Malassezia spp. and Hosts

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Malassezia is a lipid-dependent genus of yeasts known for being an important part of the skin mycobiota. These yeasts have been associated with the development of skin disorders and cataloged as a causal agent of systemic infections under specific conditions, making them opportunistic pathogens. Little is known about the host–microbe interactions of *Malassezia* spp., and unraveling this implies the implementation of infection models.

Keywords: Malassezia ; Host-microbe interaction ; Galleria mellonella

1. Introduction

Malassezia is a lipid-dependent genus of yeasts found as commensals on human and animal skin [1][2]. Under specific conditions, these yeasts have been associated with skin diseases [3], Crohn's disease, the exacerbation of colitis [4], Parkinson's disease [5], pancreatic ductal adenocarcinoma [6], and fungemia [7][8][9]. Factors determining the outcome of host–microbe interactions are multifactorial, involving environmental conditions like temperature and humidity, but also host factors and the predisposition of the host, which may be related to genetic factors and impairment in the immune response[10][11]. In addition, the virulence factors of *Malassezia* are likely to be involved. *Malassezia* spp. are generally regarded as opportunistic pathogens but how this skin commensal contributes to skin diseases remains a matter of debate. Studying the lifestyle of *Malassezia* spp. in a infection model is expected to contribute to unraveling this long-standing issue.

2. Infection Models as a Way to Understand Host–Microbe Interactions

2.1. In Vitro Models of Host-Microbe Interaction

In fungal infection research, the in vitro (*ex vivo*) models have been used to elucidate the mechanisms of interaction between fungi and their hosts. Indeed, an ex vivo model allows for the identification of the specific host tissue response to a pathogen, but it does not depict the whole host response [12][13][14]. The co-culturing of human keratinocytes with *M. furfur* yeasts was used to evaluate the activity of the cecropin A(1-8)–magainin 2(1-12) hybrid peptide analog P5 (an AMP). This research showed that this therapeutic alternative can indeed inhibit *M. furfur* growth without causing damage to keratinocytes. Moreover, AMPs can also modulate the inflammatory response of keratinocytes; this opens up the opportunity to evaluate new therapeutic alternatives in co-cultures of *Malassezia* and human keratinocytes, evaluating not just the drug effect on the pathogen but also the drug effect on and via the host [15]. Other studies have reported that *Malassezia* can induce or repress the production of cytokines in keratinocytes. The level of production depends on the species [16][17][18], the growth phase, and the hydrophobicity [19], and is affected by keratinocyte invasion and the survival of the pathogen inside the host cells [20]. In addition, it has been observed that *M. pachydermatis,* a zoophilic species, can invade human keratinocytes (12.1%) [21] and induce a strong inflammatory response during the first 24 h after coincubation[21][22]. In contrast, *M. furfur* has shown a lower induction of the inflammatory response, something that may be related to the avoidance of phagocytosis [20]. Interestingly, the presence of a capsule-like lipid layer may reduce the pro-inflammatory cytokine production in keratinocytes, as a way to evade the immune response [23].

In addition, the role of some factors that are excreted by species of *Malassezia* can be elucidated through in vitro model experiments. For example, the extracellular nanovesicles of *M. sympodialis* were co-cultured with keratinocytes and monocytes, demonstrating for the first time that these small structures are phagocytized by keratinocytes and monocytes [24]. Later, it was demonstrated that these nanovesicles play an important role in activating the keratinocytes as part of the cutaneous defense against *Malassezia* [25]. Furthermore, *M. furfur* has also been shown to secrete extracellular vesicles that can induce the production of pro-inflammatory cytokines in human keratinocytes. Additionally, similar to what was reported in *M. sympodialis*, the vesicles secreted by *M. furfur* are phagocytized by keratinocytes [26].

Another in vitro model is the skin equivalent (SE) generated from the isolation and cultivation of fibroblasts and keratinocytes. This system allowed the growth of an inoculum of 1×10^2 CFU/mL of *M. furfur*, which grew to 1×10^4 CFU/mL, which could mean that SE may produce and release the nutrients necessary for *Malassezia* to grow on this surface. This model appeared to mimic the lipid production by the host since the culturing media did not contain these lipids [27], but care must be taken that growth is not due to lipids associated with yeast cells and/or carried over from lipid-rich media used for pre-culturing. Similar to SE, there are other models that may allow for the understanding of the host response to *Malassezia*. For example, the reconstructed human epidermis (RHE) offers the opportunity to follow the progress of the infection over time and measure products of the immune response at every time point. In this case, it has been reported that *M. furfur* and *M. sympodialis* suppressed the inflammatory response after 48 h, thereby evading the host immune system. Additionally, this model showed again that the keratinocyte response pattern depends on the *Malassezia* species used, indicating that virulence properties and mechanisms of pathogenesis differ between them [28].

2.2. In Vivo Models of Host–Pathogen Interactions

2.2.1. Mammalian Models of Host–Pathogen Interactions

Mammalian in vivo fungal infection models include mice, rats, guinea pigs, dogs, and rabbits [24][27][29][30]. In fungi, these models have allowed for the elucidation of the role of virulence factors, like the formation of biofilms of *Candida albicans* using rabbits and rats as infection models [27]. For *Malassezia*, the implementation of a host model has been difficult due to the weak virulence of the species of this genus. The first attempts to develop a suitable model for *Malassezia* failed because an infection could not be established in the animal model or the infection was resolved in a short time period. In 1940, Moore et al. inoculated *M. furfur* directly on the intact skin of rabbits, guinea pigs, rats, and mice, which resulted in no establishment of the infection unless they were infected by intracutaneous or intratesticular inoculation [30]. The evaluation of the efficacy of antifungal treatments against *M. furfur* in guinea pigs was possible but required daily direct inoculation on intact skin for one week, which caused skin alteration that resembled SD [31]. Similar results were observed for *M. restricta* inoculated directly on the skin surface of guinea pigs; wherein severe inflammation was observed after repeated inoculation every 24 h over 7 days. The skin inflammation lasted for 52 days and resembled SD. Furthermore, in this study, it was possible to evaluate the antifungal activity of ketoconazole and luliconazole, showing that the efficacy of ketoconazole is correlated with clinical findings using ketoconazole as an antifungal agent against *Malassezia* spp. For luliconazole, it was observed that this antifungal significantly reduced *M. restricta* rDNA copies and skin lesions. Taken together, these results demonstrated the suitability of the guinea pig, not just as an infection model, but also to evaluate antifungal activity [32].

Dogs were also used to model external otitis caused by *M. pachydermatis*; this was done through the instillation of *M. pachydermatis* inoculum into the external ear canal. The aim of this inoculation was to evaluate the activity of antifungals on external otitis development. Dogs were examined daily and a microscopical examination of ear exudate was done. The results showed the development of external otitis with an erythematous ear canal and exudate production. Additionally, abundant *M. pachydermatis* yeasts were recovered in cultures from the samples [33].

A couple of experiments have been conducted in rabbits, inoculated directly on the surface of the skin with or without occlusion with a plastic film over the inoculated area to favor colonization; this led to the occurrence of lesions on the skin and the appearance of mycelial structures in histological studies. Again, it was observed that, as soon as inoculation with yeast cells was discontinued, spontaneous healing occurred. It was, furthermore, evident that infection only occurred when occlusion was employed [34][35][36]. The presence of *Malassezia* in healthy skin and the high development of seborrheic dermatitis (SD) infections in acquired immune deficiency syndrome (AIDS) patients led to the belief that these yeasts were opportunistic [37][38]. In that way, new strategies to mimic the conditions of susceptible hosts were implemented. In 2004, Oble et al. developed a novel transgenic T-cell model in mice, in which spontaneous SD-like disease developed. Using anti-fungal staining, ovoid structures in primary lesions were observed. Furthermore, antifungal treatment resulted in the reversion of clinical symptoms. Although fungi were not isolated and characterized from the lesions, overgrowth by *Malassezia* spp. seems plausible, suggesting that infections only occur under conditions of severe immunological impairment [39].

Starting from this point, it is clear that animal models must have some kind of predisposition or repetitive exposure to successfully develop fungal infection with *Malassezia*. Yamasaki et al. developed a new deficient Mincle mouse model for *Malassezia*. Mincle, also known as Clec4e, is a PRR that recognizes the PAMP mannosyl-fatty acid in *Malassezia*. With the Mincle-deficient mice, it was demonstrated that the recognition of this PAMP induced the release of the cytokines Il-6 and TNF in the host, similarly to that observed in *Malassezia*-induced lesions in humans [40]. Another way of causing immunosuppression in animal models is through the employment of chemical substances like hydrocortisone and cyclophosphamide, which results in a different type of immunosuppression. The latter results in neutropenic animals [41].

2.2.2. In Vivo Alternative Models of Host–Microbe Interactions

In general, in vitro studies allow for the finding of patterns that require subsequent testing and validation in in vivo infection systems; ethical considerations have especially pushed the development of new model systems. With respect to animal treatment, Russell and Burch proposed the 3Rs strategy (replacement, reduction, and refinement). This strategy leads to reducing the use of mammals and the replacement of these with alternative models; like computer, in vitro, alternative vertebrate (*Danio rerio*) [42], and invertebrate models [43]. In general, invertebrate alternatives used to model fungal infections like amoeboid models [44][45], *Caenorhabditis elegans* [46][47][48], *Drosophila melanogaster* [49][50][51], *Tenebrio molitor* [52], *Bombyx mori* [53] , and *Galleria mellonella* [54][55][56][57] (Figure 1) have gained importance, amongst others, as these present an innate immune response similar to that found in mammals. Furthermore, microbial virulence factors play similar roles in mammals and invertebrate systems [44][50][58]. The results obtained with these models correlated with results obtained in mammalian models, validating the invertebrates as infection models [50][58][59][60][61][62][63]. Furthermore, the attractive features of these models include the low cost of feeding and the higher number of organisms able to be stored in a small space and used in a single experiment [53].

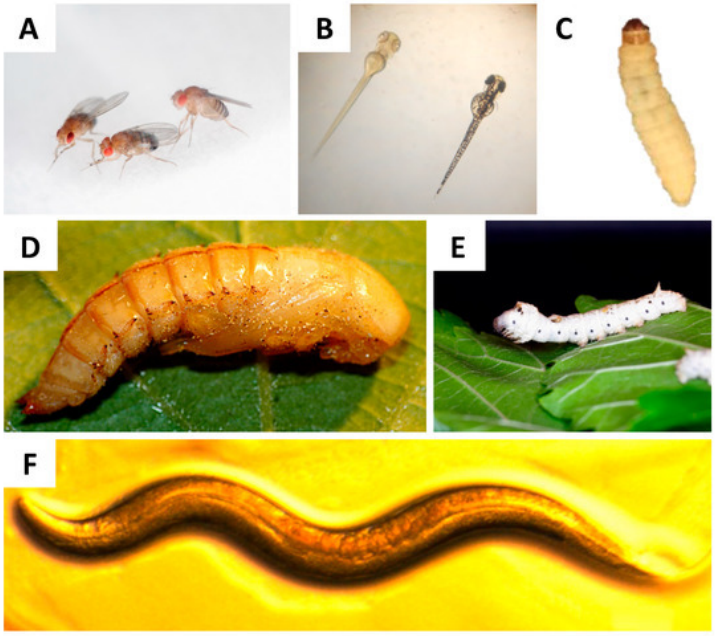

Figure 1. Alternative in vivo models for host–microbe interaction studies. (**A**) Adult *D. melanogaster* fly, whose size is approximately 3 mm. Original photograph by Flickr user NASA's Marshall Space Flight Center, CC BY-SA 2.0 license. (**B**) *Danio rerio* larval size can range from 3.5 mm to 11 mm and, as can be seen, larvae are transparent, this facilitates monitoring the progress of the infection. Original photograph by Flickr user MichianaSTEM, CC BY-SA 2.0 license. (**C**) *G. mellonella* larval size ranges from 2 cm to 3 cm and its weight ranges between 200 mg and 300 mg, making it easy to manipulate and inoculate. (**D**) *T. molitor* pupae, easy to breed and the size at the 2nd instar is similar to that of *G. mellonella*. Original photograph by Flickr user Edithvale-Australia Insects and Spiders, CC BY-SA 2.0 license. (**E**) *B. mori* larvae, these larvae are large and their weight is in the range of 900 mg to 1000 mg. Original photograph by Flickr user Gianluigi Bertin, CC BY-SA 2.0 license. (**F**) *C. elegans* nematodes, which grow to 1 mm. Original photograph by Flickr user NIH Image Gallery, CC BY-SA 2.0 license.

In the field of *Malassezia* research, hardly any work has been published with alternative in vivo models and the implementation of invertebrates as model systems is very recent. In 2018, Brilhante et al. implemented for the first time the *C. elegans* larva as an infection model for *M. pachydermatis*. In this study, *C. elegans* larvae were exposed to *M. pachydermatis* by placing the larvae in plates containing the yeasts for a period of two hours at 25 °C. The viability of the nematodes was evaluated every 24 h and the results showed that, after 96 h, the nematodes exposed to the yeast had significantly higher mortality (ranging from 48% to 95%) than the control nematodes [64]. After that, in the same year, Silva et al. also evaluated the virulence of *M. furfur*, *M. sympodialis*, and *M. yamatoensis* under different growth conditions. The implementation of *C. elegans* larvae resulted in the identification of different virulence patterns depending on the lipid supplementation of the pre-culture medium. The co-culture of larvae with *Malassezia* spp. grown in media that was not supplemented with lipids resulted in lower larval survival. In the same study, a second model was implemented. *T. molitor* larvae were inoculated with a yeast suspension, and the larvae were shown, as in the case of *C. elegans*, to have higher survival when inoculated with *M. furfur* grown in a lipid-supplemented medium [65]. These two models allowed them to assess the virulence of three species of *Malassezia* under different growth conditions. However, more research needs to be done to understand this phenomenon.

In addition to *T. molitor* larvae, other insects have been implemented recently as an infection model for *Malassezia*. That is the case for *D. melanogaster*. Wild type (*WT*) and *Toll*-deficient adult flies were inoculated with five different inoculum concentrations of *M. pachydermatis*. The results showed that *WT* flies were resistant to the infection and that *Toll*-deficient flies inoculated with the highest inoculum concentrations showed a significantly reduced survival as compared to the control. These findings were corroborated with a decrease in fungal burden in *WT* flies and an absence of yeasts in histological investigations, contrasting to what was observed in the *Toll*-deficient flies [49]. These results demonstrated the opportunistic character of *M. pachydermatis* and showed the potential of the use of immune-deficient mutant flies to study the pathogenesis of *Malassezia*.

The *G. mellonella* larva was first used as a fungal infection model in 2000. In that study, the virulence of *C. albicans* was evaluated and compared with the effect of inoculating the larvae with *Saccharomyces cerevisiae.* The results showed that inoculating the larvae with the former had a lethal effect. In contrast, *S. cerevisiae* was shown to be pathogenic. Additionally, it was found that clinical isolates of *C. albicans* were more virulent as compared to reference strains (ATCC 10231, ATCC 44990, and MEN). These results correlated with findings in mammalian models [59]. After this, *G. mellonella* has been widely implemented as a fungal infection model to evaluate virulence[58][63][66][67][68][69][70], virulence patterns related to biofilm formation [71], co-infections [60], pathogen morphogenesis [62], complex host responses [61][72][73][74][75], and antifungal susceptibility [57][76][77][78][79] at 37 °C, which is an advantage of this lepidopteran, as it can be incubated at human physiological temperatures. The results of most of these studies have been shown to correlate with results obtained in mammalian models and also in humans. Indeed, the efficacy of antifungals tested in *G. mellonella* larvae against *Cryptococcus* spp. [57] and *Candida* spp. [79] have been shown to correlate with results in the murine model, *C. elegans* larvae, and in vitro models. Even though these results are interesting, there is a need to better understand this insect. At present, there is available information related to the immune response transcriptome [80] and the miRNAs involved in the regulation of the immune response [81] that can help to evaluate the host response to a specific pathogen. All of this together makes this insect a promising tool to elucidate the complex host–microbe interactions of *Malassezia*.

G. mellonella has been standardized as an infection model for *M. furfur* CBS 1878 and *M. pachydermatis* CBS 1879, two isolates from skin lesions. The inoculation of larvae with these two species showed that larval survival depended on the inoculum concentration (higher inoculum concentration led to lower survival, compared to lower inoculum concentration). Additionally, a lower virulence was observed for *M. furfur* as compared to *M. pachydermatis* at 33 °C and 37 °C. This was evident by a decrease in larval survival, higher fungal burden, histological examination with a higher presence of hemocyte aggregates with melanin deposition, and a higher larval melanization, especially in larvae that were inoculated with *M. pachydermatis* and incubated at 37 °C. The higher virulence of *M. pachydermatis* was attributed to a high phospholipase activity and a high capacity of *M. pachydermatis* to form biofilms [82]. However, further studies are required to confirm these hypotheses. These results show that the *G. mellonella* larva is a suitable model and very useful to identify differences in virulence between species or strains.

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