Bandara, H. M. H. N., Matsubara, V. H., & Samaranayake, L. P. (2017). Future therapies targeted towards eliminating *Candida* biofilms and associated infections. *Expert Review of Anti-Infective Therapy*, 15(3), 299-318.
<https://doi.org/10.1080/14787210.2017.1268530>

Peer reviewed version

Link to published version (if available):
[10.1080/14787210.2017.1268530](https://doi.org/10.1080/14787210.2017.1268530)

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Future therapies targeted towards eliminating *Candida* biofilms and associated infections

Journal:	<i>Expert Review of Anti-infective Therapy</i>
Manuscript ID	ERI-2016-0138.R1
Manuscript Type:	Reviews
Keywords:	antifungals, candidiasis, antifungal resistance, probiotics, immunotherapy, alternative therapies, <i>Candida</i> biofilms

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Future therapies targeted towards eliminating *Candida* biofilms and associated infections

Abstract

Introduction: *Candida* species are common human commensals and cause either superficial or invasive opportunistic infections. The biofilm form of *Candida* as opposed to its suspended, planktonic form, is predominantly associated with these infections. Alternative or adjunctive therapies are urgently needed to manage *Candida* infections as the currently available short arsenal of antifungal drugs has been compromised due to their systemic toxicity, cross-reactivity with other drugs, and above all, by the emergence of drug-resistant *Candida* species due to irrational drug use.

Areas covered: Combination anti-*Candida* therapies, antifungal lock therapy, denture cleansers, and mouth rinses have all been proposed as alternatives for disrupting candidal biofilms on different substrates. Other suggested approaches for the management of candidiasis include the use of natural compounds, such as probiotics, plants extracts and oils, antifungal quorum sensing molecules, anti-*Candida* antibodies and vaccines, cytokine therapy, transfer of primed immune cells, photodynamic therapy, and nanoparticles.

Expert commentary: The sparsity of currently available antifungals and the plethora of proposed anti-candidal therapies is a distinct indication of the urgent necessity to develop efficacious therapies for candidal infections. Alternative drug delivery approaches, such as probiotics, reviewed here is likely to be a reality in clinical settings in the not too distant future.

Key words: Alternative therapies, antifungals, *Candida* biofilm, candidiasis, antifungal resistance, immunotherapy, probiotics.

1. Introduction

Candida is a dimorphic fungus commonly isolated as a member of the healthy human mycobiome (the fungal biota of the microbiome) of the gastrointestinal tract, oral cavity, skin and the vagina [1-4]. As residents of the mycobiome, they mainly coexist with bacteria, attached to either biotic or abiotic surfaces, essentially in the form of biofilms. However, when the host immunity is impaired, such as in HIV infection and AIDS, cytotoxic therapy, uncontrolled diabetes mellitus and in extremes of age (the very young and the very old) the healthy mycobiome shifts from a symbiotic existence with the resident flora, to a dysbiotic, pathogenic state [4]. Another reason for the dysbiosis is the prolonged use of broad-spectrum antimicrobials, when commensal *Candida* rapidly acquire pathogenic traits and cause a variety of infections ranging from superficial, mucosal (e.g. oral candidiasis) to haematogenously disseminated life threatening disease (e.g. candidemia) [5-7]. The incidence of *Candida* infections worldwide has been progressively rising over the last few decades mainly due to the significant increase in the population at risk, particularly, the spread of HIV, increased use of indwelling devices and immunosuppressive therapy [8].

The magnitude of *Candida* infections is alarming. For instance, *Candida* species are ranked the fourth leading agent of health care associated infections globally [9] and carry the highest crude mortality rate of all nosocomial bloodstream infections, even exceeding those caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [10]. The annual rates of incidence of *Candida*-associated nosocomial bloodstream infections at the beginning of this millennium ranged between 6.0 to 13.3, and 1.9 to 4.8 cases per 100 000 population in USA and Europe, respectively [11-15]. Hence ameliorating this disease burden is still a major healthcare priority, and the need for new antifungal therapeutic approaches rather urgent.

Although over 150 of *Candida* species have been identified thus far, only 30 of them are known to be human pathogens [16-18]. Of these *Candida albicans* by far is the most virulent, predominant cause of human infection. Over 90% of all invasive candidiasis is caused by *C. albicans* together with four major non-*albicans Candida spp.*: *Candida glabrata*, *Candida tropicalis*,

Candida parapsilosis, and *Candida krusei* [19]. An upsurge of infections caused by, hitherto lesser known non-*albicans* *Candida* species such as *Candida dubliniensis*, *Candida guilliermondii*, *Candida rugosa*, *Candida kefyr*, *Candida famata* and *Candida auris* has also been reported recently [20-22].

According to The National Institutes of Health of the United States of America, over 80% of all human microbial infections are associated with biofilms [23]. Microbial biofilms are defined as complex, structured, spatially oriented microbial communities that are encased in an extracellular matrix and attached to a surface. *Candida* species are known to build highly structured and dynamic biofilms comprising various morphological forms of the fungus (yeasts, pseudohyphae and hyphae, (Figure 1A) [24]. *C. albicans*, in particular, forms dense biofilms on indwelling devices such as catheters (urinary and central venous), joint prostheses, mechanical heart valves, pacemakers, contact lenses, and plastic dentures, resulting in significant morbidity and mortality [25,26].

The economic burden of *Candida* biofilms is alarming. In the United States alone, even with advanced therapies, over 100,000 annual deaths are reported due to catheter-associated *Candida* infections, costing over \$6.5 billion in management [25,27]. The management of such device-associated *Candida* biofilm infections remains extremely challenging due to the high rate of recalcitrance and the intrinsic resistance of biofilms to antifungals. When these devices are so infested, high doses of antifungal agents together with the removal of the device has been recommended, which inevitably results in extensive and unnecessary tissue damage, serious and life threatening risks in critically ill patients as well as antifungal therapy associated adverse side effects, such as renal and hepatic damage [28-31]. Hence, the development of alternative or adjunctive therapies against *Candida* biofilm infections is an urgent necessity.

2. *Candida* biofilm antifungal resistance

The resistance to antifungals is one of the many characteristics acquired by *Candida* during its lifestyle transition from a free-floating planktonic state to a sessile/biofilm state.

Candida biofilms exhibits more than 1000-fold greater resistance to conventional antifungals compared to their planktonic counterparts [32,33]. During biofilm development *Candida* shows alterations in its resistance to antifungals as early as from the initial, surface adherence stage, reaching a peak resistance in mature biofilms [34]. *Candida* biofilm antifungal resistance is multifactorial and a number of well-coordinated, unique mechanisms appear to generate these highly recalcitrant robust biofilms. Some of these mechanisms are briefly outlined below.

2.1 Biofilm cell density

Biofilms are densely colonized with fungal cells. Several studies have suggested that the higher concentration of densely packed *Candida* blastopores with hyphal elements is likely to generate a high degree of antifungal resistance. Perumal *et al* [35] investigated the efficacy of several antifungal agents (azoles, amphotericin B and caspofungin) on a range of fungal cell concentrations from planktonic cultures, as well as disrupted biofilms. Their observations suggested that higher fungal cell densities offered greater resistance to experimented antifungal agents regardless of the origin of the cells. Hence, the effect was more physico-chemical in nature and not biofilm specific [35].

2.2. Extracellular matrix

It is now known that the extracellular matrix of *Candida* biofilms, mainly comprising carbohydrates (glucose, rhamnose, mannose, *N*-acetylglucosamine), proteins, phosphorous, hexamine, and uronic acid plays a critical role in modulating the biofilm lifestyle [36,37]. Apart from offering succor and support for *Candida* within the matrix to provide a structural scaffold, the extracellular matrix provides nutrients, hydration, enzymes, minerals, transport media for signaling molecules, and unfettered protection from external insults [38,39]. Though the exact mechanism/s is not yet clear, multiple theories have been proposed to explain the role of biofilm matrix in antifungal resistance.

Al Fattani *et al* [37] observed a direct correlation between the degree of the production of extracellular matrix and the antifungal resistance in both *C. albicans* and *C. tropicalis* biofilms. Nett *et al* [40] investigated the role of extracellular matrix components of the biofilm in antifungal resistance and discovered a relationship between β -1,3 glucan content of the biofilms and fluconazole resistance. It appears that β -1,3 glucan inhibits fluconazole penetration into yeast cells either through drug binding or sequestration [40]. Similar observations for β -1,3 glucan-antifungal interactions have been reported for amphotericin B, flucytosine and echinocandins [41,42]. Non-*albicans Candida* such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis* biofilms have also been noted to employ similar matrix associated antifungal sequestration mechanisms [43]. Some credence to the latter hypothesis is provided by the fact that *Candida* biofilms display higher sensitivity to echinocandins, which inhibit the synthesis of matrix β -1,3 glucan [44].

The relationship of *C. albicans* glucan synthase, and its genetic regulation has been further characterized with regards to antifungal resistant biofilm phenotypes [45,46]. These, studies however indicate that the potential of drug binding or sequestration is unlikely to contribute to the degree of drug resistance, as several folds higher concentrations of the antifungal (beyond that of the respective minimum inhibitory concentrations) could be found at the distal base of the biofilms. Others have proposed that, the antifungal effect could be better related to the rate of its diffusion through the matrix, than the final concentration of the drug [47].

Martins *et al* [48] have proposed that the presence of extracellular DNA (eDNA) may contribute to biofilm drug resistance. They noted a significant augmentation of antifungal activity of polyenes and echinocandins when candidal biofilms were treated with DNases, although this effect was not seen with fluconazole [48]. The exact role of eDNA in *Candida* biofilm antifungal resistance is yet to be determined.

2.3. Drug efflux

Upregulation of drug efflux pumps in microbial cell membranes are known to be associated with resistance to many antimicrobials by limiting their intracellular accumulation,

and hence, the potency of the drug [49]. *Candida* species possess two different categories of efflux pumps: The ATP binding cassette transporters (*CDR1* and *CDR2*) and major facilitator transporter (*MDR1*) [50]. Usually, in the absence of external antifungal challenge, the expression and the activity of these pumps remains low [50]. Although upregulation of all three foregoing efflux pumps have been noted in azole resistant *Candida* particularly after long-term therapy of AIDS associated oropharyngeal candidiasis, their activity was insignificant in polyene and echinocandins resistance strains [51-53].

The activity of drug efflux pumps in biofilms appear to be phase- and time-specific. Using mutant *Candida* strains, Ramage *et al* [54] noted that drug efflux pumps play a substantive role in antifungal resistance of mature, rather than young biofilms (24-48h). On the contrary, Mukharjee *et al* [55] demonstrated that drug efflux pumps are predominantly responsible for the azole resistance in early biofilms (6h) of *Candida* [55]. In contrast, there is data to suggest that the mere attachment process of *Candida* to a surface is sufficient to trigger the upregulation of these efflux pumps and exposure to antifungals is not essential for their activation [56,57]. Similar efflux activities have been identified in non-*albicans* *Candida* species such as *C. glabrata* [58,59]. In addition, reports have indicated the presence of other potential efflux pumps, associated with fluconazole exposure e.g. those encoded by *FLU1* [60]. These data indicate that drug efflux pumps play a role in drug resistance of *Candida* biofilms, contingent upon the antifungal in question. However, the drug efflux phenomenon is likely to be overshadowed by other dominant drug resistance mechanisms operational in biofilms, and may only partly contribute to the overall resistance.

2.4. Modulation of sterol synthesis

The composition of *Candida* cell wall sterol content significantly varies at different stages of biofilm growth. For instance, the cell membrane ergosterol content is lower in *Candida* biofilms compared to planktonic counterparts, while established biofilms have a lower ergosterol content (approximately 50% less) than the early stage biofilms [55].

Candida in mature biofilms seems to utilize alternative approaches to maintain cell membrane fidelity, making them less favorable to ergosterol targeting drugs such as polyenes. Gene expression shifts may cause such alterations in the ergosterol content. *ERG11* gene, for instance, which encodes an enzyme targeted by azoles, and *ERG25* gene that plays a role in ergosterol demethylation leading to the synthesis of non ergosterol intermediates (e.g. eburicol and 14-dimethyl fecosterol) are significantly upregulated in mature biofilms [57]. Thus, the altered ergosterol biosynthetic pathway may itself confer a degree of drug resistance on *Candida* biofilms.

2.5. Persister populations

Persister cells are phenotypically variant subset of yeast cells that usually populate the basal and intermediate layers of biofilms that exhibit extremely high resistance to antimicrobial agents. Persister cells are common in both eukaryotic (*Candida*) biofilms as well as in prokaryotic (bacterial) biofilms [61,62]. Though the process of transition of regular biofilm cells to persister status is not clear, as yet, it has been speculated that their metabolic dormancy might be responsible for the elevated antimicrobial resistance [63]. Modifications in *Candida* cell wall and the membrane structure may also contribute to persister cells development. This hypothesis is supported by the transcriptome analysis of persister population showing upregulation of genes responsible for ergosterol synthesis (*ERG1* and *ERG25*) and β -1,6 glucan synthesis [64].

Up to 0.1-1% cells of the biofilm may consist of persister population, however, its concentration appear to be species and strain specific [65]. For instance, *C. albicans* SC5314 strain is prone to form less persister cells than *C. albicans* GDH2346 [65]. Whereas *C. glabrata* does not form persister cells at all, compared with either *C. albicans* or *C. krusei* [65,66].

Attempts to eradicate persister cell populations in candidal biofilms exposed to azoles have been made by Bink *et al* [67]. They reported that exposure of the biofilms to *N,N'*-didiethyldithiocarbamate (DDC), an inhibitor of superoxide dismutase, plays a major role in persister generation against azoles, and reduced the persister population by 18 fold [67].

Although persister phenomenon is unlikely to be the sole mechanism that confers antifungal resistance on *Candida* biofilms, their elimination is a promising objective that may help preclude yeast repopulation after successful antifungal therapy.

2.6 Stress response

In general, microbial biofilms inhabit extremely hostile environments. Consequently, the biofilm microbe populations are exposed to a wide variety of internal and external stresses and insults. In response to such threats, these microbes have developed an array of stress responses, several of which have been identified in *Candida* biofilms. For instance, Kumamoto *et al* [68] demonstrated, using mutant strains that MAPK pathway, which maintains the cell wall integrity and contribute to filamentation of *Candida*, plays an important role in fluconazole resistance [68]. Mutant *C. albicans* strains of *MKC1*, which encode an important component Mkc1p in MAPK pathway, exhibited 100-fold less resistance to fluconazole [68].

Similarly, a Ca^{2+} calmodulin-activated serine/threonine protein phosphatase, calcineurin, is associated with fluconazole resistance. Though the main function of calmodulin is to maintain cell homeostasis, morphogenesis and virulence, disruption of *CNB1* or *CRZ1* genes in calcineurin pathway led to increased susceptibility of *Candida* to fluconazole. When calcineurin pathway was blocked pharmacologically (using FK506), similar susceptibilities were observed suggesting the potential of calmodulin as a promising therapeutic target [69].

Additionally, the heat shock protein 90 (HSP90) that is necessary for activation of calcineurin was identified to be important in *Candida* azole and echinocandins resistance [70,71]. An investigation of the potential of using HSP90 inhibitor together with fluconazole has shown that only 7 of 260 *C. albicans* strains tested developed resistance to fluconazole with either geldanamycin (HSP90 inhibitor) or FK506, suggesting another putative approach for tackling *Candida* biofilm antifungal resistance [72].

In summary, it is clear that the transition to biofilm lifestyle triggers a variety of cellular and molecular responses in *Candida* that is quite distinct from its planktonic counterpart. Thus

far, a number of mechanisms governing antifungal resistance in *Candida* biofilms, predicated by the stage of the biofilm development, as well as the specific *Candida* species and strains have been identified. However, the global linking mechanisms of these pathways and the triggers that activate such mechanisms remain to be clarified.

3. Current armory of antifungals against *Candida*

Human beings as well as fungi are eukaryotic organisms. This evolutionary resemblance of fungal and human cells severely restricts the number of drug targets that could be exploited for the development of antifungal agents [73,74]. Therefore, only a limited group of antifungal agents are currently available for managing *Candida* biofilm associated infections (Figure 2). These include polyenes, azoles, pyrimidine analogs, allylamines, thiocarbamates, morpholines and echinocandins. The mode of action, toxicity, antifungal resistance mechanisms of these drugs are briefly outlined in Table 1.

In general, azole antifungals, classic formulations of polyenes and the pyrimidine analogs, allylamines are not very effective against *Candida* biofilms [33,55,75,76]. In contrast, echinocandins, and lipid formulations of amphotericin B have displayed promising *in vitro* and *in vivo* potential against *Candida* biofilms [44,77-80]. However, reports are emerging on the resistance of *Candida* biofilms to the newer echinocandins [81]. Thus, total eradication of candidal biofilms with the pharmaceuticals available today remains a rather daunting challenge.

4. Combined antifungal therapy

Management of fungal infections via multiple antifungal administrations has been considered due to potential advantages over monotherapy. Antifungal multi-therapy benefits include: i) lower probability of antifungal resistance, ii) potential synergy of antifungal agents lowering the effective dose, duration and associated adverse effects; iii) broader spectrum of antifungal activity compared to a single agent; iv) improvements of fungicidal properties; and v)

inversion of selective pressure to disadvantaged drug resistant mutants and collateral sensitivity [82-85].

Unlike with *Cryptococcus* and *Aspergillus* infections, attempts to investigate combined antifungal therapy against *Candida* infections appear to be limited. This is particularly due to the superior efficacy of existing agents such as polyenes and azoles against *Candida* infections. The Infectious Diseases Society of America (IDSA) in its latest guidelines have recommend that combination therapy should be avoided in treating invasive candidiasis as single agents are as effective as combined therapy [86].

As mentioned above, the HSP90 inhibitors, which suppress heat shock protein contributing to drug induced stress response, have been evaluated using *C. albicans* biofilms. *C. albicans* catheter biofilm infection simulated in a rat model further proved that HSP90 inhibitors (e.g. 17-AAG) were capable of increasing the susceptibility of *Candida* (in catheter biofilms) to fluconazole, without any toxic effects [70]. In another *in vivo* study, when caspofungin was combined with HSP90 inhibitors, the survival of the murine litter infected with *C. albicans* improved significantly [87], suggesting the potential of HSP90 as a candidate agent for combined antifungal therapy against *Candida* biofilms.

One of the well-known HSP90 client proteins, calcineurin has been receiving attention as a potential anti-fungal target due to its multiple roles in cell physiology such as cation homeostasis, cell cycle progression, virulence, cell morphogenesis and antimicrobial resistance [88,89]. Particularly, when azoles were combined with a calcineurin inhibitor, cyclosporin A or FK506, significant inhibition of *Candida* biofilms have been observed both *in vitro* and *in vivo* [69], suggesting the likely role of calcineurin in *Candida* biofilm antifungal resistance.

Recently, there has been a resurgence of interest in developing combined antifungal therapy as the world is rapidly running out of potent agents not only due to the emergence of resistant strains but also due to the scarcity of effective drugs available. According to Antifungal Synergistic Drug Combination Database (ASDCD, <http://asdcd.amss.ac.cn/>), there appears to be virtually hundreds of candidate molecules and compounds that may present potential synergy or

additive effects with existing antifungal agents. Thus, combined antifungal therapy regimens are likely to be a reality sooner than expected in managing *Candida* biofilm-associated infections.

5. Antifungal lock therapy

Candida species are the third leading cause of catheter-associated blood stream infections, and the agent for highest overall crude mortality for all nosocomial bloodstream infections [9]. Consequently the Infectious Disease Society of America (IDSA) has categorically recommended removal of the infected catheter, when associated with *Candida* [31]. These infections have many consequences. The removal and replacement of the infected catheter is likely to result in trauma as well as increased catheter-wound associated morbidity and mortality. Extremely high concentrations of antimicrobial agents are also necessary for eradicating refractory candidal biofilms within the luminal surfaces of catheters. This is particularly the case in situations requiring prolonged therapy in debilitated, bed-ridden patients with chronic illnesses. In order to prevent and improve outcomes of such catheter-related *Candida* infections, antifungal lock therapy (ALT) has been recently proposed. ALT entails installation of high concentrations of antimicrobial agents in a solution ('lock' solution) within the infected intravascular catheter for a prolonged period so as to 'sterilize' the catheter lumen [90-93].

The potential of amphotericin B lipid complexes, and liposomal amphotericin B as lock therapy against *C. albicans* as well as, *C. glabrata* and *C. parapsilosis* has been shown in catheter biofilm models. Both of the foregoing amphotericin B preparations exhibited moderate efficacy in lowering biofilm viability, although, liposomal amphotericin B failed to eliminate mature 5-day old *C. parapsilosis* biofilms when exposed to a shorter lock period (<4h) [91,92]. This is likely to be due to the slow drug release mode of liposomal preparations.

Sequential exposure of *C. albicans* biofilms (on silicone disks) to sub-inhibitory concentrations of echinocandins followed by voriconazole or posaconazole was extremely potent in biofilm destruction [94]. To date however, the most promising anti-candidal biofilm effect has

been observed with lower concentrations echinocandins, together with liposomal formulations of amphotericin B [95,96].

Various other alternative agents have also been tested for their potency against *Candida* biofilms, essentially using *in vitro* models [93]. These include antibiotics such as doxycycline, vancomycin and tigecycline, and other chemicals such as heparin, streptokinases, lactoferrins, parabens, taurolidine, chitosan, EDTA, nonsteroidal anti-inflammatory drugs (NSAIDs, e.g. Aspirin) DNases and ethanol [48,97-102]. In experimental studies all of the above agents, either alone or in combinations demonstrated moderate to strong fungicidal activity against *C. albicans* and non-*albicans Candida* biofilms on catheter luminal surfaces. These agents, however, remain to be exploited as potential antifungals for lock therapy.

There is limited information on antifungal lock therapy either in animal models or human clinical trials. In line with *in vitro* studies reported above, some have noted the superior efficacy of liposomal amphotericin B or echinocandins as antifungal lock therapy using a rabbit catheter infection model [103,104]. A few case reports indicate attempts to use, liposomal amphotericin B, caspofungin and ethanol as antifungal lock therapy against *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lipolytica* and *C. guilliermondii* in catheter-related infections, although the rate of failure was disappointing (as high as 100%,) particularly with liposomal amphotericin B [105-107]. These anecdotal reports, wanting of critical information such as pH, dosing intervals, the compatibility of the lock solutions, and the potential risk of precipitation of antifungals are inadequate to arrive at a final judgment on the *in vivo* efficacy of antifungal lock therapy in catheter-associated *Candida* biofilms. Hence further research on lock therapy is urgently required.

6. Other potential approaches for combating *Candida* biofilms

6.1. Denture cleansers

In denture wearing elderly, the tissue fitting surfaces of unhygienic, acrylic (poly methyl methacrylate) denture surfaces may act as reservoirs of candidal biofilms and cause recalcitrant *Candida*-associated denture stomatitis [108]. Various denture cleansers have been evaluated for

their potential to eliminate *Candida* in denture reservoirs. However, there are only a few reports focusing on *Candida* biofilms as most deal with the initial phase of yeast adherence to denture surfaces. Sodium hypochlorite (0.5-5.25%) was shown to be the most effective in killing adherent *C. albicans* and *C. glabrata* in some studies [109,110], despite the potential chemical damage hypochlorite may inflict upon the dentures, especially at higher concentrations. An enzyme based denture cleanser solution (Polident 3 Minute; GlaxoSmithKline, Philadelphia, USA) and another prepared from denture cleansing tablets (Corega Tabs; Block Drug Co, Jersey City, NJ) were found to effectively reduce the yeast burden in 72h *Candida* biofilms on acrylic denture materials despite their failure to remove the biofilm completely [111]. Similarly, various other commercially available cleansers e.g. Polident 3 min (GlaxoSmithKline, PA, USA), Efferdent, (Warner Lambert Co., NJ, USA), and cleansers with proteolytic/yeast lytic enzymes (e.g. Pika (Rohto Pharm Co, Japan), Liodent (Lion corporation, Tokyo, Japan), Dr. Hrealth (Sunstar Incorporation, Osaka, Japan), were also shown to have antifungal effects on adherent *Candida*, but not on *Candida* biofilms [112-114]. The active ingredients of these denture cleansers comprise antimicrobial agents including EDTA, sodium bicarbonate, sodium perborate, hydrogen peroxide, and sodium hypochlorite, with varying degrees of antifungal activity. However, an efficacious denture cleanser with anti-candidal biofilm activity remains to be elusive, as yet.

6.2. Mouth washes

Mouthwashes containing various active agents have been prescribed for daily use in reducing the microbial burden of the oral cavity, particularly in controlling dental plaque biofilms. In this context, the impact of commonly used mouth rinses on eradicating *Candida* biofilms has also been investigated. In *in vitro* studies, commercially available Corsodyl, Oraldene, and Listerine mouth rinses were found to reduce the viability of 48h *C. albicans* biofilms by approximately 80% [115]. The constituents of these mouthwashes are noteworthy. Listerine (Pfizer Consumer Health Care, UK) contains eucalyptol, thymol, methyl salicylate, menthol and ethanol as active agents, while Oraldene (Warner-Lambert Consumer Health Care, UK) contains

hexetidine, and Corsodyl (GlaxoSmithKline Consumer Health Care, UK) contains chlorhexidine gluconate and ethanol.

Similarly, another study has reported the superior efficacy of chlorhexidine gluconate (0.12%) and Listerine mouthwashes in eliminating *Candida* biofilms [116]. Interestingly, in a flow biofilm model of *C. albicans*, chlorhexidine appeared to be more effective in comparison to fluconazole and miconazole in reducing biofilm *Candida* [117].

The duration of exposure to a mouthwash appears to have a critical impact on the substantivity and the consequent reduction of the biofilm cell density. In a clinical trial, Tomas *et al* [118] noted that more than 60s exposure to chlorhexidine gluconate significantly improves *C. albicans* biofilm elimination, and the substantivity of the antiseptic. This said, most mouthwashes have significant undesirable side-effects including enamel staining, altered taste and a burning sensation (dysguesia), whilst the high alcohol content in some mouthwashes have been linked to mucosal pathology [119,120], These limitations of mouth washes detracts from their regular use in combating oral infections including candidal infestations..

6.3. Topical applications

The potential of topical applications of antifungals in the management of *Candida* biofilms and related infections have been sparsely studied. Chlorhexidine gluconate gel as a topical adjunctive therapy for oral candidiasis seem to have benefits due to its prolonged antifungal effect (i.e. substantivity) permitting longer intervals of administration, thus increasing patient compliance [121]. Similarly, another study showed that the addition of chlorhexidine gluconate (0.2%) to tooth paste had favorable effect on reducing *Candida* colonization in HIV-infected children [122]. However, *Candida* regrew within 2-8 weeks after the termination of chlorhexidine-laced toothpaste [122]. Nevertheless, the potential of topical applications of agents such as antiseptic gels and surface coatings for managing *Candida* biofilm infections is worth investigating further.

7. New anti-*Candida* therapeutics and promising future approaches

Since the invention of amphotericin B in 1950s, only a handful of antifungal agents have been successfully introduced into the general consumer market for managing fungal infections. For instance, over the last three decades, echinocandins remain as the only novel antifungal drug class that was introduced by the pharmaceutical industry, and successfully adopted by clinicians.

One major reason for this is the extreme challenge faced by the drug manufacturers when developing a new therapeutic agent, given the current stringent regulatory environment. Perhaps more importantly, in the case of antifungal agents, the challenge is magnified by the significant structural and functional similarities shared by the eukaryotic fungal cells and the human cells. This filial connectivity of humans and fungi drastically limits the number of drug targets in eukaryotic fungal cells that can be exploited for drug development leading to the current scarcity of antifungals available for the treatment of candidal infections [123].

The current arsenal of antifungal drugs is further threatened by the increasing emergence of drug-resistant *Candida* species worldwide due to i) misuse of antifungals, ii) there limited spectrum of activity, iii) failure of biofilm elimination due to the largely fungistatic properties of the majority of drugs and iv) the significant toxicity of existing antifungal compounds [124]. Hence, alternative or adjunctive therapies are urgently warranted to control and prevent superficial and invasive *Candida* infections. A diversity of alternative antifungal therapies can be found in the literature to date and we feature below the most promising.

7.1. Probiotics

Probiotics are defined as live microorganisms that, when administered or consumed in adequate quantities, confer health benefits on the host. A multiplicity of beneficial effects of probiotics are documented in the literature [125]. They are especially useful for combating pathologies of the gastrointestinal tract, such as relieving diarrhea, lactose intolerance and inflammatory bowel diseases [126].

Bacteria belonging to the genera *Lactobacillus* and *Bifidobacterium* are commonly used as

probiotics [126]. To be considered safe for human consumption, the probiotic microbe needs to be of human origin, and devoid of transmissible antibiotic resistance genes. Their functional requirements include adequate adherence and colonization on epithelial surfaces, acid and bile tolerances, immunostimulation, and antagonistic activity against pathogens [126].

The probiotics, mainly lactic acid bacteria, are deemed worthy as an alternative prophylactic and therapeutic agent for managing human candidiasis (Figure 1A and B) [127-129]. The efficacy of probiotics in relieving mucosal *Candida* infections has been extensively assessed in recent clinical investigations, *vis a vis* urogenital [130-133], gastrointestinal [127,134-136], and oral infections [137-141]. These studies demonstrate that probiotic bacteria (administration of either single or multiple strains) alone or in combination with standard antifungals (e.g. fluconazole) reduce candidal mucosal colonization, relieve signs and symptoms of candidiasis, and enhance the antifungal effect of conventional therapy. As these randomized controlled trials substantiate the antifungal activity of probiotics in humans, we believe that probiotics have potential therapeutic value against *Candida* biofilms, [129]. Indeed, Matsubara *et al* [142] have noted, using a murine model, that the effects of probiotics may surpass even those of conventional antifungals in reducing experimental oral candidiasis. The anti-*Candida* biofilm potential of probiotics is both species and strain-specific and therefore the selection of a probiotic for therapeutic purposes needs to be carefully tailored to fit the specific clinical circumstances [143].

Although the mechanisms of action of probiotics against *Candida* is unclear, as yet, the major attribute of probiotics appears to be the restoration of a natural healthy microbiome in a given habitat [129]. The re-establishment of a well-balanced and symbiotic state by probiotic bacteria, with suppression of *Candida* infection, may entail five distinct mechanisms: i) co-aggregation of probiotic and fungal cells in order to impede fungal colonization, ii) production of antagonistic antimicrobial and anti-biofilm compounds, iii) competing with the pathogen for available nutrients and adhesion sites, iv) production of quorum sensing chemicals that lead to down regulation of toxin production by the pathogens, and finally, v) modulation of the humoral and cellular immune system of the host (e.g. subduing antibody and phagocytic activity)[144].

Clearly, stringent regulatory requirements need to be fulfilled prior to establishing a treatment protocol for a probiotic that is planned to replace traditional antifungal regimens. Initial, rigorous clinical trials are needed to ascertain the activity of the probiotic formulation, determine the dosage and administration schedules, and bio-dynamics, and side effects in humans. In the longer term, the potential for selection of resistant strains, mutability, and tolerability of the probiotic on prolonged use are also a concern due to the administration of live organisms. Despite these drawbacks probiotics remain a promising, yet to be fully explored, alternative therapeutic mode against biofilm associated candidal infections.

7.1.1. -2-hydroxyisocaproic acid (HICA)

Lactobacillus species are the most widely used probiotic bacterial species.. Its probiotic activity is thought to be mainly related to the production of 2-hydroxyisocaproic acid (HICA), an α -hydroxy-amino acid [145]. The latter is found in human muscle and connective tissues and have protein synthetic and anti-inflammatory properties. Nieminen *et al* [145] investigated the effect of HICA on *C. albicans* biofilm formation and mutagenic acetaldehyde production *in vitro*. The latter chemical produced by most *Candida* spp. is an important intermediary in candidal biofilm development. HICA was found to reduce biofilms of *C. albicans*, to a greater extent than capsofungin at acidic pH, and to inhibit acetaldehyde production. Using a murine *C. albicans* biofilm model, the same group found through histopathological examination that HICA attenuated inflammatory response, together with reduced expression of matrix metalloproteinase 9 (MMP-9) and myeloperoxidase, both found in chronic inflammatory diseases and linked to the loss of soft and hard tissues [146]. This said, the precise mode of action for HICA is still unclear, and broader transcriptional and proteomic studies as well as *in vivo* studies are warranted to elucidate the mechanisms underlying its anti-microbial activity, and to evaluate its potential clinical use.

7.2. Natural compounds

A wide array of peptides and oils have natural antifungal activity [147,148] (Table 2). One

of these, saponins, produced by over one hundred plant families play a crucial role in defending the plants from extraneous microbial pathogens [149]. Coleman *et al.* [149] using a *Caenorhabditis elegans* model, identified 12 such saponins with antifungal capacity. Two of them at relatively low concentrations inhibited *C. albicans* isolates that were resistant to conventional antifungal agents. The precise mode of action of saponins is not yet clear although they appear to adversely affect filamentation and biofilm formation - two central, virulence attributes of *Candida*. Saponins are also known to bind to fungal ergosterol, and initiate pore formation in *Candida* cell membranes that lead to leakage of cellular contents and death [149]. It has also been noted that saponins act synergistically with other antifungals, *in vivo* and enhance photodynamic fungal inactivation when combined with a photosensitizer [149].

In a recent comprehensive study, Saleem *et al* [150] tested a polyphenol natural compound, lichochalcone-A, found in licorice roots of *Glycyrrhiza* species for its antifungal properties. This compound was found to significantly reduce *Candida* biofilm growth, even surpassing the effect of fluconazole, and decrease the proteinases and phospholipase activities of the yeast. The latter secretory enzymes play a significant role particularly in invasive candidal infections. In a time-kill assay, the fungal load was eradicated after 24 h, suggesting the potential efficacy of lichochalcone-A as a topical treatment. Further, in a mouse model of oral candidiasis, tongue tissues samples of lichochalcone-A treated litter presented with a lesser degree of infiltration and colonization of *C. albicans* compared with the control litter [150]. When the toxicity of this polyphenol was tested by the latter group, using a co-culture model of fibroblasts and *Candida*, no apparent toxicity was noted. Thus lichochalcone-A appears to be a promising new agent effective against *C. albicans* biofilm associated infections, although much more work is required to fully characterize its activity [150].

Other plant-derived peptide that has anti-biofilm activity against *Candida* is Tn-AFP1 [151,152]. This plant peptide purified from fruits of *Trapa natans*, inhibited *C. tropicalis* growth and disrupted biofilm formation in a concentration dependent manner, with no apparent adverse effect on red blood cells [151]. The latter workers noted Tn-AFP1 down regulated *C. tropicalis*,

MDR1 and *ERG11* genes, both of which are associated with biofilm formation and drug resistance [151].

Another novel, natural and cheap antimicrobial molecule that has been recently evaluated for its antifungal activity is carbohydrate-derived fulvic acid (CHD-FA), a derivative of humic acid [153,154]. This colloidal organic acid was found to be fungicidal *in vitro* against both planktonic and biofilm *C. albicans*. In one study, using an *in vitro* biofilm model, CHD-FA was noted to kill 92% of *C. albicans* after 24 h, including drug resistant strains, demonstrating its efficacious candidacidal potential [153]. The mechanism of its anti-candidal activity is yet unknown as the CHD-FA does not appear to adversely affect either the extracellular matrix production or its efflux pump activity. The low cost and broad-spectrum anti-biofilm activity of CHD-FA makes this natural compound a promising candidate agent worthy of further exploration.

Furthermore, ApoEdpL-W peptide, a 18-amino-acid cationic, tryptophan-rich peptide derived from human Apolipoprotein E (ApoE), demonstrated fungicidal activity against *C. albicans* planktonic cells, but remained fungistatic at lower concentrations[152]. ApoEdpL-W was also active against early-stage *C. albicans* biofilms, but less active against mature biofilms, probably due to its affinity for extracellular matrix beta-glucans [152]. The mode of action of this antimicrobial peptide is not clear as yet, but it seems to act intracellularly at a vacuolar level through a process of endocytosis [152].

Another category of natural products with antifungal properties that deserves attention is the tea polyphenols. In an *in vitro* study, three major polyphenol derivatives of green tea: epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), and epicatechin-3-gallate (ECG), were tested on their ability to nurture *C. albicans* growth and maintain a biofilm community [155]. Fungal biofilms incubated with any of these polyphenols for 48 h demonstrated a 72%, reduction of viable cells compared with untreated control cultures. Established biofilms treated with tea polyphenols were also significantly disrupted after 24 h of incubation. The mechanism of action of these natural extracts may include impairment of proteasomal activity, leading to cellular metabolic and structural disruptions that subdue biofilm formation in *C. albicans* [155]. The role

of tea polyphenols, if any, in therapy of fungal infections remains to be determined.

The potency of essential oils derived from plants against *Candida* has been investigated for over three decades [156]. However, it is only recently that some have focused and partially succeeded in identifying new essential oils with a degree of antifungal activity [157,158]. Cinnamon oil, extracted from *Cinnamomum* species, has been considered the most active out of a range of essential oils, showing candidacidal activity against both planktonic and biofilm cultures of *Candida orthopsilosis* and *C. parapsilosis* in [159], and clinical isolates *C. albicans*, *C. glabrata*, and *C. krusei* [157]. The fungicidal effect of essential oils may entail interference with the fungal cell wall development and structural modifications that render them susceptible. The hydrophobicity of these oils enable their close interaction with the lipid bilayer of fungal cell membrane, leading to increased permeability, leakage of cell contents and death [160].

Sesamol, a natural phenolic compound derived from sesame oil has been noted to be antifungal. One study investigating its mode of action noted that sesamol not only affects the cell membrane fluidity, but also inhibits yeast to hypha transition, biofilm formation, mitochondrial dysfunction, and disruption of iron transport, and DNA repair [161].

Garlic (*Allium sativum*) has been traditionally used as an antimicrobial agent against a variety of microbes. The anti-*Candida* effect of garlic extracts have been examined and these include a wide range of ultrastructural perturbations affecting cytoplasmic membranes, organelles and cytoskeletal organization [162]. Further investigations into the candidacidal activities are now focused on the purified garlic constituents to determine the targets organelles that inhibit yeast growth. The garlic extract known as allyl alcohol, for instance, seems to mainly target cytosolically located, alcohol dehydrogenases Adh1 and Adh 2, and mitochondria based Adh3. A significant decrease in NAD(P)H after addition of allyl alcohol is indicative of another mechanism of its anti-*Candida* action. The effect of garlic or its extracts on candidal biofilms is yet to be determined.

Natural products have the potential to be administered either alone or in combination with other antifungal drugs to combat *C. albicans* infections. *In vitro* tests have demonstrated

synergistic activity between natural compounds and conventional antifungal agents, such as amphotericin B, further reducing their minimal inhibitory concentrations (MIC) necessary to inhibit the biofilm formation of *Candida spp.* [163,164].

Clearly, the foregoing indicates the array of natural anti-candidal agents that appear to be promising, and awaiting further investigations. However, they are yet to be comprehensively tested in human clinical trials, and the toxicities of many of the compounds are yet to be evaluated [165]. Further clinical studies are urgently needed to establish the legitimacy of these rather promising natural compounds as alternative or supplemental therapies against biofilm associated *Candida* infections.

7.3. Boric acid

Boric acid (BA) is a versatile chemical that assists wound healing [166], and bone mineralization [167]. BA has also been used as an alternative therapeutic agent for recurrent vulvovaginal candidiasis [168]. Clinical studies that have compared fluconazole and BA in the management of vulvovaginal candidiasis indicate that it is as effective as fluconazole in managing the condition, with the added advantages such as lower cost, ready availability and safety [169,170].

The mechanism of action of BA against fungi is not yet understood, although some have suggested that it inhibits *Candida* filamentation [171]. Pointer et al have noted that the selective inhibitory effect of BA on *C. albicans* hyphae is likely be due to the disruption of apical cytoskeletal elements of growing hyphae [172]. Well controlled large scale clinical trials are necessary to validate the use of BA against *Candida* infection in humans.

7.4. Silver nanoparticles

In the past decade or so, nanotechnology has emerged as a new paradigm that could be exploited for therapeutic drug delivery. Perhaps the most promising antimicrobial nanomaterial thus far developed is the silver nanoparticles (AgNPs). When silver is in nanometric form (<10

nm), the larger total surface area is translated into higher surface reactivity that significantly enhances its antimicrobial activities with little toxicity [173]. As opposed to ionic silver, silver nanoparticles have potent, broad-spectrum antimicrobial activity with a well-tolerated tissue response, and no cytotoxic effects on either human fibroblasts or erythrocytes [174,175].

AgNPs have shown fungicidal activity against *Candida* spp., including *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* strains [157,174]. The stabilization of silver nanoparticles by surface-active agents (sodium dodecyl sulfate-SDS, Tween-80) or polymers (polyvinylpyrrolidone) significantly enhances its antifungal effects by increasing their aggregate stability [174]. This nano-sized inorganic material appear to act against fungal cells by disrupting the structure of the cell membrane and inhibiting the normal budding process due to the destruction of the membrane integrity [175]. When stabilized, with SDS, the nanoparticles, demonstrate augmented permeability of the cell wall and cytoplasmic membrane via bonding to its lipids and proteins.

As *Candida* spp. are key colonizers, and play a major role in the formation of catheter lumen and other implant biofilms, the reduction or inhibition of *Candida* colonization on the surface of these medical devices, is a key to prevent recalcitrant infections (see above) [176]. Hence, AgNPs have been suggested as a useful coating material for catheter luminal surfaces and medical titanium implants, in order to reduce the biofilm burden, and to minimize the risk of infectious complications, and implant rejections [177,178].

7.5. Immunotherapeutic approaches

7.5.1. Vaccines

Vaccines against candidiasis appear to be a promising alternative against invasive infections [23]. Their preventive effect is based primarily on the reinforcement of immunogenicity, thus eliciting a stronger immune response [179]. Various fungal cell components have been explored as an active vaccine for disseminated and mucosal candidiasis (Table 3) [180]. We discuss below the major candidate vaccines currently being explored, namely fungal cell-wall

polysaccharide, proteins, and live attenuated fungal vaccines [179].

Animal studies by Saville *et al* [181] with, genetically engineered attenuated *C. albicans* tet-NRG1 strain vaccine, and then challenged with a fully virulent *C. albicans* CAF-2 strain, demonstrated significantly higher survival rates (100% more than the controls) of immunocompetent mice compared with their non-immunized litter mates [181]. These investigators tested the same vaccination strategy in immunocompromised animals (B-cell-deficient, T-cell-deficient, and neutropenic DBA/2N mice) in order to determine the immune mechanisms involved. Vaccination fully protected B-cell-deficient and DBA/2N mice against disseminated candidiasis, but failed to protect the T-cell-deficient mice. Hence, the protection of the was conferred by a T-cell-mediated adaptive immune response, with little or no participation of B-cells and functional neutrophils [181]. Although promising, the presence of complex, poorly characterized antigens and live attenuated *C. albicans* strains in this vaccine may have intrinsic clinical disadvantages [179].

Protein vaccines are potentially safer than attenuated live organisms. A variety of proteins, from the secreted aspartyl proteinase (SAP) family, and agglutinin-like sequence (Als) family, has already been tested with successful outcomes in pre-clinical trials, demonstrating a reduction of local and systemic infection by *Candida spp.* [179]. In a rat candidal vaginitis model, SAP vaccination (via intravaginal and intranasal routes) was found to promote fungal clearance in the vagina, with no fungi 3 weeks after the *Candida* challenge [182].

Furthermore, immunization of mice with recombinant N-Terminal Domain of Als1p (rAls1p-N) improved their survival on subsequent challenge with a lethal inoculum of *C. albicans* [183]. This study also suggested that the mechanism of action of the rAls1p-N vaccine is based on the stimulation of cell-mediated, rather than the humoral, response against *C. albicans*.

Glycoconjugates vaccines are another alternative for immunization against candidiasis. The conjugates of polysaccharide and a protein elicits a T-cell response, and have the potential to directly inhibit fungal growth [179]. Using a novel methodology, Xin *et al* [184] developed a fully synthetic glycopeptide vaccine against *Candida* by combining β -mannan and a peptide epitopes.

The vaccine comprised six T cell peptides from *Candida albicans* cell wall proteins selected by algorithm peptide epitope searches and conjugated to the fungal cell wall β -mannan trisaccharide [β -(Man)₃]. This vaccine showed protection against experimental disseminated candidiasis in mice. This vaccine differed from others in that it was likely to elicit a dual immune response against both the glycan epitope as well as the peptide carrier.

One key concern, and a major distraction for the proponents of vaccines against *Candida* infections is the generally held belief that disseminated candidiasis occurs almost exclusively in immunocompromised patients, who may not respond well to an active, as opposed to a passive, vaccination process. However, a recent study has allayed this concern as active immunizations with a peptide-pulsed dendritic cell vaccine protected neutropenic mice against disseminated candidiasis [185]. On the other hand, the same group found that the administration of monoclonal antibodies also protected the animals, implying the possibility of a passive immunotherapeutic strategy to protect against disseminated candidiasis [185].

The ideal vaccine against, either planktonic or biofilm *Candida* is yet to be developed. Cell wall proteins is likely to be a candidate antigen for inducing a protective host immune response, as most virulence attributes of *Candida*, including those involving adhesion, invasion, and the yeast-to-hypha transition, are associated with cell wall compounds [179]. The use of adjuvants, such as alum adjuvant, and dendritic cells as vehicles that present the vaccine to T cells are examples of tools that may enhance the immune response of the vaccine (Table 3) [180]. Although to date, most *C. albicans* vaccines are univalent, a multivalent vaccine with multiple unrelated antigens may also provide better protection against candidal infections [179].

Finally, up to now candidal vaccine technology has been limited to animal experiments and hence comprehensive clinical trials are urgently needed to demonstrate their efficacy and safety in humans.

7.5.2. Anti-*Candida* antibodies

Anti-*Candida* antibodies are a yet another prospective avenue for immunotherapy [186].

It has the potential for application in the treatment as well as prevention of candidiasis especially in immunocompromised patients. A human recombinant monoclonal antibody (Mycograb - NeuTec Pharma) against heat shock protein 90 (Hsp90) was investigated in a double-blind, randomized study in 139 patients with invasive candidiasis [187]. Combination therapy with the monoclonal antibody and amphotericin B led to a significantly better clinical and culture-confirmed outcome compared with mono-therapy. A pre-clinical study testing the same antibody against Hsp90 together with caspofungin was also found to be synergistic in another study [188].

Other attempted therapeutic vaccine approaches include the use of human recombinant antimannan immunoglobulin G3 (IgG3) monoclonal antibody derived from immune serum of mice vaccinated with *Candida*-mannan containing liposomes [189], and human recombinant anti-mannan antibody G1 (IgG1) [190], both of which were effective against disseminated candidiasis in mice. Worth mentioning also are anti- β -glucan antibodies induced by β -glucan-conjugate vaccine [191], and idiotypic antibodies [192] which were effective experimental approaches against vaginal candidiasis in mice.

7.5.3. Cytokine therapy

Cytokines play an important role in the host defense against infections and have been investigated as a useful tool for immunomodulation [186]. The adjunctive administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to promote clinical improvement with a reduction in mycological burden without adverse events in immunocompromised patients [193]. Another cytokine, interferon gamma (IFN γ), produced by T cells and NK cells, was found to increase anti-candidal potential of macrophages, although some have queried its efficacy [186]. In one study, the administration of IFN γ reduced the fungal burden in mice with disseminated candidiasis [194], but in another it failed to improve the potency of fluconazole against oral candidiasis in mice [195]. There is also a single clinical report of four patients, that has stated the beneficial contribution of IFN γ in patients with refractory fungal infections [196].

The literature on cytokine therapy for *Candida* infections is sparse and rather contradictory. Fuller, clinical trials with adequate patient numbers are necessary to validate the use of cytokine therapy as an adjunct in the treatment of candidiasis.

7.5.4. Adoptive transfer of primed immune cells

Adoptive transfer of anti-fungal T cells has been attempted as an alternative immunotherapeutic approach to restore host immunity and as a cure for invasive fungal infections [197]. This approach utilizes infusion of patients with dendritic cells (DCs), primed *ex vivo* with antigens that induce specific cytokines, in order to induce an adaptive immune response [186]. These human T cells appear to inflict damage on the hyphal forms of *Candida* and also significantly boost the hyphal damage induced by human neutrophils [186]. The generated T cells do not seem to be impaired by cryopreservation. Hence, the anti-*Candida* T cells have the potential to be preserved, prior to the patient reaching an immune compromised state, to be subsequently infused if the infection is chronic and recalcitrant [198].

7.6. Photodynamic therapy

Antimicrobial photodynamic therapy (PDT) combines a non-toxic dye, a photosensitizer, added to microorganisms, which is activated by visible light of an appropriate wavelength, promoting a phototoxic response in the cells, usually leading to oxidative damage. This process essentially leads to disorganization of the cell wall as well as DNA damage, thus causing cell death [199,200].

Different photosensitizers, both natural and synthetic, mostly belonging to the phenothiazinium, porphyrins or phthalocyanines classes, have been investigated for their utility as antifungals. A number of studies have demonstrated the efficacy of phenothiazine dyes such as methylene blue and toluidine blue in photodynamic therapy against *Candida* [199-203]. In a recent study, a glucosamine salt of a chlorine derivative soluble in water (Photodithazine® - PDZ; a second-generation photosensitizer), and a light emitting diode (LED) light promoted significant

reduction in the viability of *Candida* isolates [202]. The same combination was also effective against treating mice with oral candidiasis [201].

The results of PDT may vary accordingly to the physiological state of the microorganisms as well as factors associated with the photosensitizer, such as the type, concentration, and period of incubation as well as the period of exposure and energy density of the laser [199]. In an *in vitro* study which investigated the effect of toluidine blue O (TBO) and 15 min of LED irradiation (energy density of 180 J/cm²) on the growth and adhesion of different *Candida* isolates to buccal epithelial cells PDT inhibited the adhesion of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* to buccal epithelial cells [203]. As the adhesion of *Candida* to host cells is an essential prerequisite for biofilm formation, colonization and infection, such regimens that inhibit *Candidal* adhesion may have useful translational potential.

In summary, the novel approaches described above appear to be promising in eliminating *Candida* biofilms and associated infections. However, caution must be exercised when inferring their future potential due to the dearth of data from large scale, prospective clinical trials.

8. Acknowledgments

The authors declare that they have no conflict of interests. No funding was received for this review.

9. Expert commentary

Microbial biofilms are robust structures that elicit extreme resistance to many antimicrobials, and *Candida* biofilms are no exception. Some of these resistance mechanisms are unique to the biofilm life style of the yeast while others they share with their planktonic counterpart. The common evolutionary resemblance between eukaryotic, fungal and human cells is yet another obstacle that impedes the development of anti-candidal agents. Further, most if not all, candidal infections are polymicrobial in nature. *C. albicans*, in particular, is known to build mixed species biofilms with other *Candida* species such as *C. glabrata*, *C. tropicalis*, *C. dubliniensis*,

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C. parapsilosis, *C. guilliermondii*, and *C. krusei* [25,204,205]. More importantly, they intimately interact and cohabit with various bacterial species in different host niches (e.g. oral cavity, vagina, gut). These include bacteria such as *Streptococcus mutans*, *Streptococcus gordonii*, *Actinomyces viscosus*, and *Fusobacterium* species inhabiting the oral cavity [23,206,207], *Enterococcus* and *Escherichia coli* in the gastrointestinal tract, lactobacilli in the genital tract, and *Pseudomonas aeruginosa* in lung and wound infections [41,208-212]. Some of these interspecies and inter-kingdom interactions, mediated particularly through quorum sensing mechanisms have been fairly well mapped [213].

Up until now investigations on *Candida* biofilms have been hampered due to the lack of good *in vitro* and *in vivo* experimental systems. The introduction of high throughput biofilm models such as nano-biofilms is therefore a welcome addition to the armamentarium of biofilm biologists [214]. These studies should focus on developing and redefining models that closely mimic the natural eco systems of bacteria and fungi.

The major drawback of the deficiency of drug targets in *Candida* can be overcome by identification of molecules that mediate yeast adhesion, regulation of persister formation, extracellular matrix secretion, and biofilm dispersal. These molecules may be useful in developing inhibitory substances that disrupt the biofilm life cycle. In addition, new approaches for drug penetration into biofilms are needed to further exploit the potential of existing antifungal agents and to minimise their side effects. One example being the echinocandins, that are considered the most efficacious against mature candidal biofilms, but are sparsely used due to their adverse effects [215,216].

Quorum sensing signalling molecules that are critical for the community life style of candidal biofilms, are likely to have considerable utility as anti-biofilm chemicals. Noteworthy here is the recently shown therapeutic benefits of inter-kingdom signalling molecules as biofilm disrupters, and antimicrobial adjuncts in eliminating bacterial biofilms in the respiratory tract [217]. Similar approaches are worth investigating for managing *Candida* biofilm infections. In

addition, discovering new molecules that interfere with biofilm signalling pathways, as designer biomimetic molecules, would also be an attractive option.

Introduction of probiotics will likely to be one of the more promising approaches for eliminating biofilms. However, these probiotic organisms need to possess a very high safety margin, and optimization of their administration regimens is essential for long lasting anti-*Candida* effect. Other unconventional approaches such as use of nanoparticles and plant products against candidal biofilms are also likely to deliver promising results.

According to Antifungal Synergistic Drug Combination Database, many studies have been conducted in combining more than one active agent to achieve superior biofilm elimination to minimize side effects, and obviate emergence of drug resistance. These approaches, though promising, their utility in biofilm eradication is yet to be proven due to the scarcity of good experimental models that faithfully mimic the clinical environs. Finally, animal studies and long term human trials are critically important to establish the veracity and efficacy of any newly developed therapeutic regimens against candidal biofilms and these remain challenges due to the high developmental costs and the stringent regulatory environment.

10. Five -year view

Improvements in drug delivery systems as well as combination antifungal therapy are likely to advance the treatment regimens against local and systemic biofilm-associated *Candida* infections. In addition, the discovery of molecules that mediate *Candida* virulence and their exploitation as new drug targets will expand the antifungal armoury against *Candida*. Alternative therapies presented in this review, especially probiotics, are likely to be a reality, once there is satisfactory clinical database to support their use.

11. Key Issues

- *Candida* is a dimorphic, opportunistic fungus/yeast commonly isolated as a member of the healthy human mycobiome.
- *Candida* species can cause mild superficial infections to lethal systemic infections particularly in susceptible compromised hosts.
- A significant portion of *Candida* infections are biofilm mediated, and exist in harmony with other bacteria and fungi, as highly specialised communities, encased in an extracellular matrix, and attached to either biotic or abiotic surfaces.
- Biofilms exhibit extreme recalcitrance to conventional antifungal agents compared with the suspended, planktonic forms of the yeast.
- The mechanisms that entail *Candida* biofilm antifungal resistance are complex and include biofilm specific mechanisms, such as the biofilm matrix associated factors and stress response, as well as those shared with their planktonic counterparts (e.g. drug efflux pumps and alterations in sterol synthesis).
- Polyenes and echinocandins remain the most efficacious antifungals against *Candida* biofilms, while azoles are predominantly effective against the planktonic forms of the yeast.
- Combinations antifungal therapy has had limited success in reducing drug toxicity and improving outcomes.
- Antifungal lock therapy, proposed for catheter-related candidal infections may have potential utility against these infections.
- Non-pharmaceutical approaches such as probiotics, silver nanoparticles and photodynamic therapy have also shown satisfactory activity against *Candida* biofilm formation and maturation, mostly in *in vitro* studies.
- Recent studies have shown that natural compounds such as plants extracts and oils may help eradicate *Candida* biofilms with minimal undesirable effects.

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- Modulations of the host immune response through anti-*Candida* antibodies, cytokine therapy and vaccines have all received considerable recent attention, but the results of these trials are inconclusive.
- Most data generated on anti-*Candida* biofilm strategies are based on *in vitro* approaches. Thus, cautions must be exercised when extrapolating their applications for clinical use.
- Further understanding of antifungal resistance mechanisms, identification of new drug targets and high throughput screening of potential therapeutic agents are necessary in combating *Candida* biofilm-related infections.

Table 1:

Antifungal class	Mode of action	Adverse effects	Resistance
Polyenes e.g. amphotericin B and nystatin	<ul style="list-style-type: none"> Bind to the ergosterol in fungal cell membrane forming pores (Figure 2). The pores compromise fungal cell wall integrity resulting ionic imbalance, intracellular contents loss and cellular death [218]. 'Sterol sponge model': polyenes (amphotericin B in particular) forms large extramembranous aggregate to extract ergosterol from the phospholipid bilayer, and sterol depletion in fungal cell membrane [219]. 	<ul style="list-style-type: none"> Dose dependent toxicity, particularly, nephrotoxicity and infusion related adverse effects [220]. Toxicity is due to the functional resemblance of fungal ergosterol to mammalian cholesterol leading to polyenes cross reactions [74]. Liposomal formulations of amphotericin B were introduced to improve the safety margin [73,74]. 	<ul style="list-style-type: none"> Candidal resistance to polyenes have been extremely rare. Possible amphotericin B resistance in <i>Candida</i> is species dependent. <i>C. glabrata</i> and <i>C. krusei</i> exhibit higher minimum inhibitory concentration (MIC) to amphotericin B than <i>C. albicans</i> [221]. <i>C. lucitaniae</i>, <i>C. guilliermondii</i> and <i>C. glabrata</i> have been identified to express resistant traits to amphotericin B [5]. Polyene resistance could be due to the mutations of <i>ERG3</i> and <i>ERG6</i> genes, resulting lower ergosterol contents in the cell membrane [222-224].
Azoles (2 subclassess) 1. Imidazoles <ul style="list-style-type: none"> Include miconazole, ketoconazole, clotrimazole, oxiconazole, econazole and tioconazole 	<ul style="list-style-type: none"> Affect the cell membrane ergosterol by inhibiting its biosynthesis Inhibits cytochrome P450 14α-lanosterol demethylase encoded by <i>ERG11</i> gene leading to suppression of 	<ul style="list-style-type: none"> Azoles toxicity is rare Nausea, minor gastrointestinal symptoms and asymptomatic transient hepatic enzyme elevations were reported [228]. 	<ul style="list-style-type: none"> Azole resistance in oropharyngeal candidiasis in HIV infected patients is related to heavy overuse and fungistatic nature [229]. Non-albicans <i>Candida</i> species e.g. <i>C. glabrata</i>, <i>C. krusei</i> and <i>C. lucitaniae</i> are intrinsically less

<ul style="list-style-type: none"> • Commonly prescribed for mucosal infections as topical applications <p>2. Triazoles</p> <ul style="list-style-type: none"> • Include fluconazole, itraconazole, posaconazole, voriconazole and terconazole • Prescribed for both tropical and systemic fungal infections [218,225,226] 	<ul style="list-style-type: none"> • lanesterol to ergosterol conversion (Figure 2). • Severe reduction of the ergosterol in the cell membrane may also affect the “sparking” functions leading to altered cell proliferation and growth [51,52,218]. • suppression of cytochrome P450 14α-lanosterol demethylase lead the cell to bypass ergosterol synthesis and accumulate toxic methylated sterols that significantly affect membrane packing and elevates membrane fluidity (Figure 2). As a result, cell membrane undergo severe stress and the functions of various cell membrane proteins will be severely affected [227]. 	<ul style="list-style-type: none"> • Rare adverse effects include hypokalaemia, pedal oedema, and testicular or adrenal steroidogenesis [228]. 	<p>susceptible to fluconazole [230,231].</p> <ul style="list-style-type: none"> • Possible causes of resistance <ul style="list-style-type: none"> • Low intracellular drug accumulation due to Upregulation of drug efflux pumps coded by <i>CDR1</i>, <i>CDR2</i> and <i>MDR1</i> • Decreased target affinity due to mutation of <i>ERG11</i> genes • Overexpression of <i>ERG11</i> • Inactivation of <i>ERG3</i> gene to reduce accumulation of intracellular toxic sterols [232-234].
<p>Pyrimidine analogs/Fluoropyrimidines</p> <p>E.g. 5-fluorocytosine (5-FC) and 5-fluorouracil.</p>	<ul style="list-style-type: none"> • Fluoropyrimidines are synthetic structural analogs of the nucleotide cytosine • These analogs incorporate in to fungal nucleic acids during their synthesis and cause 	<ul style="list-style-type: none"> • Compared to 5-fluorouracil, 5-FC results lesser side effects, though, serious adverse effects such as hepatotoxicity and bone 	<ul style="list-style-type: none"> • Resistance to 5-FC is rare (<2%) • Mainly due to the point mutations in <i>FCY1</i> or <i>FUR1</i> genes that encode cytosine deaminase and uracil phosphoribosyl transferase (Figure 2). [224,238-240].

	either inhibition of DNA replication or synthesis of corresponding proteins (Figure 2) [235].	marrow lesions have been reported [235-237].	<ul style="list-style-type: none"> • Mutations of cytosine permease coded by <i>FCY2</i> may also lower the uptake of the antifungal in to the cell (Figure 2) [238,240].
Echinocandins e.g. caspofungin, micafungin and anidulafungin	<ul style="list-style-type: none"> • Echinocandins are semisynthetic lipopeptides with a central cyclic hexapeptide structure [241]. • non-competitively inhibit a principal cell wall structural constituent, 1,3-beta-D-glucan (Figure 2) leading to static destabilization of the fungus causing osmotic cell lysis [242,243]. 	<ul style="list-style-type: none"> • Echinocandins possess very low MIC resulting high safety margin [244,245]. • Well tolerable with only mild side effects • Possible associations with hyperbilirubinaemia, acute renal failure, and haemolytic anaemia [246]. • Hepatocellular tumors has been reported in animal models. • Recommended only when other antifungal agents are contraindicated [246]. 	<ul style="list-style-type: none"> • <i>Candida</i> resistance to echinocandins is low. • Echinocandin resistant <i>C. albicans</i>, <i>C. glabrata</i>, <i>C. tropicalis</i> and <i>C. krusei</i> have been reported [247-250]. • <i>C. parapsilosis</i>, <i>C. orthopsilosis</i>, <i>C. metapsilosis</i> and <i>C. guilliermondii</i> are known to possess intrinsic resistance to echinocandins [251]. • Echinocandin resistance in <i>Candida</i> is caused by alterations of the (1,3)-β-glucan synthase complex due to point mutations in <i>FKS1</i> and/or <i>FKS2</i> [252].
Allylamines, thiocarbamates, morpholines	<ul style="list-style-type: none"> • Ergosterol synthesis inhibitors • Allylamines and thiocarbamates inhibit squalene epoxidase coded by <i>ERG1</i> gene • morpholines suppresses <i>ERG24</i> encoded $\Delta 7,8$- 	<ul style="list-style-type: none"> • Less commonly used in treating <i>Candida spp.</i> associated infections mainly due to their numerous adverse effects including gastrointestinal upset, taste disturbance, transient elevation of liver 	<ul style="list-style-type: none"> • Cross-resistance with azoles • Allylamines are used by CDR efflux pumps as substrates [255,256]

	isomerase and <i>ERG2</i> encoded C14-reductase (Figure 2) [226].	enzymes and liver toxicity. [226,253,254]
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Table 1: The classes, mode of actions, adverse effects of commonly used anti-*Candida* antifungals and *Candida* resistance mechanisms

Table 2

Natural compound	Origin	Effective against	Reference
Saponins	Different plant families	<i>C. albicans</i>	[149]
Lichochalcone-A	licorice roots of <i>Glycyrrhiza</i> species	<i>C. albicans</i>	[150]
Carbohydrate- derived fulvic acid (CHD-FA)	Humic acids (humic substances)	<i>C. albicans</i>	[153]
Plant peptide (Tn-AFP1)	<i>Trapa natans</i> fruits	<i>C. tropicalis</i>	[151]
Human peptide (ApoEdpL-W)	Human Apolipoprotein E	<i>C. albicans</i> , <i>C. parapsilosis</i> , <i>C. tropicalis</i> , <i>C. glabrata</i>	[152]
Tea polyphenols	Green tea	<i>C. albicans</i>	[155]
Cinnamon oil	<i>Cinnamomum zeylanicum</i>	<i>C. orthopsilosis</i> and <i>C. parapsilosis</i>	[159]
	<i>Cinnamomum aromaticum</i> (<i>Cinnamomum cassia</i>)	<i>C. albicans</i> , <i>C. glabrata</i> , and <i>C. krusei</i>	[157]
Sesamol	Sesame oil	<i>C. albicans</i>	[161]
Asarones	<i>Acorus calamus</i>	<i>C. albicans</i> and <i>C. tropicalis</i>	[164]
Garlic extract (Allyl alcohol)	<i>Allium sativum</i>	<i>C. albicans</i>	[162]
Acteoside (polyphenolic compounds)	<i>Colebrookea oppositifolia</i>	<i>C. albicans</i> , <i>Cryptococcus neoformans</i> , and <i>Aspergillus fumigatus</i>	[163]

Table 2: Antifungal activity of natural compounds against a variety of fungal strains

Table 3

Vaccine targets	Strategies to enhance <i>Candida</i> vaccines
Heat-killed organisms	Varied adjuvants
Attenuated live organisms	Dendritic cell presentation
<i>Candida</i> cell surface iC3b receptors	Virosome delivery
<i>Candida</i> enolase	DNA delivery
Cell wall proteins	Lipid particle delivery systems
<i>Candida</i> mannans and β -Glucans	Nasal delivery systems
Heat-shock protein 90 (Hsp90)	
Glycosyl phosphatidylinositol (GPI)-anchor mannoprotein (Hyr1p)	
SAP and ALS genes family proteins	

Table 3. Entities explored for developing vaccines against local and disseminated candidiasis, and adjuvants and delivery systems proposed to boost *Candida* vaccine efficacy [Adapted from Edwards *et al* [180].

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Figure 1: The effect of probiotic lactobacilli on *Candida albicans* biofilms. Confocal laser scanning microscopic images (Stained with Live and Dead stain, Invitrogen, USA)
A). Control *C. albicans* 24h biofilm; note the dense, spatially oriented structure that contains hyphae, pseudo hyphae and yeast cells. B) Lactobacilli treated *C. albicans* 24h biofilm; note the significant reduction of the density and the complexity of the biofilm with lower hyphal content compared to control.

138x70mm (300 x 300 DPI)

Figure 2: Mechanisms of action of the antifungal classes currently used against *Candida*!! +
 5-FC: 5-fluorocytosine, 5-FU: 5-fluorouracil, 5-FUMP: 5-fluorouridine monophosphate!! +

196x225mm (300 x 300 DPI)

Legends

Figures

Figure 1: The effect of probiotic lactobacilli on *Candida albicans* biofilms. Confocal laser scanning microscopic images (Stained with Live and Dead stain, Invitrogen, USA)

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Figure 2: Mechanisms of action of the antifungal classes currently used against *Candida*

5-FC: 5-fluorocytosine, 5-FU: 5-fluorouracil, 5-FUMP: 5-fluorouridine monophosphate

Tables

Table 1: The classes, mode of actions, adverse effects of commonly used anti-*Candida* antifungals and *Candida* resistance mechanisms

Table 2: Antifungal activity of natural compounds against a variety of fungal strains

Table 3: Entities explored for developing vaccines against local and disseminated candidiasis, and adjuvants and delivery systems proposed to boost *Candida* vaccine efficacy [Adapted from Edwards *et al* [180].

Responses to Referee(s)' Comments.

Referee 1:

The authors review the role, prevention and treatment of biofilm in *Candida* infections,

1) The manuscript is far too long and addresses numerous issues that are not directly relevant to biofilm and its role in candidiasis eg. Far too much attention to antifungal drugs and although penetration of antifungals into biofilm is important but not worth several pages on antifungals.

Response: The antifungals section was significantly shortened and compiled in to a single table. (Section 3, page 9; and Table 1, page 32 -35). The title of the manuscript is amended to represent the content more precisely.

All sections of the manuscript were edited to highlight the relevant information on *Candida* biofilms and related infections, particularly to stress their drug resistance, and therapies under development.

2) The authors use “superlatives” all the time and often inappropriately. Moderation in language use will be useful.

Response: We have reduced the use of superlatives throughout the manuscript as recommended.

3) “The vast majority of *Candida* infections are biofilm mediated” – nonsense! Perhaps correct in relation to intravascular catheters, but mucosal (superficial) and invasive candidiasis much less relevant if at all. No evidence that *Candida* biofilm forming on urinary catheters is relevant except to explain persistence of Candiduria – catheters should be immediately removed once candiduria detected.

Response: The particular statement was changed. Please see the introduction in the abstract (page 1) and under key issues (page 30)

Nobile CJ and Johnson AD have reviewed the relationship of *Candida* biofilms with superficial, device-associated and systemic candida infections in “*Candida albicans* Biofilms and Human Disease” (Annu Rev Microbiol. 2015; 69:71-92.)

4) What is the mycobiome as opposed to microbiome?

Response: Mycobiome is the fungal biome in a specific niche such as the oral cavity or the gut, as opposed to bacteriome and the virobiome.

The following reference was added to the first paragraph of the introduction section (page 2).

Please refer to “The human mycobiome in health and disease” By Cui L, Morris A, Ghedin E. (Genome Med. 2013 Jul 30;5(7):63.)

5) Authors need to acknowledge that most of the data generated recently on biofilm is based upon in vitro models which have limitations and in vivo studies are lacking.

Response: The limitations of the *in vitro* findings and the necessity of *in vivo* studies were stated at the end of Antifungal lock therapy (page 12), Probiotics (page 17), *D,L-2-hydroxyisocaproic acid* (page 18), and Natural compounds (page 21) sections.

In addition, following statement was added to “key issues”

“Key issues: Most data generated on anti-*Candida* biofilm strategies are based on *in vitro* approaches. Thus, cautions must be exercised when extrapolating their applications for clinical use. (Page 31)

6) Many of non-pharmaceutical approaches have some value *in vitro* models only and are not relevant to clinical disease.

Response: We agree with the reviewer, however, the aim of this review is to bring new therapies, products that have demonstrated anti-*Candida* properties with potential to be used against candidiasis in humans, even if they are in the early stage of development.

The short arsenal of antifungal and the increasing emergence of drug-resistant *Candida* species give more credence for the development of adjunctive approaches that may be used as supplemental therapy.

Hence the following clarification was added on Page 27:

“In summary, the novel approaches described above appear to be promising in eliminating *Candida* biofilms and associated infections. However, caution must be exercised when inferring their future potential due to the dearth of data from large scale, prospective clinical trials. (Page 27)”

7) Probiotics have never been shown to be useful in patient-related biofilm elimination or control.

Response: The following clinical studies have demonstrated the capacity of probiotics alone or in combination with conventional antifungals to reduce oral colonization of *Candida* spp. in the oral cavity (1-5), urogenital track (6-8), and gastrointestinal track (9-11). Improvement of the clinical symptoms of candidiasis were also reported after administration of probiotic bacteria. These randomized controlled trials substantiate the antifungal activity of probiotics in humans, thus we believe that probiotics has the status of a future therapy against *Candida* biofilms. (The following studies have been already cited in the manuscript).

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4. **Li D, Li Q, Liu C, Lin M, Li X, Xiao X, Zhu Z, Gong Q, Zhou H.** 2014. Efficacy and safety of probiotics in the treatment of Candida-associated stomatitis. *Mycoses* **57**:141-146.
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7. **Hu H, Merenstein DJ, Wang C, Hamilton PR, Blackmon ML, Chen H, Calderone RA, Li D.** 2013. Impact of eating probiotic yogurt on colonization by *Candida* species of the oral

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8) It might be useful to separate biofilm occurrence on catheters etc (invasive candidiasis) from superficial candidiasis (oral and vaginal) since therapy would be entirely different!

Response: The objective of this review is to evaluate current and potential future approaches to eliminate *Candida* biofilms and associated infections but not different clinical entities. Therefore, we have not separated *Candida* biofilm associated infections based on their clinical presentation to prevent potential repetition of the current and future approaches throughout the manuscript.

9) No word on vaginal candidiasis and topical boric acid advocated as anti-biofilm. Major omission!

Response: As mentioned in the response to comment 8, we refrained from discussing treatments/therapies based on the clinical presentation (vaginal, oral or gastrointestinal candidiasis) to avoid repetitions. However, different clinical variants of candidiasis were mentioned separately when necessary. Please refer to page 16 paragraph 2 – Probiotic section; page 24 paragraph 2 – Vaccines section.

A new section about boric acid was included in the review (page 21 Section 7.3.)

Referee 2:

This is a comprehensive review of the literature on therapies targeted to *Candida* biofilm-related infections. Overall, it is a good and complete review of the available data. I have some comments below that need some attention by the authors.

1. The drug efflux subsection should be re-written because the text does not always correspond to the indicated references (refs: 47-56). For example, ref. 53 (Lepak et al.,) concerns fluconazole activity against *C. albicans* planktonic cells, not biofilms. Ref 55 is a temporal global gene expression analysis of an *in vivo* *Candida albicans* biofilm, it is not about non-*albicans* *Candida* species. The conclusion of the particular subsection should also be re-phrased as authors conclude that efflux pumps play a significant role in antifungal resistance of *C. albicans* biofilms (pg 6; lines 42-43), but earlier on the authors state that the activity of efflux pumps was insignificant in polyene and echinocandins resistance strains (pg 6; lines 17-18). Furthermore, authors should exercise caution on generalizing about drug resistance, as most studies in the subsection concern data only on fluconazole resistance and there is one study on the activity of caspofungin against *Candida* displaying fluconazole resistance. It is not certain whether this is true for all azoles or all echinocandins.

Response: The drug efflux section has been extensively reviewed and the citation errors were corrected.

Conclusion was amended as follows;

“These data indicate that drug efflux pumps play a role in drug resistance of *Candida* biofilms, contingent upon the antifungal in question. However, the drug efflux phenomenon is likely to be overshadowed by other dominant drug resistance mechanisms operational in biofilms, and may only partly contribute to the overall resistance.” (Page 6 penultimate paragraph)

2. Ref 83 reports on the mode of action of antifungals and the mechanisms of drug resistance of *Candida* species without exploring drug resistance of biofilms. Therefore the statement “However, recent reports have indicate failure of fluconazole in eliminating *Candida* biofilm related infections” does not correspond to ref 83 (page 11, lines 27-28).

Response: Entire section of “Current armory of antifungals against *Candida*” is now tabulated. Please see the response to first comment from the Reviewer 1 and particular reference was removed. (Section 3, page 9; and Table 1, page 32 -35)

Correct references were added. Please see page 9 under the summarized section of “Current armory of antifungals against *Candida*”. (Section 3, page 9; and Table 1, page 32 -35)

3. Section 3. “Current armory of antifungals against *Candida*” (pg 9-14) is mostly descriptive and concerns general knowledge on the mechanism of action of each reported drug and their effectiveness on various *Candida* species, but it does not contain any relevant information on the antibiofilm activity of these drugs. The section needs to be re-written in order to highlight information relevant to biofilm eradication.

Response: Please see the responses to previous comment and first comment of the reviewer 1. (Section 3, page 9; and Table 1, page 32 -35)

4. Same holds true for section 6. “Combined antifungal therapy”. This section, except for the Hsp90 inhibitors which have already been mentioned under the subsection of “Stress response” (pg 6, line 44), offers information on the use of combined antifungals as a strategy to combat *Candida* infections, but the particular section as well as the references provided are not specific to *Candida* biofilms (pg 14-15).

Response: Particular section on “combined antifungal therapy” was refined to discuss anti biofilm approaches and nonspecific references were removed. (Pages 9-11)

5. Subsection 7.2 needs to be part of 7.1 because HICA is also considered a probiotic.

Response: The subsection 7.2 was shifted to 7.1.1 as a subsection of Probiotics section. (page. 17)

6. Section 7.5 (pg 27-32) refers to immunotherapeutic approaches as a strategy against invasive infections which is beyond the scope of this review. It is suggested to be eliminated.

Response: We beg to differ. In our review we sought to bring the relevant proposed therapies against, both superficial and invasive candidiasis. For instance, candidate vaccines have shown some promise against superficial candidiasis (Extensively reviewed by Edwards et al 2012 Ref. 180). Hence, in our opinion, the inclusion of immunotherapeutic approaches is relevant as a future therapy against *Candida*.

7. Several typos throughout the text

Response: The text has been revised for typos.

EDITOR'S COMMENTS:

1. Please clearly hand sign the disclosure form, once you have filled it in (see attached).

Response: Disclosure form was signed.

2. Please confirm whether your figures and tables are original, or provide a copy of permission to use them.

Response: We hereby confirm that all figures and tables are original.

3. Please include some reference annotations in your end-text reference list (see attached guide).

Response: Reference annotations were included.