assay, which was performed as described in chapter 2 (2.3.2.2).

4.3.6 Measurement of surface properties of the materials tested

Both of the materials tested (polycarbonate and PMMA denture material) were examined for their surface roughness and wettability. Non-contact 3D optical profiling was performed for the flat sections of both materials using Contour GT-X 3D optical profiler microscope (Bruker, UK), which is based on white light interferometry, to measure the surface roughness metrological property. Five samples were interrogated and average values were obtained from two measurements. The images were corrected to a line-wise plane and the average surface roughness (R_a), which is the most popular parameter typically tested (Choi *et al.*, 2014), was calculated from 180×180 μm acquired images via Vision 64^{TM} analysis software.

For wettability evaluation purposes the measurement of static water contact angle (WCA) was conducted for the patterned and flat sections using a Theta optical tensiometer (Biolin Scientific, Stockholm, Sweden). At room temperature, a drop of 3 μl water was dispensed on the surface of the section on a vibration-free stage through an automated flat tipped needle, as illustrated in Figure 4.5. The WCA was calculated by averaging the measurement of 12 WCA/sec within 30 sec for the right and left sides of the drop. The experiment was performed for 4 different samples with at least 3 measurements.

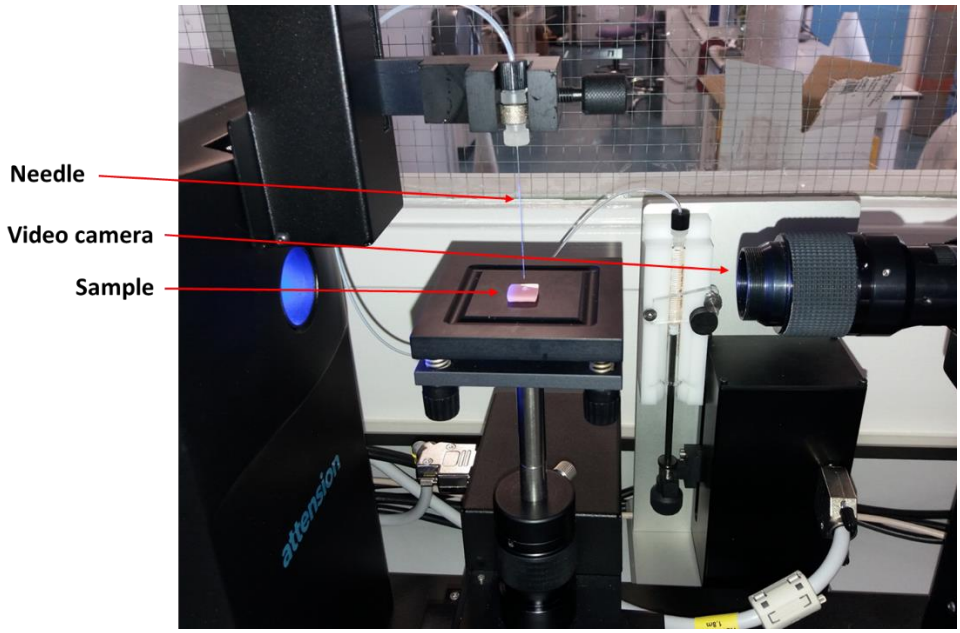


Figure 4.5: Tensiometer device loaded with sample.

4.3.7 Candidal colonisation on CUR-adsorbed nano surfaces

It was a subsequent aim to interrogate the effect of combining the nano scale modified surface with CUR adsorption of sub-inhibitory concentration on the adherence and biofilm formation of *C. albicans*. Polycarbonate nano-pit patterned sections of the SQ form of arrangement were used and flat surfaces were used as controls.

In the adherence experiment, *C. albicans* were propagated and standardised to 1×10^6 CFU/ml in PBS. Samples were distributed in 35 mm petri dishes and five ml of CUR (800 µg/ml and diluted with RPMI-1640 medium) was allowed to adsorb to the nano patterned and flat surfaces for 10 min at room temperature and covered with aluminium foil to prevent light exposure to mimic what was conducted in chapter 3 (3.3.4). Control samples were considered that were immersed in RPMI medium only. After completing the adsorption time, the sections tested were gently dipped in five ml of distilled water to remove the non-adsorbed molecules and transferred to fresh petri dishes. Then, five ml of standardised *C. albicans*-PBS suspensions were added to all targeted sections [control flat without CUR (F-), control SQ nano pattern without CUR (SQ-), Flat with CUR (F+) and SQ nano pattern with CUR (SQ+)] and incubated for 30 min at 37 °C static situation.

Next, the incubated cellular suspension was removed, and the sections were washed twice by gentle dipping in five ml of PBS to remove the non-adherent cells. Afterwards, the cells were collected from the sections through sonication in 15 ml of PBS for 10 min at 35 kHz and vortex for 15 sec. The collected cells were counted using the Miles and Misra plate counting method, as described in chapter 3 (3.3.5) (Miles *et al.*, 1938). In this experiment, three surface replicates were considered and the work repeated in four independent occasions.

In the remainder of the experiment, biofilm formation was targeted. *C. albicans* were standardised to 1×10^6 CFU/ml in RPMI-1640 medium. Same aforementioned parameters were undertaken and the *C. albicans* loaded samples were incubated for 24 h at 37°C in static station. The developed biofilms were washed twice in five ml of PBS and transferred to fresh 35 mm petri dishes. The developed biofilms were evaluated from their metabolic activity and biomass themes using XTT metabolic and CV staining assays, respectively. These assays were previously annotated in chapter 2 (2.3.3.4) and chapter 2 (2.3.2.2), respectively. In these assays, 5 ml of the assay's reagent was used and only one ml was spectrophotometrically measured in fresh 24 well plate using plate reader (FluoStar Omega, BMG Labtech, UK). This experiment was independently repeated three times using three replicates.

4.3.8 Adsorption of CUR to SQ nano-pit topographies

To test the effect of the SQ nano-pit surfaces on the capacity of CUR adsorption, SQ nano-pit polycarbonate specimens were used in comparison to flat specimens. The CUR adsorption parameters used in the previous section were used. The adsorbed CUR was measured as explained in chapter 3 (3.3.4). The experiment was replicated using three specimens and repeated in three independent occasions.

4.3.9 Statistical analysis

GraphPad prism 5 was used to analyse data and generate associated figures. Any non-normally distributed data were analysed with non-parametric statistical tests and further were Log_{10} transformed and then analysed with parametric statistical tests, if any disagreement between them it will be reported. Proportional data were natural log transformed, then analysed. Tests used will be individually reported in the appropriate results sections.

4.4 Results

The adhesion capacity of *C. albicans* to engineered topographies was investigated, using micro-scale to nano-scale featured surfaces that can influence the size of the *C. albicans* yeast form. Therefore, two micrometre diameter feature and four micrometre pitch area (the area between the features) were selected and used as maximum dimensions.

4.4.1 Adhesion of *C. albicans* to micro-patterned topographies

At the outset, there was a tendency to explore the feasibility of using the most commonly used denture material (PMMA) to replicate features of micron scale. The importance of this investigation originates from the inherent high viscosity (dough stage) of the polymer during the replication that may impede the replication process. It was not possible to use the same injection-moulding machine used for polycarbonate replication to replicate the PMMA denture material patterned substrates, because of the PMMA being supplied in a powder/liquid form and the high viscosity of the resultant mixture. For this reason, other ways of replication were attempted. Two ways were undertaken, thumb embossing and conventional dental compression moulding technique. The capacity for replication was examined through scanning electron microscopy (Figure 4.6). Using of the dental compression moulding technique showed a well replicated topography, in contrast to the thumb embossing technique. The replication of features including the base and top was clearly affected. For this reason, the dental compression moulding technique was undertaken.

(i) **(ii)**

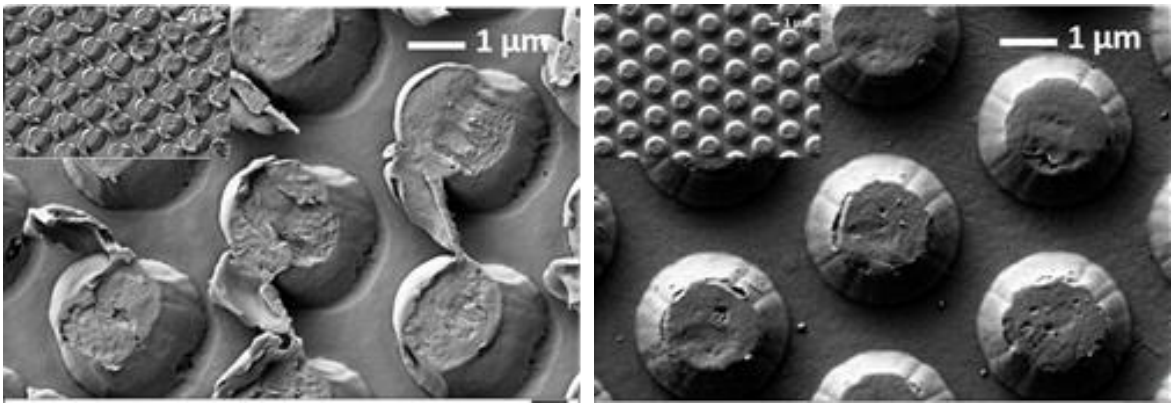


Figure 4.6: Optimisation of the micro-scale replicability of PMMA denture material. SEM micrographs of micro-pillar replicated topography using thumb embossing technique **(i)** and dental compression technique **(ii)**. Scale bar is 1 µm and images acquired at 10000 and 25000 x magnifications.

First, it was important to analyse the factors that could influence the adhesion of *C. albicans* to the micron and submicron patterned topographies. The adhered cells were visually quantified using light or scanning electron microscopy by direct counting or through images, respectively. Thirty min incubation was sufficient to ensure adherence of Y form *C. albicans*, while adhesion of 90 min in RPMI-1640 medium stimulated *C. albicans* cells to the G morphological form (Figure 1.6-Chapter 1).

SEM characterisation of the micro-pillar topographies showed the successful replication and the spatial arrangement of features (Figure 4.7). The adhesion percentage (in comparison to the flat topographies) of the Y and G morphological forms to the micro-pillar polycarbonate topographies showed opposing results, as shown in Figure 4.8. Adhesion of the Y cells to the patterned micro-pillars was approximately 4-6 times (374-645%) and significantly ($p<0.01$) greater than that of the non-patterned flat surfaces. However, G cells showed a non-significant reduced adhesion capability (66-85%) to these surfaces. These opposing results between Y and G cells were statistically significant ($p<0.0001$).

In both morphological forms of *C. albicans*, non-significant differences were observed between the two arrangement forms tested (SQ and NSQ). Similarly, the different feature sizes (0.5, 1 and 2 µm) did not show statistical differences, though there was a trend for more adhesion capacity on surfaces of larger features and inter-feature pitches, which was clear in adhesion of yeasts to the SQ arrangement.

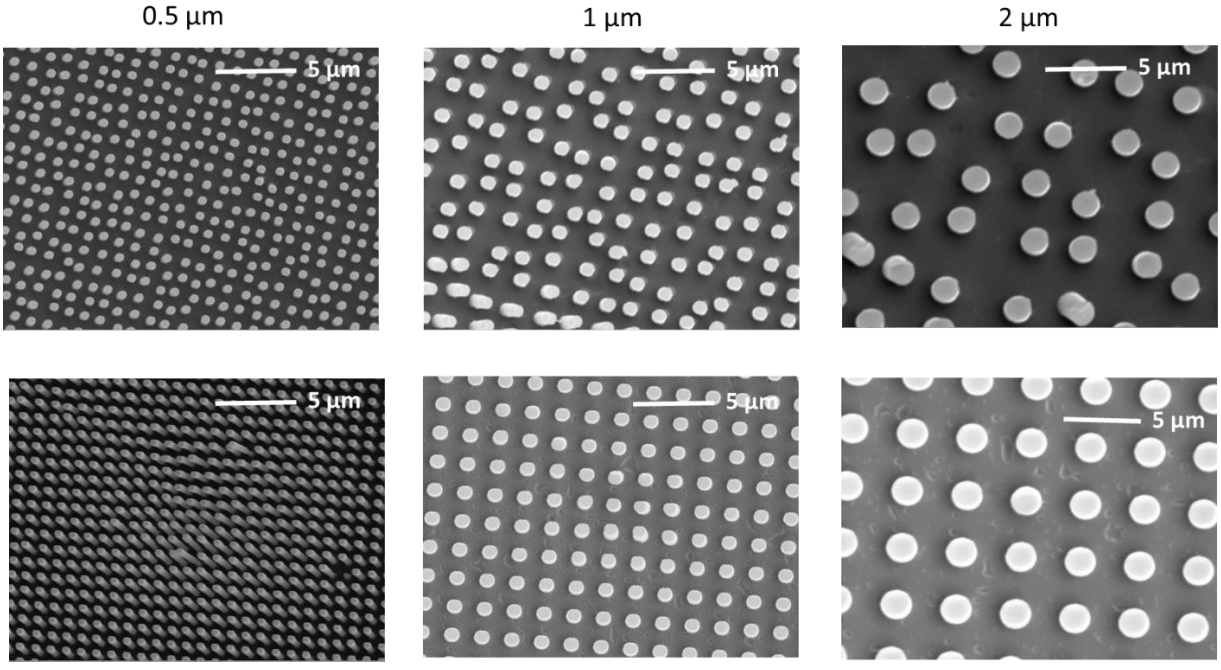
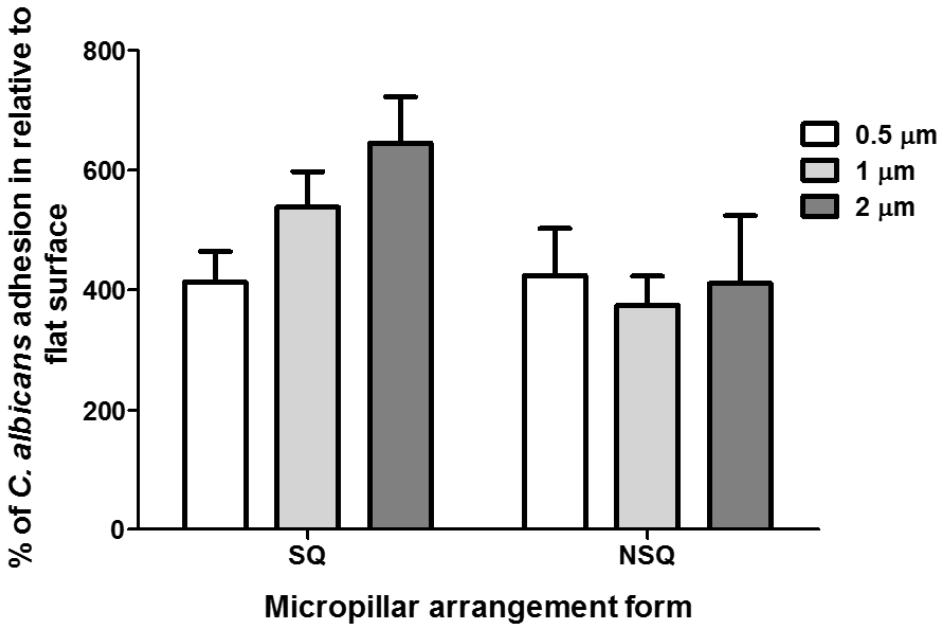


Figure 4.7: Characterisation of polycarbonate replicated micro-pillars. The upper panel of SEM micrographs represents the near square (NSQ) form of arrangement and the lower panel represents the square (SQ) form of arrangement. Scale bar is 5 µm and the magnification used was 5000 X.

(i)



(ii)

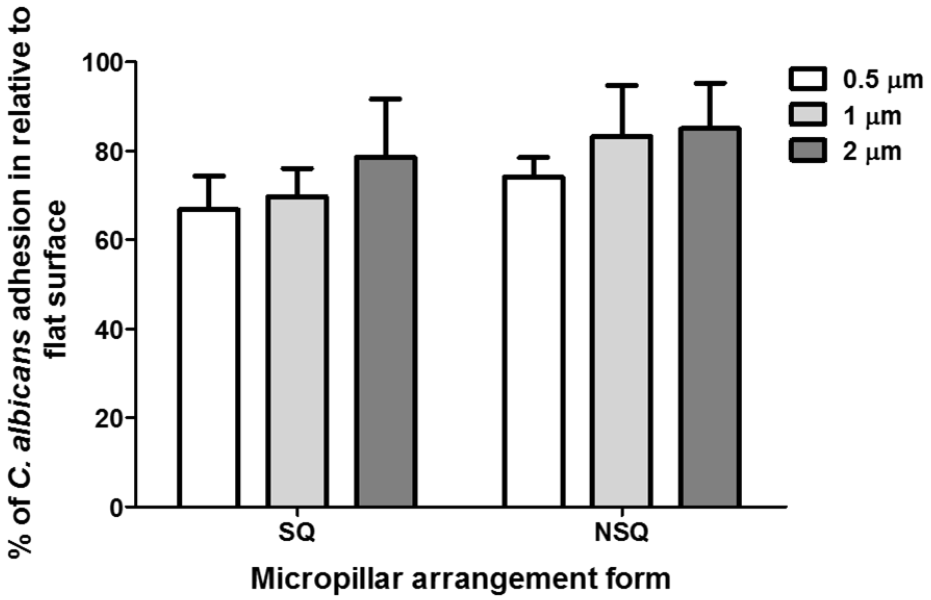


Figure 4.8: Adhesion of *C. albicans* to micro-pillar topographies. (i) Adhesion data of Y form cells. **(ii)** Adhesion data of G form cells. Topographies used were of different arrangement forms and different feature size. Data obtained from eight surfaces (2 samples in 4 independent occasions). Mann-Whitney test was used to compare patterned and non-patterned surfaces and unpaired *t* test was used after natural log transformation of proportions to analyse different arrangement forms and feature dimensions. Error bars represent standard error of mean.

Given the high levels of adhesion of the yeast cells to micro-pillar patterns, then future analysis was stopped, and studies diverted to sub-micron pit features. Two different pit-inter-distances were investigated. The topographies were successfully replicated in PMMA denture material as described in section 4.3.1.3, and characterised via scanning electron microscopy as illustrated in Figure 4.9.

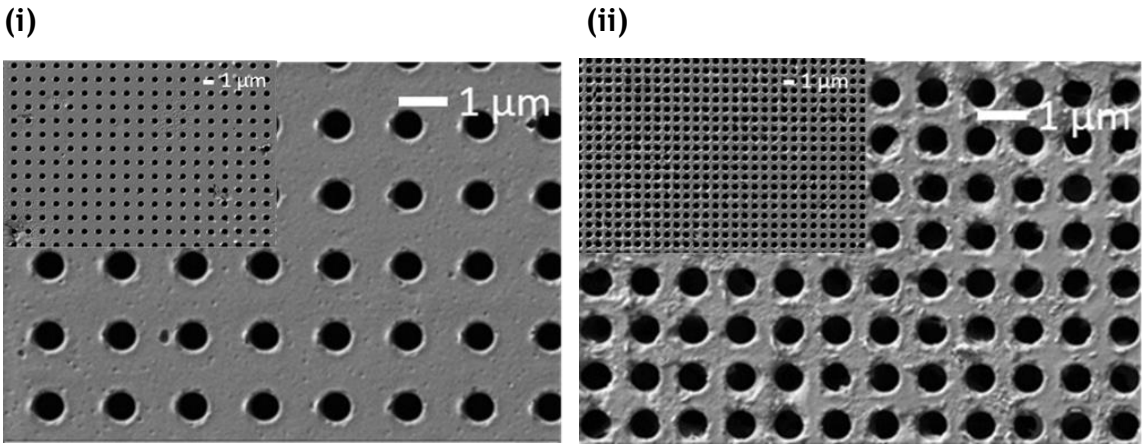
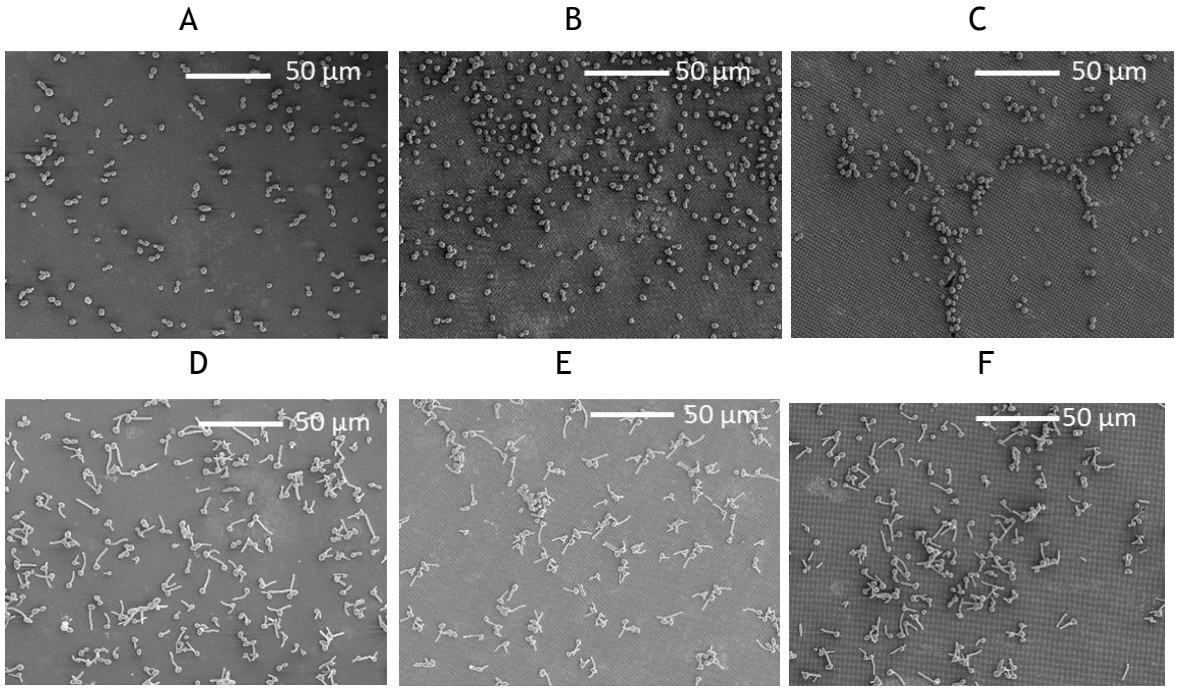


Figure 4.9: Characterisation of PMMA denture material micro-pit patterns. SEM micrographs of micro-pit replicated topography. Pit diameter is 0.63 µm and depth is 1.3 µm. **(i)** Inter-pit distance (the shortest straight line between two horizontally adjacent pits) is 0.8 µm±0.02 **(ii)** 0.3 µm±0.02. Scale bar is 1 µm and images acquired at 10000 and 25000 x magnifications.

Similar *C. albicans* morphological forms (Y and G) were allowed to adhere to the micro-pit replicated surfaces and SEM micrographs were acquired as shown in Figure 4.10(i) representative images. Dimensions of the analysed images were 225 x 165 µm. In Figure 4.11(ii), the percentages of Y form adherence onto sub-micron pit topography relative to non-patterned (flat) surfaces (128%) showed a clear drop in comparison to that of the sub-micron pillar topography shown in Figure 4.8. This reflected the relative inherent anti-adherent capacity of pit form topography in comparison to the pillar form. An interesting observation from Figure 4.10(ii) was the decrease of the percentage of Y adherence from 128% to 86% in respect of the two different pit-inter-distance topographies, with a reduction of yeast form adhesion to closely located pits. However, it was not statistically significant ($p=0.077$). Furthermore, both of the sub-micron pit topographies showed slight anti-adherence capacity to the G morphological form, with 83-84% adhesion relative to the flat topographies. Collectively, these data suggested using pit-patterned topographies of smaller dimensions.

(i)



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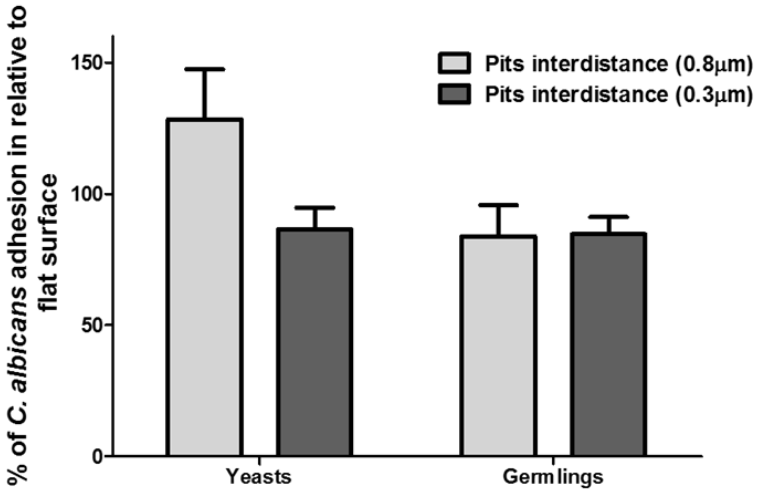


Figure 4.10: Adhesion of *C. albicans* to micro-pit PMMA denture material. (i) SEM micrographs of adhered Y (upper panel) and G (lower panel) forms on flat (A,D), 0.8 µm inter-pit distance (B,E) and 0.3 µm inter-pit distance topographies (C,F). Scale bar is 50 µm and magnification used is 500 x. **(ii)** Adhesion to the micro-pits relative to that of the flat unpatterned PMMA. Data obtained from 8 values (4 values in 2 independent occasions). Unpaired *t* test was used to compare patterned and non-patterned surfaces and natural log transformation of proportions was performed to compare different morphologies and pit inter-distances. Error bars represent standard error of mean.

4.4.2 Adhesion of *C. albicans* to nano-pit patterned topographies

Based on the previous data, pit-patterned topographies with sub-micro dimensions could potentially have anti-adhesive properties. This component of the study focussed on the impact of surface nano-pit topographies on adhesion of ovoid Y and hyphal G cells. These nanotopographies were initially characterised by SEM (Figure 4.11).

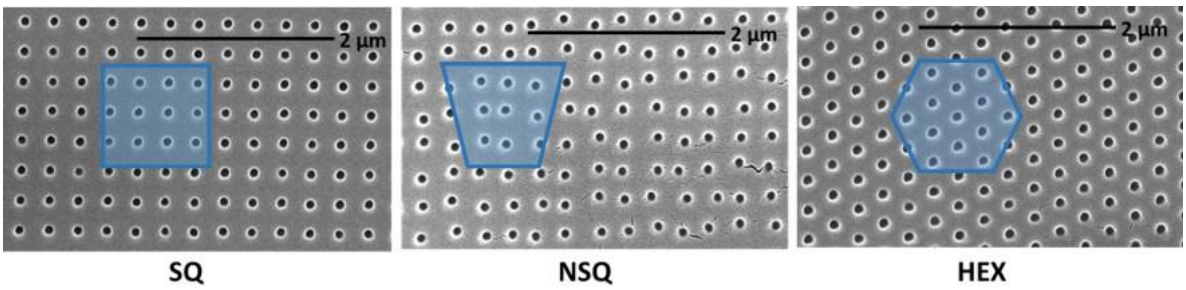
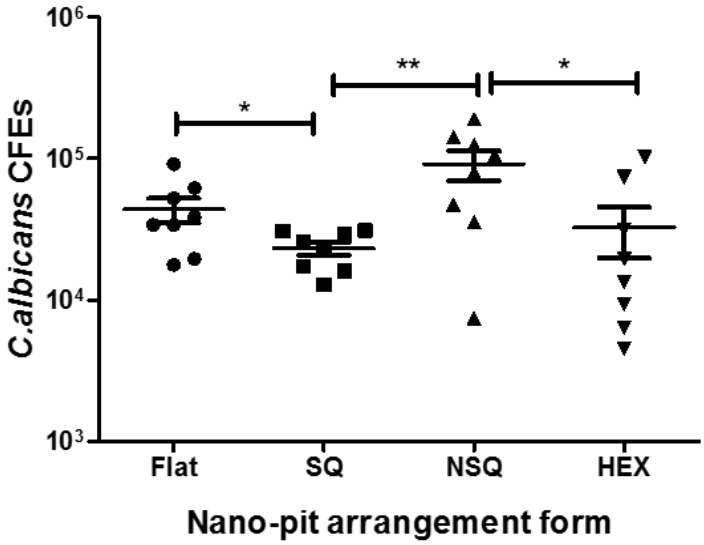


Figure 4.11: Characterisation of polycarbonate nano-pit patterns. Scanning electron micrographs of polycarbonate nano-pit showed the arrangement forms tested. Pit diameter is 120 nm and depth is 100 nm. Scale bar is 2 µm. SQ, NSQ and HEX stand for square, near square and hexagonal arrangement forms, respectively. The magnification used is 20000 x.

Assessment of these two morphologies adhering to SQ, NSQ and HEX nano-pits on polycarbonate was quantified using qPCR. The three arrangement forms tested of the nanotopographies showed different adhesive properties of *C. albicans*. NSQ nanotopography showed a significant increase in the adhesion capacity relative to the other nanotopographies tested, especially in Y morphology form (Figure 4.12 i). However, the highly ordered SQ arrangement showed an contrasting observation, where it was shown that SQ arrangement significantly reduced adhesion (relative to flat surfaces) of both Y cells ($p=0.038$), with ~48% reduction in adhesion, and G cells ($p=0.020$), with approximately 64% reduction adhesion as illustrated in Figure 4.12 i and ii, respectively. Unpaired *t* test were used for statistical analysis and any non-parametric data were Log_{10} transformed prior to analysis, which were preceded by one-way ANOVA that showed significant differences ($p<0.01$). For further analysis, non-parametric data were analysed by Mann Whitney test.

NSQ and HEX had no significant anti-adhesion properties for either cellular morphology in comparison to the flat, where NSQ showed the highest adhesion capability for *C. albicans* among the surfaces tested. Figure 4.13 shows a microscopic observation of the adhered yeasts on flat and SQ pattern, where the cells (and even the substrate surface) seemed covered with cell wall adhesins in relatively higher abundance in flat topography.

(i)



(ii)

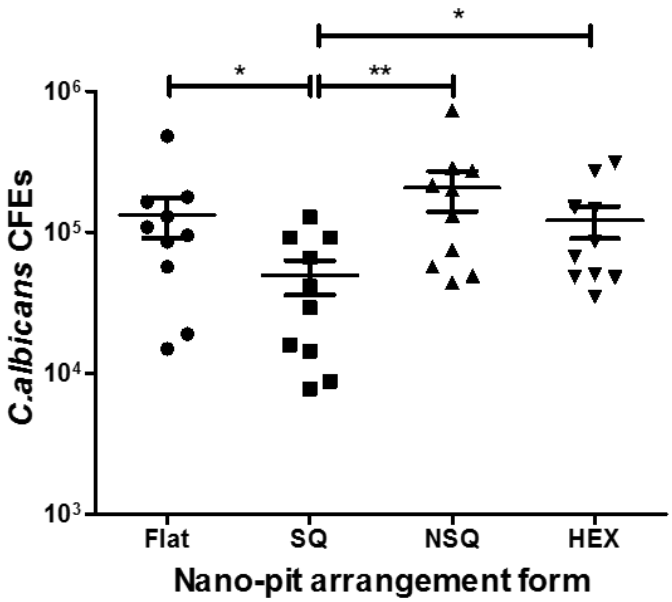


Figure 4.12: Quantification of adhered *C. albicans* to polycarbonate nanotopographies. Quantification of adherent colony forming equivalents (CFEs) of Y **(i),** where (n=8) and G **(ii),** where (n=10). The horizontal line represents mean and error bar represents standard error of mean. Data were analysed by unpaired *t* test, * and ** represent ($p<0.05$) and ($p<0.01$), respectively.

(i) **(ii)**

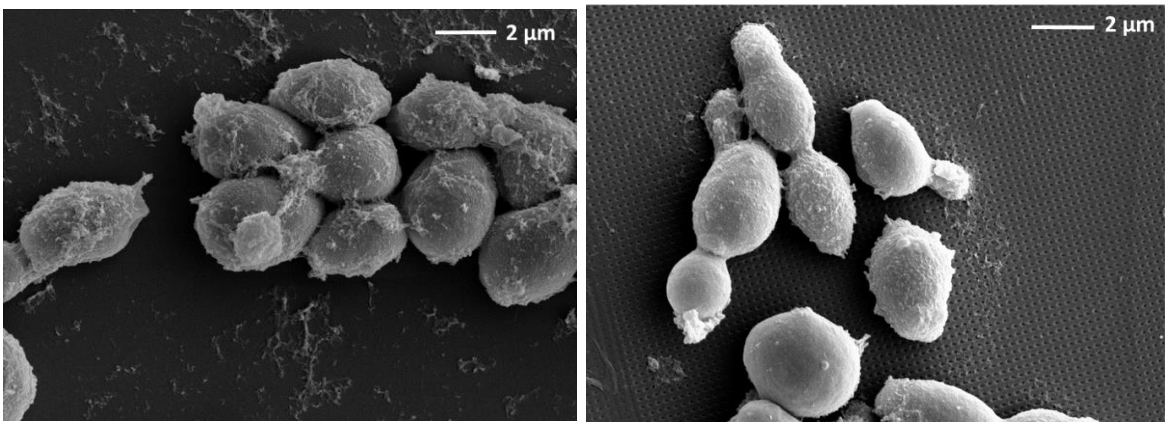


Figure 4.13: Adhered yeasts on SQ nanotopography. SEM micrographs of Y cells on flat **(i)** and SQ pattern **(ii)**. Scale bar is 2 µm and magnification used is 6000 x**.**

In parallel, expression profiles of key adhesion genes (*ALS1*, *ALS3* and *EAP1*) were assessed by real-time qPCR and presented as heat-map relative to the housekeeping gene *ACT1* (Figure 4.14). Here, it was shown that all 3 adhesion genes were down-regulated in the polycarbonate nanotopographies in comparison to the flat topographies in both Y and G cells, but notably for the SQ arrangement *ALS1* was significantly down-regulated with (p=0.043) and (p=0.036) in Y and G cells, respectively.

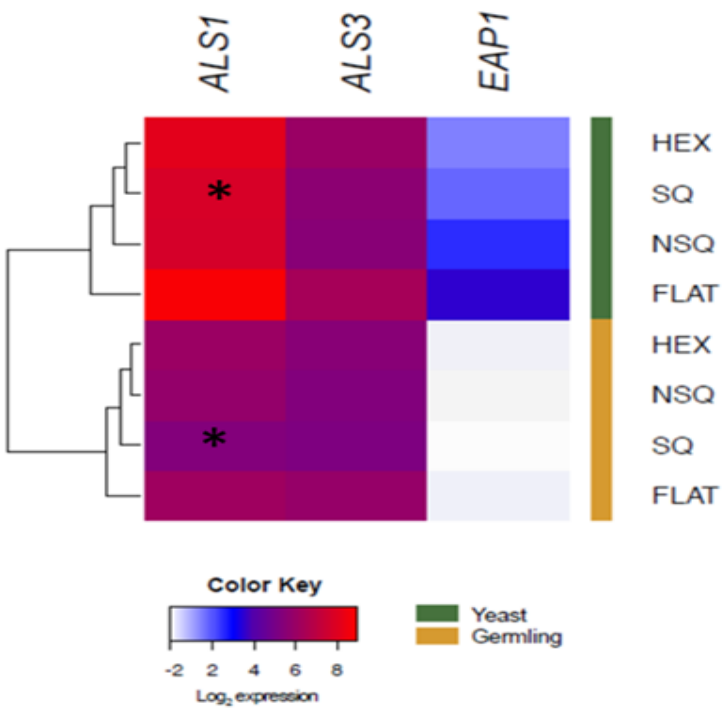


Figure 4.14: Visual transcriptional analysis of adhered *C. albicans* to polycarbonate nanotopographies. Heat map transcriptional data of selected adhesion genes (*ALS1*, *ALS3* and *EAP1*) of Y and G cells, where gene expression data are represented in Log_2 colour key values. Unpaired *t* test was used after natural log transformation of the data. * represent ($p<0.05$).

Based on these data, nanoimprinted (SQ form arrangement) PMMA denture material was developed using the dental compression moulding. The validity of this approach for replication of pits at a nano-scale was investigated by scanning electron microscopy (Figure 4.15), where the presence of imprinted nanopits were observed regularly spaced. As with polycarbonate surfaces, we demonstrated a significant reduction of adherence of both Y ($p=0.025$) and G ($p=0.001$) cells on PMMA with approximately 35 and 55% reduction in adhesion, respectively, using same abovementioned statistical tests (Figure 4.16 i& ii). Analysis of the adhesion expression profiles showed no difference between the control and SQ PMMA surface (Figure 4.17).

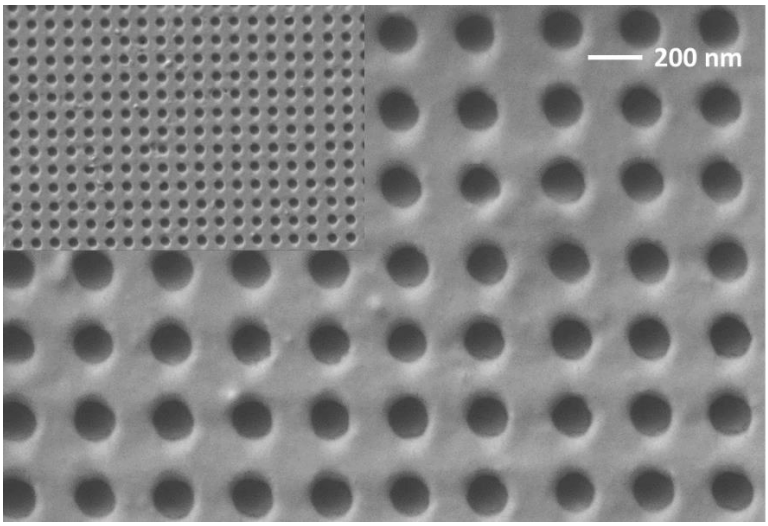


Figure 4.15. Characterisation of nanoimprinted PMMA denture material. SEM micrograph of replicated SQ nano-pit topography (scale bar is 200 nm) and magnification used was 50000 and 100000 x.

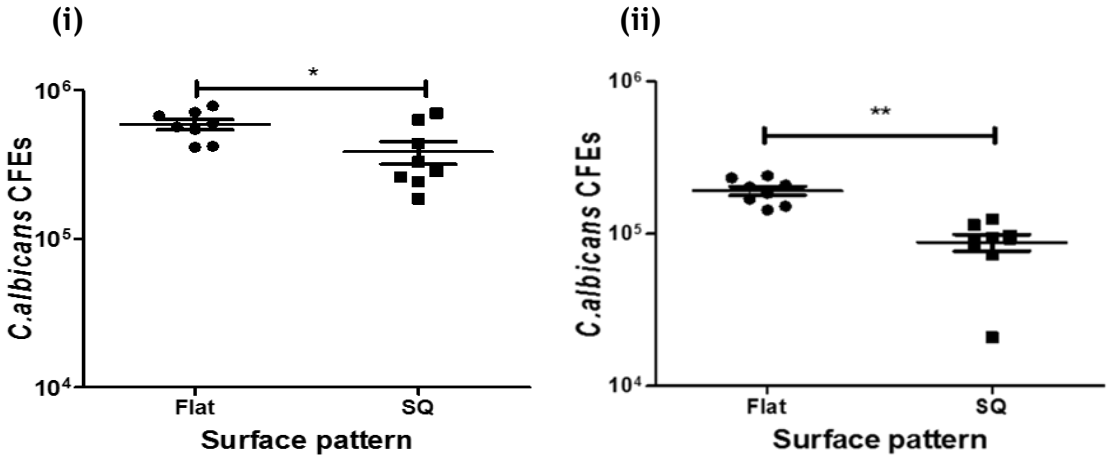


Figure 4.16. Quantification of adhered *C. albicans* to PMMA denture material nanotopographies. Quantification of adherent colony forming equivalents (CFEs) of Y **(ii)** and G **(ii).** Data obtained from four independent occasions (n=8). The horizontal line at mean and error bar represents standard error of mean). Data were analysed by unpaired *t* test, * and ** represent ($p<0.05$) and ($p<0.01$), respectively.

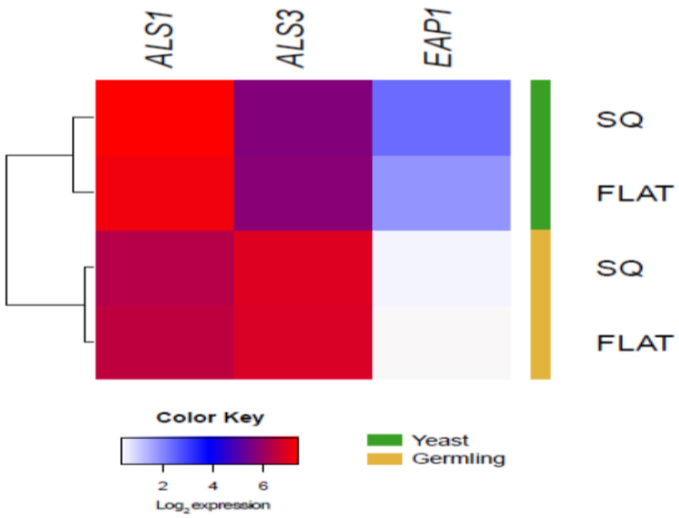


Figure 4.17: Visual transcriptional analysis of adhered *C. albicans* to PMMA denture material nanotopographies. Heat map transcriptional data of selected adhesion genes (*ALS1*, *ALS3* and *EAP1*) of Y and G cells, where gene expression data are represented in Log_2 colour key values. Unpaired *t* test was used after natural log transformation of the data.

4.4.3 Evaluation of surface roughness and wettability properties

The physical surface properties of the two materials (polycarbonate and PMMA) tested were explored with regard to surface roughness and wettability. A statistical difference of high significance ($p<0.001$) was detected between the surface roughness average of flat polycarbonate (R_a 4.1 nm) and that of the flat PMMA denture material (R_a 1549 nm) (Figure 4.18). The surface roughness of the patterned material was not measured because the depth of the nano-pits was already identified. On the other hand, when the water contact angle (WCA) was evaluated for the nanopit SQ replicated imprints a significant difference was observed in the patterned topographies relative to the flat ($p<0.05$), and a higher WCA was observed in the polycarbonate material relative to the PMMA denture material in both flat and patterned topographies ($p<0.01$) (Figure4.19).

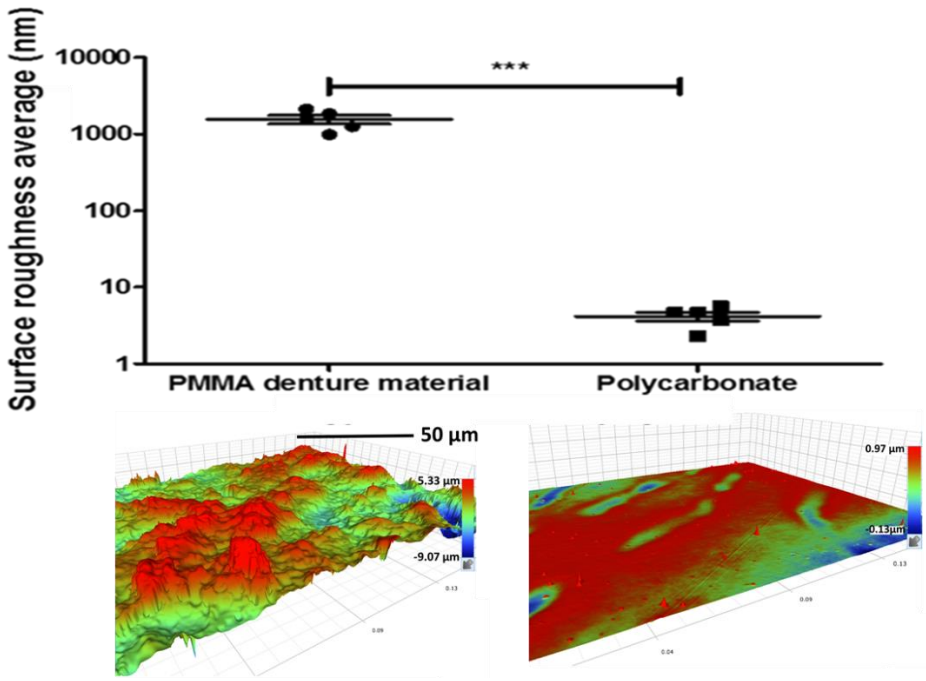


Figure 4.18: Surface roughness of flat surfaces of materials tested. Surface roughness average was measured with Contour GT-X 3D microscope and the micrograph images with scale bar of 50 µm. Data were analysed by unpaired *t* test, *** represents p< 0.001.

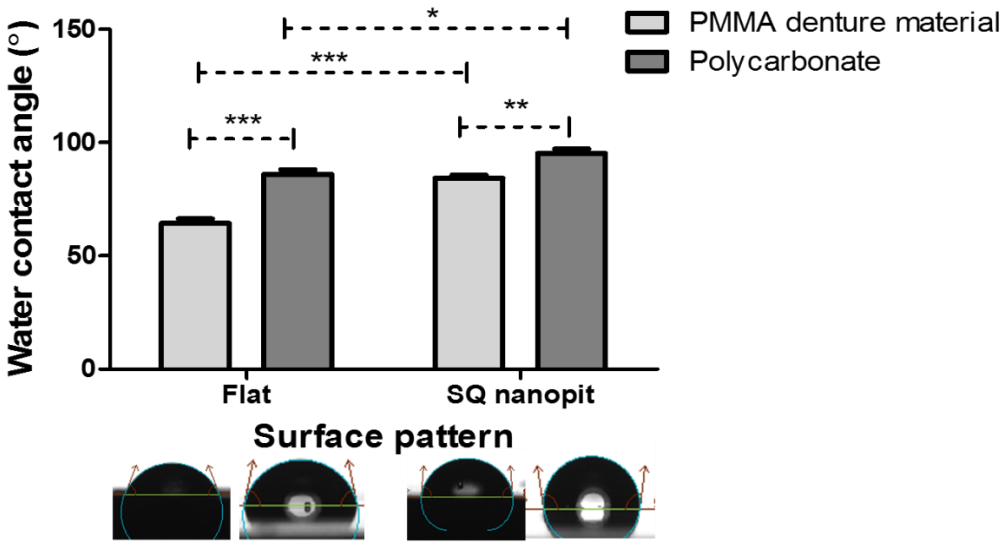


Figure 4.19: Wettability of the flat and nanopatterned materials tested. Static WCA of all investigated materials (error bar shows the standard error of mean), Images acquired at the 30th second by the tensiometer camera. Data were analysed by unpaired *t* test, *, ** and *** represent p<0.05, p<0.01 and p< 0.001, respectively.

4.4.4 Biofilm formation onto SQ nano-pit topographies

Biofilm formation on SQ nanotopographies were evaluated for metabolic activity and biofilm biomass outlooks using XTT and CV assays, respectively. Initial (4 h) and mature (24 h) *C. albicans* biofilms were tested. In both assays and in both biofilms developed, there were no significant differences (Figures 4.20, 4.21), which clarified the incapability of nanotopographies to prevent *C. albicans* from developing an interlocking hyphal recalcitrant net. This may instigate the necessity for exploration of the effect of CUR combination with nanotopographies on adhesion and biofilm formation.

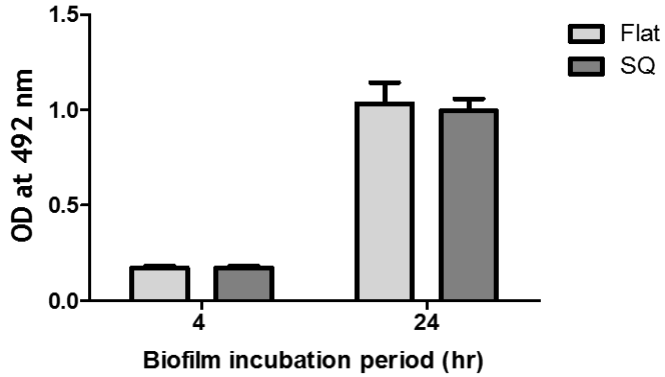


Figure 4.20: Evaluation of metabolic activity of *C. albicans* biofilms on SQ nanotopographies. Early and mature biofilms were analysed with XTT assay. Data were statistically analysed using unpaired *t* test. Error bars represent standard error of mean.

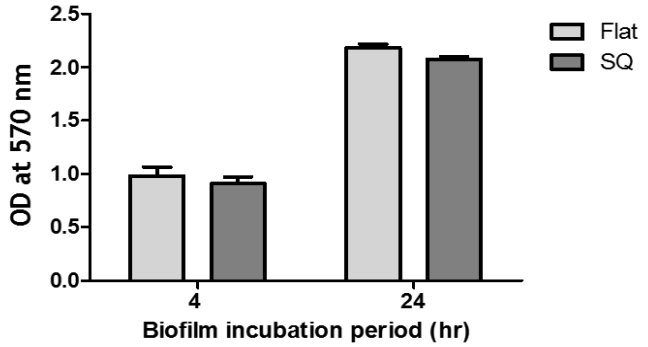


Figure 4.21: Evaluation of biomass of *C. albicans* biofilms on SQ nanotopographies. Early and mature biofilms were analysed with CV assay. Data were statistically analysed using unpaired *t* test. Error bars represent standard error of mean.

4.4.5 Combination of CUR and SQ nanotopographies

A potential synergistic effect of combining adsorption of sub inhibitory concentration of CUR with polycarbonate SQ nanotopographies on *C. albicans* adhesion and biofilm formation was assessed using colony counting method and biofilm metabolic activity and biomass assessment assays. Figure 4.22 shows a highly significant effect ($p<0.001$) of the CUR adsorption on reduction of adhesion (89-90%) and biofilm formation (73-77% metabolic activity) and (85-86% biomass) in both flat (F+CUR) and SQ (SQ+CUR) surfaces, respectively. However, a non-significant ($p>0.05$) impact was observed for combining the adsorbed CUR with nanotopographies over the CUR adsorbed flat topographies that might indicate the absence of synergism between CUR and nanotopographies.

Next, when the impact of nanotopographies on capacity of CUR adsorption was assessed, a non-significant difference was observed ($p>0.05$), though adsorption to nanotopographies (33.1 µg/ml) was slightly lower than that to flat topographies (36.7 µg/ml) (Figure 4.23).

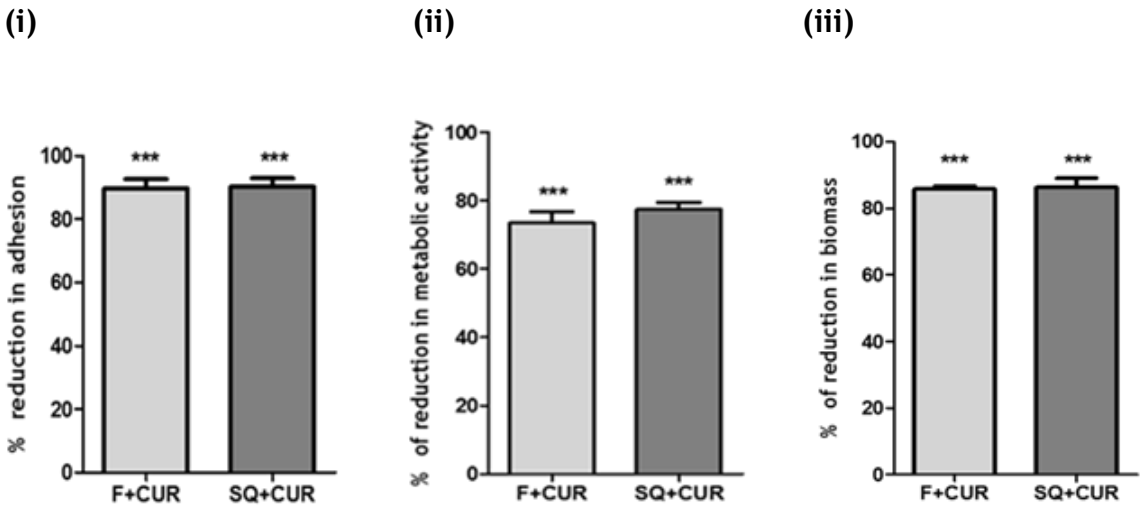


Figure 4.22: Effect of combining SQ nanotopographies with CUR on *C. albicans* colonisation. (i) Reduction in adhesion. Reduction in biofilm metabolic activity **(ii)** and biomass **(iii)**. F+CUR and SQ+CUR represent reduction in *C. albicans* colonisation of CUR-adsorbed flat relative to CUR-free flat surfaces and CUR-adsorbed nanotopographies relative to CUR-free nanotopographies, respectively. Error bars represent standard error of mean and *** asterisks represents $p<0.001$.

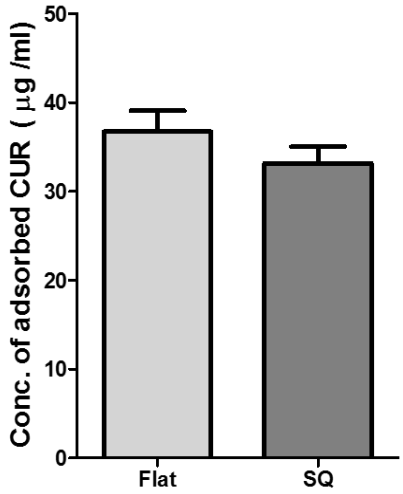


Figure 4.23: Adsorption of CUR to SQ nanotopography. The concentration of adsorbed CUR (μg/ml) was measured spectrophotometrically. Unpaired *t* test was used to analyse the data statistically ($p>0.05$). Error bars represent standard error of mean.

4.5 Discussion

Adherence of fungi and consequent biofilm formation is an important clinical problem (Rajendran *et al.*, 2016a), though these properties can also be exploited for novel therapeutic targets of fungi. Fungal attachment to biotic and abiotic surfaces can trigger a physicochemical interaction, and this contact sensing interaction can be ascribed to fungal-specific mechanisms permitting *C. albicans* to respond to different topographies in discriminative ways (Kumamoto, 2008). One promising approach is to reduce the available contact area to the *C. albicans* attachment through engineering of the surfaces. The "attachment point" theory (Hoipkemeier-Wilson *et al.*, 2004;Kearns *et al.*, 2011) might provide a support for explanation of the mechanical impact of the topographies tested on the capability of *C. albicans* adhesion. This theory assumes a stronger adherence of the microorganism if it is smaller than the topography feature and vice versa. Therefore, the aim of this chapter was to allow *C. albicans* adherence to topographies with relatively smaller features. Here, it was shown for the first time the ability to replicate sub-micro/nano-scale features using compression moulding technique on PMMA denture material to reduce candidal adhesion.

According to this study, for *C. albicans* Y form, micro-pillar and micro-pit topographies increased the adhesion capacity, though it was slight for the latter. A trend of higher adhesion percentages was observed onto the larger features of the highly ordered pillars only, which could be attributed to the consistent increase in the dimensions of the pitches (inter-pillar space). These pitches could offer shelter for the Y form cells.

For G form, the adhesion capacity was slightly decreased. In detail, the micro-pillar data disagree with studies performed on *Staphylococcus epidermidis/aureus* that showed reduction in adhesion with micron and sub-micron pillars (Xu & Siedlecki, 2012a;Xu & Siedlecki, 2014), which could be attributed to the difference in the adhesion methodology used or kind of the microorganism, but particularly, the massive difference in size and mass between bacteria and yeast (Salerno *et al.*, 2011).

The difference between the adhesion capacity of oval-shaped yeasts on micro-scale pillar and pit topographies can be explained by understanding these two topographies using simple geometric and arithmetic analysis. As an analogy, the inter-pillar field works in a manner similar to an egg box, where the area of the inter-pillar field is four times larger in terms of surface area compared to pit topography. Thus, pit featured topography has superior anti-adhesive potential relative to pillar topography, which was endorsed with the data presented above, suggesting the importance of spatial arrangement.

Cell morphology also plays an important role, as hyphal-shaped germling cells adhered to both micro-scale pillar and pit topographies with slightly reduced capacities. This may be in line with Whitehead & Verran (2006) and Perera-Costa *et al.* (2014), where they revealed the importance of shape and even the size of bacteria on retention on micro and sub-microtopographies. It is noteworthy to mention that G form has more adhesion capacity than Y form due to over-expression of some cell wall adhesins that support adherence and biofilm integrity (Nobile & Mitchell, 2005).

Nevertheless, microtopographies of maximum 2 μm features reduced their adhesion relative to that on flat indicating the importance of microorganism dimensions and shape on adhesion to engineered topographies. It was also shown that smaller feature dimensions and pit form topographies showed lower adhesion capacities relative to their larger and micro-pillar counterparts, which was the main driver for exploring smaller dimension materials. The highly order arrays of nanopits with a SQ form arrangement that were replicated on two different substrates showed the capacity to significantly reduce the adhesion of the pathogenic yeast *C. albicans* in both Y and G morphological forms. The disparity in the data presented in this chapter and that reported by Whitehead *et* al (2005), may simply be because of an order of scale from micro- to nano-topographies (Whitehead *et al.*, 2005).

An explanation for the anti-adhesive capacity of nanopits may be related to fluid dynamics, where air entrapment in the pit features may cause the surge in the hydrophobicity of the nanoimprinted topographies relative to their flat counterparts (Martines *et al.*, 2005), and given the dynamic lower resistance of fluid flow on hydrophobic surfaces (low fluid drag) (Rothstein, 2010). Accordingly, the nanoimprinted topographies might permit for larger removal of adherent *C. albicans* due to the lower resistance to fluids flow. This explanation coincides with the Whitehead & Verran (2006) concept about the role of hydrophobic surfaces in reduction of bacterial adhesion.

Furthermore, a recent study showed the anti-adhesive capacity to *C. albicans* of PMMA denture material coated with trimethylsilane hydrophobic film that increased the surface energy (Liu *et al.*, 2017). Moreover, the anti-adhesive capacity of nano-pit surfaces was not influenced by the wettability differences of the polycarbonate and PMMA denture material surfaces tested, where both nanoimprinted polymer types showed an anti-adhesive capability. This is in agreement with Perera-Costa *et al.* (2014) study that showed an anti-adhesive capacity of spatially organised micro engineered surfaces relative to flat surfaces on bacterial adhesion regardless of the surfaces hydrophilicity/ hydrophobicity of the microtopographies. Therefore, the trapped air in the nano-pits increased the hydrophobicity of the surface and it is one of the probable mechanisms for the antiadhesive capacity. While, we can suggest that the primary antiadhesive mechanism of the nano-pits is the physical mechanism where less surface area available for the adhesins to attach.

When the data of gene expression were analysed, interesting observations have confirmed the substrate differential impact on the expression of key adhesins. This may propose that the biology-related physical characteristics of the SQ nano-pit topographies were sufficient to challenge bio-molecular interactions between *C. albicans* morphological types, their adhesins, and the topography. The absence of total resemblance observed in gene expression profiles between both substrates tested might be clarified by the fact that Y and G cells are hydrophilic and hydrophobic, respectively (Rodrigues *et al.*, 1999), and from our data that PMMA and polycarbonate are hydrophilic and hydrophobic relative to each other,

respectively. Therefore, in a selective manner, induction of stress and differential induction of adhesins might be attributed to these physicochemical interactions (Fox *et al.*, 2015). Indeed, Krasowska & Sigler (2014) emphasised that hydrophobic microorganisms favourably adhere to hydrophobic substrates and the hydrophilic microorganisms adhere to hydrophilic substrates.

We could not explain the differences among the different nano-pit spatial arrangements tested using the gene expression data, though the highly ordered SQ and HEX arrangements showed relatively similar results. Nevertheless, several studies showed the potential inherent anti-adhesive capacity of nano-pit substrates to *in-vitro* mammalian cells adhesion formation (Martines *et al.*, 2004;Biggs *et al.*, 2007;Biggs *et al.*, 2009).

The SQ nanotopography was tested against harsh biofilm environments characterised with a dense interlocking network of candidal hyphae. These nanotopographies did not show significant anti-biofilm capability, that may be caused by the entangling filamental nature of the biofilms tested and the static environments that permit the candidal cells to adhere, settle, and form a cohesive community for a sufficient time prior to submitting to dislodging challenges. This stimulated investigation of the dual effect of combination the adsorbed CUR molecules with nano-surfaces. Additionally, given the proposed relative increase in the available surface area within nanotopographies for CUR adsorption that may suggest a synergistic effect. However, a nanotopography-CUR adsorption synergistic effect was not observed. The absence of a topography effect could be attributed to the larger effect of CUR that may preclude quantification of the topography effect. Besides, the adsorbed molecules may reduced the topography anti-adhesive influence. Furthermore, the nanotopographies did not adsorbed larger quantities of CUR as expected, which might be ascribed to the molecule size of the CUR used.

Non-patterned PMMA denture material showed high R_a, while the R_a of the nanopatterned PMMA was not measured because of the specific nanofabrication technique used in this study and the known depth of the nano-pits, nevertheless, it can be suggested to measure the R_a of nano-pit topographies in further studies.

Translationally, fabricating topography with SQ nanoimprinted pits onto PMMA denture material has its advantages over unpatterned surfaces, whose associated large surface roughness becomes negligible when nanoimprinted, taking into consideration the fabrication method of the fitting surface of the denture that is characterised by a non-polished surface. Instead, crucial biophysical characteristics are induced through the SQ pit nanotopography, where adhesion of *C. albicans* is significantly decreased. CUR adsorption has a substantial effect on candidal colonisation as shown in the last chapter. Indeed, the adsorption of CUR onto nanotopographies may add an advantage, especially if nanonisation technologies is considered, where nano-scale CUR molecules are harnessed. Regarding denture production, this has a potential value where the denture stomatitis affecting millions of denture wearing individuals globally. Overcoming the obstacles to manufacturer these will prove challenging, yet the potential impact to oral health is enormous.

Chapter findings

- PMMA denture material can replicate sub-micro and nano features.
- Adhesion of *C. albicans* to micro and sub-micro featured topographies depends on the candidal morphological form and the shape of the features.
- *C. albicans* variably respond to the spatial arrangement of nano-pit topographies, where SQ arrangement showed an anti-adhesive capacity for both candidal morphological forms tested.
- Hyphal candidal biofilms were not affected by the SQ nanotopography.
- The Molecular response to nanotopographies is complex and can be affected by the hydrophobicity/hydrophilicity and surfaces roughness of surfaces tested.

- Nano-pit surfaces showed higher water contact angle. Flat PMMA tested showed a rougher surface and lower water contact angle than their polycarbonate counterpart.

- Combination of natural antimicrobials with surface topography through adsorption of CUR to SQ nanotopographies did not significantly enhance the anti-adhesive and anti-biofilm capability. Similarly, SQ nanotopographies did not improve the CUR adsorption capacity to the surfaces.

5 Final discussion

5.1 Introduction

Removable dentures are beneficial prosthetic devices, which improve dental aesthetics and function although they can be detrimental and associated with oral and systemic diseases. PMMA denture materials have many beneficial features, such as good mechanical and physical properties and a relatively low production cost yet they are highly susceptible to accumulation of microbial biofilms (Sivakumar *et al.*, 2014).

Denture stomatitis is a multifactorial disease that affects more than half of all denture wearers who form a considerable percentage of the population due to the fact that one-fifth (20%) of the UK population have removable dentures (Hannah *et al.*, 2017) and given increases in life expectancy, this percentage is likely to rise. Indeed, the World Health Organisation predict that the percentage of people over 60 years old will approximately double from 11.7 in 2013 to 21.1% in 2050 (UN, 2013). Control of prosthesis associated biofilms is an essential strategy in the management of DS (Yarborough *et al.*, 2016). *Candida* species, and especially *C. albicans*, has been identified as a pivotal microbial contributing factor in DS (Gendreau & Loewy, 2011). Candidal denture carriage in health and disease has previously been investigated using traditional microbiological methods (Barbeau *et al.*, 2003;Coco *et al.*, 2008b;Dagistan *et al.*, 2009;Salerno *et al.*, 2011). In this study, a molecular-based qPCR was employed to obtain data and quantify adherent cells. Furthermore, given the heterogeneity (strain variability) of *C. albicans* biofilm formation, it was uncertain whether this property is associated with DS (Kean *et al.*, 2018).

Reports have shown that dentures are a potential reservoir for pathogenic respiratory bacteria that cause pneumonia and are associated with a high mortality rate (Przybylowska *et al.*, 2014;O'Donnell *et al.*, 2016). In polymicrobial environments, such as the oral cavity, *C. albicans* acts as a scaffold and via cell-cell communication can modify pathogenicity of either cell (Kean *et al.*, 2017). Therefore, discovering innovative strategies to control adhesion and biofilm formation of *C. albicans* is a worthwhile goal.

The development of novel therapeutic strategies that could augment the current strategies used in prevention and treating DS could improve the clinical management of denture-associated diseases.

5.2 Candidal denture biofilm and DS: Impacts of quantity and heterogeneity

Many studies have reported the association between *Candida* and DS, though, often denture carriage importance was not the specific focus. This study for the first time according to our knowledge reports the use of a molecular quantitative method (Real-time qPCR) to quantify the denture adherent candida cells, though previous studies used this molecular method to quantify bacteria in dental-related research (Blome *et al.*, 2008;Kim *et al.*, 2013;O'Donnell *et al.*, 2016). Here we confirm the significance of the candidal load on dentures in DS, though our data showed several dentures of healthy and diseased patients shared approximately similar candidal carriage quantities. These data, alongside the recently reported concepts of *C. albicans* biofilm formation heterogeneity (strain variability) associated with increasing resistance, pathogenicity and mortality (Sherry *et al.*, 2014;Rajendran *et al.*, 2016a), have driven this research to investigate the potential association between biofilm formation heterogeneity and DS.

The data described in this thesis did not demonstrate an active role for *C. albicans* biofilm forming heterogeneity in DS, which could be attributed to the relatively mild nature of DS in comparison to the systemic candedemia, which is often associated with immunocompromised patients, rather than the immunocompetent patient cohort examined in this study. Figure 5.1 summarises the obtained data regarding the candidal denture load, DS and biofilm formation heterogeneity.

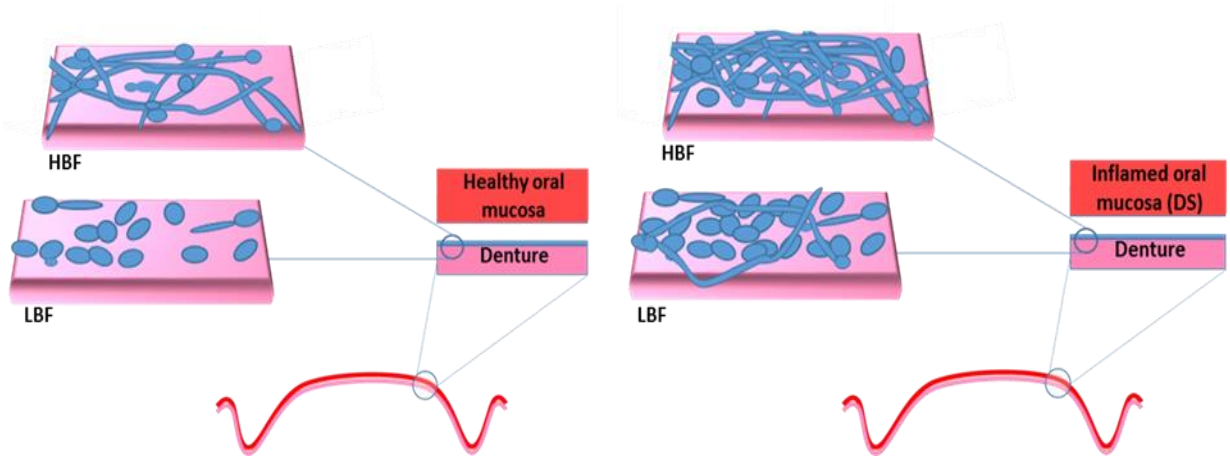


Figure 5.1: Schematic representation of the relationship of candidal denture load, denture stomatitis and *C. albicans* heterogeneity. Higher candidal load is associated with denture stomatitis. However, *C. albicans* biofilm formation heterogeneity does not represent a key determinant, where both HBF and LBF are observed similarly in isolates from DS and healthy participants. HBF and LBF represent high and low biofilm formers.

Challenges in the development of a globally standardised *in vitro* candidal biofilm model are numerous due to the adaptability of *C. albicans* to various environmental conditions (Soll & Daniels, 2016), therefore experiments to optimise and characterise biofilms were first undertaken in this thesis. This research mainly concentrated on the use of RPMI-1640 as a well-established medium to test the heterogeneity of biofilm formation and to develop recalcitrant biofilms. RPMI-1640 is capable of inducing different candidal morphological forms and subsequently it will offer the capability to investigate adhesion capacities of these various forms. However, this research also demonstrates that artificial saliva has the possibility of being used to recreate clinically relevant conditions in future studies.

Given that candidal carriage was found to be significant in DS, the question remained whether denture material has an impact on the early and mature stages of biofilm formation? There is little reliable evidence to show whether the denture material has an impact on early and mature biofilm formation (Pereira-Cenci *et al.*, 2008b). Therefore, a qPCR based methodology was used alongside representative clinical isolates with defined biofilm formation heterogenic capabilities. For all isolates, PMMA showed a greater capacity than soft liners tested to induce the formation of mature biofilms, but not early stage biofilms,

suggesting that the adhesion stage of biofilm formation is not influenced by denture material, though only limited number and type of denture base materials were tested. For this reason, studying the adhesion of *C. albicans* using representative denture related strains may provide a more clinically relevant approach to studying the capability of *C. albicans* to colonise denture surfaces.

5.3 Why CUR could be a promising polyphenol for denture wearers?

As of 2015, over 125 clinical trials were registered with the US National Institutes of Health investigating CUR biological activity (Ahmad *et al.*, 2017), which indicates the intriguing nature of this molecule in modern research. Cancer, Alzheimer and cardiovascular malfunction are destructive age-related diseases (Bennett & Leurgans, 2010). Given its potential anti-cancerous, anti-Alzheimer and beneficial cardiovascular-related CUR is a rational target for geriatric-related research (Hamaguchi *et al.*, 2010;Marchiani *et al.*, 2014;Hu *et al.*, 2015;Venigalla *et al.*, 2016;Yao *et al.*, 2016;Sun *et al.*, 2017;Larasati *et al.*, 2018;Reddy *et al.*, 2018). Indeed, most denture wearers are classified as being within the older population, thus introducing CUR to oral healthcare and denture-related research seems interesting on multiple levels.

Given the reported broad-spectrum antimicrobial effect of CUR (Moghadamtousi *et al.*, 2014;Vaughn *et al.*, 2017), one focus of this thesis was to investigate the potential interaction between CUR and one of the most common opportunistic microorganisms related to denture-associated diseases, i.e. *C. albicans*. The overarching hypothesis was that CUR could be used in a clinical context to manage and minimise the attachment of the organism and impact on downstream denture plaque development. The studies presented in this thesis showed that CUR effectively inhibited *C. albicans* in both modes of growth (planktonic and biofilm) in a concentration dependent manner. Nonetheless, biofilms showed signs of surviving even in the presence of high CUR concentrations, suggesting that CUR could be better employed in prevention rather than treatment of established denture biofilms.

Data presented in this thesis showed the affinity of CUR to adsorb to denture material polymers, and these adsorbed sub-inhibitory concentrations showed an anti-adherent impact on *C. albicans*. This impact was augmented by CUR pre-exposure to create a significant synergistic-like effect (Figure 5.2i). Therefore, using CUR in denture care soaks or direct oral consumption of CUR either as a dietary supplement, or through normal daily ingestion, may allow CUR to adsorb on to the denture material and exert its biological effect. The affinity of CUR to PMMA denture material could be driven by the hydrophobic interaction theory, where the hydrophobic substance is attracted to the hydrophobic surface of the denture in water-based biological environments (Israelachvili & Pashley, 1984). Furthermore, given the bonding affinity between salivary proteins/peptides and polyphenols, specifically proteins rich in the amino acid proline (Charlton *et al.*, 2002), the opportunity of interaction between the denture material and adsorbed CUR could be enhanced in natural (oral) environments due to deposition of the bonded complexes upon the denture material.

The dimorphic capacity of *C. albicans* is an important virulence factor expressed by this opportunistic microorganism and interrogating CUR capacity against the different forms appeared intriguing. Data presented in this thesis showed a greater effect on the immature form (yeast) in comparison to the mature form (hyphae). Mechanistically, the biological effects of CUR are many and complex (Zhou *et al.*, 2011). Amongst others, CUR acts as an iron chelating agent and this suggests that CUR has the potential to act as a divalent cation chelator such as ethylene diamine tetraacetic acid (EDTA) (Jiao *et al.*, 2006). The inhibitory effect of EDTA on a *C. albicans* biofilms was demonstrated through inhibition of filamentation and depletion of metal ions that are important to candidal biofilms integrity (Ramage *et al.*, 2007).

CUR has the capacity to modulate various molecular targets through alteration of gene expression and signalling pathways or by direct interaction with biological molecules (Shishodia, 2013). Indeed, CUR modulated some of the key adhesion and aggregation-associated genes in both yeast and hyphae morphological forms of *C. albicans*. The *ALS3* gene, which is involved in adhesion, dimorphism, invasion (Liu & Filler, 2011) and aggregation with bacteria (Peters *et al.*, 2012;Bamford *et*

al., 2015;O'Donnell *et al.*, 2015a), was down-regulated in the presence of CUR. This suggests a possible vital role for CUR in interfering with biofilm formation and therefore pathogenicity crucial for *in vivo* polymicrobial biofilm formation. However, *ALS5* and *AAF1* are aggregation associated genes that showed a considerable up-regulation. This coincides with the observed quantified aggregation that was elicited in response to CUR. *ALS1* gene expression was also affected, but the data was found to be inconclusive and varied depending on length of exposure to CUR. These data indicate the high adaptability of *C. albicans* to external modulating molecules. Previously, *ALS5* and *ALS1* have been shown to play a role in aggregation of *C. albicans* cells to themselves and to other bacteria (Klotz *et al.*, 2007). Therefore, the data here in could suggest a compensatory function to the down-regulated powerful *ALS3*. Unexpectedly, *Eap1*, which has an adhesin function and is regulated by hyphal inducing *EFG1* gene (Li & Palecek, 2003) showed a considerable up-regulation suggesting a noticeable cell-cell aggregation role (Li & Palecek, 2008) or potential compensatory regulative role due to the down-regulation of important hyphal inducing genes.

CUR molecules that coat the exposed cells may act as glue that keep the cells aggregated in a direct way and this direct physical aggregation could drive gene expression targets to elicit an indirect biological aggregation. Overall, CUR could contribute to cell-cell aggregation and less to cell-substrate adhesion, but this requires further studies to confirm (Figure 5.2).

CUR has been reported to have anti-amyloid effects through producing non-effective amyloid aggregates with less density and toxicity. Mechanistically, CUR can bind to the amyloid beta monomers or penetrate the amyloid beta polymerised sheets leading to weaker amyloid aggregates (Gupta *et al.*, 2011;Rao *et al.*, 2015;Reddy *et al.*, 2018). Given the amyloid-like clustering of the ALS adhesins on the cell wall (Lipke *et al.*, 2012) occurrence of these potential physico-biological events in the interaction between *C. albicans* and CUR is possible. Moreover, the amyloidogenic domain sequence that exists in several members of the ALS gene family (Garcia *et al.*, 2011) suggests the possibility of formation of weak aggregates due to the potential conformational changes in the expressed proteins. These weak cellular aggregates reduce the capability of *C.*

albicans to withstand shear stresses. This notion could explain why the cellular aggregates did not outweigh the anti-adherence capacity. Additionally, the relative hydrophobicity of CUR could prevent formation of the amyloid-like ALS clusters on the cell wall by acting as a macromolecular crowder (Latshaw & Hall, 2015). The inability to form these macromolecular adhesin clusters could reduce cell wall hydrophobicity, which indeed, was observed in the data shown in this thesis.

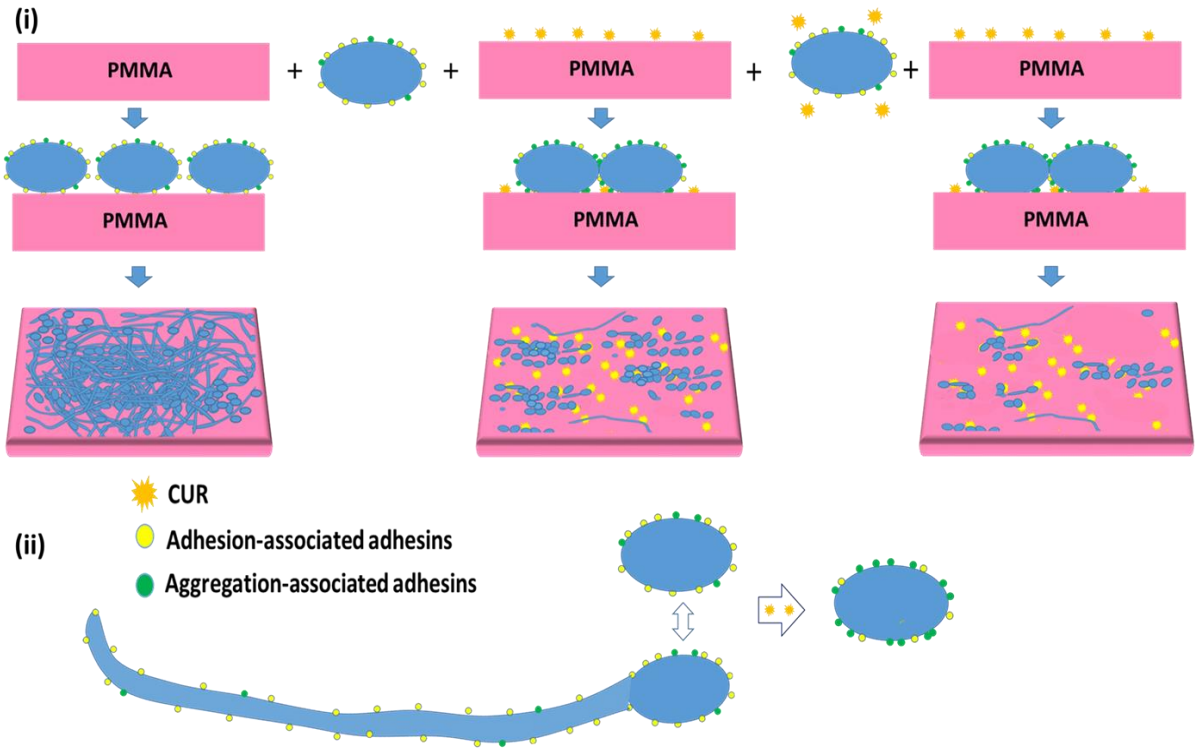


Figure 5.2: Impact of CUR on *C. albicans*. (i) Adsorption of CUR to PMMA reduces the number of adhered cells, elicits aggregation, reduces filamentation and induces a synergistic effect from the pre-treatment of the cells with sub-inhibitory concentrations. **(ii)** The expressed molecular response to CUR treatment.

Modification of denture acrylics with active and antimicrobial macromolecules is an important field for research but it is essential that researchers are mindful of the risks of unwanted sequelae (Sivakumar *et al.*, 2014). In spite of the expected colour alteration and the potential weakening effect on the mechanical properties of PMMA, it was interesting to investigate the opportunity of modifying the PMMA denture material with a safe and active molecule through creating simple CUR-incorporated PMMA to determine the subsequent effect on *C. albicans* adhesion.

The data showed no effect on the adhesion capacity. Investigation of CUR release from the polymerised PMMA in the appropriate solvent showed lack of detectable CUR release, suggest that there is a strong chemical bonding between CUR and PMMA. Nonetheless, using colourless curcuminoid analogues or derivatives and using conjugate chemistry needs further investigation (Tang *et al.*, 2010;Requejo-Aguilar *et al.*, 2016). In the context of the reported safety and multiple beneficial effects of CUR, designing clinical trials for DS-related research that include CUR as main component of a mouth wash, food supplement or denture soak are rational suggestions.

5.4 Micro and nanopatterned surfaces: the quest for antifouling denture surface

In the development of a new generation of biomedical materials, researchers must give consideration to the topography of surface as vital interactions take place with the biological environment at that interface (Li *et al.*, 2014). Biomedical material surfaces with a micro- and nano- fabricated surface have shown promise in a range of beneficial areas (Ploux *et al.*, 2009;Kearns *et al.*, 2011), such as antimicrobial properties (Xu & Siedlecki, 2012b) and the capacity to control stem cell adhesion and fate, which contributes to the evolution of the next generation of regenerative therapy (Dalby *et al.*, 2014). This significant impact on cellular behaviour has boosted the potential of fabrication of denture material surfaces to control the microbial fouling process. Micro- and nano-patterned denture surfaces could interfere with colonisation of *C. albicans* on dentures.

To this end, several micro- and nano-patterned surfaces were investigated in a rational manner, where the focus was on the first crucial stage of microbial biofilm formation 'adhesion'. The main morphological forms of *C. albicans* within the adhesion stage (yeast and germling) were tested because these forms have different shapes (ovoid and hyphal-like) and dimensions that could influence the interaction between cells and surface topography. In addition to the already successfully used injection moulding technique to replicate micro- and nano-scale features in polycarbonates (Reynolds *et al.*, 2012), data presented in this thesis showed the capacity of dental compression moulding to replicate micro, sub-micro

and nano-scale features in a heat cured PMMA denture. These data have paved the way to interrogate the adherence of *C. albicans* to these replicated topographies.

Review of the literature showed the potential antimicrobial properties of pillar shaped micro, sub-micro and nano-features (Ma *et al.*, 2011;Ivanova *et al.*, 2012;Xu & Siedlecki, 2012b;Xu & Siedlecki, 2014). Therefore, it was interesting to investigate adhesion to micro and sub-micro pillar topographies. In contrast to the literature, the data showed a considerable increase in the adhesion capability of the yeast morphological form to the micron and sub-micron pillars. This may be explained by differences in feature dimensions, material types, microorganisms and methodologies used. Nevertheless, germling forms of *C. albicans* showed a reduced adhesion capability. The form and dimensions of the adhered cells could be a potential explanation for this difference.

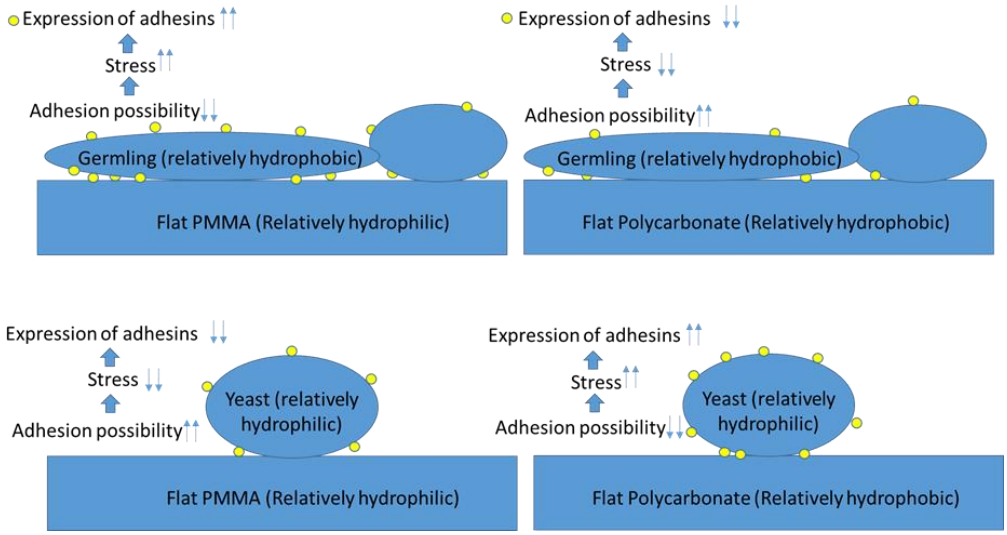
The data described above indirectly suggested that using the opposite of pillar topography, the 'pit form', may have more beneficial effects. Indeed, pit form microtopography displayed an anti-adhesive capability indicating an influential impact for this geometric form of patterned topography on adhesion of *C. albicans*. In light of the potential relative anti-adherent nature of pit microtopographies to *C. albicans*, nano-pit topographies in various arrangements were investigated, where the highly ordered arrangement forms, especially the SQ form, manifested anti-adhesive capability to both yeast and germling morphological forms in both denture materials (polycarbonate and PMMA denture material).

The gene expression profile of adherent cells on the nano-polycarbonate surfaces showed a down-regulation in the genes investigated, though such down-regulation was not observed in PMMA suggesting a greater role for physical mechanism in candidal adhesion. Investigation of the surface properties of the materials tested helped to understand the molecular response of the *C. albicans* and the differences observed between the materials, where relative hydrophobicity/hydrophilicity of the material and the adherent cells may elicit different stresses and interactions (Figure 5.3). PMMA denture material, which

was fabricated in a way that is relevant to prosthodontic clinical practice, showed high roughness average. Therefore, imprinting of the denture surface material with 100 nm depth pits appeared to be relatively negligible to the surface roughness effect, and theoretically produces PMMA denture surface with lower surface roughness that reduces the adhesion potential.

These data force us to consider the clinical feasibility of producing dentures with nanoimprinted features. Production of nanoimprinted polymer extra-thin sheets and the possibility of using them as a denture surface lining could be a promising suggestion. Indeed, production of nanoimprinted polymer sheets is interesting and reveals significant implications (Gadegaard *et al.*, 2006a). Another potential suggested idea is harnessing of direct nanoimprinting, where it was proven as a successful method to generate nanoimprinted topographies, even using much harder metal surfaces whether planar or curved (Greer *et al.*, 2013).

(i)



(ii)

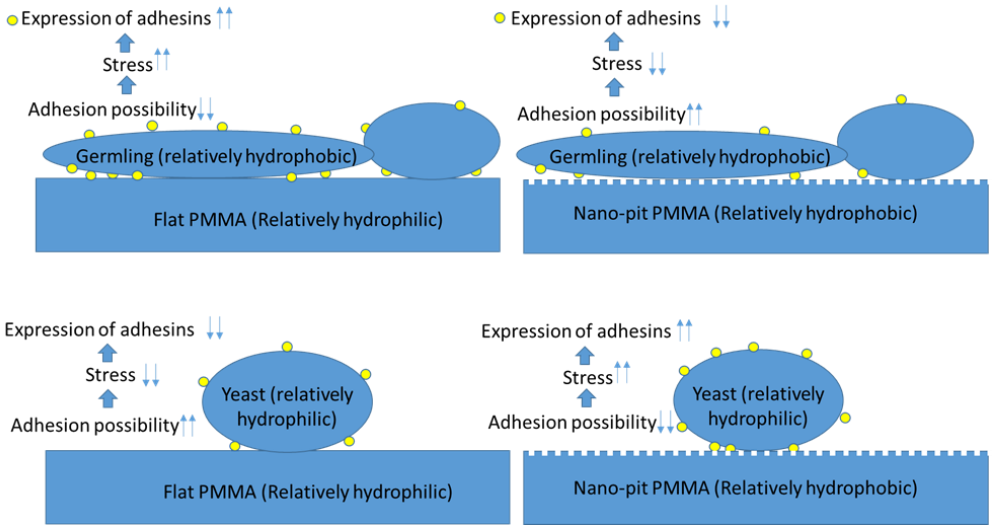


Figure 5.3: Schematic representation of adhesion of yeast and hyphae cells on relatively hydrophobic and hydrophilic surfaces. A simplification of our notion of the interactive adhesion between the surfaces and *C. albicans* morphological forms, and how this could shape the expression of adhesins in flat **(i)** and nanoimprinted surfaces **(ii)**. Yellow circles represent adhesins.

5.5 Combining nanotopography with adsorbing CUR

Given the anti-adhesive capability that was separately observed against *C. albicans* from CUR adsorbed and nanoimprinted surfaces, it was intriguing to combine these different anti-adhesive strategies in an endeavour to obtain a synergistic-like effect. Indeed, this idea has been inspired by nature, where anti-fouling surfaces manifest both chemical and physical properties (Magin *et al.*, 2010). For instance, shark skin and lotus leaves harness the combination of surface topography and surface chemicals to synergise their anti-fouling effect (Sun *et al.*, 2005;Ma *et al.*, 2011;Bixler & Bhushan, 2012). A research group from China reported the potential biological diverse benefits of combining patterned topographies with chemically modified polymers and the possibility of obtaining optimal results (Li *et al.*, 2014). Therefore, CUR was adsorbed onto SQ nano-pit topographies, and adhesion and biofilm formation was investigated. Anti-adhesive or anti-biofilm synergism was not observed, and the CUR effect may control the combination data. The nanotopographies showed a trend of reduced adsorption of CUR, which may explain this observation. Furthermore, the concealed role of the nanotopography might be attributed to the size and accumulation of adsorbed CUR molecules that may reduce the topography effect suggesting using nano-sized CUR particles as a promising strategy.

5.6 Future work

The structure of this thesis was focused on the interaction between DS and candidal biofilms on denture material, besides interrogation of novel strategies to control these tenacious biofilms. The studies presented herein potentially show that reducing denture candidal carriage through nature-inspired concepts of combining physical or chemical approaches could reduce the likelihood of patients developing DS. Accordingly, further investigation and development of these strategies will increase their efficacy to reduce microbial biofilms and this will form the basis of future work. It would be interesting to investigate the nano-pits of SQ arrangement of different dimensions such as smaller diameter or smaller pitches (pit centre to pit centre). Another suggestion is to design and investigate SQ nano-pits of extreme hydrophilic and hydrophobic nature.

Taking the research into different dimensions could reveal a promising field of research, such as challenging the strategies used in this research with relevant bacterial biofilms (*Streptococcus mutans*, *Staphylococcus aureus*) and more intricate polymicrobial biofilms to investigate the potential possibility of CUR molecules and highly ordered nano-pit topographies to control diverse biofilms. Indeed, Ramage's lab has a previous experience with similar diverse Harnessing polymicrobial biofilms (Sherry *et al.*, 2013;Millhouse *et al.*, 2014;Sherry *et al.*, 2016) and this lab showed the potential antibacterial efficacy of CUR against periodontal pathogens (Shahzad *et al.*, 2015). Furthermore, several reports showed the potential antibacterial capacity of submicron and nanopatterned topographies against polymicrobial biofilms (Ploux *et al.*, 2009;Xu & Siedlecki, 2012b). Thus, investigation of polymicrobial biofilms is a worthwhile future exercise.

CUR sensitivity to light can be utilised to increase its efficacy (photodynamic therapy) and to reduce its staining effect. Given the safety of CUR, there is an opportunity to design a clinical trial to investigate the preventive effect of CUR on the adhesion of *C. albican* and the possible incidence of DS in healthy denture wearers using various ways of delivery to the oral cavity.

The use of nano-sized modified CUR, conjugated CUR, CUR modified through liposomes or polymeric micelles could amplify the biological efficacy and enhance the low oral bioavailability that are observed in clinical trials using free non-modified CUR. Indeed, a recent study showed the effectivity of functionalising the fitting surface of the PMMA denture with polycaprolactone microspheres containing Amphotericin-B for anti-candidal therapy using a 3D printing (fused filament fabrication) technique (Nagrath *et al.*, 2018). In addition, using CUR analogues or derivatives characterised with colourless or neutral colours and investigating their biological activity on adhesion and biofilm formation of *C. albicans* on denture materials is also worthy of future investigation. If this strategy reveals promising results there will be a chance to integrate these analogues or derivatives in the denture materials through incorporation of conjugates or coating strategies, where the resultant denture material could be nanoimprinted to maximise the beneficial anti-biofouling impact.

Finally, in light of increasingly emerging multi drug-resistant candida pathogens, even against the potent echinochandins (Arendrup & Patterson, 2017;Xiao *et al.*, 2018) the search for new preventative strategies takes on even greater importance. Failure to prevent unnecessary use of antifungals and reduce investments in searching for new antifungals are critical issues that enhance the growth of resistant pathogens. Accordingly, focusing on development of preventive strategies is important. Using host-derived molecules for instance, acetylcholine (Rajendran *et al.*, 2015) alongside micronutrients to control these complex biofilms as an alternative to traditional antimicrobials appears to be a potential strategy.

Thesis findings

- The multifactorial nature of DS cannot conceal the impact of denture candidal carriage that shows superior significance relative to the candidal heterogeneity, thus reducing that carriage is a worthwhile target.

- Nature inspired strategies of combining safe, natural, chemical molecules with nanofabrication physical approaches to control microbial biofilms are intriguing and worth further interrogative work. Using CUR molecules with sub-inhibitory concentration and SQ arrangement form of nano-pit topographies reduced adhesion of *C. albicans* to PMMA denture material. However, adsorbing CUR to the nano-pit topographies did not show significant synergistic-like effect.

- The obtained data from the strategies used showed antifouling capability against *C. albicans* biofilms and encourage investigation against more complicated polymicrobial biofilms.

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The Anti-Adhesive Effect of Curcumin on *Candida albicans* Biofilms on Denture Materials

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The use of natural compounds as an alternative source of antimicrobials has become a necessity given the growing concern over global antimicrobial resistance. Polyphenols, found in various edible plants, offers one potential solution to this. We aimed to investigate the possibility of using curcumin within the context of oral health as a way of inhibiting and preventing the harmful development of *Candida albicans* biofilms. We undertook a series of adsorption experiments with varying concentrations of curcumin, showing that 50 μg/ml could prevent adhesion. This effect could be further synergized by the curcumin pre-treatment of yeast cells to obtain significantly greater inhibition (>90%, $p < 0.001$). Investigation of the biological impact of curcumin showed that it preferentially affected immature morphological forms (yeast and germlings), and actively promoted aggregation of the cells. Transcriptional analyses showed that key adhesins were down-regulated (ALS1 and ALS3), whereas aggregation related genes (ALS5 and AAF1) were up-regulated. Collectively, these data demonstrated that curcumin elicits anti-adhesive effects and that induces transcription of genes integrally involved in the processes related to biofilm formation. Curcumin and associated polyphenols therefore have the capacity to be developed for use in oral healthcare to augment existing preventative strategies for candidal biofilms on the denture surface.

Keywords: Curcumin, polyphenol, *Candida albicans*, adhesion, adsorption

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INTRODUCTION

Increasingly there are fewer antimicrobial options available to treat life-threatening infections, due largely to therapeutic mismanagement actively driving resistance, coupled with disinvestment in antimicrobial drug development from the pharmaceutical industry. The antimicrobial resistance debate suggests an imminent return to an era of uncertainty and limited therapeutic options, suggesting that we should stop our wavering and tackle this threat head on.

Naturally derived chemotherapeutic agents are an attractive option, particularly those botanically derived molecules, which offer advantages over synthetic derivatives due to their natural evolution and diminished likelihood of resistance. An interesting active plant extract worth consideration is the polyphenol curcumin (diferuloylmethane), extracted from the

rhizomes of the *Curcuma longa* plant (Mahmood et al., 2015). It is the active ingredient of turmeric and copiously used in Asia as a food additive or dietary supplement, though it forms typically <5% composition (Esatbeyoglu et al., 2012; Kwon, 2014). Curcumin (CUR) shows safe and effective biological activities such as anti-inflammatory, antioxidant, anti-proliferation, with potential efficacy against many human diseases as suggested by animal studies (Gupta et al., 2012). Importantly, CUR displays broad-spectrum antimicrobial properties (Moghadamtousi et al., 2014), including antibacterial (Shahzad et al., 2015; Tyagi et al., 2015) and antifungal properties (Martins et al., 2009; Khan et al., 2012), as well as the ability to influence adhesive and biofilm properties (Shahzad et al., 2014, 2015).

Studies by our group have shown that CUR has the capacity to alter the adhesion of key periodontal pathogens, and impact overall biofilm formation (Shahzad et al., 2015). Parallel studies on the primary denture pathogen *Candida albicans* demonstrated that CUR exhibited anti-biofilm properties at high concentrations, as well as antifungal activity against planktonic and biofilm cells (Shahzad et al., 2014). It is thought that these elevated concentrations directly impact cell wall permeability through signaling of the MAP kinase and calcineurin-mediated signaling, pathways which maintains cell wall integrity (Kumar et al., 2014). *C. albicans* is a major global opportunistic pathogen, armed with recognized virulence determinants that include colonization factors (adhesins, hyphae and thigmotropic properties), as well as the release of invasins, such as hydrolytic proteins that facilitate invasion into the host (O'Donnell et al., 2015c). The ability to adhere to both biological and inert substrates and form biofilms makes this organism of particular interest in the context of oral disease (O'Donnell et al., 2016). Biofilm etiology in this environment is a primary mechanism of persistence and survival in the oral cavity, providing physical protection from endogenous and exogenous antimicrobial factors (Ramage et al., 2014). Most significantly, *C. albicans* prominent role in inducing inflammation to cause denture induced stomatitis means we have a keen interest in developing ways to manipulate and interfere with biofilm development, as this is critical in preventing this disease. Therefore, this study aimed to investigate whether CUR could be used through direct interaction with materials and the yeast *C. albicans* to interfere with early adhesion events on a clinically relevant substrate.

MATERIALS AND METHODS

Culture Conditions and Standardization

The laboratory based *C. albicans* SC5314 was used in this study (O'Donnell et al., 2016). Yeast cells were cultivated as working stocks on fresh Sabouraud agar (Sigma-Aldrich, UK) for 48 h at 30°C and maintained at 4°C. One unique colony was used to grow the cells in yeast-peptone-dextrose (YPD) medium (Sigma-Aldrich) for 18 h at 30°C and 150 rpm orbital shaker. The cells were washed twice by centrifuging in sterile phosphate buffered saline (PBS; Sigma-Aldrich, UK) and standardized using a Neubauer haemocytometer.

Antifungal Susceptibility Testing

Planktonic and sessile cells were first investigated for their susceptibility to the polyphenol CUR (HPLC grade, Acros Organics, Belgium). Stock CUR was prepared immediately preceding the experiment using an non-antimicrobial concentration of dimethyl sulfoxide (DMSO) as a solvent and adjusted to <5% v/v in RPMI-1640 medium (Sigma-Aldrich, UK; Shahzad et al., 2015). Standardized CLSI M-27A broth microdilution methodology was initially undertaken for planktonic yeast cultures in 96 well round bottomed microtitre plates (CLSI-M27-A, 2008). Clear wells with no visible growth were considered as the minimum inhibitory concentration (MIC). For sessile susceptibility testing, pre-formed 24 h biofilms were challenged with CUR using standardized sessile antifungal testing (Ramage et al., 2001; Pierce et al., 2008). Reduction of tetrazolium to formazan through an XTT assay was used, and the optical densities quantified at 492 nm using a microtitre plate reader (FluoStar Omega, BMG Labtech, UK). Negative and positive controls were included, and the experimental wells data were compared to the positive control data to reveal the $SMIC_{80}$, where the optical density is reduced more than 80% in comparison to the positive control optical density, reflecting significant bioactivity against the biofilm. These procedures were repeated in three independent occasions where three replicates have been considered.

Investigating the Effect of CUR Adsorption on Adhesion

The potential capability of CUR to be adsorbed to denture material was investigated. Heat cure poly methyl methacrylate (PMMA) denture base material (Chaperlin and Jacobs Ltd, Surrey, UK) was used to fabricate 12 mm diameter discs using the dental compression molding technique. These discs were immersed in ddH_2O for 7 days to ensure excess toxic monomers were removed. CUR was diluted in RPMI-1640 medium to 200, 400, and 800 μg/ml. Discs were distributed in 24 wells plates (Costar, Corning Incorporated, USA) and 1 ml of the CUR suspension was added. The plates were incubated at room temperature for a series of time points (1, 5, 10, 30, 60, 120, 240, and 1,440 min). Next, discs were transferred to fresh wells and washed with 1 ml of distilled water and then 1 ml of DMSO was added to dissolve the adsorbed CUR. To quantify the released CUR, a standard curve (0.39–100 μg/ml serially double diluted) was developed and measured at 436 nm using the spectrophotometer. Based on these data, PMMA discs were immersed in 1 ml of 800 μg/ml of CUR for 10 min (equivalent to 50 μg/ml) then washed with PBS to remove the unabsorbed molecules. These treated discs were inoculated with 1 ml of 5×10^5 CFU of *C. albicans* SC5314 cells and incubated for 30 min at 37°C. Following the initial adhesion, cells were washed in PBS and the adherent cells removed by sonication at 35 kHz for 10 min (Ultrasonic bath, Fisher scientific, UK), and enumerated using the Miles and Misra plate counting method (Miles et al., 1938). The final cell number was expressed per cm^2 PMMA, which was compared to a CUR negative control. All experiments were performed

with three independent sections on three independent occasions. Scanning electron microscopy (SEM) was also performed using the same experimental parameters, then processed and imaged, as described previously by our group. Briefly, biofilms were grown on Thermanox™ coverslips or hydrogel cellulose matrix and treated, as previously described. Biofilms were washed twice with PBS, before being fixed in 2% para-formaldehyde, 2% glutaraldehyde, 0.15M sodium cacodylate, and 0.15% w/v alcian blue, at pH 7.4, and prepared for SEM as previously described (Erlandsen et al., 2004). The specimens were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope.

Investigating the Biological Effect of CUR on Adhesion and Biofilm Formation

Discs were adsorbed with CUR as described above, then the discs (untreated and treated) were inoculated with either 1 ml of 5×10^5 CFU + 3 min CUR (50 μg/ml) or PBS (negative control) treated *C. albicans* SC5314 cells, and incubated for 30 min at 37°C. Adhesion of *C. albicans* was then assessed and quantified as described in the previous section, with the levels of adhesion expressed as a proportion of the negative control (PMMA-/*C. albicans*-). In parallel we then assessed whether longer CUR exposure time negatively impacted adhesion by treating cells for 3, 30, and 90 min, and the levels of adhesion to PMMA quantified. Finally, we assessed whether or not CUR exposure to *C. albicans* cells at different growth phases played a role, with the hypothesis that there may be differences in how yeast (Y), germlings (G), or hyphae (H) responded to this molecule. Briefly, cells were grown overnight, standardized to 1×10^6 CFU in RPMI and inoculated into a 96-well microtitre plate. Cells were then exposed to 50, 100, or 200 μg/ml CUR at either 0 h (Y), 2 h (G), or 4 h (H) post-inoculation and incubated for a further 24 h at 37°C. Thereafter, the cells were washed in PBS and the resultant biofilm quantified using an XTT metabolic reduction assay. All experiments were performed in duplicate on three independent occasions.

Investigating the Aggregative Effect of CUR

In order to assess whether CUR has additional effects on the physicality of these yeasts, we assessed its impact on aggregation. *C. albicans* SC5314 cells (Y) were standardized (1×10^6 cells) in PBS and exposed ± to subinhibitory concentrations of CUR (50 μg/ml) for 90 min at 37°C under constant agitation (200 rpm). Following incubation the cells were serially 10 diluted in PBS diluent and plated onto Sabouraud agar using the Miles and Misra methodology. The plates were then incubated overnight at 37°C and the colonies enumerated. In parallel, cells were examined under a light microscope to evaluate aggregation visually. All experiments were performed in triplicate on three independent occasions.

Investigating the Molecular Effect of CUR on Adhesion and Biofilm Formation

Preparation of Y and H cells was performed using an initial inoculum of 1×10^8 cells and 5×10^5 cells of *C. albicans* SC5314 in RPMI, respectively. For H cells these were incubated on PMMA sections within 24 wells plates for 4 h. Both Y and H cells were then treated ± CUR (50 μg/ml) in RPMI for 3, 30, and 90 min, after which they were prepared for RNA extraction. Cells were either centrifuged or sonicated in a 35 kHz for 10 min (Ultrasonic bath, Fisher scientific, UK) to harvest the cells. These were then washed by centrifugation prior to RNA extraction using a combined mechanical disruption (0.5 mm glass beads) and chemical TRIzol™ method (Invitrogen, Paisley, UK). After DNase treatment (Qiagen, Crawley, UK) and purification (RNeasy MinElute clean up kit, Qiagen, Crawley, UK), cDNA was synthesized using a High Capacity RNA to cDNA kit (Life Technologies, Paisley, UK), and quantitative PCR performed using a SYBR® GreenER™ assay (Life Technologies Ltd, Paisley, UK). The primers used for quantitative PCR were ALS1, ALS3, ALS5 (agglutanin-like sequence 1, 3, and 5), EAP1 (epithelial adhesion protein 1), and AAF1 (adhesion and aggregation factor 1). Each parameter was analyzed in duplicate using MxProP Quantitative PCR machine and MxProP 3000 software (Stratagene, Amsterdam, Netherlands). Gene expression was normalized to the housekeeping gene ACT1 according to $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). **Table 1** summarizes all the primer details used in this study. A heatmap was created for the differential expression of genes ($\log_2$) over the period of 3–90 min from the untreated control compared to CUR exposed cells. Maps and clusters were generated in R with the use of heatmap 0.2 function from the gplots package. All experimnets were performed in triplicate on three independent occasions.

Statistical Analysis

As we were unable to ascertain that the data conformed to a Gaussian distribution data analysis was performed on non-parametric data using either a Mann–Whitney test or a Kruskal–Wallis test with Dunn's multiple comparison post-test. All independant data points are presented, with error bars representing the median with interquartile range. Where

TABLE 1 | Primers used for real time qPCR transcriptional analysis of *Candida albicans*.

Gene	Sequence (5′–3′)	References
ALS1	F—TTCTCATGAATCAGCATCCACAA	Nailis et al., 2009
	R—CAGAATTTTCACCCATACTTGGTTTC	
ALS3	F—CAACTTGGGTTATTGAAACAAAAACA	Nailis et al., 2009
	R—AGAAACAGAAACCCAAGAACAACCT	
ALS5	F—CTGCCGGTTATCGTCCATTTA	Green et al., 2005
	R—ATTGATACTGGTTATTATCTGAGGGAGAAA	
EAP1	F—ACCACCACCGGGTATACAAA	Sherry et al., 2014
	R—GCCATCACATTTGGTGACAG	
AAF1	F—CTGCCCTTGTTGGTACATCT	This study
	R—TGGGATAGTTGGTGGAGGAG	
ACT1	F—AAGAATTGATTTGGCTGGTAGAGA	Ricardo et al., 2009
	R—TGGCAGAAGATTGAGAAGAAGTTT	

proportional data is presented, analysis was performed on the original data sets. All statistics and figures were produced using GraphPad Prism v.5 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Curcumin Adsorption Reduces *Candida albicans* Adhesion

First, we tested the potential of CUR to inhibit and kill planktonic and biofilm cells to establish biologically active working concentrations suitable for use in downstream analyses. The planktonic MIC (PMIC) was shown to be 100 μg/ml of CUR for SC5314 and two other clinical strains tested (data not shown), whereas the sessile (biofilm) MIC that caused an 80% reduced metabolic activity ($SMIC_{80}$) was ≥ 200 μg/ml, demonstrating that the biofilm's activity and/or viabilty are significantly reduced.

Based on these data we wanted to evaluate whether these levels of CUR could be adsorbed to PMMA material to prevent *C. albicans* adhesion. We therefore adsorbed 200, 400, and 800 μg/ml of CUR to PMMA sections over different time periods and quantified these adsorbed concentrations using an elution method alongside an optimized standard curve. **Figure 1A** illustrates the kinetics of adsorption for each concentration. It was shown that 800 μg/ml CUR was required to achieve concentrations with anti-biofilm activity (200 μg/ml), though 90 min adsorption was required to achieve this. Nevertheless, after 10 and 30 min adsorption, 50 and 100 μg/ml concentrations were achieved from this initial concentration, respectively. The lower concentration of 400 μg/ml was able to achieve PMIC levels, though this took ∼90 min adsorption. Finally, 200 μg/ml was not able to achieve any antimicrobial level concentrations, even after 24 h adsorption. Based on these data we focussed on adsorption of 800 μg/ml for 10 min, which was able to achieve 50 μg/ml on the surface of the PMMA for downstream analysis. Next, we evaluated the capacity of *C. albicans* to attach to PMMA for 30 min adsorbed with CUR (50 μg/ml) and compared a control (**Figure 1B**). Here we showed a significant three-fold reduction in adhesion of *C. albicans* was observed ($p < 0.004$). When analyzed by SEM the visible reduction of yeasts cells can be shown on the PMMA surfaces (**Figure 1C**).

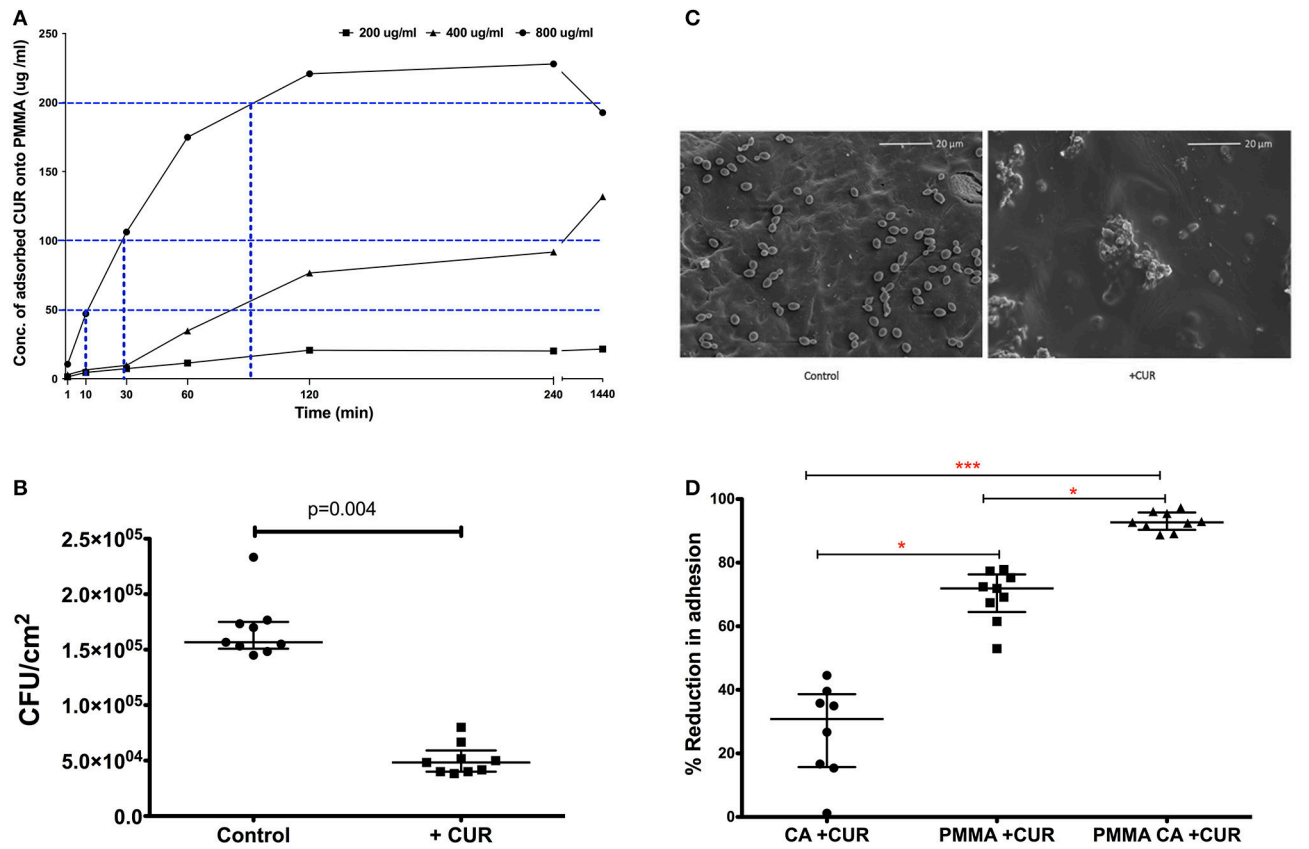

FIGURE 1 | The impact of CUR adsorption to PMMA and its impact on *Candida albicans* SC5314 adhesion. (A) Time and concentration dependant adsorption of CUR to PMMA (blue dotted line) at half the MIC (50 μg/ml), MIC (100 μg/ml), and $SMIC_{80}$ 200 μg/ml. **(B)** *C. albicans* inhibitory capability of 50 μg/ml adsorbed CUR onto PMMA compared to untreated control and a Mann–Whitney test was performed on data from nine independent experiments. **(C)** SEM images of 30 min adherent *C. albicans* cells onto CUR adsorbed (+CUR) and non-adsorbed PMMA control. **(D)** Single and dual-treatment of CUR on *C. albicans* adhesion, which were analyzed using a Kruskal–Wallis test with Dunn's multiple comparison post-test performed on data from nine independent experiments. All independant data points are presented, with error bars representing the median with interquartile range (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Given that CUR was shown to elicit antimicrobial effects, both planktonically and to a lesser extent against sessile biofilms cells, and that adsorption appeared to influence adhesive capacity, we decided to investigate whether combining an early direct effect on *C. albicans* with that of adsorption to PMMA would synergize CUR activity in prevention of adhesion and colonization. *C. albicans* was treated for 3 min ± CUR 50 μg/ml (CA + CUR), which was compared to both PMMA adsorbed with CUR (PMMA + CUR), or a combination of CUR treated *C. albicans* and CUR adsorbed PMMA (PMMA CA + CUR). **Figure 1D** demonstrates that the direct treatment of *C. albicans* alone only inhibited 30 min adhesion by 27%, which was significantly lower than CUR adsorption alone (70%, $p < 0.05$). However, combining the effect on *C. albicans* with adsorbed CUR resulted in a significant reduction than adsorption alone of 93% ($p < 0.001$), thus improving the anti-adhesive capacity of *C. albicans*.

Curcumin Prevents Biofilm Formation and Promotes *Candida albicans* Aggregation

Our data above showed a positive anti-candidal effect with respect to surface adsorption, but also showed that a brief CUR pre-exposure (3 min) resulted in reduces adhesion of *C. albicans*, suggesting sub-inhibitory concentrations elicited some biological activity. We therefore sought to further investigate this effect on *C. albicans* by extending the CUR pre-exposure time. We were able to show that extending the time from 3 to 30 and 90 min significantly enhanced anti-adhesion ($p > 0.01$). Though, between 30 and 90 min there were no significant improvements in anti-adhesion properties (**Figure 2A**).

In order to understand the longer term effects of CUR treatment on *C. albicans* and how this could impact biofilm formation, we prepared yeast cells (Y = 0 min), germlings (G = 120 min) and hyphae (H = 240 min) prior to CUR exposure at 50, 100, and 200 μg/ml, which were then allowed to develop for biofilm for 24 h (**Figure 2B**). The resultant data showed that at lower sub-inhibitory concentrations (50 μg/ml) and PMIC levels (100 μg/ml) the anti-biofilm effects moderately impacted the overall biofilm metabolism, though at SMIC levels (200 μg/ml) a significant reduction in biofilm formation was observed for Y and G to approximately >90% of the control ($p < 0.01$), though H cells were least impacted ($p < 0.05$).

Next, we sought to determine whether CUR exhibited any additional effects on *C. albicans*. The premise of the experiment was to assess whether CUR induced aggregation through

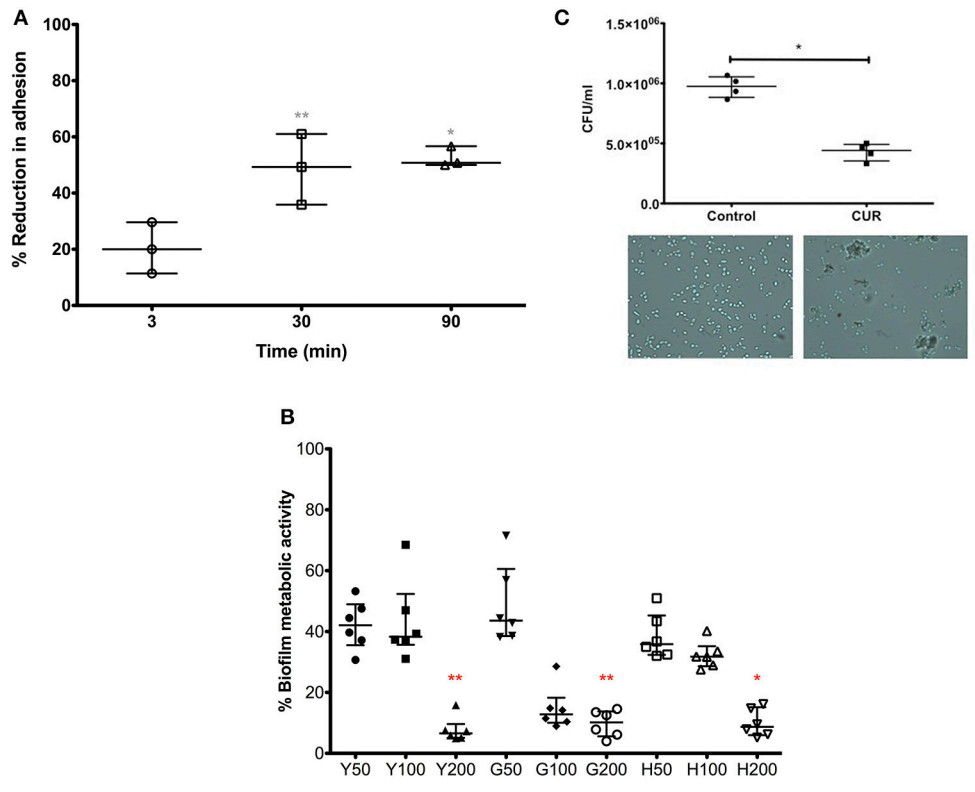

FIGURE 2 | The impact of CUR on *C. albicans* adhesion, biofilm formation, and aggregation. (A) *C. albicans* was pre-treatment with sub-inhibitory concentration of CUR (50 μg/ml) for 3, 30, and 90 min and adhesion to PMMA assessed on data from triplicate data from three independent experiments. **(B)** Different morphological forms of *C. albicans* (Y, yeast; G, germlings; H, hyphae) were pre-treated with CUR at 50, 100, and 200 μg/ml, and the resultant biofilm formation assessed metabolically after 24 h. Data represents six independent experiments, which was analyzed using a Kruskal–Wallis test with Dunn's multiple comparison post-test. **(C)** Aggregation of *C. albicans* exposed to CUR (50 μg/ml) was assessed by total viable cell counts, analyzed using a Mann–Whitney test on triplicate data from four independent experiments, and the phenotype validated by light microscopy (400 × magnification). All independent data points are presented, with error bars representing the median with interquartile range (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

alteration of the cell wall surface. We reasoned that using a plate counting based approach of cells treated at sub-inhibitory concentrations, then if aggregation occurred then the CFU would be lower than the respective control as each aggregate would result in only one CFU due to a heterogeneous population of cells. Indeed, we demonstrated a significant reduction in cell counts in the CUR group ($p < 0.01$), which was further confirmed through light microscopy observations (**Figure 2C**).

Curcumin Affects the Temporal Expression of *Candida albicans* Adhesins

Our data above suggest that CUR elicits biological effects on *C. albicans*, most significantly on preventing biofilm formation and impacting aggregation. We therefore aimed to assess a panel of associated genes through transcriptional analysis. To do this we focussed on Y and H cells at 3, 30, and 90 min post CUR exposure. Visual representation of these patterns was illustrated using heat map analysis and hierarchical clustering (**Figure 3**; raw C_T data profiles are presented in Supplementary Figure 1). We demonstrated that Y cells treated with CUR showed temporal changes in gene expression, most notably the down-regulation of the adhesin ALS3, and minimal impact on its related ALS1. Whereas, the clustered aggregative and flocculation genes AAF1, EAP1, and ALS5 transcripts were all up-regulated in a time dependant manner. Similar patterns and clustering of expression were also observed for the H cells, with AAF1 showing the highest levels of expression at 30 min compared to the control, and reciprocally ALS3 being the most down-regulated. Overall though the levels of differential expression were consistently lower in the H cells (**Figure 3B**) than Y cells (**Figure 3A**).

DISCUSSION

CUR is a polyphenol with potent biological effects, and has been described as a modern biological regulator (Esatbeyoglu et al., 2012). The data presented herein demonstrate the potential for its use within the context of oral health for denture wearers. We have shown that CUR has the capacity to adsorb to denture relevant substrates and to inhibit *C. albicans* adhesion, rather than actively kill or inhibit the microorganisms. Interestingly, *C. albicans* exposed to CUR induced cellular aggregation, and effect that also reduced its adhesion capacity. Transcriptional analyses revealed that key adhesins were negatively impacted whereas genes associated with aggregation were positively impacted. Collectively, these data demonstrate that CUR has the potential to be used in denture care as a means of preventing denture-induced stomatitis, a disease associated with *C. albicans* biofilms (O'Donnell et al., 2015a).

Initially, we wanted to evaluate and confirm the antimicrobial properties of CUR. Our data is in general agreement with others, showing that concentrations around 100 μg/ml are required to inhibit cellular growth (Martins et al., 2009; Sharma et al., 2010; Khan et al., 2012), and 200 μg/ml to elicit any anti-biofilm activity (Shahzad et al., 2014). Any deviations can be accounted for in terms of variability of CUR source and purity, ratios of curcuminiods involved, and protocols used for broth microdilution method and the strains used. Given that our primary interest was in preventing *C. albicans* opposed to actively inhibiting and killing *C. albicans* then we focussed on lower sub-inhibitory concentrations of 50 μg/ml. This was driven through the translational possibility that CUR could be taken as part of a diet or supplement, so could be maintained within saliva as well as adsorbing to hard tissues and prostheses in the oral cavity. Here, we showed that ∼6% of the CUR provided adsorped to the surface within 10 min. Conceptually, CUR could be delivered directly during ingestion, and then back through the blood into saliva indirectly. In Nepal and India for example, daily CUR consumption can reach up to 100 mg in Nepal and India (Shahzad et al., 2015), whereas in South Korea this may only reach 15 mg (Kwon, 2014).

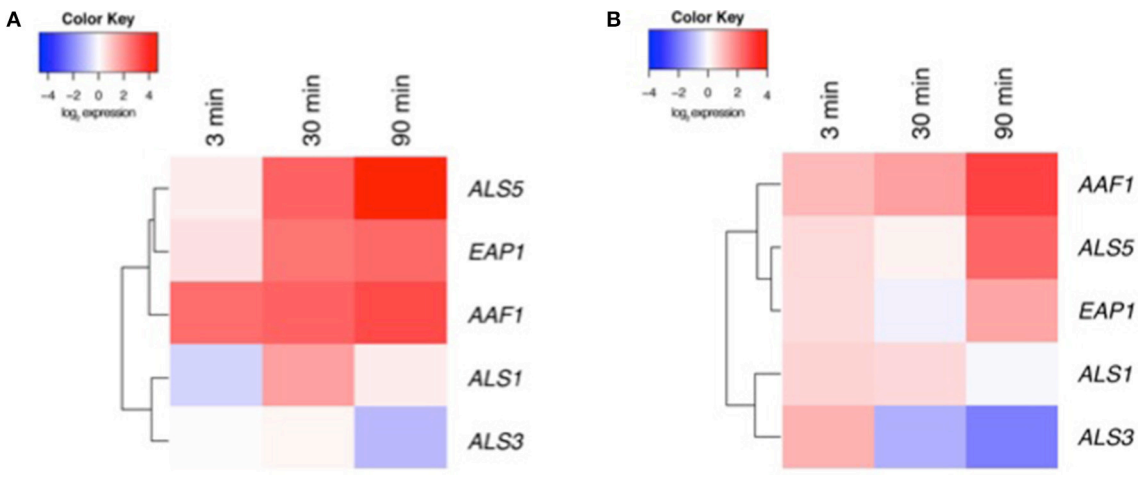

FIGURE 3 | Transcriptional analysis of CUR treated *C. albicans*. (A) Y and **(B)** H cells were prepared and exposed ± CUR for 3, 30, and 90 min. Expression of ALS1, ALS3, ALS5, EAP1, and AAF1 were then assessed using qPCR and relative gene expression assessed the ACT1 housekeeping gene. A heatmap and clustering was created for the differential expression of genes (log_2).

So delivering an antimicrobial concentration solely through a diet is not without challenges, though reaching an anti-adhesive concentration is possible. To this end we were able to demonstrate that 50 μg/ml could be readily adsorbed onto PMMA, the polymer used to construct denture prostheses. The optimized PMMA adsorbed with CUR was shown to reduce *C. albicans* adhesion by up to 70%, which was further reduced by 93% when a 3 min pre-treated *C. albicans* were inoculated on the optimized CUR adsorbed PMMA denture material. This synergized effect demonstrated that CUR has dual functionality, through surface adsorption and directly against *C. albicans*.

To further understand the biological basis of the sub-inhibitory CUR effect, we undertook a series of experiments to determine its effects on the kinetics of adhesion, whilst also evaluating how its impact on different morphological forms of *C. albicans*. We have previously shown that other natural compounds, such as tea tree oil (TTO) derivatives and carbohydrate-derived fulvic acid (CHD-FA) affect *C. albicans* development depending on the stage of biofilm growth (Ramage et al., 2012; Sherry et al., 2012), therefore we reasoned that CUR could also interact with *C. albicans* in a similar manner. We were able to show that prolonged exposure (30 min) of *C. albicans* yeast cells significantly reduced its adhesive capacity onto PMMA, suggesting that CUR was able to modify its adhesive capacity in some way. We further investigated this through looking at cells grown to different stages of morphological maturation, namely Y, G, and H cells. We hypothesized that depending on the stage of growth that the cells were exposed to CUR then this may affect overall biofilm development. Indeed, we showed that all morphological forms displayed reduced overall biofilm formation, though only the 200 μg/ml significantly reduced the Y and G cells in comparison to the other concentrations. H cells were not affected in a concentration dependant manner, suggesting that CUR was more effective against immature morphological forms. Interestingly, although biofilm formation was generally inhibited compared to control levels, there was still significant biofilm remaining, again suggesting early preventative intervention was most beneficial. The major limitation of this interpretation is the sample sizes used during these analyses (Vaux, 2012). Indeed, it begs the question whether the statistical analyses are worthwhile, hence why individual data-points are presented. Nevertheless, when we look at the data in its entirety, there are certainly trends suggestive that CUR exhibits positive biological effects, though further studies are required to confirm our observations.

CUR is a polyphenol with both antioxidant and hydrophobic properties (Priyadarsini, 2013; Mirzaei et al., 2017), which may explain why it preferentially adsorbs to PMMA and the cell wall of *C. albicans*. We hypothesized that the hydrophobic nature of the molecule could drive the coated *C. albicans* cells to aggregate with one another, and if we consider this within the context oral delivery within saliva then there is the possibility of creating complexes of cells that minimize their interaction with the denture surface. Indeed, we were able to demonstrate this both quantitatively and visually, which may explain why we observe synergized inhibition of adhesion at sub-inhibitory concentrations. *C. albicans* possesses a range of morphological and genetic attributes suited to colonization and biofilm formation (Blankenship and Mitchell, 2006). Finding ways of impacting these offers possibilities for novel anti-candidal therapeutics. Mechanistically, we were intrigued to understand how CUR induced *C. albicans* specific effects. Previous studies have shown that CUR has the ability to modulate of global repressor of filamentation TUP1 (Sharma et al., 2010). Indeed, our own focussed studies on mature biofilms showed that HWP1, a key hyphal wall associated element, was down-regulated (Shahzad et al., 2014). To this end we employed a transcriptional approach to assess genes implicated in adhesion and aggregation. CUR appeared to down-regulate the ALS agglutanins (ALS1 and ALS3), in both Y and H cells, suggesting it minimized their adhesive capacity. Both genes have been shown to be important in early biofilm events (Nailis et al., 2009; Fox et al., 2015). Of these ALS3 appeared to the most affected in these cells, which encodes the protein with a superior adhesive role and pivotal role in biofilm formation (Zhao et al., 2004; Nobile and Mitchell, 2005; Hoyer et al., 2008). ALS5 is a less well-defined member of this family, and although defined as an adhesin, functionally it appears to have amyloid properties and the capacity to improve aggregation (Rauceo et al., 2004; Garcia et al., 2011). This may explain why it is up-regulated following CUR treatment and this fits with the phenotype we observe. Moreover, AAF1 was also up-regulated by CUR in both Y and H cells, which is a gene highly related to the aggregation and flocculation (Fu et al., 1998). Interestingly it appears to have a minimal role in adhesion (Rieg et al., 1999), so further supports the notion of the phenotypes and anti-adhesive properties observed following induction by CUR. EAP1 showed similar trends to ALS5, though this encodes a protein known to enhance adhesion and biofilm formation (Li et al., 2007; Fox et al., 2015). This data is somewhat surprising, as we would have expected a similar level of down-regulation to that of ALS3. This suggests that EAP1, while exhibiting these adhesive properties, may have supplementary roles in cell-cell adhesion, though this requires further investigation. Collectively, these data demonstrate that CUR has the capacity at low concentrations to induce meaningful biological effects beneficial to minimizing candidal colonization.

In summary, given the strict denture hygiene regimen of brushing and cleansing required by an aging denture wearing global population, then this research provides opportunities to augment existing oral health strategies through dietary intake of important polyphenols like CUR. Not only is CUR effective against *C. albicans*, but also other oral pathogens (Shahzad et al., 2015). Given that the microbiome and mycobiome of denture wearers is highly diverse (O'Donnell et al., 2015b), then the broad-spectrum profile increases the overall appeal of this oral healthcare strategy. Indeed, there is merit to consider using a CUR based solution as a denture soak that has the potential to act and minimize further adhesion of these pathogenic biofilm related microorganisms, which with the enhanced antimicrobial activity induced through photactivation (Cieplik et al., 2015), may provide both a dual decontamination and preventative strategy. Indeed, clinical studies have already shown an additional benefit to photodynamic therapy (PDT) alone (Pereira et al.,

2015), as well as alongside CUR in the context of oral health (Leite et al., 2014). Mechanistically PTD works through locally acting light-activated photoantimicrobials molecules that produce highly reactive oxygen species, which are harmful to the site of action (Wainwright et al., 2017). This approach could therefore provide an augmentative benefit in enhancing lower concentrations of orally delivered antimicrobials, which could be light-activated bi-daily or more frequently. The hurdles will be in establishing and maintaining activatable concentrations that continue to exert an anti-adhesive effect. Careful consideration on the delivery of these molecules is required, and partnering up with nanotechnological approaches seems an obvious avenue of investigation, such as the creation of nanosized curcumin, which has already been shown to improve cellular interaction (Gopal et al., 2016), and would optimize our ability to deliver biologically relevant concentrations.

AUTHOR CONTRIBUTIONS

HA, RR, and MS participated in study design and experimental procedures and were responsible for preparation of the manuscript. DL consulted and performed the statistical analysis CW, DR and CN contributed to study design and manuscript preparation. GR and EC conceived the study, participated in study design and was responsible for producing the final manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00659/full#supplementary-material

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