Chapter 10
Animal Infections: The Role of Fungal Biofilms

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10.1 Introduction

Microorganisms, throughout their evolution, modify their physical and metabolic habitats to adapt to the environmental conditions. Bacteria were initially believed to be unicellular; however, this was not accurate, as the pure planktonic mode of growth is uncommon and the bacteria frequently exist in complex communities (Marsh 1999). One of the biggest paradigms among microbiologists is the concept that bacteria are an asocial organism, which divide and reproduce identical copy progeny. However, it has been known for years that bacteria may demonstrate group behaviour, in which bacteria cross-talk with each other and act as a community (Aguilar-Romero et al. 2011). Now, with the use of advanced techniques, direct observation indicates that most microbes remain adherent to surfaces inside a structured ecosystem, called biofilms (Costerton et al. 1987). Microbial biofilm is defined as adherent microbial communities surrounded by self-produced extracellular polymeric substances (EPS) matrix. The microbial biofilm could be formed on biotic or abiotic surfaces, and could consist of a single microbial species or polymicrobial species, such as biofilms of bacteria and fungi. Biofilm formation is an important aspect of many bacteria diseases; however, many pathogenic filamentous fungi and yeasts, such as *Candida* spp., form biofilms. The composition and architecture of the fungal biofilms attribute tolerance to antifungal agents and require up to 10–1000 times greater concentrations of antifungal agents than planktonic cells to eradicate biofilms. In mature biofilms, the cell metabolism is slow and demonstrates differential gene expressions compared to the counterpart planktonic cells; therefore, the

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common antifungal agents are ineffective on active growing cells. In addition, the biofilm microbes are resistant to phagocytosis and, thus, are resistant to host responses (Amorena et al. 1999; Jefferson 2004). The biofilm increases the frequency of spread of resistant genes and gives rise to persister cells. As a result, biofilm infections are difficult to treat and increase the cost of treatment and recovery time.

Although filamentous fungi and yeast are also known to form biofilms, most of the fungi biofilms have been studied using a yeast like *Candida* spp. According to some researchers, the biofilm formation stages in yeast and filamentous fungi are not similar. The biofilm formation by yeast is similar to bacteria. However, the filamentous fungi form biofilms with some additional stages (Harding et al. 2009). The filamentous fungi secrete small proteins called hydrophobins, which facilitate the adhesion of hyphae to hydrophobic surfaces during biofilm formation (Harding et al. 2009; Wosten 2001). In animals, various fungal species, including *Candida* spp., *Cryptococcus* spp. (Ajesh and Sreejith 2012; Martinez and Casadevall 2006; Pettit et al. 2010; Martinez et al. 2010), *Malassezia* spp. (Bumroongthai et al. 2016), *Trichosporon* spp. (Iturrieta-Gonzalez et al. 2014), *Fusarium* spp. (Imamura et al. 2008; Mukherjee et al. 2012; Peiqian et al. 2013), *Scedosporium* spp., *Lomentospora prolificans* (Mello et al. 2016) and *Coccidioides* spp. (Davis et al. 2002), cause various biofilm-related infections, such as otomycosis, dermatitis, stomatitis, onychomycosis, vulvovaginitis, urinary tract infection and respiratory tract infection. In addition, the multi-species biofilm of fungi and bacteria is common in animal infections. In this review chapter, we present an overview of fungal biofilm-related infections in animals.

10.2 Fungal Biofilms and Infectious Disease

Although biofilm formation by *C. albicans* has been known for many years, recently, many studies have reported that various species of filamentous fungi possess the ability to form biofilms. The biofilm mode of growth plays an important role in infectious disease in both humans and animals. Paracoccidioidomycosis is a systemic, endemic mycosis caused by a dimorphic fungus *Paracoccidioides brasiliensis*. In the yeast phase, *P. brasiliensis* forms biofilm and increases the gene expressions of GP43, enolase, GAPDH and aspartyl proteinase, and decreases the expression of phospholipase, which are required for adhesion and biofilm formation (Sardi Jde et al. 2015). Histoplasmosis is a respiratory systemic mycosis caused by *Histoplasma capsulatum*, a dimorphic fungus that exists as biofilm in the yeast phase (Pitangui et al. 2012). In humans and animals, dermatophytes fungi invade keratinised tissues, producing dermatophytosis (Weitzman and Summerbell 1995; Costa-Orlandi et al. 2012). It has been reported that the fungi *Trichophyton rubrum* and *T. mentagrophytes* form biofilms that result in dermatophytosis and onychomycosis, which often relapse and show non-response to ward treatment (Burkhart et al. 2002; Costa-Orlandi et al. 2012). Moreover, the biofilms of *Histoplasma*,

Paracoccidioides and *Trichophyton* are resistant to antibiotics. The saprophytic fungi *Aspergillus* causes severe superficial and systemic infections (Gonzalez-Ramirez et al. 2016; Müller et al. 2011; Kaur and Singh 2014) in humans and animals. Aspergillosis caused by *Aspergillus* is the second major cause of nosocomial infection, which has a higher mortality rate (Kaur and Singh 2014; Ramage et al. 2011). *Aspergillus* is an opportunistic fungi and, in immune-suppressed conditions, causes aspergilloma, invasive pulmonary aspergillosis, allergic bronchopulmonary aspergillosis and even systemic dissemination (Kaur and Singh 2014; Ramage et al. 2011; Williams et al. 2016). The aspergilloma caused by *Aspergillus* is a fungal mass with biofilm characteristics (Ramage et al. 2011). The biofilm formations by *Aspergillus* take place within 24 h, and the mature biofilms demonstrate increased biofilm biomass, with channels developed between hyphae through which nutrients and fluid transportation occurs (Ramage et al. 2011; Villena et al. 2010). The extracellular matrix (ECM) composition of the biofilms has been detected and is composed of hydrophobins, α-1,3-glucans, galactomannan, polyols, melanin, monosaccharides and antigens (Ramage et al. 2011; Beauvais et al. 2007).

Cryptococcosis is an important fungal infection of animals and humans. It is one of the common infections of various domestic animals, including cats, dogs, ferrets, horses, camelids, goats, sheep, cattle, dolphins, birds, koalas and other marsupials (Malik et al. 2002; Sykes et al. 2010). It is caused by fungi belonging to *Cryptococcus* spp., *C. laurentii* and *C. albidus*, which, together, cause almost 80% of infections in humans and animals (Khawcharoenporn et al. 2007). *Cryptococcus laurentii* is an encapsulated saprophytic yeast and causes superficial infections like keratitis and deep-seated infections such as fungaemia and meningitis (Cheng et al. 2001; Shankar et al. 2006; Khawcharoenporn et al. 2006). *Cryptococcus neoformans* forms biofilms, has a worldwide distribution and infects immune-suppressed patients (Walsh et al. 1986; Ingram et al. 1993).

10.3 *Candida* spp. Biofilms

In animals, *C. albicans* causes otomycosis, dermatitis, stomatitis, onychomycosis, vulvovaginitis, subclinical mastitis, stomatitis, dermatitis and otitis, urinary tract infection and respiratory tract infection (Vijay and Pal 2013). The biofilm formation by *Candida* spp. is the most studied fungal biofilm and has been known since 1990. Many in vitro (Marcos-Zambrano et al. 2016) and in vivo studies revealed that *Candida* biofilms are heterogeneous and composed of hyphal, pseudohyphal, yeast blastospores and ECM (Henriques et al. 2006; Pires et al. 2011). In *Candida* biofilms, the yeast and hyphae are important structural components (Finkel and Mitchell 2011). The filamentation of fungi increases the suppression of biofilms and increases resistance to adverse conditions, such as sonication and vortexing. *Candida* biofilms express many genes involved in fungal adhesion, quorum sensing, ECM production and morphogenesis (Blankenship and Mitchell 2006; Bonhomme

and d'Enfert 2013; Finkel and Mitchell 2011). Within the genus *Candida*, the species *C. albicans*, *C.glabrata*, *C. parapsilosis* and *C. tropicalis* form biofilms; however, they vary in the carbohydrates and protein compositions in the biofilms' ECM and in their morphology. The biofilms of *C. albicans* and *C. parapsilosis* contain both yeast hyphae and pseudohyphae, while the biofilms of *C. glabrata* and *C. tropicalis* contain only yeast cells (Silva et al. 2009).

10.4 *Candida albicans* Multi-species Biofilms with *Streptococcus mutans*

Candida albicans is an opportunistic fungus found on various body sites in humans and on animals. However, it causes localised and systemic infection in immune-suppressed conditions (Tsui et al. 2016). *Candida* can form biofilms on biotic and abiotic surfaces, and frequently form multi-species biofilms with bacteria, which increases the virulence (Kojic and Darouiche 2004; Ramage et al. 2005). *Candida albicans* causes oral mucosal infections in both animals and humans, where the fungal interacts with the commensal viridans streptococci (Xu et al. 2014; Thein et al. 2009). Various researchers reported that *C. albicans* is usually absent in dental plaque biofilms of healthy individuals and it neither colonizes alone on the teeth of rodents nor co-colonizes with *S. mutans* in the absence of sucrose (Gregoire et al. 2011; Xiao et al. 2016). However, a *C albicans* and *S. mutans* interaction has been detected in plaque biofilms, with a high number of *S. mutans* causing aggressive tooth decay and rampant carious lesions (Falsetta et al. 2014; De Carvalho et al. 2006; Xiao et al. 2016). In this interaction, a secretary bacteria exo-enzyme called glucosyltransferase (Gtfs) plays an important role. The *S. mutans* can utilise host dietary sucrose through Gtfs and produce biofilm extracellular polymeric matrix, which mainly consists of α-glucans. Furthermore, the Gtfs can bind on the *C. albicans* cell surface and could produce EPS on the fungal surface, which results in enhanced bacterial binding and multi-species biofilm formation (Falsetta et al. 2014; Pereira-Cenci et al. 2008).

Candida albicans forms polymicrobial biofilms with *Staphylococcus aureus*. The fungus and bacteria has been detected in nosocomial and blood infections (Klotz et al. 2007). Many studies suggested that the polymicrobial interaction of *C. albicans* and *S. aureus* give rise to synergism and results in increased mortality in mice (Carlson 1982; Adam et al. 2002). Furthermore, the treatment and eradication of biofilms require a higher concentration of antibiotics. Indeed, *C. albicans* serves as a scaffold for the biofilm formation of *S. aureus*, and the bacterial cells were coated with matrix produced by *C. albicans* (Melphine and Mairi 2009).

10.5 Mode of Biofilm Formation

Both filamentous fungi and yeasts can form biofilms on biotic and abiotic surfaces; however, many reports are available for yeast biofilms but very few for filamentous fungi (Harding et al. 2009; Blankenship and Mitchell 2006). Many authors suggest that the definition of bacterial biofilms does not fit for filamentous fungi; therefore, fungal biofilms need to be revisited. Harding et al. proposed a model for filamentous fungi biofilms and suggested that the basic steps of biofilms formation are similar in filamentous fungi and bacteria, despite their distinct morphology (Harding et al. 2009). In addition, the filamentous fungi secrete small proteins called hydrophobins, which facilities the adhesion of hyphae to a hydrophobic surface during biofilm formation (Harding et al. 2009; Wosten 2001).

As per Harding et al. (2009), the biofilm formations of filamentous fungi are divided into various stages, as described in Fig. 10.1:

A. *Propagule adsorption*: involving the contact of spores, hyphal fragments or sporangia with a surface;
B. *Active adhesion*: in which adhesins are secreted by spores during germination and other reproductive structures;
C. *First microcolony formation*: which involves elongation and hyphal branching, forming a monolayer with the production of ECM;
D. *Second microcolony formation or initial maturation*: in which compact hyphae networks form in three dimensions, are covered by an ECM and the formation of water channels occurs;
E. *Final maturation*: in which fruiting bodies and other survivor structures are formed, depending on the fungi;
F. *And, finally, the dispersion or planktonic phase*: in which conidia and/or hyphae fragments are released, beginning a new cycle.

In yeasts, the *C. albicans* biofilms are the most often studied and the biofilm development stages are shorter than those of filamentous fungi and bear a close resemblance to those of bacterial biofilms (Chandra et al. 2001; Costa-Orlandi et al.

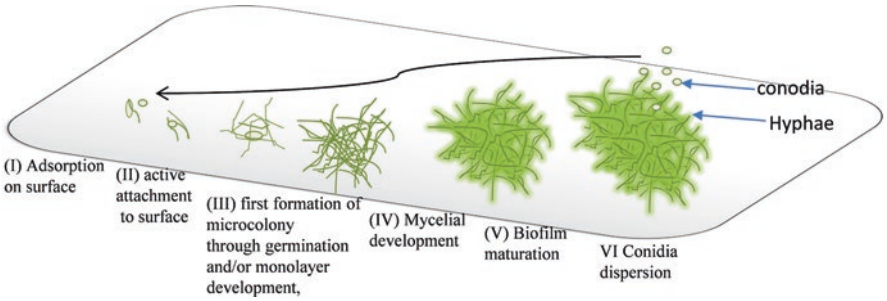

Fig. 10.1 Biofilm formation stages in filamentous fungi. (Figure 10.1 was reproduced from the original article published by Harding et al. (2009))

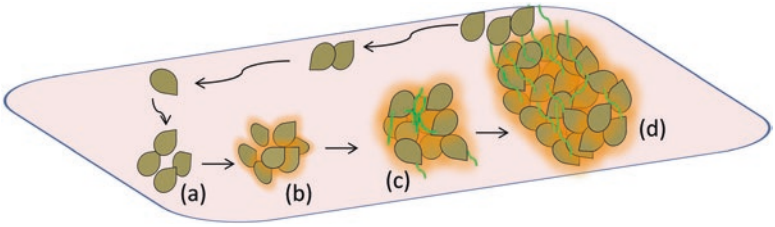

Fig. 10.2 Biofilm formation stages in yeast fungi. (Figure 10.2 was reproduced from the original article published by Harding et al. (2009))

2017). Harding et al. (2009) reported that the *C. albicans* biofilm development involves fewer stages compared to the filamentous fungi, as shown in Fig. 10.2:

(a) The adsorption of yeast cells to a surface
(b) Followed by initial adhesion
(c) Formation of basal layers of yeast with early development of hyphae and ECM
(d) Biofilm maturation containing a significant number of yeasts, hyphae, pseudohyphae, ECM and water channels that allow the movement of nutrients
(e) And cell dispersion.

10.6 Mechanism of Resistance in Fungal Biofilms

The biofilms of filamentous fungi and yeasts are difficult to eradicate, as they demonstrate increased resistance to antifungal agents (Scorzoni et al. 2017). The fungal biofilms demonstrate up to 1000-fold more resistance to antifungal agents than the planktonic form (Di Bonaventura et al. 2006; Tre-Hardy et al. 2008). The adaptive resistance acquired by fungal biofilms imparts resistance to antifungal agents (Ramage et al. 2012). The biofilms facilitate the adaptation of fungi to environmental conditions, the structure of biofilms protects cells and obstructs antifungal agent diffusion, the biofilm cells demonstrate altered metabolism and gene expression results in persister cells (Niimi et al. 2010; Rajendran et al. 2013). The adaptive factors which play a vital role in fungal biofilm resistance are shown in Fig. 10.3.

10.6.1 Biofilm Resistance Due to Extracellular Matrix

The bacterial biofilm ECM has been studied extensively. However, there is limited information on fungal biofilms' ECM. The main characteristic of all mature fungi biofilms, irrespective of the genus, is the presence of extracellular polymer matrix, which confers resistance to antifungal agents (Ramage et al. 2009). ECM production is a highly regulated process and the resistance to antimicrobial agents depends

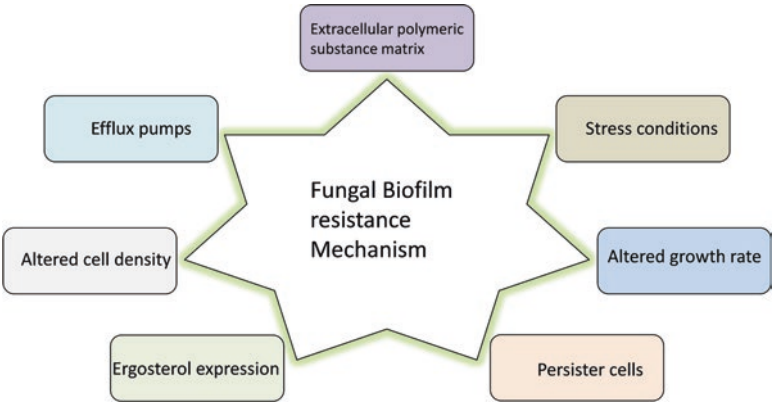

Fig. 10.3 Factors contributing resistance to fungal biofilms

on the diffusion and chemical compositions of the ECM. The ECM of *Candida* spp., including *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *C. albicans* has been characterised (Silva et al. 2009, 2011). The ECM of *C. albicans* and *C. tropicalis* biofilms is composed of protein, uronic acid, phosphorous, hexosamine and carbohydrates (Al-Fattani and Douglas 2006). It was detected that the biofilms were detached when treated with glucanase, indicating that β-1-3 glucans are the main carbohydrates components. β-Glucans also prevent neutrophil activation and release reactive oxygen species (ROS), which decreases the host defence response (Xie et al. 2012). In *C. albicans*, the ECM production is regulated by zinc regulator ZAP1 (Nobile et al. 2009). The other fungi which form biofilms and produce a significant amount of ECM are *C. neoformans*, *Apophysomyces elegans*, *Rhizopus oryzae*, *Aspergillus* species, *Blastoschizomyces capitatus*, *Saccharomyces cerevisiae*, *Lichtheimia corymbifera*, *Malassezia pachydermatis*, *Pneumocystis* species and *Rhizomucor pusillus* (Beauvais et al. 2009; Cannizzo et al. 2007; Cushion et al. 2009; D'Antonio et al. 2004; Martinez and Casadevall 2006; Singh et al. 2011).

10.6.2 Biofilm Resistance Due to Efflux Pump

The resistance of *C. albicans* against high-dose azoles is due to the increased efflux of drug mediated by the ATP-binding cassette and the major facilitator's superfamily transporters (Albertson et al. 1996; Lopez-Ribot et al. 1999; Sanglard et al. 1997). The primary function of efflux pumps is to maintain homeostasis during harsh environmental conditions; however, the exposure to high doses of antifungal agents increase efflux pump expression, resulting in resistance to antifungal agents (Piddock 2006; Bueid et al. 2010). Elevated levels of efflux pump expression have been detected in implanted catheters in animal models and in the *Candida* biofilm model (Andes et al. 2004; Nett et al. 2009; Bizerra et al. 2008).

10.6.3 Biofilm Resistance Due to Stress Responses

The phenotypic modulation of fungi in response to stress due to micro-environmental conditions and the adaptation of heterogeneity within fungi play vital roles in increasing resistance to antifungal agents (Shapiro et al. 2011). Thermal stress, oxidative stress, ionic stress and osmolality are micro-environmental stresses involved in fungal biofilms. Thermal, ionic and oxidative stressors, in addition to osmolality, are likely to be involved within the micro-environment of a biofilm, particularly in vivo. The adaptation of fungi to stress and biofilm development is controlled by the MAPK signal transduction network (Cannon et al. 2007). In *C. albicans*, biofilms development involves Mck1p and the mutation of this gene increased the sensitivity of biofilms towards azoles (Cannon et al. 2007; Kumamoto 2005). The calcineurin pathway is also involved in the stress tolerance response employed in fungi and plays an important role in biofilms and increases resistance (Steinbach et al. 2007).

10.6.4 Altered Synthesis of Ergosterol Increases Resistance

Azoles are fungicidal for moulds such as *Aspergillus* species and are fungi-static for yeasts, including *Candida* spp. However, gradually, *C. albicans* can acquire resistance to high levels of azoles due to the alteration of Erg11 (Anderson 2005). In the yeast, the azoles inhibit ergosterol biosynthesis, though blocking enzyme 14 α-demethylase encoded by ERG11 results in the accumulation of toxic sterol pathway intermediates (Akins 2005; Cannon et al. 2007). The main gene Erg11 could be overexpressed or develop mutation. A high level of ergosterol has been detected in early-phase biofilms, whereas it was lower in intermediate or mature biofilms (Mukherjee et al. 2003). Also, the ergosterol fluctuation in biofilms confers resistance against both azole- and polyene-derived antifungal agents (Khot et al. 2006).

10.6.5 Biofilm Resistance Due to Growth Rate

Most of the antimicrobial agents developed in the past are effective on the planktonic cells, which act on the active, rapidly growing cells. However, the cells in biofilms grow slowly and with altered metabolism in nutrient-limited, adverse conditions. For example, the azole inhibits actively growing cells; however, it is ineffective on slowly growing biofilm cells.

10.6.6 Biofilm Resistance Due to Cell Density

The fungal biofilms are characterised by high cell density, which confers resistance to biofilms. The fungal biofilms have a dynamic structure, which consists of high cell density arranged in a stack, micro-colonies and water channels for nutrients supply, aeration and waste transportation. The specialised architecture of biofilms prevents the diffusion of antifungal agents, which results in increasing resistance for antifungal agents (Chandra et al. 2001; De Beer et al. 1994; Lawrence et al. 1991). The high cell density facilitates the communication and coordination behaviours. It is well documented that fungi in biofilms communicate and coordinate their activities through a mechanism called quorum sensing, which is mediated by small molecules called auto-inducers (Miller and Bassler 2001). In *C. albicans*, farnesol is a quorum sensing molecule (Hornby et al. 2001).

10.6.7 Biofilms are a Source of Persister Cells

The biofilms' mode of growth prevents the antimicrobial agents from diffusing uniformly inside biofilms; therefore, cells deep inside the biofilm remain unaffected by antifungal agents. A subset of cell populations spontaneously enter in the dormant, non-dividing state, resulting in persister cells. The persister cells are a population of cells that are dormant and highly recalcitrant to antimicrobial challenge (Lewis 2010). Upon antimicrobial treatment, the persister cells survive and, on discontinuation of the antimicrobial therapy, the persistent cells can restore the biofilms. The persister cells with altered cell membrane and independence from efflux pumps have been detected in the *Candida* biofilms treated with amphotericin B (Khot et al. 2006; LaFleur et al. 2006; Al-Dhaheri and Douglas 2008).

10.7 Conclusions

Filamentous fungi and yeast both form biofilms on biotic or abiotic surfaces. Fungal biofilms are highly organised communities involved in various infections of both humans and animals. The filamentous fungi and yeasts form biofilms as single-species biofilms or in combination with bacteria, called multi-species biofilms. In multi-species biofilms, a cooperativity between bacteria and fungi has been detected, which enhances the survival opportunity of both species. Fungal biofilms are resistant to antifungal agents and the host defence. The ECM constituents and the architecture of the fungal biofilm contribute to the resistance of biofilms against antifungal agents. The biofilm matrix consists of polymer substances that hinder the diffusion of antifungal agents into the biofilms. As a result, the microbial cells deep inside the

biofilms are relatively unaffected by antimicrobial agents and give rise to persister cells. Upon antimicrobial treatment, the persister cells survive and, on discontinuation of the antimicrobial therapy, the persister cells can restore the biofilms. The persister cells, with altered cell membrane and independence from efflux pumps, have been detected in the *Candida* biofilms treated with amphotericin B. The biofilm mode of growth plays an important role in infectious disease in animals and a source of persister cells gives rise to difficult-to-treat infections, resulting in increased treatment cost and recovery time.

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