Polymicrobial infections involving clinically relevant Gram-negative bacteria and fungi

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Abstract
Interactions between fungi and bacteria and their relevance to human health and disease have recently attracted increased attention in biomedical fields. Emerging evidence shows that bacteria and fungi can have synergistic or antagonistic interactions, each with important implications for human colonization and disease. It is now appreciated that some of these interactions may be strategic and helps promote the survival of one or both microorganisms within the host. This review will shed light on clinically relevant interactions between fungi and Gram-negative bacteria. Mechanism of interaction, host immune responses, and preventive measures will also be reviewed.

1 | INTRODUCTION

In their natural habitat, microorganisms often exist in close association with a diversity of other species. These interactions drive evolutionary development and adaptation in each microorganism, which promotes the survival of the species within a given environmental or host-specific niche. To promote survival, the interactions between microorganisms can either be synergistic or antagonistic and are often mediated by metabolic factors, secretion of antimicrobial molecules, or structural properties related to cell–cell interactions (Hogan & Kolter, 2002; Gaddy, Tomaras, & Actis, 2009; Gibson, Sood, & Hogan, 2009; Peleg, Hogan, & Mylonakis, 2010). Within humans or in the natural environment, microorganisms predominate live as biofilms, which are complex three-dimensional structures comprising of cell aggregates encased within a self-produced matrix of extracellular polymeric substances (Bjarnsholt et al., 2013; Flemming et al., 2016). In reality, these biofilms are often polymicrobial (Partida-Martinez, Monajembashi, Greulich, & Hertweck, 2007). The focus of this review is on bacterial–fungal interactions and infections involving human pathogens, more specifically clinically relevant Gram-negative bacteria. Developing an understanding of how these microorganisms interact and how the host immune system responds will help elucidate disease mechanisms and uncover new insights for novel therapeutic or preventative strategies (Kobayashi & Crouch, 2009).

Clinically, the role of polymicrobial infections on patient outcomes remains poorly studied. With respect to Gram-negative bacteria, the most common sites for mixed-species infections with fungi are the respiratory tract, the gastrointestinal system, the skin, and the urinary tract. This is predicted based on the resident microflora of these parts of the human body and is now well supported by microbiome studies of healthy humans. The human microbiome project recently analyzed 242 healthy human subjects and revealed that the signature microbes of a particular niche differ greatly among individuals (Human Microbiome Project, 2012). More recent studies have also focused on fungal diversity in different parts of the body, also termed the mycobiome (Ghannoum et al., 2010; Cui, Morris, & Ghedin, 2013). These studies highlight the dominance of Candida albicans as a resident fungus but also highlight the diversity in fungal species that may be found in on the human body including Malassezia, Cladosporium, Aureobasidium, Saccharomyces, Aspergillus, Fusarium, Alternaria, Penicillium, and Cryptococcus (Ghannoum et al., 2010; Charleston et al., 2012; Findley et al., 2013; Diaz, Strausbaugh, & Dongari-Bagtzoglou, 2014; Goral ska, 2014). These fungal species are ubiquitous in the environment, and therefore, it is not surprising that they are found in human body sites exposed to the environment (e.g., skin and respiratory tract). In a healthy human host, these fungi pose negligible risk of acute disease, but when the host immune system is impaired in some way such as post-chemotherapy or transplant-related immunosuppression, several of these fungi can become invasive and cause serious infection. Of all the human fungal pathogens, the most common one that is in constant interaction with a diverse range of bacteria is C. albicans. Hospitalized patients are often heavily colonized with C. albicans at many body sites and Candida thrives off forming complex biofilms on almost all medical devices, including mechanical ventilator
tubing used in the intensive care unit, vascular and urinary catheters, and intra-abdominal draining tubes (Kojic & Darouiche, 2004). Gram-negative bacteria also heavily populate these sites, and the mechanisms and proposed impact of their interactions are discussed below and shown in Figure 1.

2 | CLINICALLY IMPORTANT BACTERIAL–FUNGAL INTERACTIONS

2.1 | *C. albicans* and *Escherichia coli*

*E. coli* is a Gram-negative bacterium that specializes in transitioning from a commensal to an opportunistic pathogen of humans. It is one of the dominant bacterial species found in the gastrointestinal tract of warm blooded animals (Tenaillon, Skurnik, Picard, & Denamur, 2010) and is one of the most common causes of urinary tract and intra-abdominal infections, and community-acquired bloodstream infections. Given that *Candida* is a commensal of the human gut, studies have assessed *Candida–E. coli* interactions in the context of peritonitis (Gale & Sandoval, 1957; Burd, Raymond, & Dunn, 1992; Akagawa, Abe, & Yamaguchi, 1995; Klaerner et al., 1997; Ikeda, Suegara, Abe, & Yamaguchi, 1999). Klaerner et al. showed that the interaction between *E. coli* and *C. albicans* was synergistic and increased lethality in mice when infected intraperitoneally compared to mono-infection with either microorganism (Klaerner et al., 1997). Similarly, in the urinary tract, *E. coli* contributed to the establishment of *C. albicans* infection in which *C. albicans* alone failed to cause infection due to its inability

![FIGURE 1](image)

Mechanisms and impact of Gram-negative bacteria and fungal interaction I. Direct cell-cell contact: *P. aeruginosa* preferentially attached to *C. albicans* hyphae forming biofilms on the filaments (Hogan & Kolter, 2002; Gibson et al., 2009) II. Facilitation of tissue attachment: *C. albicans* attachment to the bladder mucosa is facilitated by the presence of *E. coli* (Levison & Pitsakis, 1987). III. Interaction through secretory metabolites: (a) a feedback mechanism where phenazines from *P. aeruginosa* stimulate production of ethanol by *C. albicans* that subsequently benefits *P. aeruginosa* (Chen et al., 2014). (b) *Candida* QS molecule, farnesol inhibits *A. baumannii* growth and viability (Peleg et al., 2008) and (c) *A. baumannii* secretory factor and *Pseudomonas* QS molecule (C12-acyl homoserine lactone) inhibits *C. albicans* yeast to hyphae transition (Cugini et al., 2007; Gaddy et al., 2009)
to attach to the bladder mucosal lining (Levison & Pitsakis, 1987). The mechanism was shown to be agglutination of *C. albicans* to the man-
ose-binding *E. coli*, which facilitated the attachment of *Candida* to the bladder mucosa (Figure 1; Levison & Pitsakis, 1987). It has also been reported that *E. coli* positively modulates biofilm growth of *C. albicans* in vitro, and this was due to bacterial lipopolysaccharide (LPS; Bandara, Yau, Watt, Jin, & Samaranayake, 2009). The mechanism by which *E. coli* LPS facilitates *Candida* biofilm growth is not known, but a hypothesis is that it is secondary to LPS-mediated glucocorticoid production leading to immunosuppression (Besedovsky, del Rey, Sorkin, & Dinarello, 1986; Chang, Feddersen, Henson, & Voelkel, 1987; Burd et al., 1992; Akagawa et al., 1995). Despite this positive association between *E. coli* and *C. albicans* in the urinary tract, *E. coli* negatively modulated the growth and biofilm formation of other *Candida* species, including *C. tropicalis*, *C. dubliniensis*, *C. krusei*, and *C. parapsilosis* (Bandara et al., 2009). Thus far, the clinical implications of this antagonism in a biofilm setting remain unknown, but further work is welcomed and may uncover relevant mechanisms that could be exploited as future novel therapeutics.

### 2.2 *C. albicans* and *Pseudomonas aeruginosa*

The respiratory tract and the skin are the major human body sites harboring interactions between *C. albicans* and *P. aeruginosa*. The clinical impact of a polymicrobial infection involving these two microorganisms has been studied in acute ventilator-associated pneumonia (VAP), as well as in chronic supplicative lung disease such as cystic fibrosis. *C. albicans* has been frequently isolated from the airways of patients on mechanical ventilatory support (Hamet et al., 2012) and in general is clinically ignored. It has now been shown that *Candida* colonization of the respiratory tract may promote the development of pseudomonal VAP and has been associated with the presence of multidrug-resistant bacteria (Azoulay et al., 2006; Hamet et al., 2012). Furthermore, more severe clinical outcomes have been observed in patients with VAP and cystic fibrosis infected with *P. aeruginosa* and *Candida* spp. simultaneously, relative to *P. aeruginosa* infection alone (Navarro et al., 2001; Hamet et al., 2012). These human data have also been supported by findings from a rat pneumonia model (Roux et al., 2009) and by another clinical study showing that antifungal treatment can decrease the risk of developing *P. aeruginosa* infection in patients on mechanical ventilation (Nseir et al., 2007). These studies highlight that *Candida* colonization in the airways may in itself not cause disease, but it may be playing an underappreciated role in facilitating bacterial infection.

Patients with burn wounds are also confronted with *Candida–P. aeruginosa* co-colonization and infection (Gupta, Haque, Mukhopadhyay, Narayan, & Prasad, 2005). Clinical outcome data are sparse in this area, but a previous study using a murine burn wound infection model has shown that co-infection led to greater mortality than monomicrobial infection, and greater fungal burdens were observed at the burn wound site and internal organs (Neely, Law, & Holder, 1986).

The enhanced virulence observed with *Candida–Pseudomonas* co-infection may be partly explained by a recent proteomics study, which found that when co-cultured with *C. albicans* in mixed biofilms, *P. aeruginosa* regulated its own production of various quorum-sensing (QS) molecules that consequentially induced the production of several *P. aeruginosa* virulence factors including pyoverdine, rhamnolipids, and pyocyanin (Trejo-Hernandez, Andrade-Dominguez, Hernandez, & Encarnacion, 2014). There have now been several studies characterizing the complex mechanisms of interaction between *Candida* and *Pseudomonas* (Peleg et al., 2010). These studies have shown both antagonistic and synergistic interactions, depending on diverse environmental factors, timing of interaction, and growth state at the time of interaction. For example, co-culturing *P. aeruginosa* and *C. albicans* in liquid culture showed that *P. aeruginosa* preferentially adhered to and formed biofilms on *C. albicans* hyphae rather than yeast cells, and through the secretion of phospholipase C and phenazines, caused hyphal death (Figure 1; Hogan & Kolter, 2002; Gibson et al., 2009). A high concentration of phenazines triggered the production of toxic reactive oxygen species that killed *C. albicans* hyphal cells (Hogan & Kolter, 2002; Nseir et al., 2007; Morales et al., 2013). A lower concentration of phenazines impaired hyphal growth of *C. albicans* and more importantly switched fungal respiration to fermentation leading to the production of ethanol, glyc erol, and acetate by *C. albicans* in glucose containing media (Morales et al., 2013). It was shown that *C. albicans* ethanol production not only influenced biofilm maturation but also promoted more phenazine production by *P. aeruginosa* through WspR-dependent activation of Pel exopolysaccharide (Chen et al., 2014). The spectrum of *P. aeruginosa* phenazines produced was in favor of those most effective against fungal cells and led to greater production of ethanol by *C. albicans*, forming a feedback loop driving the polymicrobial interaction towards the protection of *P. aeruginosa* (Chen et al., 2014).

Other well-studied molecular factors governing the interactions between *P. aeruginosa* and *C. albicans* are their QS systems. QS molecules play a crucial role in inter-cellular communication within a given population of cells, and there has been greater appreciation of their cross-kingdom impact. *P. aeruginosa* produces acyl homoserine lactones, such as 3-oxo-C12-homoserine lactone that inhibits the Ras1–cyclic AMP–protein kinase A pathway required for hyphal growth in *C. albicans*, thereby inhibiting filamentation of the fungus (Hogan, Vik, & Kolter, 2004; Davis-Hanna, Plispanen, Stateva, & Hogan, 2008; McAlester, O’Gara, & Morrissey, 2008). On the other hand, *C. albicans* modulates *P. aeruginosa* biological activities through the production of farnesol, a well-known eukaryotic QS molecule. Cugini et al. initially showed that farnesol produced by *C. albicans* reduced the production of *Pseudomonas* quinolone signal and pyocyanin in *P. aeruginosa* (Cugini, Calfee, Morales, Pesci, & Hogan, 2007). In a later study, they reported that farnesol stimulated the production of *Pseudomonas* quinolone signal, N-butanol-L-homoserine lactone and phenazine in *P. aeruginosa* when the growth environment changed from a liquid culture to a colony biofilm grown on solid medium (Cugini, Morales, & Hogan, 2010). The authors speculated that the varied effects of farnesol on *P. aeruginosa* QS systems were associated with different pathways it activated, either through interaction with specific targets such as QS transcription factors (Cugini et al., 2007), or through promoting the production of reactive oxygen species, which is linked to the activation of *P. aeruginosa* QS systems (Cugini et al., 2010). Overall, the laboratory studies characterizing the diverse interactions
between Candida and Pseudomonas highlight the real complexities of their interaction, and questions still remain as to how they apply during human infection.

2.3 | C. albicans and Acinetobacter baumannii

A. baumannii has gained considerable importance as a multidrug-resistant, hospital-associated pathogen (Howard, O’Donoghue, Feeney, & Sleator, 2012). It has a particular predilection in causing infections in patients in intensive care units with VAP and bloodstream infection being the most life-threatening. A. baumannii is able to survive in the hospital environment for prolonged periods of time, leading to problematic hospital outbreaks (Garnacho-Montero et al., 2005). A. baumannii interacts with fungal species, most importantly C. albicans, in critically-ill patients who are often on mechanical ventilation and have vascular and urinary catheters in situ. The first descriptions of A. baumannii-fungal interactions were with the model yeast Saccharomyces cerevisiae, where Acinetobacter was able to utilize ethanol produced by the yeast as a carbon source for improved growth (Smith, Des Etages, & Snyder, 2004). Interestingly, these findings led to subsequent studies showing that ethanol mediated increased A. baumannii resistance to salt stress, increased growth, virulence gene expression, and virulence in multiple non-mammalian infection model systems (Smith et al., 2004; Smith et al., 2007; Camarena, Bruno, Eusirkeren, Poggio, & Snyder, 2010). In contrast, laboratory studies assessing interactions between A. baumannii and C. albicans have demonstrated an antagonistic relationship, whereby A. baumannii preferentially targets the key virulence characteristic of C. albicans; hyphal development (Peleg et al., 2008). The A. baumannii outer membrane protein was implicated as a mechanism of attachment and killing of the filamentous form of Candida by triggering apoptosis (Gaddy et al., 2009). However, a secretory factor was also thought to be at play (Gaddy et al., 2009). This polymicrobial interaction was also studied within the nematode Caenorhabditis elegans, which showed that A. baumannii prevented the morphological transition of C. albicans from a yeast to hyphal cell, and this in turn led to reduced severity of disease (Peleg et al., 2008). Conversely, when C. albicans was allowed to establish a mature biofilm before co-infection with A. baumannii, the growth of the bacteria was impaired, and this was shown to be secondary to the secretion of the Candida QS molecule farnesol (Peleg et al., 2008). Farnesol appears to mediate its effect on A. baumannii by interfering with bacterial membrane integrity (Kostoulias et al., 2016). Similar to the Candida–Pseudomonas interactions, the nature of Candida–A. baumannii interactions are diverse and are determined by environmental conditions, initial inoculum, and growth state of the individual microorganisms.

2.4 | Cryptococcus neoformans and Gram-negative bacteria

Apart from Candida, bacterial–fungal interactions with another yeast known as C. neoformans have also been reported. C. neoformans is an encapsulated soil fungus that is capable of infecting humans through the respiratory tract. It is particularly dangerous in individuals with impaired immunity, causing life-threatening meningitis (Howard et al., 2012). As a survival mechanism, C. neoformans synthesizes melanin to protect itself from various environmental insults such as UV rays, temperature shocks, predation by amoebae, and heavy metal toxicity (Nosanchuk & Casadevall, 2003; Frases, Chaskes, Dadachova, & Casadevall, 2006; Kronstad et al., 2012). Melanin production by C. neoformans is normally dependent on an environmental precursor. A remarkable interaction between C. neoformans and a Gram-negative bacterium, Klebsiella aerogenes, was observed in a laboratory setting where C. neoformans was able to produce melanin by converting tyrosine to L-3,4-dihydroxyphenylalanine utilizing a bacterial enzyme during combined growth (Frases et al., 2006). C. neoformans has also been shown to interact with A. baumannii, whereby enhanced production of the polysaccharide capsule was observed for certain serotypes (Abdulkareem, Lee, Ahmadi, & Martinez, 2015). It would appear that through evolution, C. neoformans has developed multiple strategies for survival, including taking advantage of bacterial neighbors for self-preservation. However, the clinical implications of these findings are not clear, as co-infections with these Gram-negative bacteria would be extremely rare.

2.5 | Aspergillus fumigatus and Gram-negative bacteria

Apart from yeast–bacterial interactions, interactions with Aspergillus, the most common mould pathogen of humans, have also been studied (Brandl et al., 2011; Kwon-Chung & Sugui, 2013). The clinical relevance of these interactions predominately relates to the respiratory tract and is especially evident in those with suppurative lung disease such as patients with cystic fibrosis (Kwon-Chung & Sugui, 2013). In the context of cystic fibrosis, Aspergillus interacts with a multitude of respiratory Gram-negative bacteria, including P. aeruginosa, Burkholderia cepacia complex, Stenotrophomonas maltophilia, and Achromobacter spp. (Brandl et al., 2011; de Vrankrijker et al., 2011; Hauser, Jain, Bar-Meir, & McCollney, 2011; Lambiase et al., 2011). P. aeruginosa exerts an inhibitory effect on the establishment of A. fumigatus biofilms by secreting a small diffusible heat-stable molecule (Mowat et al., 2010). The effect of this molecule however was rendered ineffective once the biofilm was established. Notably, a P. aeruginosa QS mutant did not show inhibitory effects on A. fumigatus biofilm formation, indicating involvement of the QS system in the inhibition, but further investigations are required to identify the exact molecule (Mowat et al., 2010). Whether these interactions impact on the co-existence of these microorganisms and the magnitude of lung destruction in patients with cystic fibrosis remains to be determined.

3 | HOST IMMUNE RESPONSES TO BACTERIAL–FUNGAL INFECTIONS

Very little is known about host immune responses to polymicrobial infections. It is clear that immune responses against bacteria or fungi alone are dissimilar (Allard et al., 2009), but whether dual infection stimulates both immune pathways or is dominated by one has only recently been explored. In a mouse model to investigate lung adaptive immune responses, fungal lysate alone from C. albicans and A. fumigatus led to airway eosinophilia, release of Th2 type cytokines (IL-4, IL-5 and
IL-13), and changes in mucus production, whereas bacterial antigens from P. aeruginosa evoked an inflammatory response dominated by neutrophils, secretion of Th1 type cytokines, and minimal mucus production (Allard et al., 2009). Interestingly, co-administration of bacterial and fungal antigens activated immune responses characteristic of bacteria alone, suggesting that at least in this mouse model, bacterial antigens were able to immunomodulate the response towards fungal antigens (Allard et al., 2009). How Pseudomonas antigens direct the immune response towards fungal lysates from Th2 to Th1 is not defined, but it appears to be independent of bacterial LPS and TLR4 (Allard et al., 2009). A subsequent report suggested that Candida can also modulate the host immune response, which may promote pseudomonal infection (Roux et al., 2009). Using a rat pneumonia model, pre-infection with Candida helped establish P. aeruginosa pneumonia by impairing reactive oxygen species generation by alveolar macrophages (Roux et al., 2009). These studies highlight the potential clinical implications of understanding how the host immune system reacts to mixed bacterial–fungal infections and further studies may provide important insights for the development of future preventative or therapeutic strategies against polymicrobial infections.

4 | TREATMENT AND PREVENTATIVE STRATEGIES AGAINST BACTERIAL–FUNGAL INFECTIONS

Very little is known about the most effective antimicrobial treatment of polymicrobial infections. Standard therapy includes use of conventional antifungal and antibacterial agents based on antimicrobial susceptibility results for individual microorganisms, but reports of greater severity of illness, longer duration of symptoms, and greater modification of treatment antimicrobials in polymicrobial infections exist (Dyess, Garrison, & Fry, 1985; Tsai et al., 2014). Though C. albicans was previously considered a colonizer in the respiratory tract, it may actually play an important role in facilitating Gram-negative bacterial infection through protection within biofilms or alteration of local host innate immune defenses. It is well established that organisms are more resistant to antimicrobials in biofilms, and this appears to be an even greater problem with polymicrobial biofilms (Harriott & Noverr, 2009; Chotirmall et al., 2010; Filkins & O’Toole, 2015). The fungal scaffold may provide a shield for the interacting bacteria, and therefore, combining a biofilm-active antifungal with an effective antibacterial may provide a potential therapeutic strategy for these difficult infections (Qu et al., 2016).

Polymicrobial biofilms have been implicated in the pathogenesis of cystic fibrosis and many hospital acquired infections related to implanted medical devices such as central venous or urinary catheters (Coad, Griesser, Peleg, & Traven, 2016). Developing effective and durable medical surface-attached antimicrobials that have activity against Candida and Gram-negative or -positive bacteria forms an unmet clinical need. A recent example targeting the Candida biofilm structure is the covalent surface attachment of the echinocandin antifungal, caspofungin (Coad et al., 2015; Kucharikova et al., 2016). These data hold promise for the prevention of the supporting Candida biofilm structure that may be an important driver of polymicrobial biofilm-related infections. It will clearly take a combination of innovative strategies to prevent the burgeoning problem of biofilm-related infections, in particular those that are polymicrobial (Salwiczek et al., 2014).

5 | CONCLUSIONS

Although the seriousness and the impact of bacterial–fungal interactions have been recognized globally, effective measures to deal with the problem are lacking. From a mechanistic point of view, opportunities exist for identifying novel therapeutic strategies by exploiting the ability of one microorganism to inhibit the growth and viability of another. Also, new combination therapies and surface-attached antimicrobials may help to target these interactions. Our understanding of polymicrobial infections and their mechanisms of interaction are evolving, but it is clear that we still have a great deal to learn about this complex and diverse problem.

ACKNOWLEDGMENT

A.Y.P was supported by an Australian National Health and Medical Research Council Career Development Fellowship and Project Grant.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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How to cite this article: Dhamgaye, S., Qu, Y., and Peleg, A. Y. (2016), Polymicrobial infections involving clinically relevant Gram-negative bacteria and fungi, Cellular Microbiology, 18, 1716–1722. doi: 10.1111/cmi.12674