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In vitro or *in vivo* models, the next frontier for unraveling interactions between *Malassezia* spp. and hosts. How much do we know?

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Abstract:

Malassezia is a lipid-dependent genus of yeasts known for being an important part of the skin mycobiota. These yeasts have been associated in 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 interaction of *Malassezia* spp., and unraveling this implies the implementation of infection models. In this mini review we present different models that have been implemented in the fungal infections study with greater attention in *Malassezia* spp. infections. These models range from *in vitro* (cell cultures and *ex vivo* tissue), to *in vivo* (murine models, rabbits, guinea pigs, insects, nematodes, and amoebas). We additionally highlight the alternative models that reduce the use of mammals as model organisms, which have been gaining importance in the study of fungal host-microbe interactions. This is due to the fact that these systems have shown to have reliable results, which correlate with those obtained from mammalian models. Example of alternative models are *Caenorhabditis elegans*, *Drosophila melanogaster*, *Tenebrio molitor*, and *Galleria mellonella*. These are invertebrates that have been implemented in the study of *Malassezia* spp. infections in order to identify differences in virulence between *Malassezia* species.

Keywords: *In vitro, in vivo,* **animal model,** *Malassezia,* **infection, host-pathogen interaction,** *Galleria mellonella.*

1. Introduction

Malassezia is a lipid-dependent genus of yeasts found as commensal on human and animals skin [1,2]. Under specific conditions, these yeasts have been associated with skin diseases [3], Crohn´s disease, exacerbation of colitis [4], Parkinson disease [5] pancreatic ductal adenocarcinoma, [6] and fungemia [7–9] (**Table 1**). Factors determining the outcome of host-microbe interactions are multifactorial, involving environmental conditions like temperature, humidity, but also host factors and predisposition of the host, which may be related to genetic factors and impairment in the immune response [10,11]. In addition, 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 life style of *Malassezia* spp., in model organisms is expected to contribute to unravel this long-standing issue.

Even though *Malassezia* was described for the first time in 1846 and studied for a long time, relatively little is known about interactions with the host. In part, this is due to the specific nutritional requirements of the yeasts [1,2]. The fact that *Malassezia* requires fatty acids in media for growth has

complicated the development of in *vitro* and *in vivo* models [12,13]. Many studies have addressed and compared the relative abundances of *Malassezia* species on healthy and diseased skin of hosts [2]. Clearly, these species are regarded as skin commensals, which makes it more complex to determine their direct role in disease development. They were proposed to modulate the immune response through different mechanisms. For example, the composition of the cell wall contributes to evade phagocytosis and decrease the release of proinflammatory cytokines by immune cells (IL-1β, IL-6 and TNF-α) [14], the induction of IL-17 that leads to skin inflammation [15,16], the indolic compounds that may inhibit de respiratory burst of neutrophils [17–21]. Furthermore, the nutritional requirement may lead to the release of fatty acids that can contribute to skin irritation [10]. But how these properties contribute to virulence has not been studies in depth in different infection models.

Malassezia is a prominent members of the skin mycobiota and considered a commensal. Understanding the transition from a commensal microorganism to a pathogen in skin and systemic diseases is a major aim in current research. Besides, identifying predisposing and risk factor of the host that contribute to this transition and also the response of the yeasts to these changing conditions can be studied and may help in the development of new therapeutic alternatives. The aforementioned goals require the implementation of infection models in which virulence properties of *Malassezia* spp. can be studied. Depending on the formulation of the research question, different types of infection models might be used. However, to unravel host-microbe interaction, it is necessary to study infections in more than one model since each model system has its properties and limitations. This review aims to show the importance of different infection models in the study of the *Malassezia* genus to understand the virulence properties of these yeasts, and we will describe novel alternative models that are gaining importance in this field.

Table 1. Diseases associated with *Malassezia* spp.

Disease	Clinical findings	Species involve	Most commonly affected population	References
Pityriasis Versicolor (PV)	Macules in the trunk and arms; the skin lesions are hypopigmented and hyperpigmented	*Malassezia globosa, Malassezia sympodialis,* and *Malassezia furfur*	Young adults and rarely children and older adults	[3,22–27]
Dandruff/ seborrheic dermatitis (D/SD)	Flaking and erythema in sebum rich areas like the scalp, nostrils, chest and eyebrows	*M. globosa, Malassezia restricta, M. furfur* and *Malassezia obtusa*	Elders, infants, children in puberty and HIV patients	[3,25,27–33]
Atopic dermatitis (AD)	Chronic inflammatory illness with pruritic eczematous lesions. *Malassezia* has been proposed to act as an exacerbator	*M sympodialis, M. globosa, M. furfur, M. restricta, Malassezia japonica, Malassezia yamatoensis* and *M. slooffiae*	Adults with genetic and environmental predisposing factors	[3,27,34–37]
Folliculitis	Small dome-shaped papules localized around follicular area, mainly in the back, chest and shoulders. The papules can evolve into pustules	*M. globosa, M. restricta, M. sympodialis, M. furfur* and *M. pachydermatis*	Teenagers and young adult males	[3,25,26,31,32]

Psoriasis	Chronic skin disease, characterized by hyperproliferation and hyperkeratinization of the epidermis. *Malassezia* may augment inflammation and severity of the disorder	*M. globosa, M. furfur, M. sympodialis, M. restricta* and *M. slooffiae*	Patients with psoriasis, mainly in scalp of young male adults	[3,27,37–39]
Crohn´s disease	Inflammatory bowel disease characterized by altered immune response to intestinal microbiota. *Malassezia* yeasts in the gut may increase the severity of the disease	*M. restricta*	Crohn´s disease patients carrying the CARD9^{S12N} risk allele.	[4]
Parkinson disease	Neurodegenerative disease. Seborrheic dermatitis has been strongly associated with this disease.	*M. globosa, M. restricta, M. furfur* and *M. obtusa*	Elders. Risk increases after a seborrheic dermatis diagnosis	[5]
Pancreatic ductal adenocarcinoma	Carcinoma due to fungal dysbiosis	*M. globosa*	Individuals with oncogenic *Kras* that induces inflammation resulting in fungal dysbiosis	[6]
Invasive infections	Fungemia, endocarditis, bronchopneumonia, respiratory distress, splenic lesion etc.	*M. furfur, M. pachydermatis, M. sympodialis* and *M. restricta*	Low weight neonates and immunocompromised patients	[3,7,8,26,40–47]

2. Infection models as a way to understand host-microbe interaction

Little is known about the virulence properties and infection mechanisms of *Malassezia* spp., and the implementation of infection models may allow for evaluation of the interaction of these yeasts with the hosts, virulence of different species or strains of a specific species and antifungal activity. There are different types of suitable models in which virulence and infection can be studied, but it is critical to realize that results obtained in each model provide partial answers, as was mentioned before. It is therefore important to study virulence properties in different *in vitro* and *in vivo* models and the results obtained can provide complementing answers [48–51].

One of the infection models that may help to unravel host-microbe interactions are *in vitro* models, which have been used since the 1960s [51]. *In vitro* models are generally easier to handle, the majority of factors can be controlled, the evaluation in drug activity evaluation is more accurate and in some cases are cheaper than using animal models. These models can also be cataloged *as ex vivo* models [48,52,53], like cultured cells, removed organs, skin equivalent or dermis equivalent [50,54,55]. As good as the *in vitro* models are, they do not fully reproduce the host-microbe interaction as occurring for example on the skin.

Contrary to *in vitro* models, the *in vivo* models mimic the complexity of host response better [49,50,53,56]. These are rather diverse and can vary from mammalian models to insect models. Mammalian models are phylogenetically the closest to human beings and, generally, regarded to reproduce more accurately the host-microbe interaction, designed as fidelity [48,50,53]. Also, many of these models are well characterized allowing genetic modifications to a desirable condition. The drawbacks of these models are the high cost of feeding and maintenance, the limited number of individuals, the ethical implications, and the need for trained personnel to handle the animals [50]. These drawbacks can be solved by the implementation of alternative animal models, like invertebrates.

Invertebrate animal models have recently gained importance in fungal research since studies have shown that microbial virulence factors involved in infections in mammals are the same as those involved in invertebrate infections [49]. In fact, it seems that different aspects of the innate immune response in vertebrates and invertebrates are shared and represent a conserved trait, which means that human pathogens interact, at least in part, similarly with both immune systems [49,57]. The innate immune responses in invertebrate models are comparable to for example the human immune response to fungi via Toll-like receptors, which were originally discovered in *Drosophila melanogaster* [58], a model system already used with *Malassezia* [59], and also present in *Caenorhabditis elegans* [60]. Besides, the well-developed phagocytic system in lepidopterous and coleopterous larvae parallels the process of phagocytosis in mammalian systems [49,56,61–65].

2.1. *In vitro* models of host-microbe interaction

In fungal infections research, the *in vitro* (*ex vivo*) models have been used to elucidate the mechanisms of interaction between fungi and their host. Indeed, *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 [48,50,53]. An example of this is the implementation of keratinocytes to evaluate the response of these cells to skin-related fungal infections. *Trichophyton rubrum* was shown to induce the production of skin-derived antimicrobial peptides (AMP) in primary keratinocytes which may help the host to control dermatophytes infection [66]. Similarly, this model has been used as an infection model for *Candida albicans,* identifying the induction of proinflammatory cytokines production [67] and proteins involved in fungal adhesion to keratinocytes and interaction with the host [68] .

Co-culturing of human keratinocytes with *M. furfur* yeasts was used to evaluate the activity of cecropin A(1-8)–magainin 2(1-12) hybrid peptide analog P5 (AMP). This research showed that this therapeutic alternative can indeed inhibit *M. furfur* growth without causing damage to keratinocytes. Moreover, AMP can also modulate the inflammatory response of keratinocytes; this opens 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 [69]. Other studies have reported that *Malassezia* can induce or repress the production of cytokines in keratinocytes. The level of production depends on the species [70–72], the growth phase and hydrophobicity [73], and be affected by keratinocytes invasion and survival of the pathogen inside the host cells [74]. In addition, it has been observed that *M. pachydermatis,* a zoophilic specie, can invade human keratinocytes (12.1%) [75] and induce a strong inflammatory response during the first 24 hours after coincubation [75,76]. In contrast, *M. furfur* has shown a lower induction of an inflammatory response, something that may be related to the avoidance of phagocytosis [74]. Interestingly, the presence of a capsule-like lipid layer that may reduce the pro-inflammatory cytokines production in keratinocytes, as a way to evade de immune response [77].

In addition, the role of some factors that are excreted by *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 [34]. Later, it was demonstrated that these

nanovesicles play an important role in activating the keratinocytes as part of the cutaneous defense against *Malassezia* [78]. Furthermore, *M. furfur* has also been shown to secrete extracellular vesicles that can induce the production of pro-inflammatory cytokines in human keratinocytes. Also, similar to what was reported in *M. sympodialis*, the vesicles secreted by *M. furfur* are phagocytized by keratinocytes [79]

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 mimics the lipid production by the host since the culturing media did not contain these lipids [54], 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), which offers the opportunity to follow the progress of the infection over time and measuring 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 hours, thereby evading the host immune system. Also, this model showed again that the keratinocytes response pattern depends on the *Malassezia* specie used indicating that virulence properties and mechanisms of pathogenesis differ between them [55].

2.2   *In vivo* models of host-pathogen interaction

2.2.1   Mammalian models of host-pathogen interaction

Mammalian *in vivo* fungal infection models include mice, rats, guinea pigs, dogs, and rabbits [50,53,80,81]. In fungi, these models have allowed for the elucidation of the role of virulence factors, like formation of biofilms of *Candida albicans* using rabbits and rats as infection models [53]. Immunosuppressed rats and mice have been also used as animal model to study invasive rhinosinusitis caused by *Aspergillus fumigatus* [82], drug evaluation in pulmonary aspergillosis [83]. Furthermore, mice models were used to establish keratitis infections with fluorescently labeled *Fusarium solani* allowing for the *in vivo* observation of the pathogens during infection [84].

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 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 [81]. Evaluation of the efficacy of antifungal treatments against *M. furfur* in guinea pig was possible but required daily direct inoculation on the intact skin for one week which caused skin alteration that resembled SD [85]. 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 hour during 7 day. The skin inflammation lasted for 52 days and resembled SD. Furthermore, in this study it was possible to evaluate the antifungal activity or ketoconazole and luliconazole, showing that the efficacy of ketoconazole correlates with clinical findings using Ketoconazole as antifungal agent against *Malassezia* spp. For luliconazole, it was observed that this antifungal significantly reduced *M. restricta* rDNA copies and skin lesions. Taking together, these results demonstrated the suitability of the guinea pig, not just as an infection model, but also to evaluate antifungal activity [86].

Dogs were also used to resemble external otitis caused by *M. pachydermatis*; this was done through 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. Dog were examined daily and

microscopical examination of ear exudate was done. The results showed the development of external otitis with erythematous ear canal and exudate production. Also, abundant *M. pachydermatis* yeasts were recovered in culture from the samples [87].

A couple of experiments have been conducted in rabbits, inoculating directly on the surface of the skin with or without occlusion with a plastic film over the inoculated area to favor the colonization; leading 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 [88–90]. 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 [91,92]. In that way, new strategies to mimic the conditions of susceptible hosts were implemented. Oble *et al.* in 2004 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 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 [93].

Starting from this point, it is clear that animal models must have some kind of predisposition or repetitive exposure to successfully develop the fungal infection with *Malassezia*. Yamasaki *el al*. developed a new deficient Micle mouse model for *Malassezia*. Micle (CLEC4e) is a PRR that recognizes the PAMP mannosyl-fatty acid in *Malassezia*. With the Micle-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 as observed in *Malassezia*-induced lesions in humans [94]. Other ways of 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 [95].

Predisposing factors include skin barrier disruption. In 2019, Sparber *et al.* demonstrated that epicutaneous infection by *Malassezia* spp. can be established by disrupting skin integrity using an adhesive tape on the dorsal skin of the ear of a mouse. This study showed that *Malassezia* induces the release of IL-17, which stimulates the tissue inflammation, agreeing with findings in atopic dermatitis [15]. With respect to mammal models, new *in vivo* alternatives have now been proposed that facilitate *Malassezia* infection in animals.

2.2.2 *In vivo* alternative models of host-microbe interaction

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 way in 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 vertebrates (*Danio rerio*) [96] and invertebrates models [97]. In general, invertebrate alternatives used to model fungal infections like amoeboid models [49,98], *Caenorhabditis elegans* [99–101]*, Drosophila melanogaster* [59,102,103]*, Tenebrio molitor* [104]*, Bombyx mori* [56] and *Galleria mellonella* [61,63,105,106] (**figure 1 and table 1**) have gained importance amongst others as these present an innate immune response similar to that found in mammals. Furthermore, microbial virulence factors play in mammals or invertebrate systems similar roles [49,102,107]. The results obtained with these models correlated with results obtained in mammalian models, validating the invertebrates as infection models [102,107–112]. Furthermore, 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 [56].
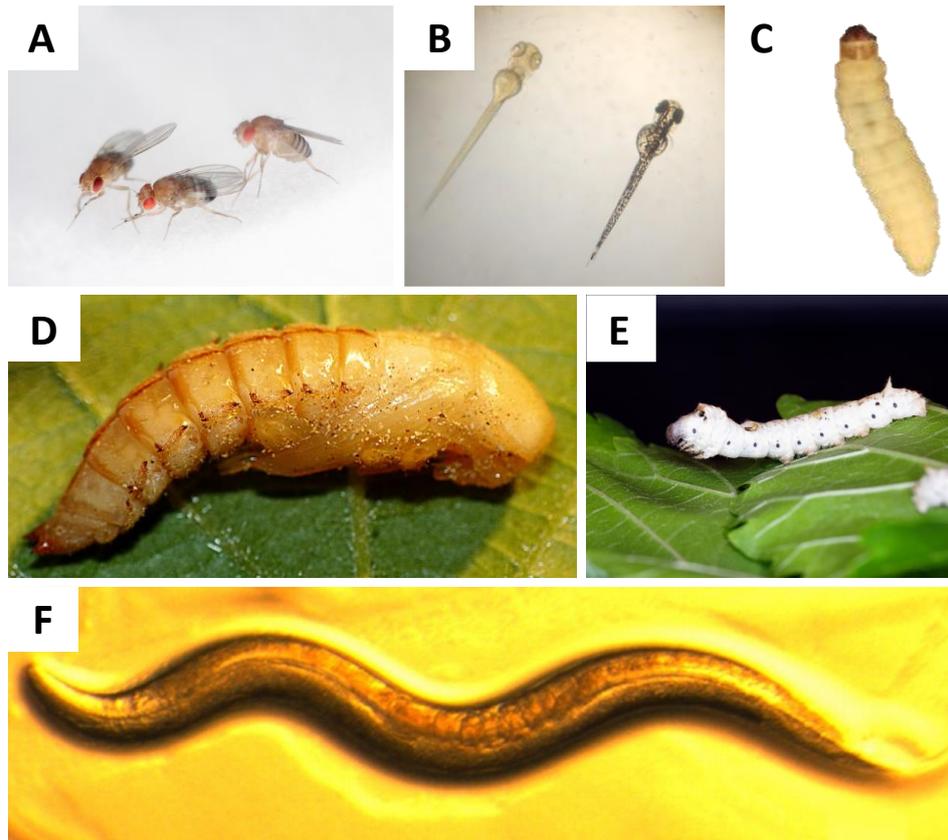
Figure 1. **Alternative *in vivo* models for host-microbe interaction studies.** (**A**) Adult *D. melanogaster* fly, whose size is approximately 3 mm. Original photograph by flickr user NASA's Marshall Space Flight Center, CC BY-SA 2.0 license. (**B**) *Danio rerio* larvae size can ranges from 3.5 mm to 11 mm and as can be seen larvae are transparent, this facilitates to monitor the progress of the infection. Original photograph by flickr user MichianaSTEM, CC BY-SA 2.0 license. (**C**) *G. mellonella* larvae size ranges from 2 cm to 3 cm and weight 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 the 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.

Table 2. *In vitro* and *in vivo* models available for the *Malassezia* spp. infections study

Infection model	cost	Inoculation	Advantages	Disavantage	References
Keratinocytes culture	High	-Co-culturing	-Controlled conditions -Just one type o cell	-It does not represent the complex interactions with the host	[48,50,70–77,79]

Model		Inoculation route	Advantages	Disadvantages	References
Murine model	High	-Oral gavage - Inoculation through the tail vain -Inhalation and intranasal administration -Direct inoculation -Ocular -Intracranial -Intraperitoneal	-Well defined inoculation routes -Immune response is similar to the human´s, with innate and adaptative immune response -Mimics human infection disease - Annotated genome - Available mutants	-Ethical issues -Bigger space to storage -Longer generation time -Trained personnel to handle the models - immune suppression required	[53,56,113,114]
Amoeboid model (*Acanthamoeba castellani)i*	Low	-Co-incubation	-Controlled conditions -Inoculum quantification -Available mutants -Incubation at 37 °C -Phagocytosis assays -Short life cycle -Annotated genome	-Undesired mutation and loss of phagocytic abilities in long cultured strains	[49,50,98,115,116]
Zebrafish larvae (*Danio rerio*)	Low	-Microinjection into the caudal vein, notochord, duct of Cuvier, hindbrain ventricle, eye, peritoneal cavity or muscle - exposure by immersion	-Short generation time -Annotated genome sequence -Available mutants -Transparency - High-throughput screening -Innate immune response similar to that of humans	-In larval stage there in no adaptative immune response -Ethical issues in some countries -Difficult to handle	[96,114,117–122]
Caenorhabditis elegans	Low	-Exposure of larvae by immersion (feeding and contact with the cuticle)	-Short generation time -Small size -Easy to grow - Annotated genome sequence -Available mutants -Innate immune response similar to that of humans -Results correlated with result from mammals	-No adaptative immune response -Difficult to inoculated and quantify the inoculum	[83,123]
Silk worm (*Bombyx mori*)	Low	-Microinjection into the haemocoel -Oral (puncture)	-Inoculum quantification -High inoculum volume -Results correlated with result from mammals -Innate immune response similar to that of humans	-No adaptative immune response	[56,114,124]

Drosophila melanogaster	Low	-Puncture in the dorsal side of the thorax	- Annotated genome sequence - Available mutants -Innate immune response similar to that of humans	-No adaptive immune response -Difficult to inoculated and quantify the inoculum	[59,102,125]
Galleria mellonella	Low	Microinjection directly to the haemocoel -Topical -Oral	-Inoculum quantification -Wide range of temperature - Innate immune response similar to that of humans -Available immune response transcriptome -Results correlated with result from mammals	-No adaptive immune response	[63,105,114,115,126–129]

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 *C. elegans* 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 during a period of two hours at 25 °C. The viability of the nematodes was evaluated every 24 hours and the results showed that after 96 hours the nematodes exposed to the yeast had significantly higher mortality (ranging from 48% to 95%) than the control nematodes [130]. 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 pattern 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, *T. molitor* showed, as in the case of *C. elegans*, to have higher survival when inoculated with *M. furfur* grown in lipid supplemented medium [131]. 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*, other insects have been implemented recently as an infection model for *Malassezia*. That's the case of *D. melanogaster*. Wild type (*WT*) and *Toll*-deficient adult flies were inoculated with five different inoculum concentrations of *M. pachydermatis*. Results showed that *WT* flies were resistant to the infection and that *Toll*-deficient flies inoculated with the highest inoculum concentrations showed a significant reduced survival as compared to control. These findings were corroborated with a decrease of fungal burden in *WT* flies and an absence of yeasts in histological investigations, contrasting to what was observe in the *Toll*-deficient flies [59]. 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 to *S. cerevisiae* did not show to be pathogenic. Also, 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 [108]. After these, *G. mellonella* has been widely implemented as a fungal infection model to evaluated virulence [107,112,115,127,132–134], virulence patterns related to biofilm formation [129], co-infections [109], pathogens morphogenesis [111,135], complex host response [110,136–139], and antifungal susceptibility [106,140–142] at 37 °C, which is an advantage of this Lepidoptera, that can be incubated at human physiological temperatures. The results of most of these studies have shown to correlate with results obtained in mammalian models and also in humans. 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 [143] and the miRNAs involved in the regulation of the immune response [144] 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 interaction 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 lead to lower survival, compared to lower inoculum concentration). Also, 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 hemocytes aggregates with melanin deposition and a higher larval melanization, especially, 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 [145]. However, further studies are required to confirm these hypotheses. These results show that *G. mellonella* larva is a suitable model and very useful to identify differences in the virulence between species or strains.

3. Conclusion

The use of both *in vitro* and *in vivo* models is important in unraveling the interactions between microbes and hosts, and a variety of models are indeed available (**Figure 1** and **Table 2**). *In vivo* models clearly allow for direct comparison of virulence and studies of pathogenic developments in the host, and are in that respect most attractive. Alternative models that can replace mammalian models on ethical grounds are favored to reduce the number of animals used in research. However, it is important to keep in mind that alternative models do not completely replace mammalian models. In short, insect larvae like *G. mellonella* larva have proven to be reliable models and with results similar to those reported in the murine models, making it an interesting tool to decipher aspects of the host-microbe interaction of *Malassezia.*

4. Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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6. Author Contributions

M.T. and A.M.C.R; Writing-Original draft preparation, A.M.C.R and H.C. Writing - Review and editing. All authors have approved the final version.

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