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Christine Imbert *Editor*

Fungal Biofilms and Related Infections

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Aspergillus Biofilms in Human Disease

Craig Williams, Ranjith Rajendran, and Gordon Ramage

Abstract

The biofilm phenotype of *Aspergillus* species is an important and accepted clinical entity. While industrially these biofilms have been used extensively in important biofermentations, their role in clinical infection is less well defined. A recent flurry of activity has demonstrated that these interesting filamentous moulds have the capacity to form biofilms both *in vitro* and *in vivo*, and through various investigations have shown that these are exquisitely resistant to antifungal therapies through a range of adaptive resistance mechanisms independent of defined genetic changes. This review will explore the clinical importance of these biofilms and provide contemporary information with respect to their clinical management.

Keywords

Aspergillus biofilm • Filamentous moulds • Fungal infections • Aspergillosis • Antifungal drugs

1 Introduction

Fungal biofilms are an important clinical problem (Ramage and Williams. 2013). The widespread use of indwelling medical devices, broad-spectrum antibiotics, and an aging and more immuno-compromised patient population, has created an opportunity for yeasts and moulds to form infections in the form of biofilms. This chapter will discuss the anatomical areas where A*spergillus* biofilms may be important and the evidence that they exist and discuss the treatment of aspergillus infection.

A biofilm is composed of microorganisms attached to surfaces or one another and enclosed within an extrapolymeric matrix. The biofilm mode of growth is the preferred form of growth of microorganisms and accounts for up to 65 % of all clinical infections. This mode of growth

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gives the organism a number of advantages including high level antimicrobial resistance which may cause problems for the clinician attempting to treat such infections (Donlan and Costerton. 2002). Over recent years there has been a growing appreciation that pathogenic fungal species both have the ability to form biofilms and that these biofilms may impact clinical practice (Ramage et al. 2009; Sayed et al. 2012; Fanning and Mitchell. 2012; Williams and Ramage. 2015).

Fungi can be broadly divided into yeasts and moulds. In terms of the number of infections, *C. albicans*, a normal commensal of human mucosal surfaces and opportunistic pathogen in immunocompromised patients, is the most clinically important of fungi species in terms of the production of clinically relevant biofilms. These biofilms have much in common with *Aspergillus* biofilms, which are the subject of this chapter. A candidal biofilm begins with yeast cells attaching to a relevant surface using defined adhesins, followed by the formation of a microcolony with yeast cells undergoing morphological switching to pseudo- and true-hyphae, which results in the rapid formation of a meshwork of hyphae interspersed with budding yeast cells (Ramage et al. 2002). As the biofilm matures it becomes enclosed in a glucan rich polymeric matrix (Nett et al. 2010) which provides protection from host defenses and treatment with antifungal agents. Within the biofilm there are a range of niches and hypoxic areas, which influence filamentation (Bonhomme et al. 2011). Flow of fluids across the surface of the biofilm may then result in the dispersion of daughter cells, which attach to a new substrate and the cycle starts again (Uppuluri et al. 2010). This entire process is controlled by various transcription factors, such as Bcr1p, Ace2p, Efg1p and Zap1p, which are involved in precisely regulated molecular pathways (Finkel and Mitchell 2011; Nobile et al. 2006; Zhao et al. 2006; Fanning et al. 2012). The molecular biology of *Aspergillus* biofilms is less well understood, but it is a rapidly developing area of microbiology (Beauvais et al. 2007; Bom et al. 2015; Gravelat et al. 2008; Gravelat et al. 2010; Mowat et al. 2007; Ramage et al. 2011; Winkelstroter et al. 2015). Figure 1 illustrates the morphological complexity of these filamentous moulds.

2 Where May Aspergillus Biofilms Be Important?

2.1 Upper Airways

Sinusitis (or rhinosinusitis) is defined as an inflammation of the mucous membrane lining the paranasal sinuses. It may be acute or chronic, however, subacute and acute exacerbation of

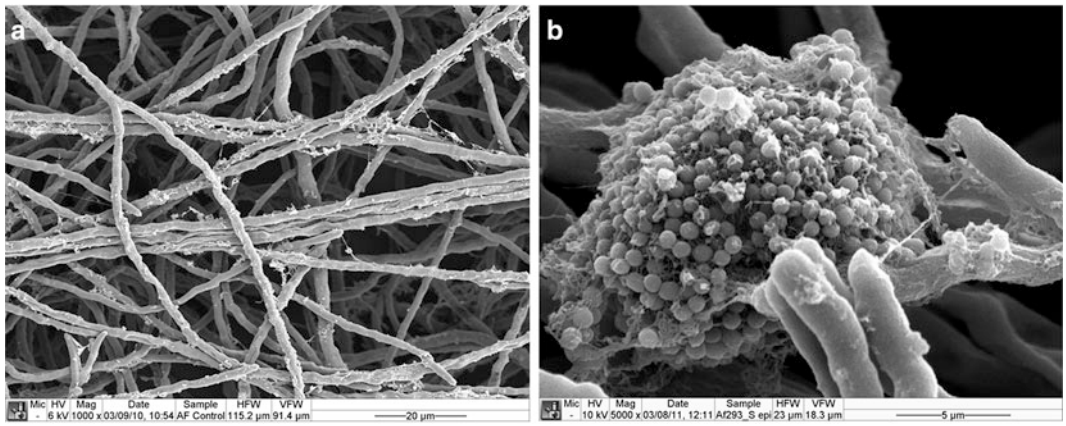

Fig. 1 Scanning electron micrographs of biofilms formed by (**a**) an *Aspergillus fumigatus* and (**b**) an co-culture of *Aspergillus fumigatus* and *Staphylococcus epidermidis*

chronic diseases has also been described and as all types have similar symptoms it is often clinically difficult to distinguish these. Around ninety percent of adults have had some symptoms of sinusitis at some time. There is a growing appreciation that chronic rhinosinusitis is typified by biofilm growth (Foreman et al. 2011; Keir et al. 2011; Ebbens et al. 2009a). While there is increasing evidence for the role of bacterial biofilms in this infection, there role of fungi remains controversial. (Ebbens et al. 2009b). Paranasal sinus fungus balls have been described (Grosjean and Weber. 2007; Karkas et al. 2013), which share some of the features of fungal biofilms (Harding et al. 2009; Mowat et al. 2008a, b). In a recent study of 118 patients with chronic sinusitis, nasal discharge, headache and visual disturbance, over a 14 year period 23.7 % had a sphenoidal fungus ball in which Aspergillus *fumigatus* and *Aspergillus nidulans* hyphae were observed microscopically (Karkas et al. 2013). In terms of infections associated with foreign bodies *A. fumigatus* infection within the maxillary sinus associated with a zygomatic implant has been reported (Sato et al. 2010). Experimental studies have shown that *A. fumigatus* biofilms form in a primary human sinonasal epithelial model (Singhal et al. 2011) and in a sheep model of induced sinus biofilms *A. fumigatus* readily forms biofilms often associated with *Staphylococcus aureus* (Boase et al. 2011). These data suggest that fungal biofilms, alone or more likely in mixed species biofilms with other organisms, may play a role in sinus infection however there is little evidence to support the role of fungi in other upper airway biofilm infections such as otitis media (Bakaletz. 2007; Martin et al. 2005; Yao and Messner 2001).

2.2 Lower Airways

Lower respiratory tract infection may be due to biofilm infection, the archetype of which is *Pseudomonas aeruginosa* in cystic fibrosis patients (Singh et al. 2000). It is also now recognised however that fungal biofilms present in the lung may also contribute to infection.

Filamentous fungi, mainly *A. fumigatus,* may cause a spectrum of respiratory disease including a discrete lesion in a pre-existing cavity, aspergilloma, wheezing mediated by an immune response, allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis (IA) (Denning. 1998). A bronchopulmonary lavage (BAL) of these individuals often reveals the presence of numerous intertwined hyphae in the form of a complex multicellular structure when examined histologically (Jayshree et al. 2006), this is indicative of a biofilm phenotype (Harding et al. 2009; Mowat et al. 2008a, b). The recently described *Aspergillus* bronchitis may also be biofilm associated and is characterized by bronchial casts containing mycelia forming compact masses (Young et al. 1970). It is clear that *Aspergillus* species form medically important biofilms (Ramage et al. 2011; Gutierrez-Correa et al. 2012) and understanding their clinical role in is crucial, as with all biofilms, these structures are highly resistant to antifungal therapy (Mowat et al. 2008a, b; Seidler et al. 2008).

Infection in CF patients is also commonly associated with *S. aureus and Haemophilus influenzae*, and recent advances in culture-independent, next generation sequencing technologies, have revealed that the microbiome of the CF lung is much richer than previously appreciated comprising of a diverse range of bacterial and fungal pathogens, of which *A. fumigatus* is the most prevalent filamentous fungi (Ramage et al. 2011). *A. fumigatus* has a prevalence rate of between 10 and 57 % (Pihet et al. 2009; Bauernfeind et al. 1987), though other fungi have been isolated from the lungs including, *Scedosporium* species, *Aspergillus niger, Aspergillus flavus, Aspergillus nidulans, Aspergillus terreus* (Sudfeld et al. 2010; Cimon et al. 2003). Lungs of CF sufferers are lined with a thick viscous mucus layer susceptible to polymicrobial infections, leading to recurrent infections and continuous inflammation (Rowe et al. 2005). The interplay between the pathogens residing in the lung may be responsible for the

acute exacerbations associated with CF, where the balance is tipped towards an environment with excess inflammatory, oxidative and proteolytic activity. Several studies have identified an association between *A. fumigatus* and *P. aeruginosa,* whereby co-infection saw decreased pulmonary function in comparison to those with a mono-infection (Amin et al. 2010), a phenomenon also reported with *Candida* species and *P. aeruginosa* (Chotirmall et al. 2010). Evidence is therefore increasing for the improved clinical management of these patients (Delhaes et al. 2012). Indeed, interkingdom interactions of the CF lung, and elsewhere, may lead to adverse clinical outcomes (Leclair and Hogan 2010). The ability of these microbes to form strong mixed species biofilms likely contributes towards their persistence, making it extremely difficult to eradicate the infection (Seidler et al. 2008; Lutz et al. 2012). *P. aeruginosa* has also been shown to inhibit *A. fumigatus* filamentation via the release of molecules involved in intra-cellular communication (Mowat et al. 2010). Investigations into the interactions between these two are limited, however the release of small molecules designed to inhibit fungal growth appear to be the primary form of interaction. One particular group of metabolites known as phenazines, have been reported to inhibit *A. fumigatus* biofilm formation, however it was also found that *A. fumigatus* was able to convert these metabolites released by *P. aeruginosa* to produce fungal siderophores, which may in turn influence CF progression (Moree et al. 2012). Furthermore, *P. aeruginosa* releases the metalloprotease elastase, which has been shown to be toxic to host cells (Smith et al. 2015). It was found that elastase production was constitutive, but became significantly increased in the presence of *A. fumigatus* during biofilm co-culture. Furthermore, elastase was cytotoxic to human lung adenocarcinoma cells, and therefore the presence of both of these pathogens could contribute towards enhanced pathogenicity (Smith et al. 2015). Thus, in general, evidence suggests that the co-isolation of both of these organisms indicates a poorer prognosis; however the relationship between the two remains poorly understood and requires further investigation into their polymicrobial interactions. Indeed, these studies highlight potential battles going on within a polymicrobial environment such as the CF lung, which plays a crucial role in the overall pathogenesis of disease (Peters et al. 2012), exemplified by studies in a Drosophila model of polymicrobial infection in which microorganisms from CF showed a different outcome depending on the presence or absence of *P. aeruginosa* (Sibley et al. 2008a, b). Collectively, these provide evidence for the need to consider earlier use of antifungals to improve clinical management of these patients (Delhaes et al. 2012).

3 Wounds

Non-healing wounds, such as diabetic foot ulcers (Seth et al. 2012) represent a significant clinical burden to patients, and are associated with the presence of microbial biofilms. *S. aureus* and *P. aeruginosa* are often isolated together in these patients and have been shown to have a non-random association within the wound site (Fazli et al. 2009). Evidence is emerging that pathogenic fungal species may play a role in these infections (Branski et al. 2009).

Wounds acquired in combat situations especially with persistent evidence of wound necrosis often contain fungi with mould isolates found in 83 % of cases (*Mucorales*, n = 16; *Aspergillus* spp, n = 16; *Fusarium* spp, n = 9), commonly with multiple mould species among infected wounds (28 %). Clinical outcomes included 3 related deaths (8.1 %), frequent debridements and amputation revisions (58 %) (Warkentien et al. 2012).

A next generation sequencing approach to venous leg ulcers reveals that *C. albicans, C. glabrata* and *Aspergillus* species are present, but intriguingly the authors report that individuals seem to have unique microbial profiles, (Wolcott et al. 2009). A further retrospective molecular analysis of 915 chronic wound infections, pressure ulcers, diabetic foot ulcers, non-healing surgical wounds and venous

leg ulcers, showed that 208 (23 %) of these contained pathogenic fungi (Dowd et al. 2011). Yeasts were the most abundant fungi (*Candida* spp.), but *Aureobasidium, Cladosporium, Curvularia*, *Engodontium, Malessezia*, *Trichtophyton,* and *Ulocladium* were also present. Overall, fungal species represented over 50 % of the microbial burden in the majority of specimens examined but direct evidence that the fungi were present as biofilms is lacking.

4 Medical Devices

Broad-spectrum antibiotics, parenteral nutrition, immuno-suppression due to chemotherapy and radiotherapy, and disruption of mucosal barriers due to surgery, are among the most important predisposing factors for invasive fungal infection (Odds 1988). *Candida* species predominate and are the fourth most common cause of bloodstream infection in patients requiring intensive care and the most common etiologic agent of fungal related biofilm infection. However, other filamentous fungi biofilm related infections have also been increasingly described, including *Aspergillus* (Escande et al. 2011). *Aspergillus* species have been reported to cause serious biomaterial related biofilm infections, involving catheters, joint replacements, cardiac pace makers, heart valves, and breast augmentation implants (Escande et al. 2011; Langer et al. 2003; Rosenblatt and Pollock 1997; Jeloka et al. 2011; Golmia et al. 2011). Fungal biofilms are also associated with building fabrics and hospital infrastructure (Short et al. 2011; Siqueira et al. 2011; Richardson 2009; Anaissie et al. 2002).

5 Antifungal Treatments

Guidelines exist for a number of Aspergillus infections. The guidance for Chronic Pulmonary aspergillosis (Denning et al. 2016), which does not mention biofilms, recommends, a minimum of 4–6 months oral triazole therapy initially and patients who deteriorate in this period should be deemed failures and an alternative regimen used. Micafungin, Caspofungin Liposomal Amphotericin and Amphotericin B (AmB) deoxycholate are also recommended.

For Invasive aspergillus three major sets of guidelines are available, ECIL (Maertens et al. 2011), ESCMID (Ullmann et al. 2012), and IDSA (Walsh et al. 2008). Again, none specifically reference the presence of biofilm and they all recommend either azoles, AmB or echinocandins with different grades of evidence. A useful review by Leroux and Ullmann (2013) highlight the methodological differences between these studies and tabulates the recommendations (Leroux and Ullmann 2013). So when considering aspergillus infection it is clear that AmB, including a variety of lipid formulations and members of the azole class, which includes fluconazole, itraconazole, voriconazole (VRZ) and posaconazole, are effective in the treatment of invasive aspergillosis (IA) and are the mainstay treatment for the disease (Denning et al. 1989; Oren et al. 2006; Raad et al. 2008; Sambatakou et al. 2006).

One consideration in the choice of treatment is the presence of triazole resistance. High rates of triazole resistance in *A. fumigatus* were first reported in the Netherlands and in the UK. Subsequently the rate of triazole resistance in *A. fumigatus* has been reported in Europe at rates from 1.7 to 29.6 %. More worrying is a reported increase in the yearly rate of resistance by 6 % per annum in patients without prior exposure to antifungal therapy (Goncalves et al. 2016).

Although the triazoles have proven efficacy with good safety profiles, they have been shown to be associated with resistance through their continuous use (Howard et al. 2009; Meneau et al. 2005; Mosquera and Denning 2002). Azoles actively target the 14-α-demethylase enzyme, blocking ergosterol biosynthesis and destabilizing the cell membranes of actively growing cells. Mutations within the ergosterol biosynthesis pathway have been reported to cause azole cross-resistance through mutations within the *cyp51A* gene (Howard et al. 2009; Mellado et al. 2007; Snelders et al. 2008;

Snelders et al. 2010). However, a recent study reported that 43 % of azole-resistant isolates did not carry the *cyp51A* mutation, indicating that other mechanisms of resistance were responsible (Bueid et al. 2010). In addition to this mechanism of resistance the presence of biofilm may require consideration of other mechanisms of resistance (Ramage et al. 2011; Rajendran et al. 2013; Ramage et al. 2012; Robbins et al. 2011).

6 Efflux Pumps

Azole resistance may be mediated by multidrug resistance (MDR) pumps, which are involved in the active extrusion of antimicrobial molecules, including azole (Rajendran 2011). MDR efflux transporter genes of the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) classes have been shown to be clinically important in different pathogenic fungi (Cannon et al. 2009; Morschhauser 2010). Sequence analysis suggests that *A. fumigatus* has 278 different MFS and 49 ABC transporters (Nierman et al. 2005). *A. fumigatus MDR* (*AfuMDR*) pumps have been described in several studies and have been shown to be associated with increased resistance to itraconazole (da Silva Ferreira et al. 2004; Nascimento et al. 2003). Recently, it has been shown that non-cyp51a mediated itraconazole resistance may be associated with efflux pump activity through cdr1B (Fraczek et al. 2013).

In an *A fumigatus* biofilm model azole resistance was shown to increase 16–128-fold in the 12 h phase and >512-fold at the 24 h phase compared to 8-h germlings (Rajendran et al. 2013). An Ala-Nap uptake assay demonstrated a significant increase in efflux pump activity in the 12-h and 24-h phases ($P < 0.0001$). In addition efflux pump activity of the 8-h germling cells was significantly induced by VRZ. Inhibition of efflux pump activity with the competitive substrate MC-207,110 reduced the VRZ MIC values for the *A. fumigatus* germling cells by 2–8-fold . Quantitative expression analysis of AfuMDR4 mRNA transcripts also showed a phase-dependent increase as the mycelial complexity increased, which was coincidental with a strain-dependent increase in azole resistance. This demonstrates that efflux pumps are expressed in complex *A. fumigatus* biofilm populations and that they contribute to azole resistance. Moreover, VRZ treatment induces efflux pump expression (Rajendran et al. 2013).

7 Extracellular Matrix

Biofilms are encased in an extracellular matrix (ECM). In *A fumigatus* the ECM is composed mainly of polysaccharides, hydrophobin, and melanin (Beauvais et al. 2014). This matrix is considered to be an important virulence factor and is also related to their high resistance to antifungal agents. Previous studies with cultures of *A. fumigatus* maintained under static aerial conditions have demonstrated the presence of an ECM on the colony surface of colonial mycelia that colonies encased with ECM are extremely hydrophobic and display more resistance to antifungal polyenes AmB and nystatin (Beauvais et al. 2007). It is thought that ECM may act as a physical barrier that decreases the access of antifungals to cells embedded in the biofilm community. The penetration of the drugs is a function of the amount and nature of ECM, as well as the physicochemical properties of the antifungal agents. Thus anything that disrupts the biofilm may increase the sensitivity to antifungal agents. cspA encodes a repeat-rich glycophosphatidylinositol-anchored cell wall protein in *A. fumigatus*. A deletion of cspA resulted in a rougher conidial surface, reduced biofilm formation and decreased resistance to antifungal agents (Fan et al. 2015). Another example is the oligosaccharide OligoG which is an alginate derived from seaweed, SEM and AFM both showed that OligoG (≥2 %) markedly disrupted fungal biofilm formation, both alone, and in combination with fluconazole. Calculation of Fractional Inhibitory Concentration Index showed that for *A. fumigatus* at the higher concentrations of OligoG used synergy occurred between the compound and AmB and VRZ (Tondervik et al. 2014).

Aspergillus ECM was initially thought to be composed of galactomannan, alpha-1,3 glucans, melanin and other proteins including hydrophobins (Beauvais et al. 2007). However our work has demonstrated the presence of extracellular DNA (eDNA) in *A. fumigatus* biofilm ECM, which is released upon fungal autolysis (Rajendran et al. 2013). We suggested that the role of eDNA is maintenance and stability of biofilms and biofilm resistance to antifungal drugs (Krappmann and Ramage 2013). When *A. fumigatus* biofilms are treated with DNAse there is improved antifungal susceptibility to AmB or caspofungin. These findings together demonstrated the important role of eDNA in *Aspergillus* ECM biofilm. The role of eDNA in biofilm formation and stability has been confirmed by another group who added exogenous eDNA in an *in vitro* biofilm model (Shopova et al. 2013). They also showed that eDNA improved surface adhesion of fungal spores and also co-localised with ECM biofilm polysaccharides, becoming part of the ECM surrounding the biofilm cells.

Other components of the ECM may also be targeted to improve therapeutic response to antifungals. When biofilms are treated with alginate lyase (AlgL) both fractional inhibitory concentration index values and time kill analyses show synergy between AlgL and amphotericin. In addition a combination of AlgL and amphotericin showed a reduction in hyphal thicknesses (Bugli et al. 2013).

8 Previous Therapy

As described earlier triazoles are the mainstay of treatment for aspergillosis. Failure to respond clinically or refractory infections may necessitate a switch to other antifungal agents, including AmB. One study explored the possibility that in *A. fumigatus* biofilms sequential antifungal therapy may impact adaptive resistance mechanisms (Rajendran et al. 2015). *A. fumigatus* sensitivity to AmB was decreased when it was tested in combination with VRZ. The mechanism of this increase may be twofold. Depletion of eDNA by DNase treatment enhanced AmB activity against VRZ-exposed cells by eight-fold, which visually could be explained by destabilisation of the biofilm when examined microscopically. Pharmacological inhibition of Hsp90 by GDA also significantly improved biofilm susceptibility to AmB by 4–8-fold. Suggesting that *A. fumigatus* pre-exposure to VRZ concomitantly induces eDNA release and activates the stress response, which collectively confers AmB resistance in vitro (Rajendran et al. 2013).

9 Other Approaches

Undoubtedly the most effective and logical way of dealing with clinically important fungal biofilms is to either inhibit their development, use mechanical force to disrupt them or simply remove and replace an implicated medical device. Wound fungal biofilms are managed with surgical debridement (Warkentien et al. 2012). In severe wounds, such as those occurring from combat trauma, liposomal AmB, VRZ and posaconazole have been used, often as combinational therapy, although the clinical outcomes were variable. Nevertheless, it has been reported that in the management of a case of fungal osteomyelitis combined use of VRZ and terbinafine along with surgical debridement was able to successfully control a *Scedosporium inflatum* infection and salvage the limb (Cetrulo et al. 2012).

These studies suggest that wound fungal biofilms may have a different structural composition, as they respond to azoles more effectively than other fungal biofilms. Many of these infections are polymicrobial, and undergo repeated debridement with topical antiseptics. Moreover, wound dressings containing antimicrobial molecules are used, so it is not surprising that fungal wound biofilms respond to azole therapy in this context.

10 Concluding Remarks

From review of the available literature it is evident that *Aspergillus* biofilms may play a significant role in clinical medicine. These fungi have

been shown to form biofilms in both hard and soft tissue, and upon implanted medical devices. Diagnosing the presence of a fungal biofilm is difficult, however recently published guidelines hope to improve this situation (Hoiby et al. 2015). In general clinical awareness of the possibility of the presence of biofilm is important in making the diagnosis, which could be supported by diagnostic testing, but at present there is no definitive test for an *Aspergillus* biofilm. Promising avenues of research include transcriptional and metabolomics, but this is someway off finding its way to the clinical laboratories.

Removal and replacement of medical devices, or surgical debridement of soft tissue, where appropriate, represents the first line in clinical management, followed by antifungal management. Treatment is with conventional antifungal agents the choice of which is dictated on by the site of infection.

Liposomal formulations of AmB and azole agents show the greatest efficacy against aspergillius biofilms. Methods to augment antifungal activity, such as matrix degrading molecules, natural products and microbially derived molecules have been demonstrate experimentally, however, their use in clinical situations is anecdotal. Nonetheless, our knowledge of the adaptive resistance within the biofilm has revealed potential therapeutic targets. Clearly more work needs to be done in order to provide a guarantee of successful management of *Aspergillus* biofilms. In addition, further consideration needs to be given to how interactions between prokaryote and eukaryote in polymicrobial biofilm infections impact clinical management (O'Donnell et al. 2015).

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Candida albicans in Multispecies Oral Communities; A Keystone Commensal?

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Abstract

The complexity of the oral cavity, in which many hundreds of microbial species interact represents a challenge for modern microbiologists. What are all these species doing there? And why do we accept so many opportunistic pathogens to be part of our health (commensal) microflora? While the role of bacteria are often being studied, the role of fungi in the interactions within the oral cavity are understudied. This is partly because fungi in the oral cavity are generally considered as pathogens and related to diseases. In this chapter we will explore mechanisms of interaction between bacteria and fungi in the oral cavity that are involved in maintenance of oral health. We will argue that fungi in general and *C. albicans* specifically, should be regarded a keystone commensal in the oral cavity.

1 Introduction

1.1 The Oral Cavity

The oral cavity is the beginning of the gastrointestinal tract and digestion of our food starts here. Like other parts of this digestive system, the oral cavity is colonized by billions of microorganisms ranging from viruses, bacteria to eukaryotes such as fungi and protozoa (Filoche et al. 2010). These microorganisms mostly reside in 3-dimensional communities termed biofilms with large species diversity. The oral cavity is a very diverse environment with many distinct ecological niches (Simon-Soro et al. 2013) that probably

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differentiate based on the nature of the surface (e.g. hard tooth surfaces or softer mucosal surfaces), but also on varying nutrient availability and dynamic occurrence of oxygen limitation.

1.2 Oral Ecology

Upon birth humans are colonized by bacteria, starting in the oral cavity. While passing through the birth canal the infant gets inoculated with resident bacteria. However, recently bacterial presence was shown in the placenta and it is possible that the fetus is trained to recognize commensal flora during its time in the womb (Zaura et al. 2015). Surprisingly, these placental bacteria were closely related to oral bacteria. After initial colonization of the oral cavity, the downstream parts of the gastrointestinal tracts are slowly colonized, initially with these oral bacteria. After some time the gut ecology differentiates into a specific composition and remains relatively stable over time. The oral ecosystem however is relatively dynamic; it changes over time with its ever-changing host. Hour-to-hour variation, monthly cycles as well as lifetime changes such as tooth eruption and tooth loss change the playing field for adhesion. Also hormonal changes during puberty and pregnancy are reflected in the composition of the saliva and strongly influences oral composition (Kumar 2013). In general it is believed that a healthy oral ecology is highly diverse in species composition. This diversity is lost when pathology arises and/or *vice versa*, pathology arises when this diversity is lost. The role of oral bacteria in oral pathologies is well studied. This is not surprising as they are the cause of the most common infectious diseases of today: caries and gingivitis. In contrast, the role of bacteria in maintaining health has only recently attracted attention.

1.3 Oral Fungi Focussed on Disease

In contrast to the role of bacteria in oral diseases, the role of fungi has received considerably less attention (Krom et al. 2014). *Candida albicans* is probably the most commonly encounter oral fungus. It is linked to common diseases such as oral thrush in infants and HIV-positive patients. In addition, it is commonly found associated with biomaterials such as dentures where it causes denture stomatitis (O'Donnell et al. 2015). More recently it has been suggested that *C. albicans* is related to development of tooth caries (Metwalli et al. 2013) and might be linked to endodontitis (Waltimo et al. 2003). In all cases *C. albicans* is rarely found alone; there is strong evidence for complicated physical interactions with a range of oral bacteria. For instance, *S. mutans* and *C. albicans* interact physically and are found in high numbers in caries, especially in early childhood caries (Metwalli et al. 2013).

1.4 Oral Fungi in Health

Compared to the attention given to the role of bacteria in the healthy oral cavity and compared to the involvement of fungi in pathology, there is little to no information on the role of fungi in healthy oral ecologies. Ghannoum and coworkers for the first time described the mycobiome (the total of all fungi) of healthy volunteers (Ghannoum et al. 2010). Subsequently several other studies have shown that health oral ecologies contain many fungal species (Dupuy et al. 2014; Monteiro-da-Silva et al. 2013; Monteiro-da-Silva et al. 2014). It is becoming clear that over 100 fungal species can be found as part of the oral flora. Due to the highly mobile nature of fungal spores some of these species might be environmental contaminants and be transiently present, but many others are likely to be part of the resident oral flora. It should be taken into account that while fungal counts might remain extremely low compared to bacterial counts (less than 0.1 %), their size compensates for this. Lets considered bacteria to be spheres with a radius of 0.5 μm (very realistic) and fungi to have a radius of 2.5 μm (realistic for yeasts, but certainly not for filamentous forms). The calculated volumes according to the formula

$V = 4/3*\pi*r^3$ are 0.5 μm^3 and 65.4 μm^3, respectively. This means that if the total number of fungi is only 0.1 % of the total microbial load, they represent at least 10 % of the biovolume (*ergo* biomass). **Therefor they are to be considered a significant part of the healthy oral ecology and should not be disregarded!**

In this chapter we will explore mechanisms of interaction between bacteria and fungi in the oral cavity that are involved in maintenance of oral health. We will argue that fungi in general and *C. albicans* specifically, should be regarded a keystone commensal in the oral cavity.

2 Type of Bacterium-Fungus Interactions Related to Health

It is generally accepted that a healthy ecosystem is a balanced ecosystem. Such balance is maintained by the interaction of forces within the ecosystem. These forces are derived from interactions between individuals within the ecosystem and balance is the overall result of all of these interactions (Krom and Oskam 2014). The interactions can occur on different levels; mainly physical, metabolic and chemical interactions (Fig. 2.1).

2.1 Physical Interactions

Within the biofilm microbes are by definition closely packed. However, on a single cell level, there are positive and negative interactions resulting in attraction and repulsion of individual cells. As a consequence of non-specific (mostly hydrophobic and electrostatic) and specific (protein-protein) interactions certain species are commonly found together. Both mechanisms play a role in the adhesion of bacteria to fungi, as has been illustrated recently for the interactions between the Gram-negative bacterium *Pseudomonas aeruginosa* and *C. albicans*. *P. aeruginosa* strongly adheres to and kills hyphae of *C. albicans*, but not yeasts cells. This difference in adhesion to and subsequent killing of the hyhal and yeast morphology is utilized by *C. albicans* as a defense mechanism (for more details (Jarosz et al. 2011)). Using physic-

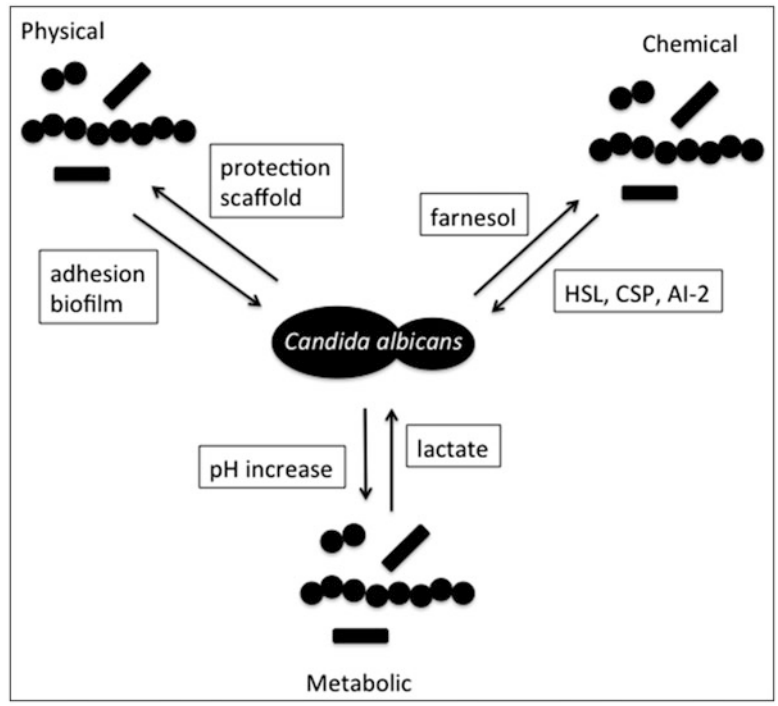

Fig. 2.1 Schematic overview of the three types of interactions between *C. albicans* and bacteria described in this chapter. This overview is far from complete. See text for more details

chemical and force microscopic analyses, it was shown that the strong hydrophobic nature of the hyphal cell wall attracts cells of *P. aeruginosa* (Ovchinnikova et al. 2012a, b). By virtue of their hydrophobic nature, surface proteins of the hyphal cell wall were shown to be mostly responsible for the non-specific interactions mediating this adhesion. When the bacterium approaches the fungus close enough, a specific chitin-binding protein interacts with chitin in the fungal cell wall (Ovchinnikova et al. 2012a, b). The role of non-specific interactions and a specific bacterial binding protein, but absence of a specific fungal binding protein might illustrate the antagonistic nature of this interactions; beneficial for *P. aeruginosa*, but disadvantageous for *C. albicans* (Hogan and Kolter 2002; Hogan et al. 2004). In contrast, the interaction between *S. aureus* and *C. albicans* is probably a benign, if not mutually advantageous interaction for both species. Using a similar approach as described for *P. aeruginosa*, interactions between *S. aureus* and *C. albicans* were investigated. It was shown that a specific hyphal wall protein (Als3p) was involved in strong interaction forces (Peters et al. 2012). A *S. aureus* counterpart has not yet been identified, but considering the extend of the interaction forces determined using force microscopy, this will only be a matter of time. Similar interactions have been observed between *Streptococcus mutans* and *C. albicans* as well as for *C. albicans* and a range of other oral bacteria (Nobbs et al. 2009; Silverman et al. 2010; Wright et al. 2013; Bachtiar et al. 2014). The potential relevance of such interactions in the oral cavity follows from two independent observations. Firstly, upon adhesion to the hyphae of *C. albicans*, *S. aureus* become more tolerant towards antibiotics (Harriott and Noverr 2010). Secondly, using a murine gut-model, it was shown that when *C. albicans* was present, bacterial recolonization following antibiotic treatment was enhanced (Mason et al. 2012b; Mason et al. 2012a; Erb Downward et al. 2013). When these two observations are combined, it is very well possible that adhesion to and biofilm formation on *C. albicans* protects many bacteria against antibiotic challenges and such surviving biofilms are the source of recolonization. In the oral cavity, environmental challenges are constantly present; at least twice a day high levels of antimicrobial compounds are washed through the oral cavity. The mixed-species *C. albicans* – bacteria biofilms could therefor be instrumental to healthy recolonization after routine oral hygiene. Healthy recolonization is of great importance to maintenance of overall health. There is a growing body of evidence suggesting that the oral ecology is involved in regulation of important physiological functions, such as blood pressure (see Box 1). Consequently, this scaffold function of *C. albicans* in the oral cavity could assist in stability of the oral ecology and thus in maintenance of overall health.

Box 1: How oral bacteria impact healthy physiology

Our blood pressure is regulated by a small diffusible molecule: NO (nitric oxide). Dietary components with high nitrate content, such as spinach and other green vegetables, are able to lower the blood pressure (Larsen et al. 2006). Nitrate (NO_3) is converted to nitrite (NO_2) and subsequently to nitric oxide (NO). We humans have enzymes capable of converting NO_2 to NO, but we lack enzymes that convert NO_3 to NO_2. Upon digestion of dietary NO_3, our body feeds back the NO_3 to the oral cavity through the salivary glands. In the oral cavity bacteria convert the NO_3 to NO_2 that is taken up by the host. Oral disinfection using chlorhexidine prior to the consumption of dietary NO_3 prevented the conversion to NO_2 and subsequent lowering of the blood pressure (Govoni et al. 2008). Thus, oral bacteria are truly important for a healthy physiology!

2.2 Chemical Interactions

Chemical interaction within a species is commonly referred to as intraspecies signaling or quorum sensing. A small diffusible molecule is synthesized and accumulates in the external

medium. Upon reaching a threshold concentration, gene expression is induced and a concerted response of the population is given. Due to the external nature of the quorum sensing molecules they are commonly detected by other species than the ones producing them (Jarosz et al. 2011). Several bacterial QS molecules have been shown to affect *C. albicans* behavior (Fig. 2.1), amongst others Homoserine lactone (HSL) produced by *P. aeruginosa* (Hogan et al. 2004), Competence Stimulating Peptide (CSP) produced by *S. mutans* (Jarosz et al. 2009) and autoinducer-2 (AI-2) of *Aggregatibacter actinomycetemcomitans* (Bachtiar et al. 2014). This interaction is bi-directional as *C. albicans* secretes the QS molecule farnesol (Hornby et al. 2001) which, inhibits *Pseudomonas* quinolone signal (PQS) production (Cugini et al. 2007; Cugini et al. 2010). PQS is needed for the expression of several virulence factors (Bjarnsholt et al. 2010) and *C. albicans* thus decreases the virulence of *P. aeruginosa* using chemical interactions.

2.3 Metabolic Interactions

In addition to physical and chemical interactions, metabolic interactions probably play a role in the oral cavity. For example, recently it was shown that *C. albicans* allows growth and biofilm formation of anaerobic bacteria under aerobic conditions (Fox et al. 2014). In the oral cavity the presence of anaerobic bacteria is well-established, also in health. For example, the strict anaerobic *Veillonella spp.* are related to decreased caries risk because they consume lactate (the main acid involved in caries development). How such strict anaerobes can survive in an aerobic niche as the oral cavity is still not entirely known. Recently it was shown that *C. albicans* allows growth and biofilm formation of several strict anaerobic (mainly gut) bacteria under aerobic condition (Fox et al. 2014). We have shown *in vitro* that in the presence of *C. albicans* many strict anaerobic oral bacteria can also grow under aerobic culture conditions (Janus et al., submitted for publication). The mechanism by which *C. albicans* creates an anaerobic micro-niche is unknown, but could be related to high level of O_2 consumption typical for yeasts (Wesolowski et al. 2008). It is known that there is enormous niche-diversity in the oral cavity (Jenkinson 2011) and conceptually the observation that *C. albicans* creates an anaerobic micro-niche explains the niche-diversity that is so typical for the oral cavity; aerobic and anaerobic niches exist side by side, depending on the microbial species composition.

A metabolic interaction at the substrate level has been shown for the interaction between *S. mutans* and *C. albicans*. Caries is a long-term process in which lactic acid, produced by bacteria, result in demineralization of tooth surfaces. Under healthy conditions, lactic acid is removed by saliva flow and microbial metabolism. Health-related bacteria such as *Veillonella spp.* grow on the lactic acid produced mainly by *S. mutans*. Several studies have described *C. albicans* as an organism capable of synergizing the onset and progression of caries induced by *S. mutans* (Metwalli et al. 2013; Falsetta et al. 2014; Jarosz et al. 2009). We have recently shown that the presence of *C. albicans* in a dual-species biofilm with *S. mutans* does not synergize the cariogenic capacity in terms of acidity and calcium release, the two main surrogates for caries (Willems et al., Pathogens and Disease, accepted for publication). In contrast, the presence of *C. albicans* appears to decrease the cariogenic potential of the biofilm. Mixed biofilms become less acidic over time, and after 72 h of biofilm growth the bulk pH remains above the critical pH of 5.5 (Matsui and Cvitkovitch 2010). Since lactic acid production potential was increased in our mixed biofilms, it is possible that *C. albicans* influences the pH in an independent fashion of *S. mutans*. There are several possible explanations for this observation. A study by Ene et al. (2012) showed that *C. albicans* is able to grow using lactic acid as a carbon source (Ene et al. 2012). Furthermore, lactic acid is the most preferred source of carbon in hypoxic conditions for the fungus and hypoxic conditions are being created by *C. albicans* (Fox et al. 2014). Logically, consumption of lactic

acid by *C. albicans* would cause the environment to become less acidic. This effect is enlarged as *S. mutans* increases in numbers, since sucrose is then consumed even faster. At an early time point when sucrose is not limited, *C. albicans* favors the consumption of sucrose over lactic acid, producing ethanol. When sucrose becomes limited, *C. albicans* is obligated to consume the lactic acid. Alternatively, *C. albicans* can regulate its external pH in response to low pH (Vylkova et al. 2011). This has mainly been studied in relation to phagocytosis (Vylkova and Lorenz 2014). Production of NH_4^+ from amino acids result in increase of the bulk pH. This phenomenon would resemble the therapeutic effect described for arginine-containing toothpastes and arginine has been shown to increases *C. albicans* presence in *in vitro* oral biofilms (Koopman et al. 2015).

3 Keystone Organisms' Theory

Originating from architecture where the keystone represents the top stone of an arch, the keystone paradigm has made its entry into ecology near the end of the last century. When a certain species has disproportionately large effects on their communities, given their abundance, it is thought to form the 'keystone' of the community's structure (Hajishengallis et al. 2012). Traditional infections are based on accumulation of a specific pathogen to levels unacceptable to the host (dominant pathogen). Dysbiosis with the host is a result of this accumulation in microbial load. Keystone pathogens modulate the host and/or the ecology in such a way that is unacceptable to the host, without increasing its own presence significantly.

3.1 Keystone Pathogens in Oral Diseases

In oral ecology, the keystone hypothesis is largely used to describe the role of keystone pathogens, most notably *Porphyromonas gingivalis* (reviewed in (Hajishengallis et al. 2012)). Briefly, periodontitis is a very common oral disease related to chronic inflammation of the tooth-supporting tissues. While the microbial biofilms is certainly involved in the onset of the disease, it is the inflammatory response by the host that causes irreversible damage to the tooth-supporting tissues. One of the bacteria involved in periodontitis is *P. gingivalis*. Important virulence factors of *P. gingivalis* are secreted proteases, the gingipains. These proteases activate the complement factor C5, generating C5a that binds to the C5a-receptor leading to activation of inflammation and impairs leukocyte killing. Together this allows other bacteria to thrive. So by the mere production of a protease a low-level presence of *P. gingivalis* can orchestrate inflammation.

4 Conclusion

The role of fungi in the complex oral ecosystem that is dominated by 400+ bacterial species has remained enigmatic. Recent studies on the role of *C. albicans* in the healthy oral cavity has started to shed a light on several key functions of fungi in maintaining a healthy balance. However, unmistakably, *C. albicans* are a minority when cell numbers are considered. The overview provided above however indicates that even when present in low numbers a considerable effect on the total ecology is exerted. For instance by rapid consumption of molecular oxygen, rapid increase of local pH and by providing a physical scaffold for oral bacteria to adhere to. We therefor present the hypothesis that ***C. albicans* functions as a keystone commensal in the healthy oral cavity**.

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The Extracellular Matrix of Fungal Biofilms

Kaitlin F. Mitchell, Robert Zarnowski, and David R. Andes

Abstract

A key feature of biofilms is their production of an extracellular matrix. This material covers the biofilm cells, providing a protective barrier to the surrounding environment. During an infection setting, this can include such offenses as host cells and products of the immune system as well as drugs used for treatment. Studies over the past two decades have revealed the matrix from different biofilm species to be as diverse as the microbes themselves. This chapter will review the composition and roles of matrix from fungal biofilms, with primary focus on *Candida* species, *Saccharomyces cerevisiae, Aspergillus fumigatus*, and *Cryptococcus neoformans*. Additional coverage will be provided on the antifungal resistance proffered by the *Candida albicans* matrix, which has been studied in the most depth. A brief section on the matrix produced by bacterial biofilms will be provided for comparison. Current tools for studying the matrix will also be discussed, as well as suggestions for areas of future study in this field.

Keywords

Extracellular matrix • Biofilm • Antifungal resistance • *Candida albicans* • Device-associated infections

1 Introduction

A defining feature of the biofilm lifestyle is production of an extracellular matrix. The matrix surrounds the cells within the biofilm, providing a structural scaffold for both adhesion to surfaces and cohesion between cells (Flemming and Wingender 2010; O'Toole 2003). Additionally, the matrix of certain organisms can contribute to the retention of water and nutrients, and in some cases these nutrients are thought to derive from matrix materials hydrolyzed by microbial-produced enzymes (Flemming and Wingender 2010). Perhaps the most medically-relevant function of the extracellular matrix is its ability to provide a physical barrier between biofilm cells

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and the surrounding environment. In a human host, this includes cells and products of the immune system and often drugs used for treatment (Costerton et al. 1999; Donlan 2001).

The composition of the matrix has been comprehensively studied in only a handful of biofilm species. Most of these studies have occurred in bacteria, though the fungi *Saccharomyces cerevisiae, Aspergillus fumigatus*, *Cryptococcus neoformans* and *Candida albicans* serve as exceptions. The diversity of biochemical entities discovered, and the observation that matrix components can be similar to components of the fungal cell wall, suggests that the composition of biofilm matrices can be as diverse as the microbes that produce them (Branda et al. 2005; Zarnowski et al. 2014). This chapter will review the composition and roles of matrix from fungal biofilms, with a brief coverage on bacterial matrices for comparison. Current tools for studying the matrix will also be discussed, as well as suggestions for areas of future study in this field.

2 Matrix of Bacterial Biofilms

Most studies of biofilm extracellular matrix components to date have utilized bacterial species. One well-characterized model system involves the gram-positive, spore-forming bacterium *Bacillus subtilis*, which can form pellicles or submerged biofilms depending on the strain and environmental conditions (Vlamakis et al. 2013). Several polymeric components of the *B. subtilis* matrix have been described, such as poly-DL-glutamic acid (PGA) and the proteins TapA, TasA, and BslA. This matrix also contains exopolysaccharide (EPS), which is produced by the *eps* operon (Marvasi et al. 2010). Notably, the EPS component was recently discovered to be at least partially composed of poly-N-acetyl glucosamine (PNAG), a widely conserved bacterial polysaccharide (Roux et al. 2015), and had previously been shown to act as a positive regulator of its own synthesis (Elsholz et al. 2014).

A second model organism for the study of biofilm formation is *Pseudomonas aeruginosa*, which produces an extracellular matrix containing polysaccharides, extracellular DNA (eDNA), lipids (namely rhamnolipids), and proteins (Mann and Wozniak 2012). Polysaccharides include Psl, Pel, levan, and alginate, whose quantity depends on the strain in question, the stage of biofilm development, and the site of biofilm formation (Ma et al. 2009). For example, strains designated mucoid or non-mucoid differ in their overexpression of alginate, which occurs during infections of the lungs of cystic fibrosis patients (Franklin et al. 2011; Mann and Wozniak 2012). The other polysaccharides are typically produced by environmental strains or those isolated from other types of infections. Pel has been extensively studied, and found to lend a protective role against aminoglycoside antibiotics (Colvin et al. 2011). DNA in the *Pseudomonas* matrix also contributes to structural stability, and has been found to induce antibiotic resistance (Yang et al. 2011; Mulcahy et al. 2008).

Other molecules have been identified in the matrices of multiple bacterial biofilms. Namely, cellulose is a critical component for *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter* spp., *Citrobacter* spp., and *Salmonella typhimurium* (Hobley et al. 2015; Branda et al. 2005; Hung et al. 2013). PNAG, identified in *B. subtilis*, is also found in *Staphylococcus aureus* and *Staphylococcus epidermidis*, as well as the related molecule polysaccharide intercellular adhesin (PIA) (Branda et al. 2005).

In addition to the composition and production of individual matrix components, assembly of the entire matrix over the course of bacterial biofilm development has been explored in two species. The first of these is *Vibrio cholerae*, which produces a matrix of *Vibrio* polysaccharide (VPS) and the proteins RbmA, RbmC, and Bap1 (Teschler et al. 2015; Fong et al. 2010; Reichhardt et al. 2015b). A 2012 study by Berk et al. revealed distinct stages of cellular clustering during biofilm formation, with RbmA providing initial adhesion between cells and Bap1 aiding in surface adhesion. Cell clusters were surrounded by mixtures of VPS, RbmC, and Bap1 in the later stages of biofilm development (Berk et al. 2012). Similarly, “spatially

segregated" subpopulations of cells surrounded by extracellular matrix were observed within *E. coli* pellicles in a 2013 publication by Hung et al. In addition, this study confirmed that amyloid fibers formed by curli as a major matrix constituent, with cellulose, flagella, and type 1 pili also involved (Hung et al. 2013). Together, these recent findings support the notion that biofilm matrix is not simply a disorganized conglomerate, but rather specific microenvironments of components that emerge in time and space.

3 Fungal Matrix

Multiple types of fungi form biofilms, found both in the environment and in infection settings. The first investigations of the extracellular matrix of fungal biofilm species were performed by the Douglas group, with *Candida albicans*, nearly two decades ago (Hawser and Douglas 1994; Hawser et al. 1998). Since that point, several groups have investigated basic aspects of matrix production and its individual components. However, not until the past few years has a larger picture of the matrix emerged, using multiple approaches to examine how its various entities collectively function to provide structural integrity and protection to the cells of biofilms.

3.1 Tools for Studying the Matrix

It has been proposed that successful quantification of the entire extracellular matrix is nearly impossible, as matrix isolation methods could potentially disrupt the cell wall (Flemming and Wingender 2010). However, recent protocols have demonstrated no change in the chemical composition of the cell wall following matrix extraction. These methods have used either mild detergents (or none at all), combined with gentle sonication to separate the cells from the matrix material (Zarnowski et al. 2014; Reichhardt et al. 2015a; Lattif et al. 2008).

Biochemical approaches have been used to analyze the different classes of macromolecules found in extracellular matrices. To quantify the gross amount of protein, colorimetric assays such as bicinchoninic acid (BCA), Bradford, Folin-Lowry, Kjeldahl or ultraviolet absorption can be employed. For identification of specific proteins, chromatographic and electrophoretic separation strategies have been extensively used followed by various mass spectrometry (MS) analytical techniques (Zarnowski et al. 2014; Reichhardt et al. 2015a). Recent advances in bottom-up proteomics technologies have been especially important to the study of extracellular matrix, as they allow for in-depth relative and absolute quantification of proteins in intact or minimally-processed biological samples (Zhang et al. 2013). For example, our group recently utilized shotgun proteomics to achieve global protein identification in the *C. albicans* biofilm matrix (Zarnowski et al. 2014).

Overall measurement of monosaccharides can be accomplished using the phenol-sulfuric assay (DuBois et al. 1951). A specific assay for β-1,3 glucan quantification is the limulus assay, which has been used both in vitro and as a diagnostic in clinical settings (Nett et al. 2007b; Nett et al. 2007a). For the most precise quantification of carbohydrates gas chromatography is the gold standard, and is typically coupled with MS analysis for qualitative purposes. For structural analysis, complementary approaches utilizing GC, MS, and multiple nuclear magnetic resonance (NMR) procedures provide the granularity needed for accurate structural assignments. Similar to techniques previously used to analyze the *C. albicans* cell wall, a combination of several solution-state ^{1}H and ^{13}C NMR techniques have been used to examine the matrix (Shibata et al. 2007; Zarnowski et al. 2014). Recently, solid-state ^{13}C, ^{15}N, ^{31}P NMR spectroscopy has also proven a powerful ally in generating more complete descriptions of macromolecular assemblies (Reichhardt et al. 2015a; Cegelski 2015). For more detailed information on the molecular size and shape of individual matrix polysaccharides, small-angle X-ray scattering (SAXS) has also been employed.

Lipids in the extracellular matrix have also been quantified using gas chromatography. For

crude separation of different lipids, TLC can be used, though the most detailed data can be achieved using MS-based shotgun lipidomics. The system recently employed by our group involved liquid chromatography (LC)/mass selective detector (MSD) time of flight (TOF) with electrospray ionization (Zarnowski et al. 2014). This technique has also been employed for analysis of small molecular weight lipophilic molecules, such as ergosterol and other sterols.

The final class of macromolecule, nucleic acids, can be measured in crude matrix material spectrophotometrically or with use of specific dyes (Zarnowski et al. 2014; Martins et al. 2010). In our group's recent study, the potential presence of coding regions was examined by creating a clone micro-library of random regions of matrix DNA, followed by sequence homology analysis to the Candida Genome Database.

For measurement of known matrix entities, monoclonal antibodies have been produced for both imaging purposes and quantification in microtiter based assays such as ELISA (Zarnowski et al. 2014; Martinez and Casadevall 2015). For further characterization of the matrix material, both SEM and TEM have been used, as well as atomic force microscopy (AFM) (Yang et al. 2011; Lal et al. 2010).

3.2 *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* has long been used as a model organism for many basic aspects of eukaryotic biology, and as a means to investigate fungal biology in a non-pathogenic system. In the last fifteen years, it has also been explored as a model for the biofilm lifestyle. Certain strains of *S. cerevisiae* exhibit flocculation, or clumping of cells, and others can form true surface-adherent biofilms (Verstrepen and Klis 2006; Bojsen et al. 2012). Both flocculation and biofilm formation rely on the FLO family of cell surface adhesins, or flocculins, which are related to the ALS family and the Hwp1p adhesins in *Candida albicans* (Nobile et al. 2008; Guo et al. 2000; Reynolds and Fink 2001). Of these, FLO11 is required for *S. cerevisiae* biofilm formation (Reynolds and Fink 2001; Guo et al. 2000; Ishigami et al. 2004).

S. cerevisiae can produce extracellular matrix in both the flocculating and biofilm forms, as well as in structured colonies formed by environmental isolates. This matrix material has been visualized using electron microscopy (Zara et al. 2009; Kuthan et al. 2003; Vachova et al. 2011; Beauvais et al. 2009). The 2009 study by Beauvais et al. examined the composition and role of the matrix in FLO1-expressing cells, which exhibit strong flocculation and higher resistance to stress and drugs (Beauvais et al. 2009). Flo1p aids in flocculation by interacting with sugars on the cell walls of neighboring yeast, as its exposed N-terminus possesses lectin-like properties (de Groot and Klis 2008; Dranginis et al. 2007). Matrix from the 'floc' was extracted using EDTA and shown to be loosely attached to the cell surface. The material contained mainly glucose and mannose, with a negligible amount of protein. GC-MS analysis revealed the mannose portion to be a chain of (1–6) mannan with (1–2) and (1–3) linked branches. Beauvais et al. also showed the matrix from flocculated *S. cerevisiae* was able to exclude high molecular weight molecules such as antibodies or concanavalin A. However, smaller entities, namely amphotericin B and ethanol, were not blocked in this case.

Additional studies have described components of the *S. cerevisiae* matrix produced under different growth conditions. A study with environmental isolates formed 'fluffy' colonies, as opposed to the smooth surface produced by laboratory strains. Matrix from the environmental strains contained an unidentified protein unrelated to flocculins. The matrix material also reacted with concanavalin A, indicating the presence of exposed terminal mannose or glucose residues (Kuthan et al. 2003). More recent investigations involved cells grown in a three-dimensional mat on solid medium, and used multiple analytical techniques to identify protein and carbohydrate matrix components in both *S. cerevisiae* and *C. albicans* (Faria-Oliveira

et al. 2014; Faria-Oliveira et al. 2015). A number of proteins were identified with 2-DE and MALDI-TOF MS, including the dehydrogenase Tdh3p for both species. For *S. cerevisiae*, two different molecular weight polysaccharides were identified, which contained glucose, mannose, and smaller amounts of galactose. The different matrix components identified in each of these studies, especially the relative abundance of proteins between flocculating and mat forms, highlights the impact of growth conditions on the production and content of extracellular matrix. This, in addition to the methods used for analysis, must be carefully considered when comparing information from different investigations.

3.3 *Aspergillus fumigatus*

Aspergillus biofilms can form during chronic lung infections, such as aspergilloma, and more rarely form on medical devices in humans (Ramage et al. 2011; Loussert et al. 2010). Extracellular matrix has been found to surround these three-dimensional hyphal structures during both in vitro and in vivo in studies with *A. fumigatus*, the most common species of *Aspergillus*, with thick material covering the surface of the cells (Loussert et al. 2010; Beauvais et al. 2007; Beauvais et al. 2014). Extracellular matrix levels increase during the maturation of *Aspergillus* biofilms, which form after adhesion of conidia to a substrate (Beauvais et al. 2007; Kaur and Singh 2014).

The initial study of *Aspergillus* biofilm matrix content utilized biofilms grown under aerial-static conditions in glucose yeast extract (GYE) medium (Beauvais et al. 2007). The matrix was found to contain galactomannan, α-1,3 glucan, proteins, polyols, and melanin. This study, using mainly immunoassays, revealed the presence of galactomannan in both the cell wall and matrix, while α-1,3 glucan was primarily located extracellularly near the surface of hyphae. The protein content, which represented 2 % of the total matrix, included three major secreted antigens: dipeptidylpeptidase V (DPPV), catalase B (CatB), and ribotoxin (ASPF1). A group of hydrophobin proteins was also identified, with purported roles in intercellular adhesion during aerial growth. Additionally, eDNA that matches genomic DNA sequence has been identified in the *A. fumigatus* biofilm matrix (Rajendran et al. 2013). Interestingly, host DNA may also play a role in the *Aspergillus* matrix, as addition of exogenous DNA resulted in greater structural integrity and matrix carbohydrate production. This could reflect the conditions of the cystic fibrosis (CF) lung, where concentrations of human DNA are relatively high, and provides an interesting glimpse into the potential role of host factors in the matrix of biofilm infections.

A more recent study grew *A. fumigatus* biofilms in RPMI 1640 medium and utilized solid-state NMR to define matrix composition (Reichhardt et al. 2015a). Multiple approaches were combined to examine facets of the entire matrix: ^{13}C, ^{15}N, and ^{31}P-NMR, electron microscopy, and protein identification using PAGE and mass spectrometry. Overall, this investigation found the matrix to contain roughly 40 % protein, 43 % polysaccharides, up to 14 % lipids, and 3 % aromatic-containing components such as melanin. Combined with existing studies that have identified specific matrix components, work such as this provides powerful quantitative data to frame future investigations of matrix structure and function.

In vivo *Aspergillus* biofilm matrix has also been investigated, using both aspergillomas from human patients and a murine model of invasive pulmonary aspergillosis (Loussert et al. 2010). Similarly to in vitro *A. fumigatus* biofilms, both biofilm models contained galactomannan within the cell wall and matrix. One observed difference between the in vivo models was the lack of matrix α-1,3 glucan in the pulmonary aspergillosis biofilms. However, they both differed from the in vitro model in their relatively higher levels of galactosaminogalactan (GAG), a cell wall exopolysaccharide that has been characterized by the Sheppard group (Lee et al. 2014; Bamford et al. 2015). GAG has been found to mediate adherence, and plays a role in the host immune response partially by masking

β-1,3 glucan and also through inducing interleukin-1 receptor antagonist (IL-1Ra), which blocks proinflammatory IL-1 signaling (Gravelat et al. 2013; Gresnigt et al. 2014).

Like the *C. albicans* matrix, the matrix of *Aspergillus* biofilms is thought to contribute to antifungal resistance, either through slowing drug diffusion or perhaps through a more specific sequestration mechanism (Beauvais et al. 2007; Bugli et al. 2013; Rajendran et al. 2013). One matrix material that has been identified to have a role in *A. fumigatus* biofilm resistance is eDNA (Rajendran et al. 2013; Shopova et al. 2013). An in vitro study by Rajendran et al. showed that the eDNA, which accumulates over biofilm maturation, can be degraded by DNase to result in higher biofilm susceptibility to amphotericin B and caspofungin. A possible mechanism by which eDNA enters the matrix is autolysis, as chitinase activity was found to increase DNA release. An additional study yielded increased *A. fumigatus* biofilm susceptibility to amphotericin B after treatment with alginate lyase, which degrades uronic acid-containing carbohydrates (Bugli et al. 2013). One explanation for this result is that a currently unknown carbohydrate is also contributing to antifungal resistance in the *Aspergillus* matrix.

3.4 *Cryptococcus neoformans*

Cryptococcus neoformans, which can cause meningoencephalitis in humans, has the ability to form biofilms on polystyrene plates and on the surfaces of medical devices such as the shunts used to treat intracranial hypertension (Martinez and Casadevall 2015). During biofilm formation, extracellular matrix increases over time and provides structure and complexity to groups of adherent cells (Martinez et al. 2010; Martinez and Casadevall 2007).

This basidiomycete grows in a yeast form covered by a polysaccharide capsule composed of glucuronoxylomannan (GXM), a structure required for virulence. GXM is shed into the environment, and the capsule structure is required for biofilm formation (Martinez and Casadevall 2005). Initial studies of the *C. neoformans* biofilm matrix sought to identify GXM using monoclonal antibodies. This confirmed the presence of GXM surrounding the cells of the biofilm, and GC/MS analysis detected monosaccharide content consistent with the presence of GXM. However, this study also detected monosaccharides not found in GXM (glucose, ribose, and fucose) suggesting the matrix contains additional types of polysaccharides (Martinez and Casadevall 2007).

The *Cryptococcus* matrix is thought to protect the biofilm cells from surrounding stressors in both an infection setting and in the environment, where the fungus is associated with pigeon excreta (Martinez and Casadevall 2006b, 2007; Alvarez et al. 2008). *C. neoformans* biofilms are more resistant to changes in temperature, pH, and UV light compared to planktonic cells, lending evidence to the idea that biofilm growth in nature provides a protective advantage (Martinez and Casadevall 2007). Similar to biofilms formed by other infectious fungi, those of *C. neoformans* display higher levels of antifungal resistance compared to planktonically-grown cells (Martinez and Casadevall 2006b), and are also more resistant to antimicrobial molecules produced by cells of the innate immune system (Martinez and Casadevall 2006a). Interestingly, a study with both *C. neoformans* and *C. gattii* showed the formation of biofilm-like microcolonies to be associated with successful resistance to phagocytosis, as well as escape from macrophages. These microcolonies were surrounded by a polysaccharide matrix containing antibody, suggesting utilization of antibody-mediated agglutination in favor of the fungus (Alvarez et al. 2008).

3.5 *Candida*

Candida albicans is the most common fungal pathogen, and frequently forms biofilms on implanted medical devices. The first studies describing the *C. albicans* biofilm matrix were conducted by the Douglas group, and demonstrated the importance of environmental

conditions on overall matrix production (Hawser et al. 1998). Conditions of continuous flow, which most closely resemble those of in vivo biofilm growth, resulted in biofilms with the most abundant matrix. In support of this, in vivo biofilms from both the catheters of patients as well as multiple animal models produce visibly ample matrix (Paulitsch et al. 2009; Andes et al. 2004; Nett et al. 2010b; Johnson et al. 2012).

The content of the *C. albicans* matrix initially identifed carbohydrates, hexosamine, phosphorus, protein, uronic acid, and eDNA (Baillie and Douglas 2000; Al-Fattani and Douglas 2006). Subsequent studies identified β-1,3 glucan as one specific carbohydrate component (Nett et al. 2007a; Nett et al. 2007b). Several groups have also investigated variations in matrix content between other *Candida* species, and have noted differences in overall carbohydrate and protein content (Al-Fattani and Douglas 2006; Silva et al. 2009). Notably, the major matrix component identified in *C. tropicalis* was hexosamine, and biofilms of this species were also disrupted by different enzymatic treatments than those of *C. albicans*.

A comprehensive analysis of the *C. albicans* matrix was recently performed by our group (Zarnowski et al. 2014). All four classes of macromolecules were analyzed, and by dry weight was comprised of 55 % protein, 25 % carbohydrate, 15 % lipid, and 5 % nucleic acid. The relative abundance of protein was much greater than previously reported (Al-Fattani and Douglas 2006), and proteomic analysis indicated 458 distinct entities. This revealed many similar protein classes to those previously identified, including those implicated in carbohydrate and amino acid metabolism (Thomas et al. 2006; Faria-Oliveira et al. 2014). Lipids identified in the *C. albicans* matrix included both neutral and polar glycerolipids, and a small proportion of sphingolipids. The nucleic acids identified were mainly non-coding sequences of DNA.

Recent study included further detailed analysis of the matrix polysaccharides. Surprisingly, a previously identified carbohydrate with functional roles in the matrix, β-1,3 glucan, was found in much smaller quantities compared to two other polysaccharides, β-1,6 glucan and α-1,6 mannan with α-1,2 linked branches. While these have some similarity to the polysaccharides found in the *C. albicans* cell wall, several lines of evidence suggest that matrix and cell wall carbohydrates are produced or assembled in a distinct manner (Chaffin 2008). First, the three polysaccharides were found to physically interact in a mannan-glucan complex (MGCx), a structure not described in the cell wall. Second, the cell wall and matrix polysaccharides are found in different proportions to one another and with different linkages. For example, the length of mannan chains in the matrix can reach up to 12,000 mannose residues, whereas those in the cell wall have only been reported up to 200 residues. Finally, the cell wall polysaccharide chitin was not detected in the extracellular matrix, further suggesting that cell wall and matrix are distinct.

Subsequent investigations explored the genetic basis for each of these matrix polysaccharides by screening a library of mutant strains lacking enzymes in each of the carbohydrate production pathways (Mitchell et al. 2015). A subset of mutants was found to have lower levels of total matrix, lower quantities of each polysaccharide, as well as increased susceptibility to antifungal treatment (detailed below). Seven genes were identified that govern levels of matrix mannan (*ALG11, MNN9, VAN1, MNN4-4, PMR1,* and *VRG4*), while two genes govern levels of matrix β-1,6 glucan (*BIG1* and *KRE5*). The previously studied gene encoding the β-1,3 glucan synthase, *FKS1*, was included in order to study this third matrix polysaccharide. Strikingly, when mixed biofilms containing mutants from the different pathways were grown together, matrix of normal structure and function was restored. This suggested that the MGCx components assemble extracellularly, as the mutants lacking one polysaccharide could be complemented by neighboring cells lacking a different polysaccharide. These findings are represented in Fig. 1. Reflecting a community behavior, the assembly of matrix materials by the biofilm constitutes an exciting area for further studies.

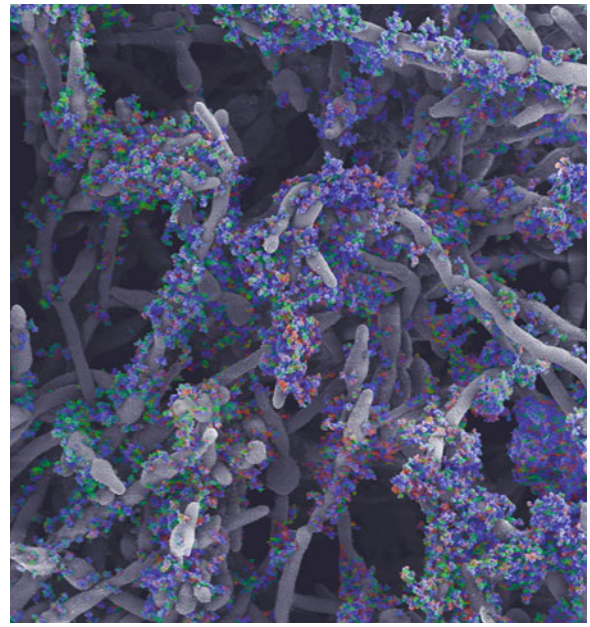

Fig. 1 Pictured is a scanning electron micrograph of a *Candida albicans* biofilm. The extracellular matrix is false colored in blue, red, and green, representing its three major polysaccharides. Work by Mitchell et al. reveals that these components physically interact, and are each required for matrix structure and function (Mitchell et al. 2015)

3.6 Role of *Candida* Matrix in Drug Resistance

The early biofilm studies by the Douglas group began to explore the role of *Candida* biofilm matrix in resistance to antifungal therapies. The growth conditions of continuous flow, associated with increased matrix, provided the highest level of protection from drug treatment (Baillie and Douglas 2000; Al-Fattani and Douglas 2006). It should be noted that antifungal resistance in *C. albicans* is multifactorial, with the matrix emerging as a relevant mechanism during the mature stage of biofilm growth (Taff et al. 2013; Ramage et al. 2012). Namely, efflux pumps reduce intracellular accumulation of triazole antifungals during the early phases of biofilm growth (Mukherjee et al. 2003; Ramage et al. 2002). Additional mechanisms thought to impact biofilm drug resistance include the presence of drug-tolerant persister cells and changes in the sterol content of the cell membrane (Mukherjee et al. 2003; Nett et al. 2009; LaFleur et al. 2006).

Matrix contribution to biofilm resistance was tested even more directly by Nett et al. (Nett et al. 2007b). Extracellular matrix was isolated from biofilms and added to planktonic cells prior to antifungal susceptibility testing. The planktonic cells with matrix added were found to gain levels of resistance similar to mature biofilms. Further, an additional experiment tracked the penetration of radiolabeled fluconazole within biofilms, and found the majority to be retained within the matrix. This work also first implicated the matrix carbohydrate β-1,3 glucan in the resistance phenotype: biofilms treated with β-1,3 glucanase greatly increased susceptibility to fluconazole treatment both in vitro and in vivo. A 2010 study by the d'Enfert group corroborated the importance of matrix for antifungal protection from the polyene antifungal, amphotericin B (Vediyappan et al. 2010). Further studies have demonstrated a role for β-1,3 glucan in the resistance against additional classes of antifungals, and in the species *C. glabrata, C. parapsilosis,* and *C. tropicalis* (Nett et al. 2010a; Mitchell et al. 2013; Yi et al. 2011; Fernandes et al. 2015).

Several studies have investigated the production and regulation of β-1,3 glucan in the matrix. The β-1,3 glucan synthase Fks1p is clearly involved directly in production and control of the levels of this matrix material, and the glucan modifier proteins Bgl2p, Phr1p, and Xog1p were

found to impact delivery of β-1,3 glucan from the cell surface to the extracellular space (Nett et al. 2010a, c; Taff et al. 2012). A separate regulatory mechanism for production β-1,3 glucan involves the transcription factor Zap1p, which negatively impacts the production of this material (Nobile et al. 2009). Nobile et al. found relevant targets of Zap1p to be two glucoamylases, *GCA*1 and *GCA*2, which may contribute to the hydrolysis of matrix carbohydrates. Other Zap1p targets include alcohol dehydrogenases ADH5, CSH1, and LFD6, which could perhaps impact the biofilm and matrix through quorum sensing pathways. Recent work found that β-1,3 glucan, while present in relatively low quantities, works in conjunction with the other matrix polysaccharides to provide the biofilm resistance phenotype (Mitchell et al. 2015).

Several other pathways have been implicated in the resistance of *C. albicans* biofilms. The transcription factor Bcr1p, a regulator of overall biofilm formation, has been found to influence permeability to dyes and neutrophils, as well as sensitivity to fluconazole. This was suggested by the finding of different expression levels of Bcr1p in mating competent and mating incompetent biofilms, as well as the finding that Bcr1p was required for abundant matrix levels in a vaginal colonization model (Srikantha et al. 2013; Harriott et al. 2010). The heat shock protein Hsp90p has also been found to be a regulator of matrix production, and therefore drug resistance, as disrupting this pathway results in azole susceptibility and also lower levels of β-1,3 glucan in the matrix (Robbins et al. 2011). Similar phenotypes were yielded by disrupting either *SMI*1 or *RLM*1, members of the protein kinase C (PKC) pathway (Nett et al. 2011). These defects were restored by overexpression of *FKS*1 in the mutants, though no effect was seen when upstream components of the PKC pathway were disrupted. Thus, the pathway members that impact drug resistance and the integrity of the cell wall are thought to overlap in a distinct manner from the whole PKC pathway.

An additional matrix component found to impact antifungal resistance is extracellular DNA (eDNA). Addition of DNase to biofilms increases the efficacy of some but not all antifungal drug classes (Martins et al. 2010; Martins et al. 2012). As described above, eDNA is an important structural component of other biofilm species, and in bacterial species is related to the exchange of genetic material (Rajendran et al. 2013; Mulcahy et al. 2008). However, in *Candida* biofilms the exact mechanism of how eDNA contributes to drug resistance remains unclear, especially in light of these sequences being largely non-coding (Zarnowski et al. 2014).

4 Conclusions

The studies outlined here have provided insight into key components of the fungal biofilm extracellular matrix, as summarized in Table 1. However, continued elucidation of matrix composition, production, and function is critical for several reasons. Besides providing fundamental information on the biofilm lifestyle, characterization of new matrix materials may identify novel targets for antifungal therapies. Based on this information, if methods of matrix inhibition are developed, they might prove most useful in combination with existing antifungals that, alone, are ineffective against biofilms. As proof of principle, adding α-mannosidase or β-1,6 glucanase to biofilms in combination with fluconazole results in a greater reduction of biofilm growth in comparison to either treatment alone (Mitchell et al. 2015). Additionally, specific matrix components are upregulated during biofilm growth in contrast to planktonically grown cells, with some present only during the biofilm lifestyle. These could serve as diagnostic markers for biofilm infections of medical devices, which are often difficult to differentiate from other types of fungal infection.

To best advance our existing knowledge of the extracellular matrix, future studies should focus on several topics. First, the roles of specific matrix components should continue to be investigated as the components themselves are identified. While our image of the matrix is becoming that of a complex structure, with

Table 1 Extracellular matrix components of fungal biofilms

Biofilm species	Carbohydrate	Protein	Lipid	Nucleic acid	Other
Saccharomyces cerevisiae	Glucose, mannose in α-1,6 chains with α-1,2 and α-1,3 branches (Beauvais et al. 2009); two different molecular weight polysaccharides containing glucose, mannose, and galactose (Faria-Oliveira et al. 2015)	Relatively low protein levels (Beauvais et al. 2009); multiple proteins identified including Tdh3, Hsp26, and Sod2 (Faria-Oliveira et al. 2014)	–	–	–
Aspergillus fumigatus	Galactomannan, α-1,3 glucan, monosaccharides (Beauvais et al. 2007); cell wall exopolysaccharide galactosaminogalactan (Gravelat et al. 2013); 43 % of total matrix (Reichhardt et al. 2015a)	Proteins, including hydrophobins (Beauvais et al. 2007); 40 % of total matrix (Reichhardt et al. 2015a)	Comprises up to 14 % of total matrix (Reichhardt et al. 2015a)	eDNA (Rajendran et al. 2013)	Melanin, polyols (Beauvais et al. 2007)
Cryptococcus neoformans	Glucurunoxylomannan, xylose, mannose, glucose, galactoxylomannan (Martinez and Casadevall 2007)	–	–	–	–
Candida albicans	25 % of total matrix, includes α-1,6 mannan with α-1,2 branches, β-1,6 glucan, and β-1,3 glucan (Zarnowski et al. 2014)	55 % of total matrix, with 458 distinct entities (Zarnowski et al. 2014)	15 % of total matrix, includes neutral and polar glycerolipids, sphingolipids (Zarnowski et al. 2014)	5 % of total matrix, primarily non-coding sequences (Zarnowski et al. 2014); eDNA (Al-Fattani and Douglas 2006)	Phosphorus, uronic acid, hexosamine (Al-Fattani and Douglas 2006)
Candida glabrata	Higher concentration of carbohydrate and protein than *C. parapsilosis* matrix (Silva et al. 2009)		–	–	–
Candida parapsilosis	Higher levels of carbohydrate than protein (Silva et al. 2009)		–	–	–
Candida tropicalis	Higher levels of carbohydrate than protein (Fernandes et al. 2015); lower levels of protein than *C. albicans* matrix (Al-Fattani and Douglas 2006)		–	–	Phosphorus, uronic acid, hexosamine (Al-Fattani and Douglas 2006)

individual components acting in concert, some of these components may be more critical than others in conferring certain physical properties. These queries will expand as studies involving mixed species biofilms become more prevalent: do biofilms with multiple microbes contain interacting matrix components? Do these inhibit or promote the overall integrity of that combined matrix, or perhaps lead to the alteration of cellular processes? Further, what is the role of host components that interact with the fungal matrix during biofilm infections?

The specific environmental triggers that either promote or inhibit matrix production should also be further characterized. The earliest studies of *Candida* biofilm matrix showed that continuous flow, the condition most similar to those encountered by an in vivo biofilm, were most conducive to matrix accumulation (Baillie and Douglas 2000). Variation in growth conditions can also drastically change the proportions of individual matrix materials, as with the case of eDNA levels from biofilms grown in different types of medium (Martins et al. 2010). Ultimately, those conditions which best mimic the relevant infection niche should be used when possible, at least as validation for other studies. This will best inform the development of future therapeutic approaches, and will prove critical as the use of implanted medical devices and the associated biofilm infections continues to rise.

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Fungal Biofilms: Update on Resistance

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Abstract

Over the past decade, the emergence of biofilm-related invasive fungal diseases has been the subject of numerous studies focused on antifungal resistance and its impact on antifungal therapy in severely ill patients. The majority of the studies investigated the molecular mechanisms involved in antifungal resistance and pathogenicity of biofilm production by *Candida albicans* and *Aspergillus fumigatus*, the most common etiologic agents of yeast and mold invasive infections. The main mechanism characterizing biofilm-related antifungal resistance is the production of extracellular matrix, a physical barrier preventing the drugs from entering and expressing their activity. However, over-expression of efflux pumps, genetic changes of drug targets, persister cells, biofilm-host immune system interaction, proteins leading to filamentation, all together contribute to the onset of biofilm antifungal resistance. Some of these mechanisms are shared with planktonic cells and are often related to developmental phases of biofilm formation. All physical and genetic factors leading to biofilm-related antifungal resistance have been briefly discussed.

1 Introduction

Fungal biofilms are complex communities of cells and hyphae attached to a substrate and protected by an extracellular polymeric matrix. Biofilm formation is involved in the pathogenic process of invasive fungal infections and therefore it is considered an important virulence factor. However, the most intriguing feature of fungal biofilms is their marked resistance to both host immune cells and antifungal drugs commonly used to treat invasive fungal diseases. Fungi are able to form biofilms on both abiotic surfaces and animal tissues, having broad implications for healthcare. The resistance displayed by biofilms to antifungals represents a considerable therapeutic problem as it may

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involve all the main classes of antifungal agents currently available.

Over the past decade, the emergence of biofilm-related invasive fungal diseases has been the subject of numerous studies with emphasis on antifungal resistance and its impact on the treatment of severely ill patients (Pierce et al. 2013, 2015; Cowen et al. 2014; Nett 2014; Mathé and Van Dijck 2013; Mitchell et al. 2013; Rajendran et al. 2013; Srikantha et al. 2013; Taff et al. 2013; Ramage et al. 2012; Shapiro et al. 2012; Nett et al. 2007, 2010a, b, 2011; Watamoto et al. 2009; Bizerra et al. 2008; Al-Fattani and Douglas 2006).

Candida albicans and *Aspergillus fumigatus*, the major etiologic agents of invasive fungal diseases among yeasts and molds respectively, have been the fungal species more extensively studied for investigating the mechanisms involved in pathogenicity and biofilm-related antifungal resistance, two aspects closely correlated (Fan et al. 2015; Nobile et al. 2014; Sherry et al. 2014; Mathé and Van Dijck 2013; Rajendran et al. 2011, 2013; Srikantha et al. 2012, 2013; Taff et al. 2013; Bink et al. 2012; Shapiro et al. 2012; Lattif et al. 2011; Robbins et al. 2011; Ferreira et al. 2010; Nett et al. 2010a, 2010b; Vediyappan et al. 2010).

As Ramage et al. (2012) pointed out, fungal biofilm resistance involves both physical barriers and regulatory processes, some of the latter already shown for planktonic cells. Thus, antifungal resistance of biofilms is multifactorial and heterogeneous, often related to developmental phases of biofilm formation (Taff et al. 2013; Lattif et al. 2011) (Fig. 1).

In the following sections the different physical and genetic factors leading to antifungal resistance of biofilms will be briefly discussed.

2 The Extracellular Matrix

Microbial biofilms are characterized by the presence of an extracellular matrix (ECM) that embeds the cells contributing to the complex tridimensional architecture of the community. ECM presence is considered a defining feature of biofilms and its composition varies among different species of microorganisms (Flemming and Wingender 2010; Al-Fattani and Douglas 2006; Reichhardt et al. 2015). Despite several differences, carbohydrates, proteins and nucleic acids are the main constituents in both bacterial and fungal biofilms (Van Acker et al. 2014).

The ECM is alternatively named extracellular polymeric substance (EPS) and confers to sessile

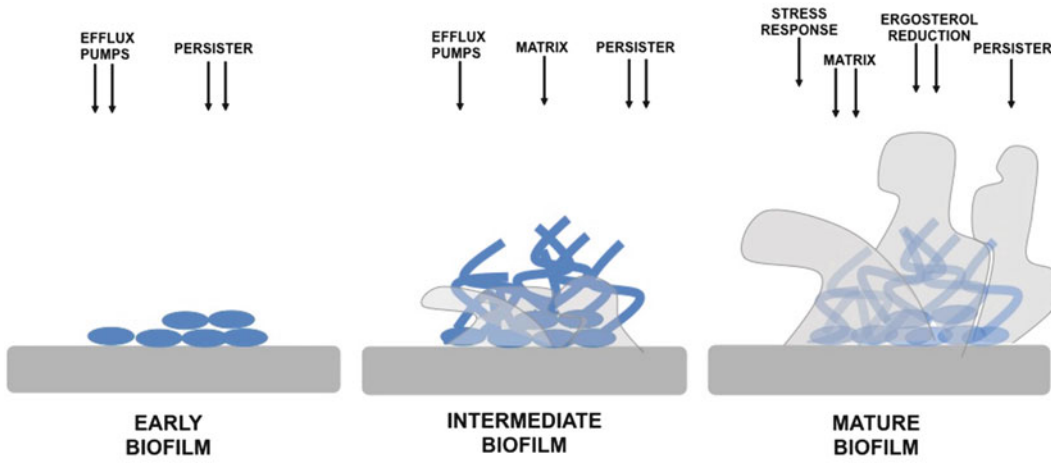

Fig. 1 Schematic overview of fungal resistance mechanisms involved in various biofilm phases. The arrows represent the different factors that contribute to intrinsic resistance of fungal biofilms. The number of arrows for each factor indicates the greater (*two arrows*) or less impact (*one arrow*) in each developmental phase

cells a broad range of advantages such as adhesion and cohesion properties, mechanical properties, nutritional sources, enzymatic activities, and protection (Flemming and Wingender 2010).

Biofilm forming-ability and subsequent matrix production has been demonstrated for almost all pathogenic fungi (Ramage et al. 2012). However, extensive studies have been made mostly for *Candida albicans*, by Andes group (Zarnowski et al. 2014; Nett and Andes 2006; Mitchell et al 2015), and to a lesser extent for other *Candida* spp. (Fernandes et al. 2015; Silva et al. 2009) and *Aspergillus fumigatus* (Wuren et al. 2014; Loussert et al. 2010; Reichhardt et al. 2015).

The chemical composition of ECM produced has been recently reported for some fungal species. Four macromolecular classes were found to be the major constituents of *C. albicans* EPS: proteins and glycoproteins (55 % [wt/wt]), carbohydrates (~25 % [wt/wt]), lipids (~15 % [wt/wt]), and nucleic acids (~5 % [wt/wt]) (Zarnowski et al. 2014). Concerning the polysaccharides, the main cell wall constituents, β-1,3-glucans, were found to be poorly represented in the matrix, whereas the most abundant were α-1,2-brached, and α-1,6-mannans.

C. tropicalis matrix contains mainly hexosamine (27.4 %), with smaller amounts of carbohydrates (3.3 %, including 0.5 % glucose), and proteins (3.3 %) (Al-Fattani and Douglas 2006).

Less is known about matrix composition in other *Candida* species, but according to Silva et al. (2012) *C. parapsilosis* biofilms consist of high amounts of carbohydrates and small amounts of proteins, whereas *C. glabrata* display high amounts of both proteins and carbohydrates.

In *Cryptococcus neoformans*, the capsular component glucurunoxylomannan is the predominant component, together with xylose, mannose, and glucose (Martinez and Casadevall 2007).

The hydrophobic matrix of *A. fumigatus* is composed of galactomannan, galactosaminogalactan, α-1,3 glucans, monosaccharides, polyols, melanin, proteins, and DNA (Beauvais et al 2014; Loussert et al. 2010).

2.1 Matrix-Related Antifugal Resistance

Despite differences in the composition, ECM actively participates in the drug resistance phenotype of biofilm-embedded cells. The confirmation of its role has been clearly demonstrated by Nett et al. that added biofilm matrix to planktonic cells obtaining a drug-resistant phenotype (Nett et al. 2007).

The most intuitive mechanism, although not the most relevant, is the capability of reducing penetrance of drugs throughout biofilm layers. Despite biofilm spatial heterogeneity and the presence of water channels that facilitate nutrient influx and waste efflux (Chandra et al. 2001; Ramage et al. 2005), the penetration of antimicrobial drugs could be not sufficient to achieve an appropriate distribution in all the biofilm areas (Van Acker et al. 2014). Although some authors (Al-Fattani and Douglas 2004) observed, by using a filter disk assay, a good biofilm penetration by azoles, amphotericin B and flucytosine, the used technique as well as the lower presence of matrix in the *in vitro* growth of *Candida* biofilms could have overestimated the distribution into small niches present into the biofilm (Van Acker et al. 2014). Many studies demonstrated that static growth of biofilms resulted in a lower matrix production in comparison to flow-through or *in vivo* models (Baillie and Douglas 2000; Nett and Andes 2006; Uppuluri et al. 2009).

Poor or delayed drug diffusion could in turn promote a secondary mechanism of resistance. Cells exposed to low and inadequate concentrations of drugs, in fact, could develop or put in place defensive strategies to survive the action of the compound.

However, recent studies suggested that specific constituents of ECM play a crucial role in drug resistance.

2.1.1 *Candida albicans* and other *Candida* spp.

C. albicans biofilm resistance is multifactorial and different mechanisms participate in a stage-dependent manner (Ramage et al. 2012). However, a matrix-dependent mechanism has been fully elucidated by Andes group (Nett et al. 2007 and 2010b; Taff et al. 2012). β-1,3-glucans have been shown to be able to sequester azoles, echinocandins and polyenes, resulting in a multidrug resistant mechanism (Nett et al. 2010a). It relies on the expression of *FKS1*, encoding a β-1,3 glucan synthase, and has been demonstrated to be biofilm-specific, as overexpression or heterozygous disruption of *FKS1* does not alter susceptibility of planktonic cells (Nett et al. 2010b). Moreover, the crucial role of β-1,3 glucans has been corroborated by studies from mutants of three genes controlling β-1,3 glucans delivery into the matrix, two glucan transferases and an exo-glucanase (*BGL2* and *PHR1*, and *XOG1*, respectively). These mutants, with reduced glucans content, displayed enhanced susceptibility to the above-mentioned antifungals (Taff et al. 2012).

Recently, another *C. albicans* matrix component, the extracellular DNA (eDNA), has been observed to mediate, at least partially and with unknown mechanism, the resistance to echinocandins and polyenes, but not to azoles (Martins et al. 2012). Accordingly, DNAse treatment was shown to enhance drug susceptibility.

Extracellular DNA (eDNA) and its role in antimicrobial resistance have been first identified in bacterial biofilms (Whitchurch et al. 2002; Tetz et al. 2009). Bacterial eDNA exerts other roles, such as nutrient source, genetic information exchange, and biofilm increased stability.

C. albicans eDNA has been shown improve the stability of mature biofilms, without affecting it establishment (Martins et al. 2010).

eDNA has been demonstrated also in other *Candida* species (Al-Fattani and Douglas 2006; Sapaar et al. 2014).

Furthermore, a recent study on *C. tropicalis* biofilm demonstrated a matrix involvement in the amphotericin B resistance (Fernandes et al. 2015). The authors observed that amphotericin B administration to preformed biofilm dramatically increased the carbohydrate and protein contents of ECM, resulting in a thicker and drug-proof biofilm.

2.1.2 *Aspergillus fumigatus*

In vivo, in both aspergilloma and invasive aspergillosis, *A. fumigatus* has been observed to produce ECM (Loussert et al. 2010; Seidler et al. 2008). The *in vivo* ECM composition has been demonstrated to be highly similar to those obtained *in vitro* (Loussert et al. 2010), and its production is stimulated by the presence of serum or its proteins (Shopova et al. 2013; Wuren et al. 2014).

Galactomannan and galactosaminogalactan (GAG) are the major components of ECM and constitute its scaffold; α-1,3 glucan, found in lower proportion, mediate hyphae cohesion (Loussert et al. 2010).

Rajendran et al. recently demonstrated that eDNA plays a crucial role in *A. fumigatus* biofilm production (2013). eDNA matrix content increases with the maturation of the biofilm, released by autolytic processes catalyzed by chitinases. This increase goes in parallel with the increase in antifungal resistance. Moreover, the authors, by adding exogenous DNA to early-phase biofilms, observed an increase of biofilm biomass elucidating a role for eDNA in promoting and maintaining biofilm architectural integrity. Similarly, DNase I treatment drastically altered the structural integrity of mature biofilms. The eDNA role in antifungal resistance was demonstrated by the same group by treating preformed *A. fumigatus* biofilms with amphotericin B, caspofungin and azoles in combination with DNase. An increase in susceptibility to echinocandins and polyenes was observed. This behavior was not shown analyzing azoles (Rajendran et al. 2013), probably due to the lack of active cell proliferation, and thus of ergosterol biosynthesis, in mature biofilm.

3 Efflux-Pumps Activity in Fungal Biofilm and Expression of Drugs Targets

Genes encoding for efflux pumps have been identified and extensively studied in *C. albicans*, and several authors reported an increasing expression during sessile growth (Sanglard and Odds 2002; Ramage et al. 2012).

In *C. albicans* planktonic cells, resistant phenotypes are due to altered expression of three genes: *CDR1* and *CDR2*, encoding for ATP-binding cassette (ABC) transporters, and *MDR1*, encoding for major facilitator superfamily (MFS) transporters (Sanglard and Odds 2002).

By using single, double and triple mutants of *C. albicans* for these genes, Mukherjee et al. (2003) demonstrated that efflux pumps-mediated fluconazole resistance is dependent on the phase of biofilm formation, being evident in early phases of the process but not in mature biofilms. Accordingly, Ramage group showed that 24 h- and 48 h-old *Candida* biofilms do not display altered efflux pumps activity (Ramage et al. 2002).

CDR1/2 and MDR1 homologs have been identified also in other pathogenic *Candida* species and in *C. neoformans* (Morschhäuser 2010). *C. tropicalis* CtMDR overexpression (Bizerra et al. 2008) has been shown in 24 h grown biofilms compared with their planktonic counterparts. Similar results were obtained in *C. glabrata* for CgCDR1 and CgCDR2, with a maximum of overexpression in early or intermediate phases of biofilm formation (Song et al. 2009).

Genomic sequence analysis showed in *A. fumigatus* the presence of putative 278 different MFS and 49 ABC transporters (Nierman et al. 2005). Rajendran et al. (2011) demonstrated that *A. fumigatus* voriconazole resistance dramatically increases as germlings proliferate to mycelia. Moreover, by assessing mRNA expression of efflux pump AfMDR4, the authors showed a phase-dependent increase, higher at 24 h, and strain-dependent increase, further enhanced by voriconazole administration. *AfMDR4* overexpression was also confirmed in *A. fumigatus in vivo* biofilms. Due to the high number of efflux pumps in *A. fumigatus*, most of them yet to be characterized, it is possible that other genes could compensate the lack of *AfMDR4* overexpression resulting in an equal resistant phenotype. Indeed, da Silva Ferreira and colleagues (2006) by performing a global transcriptomic analysis reported *mdr1* gene to be overexpressed in mycelial growth during voriconazole exposition.

Thus, efflux pumps-mediated resistance is relevant in the first phases of fungal biofilm whereas in mature biofilms other mechanisms should prevail.

The ergosterol biosynthetic pathway is the target of both polyenes and azoles. An overall decreased content in ergosterol has been observed in *C. albicans* sessile cells in comparison to the planktonic counterpart (Mukherjee et al. 2003), being more pronounced in mature biofilms.

Recent *in vitro* and *in vivo* biofilm models (Khot et al. 2006; Nett et al. 2009) showed an overexpression in *C. albicans* biofilms of *ERG25*, a C4 methyl sterol oxidase with a role in C4- demethylation of ergosterol biosynthesis intermediates. *ERG25* promotes the conversion of lanosterol to non-ergosterol intermediates, reducing the ergosterol content in biofilm membranes.

Hence, it is possible to hypothesize that a modulation of ergosterol could contribute to resistance in later phases of biofilm formation.

4 Other Mechanisms Promoting Biofilm Resistance

Adhesion to abiotic surfaces, cell to cell interaction as well as hyphal development are crucial events for biofilm formation. Therefore, cell wall proteins involved in the processes of adhesion and/or filamentation, and transcription factors regulating their expression have been deeply investigated as potential players in biofilm antifungal resistance. Although many studies are focused on *C. albicans* (Blankenship and Mitchell 2006), in a recent work, Fan and

colleagues (2015) demonstrated that similar processes could be present also in *A. fumigatus*. By deleting *cspA* gene of *A. fumigatus*, encoding for the cell surface protein A, the authors achieved a reduction in biofilm formation that led to enhanced sensitivity to amphotericin B and itraconazole.

In patients with invasive fungal infection, dispersion of biofilm cells and interaction of sessile cells with the ECM play an important role in persistence and antifungal treatment failure.

Hsp90 is an important protein involved in fungal morphogenesis and virulence, and in development of biofilm and drug resistance (Shapiro et al 2012). Robbins and colleagues (2011) found that genetic depletion of Hsp90 impaired dispersion of *C. albicans* biofilm cells *in vitro*, while compromised function of Hsp90 abrogated azole resistance in *C. albicans* biofilm, probably by reducing the levels of ECM glucans. The same authors demonstrated that inhibition of Hsp90 with geldanamycin reduced echinocandins resistance in *A. fumigatus* biofilm.

5 Unconventional Compounds to Implement Fungal Biofilm Treatment

Studies addressing the multifaceted resistance of fungal biofilms make clear the need to develop new strategies to treat such infections.

Due to the crucial role of ECM in biofilm resistance, behaving as a "drug sponge" for many of the available antifungals (Nett et al. 2010a and 2010b), new compounds targeting matrix production as well as promoting its disaggregation could help in eradicating sessile pathogens.

DNase treatment was shown to be effective in enhancing the activity of polyenes and echinocandins (Martins et al. 2012; Rajendran et al. 2013) that, whilst recognized as the most active classes against biofilm, are unable to completely kill biofilms (Ramage et al. 2014).

Alginate lyase (Bugli et al. 2013) and the alginate oligomer, OligoG, (TØndervik et al. 2014) have been recently shown to enhance polyene and azole susceptibility, respectively. In particular, Oligo G was demonstrated to modulate *per se* both fungal growth, by inhibiting filamentation, and biofilm formation, by altering biofilm morphology.

Mucolytic agents are also under investigation. Ambroxol (2-amino-3,5-dibromo-N-[trans-4-hydroxycyclohexyl] benzylamine), an expectorant used in many lung diseases, showed a good activity against *C. parapsilosis* biofilms (Pulcrano et al. 2012) improving fungal susceptibility to voriconalole. As for alginate lyase, this effect was biofilm-specific, as drug combination lacked to show a synergistic effect on planktonic cells.

Many natural compounds have also been demonstrated to exert an anti-biofilm effect, such as chitosan (Cobrado et al. 2013), polyphenols (Sardi et al. 2013), and fulvic acid (Sherry et al. 2012). In particular, a carbohydrate derived fulvic acid (CHD-FA), already known to be safe for topic and oral administration (Gandy et al. 2012), was shown to exert a rapid activity on both planktonic and sessile cells of *C. albicans* without differences in the minimal inhibitory concentrations (MICs).

6 Drug Tolerance

Besides multifactorial biofilm-mediated resistance, drug tolerant cells, named persisters, have been shown to characterize *Candida* biofilm communities (Lewis 2010; Li et al. 2015). As in bacterial biofilms, these cells represent a sub-population that spontaneously and stochastically arise and are genetically identical to the clonal population, representing thus a phenotypic variant (Lewis 2010; LaFleur et al. 2006).

Chronic carriage of *Candida*, an important risk factor for subsequent infection, has been shown to increase the number of persister cells in the microbial population (LaFleur et al. 2010). Periodic application of antifungals may in fact select for strains with increased levels of persister cells. This aspect has broader implications in the clinical setting of susceptible

patients, as prolonged drug treatments and prophylaxis could favor drug tolerance and infection recurrence.

Biofilm communities are enriched in persisters, although their percentage is strain-dependent (Li et al. 2015). Studies performed on several strains mutated in their capability to form biofilm, e.g., strains lacking the transcription factors, led to the conclusion that attachment to substrates, rather than complex biofilm architectures, is the crucial step promoting persisters selection (LaFleur et al. 2006).

Persisters are dormant cells that exhibit tolerance to multiple drug classes, including amphotericin B and azoles (LaFleur et al. 2006; Bink et al. 2012; Li et al. 2015).

The presence of persister cells results in a biphasic killing curve, with the majority of cells dying rapidly at concentration of antimicrobial close to the MIC, and a small fraction surviving even at the highest concentrations (LaFleur et al. 2006).

The mechanism responsible of antimicrobial tolerance is still not completely understood.

Transcriptional and proteomic analysis of persisters showed a differential regulation of genes that could explain the dormant status of these cells. In particular, glycolysis, tricarboxylic acid cycle (TCA) and protein synthesis have been demonstrated to be down-regulated, whereas the alternative glyoxylate shunt enzymes were over-activated (Li et al. 2015). This switch resulted in a lower production of reactive oxygen species (ROS), avoiding ROS-enhanced killing. Moreover, expression of proteins involved in stress response, as heat-shock proteins HSP90, HSP120, and HSP70, were also induced (Li et al. 2015). Accordingly, superoxide dismutase mutants biofilms (Δ*sod4* Δsod5) were shown to form less miconazole-tolerant persisters (Bink et al. 2011). Up to date, miconazole, echinocandins and liposomal formulations of amphotericin B showed a better efficacy against fungal biofilms. All these compounds, besides their specific activity, induce ROS production, improving microbial killing as already reported for bacterial biofilms (Kohanski et al. 2007).

Furthermore, persisters show a different regulation of genes involved in both ergosterol and β-1,6 glucan pathways (Khot et al. 2006). Modifications in membrane and cell wall compositions could represent a complementary pathway leading to drug tolerance.

7 Conclusions

The resistance to antifungal drugs is a well-established health problem and the mechanisms underlying this phenomenon are well known, mainly through *in vitro* studies on planktonic cells (Perlin et al 2015; Cowen et al 2014; Perlin 2014; Bowyer et al 2011; Marie and White 2009). The antifungal resistance, which often results in a therapeutic failure, has been progressively more associated to the ability of fungi to develop biofilms. Several studies have shown that the physical barrier of biofilm extracellular matrix is the major mechanism allowing embedded cells to prevent drugs from entering and expressing their activity. However, biofilm antifungal resistance is complex, involving different genetic mechanisms some of them in common with planktonic cells. Over-expression of efflux pumps, genetic changes of drug targets, persister cells, biofilm-host immune system interaction, proteins leading to filamentation, all together contribute to the onset of biofilm antifungal resistance at early, intermediate or mature phases of biofilm development.

Overcoming biofilm-related resistance should represent an important progress in antifungal therapy and would have broad implications for healthcare applications. Different approaches, such as targeting stress responses, matrix production, or other biofilm-specific mechanisms of resistance, are currently under investigation.

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Fungi, Water Supply and Biofilms

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Abstract

Even though it has been studied for many years, water-related infectious risk still exists in both care and community environments due to the possible presence of numerous microorganisms such as bacteria, fungi and protists. People can be exposed directly to these microorganisms either through aerosols and water, after ingestion, inhalation, skin contact and entry through mucosal membranes, or indirectly usually due to pre-treatment of some medical devices. Species belonging to genera such as *Aspergillus, Penicillium, Pseudallesheria*, *Fusarium, Cuninghamella*, *Mucor* and in some particular cases *Candida* have been isolated in water from health facilities and their presence is particularly related to the unavoidable formation of a polymicrobial biofilm in waterlines. Fungi isolation methods are based on water filtration combined with conventional microbiology cultures and/or molecular approaches; unfortunately, these are still poorly standardized. Moreover, due to inappropriate culture media and inadequate sampling volumes, the current standardized methods used for bacterial research are not suitable for fungal search. In order to prevent water-related fungal risk, health facilities have implemented measures such as ultraviolet radiation to treat the input network, continuous chemical treatment, chemical or thermal shock treatments, or microfiltration at points of use. This article aims to provide an overview of fungal colonization of water (especially in hospitals), involvement of biofilms that develop in waterlines and application of preventive strategies.

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1 Introduction

Numerous studies have shown that water could be contaminated by many microorganisms, both in hospital and in community environments: virus, bacteria, protists and also microscopic fungi, such as single-celled yeasts and multicellular filamentous fungi. People can be exposed to microorganisms present in water after ingestion (drinking), inhalation (showering), skin contact (showering or bathing) and entry through mucosal membranes (showering and bathing). The fungal risk related to water has been highlighted in some nosocomial outbreaks due to *Fusarium* sp, *Aspergillus* sp or *Exophiala janselmei* sp and also in drowning-related infections due to *Pseudallescheria* sp (Ao et al. 2014; Figel et al. 2013; Gonçalves et al. 2006; Heinrichs et al. 2013; Nucci et al. 2002; Pires-Gonçalves et al. 2008; Warris et al. 2001a; Warris et al. 2002). Historically, hydric fungal contamination has been considered as a minor health issue in health facilities because of the rather opportunistic behavior of related infections. However, fungi can also be responsible for severe systemic infections and given an increasing number of fragile or immunocompromised patients, effective control of the microbiological water quality, including fungi, has become of increasing interest. Fungi of environmental origin can contaminate neutropenic patients in hematology units, patients receiving a solid-organ transplant and patients from intensive care units receiving high-dose corticosteroids who should benefit from prophylactic measures against bio-aero contamination-related risk. Fungi can cause candidemia or, more frequently and more worrisome in this context, fatal diseases due to filamentous fungi infection. Airborne fungi responsible for nosocomial infections usually belong to the *Aspergillus* genus. *Aspergillus* conidia – potentially responsible for nosocomial aspergillosis – can also be airborne from a water source, thereby establishing a potential link between aero- and water-contamination (Anaissie and Costa 2001). Mold aerocontamination in health facilities is well documented, especially regarding the "*Aspergillus*-related risk" (Ao et al. 2014; Warris et al. 2002). However, it has been shown that air control may not be sufficient to ensure the prevention of aspergillosis in units receiving immunocompromised patients (VandenBergh et al. 1999; Warris et al. 2001b). Actually, this contamination may occasionally originate from the aerosolisation of a mouldy aquatic niche (Anaissie et al. 2003). Rigorous control of both air and water supply is essential to control the related infectious risk. Studies reported in this article focused on the fungal presence in water and were generally conducted in the context of microbial investigations in health facility networks subsequent to an outbreak or were designed to search for specific fungi such as *Pseudallesheria* sp or black yeasts. Available data concerning investigations on hospital water networks further to bacterial outbreaks (*Pseudomonas aeruginosa*, *Legionella pneumophilla*) contributed to the development of an analytical method applied to fungi (Kauffmann-Lacroix et al. 2008). Current knowledge on water as a fungal biotope and on interactions established between fungi and other microorganisms potentially present in water still remain incompletely understood. This article deals with the fungal human infectious risk related to water exposure.

2 Biofilms in Water

One of the major issues related to the presence of fungi in water is their possible growth, together with other microorganisms such as bacteria and protists, in complex structures called biofilms. Indeed, water networks particularly favor biofilm formation. Flemming has reported that 95 % of the bacterial biomass occurred as biofilms on the inner surface of the drinking water distribution system whereas less than 5 % existed in the planktonic form (Flemming 2002). If current knowledge on drinking water biofilms has been obtained mainly from studies on bacterial biofilms, it has been also shown that *Penicillium expansum* was able to develop a mature biofilm in around 48 h in a model mimicking drinking

water distribution system conditions (Simões et al. 2015). The development of a biofilm in drinking water typically involves seven successive phases: initial adhesion (4 h), germlings (8 h), initial monolayers (12 h), a monolayer of intertwined hyphae (24 h), mycelial development, hyphal layering and bundling, and finally the development of the mature biofilm (≥48 h) (Simões et al. 2015). In the context of dental unit waterlines, it has been shown that biofilms start to develop in the early hours following water supplying for a newly installed dental unit (Williams et al. 1995). Actually, by developing within a biofilm, sessile microorganisms become less susceptible or sometimes totally resistant to different antimicrobial treatments, compared to microorganisms growing planktonically. This resistance is partially explained by the presence of persisters which are highly tolerant to treatments, especially in the deeper layers of the biofilms. Other factors can contribute to susceptibility loss: the matrix produced by sessile microorganisms and consisting of exopolymers containing open water channels limits the penetration of antimicrobial agents, thereby constituting a physical barrier to antimicrobial agent efficacy and strongly limiting the diffusion of oxygen and nutrients inside the biofilm (Brown et al. 1988; Costerton et al. 1995). In addition, the process of biofilm formation is regulated by specific genes of which expression is triggered by adhesion; regulation of the genes encoding efflux pumps can significantly limit the activity of some antifungal agents (Costerton et al. 1995; Ramage et al. 2012). Even though recent years have been marked by many advances, comprehension of the mechanisms that govern the resistance of fungal cells within biofilms has remained incomplete. That much said, biofilms can be considered as reservoirs of microbial contamination of water systems by periodic release of fungal cells, isolated or in clusters with matrix and other microorganisms. Biofilms grow at an interface such as the one existing between air and water, and are promoted by flux, especially in cases of slow flux alternating with stagnation periods. These conditions are found in water systems, in community and hospital environments, for example at the level of shower heads and siphons. This is also the case in endoscopy reprocessing units, dialysis units and dental units.

3 Methods Dedicated to Fungi Isolation from Water

The filtration method applied to bacteria in health facilities has been helpful for developing a specific method adapted to fungi: samplings are performed on cold water and filtered volumes are larger than the ones recommended for bacterial controls (Kauffmann-Lacroix et al. 2008). Indeed, fungi are usually inactivated in hot water samples (>55 °C), highlighting the fact that *Legionella* and fungi tests cannot be performed using the same water sample. According to the literature, sampled volumes usually range between 50 mL and 500 mL. However, this volume varies considerably according to the different studies: 50 mL (Arvanitidou et al. 2000), 100 mL (Schiavano et al. 2014), 250 mL (Kadaifciler et al. 2013), 500 mL (Sammon et al. 2010; Warris et al. 2001a; Warris et al. 2010) or 1 L (Gonçalves et al. 2006; Mesquita-Rocha et al. 2013). Such major differences in sampling volumes are probably explained by the variable fungal contamination level in the water studied but also by differing laboratory customs and means. For example, in France, the water delivered to the point of use is only lightly contaminated by fungi because of preliminary treatments to make it drinkable. In such cases, the recommended sampling volume for fungal analysis could be 1 L (Kauffmann-Lacroix et al. 2008). The lack of standardized methods of search for fungal contaminations taking into consideration volume, culture medium and incubation temperature could explain some failures to conclusively prove the water origin of healthcare associated infections: due to fail in fungal isolation or misidentification of co-existing different phenotypes. In addition, the fact that some fungi cannot be isolated from some samples does not prove that these species were not previously present (Anaissie et al. 2003). Regarding conditions for experimental cultivation, use of a culture medium

supplemented with an antibiotic is recommended. However, various culture media (Figs. 1 and 2) are used to isolate fungi from water samples and the fact that they vary from one laboratory to another may be an issue. For example, depending on studies, authors used Sabouraud dextrose agar supplemented with chloramphenicol (Arvanitidou et al. 2000; Mesquita-Rocha et al. 2013; Schiavano et al. 2014) or sometimes with penicillin (Warris et al. 2001a; Warris et al. 2010) ; tap water agar, half-strength corn meal agar, neopeptone-glucose rose Bengal aureomycin and oomycete selective medium were also used (Gonçalves et al. 2006) as well as malt extract agar with chloramphenicol (Sammon et al. 2010). Incubation temperature also varies according to the studies: 25 °C (Gonçalves et al. 2006; Sammon et al. 2010) ; 25 °C and 37 °C (Mesquita-Rocha et al. 2013); 23.5 ± 1.5 °C (Schiavano et al. 2014) ; 35 °C and 42 °C (Warris et al. 2010), 35 °C (Warris et al. 2001a). Importantly, an incubation temperature close to 30 °C should be preferred as it would support biodiversity, whereas a warmer temperature, such as 37 °C or 40 °C could promote some specific species such as *A. fumigatus complex* (Oliveira et al. 2013). Finally, most of the time, incubation was performed for one week (Gonçalves et al. 2006; Sammon et al. 2010; Schiavano et al. 2014; Warris et al. 2001a; Warris et al. 2010) but it could be extended to 15 days (Mesquita-Rocha et al. 2013); some authors incubated water samples for three or four weeks

Fig. 1 *A. fumigatus* on Czapek, Sabouraud-Gentamicin and Sabouraud medium respectively (*up to down*)

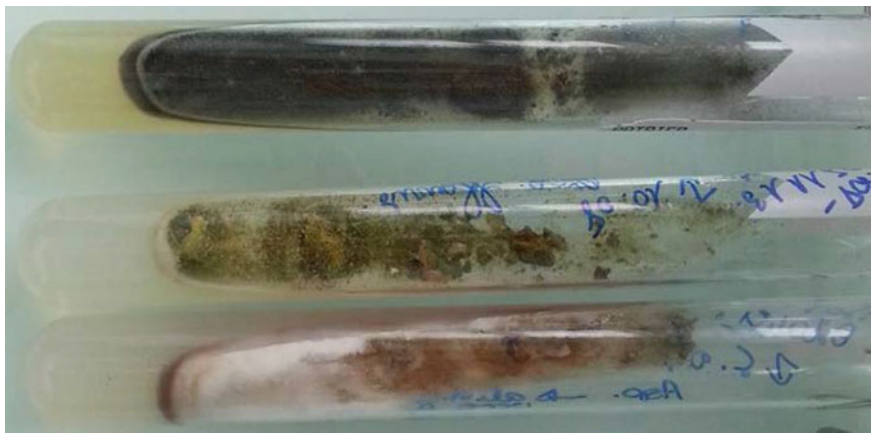

Fig. 2 *A. fumigatus*, *A. flavus* and *A. nidulans* respectively (up to down) on Czapek medium

at room temperature (Arvanitidou et al. 2000). Only a few laboratories currently implement the new molecular methods for routine analyses; in the future, however, the fungal ecology of water will be certainly more and more extensively explored thanks to these methodological advances.

4 Fungi Isolated from Water

Fungi are eukaryotic and aerobic microorganisms, nonchlorophyllous heterotrophs, and they are characterized by two different forms: the asexual or anamorph one, which is observed in culture (for example *A. fumigatus*) and the sexual one, which is not always known (for example *Neosartorya fumigata*) (Kwon-Chung and Sugui 2009). Less than 10 % of an estimated 1.5 million of fungal species have been described to date; consequently, the identification of fungi up to the species is quite difficult and lacking in most of the studies. *Aspergillus* is a genus bringing together different species based on morphological, physiological and phylogenetic characteristics (Balajee et al. 2007). *Aspergillus* has been associated with nine teleomorph genera, but phylogenetic data suggest that it is linked together with other genera. After the recent changes in the International Code of Nomenclature for algae, fungi and plants, the *Aspergillus* genus now contains 339 species (Samson et al. 2014). Recent molecular approaches have offered prominent alternative methods compared to conventional approaches based on macroscopic and microscopic characteristics, and multiple recent studies have demonstrated the limited utility of morphological methods used singly for identification of clinically relevant species. It is increasingly recognized that comparative sequence-based methods used in conjunction with traditional phenotype-based methods can offer better resolution of species within this genus. Various diagnostic tools have been developed for medically important groups of fungi but are still lacking in standardization (Roe et al. 2010); however very recent publications of standardized methods for DNA extraction, amplification and sequence analysis can be applied to fungal species identification (Schelenz et al. 2015). They are currently based on the use of DNA sequences variation designed to distinguish species or strains. Multiple genes ranging from the universal ribosomal DNA region ITS and the large ribosomal subunit D1–D2 to protein encoding genes such as the β-tubulin and calmodulin gene regions are now used to delimit species within aspergilli.

Numerous genera of filamenteous fungi with a number of different species, (*Acremonium, Alternaria, Aspergillus, Cladosporium, Exophiala, Fusarium, Penicillium, Trichoderma*…) and yeasts (*Candida*) can be isolated from water, notwithstanding the hydrophobic nature of certain structures (spores, blastospores). Fungi infect many organic substrates and are responsible for their degradation or transformation. Their growth is promoted by heat and moisture. As regards filamentous fungi that are pathogenic for humans, we do not currently know in which cases water is the original biotope or else represents a habitat that could be described as transitional. The recent identification of certain fungi to clarify the species by means of molecular biology appears to support the idea that water could be the preferred habitat of certain species. Quantitative evaluation of the different fungi isolated from water was shown to depend on its origin: surface water presents a higher risk of colonization compared to deeper samples such as groundwater (Göttlich et al. 2002). A recent study (Oliveira et al. 2013) identified different species in waters with different origins – from surface source and groundwater – in Portugal. These authors underscored the great biodiversity of fungi by isolating 27 different genera. Several pathogenic species were found, including *Aspergillus species, A. fumigatus, A. calidoustus* (Hageskal et al. 2007) and *A. viridinutans, Fusarium sp,* and Zygomycetes, *Mucor racemosus*, *Cuninghamella bertholletiae*. Interestingly, the presence of *Penicillium brevicompactum* was reported in water in another study, even if *Penicillium sp* are only exceptionally responsible for human infections in Europe (only one case of infection associated with *P. brevicompactum*) (De la Cámara et al. 1996).

However, *A. calidoustus* (group *A. usti*) is emerging as a pathogen reported in immunocompromised patients. In a recent study of the section *A. usti,* strains isolated in immunocompromised patients were investigated showing the emergence of *A. calidoustus* among lung-transplanted patients with pulmonary aspergillosis resistant to azole antifungals of the third generation (Egli et al. 2012). Furthermore, frequent isolation of fungi belonging to the genus *Pseudallesheria* (*Scedoporium*) in patients having escaped drowning was reported in the 2000s (Tintelnot et al. 2008) and is another example of exposure to water-related risk of fungal infection. This observation was confirmed by reported cases in patients who had been injured during a tsunami in Thailand or Japan. Finally, black yeasts were studied because of their general availability in wetlands; they are characterized by their resistance in very hostile environments such as hypersaline water, sea or ice from icebergs (Heinrichs et al. 2013).

5 The Fungi Isolated from Water Supply in Health Facilities

According to some studies, filamentous fungi can be isolated in water from both hospital and community environments, and yeasts would resultantly be less frequently found (Fig. 3). In a pioneer French study in 1985, the fungal microflora was investigated in 38 samples of chlorinated tap drinking water. Interestingly, filamentous fungi and yeasts were isolated in 81 % and 50 % of the water samples respectively; as regards yeasts, *Candida* genus was the most isolated, and half of the filamentous fungi belonged to three genera: *Penicillium*, *Aspergillus* and *Rhizopus* (Hinzelina and Block 1985). From that time, other studies devoted to air and water analyses have highlighted the presence of fungi in drinking water and reported the almost systematic presence of *Penicillium* and *Aspergillus* genera in positive samples. Their presence may be related to their high frequency in air (Gangneux et al. 2002; Siqueira et al. 2011). Aside from *Penicillium* and *Aspergillus*, European studies have often reported the presence of *Acremonium, Cladosporium*, *Epicoccum*, *Exophiala*, *Fusarium*, *Phoma*, *Trichoderma* and *Phialophora* in drinking water (Arvanitidou et al. 1999; Arvanitidou et al. 2000; Gonçalves et al. 2006; Göttlich et al. 2002; Hageskal et al. 2006; Hageskal et al. 2007). More recently, black yeasts and filamenteous fungi were reported as coming from water in hemodialysis units (Arvanitidou et al. 2000; Figel et al. 2013; Pires-Gonçalves et al. 2008; Rao et al. 2009; Schiavano et al. 2014; Varo et al. 2007). Patients with chronic renal insufficiency undergoing haemodialysis or peritoneal dialysis may be

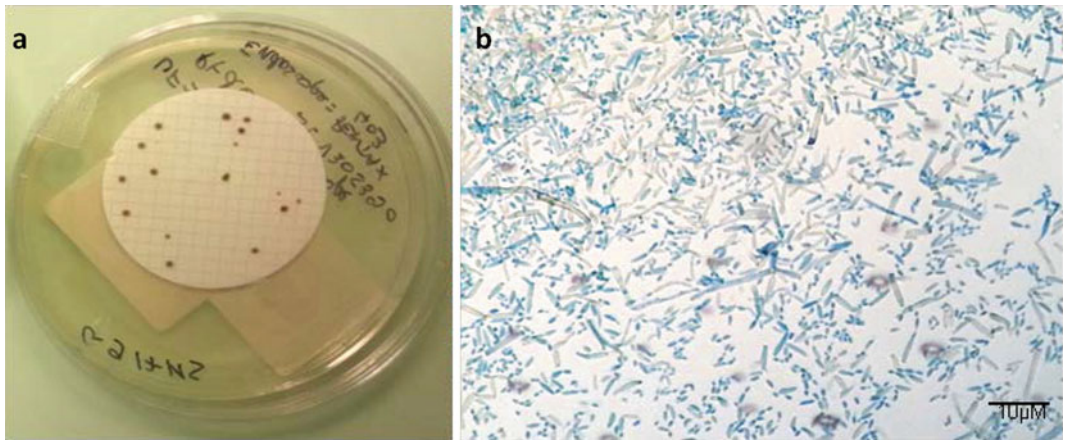

Fig. 3 *Cladosporium* sp isolated from an endoscopic rinsing solution filtration. (**a**) macroscopic aspect on malt agar (**b**) microscopic aspect (conidiophores and conidia)

susceptible to a number of primary or opportunistic pathogens including fungi from water and dialysate in haemodialyse units (Arvanitidou et al. 2000; Unal et al. 2011); at times the link between the causal agent and water remains missing (Proia et al. 2004). In hemodiafiltration procedures, dialysis water is used in the form of dialysate and infusate up to 3400–6800 L directly following ultrafiltration through two or three dialysis water monitor ultrafilters (Locatelli et al. 2010). Microbiological water quality is consequently an important issue in dialysis and the water supply used to prepare the dialysate can be one of the sources of contamination (Rao et al. 2009). In Brazil, legislation regulating water microbiological quality for dialysis does not cover waterborne microbes such as fungi (Figel et al. 2013). Likewise in Brazil, the fungal contamination in water distribution systems of a tertiary care hospital devoted to medical assistance of children with cancer was recently investigated over a period of 12 months (Mesquita-Rocha et al. 2013). These authors showed that the highest concentrations of fungi in the water system occurred in autumn and summer. Generally, along with authors from other countries, they have isolated a wide variety of filamentous fungi from the water studied, but the isolated strains were hardly in any way related to invasive infection.

Health facilities do not react in the same way to water fungal contamination, which has many sources and causes: surface water or groundwater, storage tank, hot water tank, nature and age of the pipes in the network... It has been shown that the differing conditions depending on the differing origins of the water may lead to qualitative and quantitative differences of fungal colonization, which is extensively increased in water originating from superficial water or having previously stagnated in a storage tank (Anaissie et al. 2002; Warris et al. 2001a). Studies performed in hospital environments have particularly targeted *A. fumigatus* of which the contamination level tended to differ according to geographical location: the United States (Anaissie et al. 2002; Squier et al. 2000), Norway (Hageskal et al. 2006; Hageskal et al. 2007), the Netherlands (Warris et al. 2003) or Greece (Panagopoulou et al. 2007). The observed differences are correspondingly related to geography, climate, and culture procedures but also, as previously mentioned, to non-standardized methods leading to the use of different culture media, sampling volumes... Even if the results of these studies are difficult to compare, the fungal composition of water seems to vary between hospitals according to water supply conditions, and probably seasons and network features (material, age...). Overall, available studies have evaluated the fungal colonization of water but have only exceptionally shown a direct link between water colonization level and infections. Finally, in hospitals, water must be considered as an environmental source of fungi that is airborne, and transmitted through cardboard and food.

6 Transmission of Fungi Responsible for Water-related Infections in Health Facilities

In France and in many other countries, there exist numerous standards and rules to oblige hospitals to monitor and control the large water volumes used for food, health care (hygiene, toilets...), hemodialysis, medical device disinfection, air conditioning systems, etc. The control of microbiological water quality consequently requires the implementation of organizational, technical and practical actions, including prophylactic approaches. Nevertheless, water can remain a reservoir for hospital-acquired infections.

Regarding fungal risk, patients are potentially exposed to infections, especially lung infections, following aerosol inhalation (humidifier, shower, dental care) and also direct contact with medical devices that were previously washed with water. Direct contact with water or aerosols may also be responsible for wounds or mucosa membrane infections. Finally, water especially for peritoneal dialysis, drinking water and ice may be sources of local infections, even if reported cases nowadays remain the exception. When

water-related fungal infections are suspected, microbiologists must prove, in order to establish the environmental source of infection, that the pathogenic fungi isolated from a patient and those isolated from water are the same. Unfortunately, the genotyping approaches needed to associate them are only rarely performed (Anaissie et al. 2002; Squier et al. 2000). In a study reporting an outbreak of *A. fumigatus* in a neonatal intensive care unit, the authors used microsatellite strain typing to prove that *A. fumigatus* strains isolated from neonates and those isolated from environment were genotypically related. The source of infection may have been the humidity chambers of neonate incubators (Etienne et al. 2011).

7 Specific Case of Dental Unit Waterlines

Conditions encountered in dental unit waterlines strongly favor biofilm formation: significant water stagnation periods between patients, or during nights and weekends, together with the high surface ratio – water volume, water circulation in laminar flow (maximum flow at the center and minimum at the periphery). The presence of multiple and complex flora in these waterlines, of both environmental and human origins, is related to a potential health risk for both patients and dental staff exposed to the water drops and aerosols generated during dental care (Coleman et al. 2014; Szymanska 2003). Nowadays, the microbial contamination of dental unit waterlines is well documented especially as regards bacterial identification (Barbot et al. 2012; Kumar et al. 2010; O'Donnell et al. 2011; Szymanska 2003; Szymańska 2005b). The presence of bacteria in biofilms formed in dental unit waterlines is likewise well-documented but fungi are also involved. In 2015 a Polish study investigated 25 dental units and independent water tanks and revealed the presence of fungi in 12 units: 11 of the 25 collected biofilm samples were contaminated by fungi, as well as 16 of the output water samples from rotary instruments (Szymańska 2005a). Interestingly, in this study, fungi of environmental origin such as *Aspergillus* sp were among the most frequent species. However, *Candida* sp. yeasts were also present; they most probably originated from the saliva reflux occurring due to negative pressure when the rotary dental instruments stop. Moreover, the variety of fungal species was two times higher in both biofilm samples and in the output water sampled from rotary dental instruments as compared to water sampled from tanks. These data suggest that biofilm dispersal could contribute to the output water fungal contamination. In addition, other studies have shown the presence of fungi in the same context. For example, in Brazil, filamentous fungi were isolated in 70 % of the overall water sampled from either three-water syringe, handpieces, water reservoir or the water supply of dental units (Lisboa et al. 2014). Overall, 16 filamentous fungi genera were isolated and among them the most represented were: *Acremonium* (46.7 %), *Exophiala* (14.7 %), *Penicillium* (9.4 %) and *Aspergillus* (8.9 %) (Lisboa et al. 2014). In Istanbul, Turkey, similar investigations revealed the presence of *Penicillium sp*, *Cladosporium sp*, *Candida sp*, *Cryptococcus sp* and *Aspergillus sp* in dental unit waterlines (Kadaifciler et al. 2013). All in all, many studies have highlighted fungal presence in dental unit waterlines, but the fungal community seemed variable, probably depending on the sampling location (i. e handpieces, air/water syringe, water tanks, incoming water). Development of specific biofilms in each sampled condition could explain the variability of the fungal community. Polymicrobial biofilms are able to grow in rather poor nutritional conditions and to promote the survival of numerous microbial species such as *Candida* sp yeasts, which normally exist under more nutritional conditions. Laboratory experiments have also shown that human microbial species such as *Streptococcus gordonii*, *C. albicans*, *C. glabrata* and *C. parapsilosis* have been able to survive and sometimes to proliferate in water as soon as it contained a small concentration of saliva (1 or 2 % v/v) (Barbot et al. 2011; Costa et al. 2013). Furthermore, it has been shown that some species of free-living

amoebae have been able to help the survival or even promote the proliferation of *Candida albicans*, *C. glabrata* and *C. parapsilosis* in water (Vanessa et al. 2012). Free-living amoebae are commonly found in water systems and can contribute to the fungal risk associated with water by the biofilm.

These considerations once again raise the issue of the prevention of infectious risk related to water and at this time, the one radical action able to eliminate a preformed biofilm requires the replacement of contaminated surfaces; in the case of dental unit waterlines, chemical agents (based on chlorine or hydrogen peroxide. . .) and physical measures (pipe flushing) are only partially active on biofilms, even when combined. Up to now none of the cases of severe fungal infection have been definitively linked to dental unit waterline exposure, but the presence of such fungi, at times including opportunistic pathogens, may expose patients and dental staff to diseases as asthma, rhinitis, other respiratory problems, etc. Epidemiological research should be planned to evaluate this potential infectious risk. Once again, all these data reinforce the need for better monitoring and control of the microbial quality of dental unit waterlines in order to limit the infectious risk related to dental care.

8 Prophylactic and Curative Actions Developed in Hospitals

Hospital water networks are a potential source of hospital-acquired infections. Currently infectious risk control is mainly related to bacterial risk and is based on the implementation of a program of both technical improvement and monitoring of water quality. In many countries, health facilities have implemented some preventive measures such as ultraviolet radiation to treat the input network or continuous chemical treatment of cold water, usually using chlorine (hypochlorite or chlorine dioxide), or at the point of use with microfiltration. Hot water can be treated by similar processes (Ministères des solidarités and DGS/DHOS 2005). In care units with immunocompromised patients, end-filters (0.2 μm) applied to tap and showerhead allow a sterilizing filtration at the point of use, which is considered efficient to obtain microbiologically controlled water (Fig. 4). Warris and collaborators recently evaluated the efficacy of point of use filter devices on taps and showerheads in view of reducing patient exposure to filamentous fungi by developing a laboratory test (simulated test rig) and monitoring filter efficiency in real conditions in a pediatric bone marrow transplantation unit (Warris et al. 2010). Their results -obtained in laboratory conditions – showed that filters were highly effective in reducing the number of colony-forming units of *A. fumigatus* alone or in combination with *Fusarium solani* from contaminated water for a period of at least 15 days. Finally, the use of sterile water in humidifiers and in the rinsing of critical medical devices represents a significant improvement. In a bone marrow transplantation unit, authors have shown that point of use filters completely retain mould contamination on the first day; however high concentration of particles in the water occluded filters, thereby putting a premature end to the study (Warris et al. 2010). That shows why point of use filters must be changed regularly, according to supplier recommendations.

Numerous factors are involved in the degradation of network water-quality, including water stagnation, corrosion, furring up, retrograde contamination and biofilm formation. Measures for the technical monitoring of plumbing and of some other critical points, combined with regular microbiological tests, contribute to insure the water quality. Health facilities can now archive all of these data. Bacterial monitoring is a very sensitive indicator of microbiological pollution; however due to inappropriate culture media and inadequate sampling volumes, the current standardized methods used in bacterial research are not suitable for fungal research.

Curative methods are based on the different types of maintenance applied to non-operating networks; shock chlorination as well as thermal shocks can be carried out. Treatments using peracetic acid or hydrogen peroxide can also be performed and are allowed by some legislations

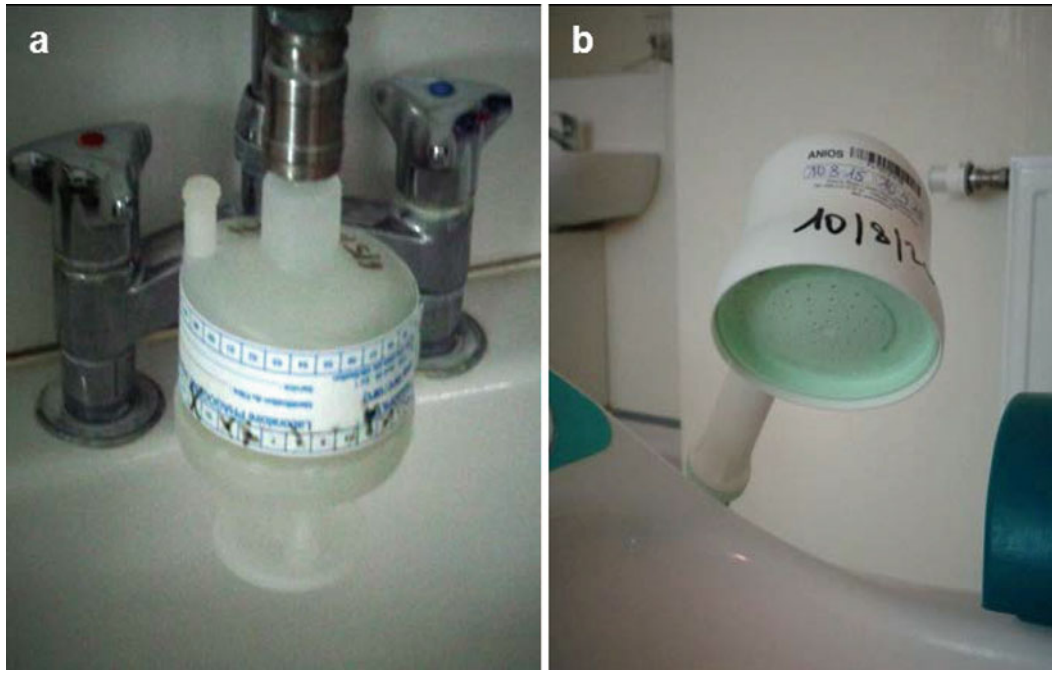

Fig. 4 End-filters (**a**) Tap, (**b**) Showerhead

(for example in France: Ministères des solidarités and DGS/DHOS 2005). Hydrogen peroxide would be more efficient against bacteria than against yeasts and viruses (Ministères des solidarités and DGS/DHOS 2005). In some cases, maintenance on the network has to be planned, at times requiring partial replacement of pipes. Regarding the technological strategies deployed for the prevention of dialysis water pollution, including fungal contamination, daily overnight thermal disinfection procedures have proved at times to be more effective than frequent chemical disinfection (Bolasco et al. 2012). In addition, these authors showed the interest of two-stage reverse osmosis for dialysis procedures.

9 Conclusion

A number of studies clearly show the frequent presence of fungi in hospital and community water. In the future, identification by sequencing isolated species may provide us with valuable epidemiological information and will allow better evaluation of the risk of developing invasive water-related fungal infection, especially for elderly, dialyzed, transplanted and more generally for all immunocompromised patients. Currently, in numerous health facilities, for example in French hospitals, the prevention of water-related risks is more focused on bacteriological risk. There are many types of water in health facilities, including drinking water, dialysis water, sanitary water and water supply related to health care. Through current regulations, bacterial environmental risk is well-controlled. Hot water is not usually a vector of the molds responsible for fungal infections; on the other hand, cold water is a potential vector which must be taken into account. Although the fungal risk has not been systematically evaluated, and even though it is quite low, at least in Europe (low number of colony forming units reported by liter), it remains a possibility that should not be neglected. Despite the measures for air quality control in hospitals, control of invasive fungal infection risk in immunocompromised patients can at times be insufficient and we must remain attentive to other potential sources of fungal

contamination, such as water, whose impact needs to be monitored. Finally, the development of new anti-biofilm strategies may also help to limit the presence of fungi in water and, consequently, water-related fungal risk.

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Diagnostic of Fungal Infections Related to Biofilms

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Abstract

Fungal biofilm-related infections, most notably those caused by the *Candida* and *Aspergillus* genera, need to be diagnosed accurately and rapidly to avoid often unfavorable outcomes. Despite diagnosis of these infections is still based on the traditional histopathology and culture, the use of newer, rapid methods has enormously enhanced the diagnostic capability of a modern clinical mycology laboratory. Thus, while accurate species-level identification of fungal isolates can be achieved with turnaround times considerably shortened, nucleic acid-based or antigen-based detection methods can be considered useful adjuncts for the diagnosis of invasive forms of candidiasis and aspergillosis. Furthermore, simple, reproducible, and fast methods have been developed to quantify biofilm production by fungal isolates in vitro. In this end, isolates can be categorized as low, moderate, or high biofilm-forming, and this categorization may reflect their differential response to the conventional antifungal therapy. By means of drug susceptibility testing performed on fungal biofilm-growing isolates, it is now possible to evaluate not only the activity of conventional antifungal agents, but also of novel anti-biofilm agents. Despite this, future diagnostic methods need to target specific biofilm components/molecules, in order to provide a direct proof of the presence of this growth phenotype on the site of infection. In the meantime, our knowledge of the processes underlying the adaptive drug resistance within the biofilm has put into evidence biofilm-specific molecules that could be potentially helpful as therapeutic targets. Surely, the successful management of clinically relevant fungal biofilms will rely upon the advancement and/or refinement of these approaches.

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1 Introduction

Rapid and accurate diagnosis of fungal infections, especially those invading bloodstream and deep tissues (i.e., lung, abdomen, peritoneum, meninges, endocardium, sinus, orbit, etc.) and thus termed "invasive", is paramount to improve the clinical and therapeutic management of infected patients in many hospital settings (Perfect 2013). For example, how much early is the diagnosis of candidemia/invasive candidiasis (IC) or invasive aspergillosis (IA), and the consequent initiation of appropriate antifungal treatment (Garey et al. 2006; Parkins et al. 2007; Upton et al. 2007), so much favorable will be the outcome of these infections especially when they are associated with biofilm formation (Tumbarello et al. 2007; Rajendran et al. 2015).

In the last years, the clinical mycology laboratory has enhanced its diagnostic capability thanks to the acquisition of new methods, which have been progressively introduced in the routine analysis of clinical specimens so as to replace conventional methods (Arvanitis et al. 2014). In one case, use of new methods has changed the mycological diagnostic workflow; in another case, they are adjunct diagnostics for invasive fungal infections (IFIs). In this context, a growing number of pathogenic *Candida* or *Aspergillus* species can be identified successfully, thus encompassing species that differ with respect to the virulence potential and the antifungal susceptibility profile (Hadrich et al. 2012; Ziccardi et al. 2015).

Biofilm formation is a common feature to many clinically encountered fungi, including not only *Candida* and other yeasts (*Cryptococcus neoformans*, *Cryptococcus gattii*, *Rhodotorula* species, *Malassezia pachydermatis*, *Saccharomyces cerevisiae*, and *Trichosporon asahii*) but also fungi such as *Aspergillus fumigatus*, *Fusarium* species, *Mucorales*, *Histoplasma capsulatum*, *Coccidioides immitis*, and *Pneumocystis* species (Sardi et al. 2014). Nevertheless, while there is growing interest in unveiling the true involvement of fungal biofilms in human disease (Williams and Ramage 2015), this goal has been primarily achieved with the pathogenic fungi *Candida albicans* (and, at lesser extent, non-*albicans Candida* species) and *Aspergillus fumigatus* (Herwald and Kumamoto 2014; Müller et al. 2011).

This chapter is, therefore, aimed at reviewing the current state of the clinical laboratory diagnosis of IFIs, with particular focus on the IFIs caused by species of *Candida* and *Aspergillus*. Note that the two genera were found to cause approximately 85 % of IFIs in large prospective surveillance studies that were conducted recently in North America (Neofytos et al. 2009; Azie et al. 2012). Not surprisingly, major progress in the IFI diagnosis has regarded the development of new laboratory assays for rapid detection of five most common *Candida* species—*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* account for 92 % of cases of candidemia globally (Guinea 2014)—as well as of *A. fumigatus* that alone accounts for 90 % of cases of IA—a severe invasive fungal disease (IFD) characterized by high mortality rate (Abad et al. 2010). The role of newly developed assays for the diagnosis of candidiasis and aspergillosis, including medical device-associated infections, will also be discussed. Above all, it is important to note that recently published international guidelines underscore basic aspects to be taken into consideration when managing patients with the suspect of IFI (Arendrup et al. 2012a; Cuenca-Estrella et al. 2012; Cornely et al. 2014; Tortorano et al. 2014; Chowdhary et al. 2014; Arendrup et al. 2014a). Thus, it is desirable that clinical microbiology laboratories serving hospitals with patients at risk of fungal infection are complying with these guidelines. For brevity, only some of these aspects will be addressed in this chapter.

2 Diagnostics for *Candida* Infections

2.1 Culture Based Diagnostics

Three entities need to be considered when diagnosing IC: (i) candidemia in the absence of

deep-seated candidiasis (DSC; i.e., infections of tissue sites beneath mucosal surfaces), (ii) candidemia associated with DSC, and (iii) DSC in the absence of candidemia (Clancy and Nguyen 2013). Approximately one-third of patients with IC fall into each of above categories (Leroy et al. 2009). Blood cultures (BCs) remain the gold standard for the diagnosis of invasive *Candida* infections, and should be the initial diagnostic test when candidemia is suspected (Cuenca-Estrella et al. 2012). Conversely, the gold standard tests for DSC are sterilely collected cultures of infected fluids or tissues, but the sensitivity of these cultures is low, which may reflect difficulties in identifying optimal sampling sites or uneven distributions/low burdens of viable organisms (Clancy and Nguyen 2013). Furthermore, collecting samples from deep-seated sites through surgery or other invasive procedures is often precluded by underlying medical conditions. In keeping with these observations, it was estimated that around 30–50 % of patients with IC are actually not diagnosed by BCs (Clancy and Nguyen 2013).

Cultures take 2–5 days to complete—1–3 days to grow and an additional 1–2 days for identification of the organism (Arvanitis et al. 2014). However, the accurate species identification is pivotal for initiating targeted (though delayed) antifungal treatment (Chandrasekar 2010; Ostrosky-Zeichner 2012), especially when antifungal susceptibility testing (AFST) results are not promptly available (Sanguinetti et al. 2015). It is considerable that the time to identification can now be accelerated through the use of new technologies directly on positive BCs. These include pathogen-specific real-time PCR, peptide nucleic acid fluorescence in situ hybridization (PNA FISH), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Borman and Johnson 2013; Arvanitis et al. 2014).

Despite its well-documented good performance (Lagacé-Wiens et al. 2012; Spanu et al. 2012), MALDI-TOF MS often fails to identify polymicrobial BCs in their entirety. Alternatively, the multiplex PCR-based FilmArray BCID panel, that detects simultaneously 24 microbial pathogens (including 5 *Candida* species, already mentioned in Sect. 1), proved to be accurate not only with monomicrobial BCs (Altun et al. 2013). Using a combined testing with both the methods, approximately 98 % of bloodstream infections were correctly diagnosed during a 17-month period of clinical routine in our lab. Specifically, 65 (5.3 %) of 1223 monomicrobial BCs, that provided unreliable results with the MALDI-TOF MS system, were accurately detected with the FilmArray BCID panel; 9 of 65 microorganisms were *Candida* yeasts. Of note, 153 (89.5 %) of 171 polymicrobial BCs had complete identification results with FilmArray BCID, including all but 1 of 29 BCs that grew *Candida* species together with bacteria (unpublished data). Also, by our approach, the median time to identification was shortened (19.5 h vs 41.7 h with the culture-based reference method; $P < 0.001$), whereas a minimized use of FilmArray BCID led to significant cost savings (unpublished data).

Similarly, the Luminex xTAG fungal assay—a multiplex-PCR Luminex xMAP bead probe fluid array using xTAG analyte-specific reagents—that was developed to detect clinically significant *Candida* species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. lusitaniae*, *C. guilliermondii*), *C. neoformans*, *H. capsulatum*, and *Blastomyces dermatitidis* from BCs, was shown to have sensitivity and specificity of 100 % and 99 %, respectively (Balada-Llasat et al. 2012). As expected, turnaround times with the assay were considerably faster than conventional culture-based identifications, as well as the assay was able to detect mixed *Candida* infections that were observed in 5.8 % of the cases (Balada-Llasat et al. 2012). However, as in its current format the assay takes 5 h to complete, as well as a meticulous technique to prevent cross-contamination in the open system, it is expected that the DNA extraction protocol will be optimized to enhance the usability of the Luminex xTAG fungal assay in clinical practice.

However, both the PNA FISH and multiplex approaches are currently limited by the relatively

low number of fungal species that the systems are designed to detect, with the consequence that infections with uncommon species will be not diagnosed, as well by the inability of the systems to discriminate between cryptic *Candida* species. For example, the inclusion of fungal probes for detecting *Candida dubliniensis, Candida orthopsilosis*/*C. metapsilosis*, and *Candida nivariensis*/*C. bracarensis* would be beneficial since these species can be recovered from BCs and should be distinguished from *C. albicans*, *C. parapsilosis*, and *C. glabrata*, respectively (Cornet et al. 2011).

The growing diversity of infecting species/strain makes the identification of clinical yeasts increasingly challenging. Results from a recent meta-analysis of published articles on the Vitek 2 and other two major systems, namely the AuxaColor and the API ID32C, showed that the accuracy of identification of clinically important yeasts using conventional commercial systems is currently not optimal, particularly for less common species (Posteraro et al. 2015). So, clinical microbiologists should reconsider the clinical utility of these systems, particularly in view of new diagnostic tools such as MALDI-TOF MS (Posteraro et al. 2013).

With the advent of MALDI-TOF MS in the mycological field (Santos et al. 2011), it is now possible to identify all the closely related species within the aforementioned *Candida* species complexes, that, except for *C. dubliniensis*, are predictably not identifiable by any conventional method that is commercially available for routine clinical use. Following the progressive introduction of MALDI-TOF MS in the diagnostic workflow in our laboratory and other European laboratories (Ling et al. 2014), automated systems such as the Vitek 2 are almost exclusively used to perform antimicrobial susceptibility testing of clinical isolates. On the other hand, the PNA FISH assay showed a 100 % agreement with the result of culture-based MALDI-TOF MS identifications on 35 blood samples positive for yeasts (Calderaro et al. 2014).

Apart from the limitation of the growth of organism in culture, the MALDI-TOF MS system, when applied on the isolated yeast, has an indisputable value so as to uniquely compete with other molecular, usually genomic, methods (Borman and Johnson 2013). It is important to note that very few false identifications occur with MALDI-TOF MS systems, as the absence of a match with a suitable spectrum in the reference database leads to log(score) values that are too low to be accepted (<1.7). Thus, a continuous updating of the fungal (yeast) database, by adding the spectra from those same isolates that have been ultimately identified by other methods, greatly enhance the identification capability of MALDI-TOF MS. In this way, recurring to longer and labor-intensive molecular techniques in the clinical routine practice will be restricted to very rare occasions (Posteraro et al. 2013).

2.2 Nonculture Based Diagnostics

Beside to classical techniques, nonculture approaches are noninvasive and promise more rapid diagnosis than conventional approaches (Perfect 2013). Newer nucleic acid (NA)-based or antigen-based diagnostics are able to identify cases of IC, particularly DSC, by detecting *Candida* NA and cellular components (i.e., β-D-glucan [BDG], mannan [MN]) that persist in the blood or that are released from deep fluid or tissue sites. As a major cell wall component of most fungal pathogens—two unique, but notable exceptions are *Mucor* and *Cryptococcus*—the presence of BDG in blood serves as pan-fungal marker for most IFIs, including IC (Marty and Koo 2009). In clinical settings, BDG tests have a sensitivity and specificity of 64–90 % and 73–100 % for detection of candidemia, with very high negative predictive (73–97 %), and there multiple factors that contributed to false-positive results, such as surgical gauze or sponges, administration of immunoglobulin or albumin, hemodialysis, and serious bacterial infections or mucositis, in addition to those recognized for the *Aspergillus* galactomannan (GM) test (e.g., amoxicillin-clavulanate, piperacillin-tazobactam, platelet transfusion, etc.) (Arendrup et al. 2015). In *C. albicans*, biofilm (pathogenic) cells differ from commensal

cells in that they are surrounded by an exopolymeric matrix, also termed extracellular matrix (ECM), that is mostly composed of α-mannan, β-1,6 glucan, and β-1,3 glucan (i.e., BDG) (Taff et al. 2013). Compared to commensals, cells in biofilms produce higher levels of soluble BDG, which can be detected in culture supernatants and in the serum of rats carrying a *C. albicans*-infected central venous catheter (CVC) (Nett et al. 2007). In addition to promote biofilm resistance to multiple antifungals (Taff et al. 2013), glucan modification enzymes (encoded by *BGL2*, *PHR1*, and *XOG1* genes) may play a biofilm-specific role in mediating the delivery and organization of mature ECM (Taff et al. 2012b). Thus, these enzymes may represent potentially attractive targets for detection of, and for therapeutic interventions against candidiasis (Herwald and Kumamoto 2014).

In a recent study, Nguyen et al. (2012) found that the sensitivity of a real-time quantitative PCR assay (ViraCor-IBT) was superior to BDG in diagnosing candidemia and DSC. In addition, both PCR and BDG tests were significantly more sensitive than traditional BCs (the respective sensitivities were 88 %, 62 %, and 17 %; $P = 0.005$ and 0.003 for BCs vs BDG and PCR, respectively), whereas the combination of BC and BDG or PCR had sensitivity of 79 % and 98 %, respectively. According to data from a recent meta-analysis—sensitivity and specificity of BDG for the diagnosis of IC were shown to be 57–97 % and 56–93 %, respectively (Karageorgopoulos et al. 2011)—the serum BDG assay is currently recommended in guidelines from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) (De Pauw et al. 2008) and from the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (Cuenca-Estrella et al. 2012). In another study, the diagnostic accuracy of BDG not only was higher than that of *Candida* score and colonization index (both predictive rules of IC; León et al. 2014), but also BG positivity anticipated the intra-abdominal candidiasis diagnosis (5 days) and antifungal therapy (6 days) (Tissot et al. 2013). Two populations are shown to consistently benefit from BDG testing, namely patients with hematological malignancies and those who have undergone allogeneic hematopoietic stem cell transplants, particularly during episodes of neutropenia (Theel and Doern 2013). Nonetheless, careful dissection of published data reveals certain scenarios (e.g., the ICU) where BDG testing is relevant and may lead to improved patient outcome (Posteraro et al. 2011). With regard to serial testing of high-risk patients, neither the ECIL-3—the 3rd European Conference on Infections in Leukemia guidelines categorized BDG testing as "B II", indicating that there is "moderate evidence to support recommendation for use" in patients with leukemia (Marchetti et al. 2012)—nor the EORTC/MSG provides BDG timing or interval testing guidelines. Intriguingly, a recent meta-analysis of cohort studies from the ECIL-3 reported a diagnostic odds ratio of 111.8 vs 16.3 for the presence of IFDs in hematological patients following two consecutively positive BDG results compared to a single positive BDG result (Lamoth et al. 2012). This meta-analysis also reported a pooled sensitivity of 49.6 %, alongside a PPV and NPV of 83.5 % and 94.6 %, respectively. Nevertheless, despite the strong NPV, and the consistently low sensitivity reported among studies, a negative BDG test cannot exclude the diagnosis of IFD, as well the value of BDG as a screening test is questionable (Theel and Doern 2013). In the last context, findings from a very recent meta-analysis of prospective cohort studies suggest that the BDG assay has high sensitivity and specificity for discriminating between patients with and without IFD, but that, in clinical practice, BDG assay results should be evaluated together with clinical and microbiological (e.g., other surrogate marker (s)) findings (Hou et al. 2015).

While NA amplification (i.e., PCR) techniques are still lacking standardization (Alanio and Bretagne 2014), commercial tests are also available for measurement of MN, anti-mannan antibody (AMN), and Cand-Tec

Candida antigen. Of the four (including BDG) tests compared in one study, BDG and the combinations of BDG plus MN and of MN plus AMN showed the highest sensitivity (Held et al. 2013). Finally, the *C. albicans* germ tube antibody (CAGTA) assay, that detects antibodies against the surfaces of *C. albicans* germ tubes by indirect immunofluorescence, was shown to be helpful in diagnosing candidemia associated with deep-seated infection (Martínez-Jiménez et al. 2014). In an effort to establish the place of CAGTA test in routine clinical practice, a new diagnostic tool for IC that was based on positive CAGTA and BDG assays was introduced by León et al. (2012). Later, the same group evaluated the performance of BDG and CAGTA for the diagnosis of IC in a prospective cohort of 107 unselected, non-neutropenic ICU patients, and showed that two consecutive BDG levels $\geq$80 pg/mL allowed discrimination among IC and high-grade colonization (Martín-Mazuelos et al. 2015). Furthermore, the best combinations found to diagnose candidemia in 31 patients were CAGTA and BDG using cutoffs of 1/80 and 80 pg/mL, respectively (sensitivity 96.8 % and specificity 84 %), and CAGTA and MN using cutoffs of 1/80 and 75 pg/mL, respectively (sensitivity 93.5 % and specificity 86.0 %) (Martínez-Jiménez et al. 2015a). An approach to improve the performance of antigen assays is the combination with other diagnostic tools. One example is a study by us, who used BDG measurement together with the *Candida* score in 14 IC patients and increased the sensitivity from 92.9 to 100 % (Posteraro et al. 2011). However, this gain in sensitivity came at the price of a loss of specificity from 93.7 to 83.5 %. Similarly, Levesque et al. (2015) showed that, on the day the clinical diagnosis of IC was made after liver transplantation, the combination of two sequential BG-positive samples (>146 pg/mL) and a colonization index of $\geq$0.5 resulted in sensitivity, specificity, PPV, and NPV of 83 %, 89 %, 50 %, and 97.6 %, respectively. Interestingly, the BDG assay, used in combination with the CAGTA test, provided NPV as high as to justify the use in the therapy decision-making process and the discontinuation of empirical antifungal therapy (Martínez-Jiménez et al. 2015b).

The primary testing goal should be that of identifying patients early in the course of IC. To this regard, a novel technology, based on the application of T2 magnetic resonance (T2MR), was exploited to develop an automated instrument platform, T2Dx, which provides a 'patient sample-to-answer' clinical diagnostic test, namely the T2Candida (Pfaller et al. 2015). In this FDA-cleared test, whole blood is subjected to PCR amplification of *Candida* sequences, followed by hybridization to nanoparticles that elicit a T2MR signal to detect *Candida* species—this without the need for culture or nucleic acid extraction steps. Exciting results from a preliminary study of the T2MR method showed that it is able to reduce the time to result to an average of 2 h, in contrast to the 48-h average of BCs (Neely et al. 2013). Next, in a clinical trial, the T2MR method was found to have an overall sensitivity per patient of 91.0 %, with a mean time to species identification of only 4.4 h, compared to the 2–5 days typically needed by the automated BC systems (Mylonakis et al. 2015). Across all studies to date, T2Candida has an overall specificity of >99.4 % from >1560 patients, and 12 proven cases of IC have been detected with T2Candida in patients with negative BCs (Pfaller et al. 2015). As the 5 target *Candida* species of the T2MR method account for the majority of cases of candidemia (Guinea 2014), and have distinct antifungal susceptibility profiles (Miceli et al. 2011), the rapid speciation allowed by this method may drive the appropriate choice of antifungal therapy. In 5000-patient Monte Carlo simulations, the average time to initiation of antifungal therapy was 0.6 ± 0.2 days for T2Candida (used for detection and species-level identification directly from whole blood) vs 2.6 ± 1.3 days and 2.5 ± 1.4 days, respectively, when either PNA FISH or MALDI-TOF MS methods were used to identify *Candida* species from positive BCs (Aitken et al. 2014).

3 Diagnostics for *Aspergillus* Infections

3.1 Culture Based Diagnostics

As with IC, proven diagnosis of IA is usually made by demonstrating fungal hyphae in tissue biopsy specimens (De Pauw et al. 2008). However, one of major problems to the regard is obtaining tissue for standard histopathological analysis, because of the contraindication to perform biopsy in severely ill patients, who could be at risk of bleeding due to thrombocytopenia (Chandrasekar 2010; Ostrosky-Zeichner 2012). Even when tissue is available, several filamentous fungi appear morphologically identical at the microscopic observation (Perfect 2013), so that uniform, septate, narrow-angle-branching hyphae of *Aspergillus* are undistinguishable from those of the pathogenic *Fusarium* or *Scedosporium* (Cuenca-Estrella et al. 2011). Also with the pathogenic *Mucorales*—unique features to these fungi are broad, sparsely septate, ribbon-like hyphae, with open angle (90 °) branches—morphology of fungi in tissue is not definitive criterion because it can be difficult for even experienced pathologists (Lanternier et al. 2012). Nevertheless, histopathology allows for both the tissue invasion by fungi and the host response or tissue necrosis to be detected, as well as for clarifying if a positive culture is the result of infection, colonization, or contamination (Arvanitis et al. 2014).

In general, the sensitivity of culture for the diagnosis of aspergillosis is low, and *Aspergillus* species are almost never recovered in BCs even in disseminated disease (Chandrasekar 2010; Ostrosky-Zeichner 2012). In two large multicenter surveillance studies, among transplant recipients with a positive molecular test for IA, only 25–50 % had a positive culture result (Neofytos et al. 2009; Kontoyiannis et al. 2010). However, one study, published in 2005, showed that incubation of cultures from *Aspergillus*-infected tissue specimens at 35 °C (in room air) led to a 31 % increase in sensitivity compared to incubation at 25 °C, suggesting that the yield of cultures may improve under laboratory conditions that mimic the physiological temperature (Tarrand et al. 2005). In agreement with these observations, it was recently argued that the fungal yield from sterile or non-sterile sites—specimens usually analyzed include sputum, bronchoalveolar lavage (BAL) fluid, aspirates from lesions, cerebrospinal fluid (CSF), and other tissues—may at least in part depend on the suboptimal sampling or the unstandardized specimen processing in the routine clinical microbiology laboratory (Arendrup et al. 2015). As specified in the aforementioned guidelines (see Sect. 1), factors argued to improve respiratory fungal diagnostics include (i) rapid, direct microscopy with optical brighteners such as Calcofluor white and Blankophor, (ii) increasing the volume of clinical specimen plated, (iii) use of both selective and nonselective media, and (iv) prolonged incubation of primary culture plates (Hamer et al. 2006; Fraczek et al. 2014; Arendrup et al. 2015). Most stains are inexpensive and can be performed easily in various specimens, as well as microscopy performed in conjunction with cultures improves their positive predictive value by confirming positive culture results (Arvanitis et al. 2014). Although contamination of clinical specimens by *Aspergillus* may occur (Burco et al. 2012; Kanamori et al. 2015), positive culture results, especially when repetitive and associated with positive smear results, are strongly suggestive of aspergillosis (Arvanitis et al. 2014).

Despite being still essential, cultures provide results after too long a delay for optimal IFI management (Ostrosky-Zeichner 2012). Species identification may take many days, especially with fungi that are slow to sporulate, which may delay the selection of the appropriate antifungal (Van Der Linden et al. 2011). However, while accurate identification of the infecting pathogen remains a fundamental step in the mycological diagnostic process also for aspergillosis, the differentiation to the species level within the *Aspergillus* species complexes is very problematic, at least with traditional macroscopic and microscopic analyses (Balajee

et al. 2007). Although a polyphasic approach (Samson and Varga 2009)—it combined phenotypic (morphology and extrolite profiles) and molecular (e.g., ITS, calmodulin, β-tubulin, actin) characters—should be used, a robust *Aspergillus* species delineation is possible simply using molecular methods (Balajee et al. 2009b). In this end, partial β-tubulin or calmodulin gene sequences are currently utilized to identify a species within a given *Aspergillus* species complex (Balajee et al. 2009a), because conventional loci—the nuclear ribosomal ITS regions (ITS1, 5.8S rRNA, and ITS2)—are not variable enough to allow resolution of closely related species of fungi (Borman and Johnson 2013). So, using these additional loci, a number of cryptic (or sibling) species—morphologically indistinguishable from each other but separable with only DNA-based molecular methods (Hawksworth 2006)—are nowadays identified within *Aspergillus* section *Fumigati*, and it is relevant in view of that some species have in vitro antifungal susceptibility profiles which differ significantly from that of *A. fumigatus* sensu stricto (Van Der Linden et al. 2011).

As with *Candida* species, the time to identification for colony-growing aspergilli from clinical specimens can be considerably shortened using MALDI-TOF MS. However, unlike yeasts, MALDI-TOF MS-based identification of filamentous fungi, including aspergilli, fusaria, *Mucorales*, and dermatophytes is more challenging (Posteraro et al. 2013). Inconclusive MALDI-TOF MS results often occur due to discrepancies between the methods used for routine testing and for spectral database construction, and several research groups have worked to develop and validate sample preparation protocols and fungus-specific databases (Sanguinetti and Posteraro 2014). Since early efforts by us (De Carolis et al. 2012) and others (Alanio et al. 2011) to date, two clinically comprehensive mold databases have been described, one comprising 294 reference spectra—which correspond to 294 strains from 152 species (Lau et al. 2013)—and the other one comprising 2832 reference spectra (four replicas for each strain)—which correspond to 708 strains from 347 species of molds (Gautier et al. 2014). In our study (De Carolis et al. 2012), using water suspensions of fungal mycelia and/or conidia and a home-made reference database encompassing spectra from *Aspergillus* (33 species), *Fusarium* (12 species), and *Mucorales* (10 species), MALDI-TOF MS accurately identified 91 (96.8 %) of 94 fungal isolates tested. Interestingly, the log(score) values of the 91 isolates with correct results were all higher than 2.0; the remaining 3 isolates (1 *Emericella nidulans*, 1 *Aspergillus niger*, and 1 *Aspergillus versicolor*) could be identified only to the genus level, as their log(score) value was lesser than 2.0 (1.817, 1.874, and 1.796, respectively), but had concordant species designations as compared with the multilocus sequence analysis results. In another study (Alanio et al. 2011), using a chemical (acid formic and acetonitrile) extraction of the fungal colonies and a database built with the reference spectra from 146 mold strains, mold isolates obtained from sequential clinical samples were prospectively analyzed. Eighty-seven percent (154/177) of isolates, including 86 *A. fumigatus*, 38 other aspergilli (9 species), and 53 other molds (23 species), were identified to the species level, whereas the MALDI-TOF MS-based approach failed in 12 % (21/177) of isolates belonging to species not represented in the reference library. Although the methods employed by investigators at each single center proved to work well in their own evaluations, multicenter studies are necessary for the standardization of MALDI-TOF MS use into routine mold identification.

3.2 Nonculture Based Diagnostics

The development and validation of newer diagnostic approaches are currently in the spotlight of scientific research, but the lack of a reliable gold standard to be used as a comparator makes very difficult to estimate the performance of a new diagnostic test for IA (Arvanitis and Mylonakis 2015). For most trials and guidelines, this problem has been in part circumvented by using the aforementioned EORTC/MSG criteria (De Pauw et al. 2008), which divide the patients suspected

to have IA into four categories—proven, probable, possible, and unlikely IA—based on risk factors, radiological, and microbiological criteria. However, an unavoidable number of patient cases classified as possible IA, due to the absence of one of the microbiological criteria that define a probable IA case, will in fact have the disease, and their misclassification will in turn lead to erroneous calculation of the performance estimates of a diagnostic test in the development (Arvanitis and Mylonakis 2015).

Among the newer molecular and serologic tests for IA, some of them such as GM and BDG are now widely used in clinical practice, while others such as the *Aspergillus* PCR have yet to be implemented in clinical laboratories (Arvanitis et al. 2014). As a component of the cell wall of *Aspergillus* spp. (Leeflang et al. 2008), the GM antigen can be detected in clinical specimens, and the serum and BAL GM tests, both using the Platelia GM enzyme-linked immunoassay, were approved by the FDA, and are incorporated into clinical guidelines (see Sect. 1) and routinely used for discriminating between patients with possible or probable IA (De Pauw et al. 2008). False-positive results have been reported for patients receiving piperacillin-tazobactam antibacterial agents (Viscoli et al. 2004), although this association seems to be now confuted (Mikulska et al. 2012). While the sensitivity and specificity of GM on serum—using 0.5 optical density (OD) index as the cutoff for positivity—was estimated to be 79 % and 81–86 %, respectively, in two large meta-analyses (Leeflang et al. 2008; Pfeiffer et al. 2006), GM performed on BAL was shown to have a pooled sensitivity and specificity of 85 % and 94 %, respectively, in an earlier meta-analysis of studies that used an OD index of 1 (Guo et al. 2010), and, impressively, of 92 % and 98 %, respectively, in a more recent meta-analysis of studies that used an OD index of 1.5 (Heng et al. 2015). In that study, comparing serum GM and *Aspergillus* PCR testing on BAL fluid, BAL-GM conferred greater sensitivity, but lower specificity than the serum GM test, and similar specificity as the PCR assay (Heng et al. 2015). With regards to the BDG assay, in the aforementioned meta-analysis by Karageorgopoulos et al. (2011), the pooled sensitivity (75.6 %) and specificity (85.3 %) among patients with IFIs (including IA) did not differ significantly in the subgroup of studies that evaluated the performance of the assay in IA. Unfortunately, unlike GM, the BDG test cannot differentiate between IFIs due to *Aspergillus* spp. and IFIs due to other fungi (Marty and Koo 2009).

As a potential alternative to the Platelia GM assay (Arvanitis and Mylonakis 2015), a lateral flow device (LFD) that uses the JF5 monoclonal antibody was shown to detect a glycoprotein antigen in the serum and BAL of patients with IA, holding promise to be used as rapid point-of-care test for IA (Thornton 2008). In an early trial (White et al. 2013), the performance of this LFD was compared to real-time PCR (targeting the 28S rRNA gene) and GM detection on serum from an EORTC/MSG-defined hematological population. In a proven/probable-IA population versus a no-IA population, the LFD performance parameters were comparable to those of both PCR and GM, with a sensitivity and specificity of 81.8 % and 98 %, respectively (White et al. 2013). In combination with PCR, the LFD provided both 100 % sensitivity and 100 % specificity, besides to being easy to use and to allowing for rapid testing of easily obtainable specimens (White et al. 2013). Likewise, a small pilot study conducted at two university hospitals in Austria and Germany, on BAL fluid samples from 78 patients at risk for invasive pulmonary aspergillosis (IPA), showed that the combination of the GM assay and PCR or, if PCR is not available, the LFD test, allows for sensitive and specific diagnosis of IPA (Hoenigl et al. 2012). Furthermore, in a recent prospective study of 221 patients with underlying respiratory diseases (and without hematological malignancy or previous solid organ transplantation), BAL LFD for IA provided sensitivity and specificity (77 % and 92 %, respectively) that differed markedly from those of GM (OD index 0.5, 97 % and 81 %, respectively; OD index 1.0, 97 % and 93 %, respectively; OD index 3.0, 61 % and 99 %, respectively), BDG (cutoff

80 pg/mL, 90 % and 42 %, respectively; cutoff 200 pg/mL, 70 % and 61 %, respectively), and mycological culture (29 % and 97 %, respectively) (Prattes et al. 2014). These findings were confirmed by a more recent semiprospective multicenter study that evaluated the performance of the LFD test for BAL IPA detection in 133 critically ill patients. Sensitivity and specificity were 80 % and 81 %, respectively, that contrasted with a sensitivity of 50 % and a specificity of 85 % exhibited by the fungal BAL culture, thereby suggesting that LFD may be a reliable alternative for IPA diagnosis in ICU patients if GM results are not rapidly available (Eigl et al. 2015).

Primarily due to the lack of standardization, PCR for the diagnosis of IA has shown in individual studies sensitivity and specificity which ranged from 43 to 100 % and 64 to 100 %, respectively, and from 36 to 100 % and 70 to 100 %, respectively, depending on whether serum and whole blood or BAL fluid were tested (Arvanitis et al. 2014). According to this, the results from two comprehensive meta-analyses of studies where PCR tests were performed on blood specimens showed a pooled sensitivity and specificity of 84–88 % and 75–76 %, respectively, which led to conclude that PCR is unable on its own to confirm or exclude IA (Mengoli et al. 2009; Arvanitis et al. 2014). A multicenter effort was recently completed by the European *Aspergillus* PCR Initiative (EAPCRI) to provide guidelines and specific recommendations, that should improve the in vitro performance of *Aspergillus* spp. PCR using whole blood and serum specimens (White et al. 2010; White et al. 2011). Likewise, when PCR was used to detect the *Aspergillus* spp. DNA in BAL specimens, this test exhibited a pooled sensitivity and specificity of >90 % in two recent meta-analyses (Sun et al. 2011; Avni et al. 2012). Excluding BAL, it is clear that, if considering whole blood and serum PCR equivalent in terms of performance, the serum PCR might be used preferentially, as serum is easier to process and allows for other serological tests to be performed at the same time (Arvanitis and Mylonakis 2015). However, a very recent study by the EAPCRI was conducted with the aim to evaluate if previous recommendations for serum are also applicable to plasma PCR and to compare the analytical performance of plasma PCR with that of serum PCR (Loeffler et al. 2015). This study showed that, despite the absence of major differences in the molecular processing of serum and plasma, the formation of clot material potentially reduces available DNA in serum. Interestingly, in a companion study by the EAPCRI (White et al. 2015), clinical performance and clinical utility (time to positivity) of the standardized *Aspergillus* PCR were calculated for both plasma and serum specimens. These specimens were concomitantly obtained from hematological patients in a multicenter retrospective anonymous case-control study, with cases diagnosed according to the EORTC/MSG criteria. While confirming the analytical finding that the sensitivity of *Aspergillus* PCR using plasma (94.7 %) is superior to that using serum (68.4 %), it was shown that PCR positivity occurred earlier when testing plasma, providing sufficient sensitivity for the screening of IA (White et al. 2015).

4 Biofilm Production in Fungal Isolates

It is estimated that 80–90 % of hospital-acquired bloodstream and deep tissue infections are associated with the use of indwelling medical devices such as CVC and prosthesis (Chandra et al. 2014). Most cases of central line-associated BSI (CLABSI)—that occur, in the United States, at a rate of 1.5 per 1000 CVC days and a mortality rate of 12–25 %—involve microbial biofilms on the catheter surfaces (Yousif et al. 2015). Once a mature biofilm is formed within the human host through a medical device, the infection becomes recalcitrant to antimicrobial treatment (Ramage et al. 2012) and can develop into a chronic condition (Yousif et al. 2015). Although catheter removal is the conventional management of catheter-associated fungemia (Walraven and Lee 2013), salvaging the catheter can often help to minimize mechanical complications that the procedure itself carries; so, many techniques

have been used to prevent the biofilm formation by targeting different stages of biofilm maturation (Yousif et al. 2015).

A plenty of studies reproducing a fungal sessile community in vitro has allowed not only to study the development of a biofilm, its interaction with other microorganisms and the environment, and its susceptibility to available antifungal agents, but also to search for new therapies (Williams and Ramage 2015). In the meantime, these studies have revealed biofilm features which led to the development of several methodologies strategies (Williams and Ramage 2015). For example, published work indicates that biofilms grown in microtiter dishes do develop some properties of mature biofilms, such as antibiotic tolerance and resistance to immune system effectors (Ramage et al. 2012).

Two methods are mostly used to quantify biofilm production by fungal isolates in vitro (Costa et al. 2013). One method quantifies biomass production using crystal violet stain (CV) (O'Toole 2011) and the other one, the metabolic activity of viable embedded biofilm cells based on reduction of tetrazolium salt XTT to formazan dye XTT (Pierce et al. 2008). However, poor agreement between the both methods was observed in earlier studies, which were performed exclusively on *C. albicans* isolates and with a small number of isolates (Alnuaimi et al. 2013; Taff et al. 2012a). Furthermore, as variability in biofilm formation can be observed among *C. albicans* isolates, the categorization of isolates based on this can be used to predict how pathogenic the isolate will behave clinically (Sherry et al. 2014). By measuring the biomass production by CV and the metabolic activity by XTT, Marcos-Zambrano et al. (2014) studied 577 yeast isolates causing fungemia and established tentative cutoff values to classify the isolates as being low (<0.097), moderate (0.097–0.2), or high (>0.2) biofilm-forming—LBF, MBF, or HBF, respectively—and as having low (<0.44), moderate (0.44–1.17), or high (>1.17) metabolic activity—LMA, MMA, or HMA, respectively. Consistent with the findings by scanning electron microscopy analysis, LBF strains had less tick and compact structures, whereas MBF and HBF strains had dense structures which covered the whole surface of the assay discs. When the procedures were compared, species-specific biofilm production patterns were noted among the isolates, with an overall categorical agreement between the procedures of only 44 %. Interestingly, all the four *Candida* species—*C. albicans*, *C. parapsilosis*, *C. glabrata*, and *C. tropicalis*—known to be the most common causes of catheter-related BSI, produced biofilms with high biomass or high metabolic activity, suggesting that both the features may play an important role in colonization (Marcos-Zambrano et al. 2014). Finally, the authors showed that XTT and CV can be used as complementary procedures for studying biofilm production, and they claimed the necessity of studies that investigate the outcome of patients infected by HBF or HMA isolates of pathogenic yeasts (Marcos-Zambrano et al. 2014).

With regards to *Aspergillus*, the well-established behavior of the fungus to form biofilm in vitro (Mowat et al. 2007, 2008; Seidler et al. 2008) was shown to correlate with localized and invasive forms of aspergillosis, which are both characterized by the mycelial development of *A. fumigatus* that arises from inhaled conidia (Loussert et al. 2010; Müller et al. 2011). In addition, *Aspergillus* species have been reported to cause biomaterial-related biofilm infections, which involve catheters, cardiac pace makers, heart valves, and joint replacements (Williams and Ramage 2015). Like in *C. albicans* biofilms, the ECM that surrounds the intricate hyphal network of *A. fumigatus* (Beauvais et al. 2007) in either IA or aspergilloma may account for the resistance of the fungus against phagocytic and antimicrobial attacks (Ramage et al. 2012).

Clinical biofilm-associated infections are difficult to diagnose (and treat), but the differences that exist between planktonic and sessile microbial cells could be exploited into the search for new diagnostic targets. While advanced molecular in situ or imaging techniques may be effective to demonstrate biofilms in vivo, older immunohistochemical or immunofluorescent techniques, that utilize specific polyclonal or monoclonal

sera, represent a still useful tool to targeting pathogens in host tissues (Hall-Stoodley et al. 2012). However, use of these antibodies is often limited by that they are thought to bind to the ECM non-specifically and by almost overall lack of commercially available antibodies specific for fungal pathogens (Loussert et al. 2010; Hall-Stoodley et al. 2012). In a study (Bugli et al. 2014), we generated an anti-gliotoxin mouse polyclonal antibody that was used, in an immunofluorescent assay, for detecting the mycotoxin in *A. fumigatus* biofilms. This assay proved to be able to demonstrate *A. fumigatus* gliotoxin not only on the hyphae cultured onto the human lung epithelial cell line A549, but also, directly, on the hyphae into respiratory tract specimens or lung tissues from patients with IA.

5 Antifungal Susceptibility Testing Assays

5.1 Planktonically-Growing Isolate Testing

Two standardized microdilution-based procedures by the Clinical and Laboratory Standards Institute (CLSI 2008a, 2008b) and the European Committee on Antibiotic Susceptibility Testing (Arendrup et al. 2012b, 2014b) are universally accepted for performing AFST, but these procedures are complex, time-consuming, and not intended for routine use (Alastruey-Izquierdo and Cuenca-Estrella 2012).

Following a multistep process based on the analysis of MIC distribution curves for wild-type populations and the clinical relationship between MIC values and efficacy, CLSI/EUCAST MIC breakpoints (i.e., obtained with the aforementioned reference methods in specialized mycology laboratories) are, to date, available to interpret the AFST results of amphotericin B, azoles, and echinocandins for *Candida*, and amphotericin B and azoles for *Aspergillus* (Cuenca-Estrella 2014). Therefore, besides to be an important step in establishing fungal clinical breakpoints (CBPs), the MIC distributions of wild-type fungal populations provide a measure of the epidemiological cutoff values (ECVs). These values, in the absence of specific CBPs, may be very useful in antifungal resistance surveillance to monitor the emergence of resistant isolates (i.e., those with gene mutations associated with reduced therapeutic responses) (Pfaller 2012). In particular, while breakpoints are not yet available for the CLSI, the EUCAST CBPs have been established for *A. fumigatus* (and for some *Aspergillus* species) for the majority of the licensed antifungal agents (Arendrup et al. 2012c; Hope et al. 2013). Therefore, EUCAST testing is somewhat more appealing for guiding antifungal therapy (Arendrup et al. 2015), whereas both the EUCAST and CLSI reference methods are equivalent for epidemiological surveillance purposes (Arendrup et al. 2013; Pfaller et al. 2009).

However, commercial techniques such as the Vitek 2 yeast susceptibility test, Etest, and Sensititre YeastOne are currently approved by the US FDA for clinical testing (Posteraro and Sanguinetti 2014). These techniques, all easy-to-use modifications from the CLSI/EUCAST reference methods, show a good essential agreement (defined as MICs within 2 dilutions) with the reference methods, but the categorical agreement (i.e., agreement in categorizing an isolate as susceptible, intermediate, or resistant) may be lower, especially for the echinocandin class of antifungal agents (Pfaller et al. 2012; Arendrup and Pfaller 2012; Astvad et al. 2013). Thus, *Candida* isolates with MIC values around or above the breakpoints should be referred for reference testing, particularly for isolates with possible echinocandin resistance which is emerging (Arendrup and Perlin 2014).

As MIC determination is strongly recommended for the patient management (Cuenca-Estrella 2014), epidemiological surveys of deep, blood, and mucosal infections should be done to monitor antifungal susceptibilities of *Candida* and *Aspergillus* isolates. Therefore, it is mandatory that the laboratories that serve patients at risk of IFIs are able to perform AFST for *Candida* and to screen for azole resistance in *A. fumigatus*. In this end, a multi-dish

azole-agar dilution plate (itraconazole 4 μg/mL, posaconazole 0.5 μg/mL, voriconazole 1 μg/mL) was developed as a screening test for identifying potentially resistant *A. fumigatus* isolates (Howard and Arendrup 2011). While this test can be performed directly from primary plates in parallel with the subculturing of the fungus, in order to provide an early indication of possible azole resistance (Astvad et al. 2014), it is important that more than one colony are eventually tested to take into account the coexistence of susceptible and resistant isolates (Arendrup et al. 2015). As up to 10 % of the *A. fumigatus* isolates will be positive at the azole-plate screening (Escribano et al. 2012), isolates need to be confirmed as resistant by means of AFST reference method and also by *cyp51A* gene sequencing (Astvad et al. 2014).

Finally, through the AFST methods coupled with detection of molecular fungal alterations conferring reduced antifungal drug susceptibility (Perlin 2009), often directly from clinical specimens (Denning et al. 2011; Zhao et al. 2013) it is now possible to ensure a close antifungal resistance surveillance in many clinical settings. In most IA cases, the detection of *cyp51A* gene mutations in primary clinical specimens may be the sole strategy for detecting *Aspergillus* resistance to (tri)azoles due to the absence of culture growth, which makes an MIC determination impossible (Verweij et al. 2009; Howard and Arendrup 2011). However, NA-based assays, though allowing for quicker detection of azole-resistance in culture positive samples, are to date not standardized or practical for most clinical laboratories (Cuenca-Estrella 2014), besides to be unable to reveal the influence from other resistance mechanisms (Verweij et al. 2009; Howard and Arendrup 2011).

Novel assays, such those based on flow cytometry or MALDI-TOF MS are upcoming tools for AFST (Posteraro and Sanguinetti 2014). Based on short-time antifungal drug exposure of fungal isolates, these assays could provide a reliable means for quicker and sensitive assessment of AFST, through the determination of new endpoints in alternative to the classical MIC. Whether these endpoints held the promise to potentiate the practicability and the clinical utility of AFST, it needs to be determined in future studies that are specifically aimed at better defining reproducibility and standardization of these advanced AFST assays.

5.2 Sessile/Biofilm-Growing Isolate Testing

As aforementioned, cells in biofilms display phenotypic properties that are radically different from their planktonic counterparts, including their resistance to antimicrobial agents (Ramage et al. 2012). Many catheter-associated infections are related to intraluminal biofilms, which are notoriously difficult to treat, as biofilm-related (sessile) MICs (SMICs) are often dramatically elevated than MICs in planktonic (non-biofilm) form (Tobudic et al. 2012). Several methods for fungal susceptibility testing under biofilm growth conditions have been developed (Ramage and López-Ribot 2005; Pierce et al. 2010; Sternberg et al. 2014; El-Azizi et al. 2015) and validated in a variety of studies published recently (Tobudic et al. 2012). In general, these methods rely upon the XTT-based approach that provides the most reproducible, accurate, and easy modality for the antifungal drug testing of fungal biofilms (Pierce et al. 2008; Nett et al. 2011). In the static model, fungal biofilms formed into 96-well microtiter plates can be directly used for the biofilm AFST assay. The readout of this assay is colorimetric, based on the reduction of XTT by metabolically active fungal biofilm cells. A typical experiment takes approximately 48 h, of which 24 for biofilm formation and an additional 24 for AFST (Pierce et al. 2010). In a catheter continuous flow model, *C. albicans* biofilm can be eradicated by liposomal amphotericin B at the MIC, as documented by a 20 % reduction in the growth of hyphal network that was observed after 24-h biofilm formation and a 24-h treatment with the drug, in comparison with the untreated control (Seidler et al. 2010). In our study (Fiori et al. 2011), in vitro activities of anidulafungin

and caspofungin against biofilms of *Candida* and *Aspergillus* clinical isolates were evaluated and compared with those of the older antifungals amphotericin B, fluconazole (only for *Candida* isolates), and voriconazole. The MICs and MECs were recorded after incubation for 24–48 h, while the SMICs were determined by the 96-well microtiter assay. We found that *Candida* species exhibited high $SMIC_{90}$s against fluconazole, voriconazole, and amphotericin B, whereas the anidulafungin $SMIC_{90}$s were very low, as were those for caspofungin. In comparison, *Aspergillus* species exhibited higher $SMIC_{90}$ not only against amphotericin B and voriconazole but also against the echinocandins.

In a more recent study (Rajendran et al. 2015), *C. albicans* LBF and HBF were tested for their response to voriconazole, amphotericin B, and caspofungin at low (clinically relevant; 2 mg/L) concentration and high (potentially useful in antifungal lock therapy; 200 mg/L) concentration. The results show that LBF and HBF isolates were significantly different in susceptibility to voriconazole and caspofungin at both 2 mg/L ($P < 0.05$) and 200 mg/L ($P < 0.001$), whereas amphotericin B was shown to be equally effective against LBF and HBF isolates, although a significant difference was found between 2 and 200 mg/L (HBF, $P < 0.0005$; LBF, $P < 0.005$). This study would reinforce the concept that the biofilm phenotype represents a significant clinical entity, and that isolates with this phenotype differentially respond to antifungal therapy based on in vitro evidence (Rajendran et al. 2015).

6 Concluding Remarks/Future Perspectives

Despite newer and innovative approaches, the diagnosis of fungal infections related to biofilms, most notably those caused by the *Candida* and *Aspergillus* genera, remains difficult. This is particularly because of challenges in identifying the presence of a fungal biofilm in vivo, either on both hard and soft tissue or upon implanted medical devices. As for biofilm-associated bacterial infections, there is the need for novel diagnostic methods that targeting specifically biofilm molecules can provide a direct proof of the real presence of a biofilm on the site of infection. In the meantime, removal or replacement of medical devices, where appropriate, represents the first line in clinical management, followed by antifungal management, of biofilm infections. While the treatment with conventional antifungal agents, such as liposomal amphotericin B and echinocandins, is fundamental, experimental evidences show that it is possible to enhance antifungal activity through ECM degrading molecules, natural compounds, and microbially derived substances. In addition, our understanding of the processes underlying the adaptive drug resistance within the biofilm has shed light on specific molecules that could be potentially useful as therapeutic targets. Further progress and refinement of diagnostic and therapeutic approaches will be expected, in the next future, in order to provide a reliable means of successfully managing fungal biofilms of clinical importance.

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Disinfectants to Fight Oral *Candida* Biofilms

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Abstract

Oral biofilms, especially those caused by oral mycobiota, which include *Candida* species, are very difficult to eradicate, due to their complex structure and recalcitrance. Moreover, the mouth is prone to be colonized since it presents different types of surfaces, especially biomaterials and dental implants, often associated with a high rate of infections. Therefore, although disinfection of the oral cavity is of major importance, the number of commercially available disinfectants is not high. However, new solutions, as silver nanoparticles are being developed to help oral biofilms' eradication.

1 Introduction

The oral cavity is one of the most complex cavities of the human body, comprising hundreds of different bacterial, viral, and fungal species. The complexity of the oral cavity is in fact increased by the high number of different surfaces that can co-exist there, namely teeth, gingival sulcus, tongue, cheeks, hard and soft palates and medical devices (prosthetic devices or implants). In addition to the oral specific environment (variations on pH and sugar concentration, etc.), biomaterials are very prone to infections, specially due to its heterogeneous composition (from ceramics to metals) and structure (from very smooth, as the crown surface, to very rough, as the implant). These infections can be less complex as caries, periodontitis and thrush or can evolve to severe diseases, as endocarditis or glomerulonephritis.

Several microorganisms can be found in the oral cavity, including the well-known bacteria, such as streptococci, lactobacilli, staphylococci and corynebacteria, as well as fungi. *Candida* species belong to the latter and are the most frequent (isolated from 75 % of participants), followed by *Cladosporium* (65 %), *Aureobasidium*, *Saccharomycetales* (50 % for both), *Aspergillus* (35 %), *Fusarium* (30 %) and *Cryptococcus* (20 %). Moreover, four of these predominant genera are known to be pathogenic in humans (Ghannoum et al. 2010). So, the

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mycobiota is a medically important component of the oral microbiota, since opportunistic fungal infections commonly afflict the oral mucosa, being of special importance on immunocompromised hosts.

2 Oral Candida Species' Biofilms

The genus *Candida* contains over 200 different species that are ubiquitously distributed. However, only a few of these have been implicated in human oral infection (Calderone 2002). Oral candidoses have been recognized throughout human history and are often described as being 'the disease of the diseased', reflecting the opportunistic pathogenic nature of *Candida*. Whilst *Candida* species are generally regarded as harmless members of humans, infection can arise if a colonized individual becomes immunocompromised (Silva et al. 2012). The most prevalent *Candida* species recovered from the human mouth, in both commensal state and cases of oral candidoses, is *Candida albicans* (Pfaller and Diekema 2007; Samaranayake et al. 2009). However, the so–called non-*Candida albicans Candida* (NCAC) species are increasingly recognized as important agents of oral infections (Pfaller and Diekema 2007). In terms of oral prevalence, *C. albicans* is followed by *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* (Weems 1992; Williams et al. 2011). The apparently increased involvement of NCAC species in oral candidoses may partly be related to improvements in diagnostic methods, such as the use of primary agars with ability to differentiate species, and the introduction of molecular techniques in the routine diagnosis of fungaemia (Liguori et al. 2009). However, the increased prevalence of NCAC species in disease could also be a reflection of the inherently higher level of antifungal drug resistance in some NCAC species (González et al. 2008) compared to *C. albicans*, as this would promote their persistence in mixed-species infections treated with traditional antifungal agents. *Candida* species virulence has been attributed to their ability to form structured aggregates of cells called biofilms (Silva et al. 2012). *Candida* biofilms can play a significant role in clinical oral infections because of their resilience and resistance to normal host removal mechanisms and also antifungal therapy. In general, for *Candida* species, biofilm formation occurs as a result of a sequence of events: (i) adherence/colonization to the surface; (ii) cell proliferation/invasion and well organized colony formation; (iii) matrix production with differentiation into a mature three-dimensional structure consisting of yeast, pseudohyphae, and/or hyphae embedded within extracellular matrix and finally (iv) detachment of biofilm cells to promote colonization and infection of distal sites (Seneviratne et al. 2008; Silva et al. 2012). Involvement of *Candida* biofilms in human oral infections is well recognized, particularly when occurring on biomaterials, such as acrylic and metals, used for implanted medical devices. Silva et al. (2009) confirmed that NCAC clinical isolates, as it is described for *C. albicans* (Chandra et al. 2001), are also able to produce biofilms, although these were less extensive for *C. parapsilosis* comparatively to *C. tropicalis* and *C. glabrata* (Fig. 1a) (Silva et al. 2009).

As it is well known, biofilms' formation on denture surfaces is promoted by poor oral hygiene and practices, such as failure to remove the denture whilst sleeping and reduced denture cleaning. In the event of host debilitation, which causes an ecological shift in favour of *Candida* growth, candidal biofilms may develop also on the mucosa itself, as it is possible to observe in Fig. 1b (Silva et al. 2011).

As described previously, the presence of candidal biofilms reduce the likelihood of removal of organisms by the host defense mechanism and antifungal agents. From the clinical perspective, the most important feature of *Candida* biofilms is, in fact, their role in resistance to conventional antifungal therapy (Hawser and Douglas 1995). Several groups have demonstrated that *Candida* cells present in biofilms show a dramatic increase in the levels of resistance to the most commonly used antifungal agents (Ramage et al. 2001). Although the mechanisms of biofilm resistance to antifungal agents are not fully understood, the

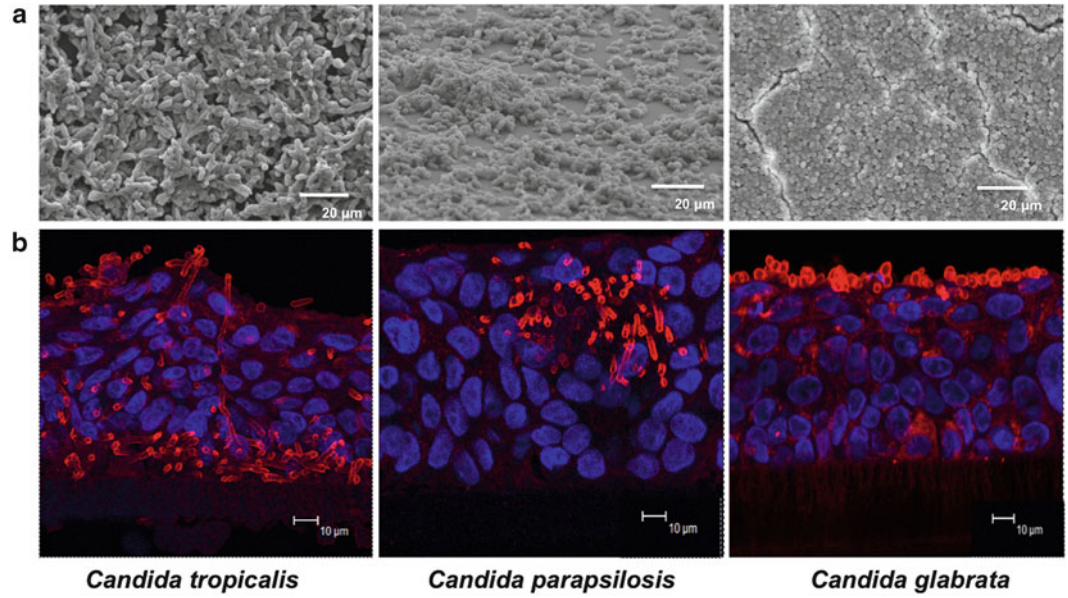

Fig. 1 (**a**) Scanning electron microscopy images of *Candida* species colonizing acrylic surfaces and (**b**) Confocal laser scanning microscopy images of *Candida* species colonizing and invading human oral epithelium after 24 h. *Candida* cells are shown in *red*, and the nucleic of the epithelial cells appear in *blue* in confocal microscopy images; 3000 X corresponds to original magnification of scanner electron microscopy images

current consensus is that biofilm resistance to drugs is a complex multifactorial phenomenon involving different molecular mechanisms of resistance, compared to those displayed by planktonic cells (Silva et al. 2012). So, it is of major importance to know which oral disinfectants are available to fight these biofilms.

3 Oral Disinfectants

As described, *Candida* biofilms are very complex structures that can be formed on both biotic and abiotic surfaces, and are associated with important and recalcitrant infections. Therefore, disinfection of oral biomaterials is of major importance.

As well as the antifungal agents available, the number of disinfectants used for elimination of fungal colonization is very low. The most common are chlorhexidine, benzalkonium chloride and nystatin, among others. However, as their effect on biofilms is still reduced, other alternatives, as silver nanoparticles, are arising.

3.1 Chlorhexidine

Chlorhexidine is a highly cationic polybiguanide (bisbiguanide) with a broad spectrum of antimicrobial activity, including *C. albicans* and other common NCAC species (Suci and Tyler 2002; Ellepola and Samaranayake 2001). It is considered one of the most important medication in a basic health system (WHO 2013).

Additionally, chlorohexidine acts both as fungicide and fungistatic agent, but the specific mode of action is not yet fully understood. Nevertheless, several studies have assessed the antimicrobial effects of chlorohexidine identifying effects on *Candida* viability and structural integrity such as: (i) disruption of the attachment to the surface substrate; (ii) cell wall changes as retraction of the plasmalemma from the cell wall, and condensation and fragmentation of the cell wall (indicating possible lysis and escape of cytoplasmic components through the plasmalemma); (iii) increased vacuolation, aggregation and clumping of the cytoplasm contents; and (iv) inhibition of budding (MacNeill et al. 1997;

Bobichon and Bouchet 1987; Ellepola and Samaranayake 2001).

Chlorhexidine has also been shown to bind avidly to negatively charged surfaces such as epithelial cells (Audus et al. 1992), and to adsorb to enamel and salivary proteins (Hjeljord et al. 1973). Because of this, it has been suggested that a crucial feature of this compound is its high substantivity in the oral cavity, i.e. the capacity to be retained in the mouth (Bonesvoll et al. 1974a, b). The slow and continuous release of chlorhexidine from pellicle-covered oral surfaces seems to be a relevant pharmacodynamic feature, prolonging the therapeutic effect in the oral environment (Ellepola and Samaranayake 2001). However, this characteristic has also been associated with the destruction of the normal ultrastructure of epithelial cells and their receptors for microbes (Vaahtoniemi 1997), as well as with denaturation and precipitation of salivary mucinous proteins (Gjermo 1989). This may lead to side effects such as interference with taste sensations, soreness of the oral mucosa, and discoloration of the teeth (Gjermo 1989).

This antimicrobial agent is widely prescribed as an adjunctive therapeutic supplement due to its antimicrobial activity against a broad spectrum of organisms that includes *Candida* (Epstein 1990; Zegarelli 1993; Suci and Tyler 2002; Ellepola and Samaranayake 2001; Torres et al. 2007). In particular, it is used in mouth rinse formulations to reduce microbial burden in the oral cavity, related to biofilms, having an improved action compared to washes with antifungal agents as nystatin and clotrimazole (Treister and Woo 2010). However, the effectiveness of chlorhexidine as an antimicrobial agent is influenced by its concentration and exposure time, and no firm conclusions have been taken regarding the best method to prevent infection in the oral cavity (Fathilah et al. 2012). Nevertheless, a 0.2 % chlorhexidine gluconate solution is typically used in clinical practice (Salem et al. 1987) against *Candida*-associated denture stomatitis and in acute pseudomembranous candidosis; and a 2 % chlorhexidine gluconate is used as an overnight denture disinfectant (Ellepola and Samaranayake 2000; Budtz-Jorgensen 1990). Chlorhexidine is also used as prophylaxis for both chemotherapy- and radiotherapy-induced mucositis (Kowanko et al. 1998). It has been suggested that chlorhexidine may be used as a pre-treatment on denture acrylic to reduce subsequent adherence of *C. albicans* (Spiechowicz et al. 1990; McCourtie et al. 1985; McCourtie et al. 1986).

However, there are some problems that lead to therapeutic failure of chlorhexidine: both the diluent effect of saliva and the cleansing action of the oral musculature reduce the availability of the compound below the effective therapeutic concentration (Ellepola and Samaranayake 2001); and the reduced susceptibility to antifungals of *Candida* biofilms (Suci and Tyler 2002; Baillie and Douglas 1999; Hawser and Douglas 1995).

3.2 Benzalkonium Chloride

Benzalkonium chloride is a quaternary ammonium compound consisting of a mixture of alkylbenzyldimethylammonium chlorides whose alkyl group has different even-numbered alkyl chain lengths. This compound has been used as a biocide, a cationic surfactant and a phage transfer agent.

The amphiphilicity of benzalkonium chloride is essential for its antimicrobial activity. The action of this compound begins with the interaction of the hydrophilic cationic region with the negatively charged components of the surface of the pathogen, establishing electrostatic interactions. These outcompete the divalent cations that commonly stabilize the structure of the pathogen's surface. After this contact, the hydrophobic region of benzalkonium chloride penetrates the hydrophobic bilayer, compromising cellular permeability controls and causing cell leakage and lysis (Coughlin et al. 1983; Fazlara and Ekhtelat 2012; Mangalannalli-Illathu and Korber 2006; McDonnell and Russell 1999).

The main antimicrobial activity of benzalkonium chloride – membrane disruption, is most effective against Gram-positive bacteria,

having an effect also on Gram-negative bacteria, some enveloped virus, fungi, yeasts and protozoa (Fazlara and Ekhtelat 2012).

It is an active ingredient of biocides used in different applications, from clinical, food line to domestic household (Mangalannalli-Illathu and Korber 2006). Its main use is as a preservative in ophthalmic solutions (as eye drops), in concentrations ranging from 0.004 to 0.01 % since higher values can be caustic and cause irreversible damage to the cornea (Nelson and Goldfrank 2011; Baudouin et al. 2001). Additionally, it has also been tested against *Candida* in plastic surfaces of medical devices (e.g. denture materials), achieving a pathogen reduction when used at least at a concentration of 5000 mg/L. In opposition, benzalkonium chloride was shown to be unable to prevent fungal adherence to extracellular matrix proteins (Imbert et al. 2003b).

3.3 Nystatin

Nystatin belongs to the polyene class, fungicidal due to the ability to interact with the ergosterol component within the fungal cell membranes to generate pores causing cell leakage and loss of cytoplasmatic content. This agent is generally regarded to have the broadest spectrum of antifungal activity (Silva et al. 2001). Nystatin is frequently used topically and can be administrated in a variety of oral formulations in the treatment of oral candidoses including suspensions and pastilles (Williams et al. 2011). Silva et al. (2013) revealed that on single pre-formed biofilms, nystatin was able to significantly inhibit *C. glabrata* and *C. albicans* biofilms. Thus, one strategy that has been the focus of recent research is to actually modify the medical surfaces so they are less prone to biofilm formation by microorganisms, including *Candida* species (Price et al. 2002; Price et al. 2005). In addition, thin-film polymer formulations with incorporated antifungal agents, such as chlorhexidine and nystatin have also recently been shown to inhibit *C. albicans* biofilms growth on denture materials (Redding et al. 2009). In fact, the incorporation of nystatin into the thin-film polymers was able to reduce biofilm formation between 70 % and 80 % (Redding et al. 2009). In studies in which the concentration of nystatin was evaluated, the higher concentration tested had the highest effect, being time dependent, and decreasing and/or disappearing after 1 week, 15 days or 1 month depending on the concentrations (Skupien et al. 2013).

Despite polyenes being very poorly absorbed through the gut and their use being relatively limited, in view of their efficacy on the control of *Candida* growth and biofilm development it is possible to consider that nystatin can be used as an alternative disinfectant to fight *Candida* species.

3.4 Silver Nanoparticles

Silver nanoparticles (SN), due to their unique properties, are starting to be used in many day-to-day applications, including medical applications. Although there are various theories about the microbicidal action of SN, the exact mechanism is not clearly known and is yet a debate topic. SN has the ability to anchor to the microorganism's cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane integrity. It has also been proposed that the microbicidal activity can be due to the release of silver ions by nanoparticles, which can interact with important enzymes and inactivate them. It has also been postulated that nanoparticles can even modulate the signal transduction in microorganisms (Prabhu and Poulose 2012).

The effect of SN against *Candida* biofilms was first reported by Monteiro et al. (2011). In this study, SNs were synthesized by silver nitrate reduction with sodium citrate and stabilized with ammonia. Then, these nanoparticles were applied (during 24 h) to adherent cells (2 h) or biofilms (48 h) of *C. albicans* and *C. glabrata* previously developed (Fig. 2).

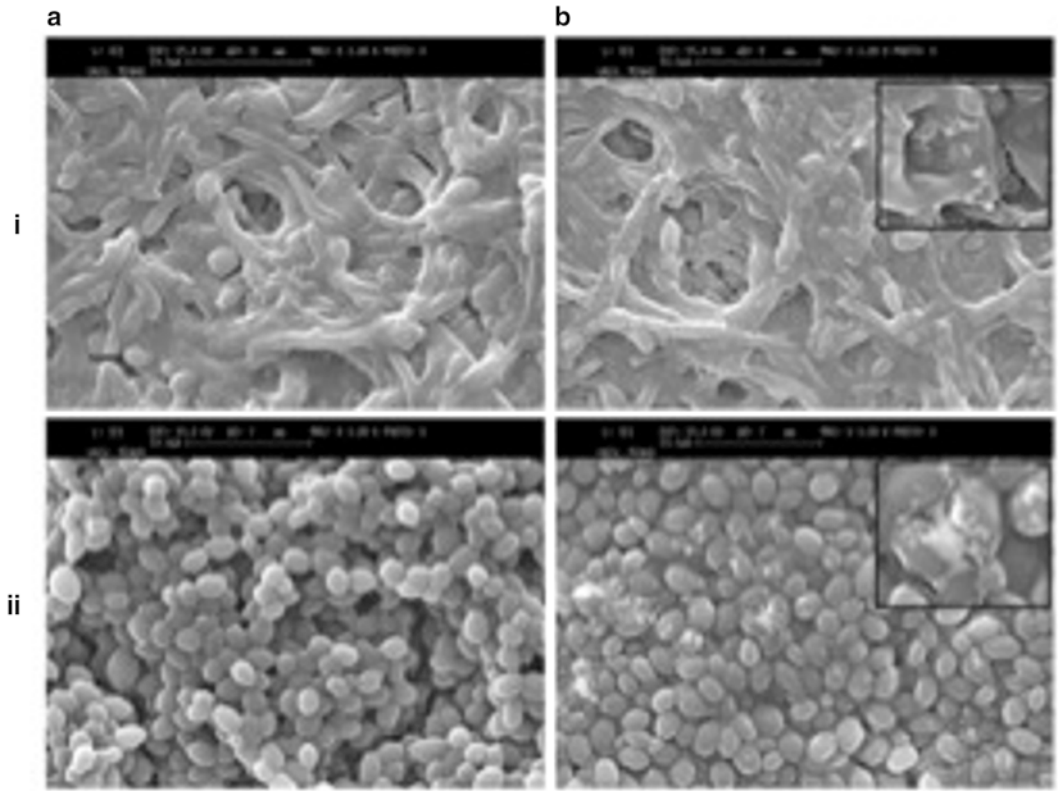

Fig. 2 Scanning electron microscopy images showing the structure of *Candida albicans* (i) and *Candida glabrata* (ii) mature biofilms under different experimental conditions: (**a**) control and (**b**) biofilms treated with SN (Note agglomerated SN in an enlarged view of part of biofilms (Monteiro et al. 2013))

The results for adhered cells showed a significant reduction in the total biomass and in the number of cultivable cells for SN concentration at/or higher than 3.3 μg/mL for both species. So, SNs showed the ability to inhibit biofilm formation when applied to adherent cells. Importantly, SNs were more effective in the reduction of the number of cells and total biomass when applied on adhered cells than pre-formed biofilms (Monteiro et al. 2011). Regarding pre-formed *Candida* biofilms, the highest SN concentration tested (54 μg/mL) significantly reduced biofilms of *C. glabrata*. In general, *C. glabrata* biofilms have reduced thickness and are devoid of hyphae compared with *C. albicans* biofilms (Silva et al. 2009; Samaranayake et al. 2005). Consequently, all these features can help explain the better effect of SN against *C. glabrata* biofilms, which is still of major clinical importance since this species is known to be resistant to conventional antifungal drugs, making it very difficult to eliminate. Furthermore, the effective SN concentration against *C. glabrata* biofilms can be considered low when compared with the concentrations of conventional antifungal used. For instance, fluconazole at concentrations ranging from 50 to 1250 μg/mL was ineffective against *C. glabrata* biofilms (Fonseca et al. 2014).

In the view of the SN application as disinfectant of medical devices against *Candida* biofilms proliferation, and considering that the loss of the chemical stability might reduce their effectiveness, a study was conducted to verify whether heating or changing pH of a SN stock solution, as well as the treatment period, would affect the anti-biofilm activity (Monteiro et al. 2014). Several parameters were evaluated regarding SN

(54 μg/mL) stability, namely temperature (50 °C, 70 °C, and 100 °C) and pH (5.0 and 9.0) in mature biofilms grown on acrylic resin specimens. Surprisingly, SN colloidal suspensions heated at 50 °C and 70 °C for 30 min did not display modifications in their absorption spectra as compared with SN suspension that was not heated. In addition, heating SN at 50 °C and 70 °C did not compromise the effectiveness of SN against *Candida* biofilms (Monteiro et al. 2014). Regarding pH variations and despite the appearance of dark aggregates, evidencing instability of SN at both acid and basic pH, the results also showed that the variations in pH did not impair the effectiveness of SN in reducing *Candida* species biofilms. One of the factors that may contribute to *Candida* biofilms' antifungal resistance is the presence of extracellular matrix (Silva et al. 2001). The matrix of *Candida* biofilms is constituted by several proteins, carbohydrates (Silva et al. 2009) and DNA (Martins et al. 2010). Antifungal drugs may bind to constituents of the extracellular matrix, preventing the drugs from reaching the cells in the deeper layers of the biofilm (Vediyappan et al. 2010). Therefore, strategies focusing on eradication of extracellular matrix may collaborate to fight *Candida* infections associated with biofilms. Monteiro *et al*. (2013) applied SNs on *C*. *glabrata* mature biofilms and it was possible to observe a significant reduction in matrix protein and DNA contents (Monteiro et al. 2013). Moreover, an *in vitro* study compared the antifungal effects of nystatin and SN on pre-formed single and dual species (*C*. *albicans* and *C*. *glabrata*) biofilms on acrylic resin surfaces under conditions that attempted to mimic the oral environment (Silva et al. 2013). This work revealed that *C*. *albicans* and *C*. *glabrata* colonized acrylic surfaces in the presence of artificial saliva and co-existed in dual species biofilms without antagonism and that both nystatin and SN were able to inhibit single and dual species biofilms. SNs were also tested in combination with chlorhexidine and a synergetic activity was observed for *C*. *glabrata* biofilms, achieving a reduction in biofilm biomass of more than 84 % (Monteiro et al. 2013). Recently, Longhi et al. (2015) revealed that the combination of SN and fluconazole reduced the concentration of the latter around 16–64 times against planktonic cells of *C*. *albicans* and a significant dose-dependent decrease in the viability of both initial and mature *C*. *albicans* biofilm (Longhi et al. 2015). In a clinical context, these combinations are very important to decrease the concentrations used on traditional antifungal therapy and reduce the probability of their increased resistance.

In view of all these attributes it is possible to consider that SN can be used as an alternative disinfectant to fight *Candida* biofilms; however, further research is needed to elucidate the mode of action, cytotoxicity and the longevity of SN formulations and microbial properties of SN generated by the incorporation of nanoparticles for example into denture acrylic.

3.5 Other Alternative Disinfectants

Besides the previously described disinfectants, the literature suggests other alternatives, as the use of 0.5 % sodium hypoclorite concentration that can help disinfect tissue conditioners and denture lines (Skupien et al. 2013).

In addition, povidone iodine is recognized as an effective broad spectrum biocidal agent, whose *in vitro* biocidal activity has been studied for years against bacteria, yeast, moulds, viruses, fungi, protozoa, actinomycetes and rickettsia (Duarte and Hamdan 2006; Zamora 1986; Amend and Pietsch 1972). Typical iodine solutions present significant oral toxicity, but this complex exhibits markedly lower toxicity, being less hazardous in case of accidental ingestion (Kumar et al. 2009). Povidone iodine has been mostly used for surgical scrubbing and as a prophylactic irrigation solution against surgical site infection (Chundamala and Wright 2007). A study has also demonstrated that when used at 2.5 %, povidone iodine is able to completely inhibit yeast adherence, suggesting that it could be a good candidate for prevention of candidoses related to medical devices (Imbert *et al* 2003a). However, it has only been applied: in

gynaecology for vaginitis associated with *Candida* (Dugal and Chaudhary 2013); as an antiseptic for infected inflammatory conditions of the mouth and pharynx caused by *C. albicans* (Silva et al. 2009); in reducing odor causing bacteria in mouth wash products at a small concentration (0.5 %) (Addy et al. 1977; Becker and Nel 1976).

Moreover, microwave irradiation has also been described as an alternative method of disinfection (Skupien et al. 2013).

Other option of disinfection is the low-level laser therapy (LLLT) and photodynamic therapy (PDT), which are also emerging as promising alternatives to conventional treatments for *C. albicans* infections (Dougherty 2002; Dougherty et al. 1998). LLLT uses low doses of visible light of appropriate wavelength to activate existing biological chromophores in cells, leading to the generation of reactive oxygen species (Souza et al. 2010). For its turn, PDT uses photosensitizing agents that are also activated by an appropriate wavelength of light in the presence of oxygen, promoting a phototoxic response of the cells, usually via oxidative damage (Dougherty et al. 1998; Mima et al. 2010). In consequence of the use of nonspecific oxidizing agents, organisms resistant to conventional antifungals may be successfully killed by PDT and the development of resistance to this therapy seems unlikely (Mima et al. 2010). A few *in vitro* studies have shown that *C. albicans* is susceptible to photoinactivation, but the effects on its growth, adherence and virulence factors are far from being defined (Mima et al. 2010; Sennhenn-Kirchner et al. 2009). Evidently, the knowledge about the application of LLLT and PDT applied to NCAC species is even scarcer.

4 Conclusions

Although there are some alternatives to combat oral infections, they are not always efficient on biofilms, especially those from *Candida* species. So, more research should be developed in this field to better treat oral *Candida* infections associated to biofilms, which can lead to serious infections, with special relevance in immunocompromised patients.

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Updates on Therapeutic Strategies Against *Candida* (and *Aspergillus*) Biofilm Related Infections

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Abstract

Fungal biofilm related infections are commonly associated with medical devices with biofilms contributing to the virulence of the involved fungal species. If infection does occur, removal of medical device is often warranted. However, this is not always possible. Moreover, biofilm associated infections are often resistant to antifungals and host immunity. Therefore, a need for new agents and strategies to combat these devastating infections is needed. Although no randomized clinical trials have been conducted or are likely to be conducted in the future, the Infectious Disease Society of America (IDSA) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) utilized available published data and clinical experience of the infectious disease community to propose strategies to treat biofilm associated devise infections. In this chapter we describe the emerging therapies for biofilm related infections.

1 Introduction

The *Candida* genus, a eukaryotic organism, is one of the relatively few fungi that can cause severe human infection and disease. Despite its known role as a human commensal organism, it has the ability to inflict a spectrum of infections ranging from lesser superficial skin and mucosal infections to life-threatening systemic infections. *Candida's* growth and virulence arises from multiple factors including dimorphism (converting between budding yeast and branching septal hyphal forms), ability to adhere via adhesion proteins (agglutinin-like sequence (ALS) family), binding proteins (invasins), secretion of hydrolases (e.g. phospholipases and aspartic proteinases), pH regulation mechanisms, metabolic adaptations, environmental stress reactions, heat shock proteins, trace metal and nutrient acquisition, and biofilm formation (Mayer et al. 2013). Medically relevant fungi that

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produce biofilms include *Candida*, *Aspergillus*, *Cryptococcus, Trichosporon*, *Coccidioides*, and *Pneumocystis* (Fanning and Mitchell 2012). Biofilm infections in general, including both fungal and bacterial, can affect any of the organ systems in humans, and the Center for Disease Control and Prevention estimates over 60 % of chronic infections are related to biofilms (Ehrlich et al. 2004).

As of 2004, *Candida* spp. are the fourth most common cause of nosocomial blood stream infection (BSI), with *Candida albicans*, specifically, being the most common cause of invasive candidiasis (IC), followed by *Candida glabrata* (Pfaller and Diekema 2007). Dispersion of yeast, by detachment from infected indwelling devices, can result in hematogenous spread of yeast from biofilms surfaces, resulting in disseminated infections. *Candida* is the third leading cause of infection related to catheters, and if disseminated, it can cause up to 40 % mortality (Vila et al. 2015). Biofilms may form on a variety of surfaces with catheter, dental, and mucosal surfaces being the most common (Mayer et al. 2013). The most commonly inserted medical devices in the United States are urinary and intravascular catheters, which are also the two most common causes of nosocomial bloodstream infections (Trautner and Darouiche 2004). This poses a significant problem because there has been an increase in the use of indwelling catheters in medicine. More than 200 million catheters of all types are used each year in the United States (Thomas et al. 2004). The biofilm, composed mainly of the polysaccharide β-1,3 glucan, forms in sequential steps—adhesion of initial yeast cells, proliferation, formation of hyphal cells, accumulation of extracellular matrix, and finally dispersion of yeast cells (Mayer et al. 2013). The biofilm matrix is comprised of biopolymers, mainly polysaccharides and proteins, however lipids and nucleic acid are also commonly found. This matrix is often used in a combined term, exopolymeric substances (EPS). The EPS is a slimy layer that protects the fungus from outside forces (Purevdorj-Gage and Stoodley 2004). *Candida albicans* has shown to produce larger and more complex biofilms than other fungal species (Kuhn et al. 2002a). This may contribute to *C. albicans* common pathogenicity in humans, especially when introduced through implantable devices.

In the past decades there has been an increase in candidiasis, paralleling the increased use of medical implants, which are common substrates for biofilm formation (Lopez-Ribot 2014). In contrast to the planktonic form (free floating cells), the organized structure of biofilms combines metabolic plasticity, increased concentration of drug efflux pumps, and architectural formation of the biofilm matrix. The biofilm matrix increases the organism's resistance to chemical, immunological, and mechanical forces. Contact sensing through cell wall molecules and thigmotropism (directional hyphal growth on uneven surfaces) help the fungus grow and adhere to surfaces touching their biofilm, and invade into substratum layers (Mayer et al. 2013). Thigmotropism takes place secondary to complex signaling pathways that recruit actin and microtubules at the apex of filamentous fungi. Calcium channels, GTPases, and protein complexes all play a role in signaling. This gives the fungus an advantage, orienting and guiding the organism towards the most suitable environment (Brand and Gow 2009). Due to a transcriptional activator (required for biofilm formation), *GCN4*, many metabolism processes and substrates are at higher levels in biofilms as well. Biofilm cells have an increased turnover of protein due to an overexpression of protein synthesis genes and higher concentrations of translation factors, ribosomal proteins. If nutrient deficient, this may play a role in recycling of nutrients in the biofilm matrix furthering their persistence to survive (Fanning and Mitchell 2012). For example, the gene *MET3*, which encodes for assimilation of sulfur metabolism, is up-regulated in biofilm cells, and does not show such high concentrations in planktonic cells (Murillo et al. 2005). One goal of the scientific community is to further understand how biofilms function in order to properly target infectious fungi, outsmarting their mechanisms of resistance.

2 Biology and Resistance of Biofilms

As mentioned above, biofilms contribute to a variety of virulence factors for the organism. They also contribute to microbial resistance to antimicrobials. The network of EPS can hinder a drug's ability to penetrate the biofilm (Lopez-Ribot 2014). The EPS' adhesive and cohesive forces cause strong cell-cell and cell-surface interactions, enforcing this protective barrier, not solely against drugs, but also environmental factors, such as radiation, oxidation, desiccation, predators and host-immune functions (Lopez-Ribot 2014). In fact, nuclear magnetic resonance (NMR) imaging analysis has shown aggregate interaction between the biofilm matrix and the antifungal fluconazole (Zarnowski et al. 2014). Biofilm production is regulated by genes and several transcription factors. *BGL2* and *PHR1* are glucan transferases that are positive regulators of β-1,3 glucan production (Mayer et al. 2013). Fluconazole was more effective against biofilms produced by fungal mutants lacking *BGL2, PHR1*, and *XOG1* (a gene encoding exo-gluconase) *in vivo* and *in vitro*, during biofilm growth (Taff et al. 2012). Although fluconazole is not commonly used to treat biofilm related infections, this demonstrates the role enzymes involved in the fungal biofilm play in their reaction to medications. *C. albicans* has multidrug resistant transporter genes (*MDR1, CDR2, and PDR16*) which are overexpressed in *in vivo* biofilms (Fanning and Mitchell 2012). Biofilms demonstrate up-regulation of other virulence factors as well. ALS-1 is the most up-regulated adhesion protein in candidal biofilms (Garcia-Sanchez et al. 2004). Sterol biosynthesis genes are also up-regulated in *C. albicans* and contribute to resistance to azole drugs as well (Nett et al. 2009). Mukherjee et al. showed that the mechanism underlying fluconazole resistance depends on the biofilm phase. Efflux pumps were shown to be responsible for resistance at early phase; while sterol synthesis is responsible for resistance at the mature phase. The early phase is defined as the adhesion of fungal cells to the substrate in the first 11 h. The mature phase is between hour 31 and 72, at which the cells are encased in a thick ECM. These studies were done using wild-type and knock-out strains of the efflux pump genes: CDR and MDR1. The knock-out strains were more susceptible to fluconazole (Mukherjee et al. 2003). One study has shown that a family of genes called the quinidine drug resistance (QDR) family, in fact, changes functionality of the biofilm regulation, and thus, inhibits the virulence of *C. albicans* (Shah et al. 2014). This is a great example of how the role of biofilms, resistance mechanisms, and overall virulence of the fungus are intertwined and all play a role in how we may target and prevent infection.

3 Approach to Discovery and Novel Treatment

In the previous section we discussed the role of biofilms, and how the structure, metabolism, and gene expression of biofilms increase *Candida*'s virulence. Here we will discuss how we came to establish certain treatment guidelines, and new treatments on the horizon. In the next section we present tables summarizing the *Clinical Practice Guidelines for the Management of Candidiasis: 2009 Update by the Infectious Disease Society of America* and the *European Society of Clinical Microbiology and Infectious Diseases Guideline for the Diagnosis and Management of Candida Diseases 2012: Non-neutropenic Adult Patients*. These are guidelines based on clinical practice and research. Physicians are to use their judgment in following these guidelines case by case. Each patient has a unique medical history and presentation of illness, for which these guidelines may do just as the name suggests and guide the physician in his or her therapeutic choices. It is important to become familiar with these current treatment recommendations. It is equally important to understand the upcoming practices and emerging therapeutics in candidal infection control. First and foremost, efforts must be made to prevent invasive candidiasis from occurring. Steps to insure candidal biofilms from emerging

and subsequent dissemination are crucial. Nonpharmacologic methods of prevention include hand hygiene, judicious use of antibiotics in the community (to prevent resistance), and proper use of catheters and other implanted devices (Pfaller and Diekema 2007). It is common practice to remove or replace any foreign device from the patient if it were to harbor biofilm producing fungi or cause infection. Data from 1915 patients from seven trials demonstrated that two factors correlated with greater clinical success in patients with candidemia: use of an echinocandin and the removal of central venous catheters (CVC) (Andes et al. 2012). Interestingly, Nucci et al. showed in an analysis of 842 patients, that *early* CVC removal did not show any clinical benefit in adults who developed candidemia (Nucci et al. 2010), potentially pointing out that time of removal of catheter may be irrelevant. At times, however, patients require the use of such devices. For example, in the intensive care unit, a hypotensive patient whose venous access is poor may only have one source (a central venous catheter) for crucial fluid replacement and vasopressors (medicines that raise blood pressure). In such cases, we must rely on anti-fungal medications. A 2004, *in vivo*, study inserted catheters in rabbits and subsequently infected the, with *C. albicans*. This showed that amphotericin B almost cleared fungal growth and fungal biofilms when assessed with cultures and scanning electron microscopy. These results were significant when compared to the control arm (untreated rabbits) and fluconazole treated arm of the study. This may give insight into the salvation of infected catheters in humans (Schinabeck et al. 2004). As of yet we have no Food and Drug Administration (FDA) approved medicines that are biofilm specific. We must rely on ways to inhibit growth of or disruption of biofilms to render the microbe susceptible to current antifungal medications (Nobile and Johnson 2015). Further proof that biofilms are more resistant to anti-fungals then their planktonic structural counterpart, is demonstrated in an *in vitro* study by Chandra et al. using denture model biofilms; *C. albicans* with biofilm required higher concentrations to reach susceptibility in four antifungals: amphotericin B, nystatin, chlorhexidine, and fluconazole (Chandra et al. 2001). In a follow up study, new formulations of drugs were shown to have activity against candidal biofilms. These included lipid formulations of amphotericin B and echinocandins (caspofungin and micafungin) (Kuhn et al. 2002b).

Models for bench-work research on biofilms have come a long way, with the use of high-throughput screening (a method of testing high numbers of compounds with machinery and data processing software). One example of this is a recent study using a microarray platform of nano-scale biofilms encapsulated in different matrices. This research design puts in perspective how biofilm growth is affected by drugs when grown in different mediums. This can potentially be translated from *in vitro* results to patient care, if we were to take into consideration all the different materials that are used in prosthetics and other implantable medical devices (Srinivasan et al. 2014). A previous *in vivo* study tested the medium of the drug rather than the fungus. The authors implanted a material called amphogel (a dextran based hydro-gel), which absorbs amphotericin B, in mice. The gel was inoculated with *C. albicans* and with time showed decreased surviving fungal cells and less biofilm formation. This matrix for drug may be used in the future to coat implantable devices in humans (Zumbuehl et al. 2007).

There have been recent advances in achieving such mechanistic methods of biofilm inhibition. One study found that in combination with standard antifungals (fluconazole and capsofungin), a non-steroidal anti-inflammatory drug, flufenamic acid (FFA), had up to 99 % prevention of *C. albicans* biofilm formation. FFA alone had up to 80–85 % reduction of biofilm formation, dependent upon concentration of FFA (Chavez-Dozal et al. 2014).

Miltefosine, an alkylphospholipid, has shown efficacy against *C. albicans* biofilm formation and against preformed biofilms, in azole-resistant pathogens. The efficacy was demonstrated *in vitro* (planktonic forms) and topically, *in vivo*

(murine oropharangeal models) (Vila et al. 2015). Furthermore, immunomodulatory properties of statins (medication for high cholesterol in humans) has been shown to slow Candidal growth. A follow up multicenter study has demonstrated a potential benefit of use of statins in hospital patients who developed candidemia (Cuervo et al. 2013). New tools to defend against biofilm fungal infections are yet to be discovered; however, some tools may be right under our nose, such as these statins.

Certain biofilms may be polymicrobial, meaning different species of fungi are involved, or perhaps fungi mixed with bacterial biofilm. This poses a treatment challenge, because of the large evolutionary difference between prokaryotic bacteria and eukaryotic fungi. One study has found that Guanylated polymethacrylates, a copolymers of 2-guanidinoethyl methacrylate (2-GEMA) and methyl methacrylate (MMA), which are structural imitators of the amino acids arginine and alanine, respectively, has strong activity against *C. albicans* and *Staphylococcus aureus* (a Gram positive bacteria) polymicrobial biofilms (Qu et al. 2015). This presents a great opportunity for the future of medicine as monotherapy is usually preferred, to avoid further side effects and other dangers of polypharmacy.

Lastly, another study has identified 19 other compounds that inhibit over 30 % metabolic function of biofilms versus clotrimazole alone (LaFleur et al. 2011). Understanding that this study is conducted *in vitro*, we have a long way to go before such molecules can be formed into drugs to be used for management of biofilm-associated infections in humans, but the prospect for the future is exciting.

4 American and European Guidelines to Therapy

Details on the treatment of candidal infections in general can be found in the *Clinical Practice Guidelines for the Management of Candidiasis: 2009 Update by the Infectious Disease Society of America* and the *European Society of Clinical Microbiology and Infectious Diseases Guideline for the Diagnosis and Management of Candida Diseases 2012: Non-neutropenic Adult Patients*. In this section we present summary tables (Tables 1 and 2) of treatment recommendations for device related candidal infections. These infections are more likely to have been biofilm related. The treatment of biofilms specifically, however, is trickier and more complicated. There are no standard set of guidelines for these treatments. Research has suggested that removal of catheter, when warranted, improves survival in *C. albicans* biofilm related infections. When removal is not possible, antifungal lock therapy may be beneficial. Antifungal lock therapy uses high dose concentrations in medical devices (mainly catheters) to sterilize the equipment. To enforce what has been discovered and discussed in the previous section, the drugs that most often work for biofilm related infections are liposomal amphotericin B and echinocandins, while azoles are less reliable.

5 Moulds: *Apergillus* and *Fusarium*

Lastly, we will briefly review other clinically important fungal species, besides *Candida*. *Aspergillus* also forms biofilms on either biotic or abiotic surfaces. The processes are similar to other fungi. Initial cells (condia) adhere to substrates, multiplying and forming hyphal forms (mycelia). Extracellular matrix binds the biofilm together (Fanning and Mitchell 2012). The *in vivo* biofilm, which invasive *Apergillus fumigatus* forms, is composed of hyphae with an extracellular matrix of polysaccharides, galactomannan, and galactosaminogalactan. There is a growing need for new therapies against *Aspergillus*, due to recent resistant forms (Beauvais and Latge 2015). A recent prospective multicenter international surveillance study of azoles found 3.2 % prevalence (increased from previous reports) of azole resistant *A. fumigatus* (van der Linden et al. 2015) Just as fungi form new resistance characteristics, new therapies are always on the horizon. A study in 2015,

Table 1 Abridged summary of the European Society for Clinical Microbiology and Infectious Diseases selected implantable device related infections

Population	Intention	Intervention
Ventilated patients >48 h with another ≥72 h expected	Prophylaxis against candidiasis/ candidaemia	Fluconazole 100 mg/day
Ventilated ≥ 3 days, received antibiotics, CVC, and any of the following: parenteral nutrition, dialysis, major surgery, pancreatitis, systemic steroids, or immunosuppression	Prophylaxis against candidiasis/ candidaemia	Caspofungin 50 mg/day
Candidaemia/invasive candidiasis	Treatment	Anidulafungin 200/100 mg, Capsofungin 70/50 mg, Micafungin 100 mg, Amphotericin B liposomal 3 mg/kg
Candidemia with CVC	Catheter management	Remove if possible (not over a guidewire). If not possible treat with Echinocandin, liposomal amphotericin B or amphotericin B lipid complex
Asymptomatic candiduria	To clear *candida*	None, Fluconazole 200 mg for 14 days, if susceptible, removal of urinary catheter, Amphotericin B deoxycholate bladder irrigation
Cystitis	To cure	Fluconazole, if susceptible, Amphotericin B deoxycholate +/− flucytosine
Prosthetic valve endocarditis	To cure	Surgery within days, if surgery not possible, liposomal amphotericin B 5 mg/kg or capsofungin 70/50 mg
Pacemaker, ICD, or VAD infection	To cure	Removal
Prosthetic joint infection	To cure	Removal, if prosthesis needed, Fluconazole 400 mg for life

Cornely et al. (2012)
ICD implantable cardiac defibrillator, *VAD* ventricular assist device

Table 2 Abridged summary of the Infectious Disease Society of America selected implantable device related infections

Population	Primary therapy	Alternative therapy	Comments
Candidemia (nonneutropenic adults)	Fluconazole 800 mg	LFAmB 3–5 mg/kg daily or AmB-d 0.5–1 mg/kg	Choose an echinocandin for moderate to severe illness or recent azole exposure
Asymptomatic cystitis	None		Eliminate predisposing factors
Symptomatic cystitis	Fluconazole 200 mg daily for 2 weeks	AmB-d 0.3–0.6 mg/kg for 1–7 days	
Endocarditis	LFAmB 3–5 mg/kg	Step-down therapy to fluconazole 400–800 mg daily until negative blood culture results	Valve replacement is strongly recommended, if unable, chronic suppression with fluconazole
Infective pacemaker, ICD, or VAD	LFAmB 3–5 mg/kg	Step-down therapy to fluconazole 400–800 mg daily until negative blood culture results	Removal of pacemakers and ICDs strongly recommended, if unable to remove VAD, chronic suppression with fluconazole

Pappas et al. (2009)
LFAmB lipid formulation of amphotericin B, *AmB-d* amphotericin B deoxycholate, *ICD* implantable cardiac defibrillator, *VAD* ventricular assist device

determined that phenzines (aromatic ring compounds), suppress fungal growth in low iron environments, while some phenzines produce reactive oxygen species to cause dysfunction in mitochondria of the microbe, which may have infiltrated, and caused infection from a persistent biofilm formation (Briard et al. 2015). Interestingly, phenzine is produced by the bacteria *Psuedomonas aeruginosa*. These two pathogens often co-exist in cystic fibrosis patients, who often have invasive devices such as endotracheal tubes, in close proximity to the lungs. This is feasible because of resistance mutations in Aspergillus' mitochondrial enzymes of metabolism, that do not allow phenzines to disrupt function (Briard et al. 2015). This may lead to better understanding of symbiotic and antagonistic relationships of organisms *in vivo*, and future strategies for treatment. One example of a potential synergistic effect of *therapy* for Aspergillus biofilms was seen *in vitro* when tacrolimus (used as a T-cell activator in humans) was combined with certain azoles and amphotericin B (Gao and Sun 2015).

Recent studies are looking at structural properties as well as biochemical pathways leading to formation of galactosaminogalactan (GAG) in *Aspergillus* biofilms—a strong virulence factor that may be the target of newer therapies (Bamford et al. 2015; Beaussart et al. 2015). Considering all the above, we still have a limited armamentarium. We are in need of randomized controlled trials, but these are hard to come across.

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Natural Sources as Innovative Solutions Against Fungal Biofilms

Marion Girardot and Christine Imbert

Abstract

Fungal cells are capable of adhering to biotic and abiotic surfaces and form biofilms containing one or more microbial species that are microbial reservoirs. These biofilms may cause chronic and acute infections. Fungal biofilms related to medical devices are particularly responsible for serious infections such as candidemia. Nowadays, only a few therapeutic agents have demonstrated activities against fungal biofilms in vitro and/or in vivo. So the discovery of new anti-biofilm molecules is definitely needed. In this context, biodiversity is a large source of original active compounds including some that have already proven effective in therapies such as antimicrobial compounds (antibacterial or antifungal agents). Bioactive metabolites from natural sources, useful for developing new anti-biofilm drugs, are of interest. In this chapter, the role of molecules isolated from plants, lichens, algae, microorganisms, or from animal or human origin in inhibition and/or dispersion of fungal biofilms (especially *Candida* and *Aspergillus* biofilms) is discussed. Some essential oils, phenolic compounds, saponins, peptides and proteins and alkaloids could be of particular interest in fighting fungal biofilms.

Keywords

Biofilm • *Candida* • *Aspergillus* • Natural substances • Bioactive metabolites

1 Introduction

Current available antifungal agents have usually little effect on fungal biofilms. The biodiversity represents a huge reservoir of natural components with variable and not yet described activities on human infectious diseases,

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including fungal diseases. Our goal was to review the already known components active against fungal biofilms. Most available studies focus on *Candida* yeasts and to a lesser extent *Aspergillus* molds. Active components will be described depending on their origin stemming from plants, lichens, algae, microbes (fungi or bacteria), animals as well as Humans. In each case, they will be classified depending on their role (for example biosurfactants or antibodies) or chemical features (for example essential oils (EO), saponins, phenolic compounds, peptides, alkaloids…). The development of fungal biofilms involves three different steps: early (adhesion), maturation and dispersion phases. The specific activity of natural components against these phases will be clarified when data are available. Especially, efficiency against early phase biofilms would suggest a prophylactic benefit against biofilm-related fungal infections and efficiency against already formed mature biofilms would suggest a both prophylactic and curative benefit depending on the infection location. Thus, all these data are developed below and summarized in Table 1.

2 Anti-Biofilm Compounds of Plant Origin

Considering literature data, plants currently constitute the largest source of active compounds against fungal biofilms. These compounds can be classified into six groups: terpenoids; phenolic compounds; peptides; alkaloids; biosurfactants; compounds with unknown or mixed composition.

2.1 Terpenoids

This group constitutes, with steroid group, the largest known ensemble of plant secondary metabolites. It especially includes EO, oleoresin, iridoids, pyrethrins, sesquiterpene lactones, saponins, cardiac glycosides, phytosterols, carotenes, polyisoprenes (Bruneton 2008). At this point only some active compounds belonging to EO or terpenes and to saponins will be described.

2.1.1 Essential Oils and Terpenes

Among substances obtained from plants, EO stand out as natural, complex and variable mixtures mainly composed of terpenes and aromatic compounds derived from phenylpropane and characterized by strong odor and volatility. They are produced by aromatic plants, as secondary metabolites. Numerous studies have already been performed to investigate the anti-biofilm activity of EO. For example, Furletti et al. reported that *Coriandrum sativum* EO affected the formation of *Candida albicans* biofilm causing fungal cells deformation, presenting a withering appearance, that finally resulted in a decreased biofilm growth (Furletti et al. 2011).

Furthermore, *Cinnamomum zeylanicum* EO, predominately composed of cinnamaldehyde (54 %), inhibited the formation of *Candida orthopsilosis* or *C. parapsilosis* biofilm at concentrations above 250 μg/ml and was also active against 24 h old biofilms of these species (minimum biofilm reduction concentration: 1000 and 2000 μg/ml respectively). A possible explanation was that the hydrophobicity of their volatile compounds allowed for their insertion into the lipid bilayer of the cell membrane, causing a disturbance and increasing its permeability to protons. Scanning electron microscope (SEM) observations supported this hypothesis and showed a marked decrease in cell aggregates and the complete absence of an extracellular matrix in *Candida* biofilms (Pires et al. 2011).

The EO extracted from leaves of *Ocimum americanum*, an aromatic herbaceous plant native of Asia and Africa, also demonstrated effectiveness against yeast biofilms. A 5-min exposure of 24 h old *C. albicans* biofilms to 3 % v/v EO eliminated 3 $\log_{10}$ of biofilm cells. At a lower concentration (0.3 % v/v), a 2 $\log_{10}$ reduction in *C. albicans* was observed. Authors suggested the possibility of using this EO in oral health care products (Thaweboon and Thaweboon 2009).

The EO obtained from leaves of *Croton cajucara* also demonstrated anti-biofilm activity,

Table 1 Chemical or functional groups of natural origins with highlighted anti-biofilm activities against fungi

Origins	Chemical or functional groups		Studied phases of biofilm development			Tested fungal species	References
			Adhesion and/or Maturation	Mature biofilm	Dispersion		
Plant	Terpenoids	Essential oils/Terpenes	x	x		*C. albicans* *C. dubliniensis* *C. glabrata* *C. orthopsilosis* *C. parapsilosis* *C. tropicalis*	Alviano et al. (2005), Anghel et al. (2013a), (2013b), Bersan et al. (2014), Braga et al. (2008), Carneiro et al. (2011), Chifiriuc et al.(2012), Curvelo et al. (2014), Da Silva et al. (2012), Dalleau et al. (2008), De Campos Rasteiro et al. (2014), Doke et al. (2014), Furletti et al. (2011), Grumezescu et al. (2012), Hsu et al. (2013), Khan and Ahmad (2012a), (2012b), Palmeira-de-Oliveira et al. (2012), Pemmaraju et al. (2013), Pires et al. (2011), Ramage et al. (2012), Raut et al. (2013), Saharkhiz et al. (2012), Sroisiri and Boonyanit (2010), Stringaro et al. (2014), Sudjana et al. (2012), Sun et al. (2012), Taweechaisupapong et al. (2010), Thaweboon and Thaweboon (2009), Trindade et al. (2015)
		Saponins	x	x		*Aspergillus sp.* *C. albicans*	Chevalier et al. (2012), Coleman et al. (2010), Sadowska et al. (2014), Vediyappan et al. (2013)
	Phenolic compounds	Stilbenoïds	x			*C. albicans*	Cheng et al. (2009), Li et al. (2012), Zhang et al. (2011)
		Tannins	x	x	x	*C. albicans* *C. glabrata*	Bakkiyaraj et al. (2013), Evensen and Braun (2009), Feldman et al. (2012), Girardot et al. (2014), Luiz et al. (2015), Rane et al. (2014)
		Quinones	x	x		*C. albicans* *C. dubliniensis*	Tsang et al. (2012), Tsang et al. (2013)
		Flavonoids	x	x		*C. albicans*	Cao et al. (2008), Messier et al. (2011), Messier and Grenier (2011), Onsare and Arora (2015)
	Peptides		x	x		*C. albicans* *C. tropicalis*	Delattin et al. (2014), Mandal et al. (2011), Mandal, (2012)
	Alkaloids		x	x		*A. fumigatus* *C. albicans* *C. neoformans*	Shao et al. (2014), Wei et al. (2011), Zhao et al. (2013)
	Biosurfactants		x			*C. albicans*	Cochis et al. (2012), Rodrigues et al. (2006)
Lichen	Phenolic compounds	Dibenzofuran		x		*C. orthopsilosis* *C. parapsilosis*	Pires et al. (2012)
		Flavonoids	x			*C. albicans*	Singh et al. (2015)
		Pyridins	x			*C. albicans*	Chang et al. (2015)
		Benzenediols		x		*C. albicans*	Li et al. (2015)
	Terpenoids	Triterpens	x			*C. albicans*	Chang et al. (2012)

(continued)

Table 1 (continued)

Origins	Chemical or functional groups		Studied phases of biofilm development			Tested fungal species	References
			Adhesion and/or Maturation	Mature biofilm	Dispersion		
Alga	Fatty acids		x			*C. albicans* *C. dubliniensis*	Thibane et al. (2010)
Fungus	Alkaloids		x			*C. albicans* *C. glabrata* *C. kefyr* *C. parapsilosis,* *C. tropicalis*	Wang et al. (2012b), You et al. (2013)
	Terpenoids	Terpenes	x	x		*C. albicans* *C. dubliniensis* *C. metapsilosis* *C. orthopsilosis* *C. parapsilosis* *C. tropicalis*	Cordeiro et al. (2014), Hornby et al. (2001), Ramage et al. (2002), Weber et al. (2010)
	Biosurfactants		x			*C. albicans*	Monteiro et al. (2011)
	Glucosides		x			*C. albicans*	Wang et al. (2014a)
Bacteria	Peptides/ Proteins		x	x		*C. albicans*	Dusane et al. (2013), Troskie et al. (2014), Wang et al. (2012a), Yala et al. (2011)
	Biosurfactants		x	x		*C. albicans* *C. tropicalis*	Busscher et al. (1997), Ceresa et al. (2015), Dusane et al. (2011), Janek et al. (2012), Rautela et al. (2014), Rodrigues et al. (2006), Singh et al. (2013), Zakaria Gomaa (2013)
Animal	Resins		x			*C. albicans*	Capoci et al. (2015), De Castro et al. (2013)
	Peptides/ Proteins		x	x		*A. fumigatus* *C. albicans* *C. glabrata* *F. solani*	De Brucker et al. (2014), Kavanaugh et al. (2014), Sengupta et al. (2012), Wang et al. (2014b)
	Antibodies (proteins)		x			*C. albicans* *C. dubliniensis* *C. glabrata* *C. tropicalis*	Bujdáková et al. (2008), Fujibayashi et al. (2009), Hodgkinson et al. (2007), Holmes et al. (2012), Ibrahim et al. (2008), Kamikawa et al. (2014), Mishra et al. (2015)
Human	Peptides/ Proteins		x	x		*C. albicans* *C. glabrata*	Konopka et al. (2010), Pusateri et al. (2009), Rossignol et al. (2011), Tati et al. (2014)

which seemed especially linked to its main compound: linalool. This compound induced a reduction in cell size and abnormal germination. The EO inhibited the growth of *C. albicans* biofilm similar to chlorhexidine (Alviano et al. 2005).

The EO obtained from rhizomes of *Boesenbergia pandurata* demonstrated a potent anti-*Candida* biofilm activity in vitro (Taweechaisupapong et al. 2010). In addition, rhizome extract of *B. pandurata* inhibited *C. albicans* adhesion to denture acrylic surfaces in a dose-dependent manner: a pretreatment of dentures with this extract at 25, 50 and 100 mg/ml inhibited candidal adhesion by approximately 47 %, 66 %, and 75 %, respectively (Sroisiri and Boonyanit 2010).

Palmeira-de-Oliveira et al. investigated the EO obtained from aerial parts of *Thymbra capitata*, a circum-Mediterranean plant; this EO was rich in carvacrol (75 %). Authors showed that *T. capitata* EO disrupted the biomass and inhibited the metabolic activity of *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis and C. guilliermondii* pre-formed 24 h old biofilms. Concentrations about 0.32 µl/ml (which also corresponded to the EO minimal inhibitory concentration (MIC)) disrupted the biofilm matrix and inhibited the yeast metabolism up to 50 %. A marked metabolic inhibition (>80 %) was observed at 0.64 µl/ml, except for *C. albicans* that presented a more resistant profile. Biofilm biomass was also widely reduced at this concentration, except for *C. glabrata* (no more than 40 % disruption) (Palmeira-de-Oliveira et al. 2012).

Bersan et al. highlighted the interest of *Cyperus articulatus* EO acting against the formation of a *C. albicans* biofilm and inhibiting a 72 h old fungal biofilm (Bersan et al. 2014).

Mentha piperita EO obtained from aerial parts and rich in menthol (53.28 %), menthyl acetate (15.1 %) and menthofuran (11.18 %) was reported to completely inhibit the biofilm formation of *C. albicans* and *C. dubliniensis* at concentrations up to 2 µl/ml in a dose-dependent manner (Saharkhiz et al. 2012). In the same way, EO extracted from leaves of another *Mentha* species, *M. suaveolens*, was recently shown to be rich in piperitenone oxide (90 %) and to reduce both adherence (decrease of about 40 %) and biofilm formation (decrease of about 70–80 % of the metabolic activity) of *C. albicans* (Stringaro et al. 2014). The inhibitory effect on biofilm formation was confirmed by SEM observations. Besides, a synergistic effect was observed between this EO and fluconazole. It was supposed that monoterpens of this EO passed across the cell wall, damaged lipid bilayer of cell membrane increasing the *Candida* cell membranes permeability and promoting fluconazole action on ergosterol, finally causing cell damage (Stringaro et al. 2014). Recently, a novel nerolidol-rich EO derived from leaves and inflorescences of *Piper claussenianum* was tested on *C. albicans* yeast-to-hypha transition and on biofilm formation and stability. Leaf EO managed to downregulate the yeast-to-hypha transition by 81 %, and reduced biofilm formation by about 30 and 50 % after incubation for 24 and 48 h, respectively. This EO also reduced the viability of pre-formed mature biofilm (48 h) by 63.9 %. Finally, synergistic effect was highlighted using association between the leaf EO and fluconazole (Curvelo et al. 2014).

Melaleuca alternifolia (tea tree) EO, rich in terpinen-4-ol (42.8 %) and γ-terpinene (20.4 %), used at a 12.5 % was effective in vitro to eradicate a 48 h old *C. albicans* biofilm; it also reduced the *C. albicans* yeast number in an immunosuppressed mouse model (de Campos Rasteiro et al. 2014). Another study demonstrated that sub-inhibitory concentrations of this oil reduced *C. albicans* adhesion to both human cells and polystyrene, inhibited biofilm formation and decreased cell surface hydrophobicity (Sudjana et al. 2012). Complementarily, tee trea EO and two of its components: terpinen-4-ol and α-terpineol, displayed a potent activity against 69 biofilm-forming *C. albicans* tested strains. *M. alternifolia* EO used at 50 % minimal inhibitory concentration value (MIC_{50}) effectively inhibited biofilm growth when *C. albicans* cells were treated at 0, 1, and 2 h post-adhesion. Interestingly, terpinen-4-ol displayed no significant toxicity at 0.5 × MIC_{50} which gave it safety advantages over the

complete EO; authors' results suggested that terpinen-4-ol may be suitable for prophylaxis and treatment of established oropharyngeal candidosis (Ramage et al. 2012).

Cymbopogon nardus EO and its main compound (citronellal: 38 %) were recently investigated for their anti-adherent properties. The EO significantly inhibited *C. albicans* adherence to both dental implants and cover screws ($p < 0.001$) whereas citronellal only inhibited adherence to dental implants ($p < 0.001$) (Trindade et al. 2015).

EOs of another species of *Cymbopogon* : *C. citratus* and of *Syzygium aromaticum* were investigated by Khan and Ahmad and both demonstrated *C. albicans* anti-biofilm activities. These two oils were more active than amphotericin B and fluconazole against preformed biofilms. At 0.5 × MIC, *C. citratus* EO followed by *Syzygium aromaticum* EO were most inhibitory against biofilm formation. Deformations of the biofilm's three dimensional structure were observed by SEM after treatment with *C. citratus* oil (Khan and Ahmad 2012b).

Raut et al. recently investigated twenty-eight terpenoids of plant origin for their activity against *C. albicans* biofilms. Some of these molecules inhibited yeast-to-hypha transition at low concentrations (0.031–0.5 mg/ml), while adhesion to a solid surface was prevented at 0.5–2 mg/ml. Treatment with 14 terpenoids resulted in significant ($p < 0.05$) inhibition of *C. albicans* biofilm formation, and of these, linalool, nerol, isopulegol, menthol, carvone, α-thujone, and farnesol exhibited biofilm-specific activity. Finally, thymol, carvacrol, eugenol, citral, citronellol, geraniol, linalool, and ionone inhibited 24 h old biofilms (Raut et al. 2013). Other studies confirmed the interest of carvacrol, linalool, geraniol, thymol, eugenol, cinnamaldehyde, α-pinene and β-pinene or menthol against *C. albicans*, *C. glabrata* or *C. parapsilosis* biofilms alone or in association (one or two terpenes) with fluconazole (Braga et al. 2008; da Silva et al. 2012; Dalleau et al. 2008; Doke et al. 2014; Hsu et al. 2013; Khan and Ahmad 2012a; Pemmaraju et al. 2013).

Casbane diterpene was isolated from the ethanolic extract of *Croton nepetaefolius,* an aromatic plant used in folk medicine, native of the Northeast of Brazil. This compound was shown to be effective against *C. albicans* and *C. tropicalis* biofilm formation, causing a decrease of more than 50 % of the ability to form biofilm at 62.5 μg/ml and 15.6 μg/ml respectively after 24 h of treatment (Carneiro et al. 2011).

Overall, these data show the anti-adherent and anti-biofilm interest of numerous EO. However, EO are nonwettable, highly volatile and consequently unstable. There is thus a challenge for EO applications in the biomedical field. In this context, some galenical studies have been performed in recent years. Literature data report for example the fabrication and characterization of a novel nanostructured phyto-bioactive coated rayon/ polyester wound dressing surface refractory to *C. albicans* adhesion, colonization and biofilm formation, based on functionalized magnetite nanoparticles and EO from *Anethum graveolens* and *Salvia officinalis*. This complex preparation exhibited a long term anti-biofilm effect, maintained for at least 72 h. Authors concluded that the combination of stabilizing carrier properties of magnetite nanoparticles with antimicrobial features of natural substances could be a successful approach to control and prevent fungal biofilm associated infections (Anghel et al. 2013b). The same kind of structure constituted by biohybrid nanostructured magnetite nanoparticles/ *Satureja hortensis* EO was reported as being active against *C. albicans* adherence and biofilm development (Anghel et al. 2013a).

An hybrid magnetite nanoparticles/ *Rosmarinus officinalis* EO nanobiosystem or hybrid nanomaterial/ *Eugenia carryophyllata* EO also displayed anti-biofilm activity against *C. albicans* or *C. tropicalis* and authors suggested that they may be pelliculised on the surface of catheter pieces (Chifiriuc et al. 2012; Grumezescu et al. 2012). Finally, polyethylene glycol (PEG)-stabilized lipid nanoparticles sustained releasing terpinen-4-ol were also reported. The anti-biofilm activity of these

nanoparticles was shown to result from their ability to disrupt cell membrane structure and blocking of respiration chain by the inhibition of succinate dehydrogenase which is bound to the inner mitochondrial membrane of cells (Sun et al. 2012).

2.1.2 Saponins

Saponins are glycosides commonly found in plants. Their aglycone part is a triterpen or a steroid group. In addition to their surface-active properties and their common but more problematic hemolytic properties, some saponins (for example ivy or goldenrod saponins) previously demonstrated antifungal activity against phytopathogens, Human pathogens (such as *Candida)* or some dermatophytes in vitro (Bruneton 2008). Thus, in this context, two saponins, one of arginoside type and another of barrigenol family were screened from the Analyiticon Discovery compound collection housed at the Broad Institute of Harvard and MIT; they inhibited *C. albicans* biofilm formation at concentrations below the MIC (10 and 20 μg/ml respectively); the obtained inhibition level was comparable to that caused by caspofungin and one of the tested compounds visibly reduced the number of *C. albicans* hyphae in the culture. Without hemolytic activity at 100 μg/ml, saponins may provide a promising source of new *C. albicans* anti-biofilm agents (Coleman et al. 2010).

Saponin-rich fractions from *Medicago sativa* (aerial parts and roots) and *Saponaria officinalis* (used as a well-known source of plant saponins) inhibited *C. albicans* yeast-to-hypha transition, limited hyphal development, reduced yeast adhesion and biofilm formation, and eradicated mature (24 h) *Candida* biofilms (Sadowska et al. 2014).

Another plant showed an interest against *C. albicans*: *Solidago virgaurea* (Goldenrod). Its water extract, rich in saponins (0.7 or 0.95 mg/ml depending the subsp *virgaurea* or *alpestris*), decreased *C. albicans* yeast-to-hypha transition and strongly inhibited both biofilm growth and already formed 18 h old biofilms (Chevalier et al. 2012).

Small molecules belonging to the triterpenoid saponin family of gymnemic acids, isolated from *Gymnema sylvestre* leaves, inhibited *C. albicans* yeast-to-hypha transition and conidial germination and hyphal development of *Aspergillus* sp. They also inhibited the formation of invasive hyphae from *C. albicans*-infected *Caenorhabditis elegans* worms and rescued them from being killed by *C. albicans*. According to their results, authors suggested that these compounds may represent a valuable source for the development of novel anti-biofilm agents (Vediyappan et al. 2013).

2.2 Phenolic Compounds

Phenolic compounds form an abundant and large group containing in their structure at least one phenolic hydroxyl ring, exclusively derived from the shikimate/phenylpropanoid and/or the polyketide pathway, and deprived of nitrogen-based functions. This group is divided in numerous classes such as phenols, coumarins, lignans, flavonoids, anthocyanins, tannins, quinines, stilbenoïds (Bruneton 2008). Their activity against fungal biofilms has been widely investigated. For example, Shahzad et al. recently investigated the anti-biofilm activity of 14 polyphenols. Results showed that both pyrogallol, available in low amounts in cocoa products, coffee and beer and curcumin, available in common foods such as dried turmeric and curry power, displayed activity against *C. albicans* biofilms, without disrupting the biofilm or directly affecting the cellular structure. Curcumin displayed superior anti-biofilm activity, significantly inhibiting initial cell adhesion following pre-coating ($p < 0.01$), biofilm growth ($p < 0.05$) and gene expression ($p < 0.05$) and could constitute an opportunity for the development of oral care products (Shahzad et al. 2014).

The freeze-dried fruit rich in polyphenols of *Lonicera caerulea* (blue honeysuckle) and its phenolic fraction reduced biofilm formation and cell adhesion to artificial surfaces of *C. parapsilosis* (Palíková et al. 2008).

2.2.1 Stilbenoïds

Stilbenoïds are phenolic compounds with two benzenes separated by an ethane or ethene bridge (stilbene, bibenzyls, bisbibenzyls) (Bruneton 2008). As they often displayed activity against planktonic cells, their anti-biofilm activity was studied. Some bibenzyls and bisbibenzyls extracted from liverwort plants (bryophytes) have been screened for their anti-*C. albicans* properties. Among them, two natural compounds, isoriccardin C and BS-34 were found to inhibit yeast-to-hypha transition and biofilm formation in a dose-dependent manner which was consistent with an elevated farnesol production and Dpp3 expression (Zhang et al. 2011). In the same way, riccardin D, a macrocyclic bisbibenzyl isolated from Chinese liverwort *Dumortiera hirsute* was shown to display both prophylactic and therapeutic effects against *C. albicans* biofilm formation in a dose-dependent manner in vitro and in vivo; SEM and confocal laser scanning microscope (CLSM) showed that biofilm morphology was remarkably altered after riccardin D treatment, especially hyphal development by inhibiting the Ras-cAMP-Efg pathway which delayed filamentation (Cheng et al. 2009; Li et al. 2012).

2.2.2 Tannins

Tannins were defined as "phenolic natural products that precipitate proteins from their aqueous solutions" (Mole and Waterman 1987). In plants, two groups of tannins are distinguished: hydrolyzable tannins (esters of a sugar and of a variable number of phenolic acid molecules; can be quoted ellagitannins or gallotannins) and condensed tannins (or proanthocyanidins which are polymeric flavans). These compounds have been shown to present antioxidant, enzymatic inhibition, antidiarrheal and mostly antiseptic properties hence their likely interest against fungal biofilm (Bruneton 2008).

A study conducted by Evensen and Braun, highlighted that physiologic concentrations of tannins not only prevented biofilm formation, but also reduced the *C. albicans* population of a mature 48 h old biofilm. Green tea polyphenols, especially tannins, have been shown to significantly decrease *C. albicans' s* ability to grow and sustain biofilms (Evensen and Braun 2009). These authors showed that the treatment of yeasts with 1.0 µmol/l of –(−)epigallocatechin-3-gallate (EGCG), the most abundant tannin, caused a 75 % reduction of viable cells during 48 h of biofilm formation. Already formed biofilms treated with EGCG were also reduced by 80 %. The same concentrations of epigallocatechin and epicatechin-3-gallate also demonstrated similar biofilm inhibition. Regarding the mechanism of action, these authors showed that yeast treatment with catechine induced a reduction of in vivo proteasome activity contributing to cellular metabolic and structural disruptions causing anti-biofilm activity (Evensen and Braun 2009).

Some proanthocyanidins, demonstrated an efficacy against biofilms of *Candida* sp. For instance, several studies reported the anti-biofilm activity of A-type proanthocyanidins obtained from Cranberry (*Vaccinium macrocarpon*). These compounds prevented biofilm formation and reduced *C. albicans* adhesion to oral epithelial cells, saliva-coated acrylic resin discs, polystyrene and silicone. They also reduced *C. glabrata* adhesion to polystyrene. At concentrations of at least 16 mg/l, they were shown to significantly reduce biofilm formation of *C. albicans* and be additive in combination with conventional antifungal agents (fluconazole, amphotericin B and caspofungin) (Feldman et al. 2012; Girardot et al. 2014; Rane et al. 2014).

The anti-biofilm activity of proantocyanidin fractions obtained from *Stryphnodendron adstringens* stem bark was also recently shown against *C. albicans*. At doses considered as safe (>31.25– > 1000 µg/ml), some proantocyanidin fractions inhibited biofilm formation and reduced the metabolic activity of 24 h old biofilm cells. Interestingly, these fractions also reduced the metabolic activity of free cells detached from the biofilm, indicating that they could contribute to inhibit infection dissemination (Luiz et al. 2015).

Finally, the methanolic extract of pomegranate (*Punica granatum* L.) rich in ellagic acid displayed anti-biofilm activity against *C. albicans*: it was shown to both inhibit biofilm formation and disrupt pre-formed biofilms when used at 250 μg/ml; it also inhibited yeast-to-hypha transition. Other results showed that pure ellagic acid was active against biofilm formation at concentrations lower than 40 μg/ml which could explain pomegranate extract activity (Bakkiyaraj et al. 2013).

2.2.3 Quinones

Some quinones could also help to fight fungal biofilms. These compounds that contain oxygen are essentially the oxidized homologs of aromatic derivatives. Some of them previously demonstrated laxative or antibacterial and fungicidal properties (Bruneton 2008). Purpurin which is a natural red anthraquinone pigment commonly found in madder root (*Rubia tinctorum* L.), blocked *C. albicans* yeast-to-hypha transition when used at a sub-lethal concentration (3 μg/ml) (Tsang et al. 2012; 2013). This non-toxic compound to Human cells also inhibited *C. albicans* and *C. dubliniensis* biofilm formation and reduced the metabolic activity of 24 h old biofilms in a concentration-dependent manner. *C. albicans* or *C. dubliniensis* biofilms treated by purpurin appeared scanty and exclusively consisted of blastospore aggregates (Tsang et al. 2012; 2013). Regarding the mechanism of action, purpurin was shown to downregulate the expression of hypha-specific genes (ALS3, ECE1, HWP1, HYR1) and the hyphal regulator RAS1. Purpurin triggered metacaspase-independent apoptosis in *C. dubliniensis* biofilms (Tsang et al. 2012; 2013).

2.2.4 Flavonoids

Some universal plant pigments, called flavonoids and possessing a basic structural element namely the 2-phenylchromane, previously demonstrated venoactive, antioxidant, enzymatic inhibitor, antimicrobial properties (Bruneton 2008). Concerning an anti-biofilm activity of plant flavonoids, some examples were quoted in literature data. Baicalein, a flavone present in the Chinese herb *Scutellaria baicalensis*, inhibited the formation of *C. albicans* biofilms in a dose-dependent manner (inhibition of 70 % at concentrations between 4 μg/ml and 32 μg/ml). It was active against different biofilm growth stages, with 89 % and 52 % inhibition when added at 0 h and 24 h of the incubation period, respectively. Baicalein may act by decreasing the cell surface hydrophobicity as suggested by results showing a downregulation of *CSH1* (a gene encoding the cell surface hydrophobicity-associated protein) in baicalein treated fungal cells (Cao et al. 2008).

A synthetic isopentenyloxychalcone of natural origin, 4-hydroxycordoin, was shown to inhibit two important virulence factors of *C. albicans*. Indeed it strongly inhibited biofilm formation at 20 μg/ml, while concentrations ranging from 50 to 200 μg/ml significantly reduced yeast-to-hypha transition (Messier et al. 2011). More recently the same research team investigated licochalcone A which is predominantly present in licorice. This flavonoid used at 0.2 μg/ml induced a 59 % inhibition of the *C. albicans* biofilm formation and a cytotoxicity study showed that no toxicity was observed at this concentration (Messier and Grenier 2011).

Very interestingly, other flavonoids, extracted from *Moringa oleifera* seed coat, were recently showed to inhibit early attachment, promote disruption of 14 h old preformed biofilms and decrease metabolic activity of *C. albicans* biofilms, all of that being obtained at concentrations without toxicity or mutagenicity (Onsare and Arora 2015).

2.3 Peptides

Proteins and peptides, primary metabolites, with anti-microbial activity are produced by a wide variety of organisms in order to protect themselves from infection (Mandal et al. 2011). Some studies reported peptides with activities against fungal biofilms, such as Tn-AFP1 which is a plant peptide, with molecular mass of 1230 Da. This peptide was purified from fruits of *Trapa natans,* a floating annual aquatic plant

and was able to disrupt the formation of *C. tropicalis* biofilm in a concentration dependent manner; a 50 % inhibition of the metabolic activity was observed using Tn-AFP1 at 16 µg/ml. This peptide also downregulated MDR1 and ERG11 gene expression (Mandal et al. 2011).

Recent results have shown that a decapeptide from the model plant *Arabidopsis thaliana,* OSIP108, inhibited the biofilm formation process of *C. albicans* without toxicity against various human cell lines such as osteoblasts, mesenchymal stem cells and endothelial cells. Besides, OSIP108 (12.5–100 µM) synergistically enhanced the activity of conventional antifungal agents such as amphothericin B and caspofungin against mature *C. albicans* biofilms (Delattin et al. 2014).

Finally, a hydroxyprolin rich glycopeptide extracted from pericarp of *Datura stramonium* and called datucin also displayed an anti-biofilm activity against *C. albicans*: the minimum biofilm eradication concentration was 2 µM (Mandal 2012).

2.4 Alkaloids

Alkaloids are defined as "an organic compound of natural origin, which contains a nitrogen atom, is more or less basic, is of limited distribution, and has, at low doses, marked pharmacological properties" (Bruneton 2008). These compounds have been shown to perform various pharmacological activities such as a depressant or stimulant of the central nervous system, sympathomimetic or sympatholytic of the autonomic nervous system, antimalarial, antifibrillant, antitumor, local anesthetic, antimicrobial (Bruneton 2008). Some alkaloids are considered to be also active against fungal biofilms.

Tetrandrine is a bis-benzylisoquinoline alkaloid compound originating from several natural plant sources, including *Stephania tetrandra*. Results of Zhao et al. showed that the addition of tetrandrine (concentration $\geq$16 mg/l) just after adhesion significantly inhibited ($p < 0.05$) the formation of *C. albicans* biofilms in a dose-dependent manner. Interestingly tetrandrine at 32 mg/l was able to significantly inhibit mature biofilms ($p < 0.05$), and here again activity increased depending on this compound concentration. These authors also investigated the anti-biofilm capacity against other fungal species, and observed only a weak anti-biofilm activity against *C. neoformans* and no effect against *A. fumigatus*. They hypothesized that the mechanism of action of tetrandrine could possibly be partly linked to its ability to decrease the cell surface hydrophobicity and to inhibit *C. albicans* hyphal development through the Ras1p-cAMP-PKA pathway; this alkaloid compound would thus present an interest in *C. albicans* biofilm-associated infections (Zhao et al. 2013).

Some combinations could be interesting. For example, berberine, a plant alkaloid from *Hydrastis canadensis* (goldenseal) combined with miconazole were able to reduce *C. albicans* biofilm formation by >91 % after 24 h whereas neither berberine nor miconazole alone were active. This combination also exhibited synergy against pre-formed *C. albicans* biofilms (Wei et al. 2011).

Finally, matrine, one of the major alkaloid components found in sophora roots, could reduce *C. albicans* yeast-to-hypha transition; additionally this compound used at 2 mg/ml would cause a 50 % decrease of the metabolic activity of *C. albicans* biofilms in development after a 24 h co-incubation of matrine with *C. albicans* cells (Shao et al. 2014).

2.5 Biosurfactants

Biosurfactants are a surface-active heterogeneous group of amphiphilic molecules released by microorganisms that exhibit surface activity (Rodrigues et al. 2006). Various chemical structures of primary metabolites, such as glycolipids, lipopeptides, polysaccharide–protein complexes, protein-like substances, lipopolysaccharides, phospholipids, fatty acids and neutral lipids, have been attributed to

biosurfactants. Due to their affinity to adhere to surfaces, these antimicrobial compounds are being used as a new strategy for inhibiting the adhesion of *C. albicans* in non-biological surfaces (Rodrigues et al. 2006). Microbial flora associated to plants could also be a source of biosurfactants with anti-biofilm activity. Endophytes help to protect their host plants by producing substances, including biosurfactants, which improve their natural protection against many environmental agents. As they are closely linked to plants, they are included in this section instead of Sect. 4.

For example, Cochis et al. demonstrated the anti-biofilm efficacy of three biosurfactants obtained from endophyte biofilms selected from *Robinia pseudoacacia* and from *Nerium oleander*. Actually, *C. albicans* biofilm formation on elastomer silicon or resin surfaces pre-coated with these biosurfactants used at low concentrations (78.1 or 156 μg/ml) was significantly reduced ($p < 0.01$) thanks to their good anti-adhesion activity. Interestingly these biosurfactants displayed a higher anti-*Candida* activity than that of chlorhexidine and a cytocompatibility with epithelial cells and fibroblasts according to what was observed (Cochis et al. 2012).

2.6 Unknown or Mixed Composition

Some literature data pointed out anti-biofilm activities of plant extracts with various or unspecified composition. For example, Shuford et al. investigated the inhibitory activity of a fresh garlic extract against *C. albicans* biofilm formation (treatment immediately after adherence by concentrations ranging between 0.5 and 1 mg/ml) and against 48 h old mature biofilms treated for 1 h or 48 h (concentrations ranging between 2 and 4 mg/ml). Results showed that fresh garlic extract was significantly active in all cases ($p < 0.001$). The superior activity observed with 1 h versus 48 h of treatment against pre-formed biofilms probably related to the half-life of the extract at 37 °C and should be taken into account for an eventual future therapeutic use (Shuford et al. 2005).

In another study, the activity of *Cassia spectabilis* methanol leaf extract against biofilm formation of *C. albicans* was investigated using transmission electron microscopy (TEM) and SEM approaches. This extract used at 6.25 mg/ml significantly prevented biofilm formation, SEM analysis revealing a reduction in adhering cells and biofilm development in presence of the extract. In addition TEM analysis highlighted some alterations in morphology and complete collapse of yeast cells after 36 h of exposure to the extract (Sangetha et al. 2009).

Barbieri et al. evaluated the anti-adherent property of crude, methanol and ethyl acetate-methanol extracts from *Schinus terebinthifolius* and *Croton urucurana* on *C. albicans* biofilms in vitro. Results showed that *S. terebinthifolius* ethyl acetate–methanol and methanol extracts were the most efficient against *C. albicans* biofilms ($p < 0.05$). These extracts were constituted by several active compounds, including phenolic compounds, anthraquinones, terpenoids, and alkaloids. Concerning *C. urucurana* extracts, higher concentrations were needed to achieve anti-adherent activity (Barbieri et al. 2014).

3 Anti-biofilm Compounds of Lichen and Algual Origin

3.1 Lichen Origin

Lichens are constituted by the association of a fungus (usually an ascomycete, called the mycobionte) and a green alga or a cyanobacteria (called the photobionte). This symbiotic organization leads to the formation of lichen thalli. Besides, recent molecular studies have revealed that lichens also present diverse microbial communities which colonize surfaces and the inside of lichens in a biofilm-like manner. This complex organization is at the origin of a great variety of secondary metabolites, many of which are unique such as some depsides, despidones, dibenzofuranes or pulvinic derivatives (Grube

and Berg 2009). Besides quinones, anthraquinones, terpenes and steroids were also found (Rundel 1978). Many secondary lichen metabolites offer protection to lichen communities against other microorganisms. And some of these compounds have previously demonstrated some biological activity such as antimicrobial (physodic, chloroatranorin, lobastin, atranorin, olivetoric, usnic, norstictic, protocetraric and vulpinic acids, anthraquinones), antitumoral (usnic, D-protolichesterinic and nephrosternic acids), UV protectors (usnic, diffractic, barbatic acids, parietine), antioxidant (despidones, usnic, lecanoric acid, atranorin, orsenillic acid, orcinol...), antiviral (usnic acid), antiprotozoal (5-propylresorcinol, isodivaricatic, divaricatic, usnic acids), smooth muscle relaxant (fumaroprotocetraric, usnic acids, depsidones), inhibitors of tyrosinases (resorcinol derivatives), allergenic (D-usnic and evernic acids and atranorin), analgesic, antipyretic and anti-inflammatory (diffractaic, usnic acids) (Shukla et al. 2010; White et al. 2014). Antimicrobial activity was mainly evaluated on planktonic cells even if a few studies also focused on sessile cells. Indeed some lichen metabolites may be useful to control biofilms formed by human pathogens as they offer protection to lichen communities against adherent microorganisms (Francolini et al. 2004; Lazăr and Chifiriuc 2010). Concretely, Pires et al. evaluated the activity of a 48 h treatment with usnic acid against 24 h old *C. orthopsilosis* or *C. parapsilosis* biofilms. They observed a 50 % reduction in the metabolic activities of the two species biofilms using usnic acid at 3.9 μg/ml; furthermore 31.2 and 62.5 μg/ml reduced the growth of the cells in 80 % respectively (Pires et al. 2012).

Retigeric acid B, a pentacyclic triterpenoid was isolated from the lichen *Lobaria kurokawae*. It demonstrated in vivo ability to inhibit *C. albicans* yeast-to-hypha transition in infected mice; it also inhibited in vitro adherence of *C. albicans* to KB/VCR cells in a dose-dependent manner using concentrations ranging between 8 and 32 μg/ml. Finally, retigeric acid B and azoles interacted synergistically to block *C. albicans* biofilm formation (Chang et al. 2012).

Recently, quercetin, a dietary flavonoid isolated from an edible lichen (*Usnea longissima*), was demonstrated as strongly suppressing the production of virulence weapons such as biofilm formation or hyphal development. Interestingly, treatment with quercetin also increased fluconazole mediated cell death in fluconazole resistant *C. albicans* biofilms. Quercetin activity could be related to an increase of farnesol production, which is known to coregulate hyphal development, biofilm formation, and virulence factor production (Singh et al. 2015).

The fungal flora inside lichens is also a source of anti-biofilm metabolites. Pyridoxatin, a small natural product isolated from an endolichenic fungus: *Tolypocladium cylindrosporum*, derived from the lichen *Lethariella zahlbruckner,* could prevent the formation of biofilm. Investigations to clarify the mechanism of action revealed that pyridoxatin interfered with the biosynthesis of ergosterol, which probably inhibits the cell growth of *C. albicans* (Chang et al. 2015).

Similarly, diorcinol D is a natural product obtained from an endophytic fungus: *Aspergillus versicolor* derived from the lichen *Lobaria quercizan*. This compound demonstrated anti-biofilm activity against 24 h old *C. albicans* biofilms at 32 μg/ml, and its effect was enhanced by adding fluconazole (8 μg/ml). In the same way, more than 250-fold reductions of the MICs of fluconazole were observed when combined with diorcinol against these mature biofilms. CLSM images confirmed this activity (Li et al. 2015).

3.2 Algual Origin

Algae, photosynthetic marine or aquatic organisms, also produce active metabolites that are especially active against biofilms. For example, Thibane et al. investigated the effectiveness against *Candida* biofilms of marine long chain polyunsaturated fatty acids contained in algae. It

was found that stearidonic acid (18:4 n-3), eicosapentaenoic acid (20:5 n-3), docosapentaenoic acid (22:5 n-3) and docosahexaenoic acid (22:6 n-3) had an inhibitory effect on mitochondrial metabolism, affected cellular morphology in biofilms of both *C. albicans* and *C. dubliniensis* and significantly inhibited biofilm biomass of *C. dubliniensis* (Thibane et al. 2010).

4 Anti-Biofilm Compounds of Microbial Origin

Fungi and Bacteria also play an important role as producers of active compounds. They are able to synthesize active primary metabolites such as protein and peptides or glycolipids and active secondary metabolites such as alkaloids or terpenes. Some literature data highlight the anti-biofilm efficacy of some microbial compounds.

4.1 Fungal Origin

For a long time, fungi showed their interest as a main source of antimicrobial substances. Major antifungal drugs currently employed as echinocandins (caspofungin, micafungin and anidulafungine) were initially obtained by fermentation of filamentous fungi including *Aspergillus* sp. These fungi are also a source for new anti-biofilm compounds presented here in three non-exhaustive groups: alkaloids, terpenes and biosurfactants/glucosides.

4.1.1 Alkaloids

A complex prenylated indole alkaloid named waikialoid A and a polyketide metabolite, waikialide A, produced by a Hawaiian-soil-derived *Aspergillus* sp have demonstrated activity against *C. albicans* biofilm formation: their presence during both adherence step and biofilm growth inhibited the metabolic activity of 48 h old biofilms with IC_{50} values of 1.4 µM and 32.4 µM respectively. Results also showed that waikialoid A inhibited yeast hyphal development, suggesting an effect on the early phase of biofilm formation, and displayed no toxicity against humain cells up to 200 µM (Wang et al. 2012b).

More recently, this research team identified two other alkaloid metabolites named shearinines D and E and produced by an Alaskan-soil-derived *Penicillium* sp which also exhibited anti-biofilm activity against *C. albicans*. Here again the presence of these alkaloids during both adherence step and biofilm growth inhibited the metabolic activity of 48 h old biofilms (IC_{50} = 8.5 and 7.6 µM, respectively) but they blocked the outgrowth of hyphae suggesting an effect on a relatively late stage of biofilm development; interestingly shearinines D and E exhibited synergistic activities with amphotericin B against different species of *Candida* (*C. albicans*, *C. glabrata, C. parapsilosis, C. tropicalis, C. kefyr*) (You et al. 2013).

4.1.2 Terpene

Here we can again quote farnesol, a sesquiterpene alcohol acting as a quorum sensing molecule which inhibits the yeast-to-hypha transition and compromises *C. albicans* biofilm formation (Hornby et al. 2001; Ramage et al. 2002). Farnesol has also been shown to inhibit the formation of *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* biofilms (Weber et al. 2010).

Finally, cyclosporine A, a calcineurin inhibitor with a fungal origin, was shown to exhibit synergism with conventional antifungals (amphotericin, fluconazole, voriconazole, caspofungin) against *C. parapsilosis* species complex. The tested combinations involving cyclosporine A prevented biofilm formation and showed an inhibitory effect against mature biofilms (48 h old) of *C. parapsilosis sensu stricto*, *C. metapsilosis* and *C. orthopsilosis* (Cordeiro et al. 2014).

4.1.3 Biosurfactants and Glucosides

As previously mentioned, biosurfactants could be used to prevent fungal adherence to non biological surfaces such as medical devices. Monteiro et al. showed that a glycolipid-type biosurfactant produced by the yeast *Trichosporon montevideense* CLOA72 used at

16 mg/ml reduced up to 87.4 % the biofilm formation of a *C. albicans* strain isolated from the apical tooth canal. Changes of cell surface characteristics induced by this compound could contribute to the inhibition of initial adherence of *C. albicans* cells. Finally this compound presented no cytotoxic effect in mammalian cells (Monteiro et al. 2011).

Other primary metabolites: polyketide glycosides from *Bionectria ochroleuca*, a fungus cultured on Cheerios breakfast cereal exhibited potent biofilm inhibition against *C. albicans* (Wang et al. 2014a).

4.2 Bacterial Origin

Similarly to fungi, bacteria also demonstrated their importance as producers of antimicrobial substances. For example a current major antifungal drug: amphotericin B was initially produced by filamentous bacteria of *Streptomyces* sp genus. Bacteria can also synthesize new anti-biofilm compounds as active peptides, proteins and biosurfactants. A study conducted by Sharma and Srivastava focused on the anti-biofilm activity of a spent culture filtrate of an environmental isolate of lactic acid bacterium, *Lactobacillus plantarum*. They observed that *C. albicans* biofilm formation was significantly reduced ($p < 0.05$) when the filtrate was present (Sharma and Srivastava 2014).

Tyrocidines are cationic cyclodecapeptides produced by *Bacillus aneurinolyticus*. Troskie et al. observed that the formation of *C. albicans* biofilm in the presence of tyrocidines for 24 h caused a biofilm reduction suggesting a preventive interest. They also observed that tyrocidines disrupted the membrane integrity of 24 h old *C. albicans* biofilm cells and demonstrated pronounced synergistic biofilm-eradicating activity in combination with amphotericin B and caspofungin (Troskie et al. 2014).

A protein designed as BL-DZ1 was obtained from a tropical marine strain of *B. licheniformis* isolated from the surface of a green mussel, *Perna viridis*. The characterization of BL-DZ1 demonstrated its ability to decrease biofilm formation (87 % inhibition in presence of BL-DZ1 at 1.60 µg/ml) and to disperse *C. albicans* 24 h old biofilms pre-formed onto polystyrene surfaces (up to 67.2 % inhibition by BL-DZ1 at 1.60 µg/ml) (Dusane et al. 2013).

A cyclic lipopeptide biosurfactant constituted by a mixture of iturin and fengycin and produced by another *Bacillus: B. amyloliquefaciens* reduced *C. albicans* cell surface hydrophobicity. It hindered yeast-to-hypha transition and reduced the mRNA expression of hyphae-specific genes HWP1 and ALS3. The presence of this biosurfactant during biofilm growth (for 48 h) inhibited *C. albicans* biofilm formation in the range of 46–100 % in a concentration-dependent manner (between 1 and 6 mg/ml). Furthermore, this compound used at similar concentrations dislodged 25–100 % of 48 h old biofilm preformed on polystyrene surfaces (Rautela et al. 2014).

Gramicidins were first identified as antibiotic molecules produced by a soil-derived *B. brevis* strain but could also have an anti-adherent potential. In a study of Yala et al. gramicin A was covalently bound to cystamine self-assembled monolayers on gold surface. Authors showed that the adsorbed gramicidin was able to reduce by 90 % the number of culturable *C. albicans* cells attached to the surface (Yala et al. 2011).

Mutanobactin A is a hybrid polyketide-nonribosomal peptide metabolite produced by *Streptococcus mutans*. This compound and two new analogues, mutanobactins B and D have been investigated and their ability to inhibit the formation of *C. albicans* biofilms was highlighted (IC_{50} up to 5.3 µM) (Wang et al. 2012a).

A study performed by Busscher et al. then completed by Rodrigues et al. reported that biosurfactants produced by *S. thermophilus* significantly reduced initial *C. albicans* and *C. tropicalis* adhesion. These biosurfactants were mixtures of various compounds with a glycolipid-like component being the most active (Busscher et al. 1997; Rodrigues et al. 2006).

A crude biosurfactant from ten lactobacilli isolated from Egyptian dairy products also exhibited anti-adhesive activity against

C. albicans (Zakaria Gomaa 2013) as well as a biosurfactant produced by *Lactobacillus brevis*, which was shown to reduce both fungal adhesion and biofilm formation of *C. albicans* on silicone elastomer surfaces (Ceresa et al. 2015).

A di-rhamnolipid biosurfactant produced from *Pseudomonas aeruginosa* was able to disrupt *C. albicans* biofilm formed on polystyrene surfaces by modifying the extracellular matrix (Singh et al. 2013).

Pseudofactin II is a biosurfactant cyclic lipopeptide secreted by *P. fluorescens* and obtained from freshwater from the Arctic Archipelago of Svalbard. This biosurfactant at 0.25 mg/ml has been shown by CLSM observations to reduce *C. albicans* adhesion to glass, polystyrene and silicone surfaces. On polystyrene surfaces, this surfactant used at 0.035–0.500 mg/ml caused adhesion inhibition in the range of 45–99 % (Janek et al. 2012).

Finally, another biosurfactant, a glycolipid composed of glucose and palmitic acid and produced by a tropical marine strain of *Serratia marcescens* isolated from the hard coral, *Symphyllia* sp. was also investigated; its ability to both prevent adhesion (up to 76 % and 82 % at 50 and 100 µg/ml) and disrupt 24 h old preformed biofilms of *C. albicans* (polystyrene surfaces; up to 55 % at 50 µg/ml) was demonstrated. CLSM and SEM confirmed the effective removal of biofilms from glass surfaces (Dusane et al. 2011).

5 Anti-Biofilm Compounds of Animal Origin

Literature data highlighted that insects such as bees or wasps and mammals such as pigs, mice, cows or chickens also produced anti-biofilm compounds. These compounds are of natural origins meaning without human intervention (resin, proteins and peptides) or of artificial origins requiring human action (production of antibodies). Some of them will be developed below.

5.1 Resin, Proteins and Peptides

Numerous properties including antimicrobial, anti-inflammatory, immunostimulatory or antiseptic effects have been previously described in literature for a complex resin produced by honey bees and called propolis (Capoci et al. 2015). Besides a study performed using 30 clinical *C. albicans* strains demonstrated that an ethanol extract of propolis used at 273 µg/ml inhibited ($p \leq 0.05$) the biofilm formation of 93.34 % of strains (Capoci et al. 2015). Another study confirmed the ability of propolis to reduce *C. albicans* biofilm formation in vitro and also identified some genes involved in cell adhesion, biofilm formation or filamentous growth that contributed to propolis tolerance (de Castro et al. 2013).

Other compounds of interest were mucins obtained from pig stomachs. These biopolymers, heavily glycosylated proteins, were the main gel-forming constituents of mucus. Mucins may suppress *C. albicans* surface adhesion by downregulating ALS1 and ALS3 genes and decreasing its biofilm formation. Besides, a new oval-shaped form of cells was observed which was less able of stably integrating into an emerging biofilm. The yeast-to-hypha transition appeared to be also perturbed (Kavanaugh et al. 2014).

A 26-amino-acid truncated form of the 34-amino-acid cathelicidin-related peptide was produced by murine pancreas and named P318; its use at 0.15 µM was shown to inhibit *C. albicans* biofilm formation (De Brucker et al. 2014).

A study performed by Sengupta et al. revealed that lactoferricin B, an antimicrobial peptide derived from a milk protein, could improve biofilm susceptibility to antifungal agents and be a promising candidate as an antibiofilm-antifungal additive in lens care solution against three common keratitis-associated fungal pathogens: *C. albicans*, *Fusarium solani* and *Aspergillus fumigatus* (Sengupta et al. 2012).

Finally, polybia-MPI, an amphipathic cationic peptide (14 amino-acids) originally isolated from

the venom of a social wasp *Polybia paulista* was recently investigated for its anti-*C. glabrata* activity (Wang et al. 2014b). These authors underscored the anti-biofilm activity of polybia-MPI : the first 24 h of *C. glabrata* biofilm formation were inhibited in a dose-dependent manner in presence of polybia-MPI (8–64 μM) (Wang et al. 2014b).

5.2 Antibodies

Another strategy to fight *Candida* biofilm may be the use of antibodies. Nowadays, cell-surfaces, cell walls, and secreted proteins are targeted by many antifungal therapies (Mishra et al. 2015). Antibodies are believed to stimulate anti-*Candida* activity by different mechanisms, such as inhibition of adhesion and neutralization of virulence-related antigens (Mishra et al. 2015). Recently, these authors found that a monoclonal antibody (MAb 7D7) generated in mice against *C. albicans* biofilm cell surface antigen (47.2 kDa) could prevent *C. albicans* adhesion and biofilm formation, although MAb 7D7 activity appeared to be strain dependent (Mishra et al. 2015).

Another study presented the activity of a polyclonal serum obtained after immunization of rabbits with a peptide corresponding to a fragment of the *Candida* antigen CR3-RP. Authors observed that ability of *C. albicans* to adhere to buccal epithelial cells and to form biofilms were reduced by up to 35 % ($p < 0.001$), and 28 % ($p < 0.001$) respectively, in presence of the studied serum. Changes in biofilm thickness and integrity were also observed. The *Candida* antigen CR3-RP may have the potential for vaccine development (Bujdáková et al. 2008).

Additionally, polyclonal anti-*C. albicans* antibodies (anti-CA IgY) in chicken egg yolks were proved to significantly reduce the adherence of *C. albicans*, *C. dubliniensis* and *C. tropicalis* to human buccal epithelial cells and human pharynx carcinoma cells in a dose-dependent manner (Fujibayashi et al. 2009; Ibrahim et al. 2008). Fujibayashi et al. also observed that anti-CA IgY (2 mg/ml) inhibited biofilm formation of *C. albicans* in nutrient-poor condition (medium without serum), but inhibition was slightly restored in medium containing 10 % serum (Fujibayashi et al. 2009). According to authors, anti-CA IgY may be considered as a prophylactic immunotherapy under limited conditions (Fujibayashi et al. 2009; Ibrahim et al. 2008). A study has recently completed these results, demonstrating an anti-adhesion activity of anti-CA and anti-*C.* glabrata IgY against *C. albicans* and *C. glabrata* cells to denture base material (Kamikawa et al. 2014).

Furthermore, bovine milk produced by immunized cow was studied, and contained anti-*C. albicans* immunoglobulin A antibodies. This milk decreased adherence of *C. albicans* to silicone in vitro. However, this effect was not replicated in a small clinical pilot study (Hodgkinson et al. 2007; Holmes et al. 2012).

6 Anti-Biofilm Compounds of Human Origin

Finally, even Humans appear to be a promising source of compounds with anti-biofilm activity especially Human-derived peptides and proteins.

First, intuitively, Human-derived antimicrobial peptides should have reduced toxicity. Rossignol et al. investigated a 18-amino-acid cationic, tryptophan-rich ApoEdpL-W peptide derived from human ApoE apolipoprotein (Rossignol et al. 2011). This peptide (concentrations ranging between 2.5 and 20 μM) added once *C. albicans* was adhered to polystyrene (early-stage biofilm), inhibited the biofilm development. In addition, ApoEdpL-W adsorbed on polyurethane and polydimethylsiloxane substrates partially prevented the formation of biofilms by promoting biofilms that had a strong tendency to detach from polymer, suggesting that this peptide could be promising coating for medical devices. Unfortunately, a significantly lower activity was observed against mature biofilms. This study also suggested that ApoEdpL-W mode of action was dependent upon vacuolar targeting (Rossignol et al. 2011).

Histatins, a family of basic peptides secreted by the major salivary glands in humans, especially histatin 5, were investigated and demonstrated an ability to inhibit both *C. albicans* biofilm formation and *C. albicans* and *C. glabrata* mature 48 h old biofilms developed on denture acrylic (Konopka et al. 2010; Pusateri et al. 2009).

However a recent study also reported that a large glycofragment of the Msb2 surface protein was released by *C. albicans* into the growth environment, and protected against histatin-5 activity (Swidergall et al. 2013). Besides, it has been shown that histatins were rapidly degraded in vivo, limiting their interest as therapeutic agents (Tati et al. 2014). A conjugate peptide was constructed using spermidine linked to the active fragment of histatin-5 (Tati et al. 2014). This component appeared significantly more effective in killing *C. albicans* and *C. glabrata* through the biofilm development than histatin-5 used alone. Finally, topical application to tongue surfaces of immunocompromised mices caused reduction of *C. albicans* colonies recovered from tongue tissues, highlighting the potential of this new class of peptides (Tati et al. 2014).

7 Conclusion

Nature shows a crucial interest in the fight against fungal biofilm. Literature data are numerous concerning original compounds of various natural origins such as plants, lichens, algae, fungi, bacteria, animals as well as humans demonstrating activity against fungal biofilms. The chemical structure of these active compounds is variable: the activity can be attributed to primary metabolites such as peptides, proteins, glycolipids, lipopeptides, polysaccharides, lipopolysaccharides, phospholipids, fatty acids or to secondary metabolites such as essential oils, terpenes, phenolic compounds, saponins and alkaloids. Available data mainly concern *Candida* sp and to a lesser extent *Aspergillus* sp biofilms. To our knowledge, little data is available concerning natural compounds active against biofilms involving dermatophyte fungi as *Trichophyton* or yeasts such as *Malassezia* sp also involved in some infection. Up to now, investigations have mainly focused on the anti-adhesion or anti-maturation activity of natural compounds. It would be of course essential to characterize components able to inhibit dispersion or to eradicate already formed biofilms. Anyway, all these natural sources are a huge reserve for new drugs and are worth exploring in order to develop new anti-biofilm strategies.

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