Targeting Candida albicans in dual-species biofilms with antifungal treatment reduces Staphylococcus aureus and MRSA in vitro

Luo, Y., McAuley, D. F., Fulton, C. R., Sá Pessoa, J., McMullan, R., & Lundy, F. T. (2021). Targeting Candida albicans in dual-species biofilms with antifungal treatment reduces Staphylococcus aureus and MRSA in vitro. PLoS ONE, 16(4), e0249547. https://doi.org/10.1371/journal.pone.0249547

Published in:
PLoS ONE

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
© 2021 The Authors.
This is an open access article published under a Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution and reproduction in any medium, provided the author and source are cited.

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.
RESEARCH ARTICLE

Targeting *Candida albicans* in dual-species biofilms with antifungal treatment reduces *Staphylococcus aureus* and MRSA *in vitro*

Yu Luo1, Daniel F. McAuley1,2, Catherine R. Fulton1, Joana Sá Pessoa1, Ronan McMullan1, Fionnuala T. Lundy1*  

1 Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen’s University Belfast, Belfast, United Kingdom, 2 Belfast Health & Social Care Trust, Regional Intensive Care Unit, Royal Victoria Hospital, Belfast, United Kingdom  

* f.lundy@qub.ac.uk

Abstract

Polymicrobial biofilms consisting of fungi and bacteria are frequently formed on endotracheal tubes and may contribute to development of ventilator associated pneumonia (VAP) in critically ill patients. This study aimed to determine the role of early *Candida albicans* biofilms in supporting dual-species (dual-kingdom) biofilm formation with respiratory pathogens *in vitro*, and investigated the effect of targeted antifungal treatment on bacterial cells within the biofilms. Dual-species biofilm formation between *C. albicans* and three respiratory pathogens commonly associated with VAP (*Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*) was studied using quantitative PCR. It was shown that early *C. albicans* biofilms enhanced the numbers of *E. coli* and *S. aureus* (including methicillin resistant *S. aureus*; MRSA) but not *P. aeruginosa* within dual-species biofilms. Transwell assays demonstrated that contact with *C. albicans* was required for the increased bacterial cell numbers observed. Total Internal Reflection Fluorescence microscopy showed that both wild type and hyphal-deficient *C. albicans* provided a scaffold for initial bacterial adhesion in dual species biofilms. qPCR results suggested that further maturation of the dual-species biofilm significantly increased bacterial cell numbers, except in the case of *E. coli* with hyphal-deficient *C. albicans* (*Ca_gcn5Δ/Δ*). A targeted preventative approach with liposomal amphotericin (AmBisome®) resulted in significantly decreased numbers of *S. aureus* *in vitro* significantly increased bacterial cell numbers, except in the case of *E. coli* with hyphal-deficient *C. albicans* (*Ca_gcn5Δ/Δ*). A targeted preventative approach with liposomal amphotericin (AmBisome®) resulted in significantly decreased numbers of *S. aureus* in dual-species biofilms, as determined by propidium monoazide-modified qPCR. Similar results were observed when dual-species biofilms consisting of clinical isolates of *C. albicans* and MRSA were treated with liposomal amphotericin. However, reductions in *E. coli* numbers were not observed following liposomal amphotericin treatment. We conclude that early *C. albicans* biofilms have a key supporting role in dual-species biofilms by enhancing bacterial cell numbers during biofilm maturation. In the setting of increasing antibiotic resistance, an important and unexpected consequence of antifungal treatment of dual-species biofilms, is the additional benefit of decreased growth of multi-drug resistant bacteria such as MRSA, which could represent a novel future preventive strategy.
**Introduction**

Biofilm formation is a phenotype of many microorganisms, linked to the ability to survive in a hostile host environment [1]. Biofilm-related infections are considered a significant and increasingly prevalent source of morbidity and mortality within the healthcare system and thus much research effort is required towards aiding their prevention and management [2]. Indeed the polymicrobial nature of biofilms associated with infections such as cystic fibrosis [3,4] and chronic wounds [5] is increasingly recognised as an important contributor to disease pathogenesis. Despite our knowledge that biofilms in vivo generally contain multiple species encased within an extracellular polymeric matrix, much remains to be learned about the development of mixed species biofilms and the beneficial, parasitic or antagonistic interactions that exist between the microorganisms within them [6–9].

In-dwelling medical devices, many of which are used in critically ill patients, have also been well documented to support polymicrobial biofilm growth [10,11], leading to infections ranging from catheter-related urinary tract infections [10], to ventilator associated pneumonia (VAP) [11,12]. Biofilm colonisation of the endotracheal tube in VAP patients has been reported to occur rapidly after intubation [13], with biofilms acting as reservoirs for pathogenic bacteria [14]. Indeed it has been shown that there is microbiological continuity between airway colonization, biofilm formation and VAP development [15]. Emerging technologies to detect pathogens associated with VAP have identified a broad range of Gram positive and Gram negative bacteria including, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, amongst the common bacterial pathogens associated with VAP [16]. Of particular interest is the finding that *Candida albicans* [17], is often present in endotracheal tubes [11], and is associated with an increased risk of VAP (although not as the pathogen causing VAP), as well as prolonged intensive care unit and hospital stays [18]. Given that oral carriage of *Candida* is reported in up to 40–60% of the population [19,20], it is plausible that *C. albicans* could adhere to endotracheal tubes on their insertion through the mouth. Thus, establishment of an early *C. albicans* biofilm could facilitate respiratory pathogen integration, leading to subsequent polymicrobial biofilm formation and ultimately drive the development of VAP.

An emerging interest in interactions between fungi and bacteria has highlighted the complexity of fungal-bacterial interactions in polymicrobial and dual-kingdom biofilms, particularly those involving dimorphic fungi. Polymicrobial biofilms are notoriously difficult to eradicate and tend to be recalcitrant to both antimicrobials and host defences [21]. It has been suggested that the morphological plasticity of *C. albicans* between yeast and hyphal forms has a major influence on its virulence [22] and that the hyphal form of *C. albicans* could provide architecture to the developing biofilm [8,23].

In this study, we investigated dual-species biofilms between *C. albicans* and three bacterial species with a view to determining if *C. albicans* could enhance respiratory bacterial pathogen numbers in dual-species biofilms, using qPCR for bacterial enumeration. We used a biofilm model in which *C. albicans* was inoculated first, to develop an early biofilm before the addition of the bacterial species. We also investigated whether contact was needed between the early *C. albicans* biofilm and bacterial cells to enhance bacterial cell numbers. Using Total Internal Reflection Fluorescence (TIRF) microscopy, we imaged bacterial attachment to wild type and hyphal-deficient *C. albicans* and by qPCR determined bacterial cell numbers during dual-species biofilm maturation. Furthermore, we examined whether a targeted preventative approach with an antifungal could reduce bacterial cell numbers in dual-species biofilms. The current work has potential clinical implications as it could open up new preventive and therapeutic targets for the management of polymicrobial biofilms infections such as VAP, with the
possibility of decreasing bacterial numbers of S. aureus and MRSA without antibiotics, thereby lowering the risk of developing antibiotic resistance.

**Materials and methods**

**Micro-organism strains and growth conditions**

*C. albicans* (NCTC 3179) was sub-cultured aerobically on Sabouraud agar plates and propagated in yeast peptone dextrose (YPD) broth (US Biological). *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 29522), *S. aureus* (NCTC 6571) and methicillin resistant *S. aureus* (MRSA; MRSA 4D) were grown on blood agar plates and propagated in brain heart infusion (BHI) broth. The laboratory strain (wild type) of *C. albicans* (NCTC 3179) was used in all experiments, except where otherwise stated, such as dual-species biofilm treatment with liposomal amphotericin, in which a clinical isolate of *C. albicans* (CA 239SB) was also studied. In experiments on dual-species biofilm maturation, a *C. albicans* hyphal-deficient mutant (*Ca_gcn5Δ/Δ*) [24] (gift from Prof Karl Kuchner, Medical University of Vienna) was studied along with the laboratory strain of *C. albicans*. The clinical isolate and hyphal-deficient mutant of *C. albicans* were subcultured as described for the laboratory strain.

**Preparation of single species bacterial biofilms**

Overnight cultures (18 hours) of *P. aeruginosa*, *E. coli*, *S. aureus* and MRSA were resuspended in BHI to yield inocula of 5.0 x 10^6 cells/ml. A total volume of 100 μl of each inoculum in BHI was added to microtitre plate wells (Thermo Fisher Scientific, Roskilde, Denmark), and the plates were incubated at 37˚C for 4 hours to allow initial biofilm formation under static growth conditions. Wells were washed carefully, three times with PBS, to remove planktonic cells and the biofilms incubated with 100 μl fresh broth for a further 24 hours to allow biofilm maturation. Biofilms were then washed to remove planktonic cells and quantified by qPCR as outlined below. Single species bacterial biofilms were grown in BHI throughout (4 hours for initial biofilm formation, plus an additional 24 hours for early biofilm maturation).

**Preparation of dual-species biofilms**

A schematic outline of dual-species biofilm formation, treatment and quantification is shown in S1 Fig. Overnight cultures (18 hours) of *C. albicans* were washed with phosphate-buffered saline (PBS) and resuspended in a modified Roswell Park Memorial (RPMI) medium (RPMI-1640; Sigma-Aldrich, St Louis, USA), referred to subsequently as RPMI, to yield an inoculum of 1.0 x 10^6 cells/ml [25]. The *C. albicans* inoculum (100 μl) was added to microtitre plate wells (Thermo Fisher Scientific, Roskilde, Denmark) and incubated under static growth conditions at 37˚C for 4 hours to allow initial biofilm formation. The biofilm was washed three times with PBS to remove planktonic *C. albicans* cells and spent RPMI, prior to inoculation with 100 μl of *P. aeruginosa*, *E. coli*, *S. aureus*, or MRSA (5 x 10^6 cells/ml) in BHI. Bacteria were allowed to adhere to the initial *C. albicans* biofilms for 4 hours to facilitate dual-species biofilm formation. Following washing to remove planktonic cells and spent media as outlined above, biofilms were incubated in BHI for a further 24 hours to allow dual-species biofilm maturation. Wells were then washed with PBS as outlined above and the biofilms were quantified by qPCR (S1A Fig). Single species bacterial biofilms served as controls.

In selected experiments, to further investigate the role of *C. albicans* in enhancing bacterial cell numbers during biofilm maturation, we prepared dual-species biofilms as outlined above with wild type *C. albicans* or *C. albicans gcn5Δ/Δ* hyphal-deficient mutant. We then quantified bacterial numbers after initial adhesion (4 hours), or following a combined initial adhesion
period (4 hours) and further maturation period (24 hours). Quantification of bacterial cell numbers was undertaken by qPCR as described below.

**Bacterial cell quantification in biofilms by qPCR**

To quantify cell numbers in single species or dual-species biofilms, the biofilms were detached from microtitre wells into 100 µl of broth by sonicating for 5 min in an ultrasonic bath (Dawe, Middlesex, UK). Any remaining cells were then collected into a further 100 µl broth. DNA was extracted using the microLYSIS®-Plus kit (Microzone, Haywards Heath, UK) as per the manufacturer’s instructions and individual monoplex qPCR assays were performed using an Mx3005P qPCR System (Agilent Technologies, California USA) as detailed in S1–S5 Tables (Supporting information). The candidate genes selected were expressed by the specific microorganisms used in this study and the primers employed for their quantification had been designed and published previously: Primers for *P. aeruginosa* were against the *oprL* gene [26]; Primers for *E. coli* quantification were against the 16S rRNA gene [27]; Primers for *S. aureus* quantification were against the Panton-Valentine leucocidin (PVL) gene [28]. The *S. aureus* strains NCTC 6571 and MRSA 4D used in this work both harboured the PVL gene.

**Generation of standard curves for qPCR**

To allow for quantification of bacterial cell numbers, DNA standards were prepared by extraction of DNA from planktonic organisms using the microLYSIS®-Plus kit and purified using the DNeasy kit (Qiagen, Manchester, UK). DNA standards, as previously described by us [29], were used in all qPCR assays to generate standard curves from which the numbers of organisms within the biofilms could be determined. Cell numbers within the biofilm were thus generated from and were equivalent to cell numbers from planktonic cultures used in the standard curves.

**Transwell assays for determining the influence of contact with *C. albicans* biofilms on *E. coli*, *S. aureus* and MRSA cell numbers**

To determine whether contact with early *C. albicans* biofilms was required to enhance respiratory pathogen cell numbers, Transwell assays were employed in which *C. albicans* biofilms grown in the upper chamber, were physically separated from bacteria (*E. coli*, *S. aureus*, or MRSA) in the lower chamber. A 1 ml inoculum of *C. albicans* (1.0 x 10⁶ cells/ml in RPMI) was added to the upper chamber (Costar Transwell 12 mm, 0.4 µm Polyester Membrane, Corning NY) and incubated for 4 hours to allow initial *C. albicans* biofilm formation. The *C. albicans* biofilm was washed three times with PBS and the lower chamber was inoculated with 2 ml of bacteria (5 x 10⁶ cells/ml in BHI) before replacing the *C. albicans*-coated upper chamber containing 1 ml BHI. Transwells were incubated for a further 4 hours before washing both chambers three times with PBS and adding fresh BHI (1 ml upper chamber and 2 ml lower chamber). Biofilms were incubated for a further 24 hours to allow maturation before bacterial biofilm quantification by qPCR as outlined above. For control experiments, the upper chamber was not inoculated with *C. albicans*, but all other steps were undertaken as outlined above. No Transwell experiments were undertaken with *P. aeruginosa*, as cell numbers of *P. aeruginosa* did not increase in the dual-species *C. albicans*-P. aeruginosa biofilms that we studied.

**Electroporation of reporter plasmids into *E. coli*, *S. aureus* and MRSA**

*S. aureus* (NCTC 6571) and MRSA (4D) were grown in BHI overnight, centrifuged for 10 minutes, resuspended in 300 mM sucrose (to 1/10 volume) and 100 µL of this mixture was
electroporated (25 μF, 200 Ω, 2.5 kV) with 100 ng of a derivative of pCN47 containing the phy-
per promoter and GFP [30] (a gift from Prof Iñigo Lasa, Universidad Pública de Navarra). E. coli
(ATCC 29522) was prepared in the same manner and electroporated (25 μF, 200 Ω, 2.5
kV) with 100 ng of pUC18T-mini-Tn7T-Apr-mCherry [31] (a gift from Dr Ayush Kumar,
University of Manitoba).

**Dual-species biofilm preparation for TIRF microscopy**
Overnight cultures of *C. albicans* and mCherry-labelled *E. coli*, GFP-labelled *S. aureus*, or
GFP-labelled MRSA were prepared as outlined above and biofilms were formed in μ-Slide 8
well ibiTreat chamber slides (ibidi, Germany) by inoculating wells with 100 μl *C. albicans labo-
ratory strain or *C. albicans gcn5Δ/Δ* hyphal-deficient mutant (1.0 x 10⁶ cells/ml in RPMI).
Wells were incubated for 4 hours to allow initial *C. albicans* biofilm formation. The biofilm
was washed with PBS to remove planktonic cells and RPMI, prior to inoculation with 100 μl
mCherry- or GFP-labelled bacteria (5.0 x 10⁶ cells/ml in BHI). Bacteria were allowed to adhere
to the *C. albicans* biofilms for 4 hours to facilitate initial dual-species biofilm formation.
Images were acquired by TIRF microscopy (Leica UK) in the epifluorescence mode using a
Leica EL6000 external light source for fluorescent images and LED lamp for bright field
images. Fluorescent and bright field images were overlaid using LAS-X software (Leica Appli-
cation suite).

**Dual-species biofilm treatment with liposomal amphotericin and
subsequent quantification by PMA-qPCR**
To determine the effect of antifungal treatment on bacterial numbers in dual species biofilms,
liposomal amphotericin (1 μg/ml; reflecting the clinical breakpoint recommended by
EUCAST) was added to *C. albicans* (NCTC 3179) or clinical isolate of *C. albicans* (CA 239SB)
inoculum preparations, before addition to the wells of the microtitre plates (S1B Fig). The
remaining steps for dual-species biofilm were as outlined above, the only exception being that
after the last washing step at each stage of the protocol, 1 μg/ml liposomal amphotericin was
added to the BHI media (S1B Fig). Following biofilm treatment with liposomal amphotericin,
it was important to quantify only living cells within the biofilm and thus a PMA (Biotium Inc.,
California, USA) qPCR protocol was employed as previously described by us [29]. Briefly, fol-
lowing biofilm detachment from microtitre plates, as outlined above, the DNA-intercalating
agent PMA (200 μM) was added to each tube and incubated at 37˚C for 5 min, prior to photo-
activation with a broad-spectrum LED flood light [32]. PMA binds to DNA from dead cells
and prevent its amplification by qPCR, thereby allowing quantification of DNA from living
cells only. DNA was then extracted and quantified by qPCR using an Mx3005P qPCR System
(Agilent Technologies, California USA) with the reaction conditions outlined in S1–S5 Tables
(Supporting information).

**Statistical analysis**
Datasets were analysed by nonparametric Mann-Whitney statistical tests or Kruskal-Wallis
tests for multiple comparisons, as detailed in figure legends. Data from a total of three inde-
pendent experiments were analysed for each dataset. Statistical analysis and graphing of data
was performed using GraphPad Prism version 8 for Windows. A p value of <0.05 was consid-
ered statistically significant.
Results

*C. albicans* early biofilms enhance cell numbers of selected respiratory pathogens

We initially studied whether the presence of early biofilms of *C. albicans* could enhance cell numbers of *P. aeruginosa*, *E. coli*, *S. aureus* or MRSA ([S2 Fig](#)). In *C. albicans*-*P. aeruginosa* dual-species biofilms, *P. aeruginosa* cell numbers (4.3x10⁶) were not significantly different to those in axenic *P. aeruginosa* biofilms (3.4x10⁶) ([S2A Fig](#)) and it was not studied further. However, numbers of *E. coli* (8.9x10⁶) were significantly increased in the presence of early *C. albicans* biofilms compared with axenic *E. coli* biofilms (3.0x10⁶) ([S2B Fig](#)). Likewise, *S. aureus* numbers were significantly increased in dual-species (2.6x10⁷) compared with axenic biofilms (1.2x10⁷) ([S2C Fig](#)). To demonstrate that results obtained with laboratory strains were also applicable to clinical isolates we showed that cell numbers of a clinical isolate of MRSA were significantly increased when grown in the presence of a clinical isolate of *C. albicans* (2.4x10⁷) compared with axenic MRSA biofilms (1.0x10⁶) ([S2D Fig](#)).

Contact with *C. albicans* early biofilms is required to enhance cell numbers of respiratory pathogens

To determine whether contact between *C. albicans* and bacteria was required for the increased bacterial cell numbers observed, *C. albicans* early biofilms were separated from respiratory pathogenic bacteria in Transwell assay experiments. In the absence of contact between *C. albicans* and bacteria, no increase in *E. coli*, *S. aureus* or MRSA cell numbers was observed ([Fig 1](#)) and indeed MRSA numbers were shown to decrease.

Dual species biofilm formation with *C. albicans* wild type and hyphal-deficient mutant

To confirm the presence of *C. albicans* biofilms after 4 hours initial biofilm formation, we demonstrated the presence of extracellular polymeric matrix, using SYPRO™ Ruby Biofilm matrix stain ([S3 Fig](#)). Having established the presence of any early biofilm, we wanted to determine if hyphae had a role in enhancing bacterial numbers following inoculation of respiratory pathogens on the pre-formed *C. albicans* biofilm. TIRF microscopy of wild type and hyphal-deficient *C. albicans* showed attachment of *E. coli*, *S. aureus* and MRSA following 4 hours initial adhesion ([Fig 2A–2F](#)). To further elucidate the role of *C. albicans* hyphae, we quantified cell numbers of *E. coli*, *S. aureus* and MRSA after 4 hours initial adhesion to wild type or hyphal-deficient mutant *C. albicans* biofilms and then after an additional 24 hours (corresponding to further maturation of the dual-species biofilms). Following the initial bacterial

![Fig 1. Increased bacterial cell numbers in dual species biofilms is contact dependent with early *C. albicans* biofilm. A two chamber Transwell assay was used to determine whether contact between early *C. albicans* biofilms was required for enhanced bacterial biofilm cell numbers.](https://doi.org/10.1371/journal.pone.0249547.g001)
adhesion period to the pre-formed C. albicans biofilms, no significant differences in bacterial numbers attached to wild type or hyphal-deficient mutant C. albicans were observed. Following dual species biofilm maturation for a further 24 hours E. coli, S. aureus and MRSA numbers increase significantly in biofilms with wild type C. albicans. However, during the maturation phase with the hyphal-deficient C. albicans only S. aureus and MRSA increased significantly, with E. coli numbers failing to do so (Fig 2G–2I).

**Targeting C. albicans with liposomal amphotericin in dual-species biofilms decreased S. aureus and MRSA cell numbers**

In view of our results showing that C. albicans early biofilms enhanced E.coli, S. aureus and MRSA numbers, we were prompted to test a novel approach aimed at decreasing pathogenic bacterial cell numbers by targeting the architecturally supporting micro-organism, C. albicans, with the antifungal drug liposomal amphotericin. Following treatment of dual-species with liposomal amphotericin, no significant reduction in E.coli cell numbers was observed (Fig 3A). However, liposomal amphotericin treatment of dual species C. albicans-S. aureus biofilms resulted in significant reduction of S. aureus (Fig 3B) cell numbers. Moreover, in dual-species biofilms containing clinical isolates of C. albicans and MRSA, liposomal amphotericin treatment significantly reduced MRSA numbers (Fig 3C). Cell numbers in axenic S. aureus biofilms were not significantly altered by liposomal amphotericin treatment (Fig 3D), suggesting it did not have a direct effect on S. aureus within the biofilms.

**Discussion**

C. albicans is often overlooked as a bystander in polymicrobial biofilm-associated infections. However, using qPCR to determine bacterial cell numbers within dual-species biofilms [29] we showed that C. albicans early biofilms enhanced cell numbers of S. aureus, MRSA and E.
coli, but not P. aeruginosa. Our results, which focus on quantifying bacterial cell numbers, agree with previous studies in which C. albicans was shown to enhance S. aureus dual-species biofilm formation in the presence of serum [33,34] and that an antagonistic relationship exists between P. aeruginosa and C. albicans [35,36].

Using a Transwell assay, we demonstrated that contact between early C. albicans biofilms and E. coli, S. aureus or MRSA was required to enhance bacterial cell numbers. Previously, using a Transwell assay Harriott & Noverr [33] showed contact requirement when C. albicans and S. aureus were inoculated simultaneously in Transwell upper and lower chambers respectively. In our Transwell assay (as in our biofilm model) C. albicans was inoculated first (upper chamber) to allow early biofilm development before bacterial inoculation (lower chamber). Despite the presence of an early C. albicans biofilm, soluble factors from the early biofilm did not support increased numbers of bacteria. Our results therefore concur that contact is required between C. albicans and S. aureus [33] and also between C. albicans and E. coli or MRSA, even when an early C. albicans biofilm is present. The decreased cell numbers of MRSA observed in the Transwell assay results, highlight that additional factors, such as the production of farnesol by C. albicans [37], which is recognised to have inhibitory effects against MRSA [38], may be more evident in the absence of direct fungal-bacterial contact.

In the presence of direct contact, the architecture of C. albicans has been reported to have an important role in polymicrobial biofilms [8,23,34,39,40]. C. albicans adhesion proteins such as the agglutinin-like sequence 3 protein (Als3p) [41,42], as well as Staphylococcal adhesins such as fibronectin binding protein B (FnPB), S. aureus surface protein F (SasF) or a putative N-acetyl muramoul-1-alanine amidase (Atl) [43,44] may contribute to physical interactions in dual-species biofilm formation. Although our results do not appear to concur with previous work on the importance of the hyphal adhesion protein Als3p in S. aureus adhesion, multiple additional C. albicans adhesins are likely to have roles in biofilm formation [45]. Moreover, yeast wall protein 1 (Ywp1), a protein downregulated during filamentation [46] and previously
reported to regulate dispersion in *C. albicans*, has recently been shown to function in maintaining adhesion following initial attachment [45]. Thus, biofilm formation is likely to involve multiple adhesins, with complex temporal and spatial roles in the initial attachment and biofilm maturation stages. Furthermore, although mediators such as prostaglandin E2 (PGE2) have been reported to enhance *C. albicans-S. aureus* biofilm formation [47], the concentration of PGE2 produced by a 24 hour culture of *C. albicans* has been shown to be insufficient to do so [47]. Thus, while soluble factors may enhance bacterial numbers in maturing dual-species biofilms, our results suggest that during the initial stages of dual-species biofilm formation, contact is essential.

TIRF microscopy of wild type or hyphal-deficient mutant *C. albicans* showed attachment of *E. coli*, *S. aureus* and MRSA in early dual-species biofilms. Quantification of bacterial numbers in early and maturing dual-species biofilms showed that hyphal-deficient *C. albicans* was capable of significantly enhancing *S. aureus* and MRSA, but not *E. coli* numbers, suggesting that subtle differences may exist in the ability of the *C. albicans* strains to increase bacterial numbers in the maturing biofilms.

It is well recognised that liposomal amphotericin has enhanced efficacy against *C. albicans* biofilms [48,49] and as expected, *C. albicans* numbers were decreased in all dual species biofilms studied, following liposomal amphotericin treatment (results not shown). Interestingly, we found that liposomal amphotericin treatment of dual-species *C. albicans-S. aureus* or *C. albicans-MRSA* biofilms also resulted in a significant reduction in *S. aureus* and MRSA cell numbers, despite their being no direct effect of the antifungal on *S. aureus* axenic biofilms. Notwithstanding some previous indications of potential synergistic relationships between *C. albicans* and *S. aureus* [35,50,51], antifungal treatments have not been actively pursued as a potential mechanism to reduce bacterial burden. Several elegant studies on *C. albicans* dual-species biofilms have previously reported that fungal cells may modulate the action of antibiotics [33,39,40,52]. However, no studies to date have successfully targeted *C. albicans* as a means of reducing bacterial cell numbers in dual-species biofilms. It had previously been suggested that in the absence of a known antibacterial effect of miconazole against *S. aureus*, the clinical success of topical treatment with the antifungal, miconazole, could potentially be attributed to direct activity against the *C. albicans* biofilm meshwork, which could destabilise *S. aureus* colonization [42]. However, more recently, the imidazole antifungal miconazole has indeed been shown to have direct efficacy against *S. aureus* [53] and therefore the clinical efficacy of topical miconazole could involve direction antibacterial action. What we show in the current study, which has not been reported previously, is that targeting *C. albicans* with liposomal amphotericin in dual-species biofilms reduces *S. aureus* and MRSA numbers, without direct antibacterial/off-target effects on *S. aureus*.

Interesting, liposomal amphotericin treatment of dual-species *C. albicans-E.coli* did not reduce *E. coli* numbers. Our data on dual-species biofilm development in either wild type or hyphal-deficient *C. albicans*, showed that *E.coli* numbers failed to increase significantly during biofilm maturation with the hyphal-deficient mutant, suggesting that hyphae may have a more prominent role in dual-species *C. albicans-E.coli* biofilm formation. With this in mind, it is possible that, following liposomal-treatment, dead hyphae may still be able to provide architectural support for *E. coli*, a phenomenon that has been described in other fungal species [54]. Such an effect could potentially contribute to the lack of reduction in *E. coli* numbers that we observed following liposomal-treatment of *C. albicans-E.coli* biofilms.

**Conclusions**

In conclusion, we demonstrate that early *C. albicans* biofilms facilitated increased numbers of *E. coli, S. aureus* and MRSA in dual-species biofilms via direct contact. Furthermore, treatment
of dual-species biofilms with liposomal amphotericin significantly reduced \textit{S. aureus} and MRSA cell numbers but not \textit{E. coli} numbers. Given the importance of fungal-bacterial biofilms in a wide range of human diseases \cite{55}, the identification of \textit{C. albicans} as a target microorganism in polymicrobial biofilm infections may have important clinical consequences. Targeted treatment of early \textit{C. albicans} biofilms could be developed to reduce not only fungal but also bacterial burdens in polymicrobial biofilm infections. The controlled release \cite{56,57} of surface-bound antifungal drugs may prove useful in this respect.

\textbf{Supporting information}

\textbf{S1 Fig.} Schematic outline for polymicrobial biofilm formation, treatment and quantification. (A) Polymicrobial biofilm formation and quantification by qPCR. Inoculation of \textit{C. albicans} in Roswell Park Memorial Institute (RPMI) broth, followed by inoculation and subsequent growth of \textit{S. aureus}, MRSA, \textit{E. coli} or \textit{P. aeruginosa} in brain heart infusion (BHI) broth. (B) Treatment of polymicrobial biofilms with liposomal amphotericin (1μg/ml; EUCAST clinical breakpoint) and subsequent quantification by propidium monoazide (PMA)-qPCR.

\textbf{S2 Fig.} qPCR quantification of respiratory pathogens in axenic and polymicrobial biofilms (consisting of \textit{C. albicans} (Ca) and respiratory pathogen). (A) Cell numbers of \textit{P. aeruginosa} (Pa). (B) Cell numbers of \textit{E. coli} (Ec). (C) Cell numbers of \textit{S. aureus} (Sa). (D) Cell numbers of MRSA. All respiratory pathogenic bacteria were detected in axenic and polymicrobial biofilms by qPCR using specific primers. Data from a total of three independent experiments (Mann Whitney; ns: p > 0.05; **: p < 0.01, ***: p < 0.001, error bars SD).

\textbf{S3 Fig.} Confocal fluorescent microscopy of \textit{C. albicans} 4 hour biofilm. To demonstrate the presence of an early biofilm containing extracellular matrix, chamber slides were stained with SYPRO \textsuperscript{TM} Ruby Biofilm Matrix Stain following 4 hours incubation with \textit{C. albicans} (see \textbf{S1 Methods}). Image acquired using a Leica TCS SP8 confocal laser scanning microscope (Leica, UK). To preserve the image data (without modification) images were processed in 3D, using LAS-X software (Leica Application suite), for addition of a 3D scale.

\textbf{S1 Table.} qPCR reaction formulation for \textit{S. aureus}.

\textbf{S2 Table.} qPCR reaction formulation for \textit{E. coli}.

\textbf{S3 Table.} qPCR reaction formulation for \textit{P. aeruginosa}.

\textbf{S4 Table.} qPCR conditions for \textit{C. albicans} and \textit{E. coli} (instructions provided with FastSart kit, Roche).

\textbf{S5 Table.} qPCR conditions for \textit{S. aureus} and \textit{P. aeruginosa} (adapted from instructions provided with Platinum\textsuperscript{©} quantitative PCR SuperMix-UDG).

\textbf{S1 Methods.}
Acknowledgments
The authors thank Dr Amanda Willis (Queen’s University Belfast) for providing the clinical isolate of \textit{C. albicans}, Prof Michael Tunney (Queen’s University Belfast) for providing the clinical isolate of MRSA, Prof Karl Kuchler (Medical University of Vienna) for gifting the \textit{gcn5Δ/Δ C. albicans} hyphal-deficient strain, Prof Iñigo Lasa (Universidad Pública de Navarra) for gifting the GFP plasmid and Dr Ayush Kumar (University of Manitoba) for gifting the mCherry plasmid.

Author Contributions

Conceptualization: Daniel F. McAuley, Ronan McMullan, Fionnuala T. Lundy.

Data curation: Yu Luo, Catherine R. Fulton, Joana Sá Pessoa.

Formal analysis: Yu Luo, Catherine R. Fulton, Joana Sá Pessoa, Fionnuala T. Lundy.

Funding acquisition: Daniel F. McAuley, Ronan McMullan, Fionnuala T. Lundy.

Investigation: Yu Luo.

Methodology: Catherine R. Fulton, Joana Sá Pessoa.

Project administration: Daniel F. McAuley, Ronan McMullan, Fionnuala T. Lundy.

Resources: Daniel F. McAuley, Ronan McMullan, Fionnuala T. Lundy.

Supervision: Daniel F. McAuley, Ronan McMullan, Fionnuala T. Lundy.

Writing – original draft: Yu Luo, Fionnuala T. Lundy.

Writing – review & editing: Yu Luo, Daniel F. McAuley, Catherine R. Fulton, Joana Sá Pessoa, Ronan McMullan, Fionnuala T. Lundy.

References

1. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002; 15: 167–193. https://doi.org/10.1128/cmrr.15.2.167-193.2002 PMID: 11932229

2. Percival SL, Suleman L, Vuotto C, Donelli G. Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. J Med Microbiol. 2015; 64: 323–334. https://doi.org/10.1099/jmm.0.000032 PMID: 25670813

3. Quinn RA, Lim YW, Maughan H, Conrad D, Rohwer F, Whiteson KL. Biogeochemical forces shape the composition and physiology of polymicrobial communities in the cystic fibrosis lung. mBio 2014; 5: e00956–13. https://doi.org/10.1128/mBio.00956-13 PMID: 24643867

4. Filkins LM, O'Toole GA. Cystic fibrosis lung infections: Polymicrobial, complex, and hard to treat. PLoS Pathog. 2015; 11: e1005258. https://doi.org/10.1371/journal.ppat.1005258 PMID: 26719892

5. Kalan L, Loesche M, Hodkinson BP, Heilmann K, Ruthel G, Gardiner SE, et al. Redefining the chronic-wound Microbiome: fungal communities are prevalent, dynamic, and associated with delayed healing. mBio 2016; 7: e01058–16. https://doi.org/10.1128/mBio.01058-16 PMID: 27601572

6. Brand A, Barnes JD, Mackenzie KS, Odds FC, Gow NA. Cell wall glycans and soluble factors determine the interactions between the hyphae of \textit{Candida albicans} and \textit{Pseudomonas aeruginosa}. FEMS Microbiol Lett. 2008; 287: 48–55. https://doi.org/10.1111/j.1574-6968.2008.01301.x PMID: 18680523

7. Shirliff ME, Peters BM, Jabra-Rizk MA. Crosskingdom interactions: \textit{Candida albicans} and bacteria. FEMS Microbiol Lett. 2009; 299: 1–8. https://doi.org/10.1111/j.1574-6968.2009.01688.x PMID: 19552706

8. Peters BM, Jabra-Rizk MA, Schepers MA, Leid JG, Costerton JW, Shirliff ME. Microbial interactions and differential protein expression in \textit{Staphylococcus aureus} -\textit{Candida albicans} dual-species biofilms. FEMS Immunol Med Microbiol. 2010; 59: 493–503. https://doi.org/10.1111/j.1574-6968.2010.01371.x PMID: 20608978
9. Bor B, Cen L, Agnello M, Shi W, He X. Morphological and physiological changes induced by contact-dependent interaction between Candida albicans and Fusobacterium nucleatum. Sci Rep. 2016; 6: 27956. https://doi.org/10.1038/srep27956 PMID: 27295972

10. Hola V, Ruzicka F, Horka M. Microbial diversity in biofilm infections of the urinary tract with the use of sonication techniques. FEMS Immunol Med Microbiol. 2010; 59: 525–528. https://doi.org/10.1111/j.1574-695X.2010.00703.x PMID: 20602639

11. Cairns S, Thomas J, Hooper S, Wise MP, Wilson MJ, et al. Molecular Analysis of Microbial Communities in Endotracheal Tube Biofilms. PLoS One 2011; 6: e14759. https://doi.org/10.1371/journal.pone.0014759 PMID: 21423727

12. Hellyer TP, Morris AC, McAuley DF, Walsh TS, Anderson NH, Singh S, et al. Diagnostic accuracy of pulmonary host inflammatory mediators in the exclusion of ventilator-acquired pneumonia. Thorax 2015; 70: 41–47. https://doi.org/10.1136/thoraxjnл-2014-205766 PMID: 25298325

13. Danin P, Girou E, Legrand P, Louis B, Fodil R, Christov C, et al. Description and microbiology of endotracheal tube biofilm in mechanically ventilated subjects. Resp Care 2014; 60: 21–29. https://doi.org/10.4187/respcare.02722 PMID: 25371399

14. Pneumatomikos I, Dragoumanis C, Bouros D. Ventilator-associated pneumonia or endotracheal tube-associated pneumonia? Anesthesiol. 2009: 110: 673–680. https://doi.org/10.1097/ALN.0b013e31819866e0 PMID: 19212256

15. Gil-Perotín S, Ramírez P, Martí V, Sahuquillo JM, Gonzalez E, Calleja I, et al. Implications of endotracheal tube biofilm in ventilator-associated pneumonia response: a state of concept. Crit Care 2012; 16: R93. https://doi.org/10.1186/cc11357 PMID: 22621676

16. Kabak E, Hudcova J, Magaryciz S, Stulik L, Goggin M, Szijártó V, et al. The utility of endotracheal aspirate bacteriology in identifying mechanically ventilated patients at risk for ventilator-associated pneumonia: a single-center prospective observational study. BMC Infect Dis. 2019; 19: 756. https://doi.org/10.1186/s12879-019-4367-7 PMID: 31464593

17. Chauvel M, Nessey A, Cabral V, Znadi S, Goyard S, Bachellier-Bassi S, et al. A versatile overexpression strategy in the pathogenic yeast Candida albicans: identification of regulators of morphogenesis and fitness. PLoS One 2012; 7: e45912. https://doi.org/10.1371/journal.pone.0045912 PMID: 23049891

18. Azoulay E, Timis J, Tafiet M, de Lassence A, Darmon M, Zahar JR, et al. Candida colonization of the respiratory tract and subsequent Pseudomonas ventilator-associated pneumonia. Chest 2006; 129: 110–117. https://doi.org/10.1378/chest.129.1.110 PMID: 16424420

19. Samararayake LP, Keung Leung W, Jin L. Oral mucosal fungal infections. Periodontol 2009; 49: 39–59. https://doi.org/10.1111/j.1600-0757.2008.00291.x PMID: 19152525

20. Lockhart SR, Joly S, Vargas K, Swalis-Wenger J, Enger L, Soll DR. Natural defenses against Candida colonization breakdown in the oral cavities of the elderly. J Dent Res. 1999; 78: 857–868. https://doi.org/10.1177/00220345990780040601 PMID: 10326730

21. Harriott MM, Noverr MC. Importance of Candida-bacterial polymicrobial biofilms in disease. Trends Microbiol. 2011; 19: 557–563. https://doi.org/10.1016/j.tim.2011.07.004 PMID: 21855346

22. Gil-Bona A, Parra-Giraldo CM, Hernández ML, Reales-Calderón JA, Solís NV, Filler SG, et al. Candida albicans cells having uncover new proteins involved in cell wall integrity, yeast to hypha transition, stress response and host–pathogen interaction. J Prot. 2015; 127: 340–351.

23. López-Ribot J. Candida albicans Biofilms: More Than Filamentation. Current Biol. 2005; 15: R453–455. https://doi.org/10.1016/j.cub.2005.06.020 PMID: 15964263

24. Shivaratih R, Tschermer M, Zwolanek F, Singh NK, Chauhan N, Kuchler K. The Fungal Histone Acetyl Transferase Gcn5 Controls Virulence of the Human Pathogen Candida albicans through Multiple Pathways. Sci Rep. 2019; 9: 9445. https://doi.org/10.1038/s41598-019-45817-5 PMID: 31263212

25. Pierce C, Uppuluri P, Tristan A, Wormley FL Jr, Mowat E, Ramage G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc. 2008; 3: 1494–1500. https://doi.org/10.1038/nprot.2008.141 PMID: 18772877

26. Billard-Pomares T, Herwegh S, Wizla-Derambure N, Turck D, Courcol R, Husson M-O. Application of quantitative PCR to the diagnosis and monitoring of Pseudomonas aeruginosa colonization in 5-18-year-old cystic fibrosis patients. J Med Microbiol. 2011; 60: 157–161. https://doi.org/10.1099/jmm.0.023838-0 PMID: 20947668

27. Lee DH, Bae JE, Lee JH, Shin JS, Kim IS. Quantitative detection of residual E. coli host cell DNA by real-time PCR. J Microbiol Biotechnol. 2010; 20: 1463–1470. https://doi.org/10.4041/jmb.1004.04035 PMID: 21030834

28. McDonald RR, Antonishyn NA, Hansen T, Snook LA, Nagle E, Mulvey MR, et al. Development of a triplex real-time PCR assay for detection of Panton-Valentine leukocidin toxin genes in clinical isolates of
methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol. 2005; 43: 6147–6149. https://doi.org/10.1128/JCM.43.12.6147-6149.2005 PMID: 16331116

29. Luo Y, Bolt HL, Eggimann GA, McAuley DF, McMullan R, Curran T, et al. Peptoid efficacy against polymicrobial biofilms determined by using propidium monoazide-modified quantitative PCR. ChemBiochem 2017; 18: 111–118. https://doi.org/10.1002/cbic.201600381 PMID: 27900840

30. Valle J, Echeverz M, Lasa I. α5 Inhibits Poly-N-Acetylglycosamine Exopolysaccharide Synthesis and Biofilm Formation in *Staphylococcus aureus*. J Bacteriol 2019; 201: e00098–19. https://doi.org/10.1128/JB.00098-19 PMID: 30928589

31. Ducas-Mowchun K, De Silva PM, Crisostomo L, Fernando DM, Chao TC, Pelka P, et al. Next Generation of Tn7-Based Single-Copy Insertion Elements for Use in Multi- and Pan-Drug-Resistant Strains of Acinetobacter baumannii. Appl Environ Microbiol. 2019; 85: e00066–19. https://doi.org/10.1128/AEM.00066-19 PMID: 30928589

32. van Frankenhuysen J, Trevors J, Flemming C, Lee H, Habash MB. Optimization, validation, and application of a real-time PCR protocol for quantification of viable bacterial cells in municipal sewage sludge and biosolids using reporter genes and *Escherichia coli*. J Ind Microbiol Biotechnol. 2013; 40: 1251–1261.

33. Harriott M, Noverr M. *Candida albicans* and *Staphylococcus aureus* Form Polymicrobial Biofilms: Effects on Antimicrobial Resistance. Antimicrob Agents Chemother. 2009; 53: 3914–3922. https://doi.org/10.1128/AAC.00657-09 PMID: 19564370

34. Kean R, Rajendran R, Hagarty J, Townsend EM, Short B, Burgess KE, et al. *Candida albicans* mycofilms support *Staphylococcus aureus* colonization and enhances miconazole resistance in dual-species interactions. Front Microbiol. 2017; 8: 258. https://doi.org/10.3389/fmicb.2017.00258 PMID: 28280487

35. Hogan D, Kolter R. Pseudomonas-Candida Interactions: An Ecological Role for Virulence Factors. Science 2002; 296: 2229–2232. https://doi.org/10.1126/science.1070784 PMID: 12077418

36. Bandara HM, Yau JY, Watt RM, Jin LJ, Samarayake LP. *Pseudomonas aeruginosa* inhibits in-vitro Candida biofilm development. BMC Microbiol. 2010; 10: 125. https://doi.org/10.1186/1471-2180-10-125 PMID: 20416106

37. Jabra-Rizk MA, Shirliff M, James C, Meiller T. Effect of farnesol on Candida dubliniensis biofilm formation and fluconazole resistance. FEMS Yeast Res. 2006; 6: 1063–73. https://doi.org/10.1111/j.1567-1364.2006.00121.x PMID: 17042756

38. Kuroda M, Nagasaki S, Ito R, Ohta T. Sesquiterpene farnesol as a competitive inhibitor of lipase activity of *Staphylococcus aureus*. FEMS Microbiol Lett. 2007; 273: 28–34. https://doi.org/10.1111/j.1574-6968.2007.00772.x PMID: 17559400

39. Harriott M, Noverr M. Ability of *Candida albicans* mutants to induce *Staphylococcus aureus* vancomycin resistance during polymicrobial biofilm formation. Antimicrob Agents Chemother. 2010; 54: 3746–3755. https://doi.org/10.1128/AAC.00657-09 PMID: 20566760

40. Jenkinson HF, Douglas LJ. Interactions between Candida Species and Bacteria in Mixed Infections. In: Brogden KA, Guthmiller JM, editors. Polymicrobial Diseases. Washington, DC: ASM Press; 2002. pp357–374.

41. Peters BM, Ovchinnikova ES, Krom BP, Schlecht LM, Zhou H, Hoyer LL, et al. *Staphylococcus aureus* adherence to *Candida albicans* hyphae is mediated by the hyphal adhesin Als3p. Microbiology 2012; 158: 2975–2986. https://doi.org/10.1099/mic.0.062109-0 PMID: 22918893

42. O’Donnell LE, Millhouse E, Sherry L, Kean R, Malcolm J, Nile CJ, et al. Polymicrobial Candida biofilms: friends and foe in the oral cavity. FEMS Yeast Res. 2015; 15: fov077. https://doi.org/10.1093/femsyr/fov077 PMID: 26298018

43. Schlecht LM, Peters BM, Krom BP, Freiberg JA, Hänisch GM, Filler SG, et al. Systemic *Staphylococcus aureus* infection mediated by Candida albicans hyphal invasion of mucosal tissue. Microbiology. 2015; 161: 168–181. https://doi.org/10.1099/mic.0.083485-0 PMID: 25332378

44. Negrii TC, Koo H, Arthur RA. *Candida*—Bacterial Biofilms and Host—Microbe Interactions in Oral Diseases. In: Belbasakis GA, Hajishengallis G, Bostanci N, Curtis M.A, editors. Oral Mucosal Immunity and Microbiome. Cham Switzerland: Springer Nature Switzerland; 2019. pp119–141.

45. Granger BL, Flenniken ML, Davis DA, Mitchell AP, Cutler JE. Yeast wall protein 1 of *Candida albicans*. Microbiology (Reading). 2005; 151:1631–1644. https://doi.org/10.1099/mic.0.27663-0 PMID: 15870471

46. McCall AD, Pathirana RU, Prabhakar A, Cullen PJ, Edgerton M. Candida albicans biofilm development is governed by cooperative attachment and adhesion maintenance proteins. NPJ Biofilms Microbiomes. 2019; 5:21. https://doi.org/10.1038/s41522-019-0094-5 PMID: 31452924
47. Krause J, Geginat G, Tammer I. Prostaglandin E2 from Candida albicans stimulates the growth of Staphylococcus aureus in mixed biofilms. PLoS One 2015; 10: e0135404. https://doi.org/10.1371/journal.pone.0135404 PMID: 26262843

48. Kuhn DM, George T, Chandra J, Mukherjee PK, Ghannoum MA. Antifungal susceptibility of candida biofilms: Unique efficacy of amphotericin B lipid formulations and echinocandins. Antimicrob Agents Chemother. 2002; 46: 1773–1780. https://doi.org/10.1128/aac.46.6.1773-1780.2002 PMID: 12019089

49. Ramage G, Jose A, Sherry L, Lappin DF, Jones B, Williams C. Liposomal amphotericin B displays rapid dose-dependent activity against Candida albicans Biofilms. Antimicrob Agents Chemother. 2013; 57: 2369–2371. https://doi.org/10.1128/AAC.02344-12 PMID: 23422915

50. Carlson E. Enhancement by Candida albicans of Staphylococcus aureus, Serratia marcescens, and Streptococcus faecalis in the establishment of infection in mice. Infect Immun. 1983; 39: 193–197. https://doi.org/10.1128/IAI.39.1.193-197.1983 PMID: 6401691

51. Carlson E. Effect of strain of Staphylococcus aureus on synergism with Candida albicans resulting in mouse mortality and morbidity. Infect Immun. 1983; 42: 285–292. https://doi.org/10.1128/IAI.42.1.285-292.1983 PMID: 6352497

52. Kong EF, Kuchariková S, Van Dijck P, Peters BM, Shirtliff ME, Jabra-Rizk MA. Clinical implications of oral candidiasis: host tissue damage and disseminated bacterial disease. Infect Immun. 2015; 83: 604–613. https://doi.org/10.1128/IAI.02843-14 PMID: 25422264

53. Nenoff P, Koch D, Krüger C, Drechsel C, Mayser P. New insights on the antibacterial efficacy of miconazole in vitro. Mycoses 2017; 60: 552–557. https://doi.org/10.1111/myc.12620 PMID: 28370366

54. Miquel Guennoc C, Rose C, Labbé J, Deveau A. Bacterial biofilm formation on the hyphae of ectomycorrhizal fungi: a widespread ability under controls? FEMS Microbiol Ecol. 2018; 94. https://doi.org/10.1093/femsec/fiy093 PMID: 29788056

55. Krüger W, Vielreicher S, Kapitan M, Jacobsen ID, Niemie MJ. Fungal-bacterial interactions in health and disease. Pathogens. 2019; 8: 70. https://doi.org/10.3390/pathogens8020070 PMID: 3117285

56. McCoy CP, Irwin NJ, Brady C, Jones DS, Carson L, Andrews GP, et al. An infection-responsive approach to reduce bacterial adhesion in urinary biomaterials. Mol Pharmaceutics 2016; 13: 2817–2822. https://doi.org/10.1021/acs.molpharmaceut.6b00402 PMID: 27359363

57. Donnelly L, Hardy JG, Gorman SP, Jones DS, Irwin NJ, McCoy CP. Photochemically controlled drug dosing from a polymeric scaffold. Mol Pharmaceutics 2017; 34: 1469–1476. https://doi.org/10.1007/s11095-017-2164-9 PMID: 28508123