Review Article

Candida Biofilms and the Host: Models and New Concepts for Eradication

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Biofilms define mono- or multispecies communities embedded in a self-produced protective matrix, which is strongly attached to surfaces. They often are considered a general threat not only in industry but also in medicine. They constitute a permanent source of contamination, and they can disturb the proper usage of the material onto which they develop. This paper relates to some of the most recent approaches that have been elaborated to eradicate *Candida* biofilms, based on the vast effort put in ever-improving models of biofilm formation *in vitro* and *in vivo*, including novel flow systems, high-throughput techniques and mucosal models. Mixed biofilms, sustaining antagonist or beneficial cooperation between species, and their interplay with the host immune system are also prevalent topics. Alternative strategies against biofilms include the lock therapy and immunotherapy approaches, and material coating and improvements. The host-biofilm interactions are also discussed, together with their potential applications in *Candida* biofilm elimination.

1. Introduction

Biofilms, adherent microbial communities embedded in a polymer matrix, are common in nature. However, they are also a persistent cause of hygiene problems in the food industry and in the medical field [1]. Biofilms result from a natural tendency of microbes to attach to biotic or abiotic surfaces, which can vary from mineral surfaces and mammalian tissues to synthetic polymers and indwelling medical devices, and to further grow on these substrates [2–4]. Candidiasis, caused most frequently by *Candida albicans*, and to a lesser extent by *C. glabrata*, *C. tropicalis,* or *C. parapsilosis*, is often associated with the formation of biofilms on the surface of medical devices and tissues [5]. *Candida albicans* is a dimorphic fungus and is part of the commensal human micoflora. It is also an opportunistic pathogen of the human body when its proliferation is not controlled by the host immune system. It is one of the most often identified agents in nosocomial infections and is capable of invading virtually any site of the human host, from deep tissues and organs, to superficial sites such as skin and nails, to medical implants and catheters [6]. *C. albicans* biofilm development has been characterized in various model systems both *in vitro* and *in vivo* [7–9] and consists of distinct phases. The initial step consists of the adhesion of fungal cells of the yeast form to the substrate. It is followed by a phase of cell filamentation and proliferation, which results in the formation of multiple layers of sessile cells of different morphologies, including pseudohyphal and hyphal cells. The next step of maturation results in a complex network of cells embedded in extracellular polymeric material, composed of carbohydrates, proteins, hexosamine, phosphorus and uronic acid, as well as host constituents in natural settings [10]. There is indeed evidence that host glycoproteins, nucleic acids, and cells, such as neutrophils, may participate in the maturity of the matrix, in particular on mucosal sites [11–13]. The establishment of the biofilm extracellular matrix (ECM) represents a unique characteristic of biofilms. Quantity and composition of the matrix vary from one species to another and in different sites of infection depending on environmental cues,

Table 1: Examples of *Candida* biofilm models *in vitro*.

Models *in vitro*	Device	Used for
Closed systems (discontinuous growth conditions over time (nutrient depletion, accumulation of secondary metabolites))	(i) 96-well polystyrene microtiter plate	Easy and widespread use: comparative analyses between strains and species [33–39] to antifungal susceptibility tests [40]
	(ii) Discs/pieces of catheter in 6- to 24-well plate (discs made of silicone, polyurethane, polycarbonate, polystyrene, stainless steel, Teflon, polyvinyl chloride, hydroxyapatite, and porcelain)	
	(iii) Calgary biofilm device (80 pegs immersed into a standard 96-well plate)	Biofilm formation studies by different *Candida* species [41]
	(iv) *Candida* biofilm chip (several hundreds nanobiofilms encapsulated in collagen and formed on a glass slide treated to obtained a monolayer of hydrophobic coating)	High-throughput biofilm studies [42]
Flow systems (Continuous growth conditions)	(i) CDC biofilm reactor (24 biofilms can be formed simultaneously)	Comparative analysis of biofilm quantification methods [43]
	(ii) Microfermentors (biofilms formed on a Thermanox slide glued to a glass spatula)	Gene expression analyses [44]
	(iii) Modified Robbins device (adapted to hold several individual discs)	Study of the effects of shear forces and nutrient supplies on *C. albicans* biofilm formation [45]
	(iv) Flow biofilm model (silicone elastomer strip placed into a polypropylene conical tube)	Study of *C. albicans* biofilm development, architecture, and drug resistance [46]
Shear stress conditions	Rotating disc system (silicone catheter devices placed under a shear force of 350 revolutions per minute)	*C. albicans* biofilm architecture and development [47]

such as nutrient availability and mechanical stimuli [14–17]. Matrix synthesis by *Candida* biofilm cells has been shown to be minimal in static conditions in comparison to dynamic environments [10], aggravating biofilm formation on mucosal and abiotic sites where there is a fluid flow, such as on the oral mucosa, the urethra, or central venous catheters. The last step, dispersion of cells from a biofilm, plays a key part in the biofilm developmental cycle as it is associated with candidemia and disseminated invasive disease [18].

Pathogenic microbes that build biofilms are potential causes of constant infections that defy the immune system and resist antimicrobial treatment, partly due to the matrix-inherent limited exposure of the cells within a biofilm to these types of immunological and medical arsenals [19–22]. Other mechanisms of biofilm resistance have been suggested, such as slow growth, differential regulation of the cell metabolic activity caused by nutrient limitation and stress conditions, and cell density [23–25]. In addition, the ability to adhere, as a unique prerequisite to form a biofilm, is a fast process, which makes the prevention of biofilm development difficult with the current antimicrobial tools and strategies.

Biofilms are diverse communities and therefore vary depending on the microbe, the surface, and the colonization niche [5, 26–30]. This paper gives an update on the recent efforts made in establishing alternative means of eradication and also prevention of *Candida* spp. biofilms, by developing new models of biofilm formation in flow conditions, as

well as high-throughput rapid screening analyses *in vitro*. Newly developed *in vivo* models anticipate a shift of interest towards mixed fungal-bacterial biofilms and their role in pathogenesis in mucosal infections in particular. Keeping in mind that there is no unique model representative of all biofilms, it remains quite a challenge to tackle biofilm inhibition. One of the most attractive perspectives is the development of antimicrobe materials, and the latest findings are presented here.

2. *Candida* Biofilm Models

2.1. Models In Vitro. Biofilm formation is a multistep growth behaviour that results from complex physical, chemical, and biological processes [31, 32]. Because of the versatility of the milieu in which *Candida* biofilms can develop in the human host, from the oral cavity contributing to dental plaque formation to the blood stream in intravenous catheters and the urinary tract, it seemed necessary to reproduce *in vitro* as many conditions as possible to establish common and specific characteristics of *Candida* biofilm formation. In that respect, a multitude of *in vitro* studies has been described that relates to the impact of different types of substrate, nutritional supplies, in flow or static conditions, on adhesion and biofilm properties of several *Candida* species, and recent findings are presented next. An overview of the *in vitro* models available to study *Candida* biofilms is provided in Table 1.

2.1.1. Candida Species and Substrates Specificities. While biofilm formation is a general characteristic of many microbes, biofilm features such as architecture, matrix composition, and resistance to antifungal drugs are species and substrate dependent. And examples that demonstrate variation in biofilm ability and structure are numerous. Some studies are discussed below, and in particular studies related to *Candida* biofilms formed on dental materials. Interest has indeed grown in investigating the role of *Candida* species and the effect of the type of material in the development of denture stomatitis [48]. For example, in a comparative study, cell counts analyses showed that saliva-coated discs harboured less *C. glabrata* cells than untreated discs, while the number of *C. albicans* cells was not affected by the saliva coating [33]. However, both species adhered better on hydroxyapatite (HA) surface than on two other types of dental material, polymethylmetacrylate and soft denture liner. Surprisingly, dual species experiments showed that *C. glabrata* displayed higher cell counts when grown in the presence of *C. albicans* than when grown alone. In contrast, hyphal development by *C. albicans* seemed to be reduced in the presence of *C. glabrata* in most of the conditions tested. These data may help understand the impact that *Candida* species may have on each other, as mixed species communities are being identified in clinical samples [49]. In another case study, using discs as support for biofilm formation *in vitro*, HA substrate appeared to be less prone to *Candida* adherence than acrylic denture, porcelain, or polystyrene when not coated with saliva [34]. In addition, the effect of serum and similar materials on biofilm development of *C. albicans* clinical isolates was also evaluated *in vitro* [35]. Disc coupons made of polycarbonate, polystyrene, stainless steel, polytetrafluoroethylene (also known as Teflon), polyvinyl chloride (PVC), or HA were used in a high throughput assay. For all surfaces tested, the presence of serum increased biofilm formation. However, in absence of serum, Teflon supported higher biofilm production than any other material, likely due to its high roughness and hydrophobicity properties.

The differential ability to form biofilm of 84 strains from several *Candida* species, including *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis,* was assessed on clinical materials, such as Teflon and PVC. All species, with the exception of *Candida glabrata,* favoured Teflon [50]. In this study, *C. glabrata* together with *C. krusei* strains were not highly proficient in forming dense biofilms, as quantified by colony-forming units. Moreover, *C. parapsilosis* strains showed the least uniformity in the ability to form biofilm, followed by *C. tropicalis* and *C. albicans*. While some variability in the ability to form biofilms between strains of *C. albicans* has been documented *in vitro*, a study by MacCallum et al. [51] revealed that biofilm formation *in vitro* did not significantly vary between strains of the four major clades of *C. albicans*, classified according to single-nucleotide polymorphisms determinations and analysis of DNA repeat sequences [52]. However, high variation in the ability to form biofilm among strains of *C. parapsilosis* and less extensive biofilm formation by *C. glabrata* specimens has been illustrated in a few studies by crystal violet staining

and confocal laser scanning microscopy [36–38]. Strain-dependent variation in biofilm formation was also observed among isolates of two genetically nonidentical classes of *C. parapsilosis*, namely, *C. orthopsilosis* and *C. metapsilosis* [39, 53]. All three species could form biofilms, but metabolic activity of biofilm cells differed between strains of the same species. However, conflicting data with different isolates reported the inability of *C. orthopsilosis* and *C. metapsilosis* to form biofilm in polystyrene 96-well plate assay *in vitro* [54, 55]. Biofilm formation among *C. parapsilosis sensu strictu* strains was also found to vary according to the geographical regions and the body sites from which the isolates came from [56]. Isolates from blood and cerebrospinal fluid seemed more prone to form biofilms than isolates from nails, catheters, and mucosa. Overall, these data suggest a high variability in biofilm ability of strains of *C. parapsilosis* and related species, perhaps due to inadequate models or to an intrinsic poor ability to establish the biofilm growth by these species.

In a Calgary biofilm model adapted to *Candida* spp., *C. krusei* developed the largest biofilm mass in comparison to *C. albicans*, *C. glabrata*, *C. dubliensis,* and *C. tropicalis* [41]. This model, allowing 80 biofilms to be formed at once, seemed to be very favourable to *C. krusei* biofilm development as biofilms of that species constituted of thick multilayered structures composed of pseudohyphal cells, while the other species formed sparse biofilms.

In a last example of novel *in vitro* models of biofilm formation on various soft contact lenses, analyses revealed differences in hyphal content and architecture of the fungal keratitis causative agents *Fusarium* and *C. albicans* [57]. Polymers such as balafilcon A and galyfilcon A were favourable to filamentous growth of *C. albicans*, while others such as etafilcon A and lotrafilcon A sustained biofilms formed mainly of yeast cells. In addition, differences in biofilm formation were also observed between peripheral and central regions of the lenses, with dense biofilms formed preferentially in the centres of the lenses. Although a direct relationship between the lens ionic charge and water content and the ability of fungi to form biofilm could not be established, these data confirm previous findings that irregular surface texture of materials affect both cellular morphology and biofilm mass [58].

2.1.2. Synthetic Media and Flow Systems Mimicking In Vivo Conditions. The physiological specificity of infection sites is also an important factor, and efforts have been made to reproduce some major environmental cues *in vitro*, such as mimicking the blood flow or the urine. Biofilms grown in synthetic urine medium were comparable to those grown in the commonly used cell culture RPMI medium [59]. And time course studies revealed that the development of both types of biofilm followed a similar pattern, with an initial adherence phase, followed by growth, proliferation, and maturation. The biofilms differed slightly in their architecture, as biofilms grown in synthetic urine medium seemed to be less complex and less dense, with a larger proportion of yeast cells rather than elongated cells. Increased nutritional supply promoted biofilm formation in

another model of artificial urine medium, highlighting once again the importance of reproducing as closely as possible the physiological conditions to gain relevant information [60]. *C. tropicalis* biofilms were also characterized in artificial urine medium, on urinary catheters in a flow model [61]. Cells were able to colonize the catheters in the presence of the artificial urine medium and to detach from these silicone catheters, illustrating their capacity to colonize distal sites.

Biofilms grown in static conditions have been predominantly studied, in comparison to flow-based systems, due to a low cost, a rapid processing of large number of samples, and limited technical requirements. However, in order to maintain their niches in dynamic environments, biofilms *in vivo* endure shear forces generated by the constant flow of physiological fluids [62]. Gene expression analyses revealed only a marginal difference between biofilms grown in static conditions, such as microtiter plates or serum-treated catheters, and those grown in a flow system in microfermentors [44]. Interestingly, the biofilm transcriptomes were not strongly affected by factors such as nutrient flow and aerobiosis, in contrast to the gene expression of free-living cells. However, a few studies indicated that biofilms grown under flow conditions, in CDC reactors or modified Robbins devices, contain more extracellular matrix and more biomass [10, 43, 45]. Mature biofilms formed in a flow of replenishing nutrients consist of a dense network of yeast cells, pseudohyphae, and hyphal cells. In a simple flow model, using a silicone strip placed in a conical tube, *C. albicans* biofilms grew thicker than biofilms grown in static conditions, and grew faster as an 8-hour-grown flow biofilm had similar biomass as a 24-hour-grown static biofilm [46]. The authors speculated that uninterrupted food supply prohibited adverse conditions, such as nutrient starvation and toxic accumulation, and hence promoted rapid cell proliferation. A parallel study, using a rotating disc system (RDS) to impose shear forces at physiological levels to biofilms developed on catheter pieces, illustrated similar results as biofilms under shear stress grew thinner but denser than those in no-flow conditions [47]. In the RDS model, less cells adhered at first, but by 24 h biofilms displayed similar metabolic activity and dry weight as those obtained in the static model. Suggestions that explained the increased growth rate in shear conditions included an increased rate of maturation in these conditions and a natural selection of more robust cells capable of withstanding the fluid friction by growing faster.

2.1.3. High-Throughput Biofilm Models. Another important aspect of *in vitro* biofilm modelling is the development of high-throughput systems of particular interest in the large-scale screening of antibiofilm molecules. Most studies so far have made use of the 96-well microtiter plate assay [40]. In this model, biofilms are formed directly on the bottom of the wells, and the quantification method is based on the ability of sessile living cells to reduce tetrazolium salt (XTT) to water-soluble orange formazan compounds. In an effort to upscale biofilm production, a *C. albicans* biofilm chip system (*Ca*BChip) has recently been developed by Srinivasan et al. [42]. The high-density microarray platform

is composed of more than 700 independent and uniform nanobiofilms encapsulated in a collagen matrix and provides the first miniature biofilm model for *C. albicans*. Despite the several-thousand-fold miniaturization, the biofilms formed on the chip displayed phenotypic characteristics, such as a multilayer of yeast, pseudohyphae and hyphal cells, and a high level of antifungal drug resistance, consistent with those of biofilms formed by standard methods. However, echinocandins were not proficient to eradicate biofilm in this system, potentially due to their binding to the collagen matrix. In a second generation of the biofilm chip, other nonprotein matrices will be investigated. While this system steps-up the number of biofilms that can be produced at once in static conditions, the next step may be to develop high-throughput flow biofilm systems adapted to *Candida* spp. Such a tool has been described based on a device comprised of microfluidic channels that provide fluid flow to 96 individual bacterial biofilms [63]. The effects of antimicrobial agents on the biofilms were rapidly screened, and viability was quantified by fluorescence measurements. These high-throughput techniques will certainly contribute greatly to the discovery of novel antibiofilm molecules.

2.2. In Vivo Models of Candida Biofilms

2.2.1. Biofilm Models on Inert Substrates. *In vivo* models are undisputedly required to appreciate the hostile environment that conditions biofilm formation (Table 2). A few *Candida* biofilm models, mostly associated to catheter infections, have been developed in several rodents, giving insights on the *in vivo* biofilm structure and the efficacy of various antifungal agents [70]. The catheter-related *in vivo* biofilm models resulted in biofilm formation within 24 h and consisted of complex structures of yeast and elongated cells embedded in extracellular matrix, similar to those observed in *in vitro* model systems [8]. While susceptibility to azoles was reduced in these models, liposomal amphotericin B lock therapy and treatment with caspofungin or chitosan proved to be efficient against *in vivo* biofilms [64, 65, 71]. Central venous catheter models (CVCs) are also useful for the investigation of the kinetics and occurrence of dissemination of the microorganisms to other organs, demonstrated by colonisation by *C. albicans* of the kidneys in the rat model [8]. In addition, the development of a CVC model in mice will allow comparison to other modes of infection, in particular to the commonly used disseminated candidiasis by tail vein infection. A murine model for catheter-associated candiduria was recently developed and illustrated the role of *Candida* biofilms in the persistence of the urinary tract infection [66]. It also outlined differences between murine and human catheter-related candiduria in terms of bladder inflammation and fungal burden in the urine. In another catheter-related *Candida*-associated infection model, we developed a subcutaneous foreign body system suitable for *C. albicans* [9]. This model, of nondisseminated nature, allowed the study of biofilm development for long periods of time (Figure 1) but required the use of immunosuppression treatment of the animals due to the high inflammatory response associated with implant of foreign devices. However, efficacy of the

TABLE 2: *Candida* biofilms *in vivo* models.

Models *in vivo*	Device	Developed in
	(i) Central venous system	Rat [8], rabbit [64], mouse [65]
Catheter-associated models	(ii) Candiduria model	Mouse [66]
	(iii) Subcutaneous foreign body system (biofilms developed after 2 to 6 days in infected implated catheter fragments)	Rat (immunosuppressed before and during biofilm development) [9]
Candida-associated denture stomatitis models	(i) Acrylic denture material attached to the hard palate (biofilms developed between the hard palate and the device)	Rat (immunosuppressed on day of infection) [67]
	(ii) Custom fitted denture system (cast fabrication of a fixed part that is attached to the posterior palate and a removable part fitted to the anterior palate)	Rat [68]
Mucosal model of oropharyngeal candidiasis	Biofilms developed on the tongue after infection by swabbing and drinking water contaminated with *Candida* cells	Mouse (immunosuppressed on day of infection) [12]
Vaginitis model	*In vivo* and *ex vivo* models	Mouse (treated with estradiol prior infection) [69]
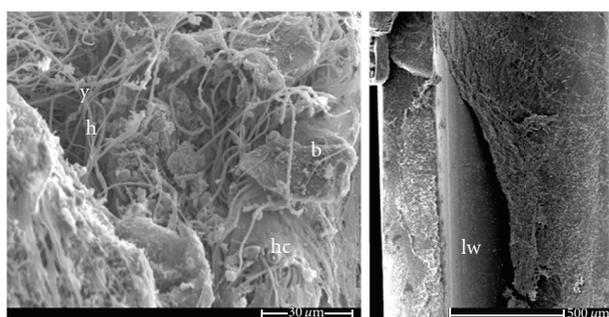
FIGURE 1: Scanning electron microscopy images of wild type *Candida albicans* biofilms developed *in vivo* in the denture model (left panel) and in the subcutaneous model (right panel). Elements such as hyphal cells (h), yeast cells (y), bacterial cells (b), host cells (hc) and catheter lumen wall (lw) are highlighted. Images were adapted from the work of Nett et al. [67], and S. Kucharíková and P. Van Dijck (MCB Laboratory, VIB, K.U. Leuven, unpublished data), respectively.

echinocandin anidulafungin, by intraperitoneal injections, was demonstrated against *C. albicans* biofilm in this *in vivo* system [72]. These *in vivo* models are all suited for further study of novel antifungal therapies and for the use of novel material technologies, including less adherent surfaces and material coating with fixed or releasing antifungal agents (see the next section).

A relatively cost- and time-effective *Candida* biofilm model on acrylic denture material, which does not require the *ex vivo* mold process, was illustrated recently [67]. In this rat model, biofilms developed between the hard palate and the denture material, following *Candida* inoculation in that 1 mm space (Figure 1). Fungal invasion of the palate and the tongue and neutrophils infiltration also occurred, indicating that the model was consistent with that of acute human denture stomatitis. Interestingly, the denture model offers the

possibility to study mixed biofilm structure and behaviour in response to antimicrobial treatments, as the biofilms were composed of both bacterial and fungal cells. Finally, biofilms developed on the denture model were inherently resistant to fluconazole, in accordance with previous findings [8, 72], but also to the echinocandin micafungin, in contrast with previous investigations performed in a different model [73]. A plausible explanation suggested by the authors is that the mixed biofilm nature combined with the specific site of infection, the oral cavity, is the cause of that antifungal resistance. An alternative rat model of *Candida*-associated denture stomatitis recently described differs by the use of animal-fitted devices [68]. In this system, a removable part of the device makes the replacement of the infected device a relatively easy step. These models promise to deliver an alternative mean of testing novel antibiofilm molecules.

2.2.2. Biofilm Models on Biotic Surfaces. Tools and models to study biofilm formation developed on implanted materials are numerous and indicative of the increased medicinal use of such implants. Biofilms formed on live surfaces are much less characterized, yet they are recognized as causing or aggravating numerous chronic diseases [74]. Besides dental plaques, few reports have investigated biofilm development in clinical samples. Biotic biofilms are poorly understood as tissue samples are sparse and not easily accessible. The oral cavity is an accessible *in vivo* model for studying protein-surface interactions and has been well characterized for bacterial biofilm [75]. A mucosal model of oropharyngeal candidiasis was recently proposed to characterise *C. albicans* mucosal biofilms *in situ* in mice [12]. Keratin, originating from desquamating epithelial cells, constituted a large proportion of the biofilm matrix. First evidence was given that epithelial cells, neutrophils, and commensal oral bacteria co-exist within the fungal mucosal biofilm developed on mouse tongue. Bacteria were mostly found on the apical part of the biofilm, and very few were seen to

invade the tongue epithelium layer. This model highlights the complexity of mucosal biofilms, as host elements and commensal organisms contribute in an active or passive manner to the structure of the biofilms.

C. albicans can also form biofilms on the vaginal mucosa, illustrated by two *in vivo* and *ex vivo* models in immunocompetent estradiol-treated mice [69]. *C. albicans* vaginal biofilms consisted of yeast and hyphal cells embedded in extracellular material, illustrated by ConA staining of the interspersed matrix. In the *ex vivo* model using vaginal explants, no exogenous nutrients were provided, yet biofilms were formed most likely by scavenging host nutrients.

Host-pathogen interactions in biofilm settings have not yet been elucidated, but comparison between these models promises to identify model-specific fungal and host elements.

2.3. Mixed Species Candida Biofilms. The relative contribution and the role of bacteria-*Candida* interactions in the pathogenesis of mucosal infections are yet to be established. However, there is clear evidence that multimicrobial interactions have a central role in the context of human disease [76]. For example, microbial diversity was illustrated in a biofilm-related infection of the urinary tract [77]. Out of 535 clinical samples of urinary catheters, *Candida* spp. were identified among the 39 different microbial taxa isolated. Single-species samples represented 12.5% only. *C. albicans* was isolated in 141 samples, and other *Candida* species were present in other 82 samples. Biofilm formation ability of each isolated strain was quantified *in vitro*, yet not in an artificial urine medium, and cut-off values were used to define no, weak, intermediate, and strong biofilm producers. *C. tropicalis* isolates were the strongest biofilm producers among the *Candida* species. Certain species of bacteria did not show biofilm formation ability in this study. These data illustrates the fact that, in multispecies biofilms, some have a great potential to cause biofilm-based infections, while others may be more passive members of the structured community. Commensalism, mutual cooperation, and antagonism make the interactions within mixed biofilms complex [78, 79]. A summary of bacteria-*Candida* interactions and their effect on fungal development is provided in Table 3. Bacteria can interact with *C. albicans* cells within mixed biofilms, and in particular with hyphal cells. The methicillin-resistant Gram-positive *Staphylococcus aureus* had the highest hyphal association, in comparison to *S. epidermidis*, *Strepococcus pyogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis,* and *Escherichia coli* in decreasing order, respectively [80]. However, interaction between *S. aureus* and *C. albicans* did not result in reduced or altered biofilm viability. In another study, addition of bacteria to preformed *Candida* biofilms *in vitro* had an antagonistic effect on biofilm cell mass, often in a cell-density-dependent manner [81]. With all inoculums tested, *P. aeruginosa* reduced significantly the fungal biofilm mass when added during the first few hours of biofilm development. In a different experimental assay, preformed bacterial biofilms significantly reduced adhesion and biofilm growth of *C. albicans* [82]. Moreover, simultaneous addition of bacteria and *C. albicans* cells showed that in all cases fungal adhesion was decreased, whereas bacterial biomasses were not affected.

Hypotheses of synergistic relationships between microbes have been suggested, and in particular within mixed biofilm communities [83]. For example, bacterial adhesion was observed on the tongue mucosa of *C. albicans*-infected animals but not of noninfected animals, in a mucosal model of oropharyngeal candidiasis [12]. Synergistic cooperation can also perturb susceptibility to antimicrobial treatment. For example, *S. aureus* resistance to vancomycin was enhanced in mixed biofilms with viable *C. albicans* cells, whereas susceptibility of the fungal cells to the antifungal amphotericin B was not altered [84]. Binding of the fungus to the bacterial cells occurs via the *Candida*-specific adhesin proteins, including Als3, Eap1, and Hwp1, as demonstrated by heterologous expression of these cell wall proteins in the model yeast *Saccharomyces cerevisiae* [85]. The role of adhesins in single- and multispecies biofilm formation is not discussed here but can be found in previous reports [86–88].

3. Antibiofilm Strategies: Research and Development

The current therapies against fungal diseases [96], employing one of the five classes of antifungals (polyenes, pyrimidine analogues, allylamines, azoles, and echinocandins) administrated orally or intravenously, are not discussed in this paper. Each antifungal compound has advantages and limitations related to its spectrum of activity and mode of action. The susceptibility of *Candida* biofilms to the current therapeutic agents remains low, with the exception of the echinocandins [97, 98]. However, these compounds have been employed in different approaches, such as lock therapy or material coating as releasing agent. These alternative methods and their perspective of usage are discussed below.

3.1. Lock Therapy Approach and Prevention against Catheter-Related Blood Stream Infections. Nosocomial infections associated with medical devices represent a large proportion of all cases of hospital-acquired infections [99]. In particular, insertion of any vascular catheter can result in a catheter-related infection, as microorganisms can colonise catheter external and internal surfaces. Some of the favourite niches of colonisation of *Candida* spp. include indeed vascular and urinary catheters and ventricular assist devices, which can be accompanied with high mortality rates [100]. Adherence to the catheter surface, facilitated by host proteins such as fibronectin and fibrinogen, can then lead to biofilm formation [101]. The antimycotic lock therapy approach is currently recommended and employed in treating catheter-related bloodstream infections (CRBSI), in particular for long-term catheters, according to the Infectious Diseases Society of America guidelines [102]. However, lock therapeutic treatment is pathogen– specific as catheter removal is recommended for CRBSI caused by *Candida* species and *Staphylococcus aureus*. The lock therapy involves the instillation of high doses of an antimicrobial agent (from 100- to 1000-fold the minimal inhibitory concentration,

TABLE 3: Interspecies relationship with *Candida* spp. growth and biofilm development.

Bacterial species	Effect on *C. albicans* hyphal growth	Effect on *Candida* biofilm
Staphylococcus aureus (+)	Associates to hyphal cells (56%) [80]	No antagonistic effect in dual biofilms with *C. albicans* (BacLight LIVE/DEAD assay) [80]
Staphylococcus epidermidis (+)	Associates to hyphal cells (25%) [80]	Reduced adhesion and biofilm formation by a glycocalyx producer strain (CFU counts) [82]
Streptococcus pyogenes (+)	Associates to hyphal cells (25%) [80]	
Streptococcus mutans and *Streptococcus intermedius*	*S. mutans* inhibits hyphal formation [89, 90]	No significant effect on biofilm viability at densities ranging from $6.25 \cdot 10^5$ to $1 \cdot 10^7$ cells/mL (bacteria added to preformed 3-hour-old biofilms; polystyrene *in vitro* model; CFUs analyses) [81]
Streptococcus gordonii (+)	Stimulates hyphal growth [91]	Promotes mixed biofilms with *C. albicans* [91]
Pseudomonas aeruginosa (−)	(i) Associates to hyphal cells (17%) [80] (ii) Reduced hyphal growth in *C. albicans-P. aeruginosa* dual biofilms [81] (iii) Binds hyphae and kill *C. albicans* [92]	(i) Reduced adhesion and biofilm formation by a nonglycocalyx producer strain (CFU counts) [82] (ii) Reduction of biofilm mass ranging from 40% to 80% in a density-dependent manner [90] (iii) Mutual biofilm inhibition between *Pa* and *C. albicans*, *C. krusei* and *C. glabrata*; decreased biofilm formation of *C. parapsilosis* and *C. tropicalis* in presence of *Pa*; increased CFUs of *Pa* in presence of *C. tropicalis* [93]
Escherichia coli (−)	Associates to hyphal cells (5.7%) [80]	(i) Reduction of biofilm mass ranging from 50% to 80% [81] (ii) Mutual decrease in biofilm cell mass between *Ec* and *C. albicans*; inhibition of biofilm development by *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *C. dubliniensis*; increased *Ec* cell numbers within *C. tropicalis* and *C. dubliniensis* biofilms [94]
Lactobacillus acidophilus		Inhibition of viable biofilm cell mass by 40% [81]
Bacillus subtilis	Associates to hyphal cells (2.5%) [80]	
Actinomyces israelii (+)		Some inhibition of biofilm at high densities [81]
Prevotella nigrescens and *Porphyromonas gingivalis*	Inhibition of *C. albicans* hyphal development [95]	Reduction of *C. albicans* biofilms, only at high densities [81]
Klebsiella pneumoniae, Serratia marcescens, and *Enterobacter cloacae*		Decreased biofilm formation (CFU counts) [82]

(MIC)) directly into the catheter in order to "lock" it for a certain period of time (from hours to days) [103].

Few reports are currently available on the usage of antifungal lock solutions in clinical practice, but they seem to indicate the curative effect of this kind of treatment [104, 105]. *In vitro* studies are more prevalent at the moment and seem to also favour the use of antifungal lock therapy to eliminate *Candida* spp. biofilms, and in particular with the usage of echinocandins [106]. For example, biofilm metabolic activity formed on silicone by *C. albicans* and *C. glabrata* could be effectively reduced by a 12 h lock treatment with micafungin (at 100–500x MIC), which was shown to persist for up to 3 days [107]. Caspofungin had an intermediate effectiveness in the same study, as its activity did not persist as long against *C. glabrata* biofilms. While these results are promising for potential use of the lock technique to treat infected catheters, 100% biofilm inhibition could not be achieved. Sterilization of catheters was obtained *in vivo* by lock treatment with amphotericin B lipid complex (ABLC) in a rabbit model of catheter-

associated *C. albicans* biofilm [108]. However, in this study, the lock solution was administrated a few hours a day for a prolonged period of time (7 days). Synergistic antibiofilm combinations, used as lock solutions, between classical antimicrobial agents and other compounds such as the mucolytic agent N-acetylcysteine, ethanol, or the chelating agent EDTA, are also effective against *S. epidermidis* and *C. albicans* individual and mixed biofilms [109]. In a similar approach, recent results suggest that the combination of antibacterial agents with Gram-positive activity, including doxycycline and tigecycline, with known antifungals, such as AMB, caspofungin, and fluconazole, can be useful for the treatment of *C. albicans* biofilms [110, 111].

The prevention of CRBSI has also been the focus of research and randomized controlled trials [112]. In a systematic assessment, Hockenhull et al. [113] showed the clinical effectiveness of CVCs treated with anti-infective agents (AI-CVC) in preventing CRBSI. While trials are still required to determine the most cost and clinical-effective anti-infective product, the routine usage of AI-CVC

will often be limited if appropriate use of other practical care behaviour is not employed in intensive care units. Antifungal impregnated CVCs have been tested in animal models. The echinocandin caspofungin was employed to prevent *C. albicans* biofilm formation in a biofilm model in mice. *C. albicans* biofilm formation was greatly reduced in CVCs that had been pretreated for 24 h with high doses of caspofungin. The dissemination to the kidneys was also reduced by such therapy [65]. Similarly, the use of chitosan, a polymer isolated from crustacean exoskeletons, as a pretreatment of catheters to prevent *C. albicans* biofilm formation was validated in a CVC biofilm *in vivo* model [71]. The use of lock technique or preventive impregnation of antifungals in combating catheter-associated infection seems promising, but not yet convincing on a cost effective point of view as huge doses are still needed to eradicate fungal growth.

3.2. Material Coating and Novel Antibiofilm Surfaces. A developing field of research focuses on the usage of modified materials or coated surfaces to prevent adherence and biofilm development. Implant materials are prone to biofilm formation affecting health in general and duration of the implant in particular. Surface characteristics, such as surface roughness, surface free energy, and chemistry, can influence the type and the feature of the biofilms [114, 115]. For example, *C. albicans* adhesion is enhanced if the roughness of the denture materials is increased [116]. It is nowadays conceivable that coatings may be engineered to promote selective adhesion, with possible attachment to cell tissue (for implant in bone contact) but not to microbes. They may also address the second phase of biofilm development involving quorum sensing, by inhibiting cell-cell communication signals [117, 118]. Biomaterial modifications as a way to prevent biofilm development have been the focus of intense research, in particular in the field of bacterial biofilms [119], but the latest findings on their impact on *Candida* biofilms are discussed next.

3.2.1. Surface Modifications. Surface properties of medical devices constitute a major factor contributing not only to the stability in the body but also to their performance and lifetime *in vivo* and their colonization by microorganisms. In that matter, albumin adhesion is beneficial since it has been shown to prevent binding of microorganisms, while fibrinogen has the opposite effect [120]. Chemical grafting of polyethylene and polypropylene surfaces, functionalized with cyclodextrins, yielded a change in protein adsorption profile of these polymers, by promoting adsorption of albumin and reducing adhesion of fibrinogen to the material surface [121]. In addition, these modified substrates incorporated well the antifungal agent miconazole, leading to reduced biofilm formation by *C. albicans in vitro*. Modified polyethylene and silicone rubbers proved to be very efficient in inhibiting *C. albicans* biofilm formation *in vitro* [122]. These cytocompatible materials were also capable of releasing for several hours considerable amount of an anionic antimicrobial drug, nalidixic acid, suggesting their use as drug-eluting systems.

Modifications of polyurethanes dental biomaterials by addition of surface-modifying end groups were successfully employed to manage *C. albicans* biofilm formation [123]. In addition, correlation between contact angle and biofilm formation was surface dependent. Increased hydrophobicity resulted in increased metabolic activity of the biofilms grown on polyetherurethane, while they inversely correlated for biofilms formed on polycarbonate surfaces. Addition of 6% polyethylene oxide to Elastane 80A showed to be the best combination as no biofilm could be observed on that surface. Biofilms on voice prostheses consist of mixed populations that can include *C. albicans*. Modification of the silicone surface of the prostheses has been employed to limit *C. albicans* colonization, as opposed to incorporation of antimicrobial agents in order to avoid the occurrence of resistance [124]. Silicone disks grafted with C1 and C8 alkyl side chains reduced adherence and biofilm formation of *C. albicans* by up to 92%. Longer side chains did not show as good results, and combinations of quaternizing agents did not work synergistically either. Similarly, grafting of cationic peptides, such as the salivary peptide Hst5 and synthetic variants, onto silicone rubber, inhibited biofilm formation by up to 93%, in a peptide-dependent manner [125].

3.2.2. Surface Coatings. Fungicidal or fungistatic materials have been employed to fabricate or coat the surfaces of medical devices and have a great potential in reducing or eliminating the incidence of biofilm-related infections. Dental resin material coated with thin-film polymer formulations containing the polyene antifungals nystatin, amphotericin B, or the antiseptic agent chlorhexidine, were used in *C. albicans* biofilm assays [126]. Biofilm reduction was the greatest on chlorhexidine containing polymers, while the other formulations were much less efficient. Similarly, multilayered polyelectrolyte thin films containing an antifungal β-peptide incorporated within the layers of the films inhibited the growth (and hyphal formation) of *C. albicans* by 74% after 2 h of contact [127].

The polysaccharide dextran is widely used in medicine and is also one of the main components of dental plaque. Cross-linked dextran disks soaked with amphotericin B solutions, described as amphogel, kills fungi within 2 hours of contact and can be reused for almost 2 months without losing its efficacy against *C. albicans* [128]. This antifungal material is biocompatible and could be used to coat medical devices to prevent microbial attachment. It was recently used for local antifungal therapy in the form of injectable cross-linking hydrogels [129]. Nitric oxide can antagonise cell proliferation by signalling rather than by toxic effect. It regulates bacterial biofilm dispersal and has also been employed in releasing xerogel to attenuate *C. albicans* adherence and biofilm formation [130]. The nitric-oxide-based method is still at the experimental level, due to poor water solubility and stability.

Coating of medical material surfaces has been employed and tested with several types of coating molecules, including the naturally occurring polymer chitosan and antimicrobial peptides such as Histatin 5 (Hst5). Surfaces coated with the polymer reduced the viable cell number in biofilms by

more than 95%, in the case of *C. albicans* and also for many bacteria such as *Staphylococcus aureus* [131]. Chitosan, which is proficient against a wide range of pathogenic microbes, disrupts cell membranes as cells settle on the surface. The use of such polymer offers a biocompatible tool for further coating design of medical devices. Acrylic disks precoated with Hst5 prove to be efficient in inhibiting biofilm formation of *C. albicans*, especially in the later stage of development, while biofilm sensitivity to the antimicrobial peptide was the same as the one of free-living cells [132]. The utility and potential of selected peptides, as therapeutic molecules, including the β-glucan synthesis inhibitors, the histidine-rich peptides, and the LL-37 cathelicidin family are being determined and could be used as coating compounds against adherence and biofilm formation [133, 134].

The possible applications of biomaterial modification remain to be clearly established and approved. Shift from a commensal bacterial biofilm to a more pathogenic biofilm involving *Candida* spp. in the oral cavity for instance is believed to be more influenced by mucosal inflammation and the general well-being of the host than on the nature and surface properties of the material itself [135]. However, development of materials that can fully abolish microbial adherence is a promising perspective against biofilm formation. The discrepancy between antimicrobial coatings killing the biofilm-proficient organisms and antimicrobial releasing coatings to prevent biofilm formation is a current issue.

3.3. Quorum Sensing Molecules and Natural Byproducts.

Adhesion and biofilm formation by *C. albicans* cells can be modulated by physical and chemical signals from the oral bacterium *Streptococcus gordonii* [91]. Indeed, most *Streptococcus* species possess the antigen I/II, a cell-wall-anchored protein receptor that mediates binding to *C. albicans*. Moreover, *C. albicans* hyphal and biofilm development are greatly enhanced by *S. gordonii*, which also relieved the fungal cells from the repressing effect of the quorum sensing molecule farnesol [91]. Farnesol, a sesquiterpene and signalling molecule produced by *C. albicans*, represses biofilm formation *in vitro* [136]. Conversely, tyrosol, a 2-(4-hydroxyphenyl) ethanol derivative of tyrosine, accelerates hypha production in the early stages of biofilm development and is secreted at least 50% more by biofilm cells than by planktonic cells [137]. Several studies demonstrated that farnesol actually increases fungal pathogenicity in animal models, potentially by interfering with normal progression of cytokine induction [138–140]. Analogs of farnesol have been identified that fail to induce pathogenicity and yet retain farnesol ability to block hyphal development [141]. While these analogs did not protect mice from candidiasis, they may be of interest in biofilm inhibition. Indeed, a number of molecules with farnesol-like activity, that can induce the shift to the yeast form of growth, have been identified in Gram-negative bacteria. For instance, the signalling molecule, homoserine lactone, produced by *Pseudomonas aeruginosa* represses *C. albicans* filamentation [142]. *P. aeruginosa* also produces several phenazines that exhibit antifungal activity against *C. albicans* [143]. Uptake of the phenazines generated reactive oxygen species production and led to fungal cell death. In mixed biofilms, binding of the toxins to the fungal cells has a negative influence on *C. albicans* growth.

In a different approach, Valle et al. [144] demonstrated that the use of nonantibiotic molecules, such as polysaccharides, produced by competitive commensal organisms can antagonize biofilm formation. A better knowledge of the microbial community behaviour and in particular of the interaction between commensal and pathogen organisms would help to combat predominance of the infectious or disease causative agents. In this scheme, natural products produced by cells within a biofilm contribute to the dynamic of the community and may play an antiadhesion role for nonwanted other microorganisms [145]. Bacterial lipopolysaccharides also modulate adhesion and biofilm ability of several *Candida* species, in an interspecies-dependent manner [146]. It is not known how mixed populations affect the host immune response in response to infection. The overall population behaviour results from a potential selective advantage to either or both species. While communication is the key, interpretation is the code. Identification and alterations of the communication signals would certainly result in a better understanding of how species coexist and permit a better control of biofilm formation [147]. Targeting quorum sensing molecules or associated signalling mechanisms is an open field of research at present, but the use of quorum quenching enzymes or quorum sensing inhibitors naturally produced by other species could help in the finding of novel antibiofilm agents [148, 149].

3.4. Host Responses to Biofilms: Perspective of Immunotherapy.

With the number of people considered at high risk for microbial infections constantly increasing, immunotherapy seems to offer a great potential despite the complexity of the interaction between the host defence system and the pathogen [150]. The ability of human pathogens, such as *Candida* spp., to cause infections depends on a constant and sometimes discontinuous battle between the pathogen and the host immune system [151]. Recognition of *Candida*-specific pathogen-associated molecular patterns (PAMPs) by dedicated pattern recognition receptors (PRRs) such as Toll-like receptors and lectins activates the innate effector cells (macrophages, dendritic cells, and neutrophils), which in turn produce a variety of soluble factors, including cytokines and chemokines [152]. However, little is yet known about the interactions between human phagocytes and *Candida* spp. biofilms, while immunotherapeutic treatment against candidiasis has been undertaken [153, 154]. Chandra et al. [155] demonstrated that adherent peripheral blood mononuclear cells (PBMCs) enhanced the ability of *C. albicans* to form biofilm. They also observed that phagocytosis of the fungal cells within a biofilm did not occur while their free-living counterparts were phagocytosed. These data defined the novel concept that *Candida* biofilms seem to have an immunosuppressive effect. Inactivated PBMCs on the other hand did not induce this enhanced growth behaviour, nor did lipopolysaccharide-activated PBMCs, suggesting that the stimulated biofilm formation resulted from (a) *Candida*-biofilm-induced secretory factor(s). Indeed, the cytokine profile of PBMCs following coculture with planktonic or

biofilm cells of *C. albicans* differed greatly, with IL-1β as the cytokine most highly overexpressed by contact with biofilms. Supporting these data, a recent study showed that phagocytes alone induced much less damage to biofilms than they did to free-living cells or to resuspended biofilm cells, which lacked the overall structure of biofilms and most of the matrix [156]. Using confocal laser scanning microscopy, Katragkou and coworkers deducted that human phagocytes looked like unstimulated cells, presenting a rounded shape when in presence of biofilms. This was also confirmed by a reduced cytokine production in a biofilm-phagocyte coculture, compared to a planktonic cells-phagocytes mix. Phagocytes appeared entrapped within the structured network of cells and matrix and were unable to internalize cells within biofilms. Moreover, *C. albicans* and *C. parapsilosis* biofilms were more susceptible to the additive effects between phagocytic host defence and the echinocandin anidulafungin than to each separately and to the combination of the azole voriconazole with phagocytes [156, 157]. These data validate the findings that echinocandins can influence host cell interactions with biofilm [158].

Pathogens have evolved many mechanisms of defence to avoid being recognized by the host environment [159–161]. *C. albicans* can evade immune attack by masking its cell wall β-glucan component, a potent pro-inflammatory signature carbohydrate, under a thick layer of mannoproteins. Clear evidence showed that exposing the β-glucans by treatment with the antifungal drug caspofungin elicited a stronger immune response [158]. These data suggest that echinocandin treatment may enhance immunity [162]. Masking of β-glucans depends on a complex network of cell wall remodelling, and targeting these regulatory processes may identify novel antifungal possibilities. For example, disruption of the MAPK pathway regulated by the extracellular signal-induced Cek1 kinase triggered a greater β-glucan exposure, which resulted in an enhanced immune response compared to the wild-type strain [163]. There are conflicting data regarding the role of the β-glucan receptor Dectin-1, expressed widely on phagocytes, in antifungal immunity [164]. However, studies suggested that Dectin-1 is required for fungal killing and induction of early inflammatory responses. These findings are of interest for biofilm recognition by the immune system, as β-1,3-glucans are found in high amounts in the extracellular matrix of *Candida* biofilms *in vitro* and *in vivo* [10, 12, 165]. Biofilms developed on soft tissue are associated with infiltration of the infected sites by neutrophils, which can then confer innate immune protection [166]. In *C. albicans*, Hyr1, encoding a GPI-anchored cell wall protein, has been shown to confer resistance to neutrophil killing *in vitro* and in the oral mucosal tissue biofilm model [12, 167]. In addition, vaccination with a recombinant Hyr1p protected mice against hematogenously disseminated candidiasis. Immunotherapeutic strategies, such as vaccination, anti-*Candida* antibodies, and cytokine therapy, are under investigation to treat *Candida* infections [168]. However, their applicability in treating biofilm-related infections is still in a preliminary state. In that framework, recent data showed that pretreatment of *C. albicans* cells with antibodies targeting the complement receptor 3-related protein led to reduced adhesion and biofilm formation *in vitro* [169]. In another study, anti-*C. albicans* antibodies from chicken egg yolk were employed as antiadherent molecules [170]. While the adherence of *C. albicans* was reduced, biofilm inhibition was only observed in absence of serum, as the activity of the antibody was very much reduced against germ tubes, of which the formation is induced in the presence of serum. *In vivo* studies of the antibody-based approach remain to be investigated in the context of biofilms.

4. Concluding Remarks

The large panel of biofilm models suitable for *Candida* research highlights the diversity of niches in which the fungus can develop ranging from biotic to abiotic surfaces. However, the role and nature of host-pathogen interactions during biofilm formation are only starting to get unveiled. The search for an antibiofilm treatment is a complex subject which requires improved knowledge of the pathogen itself, and also of the host response to adhesion and biofilm formation, the properties of the substrates onto which biofilm develop, and the interactions within microbial communities. The field of chemoinformatics may assist the development of novel antibiofilm compounds, based on already identified good candidate molecules [171]. This approach may also reveal better coating agents for material surfaces that would persist long periods of time *in vivo*. The use of natural compounds, from dietary plants or probiotics, may also be considered as they are better tolerated by humans.

Acknowledgments

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