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FUNGAL PATHOGENESIS (S DIEZMANN, SECTION EDITOR)

There Is More to Wounds than Bacteria: Fungal Biofilms in Chronic Wounds

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Abstract

Purpose of Review The management of chronic wounds, a debilitating condition, presents a considerable challenge to healthcare professionals and a significant burden on services. When these wounds are exposed to the external environment, they are susceptible to microbial infection, which further complicates their management and worsens clinical outcomes.

Recent Findings Bacteria typically exist in wounds as part of a biofilm, which is often polymicrobial in nature, alongside bacteria and fungi that are described as being more virulent and tolerant towards antimicrobials and antiseptics. Despite advancing knowledge in polymicrobial biofilm wound infections with respect to bacteria, the role of fungi is largely ignored, and their influence in chronicity and clinical management is not fully appreciated or understood.

Summary The purpose of this review is to explore the significance of fungi within chronic wound environments and, in doing so, understand the importance of interkingdom interactions in wound management.

Keywords Fungi · Wounds · Biofilm

Introduction

Chronic wounds are simply acute wounds that do not follow traditional healing processes. Although their definition is somewhat simple, the management of chronic wounds can be complex, resulting in wound management costing an estimated £8.3 billion in the UK [1]. This failure to properly heal can arise due to several factors, such as a dysregulated immune system or microbial infection [2]. Chronic wounds encompass a number of different wounds, such as diabetic foot ulcers (DFUs), venous leg ulcers, and pressure ulcers [2]. DFUs, in particular, are associated with alarmingly high mortality rates that are comparable to cancer, and in some cases, mortality rates can be higher, such as in the case of pancreatic and breast cancer [3, 4]. Although genetic and environmental factors contribute towards healing failure and mortality rates, wound infection also contributes significantly. However, administering effective treatments for these infections is accompanied by the problem of the discerning commensal, opportunist from a pathogen. Therefore, understanding who the 'usual suspects' are expected to be present as part of the skin microflora can aid in solving this puzzle.

With the ever-increasing accessibility of next-generation sequencing (NGS) methods, our ability to delve into the world of the human microbiome to identify microorganisms that are important in health and disease has increased in tandem. This has meant that our knowledge of organisms presents within the microbiome of infected wound beds has increased in recent years. The bacterial microbiome of the skin and chronic wounds is well-defined. This largely depends on the environment of the skin site. For example, *Cutibacterium* spp. was more abundant in sebaceous sites and genera such as *Staphylococcus* and *Corynebacterium* spp. dominated more moist sites such as the feet and inside of knee and elbow creases [5].

Studying the microbiome of chronic wounds has been a subject of great interest in recent years, with 32 studies being identified covering 4880 patients as identified in our own

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PubMed search in March 2022 (data not shown) using the following terms adapted from a recent microbiome meta-analysis [6]: ((wound OR diabet* foot OR diabet* foot ulcer OR DFU OR laceration OR ischemic OR neuropathic OR pressure) NOT (review)) AND ((microb* OR bacteri* OR archea* OR fung* OR mycob*) AND (structure OR composition OR diversity OR community) AND (sequencing OR metabarcoding OR amplicon OR metagenom* OR 16S OR "ITS")). In a large-scale microbiome study performed by Wolcott and colleagues (2016), the microbiome of 2963 chronic wound samples from DFUs, nonhealing surgical wounds, venous leg ulcers, and decubitus ulcers was defined [7]. Results from these analyses identified *Staphylococcus, Pseudomonas, Corynebacterium,* and *Streptococcus* as the most abundant genera in all wound types. Additionally, the top 20 most abundant genera in all samples showed comparable levels of diversity and abundance across each wound type. Based on the findings of this study, neither wound type nor patient demographics influence the microbial composition of the chronic wound microbiome. However, a feature of these analyses that is missing is a consideration of how these organisms grow together within the wound environment. Indeed, there is unequivocal evidence that the biofilm phenotype is a dominant feature of chronic wounds, with a meta-analysis identifying nearly 80% of cases with biofilms, accompanied by increased antimicrobial tolerance and virulence [8•].

Wound Biofilms: a Limited Viewpoint on Bacteria

Biofilms have been classically defined as a community of cells adhered to a surface, encased in a self-produced extracellular matrix (ECM). Microorganisms that transition from free-floating to sessile, biofilm cells exhibit increased antimicrobial tolerance and virulence compared to their planktonic counterparts [9, 10]. This biofilm-associated phenotype can often complicate the management of DFUs and chronic wounds. However, it is worth noting that biofilms in vivo, particularly those in chronic wounds, differ from the traditional 'mushroom-like' structure that was first described in *Pseudomonas* grown under continuous-flow conditions, whereby bacterial cells adhere and multiply, forming a 'stalk' that then blooms outwards, creating a shape reminiscent of a closed-cup mushroom. Despite this, they still possess the traits normally associated with biofilms, such as increased virulence and antimicrobial tolerance, which come as a result of ECM production [11]. Biofilms in chronic wounds have adhered to one another more so than they are bound to the host or one another, and this creates a smaller, aggregation of cells between 5 and 200 µm in diameter [12]. These non-surface-attached aggregates are now well described and are part of a reconceptualised thinking of the biofilm lifecycle, though notably excluding the role of fungi [13].

Early studies that focused on biofilm infections in chronic wounds gave particular attention to bacteria such as *Pseudomonas aeruginosa,* an opportunistic pathogen that is not often found as part of the healthy skin microbiome, but can be readily isolated from chronic wounds [5, 14]. These studies showed that *P. aeruginosa* also formed bacterial aggregates within the host and utilised an arsenal of virulence factors such as the LasR quorum sensing system [11]. While many studies have focused on single-species biofilms, it is important to note that the chronic wound microbiome is a complex entity; therefore, studies must give attention to multi-species biofilms. To date, several multi-species biofilm models exist to study chronic wounds, all of which favour *S. aureus* and *P. aeruginosa* (Table 1). A number of these make use of the Lubbock chronic wound biofilm (LCWM) model, which utilises Bolton broth, plasma, and lysed blood [15]. The benefit of this growth media is that biofilms formed by coagulase-positive organisms such as *S. aureus* result in the formation of aggregates that mimic the biofilm phenotype observed in vivo [15].

Previous studies have highlighted the increased recalcitrance to antimicrobials of multi-species biofilms compared to single-species biofilms. In a rat model, higher rates of infection were observed from a dual-species inoculum consisting of *S. aureus* and *P. aeruginosa* [16]. Similarly, the anaerobic bacteria, *Prevotella bivia* increases *S. aureus* pathogenicity in a murine infection model [17]. A study by Dalton and colleagues showed similar findings when using a multi-species bacterial biofilm model to interrogate inter-species interactions. These complex multi-species biofilms, containing *Enterococcus faecalis*, *Finegoldia magna*, *P. aeruginosa*, and *S. aureus*, resulted in healing impairment while remaining viable over a period of 12 days. These authors reported a decrease in wound healing and increased antimicrobial tolerance to treatments compared to single-species biofilm counterparts [18].

Although the addition of multiple species to biofilm models increases their relevance, it is important to note the utilisation of appropriate growth media and substrates to effectively mimic in vivo conditions [19]. A recent publication evaluated the role of dual-species biofilms formed by *P. aeruginosa* and *Staphylococcus aureus* in chronic wounds using a novel, layered substrate [20•]. To create this model, firstly, a subcutaneous fat layer was created and this was followed by a surrogate dermis layer, before the addition of bacterial inoculum. Following bacteria growth, this model more accurately represented the biofilm phenotype often seen in vivo and supported viable bacteria for up to 9 days, which could be used to test antimicrobial washes and dressings. Authors showed these dressings only exhibited a mild anti-biofilm effect, which agrees with clinical findings and highlights the importance of

Table 1 Multi-species wound biofilm models and their respective growth media and substrates

Authors	Bacterial/fungal spp.	Substratum	Media	Reference
Ammons, Wards and James 2011	MRSA and *Pseudomonas aeruginosa*	Porous membrane	10% brain heart infusion broth	[1]
Brown et al. 2022	*Candida albicans, Staphylococcus hominis, Peptoniphilus gorbachii, Corynebacterium simulans, Streptococcus agalactiae, Anaerococcus vaginalis, Pseudomonas aeruginosa, Staphylococcus aureus, Prevotella buccalis, Finegoldia magna,* and *Porphyromonas asaccharolytica*	Cellulose matrix	50% horse serum hydrogel	[2]
Chen et al. 2021	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Layered chronic wound biofilm model	25% tryptic soy broth and 0.5% agar	[3]
Dalton et al. 2011	*Staphylococcus aureus, Pseudomonas aeruginosa, Finegoldia magna*, and *Enterococcus faecalis*	Pipette tip	Bolton broth, 50% plasma, and 5% lysed horse blood	[4]
Di Giulio et al. 2020	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Pipette tip	Brucella broth, 0.1% agar, 50% plasma, 5% horse erythrocytes, and 2% foetal bovine serum	[5]
Gounani et al. 2020	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Cell-derived matrix	Tryptic soy broth + glucose + NaCl + foetal bovine serum	[6]
He et al. 2021	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Pipette tip	Tryptic soy broth, 50% plasma, and 5% lysed horse blood	[7]
Kim and Izadjoo 2015	*Staphylococcus aureus* and *Pseudomonas aeruginosa*	Glass cover slip	Poloxamer hydrogel	[8•]
Kucera et al. 2014	*Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtillis*, and *Enterococcus faecalis*	Pipette tip	Bolton broth, 1% gelatine, 50% plasma, and 5% freeze-thawed porcine erythrocytes	[9]
Sojka et al. 2016	*Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus agalacticae*, and *Enterococcus faecalis*	Pipette tip	Bolton broth, 1% gelatine, 50% plasma, and 5% freeze-thawed porcine erythrocytes	[10]
Su et al. 2020	MRSA and *Pseudomonas aeruginosa*	Human skin =	Tryptic soy broth	[11]
Sun et al. 2008	*Staphylococcus aureus, Pseudomonas aeruginosa*, and *Enterococcus faecalis*	Pipette tip	Bolton broth, 50% plasma, and 5% lysed horse blood	[12]
Touzel, Sutton, and Wand 2016	*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsellia pneumoniae*, and *Enterococcus faecalis*	CDC biofilm reactor (polypropylene coupons)	Bolton broth, 50% plasma, and 5% lysed horse blood	[13]
Townsend et al. 2016	*Candida albicans, Pseudomonas aeruginosa*, and *Staphylococcus aureus*	Cellulose matrix	50% horse serum hydrogel	[14]
Woods et al. 2012	*Staphylococcus aureus, Pseudomonas aeruginosa*, and *Clostridium perfringens*	Glass	Brain heart infusion broth and 5% adult bovine serum	[15]

MRSA, methicillin resistant *Staphylococcus aureus*

using appropriate substrates and conditions when studying disease biology in vitro [20•, 21]. This study makes a large step in the right direction concerning the development of accurate and reproducible chronic wound biofilms, though remarkably, these models fail to take into account the role of fungi.

Deciphering the Role of Fungi in Chronic Wounds

Despite having a general definition that covers all microorganisms within a biological niche, the word 'microbiome' is typically used to specifically reference bacteria,

and separate terms such as virome and archaeome are now employed to specify between viruses and archaea, respectively. The mycobiome, which is specific to fungi, is an under-represented and under-appreciated area of microbiome research. For example, in the gut, fungi comprise less than 1% of all microorganisms [22]. However, there are several arguments to suggest that fungi are more important than previously thought. Being more than 100 times larger than bacteria, fungi make up a considerable part of the collective microbiota biomass in addition to causing infections with high levels of morbidity and mortality.

As the first line of defence against foreign microorganisms, the skin is home to a myriad of bacteria, fungi, and viruses [23]. Using culture-dependant techniques, *Malassezia*, *Aspergillus*, and *Candida* species are recognised as some of the most cultured fungi from the skin. This has then been confirmed using NGS [24, 25]. Despite being readily identified and isolated from healthy skin, the role that fungi play in chronic wounds and how they alter regular wound healing mechanisms is still debated within the literature. The role of fungi in health and disease is subject to debate, not just in chronic wounds but also in respiratory and oral infections [26]. With this being said, Chellan and colleagues identified fungi infecting diabetic foot ulcers (DFUs) in 22% of patients, with *Candida* spp. being the most abundant [27]. More recently, culture-independent studies have identified fungi in up to 80% of samples [25]. Not only does this reinforce previous reports that culture-dependant techniques underestimate microbial colonisation and infection rates, but also indicates that previous predictions stating fungi are mere 'bystanders' to chronic wound infections are worth rethinking as they likely play a more active role in infection.

The mycobiome composition is often determined by the body site, much like its bacterial counterpart, with *Malassezia* spp., dominating most sites. However, the mycobiome of the foot and more moist areas is far more diverse and is comprised of genera such as *Candida, Aspergillus,* and *Penicillium* [23]. Findings by Kalan and co-workers (2016) showed that an increased abundance of *Ascomycota* is significantly associated with longer healing times [25]. These reports show that the mycobiome may influence wound healing in a similar way to that of the bacterial microbiota, where increased bacterial diversity is associated with longer healing times [28].

Fungi have a reputation for being opportunistic pathogens, so combining an open wound with antibiotics (given as a first-line treatment option) and fungi colonising the surrounding skin creates an ideal environment for fungal infection. Despite this obvious logic, fungi are often the subject of debate in disease biology as they are often thought to not play any active role in infection, though there is substantial evidence from the oral cavity that this is not the case [29]. An initial study in 2010 with the intention of identifying fungal infection in wounds in diabetes patients found fungal infections in nearly 30% of cases, with *Candida* spp. being the most prevalent, followed by members of the *Aspergillus* and *Trichosporon* genera [27]. For *Candida* spp., in particular, it has been shown that conditions in diabetic wounds and ulcers are optimal for inducing a shift from commensal to a pathogen. Higher blood glucose levels result in *Candida* isolates displaying a higher degree of enzyme activity, which is hypothesised to make these organisms more virulent [30]. These clinical studies highlight the importance of considering fungi in chronic wounds and should also drive consideration for antifungal therapy.

Challenges of Studying the Mycobiome

As previously stated, there are significant discrepancies between culture-dependent and independent methodologies in microorganism identification [31]. Despite the increased sensitivity that comes with molecular diagnostics such as NGS, the application of this to the mycobiome as a diagnostic method does not come without its downfalls. Challenges in mycobiome research come at many stages, from sample processing to the final data analysis stages. For example, some challenges are common across micro- and mycobiome studies, such as untimely processing or freezing of samples, and repeated freeze-thawing of samples can influence microbiota diversity [32, 33]. Additionally, harsher methods of cell lysis are required due to the robust nature of the fungal cell wall; therefore, the choice of DNA extraction method is important. For instance, chemical/enzymatic lysis can increase DNA yields while favouring the lysis of yeasts (e.g. *C. albicans*), whereas physical lysis produces higher DNA yields in filamentous fungi such as *Aspergillus fumigatus* [34, 35]. Issues can also arise in the data analysis and bioinformatic stages with incomplete fungal reference databases, leading to large numbers of unclassified operational taxonomic units (OTUs) [36]. Several other factors contribute towards the difficulties of mycobiome research. However, the minutia of details behind these which fall out with the scope of this review has been extensively reviewed by Tiew and colleagues [37].

Modelling Interkingdom Wound Biofilms

There is growing evidence to support the notion that bacteria and fungi influence one another's behaviour, which in turn can have a clinical impact [38]. However, these interactions are best studied in vitro to gain a deeper understanding of the antagonistic and synergistic virulence potential

of interkingdom interactions. Therefore, it is important to model these infections to accurately study the functionality of the chronic wound microbiome. Although a number of research groups have developed multi-species biofilm models to study microbial dynamics within chronic wounds (Table 1), these are largely devoid of fungi within their composition.

A multi-species biofilm model containing *C. albicans* and the prolific wound pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginos*a, was initially described by our group, and was one of the first to consider fungi in these models. The data showed that the presence of *C. albicans* was responsible for driving the recalcitrant nature of the biofilm, where antimicrobial treatments merely influenced biofilm composition rather than reducing overall biofilm biomass [39]. Although these data highlight the importance of fungi within wound infections, it is limited to a small number of species. We therefore enhanced the complexity of the model to a complex, 11-species interkingdom biofilm model adjacent to 3-dimensional tissue [40•]. This biofilm consortium more accurately models wound conditions by the inclusion of additional aerobic and anaerobic bacteria. Data from this study showed that although challenging wound biofilms with antiseptics can significantly reduce viable biofilm cells, a considerable portion of the biofilm remains. The residual biofilm cells that were able to persist following antiseptic exposure presented differential stimulatory effects within the epidermis model, with H_2O_2 and povidone-iodine being perhaps the more appropriate antiseptics due to their more effective immune-modulatory effects [40•]. Additionally, this study highlighted how differing atmospheric O_2 concentrations can influence the overall composition of the biofilm, with *C. albicans* dominating biofilms grown in O_2 and CO_2 conditions, whereas *Staphylococcus hominis* dominated biofilms growing in anaerobic environments. These data further stress the point made above, in that the conditions in that biofilm models are constructed should be carefully considered to effectively replicate in vivo conditions.

Fungal-Bacterial Biofilm Interactions

With a myriad of different organisms inhabiting chronic wounds, understanding the interactions between these organisms is crucial in understanding their roles in disease. There are numerous bacteria-bacteria interactions that take place within wound environments, which have been well documented elsewhere [41]. However, fungal-bacterial interactions are less well known. Many studies focusing on these interactions do so in the context of oral or respiratory disease, meaning not only should findings be translated to chronic wounds with caution, but also more studies must study interkingdom dynamics in a chronic wound model.

Candida—Staphylococcus Interactions

Interactions between fungi and bacteria found in DFU infections may drive antimicrobial tolerance and virulence [40•]. For example, a well-studied interkingdom relationship between *C. albicans* and *S. aureus,* two organisms often found in DFUs and chronic wounds are known to increase *S. aureus* tolerance to antibiotics by increasing extracellular DNA production and fungal ECM components, as well as increasing virulence by upregulating the *agr* quorum sensing pathway, resulting in increased toxin production [42–44]. This increase in tolerance and virulence is reciprocal, which has been confirmed by *S. aureus* upregulating *C. albicans* biofilm and virulence genes (Fig. 1) [45]. The presence of *C. albicans* within an interkingdom chronic wound biofilm was identified as a driving force behind antimicrobial tolerance, highlighting the importance of fungi in wound biofilms and why targeting the fungal scaffold within these biofilms may yield more positive treatment outcomes [39].

Candida—Streptococcus Interactions

Another bacterial genus commonly found in the chronic wound microbiome is *Streptococcus* [14]. *Streptococcus agalactiae* is the most abundant species of *Streptococcus* found in chronic wounds, and unlike many other members of the *Streptococcus* genus, interactions between this bacterium and *C. albicans* are still subject to debate, with some reports stating *Strep. agalactiae* inhibiting *C. albicans* hyphal formation by repressing expression of *HWP* and *EFG* [46]. However, others report that *C. albicans* increases *Strep. agalactiae* colonisation in a murine infection model, while also documenting the presence of hyphae in these infections [47]. Additional studies are in agreement that interactions between *C. albicans* and group B *Streptococci*, such as *Strep. agalactiae*, are beneficial for organisms, with close binding occurring between fungus and bacterium, which likely promotes bacterial colonisation and virulence (Fig. 1) [48]. It is important to note that the absence of hyphae may not necessarily come as a detriment to *C. albicans.* For example, as hyphae are highly immunogenic, maintaining a budding yeast phenotype may help promote chronic colonisation in the wound bed. This is in line with a recent study whereby *P. aeruginosa* wound isolates were defective in virulence functions, suggesting such factors are not required for microbial fitness in wounds [49].

Candida—Pseudomonas Interactions

An interkingdom consortium that is also commonplace within wound environments is that of *C. albicans* and *P. aeruginosa* [50]. The interactions that occur between these two organisms are more complex than that of

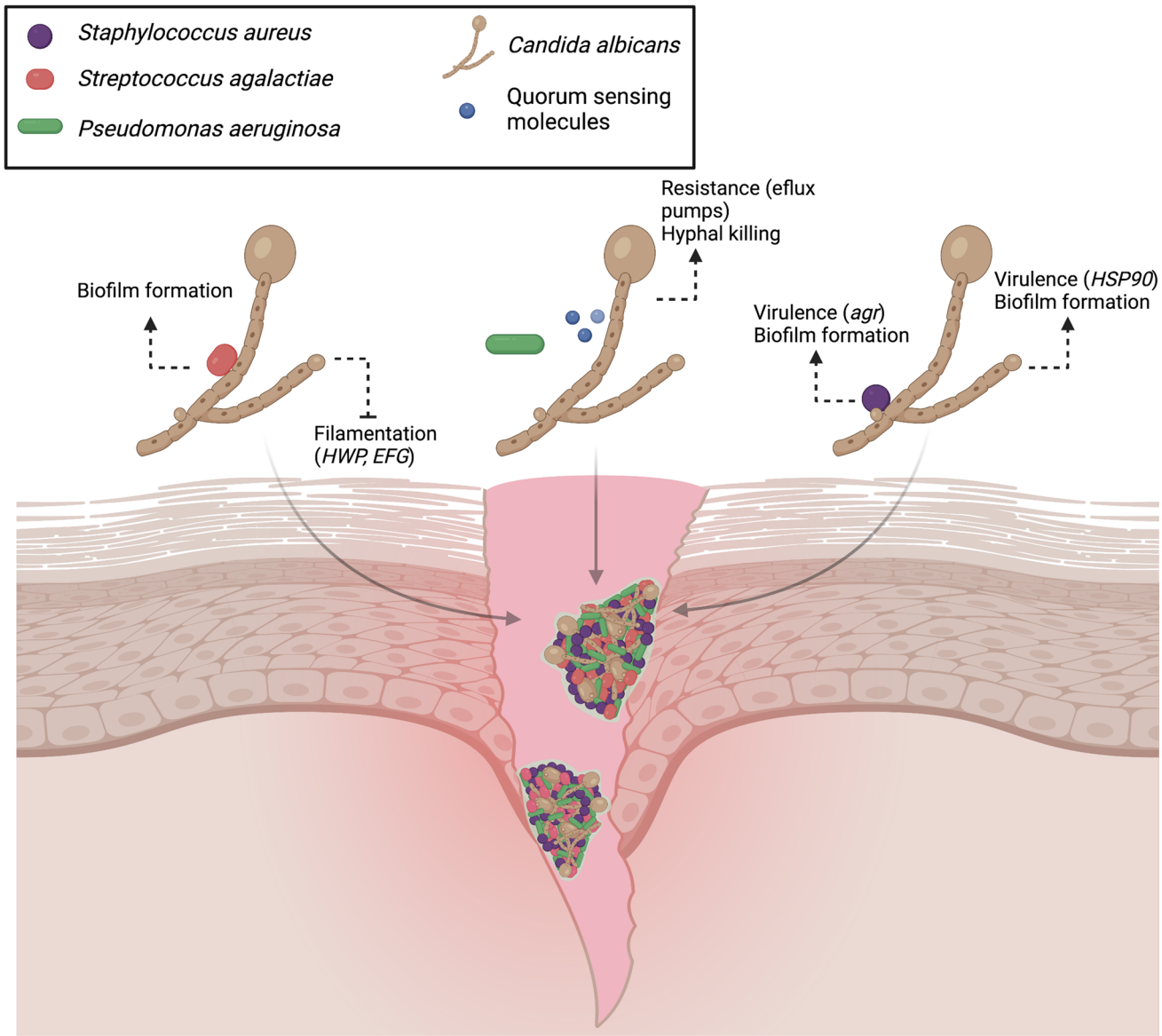

Fig. 1 Interkingdom biofilm interactions. Interactions between *C. albicans* and bacteria of interest have been summarised to highlight their implications. *S. aureus and C. albicans* possess a synergistic interaction with the fungus, driving bacterial tolerance and biofilm formation, while *S. aureus* returns the favour by increasing fungal biofilm formation and virulence. However, *C. albicans* interactions with *P. aeruginosa* and *Strep. agalactiae* are more complex, with some beneficial and some antagonistic interactions occurring. For example, *P. aeruginosa* kills hyphal cells while quorum-sensing molecules drive efflux pump activity. Similarly, on one hand, *Strep. agalactiae* represses filamentation by downregulating HWP and EFG, whereas on the other, it binds directly to *C. albicans* hyphae to promote its own growth

Staphylococcus, as interactions primarily happen indirectly via quorum-sensing molecules rather than direct binding, and antagonistic and synergistic interactions can seemingly take place simultaneously. For example, *P. aeruginosa* induces upregulation of *C. albicans* stress pathways, killing hyphal cells [51, 52]. While on the other hand, using transcriptomic and proteomic approaches, Bandara and colleagues showed that *P. aeruginosa* quorum sensing also promotes fluconazole resistance in *C. albicans* through upregulation of efflux pumps and ergosterol biosynthesis (Fig. 1) [53]. Despite many antagonistic interactions taking place in vitro, their behaviour in vivo appears more synergistic with ventilator-associated pneumonia patients; colonised by *Candida,* who are at a much greater risk of *P. aeruginosa* infection [54].

Conclusion

Although reports describing the importance of fungi within biofilms are limited, there exists growing evidence suggesting that they play an active role within infected wounds to be considered when advising treatment regimens. However, to acquire a better understanding of the role of fungal and polymicrobial biofilms within chronic wounds and to develop more effective treatment strategies, additional studies that acknowledge the fungal component of interkingdom biofilms are required.

Declarations

Conflict of Interest The authors declare no competing interests.

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