Polymicrobial *Candida* biofilms: friends and foe in the oral cavity

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ABSTRACT

The role of polymicrobial biofilm infections in medicine is becoming more apparent. Increasing numbers of microbiome studies and deep sequencing has enabled us to develop a greater understanding of how positive and negative microbial interactions influence disease outcomes. An environment where this is particularly pertinent is within the oral cavity, a rich and diverse ecosystem inhabited by both bacteria and yeasts, which collectively occupy and coexist within various niches as biofilm communities. Studies within this environment have however tended to be subject to extensive independent investigation, in the context of either polymicrobial bacterial communities or yeast biofilms, but rarely both together. It is clear however that they are not mutually exclusive. Therefore, this review aims to explore the influence of candidal populations on the composition of these complex aggregates and biofilm communities, to investigate their mechanistic interactions to understand how these impact clinical outcomes, and determine whether we can translate how this knowledge can be used to improve patient management.

Introduction

Candida biofilms, in particular *C. albicans*, are an important healthcare issue due to ineffective clinical management strategies. Over the past 20 years we have learned a great deal about their clinical importance, including the mechanisms used by members of the genus to form biofilms and resist the challenge of host and antimicrobial molecules (Nett, 2014, Ramage *et al.*, 2014). However, as our levels of knowledge have increased, in part through the development of more sophisticated technologies, there has been a growing awareness that *Candida* rarely exist within a mono-species environment, and that heterogeneous biofilm populations consisting of aggregates of other fungi and bacteria (Gram-positive and Gram-negative) are in fact a highly prevalent and clinically important entity (Figure 1).

One location within the body where *Candida* species are readily isolated is within the oral cavity. Traditionally oral microbiologists have invested significant time and effort unravelling the importance of specific bacterial-bacterial interactions, while investigations of polymicrobial interactions have not received the same level of attention. This has led to a disparity of fundamental knowledge on the significance of candidal-bacterial interactions within the oral environment. The clinical implications of these polymicrobial biofilm interactions, primarily relates to recalcitrance to antimicrobial treatment strategies. Moreover, there is growing evidence from the literature that polymicrobial interactions may synergise the pathogenic potential of one or other microorganism (Stacy *et al.*, 2014). This only serves to highlight the importance of a dual approach to microbial analysis, where mycological and bacteriological analysis can have an equal contribution through interdisciplinary collaboration (Holmes *et al.*, 1995). This review aims to critically evaluate the available evidence as a means of appraising the clinical importance of *Candida* biofilms in polymicrobial environments, using key oral diseases and groups of microorganisms to illustrate these points.

Polymicrobial candidal interactions in the oral cavity

Oral candidosis is one of the most well-defined fungal biofilm infections of both soft and hard tissue and is characterised by complex biofilms which interact

with bacteria and the host (Dongari-Bagtzoglou *et al.*, 2009, Rautemaa & Ramage, 2011). The oral cavity provides a key portal of entry within the human host, and is home to a rich and diverse microbial flora. Despite being bathed in saliva, an important innate defence mechanisms containing numerous antimicrobial molecules, the oral cavity is a favourable habitat for both prokaryotes and eukaryotes. Within this, it is suggested that up to 10^8 microbes per millilitre of saliva are present (Guo & Shi, 2013). The oral cavity, therefore, acts as an important incubator for a complex '*microbial soup'*, in which yeasts such as *Candida* interact with one-another and with a plethora of cultivatable and non-cultivatable bacterial species, primarily within biofilm communities. Advances in genome sequencing are only now beginning to shed light on the importance of *Candida* within these complex communities (Nobbs & Jenkinson, 2015). Microbiome analysis of the saliva from elderly Dutch patients showed that an increased candidal load was associated with a dysbiotic bacterial flora that favoured the co-existence with oral streptococci to the exclusion of pathogenic anaerobic species (Kraneveld *et al.*, 2012). *Candida* species have been isolated from a range of oral environments involving both soft and hard tissue of biological and non-biological origin, illustrating the adaptability of candidal yeasts (Figure 2). The sites from which *C. albicans* has been isolated include periodontal pockets, root canals, orthodontic appliances, enamel, dentures and mucosal surfaces (Ramage *et al.*, 2004, de Carvalho *et al.*, 2006, Arslan *et al.*, 2008, Dongari-Bagtzoglou *et al.*, 2009, Sardi *et al.*, 2010, Freitas *et al.*, 2014). In order for candidal biofilms to flourish in these environments, moisture, nutrients, hyphal growth and the presence of commensal bacteria are all required which influence *C. albicans* architecture and virulence (Bertolini *et al.*, 2015).

Caries

Dental caries is one of the most common diseases worldwide, impacting 2.43 billion (36% of the global population) (Vos *et al.*, 2012). Largely influenced by diet caries has a multifactorial aetiology involving behavioural, environmental and immunological factors. Microbial dental plaque biofilms adherent to tooth surfaces, play a key role in the development of dental caries, through carbohydrate metabolism (predominantly sucrose) leading to production of

large quantities of lactic acid, and ultimately the dissolution of tooth surfaces. Typically, caries has been associated primarily with *Streptococcus mutans* and *Lactobacillus* species (Loesche, 1986, Badet & Thebaud, 2008), although more recently, oral microbiome studies have highlighted the polymicrobial aetiology of carious lesions (Belda-Ferre *et al.*, 2012, Simon-Soro *et al.*, 2014). Historically, candidal yeasts have been isolated in patients with caries (Krasse, 1954, Koo & Bowen, 2014), though the evidence for their direct role has yet been shown directly. There is now growing evidence that *C. albicans* actively participates in cariogenic biofilms, through synergistic interaction with *S. mutans* (Metwalli *et al.*, 2013, Koo & Bowen, 2014). Evidence of enhanced exopolymeric matrix production, facilitated by the increased surface area associated with hyphal networks, supports mixed biofilm growth of dense communities cemented to tooth enamel. Based on this and other studies, the interaction between candidal yeasts and streptococci is an important area requiring further extensive investigation.

Periodontal disease

Periodontal disease (PD) is a complex disease orchestrated by host-pathogen interactions. It affects almost 50% of the US population under 30 years old, and by the time they reach 65 years of age approximately 70% are affected (Eke *et al.*, 2012). In its mild and reversible form (gingivitis) the gingival tissues are characterised by swelling, an inflamed gum line and bleeding, whereas in its severe and irreversible form (periodontitis) there is destruction of the supporting periodontal ligaments and progressive bone resorption. While dysregulated inflammatory responses are pivotal with respect to periodontitis, the initial catalytic stimuli common to both forms of the disease comes from complex microbial biofilms. These initially establish themselves above the gum line (supra-gingival plaque), alter the microenvironment and drive a lower redox and pH, thus enabling capnophiles and anaerobes to colonise and produce sub-gingival plaque biofilms. The microbiology of supra- and sub-gingival plaque is extremely well characterised, with the influence of defined groupings of commensal and pathogenic species accurately mapped to clinical outcomes (Ximenez-Fyvie *et al.*, 2000, Shi *et al.*, 2015). With this historical focus on defined bacterial groupings defined by Socransky's traffic light

analogy (Socransky *et al.*, 1998), there has been minimal interest with respect to the influence of candidal species (Holmstrup, 1999). This is surprising given that *Candida* species have also been isolated from subgingival mixed biofilm consortia in patients with severe chronic periodontitis, where quantitatively high levels of *C. albicans* were shown to correlate with moderate and severe chronic periodontitis (Canabarro *et al.*, 2012). There is, however, a lack of direct evidence for causality, although in diabetes patients the relationship between subgingival candidal colonisation and periodontitis is more apparent (Sardi *et al.*, 2012, Hammad *et al.*, 2013). This relationship maybe a consequence of metabolic requirements, with elevated blood sugar levels supporting the growth of *Candida* species. Further evidence for *Candida's* involvement follows the use of oral contraceptives (OC), by which several studies have found an increased prevalence of *Candida spp*. carriage, as well as higher incidences of oral and vaginal candidiasis amongst OC users (Spinillo *et al.*, 1995, Kazi *et al.*, 2012, Zakout *et al.*, 2012). Furthermore, the prevalence of severe periodontitis is higher amongst OC users, suggesting that the hormones lead to the development of a dysbiotic biofilm, enabling *Candida* yeast to colonise (Brusca *et al.*, 2010). Irrespective of why *Candida* spp. are present in this environment, we do know that the subgingival environment represents a highly diverse microbial ecosystem comprised of a variety of commensals and pathogens, ranging from benign streptococcal species to virulent *Porphyromonas gingivalis* (Socransky & Haffajee, 2005, Haffajee & Socransky, 2006). Here, competition for nutrients, gases and space, dictate biofilm structure, and it is likely that the larger *Candida* cells play a significant physical and chemical role.

Endodontic infection

Endodontitis can result from direct tooth trauma, carious lesions on the enamel surface, or from periodontal infection progressing to the root apex. It is characterised by an infection of the pulp within the dental root canal system and is the major aetiologic agent of apical periodontitis. The American Association of Endodontists estimate that over 15 million root canal treatments are performed annually in the US, and these are primarily of an infectious origin. Endodontic infections are typically of biofilm aetiology and

are associated with key oral bacterial pathogens from up to 100 different bacterial genera (Siqueira & Rocas, 2009), by and large from 4 key phyla (*Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria*), although *Enterococcus faecalis* is considered the primary aetiological agent. Nonetheless, due consideration should be made to the method employed (culture versus non-culture) when assembling the snapshot of the dominant microbiota, as this heavily biases our perception of which species are important. In fact, this is a pertinent point to all oral diseases. Endodontic biofilms tend to reflect their origin, i.e. those from cariogenic lesion on occlusal surfaces may be more similar to supra-gingival plaque whereas those in periapical infection may reflect a predominantly anaerobic environment. There is increasing evidence for the involvement of *Candida* species in endodontic infections (Siqueira & Sen, 2004). Its' role as a dentophilic pathogen are highlighted through *in vitro* studies of dentine, where penetration of dentine tubules with *C. albicans* was demonstrated (Sen *et al.*, 1997). Subsequent studies have confirmed the presence of *C. albicans* from clinical root canal specimens (Baumgartner *et al.*, 2000), with subsequent studies showing an association between C. albicans and *E. faecalis* (Peciuliene *et al.*, 2001). In spite of this evidence of polymicrobiality there are no studies describing the candidal-bacterial interactions in the root canal environment.

Denture stomatitis

Edentulousness is an irreversible clinical condition that can be described as an ultimate marker of oral disease burden and is often associated with socioeconomic factors (Jeganathan & Lin, 1992, Cunha-Cruz *et al.*, 2007). Denture stomatitis (DS) refers to inflammation of the oral mucosa and pathological changes associated with denture surfaces adjacent to tissue (Jeganathan & Lin, 1992). Approximately two thirds of individuals who wear removable complete dentures suffer from DS, though most individuals are asymptomatic (Gendreau & Loewy, 2011). With 15 million dentures wearers in the UK this is not an inconsequential disease (Coulthwaite & Verran, 2007). Many factors influence its onset and severity, including salivary flow and denture cleanliness amongst others (Oksala, 1990, Soysa *et al.*, 2004, Soysa & Ellepola, 2005, Soysa *et al.*, 2006), although microbial factors remain one of

the most important. Dentures support the growth of microbial biofilms (denture plaque) within tiny cracks and fissures. These polymicrobial biofilm communities dominate the denture surface, with up to 10^{11} microbes per milligram of denture plaque (Nikawa *et al.*, 1998), which take advantage of the varied topography associated with denture acrylics and resins (Ramage *et al.*, 2004). Some of the bacterial species isolated include periodontal pathogens such as *Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (Sachdeo *et al.*, 2008, Yasui *et al.*, 2012), although caries-associated species such as *Streptococcus* and *Lactobacillus* species predominate (Teles *et al.*, 2012), possibly through their ability to coaggregate with *C. albicans* hyphae (Bilhan *et al.*, 2009, Ribeiro *et al.*, 2012). Here they form biofilms analogous to that of the enamel surface through pioneer species, followed by coaggregation and maturation of complex polymicrobial biofilms (Figure 4).

Unlike the oral diseases described above, DS is generally considered to be of yeast aetiology, with the literature disproportionately focussed on *Candida* spp. (Coleman *et al.*, 1997, Bagg *et al.*, 2003, Redding *et al.*, 2004, Li *et al.*, 2007). *C. albicans* is the most frequently isolated yeast from the denture, but *C. glabrata*, *C. dubliniensis*, *C. tropicalis*, *C. krusei* and a range of other *Candida* species have been frequently isolated (Coco *et al.*, 2008, Williams *et al.*, 2011). *C. albicans* accounts for the majority of the inflammatory pathology observed clinically (Salerno *et al.*, 2011). It exists as a commensal in the oral cavity of 25-50% of the healthy population, and can become pathogenic under optimal conditions, such as when the immune response is compromised (Dagistan *et al.*, 2009). This is not surprising given its dimorphic capabilities, i.e. the ability to form hyphae and yeast interchangeably, a requisite of biofilm formation (Ramage *et al.*, 2002). The hyphal form has been more commonly isolated in DS sufferers and is assumed to be the more invasive form of the organism, with an enhanced ability to adhere to and colonise the prosthesis surface (Gendreau & Loewy, 2011, Verran *et al.*, 2014). Collectively, these polymicrobial biofilms actively release proteolytic and lipolytic enzymes that induce inflammation of the palatal surface (Marcos-Arias *et al.*, 2011, Ramage *et al.*, 2012), ultimately leading to DS. The scanning electron micrograph

(SEM) in Figure 3 illustrates *C. albicans* interacting with bacteria on the surface of denture acrylic, with the associated confocal micrograph, showing bacteria coaggregating with *C. albicans* hyphae.

Angular cheilitis

Angular cheilitis is an inflammation of one, or more commonly both, corners of the mouth. It is a disease of multifactorial aetiology that includes anatomical issues, dry mouth, immunosuppression, and the wearing of poor fitting dentures, amongst many others. Although not particularly common *per se*, this disease is of interest as it is often associated with the co-isolation of *Candida* species with *Staphylococcus aureus*, microorganisms not unaccustomed to one another within the human host (Tawara *et al.*, 1996, Adam *et al.*, 2002, Baena-Monroy *et al.*, 2005). Both species are leading pathogens in blood borne and systemic infections, a major cause of morbidity and mortality in hospitalized patients. These species are of significant interest because of the escalating development of antimicrobial resistance and their increasing involvement in chronic and systemic polymicrobial biofilm infections (Perlroth *et al.*, 2007), and have been shown to co-aggregate together and exist within a dynamic and interactive state (Peters *et al.*, 2012, Peters *et al.*, 2012) (Shirtliff *et al.*, 2009). The relationship between these two has been described as mutualistic, synergistic and antagonistic, yet most of the evidence indicates synergy, as the majority of their interactions are associated with enhanced pathogenicity and disease severity (Peters & Noverr, 2013, Schlecht *et al.*, 2015).

Oropharyngeal and respiratory infection

As described, *Candida* is one of the main colonisers of the oral cavity and plays an important role in many oral diseases. However, there is thought to be a potential link between oral and pulmonary colonisation of *Candida*, which could contribute towards respiratory infection. Studies have identified respiratory pathogens colonising the oral cavity, as well as oral pathogens colonising the lungs (El-Solh, 2011, Bansal et al., 2013, Vadiraj et al., 2013, Przybylowska et al., 2015). Amongst these, *Candida* has been found to be one of the most predominant pathogens in the lungs, particularly in those suffering

from lung cancer and chronic pulmonary disease (Biswas et al., 2010, Laroumagne et al., 2011, Laroumagne et al., 2013). Aspiration of oral material into the lungs is thought to be the primary entry route of oral pathogens. Therefore, given that the oral carriage rate of *Candida* is approximately 50% (Darwazeh et al., 2010), and that roughly 45% of healthy individuals aspirate oropharyngeal contents into their lungs whilst sleeping, this puts a high number at risk of pulmonary colonisation by *Candida* (Gleeson et al., 1997). Yet, despite the potential to cause infection, *Candida* colonisation of the lungs is not necessarily detrimental, particularly when *P. aeruginosa* is also isolated (Ader et al., 2011). *P. aeruginosa* is frequently related with ventilator associated pneumonia and cystic fibrosis, with *C. albicans* often co-isolated. Many studies have investigated their interactions, yet have produced conflicting results with some identifying a synergistic relationship (Roux et al., 2009); however the vast majority provide stronger evidence for an antagonistic relationship (Morales et al., 2010, Bandara et al., 2013). *P. aeruginosa* gains the upper hand the majority of the time by preventing biofilm formation via killing of *C. albicans* hyphal filaments (Hogan & Kolter, 2002, Hogan et al., 2004). Nonetheless, recently it has been shown in a murine model that lung injury caused by *P. aeruginosa* infection is alleviated if preceded by a short term *C. albicans* colonisation (Ader et al., 2011). This was due to *C. albicans* activation of innate lymphoid cells, which produced IL-22, providing protection against *P. aeruginosa* induced injury (Mear et al., 2014).

Candida polymicrobial biofilm formation is the predominant problem associated with voice box prosthesis (VP) (Talpaert et al., 2015). Silicone is the most commonly used material used for VP, however, silicone is a favourable material for microbial attachment and can very quickly become colonised (Busscher et al., 1997). Biofilm formation can lead to valve malfunctioning, causing seepage of oesophageal contents into the trachea, which could potentially cause aspiration pneumonia (van Weissenbruch et al., 1997a, van Weissenbruch et al., 1997b). *C. albicans* is the most common yeast associated with VP colonisation, though *C. glabrata* and *C. tropicalis* are also frequently isolated (Bauters et al., 2002). *Streptococcus* spp and *Lactobacillus* spp are the predominant bacterial species isolated (Neu et al.,

1994), however the majority of mature biofilms had *Candida* and lactobacilli as their primary components (Buijssen et al., 2007). The success of polymicrobial biofilms forming on VP is likely due to the location, which is difficult for host immune defences to access. For the most part, it is very unusual to find a biofilm from a VP that is not comprised of both fungal and bacterial components. Before *Candida* can colonise the VP, there is strong evidence that bacteria must be adhered first, thus such fungal-bacteria interactions are critical for biofilm formation (Millsap et al., 2001). The more intricate details involved in these interactions requires further investigation, however what is clear is that disease resulting from microbial colonisation of a VP is very much polymicrobial in nature.

Mechanisms of polymicrobial biofilm interaction

Staphylococcal interactions

The interaction between *C. albicans* and *S. aureus* has been associated with enhanced pathogenic behaviour, disease severity and morbidity (Nair *et al.*, 2014). They form mixed polymicrobial biofilms in which *S. aureus* cells are found attached to *C. albicans* hyphal filaments (Peters *et al.*, 2010, Yi Jey Lin, 2013) (Figure 5). Their co-localisation within biofilms is still unclear, as some describe them interspersed throughout the biofilm three-dimensional structure (Peters *et al.*, 2010), whereas others describe them as only found attached within the upper layers of the biofilm (Harriott & Noverr, 2009). This disparity could be explained by different experimental conditions (e.g. growth medium). The initial colonising species plays a key role in dictating their interaction, as it has been shown that *C. albicans* biofilm formation was delayed when *S. aureus* colonised first, yet when added simultaneously biofilms formed rapidly (Yi Jey Lin, 2013). The reason for this inhibition is unknown; perhaps *S. aureus* secretes an inhibitory molecule preventing *Candida* adhesion.

Studies in *S. epidermidis* have shown that extracellular DNA (eDNA) release through autolysis is an important entity in supporting mixed biofilm growth (Pammi *et al.*, 2013), and is a featurenalso critical for *C. albicans* biofilm extracellular matrix (ECM) integrity (Rajendran *et al.*, 2014, Sapaar *et al.*, 2014). Therefore, it is not surprising that eDNA and the ECM from both *C.*

albicans and *S. aureus* biofilms are both involved in affecting the action of antibacterial agents. In fact, it has been shown that *S. aureus* is protected against vancomycin treatment using concentrations as high as 1600mg/mL within the mixed biofilm environment, through *C. albicans* ECM preventing diffusion and access to *S. aureus* (Harriott & Noverr, 2009). There are, however, other adaptive resistance mechanisms that play a role in this resistance phenotype (Harriott & Noverr, 2010).

It has also been shown that *S. aureus* preferentially adhere to hyphal filaments by relying on the adhesion to the *C. albicans* agglutanin-like sequence 3 protein (Als3p) (Peters *et al.*, 2010, Peters *et al.*, 2012), though it is likely that other proteins are involved. *S. epidermidis* have also been shown to preferentially adhere to hyphae, with forces between single bacterial and fungal germ tubes showing large adhesion forces (~5 nN) (Beaussart *et al.*, 2013). Studies have shown that *S. aureus* binding to *C. albicans* hyphae was significantly stronger than all other bacteria tested, including *P. aeruginosa* (Peters *et al.*, 2010). Interestingly, it was reported that none of the members of the ALS family of adhesins, (*ALS*1-7 and *ALS*9), including *ALS*3, are involved in interspecies adhesion (Harriott & Noverr, 2010). Thus further insight is required before we can fully understand the mechanisms responsible for adherence, yet it is likely that this is a complex process in which a multitude of proteins are involved. Nevertheless, it is thought that adhesion to hyphae may assist *S. aureus* in penetrating into the host (Schlecht *et al.*, 2015), a manner analogous to injection from a needle-stick injury. This has been demonstrated in mice studies, in which mixed infections with *C. albicans als3Δ* strains together with *S. aureus* were unable to invade the tongue, whereas the wild type infections demonstrated co-infection (Peters *et al.*, 2012). The ramifications of this enhanced invasive capacity have been shown historically to impact mortality, where synergism between the co-infected species administered intraperitoneally in a mouse model, lead to 100% mortality, whereas mono-species infections caused no mortality whatsoever (Carlson, 1983). Whether or not the relationship between the two organisms is physical or chemical remains to be determined, although there is

evidence that growth related synergy is an important factor in their co-habitation of micro-niches (Carlson & Johnson, 1985). Indeed, the physical relationship between the organisms is important, but not fundamental. Recent studies indicated that morphogenesis, i.e. the presence of hyphae, is not critical for their pathogenic potential, as demonstrated in some intricate murine studies using *C. albicans* genetically locked into the yeast state (Nash *et al.*, 2014). This suggests that physical cellular interactions are not solely responsible.

Metabolic signalling between *C. albicans* and *S. aureus* may play an important role in orchestrating this relationship. Chemically mediated signalling in the form of quorum sensing (QS), could potentiate both positive and negative interactions between these two microorganisms, which may inadvertently impact clinical outcomes. *C. albicans* secretion of farnesol, a QS molecule, decreases *S. aureus* biofilm formation, as well as increasing its susceptibility to antibiotics (Akiyama *et al.*, 2002, Jabra-Rizk *et al.*, 2006, Unnanuntana *et al.*, 2009). Moreover, it was shown to competitively inhibit *S. aureus* lipase activity (Kuroda *et al.*, 2007). However, Lin et al (2013) found that *S. aureus* conditioned media had a striking impact on *C. albicans* biofilm growth rate, indicating that *S. aureus* secretes a reciprocal quorum sensing molecule that stimulates *C. albicans* growth (Lin *et al.*, 2013). Nonetheless, whether *C. albicans* secretes sufficient farnesol *in vivo* to have an effect on *S. aureus*, remains unknown. Yet despite these conflicting results, the majority of studies support the idea of a synergistic relationship between the two.

Indeed, affinity panning of a *S. aureus* phage display library against *C. albicans* biofilms demonstrated that *S. aureus* released extracellular fibrinogen binding protein (Efb) during the interaction. This was shown to coat *C. albicans* yeast cells and reduce phagocytosis by granulocytes (Fehrmann *et al.*, 2013). In order to gain a better understanding of the molecular interaction between C*. albicans* and *S. aureus*, Peters and colleagues (2010) undertook a proteomics approach to identify proteins up-regulated during their interaction (Peters *et al.*, 2010). The majority of the 27 proteins that were up-regulated were involved in processes, including, stress and growth responses,

and metabolism. *S. aureus* up-regulated stress-related genes in response to both yeast and hyphae, yet, interestingly most of these genes were up-regulated in response to yeast rather than hyphal biofilms. As for *C. albicans*, yeast cells increased a number of stress related proteins such as Tsa1p and aconitate hydratase, yet *C. albicans* in hyphal formation showed minimal changes in gene expression in response to *S. aureus*. These results suggest that both organisms induce a stress response on their initial encounter with one another, particularly whilst *Candida* exists in yeast form. However as they mature and develop into a hyphal biofilm, they may down regulate these genes as a survival strategy, facilitating survival within the host.

Clearly, these two pathogens have the ability to influence one another's behaviour, so care must be taken in their clinical management. Broad-spectrum antimicrobial activity is crucial, accounting for both prokaryote and eukaryote. The use of ethanol has been shown to be effective at preventing both mono-and poly-microbial biofilms (Peters *et al.*, 2013). However, the successful use of miconazole in angular cheilitis is interesting given no precise mechanism of action for this azole to *S. aureus* (Sud & Feingold, 1982). It could therefore be hypothesised that given the polymicrobiality of the disease miconazole acts by exhibiting *C. albicans* activity, thereby destabilising *S. aureus* colonisation, which is physically supported by the hyphal biofilm meshwork. What is clear though is that these organisms are no strangers to one another.

Streptococcal interactions

Streptococci are amongst the primary colonisers of the oral cavity and compromise a large proportion of the overall flora (Syed & Loesche, 1978, Moore *et al.*, 1982). Oral streptococcal species are often termed as the mitis group streptococci (MGS), which include *S. mitis, S. oralis, S. gordonii, S. sanguinis and S. parasanguinis* species (Kawamura *et al.*, 1995). MGS streptococci are traditionally known to be early colonisers of dental surfaces, comprising approximately 60-80% of the flora (Diaz *et al.*, 2012), although use of high throughput gene sequencing technology has revealed them to also be predominant colonisers of oral mucosal surfaces (Diaz *et al.*, 2012).

The relationship between *Candida* and streptococci is generally considered to be synergistic, with advanced microscopy showing streptococcal interactions with the hyphal filaments of *Candida* (Dutton *et al.*, 2014). Streptococci provide *Candida* with nutrients from the salivary pellicle, such as lactate and glucose, which *Candida* utilise as a source of carbon(Holmes *et al.*, 2006). Furthermore, streptococci are aciduric and thus create an acid environment through the fermentation of carbohydrates (Takahashi & Nyvad, 2011). At low pH *Candida* grows in its yeast form. However, when co-colonised with streptococci, *Candida* can grow and survive at a lower pH (<4.5), and the H_2O_2 produced by streptococci can induce hyphal growth by inducing oxidative stress (Jenkinson *et al.*, 1990, Nasution *et al.*, 2008). This interaction is bidirectional, as *C. albicans* can promote the survival of streptococci by lowering oxygen tension levels to that more acceptable for streptococcal growth, as well as providing nutrients to stimulate bacterial growth (Douglas, 2002). This synergistic relationship can prove disparaging for the host. Studies have shown that streptococci augment the persistence of *Candida* spp. Xu and colleagues (2014) demonstrated that co-infection with *C. albicans* and *S. oralis* resulted in a more pathogenic inflammatory response compared with infection with either microorganism alone, as demonstrated through an exaggerated up-regulation of TLR2 dependant inflammatory genes (Dutton *et al.*, 2014, Xu *et al.*, 2014).

Adherence to mucosal surfaces occurs through binding interactions with components of the salivary pellicle, however, there is a limited number of niches for *C. albicans* to inhabit. Thus, *C. albicans* has to compete with other microbes (Kolenbrander *et al.*, 2002). To overcome this problem *C. albicans* has evolved a mechanism allowing it to bind directly to MGS species, including *S. oralis, S. mitis* and *S. gordonii* (Jenkinson *et al.*, 1990). This interaction is mutually beneficial as *C. albicans* can support the outgrowth of streptococci by enabling them to form more robust oral biofilms (Xu *et al.*, 2014). Adherence between these two species occurs via interactions of the *C. albicans* hyphal cell wall protein Als3, and the streptococcal cell surface adhesins SspA and SspB (Holmes *et al.*, 1996), proteins that belong to the antigen I/II polypeptide

family (Bamford *et al.*, 2009). Als3p is one of eight Als protein family members expressed in *C. albicans* (Als1p-7p, Als9p). Direct binding of SspB and Als3 is required for bacterial-fungal attachment. Interaction between these molecules is associated with the N-terminal domain of Als3 (Bamford *et al.*, 2015), as deletions at the N-terminus abrogated binding to *S. gordonii*. Hoyer and colleagues (2014) have demonstrated that this interaction may be more complex than originally thought by showing that the peptide-binding domain (PBD) of *C. albicans* is essential for *C. albicans-S. gordonii* adherence. The PBD functions by binding to the free C-terminus, however, in *S. gordonii* the SspB C-terminus is covalently linked to peptidoglycan, and is thus unavailable to bind. Further investigation is required before we can fully understand the mechanism behind this interaction, though recent studies suggest that the early stage of cell wall *O*-mannosylation may be important in the development of these polymicrobial communities (Dutton *et al.*, 2014).

An important component of a biofilm is the extracellular matrix (ECM), which confers protection to antimicrobials (Xu *et al.*, 2014). The ECM of streptococcal biofilms is composed of α-glucans (Gregoire *et al.*, 2011), whereas *Candida* biofilm ECM is primarily composed of β-glucans (Al-Fattani & Douglas, 2006, Taff *et al.*, 2012). *S. mutans* utilises its ECM components to enhance adhesion to fungal cells by depositing α-glucans on the surface of hyphae (Gregoire *et al.*, 2011). Moreover, interaction between *S. mutans* and *C. albicans* is promoted by glucosyltransferase-derived ECM and expression of the *S. mutans* virulence gene gtfB (Falsetta *et al.*, 2014). It was also shown in this study that Candida-derived β1,3-glucans contribute to ECM matrix structure, whilst fungal β-glucan and mannan provide sites for GtfB binding and activity. Furthermore, β-glucans are found on the surface of hyphae as well as in the matrix (Dongari-Bagtzoglou *et al.*, 2009), thus suggesting that streptococci utilise these proteins to adhere to candidal hyphae. Collectively, this suggests the biofilm ECM contributes to this mutualistic behavior, favouring their co-existence in the oral environment to the detriment of the host.

As with *Candida – S. aureus* interactions, quorum sensing (QS), is an important factor in the relationship between *Candida* and streptococci. Farnesol, a tetraprenoid alcohol and a key intermediate in the sterol biosynthetic pathway in eukaryotic cells, represents the primary QS molecule associated with *C. albicans*, its main role being repression of hyphal growth and biofilm formation (Ramage *et al.*, 2002). However, one study has suggested that *S. gordonii* is able to suppress farnesol induced inhibition of biofilm formation, via autoinducer 2 (AI-2), as luxS mutants were less effective at permitting hyphal formation, however the mode of action has yet to be elucidated (Bamford *et al.*, 2009). Farnesol has also been shown to inhibit *S. mutans* biofilm accumulation and polysaccharide production (Koo *et al.*, 2003). Based on this and further work, it has been suggested that it may be used to control its competitiveness in mixed species biofilms and could be used as a means of a chemotherapeutic strategy (Jeon *et al.*, 2011). AI-2 is the primary QS molecule secreted by bacteria that allows inter-species communication (Vendeville *et al.*, 2005). The *luxS* gene is associated with AI-2 production and *luxS* streptococcal mutants can form monospecies biofilms. However, when co-colonised with *C. albicans*, biofilm formation becomes abrogated, suggesting this molecule is involved in cellular communication (McNab *et al.*, 2003, Bamford *et al.*, 2009). Another important signalling mechanism in streptococci, including *S. gordonii*, is through the comCDE operon, which encodes a sensor-regulator system (ComDE) activated by the comC gene product competence stimulating peptide (CSP). *S. gordonii-C. albicans* biofilms formed with ΔcomCDE or ΔcomC mutants showed increased biomass compared to wild-type biofilms. Interestingly, more eDNA was observed in the mixed ΔcomCDE mutant biofilms. Although purified CSP did not affect *C. albicans* hyphal formation. Contrary to earlier findings (Jarosz *et al.*, 2009), it did inhibit monospecies biofilm formation, suggesting that the *S. gordonii* comCDE QS-system modulates the production of eDNA (Jack *et al.*, 2015), and important component of candidal ECM (Rajendran *et al.*, 2014).

Candidal interactions

Hyphae provide *C. albicans* with an advantage over many of its competitors in terms of size and surface area, enabling them to take advantage of more sites

for adhesion and occupation of a variety of niches. This is why it is a more successful pathogen than other members of the genus. Nonetheless, there is hypothesis that *Candida spp.*, in particular *C. glabrata* benefit from *C. albicans*. There have been suggestions that DS pathology may be promoted by the synergistic interaction between these species within denture biofilms. Coco and colleagues (2008) first reported that *C. glabrata* and *C. albicans* were often co-isolated from patients, particularly those with severe inflammation. The authors hypothesised that pathogenic synergy existed between the two *Candida* species. *C. glabrata*, devoid of hyphae, forms relatively structurally poor and unstable biofilms, yet is associated with disease. Therefore, it was hypothesised to use *C. albicans* as a structural scaffold to gain entry into the host. Further studies have confirmed this, where *C. albicans* appeared to assist the invasive capacity of *C. glabrata* within an *in vitro* reconstituted epithelial biofilm model (Silva *et al.*, 2011). The mechanistic of this interaction are at present unknown, however we can speculate that tissue destruction through proteolytic and lipolytic enzymes augments the invasive capacity of the hyphae and allows co-aggregative *C. glabrata* to enter and contribute to pathogenesis. Further work by this group has shown similar data with work in a reconstituted human vaginal epithelial model, where *C. glabrata* individually caused minimal tissue damage, though there was a significant increase in *C. glabrata* colonisation and invasiveness in combination with *C. albicans* (Alves *et al.*, 2014). Damage was primarily dependent on the process of invasion, with key virulence genes upregulated (*HWP1, PLD1* and *ALS3*). Further studies using *in vivo* models to investigate the pathogenesis of denture stomatitis would be useful in this context (Nett *et al.*, 2010), although as described above there is mounting evidence that hitchhiking through adhesion to hyphae is not a limited phenomenon and may also be important with respect to *C. glabrata* using *C. albicans* to gain entry to the host (Schlecht *et al.*, 2015).

Anaerobic Gram-negative interactions

Life in subgingival plaque is highly anaerobic, favouring many obligate PD pathogens such as *P. gingivalis, F. nucleatum* and *P. intermedia*. However, given the undefined relationship between *Candida spp* and PD, then this

remains a relatively neglected area of research. Studies regarding *C. albicans* and *P. gingivalis* have produced conflicting results It was shown that *P. gingivalis* suppressed *Candida* biofilm formation through a reduction in the number of viable yeast cells coincidental with an increasing *P. gingivalis* concentration (Thein *et al.*, 2006). Conversely, it was also shown that *P. gingivalis* induces germ-tube formation in *C. albicans,* producing a more invasive phenotype, thus increasing the risk of infection (Nair *et al.*, 2001). Furthermore, both microbes appear to have an antagonistic effect on one another in relation to host cell adhesion, as *P. gingivalis* inhibited adhesion of *C. albicans* to buccal epithelial cells (Nair & Samaranayake, 1996), whilst the presence of *C. albicans* did not enhance adhesion to gingival epithelial cells or gingival fibroblasts by *P. gingivalis* (Tamai *et al.*, 2011). Yet, in the same study pre-exposure of gingival epithelial cells and fibroblasts to *C. albicans* enhanced cell invasion by *P. gingivalis*. Clearly, further studies are required to decipher how these microorganisms interact with one another.

As for *F. nucleatum*, co-aggregation studies have revealed its ability to adhere to *C. albicans* species (Grimaudo & Nesbitt, 1997), as well as *C. dubliniensis* (Jabra-Rizk *et al.*, 1999). However, the interaction with *C. albicans* may be temperature dependant as *C. albicans* grown at 37^{o}C did not co-aggregate with *F. nucleatum*, yet the two species did co-aggregate when grown at 25^{o}C and 45^{o}C (Jabra-Rizk *et al.*, 1999). The exact mechanistic behind these interactions remain unknown, however these observations indicate *C. albicans- F. nucleatum* interactions may be an important factor in oral colonisation by yeasts.

Studies using lipopolysaccharide (LPS) from a variety of Gram-negative strains have shown that hyphal formation is inhibited as is biofilm formation in a number of *Candida spp.* (Bandara *et al.*, 2010), indicating that physical interaction may be an important factor in defining their subgingival niches. Subsequent work in *Escherichia coli* demonstrated that secreted elements also play an important role in affecting hyphal formation (Bandara *et al.*, 2013). This is also true of the relationship between the capnophilic bacterium *A. actinomycetemcomitans* where it has been shown that its release of AI-2

was actively involved in inhibiting *C. albicans* hyphal growth and biofilm formation (Bachtiar *et al.*, 2014). Given the complexity of various metabolites and QS molecules in subgingival plaque, such as volatile sulphur compounds, fatty acids and AI-2 (Kurita-Ochiai *et al.*, 1995, Huang *et al.*, 2011, Jang *et al.*, 2013, Basic & Dahlen, 2014), it is likely these also impact hyphal formation and *Candida's* ability to contribute to PD (Noverr & Huffnagle, 2004). This anoxic environment has been shown to result in significant transcriptional changes in *C. albicans*, including the upregulation of *WOR1*, which is a transcriptional regulator central to phenotypic switching (Fox *et al.*, 2014). Based on the available literature it could be surmised that subgingival plaque is most likely to antagonise yeast proliferation, except in cases where there is dysbiosis of the biofilm ecology, such as following broad-spectrum antibiotic therapy or pre-existing medical conditions, including diabetes (Rams *et al.*, 1990, Sardi *et al.*, 2010, Al Mubarak *et al.*, 2013).

Facultative Gram-positive interactions

Candida species and *E. faecalis* have become increasingly noted for their co-isolation within endodontic infections, both of which play an important role in nosocomial infection. Interestingly, data from a longitudinal study carried out over two years at a German teaching hospital found that *Candida* positive patients (blood, CSF, skin, feaces or sputum) were twice as likely to be co-colonised by *E. faecalis* (Hermann *et al.*, 1999). *E. faecalis* has been found to incorporate itself into *Candida* biofilms, and is the third most predominant bacterial spp. found in mucosal fungal biofilms (Dongari-Bagtzoglou *et al.*, 2009, Fox *et al.*, 2014). It was shown to adhere to *Candida* in both hyphal and yeast forms, yet caused a reduction in the overall biofilm biomass (Fox *et al.*, 2014). However, Cruz and colleagues (2013) demonstrated that *E. faecalis* inhibited hyphal morphogenesis, which was partially dependent on the *Fsr* quorum-sensing system, a major regulator of *E. faecalis* virulence (Cruz *et al.*, 2013). Collectively, these effects both impacted virulence during co-infection when compared to mono-species infection, suggesting that they both negatively influence one another's virulence and help maintain a commensal relationship (Garsin & Lorenz, 2013). Further work has revealed that *C. albicans* releases a surface protein Msb2, which binds to host antimicrobial

peptides as well as antibiotics, thus conferring protection to both organisms (Swidergall *et al.*, 2013). Furthermore, evaluating the influence of *C. albicans* on the dynamics of the bacterial microbiome following antibiotic treatment found that bacterial re-colonisation was enhanced in the presence of *C. albicans* (Mason *et al.*, 2012). Moreover, *C. albicans* reduced *Lactobacillus* spp. whilst enhancing *E. faecalis* numbers, which led to the persistence of *E. faecalis* long term. This effect was not apparent in subjects when *C. albicans* was absent. Whether this effect was due to a synergistic relationship with *E. faecalis* or an antagonistic interaction with lactobacilli remains to be elucidated.

There is a conceived dogma that lactobacilli antagonise candidal colonisation (Young *et al.*, 1956). This forms the basis of why they play a key role in probiotics. It is well documented that probiotics reduces candidal levels at several sites, including oral cavity, bloodstream and urinary tract (Mendonca *et al.*, 2012, Kumar *et al.*, 2013). Early observations indicate that *C. albicans* decreased in the presence of lactobacilli through provision of nutrients for lactobacilli that leads to lactic acid production, thus hindering candidal growth through pH dependant inhibition. This dynamic relationship suggests that there is a close association between the two, but to date this has mainly been observed in vaginal infection. Our own microbiome studies of the denture plaque have shown that *C. albicans* and lactobacilli are positively associated in disease (unpublished work). The role of lactobacilli in maintaining homeostasis at the vaginal mucosa initially came to light due to the occurrence of vaginal candidiasis during treatment with systemic antibiotics. The mechanisms by which *Lactobacillus* species inhibits growth and virulence of *Candida* spp. are not yet fully understood, but perhaps the production of hydrogen peroxide as it has been shown to cause anti-candidal activity, albeit in some strains of lactobacilli (Strus *et al.*, 2005). This suggests that other interactive mechanisms are involved in disease, including the modulation of the host response whereby lactobacilli cells have been shown to up-regulate inflammatory cytokines when co-cultured with *C. albicans* (Martinez *et al.*, 2009), potentially assisting in the clearance of candidal infection. Despite the overwhelming evidence of an antagonistic interaction, certain species of oral *Lactobacillus*, namely *L. casei*, have demonstrated a stimulatory effect on *C.*

albicans hyphal growth (Orsi *et al.*, 2014), and in fact it has been demonstrated that candidal hyphae have the capacity to co-aggregate and support lactobacilli levels in patients with higher levels of oral disease (Bilhan *et al.*, 2009). Nevertheless, further studies are required to investigate these interactions in detail to determine the true extent of the dynamic relationship; particularly as the conceived antagonism may only exist for *C. albicans*. For example, recent studies have shown that only one of six probiotic *Lactobacillus* species had an inhibitory effect on *C. glabrata* growth (Jiang *et al.*, 2015). This suggests that the interaction between *Candida* and lactobacilli may be dependant on the particular environment they co-habit.

Conclusions

Collectively, these data demonstrate that the interaction between candidal species and other microoorganisms may be dependant on the nature of the interaction (chemical, physical, or both) and the particular environment they cohabit. It is clear from many of these studies that the interaction between *C. albicans* hyphae and different bacterial species is important in defining their interaction, whether mutualistic or antagonistic in nature. The secretion of signalling molecules from the myriad of microorganisms in the oral cavity, such as AI-2, farnesol, and other small molecules is clearly important, with recent studies supporting the notion that the metabolome plays an integral part in defining the interaction between the host, *Candida* and microbiota such as lactobacilli (Romani *et al.*, 2015). Understanding how each of these specific interactions influences one another and *Candida's* pathogenicity will enable us to target this medically important yeast rationally. Though, we must be cognisant of the negative influences of changing its role within complex oral biofilm communities and the consequences of dysbiosis (McLean, 2014), as this may support the unnecessary proliferation and overgrowth of candidal yeasts that leads to oral disease.

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Figure legends

Figure 1: Interactions between *Candida albicans* and bacteria.
Candida albicans coaggregating with either (A) Gram-negative and (B) Gram-positive bacteria, or (C) a polymicrobial biofilm aggregate consisting of Gram-positive and Gram-negative oral bacterial species interacting with *C. albicans* hyphae. White scale bars = 20um. Confocal Image taken by Dr Owain Millington, Biophotonics Unit, Strathclyde University)

Figure 2: Oral sites of polymicrobial *Candida* biofilm diseases.
The schematic diagram illustrates site within the oral cavity typically where *Candida*-bacterial polymicrobial biofilms are observed (clockwise from top position): caries, periodontitis, orthodontic, endodontic, angular cheilitis, denture stomatitis.

Figure 3: Micrographs of polymicrobial *Candida* denture related biofilms. (A) Scanning electron micrograph (SEM) and (B) confocal laser scanning micrograph (CLSM) of complex bacterial communities coaggregating with *Candida albicans* upon denture acrylic. These micrographs show low (SEM) and high (CLSM) magnifications of mixtures of *C. albicans* yeast (round) and hyphae (long filaments – white arrows) coaggregated with smaller bacterial species. Confocal Image taken by Dr Owain Millington, Biophotonics Unit, Strathclyde University).

Figure 4: Development of polymicrobial *Candida* biofilm on denture acrylic. This schematic representation of denture biofilm development shows how initial colonisation by yeast and bacterial species (white), followed by hyphal formation and co-aggregation (grey), which then enables the bacterial species to expand and grow into the spaces unoccupied on the surface of both the acrylic and *C. albicans* hyphae (black).

Figure 5: *Candida albicans* and *Staphylococcus aureus* polymicrobial biofilm. This confocal scanning laser micrograph shows the close interactions between clusters of *S. aureus* (yellow) and *C. albicans* hyphae (white/green). The appearance of these interactions demonstrates close attachment between the two in three-dimensional space, suggesting structural stability and an element of co-operation with one-another. Confocal Image taken by Dr Owain Millington, Biophotonics Unit, Strathclyde University)