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Candida albicans biofilms and polymicrobial interactions

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ABSTRACT

Candida albicans is a common fungus of the human microbiota. While generally a harmless commensal in healthy individuals, several factors can lead to its overgrowth and cause a range of complications within the host, from localized superficial infections to systemic life-threatening disseminated candidiasis. A major virulence factor of *C. albicans* is its ability to form biofilms, a closely packed community of cells that can grow on both abiotic and biotic substrates, including implanted medical devices and mucosal surfaces. These biofilms are extremely hard to eradicate, are resistant to conventional antifungal treatment and are associated with high morbidity and mortality rates, making biofilm-associated infections a major clinical challenge. Here, we review the current knowledge of the processes involved in *C. albicans* biofilm formation and development, including the central processes of adhesion, extracellular matrix production and the transcriptional network that regulates biofilm development. We also consider the advantages of the biofilm lifestyle and explore polymicrobial interactions within multispecies biofilms that are formed by *C. albicans* and selected microbial species.

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Introduction

A biofilm is a consortium of microbes attached to a biotic or abiotic substrate embedded within a matrix of extracellular polymeric substances. For many microbes, the ability to form biofilms is an important virulence factor. Biofilms are found on natural substrates including plant and mammalian tissues and form readily on synthetic polymers such as plastics found in catheters, prostheses and implanted heart valves (Nobile and Johnson 2015). Rapid medical intervention, high concentrations of antimicrobials and replacement of the infected device are required to manage a biofilm infection. However, such procedures are not without significant patient risk, as toxicity issues may arise from antimicrobial therapy and replacement of infected devices may sometimes require major surgery (for instance, when replacing coronary stents or heart valves). Thus, biofilm-related infections contribute significantly to the economic burden of global healthcare (Pittet 1994; Crump and Collignon 2000; Schierholz and Beuth 2001; Orsi et al. 2002; Yousif et al. 2015) and are associated with poor clinical outcome.

Candida species, which include the opportunistic pathogen *Candida albicans*, are one of the main causes of nosocomial infections worldwide, particularly in immunocompromised individuals (Hajjeh et al. 2004). *Candida albicans* can form complex biofilms on medical devices and, often, device removal is recommended (Kojic and Darouiche 2004; Pappas et al. 2016; Cavaleiro and Teixeira 2018; Nett and Andes 2020). Biofilms are also associated with recurrent candidaemia, which affects more than 400 000 people per year and has an alarming mortality rate of approximately 50% (Tumbarello et al. 2012; Bongomin et al. 2017; Li et al. 2018).

Candida albicans forms biofilms comprised of yeast cells, pseudohyphae and hyphae. The transition from planktonic growth to biofilm is accompanied by a complex remodelling of phenotypic behaviour underpinned by myriad changes in gene expression. The development of *C. albicans* biofilm occurs progressively, and is typically classified into four stages; (i) adsorption and adhesion of *C. albicans* yeast cells to a substrate, (ii) formation of microcolonies and production of the extracellular matrix, (iii) maturation and (iv) dispersal of cells from the mature biofilm. Once established, *C. albicans*

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biofilms are highly tolerant to antifungal therapy and can serve as a reservoir for recurrent infection. Indeed, the medical impact of *C. albicans* biofilm is underscored by a positive correlation between biofilm formation, increased virulence and higher patient mortality (Tumbarello et al. 2012; Rajendran, May, et al. 2016).

In this review, we summarise the current understanding of the processes involved in *C. albicans* biofilm formation and the regulatory circuits that are pivotal to *C. albicans* biofilm development. We explore multispecies biofilms, the advantages conferred to microbes during biofilm growth, persister cells and resistance to host immune clearance.

Biofilm formation

Biofilm formation begins with the adhesion of *C. albicans* yeast cells to a suitable surface. These early contact events comprise several non-specific factors including attractive and repulsive forces (such as van der Waals forces, hydrophobic interactions and Brownian movement forces), and electrostatic interactions that facilitate initial adherence (Douglas 1987; Hazen 1989; Hazen et al. 1990; Hobden et al. 1995; Jones et al. 1995; Williams et al. 2013). While these factors may not be the predominant mechanism for yeast cells to adhere, they are nevertheless an important prerequisite for biofilm formation (Park et al. 2008; Silva-Dias et al. 2015).

Following initial contact, cell wall-associated adhesion molecules are expressed to further strengthen fungal adhesion. There are three key families of *C. albicans* adhesins that play a major role in mediating adherence during biofilm formation: the agglutinin-like sequence (Als) family, the hyphal wall protein (Hwp) family, and the individual protein file family F/hyphally regulated (Iff/Hyr) family (de Groot et al. 2013). The most widely studied are the Als adhesins (Hoyer and Cota 2016) comprising eight members (Als1p–Als7p and Als9p), which are glycosylphosphatidylinositol (GPI)-linked to β -1,6 glucans in the fungal cell wall. The Als family of adhesins is key in both yeast and hyphal adhesion during biofilm formation and development.

During the initial adhesion of yeast cells to surfaces, Als1p and Als5p facilitate adhesion via their amyloid-forming region that is critical for yeast cell–cell aggregation and cell–substrate adhesion (Rauceo et al. 2004; Ramsook et al. 2010; Garcia et al. 2011). These yeast cell aggregates form quickly and are key to the establishment of mixed-species biofilms with other fungal species and bacteria (Klotz et al. 2007; Alsteens et al. 2010; Ramsook et al. 2010; Lipke et al. 2012, 2014). In addition

to the Als family, other adhesins are important in mediating the initial attachment of *C. albicans* yeast cells during biofilm formation (Figure 1(A)). The enhanced adhesion to polystyrene protein (Eap1p) is significant in the clinical setting due to its ability to mediate attachment of *C. albicans* to plastics found in medical devices (Chong et al. 2018; McCall et al. 2019). Deletion of *EAP1* renders *C. albicans* unable to attach to plastic surfaces and epithelial cells *in vitro*. In addition, Eap1p is required for biofilm formation in a rat central venous catheter model (Li et al. 2007).

Upon contact with a suitable surface, yeast cells germinate to form hyphae and pseudohyphae. The adhesion of these distinct morphological forms is crucial during the later stages of biofilm development. A vast array of adhesins orchestrate the adherence of yeast, pseudohyphae and hyphae, thereby contributing to biofilm maintenance. Als1p and Als3p have critical roles in facilitating hyphal adhesion during biofilm formation (Nobile et al. 2008). A *C. albicans* mutant unable to express *ALS3* forms weaker and more disorganised biofilms compared to wild-type fungi *in vitro* (Zhao et al. 2006). Furthermore, disruption of both *ALS3* and *ALS1* (*als3 $\Delta\Delta$ /als1 $\Delta\Delta$*) reduces the biofilm biomass when compared with controls (McCall et al. 2019).

Other Als family members also make significant contributions to biofilm development. Increased expression of *ALS6*, *ALS7*, and *ALS9* rescues the biofilm defect of an *als3 $\Delta\Delta$ /als1 $\Delta\Delta$* mutant (Nobile et al. 2008), while mutations in the amyloid-forming region of Als5p prevent the formation of adhesion nanodomains, cellular aggregates and biofilm development on polystyrene surfaces *in vitro* (Garcia et al. 2011). Conversely, decreased expression of *ALS2* but not *ALS4* results in impaired biofilm formation in a catheter biofilm model (Zhao et al. 2005). However, overexpression of *ALS2* or *ALS4* fails to rescue biofilm formation in an *als3 $\Delta\Delta$ /als1 $\Delta\Delta$* null mutant *in vivo* (Nobile et al. 2008). This suggests that only certain Als proteins may function equivalently to Als1p and Als3p in an *in vivo* model of biofilm formation (Nobile et al. 2008).

Hyphal wall protein 1 (Hwp1p) is the most thoroughly characterised member of the Hwp family. Hwp1p is a molecular mimic of the substrate recognised by mammalian transglutaminase enzymes which cross-link Hwp1p to the surface of the host cell (Staab 1999), facilitating adhesion. A *C. albicans* *hwp1 $\Delta\Delta$* mutant is unable to produce hyphae or form biofilms (Nobile, Nett, et al. 2006), underscoring the critical role of Hwp1p in these processes. Interestingly, overexpression of *HWP1* is unable to promote biofilm formation in an *als3 $\Delta\Delta$ /als1 $\Delta\Delta$* mutant *in vitro* or *in vivo* (Nobile

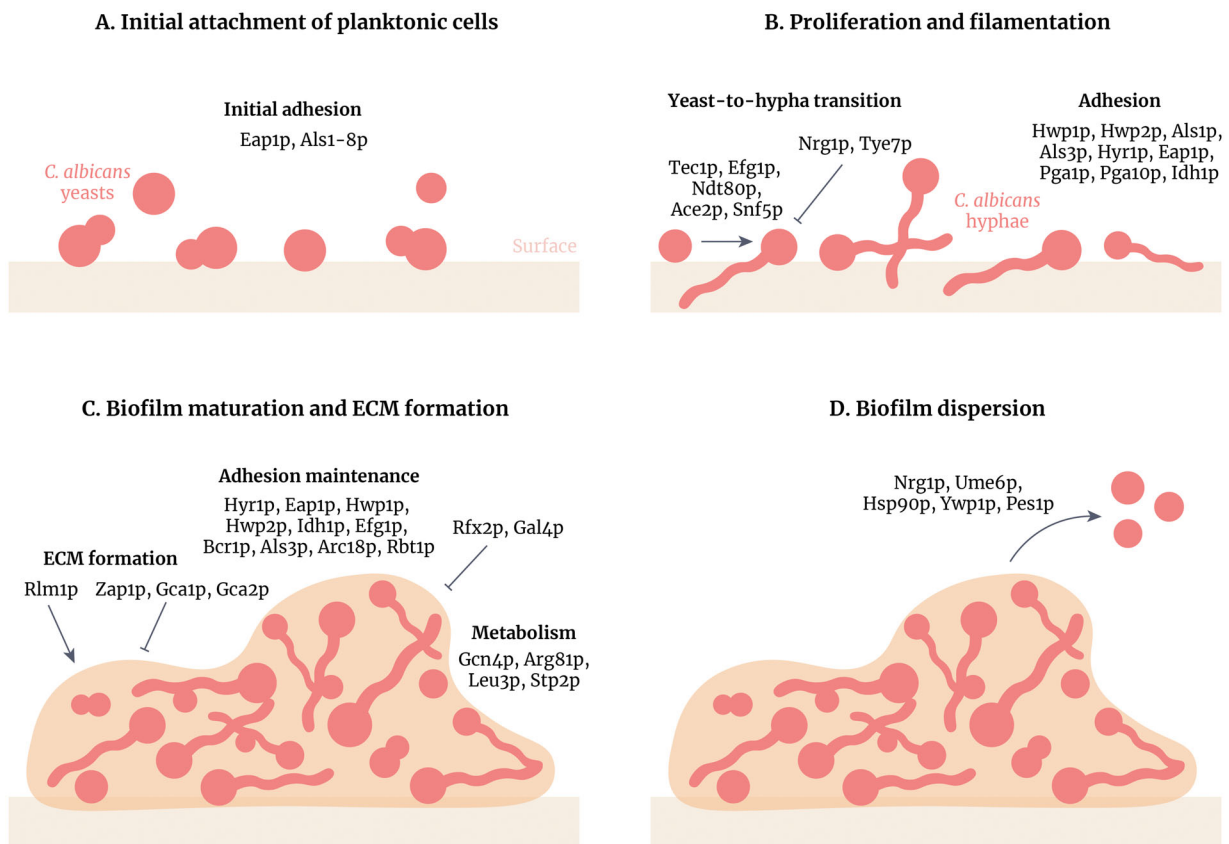


Figure 1. Stages of *Candida albicans* biofilm formation and development. *Candida albicans* biofilm formation is a multifactorial process that consists of four main stages. (A) Initial attachment of planktonic cells: *C. albicans* yeasts attach to a surface (e.g. epithelia, biomaterials or cellular aggregates) through adhesins such as Als family members. (B) Proliferation and filamentation: yeasts transition to hyphae and this process is regulated by many transcription factors (TFs) including Tec1p and Efg1p. Hyphae express specific adhesins such as Hwp1p and Hyr1p. (C) Biofilm maturation and extracellular matrix formation: the matrix forms around the *C. albicans* cells, positively regulated by the TF Rlm1p, providing structural support and protection against antifungals and the host immune system. Adhesion is maintained and amino acid metabolism is increased in the biofilm. (D) Biofilm dispersion: yeast cells disperse from the biofilm to colonise other parts of the body. These cells differ from initial planktonic cells as they are more virulent and more likely to form biofilms. Figure created with Adobe Illustrator.

et al. 2008), though a mixture of biofilm defective *hwp1ΔΔ* and *als3ΔΔ/als1ΔΔ* strains can form a hybrid biofilm. This suggests that while Hwp1p has a distinct function to the Als proteins, the three adhesins may have complementary roles in mediating adherence of *C. albicans* cells during biofilm formation (Nobile et al. 2008). Other Hwp family members including Hwp2p and Rbt1p contribute to initial cell attachment and the maintenance of adhesion (Li et al. 2007; Ene and Bennett 2009; Younes et al. 2011; Desai and Mitchell 2015; McCall et al. 2019). Mutants defective in *HWP2* are unable to adhere efficiently to epithelial cells and present a decrease in biofilm mass (Younes et al. 2011), while *RBT1* overexpressing strains exhibit an increase in cell surface hydrophobicity, which is essential for *C. albicans* adherence to polystyrene (Monniot et al. 2013).

The Iff/Hyr family of adhesins includes the hyphae specific Hyr1p (Bailey et al. 1996), which appears to be the only member of this family associated with biofilm formation and development. Mutants defective in *HYR1* exhibit reduced adhesion in a flow system during the initial stages of biofilm development, as well as a reduction in biomass due to defective attachment (McCall et al. 2019). Furthermore, a significant reduction in both initial attachment and subsequent adhesion was observed following the loss of *HYR1*, while the loss of *ALS3/ALS1* only affected the maintenance of adhesion, suggesting that Hyr1p is key in mediating adhesion during both early and late stages of biofilm development (McCall et al. 2019).

While research has focussed predominantly on the three main *C. albicans* adhesin families, several

additional factors contribute to biofilm adhesion, including the putative GPI-anchored proteins Pga1p and Pga10p (de Groot et al. 2003; Pérez et al. 2006; Hashash et al. 2011; Awad et al. 2018), as well as Ecm33p, Mp65p, Msb2p, Pbr1p, Arc18p, Pmt1p, Mnn9p, Spt7p, orf19.831 and Ihd1p (Sahni et al. 2009; Sandini et al. 2011; Puri et al. 2012; Rouabhia et al. 2012; Lee et al. 2016; McCall et al. 2019). Gene deletion studies have demonstrated decreased or impaired adhesion and fragile biofilms. Collectively, these observations highlight a clear role for adhesins and adhesin-like molecules during the initial stages of biofilm formation.

Biofilm development

Following adhesion, biofilm development is accompanied by changes in morphology, cell number, the secretion of extracellular polymeric substances and production of an extracellular matrix (ECM). Once adhered, yeast cells proliferate to form microcolonies, forming the basal layer of the biofilm. As the biofilm matures, the biomass increases with yeast cells, hyphae, pseudohyphae and the ECM accounting for a large proportion of the biofilm (Figure 1(B)). Hyphae are fundamental components that support the structural integrity of the biofilm and provide a scaffold for the attachment of additional yeast cells, pseudohyphae, other hyphae, as well as bacteria in multispecies biofilms (Chandra et al. 2001; Harriott and Noverr 2009). Mutants defective in the production of hyphae or hypha-specific adhesins are also defective in the production of substantial biofilms (Baillie and Douglas 1999; Ramage, VandeWalle, et al. 2002; Richard et al. 2005). Accordingly, the ability to form hyphae is critical for the normal development and maintenance of *C. albicans* biofilms. In a typical biofilm, an adherent layer of yeast cells is covered by an entangled mesh of hyphal filaments and pseudohyphae and encased in an ECM (Baillie and Douglas 1999; Chandra et al. 2001) (Figure 1(C)). Indeed, the ECM is essential for biofilm development and maturation. The ECM is composed of extracellular polymeric substances secreted by cells within the biofilm, including a complex mixture of macromolecules comprising proteins (55%), carbohydrates (25%), lipids (15%) and nucleic acids (5%) (Zarnowski et al. 2014). The most common polysaccharide found in the ECM of *C. albicans* biofilms are mannans, which are often associated with β -1,6 glucans (Pierce et al. 2017). Proteomic analysis of *C. albicans* extracellular polymeric substances also reveals the presence of numerous host molecules, including those found in epithelial cells and neutrophils, suggesting a potential role for host

components in biofilm development (Andes et al. 2004; Dongari-Bagtzoglou et al. 2009; Zarnowski et al. 2014; Nett et al. 2015).

The presence of nucleic acids in *C. albicans* biofilms contributes to the structural and protective properties of the ECM. Extracellular deoxyribonucleic acid (eDNA) released by *C. albicans* within the ECM may contribute to the maintenance and stability of mature biofilms, but is not required to establish a *C. albicans* biofilm (Al-Fattani and Douglas 2006; Martins et al. 2010; Rajendran et al. 2014), suggesting an important role for eDNA in ECM assembly. The release of eDNA confers heterogeneity during biofilm formation, with higher levels of eDNA observed in isolates with high biofilm-forming capacity compared to isolates with lower capacity (Rajendran et al. 2014). Moreover, eDNA may also play a role in modulating the host immune response by activating bone marrow-derived myeloid dendritic cells (Kasai et al. 2006; Miyazato et al. 2009; Martins et al. 2010). Treatment of *C. albicans* biofilm with deoxyribonuclease I (DNase) improved the efficacy of amphotericin B *in vitro* (Martins et al. 2012), particularly in the presence of the chitinase inhibitor acetazolamide (Rajendran et al. 2014), suggesting that eDNA also contributes to recalcitrance. Accordingly, targeting eDNA within the ECM may present a novel therapy to improve the activity of antifungal drugs and decrease biofilm formation (Martins et al. 2012; Sapaar et al. 2014).

The biofilm matrix has pleiotropic functionality; it contributes to biofilm structure, provides physical support (Nobile and Johnson 2015; Pierce et al. 2017) and is critical in enhancing tolerance to antifungal drugs (described below).

Biofilm dispersal

Dispersal is the final stage of the biofilm lifecycle and describes a process where cells are released from a mature biofilm and are thus free to disseminate and establish secondary sites of infection (Figure 1(D)). The majority of dispersal occurs from the uppermost layers of biofilm and is associated with yeast cells that have an elongated morphology (Uppuluri, Chaturvedi, et al. 2010). Several factors contribute to the process of dispersal. *Candida albicans* Nrg1p, Pes1p and Ume6p are known regulators of dispersal with opposing functions. Nrg1p is a negative regulator of the yeast-to-hypha morphological switch (Braun et al. 2001; Murad et al. 2001). Consistent with the release of yeast cells from biofilm during dispersal, overexpression of *NRG1* promotes dispersal from biofilms *in vitro* (Uppuluri,

Pierce, et al. 2010), while overexpression of the Pescadillo homologue *PES1* also increases dispersal (Uppuluri, Chaturvedi, et al. 2010). In contrast, overexpression of the transcriptional regulator *UME6* reduces dispersal (Uppuluri, Chaturvedi, et al. 2010), while transcriptional repression of the chaperone protein Hsp90p also reduces dispersal of *C. albicans* cells from biofilms *in vitro* (Robbins et al. 2011).

Candida albicans yeasts express Ywp1p, a cell-surface glycoprotein that is covalently linked to β -glucans in the fungal cell wall. Intriguingly, Ywp1p is reported to exhibit anti-adhesive properties consistent with a role in dispersal from biofilms. A *C. albicans* mutant unable to express *YWP1* displays enhanced adhesion and biofilm formation (Granger et al. 2005). Members of the *C. albicans* histone deacetylase complex have also been implicated in biofilm dispersal, with *C. albicans* mutants unable to express *HOS2*, *SET3*, *SIF2* or *SNT1* exhibiting increased cohesiveness and impaired dispersal over a 60 h period (Nobile et al. 2014).

Notably, dispersed cells have a number of altered characteristics when compared to planktonic cells including an increased ability to filament and form biofilms, increased adherence to biotic and abiotic substrates, reduced susceptibility to antifungals and an increased ability to damage endothelial cells *in vitro* (Uppuluri, Chaturvedi, et al. 2010). Dispersed cells also upregulate the expression of genes whose products are involved in the acquisition of micronutrients, drug resistance, and hydrolysis of host substrates (Uppuluri et al. 2018). Moreover, dispersed cells display enhanced pathogenicity *in vivo* when compared with matched planktonic controls (Uppuluri, Chaturvedi, et al. 2010). Collectively, these observations suggest that dispersed cells are “pre-conditioned” for maximum virulence. More recent work suggests that the degree of fungal dispersal is influenced by the extracellular environment. Investigations with mixed-species biofilms reveal that the Gram-positive bacterium *Rothia dentocariosa* can inhibit the release of *C. albicans* cells *in vitro* (Uppuluri et al. 2017), thus the amount of dispersal that occurs *in vivo* is likely to be influenced by biofilm composition.

Transcriptional regulation of biofilm formation and maintenance

The formation and development of *C. albicans* biofilms are underpinned by complex changes in transcriptional circuitry. While 58 transcription factors have been identified to contribute to *C. albicans* biofilm formation and

development (Table 1), an interdependent core network of transcriptional regulators has been identified (Bcr1p, Tec1p, Efg1p, Ndt80p, Rob1p and Brg1p) that is critical for biofilm formation (Nobile et al. 2012; Glazier et al. 2017; Figure 1). *Candida albicans* mutant strains unable to express *BCR1*, *TEC1*, *EFG1*, *NDT8* or *ROB1* fail to form biofilms *in vivo*, while ablation of *BRG1* results in biofilms with abnormal structure (Ramage, Vande Walle, et al. 2002; Nobile and Mitchell 2005; Nobile et al. 2012; Yano et al. 2016; Panariello et al. 2017). Collectively, this core network of transcriptional regulators influences the expression of approximately 1000 genes involved in hypha formation, adhesion, metabolism, production of extracellular polymeric substances and drug resistance (Fox and Nobile 2012; Nobile and Johnson 2015).

More recent work has identified additional master regulators of biofilm formation including Flo8p, Gal4p and Rfx2p (Fox et al. 2015). *Candida albicans* mutant strains unable to express *GAL4* or *RFX2* form thicker biofilms (Fox et al. 2015), suggesting that these factors function as negative regulators. In contrast, strains unable to express *FLO8* were markedly defective in biofilm formation in a rat central venous catheter model (Nobile et al. 2012; Fox et al. 2015). Bcr1p is critical for biofilm formation and influences the expression of several adhesins including Als3p, Hwp1p and Hyr1p (Nobile and Mitchell 2005; Nobile, Andes, et al. 2006; Dwivedi et al. 2011). A *C. albicans* *bcr1* $\Delta\Delta$ mutant produces a defective biofilm *in vitro* and *in vivo*, but overexpression of *ALS3* rescues the defective biofilm phenotype, highlighting the importance of Als3p (Nobile and Mitchell 2005; Nobile, Andes, et al. 2006; Nobile and Mitchell 2006; Liu and Filler 2011). Interestingly, Bcr1p also targets *ECE1*, which encodes the peptide toxin candidalysin (Nobile, Andes, et al. 2006; Moyes et al. 2016). RNA transcriptome analysis reveals that several genes (including *ECE1*) are upregulated in high biofilm-forming isolates of *C. albicans* (Rajendran, May, et al. 2016).

The production of biofilm ECM is predominantly controlled by two transcription factors: Zap1p and Rlm1p. Zap1p is a zinc regulator that negatively regulates the formation of β -1,3 glucan through its target genes *GCA1* and *GCA2* (Nobile et al. 2009; Mayer et al. 2013; Nobile and Johnson 2015). In contrast, Rlm1p is a positive regulator of ECM production (Nett et al. 2011; Nobile and Johnson 2015). Another transcription factor, Stp2p, is a key activator of amino acid permease genes that influences adherence and morphogenesis, adaptation to nutrients and biofilm longevity (Böttcher et al. 2020).

Table 1. Transcription factors that contribute to *C. albicans* biofilm formation, development, and regulation.

Gene	Function/role	Mutant phenotype	References
<i>ACE2</i>	Hypha formation, metabolism, and adherence	Fails to form biofilms <i>in vitro</i> and <i>in vivo</i>	(Kelly et al. 2004; Mulhern et al. 2006; Finkel et al. 2012; Nobile and Johnson 2015)
<i>ADA2</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>AHR1</i>	Adherence	Defective biofilm <i>in vitro</i>	(Askew et al. 2011; Nobile and Johnson 2015)
<i>ARG81</i>	Adherence and metabolism	Fails to form biofilm <i>in vitro</i>	(Finkel et al. 2012)
<i>BCR1*</i>	Positive regulator of adhesins such as Als3p, Hwp1p, and Hyr1p Adhesion maintenance	Fails to form biofilm <i>in vitro</i> and <i>in vivo</i>	(Nobile and Mitchell 2005; Nobile, Andes, et al. 2006; Dwivedi et al. 2011; Desai and Mitchell 2015; Nobile and Johnson 2015; Yano et al. 2016; McCall et al. 2019)
<i>BPR1</i>	Normal biofilm formation	Defective biofilm <i>in vitro</i>	(Fox et al. 2015; Nobile and Johnson 2015)
<i>BRG1*</i>	Normal biofilm formation	Defective biofilm <i>in vitro</i> and <i>in vivo</i>	(Nobile et al. 2012; Nobile and Johnson 2015)
<i>CAS5</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>CRZ2</i>	Adherence	No defect on biofilms <i>in vitro</i> but fails to form biofilms <i>in vivo</i>	(Finkel et al. 2012)
<i>CZF1</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>DAL81</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>EFG1*</i>	Hypha formation and adherence	Fails to form biofilms <i>in vitro</i> and <i>in vivo</i>	(Ramage, VandeWalle, et al. 2002; Nobile et al. 2012; Nobile and Johnson 2015; Panariello et al. 2017; McCall et al. 2019)
<i>FCR3</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>FGR27</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>FLO8*</i>	Biofilm formation at all stages	Fails to form biofilms <i>in vitro</i> and <i>in vivo</i>	(Fox et al. 2015; Nobile and Johnson 2015)
<i>GAL4*</i>	Negative regulator of biofilm formation at intermediate stages	Enhanced biofilms <i>in vitro</i> and <i>in vivo</i>	(Fox et al. 2015)
<i>GCN4</i>	Metabolism	Defective biofilm <i>in vitro</i>	(García-Sánchez et al. 2004; Desai and Mitchell 2015; Nobile and Johnson 2015)
<i>GRF10</i>	Hypha formation	Defective biofilm formation <i>in vitro</i>	(Ghosh et al., 2015)
<i>GZF3</i>	Normal biofilm formation	Defective biofilm <i>in vitro</i>	(Fox et al. 2015)
<i>LEU3</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>MET4</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>MIG1^</i>	Metabolism & hypha formation	Slightly defective biofilm <i>in vitro</i> . mig1Δ/Δ mig2Δ/Δ double mutant highly defective biofilm <i>in vitro</i> .	(Lagree et al., 2020)
<i>MIG2^</i>	Metabolism	No change in biofilm. However, mig1Δ/Δ mig2Δ/Δ double mutant highly defective biofilm <i>in vitro</i> .	(Lagree et al., 2020)
<i>MSS11</i>	Hypha formation	Defective biofilm <i>in vitro</i>	(Tsai et al. 2014)
<i>NDT80*</i>	Hypha formation Positive regulator of ALS3, HWP1, HYR1 and ECE1	Fails to form biofilms <i>in vitro</i> and <i>in vivo</i>	(Sellam et al. 2010; Nobile et al. 2012; Nobile and Johnson 2015)
<i>NOT3</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>NRG1</i>	Negative regulator of hypha formation	Defective biofilm <i>in vitro</i>	(Uppuluri, Pierce, et al. 2010; Nobile and Johnson 2015)
<i>PES1</i>	Biofilm dispersal	Defective biofilm dispersal <i>in vitro</i> and <i>in vivo</i>	(Shen et al., 2008; Uppuluri, Chaturvedi, et al. 2018, 2010)
<i>RFG1</i>	Biofilm formation	Fails to form biofilm <i>in vitro</i>	(Fox et al. 2015)
<i>RFX2*</i>	Negative regulator of biofilm formation at intermediate time point	Enhanced biofilms <i>in vitro</i> and <i>in vivo</i>	(Fox et al. 2015)
<i>RIM101</i>	Normal biofilm formation	Defective biofilm <i>in vitro</i>	(Fox et al. 2015)
<i>RLM1</i>	Glucan production of the extracellular matrix	Fails to form biofilms <i>in vitro</i> and <i>in vivo</i>	(Nett et al. 2011; Nobile and Johnson 2015)
<i>ROB1*</i>	Biofilm formation	Fails to form biofilms <i>in vitro</i> and <i>in vivo</i>	(Nobile et al. 2012; Nobile and Johnson 2015)
<i>SFP1</i>	Repressor of adhesion and biofilm development	Enhanced biofilm formation <i>in vitro</i>	(Chen and Lan, 2015)
<i>SNF5</i>	Hypha formation and adherence Target gene: ACE2	Fails to form biofilms <i>in vitro</i> and <i>in vivo</i>	(Finkel et al. 2012; Nobile and Johnson 2015)
<i>STP2^</i>	Adherence, hypha formation and metabolism	Defective biofilm <i>in vitro</i> in terms of metabolic activity	(Böttcher et al. 2020)
<i>SUC1</i>	Adherence	Defective biofilm <i>in vitro</i>	(Finkel et al. 2012)
<i>TAF14</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)

(continued)

Table 1. Continued.

Gene	Function/role	Mutant phenotype	References
<i>TEC1</i> *	Biofilm formation, hypha formation and final biofilm integrity	Fails to form biofilms <i>in vitro</i> and <i>in vivo</i>	(Nobile and Mitchell 2005; Daniels et al. 2015; Nobile and Johnson 2015; Panariello et al. 2017)
<i>TRY2</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>TRY3</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>TRY4</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>TRY5</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>TRY6</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>TYE7</i>	Negative regulator of hypha formation	Defective biofilm <i>in vitro</i>	(Bonhomme et al. 2011; Nobile and Johnson 2015; Villa et al. 2020)
<i>UGA33</i>	Activation of glycolytic genes		
<i>UME6</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
	Regulator of hypha formation, biofilm dispersion	Defective biofilm <i>in vitro</i>	(Uppuluri, Chaturvedi, et al. 2010; Nobile and Johnson 2015; Villa et al. 2020)
<i>UPC2</i> ^	Repressor of adhesion and cell wall proteins	Enhanced biofilm formation <i>in vitro</i>	(Kakade et al., 2019)
<i>WAR1</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>ZAP1</i> (ALSO CALLED <i>CSR1</i>)	Negative regulator of extracellular matrix formation, hypha formation	Enhanced biofilm formation <i>in vitro</i> and <i>in vivo</i>	(Nobile et al. 2009; Ganguly et al. 2011; Finkel et al. 2012; Nobile and Johnson 2015)
<i>ZCF28</i>	Adherence	No defect on biofilm <i>in vitro</i> but fails to form biofilm <i>in vivo</i>	(Finkel et al. 2012)
<i>ZCF31</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>ZCF32</i>	Repressor of biofilm development, involved in adhesion, hypha formation, and dispersion	Enhanced biofilm formation <i>in vitro</i>	(Kakade et al., 2019, 2016)
<i>ZCF34</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>ZCF39</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>ZCF8</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)
<i>ZFU2</i>	Adherence	No defect on biofilm <i>in vitro</i> but fails to form biofilm <i>in vivo</i>	(Finkel et al. 2012)
<i>ZNC1</i>	Adherence	No biofilm formation <i>in vitro</i>	(Finkel et al. 2012)

*Transcription factors of the core network; ^most recently identified transcription factors.

Candida albicans biofilms in vitro and in vivo: one morphology does not fit all

Most biofilm studies have been conducted *in vitro*. For the most part, data obtained from *in vitro* biofilm studies correlate well with data obtained from *in vivo* and *ex vivo* models. The structure of biofilms from rat denture stomatitis and indwelling urinary catheter models is similar to the architecture of biofilms produced *in vitro* (Andes et al. 2004; Johnson et al. 2012). The formation and structure of *C. albicans* biofilm is influenced by numerous factors including the nature of the contact surface, environmental factors, and *C. albicans* morphology. Maturation of *C. albicans* biofilms on polyvinyl chloride catheter discs occurs within 24–48 h (Hawser and Douglas 1994), whereas maturation on polymethylmethacrylate strips requires longer timeframes (38–72 h) (Chandra et al. 2001). Interestingly, Andes et al. reported differences in temporal developments between *in vivo* and *in vitro* studies. Utilising a rat central venous catheter model, the duration of the early phase and maturation of biofilm formation *in vivo* was shorter than that observed *in vitro* (Andes et al. 2004). While *C. albicans* forms biofilms readily on abiotic

substrates and on the mucosal surfaces of the oral cavity, conflicting observations have been made regarding the formation of biofilms on the vaginal mucosa (Harriott et al. 2010; Paiva et al. 2012; Sherry et al. 2017; Noverr and Fidel 2019; Swidsinski et al. 2019; Xu et al. 2019), suggesting that tissue/anatomical location and surrounding microbiota are likely to influence biofilm development. These observations have led to some contention regarding the definition of clinical diseases that specifically involve biofilms as opposed to diseases that are associated with a diverse or altered microbiota (Harriott et al. 2010; Paiva et al. 2012; Sobel 2015; Sherry et al. 2017; Noverr and Fidel 2019; Swidsinski et al. 2019; Xu et al. 2019). Rather than being a simple binary event, biofilms comprise a spectrum of morphologies, the composition of which varies between diseases. Accordingly, the provision of an unambiguous definition of *C. albicans* biofilms in the clinical setting is not without significant challenge.

Advantages of the biofilm lifestyle

The environment within a biofilm differs markedly from that encountered during planktonic growth and offers

numerous advantages to the microbes that reside within it. The morphological variation within biofilms (yeasts, pseudohyphae and hyphae) combined with the presence of other fungi and bacteria collectively confer an increased competitive fitness that is absent in planktonic cells (Lohse et al. 2018). Microbes encased within the ECM are more likely to withstand minor mechanical stress and are better protected from the external environment and hostile host immune responses (Mathé and Van Dijck 2013; Cavaleiro and Teixeira 2018; Kernien et al. 2018). Mixed-species biofilms can produce and utilise a greater variety of nutrients through the formation of metabolic consortiums in which the waste products of one species may be metabolised by another (Elias and Banin 2012). Such communities exhibit tremendous genomic diversity and, consequently, biofilms provide a suitable environment in which new genetic material can be acquired through the process of horizontal/lateral gene transfer, particularly between species of bacteria (Roberts and Kreth 2014).

Numerous differences have been reported between *C. albicans* biofilms and planktonic cells including alterations in transcriptional network activity (Yeater et al. 2007; Nobile et al. 2012; Fox et al. 2015), altered cell wall architecture (Nett et al. 2007), differences in ergosterol content of biofilm cell plasma membranes (Mukherjee et al. 2003) and, most notably, differences in sensitivities to antifungals. *Candida albicans* biofilms are substantially more difficult to eradicate with antifungals as compared to planktonic cells. Numerous mechanisms enable planktonic cells to withstand the toxicity of antifungals including constitutive expression and activation of drug efflux pumps, mutation of genes encoding drug targets, the acquisition of drug tolerance (Berman and Krysan 2020), alterations in the copy number of genes via aneuploidy (Yang et al. 2019) and adaptive mistranslation (Weil et al. 2017). Mature *C. albicans* biofilms are highly tolerant of fluconazole, amphotericin B, and caspofungin at concentrations that are lethal to planktonic cells (Vediyappan et al. 2010). Although numerous drug efflux pumps are expressed both transiently and permanently during the formation and maturation of *C. albicans* biofilm *in vitro* and *in vivo* (Mukherjee et al. 2003; Mateus et al. 2004; Yeater et al. 2007; Nett et al. 2009; Fox et al. 2015), the overall contribution of drug efflux to biofilm longevity appears to be relevant only during the early stages of biofilm formation (Mukherjee et al. 2003). Importantly, while *C. albicans* strains deleted for specific ATP-binding cassette transporters and major facilitator superfamily transporters

(*cdr1ΔΔ*, *cdr2ΔΔ*, *cdr1/cdr2ΔΔ*, *mdr1ΔΔ* and *mdr1/cdr1ΔΔ*) were highly susceptible to fluconazole during planktonic growth, the same mutants exhibited resistance to fluconazole when grown as a biofilm (Ramage, Bachmann, et al. 2002), suggesting that factors distinct from drug efflux pumps contribute to drug tolerance within a biofilm.

Specific components of the biofilm ECM are instrumental in the orchestration of tolerance to antifungals. *Candida albicans* mutant strains unable to deliver β -1,3 glucan to the biofilm ECM are susceptible to fluconazole (Nett, Sanchez, et al. 2010; Nett et al. 2011; Taff et al. 2012) while reducing *FKS1* (glucan synthase) expression renders cells susceptible to amphotericin B, anidulafungin, and flucytosine (Nett, Crawford, et al. 2010). Indeed, β -glucan is capable of binding fluconazole and amphotericin B (Nett et al. 2007; Vedyappan et al. 2010), while treatment of *C. albicans* biofilms with the enzyme β -1,3 glucanase increases sensitivity to fluconazole (Nett et al. 2007). Sequestration of fluconazole by β -glucan in the ECM combined with a reduction in antifungal penetration into the biofilm often results in fungi receiving a sub-lethal dose of the drug, which can lead to the emergence of resistance.

Collectively, these observations are consistent with the notion that the ECM functions in part, as a “molecular trap” that impedes the diffusion of antifungals into the biofilm, causing a reduction in the local concentration of antifungal that can engage with target cells. Exposure of biofilms to diminished concentrations of an antifungal is likely to impose a positive selection pressure towards the acquisition of mutations that reduce susceptibility to subsequent therapy, an outcome that may have serious clinical implications should mutated cells be dispersed from a recalcitrant biofilm.

More recently, studies have highlighted an essential role for extracellular vesicles (EVs) in conferring the drug-resistant properties of *C. albicans* biofilms. Analyses of EVs released from *C. albicans* biofilms reveal a proteome that is distinct from that present in EVs released from planktonic cells, and which contain high levels of mannan and glucan (Zarnowski et al. 2018). *Candida albicans phr1ΔΔ* and *sun41ΔΔ* null mutants are devoid of specific EV cargo proteins and form biofilms that are sensitive to fluconazole. Strikingly, EVs that were harvested from wild type *C. albicans* biofilms and applied to *phr1ΔΔ* and *sun41ΔΔ* biofilms restored the ability to grow in the presence of the drug (Zarnowski et al. 2018), demonstrating a functional role for EVs in conferring drug resistance.

The effectiveness of the host immune response to biofilms is also diminished when compared to planktonic cells. The biofilm ECM protects underlying fungal cells from the innate immune system, particularly from killing by neutrophils and monocytes. Impaired recognition of biofilms by neutrophils and monocytes is evidenced by altered patterns of cytokine secretion when compared to those produced when encountering planktonic cells (Chandra et al. 2007). Monocytes can phagocytose planktonic *C. albicans* but not fungal cells associated with biofilms, while neutrophils fail to eradicate mature biofilms (Chandra et al. 2007; Katragkou et al. 2010; Xie et al. 2012; Pierce et al. 2017). Furthermore, neutrophils release extracellular traps in response to planktonic *C. albicans* yeast and short hyphae but fail to release extracellular traps in response to biofilm ECM (Nett 2016; Johnson et al. 2016, 2017; Pierce et al. 2017; Kernien et al. 2018).

Persister cells: a disputed presence in *C. albicans* biofilms

Persister cells are a contentious topic in the context of *C. albicans* biofilms. Defined as a subpopulation of metabolically dormant biofilm cells, these cells are only detected in the presence of antifungals. Persister cells express high levels of alkyl hydroperoxide reductase 1 (Ahp1p), which is positively correlated with prolonged survival following exposure to amphotericin B (Truong et al. 2016). When antifungal therapy is discontinued, persister cells proliferate to restore the biofilm. Patients with long-term oral carriage of *C. albicans* have a higher incidence of persister cells (LaFleur et al. 2010), suggesting that persister cells may be relevant in the context of continual colonisation or recurrent infections *in vivo* (Wuyts et al. 2018). However, despite observations which support the existence of persister cells they remain a controversial topic. Indeed, some studies suggest that persister cells are not a general characteristic of *Candida* biofilms (Al-Dhaheri and Douglas 2008; Denega et al. 2019).

Heteroresistance of *C. albicans* biofilms

As previously mentioned, *C. albicans* biofilms are difficult to eradicate. An issue that clinicians are facing is biofilm heterogeneity. Indeed, clinical isolates of *C. albicans* biofilms from the bloodstream or the vaginal mucosa seem to differ in biomass and therefore isolates can be defined as either high- or low-biofilm formers (Sherry et al. 2014, 2017). This inherent biological heterogeneity has led to patients responding differently to

antifungals (Kean et al. 2018) and is thus called heteroresistance. Heteroresistance is defined as the presence of a subpopulation with lower susceptibility to specific antifungals within a larger microbial population. A recent study of *C. glabrata* clinical strains suggests that this heteroresistance might be linked to persistent infections (Ben-Ami et al. 2016).

Multispecies biofilms: *C. albicans* and polymicrobial interactions

Interactions between *C. albicans* and the microbes found on biotic and abiotic substrates are varied and complex, comprising synergistic, antagonistic, and neutral relationships (Delaney et al. 2018). These interactions involve direct cell to cell contact, chemical interactions via the secretion of small molecules, enhancement of colonisation, changes in the host environment, the use of metabolic by-products, changes in the host response or a combination of these. Unsurprisingly, these interactions are particularly relevant in the context of biofilms and are discussed below.

Adhesion in mixed species biofilms

The interactions of *C. albicans* with other microorganisms can occur via co-aggregation and co-adhesion. *Candida albicans* adhesins (e.g. Als1p, Als2p, Als3p, Hwp1p) facilitate interaction with bacterial species such as *Streptococcus gordonii* and *Staphylococcus aureus* (Peters et al. 2012; Hoyer et al. 2014; Schlecht et al. 2015; Allison et al. 2019; Figure 2(A)). The adherence of *C. albicans* to pre-attached *Streptococcus* spp. in the oral cavity is mediated by polysaccharide receptors on the streptococcal cell surface (SspA and SspB) and *C. albicans* hypha-associated adhesins (Als1p and Als3p) (Holmes et al. 1996; Bamford et al. 2009; Silverman et al. 2010). Moreover, *S. gordonii* SspA and SspB mutants exhibit reduced expression of streptococcal adhesins for *C. albicans*, with less co-aggregation of *C. albicans* with these *S. gordonii* mutants (Klotz et al. 2007).

Microbial synergism and antagonism within mixed species biofilm

Interkingdom interactions between *C. albicans* and other microbes can be synergistic, mutually beneficial or antagonistic, and these interactions can dictate the composition of the microbiota. One of the most thoroughly characterised synergistic relationships is that of

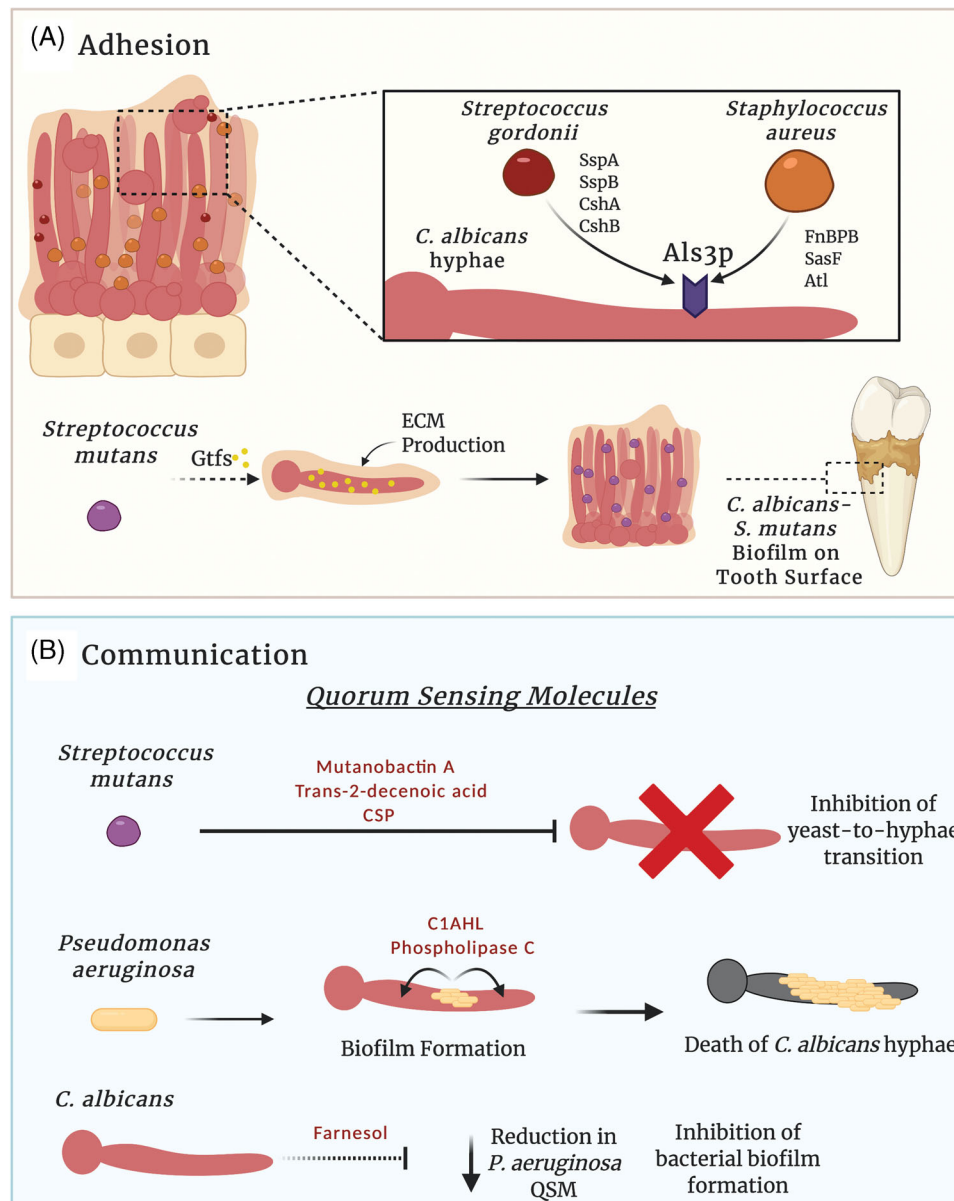


Figure 2. *Candida albicans* interactions within a multispecies biofilm. Complex physical and chemical interactions govern the development of polymicrobial biofilms. (A) Several factors influence *C. albicans*–bacterial adhesion. *Staphylococcus aureus* and *Streptococcus gordonii* can utilise *C. albicans* adhesins to directly bind to hyphae. In contrast, glycosyltransferases (Gtfs) secreted by *Streptococcus mutans* within the oral cavity can bind to *C. albicans* mannans, increasing the production of glucans and ECM. Consequently, the glucan increases the ability of the bacterium to bind to *C. albicans* and forms a *C. albicans*–*S. mutans* biofilm on the tooth surface (dental plaque). (B) Signalling molecules produced by *C. albicans* and bacterial species enable inter-kingdom communication within multispecies biofilms. For example, *S. mutans* and *Pseudomonas aeruginosa* can secrete quorum sensing molecules that influence the behaviour of *C. albicans* within the biofilm. Likewise, the *C. albicans* quorum sensing molecule farnesol, can influence the behaviour of interacting bacteria. Figure created with BioRender.

C. albicans and *Streptococcus mutans*. Within plaque biofilms, *S. mutans* glycosyltransferases bind directly to mannans on the surface of *C. albicans* yeast and hyphal cell walls, promoting the development of an extensive ECM and the formation of a mixed species biofilm (Hwang et al. 2015; Ellepola et al. 2017). Consistent with this, *S. mutans* mutants lacking glycosyltransferase genes demonstrate reduced ability to form mixed

species biofilms with *C. albicans* (Falsetta et al. 2014; Hwang et al. 2017; Figure 2(A)). The presence of both *S. mutans* and *C. albicans* in the oral microbiota significantly increases bacterial colonisation and supports the development of dental caries, suggesting that this interaction may be a pivotal factor in increasing the risk of disease (de Carvalho et al. 2006; Khoury et al. 2020). In addition to physical associations, streptococci excrete

lactate that acts as a carbon source for *C. albicans* yeast growth (Ene et al. 2012; Metwalli et al. 2013). The *C. albicans* quorum sensing (QS) molecule farnesol has known antibacterial effects, however, within a dual-species biofilm, farnesol enhances *S. mutans* cell growth and microcolony development (Kim et al. 2017). Conversely, the *S. mutans* QS molecules mutanobactin A, trans-2-decenoic acid and competence-stimulating peptide can inhibit *C. albicans* germ tube formation and the yeast-to-hypha transition (Jarosz et al. 2009; Joyner et al. 2010; Vílchez et al. 2010; Figure 2(B)). Overall, this demonstrates a mutually beneficial relationship between *C. albicans* and *S. mutans* with a synergistic partnership that can alter the oral microbiota and influence disease.

Within a dual-species biofilm, *C. albicans* can protect anaerobic bacteria from high concentrations of oxygen by providing a hypoxic microenvironment that supports the growth of *Bacteroides fragilis*, *B. vulgatus* and *Clostridium perfringens* (Fox et al. 2014; Valentine et al. 2019). Accordingly, *C. albicans* affected the biofilm composition, suggesting a role for the fungus in maintaining homeostasis within the microbiota. Additionally, *C. albicans* β -1-3-glucan facilitates bacterial tolerance to ofloxacin (De Brucker et al. 2015) and vancomycin (Kong et al. 2016) in a dual-species biofilm with *Escherichia coli* and *S. aureus* respectively, demonstrating antimicrobial tolerance and microbial persistence within multispecies biofilms. However, interactions between *C. albicans* and bacteria are not always synergistic. Antagonistic effects have been reported between *Lactobacillus* spp. and *C. albicans*, with both hypha formation and biofilm development affected by the bacteria (Orsi et al. 2014). In the normal vaginal microbiota, *Lactobacillus* spp. compete with *C. albicans* for receptors present on the surface of genitourinary epithelium for adhesion; they also secrete lactic acid and hydrogen peroxide, lowering the pH, which in turn inhibits fungal attachment to the vaginal epithelium (Boris et al. 1998; Boris and Barbés 2000; Aroutcheva et al. 2001).

The antagonistic relationship between *C. albicans* and *Pseudomonas aeruginosa* is particularly well defined. Both *P. aeruginosa* and *C. albicans* are frequently co-isolated from contaminated catheters and chronic lung infections (El-Azizi et al. 2004; Williams and Cámara 2009). Numerous interactions occur between *C. albicans* and *P. aeruginosa*, the outcomes of which are greatly influenced by the environment. *Pseudomonas aeruginosa* can form dual-species biofilms with *C. albicans* in liquid medium through a physical association with *C. albicans* hyphae and germ tubes but not yeast cells. Once formed, *C. albicans* hyphae are killed by *P.*

aeruginosa secreted virulence factors (phospholipase C and redox-active oxygen species) and quorum sensing molecules (3-oxo-C12-homoserine lactone (C12AHL)) (Hogan and Kolter 2002; Hogan et al. 2004; Gupta et al. 2005; Gibson et al. 2009; Figure 2(B)). The destruction of hyphae but not *C. albicans* yeast cells suggests a potential survival mechanism developed by the fungus that allows yeast to evade destruction by *P. aeruginosa*.

Metabolic interactions play an important role in microbial adaptation within polymicrobial environments. Metabolic dependencies between microbes can be direct or indirect and involve the production of metabolites by one species and their subsequent consumption by another, or the localised depletion of a toxic metabolite by one species, enabling a second species to persist (Krom et al. 2014). Competition for nutrients is endemic within polymicrobial communities. In a mixed-species biofilm, *P. aeruginosa* sequesters iron from *C. albicans*, reducing the metabolic activity of the fungus (Purschke et al. 2012). *Lactobacillus rhamnosus* can protect oral epithelial cells from fungal damage by inducing metabolic reprogramming of *C. albicans*. This protective effect comprises a reduction in *C. albicans* adhesion, impaired hypha formation and glucose depletion (Mailänder-Sánchez et al. 2017). As the nature of fungal metabolism is adaptable, these metabolic interactions may be significant in maintaining homeostasis.

Intra-genus interactions within *Candida* biofilms

While the establishment of mixed-species biofilms involving *C. albicans* and bacteria has been extensively described, intra-genus interactions between *Candida* species are not as well characterised. Although *C. albicans* has been studied more extensively than any other *Candida* species, other species such as *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. auris* are becoming an increasing concern in the clinical setting. Though poorly studied, *C. albicans*, and other non-*C. albicans* species have been shown to form biofilms with each other (Pereira-Cenci et al. 2008; Pathak et al. 2012). Mixed species biofilms of *C. albicans* and non-*C. albicans* species have been identified on dental acrylic resin strips in which *C. albicans* was the predominant species (Pathak et al. 2012). Moreover, aggregates of *C. albicans* and *C. dubliniensis* have been isolated from the oral cavity of immunocompromised patients (Kirkpatrick et al. 2000), while *C. albicans* and *C. glabrata* were frequently co-isolated from patients with denture-associated stomatitis (Coco et al. 2008). In another

study, the presence of *C. albicans* in a mixed biofilm facilitated the invasion of *C. glabrata* in an *in vitro* model of denture stomatitis and promoted significant tissue damage to the epithelium during vulvovaginal candidiasis (Silva et al. 2011; Alves et al. 2014). The increasing number of mixed *Candida* species biofilms poses a major problem in the clinical setting due to the resistance of *Candida* species to a variety of antifungal drugs (Al-Fattani and Douglas 2006; Bizerra et al. 2008). These interactions highlight the importance of further research into the mechanisms and consequences of *C. albicans* and non-*C. albicans* species biofilm formation.

Communication within mixed-species biofilms

Numerous chemical interactions occur between the microbes found within a mixed-species biofilm via the secretion of small molecules and the exchange of nutrients. Both bacterial and fungal populations within a biofilm can modulate their behaviour via quorum sensing; a mechanism of chemical interaction in which microbes communicate through the secretion of signalling molecules (Wongsuk et al. 2016; Figure 2(B)). Quorum sensing induces changes in transcriptional activity, which in turn influences the behaviour, survival, or virulence of either microbe. *Candida albicans* secretes farnesol, a quorum-sensing molecule that inhibits the Ras1-cyclic AMP (cAMP)-protein kinase A (PKA) (Ras1-cAMP-PKA) signalling pathway to repress hyphal development (Hornby et al. 2001; Davis-Hanna et al. 2008), inhibit biofilm formation (Ramage, Saville, et al. 2002) and upregulate the expression of *FCR1* and *PDR16*, which are involved in resistance to azole therapy (Cao et al. 2005). Notably, the addition of farnesol to cultures of *P. aeruginosa* decreases the production of *Pseudomonas* quinolone signalling (PQS) molecule, a known suppressor of *C. albicans* biofilm formation, and the PQS-controlled virulence factor pyocyanin and inhibits swarming motility (Cugini et al. 2007; McAlester et al. 2008). As these *P. aeruginosa* factors are toxic to *C. albicans*, the release of farnesol within this interaction is likely to promote *C. albicans* survival.

In the oral cavity, *S. gordonii* uses quorum sensing to suppress farnesol-mediated repression of *C. albicans* filamentation, enhancing biofilm formation (Bamford et al. 2009). Such behaviour is far from altruistic. Indeed, *S. gordonii* promotes fungal growth by providing a carbon source to *C. albicans* (Bamford et al. 2009) and, in return, *C. albicans* increases the likelihood that *S. gordonii* can survive and persist in the oral cavity by lowering oxygen tension and releasing bacterial growth factors derived from nutrient metabolism (Shirtliff et al. 2009).

Molecules secreted by microbes can have an inhibitory effect on *C. albicans*. In the gastrointestinal tract, *Bacteroides thetaiotaomicron* stimulates the host to express HIF-1 α and LL-37, which inhibit gastrointestinal colonisation by *C. albicans* (Fan et al. 2015). Similarly, *C. albicans* is susceptible to type VI secretion system (T6SS)-mediated antifungal activity perpetrated by the Gram-negative opportunistic bacterium *Serratia marcescens*, which uses its T6SS to penetrate the fungal cell wall to deliver the toxic effector proteins Tfe1 and Tfe2 that disrupt nutrient uptake, amino acid metabolism and plasma membrane potential (Trunk et al. 2018). However, whether this occurs in the context of a multispecies biofilm has yet to be investigated.

Overall, polymicrobial interactions within multispecies biofilms are of great importance and further studies may aid better understanding of *C. albicans* co-infection and possible therapeutic strategies to combat inter and intra-kingdom biofilm infections. Moreover, our understanding from next-generation sequencing studies, accompanied by greater bioinformatics power, is likely to aid our discovery of key interactions between *Candida* spp. and bacteria from an array of clinical diseases.

Clinical impact of biofilms

Biofilms are intrinsically associated with highly virulent, persistent and drug-resistant infections. The dispersal of cells from an established biofilm facilitates whole-body dissemination and reduces the likelihood of a positive clinical outcome and compounds options for effective therapeutic intervention. As such, *C. albicans* biofilms are widely considered a major virulence factor and a key determinant to the high mortality rate attributed with candidiasis (Tumbarello et al. 2007; Tumbarello et al. 2012; Rajendran, Sherry, et al. 2016). Infections that arise from mixed-species biofilms are considerably more difficult to treat than their single-species counterparts, requiring complex multi-drug treatment strategies (Costa-Orlandi et al. 2017; Montelongo-Jauregui et al. 2019). Antimicrobials that target a specific group of species often enable other microbes within the biofilm to thrive and continue infection (Pammi et al. 2014; Kean et al. 2017; Allison et al. 2019); for instance within a *S. aureus*-*C. albicans* biofilm, the efficacy of miconazole is reduced, with a much higher concentration of the drug needed to induce an effect against the biofilm (Kean et al. 2017). This increases the challenge of finding effective treatments to tackle *C. albicans* multispecies biofilms and may exacerbate issues relating to drug resistance.

While the treatment of single species *C. albicans* biofilms has been studied *in vivo*, *in vitro* and in clinical settings (Schinabeck et al. 2004; Shuford et al. 2006; Tsui et al. 2016), considerably less is known about targeted therapeutics to mixed-species biofilms. Understanding these polymicrobial interactions in mixed-species biofilms is necessary in order to improve current therapeutic strategies. Overall, we note a lack of anti-biofilm strategies, but an increasing need for efficacious therapies that specifically target *C. albicans* biofilms.

Therapeutic intervention

In recent years, attempts have been made to utilise natural or synthetic compounds with improved antimicrobial activity. The use of quaternary ammonium amphiphiles may be a useful application in preventing and treating *C. albicans* biofilms. Amphiphile screening has revealed putative antimycotic agents that target *C. albicans* membranes in biofilms (Gupta et al. 2020), and which prevent biofilm formation on acrylics and promote degradation. Natural compounds have served as a source of novel alternatives to *C. albicans* biofilm treatment (Manoharan et al. 2017). Shikonin, from the roots of the *Lithospermum erythrorhizon* plant, prevents biofilm formation and maintenance (Yan et al. 2019), while *Thymus vulgaris*, and its active compound thymol, has demonstrated efficacy against *C. albicans* biofilms *in vitro* when used alone or in combination with fluconazole, revealing disaggregation and deformity in *C. albicans* biofilms (Jafri and Ahmad 2020). Furthermore, thymol acts synergistically with standard antifungals (Sharifzadeh et al. 2018) and could be utilised in combination therapy. Recent studies have shown that micazazole combined with a chitosan-coated iron oxide nanoparticle could be used in anti-biofilm therapy against *C. albicans* (Arias, Pessan, et al. 2020), and that this was also efficacious within a range of different oral interkingdom biofilms (Arias, Brown, et al. 2020). These alternative methods of treatment may present a strategy to overcome tolerance in the treatment of *C. albicans* biofilms, but also in more complex interkingdom communities. The potential benefits of combination therapy include a broader spectrum of action, greater potency than agents used in monotherapy, reduction in the number of resistant organisms and lower tolerance to antifungal agents (Lewis and Kontoyiannis 2001; Mukherjee et al. 2005; De Cremer et al. 2015). More recently, drug combination therapy has been reported as an alternative to conventional treatment. The co-delivery of C12AHL (a quorum-sensing molecule) with

fluconazole in a liposomal drug carrier has shown promise as a strategy to eliminate *C. albicans* biofilms (Bandara et al. 2020). In recent work, key components of the host innate immune response and fungal cell wall components have been a focus for the development of new antifungals. The antimicrobial peptides ToAP2 and NDBP-5.7, increased yeast cell permeability and generated morphological alterations in *C. albicans* cells in both the early and later stages of biofilm formation (do Nascimento Dias et al. 2020). Furthermore, the combination therapy utilised within this study increased the efficacy of fluconazole and amphotericin B, decreasing their active concentrations and potential toxicity (do Nascimento Dias et al. 2020). Finally, vaccination of mice with the N-terminus of recombinant Als3p (designated NDV-3A) markedly reduced the ability of *C. albicans* to colonise a medically implanted jugular vein catheter (Alqarihi et al. 2019), suggesting that targeting key adhesins may have a positive outcome in the clinical setting.

Conclusion

The ability of *C. albicans* to form biofilms is a complex process and complicates treatment of *C. albicans* biofilm-related infections. Biofilm development, antifungal resistance and polymicrobial interactions have been a continual focus of *C. albicans* biofilm research and while recent advances have begun to highlight the complex mechanisms that underpin the formation of *C. albicans* single and mixed-species biofilms, there is still much to be discovered. The prevention of biofilm formation and the eradication of mature biofilms in the clinical setting present formidable challenges that must be overcome if the mortality associated with biofilm infection is to be addressed. The question remains as to how we can utilise this knowledge to develop more efficacious strategies to treat *C. albicans* biofilms and biofilm-related infections.

Disclosure statement

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