MALASSEZIA: A SKIN COMMENSAL YEAST IMPACTING BOTH HEALTH AND DISEASE

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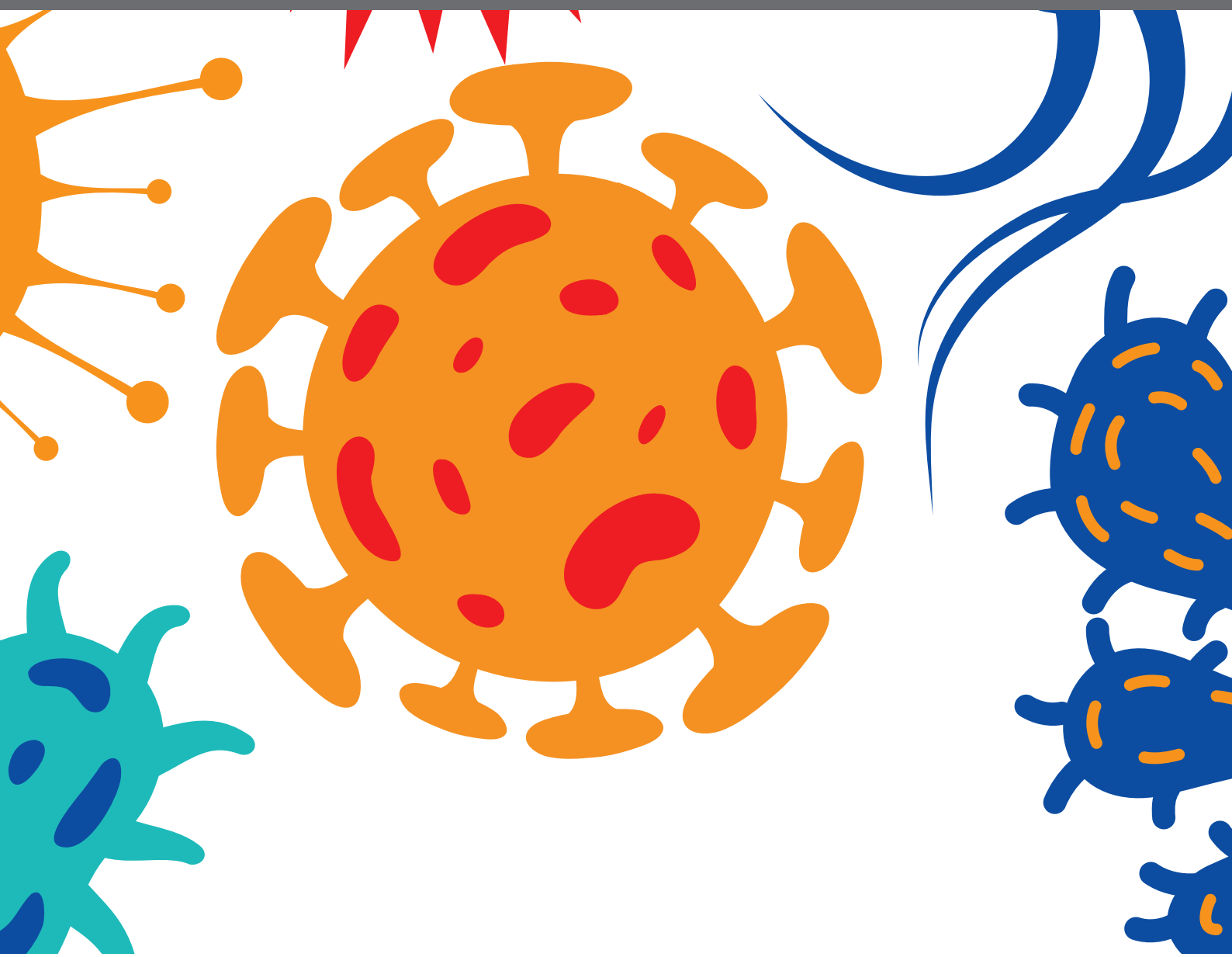

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MALASSEZIA: A SKIN COMMENSAL YEAST IMPACTING BOTH HEALTH AND DISEASE

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Table of Contents

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EDITORIAL
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Editorial: *Malassezia*: A Skin Commensal Yeast Impacting Both Health and Disease

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Editorial on the Research Topic

Malassezia: A Skin Commensal Yeast Impacting Both Health and Disease

Over the last 30 years the term "microbiome" has grown exponentially in scientific discourse. Although the human-associated microbial community consists of bacteria, fungi, viruses, and archaea, the focus has been mainly on bacteria. Interest in the fungal communities, the mycobiome, has risen only more recently. In the skin, fungi represent 5%–10% of metagenomic sequences, versus less than 1% in mucosal epithelia (gut), and is dominated by a single fungal genus, *Malassezia*. Originally thought a single species, *Malassezia* is now known to span an entire clade comprising 18 diverse species and numerous functionally distinct strains. The lipid-dependent basidiomycetous yeast is found on all humans and warm-blooded vertebrates, and in ecosystems as diverse as deep marine environments. The high proportion of *Malassezia* in the skin microbiome makes understanding this fungus' role at the intersection with the host, the environment, and the microbiota crucial. Importantly, beyond the commensal lifestyle on host skin, *Malassezia* is also associated with various pathological conditions, including dandruff, pityriasis versicolor, and common inflammatory skin disorders such as seborrheic dermatitis and atopic dermatitis. While in some cases disease is linked to the appearance of *Malassezia* hyphae and fungal overgrowth, the causal relationship between *Malassezia* and disease often remains unclear. Fungal pathogenicity is defined as a relationship where the fungus may directly harm the host by specific virulence factors, or indirectly *via* induction of a harmful host response. New pathogenic roles of *Malassezia* beyond the skin have been revealed as *Malassezia* is associated with Crohn's disease and pancreatic ductal carcinoma. The present Special Topic of Frontiers in Fungal Pathogenesis makes a significant contribution to understanding more of the above-mentioned topics. The issue contains 13 diverse scientific contributions including 7 review and 6 primary research articles.

Vijaya Chandra et al. provide an up to date, comprehensive overview of *Malassezia* in human skin from early life to adulthood, including the importance of intact epithelial barriers and local immune system activity for stable colonization. However, how the *Malassezia*-host interaction transitions from a commensal to a pathogenic relationship remains a conundrum. The authors elaborate on whether *Malassezia* is cause or consequence in the multifaceted interaction with skin in

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health and under various dermatological conditions. They also discuss evidence for a protective, mutualistic, role of *Malassezia* in healthy skin through interaction with skin bacteria, especially atopic dermatitis-associated *S. aureus*, by restricting the bacteria's pathogenicity. In addition to skin, *Malassezia* is found in other unrelated niches. The detection in healthy faecal samples by sequencing and culture indicates that viable *Malassezia* occupies the gastrointestinal niche. Spatz and Richard expand on the adaptive flexibility of *Malassezia* to grow under conditions that differ from skin with respect to lipid content, temperature, and oxygen. Fungal dysbiosis is a hallmark of intestinal inflammation and as such an increase in the abundance of *Malassezia* in the gut mycobiome is associated with IBD. Spatz and Richard also speculate on mechanisms for how *Malassezia* is involved in gut pathologies. Beyond the involvement in skin disease and to a lesser degree other barrier tissue, *Malassezia* causes severe systemic infections, especially among premature neonates and immunocompromised patients receiving parenteral nutrition. Although rare, their occurrence is underestimated and their outcome often fatal. Rhimi et al. review the latest about the epidemiology and pathogenesis of *Malassezia*-related fungemia.

Malassezia is not restricted to humans, but also found in diverse vertebrate species. Guillot and Bond discuss the relevance of *Malassezia* in veterinary dermatology, from original discovery of *M. pachydermatis* being an important canine otic pathogen to the recent discovery of *M. vespertilionis* in bats. They highlight how management of *Malassezia* otitis externa and *Malassezia* dermatitis, commonly presented to veterinarians in small animal practice, relies on both treating fungal overgrowth and restoring host immunity, as the latter is often a predisposing factor for fungal overgrowth. Successful management of *Malassezia*-related disease in humans and companion animals is hampered by poor diagnostics. Culture based isolation and enumeration of *Malassezia* remains challenging due to the lipid dependency. Saunte et al. review currently available diagnostic tools, including newer molecular methods, and treatment options for various skin pathologies. Identification of the involved *Malassezia* species is relevant for choosing the most effective antifungal drug, as different *Malassezia* spp. have varied antifungal susceptibility. The authors emphasize the need for standardization of species diagnostics and susceptibility testing. The challenges regarding robust and reproducible detection methods, treatment options and emerging resistance is also discussed by Rhimi et al. in the context of *Malassezia* fungemia and by Guillot and Bond in the context of *Malassezia*-mediated veterinary dermatitis and otitis.

Advances in *Malassezia* research are dependent on development of new tools for culture, detection, and genetic manipulation of the yeast, as well as for studying its pathogenicity and interaction with the mammalian host. Genome information is becoming available for an increasing number of species and strains, as summarized by Ianiri and Heitman and by Vijaya Chandra et al. Analysis of the available genomes contributed to resolving *Malassezia* taxonomy, shedding light on the evolutionary trajectory of *Malassezia* pathogenesis and niche adaptation, and enabling the identification of unknown genes and pathways linked to commensalism and/or pathogenicity. The availability of *Malassezia* genomes led to investigations considering emergence of *Malassezia* drug resistance. Park et al. assessed the molecular mechanisms that confer ketoconazole resistance, normally a highly effective fungistatic drug, in *M. restricta*. Comparative genome analysis of resistant strains isolated from dandruff patients suggests that multiplication of the genomic loci encoding genes involved in ergosterol synthesis and overexpression of drug efflux pumps are one mechanism underlying ketoconazole resistance in *M. restricta*. Genetic engineering of *Malassezia* was accomplished recently *via* agrobacterium-mediated transformation, as reviewed by Ianiri and Heitman. This powerful approach opens the door for both random insertional and targeted mutagenesis. The authors summarize the so far achieved mutants by either of the two approaches and discuss technical challenges and solutions. In addition, Goh et al. report generation of mCherry-expressing *M. furfur via* random insertional mutagenesis. Beyond fluorescently labelling yeast cells, this approach promises to be useful for overexpression of genes and the generation of insertional mutant libraries, which will help to deepen our understanding of *Malassezia* gene functions and host-microbes interactions.

Complementary to the new genetic tools, proteomic, metabolomic, and lipidomic approaches are essential for characterization of *Malassezia* species and strains in different environments. In this regard, Celis Ramírez et al. performed a lipidomic analysis and identified 18 lipid classes and 428 lipidic compounds, some of which segregate between different *Malassezia* species and may thus guide species discrimination and improve understanding of *Malassezia* lipid metabolism.

To study host-fungal interactions and assess antifungal immune responses, various models have become available. Studies with isolated cells in culture, including transformed keratinocyte and macrophage cell lines or primary cells isolated *ex vivo*, were recently complemented with approaches employing model hosts such as *Galleria mellonella* or experimental mice to explore the fungus-host interactions *in vivo*. In this Topic, Sparber et al. discuss the relevance of specific immune pathways for fungal control in barrier tissues. Type 17 immunity plays a central role in maintaining fungal homeostasis in healthy skin. However, IL-17 can exert pathological effects and exacerbate skin inflammation in barrier-disrupted skin. What determines the decision between protective and disease-promoting host response remains elusive. Also in this Topic, Corzo-León et al. describe an *ex vivo* human skin model, which provides a new and powerful approach to assess the host response to *Malassezia* of intact and fully differentiated human skin. The availability of diverse models opens the door for studying fungal pathogenesis and examining the functional role of putative virulence factors. As such, Vallhov et al. explored the host response to extracellular vesicles released from *M. sympodialis* and report activation of primary human keratinocytes, which strengthens the idea that extracellular vesicles play an important role in inter-organismal communication and initiation of the host response. The relevance of *Malassezia*-released compounds in the fungus-host interaction is further highlighted by Poh et al., which

on the basis of genomic information explored secreted proteases in *M. furfur* and identified secreted aspartyl protease 1 (MfSAP1) as the dominant member of a family of 14 secreted proteases. They show that MfSAP1 is capable of degrading a wide range of human skin associated extracellular matrix proteins with important implication for cutaneous wound healing.

The diversity of contributions in this Special Topic shows that we are only scratching the surface to understanding *Malassezia* biology and the complexities of *Malassezia*-host interactions. A causative relationship between the fungus and various pathologies remains a matter of debate and the mechanism of pathogenesis unclear. Increased understanding of host- and microbe-specific interactions should lead to identification of key factors that maintain healthy skin homeostasis or, in turn, initiate pathogenic changes. Recent developments have expanded our understanding of *Malassezia*'s role in the skin microbiome, with a focus on its multiple roles as commensal, pathogen, and protector through interactions with the host and inter-kingdom interactions. These approaches are leading towards development of new therapeutic targets and treatment options.

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AUTHOR CONTRIBUTIONS

SL-L drafted the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Extracellular Vesicles Released From the Skin Commensal Yeast *Malassezia sympodialis* Activate Human Primary Keratinocytes

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Extracellular vesicles (EVs) released from fungi have been shown to participate in inter-organismal communication and in cross-kingdom modulation of host defense. *Malassezia* species are the dominant commensal fungal members of the human skin microbiota. We have previously found that *Malassezia sympodialis* releases EVs. These EVs, designated MalaEx, carry *M. sympodialis* allergens and induce a different inflammatory cytokine response in peripheral blood mononuclear cells (PBMC) from patients with atopic dermatitis compared to healthy controls. In this study, we explored the host-microbe interaction between MalaEx and human keratinocytes with the hypothesis that MalaEx might be able to activate human keratinocytes to express the intercellular adhesion molecule-1 (ICAM-1, CD54). MalaEx were prepared from *M. sympodialis* (ATCC 42132) culture supernatants by a combination of centrifugation, filtration and serial ultracentrifugation. The MalaEx showed a size range of 70–580 nm with a mean of 154 nm using nanoparticle tracking analysis. MalaEx were found to induce a significant up-regulation of ICAM-1 expression on primary human keratinocytes isolated from human *ex vivo* skin ($p = 0.026$, $n = 3$), compared to the unstimulated keratinocytes. ICAM-1 is a counter ligand for the leukocyte integrins lymphocyte function-associated antigen-1 (LFA-1) and macrophage-1 antigen (Mac-1), of which induced expression on epithelial cells leads to the attraction of immune competent cells. Thus, the capacity of MalaEx to activate keratinocytes with an enhanced ICAM-1 expression indicates an important step in the cutaneous defense against *M. sympodialis*. How this modulation of host cells by a fungus is balanced between the commensal, pathogenic, or beneficial states on the skin in the interplay with the host needs to be further elucidated.

Keywords: extracellular vesicles, fungi, ICAM-1, keratinocytes, MalaEx, *Malassezia*

INTRODUCTION

Extracellular vesicles (EVs) are released not only from different mammalian cell-types but also from microorganisms, parasites, and plants (Bielska et al., 2019). EVs are heterogenous in size, 20 up to 1,000 nm in diameter, and their function differs depending on their cell of origin, different routes involved in biogenesis, release and their cargo (Soares et al., 2017). They are mainly classified into

two major groups based on size; exosomes of endosomal origin with a diameter up to 150 nm, and microvesicles bigger than 150 nm generated through outward budding of the plasma membrane (Thery et al., 2018). EVs from microorganisms, such as fungi, are usually around 100–1,000 nm, and have been associated with cytotoxicity, the invasion of host cells, and the transfer of virulence factors (Brown et al., 2015). Fungal EVs have been shown to participate in inter-organismal communication and in cross-kingdom modulation of host cells (Bielska and May, 2019) with their capacity to deliver functional (m)RNAs and micro (mi)RNA-like RNAs to recipient cells (Peres da Silva et al., 2015). Notably, EVs from the host have on their part been found to deliver host sRNA into fungal cells and induce cross-kingdom RNA interference (RNAi) to silence fungal virulence genes (Cai et al., 2018).

Malassezia species are the dominant fungal members of the human skin microbiota (Findley et al., 2013) and detectable already on the skin in 100% of newborns (Nagata et al., 2012). The genus *Malassezia* comprises a heterogenous group of increasing number of identified species, and novel culture independent methods suggest a wider-spread than previously described (Theelen et al., 2018). Although, a commensal skin colonizing yeast, *Malassezia* are also associated with a number of skin disorders including pityriasis versicolor, folliculitis, seborrheic dermatitis, dandruff, and atopic dermatitis (AD) (Saunders et al., 2012). Recently, it was found that M*alassezia restricta* is associated with the colonic mucosa in a subset of patients with Chrohn's disease who have a disease-linked polymorphism in *CARD9* (Limon et al., 2019). This observation is interesting in a host-microbe perspective indicating that host genetic factors may increase colonization with *Malassezia* and the inflammatory response.

For many years, we have utilized *Malassezia sympodialis,* the fungi most frequently colonizing the skin of both AD patients and healthy individuals in our part of the globe (Sandstrom Falk et al., 2005), as a model to investigate host-microbe interactions. We have discovered that *M. sympodialis* releases EVs, which we designated MalaEx (Gehrmann et al., 2011). These EVs are compared to their parental *M. sympodialis* whole yeast cells enriched in 110 proteins, among those two of the *M. sympodialis* allergens, Mala s 1 and Mala s 7 (Johansson et al., 2018), and they can induce inflammatory cytokine responses with a significantly higher IL-4 production in peripheral blood mononuclear cells (PBMC) from patients with AD sensitized to *Malassezia* compared to healthy controls (Gehrmann et al., 2011). Thus, MalaEx seem to play a contributing role in eliciting and maintaining eczema in patients with AD. Furthermore, we have in MalaEx detected several small RNAs with well-defined start and stop positions in a length range of 16–22 nucleotides (Rayner et al., 2017), suggesting their capacity to deliver either autocrine or paracrine signaling for *M. sympodialis* in the interplay with the host and the environment.

The first major cell population *Malassezia* interacts with on the skin is epidermal keratinocytes. Besides forming an effective mechanical barrier to the outer environment keratinocytes are also active components of the immunoregulatory network in the skin (Di Meglio et al., 2011). Keratinocytes produce and express several mediators, in response to outer signals and transmit those to immune cells in the skin thereby regulating skin immunity and inflammation (Pasparakis et al., 2014). Whole yeast cells from different *Malassezia* species have been found to induce release of a variable profile of inflammatory mediators by keratinocytes (Watanabe et al., 2001; Ishibashi et al., 2006; Donnarumma et al., 2014). Previously, we discovered an active binding and internalization of MalaEx into human keratinocytes, where MalaEx were mainly found in close proximity of the keratinocyte nuclei, suggesting a central communication with the host cell (Johansson et al., 2018). In this study, we explored the host-microbe interaction between MalaEx and human keratinocytes with the hypothesis that MalaEx might be able to activate human keratinocytes to express the intercellular adhesion molecule-1 (ICAM-1, CD54).

MATERIALS AND METHODS

Malassezia sympodialis Culture Conditions

M. sympodialis (ATCC 42132) was cultured on Dixon agar plates (Gueho et al., 1996) modified to contain 1% (vol/vol) Tween 60, 1% (wt/vol) agar, and no oleic acid (mDixon) at 32°C. After 2 or 4 days, the yeast cells were harvested, re-suspended and washed in PBS. Counting in a Bürker chamber using trypan blue exclusion showed a viability above 95%. From the 4 days cultures 6×10^7 live yeast cells/ml were added to RPMI 1640 medium supplemented with penicillin 100 units/ml, streptomycin 100 μg/ml, 2 mM L-glutamine, and 10% heat inactivated fetal calf serum (all from Gibco BRL, Life Technologies Ltd, Paisley, UK) and incubated for 48 h in 6% CO_2 at 37°C, as previously described (Gehrmann et al., 2011). Prior usage, fetal calf serum had been ultra centrifuged overnight at 100 000 × g followed by filtration through a 0.22 μm filter (Nordic Biolabs, Täby, Sweden) to remove possible EV contaminants. Each culture batch consisted of 320 ml distributed over four 175 cm^2 flasks (Falcon, Corning Inc., Tewksbury, MA, USA). At each culture step blood and Sabouraud agar plates were inoculated in parallel to exclude bacterial and *Candida* contaminations, respectively.

MalaEx Preparation

MalaEx were prepared from the 48 h *M. sympodialis* culture supernatants by using a combination of centrifugation, filtration, and serial ultracentrifugation. The culture supernatants were spun at 1 200 × g for 5 min followed by 3 000 × g for 30 min, and thereafter filtered through a 0.22 μm filter (Nordic Biolabs) and frozen to −80°C until further preparation. After thawing at RT, supernatants were centrifuged at 10 000 × g for 30 min. Thereafter, MalaEx were pelleted from the supernatants at 100 000 × g for 90 min, re-suspended in PBS and pelleted again at 100 000 × g for 90 min. The resulting pellet was carefully re-suspended in 100 μl PBS and stored frozen at −80°C. To avoid batch variations and to obtain enough MalaEx for the stimulation experiments (see below) three MalaEx preparations were pooled. The protein content was measured using a detergent compatible (DC) protein assay according to the manufacturer's instructions (BioRad, Hercules, CA, USA).

Nanoparticle-Tracking Analysis (NTA)

The particle size and concentration of the pooled MalaEx preparation was measured using a LM10 platform with sCMOS camera from NanoSight Ltd, Amesbury, UK. The system is equipped with a 405 nm laser running nanoparticle tracking analysis (NTA) 2.3 analytical software package. The samples were diluted 3,000× in 30 kDa filtered PBS and analyzed with camera level 14 and detection threshold 7. Four consecutive videos were recorded in RT while injecting the sample with a syringe pump (speed 50). The result is expressed as the mean particle size ± SD and the particle concentration of the four separate NTA runs of the pooled MalaEx sample.

Preparation of Human Primary Keratinocytes

Epidermal keratinocytes were isolated according to the manufacturer's instructions (Gibco Invitrogen Corporation, Paisley, UK) from human abdomen *ex vivo* skin received from a local plastic surgery clinic. In short, thin skin tissues were prepared by using a dermatome, which thereafter were incubated in a Dispase solution (25 caseinolytic units/ml; Gibco Invitrogen Corporation) for 18 h at 4°C. Epidermis was separated from dermis and placed into 0.05% Trypsin-EDTA (Gibco Invitrogen Corporation) for 15 min at 37°C for cell dissociation. After addition of Soybean Trypsin Inhibitor (Gibco Invitrogen Corporation) at a concentration of 10 mg/ml, cells were pelleted, washed and suspended in complete serum-free keratinocyte medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.5 μg/ml Amphotericin B (Gibco Invitrogen Corporation). Approximately 3×10^6 cells, with a viability above 95% using trypan blue exclusion, were seeded in 15 ml culture medium in each T-75 culture flask (Falcon®, Corning Life Sciences, Tweksburry, MA, USA) and cultured at 37°C, 6% CO_2. The culture medium was replaced every second or third day until the cell confluence reached ∼75%, within 19–28 days depending on skin donor. The cells were then detached with 0.05% Trypsin-EDTA treatment for 5 min at 37°C, followed by Soybean Trypsin Inhibitor 10 mg/ml, washed in culture medium, and thereafter $\sim 5 \times 10^6$ cells in 2 ml per vial were frozen using 10% DMSO and 50% heat inactivated fetal calf serum depleted from EV by ultracentrifugation and stored at −150°C.

Co-cultures of *M. sympodialis* or MalaEx With Human Primary Keratinocytes

After thawing in a 37°C water bath for 1 min, the keratinocytes were transferred to a 50 ml tube and 18 ml of culture medium was slowly added. Thereafter, the keratinocytes were spun down, 180 × g for 7 min, and re-suspended in culture medium. Approximately 3×10^6 cells, viability 80–90%, were seeded in 15 ml culture medium in each T-75 culture flask and cultured as above until the cell confluence, after 7–14 days, had reached ∼75%. The cells were detached with 0.05% Trypsin-EDTA (see above), counted and seeded into μ-slides with 8 wells, 1 cm^2 growth area per well, with a glass coverslip bottom (Cat. No. 80827, Ibidi, Martinsried, Germany). 0.6×10^5 keratinocytes in 0.35 ml culture medium were seeded into each well. The μ-slides were incubated for 2–4 days at 37°C, 6% CO_2 until 75% confluency was reached. Thereafter, the culture medium was removed and replaced with 0.35 ml fresh medium, and the stimulation agents were added to the keratinocytes; *M. sympodialis* (0.6×10^5 and 3×10^5 live yeast cells/well) harvested from the mDixon agar plates after 2 days of culture and the pooled MalaEx (1, 10, and 50 μg/ml). Lipopolysaccharides (LPS) (10 μg/ml, L8274, Sigma-Aldrich, Steinheim, Germany) was used as a positive control (Marcatili et al., 1997) and keratinocytes cultured in only the medium as a negative control. The cultures were incubated at 37°C, 6% CO_2 for 24 h. The cells in the μ-slides were used for further analyses (see below).

Confocal Laser-Scanning Microscopy (CLSM)

The keratinocytes co-cultured with *M. sympodialis*, MalaEx or LPS, or cultured alone in the 8 well μ-slides with a glass coverslip bottom were directly fixed in 4% formaldehyde for 10 min, treated with 0.5% Triton-X-100 (BDH Laboratory Supplies, Poole, UK) for 5 min, and blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) for 5 min at RT. Thereafter, Alexa Fluor 488 mouse anti-CD54/ICAM-1 or Alexa Fluor 488 IgG_1 isotype control (Biolegend, San Diego, CA, USA), both at 1:20 dilution in 5% BSA, were added for 1 h at RT. The glass coverslip bottoms were finally covered with Prolong Gold antifade mountant (Invitrogen, Thermo Fisher Scientific, MA, USA). Fluorescent images as z-scans and phase contrast images were acquired on a CLSM (TCS SP2; Leica Microsystems, Mannheim, Germany). Each florescent image was combined with each corresponding phase contrast image. Confocal images were used to manually calculate the % of ICAM-1 positive keratinocytes defined as strongly positive. For each culture condition 100 cells were analyzed.

Statistical Analysis

Statistical differences of stimulated keratinocytes compared to unstimulated was assessed by paired *t*-test using GraphPad Prism software (GraphPad Software, Inc. https://www.graphpad.com/scientific-software/prism/). Data are expressed as mean ± standard error of the mean (SEM). Differences were considered significant when $p < 0.05$.

RESULTS

Characterization of MalaEx

The pooled batch of three MalaEx batches harvested from 48 h *M. sympodialis* culture supernatants had a protein content of 1.45 mg/ml. NTA analysis indicated that the MalaEx had a size range of 70–580 nm, with a mean of 154 nm ($n = 4$ video recordings) and the total particle concentration was 1.19×10^{13} particles/ml (**Figure 1**).

MalaEx Induce ICAM-1 Expression of Keratinocytes

Co-cultures of keratinocytes with MalaEx was performed for 24 h on μ-slides with 8 wells and a glass coverslip bottom enabling direct analysis with CLSM. Unstimulated keratinocytes showed

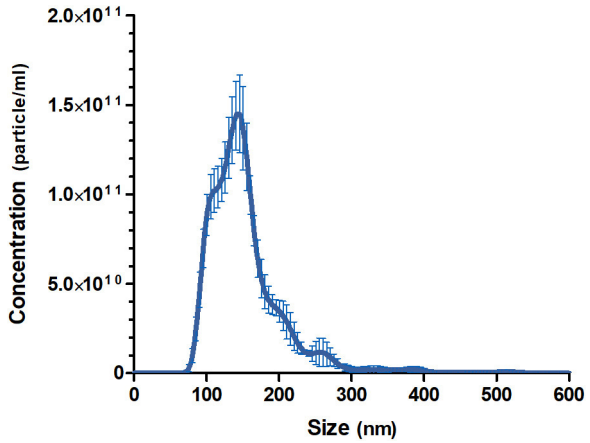

FIGURE 1 | Size distributions and particle concentration of the pooled MalaEx preparation. Three MalaEx preparations were pooled and the pooled sample was investigated by nanoparticle tracking analysis (NTA) using NanoSight. The mean particle size and the particle concentration of four separate NTA runs of the pooled MalaEx sample are plotted. Error bars represent SD.

that around 2% of the cells had a strong expression of ICAM-1 (**Figure 2**). MalaEx were found to induce an intense ICAM-1 expression on around 5–22% of the keratinocytes from the three different skin donors with a significant difference for the higher concentration of MalaEx, 10 μg/ml (mean ± SEM, 13.7 ± 1.8%, $p = 0.026$), as did the positive LPS control (mean 26 ± 3.6%, $p = 0.024$), compared to the unstimulated keratinocytes (**Figure 2**). We also tested addition of 50 μg/ml of MalaEx without any additional increase in ICAM-1 expression on the keratinocytes (data not shown). Co-culture with *M. sympodialis* whole yeast cells (0.6×10^5 cells/well) resulted in an upregulation of ICAM-1 expression to 8–15% of the keratinocytes from two of the donors; the donor who did not respond was the same who had a low response to 1 μg/ml of MalaEx (**Figure 2**). The 5-fold higher concentration, 3×10^5 live yeast cells/well, gave similar results (data not shown).

DISCUSSION

The microbial flora on the skin constantly interact with the host skin barrier including immune competent cells in a complex manner influencing local and systemic immunity (Pasparakis et al., 2014). In the current study, we addressed whether nanovesicles, MalaEx, released by a commensal yeast on the skin, *M. sympodialis,* have capacity to modify keratinocytes. We found that MalaEx can activate keratinocytes to increase the expression of ICAM-1.

The MalaEx preparation harvested by a combination of centrifugation, filtration, and serial ultracentrifugation from supernatants from *M. sympodialis* had a size range of 70–580 nm in diameter, with a mean of 154 nm, similar to our previous reports on MalaEx (Rayner et al., 2017; Johansson et al., 2018). Our data are comparable with other studies on fungal EVs considering that different protocols for isolation of EVs and the various methods used for their analysis contribute to variations in reported sizes of fungal EVs (see Table 1 in Bielska and May, 2019). With cryo-electron tomography we have previously demonstrated that MalaEx have different sizes and are morphological diverse with varying electron-dense material suggesting different amount of internal content (Johansson et al., 2018). The heterogeneity of EVs most likely reflects distinct mechanisms of biogenesis, release, and functions (Soares et al., 2017). Our previous observation that MalaEx were mainly found in close proximity of the keratinocyte nuclei after internalization in keratinocytes, using super-resolution fluorescence 3D-SIM imaging, suggested a microbe-host communication (Johansson et al., 2018). We therefore in the present study investigated the expression of the activation marker ICAM-1 on the keratinocytes after co-cultures with MalaEx.

ICAM-1 is the best characterized inducible adhesion molecule on epithelial cells and is a counter ligand for the leukocyte integrins lymphocyte function-associated antigen-1 (LFA-1) and macrophage-1 antigen (Mac-1) (Smith et al., 1989). In the skin enhanced ICAM-1 expression on keratinocytes precedes dermal T lymphocytic infiltration (Griffiths and Nickoloff, 1989), and treatment with monoclonal antibodies to ICAM-1 or LFA-1 inhibits cell infiltration in contact sensitivity reactions in sensitized mice (Scheynius et al., 1993). We here found that only around 2% of the keratinocytes had a strong expression of ICAM-1 in the unstimulated cultures, in agreement with low constitutive expression of ICAM-1 on human keratinocytes (Dustin et al., 1988). MalaEx were capable to induce a 6-fold increase of strong ICAM-1 expression on primary human keratinocytes, and two of the keratinocyte donors responded also to *M. sympodialis* whole yeast cells with increased ICAM-1 expression compared to the unstimulated keratinocytes (**Figure 2**). Inducible ICAM-1 expression on human keratinocytes is highly variable between different donors (Middleton and Norris, 1995), which can explain why one donor, nr 2, did not respond to the concentration *M. sympodialis* whole yeast cells used, nor to the lower concentration of MalaEx (**Figure 2**). Further studies will explore the donor variation in response to MalaEx.

Increased ICAM-1 expression on human keratinocytes can be induced by LPS (Marcatili et al., 1997), IFN-ɤ(Dustin et al., 1988), TNF-α, and ultraviolet radiation (Krutmann et al., 1990). We could detect significantly increased ICAM-1 expression on the keratinocytes when the keratinocytes were co-cultured with MalaEx or LPS compared to the unstimulated keratinocytes (**Figure 2**). The kinetics and molecular mechanisms underlying the MalaEx induced upregulation of ICAM-1 expression on the keratinocytes are presently unclear and also whether other EVs (Bielska et al., 2019), have similar capacity. A recent review article highlights that our knowledge of the comparability of EVs in connecting different kingdoms is limited (Woith et al., 2019). Regarding capacity to participate in an allergic immune response we have previously, however, demonstrated that *M. sympodialis* released MalaEx can carry allergens similar to human dendritic or B-cell-derived exosomes and that all three types of EVs could induce Th2-like cytokine responses in blood cells from allergic donors (Admyre et al., 2007; Gehrmann et al., 2011; Vallhov et al., 2015).

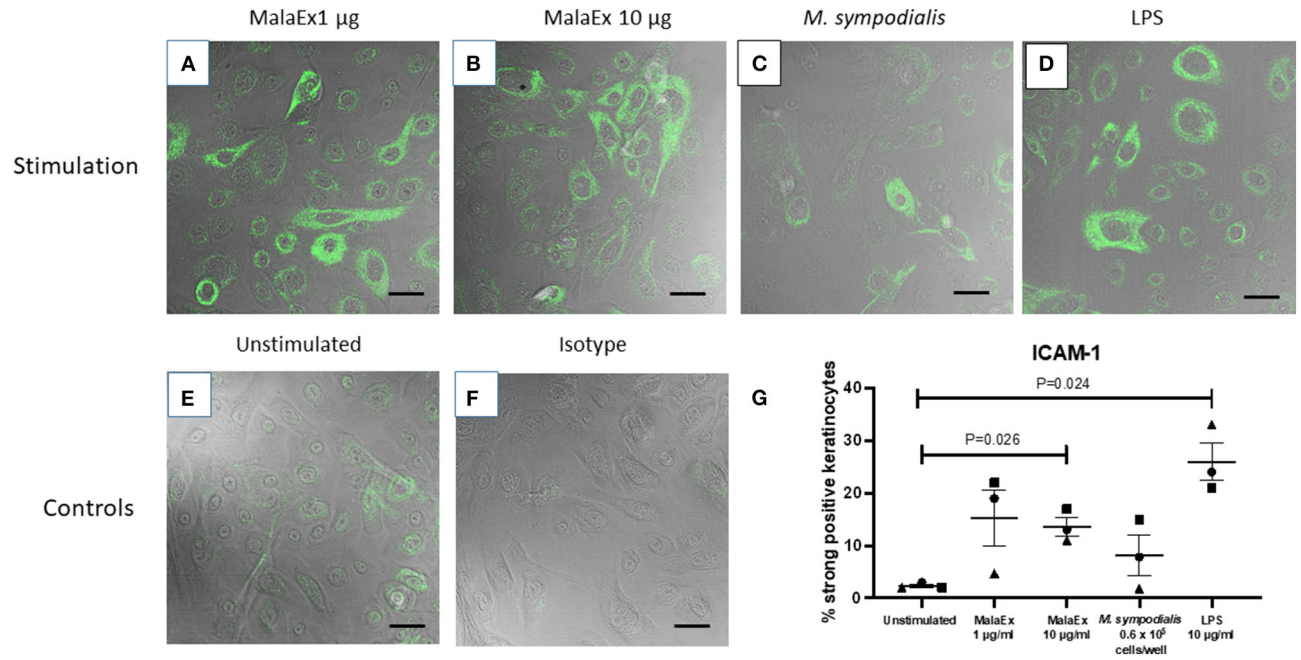

FIGURE 2 | ICAM-1 expression of keratinocytes co-cultured with MalaEx. **(A–F)** Confocal microscopy with overlapping fluorescence and phase contrast images showing ICAM-1 expression using Alexa Fluor 488 mouse anti-CD54/ICAM-1 (green) of keratinocytes from donor 1 cultured for 24 h at 37°C with MalaEx at **(A)** 1 µg/ml, **(B)** 10 µg/ml, and **(C)** with *M. sympodialis* (0.6 × 10^5 yeast cells/well), **(D)** with LPS (10 µg/ml) as a positive control, or **(E)** cultured alone. **(F)** Keratinocytes cultured alone where the isotype control Alexa Fluor 488 IgG_1 has replaced the anti-ICAM-1 antibody. Size bars are 40 µm. **(G)** The % of ICAM-1 positive keratinocytes defined as strongly positive, after cultured alone or co-cultured with MalaEx (1 or 10 µg/ml) or *M. sympodialis* (0.6 × 10^5 yeast cells/well) for 24 h at 37°C. LPS (10 µg/ml) was used as a positive control. Results are presented from three different keratinocyte donors (● Donor 1, ▲ Donor 2, and ■ Donor 3) as mean and standard error of the mean (SEM). Statistically significant differences, as calculated with paired *t*-test, are indicated in the figure.

A strength in the present study is the decision to mimic the situation on human skin as much as possible. We therefore used human primary keratinocytes from different donors, all in early passage. Cell lines, can behave considerable different compared with primary keratinocytes (Middleton and Norris, 1995), which is also the case for murine keratinocytes (Grone, 2002), and animal models with anatomical and immunological differences compared with humans (Di Meglio et al., 2011). At the same time our decision was a challenge, since the number of skin donors was limited, they were to us anonymous, and variations between human donors is expected (Middleton and Norris, 1995). For a more *in vivo* like situation, we also included the mixture of different MalaEx released into the supernatants of *M. sympodialis*, all assumed to be present on the skin. To obtain enough MalaEx we made a pool to avoid bias between different batches. Another strength is that the co-cultures of keratinocytes with MalaEx was performed on µ-slides with a glass coverslip bottom allowing direct analysis with CLSM, whereby *in vitro* manipulations to detach and separate the keratinocytes for further analysis were avoided. A limitation is, however, the use of mono-layer cultures of keratinocytes not reflecting the structure with distinct different layers of keratinocytes and appendages in normal skin. In future studies, human skin explants could be considered as a suitable model for superficial fungal infections (Corzo-Leon et al., 2019).

The ratio of *Malassezia* cells or their EVs to the number of keratinocytes in different *in vitro* studies are difficult to compare and relate to the complex *in vivo* situation in healthy vs. inflammatory skin conditions. To unravel the presence and biological significance of different EVs *in vivo* detailed studies are needed requiring development of new technologies (Coelho and Casadevall, 2019; Margolis and Sadovsky, 2019). In agreement with our observation that keratinocytes can be activated by fungal EVs *in vitro* is a study where EVs from the dermatophyte *Trichophyton interdigitale* induced the release of proinflammatory mediators by the human keratinocyte line HaCat after 24 co-culture (Bitencourt et al., 2018). Recently, EVs from *Malassezia furfur* were found capable to stimulate IL-6 production in HaCaT cells and mice epidermal keratinocytes (Zhang et al., 2019), strongly supporting our results that *Malassezia* EVs, not only their parental whole yeast cells (Watanabe et al., 2001; Ishibashi et al., 2006; Donnarumma et al., 2014), have the capacity to activate keratinocytes. Furthermore, they presented promising *in vivo* evidence in a mouse model showing that EVs from *M. furfur* topically applied could penetrate the skin and induce IL-6 expression on the keratinocytes as analyzed with immunohistochemical staining on skin sections (Zhang et al., 2019). Notably, they also confirmed our observation (Johansson et al., 2018) on internalization of EVs by keratinocytes and their perinuclear distribution pattern (Zhang et al., 2019).

Potential beneficial effects to harbor the commensal yeast *Malassezia* on the skin should not be neglected to elucidate. In that line, a unique secreted as partyl protease produced by *Malassezia globosa,* MgSAP1, was discovered to hinder *Staphylococcus aureus* biofilm formation, an established virulence attribute of *S. aureus* (Li et al., 2018). This study invites for investigations if fungal EVs have a role in release and transport of such molecules in microbial interactions and their possible beneficial effects on the host (Ianiri et al., 2018).

CONCLUSIONS

In conclusion, our data show that *M. sympodialis* released nanosized MalaEx are able to activate human keratinocytes with an enhanced ICAM-1 expression. This implies a possible cross-kingdom modulation of host cells where activation of keratinocytes by MalaEx may be an important first step in cutaneous defense to *M. sympodialis*. How this modulation of host cells by a fungus is balanced between the commensal, pathogenic or beneficial states on the skin in the interplay with the host needs to be further elucidated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study was approved by the regional ethical review board in Stockholm (2015/2082-31/1). Written informed consent was obtained at Strandkliniken, a local plastic surgery clinic in Stockholm, from all subjects donating skin. The donors were anonymous to the recipients of the skin. All experiments were performed in accordance with the Helsinki Declaration ethical principles for medical research.

AUTHOR CONTRIBUTIONS

HV, CJ, and AS conceptualized and designed the study. HV and CJ performed the co-culture experiments. RV did the NTA analysis. CJ worked on the statistical analysis. AS wrote the manuscript together with HV. CJ and RV wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Malassezia Yeasts in Veterinary Dermatology: An Updated Overview

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Lipophilic yeasts of the genus *Malassezia* are important skin commensals and opportunistic skin pathogens in a variety of animals. The species *M. pachydermatis* was first isolated from the skin of a captive Indian rhinoceros with an exfoliative dermatitis in 1925, recognized as an important otic pathogen of dogs in the 1950's, and finally accepted, after several years of controversy, as a common cause of canine dermatitis in the 1990's. Since then, there has been considerable research into the biology of *Malassezia* yeasts and their interaction with their animal hosts. In dogs and cats, *M. pachydermatis* is associated with ceruminous otitis externa and a "seborrhoeic" dermatitis, wherein pruritic, erythematous skin lesions, often with brown/black greasy, malodourous material matting hairs, preferentially develop in intertriginous areas. Skin disease is favored by folds, underlying hypersensitivity disorders, endocrinopathies, defects of cornification, and in cats, various visceral paraneoplastic syndromes. Diagnosis is based on detecting the yeast in compatible skin lesions, usually by cytology, and observing a clinical and mycological response to therapy. Treatment normally comprises topical or systemic azole therapy, often with miconazole—chlorhexidine shampoos or oral itraconazole or ketoconazole. Management of concurrent diseases is important to minimize relapses. Historically, wild-type *Malassezia* isolates from dogs and cats were typically susceptible to azoles, with the exception of fluconazole, but emerging azole resistance in field strains has recently been associated with either mutations or quadruplication of the *ERG11* gene. These observations have prompted increased interest in alternative topical antifungal drugs, such as chlorhexidine, and various essential oils. Further clinical trials are awaited with interest.

Keywords: *Malassezia*, dog, cat, diagnosis, treatment

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INTRODUCTION

The genus *Malassezia* is comprised of a group of lipophilic yeasts that have evolved as skin commensals and opportunistic cutaneous pathogens in a variety of mammals and birds (Guého-Kellerman et al., 2010). The transition from commensal to pathogen is frequent in dogs in particular, and in cats to a lesser extent, such that cases of *Malassezia* otitis externa and *Malassezia* dermatitis are commonly presented to veterinarians in small animal practice (Moraru et al., 2019). For example, the prevalence of otitis externa amongst dogs presenting to primary care practices is around 10% (O'neill et al., 2014), and up to 70% of such cases may be associated with *M. pachydermatis* (Forster et al., 2018). These cases are seldom straightforward to manage, because clinical disease often reflects yeast proliferation due to a disturbance in the normal homeostatic

balance of host immunity, on the one hand, and yeast virulence, on the other (Ashbee and Bond, 2010). Thus, successful case management is often dependent upon both treating yeast (and any concurrent bacterial) overgrowth with topical or systemic antimicrobial treatments, as well as identifying and correcting where possible, predisposing factors. Commonly identified factors include concurrent allergic or endocrine skin disease, defects in cornification, or anatomical defects such as skin folds or stenosed ear canals (Bond et al., 2010). By contrast, there are only sporadic reports of *Malassezia* associated with skin disease in large animal species. For example, *Malassezia* overgrowth in the intermammary region and preputial fossa has been implicated in tail-head pruritus and localized dermatitis in horses (White et al., 2006). Goats may present with *Malassezia*-associated seborrheic dermatitis (Pin, 2004; Eguchi-Coe et al., 2011). *Malassezia* otitis has been reported in fennec foxes (Guillot et al., 1994), ferrets (Dinsdale and Rest, 1995), pigs (Pinter et al., 2002), and camels (Kuttin and Glas, 1985).

Recently, the World Association of Veterinary Dermatology commissioned the development of clinical consensus guidelines for the diagnosis and treatment of *Malassezia* dermatitis in dogs and cats (Bond et al., 2020). A panel of specialists/diplomates in veterinary dermatology and mycology prepared a detailed literature review of publications up to mid 2018, and made recommendations on selected topics. The draft document was presented at international veterinary meetings and uploaded on the WAVD website for comment for a period of 6 months. The final version comprised a systematic review of published therapeutic studies, and current information on the ecology, pathophysiology, diagnosis, and prevention of skin diseases associated with *Malassezia* yeasts in dogs and cats.

In view of the free access availability of this recent very detailed and wide-ranging review (Bond et al., 2020), the present article aims to provide the reader with a general background summary of current understanding of the roles of *Malassezia* yeasts in animal skin disease, with emphasis on recent publications from mid 2018 to end of 2019 that expand upon previous knowledge.

THE GENUS *MALASSEZIA*: A GROWING NUMBER OF SPECIES IN A GROWING NUMBER OF ANIMAL HOSTS

Originally thought as a single species, *Malassezia* yeasts are now known to form a unique cluster consisting of 18 species living almost exclusively on the skin and mucosal sites of warm-blooded vertebrates (Lorch et al., 2018; Theelen et al., 2018) (**Table 1**). During the last decade, the analysis of the genome of *Malassezia* yeasts suggested that their ancestors were plant or soil fungal residents which progressively managed to survive and develop in the cutaneous ecosystem (Xu et al., 2007). The genus *Malassezia* (Baillon) was created in 1889 for a single species, *M. furfur*, detected in cutaneous lesions in humans (Baillon, 1889). Weidman was the first scientist to detect *Malassezia* yeasts from the skin of an animal, an Indian rhinoceros (*Rhinoceros unicornis*) with a generalized exfoliative dermatitis (Weidman, 1925). In contrast to *M. furfur* these yeasts cultivated readily on routine media without lipid supplementation. *Malassezia* yeasts were further detected from different warm-blooded vertebrates and several specific names were proposed according to the latin names of the animals on which the yeasts were initially isolated: *M. pachydermatis* (from a rhinoceros within Pachydermata, an obsolete nineteenth-century taxonomic order of mammals) (Weidman, 1925), *M. caprae* from goats (Cabanes et al., 2007), *M. equina* from horses (Cabanes et al., 2007), *M. cuniculi* from rabbits (Cabanes et al., 2011), *M. pstittaci* from parrots (Cabanes et al., 2016), and very recently *M. vespertilionis* from bats (Lorch et al., 2018). Some *Malassezia* yeasts (especially *M. pachydermatis*) appear to have a broad host range, while others are more host-specific with a close adaptation to the cutaneous ecosystem of a single animal species or a group of phylogenetically related animals (**Table 1**). The number of currently described *Malassezia* species ($n = 18$) is likely limited due to a sampling bias toward humans and domestic animals. We can imagine that the number of *Malassezia* species will increase when the skin microbiota of a broader range of wild animals is investigated.

Very recently, Lorch et al. were able to isolate a new *Malassezia* species from the skin of nine species of bats in the subfamily

TABLE 1 | *Malassezia* species and main mammalian hosts.

Malassezia species	Synonyms	Presence on healthy skin	Presence in lesions
M. furfur	*Pityrosporum ovale*	In humans Sometimes in animals	In humans (PV, FG)
M. pachydermatis	*P. pachydermatis*, *P. canis*	In dogs, cats, many others (mostly canids) Sometimes in humans (dog contact)	In dogs, cats, others (SD, OT) Sometimes in humans (FG)
M. sympodialis	*M. furfur* serovar A	In humans and animals	In humans (AD, SD) Sometimes in cats (OT)
M. globosa	*P. orbiculare* *M. furfur* serovar B	In humans and animals	In humans (PV, SD, AD) Sometimes in cats (OT)
M. obtusa		In humans	In humans
M. slooffiae		In pigs, cats (claws) In humans	In humans
M. restricta	*M. furfur* serovar C	In humans	In humans (SD)
M. dermatis		In humans	In humans (AD)
M. japonica		In humans	In humans (AD, SD)
M. nana		In cats, horses	In cats, cattle (OT)
M. yamatoensis		In humans	In humans (SD)
M. caprae		In goats	
M. equina	*M. equi*	In horses	In horses
M. cuniculi		In rabbits	
M. arunalokei		In humans	In humans
M. brasiliensis		In parrots	–
M. psittaci		In parrots	–
M. vespertilionis		In hibernating bats	–

–, not reported; PV, pityriasis versicolor; FG, fungaemia; AD, atopic dermatitis; SD, seborrheoic dermatitis; OT, otitis.

Myotinae in eastern and western United States (Lorch et al., 2018). Physiological features and molecular characterization at seven additional loci clearly demonstrated that all of the bat *Malassezia* isolates represented a single and new species which was designated as *M. vespertilionis*. Among other characteristics, the new species is able to grow over a broad range of temperatures (7–40°C), with optimal growth occurring at 24°C. The authors suggested that the wide thermal growth range may be an adaptation to survival on bat skin during both hibernation and active seasons (Lorch et al., 2018).

RECENT RE-ASSESSMENTS OF LIPID-DEPENDENCE IN *MALASSEZIA PACHYDERMATIS*

Malassezia species are lipid dependent due to an inability to synthesize long-chained (C14 or C16) fatty acids *de novo* (Shifrine and Marr, 1963). There are some differences in lipid dependence among the species and this variability has been used for the development of specific tests for their identification (Guillot et al., 1996). Historically *M. pachydermatis* was regarded as being "lipophilic but not lipid-dependent" because it was the only member of the genus to grow on Sabouraud's dextrose agar (Guillot and Bond, 1999). Recently, genome sequencing has confirmed that *M. pachydermatis* lacks a fatty acid synthase gene like the other members of the genus (Wu et al., 2015), but is uniquely able to utilize lipid fractions within the peptone component of Sabouraud's dextrose agar for growth (Puig et al., 2017). These observations explain its failure to grow on lipid-free defined media and thus *M. pachydermatis* should now also be regarded as being "lipid-dependent" (Puig et al., 2017).

ECOLOGY OF *MALASSEZIA* YEASTS IN DOGS AND CATS: COMPLEMENTARY AND CONFLICTING RESULTS FROM TRADITIONALLY CULTURE-BASED STUDIES AND MORE RECENT MOLECULAR INVESTIGATIONS

To better understand the ecology of *Malassezia* yeasts on healthy skin and in cases of cutaneous lesions, culture-based studies have been carried out both in humans (Gaitanis et al., 2012) and in animals, especially in dogs (reviewed by Bond et al., 2020). Results vary between studies because of the use of different sampling procedures, culture media, and identification techniques. However, culture-based studies clearly demonstrate that *M. pachydermatis* is the predominant cutaneous yeast in both healthy dogs and dogs with *Malassezia* dermatitis or otitis (Gustafson, 1955; Dufait, 1985; Hajsig et al., 1985; Bond and Lloyd, 1997). *M. pachydermatis* is also most important in cats but in this host other *Malassezia* species are more frequently detected (Hajsig et al., 1990; Hirai et al., 2004; Åhman et al., 2007a; Åhman and Bergstrom, 2009; Volk et al., 2010). Several investigators explored *Malassezia* colonization in various anatomical regions of different breeds of adult healthy dogs (reviewed by Bond et al., 2020). The general conclusion from these studies is that the perioral region and interdigital skin is frequently colonized (up to 80%) by *M. pachydermatis* in healthy dogs of various breeds, whereas the yeast is less-often (<25%) detected on the skin of the axilla, groin and dorsum. The skin of cats may be colonized by several *Malassezia* species. Whilst *M. pachydermatis* remains most prevalent, as in dogs, the lipid-dependent species isolated from cats include *M. sympodialis*, *M. globosa*, *M. furfur*, *M. nana*, and *M. slooffiae* (reviewed by Bond et al., 2020). *M. nana* is the most common lipid-dependent species in cats, particularly in the ear canal, and a particular *M. nana* genotype seem to predominate in this animal host (De Bellis et al., 2009; Castella et al., 2011). *M. slooffiae* is primarily but not exclusively isolated from claw folds in cats (Åhman et al., 2007a).

Recently, methods based on next generation sequencing (NGS) have allowed a better characterization of the complex microbial communities occurring on animal skin and made it possible to detect *Malassezia* species that would otherwise be missed using culture-based methods (Meason-Smith et al., 2017, 2019; Korbelik et al., 2018; Older et al., 2019). Meason-Smith et al. reported that the cutaneous mycobiota in dogs was influenced by various factors including environmental exposure, cohabitation with other pets and skin health status (Meason-Smith et al., 2015). Surprising, *Malassezia* yeasts were not the most abundant fungal organisms on healthy canine skin. Furthermore, these authors were unable to detect any significant differences in the relative abundance of *Malassezia* yeasts between healthy and allergic dogs. The discrepancy between NGS results and culture-dependent studies demonstrating increased populations of *M. pachydermatis* in allergic dogs (Bond et al., 1994; White et al., 1998) may be related to differences in methodology. Another explanation would be that dysbiosis is present at *Malassezia* species level (rather that at *M. pachydermatis* abundance) in allergic dogs. This hypothesis was very recently investigated by Meason-Smith et al. who collected skin samples from healthy, naturally affected allergic, and experimentally sensitized atopic dogs (Meason-Smith et al., 2019). Using NGS (at species level classification) and *Malassezia* species-specific quantitative real-time PCR (qPCR), they demonstrated that *M. globosa* was significantly more abundant on healthy canine skin (by both methods), *M. restricta* was significantly more abundant on healthy skin (by NGS), and *M. pachydermatis* was significantly more abundant on naturally-affected allergic skin (by NGS) and on allergen-induced atopic skin lesions (by qPCR).

The NGS method was also recently applied to better understand the mycobiota in the external ear canal of dogs (Korbelik et al., 2018). Samples were collected from six dogs with otitis externa and five clinically normal dogs. In cases of otitis externa, the mycobiota was largely dominated by *Malassezia* yeasts. Fungal species diversity, richness and evenness were all significantly reduced in samples from otitis externa when compared to healthy ears.

In cats, metagenomic analyses suggested that the skin is inhabited by bacterial communities that are distinct to each body site (Older et al., 2017) whereas fungal communities seem more unique to the individual level (Meason-Smith et al., 2017). When samples from healthy and allergic cats were collected, the

most abundant fungal sequences were identified as filamentous contaminants from the environment and not *Malassezia* yeasts, which were identified in 30 and 21% of healthy and allergic cat samples, but rarely accounted for more than 1% of the relative fungal abundance (Meason-Smith et al., 2017). The objectives of the recent study from Older et al. were to evaluate how genotype and environment can influence the bacterial and fungal microbiota of feline skin (Older et al., 2019). Using NGS and *Malassezia* qPCR, they demonstrated that *M. restricta* and *M. globosa* were the most prevalent *Malassezia* species. Sequences corresponding to *M. slooffiae*, *M. furfur*, *M. nana*, *M. pachydermatis*, *M. dermatis*, *M. sympodialis*, *M. japonica*, *M. obtusa*, and *M. yamatoensis* were also detected. *Malassezia* abundance was significantly different between cat breeds with Devon Rex cats having the highest abundance. No significant difference in abundance of any *Malassezia* species were found between the different cat breeds or when comparing indoor and outdoor animals.

Taken together, these studies demonstrate significant disparity between cultural and molecular studies in defining the *Malassezia* species component of the skin mycobiota in dogs and cats. Importantly in dogs, cultural methods seldom demonstrate *Malassezia* species other than *M. pachydermatis* in both health and disease, even when culture media reputed to support the growth of the more lipid-dependent species such as modified Dixon's agar are utilized (reviewed by Bond et al., 2020). By contrast, molecular techniques indicate the frequent presence of *M. globosa* and *M. restricta*, species that are seldom identified by culture (Meason-Smith et al., 2019), despite years of searching by the present and other authors (Guillot and Bond, 1999). Furthermore, the distinctive morphology of *M. globosa* (Guého-Kellerman et al., 2010) is not systematically observed in clinical cytological specimens from dogs with dermatitis (Bond, R., personal observations). When presented with a clinical case, it may not be strictly necessary for the attending veterinarian to know the species identity of the *Malassezia* causing the disease, provided species variation in drug susceptibility is limited (Tragiannidis et al., 2010). Further developments in both diagnostic and antifungal drug susceptibility testing are urgently required to address these aspects.

PATHOGENESIS OF *MALASSEZIA* DERMATITIS IN DOGS AND CATS: BACKGROUND AND RECENT ADVANCES

There have been significant and recent advances in understanding of the mechanisms of interaction between *Malassezia* yeasts and dogs and cats (reviewed by Bond et al., 2020). The outcome of *Malassezia* growth on the skin (commensal existence or inflammation and disease) is dependent upon the metabolic activities of the yeasts (expression of cell wall and secreted virulence attributes) and the host's innate and adaptive immune defensive responses. Interactions with other skin commensals (especially staphylococci) may also play a role in determining the outcome of colonization in animals, although this area is largely unexplored (Ianiri et al., 2018), especially in dogs and cats. All these processes should ideally result in a delicately balanced homeostatic relationship. The presence of *Malassezia* yeasts within the stratum corneum exposes the host to an array of chemicals, immunogens and allergens, comprising fungal cell wall-associated carbohydrates, proteins and lipids; secreted enzymes that generate both substrates for nutrition, and an array of irritant metabolic by-products (reviewed by Ashbee and Bond, 2010; Sparber and Leibundgut-Landmann, 2017).

In a recent investigation, Czyżewska et al. compared the protein profiles of *M. pachydermatis* isolates from 30 dogs with clinical signs of otitis externa and 34 clinically normal dogs (Czyżewska et al., 2019). The most significant finding was the presence of nicotinamide adenine dinucleotide phosphate (NADP)-dependent mannitol dehydrogenase and ketol-acid reducto isomerase (an enzyme involved in the biosynthesis of branched-chain amino acids) among *M. pachydermatis* isolates obtained from dogs with otitis externa. It is not clear whether these enzymes confer an advantage to the yeast or act as virulence factors (Czyżewska et al., 2019).

Malassezia cell wall carbohydrates are recognized as IgE binding epitopes in humans with atopic dermatitis but recent work highlighted their importance in fungal cell recognition by host phagocytic cells. C-type lectins are proteins that bind carbohydrates in a calcium-dependent manner via highly-conserved carbohydrate-recognition domains (Tada et al., 2006). Mincle, a C-type lectin expressed by activated phagocytes that binds glucosyl and mannosyl-glycolipids from *M. pachydermatis* and *M. sympodialis*, selectively recognizes *Malassezia* yeasts but not other fungi (Yamasaki et al., 2009). Recently, van der Peet et al. reported the total synthesis of a complex β-1,2-mannosyloxymannitol glycolipid from *M. pachydermatis*, which was a potent agonist of human Mincle signaling; these observations may have relevance in the further understanding of antifungal immunity (Van Der Peet et al., 2019). Whilst it is intuitive that similar mechanisms may occur in dogs and cats, species-specific studies are required to verify this.

A recent study suggested that *M. pachydermatis* is able to activate the aryl hydrocarbon receptor (AhR), a nuclear receptor and transcriptional regulator with pleiotropic effects that include down-regulation of immune stimulation, modification of melanogenesis and epidermal cell function, and inhibition of antagonistic microbes (Buommino et al., 2018). Since indole production was not detected in a study of 80 *M. pachydermatis* strains from canine otitis externa, AhR activation by *M. pachydermatis* might be associated with the release of compounds other than indolic metabolites (Kiss et al., 1996).

Experimental models have been used to better understand the pathogenesis of *Malassezia* dermatitis. Cutaneous responses to the application of viable and killed "lipid-dependent" *Malassezia* in laboratory animals (guinea pigs, mice, rabbits) generally comprised focal areas of scaling that most often resolve without treatment upon discontinuation of inoculation (Drouhet et al., 1980; Rosenberg et al., 1980; Faergemann and Fredriksson, 1981; Van Cutsem et al., 1990). Similarly, in laboratory beagle dogs, application of *M. pachydermatis* was associated histologically with epidermal hyperplasia, occasionally with parakeratosis, superficial perivascular dermal inflammation

with primarily neutrophils and lymphocytes, and sometimes mast cells (but not eosinophils); features were more severe at sites that were occluded (Bond et al., 2004). Histological changes markedly reduced within 7 days of withdrawal of yeast challenge. Inoculation of suspensions of *M. pachydermatis* into the middle ear and dermis of immunosuppressed mice led to transient infection that resolved within 21 days (Schlemmer et al., 2018). Recently, Merkel et al. developed a minihost (invertebrate) experimental model wherein the pathogenicity of *M. pachydermatis* was evaluated in wild-type (WT) and Toll-deficient *Drosophila melanogaster*. WT flies were resistant to the infection, whereas Toll-deficient flies showed inoculum-dependent mortality rates. Experimental models may prove valuable in the further elucidation of both yeast virulence and host immune factors that are important in disease processes in various species.

The presence of *Malassezia* yeasts on the skin, both in normal and excessive numbers, is known to activate the skin immune system in dogs and cats. *Malassezia* antigens can stimulate innate, antibody and cell mediated immune responses, as well as triggering hypersensitivity reactions (Bond et al., 2010). In animals in which an overgrowth of organisms has occurred, or in individuals that are predisposed to allergic sensitization, the ensuing inflammatory response can lead to clinical signs such as dermatitis and pruritus. Elevated IgE levels to *Malassezia* yeasts or *Staphylococcus* bacteria in human atopic dermatitis are related to the skin severity index. To assess whether a similar association occurs in dogs, Khantavee et al. investigated levels of allergen-specific IgE, IgG1, and IgG2 directed against *M. pachydermatis* and *S. pseudintermedius*, with total IgG levels, and correlated them with lesion severity in dogs with atopic dermatitis (Khantavee et al., 2019). They reported that specific IgE and total IgG against yeasts and bacteria were significantly increased in atopic dogs of all ages. However, no significant relationships were found between the clinical score and any specific immunoglobulin levels for both microbe types.

PREDISPOSING FACTORS FOR SKIN DISEASE IN DOGS AND CATS

Ideally commensal *Malassezia* yeasts behave as "good citizens" and occupy their ecological niche within the "transitional mantel zone" of the epidermal stratum corneum and follicular infundibulae, influenced by host skin and the external environment (Theelen et al., 2018). Normally, continuous interactions with the host immune system will maintain low numbers of the yeast without generating a clinically-appreciable inflammatory response (Bond et al., 2020). It is well-recognized that *Malassezia* dermatitis in dogs and cats is most often associated with concurrent diseases that are likely associated with altered skin immune function and/or changes in the chemical and micro-climatic conditions at the skin surface (Bond et al., 2020). Thus in dogs, hypersensitivity disorders (especially canine atopic dermatitis), defects of cornification and endocrinopathies are frequently recognized as underlying factors that must be corrected or managed as part of the therapeutic programme (Bond et al., 1996b). Skin folds commonly represent an important local factor in favoring overgrowth by *Malassezia* and or bacteria; this likely reflects local climatic differences involving factors such as reduced air movement, increased skin temperature and humidity, retained secretions, and surface frictional trauma (Jenkinson, 1992). Although not specifically studied in the dog, it is also generally recognized that *Malassezia* dermatitis is more common in tropical climates, and during warm, humid months in more temperate latitudes, reflecting external environmental effects on the skin microbiota (Theelen et al., 2018). This factor is well-documented in human medicine, with warm tropical climates favoring high positive culture rates and greater species diversity (Gaitanis et al., 2012; Leong et al., 2019).

Predisposition to *Malassezia* dermatitis in cats parallels the canine situation in many ways with one important exception. Whilst skin folds and allergic diseases are also commonly identified as factors (Ordeix et al., 2007), feline *Malassezia* dermatitis in older cats is occasionally associated with visceral neoplasia, most commonly feline (pancreatic) paraneoplastic alopecia and thymoma-associated exfoliative dermatitis (Forster-Van Hijfte et al., 1997; Mauldin et al., 2002).

Dog breeds identified to be at increased risk of *Malassezia* dermatitis include West Highland white terriers (WHWT), English setters, shih tzus, basset hounds, American cocker spaniels, boxers, dachshunds, poodles, and Australian silky terriers (Mason, 1992; Plant et al., 1992; Bond et al., 1996b; Mauldin et al., 1997). Devon rex and Sphynx cats, but not Cornish rex cats, are prone to high carriage rates of *Malassezia* yeasts (defined by culture) and a generalized seborrhoeic dermatitis that responds to oral itraconazole (Åhman et al., 2007a,b; Åhman and Bergstrom, 2009; Volk et al., 2010). These breed predilections in dogs and cats are likely analogous to the observed effects of ethnicity on *Malassezia* populations on human skin (Leong et al., 2019).

A recent study that utilized next generation sequencing (NGS) to study fungal populations on skin and mucosae in various cat breeds also reported that Devon rex cats had a high abundance of *Malassezia* species (Older et al., 2019). Interestingly, species-level analyses of the sequences identified *M. globosa* and *M. restricta* as the most abundant *Malassezia* species in the subject cats (Older et al., 2019). In contrast, previous cultural studies typically identify *M. pachydermatis* as the most abundant species in cats, even when media (for example, modified Dixon's agar) and temperatures (32–34°C) considered appropriate for the cultivation of the more demanding species are adopted (Bond et al., 1996a, 1997, 2008; Volk et al., 2010). Further investigation of these discordant results is warranted.

CLINICAL PRESENTATIONS IN DOGS AND CATS

Affected skin is usually erythematous, often with greasy brown-black material matting the lower portion of hairs; intertriginous zones are frequently involved (Bond et al., 2010). Pruritus, whilst ranging from minimal to severe, is normally a dominant feature. Concurrent hyperpigmentation,

lichenification, malodour, traumatic alopecia, and otitis externa is common. In otitis externa, the discharge from the ear canal is commonly ceruminous and rarely purulent, and inflammation commonly extends onto the pinnae. Cases of *Malassezia* paronychia present with claw fold erythema and swelling, waxy or crusty brown exudate, red-brown claw staining, and may co-exist with a wider pododermatitis of haired skin. An occasional presentation of frenzied facial pruritus in dogs with varying, sometimes subtle, erythema of chin / perioral skin, may be misdiagnosed as neurological disease (Mason, 1992, 1993).

The signs of *Malassezia* dermatitis may mimic, or complicate, those of canine atopic dermatitis. Features of concurrent diseases may be evident initially although they are commonly best appreciated once secondary *Malassezia* infection is removed. *Malassezia* dermatitis might feature in cats that present with a phenotype of allergic skin disease, idiopathic facial dermatitis (Persian/Himalayan), feline acne, and serious internal medical disorders such as feline paraneoplastic alopecia and thymoma-associated exfoliative dermatitis (Bond et al., 2010). Client expectation should be managed accordingly; residual skin disease commonly remains despite successful antifungal therapy.

DIAGNOSTIC APPROACH IN THE VETERINARY CLINIC

Following the original elegant description of tape-stripping in human dermatology (Keddie et al., 1961), this method has gained wide acceptance in veterinary clinical practice as a rapid and versatile method for recovering stratum corneum cells and their attendant adherent microbes (Maynard et al., 2011). Light microscopical examination (40–50 or 100x oil objectives) of tape-strips, or dry scrapes, stained with modified Wright Giemsa stain ("Diff-Quik" or generic equivalents) is rapid and convenient for assessment of the presence and numbers of *Malassezia* yeasts (Moraru et al., 2019). Factors such as important variations in anatomical site, breed, sampling method and host immune status commonly thwart the interpretation of the clinical significance of an observed population ("XX yeasts in YY fields"); consequently a "treat what you see" trial therapy approach with topical or systemic antifungal drugs is routinely required to establish the clinical significance of an observed population. A recent clinical consensus guideline document presents a detailed diagnostic algorithm for use in the veterinary clinic (Bond et al., 2020). The importance for investigating and correcting concurrent skin diseases and other predisposing factors, where possible, cannot be over-emphasized, if a chronic or relapsing course is to be prevented.

Cytology using swabs of lesions rolled onto glass slides is normally best restricted to use in the ear canal, as the yield of squames and yeast from the skin is inferior to that obtained by tape strips and dry scrapes (Bond and Sant, 1993; White et al., 1998; Bensignor and Carlotti, 1999). In a recent randomized, blinded prospective study of 30 dogs with otitis externa, cytological specimens obtained using a conventional cotton-tipped swab contained comparable numbers of yeasts and bacteria, but fewer inflammatory cells, when compared with samples prepared by aspiration of material from the horizontal canal with a soft rubber feeding tube (Choi et al., 2018). In an effort to improve upon the sensitivity of cytological sampling for *M. pachydermatis* in the canine ear, Puig et al. have developed a quantitative PCR method based on amplification of the single copy *ß-tubulin* gene (Puig et al., 2019). The authors judged that the results were accurate and showed improved sensitivity over cytology; this method may have useful applications in diagnosis and therapeutic monitoring, and in studies of pathogenesis and therapeutic product development.

ANTIFUNGAL DRUG SUSCEPTIBILITY TESTING FOR *M. PACHYDERMATIS*

Antimicrobial resistance has emerged globally as a serious threat to human and animal health (Fera et al., 2009). Recent publications (Brilhante et al., 2018; Schlemmer et al., 2019a) support previous observations that most wild-type *Malassezia* yeasts remain susceptible to the commonly-used azole drugs such as itraconazole, ketoconazole and miconazole, although efficacy of fluconazole is more variable (Velegraki et al., 2004; Cafarchia et al., 2012a,b; Weiler et al., 2013). In *M. pachydermatis* isolates from canine otitis externa, synergistic interactions have been reported between between caspofungin and itraconazole or fluconazole (Schlemmer et al., 2019a), whereas amphoterecin B antagonized the activity of itraconazole, but not fluconazole or posaconazole (Alvarez-Perez et al., 2019).

In view of routine susceptibility and an absence of standard methods appropriate for the *Malassezia* genus, diagnostic testing in veterinary practice tends to rely upon cytological rather than cultural methods. However, laboratory studies of *M. pachydermatis* have previously demonstrated that it is possible to select for resistance to terbinafine and azoles (Nakano et al., 2005; Jesus et al., 2011). Of greater concern are recent sporadic reports of therapeutic failure with azoles in canine *M. pachydermatis*-associated dermatitis that were associated with increased azole tolerance *in vitro*; this might reflect the chronic and relapsing course of *Malassezia* dermatitis and otitis that often necessitate frequent and lengthy treatment courses (Chiavassa et al., 2014; Watanabe et al., 2014).

Angileri et al. isolated *M. pachydermatis* from an azole-unresponsive toy poodle that had MICs that were several fold higher when compared with strains from untreated dogs (Angileri et al., 2019). Kano et al. showed that an isolate of *M. pachydermatis* with MICs of itraconazole and ketoconazole of >32 mg/L by Etest had mis-sense mutations in the *ERG11* gene that encodes lanosterol 14 –alpha-demethylase, the target site for antifungal azoles (Kano et al., 2019b). Mutations in the same gene were described in field isolates with tolerance to ravuconazole (Kano et al., 2019a) and in miconazole-resistant clones of CBS1879 (the neotype culture of *M. pachydermatis*) selected by serial passage on miconazole supplemented media (Kano and Kamata, 2019). Azole resistance in *M. pachydermatis* has also been associated with quadruplication of the *ERG11* gene (Kim et al., 2018). By contrast, mutations in drug efflux pumps, a common mechanism of azole resistance in *Candida* species (Sanguinetti et al., 2005), has not yet been reported in the genus *Malassezia*. The emergence of azole resistance amongst

Malassezia species warrants careful surveillance and product stewardship to ensure ongoing utility of this important drug class. Further data are urgently required to establish whether topical therapies are preferable to systemic treatments in this context, and to guide antimicrobial stewardship policies for antifungal therapy in small animal practice.

Concern over azole resistance has prompted heightened interest in alternative antifungal agents. There are reports of *in vitro* efficacy against *M. pachydermatis* of a honey-based gel (Oliveira et al., 2018), monensin and, to a lesser extent, narasin (polyether ionophores originally marketed as anti-coccidials and growth-promoting modifiers of the bovine rumen flora; Chan et al., 2018, 2019). Multiple recent publications have explored the potential antifungal utility of essential oils, complex mixtures of highly concentrated aromatic oils (primarily terpenes and/or phenylpropanoids) extracted from plants by steam distillation, hydrodiffusion or pressure (Manion and Widder, 2017; Bismark et al., 2019). A previous randomized clinical trial reported persistent efficacy of a commercial essential oil product (Malacalm, Flora Slr Oli essenziali, Lorenzana, Italy) applied twice daily for 1 month to dogs with *Malassezia* dermatitis (Nardoni et al., 2014), although the study is weakened by incomplete data on randomization and clinical scores (Bond et al., 2020). Publications between 2013 and 2018 were usefully reviewed by Donato et al. (2019). Most of the recent studies have been conducted *in vitro* and their utility in clinical practice remains largely untested. Comparisons between studies are hampered by an absence of agreed standard testing methods that are not yet optimized, arbitrary assignment of interpretative criteria, and likely batch variation in activities of essential oils prepared by different methods (Bismark et al., 2019). Recently, anti-*Malassezia* effects have been observed *in vitro* using winter savory, lemon grass, oregano, palmarosa and cinnamon leaf oils by agar disc diffusion and vapor assays (Bismark et al., 2019). Oregano oil and thyme oil and their major phenolic components (carvacrol, thymol) were fungicidal against *M. pachydermatis* when tested using agar dilution (Sim et al., 2019). There are recent reports of synergistic interactions between essential oil components and azoles or nystatin against *M. pachydermatis*, including carvacrol and miconazole or nystatin, thymol and nystatin (Schlemmer et al., 2019b), and between clotrimazole and essential oils of *Melaleuca alternifolia*, *Mentha piperita,* and *Origanum vulgare* (Bohmova et al., 2019).

TREATMENT OF *MALASSEZIA* DERMATITIS IN DOGS

A recent evidence-based review on the treatment of canine *Malassezia* dermatitis reported "strong" evidence for the use of a 2% miconazole and 2% chlorhexidine shampoo, used twice weekly (Bond et al., 1995, 2020; Maynard et al., 2011). "Moderate" evidence was available for a 3% chlorhexidine shampoo (Maynard et al., 2011; Bond et al., 2020). For canine cases where topical therapy is ineffective or impractical, there was "moderate" evidence for the use of ketoconazole at 5–10 mg/kg orally once or twice daily; and itraconazole at 5 mg/kg orally once daily or two consecutive days per week (reviewed by Bond et al., 2020). Recently, the clinical and cytological effects of a once daily application of a leave-on spray formulation containing zinc, ethyl lauroyl arginate, laureth-9, urea, panthenol, glycerine and butylene glycol (Aptus® Derma Spot On Concentrate™, Orion Pharma Animal Health, Sollentuna, Sweden) were evaluated in a randomized, blinded, controlled study of 18 dogs with chronic pododermatitis associated with *Malassezia* yeasts (Sjostrom et al., 2018). When compared with placebo treatment of the contralateral foot, reduced yeast counts from the actively treated foot were associated with a reduction in clinical scores.

In dogs, persistent or recurrent *Malassezia* dermatitis are usually associated with failure to identify and correct predisposing or perpetuating factors. However, the evidence that reduced susceptibility of *M. pachydermatis* to commonly used antifungal drugs may develop under both field and laboratory conditions highlights the need for surveillance and vigilance for the emergence of clinically-relevant resistance. Agreed reference methods to assess antifungal susceptibility of *M. pachydermatis* are required to assist veterinary practitioner for the management of chronic cases.

POTENTIAL TRANSMISSION OF *MALASSEZIA* YEASTS FROM ANIMALS TO HUMANS

The zoonotic potential of *Malassezia* yeasts was first defined in the context of a neonatal intensive care unit, where a cluster of low birth weight patients receiving lipid emulsions were colonized by *M. pachydermatis* that was likely introduced on the hands of health care workers transmitted by contact with pet dogs (Chang et al., 1998). Once introduced to a facility, *Malassezia* yeasts can persist on incubator surfaces for prolonged periods of time (Van Belkum et al., 1994). A subsequent case report described a facial granuloma caused by *M. pachydermatis* in a dog owner (Fan et al., 2006), and recently *M. pachydermatis*-associated fungemia has been reported in a small number of adults with various predisposing factors (Choudhury and Marte, 2014; Roman et al., 2016; Lee et al., 2019). Since hand contamination by *M. pachydermatis* is common amongst dog owners, especially in owners of allergic dogs with *Malassezia* overgrowth (Morris, 2005), there is a clear need for rigorous hand hygiene by individuals in contact with pet dogs and cats, especially when there is contact with immunocompromised individuals (Bond et al., 2020).

AUTHOR CONTRIBUTIONS

JG and RB wrote, revised, edited and approved this manuscript for submission in a collaborative process, agreed on the allocation of the review of these papers by topic, and circulated their draft reviews for the other author to edit and revise as appropriate. RB performed an initial electronic search for recent relevant publications and led the development of the remaining sections. JG led the development of the sections on the genus, ecology, and pathogenesis and uploaded the manuscript.

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REVIEW
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Malassezia-Associated Skin Diseases, the Use of Diagnostics and Treatment

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Yeasts of the genus, *Malassezia,* formerly known as *Pityrosporum,* are lipophilic yeasts, which are a part of the normal skin flora (microbiome). *Malassezia* colonize the human skin after birth and must therefore, as commensals, be normally tolerated by the human immune system. The *Malassezia* yeasts also have a pathogenic potential where they can, under appropriate conditions, invade the stratum corneum and interact with the host immune system, both directly but also through chemical mediators. The species distribution on the skin and the pathogenetic potential of the yeast varies between different *Malassezia* related diseases such as head and neck dermatitis, seborrheic dermatitis, pityriasis versicolor, and *Malassezia* folliculitis. The diagnostic methods used to confirm the presence of *Malassezia* yeasts include direct microcopy, culture based methods (often a combination of morphological features of the isolate combined with biochemical test), molecular based methods such as Polymerase Chain Reaction techniques, and Matrix Assisted Laser Desorption/Ionization—Time Of Flight mass spectrometry and the chemical imprint method Raman spectroscopy. Skin diseases caused by *Malassezia* are usually treated with antifungal therapy and if there are associated inflammatory skin mechanisms this is often supplemented by anti-inflammatory therapy. The aim of this paper is to provide an overview of *Malassezia* related skin disease, diagnostic methods and treatment options.

Keywords: *Malassezia*, folliculitis, head and neck dermatitis, seborrheic dermatitis, pityriasis versicolor

INTRODUCTION

Yeasts of the genus, *Malassezia,* formerly known as *Pityrosporum,* are lipophilic yeasts, which are a part of the normal skin flora (microbiome). The genus *Malassezia* belongs to the phylum *Basidiomycota* (class *Malasseziomycetes*) and the genus consists at present of 17 species (Grice and Dawson, 2017; Theelen et al., 2018). It is the most prevalent fungal genus of the healthy skin, but these yeasts also demonstrate a pathogenic potential where they can, under appropriate conditions, invade the stratum corneum. They interact with almost all the cellular constituents of normal epidermis, including keratinocytes, Langerhans cells, melanocytes as well as the host immune system, both directly but also through chemical mediators (Glatz et al., 2015; Grice and Dawson, 2017). *Malassezia* colonize the human skin after birth and must therefore, as a commensal,

be normally tolerated by the human immune system. Depending on sampling technique and diagnostic methods they have been isolated from 30 to 100% of newborns (Ayhan et al., 2007; Nagata et al., 2012).

Malassezia species are dependent on exogenous lipids because they lack fatty acid synthase genes, except *M. pachydermatis* (Glatz et al., 2015). This explains their distribution on seborrheic skin areas (face, scalp and thorax), but they have been detected from most body sites except the feet (Grice and Dawson, 2017). There is also a correlation between species diversity and anatomical sampling site (Grice and Dawson, 2017; Theelen et al., 2018).

The species distribution on the skin varies between different *Malassezia* related diseases, but their worldwide distribution may also differ (Grice and Dawson, 2017). For example, *M. sympodialis* considered the most prevalent species in Europe and *M. restricta* and *M. globosa* the most predominant species in Asia. The difference in the species distribution may not only be revealed by differences in geographic specificity but may also be due to a difference in diagnostic methods used. Most of the European studies used culture-based methods whereas Asian countries generally have applied molecular based methods and as some *Malassezia* species are slow-growing and more fastidious in culture, such as *M. restricta,* this particular species in culture may be overgrown by a more rapid-growing *Malassezia* species as e.g., *M. sympodialis* (Kohsaka et al., 2018).

Skin diseases caused by *Malassezia* are usually treated with antifungal therapy and if there are associated inflammatory skin mechanisms this is often supplemented by anti-inflammatory therapy. Different *Malassezia* species have shown various antifungal susceptibility patterns (Prohic et al., 2016; Theelen et al., 2018). It may therefore occasionally be important to identify the *Malassezia* species in order to choose the most sensitive antifungal drug although this poses immense practical problems in resource poor settings.

The aim of this paper is to provide an overview of the *Malassezia* related skin diseases Head and neck dermatitis, seborrheic dermatitis, pityriasis versicolor, and *Malassezia* folliculitis, their diagnostic methods and treatment options.

DIAGNOSTICS

Different sampling methods have been used to confirm the presence of *Malassezia* yeasts in skin conditions and these include tape stripping, skin scraping, swabs, and contact plates (Darabi et al., 2009). Direct microcopy is used frequently in clinical settings (Saunte et al., 2018) as it can be used to detect fungal elements after application of potassium hydroxide and adding a dye such as e.g., Parker ink, methylene blue, lactophenol blue, May-Grunwald-Giemsa, Gram staining or a fluorescence dye such as Calcofluor white and Blancophor (Rubenstein and Malerich, 2014; Tu et al., 2018). *Malassezia* is recognized by the detection of characteristic unipolar budding yeasts and in the case of pityriasis versicolor these are accompanied by short hyphae (the so-called spaghetti and meatballs appearance). Hyphae are not detected in head and neck dermatitis and rarely seen in *Malassezia* folliculitis or seborrheic dermatitis/dandruff. Even though it is possible to see differences in the shape of the *Malassezia* yeasts cells as e.g., the globose cells of *M. globosa* or the sympodial budding of *M. sympodialis,* accurate species identification is not possible by direct microscopy. For this, different *in vitro* methods have been applied.

The initial isolation usually employs Dixon's or Leeming-Notman agar and growth at 32–35°C under aerobic conditions. Daily evaluation of the cultures is required to observe the presence of mixed species colonies, which are needed to be separated using needle sampling of the colonies and/or multiple dilutions before subculturing. Identification to species level is achieved by evaluation of the different lipid assimilation profile of the *Malassezia* species (Guého et al., 1996; Mayser et al., 1997) in combination with microscopic morphological features. However, the variations revealed by this conventional mycology approach are not sufficiently specific for the identification of the current expanded *Malassezia* species, as there is a common lipid profile overlap between species (Cafarchia et al., 2011; Theelen et al., 2018). Although these culture-based methods are time-consuming and it is difficult to separate closely related species characteristics of each strain.

For this reason during the last five decades molecular based methods (Arendrup et al., 2013) as well as methods that identify the chemical imprint of the different species e.g., different Polymerase Chain Reaction (PCR) techniques, Matrix Assisted Laser Desorption/Ionization—Time Of Flight (MALDI-TOF) mass spectrometry (Kolecka et al., 2014; Diongue et al., 2018; Honnavar et al., 2018; Saunte et al., 2018) and or Raman spectroscopy (Petrokilidou et al., 2019) have been applied to achieve fast and accurate fungal identification.

Discrepancies in the epidemiological data generated by culture and molecular based *Malassezia* identification methods are well-known and probably reflect differences in growth rate, where the fast growing species may overgrowth slower ones in culture based methods and because molecular based methods are considered to be more accurate (Soares et al., 2015; Prohic et al., 2016). Additionally, species identification using molecular based methods is dependent on reliable "databases" for sequence comparison.

Antifungal susceptibility of *Malassezia* species using agar and broth dilution methods (Clinical & Laboratory Standards Institute and European Committee of Antimicrobial Susceptibility Testing assays) with lipid supplementation has been studied (Cafarchia et al., 2012; Leong et al., 2017; Peano et al., 2017; Rojas et al., 2017). *In vitro* antifungal resistance have been demonstrated in different strains, but as there is no reference procedure for antifungal susceptibility testing the strains may appear susceptible under other test conditions (Peano et al., 2017; Rojas et al., 2017).

Despite the current knowledge of *Malassezia* species' association and contribution to skin disorders, the mechanisms

Abbreviations: AD, atopic dermatitis; *M, Malassezia;* HND, Head and neck dermatitis; PCR, Polymerase Chain Reaction; PV, pityriasis versicolor; SD, seborrheic dermatitis.

TABLE 1 | *Malassezia* associated diseases and their possible pathogenesis, main diagnostics and differential diagnosis.

Disease	Possible pathogenesis	Main diagnostic	Examples of differential diagnosis
Head & neck dermatitis	Type-I hypersensitivity to *Malassezia*	Clinical Skin prick test *Malassezia* spp. specific IgE (Atopy patch test)	Contact dermatitis Steroid induced dermatitis
Seborrheic dermatitis	Colonization with *Malassezia* that triggers irritant dermatitis	Clinical Biopsy shows psoriasiform, spongiotic dermatitis without intraepidermal pustules	Rosacea Sebopsoriasis Systemic lupus erythematois Tinea capitis Zinc deficiency Contact dermatitis
Pityriasis versicolor	*Malassezia* infection	Clinical Direct microscopy with unipolar budding yeast and hyphae (spaghetti and meatballs)	Vitiligo Pityriasis alba Chloasma Nummular dermatitis
Malassezia folliculitis	Invasion of the pilo-sebaceous with *Malassezia*	Histopathology Direct microscopy with unipolar budding yeast (rarely hyphae)	Acne Steroid acne Bacterial folliculitis Eosinophilic folliculitis Pustular drug eruptions Lymphomatoid papulosis

underlying their change from a commensal to pathogen are still to be further elucidated. Furthermore, there is a need for standardization of species diagnostic methods and antifungal susceptibility testing.

MALASSEZIA-ASSOCIATED SKIN DISEASES

Even though *Malassezia* is a part of the human microbiome it is also involved in the pathogenesis of head and neck dermatitis, seborrheic dermatitis, pityriasis versicolor, and *Malassezia* folliculitis. It interacts with both the innate and acquired skin immune systems and thereby causes immune reactions under certain conditions. It is possible to detect IgG and IgM antibodies against *Malassezia* in most individuals, but healthy persons are usually not sensitized as is the cases with atopic dermatitis patients. The sensitization can in atopic dermatitis (AD) patients cause a type I hypersensitivity reaction contributing to redness, itching and further scaling in the seborrheic areas of the head and neck, the so-called head and neck dermatitis (Glatz et al., 2015; Kohsaka et al., 2018). In seborrheic dermatitis (Faergemann et al., 2001) the inflammatory reaction that leads to the development of seborrheic dermatitis seems to be an irritant non-immunogenic stimulation of the immune system that leads to complement activation and local increase in NK1+ and CD16+ cells. Pityriasis versicolor is an infection which involves proliferation of the organisms and activation of the formation of hyphae to cause superficial invasion of the stratum corneum.

In *Malassezia* folliculitis the yeasts invade the pilo-sebaceous unit leading to a dilatation of the follicles with large number of *Malassezia* cells. If the follicular walls rupture this results in a mixed inflammatory infiltrate and clinical inflammation.

HEAD AND NECK DERMATITIS

Epidemiology and Pathogenesis

Head and neck dermatitis is a subtype and difficult to treat form of atopic dermatitis, which is generally seen in post-pubertal atopic dermatitis patients. The prevalence of atopic dermatitis among adults in industrialized countries is 1–3% and it affects 10–20% of children (Brodská et al., 2014). It is thought to be due to a type I hypersensitivity reaction to *Malassezia* antigens (**Table 1**). The antigens e.g., *M. globosa* protein (MGL_1304) and its homologs from *M. sympodialis* (Mala s 8) and *M. restricta* (Mala r 8) have all been implicated in the pathogenesis of head and neck dermatitis and show different histamine releasing activity (Kohsaka et al., 2018). The *Malassezia* (antigen) proteins are found in sweat and the disease is therefore triggered by sweating (sometimes referred to as sweat allergy) (Hiragun et al., 2013; Maarouf et al., 2018). IgE antibodies against *Malassezia* is found in up to 27% of children and 65% of adults with atopic dermatitis (Glatz et al., 2015).

Malassezia's interaction with the skin immune system is thought to be both humoral and cell-mediated and it contributes to and accentuates the pre-existing skin inflammation in AD (Brodská et al., 2014). It is suggested that an increased pH, which is higher in AD patients, may contribute to allergen release by *Malassezia*. The disturbed skin barrier in AD allows both *Malassezia* allergens as well as cells to penetrate the epidermis and hereby introducing them to toll-like receptor 2 on dendritic cells and keratinocytes. A release of pro-inflammatory cytokines and *Malassezia* spp.- specific IgE antibodies is produced through T cell mediated activation of B cells and through dendritic cells and mast cells and this contributes to the skin inflammation. Furthermore, autoreactive T cells may cross react and sustain skin inflammation (Glatz et al., 2015).

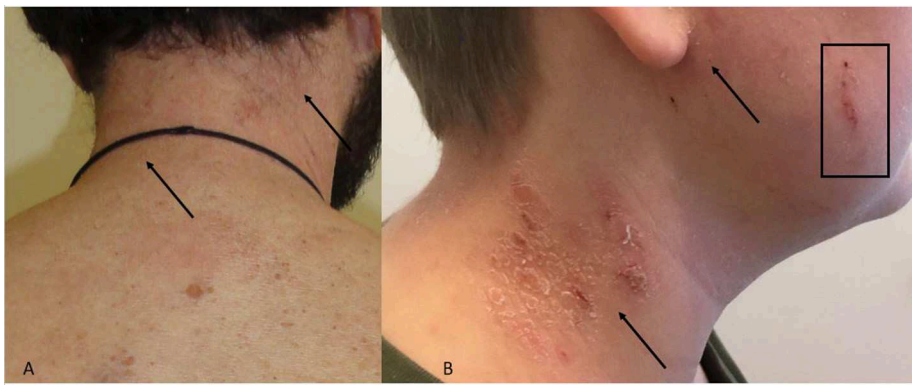

FIGURE 1 | (A,B) Head and neck dermatitis. **(A)** Neck with erythema and discrete skin scales. Arrows indicate the area. **(B)** Skin scales, erythema (arrows) and excoriation (square) of neck and cheek.

Clinical Presentation

The clinical manifestations of head and neck dermatitis are typically erythematous involvement of the eyelids, forehead and neck; sometimes the changes are wheal-like (urticarial) (Maarouf et al., 2018). Affected areas are itchy and there is often scaling giving the appearances of an eczema flare (**Figures 1A,B**).

Diagnosis

The diagnosis is based upon the clinical picture and may be supported a positive type I allergic reaction to *Malassezia* and a positive skin prick test with *Malassezia* spp. –specific extract is found in 30–80% of adult atopic dermatitis (Glatz et al., 2015). A study by Devos and van der Valk found that all AD patients with head and neck dermatitis had increased *Malassezia-spp.* specific IgE as compared with only 13.6% of AD patients without head and neck dermatitis (Devos and van der Valk, 2000). A commercial and standardized kit (ImmunoCAP® m70, Phadia) is available for measuring *Malassezia spp.*-specific serum IgE (Glatz et al., 2015). The use of atopy patch test shows diverse results (Brodská et al., 2014). In two different studies (Ramirez De Knott et al., 2006; Johansson et al., 2009) there was no correlation between IgE and atopy patch test for *Malassezia*, whereas Johansson et al. (Johansson et al., 2003) found that atopic patch test was positive in 30% of AD patients without head and neck dermatitis and in 41% of patients with head and neck dermatitis.

Treatment

Head and neck dermatitis can be treated using anti-inflammatory medications, antifungals or a combination.

The main purpose of the antifungal treatment is to reduce the skin colonization thereby reducing the amount of allergen causing the type I hypersensivity. It has been shown that AD patients with head and neck dermatitis treated with anti-fungals (itraconazole) show decreases in the total *Malassezia* specific IgE, eosinophil count as well as improving clinical severity scores (Ikezawa et al., 2004).

The clinical improvement is usually seen within the first week(s) and the daily regimen is often continued for 1–2 months followed by a twice weekly regimen to prevent relapse (Darabi et al., 2009). Systemic antifungals are useful in severe cases or when treatment failure after topical therapy.

Furthermore, in AD patients repair of the impaired skin barrier and a reduction of the inflammation with e.g., calcineurin inhibitors or topical steroids are very useful (Nowicka and Nawrot, 2019). It is not clear if the reduction of the inflammation is more important than reducing skin colonization of *Malassezia* for two reasons. First of all the treatment responses to hydrocortisone combined with placebo shampoo compared with miconazole-hydrocortisone cream and ketoconazole shampoo are not significantly different (Broberg and Faergemann, 1995). Secondly, some antifungals have anti-inflammatory properties (inhibit IL-4 and IL-5 production) (Kanda et al., 2001).

SEBORRHEIC DERMATITIS

Epidemiology and Pathogenesis

Seborrheic dermatitis is an inflammatory dermatosis with a predilection for anatomical areas with high sebaceous gland concentration such as the midface, chest, back, and scalp. Seborrheic dermatitis located on the scalp and dandruff should be considered as representing different ends of a disease severity spectrum (Grimalt, 2007). Therefore, for scalp disease the term seborrheic dermatitis/dandruff complex is suggested to encompass the scaling both with inflammation (seborrheic dermatitis) and without inflammatory component (dandruff). As dandruff is extremely common and practically all adults are affected at some point in their life, we will note only relevant data in the pathogenesis section that help us to understand seborrheic dermatitis.

Seborrheic dermatitis is a relative common dermatosis and few recent meticulous studies have addressed the point prevalence of this disease. Thus the point prevalence of seborrheic dermatitis in 161,269 working individuals in Germany (Zander et al., 2019) was recorded to be 3.2% with seborrheic dermatitis being three times more common in men than in women. Also, seborrheic dermatitis prevalence increased with

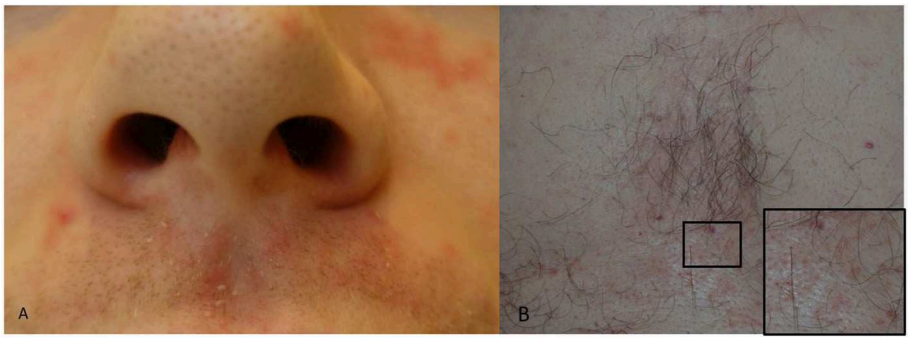

FIGURE 2 | (A,B) Seborrheic dermatitis. **(A)** Peri-nasal skin and upper lip with erythema and greasy skin scales. **(B)** Erythema and greasy skin scales of the chest and a close-up (square) of an area with erythematous lesions.

age (2.0% in <35 years; 3.6% in 35–64 years; 4.4% ≥65 years) and there was an association with other fungal diseases such as tinea pedis, onychomycosis and pityriasis versicolor. The age dependence of seborrheic dermatitis is probably responsible for the increased prevalence (14.3%) recorded in the Rotterdam study (Sanders et al., 2018a) as the median age of patients was 67.9 years. These robustly acquired data confirm the association of seborrheic dermatitis with gender (two-fold increase in men), season (increased in winter) and generalized xerosis cutis. A darker skin phenotype was a protective factor for seborrheic dermatitis. Whether this was due to difficulty in recording erythema in darker skin types or the fact that it represents a different barrier function in these skin phenotypes is a matter of debate. Nevertheless seborrheic dermatitis was also commonly diagnosed in 2.1% of young Korean male army recruits (Bae et al., 2012) (93.3% of cohort between 19 and 24 years of age), supporting the generally suggested prevalence of seborrheic dermatitis between 2 and 8% (Palamaras et al., 2012).

It well established that seborrheic dermatitis prevalence is significantly increased in subgroups of patients such as those with Human Immunodeficiency Virus (HIV) infection, where it is associated with low CD4 counts (Lifson et al., 1991) as well as neurological patients. These include those with Parkinson's disease (Skorvanek and Bhatia, 2017) patients as well as patients with spinal cord injury on which seborrheic dermatitis appears above the level of injury (Han et al., 2015), pointing toward brain-skin axis involvement. In the light of the recent implication of *Malassezia* yeasts in pancreatic ductal carcinoma development (Aykut et al., 2019), these epidemiological observations point to future research areas (Laurence et al., 2019). The understanding of the pathogenesis of seborrheic dermatitis is limited by the overlap with other conditions such as psoriasis (sebopsoriasis), the indistinct borders between seborrheic dermatitis and dandruff and the absence of a robust severity scoring system. Thus, findings in dandruff pathophysiological changes that are generated from scalp are not necessarily applicable to facial seborrheic dermatitis. Likewise only recently markers to differentiate the overlapping cases of psoriasis and seborrheic dermatitis (sebopsoriasis) have been developed. These include immunohistochemistry markers that address clinical and pathological indistinct cases of sebopsoriasis (Cohen et al., 2019). Additionally, seborrheic dermatitis patients do not share susceptibility loci with psoriasis patients (Sanders et al., 2018b). Regarding the implication of *Malassezia* yeasts in the pathogenesis of seborrheic dermatitis and dandruff there are characteristic and persistent findings that link seborrheic dermatitis or dandruff associated *Malassezia* strains with the respective conditions. Thus *M. furfur* strains isolated from seborrheic dermatitis lesions produce, *in vitro,* significantly more bioactive indolic substances as compared to strains isolated from healthy skin (Gaitanis et al., 2008). These substances [i.e., indirubin, 6-formylindolo[3,2-b]carbazole (FICZ), indolo[3,2-b]carbazole (ICZ), malassezin, and pityriacitrin] are also found on seborrheic dermatitis skin and correspond to the most active aryl-hydrocarbon receptor ligands known (Magiatis et al., 2013). As a marker of their clinical significance, indirubin is used as a potent local treatment for psoriasis (Lin et al., 2018), while there are ongoing clinical trials that evaluate aryl hydrocarbon receptor ligands applied locally for this disease (https://clinicaltrials.gov/ct2/show/NCT04053387). Likewise, the irritating effect on the skin through a compromised permeability barrier function (Turner et al., 2012) of free fatty acids (DeAngelis et al., 2005) and squalene peroxides (Jourdain et al., 2016) produced by *Malassezia* lipases as a result of its nutritional needs, are key players, at least, in the pathogenesis of dandruff. Accordingly, the skepticism expressed (Wikramanayake et al., 2019) on the implication of *Malassezia* yeasts in seborrheic dermatitis can be a useful starting point for future research toward the better understanding of seborrheic dermatitis pathogenesis.

Clinical Presentation

Seborrheic dermatitis presents with erythema, small papules and sometime pustules overlayed with greasy, white to yellow scales. The areas of predilection include the nasolabial folds and the upper lip close to the nostrils (**Figure 2A**), the eyebrows and the root of the nose, the pre- and retro auricular areas, the sternum (**Figure 2B**) and less often the back. Scalp seborrheic dermatitis/Dandruff does not involve the whole scalp, rather it appears as patchy areas of erythema

and scaling. Involvement of the eye presents as seborrheic dermatitis blepharitis.

Diagnosis

The diagnosis of seborrheic dermatitis is mostly clinical. The typical cases are straightforward in their recognition while some confusion can be created when there is co-existence with rosacea or late-onset acne. In rosacea the involvement of "convex" anatomical areas (nose, cheeks) and the evaluation of precipitating factors is of help. In acne the lesions are located in the hair follicles, scaling unless receiving therapy is not prominent and the prevailing lesions are comedones, papules and pustules.

Biopsy should be restricted to difficult to diagnose cases and the appearances are mostly described as a psoriasiform, spongiotic dermatitis without intraepidermal pustules (**Table 1**). Routine cultures for identification and characterization of *Malassezia* species involved to a case of seborrheic dermatitis are not currently suggested. Hopefully in the future, our understanding of seborrheic dermatitis pathogenesis could be associated with identification of virulence factors of *Malassezia* yeasts. This could possibly lead to the development of therapy guided by the pathogenetic mechanisms (tryptophan metabolism, enzyme production) of the case related *Malassezia* strain.

Treatment

The patient should be informed that seborrheic dermatitis can be a chronic, recurring condition and side-effects of long-term treatment should be weighed against the potential gain. This mostly pertains to topical steroids that are used in clinical practice to rapidly reduce erythema (Gupta and Versteeg, 2017). When long-term control of the inflammatory response in seborrheic dermatitis is required topical use of the calcineurin inhibitors tacrolimus and pimecrolimus is advised (Ang-Tiu et al., 2012). Safety regarding carcinogenicity of these substances is extrapolated from data in atopic dermatitis and does not seem a reason of concern (Cook and Warshaw, 2009). The use of topical antifungals (ketoconazole, ciclopirox) is supported by recent systematic reviews (Okokon et al., 2015) and given their high efficacy and improved safety they should be included in relevant therapeutic schemes. Also it should be stressed that both pimecrolimus and tacrolimus have antifungal action against *Malassezia* yeasts (Sugita et al., 2006) so at least part of their activity in seborrheic dermatitis can be attributed to this. A variety of alternative or natural product treatments are also suggested for seborrheic dermatitis (Gupta and Versteeg, 2017) while a recent suggestion is the use of formulations that restore the barrier function of the skin (Purnamawati et al., 2017) and definitely formulations that restore the barrier function of the skin will be a useful addition to treatment (Wikramanayake et al., 2019). Furthermore various salts are also efficient, like lithium succinate, which seems to interfere with the availability of the prerequisite lipids for *Malassezia* growth (Mayser and Schulz, 2016). Systemic antifungals are suggested for resistant or rapidly relapsing cases of seborrheic dermatitis (Gupta et al., 2014).

PITYRIASIS VERSICOLOR

Epidemiology and Pathogenesis

Pityriasis versicolor is a mild, chronic infection of the skin caused by *Malassezia* yeasts, characterized by discrete or confluent, scaly, dark or depigmented patches, mainly on the upper trunk but this can extend to the neck, abdomen and other sites, although the peripheries are usually spared.

Pityriasis versicolor occurs in both tropical, where it may be very common, and temperate climates and affects both genders equally. However, lesions in temperate areas are often noticed after a visit to a warmer environment. It is commonest in teenagers and young adults but can occur at any age. Data on global prevalence is not available, however in tropical climates, the condition is more common than in temperate zones, and in one study from Bahia, Brazil 40% of the population of some areas was affected (Santana et al., 2013). Although there are reports of an association between pityriasis versicolor and a number of other underlying conditions, it generally occurs in otherwise healthy individual although patients with idiopathic and iatrogenic Cushing's syndrome are more susceptible (Finding et al., 1981). It does not appear to be more common in the acquired immune deficiency syndrome (AIDS) (Mathes and Douglass, 1985).

A striking feature of most cases of pityriasis versicolor is the presence of hyphae in lesions. But the reasons for hyphal growth are still unknown. The activation of the MGL_3741 gene which encodes the enzyme Dihydroxy acid dehydratase (DHAD) in *M. globosa* has been implicated as it is present in lesional but not non-lesional skin (Aghaei Gharehbolagh et al., 2018) Lack of inflammation in lesions of pityriasis versicolor is noticeable although there is evidence of interaction between *Malassezia* species in this condition and innate and acquired immunity (Brasch et al., 2014) T-cell inhibition by a lipid component associated with the yeast cell wall has also been reported (Kesavan et al., 1998) which may partially explain the lack of clinically significant inflammation.

The mechanism for the typical pigmentary changes seen in pityriasis versicolor is still not understood, although electron microscopy shows abnormally large melanosomes in hyperpigmented lesions (**Figure 3A**), and smaller-than-normal melanosomes in hypopigmented ones (**Figure 3B**). Depigmentation has been explained on the production of dicarboxylic acids produced by *Malassezia* species (e.g., azaleic acid) causing competitive inhibition of tyrosinase and perhaps a direct cytotoxic effect on hyperactive melanocytes (Nazzaro-Porro and Passi, 1978). *M. furfur* produces pigments and fluorochromes with tryptophan as sole nitrogen source. They (i.e., malassezin, pityriacitrin, pityrialacton, pityriarubins) may explain some clinical phenomena of pityriasis versicolor (depigmentation. fluorescence, lack of sunburn in pityriasis versicolor alba) (de Hoog et al., 2017).

The *Malassezia* species mainly identified in pityriasis versicolor lesions are *M. globosa* and also *M. sympodialis* and *M. furfur*.

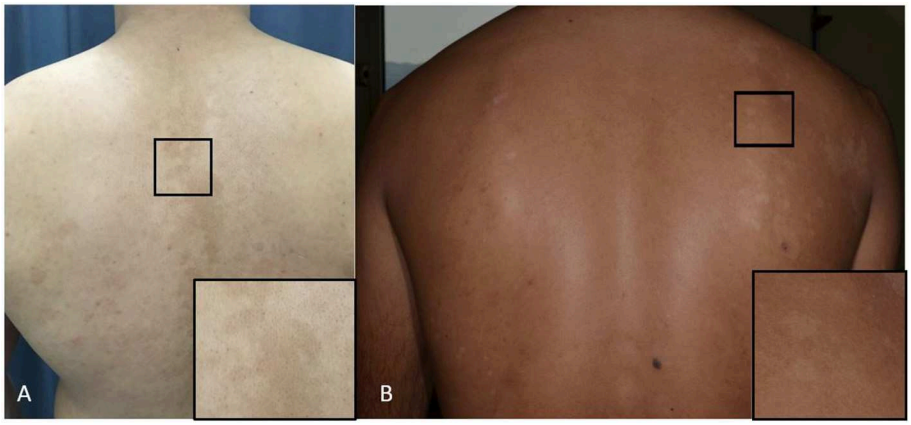

FIGURE 3 | (A,B) Pityriasis versicolor. **(A)** Hyperpigmented maculae on the back and a close-up of the lesion (square). **(B)** Hypopigmented maculae and a close-up of the lesion (square).

Clinical Presentation

The primary lesions are well demarcated macules, which may be slightly erythematous and covered by fine scales which may only be noticeable after scratching the lesional surface. These coalesce to form scattered patches of hypo- or hyperpigmentation (**Figures 3A,B**). Itching is very mild. The sites most commonly affected are the upper trunk, but there is often spread to the upper arms, the neck and the abdomen. Lesions in the axillae and groins, and on the thighs and genitalia occur, and extension down the forearms on to the backs of the hands; these atypical forms of pityriasis versicolor may be associated with oval yeast forms seen in direct microscopy. Another rare but well documented variant is one where there is marked atrophy or anetoderma-like change in the skin that follow infection (Tellechea et al., 2012). Pityriasis versicolor is a chronic infection if left untreated. In some patients, lesions recur rapidly and may not respond well to treatment. Such cases, while not common, are seen regularly. Some have been associated with the presence of the organism, *M. japonica*, and raised IgE levels (Romero-Sandoval et al., 2017).

Vitiligo and chloasma are normally distinguishable from pityriasis versicolor by their complete absence of scaling.

Diagnosis

Under filtered ultraviolet (Wood's) light, the scaly lesions may show pale yellow fluorescence. Direct microscopy shows coarse mycelium, fragmented into short filaments, together with spherical, thick-walled yeasts. Occasionally, only oval yeasts may be seen (see above). The characteristic appearance on microscopy has been described as "spaghetti and meatballs" (**Table 1**). Detection of *Malassezia* species by culture or molecular methods from skin scrapings is of no diagnostic value, and does not form part of the diagnostic investigation of pityriasis versicolor. Dermoscopy, although useful in confirming the scaling, does not identify specific diagnostic features (Mathur et al., 2019).

Treatment

The first line treatment is topical antifungal therapy. The topical azole antifungals work well in pityriasis versicolor, and there is no significant difference in results achieved by different azoles. The usual time to recovery is 2–3 weeks. A practical problem with the use of topical antifungals is the difficulty of applying creams to a wide body surface area. An alternative solution to this is ketoconazole shampoo which is lathered into the skin in a shower and then washed off after 3–4 min, and although it has not been fully evaluated in pityriasis versicolor, two or three applications of the shampoo appear to clear most infections. Terbinafine 1% cream, but not oral terbinafine, is also effective. Another approach is the application of 2.5% selenium sulfide in a detergent base (Selsun® shampoo). It is applied to all the affected areas and left overnight. Alternatives include 50: 50 propylene glycol in water. The latter has also been used intermittently as long-term suppressive therapy to prevent relapse (Faergemann and Fredriksson, 1980).

Oral itraconazole is also very effective in cases of pityriasis versicolor 100 mg daily for 10 days (Delescluse, 1990) although it is usually given in extensive or recalcitrant cases. Fluconazole has also been used.

Whatever medication is given patients should be warned that normalization of pigmentation may take several months after the end of treatment.

MALASSEZIA FOLLICULITIS

Epidemiology and Pathogenesis

Malassezia folliculitis is an inflammatory condition caused by *Malassezia* yeasts involving the pilo-sebaceous unit.

Predisposing factors includes immunosuppression (e.g., immunosuppressive medication, broad spectrum antibiotics, diabetes, HIV, hematological malignancies), occlusion and sweating (Tragiannidis et al., 2010; Prohic et al., 2016). It is more frequent in, or after visiting, tropical areas or hotter climates because of humidity and high temperatures (Tragiannidis et al., 2010).

The most prevalent species associated with *Malassezia* folliculitis are *M. globosa, M. restricta* and *M. sympodialis* (Akaza et al., 2009; Ko et al., 2011; Durdu et al., 2013; Prohic et al., 2016).

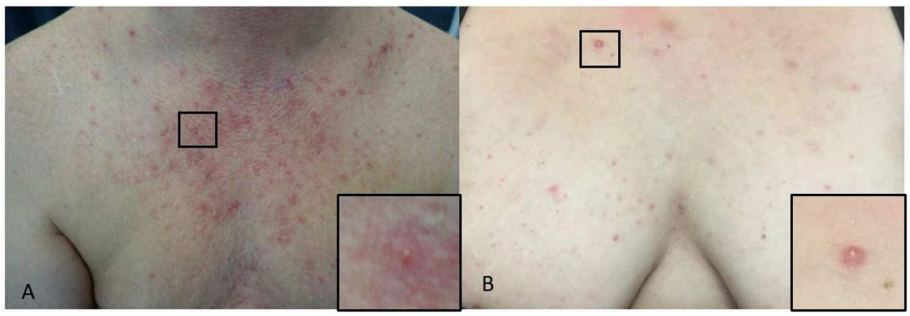

FIGURE 4 | (A,B) *Malassezia* folliculitis. **(A)** Erythematous paplues and pustules on the chest of a male and a close-up of a papule (square). **(B)** On the chest of a woman and a close-up of a papule (square).

Clinical Presentation

The typical presentation is monomorphic, approximately 2–4 mm, erythematous itchy papules or papulopustules on the chest (**Figures 4A,B**), back, upper arms, neck and face; some patients have concomitant pityriasis versicolor or seborrheic dermatitis (Hald et al., 2014). *Malassezia* folliculitis, especially in adolescent, may be misdiagnosed as acne or bacterial folliculitis, but comedones are absent and itching is a common symptom (Hald et al., 2014; Tsai et al., 2019). The itching may be less pronounced in immunosuppressed patients (Hald et al., 2014).

Diagnosis

The diagnosis is based upon the clinical picture and symptoms supported by mycological detection and response to antifungal therapy (Prohic et al., 2016). Histopathology can be used to differentiate *Malassezia* folliculitis from other types of folliculitis such as e.g., bacterial, eosinophilic or pustular drug eruptions. In *Malassezia* folliculitis invasion and dilatation of follicles with large number of *Malassezia* conidia (and rarely hyphae) is seen and inside the follicle there is a reticular pattern of keratin plugging in the majority of patients (An et al., 2019). The follicular walls may rupture resulting in a mixed inflammatory infiltrate of neutrophils, lymphocytes and histiocytes in the dermis. Direct microscopy on skin scraping and the content of pustules treated with KOH (and a dye) will detect unipolar budding yeast, rarely hyphae (**Table 1**). In a study by Tu et al. Gram staining has been shown to have a sensitivity and specificity of 84.6 and 100% as compared with a final diagnosis of *Malassezia* folliculitis when two of three criteria was met: 1. Typical clinical presentation, 2. Biopsy with *Malassezia* in inflamed hair follicle, 3. Treatment response to antifungal therapy (Tu et al., 2018). This suggests that direct microscopy which is both rapid, simple and non-invasive is an alternative to histology. Nevertheless, direct microscopy is not species specific as are culture- or molecular-based methods and it does not reveal location of the fungus in relation to the follicle.

Other diagnostic methods includes Wood's lamp which fluorescence yellow-green when the lesions is illuminated, reflectance confocal microscopy and optical coherence tomography (Rubenstein and Malerich, 2014; Andersen et al., 2018).

In clinical settings initial diagnosis based upon the combination of symptoms such as itch, clinical picture with monomorphic papulopustules without comedones supported by direct mycological detection by microscopy is sufficient to initiate therapy while awaiting histopathology results. The direct microscopy is important to differentiate *Malassezia* folliculitis from bacterial folliculitis.

Treatment

Systemic itraconazole 100–200 mg daily has been used for 1–4 weeks with a clinical treatment effect of 69–100% (Parsad et al., 1998; Durdu et al., 2013; Suzuki et al., 2016; Tsai et al., 2019) and fluconazole 100−200 mg daily for 1–4 weeks with a clinical effect of 80% (Rhie et al., 2000). Combination of systemic antifungals and topical antifungals (Abdel-Razek et al., 1995; Prindaville et al., 2018) or tretinoin/bensylperoxide (Ayers et al., 2005) is also useful. Topical therapies which have proven useful for the treatment of *Malassezia* folliculitis include azoles (Back et al., 1985; Rhie et al., 2000; Suzuki et al., 2016; Prindaville et al., 2018; Tsai et al., 2019), selenium sulfide once daily for 3 days then weekly (Back et al., 1985) and propylene glycol 50 % twice daily (Back et al., 1985). Systemic antifungal monotherapy is thought to be more efficient than topical monotherapy, but in a small study ($N = 44$) comparing ketoconazole cream twice daily with oral itraconazole 100 mg daily an improvement and treatment respond was noted in both groups although the topical treatment required a longer treatment course (Suzuki et al., 2016). Topical therapy may therefore be useful and considered in patients as a prevention measure or in patients with contraindication for systemic therapy.

Recurrence is common after treatment is completed, and maintenance therapies such as weekly topical or monthly oral antifungals have been used as prevention measures (Levy et al., 2007; Rubenstein and Malerich, 2014).

Alternative treatment options include photodynamic therapy (Lee et al., 2010, 2011).

Currently, there is no internationally approved treatment guideline for the management of *Malassezia* folliculitis.

CONCLUSION

The *Malassezia* yeasts are complex fungi which are part of the normal skin microbiome. They have pathogenic potential and are able to cause skin related diseases through different mechanisms: an activation of the immune system as in head and neck dermatitis, an eczematous/inflammatory reaction as in seborrheic dermatitis, an infection of stratum corneum as in pityriasis versicolor or a colonization (invasion) with a large number of *Malassezia* yeasts of the pilo-sebaceous unit as in *Malassezia* folliculitis. To support the clinical suspicion of the association between *Malassezia* and disease, a broad spectrum of techniques is used for the confirmation of the presence of *Malassezia* yeasts or for the detection of pathogenetic mechanisms such as *Malassezia* related type I allergy. Traditional direct microscopy, culture on lipid enriched media, biochemical tests and histopathology but also newer molecular based methods can be used for the detection of *Malassezia* yeast. For confirmation of type I allergy to *Malassezia* a specific IgE testing or prick testing is useful. A positive treatment response to antifungals, backed by reduction or temporary elimination of the organisms is highly suggestive, if not confirmatory, of a *Malassezia* etiology, but there are other variables such as the host's general condition and the species involved. Further investigative work that helps to delineate the disease mechanisms and the role, if any, of other members of the skin microbiome in the process is needed.

ETHICS STATEMENT

For patients providing clinical photos a written consent was obtained.

AUTHOR CONTRIBUTIONS

DS, GG, and RH planned, wrote, and contributed to the critical review of the manuscript.

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Identification of *Malassezia furfur* Secreted Aspartyl Protease 1 (MfSAP1) and Its Role in Extracellular Matrix Degradation

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Malassezia is the most abundant eukaryotic microbial genus on human skin. Similar to many human-residing fungi, *Malassezia* has high metabolic potential and secretes a plethora of hydrolytic enzymes that can potentially modify and structure the external skin environment. Here we show that the dominant secreted *Malassezia* protease isolated from cultured *Malassezia furfur* is an aspartyl protease that is secreted and active at all phases of culture growth. We observed that this protease, herein named as MfSAP1 (*M. furfur* secreted aspartyl protease 1) has a broader substrate cleavage profile and higher catalytic efficiency than the previously reported protease homolog in *Malassezia globosa*. We demonstrate that MfSAP1 is capable of degrading a wide range of human skin associated extracellular matrix (ECM) proteins and ECM isolated directly from keratinocytes and fibroblasts. Using a 3-D wound model with primary keratinocytes grown on human de-epidermized dermis, we show that MfSAP1 protease can potentially interfere with wound re-epithelization in an acute wound model. Taken together, our work demonstrates that *Malassezia* proteases have host-associated substrates and play important roles in cutaneous wound healing.

Keywords: skin microbiome, *Malassezia*, protease, extracellular matrix, wound healing

INTRODUCTION

The skin is our first physical barrier against the external environment and is also the residence of a rich community of microbes (Oh et al., 2014; Byrd et al., 2018). This surface is mainly colonized by diverse groups of bacteria, though intriguingly, fungal members of the skin microbiota are dominated by a single family-*Malassezia* (Findley et al., 2013; Grice and Dawson, 2017). While *Malassezia* is less abundant than skin bacteria, it has much larger biomass that allows functional significance (Ramasamy et al., 2019). This basidiomycete which mainly exists in the yeast form is highly prevalent in sebaceous areas such as scalp, back and facial skin (Prohic et al., 2016; Jo et al., 2017). Advances in sequencing technology have enabled detailed characterization of the genome sequences of skin-residing microbes isolated through both culture-dependent and

culture-independent methods (Grice, 2015; Byrd et al., 2017). Functional annotations of the *Malassezia* genome have revealed the presence of many genes encoding for hydrolytic enzymes- namely proteases, esterases (including lipases and phospholipases) and glucosyl hydrolases (Xu et al., 2007; Gioti et al., 2013; Park et al., 2017; Zhu et al., 2017). This is especially relevant for the skin environment which is nutrient-poor and enriched with lipids and proteins (Chen et al., 2018). In particular, proteases are nature's powerful tools in mediating catabolism of proteins (López-Otín and Bond, 2008), where degradation of specific protein targets can function in important processes such as nutrient acquisition and skin surface adherence (Naglik et al., 2003; Wessler et al., 2017).

In our previous work, we determined that the major secreted protease in the skin commensal *Malassezia globosa* is the aspartyl protease MgSAP1 (Li et al., 2018). This protease is readily secreted in microbial culture during exponential growth of *M. globosa* and is able to reduce *Staphylococcus aureus* biofilm formation, partially through cleavage of the *S. aureus* protein A. Genome analysis of other well-characterized *Malassezia* species such as *Malassezia furfur*, *Malassezia restricta* and *Malassezia sympodialis* further reveals that secreted proteases are well-conserved across the phylum (Wu et al., 2015). This suggests that these secretory enzymes are important for *Malassezia*'s metabolic functions. In the related human fungal opportunistic pathogen *Candida albicans*, production of secreted aspartyl proteases (SAPs) is closely associated with virulence and pathogenesis (Naglik et al., 2003). These secreted *C. albicans* SAPs are capable of degrading many human proteins and this facilitates invasion and colonization of this microbe on mucosal surfaces (Naglik et al., 2008; Winter et al., 2016).

In this study, we focused on characterizing the dominant protease secreted by *M. furfur*. *M. furfur* colonization on skin surfaces is much less abundant than *M. globosa* and *M. restricta,* but it has been associated with various dermatological conditions such as pityriasis versicolor (Gaitanis et al., 2012; Velegraki et al., 2015). More significantly, *M. furfur* is involved in certain rare systemic infections in immunosuppressed patients and in neonates on parenteral nutrition (Gupta et al., 2014; Chen et al., 2019). Functional annotation of the recently sequenced *M. furfur* CBS 14141 genome enabled us to identify different classes of secretory proteases. Using quenched fluorogenic substrates, we determined that the major secreted protease activity in the extracellular media of *M. furfur* is attributed to an aspartyl protease that is a close homolog of the previously characterized MgSAP1 protease in *M. globosa*. This protease, which we herein name *M. furfur* Secreted Aspartyl Protease 1 (MfSAP1), is highly catalytically efficient and processes a broader range of fluorogenic substrates as compared to MgSAP1. We determined that MfSAP1 rapidly cleaves a wide range of extracellular matrix (ECM) proteins associated with the dermis and epidermis. Using an acute wound model created on a 3-D human skin equivalent grown on de-cellularized human dermis, we demonstrated that a high concentration of MfSAP1 can interfere with re-epithelization after wounding.

MATERIALS AND METHODS

Annotation of the *M. furfur* CBS 14141 Secreted Proteases and Dendrogram Construction

Malassezia furfur CBS 14141 was sequenced and the genome assembled in our previous study (Wu et al., 2015) (BioProject: PRJNA286710). Putative transcripts and protein sequences were assigned using FUNAnnotate (unpublished data). Protease prediction and assignment of protease families were performed using MEROPS (Rawlings et al., 2018) (https://www.ebi.ac.uk/merops/). Secreted *M. furfur* proteases were predicted using SignalP 5.0 (Armenteros et al., 2019). The previously published list of secreted *M. globosa* CBS 7966 proteases were re-analyzed with the recently updated SignalP 5.0 to generate a revised list of secreted proteases. For dendrogram construction, protein sequences were aligned using Clustal Omega. Maximum likelihood analysis was performed with IQTree (Trifinopoulos et al., 2016) using the default settings with 1,000 bootstraps. The phylogenetic tree was constructed using Dendroscope (dendroscope.org).

M. furfur Culture and Enrichment of Aspartyl Protease

M. furfur CBS 14141 (previously named JLPK23) strain was cultured routinely in modified Dixon (mDixon) liquid (shaking at 150 rpm) or agar media at 32°C as reported previously (DeAngelis et al., 2007). Sabourad's Dextrose broth (Sigma Aldrich) was used at 30 g/l with 1% Tween-40 (Sigma Aldrich) supplementation. Minimal media culture was prepared using 3.4 g/l of yeast nitrogen broth without amino acids and ammonium sulfate (BD Difco), 5 g/l ammonium sulfate (Sigma Aldrich), 0.2% glycerol (Sigma Aldrich) and 1% Tween-40 at a final pH of 6. Culture extracellular media was obtained by spinning down the yeast culture at 5000 rpm and filtering the supernatant through 0.22 μm vacuum filter. Aspartyl proteases were enriched from the culture supernatant using pepstatin A-agarose resin (Sigma Aldrich) as previously described (Li et al., 2018). Briefly, prewashed pepstatin A-agarose beads were incubated with the culture extracellular media obtained after the specified time of growth, with shaking at 4°C for 1 h. The beads were subsequently washed with 50 mM sodium citrate and 500 mM sodium chloride at pH 4.2, further washed in 50 mM Tris-HCl pH 5.0 buffer and eluted in 100 mM sodium bicarbonate, 500 mM sodium chloride, pH 10.0. Protein concentration was determined using the Qubit Protein Assay kit (ThermoFisher Scientific).

Protease Assay With Internally Quenched Fluorogenic Peptides

Screening of protease activity with substrates S1-S19 was performed by diluting the *M. furfur* extracellular media in a final concentration of 2% (by volume) in 50 mM sodium citrate buffer, pH 4.2. The commercially available substrates S1-S19 (CPC Scientific) were used at a final concentration of 20 μM, unless otherwise indicated. The substrate sequences are listed in **Supplementary Material**. Fluorescence was monitored at Ex/Em

= 330/390 nm on a SpectraMax M2 microplate reader (Molecular Devices) and the slope over the linear range of the signal was used to calculate the proteolytic activity. For inhibition assays, each inhibitor was added at the indicated concentration and activity was determined as above, and calculated as a percentage of the control. For K_m and k_{cat} determination, enriched MfSAP1 was used at a final concentration of 5 pg/μl with varying S12 substrate concentrations and the parameters were calculated using the Michaelis Menten non-linear regression on Graphpad Prism 8.3.0.

In vitro Extracellular Matrix (ECM) Protein Degradation Assays

ECM proteins were purchased from commercial sources as follows—fibronectin (Sigma Aldrich #F0895), rat tail collagen type I (BD Bioscience, #354236), collagen IV from human placenta (Sigma Aldrich #C5533), keratin from human epidermis (Sigma Aldrich #K0253), laminin from human fibroblasts (Sigma Aldrich #L4544), vitronectin from human plasma (Sigma Aldrich #5051), and human thrombospondin-1 (Sigma Aldrich #ECM002). Degradation of the ECM proteins was assessed by incubating each protein at varying substrate to enzyme ratio of enriched MfSAP1 for 4 h at 34°C. 2X Laemmli sample buffer (Biorad) was added at the endpoint, boiled for 5 min and loaded onto NuPAGE Novex 4–12% Bis-Tris Protein gel (ThermoFisher Scientific) or 4–20% Mini-PROTEAN TGX precast gel (Biorad). The gel was stained with SimplyBlue Safestain (ThermoFisher Scientific).

Protease Treatment of Decellularized Keratinocyte and Fibroblast Cell Cultures

N/TERT-1 keratinocytes (Dickson et al., 2000) were grown to ∼60% confluency in keratinocyte serum free media (Life Technologies) on 10 cm tissue cultured treated dishes and decellularized using freshly made 20 mM ammonium hydroxide following the protocol of Hellewell et al. (2017). Primary dermal fibroblasts were cultured using the previously reported macromolecular crowding model (Lareu et al., 2007) in DMEM (Nacalai Tesque) medium containing 18.78 mg/ml Ficoll 70 (Sigma Aldrich), 12.5 mg/ml Ficoll 400 (Sigma Aldrich), and 100 μM ascorbic acid (Sigma Aldrich). Fibroblasts were decellularized using previously published protocol (Demidova-Rice et al., 2011). Briefly, cell culture media was discarded and the cells were washed with phosphate buffered saline (PBS). Decellularization buffer containing 20 mM Tris pH8 (1st Base), 15 mM sodium chloride, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (Sigma Aldrich), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Aldrich), and 0.5% w/v sodium deoxycholate (ThermoFisher Scientific) was added for 5 min at room temperature. The remaining ECM was then washed 5 times with PBS. For protease treatment, enriched MfSAP1 in 50 mM sodium citrate buffer pH 4.2 was added at the indicated concentrations and incubated at 30°C for 2 hrs. The remaining ECM was washed 3 times with PBS, hot 2X Laemmli buffer added and the plate was scraped using a cell scraper. The samples were then loaded onto NuPAGE Novex 4–12% Bis-Tris Protein gel and stained using SimplyBlue Safestain.

Preparation of 3-D Primary Keratinocyte Cultures on De-Epidermized Dermis (DED)

Primary human keratinocytes were obtained from Asian Skin Bank, A*STAR, with ethical approval IRB: B-16-135E. The cells were cultured in Full Green's media as previously described with modification (Xie et al., 2010). The cells were maintained at 37°C in an incubator with 5% CO_2/95% air, with a change of medium every 2–3 days.

The DED-HSE wound healing model was performed as previously published (Xie et al., 2010). Briefly, full thickness skin tissue samples (surgical discard) were purchased from Genoskin, France with ethical approval IRB: B-16-135E. They were trimmed into 1 cm^2 pieces and submerged in 1 M NaCl overnight. After decellularization, the epidermal layer was removed and sterile stainless-steel rings were placed onto the papillary side of each de-epidermized dermis (DED). Keratinocytes (20,000 cells) were transferred into the center of each ring placed on the DEDs and incubated for 2 days. After 2 days of incubation, the rings were removed and the reconstructed samples were elevated to the air-liquid interface and cultivated for another 9 days. To create a wound, a 4 mm biopsy punch (Integra Miltex) was used to remove the reformed epidermis layer on the samples. Topical treatments of pH 5 Full Green's media, 1 μg/ml MfSAP1, 10 μg/ml MfSAP1 (both prepared in pH 5 Full Green's media) were applied thereafter to the wound area daily for 4 days, from the day of wounding. Wound closure was visualized by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (0.5 mg/ml; Sigma Aldrich) as previously described (Xie et al., 2010), and the images were captured using Nikon SMZ745T microscope. A total of 3 independent experiments were performed. Quantification of the uncovered wound area and lateral migration was performed using ImageJ 1.52a. For each experiment, 2 technical replicates with the nearest values of each sample were used for quantification analysis. Statistical analysis was performed using the two-tailed Mann-Whitney Test on Graphpad Prism 8.3.0. The DED-HSE samples were then fixed and embedded in paraffin for further histological processing and analysis. Details on histology and immunohistochemistry is found in **Supplementary Material**.

RESULTS

Prediction of *Malassezia furfur* Secreted Proteases

Using the previously sequenced and assembled haploid *M. furfur* CBS 14141 genome, we identified 4132 putative protein-coding genes. These genes were further analyzed using the MEROPS pipeline for protease family assignment and the secreted proteases were predicted using SignalP 5.0. From this, we identified a total of 14 secreted proteases (**Figure 1A**, **Supplementary Table 1**). While the overall number of predicted secretory proteases is similar to *M. globosa* CBS 7966, one distinct

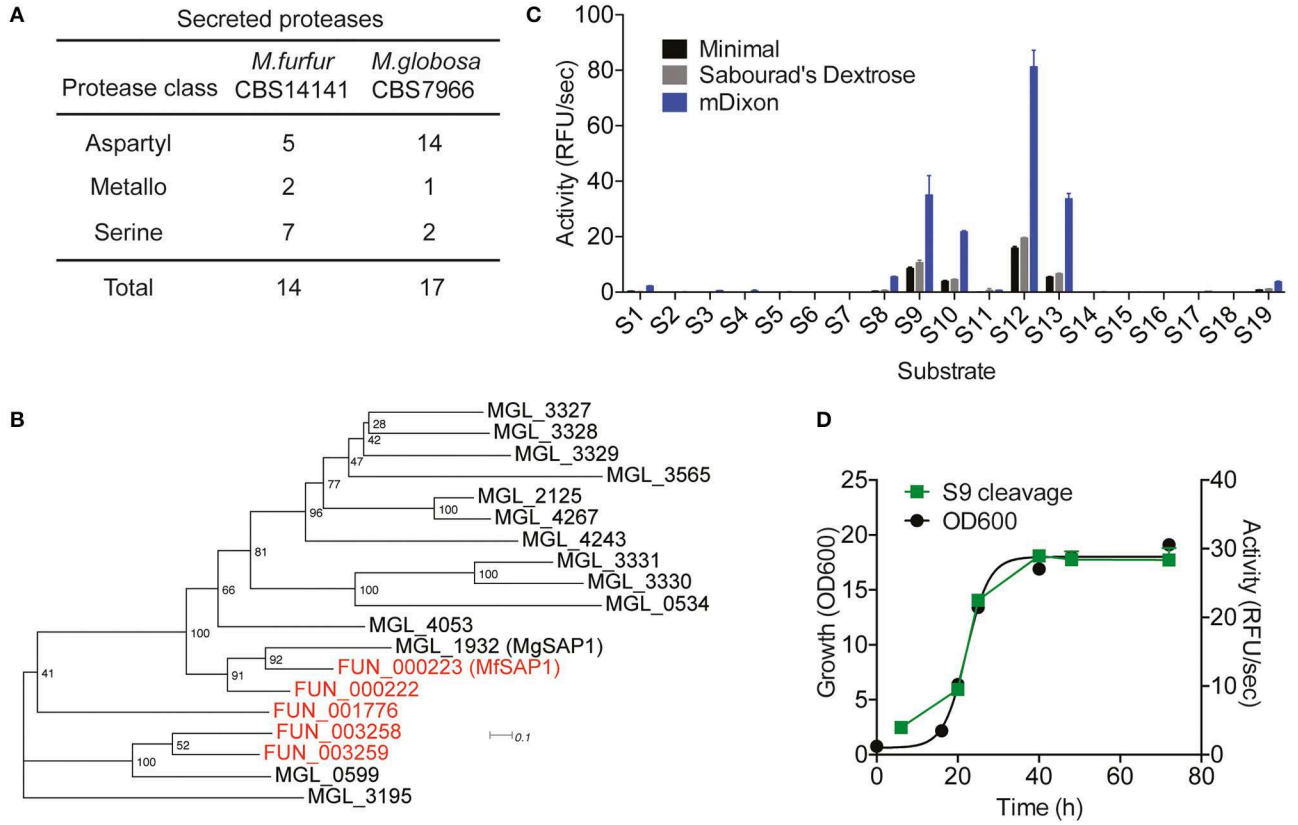

FIGURE 1 | Prediction of *Malassezia furfur* secreted proteases and assessment of extracellular protease activity in culture. **(A)** A comparison of the predicted secreted proteases in *M. furfur* CBS 14141 and *M. globosa* CBS 7966. **(B)** Unrooted phylogenetic tree of the predicted secretory aspartyl proteases in *M. globosa* (14, in black) and *M. furfur* (5, in red). Number at each node indicates the bootstrap value. **(C)** Extracellular protease activity of *M. furfur* cultured in 3 different media assessed using 19 internally quenched substrates. **(D)** Correlation of secreted protease activity using a representative substrate S9 with planktonic culture growth. Error bars represent standard deviation for $n = 3$.

difference is the expansion of the secreted serine protease family and the reduction of the aspartyl secreted protease family in *M. furfur*.

To compare the 5 *M. furfur* predicted secreted aspartyl proteases with those of *M. globosa*, we performed a multiple sequence alignment using Clustal Omega followed by an unrooted phylogenic tree analysis (**Figure 1B**). FUN_000223 clusters most closely with the previously characterized MgSAP1 (67.7% similarity), and will herein be named as *M. furfur* Secreted Aspartyl Protease 1 (MfSAP1). FUN_000222 is closely related to both MfSAP1 and MgSAP1, while FUN_003258 and FUN_003259 are more similar to MGL_0599 (**Figure 1B**).

Protease Activity in Culture Is Dominated by MfSAP1

To determine secreted protease activity, we cultured *M. furfur* CBS 14141 in 3 different media of varying nutrient richness: mDixon, Sabourad's Dextrose and minimal media. We isolated the extracellular media and performed protease activity assays using a diverse panel of 19 internally quenched fluorescent substrates (S1-S19, see **Supplementary Material**). We observed that when grown in mDixon, *M. furfur* produced the highest extracellular protease activity and this corresponds to the higher growth density obtained in this rich media (**Figure 1C**). We detected a similar pattern of substrate cleavage preference in all 3 media, with substrate S12 having the most preferred cleavage sequence. We further assessed the proteolytic activity throughout the planktonic growth phases of *M. furfur*, and observed that the protease activity correlates with growth (**Figure 1D**). This is in clear contrast with the secreted protease activity from *M. globosa* detected using the same fluorogenic substrate, which peaks at late log phase and decreases thereafter (Li et al., 2018).

To identify the class of protease responsible for the detected activity, we assessed the protease activity of the extracellular media after treatment with class specific protease inhibitors. Inhibitors of serine proteases (AEBSF), cysteine proteases (E-64), and metalloproteases (EDTA) had no significant effect on the protease activities determined using S9 and S13. In contrast, virtually no protease activity was detected in the presence of aspartyl protease inhibitor pepstatin A (**Figure 2A**). As this indicates that the major secreted enzymes activities are contributed by aspartyl proteases, we used pepstatin A-agarose as a chemical affinity tool to enrich for aspartyl proteases present in the *M. furfur* mDixon and minimal conditioned

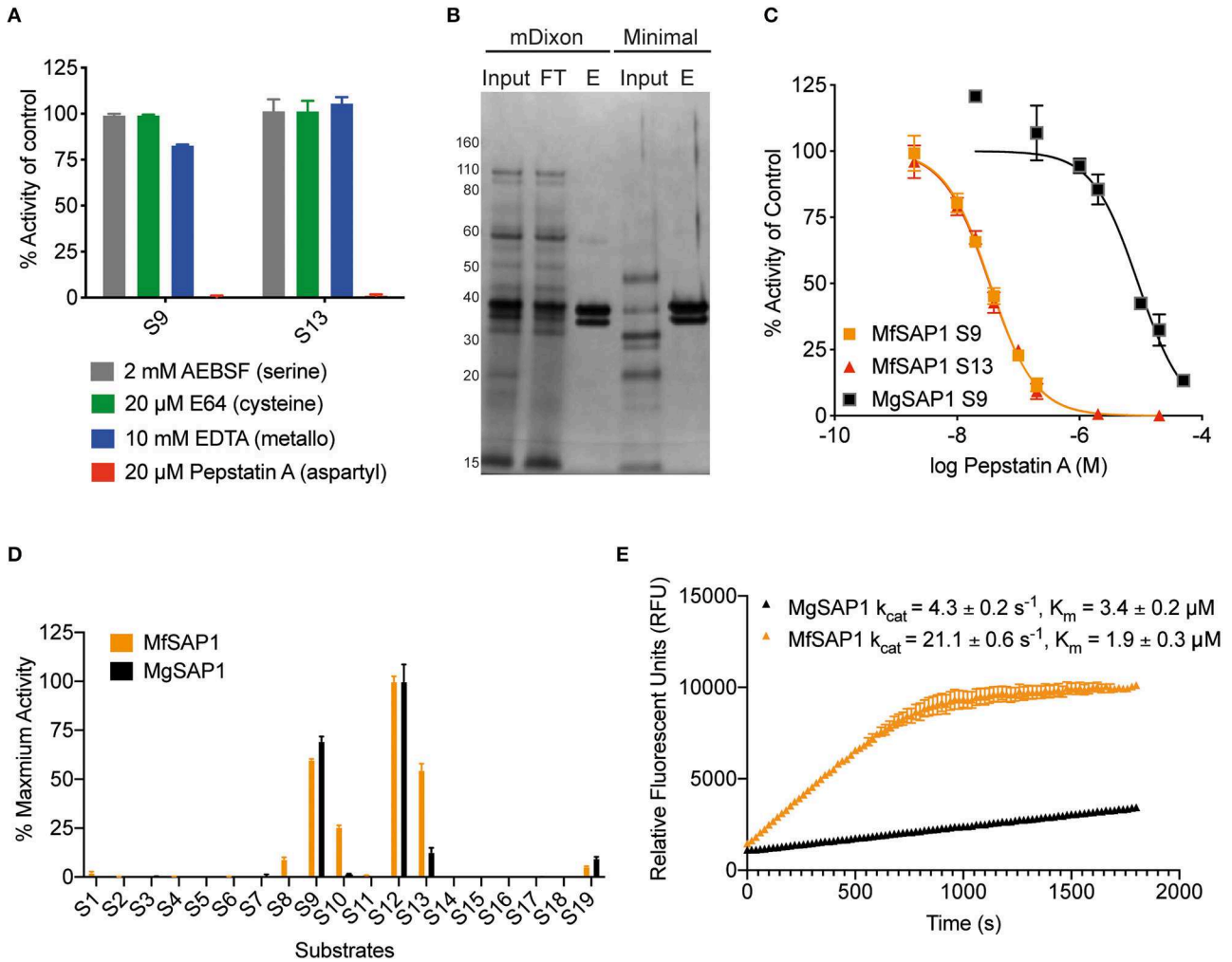

FIGURE 2 | Identification of the major aspartyl protease MfSAP1 in *M. furfur* and comparison with its *M. globosa* homolog MgSAP1. **(A)** *M. furfur* extracellular media was treated with each protease inhibitor and the remaining protease activity was assessed using S9 and S13. **(B)** Silver stain of the extracellular media (input), the enriched elute (E) and the flow-through (FT) from the pepstatin A-agarose affinity purification of the secreted proteases in *M. furfur* culture grown in mDixon and minimal media. **(C)** Inhibition curves of the enriched proteases MfSAP1 and MgSAP1 against the aspartyl protease inhibitor pepstatin A. **(D)** Comparison of the substrate cleavage preferences of MfSAP1 and MgSAP1 for the quenched substrates S1-S19. Protease activities were normalized to the maximum activity of the panel (S12) in each enzyme. **(E)** Kinetic parameters of enriched MfSAP1 and MgSAP1 for cleavage of substrate S12. A representative plot at 20 μM of S12 is shown for both enzymes. The Michaelis-Menten plot for MfSAP1 is included in **Supplementary Figure 1**. The data for MgSAP1 is previously published in Li et al. (2018). Error bars represent standard deviation for $n = 3$.

media. The major protein species we isolated are two proteins around 30–40 kDa (**Figure 2B**). Using in-gel trypsinization followed by mass spectrometry, we determined that both proteins have the same peptide sequences that corresponds to MfSAP1 (**Supplementary Figure 1**). We further confirmed that the two proteins have the same N-terminal sequence by Edman sequencing (**Supplementary Figure 1**). We interpret that the two protein species are likely different glycosylated forms of MfSAP1. We next determined the optimum pH for MfSAP1 and found it to be between pH 4 and 5 (**Supplementary Figure 1**), commensurate with the acidic pH of healthy skin. From the pepstatin A-agarose enrichment, we estimate that >95% of the protease activity in the culture conditioned media is attributed to MfSAP1, and using purified enzyme activity as an estimation, MfSAP1 is secreted at a concentration of 4.4 μg/ml in culture after late log phase in mDixon media.

Comparison of *M. furfur* MfSAP1 and *M. globosa* MgSAP1

To compare the dominant secreted protease of *M. furfur* and *M. globosa*, we first determined the IC_{50} of pepstatin A inhibition from enriched preparations of both enzymes. To our surprise, the IC_{50} for MfSAP1 as determined by inhibition of substrate S9 is 35 ± 1 nM, which is much lower than the IC_{50} of 9 ± 1 μM for MgSAP1 (**Figure 2C**). The stronger preference of pepstatin A in inhibiting MfSAP1 over MgSAP1 suggests

that there may be substantial differences in the active site binding pocket of these enzymes. To verify this, we assessed the substrate cleavage preference of both enzymes using the panel of 19 substrates. We observed that while the overall trend of preferred substrates is similar for MfSAP1 and MgSAP1, MfSAP1 cleaves an expanded repertoire of these fluorogenic substrates (**Figure 2D**). This is evident in MfSAP1's enhanced cleavage of S8, S10 and S13 compared to MgSAP1. We next determined the catalytic efficiency of both enzymes using the most preferred substrate S12 and observed that the k_{cat}/K_m for this substrate is nearly 10-fold greater for MfSAP1 than that for MgSAP1 (**Figure 2E**, **Supplementary Figure 1**).

MfSAP1 Readily Cleaves Human Skin-Associated Extracellular Matrix Proteins

Malassezia is the most abundant fungal family on the human skin at all sites except the feet (Belkaid and Segre, 2014). We therefore reasoned that the native substrates of MfSAP1 are likely extracellular proteins present on the human skin surface. To assess whether MfSAP1 processes common human extracellular matrix (ECM) proteins expressed by epidermal and dermal cells, we incubated MfSAP1 at varying enzyme to substrate ratios with purified ECM proteins at physiologically relevant condition of 34°C, pH 5 for 4 hrs. We observed strong degradation of vitronectin, human epidermal keratin, thrombospondin, and fibronectin at low enzyme to substrate ratios (**Figures 3A–E**). This is especially evident for keratin isolated from human epidermal culture, where we observed cleavage of keratin at 1,000-fold less MfSAP1. When we incubated MfSAP1 with type I collagen, the most abundant ECM protein in the dermal matrix, we observed only weak proteolysis of this matrix protein (**Supplementary Figure 2**). However, when the collagen was heat-denatured, MfSAP1 is able to degrade this ECM protein rapidly (**Figure 3D**). This indicates that MfSAP1 is unable to act on collagen I in its native, triple helix conformation. However, denatured collagen strands are accessible to MfSAP1 proteolysis. Degradation of collagen IV, a component of the epidermal-dermal junction (basement membrane) layer, proceeded similarly to that of type I collagen and denatured collagen IV was also cleaved more readily than native collagen IV (**Supplementary Figure 2**).

To assess MfSAP1's effect on intact native epidermal ECM and dermal ECM, we cultured N/TERT-1 keratinocytes and primary human dermal fibroblasts, decellularized these cultures, isolated the ECM produced *de novo*, and treated this ECM with MfSAP1 (**Figure 3F**). To increase fibroblast secretion of ECM we used macromolecular crowding to enhance collagen deposition (Lareu et al., 2007). We observed that ECM produced by both keratinocyte and fibroblast were rapidly degraded with only a short exposure to MfSAP1 at concentrations that are comparable to the concentrations of MfSAP1 secreted in culture. The sensitivity of *de novo* synthesized dermis/epidermis ECM was apparent even with low concentrations (0.1 μg/ml) of MfSAP1 (**Figure 3F**).

Assessment of MfSAP1 in Epithelial Wound Healing

Our *in vitro* experiments demonstrated that MfSAP1 is able to efficiently cleave epidermal and dermal-associated ECM proteins. In order to determine whether this activity may have any effect on epidermal wound healing, we utilized an *in vitro* reconstructed 3-D skin model to test the effects of MfSAP1 on cutaneous wound healing. Primary human keratinocytes were cultured on de-epidermized dermis (DED) obtained from human donors creating a mature, full-thickness epidermis. A biopsy punch (4 mm) was used to completely remove the epidermis (**Figure 4A**, D1) leaving a reproducible acute wound on this DED-human skin equivalent (DED-HSE). MfSAP1 in control Full Green's media adjusted to pH 5 (for compatibility with MfSAP1's acidic pH optimal) was applied onto this wound daily for 4 days at two different protease concentrations (1 and 10 μg/ml) which we demonstrated above to be efficient in processing keratinocyte and fibroblast ECM proteins. Closure of the wound is quantitatively assessed using the conversion of MTT by the viable cells in the epithelial tongue (**Figure 4A**). We observed that treatment with 1 μg/ml of MfSAP1 had little effect on the epithelial migration compared to the control samples, as determined by wound area and lateral migration of the keratinocytes (**Figure 4B**, **Supplementary Figure 3**). We observed that acute wounds exposed to 10 μg/ml MfSAP1 were significantly slower to heal, as measured by uncovered wound area and lateral migration of keratinocytes (**Figure 4B**, **Supplementary Figure 3**). These data suggest that the proteolytic activity of MfSAP1 interferes with cell attachment to underlying ECM, thus inhibiting cell migration and epidermal wound closure.

We examined the histology of the keratinocytes in the wounded DED-HSE constructs using haematoxylin and eosin (H&E) (**Figures 4A,C**). Healing wound edges were characterized by an advancing wedge-shaped "epithelial tongue" in all treatment samples (**Figure 4A**). However, samples exposed to 10 μg/ml MfSAP1 exhibited reduced lateral cell migration and immature squamous differentiation compared to the media control treated samples, and to samples exposed to 1 μg/ml MfSAP1 (**Figure 4C**). To assess the effect on keratinocyte differentiation, we performed immunohistochemistry using the transcription factor p63 (un-differentiated cells), keratin 10 (suprabasal differentiated keratinocytes), and keratin 14 (basal un-differentiated keratinocytes) (**Figure 4C**, **Supplementary Figure 3**). We observed in the 10 μg/ml MfSAP1 treated DED-HSE constructs, most of the epithelial cells in the migrating tongue were undifferentiated basal keratinocytes. This contrasts with DED-HSE constructs exposed to the control media or 1 μg/ml MfSAP1, where layers of differentiated keratinocytes with high keratin 10 expression were evident adjacent to the wound margin (**Figure 4C**).

DISCUSSION

Malassezia is an integral component of the human skin microbiome as evident from its high prevalence across mammalian species (Gaitanis et al., 2012; Theelen et al.,

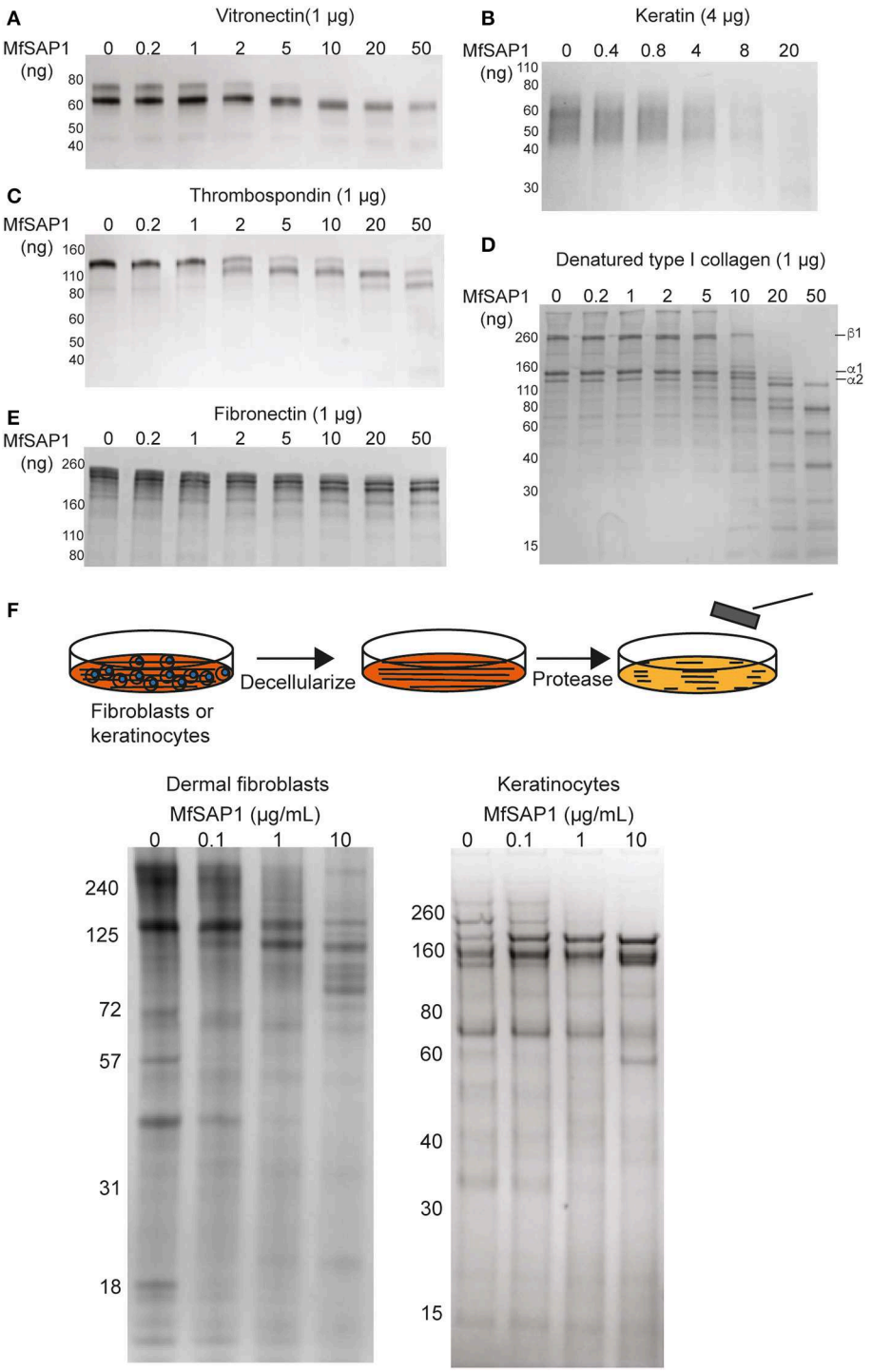

FIGURE 3 | MfSAP1 degrades human extracellular matrix proteins. **(A–E)** ECM protein degradation, as assessed by SDS-PAGE, after MfSAP1 was incubated with each purified ECM protein at varying substrate to enzyme ratios. **(F)** Cartoon of the decellularization and protease treatment (top). Degradation of whole dermal fibroblast (left) and keratinocyte (right) ECM at different MfSAP1 concentrations by SDS-PAGE analysis. Representative SDS-PAGE images are shown for a total of at least 2 independent experiments.

2018). Recent studies have revealed *Malassezia* may also impact physiological events beyond the skin environment, in particular the human gastro-intestinal microbial ecosystem (Aykut et al., 2019; Limon et al., 2019). However, our appreciation and understanding of the molecular events involved in the interactions of *Malassezia* sp. with the host and other elements

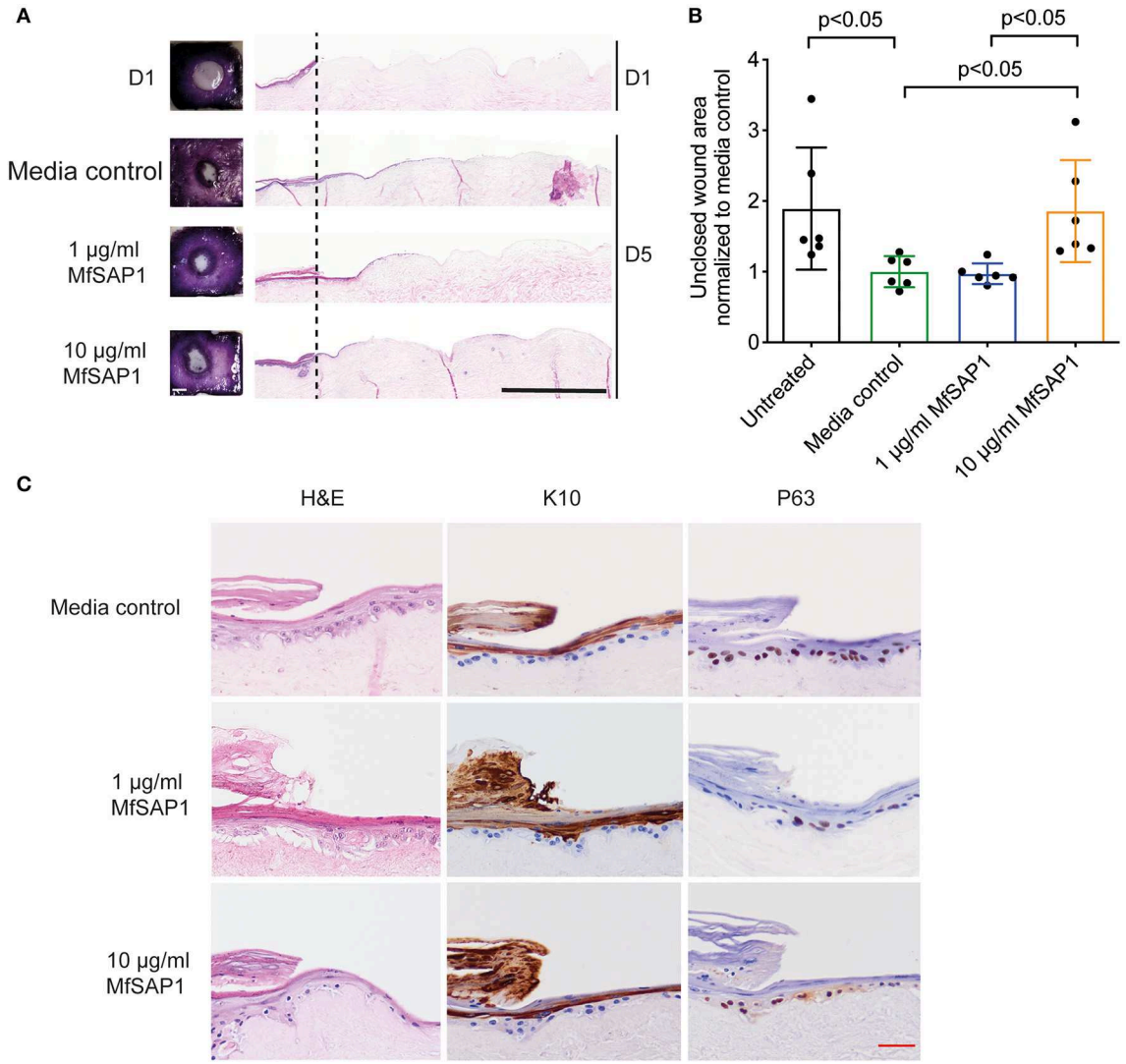

FIGURE 4 | Effect of MfSAP1 on acute wound closure. **(A)** MTT staining (left) and hematoxylin and eosin (H&E, right) staining of the DED-HSE sections on Day 1 and Day 5. The dashed line indicates the edge of the punch biopsy. Black scale bar, 1 mm. **(B)** Quantification of the uncovered wound area as determined by MTT stain using ImageJ. Each wound area sample is normalized to the media control. Error bars represent standard deviation for $n = 6$ from a total of 3 experimental replicates with technical duplicates. **(C)** H&E and immunohistochemical analysis of the wound edge on Day 5 for each treatment condition. Representative images are shown from a total of 3 independent experiments. Red scale bar, 50 µm.

of the human microbiome is poor. In this study, we have focused on a secretory enzyme that has potential to act as a mediator in these interactions. We identified that the dominant protease secreted by *M. furfur* is MfSAP1, an aspartyl protease with high catalytic efficiency. MfSAP1 exhibits specific activity against a variety of elements of the dermal and epidermal ECM, a property that has important implications for cutaneous wound healing.

Malassezia furfur is among the earliest discovered of the *Malassezia* species. As such, many seminal works were performed using this species as an exemplar of *Malassezia* sp. in skin health and disease (Ashbee and Evans, 2002). As *M. furfur* is perhaps the most easily cultivated *Malassezia* species, it is frequently isolated from human skin in culture studies even though it is much less prevalent than *M. globosa* and *M. restricta* (Wu et al., 2015; Leong et al., 2019). Importantly, *M. furfur* CBS 14141 is one of only three strains of *Malassezia* that are currently amenable to genetic manipulation (Ianiri et al., 2016; Celis et al., 2017) and understanding the functions of secretory proteases in this strain opens up future opportunities for genetic approaches for further analysis.

When studying proteases secreted by *M. furfur* and *M. globosa*, we were surprised to recognize that *M. furfur* has a reduced number of secreted aspartyl proteases, but has an expanded number of secreted serine proteases. Nonetheless, secreted protease activity from *M. furfur* is dominated by a

homolog of the previously characterized MgSAP1. The fact that the extracellular protease activity is dominated by just one secreted aspartyl protease in these two species suggests that homologs of MfSAP1 and MgSAP1 may also dominate the secreted protease activity repertoire of other *Malassezia* species. That homologous aspartyl protease has been retained in multiple species strongly implies that this protease family has integral functions across all members of the *Malassezia* family. Interestingly, while MfSAP1 has similarities with MgSAP1 in terms of protease expression and cleavage of the internally quenched substrates, MfSAP1 is catalytically more efficient and cleaves a wider range of substrates. By implication, at the same given concentration, MfSAP1 is able to process substrates at greater efficiency than MgSAP1. Furthermore, while MgSAP1 protease activity is tightly controlled at the post-translational level, and expression peaks during late log phase of planktonic growth, MfSAP1 is constitutively secreted during all stages of growth. Taken together, these observations highlight the importance of characterizing the activity of MfSAP1; despite its low abundance it is functionally relevant to the integrity and repair of human skin.

In healthy skin, the stratum corneum together with the tight junctions in the stratum granulosum forms a barrier against external agents (Matsui and Amagai, 2015). Given that *Malassezia* sp. are skin residing fungal species, we reasoned that the native substrates of MfSAP1 was likely to include molecular species present in the host's epidermal and dermal layers. As such, we considered that the relevance of MfSAP1's ECM processing activities should be considered only when the epidermal barrier is compromised, such as in an acute wound. We examined the ability of MfSAP1 to cleave a wide range of skin-associated ECM proteins *in vitro*, especially those associated with the basal keratinocytes. Key components of the epidermis and dermis ECM, including vitronectin, human epidermal keratin, thrombospondin and fibronectin are cleaved by MfSAP1 at low enzyme to substrate ratios. When present at high concentration, MfSAP1 was shown to attenuate re-epithelization, thus retarding wound healing. However, we recognize that wound healing is a complex process involving many factors contributed by the dermal layer, microvasculature, innate and adaptive immune systems; these are absent in our *in vitro* DED-HSE model. Nevertheless, in this model of partial-thickness acute wounding, we demonstrated that MfSAP1 may be part of this complex mix *in vivo*, and potentially attenuate wound healing through the degradation of key ECM components.

In conclusion, we have demonstrated in this study that *M. furfur*, a component of the human skin microbiome produces an aspartyl protease that has strong skin ECM degradation activity. Combined with our previous study of MgSAP1, it is evident that this aspartyl protease and its homologs likely modify the skin environment through degrading both human and bacteria associated substrates.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SP, DL, TD, and HL designed the experiments. HL, JG, and WC performed the protease isolation and characterization. SP, CF, SG, PL, and BS performed the DED-HSE experiments. SP, CF, and HL analyzed the data. CF and HL wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00148/full#supplementary-material

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Genomic Multiplication and Drug Efflux Influence Ketoconazole Resistance in *Malassezia restricta*

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Malassezia restricta is an opportunistic fungal pathogen on human skin; it is associated with various skin diseases, including seborrheic dermatitis and dandruff, which are usually treated using ketoconazole. In this study, we clinically isolated ketoconazole-resistant *M. restricta* strains (KCTC 27529 and KCTC 27550) from patients with dandruff. To understand the mechanisms of ketoconazole resistance in the isolates, their genomes were sequenced and compared with the susceptible reference strain *M. restricta* KCTC 27527. Using comparative genome analysis, we identified tandem multiplications of the genomic loci containing *ATM1* and *ERG11* homologs in *M. restricta* KCTC 27529 and KCTC 27550, respectively. Additionally, we found that the copy number increase of *ATM1* and *ERG11* is reflected in the increased expression of these genes; moreover, we observed that overexpression of these homologs caused ketoconazole resistance in a genetically tractable fungal pathogen, *Cryptococcus neoformans*. In addition to tandem multiplications of the genomic region containing the *ATM1* homolog, the *PDR5* homolog, which encodes the drug efflux pump protein was upregulated in *M. restricta* KCTC 27529 compared to the reference strain. Biochemical analysis confirmed that drug efflux was highly activated in *M. restricta* KCTC 27529, implying that upregulation of the *PDR5* homolog may also contribute to ketoconazole resistance in the strain. Overall, our results suggest that multiplication of the genomic loci encoding genes involved in ergosterol synthesis, mitochondrial iron metabolism, and oxidative stress response and overexpression of the drug efflux pumps are the mechanisms underlying ketoconazole resistance in *M. restricta*.

Keywords: *Malassezia restricta*, ketoconazole, resistance, genomic multiplication, efflux pump

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INTRODUCTION

The lipophilic yeast *Malassezia restricta* is the most commonly found fungus on human skin; it is implicated in skin diseases such as seborrheic dermatitis and dandruff (Clavaud et al., 2013; Findley et al., 2013; Xu et al., 2016; Park T. et al., 2017). Multiple tropical drugs with antifungal activity against *Malassezia* have been used for the treatment of skin diseases associated with *M. restricta* (Carrillo-Muñoz et al., 2013; Cafarchia et al., 2015; Rojas et al., 2017). Among these antifungal drugs, ketoconazole, an imidazole compound, exhibits highly effective fungistatic activity against *Malassezia*; the effectiveness of this azole against seborrheic dermatitis and dandruff has also been demonstrated (Danby et al., 1993; Pierard-Franchimont et al., 2001, 2002).

Azole antifungal drugs, including ketoconazole, inhibit a cytochrome P450 enzyme, lanosterol 14α-demethylase, which participates in the synthesis of ergosterol, a major constituent of the fungal cell membrane (Vanden Bossche et al., 1987; Yoshida and Aoyama, 1987). Inactivation of this enzyme results in the demethylation of lanosterol, which inhibits the process of ergosterol synthesis, leading to accumulation of the bypass product as toxic methylated sterols in the fungal cell membrane, defects in fungal plasma membrane integrity, and inhibition of cell growth (Joseph-Horne and Hollomon, 1997; Heimark et al., 2002). However, as the azole drug is fungistatic, prolonged use of antifungals offers the opportunity for pathogenic fungi to acquire drug resistance. For example, since the first reports of the emergence of miconazole-resistant *Candida albicans* in 1978, clinical isolates of azole-resistant *Candida* species have been identified continuously (Holt and Azmi, 1978; Ksiezopolska and Gabaldón, 2018). *Cryptococcus* and *Aspergillus* isolates that are resistant to azole antifungal drugs have also been reported frequently (Smith et al., 2015; Rivero-Menendez et al., 2016).

Azole resistance mechanisms involve the *ERG11* gene encoding a lanosterol 14α-demethylase, which is the direct target enzyme of azole antifungal drugs. Mutations in the coding region of the gene result in amino acid substitutions, which alter the structure of the enzyme and reduce the affinity of the target for the azole (Sanglard et al., 1998; Warrilow et al., 2010). Overexpression of *ERG11*, resulting in increased levels of the antifungal target protein, is also considered one of the mechanisms of azole resistance in fungi (Flowers et al., 2012; Feng et al., 2017).

Besides mutations and overexpression of *ERG11*, increased drug efflux resulting in decreased intracellular drug accumulation is known to be one of the major mechanisms of azole resistance. In *C. albicans*, two efflux pumps of the ATP-binding cassette (ABC) transporter family, Cdr1 (*Candida* drug resistance (1) and Cdr2 are well known to be linked with resistance to azole antifungal drugs (Prasad et al., 1995; Sanglard et al., 1997). Further, a number of azole-resistant *C. albicans* isolates showed overexpression of *CDR1* and *CDR2*; deletion of one or both genes resulted in hyper-susceptibility to azoles (White, 1997; Lyons and White, 2000; Tsao et al., 2009). Another efflux protein, Mdr1, a transporter of the major facilitator superfamily (MFS) class is involved in fluconazole resistance; this gene was found to be overexpressed in fluconazole-resistant *C. albicans* isolates (White, 1997; Lyons and White, 2000; Hiller et al., 2006; Feng et al., 2018).

Most *Malassezia* strains are sensitive to azole drugs; however, recent studies reported the emergence of azole-resistant *Malassezia* species (Jesus et al., 2011; Nijima et al., 2011; Cafarchia et al., 2012a,b; Iatta et al., 2014; Kim et al., 2018). To date, two studies have investigated the mechanisms of azole susceptibility and resistance in *Malassezia.* Iatta et al. suggested that the drug efflux pump is involved in azole resistance in *M. pachydermatis* and *M. furfur* (Iatta et al., 2017); Kim et al. demonstrated that tandem quadruplication of the genomic region containing the genes required for ergosterol synthesis contributes to azole resistance in *M. pachydermatis* (Kim et al., 2018). However, these studies were mainly focused on *M. pachydermatis* residing on canine skin, and no study, to our knowledge, has reported the isolation of azole-resistant *M. restricta*, the most predominant *Malassezia* species on human skin, and analyzed the mechanism of its resistance.

Thus, in this study, we isolated ketoconazole-resistant *M. restricta* strains from dandruff patients and aimed to elucidate their resistance mechanisms using comparative genome analysis. In the resistant isolates, we found a tandemly multiplicated genomic locus along with increased gene expression and hypothesized that the genomic multiplication contributes to azole resistance in *M. restricta*. Further, we observed increased drug efflux in a resistant isolate, suggesting that this also influences resistance in *M. restricta*.

MATERIALS AND METHODS

Strains and Growth Conditions

Malassezia restricta strains were grown on LNA medium (0.5% glucose, 1% peptone, 0.01% yeast extract, 0.8% bile salt, 0.1% glycerol, 0.05% glycerol monostearate, 0.05% Tween 60, 1.2% agar, and 0.5% whole fat cow milk) at 34°C for 3 days (Leeming and Notman, 1987; Guillot and Gueho, 1995; Park M. et al., 2017). The wild type *Cryptococcus neoformans* var. *grubii* H99 and the overexpression strain were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or YPG medium (1% yeast extract, 2% peptone, and 2% galactose) at 30°C for 1–2 days.

Drug Susceptibility Tests

Minimal inhibitory concentrations (MICs) were determined using the method described by Gupta et al. and Sugita et al. with slight modifications (Gupta et al., 2000; Sugita et al., 2005). Briefly, 20 μL of antifungal drug compounds (50× of stock solution) were serially diluted 2-fold with 980 μL of melted LNA medium in a 24-well plate. Ketoconazole, terbinafine, amphotericin B, and zinc pyrithione (ZPT) were used to test the drug susceptibility of *M. restricta.* The yeast cells were inoculated into each well of the 24-well plate and incubated at 34°C for 3 days. The MIC values were determined as the lowest concentration at which growth was invisible compared with that in the medium without any drugs. To estimate the ketoconazole susceptibility of the *C. neoformans* strain overexpressing *ATM1* and *ERG11*, 10-fold serial dilutions of cells starting at 10^4 cells were spotted onto YPD or YPG agar plates with or without ketoconazole and incubated at 30°C for 2 days. To evaluate the susceptibility of oxidative stress in *M. restricta* strains, ten-fold serial dilutions of cells starting at 10^7 cells were spotted onto LNA plates with or without H_2O_2. Plates were incubated at 34°C for 7 days.

Genome Sequencing

Malassezia restricta KCTC 27529 and KCTC 27550 were grown on LNA medium at 34°C for 3 days; genomic DNA from the cells was extracted using glass beads and vortexing as described previously (Van Burik et al., 1998). Genome sequencing of *M. restricta* KCTC 27529 and KCTC 27550 was performed

using the Illumina MiSeq and PacBio RSII platforms with C4 chemistry. Illumina libraries were constructed using the TruSeq DNA Library Prep LT Kit (Illumina, USA) according to the manufacturer's instructions. The constructed libraries were sequenced on the Illumina MiSeq instrument and 300 bp paired-end reads were generated. Raw reads were quality-trimmed using Trimmomatic v0.36 and mapped to the reference genome using bowtie2 v2.2.5 with the "–very-sensitive" option (Langmead and Salzberg, 2012; Bolger et al., 2014). The coverage of each gene and that of the whole genome were calculated using the length and the number of mapped bases. Finally, the copy number of each gene was determined by dividing its coverage with the average in the entire genome. The PacBio library, with 20-kb inserts, was prepared using the PacBio Sample Net-Shared Protocol (available at http://pacificbiosciences.com/). The constructed libraries were loaded into one SMRT cell for each sample and sequenced using the PacBio RS II instrument (PacBio, Menlo Park, CA, USA). Data from the genome sequencing were deposited in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) (Bioproject number, PRJNA592379).

Identification of Single Nucleotide Mutations in *ERG11*

The coding region of *ERG11* from the ketoconazole-susceptible *M. restricta* isolates was amplified by PCR using the primers ERG11_start1 and ERG11-4 (**Supplementary Table 1**). The resulting amplified PCR products were sequenced using ERG11_start2, ERG11-2, ERG11-3, and ERG11-4. The obtained sequences were compared with the *ERG11* sequence of the reference strain, *M. restricta* KCTC 27527.

Construction of the Overexpression Strains of *C. neoformans*

To overexpress *ATM1* in *C. neoformans*, the native promoter of the gene was substituted with the *TEF1* promoter via homologous recombination. The *TEF1* promoter was amplified by PCR using *C. neoformans* H99 genomic DNA as a template and the primers TEF1p_F_XbaI and TEF1p_R_XhoI. The PCR product was digested using XbaI and XhoI and subsequently cloned into the plasmid pJAF1 containing the neomycin resistance (NEO^R) gene; the resulting plasmid was named pWH132 (Fraser et al., 2003). The DNA fragment containing the NEO^R gene and *TEF1* promoter was amplified by PCR using the plasmid pWH132 as a template and the universal primers M13-F and M13-R. The 5′ flanking region containing the native promoter of *ATM1* and the 3′ flanking region containing the partial coding sequence of the gene from the start codon to 841 bp were amplified by PCR using the primers TEF1p_ATM1-1/TEF1p_ATM1-2 and TEF1p_ATM1-3/TEF1p_ATM1-4, respectively, with H99 genomic DNA as the template. The 5′ and 3′ flanking regions and the NEO^R-P_{TEF1} region were fused by overlapping PCR using primers TEF1p_ATM1-5 and TEF1p_ATM1-6. The resulting cassette was biolistically transformed into the H99 strain as described previously (Toffaletti et al., 1993). The used primers are listed in **Supplementary Table 1**. The *ERG11* overexpression strain that was constructed in our previous study was used in the current study (Kim et al., 2012).

RNA Isolation and cDNA Synthesis

M. restricta KCTC 27527, KCTC 27529, and KCTC 27550 were grown on LNA medium at 34°C for 3 days; *C. neoformans* H99 and strains overexpressing *ATM1* and *ERG11* were cultured at 30°C overnight in YPD or YPG medium. Total RNA was extracted from the cells using TransZol Up (TransGen Biotech, China) and used to synthesize cDNA. cDNA was synthesized using the RevertAid First Strand cDNA synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Quantitative Real-Time PCR

To validate the gene copy number and gene expression, quantitative real-time PCR (qRT-PCR) was performed. Genomic DNA and cDNA were used as templates for gene copy number and gene expression, respectively. Gene-specific primers for qRT-PCR were designed using Primer Express software 3.0 (Applied Biosystems, Foster, CA, US) and are listed in **Supplementary Table 1**. Relative quantitation of gene expression was performed using the $2^{\Delta}\Delta CT$ method on a 7500 system (Applied Biosystems, Foster, CA, US) (Livak and Schmittgen, 2001). The actin gene (MRET_1518) and *TEF2* (CNAG_00044, translation elongation factor 2) were used as endogenous control genes of *M. restricta* and *C. neoformans*, respectively.

Flow Cytometric Analysis

Cells were grown at 34°C for 3 days, and 2×10^7 cells/mL of each strain were resuspended in 20 mL of modified Dixon's (mDixon's) medium (3.6% malt extract, 0.6% peptone, 2% bile salt, 1% Tween 40, 0.2% oleic acid, and 0.2% glycerol) with or without 10 μM rhodamine 6G (R6G), followed by incubation at 34°C for 2 h (Midgley, 1989). Uptake of R6G into the cells was stopped by cooling the reaction mixture on ice. To determine R6G accumulation, samples were diluted 40-fold in cold 0.1 M phosphate-buffered saline (PBS) and directly subjected to flow cytometry using FACSAria II (BD Bioscience, San Jose, CA, USA) at a wavelength of 488 nm. The data of 5,000 cells from each sample were collected at the PE channel and analyzed using FACS DIVA (BD Bioscience, San Jose, CA, USA). For efflux of R6G, cells were washed twice with cold 0.1 M PBS and suspended in PBS, followed by incubation at 34°C for 30 min. The samples were then prepared and analyzed as described above.

Statistical Analysis

All data are presented as the arithmetic mean ± standard deviation. Differences between samples were calculated by two-tailed Student's *t*-test for unpaired data. A $p < 0.01$ was considered statistically significant.

TABLE 1 | MIC values of antifungal agents in *M. restricta*.

Strain	Ketoconazole	Terbinafine	Amphotericin B	ZPT
KCTC 27512	0.06–0.12	0.25–0.5	4	1.59
KCTC 27518	0.03–0.06	0.5–1	4–8	0.795
KCTC 27519	0.12–0.24	0.5	4–8	3.18
KCTC 27522	0.06–0.12	0.25–0.5	4	3.18
KCTC 27527	0.03	0.5	2	1.59
KCTC 27529	7.68	2	2–4	3.18
KCTC 27539	0.03–0.06	0.5–1	2–4	1.59
KCTC 27540	0.06–0.12	0.5–1	4–8	3.18
KCTC 27542	0.06–0.12	1	4–8	1.59
KCTC 27543	0.06	0.5–1	4–8	3.18
KCTC 27544	0.03–0.06	0.5–1	8	3.18
KCTC 27548	0.12	0.5–1	4–8	1.59
KCTC 27550	7.68	0.5	4	1.59
CBS 7877	0.06–0.12	1–2	4	0.795

(Unit: μg/mL).

RESULTS

Isolation of Ketoconazole-Resistant *M. restricta* Strains

Antifungal susceptibility of the clinical isolates of the *M. restricta* strains, which were obtained from Korean patients with severe dandruff previously (Park M. et al., 2017), was evaluated along with the type strain, *M. restricta* CBS 7877. MICs for ketoconazole, terbinafine, amphotericin B, and ZPT were determined as described in the Materials and Methods (**Table 1**). The MIC values of all *M. restricta* strains, including the type strain, against terbinafine, amphotericin B, and ZPT ranged from 0.25 to 2, 2 to 8, and 0.795 to 3.18 μg/mL, respectively. However, in the case of ketoconazole, two isolates, *M. restricta* KCTC 27529 and KCTC 27550, showed an MIC of 3.89–7.68 μg/mL, which is 16–256 times higher than those of the other *M. restricta* strains, including the type strain CBS 7877, which showed an MIC of 0.03–0.24 μg/mL. These results indicate that *M. restricta* KCTC 27529 and KCTC 27550 are specifically resistant to ketoconazole, an imidazole antifungal drug that inhibits ergosterol biosynthesis in fungi. These strains were thus selected for further investigation of ketoconazole resistance in *M. restricta*.

Genome Analysis of the Resistant Strains

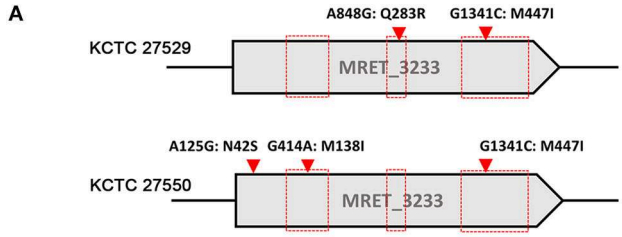
To investigate the underlying mechanism of altered ketoconazole sensitivity at the genome-wide level, genomes of *M. restricta* KCTC 27529 and KCTC 27550 were sequenced and analyzed in comparison with the genome of the susceptible reference strain, *M. restricta* KCTC 27527 (Cho et al., 2019). We used *M. restricta* KCTC 27527 as a susceptible reference strain rather than the type strain CBS 7877 because KCTC 27529 and KCTC 27550 are phylogenetically closer to KCTC 27527 than CBS 7877, and belong to the same sub-species level molecular type (Park M. et al., 2017).

The genomes of the resistant strains were sequenced using a combination of Illumina HiSeq and PacBio Sequel technologies, of which the sequencing method was principally used to increase the overall quality of genome sequencing analysis and investigate a possible copy number variation in the resistant strains. Initially, we investigated the distribution of single nucleotide mutations in the resistant strains and found that, compared to the total 7,330,907 bp in the genome of the reference strain KCTC 27527, 35,992 (0.49%) and 37,178 (0.51%) single nucleotide mutations were found in the genomes of the two resistant isolates, KCTC 27529 and KCTC 27550, respectively. Among these, 7,401 (0.10%) and 7,737 (0.11%) single nucleotide mutations resulted in amino acid substitutions in each strain, respectively. The distribution and the nature of the mutations in the genomes of the resistance strains were listed in the **Supplementary Figure 1**.

Mutations in the *ERG11* Homolog Were Identified in the Resistant Strains

One of the well-known mechanisms of azole resistance includes nonsynonymous substitution mutations within the coding region of *ERG11*, which reduces the binding affinity of lanosterol 14α-demethylase to azoles (Cowen et al., 2002). One of the examples includes the reduced affinity of the *C. albicans* Erg11 protein possessing G464S or R467K amino acid substitution to fluconazole (Kelly et al., 1999; Lamb et al., 2000). We were, therefore, particularly concerned about single nucleotide mutations in the *ERG11* homolog, MRET 3233, in the resistant strains KCTC 27529 and KCTC 27550. The results of our genome sequencing analysis revealed that KCTC 27529 and KCTC 27550 possess two (A848G:Q283R and G1341C:M447I) and three (A125G:N42S, G414A:M138I, and G1341:M447I) nonsynonymous substitution mutations, respectively, compared to the susceptible reference strain KCTC 27527. Among these mutations, G1341C: M447I was commonly observed in both KCTC 27529 and KCTC 27550 (**Figure 1A**). In KCTC 27529, all mutations were located within the hotspot regions in which mutations are frequently observed in azole-resistant *C. albicans* (Marichal et al., 1999). In KCTC 27550, all mutations except N42S were located within these regions.

We reasoned that the nonsynonymous substitution mutations observed within the *ERG11* homologs of the resistant strains would not exist in the ketoconazole-susceptible *M. restricta* strain if they influenced ketoconazole resistance. Thus, the nucleotide sequences of the *ERG11* homologs of other ketoconazole-susceptible strains were determined; we found that all mutations, except the Q283R mutation that was identified in the resistant strain KCTC 27529, were present in the *ERG11* homologs of the ketoconazole-susceptible strains (**Figure 1B**). Therefore, we ruled out the association of nonsynonymous substitution mutations identified in the *ERG11* homolog of the ketoconazole-resistant strains KCTC 27529 and KCTC 27550. The Q283R mutation was also excluded because the same mutation was identified in the fluconazole-resistant *C. albicans* strain C507 and was found not to be related to azole resistance (Chau et al., 2004). In addition, according to available crystal structures of the Erg11 protein, R283 in KCTC 27529 is corresponds to H283, C270, and V232 in *C. albicans*, *Aspergillus fumigatus*, and *Mycobacterium tuberculosis* respectively, which are located far outside of the



B

Strain		Amino acid position									
		17	42	196	137	138	208	283	447	457	507
Ketoconazole resistant	KCTC 27529	V	N	T	L	M	E	†R	I	F	D
	KCTC 27550	V	S	T	L	I	E	Q	I	F	D
Ketoconazole susceptible	CBS 7877	V	S	T	L	M	E	Q	M	F	D
	KCTC 27512	V	S	T	L	M	E	Q	I	F	D
	KCTC 27516	V	N	T	L	M	E	Q	M	F	D
	KCTC 27518	V	S	T	L	I	E	Q	I	F	D
	KCTC 27519	V	S	T	L	M	E	Q	I	L	D
	KCTC 27522	V	S	T	L	M	E	Q	I	F	D
	KCTC 27524	V	N	T	L	M	E	Q	I	F	N
	KCTC 27527	V	N	T	L	M	E	Q	M	F	D
	KCTC 27530	V	N	T	L	M	E	Q	I	F	N
	KCTC 27538	V	S	T	L	M	E	Q	M	F	D
	KCTC 27539	V	N	T	L	M	E	Q	M	F	D
	KCTC 27540	V	N	T	I	M	E	Q	M	F	D
	KCTC 27542	V	S	T	L	M	E	Q	I	F	D
	KCTC 27543	M	S	A	L	M	Q	Q	I	F	D
	KCTC 27544	V	S	T	L	M	E	Q	I	F	D
	KCTC 27768	V	N	T	L	M	E	Q	I	F	D
	KCTC 27769	V	S	T	L	M	E	Q	I	F	D
	KCTC 27879	V	S	T	L	M	E	Q	I	F	D
	KCTC 27880	V	S	T	L	I	E	Q	I	F	D
	KCTC 27881	V	S	T	L	M	E	Q	I	F	D
	KCTC 27882	V	S	T	L	M	E	Q	I	F	D
	KCTC 27883	V	N	T	L	M	E	Q	I	F	D
	KCTC 27884	V	S	T	L	M		Q	I	F	D

FIGURE 1 | Nonsynonymous substitution mutations in the *ERG11* homolog of *M. restricta* strains. **(A)** Nucleotide and amino acid substitutions in the *ERG11* homolog of ketoconazole-resistant strains, *M. restricta* KCTC 27529 and KCTC 27550, were compared with those of the susceptible strain, *M. restricta* KCTC 27527. Red boxes indicate the hotspot regions at which mutations frequently occur in the azole-resistant *C. albicans* strains (Marichal et al., 1999). **(B)** Amino acid substitutions in the *ERG11* homolog of ketoconazole susceptible *M. restricta* strains compared with that of the reference strain KCTC 27527. The colored amino acids indicate nonsynonymous mutations found in the susceptible *M. restricta* strains. †Q283R was found to be not related to azole resistance in *C. albicans* C507 (Chau et al., 2004).

heme-containing active site (Podust et al., 2001; Xiao et al., 2004). Normally, amino acid substitutions located in the region surrounding the central heme group disturb the azole binding and confer resistance (Monk et al., 2014; Flowers et al., 2015). Therefore, the effect of Q283R on the resistance of KCTC 27529 might be minimal.

Tandem Multiplications of Specific Genomic Loci Influence Ketoconazole Resistance

In addition to the identification of mutations in the *ERG11* homologs, our genome analysis revealed that multiplications of specific genomic loci occurred in the genomes of each resistant strain. We found that, in the resistant strain KCTC 27529, the genomic locus of the 2,856 bp region containing the complete coding region of MRET_4198 on chromosome 8 is tandemly repeated five times. Tandem multiplications of the genomic locus were also identified in the resistant strain KCTC 27550; moreover, the 2,241 bp region containing the complete coding region of MRET_3233 on chromosome 5 was tandemly repeated four times (**Figure 2A**). The tandem multiplications of the loci in each resistant strain were further confirmed by qRT-PCR using the genomic DNA of each resistant strain as a template; the results revealed a relative increase in the copy number of MRET_4198 and MRET_3233 in KCTC 27529 and KCTC 27550, respectively (**Figure 2B**). Genomic rearrangements such as multiplication and translocation, have been frequently observed and can lead to phenotypic differences and genome evolution in various eukaryotic organisms; further, the rearrangement event is suggested to be involved in direct or inverted repeat sequences at the boundaries of the genomic region (Koszul et al., 2004; Carvalho et al., 2011; Beck et al., 2015). However, no repeated sequence was identified in the boundaries of multiplicated loci in the genome of KCTC 27529 and KCTC 27550.

While MRET_3233 is a homolog of *ERG11*, MRET_4198 is a homolog of *ATM1* that encodes a mitochondrial inner membrane ATP-binding cassette (ABC) transporter and is required for iron metabolism such as Fe-S cluster biogenesis and heme synthesis (Kispal et al., 1997, 1999). To confirm that multiplication of the *ATM1* and *ERG11* homolog genes increased the transcript levels of each gene, the gene expression levels were determined; the results revealed significant upregulation of each gene in the resistant strain compared to the susceptible reference strain, KCTC 27527 (8.60 ± 0.52 and 8.19 ± 0.70-fold increase, respectively) (**Figure 2C**).

We next investigated whether upregulation of the tandemly multiplicated *ATM1* and *ERG11* homologs contributed to azole resistance in the ketoconazole-resistant strains KCTC 27529 and KCTC 27550. As no genetic manipulation tools have been developed yet for *M. restricta*, we utilized a genetically tractable model pathogenic fungus, *Cryptococcus neoformans* (Hull and Heitman, 2002). *ATM1* was fused with the *TEF1* promoter and integrated into its authentic locus in the genome of *C. neoformans*. Overexpression of *ATM1* in *C. neoformans* was confirmed by qRT-PCR; subsequently, the growth of fungal cells was challenged in the presence of ketoconazole (**Figures 3A,B**). The results of growth analysis showed that *ATM1* overexpression significantly reduced the sensitivity of fungal cells to ketoconazole, implying that the increased gene expression contributes to ketoconazole resistance in *M. restricta* KCTC 27529. Previously, we constructed the *C. neoformans* strain overexpressing *ERG11* under the *GAL7* promoter and demonstrated that the overexpression of gene homologs increases resistance to the azole antifungal drug, fluconazole (Kim et al., 2012). In the current study, we used the same overexpression strain and confirmed that *ERG11* overexpression reduced the sensitivity of fungal cells to

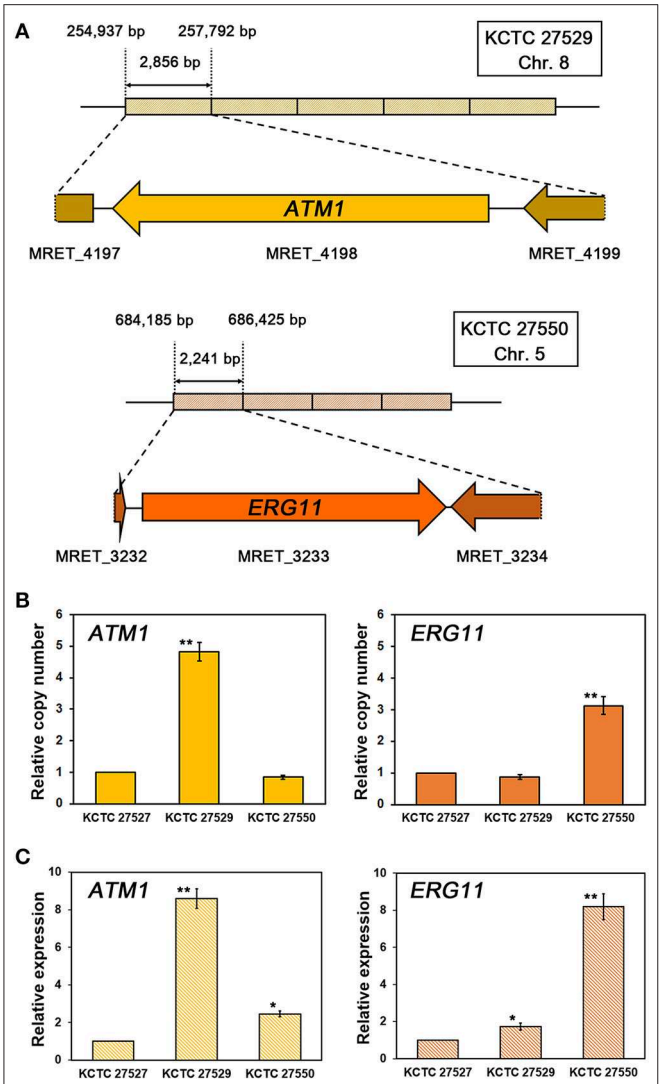


FIGURE 2 | Tandem multiplication of genomic loci and confirmation of gene copy numbers and expression. **(A)** The genomic loci containing MRET_4198, the *ATM1* homolog in chromosome 8 of *M. restricta* KCTC 27529, and MRET_3233, the *ERG11* homolog in chromosome 5 of *M. restricta* KCTC 27550, are tandemly multiplicated five and four times, respectively. The relative copy number **(B)** and expression **(C)** of the *ATM1* and *ERG11* homologs in each resistant strain were confirmed using qRT-PCR. Values were normalized using the actin gene (MRET_1518) as an endogenous control and compared to the susceptible reference strain *M. restricta* KCTC 27527. The results are averages of three biological replicates (*$p < 0.01$, **$p < 0.001$).

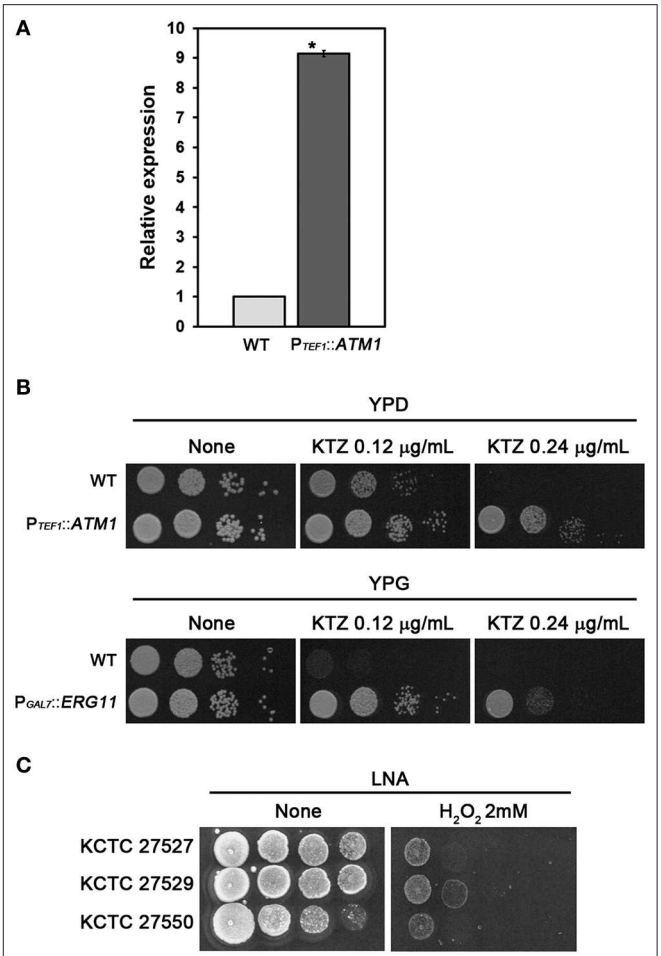


FIGURE 3 | Contribution of *ATM1* and *ERG11* overexpression to ketoconazole resistance. **(A)** Overexpression of *ATM1* in *C. neoformans* was confirmed using qRT-PCR. Values were normalized using *TEF2* as an endogenous control and compared to the wild type strain. The results are averages of three biological replicates (*$p < 0.001$). **(B)** The growth of *C. neoformans* overexpressing *ATM1* in YPD media containing ketoconazole was monitored (upper panel). The growth of the *C. neoformans* strain overexpressing *ERG11* in YPG media containing ketoconazole was monitored (lower panel). The *C. neoformans* strain harboring P_{GAL7}::*ERG11*, which was constructed and confirmed in our previous study, was used (Kim et al., 2012). Cells were serially diluted 10-fold, spotted onto the plate, and incubated at 30°C for 2 days. WT, wild type; P_{TEF1}::*ATM1*, overexpression of *ATM1*; P_{GAL7}::*ERG11*, overexpression of *ERG11*. **(C)** The growth of *M. restricta* KCTC 27527, KCTC 27529, and KCTC 27550 in LNA media containing hydrogen peroxide was monitored. Cells were serially diluted 10-fold, spotted onto the plate, and incubated at 34°C for 7 days.

ketoconazole, implying that increased expression of the gene contributes to ketoconazole resistance in *M. restricta* KCTC 27550 (**Figure 3B**).

Lack of *ATM1* in *S. cerevisiae* and *C. neoformans* induces hypersensitivity to hydrogen peroxide, which generates oxygen-derived free radicals in the cell, indicating that Atm1 is involved in the defense mechanism against oxidative stress in the fungal cells (Kispal et al., 1997; Do et al., 2018), and a study showed that ketoconazole generated reactive oxygen species in *C. albicans* (Snell et al., 2012). Therefore, we hypothesized that the resistance strain KCTC 27529 would show reduced sensitivity to oxidative stress and evaluated sensitivity of the strains to hydrogen peroxide. The results showed that *M. restricta* KCTC 27529 is less sensitive to hydrogen peroxide confirming the possible association between the increased expression of *ATM1* and ketoconazole resistance in the (**Figure 3C**). Collectively, our results suggest that the increased expression of *ATM1* and *ERG11* homologs caused by genomic tandem multiplication of each locus is one of the main

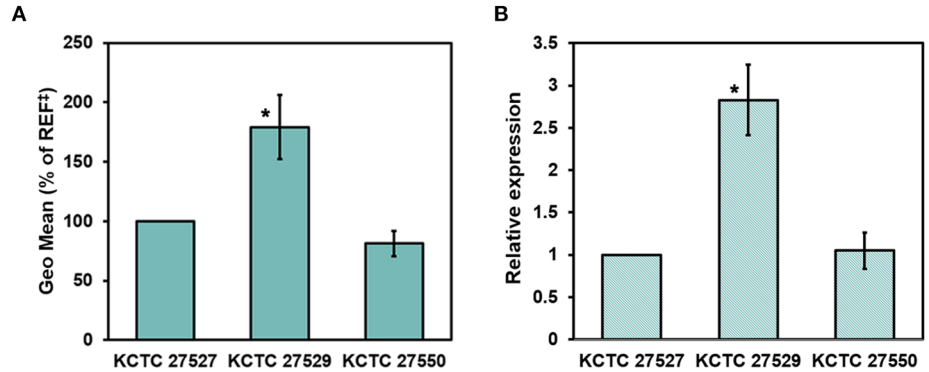


FIGURE 4 | Evaluation of drug efflux in *Malassezia restricta*. **(A)** The geometric means of differences between the efflux and accumulation of R6G in *M. restricta* KCTC 27529 and KCTC 27550 were compared to the reference strain, KCTC 27527. Values were compared to the reference strain KCTC 27527. The results are averages of three biological replicates (*$p < 0.005$). **(B)** Relative expression of the *PDR5* homolog was determined by qRT-PCR. Values were normalized using the actin gene (MRET_1518) as an endogenous control and compared to the reference strain KCTC 27527. The results are averages of three biological replicates (*$p < 0.005$). ‡REF indicates the reference strain *M. restricta* KCTC 27527.

causes of ketoconazole resistance in *M. restricta* KCTC 27529 and KCTC 27550.

Altered Expression of the *PDR5* Homolog Influences Ketoconazole Resistance

Hyperactivation of drug efflux reduces the intracellular accumulation of azoles and has been considered one of the major drug resistance mechanisms in pathogenic fungi (Morio et al., 2017). This phenomenon led us to investigate drug efflux in the resistant strains KCTC 27529 and KCTC 27550 to find an additional resistance mechanism that may exist in ketoconazole-resistant strains. Drug efflux in *M. restricta* cells was determined using rhodamine 6G (R6G) (Maesaki et al., 1999); the results revealed that drug efflux of the resistant strain KCTC 27529 was significantly increased compared with that of the susceptible reference strain KCTC 27527, whereas the drug efflux of the resistant strain KCTC 27550 was similar to that of the reference strain (**Figure 4A**). Furthermore, addition of the efflux pump inhibitor promethazine (PMZ) reduced the MIC values of KCTC 27529 significantly (Iatta et al., 2017) (**Table 2**). These results suggested that increased drug efflux also contributes to ketoconazole resistance in KCTC 27529.

To further confirm the increased drug efflux in KCTC 27529, we searched the genome for the homolog of *S. cerevisiae* Pdr5, which is involved in drug efflux, including the efflux of azole antifungal drugs (Paul and Moye-Rowley, 2014; Morio et al., 2017), analyzed its expression levels, and compared them with those in the susceptible strain. MRET_2329 was found to be the only homolog of *S. cerevisiae* Pdr5 (38.36% identity) in *M. restricta*; its expression was considerably increased (2.83 ± 0.41-fold) in the ketoconazole-resistant strain KCTC 27529 compared to the susceptible reference strain (**Figure 4B**). In *C. albicans*, an additional ABC transporter, Cdr2, plays a role in azole drug resistance along with Cdr1, the homolog of *S. cerevisiae* Pdr5 (Sanglard et al., 1997). We found that *M. restricta* possesses the homolog of *C. albicans* Cdr2, MRET_2330 (38.32% identity). However, the expression levels of the Cdr2 homolog in *M. restricta* were similar to those in the susceptible reference strain. Flr1 is involved in efflux of fluconazole in *S. cerevisiae*, and we identified the homolog in *M. restricta*, MRET_3736 (33% identity). However, the expression levels of the Flr1 homolog were also similar to that in the reference strain. These results suggested that increased drug efflux in KCTC 27529 was solely caused by hyperactivation of the *PDR5* gene MRET_2329. Collectively, our results suggested that increased drug efflux, along with genomic tandem multiplication of the *ATM1* allele, is a cause of ketoconazole resistance in *M. restricta* KCTC 27529. In *M. restricta* KCTC 27550, genomic tandem multiplication of the *ERG11* allele appeared to be the sole cause of ketoconazole resistance.

DISCUSSION

Ketoconazole is effective in the treatment of seborrheic dermatitis and dandruff, which are known to be associated with *Malassezia*. Several clinical studies have demonstrated the efficacy of ketoconazole. For example, a large-scale clinical trial showed that 575 patients suffering from scalp seborrheic dermatitis and dandruff were treated with a 2% ketoconazole shampoo for 2–4 weeks, resulting in 88% improvement (Peter and Richarz-Barthauer, 1995). The same study also showed lower disease recurrence rates in the group using the shampoo containing ketoconazole than in the placebo group (Peter and Richarz-Barthauer, 1995). Furthermore, a randomized study of 66 patients with seborrheic dermatitis and dandruff compared the efficacy of shampoo containing different concentrations of ketoconazole and showed significantly decreased flakiness and *Malassezia* density in the group treated with a higher concentration of ketoconazole (Pierard-Franchimont et al., 2001). This proven effect of ketoconazole has led to its use in the treatment of skin diseases associated with *Malassezia*. However, frequent and prolonged use of ketoconazole might

TABLE 2 | MIC values of ketoconazole with efflux inhibitor promethazine (PMZ).

	KCTC 27527	KCTC 27529	KCTC 27550
Ketoconazole	0.03–0.06	3.89-7.68	3.89-7.68
Ketoconazole + promethazine 50 μg/mL	0.03–0.06	0.98	3.89-7.68
Ketoconazole + promethazine 100 μg/mL	0.06	0.48	3.89-7.68

(Unit: μg/mL).

also cause the emergence of ketoconazole-resistant fungal strains. In the current study, we isolated ketoconazole-resistant *M. restricta* strains, KCTC 27529 and KCTC 27550; their MICs against the drug were significantly higher than that of the susceptible strain. MICs of the resistant strains against terbinafine, amphotericin B, and ZPT were similar to those of other ketoconazole-susceptible strains; thus, we considered that the drug resistance of KCTC 27529 and KCTC 27550 was specific to ketoconazole.

In the current study, we carried out comparative genome analysis to understand the mechanism of ketoconazole resistance in KCTC 27529 and KCTC 27550 and observed tandem multiplications of the genomic regions containing the genes encoding the *ATM1* and *ERG11* homologs in the genomes of the strains, respectively. Furthermore, we found that the multiplication of genes in the resistant strains resulted in significantly increased transcript levels compared with those in the susceptible reference strain KCTC 27527. In the resistant strain KCTC 27529, increased expression of *ATM1* may cause an increase in the intracellular heme contents and, in turn, increase the activity of Erg11, which contains heme as a cofactor (Balding et al., 2008; Kalb et al., 1987). Our interpretation is supported by results from previous studies showing that depletion of Atm1 in *S. cerevisiae* and *C. neoformans* resulted in decreased intracellular heme content (Hausmann et al., 2008; Do et al., 2018). Another possible explanation for the association between the increased expression of *ATM1* and ketoconazole resistance in the resistant strain KCTC 27529 is the role of Atm1 in the susceptibility of the cells to oxidative stress as shown in the current study.

Numerous studies have revealed that overexpression of *ERG11* increases the levels of the antifungal target protein and, therefore, causes resistance to azole antifungal drugs (Flowers et al., 2012; Feng et al., 2016, 2017). Moreover, a number of studies have demonstrated that overexpression is frequently induced by the increased copy number of *ERG11*, which is a result of the amplification of a segment or the entire chromosome (Marichal et al., 1997; Selmecki et al., 2008; Sionov et al., 2010; Kim et al., 2018). Studies with *C. albicans* also showed that, among fluconazole-resistant isolates, ∼20% displayed segmental aneuploidies of the left arm of chromosome 5 containing *ERG11* and increased gene expression levels (Selmecki et al., 2006, 2008). Similarly, duplication of the whole chromosome 1 carrying *ERG11* and upregulation of genes in the chromosome was observed in fluconazole-resistant *C. neoformans* (Sionov et al., 2010). Previously, we studied the mechanism of ketoconazole resistance in *M. pachydermatis*, which was isolated from a dog with otitis externa. Tandem quadruplication of the ∼64 kb genomic locus containing *ERG11* along with increased expression was identified in the strain. In the same study, we also observed segmental multiplication of the chromosomal region containing *ERG11* in the ketoconazole-resistant strains generated by *in vitro* evolution, suggesting that an increased copy number of *ERG11* in response to ketoconazole is a common resistance mechanism in *Malassezia* (Kim et al., 2018). Considering the above findings, increased expression of *ERG11* caused by the multiplication of the *ERG11* copy number might be one of the main causes of ketoconazole resistance in *M. restricta* KCTC 27550.

Previous studies with other azole-resistant fungi, including *S. cerevisiae*, *C. albicans*, and *C. neoformans*, have reported that large DNA segments ranging from segmental to the entire chromosome were amplified (Koszul et al., 2006; Selmecki et al., 2006; Sionov et al., 2010). However, we observed a tightly amplified region, including one gene in the ketoconazole-resistant *M. restricta* KCTC 27529 and KCTC 27550. Moreover, these resistant isolates were obtained from completely unrelated patients suggesting that a short segmental genomic multiplication has been evolved to develop the drug-resistant phenotype in *M. restricta*, which possesses a very compact genome.

Hyperactivation of the drug efflux pump, which exports intracellular azole, is one of the well-identified mechanisms of azole resistance (Paul and Moye-Rowley, 2014). In fluconazole-resistant *Candida species*, elevated expression of genes encoding plasma membrane efflux proteins, such as *CDR1*, *CDR2*, or *MDR1*, and increased efflux have been observed frequently (Leppert et al., 1990; Brun et al., 2004; Lamping et al., 2007; Berkow et al., 2015; Kim et al., 2017). Moreover, a recent study with fluconazole or voriconazole-resistant *M. furfur* and *M. pachydermatis* showed a significant decrease in the MICs of drugs in the presence of efflux protein inhibitors, indirectly suggesting the role of the drug efflux pump in azole-resistant *Malassezia* (Iatta et al., 2017).

We observed significant upregulation of MRET_2329, a homolog of *S. cerevisiae PDR5* and *C. albicans CDR1*, in the ketoconazole-resistant strain KCTC 27529 compared to the susceptible reference strain, which supported its significantly increased drug efflux phenotype. The regulatory mechanism of *PDR5* and *CDR1* expression in *S. cerevisiae* and *C. albicans* was studied; it was found that these genes are transcriptionally regulated by the transcription factors Pdr1/Pdr3 and Tac1, respectively (Katzmann et al., 1996; Coste et al., 2004). However, *M. restricta* lacks the homologs of genes encoding these transcription factors, implying that the regulatory mechanism underlying the expression of the *PDR5* homolog in this fungus might be different from those in *S. cerevisiae* and *C. albicans* and still needs to be explored. Collectively, we concluded that, in addition to multiplication in the genomic region containing *ATM1*, increased drug efflux mediated by increased expression

of the Pdr5 homolog is one of the resistance mechanisms in KCTC 27529.

Overall, the results of our study suggest that multiplication of the genomic loci encoding genes involved in ergosterol synthesis, and oxidative stress response and overexpression of drug efflux protein are the mechanisms underlying ketoconazole resistance in *M. restricta*. Furthermore, our data imply that a short segmental genomic rearrangement, such as a tandem multiplication, might be a common adaptive mechanism in *Malassezia* against azole antifungal drugs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) (Bioproject number, PRJNA592379).

AUTHOR CONTRIBUTIONS

MP and Y-JC performed the experiments. MP, Y-JC, YL, and WJ analyzed and interpreted the data and prepared the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00191/full#supplementary-material

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MINI REVIEW
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Host Immunity to *Malassezia* in Health and Disease

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The microbiota plays an integral role in shaping physical and functional aspects of the skin. While a healthy microbiota contributes to the maintenance of immune homeostasis, dysbiosis can result in the development of diverse skin pathologies. This dichotomous feature of the skin microbiota holds true not only for bacteria, but also for fungi that colonize the skin. As such, the yeast *Malassezia*, which is by far the most abundant component of the skin mycobiota, is associated with a variety of skin disorders, of which some can be chronic and severe and have a significant impact on the quality of life of those affected. Understanding the causative relationship between *Malassezia* and the development of such skin disorders requires in-depth knowledge of the mechanism by which the immune system interacts with and responds to the fungus. In this review, we will discuss recent advances in our understanding of the immune response to *Malassezia* and how the implicated cells and cytokine pathways prevent uncontrolled fungal growth to maintain commensalism in the mammalian skin. We also review how the antifungal response is currently thought to affect the development and severity of inflammatory disorders of the skin and at distant sites.

Keywords: mycobiota, commensalism, immunopathology, cutaneous immunity, *Malassezia*

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INTRODUCTION

The skin, one of our body's largest organs, harbors a wide variety of microbial communities, including innocuous symbiotic organisms but also potential pathogens (Findley and Grice, 2014). Advances in our understanding of the molecular mechanisms of microbial virulence and host defense have improved the current view how dysbiosis and dysregulated immune responses against commensal microbes drive pathological conditions (Chen et al., 2018).

Fungi are increasingly being recognized as common members of the microbiota. The most prevalent fungi on the mammalian skin are those of the genus *Malassezia*, with >90% of all skin fungi belonging to this genus (Findley et al., 2013). Currently, 18 species of *Malassezia* have been identified, of which 10 were found in humans and the others in a growing number of animal hosts (Guillot and Bond, 2020). Although generally viewed as a commensal, *Malassezia* has also been associated with various dermatological conditions including mild diseases, such as dandruff and pityriasis versicolor, to more severe inflammatory diseases, such as seborrheic dermatitis and atopic dermatitis (AD) (Saunte et al., 2020). In rare cases, *Malassezia* has been reported to cause blood stream infections (Iatta et al., 2014). In dogs, overgrowth of *Malassezia* is associated with otitis and dermatitis and treatment of such disorders with antifungals often improves the conditions (Bond et al., 2020). In contrast to the situation in dogs, the association of *Malassezia* with skin disorders in humans primarily relies on clinical association studies, while a causative relationship remains

a matter of debate and the mechanism of pathogenesis unclear. Dysbiosis with a higher fungal diversity and shifts in the relative abundance of certain *Malassezia* species have been implicated in AD. A large metagenomic study has reported an increase in the relative abundance of *M. dermatis* and *M. sympodialis* and a reduction of *M. globosa* on the skin of AD patients (Chng et al., 2016). Lack of consensus with other studies on the skin mycobiota in AD (Jo et al., 2017) may at least in part be due to sampling biases and discrepancies between culture- and sequence-based methods. In addition to the reported shifts in the species distribution between normal and diseased skin, intraspecies variations (Wu et al., 2015), which are known to alter phenotype and function in other fungal species (Ropars et al., 2018), may further complicate the situation. The pathogenicity of *Malassezia* spp. may also be modulated by mycoviruses that were recently identified in some isolates (Clancey et al., 2019; Park et al., 2019). Moreover, the microenvironment of the diseased skin, which is characterized by barrier disruption, lipid deficiency and elevated pH in case of AD (Weidinger and Novak, 2016), can modulate the metabolism and thereby the functional properties of the fungus (Chng et al., 2016). Inter-kingdom communications within the skin microbiota may also influence the capacity of *Malassezia* in promoting (or possibly preventing) skin disorders (Li et al., 2017). Finally, host factors such as genetics, immune status or comorbidities, can also influence the skin mycobiome composition and pathogenic potential (Jo et al., 2017) (**Figure 1**).

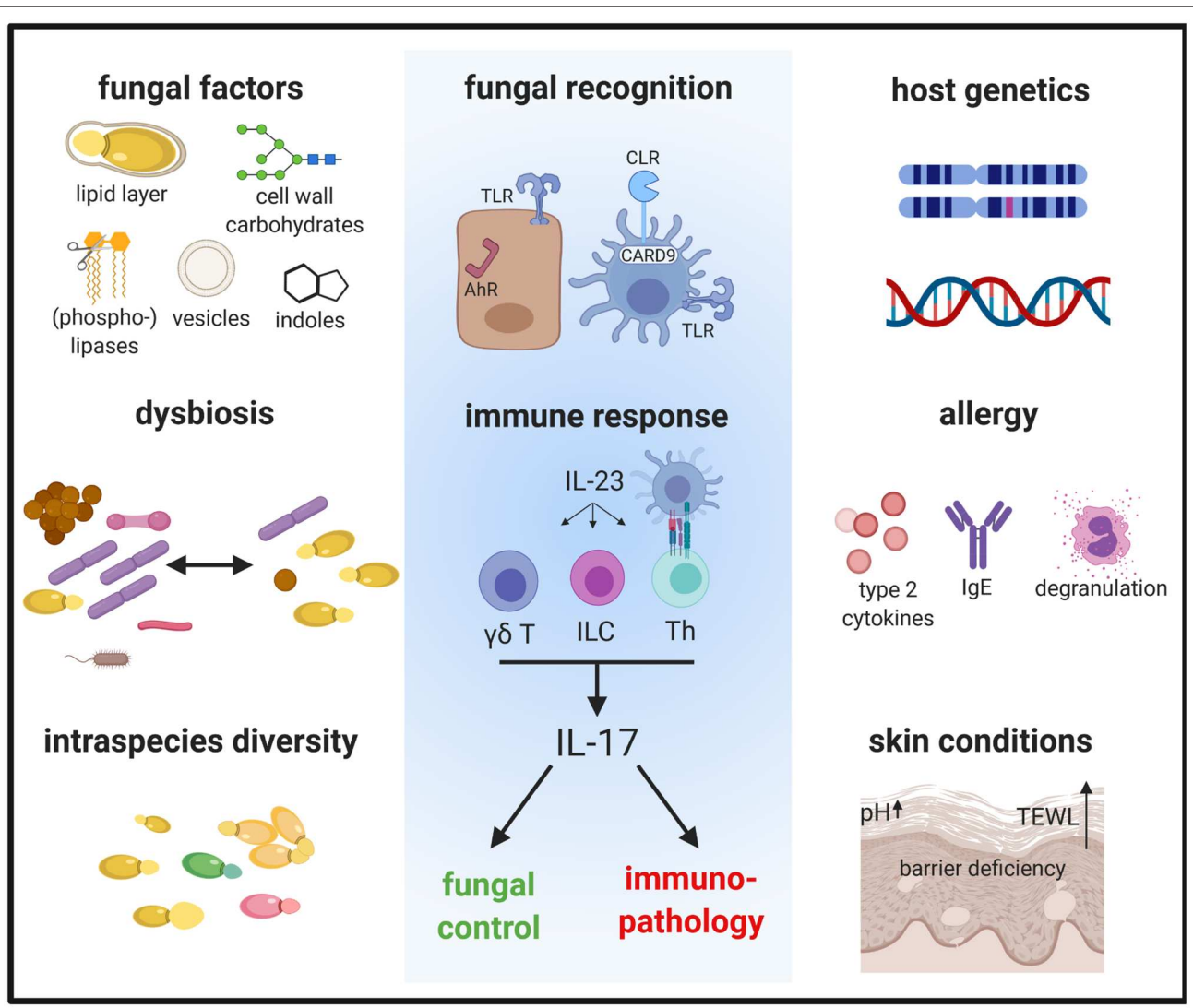

FIGURE 1 | The immune response to *Malassezia* is influenced by fungal factors including cell wall constituents and secreted components, inter- and intraspecies variations and host factors such as genetics and skin or systemic predisposing conditions. These factors determine how *Malassezia* is recognized by the host and in turn how the host responds to the fungus. The antifungal response is characterized by the activation of the IL-23/IL-17 axis, which not only controls fungal growth but can also mediate immunopathology. AhR, aryl hydrocarbon receptor; CLR, C-type lectin receptor; γδ T, γδ T cells; ILC, innate lymphoid cells; TLR, Toll-like receptor; TEWL, trans-epidermal water loss; Th, T helper cells.

To understand the role of *Malassezia* in the development and severity of skin diseases it is important to understand the mechanisms of skin-fungus interactions in the (normal) mammalian skin.

HOST RESPONSE TO THE SKIN COMMENSAL YEAST *MALASSEZIA*

Constant exposure of the skin to commensal microbes results in a continuous activation of the cutaneous immune system. Active immunosurveillance of the microbiota is critical to prevent dysbiosis, microbial overgrowth and translocation across epithelial barriers as evidenced by the frequent occurrence of opportunistic infections in immunodeficient individuals (Pellicciotta et al., 2019).

Malassezia-host interactions are mediated via direct contacts as well as indirectly via secreted factors (Velegraki et al., 2015) and extracellular vesicles released from the fungus, which may assist the delivery of soluble mediators to host cells (Johansson et al., 2018; Zhang et al., 2019; Vallhov et al., 2020). These interactions have been studied primarily *in vitro* with cultured cells (Sparber and LeibundGut-Landmann, 2017). Reconstructed human epidermis and *ex vivo* skin models, which reflect the complexity of the skin more closely, have also been developed to study the cutaneous antifungal response (Corzo-Leon et al., 2019; Pedrosa et al., 2019). More recently, a murine model has become available that provides insights into the host response against *Malassezia in vivo*. In contrast to *in vitro* systems, it allows to study the contribution of circulating immune cells, which are not normally resident in the skin, to the cutaneous host response over prolonged periods of time (Sparber et al., 2019).

Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) recognize carbohydrates in the fungal cell wall (Plato et al., 2015). Although the cell wall of *Malassezia* shows some differences to that of other fungal genera and is covered by a lipid-rich outer layer (Mittag, 1995; Kruppa et al., 2009; Stalhberger et al., 2014), CLRs are thought to play a prominent role in *Malassezia* sensing. Dectin-2 and Mincle have both been shown to bind *Malassezia* cell wall constituents resulting in *Malassezia* phagocytosis and cytokine production (Ishikawa et al., 2013; Haider et al., 2019). However, *in vivo* the two receptors do not appear to be essential for antifungal defense because knockout mice lacking either of the two receptors do not manifest an impaired immune response in the skin (Sparber et al., 2019). Functional redundancy between the receptors and/or with other CLRs may provide a likely explanation for reconciling the involvement of the downstream signaling adaptor Card9 in the cutaneous response to *Malassezia* (Sparber et al., 2019). The requirement of MyD88 for the antifungal response (Sparber et al., 2019) may be explained by the involvement of TLR2, which is also activated by *Malassezia* (Baroni et al., 2006) (**Figure 1**).

Card9-mediated signaling couples innate fungal recognition to the adaptive immune system, and importantly, it drives polarization of $CD4^+$ T cells into IL-17A-secreting effector cells (LeibundGut-Landmann et al., 2007). While this was first observed with *Candida albicans* in humans and mice (LeibundGut-Landmann et al., 2007; Glocker et al., 2009), it also applies to *Malassezia*: *Malassezia*-specific T helper cells belong preferentially to the Th17 subset in both host species and *Malassezia*-specific Th17 response is abolished in Card9-deficient mice (Sparber et al., 2019).

αβ T cells are not the only IL-17A-producing cell type. In the skin, γδ T cells and innate lymphoid cells (ILCs) constitute prominent additional sources of IL-17A and respond swiftly to cytokine stimulation in an antigen-independent manner (Cua and Tato, 2010). Indeed, both γδ T cells and ILCs secrete IL-17A in the skin of *Malassezia*-colonized mice (Sparber et al., 2019) (**Figure 1**). Whether the activation of γδ T cells and ILCs is a consequence of the experimental infection and/or whether the innate cells contribute to long-term immunosurveillance of *Malassezia* commensalism, remains to be determined. Intriguingly, the Card9 pathway does not seem to be required for induction of innate IL-17A by *Malassezia* (Sparber et al., 2019). This is reminiscent of what has been reported for innate IL-17A induction in a murine model of oropharyngeal candidiasis (Bishu et al., 2014), although the underlying cause and the Card9-independent regulatory mechanisms are unclear so far.

Complementary to classical pattern recognition receptors such as CLRs, other classes of receptors are also implicated in the recognition of *Malassezia* in the skin. They are particularly relevant in keratinocytes, which lack Syk/Card9-coupled CLRs. Examples of keratinocyte-fungal interactions are provided by studies on *C. albicans*, which implicated receptors like E-cadherin, EphA2, or EGFR in the antifungal response (Phan et al., 2007; Swidergall et al., 2018; Ho et al., 2019). In case of *Malassezia*, the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor, has gained attention because *Malassezia*-derived metabolites, in particular indoles, can trigger AhR signaling. Indole production was first reported for *M. furfur* (Gaitanis et al., 2008), but other species of *Malassezia* may also produce AhR ligands (Magiatis et al., 2013; Buommino et al., 2018) (**Figure 1**). The activation of AhR signaling can modulate skin homeostasis in manifold ways, including oxidation, epidermal barrier function, melanogenesis, and innate immunity (Furue et al., 2014). Importantly, AhR is implicated in type 17 immunity by promoting Th17 differentiation (Quintana et al., 2008; Veldhoen et al., 2008) and stimulating the production of IL-17A and related cytokines by Th17 cells, innate lymphocytes and ILCs (Veldhoen et al., 2008; Martin et al., 2009; Qiu et al., 2012). Whether and how AhR signaling modulates skin immunity and in particular the type 17 response to *Malassezia* remains an open question.

Consistently with the host-protective role of the IL-23/IL-17 immune axis in barrier tissues, IL-17 cytokines (including IL-17A and IL-17F, and possibly the epithelial cell-derived family member IL-17C, which is functionally related to IL-17A and IL-17F) prevents *Malassezia* overgrowth on the murine skin (Sparber et al., 2019). This is reminiscent of the well-known activity of IL-17 against other fungi, in particular *C. albicans*, on the skin and mucosal surfaces (Sparber and LeibundGut-Landmann, 2019). In contrast to the prominent role of IL-17 in protection from mucocutaneous candidiasis, the mechanisms

of immunosurveillance of *Malassezia* in humans are likely more complex. No case has been reported to date where a genetic defect in the IL-17 pathway or an upstream signaling element manifests in detectable *Malassezia* overgrowth or development of *Malassezia*-associated skin disorders. Of note, an increased incidence of seborrheic dermatitis was reported as an adverse event accompanying administration of the anti-IL-17A antibody Ixekuzumab (Saeki et al., 2017), and a genetic variant of *IL23R* was found associated with a decrease in dandruff (Ehm et al., 2017). These observations support a link between the IL-23/IL-17 immune axis and *Malassezia* in humans. More data are needed to confirm these findings. Moreover, additional immune mechanisms involved in the control of *Malassezia* commensalism await to be discovered.

MALASSEZIA-INDUCED IMMUNITY AND IMMUNOPATHOLOGY IN THE SKIN

While type 17 immunity is primarily host-beneficial and supports a protective state by controlling commensal fungi in barrier tissues, IL-17 can also bear pathological potential if dysregulated. The IL-17 pathway is a strong driver of inflammation in several immune-mediated diseases, the prime example of which is psoriasis (McGeachy et al., 2019). Mechanistically, IL-17 signaling induces not only the production of antimicrobial peptides and tissue repair molecules, but also chemokines that attract inflammatory myeloid cells to the tissue and it promotes hyperproliferation of keratinocytes. The causative role of IL-17 in psoriasis is demonstrated by the therapeutic efficacy of neutralizing antibodies targeting IL-17 family cytokines and receptors (Hawkes et al., 2017). Although the initial trigger of the pathological IL-17 response in psoriasis is unknown, (skin) fungal commensals are likely involved due to their prominent IL-17-inducing capacity.

IL-17 has also been linked to AD (Koga et al., 2008), although the causative relationship between the cytokine and disease pathogenesis is less clear. It appears to be of particular importance in subtypes of the disease (Leonardi et al., 2015; Noda et al., 2015; Esaki et al., 2016). *Staphylococcus aureus* colonization of the skin is among the most well-known hallmarks of AD (Kong et al., 2012), and the induction of IL-17 in response to epicutaneous *S. aureus* critically mediates skin inflammation in an experimental setting (Liu et al., 2017; Nakagawa et al., 2017). Likewise, *Malassezia* promotes skin inflammation under AD-like conditions via the IL-23/IL-17 immune axis (Sparber et al., 2019). Support for the contribution of *Malassezia*-induced IL-17 to disease pathogenesis is further provided by the observation that *Malassezia*-specific Th17 cells are enriched in AD patients (Balaji et al., 2011; Sparber et al., 2019). Beyond AD, a link between the IL-23/IL-17 immune axis and *Malassezia*-associated disorders has also been proposed in seborrheic dermatitis (Wikramanayake et al., 2018) and dandruff (Ehm et al., 2017). Besides the prominent IL-17 profile, *Malassezia*-responsive T cells in humans also produce IFN-γ (Balaji et al., 2011; Bacher et al., 2019; Sparber et al., 2019), whereby the reason underlying the discrepancy between the two host species remains unclear.

The association of *Malassezia* with AD was first based on the observation that AD patients are often sensitized to *Malassezia* with Th2 cells and IgE that are directed against the fungus (Scalabrin et al., 1999; Zargari et al., 2001; Johansson et al., 2002; Balaji et al., 2011). The *Malassezia*-specific IgE titers were found to correlate with the severity of AD (Zhang et al., 2011; Glatz et al., 2015), albeit their pathogenic role in AD is not well-understood. An increase in *Malassezia*-specific serum IgE was also documented in canine AD (Khantavee et al., 2019) (**Figure 1**).

Important questions arise as to how *Malassezia*-specific Th2 cells are primed and whether they are a cause or a consequence of the allergic response in the atopic skin environment. It also remains unclear how the mixed Th2/Th17 response develops in allergic individuals and how the Th cell subsets are related to each other. Possible non-exclusive scenarios comprise that they may result from differential polarization or selective outgrowth of specific subsets. Clonotypic analysis of *C. albicans*-specific T cell subsets revealed intraclonal functional heterogeneity in support of a scenario of preferential outgrowth of individual subsets (Becattini et al., 2015). Mixed Th2/Th17 populations may also arise from T cell plasticity with intermediate T cells co-producing IL-4 and IL-17A and co-expressing GATA3 and RORγt as they have been observed in the blood and airways of asthmatic patients (Cosmi et al., 2010; Wang et al., 2010; Irvin et al., 2014) and in the skin of atopic individuals (Roesner et al., 2015).

Regarding the mechanism how *Malassezia*-specific T cells promote the allergic inflammation, it was speculated that cross-reactivity may be involved and thereby add an autoimmune component to the pathogenicity of AD. Among the *Malassezia*-derived antigens that have been identified are some that are phylogenetically highly conserved in mammals, such as thioredoxin and manganese-dependent superoxide dismutase (Schmid-Grendelmeier et al., 2005; Glaser et al., 2006; Balaji et al., 2011). Alternatively, *Malassezia*-specific T cells may also mediate immunopathology via cross-reactivity with other microbes as recently reported for *C. albicans*-specific T cells (Bacher et al., 2019). The emerging concept of cytokine-mediated and T cell receptor-independent bystander activation of T cells may represent another putative mechanism (Lee et al., 2019).

Given the pathogenic potential of the *Malassezia*-specific immune response, tight regulation is required to maintain commensalism. Induction of immune tolerance to skin commensal microbes has been investigated in case of *Staphylococcus epidermidis*. Antigen-specific regulatory T cells (Tregs) that accumulate during a critical developmental window in neonatal life were found important to balance the antimicrobial Th17 effector cells directed against the commensal (Scharschmidt et al., 2015). In a model of fungal commensalism with *C. albicans* however, Tregs and regulatory cytokines such as IL-10 appeared not essential for immune homeostasis and stable persistence of the fungus in the oral mucosa (Kirchner et al., 2019). Whether and how immune regulation contributes to immunosurveillance and prevents immunopathology in case of *Malassezia* remains to be determined.

The pathogenic effects of *Malassezia* in case of AD and other inflammatory skin disorder may also be mediated by T cell-independent mechanisms. *Malassezia* may promote disease by triggering the production of pro-inflammatory mediators in keratinocytes and tissue-resident immune cells in the atopic skin that are more accessible to the fungus, owing to barrier defects. *Malassezia* itself produces lipases and phospholipases that generate free fatty acids, which can damage the integrity of the skin and thereby might contribute to irritation and inflammation (Dawson, 2007; Saunders et al., 2012). *Malassezia* proteases may also interfere with cutaneous wound healing by degrading extracellular matrix components (Poh et al., 2020). While these various *Malassezia*-derived components have been studied *in vitro*, their relevance for promoting inflammation in the skin remains unclear. Complementary to the above described scenarios, *Malassezia* may also influence disease by modulating the virulence of other skin microbes such as *S. aureus* via inter-kingdom interactions (Li et al., 2017). It was postulated that such interactions could possibly be host beneficial rather than disease promoting by attenuating bacterial biofilm formation (Li et al., 2017), or by mechanisms such as those reported for *Staphylococcus* interspecies interactions (Nakatsuji et al., 2017).

MALASSEZIA AND DISEASE BEYOND THE SKIN

Aside from its abundance on the skin, *Malassezia* has also been detected in extracutaneous sites. In particular, *Malassezia* sequences have been identified in the gastrointestinal tract in association with inflammatory bowel disease (IBD). Fungal dysbiosis in IBD is characterized with an enrichment of fungal taxa belonging to the Basidiomycota phylum, and this effect was balanced by an equivalent decrease in taxa belonging to the Ascomycota phylum (Sokol et al., 2017). More recently, in an ITS1 sequencing analysis of intestinal washings, *Malassezia* was associated with a subgroup of Crohn's disease patients carrying the *CARD9* risk allele (Limon et al., 2019). Among the *Malassezia* sequences, *M. restricta* was most abundant. Relevance for the role of *M. restricta* in gut inflammation was provided by the observation that gastrointestinal delivery of the fungus aggravates the outcome of DSS-induced colitis in mice in a Card9-dependent manner (Limon et al., 2019). This phenotype was further linked to the induction of Th17 cells by *M. restricta* in the colonic lamina propria (Limon et al., 2019), whereby IL-17 is involved in the pathogenesis of disease in this model (Ito et al., 2008). It remains unclear whether the detection of *Malassezia* by sequencing approaches reflects transient relocation or true colonization of the gut by the fungus. In addition, it is unknown whether *Malassezia*-specific Th17 cells are involved in the pathogenesis of Crohn's disease, and if so, whether these cells get primed locally in response to fungal dysbiosis, or whether they translocate from the skin.

That Th17 cells directed against commensal fungi act at distal sites from where they have been induced has also been observed in other contexts with either host-protective or pathogenic consequences. In gut colonization experiments, *C. albicans*-induced Th17 cells protect from systemic candidiasis in a T cell- and IL-17-dependent manner (Shao et al., 2019). The protective effect extends to other extracellular pathogens, such as *S. aureus* (Shao et al., 2019). These effects are complemented by harmful consequences such as increased susceptibility to airway inflammation and exacerbation of asthma-like symptoms (Noverr et al., 2004; Shao et al., 2019). Besides these examples from experimental models of *C. albicans* colonization in mice, *C. albicans*-specific Th17 cells in human were also shown to cross-react with *A. fumigatus* and to expand in patients with *A. fumigatus*-driven lung pathology, suggesting an involvement in allergic asthma (Bacher et al., 2019). Examples of a skin-lung axis have been provided by bacterial skin commensals (Fyhrquist et al., 2014). It is tempting to speculate that Th17 cells primed against *Malassezia* in the skin may act in a similar way to promote the progression from AD to allergic asthma and rhinitis and thereby contribute to the atopic march.

Beyond its impact on the pathogenesis of inflammatory diseases, *Malassezia* was recently implicated in carcinogenesis. Among the microbes infiltrating pancreatic ductal adenocarcinomas (PDA) tumors, *Malassezia* was markedly enriched (Aykut et al., 2019). In a murine model of PDA, *Malassezia* exerted a tumor-promoting effect via a mechanism involving mannose-binding lectin, a CLR and activator of complement and implicated in antifungal innate immunity (Aykut et al., 2019). *Malassezia* is likely a ligand of MBL, although direct binding has not been demonstrated. Whether fungal dysbiosis is cause or consequence of oncogenesis is not fully clear. The link between *Malassezia* and carcinogenesis may be more general, as fungal dysbiosis was also observed in colitis-associated cancer with an enrichment of *Malassezia* in the colonic mucosa associated fungal microbiota (Richard et al., 2018).

CONCLUDING REMARKS

Recent advances in the field have shed light on the immune response to *Malassezia* in the skin and on the divergent consequences that the antifungal response can have on the host. IL-17-dependent immunity against *Malassezia* contributes to the maintenance of fungal commensalism and skin homeostasis. The same pathway however can also have host-adverse effects in predisposed individuals, and this effect is likely not limited to the skin but also affects extracutaneous sites. The factors determining these context-dependent outcomes remain largely unclear. Moreover, it remains unknown to what extent the effects of the antifungal immunity on disease pathogenesis is shared or distinct between different *Malassezia*-associated disorders. Beyond immunological pathways, non-immunological factors such as neurotransmitters and possibly hormones affect the antifungal response, and this is likely also the case with *Malassezia*. Consistently, neuroendocrine changes are known to impact the course and severity of *Malassezia*-associated skin disorders. Future research will inform about the potential of therapeutically targeting fungal communities or the antifungal response for improving patient outcome.

AUTHOR CONTRIBUTIONS

FS and SL-L wrote the minireview. FR created the figure with BioRender.com. All authors reviewed the manuscript.

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MINI REVIEW
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Overview of the Potential Role of *Malassezia* in Gut Health and Disease

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Malassezia is the most prevalent fungus identified in the human skin microbiota; originally described at the end of the nineteenth century, this genus is composed of at least 14 species. The role of *Malassezia* on the skin remains controversial because this genus has been associated with both healthy skin and pathologies (dermatitis, eczema, etc.). However, with the recent development of next-generation sequencing methods, allowing the description of the fungal diversity of various microbiota, *Malassezia* has also been identified as a resident fungus of diverse niches such as the gut or breast milk. A potential role for *Malassezia* in gut inflammation and cancer has also been suggested by recent studies. The aim of this review is to describe the findings on *Malassezia* in these unusual niches, to investigate what is known of the adaptation of *Malassezia* to the gut environment and to speculate on the role of this yeast in the host physiology specifically related to the gastrointestinal tract.

Keywords: mycobiota, inflammatory bowel diseases, gut, cancer, *Malassezia*

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INTRODUCTION

Malassezia is the major component of the fungal skin microbiota of various mammals, representing more than 90% of the fungal population in most skin niches (Dawson, 2019). As such, *Malassezia* yeasts have been associated with human skin disorders (Gupta et al., 2004) such as dermatitis (Gaitanis et al., 2008; Darabi et al., 2009; Barac et al., 2015), pityriasis versicolor (Magiatis et al., 2013), or dandruff (Gemmer et al., 2002). The mechanisms by which *Malassezia* cells trigger such diseases are not yet clearly identified, but the current hypothesis is that the diseases can be induced either by direct invasion of the tissue by fungal filaments or indirectly through immunological and metabolic mechanisms induced by the yeast. While one can consider that *Malassezia* is perfectly adapted to the skin environment, increasing data suggest that this genus can also be found in other body compartments (Theelen et al., 2018). The development of next-generation sequencing (NGS) methods have overcome the problems associated with culture-based methods, allowing the identification of *Malassezia* in surprisingly diverse localizations, such as the digestive tract, or in very unexpected locations with the identification of *Malassezia*-like organisms in deep-sea vents, for instance (Amend, 2014). The gut microbiota is not only composed of microorganisms such as bacteria, archaea, viruses, and protozoans but also colonized by numerous fungal cells (Sekirov et al., 2010). The role of fungal gut microbiota in host homeostasis, as well as in several physio-pathologic settings, is now clearly identified (Sekirov et al., 2010; Limon et al., 2017; Richard and Sokol, 2019). However, many questions remain regarding the specific fungal species involved in this role. In this review, we will focus on recent studies

that described the identification of *Malassezia* in various non-skin environments, and we will particularly concentrate on the potential role of *Malassezia* in gut health and diseases (Chen et al., 2011; Hamad et al., 2012; Gouba et al., 2013; Suhr et al., 2016; Hallen-Adams and Suhr, 2017).

IDENTIFICATION OF *MALASSEZIA* IN NICHES UNRELATED TO THE SKIN

Although it has been long regarded as a strictly skin-located fungus, *Malassezia* is now regularly identified in various samples using NGS methods. The culture of *Malassezia* is known to be difficult, and this is probably a reason for the very low level of *Malassezia* identified using culture-dependent methods under conditions where this genus is not highly abundant. With culture-independent methods, the unexpected diversity of fungi has been unraveled, and *Malassezia* strains have been identified in unforeseen environments. Thus, *Malassezia* strains have been found in diverse localizations such as murine (Limon et al., 2019) and human (Suhr et al., 2016; Hallen-Adams and Suhr, 2017) gut, human breast milk (Boix-Amorós et al., 2017), and internal organs, including the central nervous system (Alonso et al., 2017, 2018a,b). Moreover, *Malassezia* DNA has also been found in more diverse ecosystems, such as marine environments (Amend, 2014), but this field is beyond the scope of this review, although it opens a very interesting research axis. Indeed, culture-independent studies highlighted that *Malassezia* DNA nearly identical to human-associated *Malassezia* DNA can be found in various habitats (Amend, 2014), such as in deep-sea sediments, in corals, in lobster guts or on the exoskeleton of nematodes, but more sequences and taxonomic studies are needed to allow a complete understanding of the *Malassezia* genus and its distribution on earth.

Before describing the data gathered so far on *Malassezia* identification in niches unrelated to the skin it is important to raise the question of the samples contamination. Fungi represent 30% of the skin microbiota and *Malassezia* strains are by far the majority on the skin, consequently representing a probable cause of samples contamination either during the sampling or of the samples afterward in the lab. These questions have been raised by some authors in their study and they provided several solutions:

- Contamination after sampling: This is the easiest problem to solve and a problem common to all NGS experiments. Mostly the experimentators will use internal controls, and sequenced them, like the "reagent-only" controls, but one can also see particular attention directed to the tip used, the room for extraction, the disposable supplies, etc. (Alonso et al., 2018a). Thus, the global microbial contamination would be fairly reduced and contaminating sequences can be removed from the sequences upstream the analyze pipeline.
- contamination during sampling: That matter on the other hand is much more difficult to work around. The main solutions that have been used in the experiments are: (i) the way of preparing the area before sampling like cleaning thoroughly the area (breast, rectum, etc.) prior to sample collection (Boix-Amorós et al., 2019), or (ii) getting samples directly from explant in an areas that were never in close contact with the skin like getting the mucosa-associated microbiota or from the organ itself (Liguori et al., 2016; Aykut et al., 2019). This matter, however, has not been strictly tackled especially regarding *Malassezia* in non-skin niches which is not totally surprising since this is a young area of research, but this is certainly an area of improvement.

Thus, we need to keep in mind that, a part of *Malassezia* identified might comes from the skin during samples collection however, we can also consider the fact that from the numerous reports, from very different sampling method, the identification of *Malassezia* by numerous laboratories is very unlikely to be only sample contamination. However, we will see further in this review that colonization via the skin is a very likely explanation of how *Malassezia* first enter the different niches.

Malassezia in Human Breastmilk

An analysis of the composition of the fungal microbiota present in the human breastmilk revealed two main pieces of information: (i) fungi are much more abundant in milk than bacteria compared to what is observed in many other niches. Indeed, fungi represent 30% of the total skin microbial population, while fungi represent ∼0.1% in the gut for instance. (ii) Among the fungal strains, more than 40% of the total genera identified by pyrosequencing are represented by *Malassezia* strains (Boix-Amorós et al., 2017, 2019). Interestingly, this colonization was identified worldwide with samples from different continents (Spain, China, South Africa, and Finland). *Malassezia* strains, particularly *M. globosa* and *M. restricta*, were detected in all the samples analyzed (Boix-Amorós et al., 2017). The authors ruled out the possibility that their samples were contaminated by the skin microbiota but speculated that the primary source of *Malassezia* in breast milk might be the possible transfer of fungi from the skin surrounding the breast or from the baby's mouth or skin to the breast milk. To date, no studies have followed the milk microbiota from the early production of the milk to later on in order to elaborate a possible route of colonization.

Malassezia in the Central Nervous System

In the last 3 years, a single team has raised the possibility of the involvement of fungi in different neurologic diseases, such as Alzheimer's disease, multiple sclerosis, or amyotrophic lateral sclerosis (Alonso et al., 2017, 2018a,b). Very surprisingly, using NGS methods, Alonso and coworkers identified fungi and bacteria in the brain tissue of healthy subjects and of patients and showed that in some cases, specific fungal strains seemed more abundant in the affected brains than in the brains from healthy donors. No data in these publications are provided on the actual quantities of these microorganisms in the brain, and this information would be important to have in the future. Interestingly, among the fungal strains identified, a large percentage of samples showed the presence of DNA from *Malassezia* strains, suggesting possible colonization by *Malassezia* in the central nervous system of patients (Alonso

et al., 2018b). Very importantly these data have to be confirmed by independent teams, along with the addition of quantitative data in order to evaluate a potential causal link between this *Malassezia* colonization and the diseases that has not yet been proven.

Malassezia in Other Human Body Sites

The presence of *Malassezia* in different human niches in healthy patients is controversial, particularly its colonization of the mouth (Ghannoum et al., 2010; Dupuy et al., 2014). On the other hand, in infection contexts, *Malassezia* strains have been more regularly identified in blood, urine, vagina, and lung (Theelen et al., 2018). For instance, *M. furfur* (Kaneko et al., 2012; Iatta et al., 2018) was identified in blood and in central venous catheters in 4% of neonate patients.

Malassezia as a Member of the Gut Microbiota From Healthy Subjects

In 2017, Dawson, Boekhout and coworkers asked in their review whether *Malassezia* strains were indeed part of the gut microbiota (Theelen et al., 2018). Three years later, an increasing number of publications reported the identification of *Malassezia* in healthy fecal samples (Chen et al., 2011; Hamad et al., 2012; Gouba et al., 2013; Suhr et al., 2016; Hallen-Adams and Suhr, 2017). At steady state, the *Malassezia* genus has been reported to be the second most abundant genus among all human stools analyzed by internal transcribed spacer (ITS)-gene sequencing in proportions from 2 to 4% (Gouba et al., 2013; Suhr et al., 2016; Raimondi et al., 2019), while *M. restricta* (Suhr et al., 2016; Nash et al., 2017; Auchtung et al., 2018) (more than 80% and can reach 3.8% of the total abundance Raimondi et al., 2019) and *M. globosa* (Nash et al., 2017) (36%) represent the most abundant species. Moreover, *M. pachydermatis* (Chen et al., 2011; Hamad et al., 2012; Gouba et al., 2013), *M. restricta* (Hamad et al., 2012; Gouba et al., 2013), and *M. globosa* (Hamad et al., 2012; Gouba et al., 2013) were identified by molecular detection (specific primers 18S Chen et al., 2011; Hamad et al., 2012 or JPD1/JDP2 Gouba et al., 2013) and by culture media isolation (Dixon agar medium Gouba et al., 2013). The presence of *Malassezia* living cells and a large amount of *Malassezia* DNA found in the gut content have also been associated with the intestinal mucosa (Liguori et al., 2016), which strongly suggested that this genus has the capacity to at least survive in the intestinal environment. The growth or at least survival of *Malassezia* suggested that some *Malassezia* strains found favored culture conditions within the intestine.

DIVERSITY AND GROWTH CONDITIONS OF *MALASSEZIA*

As mentioned above, *Malassezia* strains are mostly found on the skin of humans (Findley et al., 2013) and mammals (Cabañes et al., 2007; Velegraki et al., 2015) suggesting specific growth conditions *in vivo*: temperature of ∼33°C, aerobic conditions, and low nutrient availability. However, with the identification of *Malassezia* strains in niches very different from the skin (see **Table 1**), such as the gut, questions have arisen regarding how this fungus can adapt to such various environmental conditions of growth.

TABLE 1 | Compartments where *Malassezia* were identified from and the percentage of the fungal microbiota.

Compartments	*Malassezia* (% of fungal microbiota)
Brain	0.5 (Alonso et al., 2018b)
Gut	2–4 (Gouba et al., 2013; Suhr et al., 2016; Raimondi et al., 2019)
Lung	No data available in healthy subject
Milk	20–50 (Boix-Amorós et al., 2019)
Mouth	13–96 (Dupuy et al., 2014)
Skin	50–80 (Dawson, 2019)
Urine	No data available
Vagina	No data available

Malassezia Growth on the Skin

The *Malassezia* genus contains at least 14 species (Gaitanis et al., 2012; Dawson, 2019), among which *M. globosa*, *M. restricta*, *M. sympodialis,* and *M. furfur* are the most commonly identified species (Ashbee, 2007; Tajima et al., 2008) and *M. pachydermatis* is the only lipid-independent fungus (Ashbee, 2007). Indeed, one of the main physiological traits of this genus is the inability of almost all *Malassezia* strains to synthesize fatty acids (Shifrine and Marr, 1963) *de novo*. Consequently, *in vivo*, *Malassezia* strains require an external source of lipids for growth. This dependency is not a problem on the skin; indeed, skin epithelium layers can produce lipid-rich sebum mostly from sebaceous glands, which will provide a suitable source of long-chain fatty acids for optimal *Malassezia* growth (Gaitanis et al., 2012). Consequently, *Malassezia* has developed a large set of enzymes that digest these compounds with lipases and phospholipases (Velegraki et al., 2015).

This physiological constraint can explain the distribution of *Malassezia* on the body surface: sebum-rich areas such as the head, arms, legs, and torso are rich in *Malassezia* strains, while the other areas of the body show lower abundance. More specifically, *M. restricta* is predominant on the forehead and inside and outside of the ears, and *M. globosa* is predominant on the back, occiput, and groin (Theelen et al., 2018). Accordingly, on the foot, which is a very different environment by many parameters (low level of sebum, humidity, skin type, etc.), the colonization of fungi is much more diverse. As a consequence, *Malassezia* is not the predominant genus in the foot (Byrd et al., 2018), and the foot fungal community seems much more variable over time; this result possibly explains the higher frequency of fungal infections observed in environments with no true homeostasis, leaving colonization chances for opportunistic microorganisms (Findley et al., 2013).

From the Human Skin to the Gut

These observations do not explain the possible colonization of the intestinal tract by *Malassezia* strains. The first question is the source of colonization. As observed above, *Malassezia* represents a large proportion of the breastmilk mycobiota. Indeed, human

milk is composed of 39 g/L fat (Jensen, 1999), and the majority of lipids are triglycerides and fatty acids. We can assume that *Malassezia* strains can use these lipid sources for their own growth, even if no clear correlation was found between the *Malassezia* genus and fat (Boix-Amorós et al., 2017). We can consider that the breast milk can be one of the entry points during primo-colonization, but breastfeeding in the world is far below 50%, so this cannot be the only explanation (Victora et al., 2016). Logically since *Malassezia* is in high concentration on the skin, we can hypothesize that simple transfer from our own skin microbiota can be part of the colonization process.

Thus, the actual survival or even development of *Malassezia* cells within the gut remains a source of interrogations. Within the gut, three main parameters differ from the skin: (i) the absence of sebum, the source of lipids, (ii) the higher temperature, and (iii) the low level of oxygen.

Lipids

One can consider that the absence of sebum should not be a major problem since many other sources of lipids are available along the gut. For instance, bile acids synthesized by hepatocytes and stored in the gallbladder as bile salts are regularly poured into the intestine to allow the emulsion of fat, making lipids available for host cells and microorganisms. *Malassezia* fungi can thus use this source of lipids. Thus, *Malassezia* strains should be able to obtain enough sources of lipids within the gut for their growth using molecules coming from the diet or from other microorganisms in the intestinal microbiota.

Temperature

While the temperature for *Malassezia* growth is often considered to be restricted to the skin temperature (33°C), *Malassezia* isolated from hair follicles and sebaceous glands can grow in an environment slightly different from the surface of the cutaneous layer, with a slightly higher temperature and lower oxygen concentration. In accordance with this observation, numerous data have shown that most *Malassezia* strains can grow at 37°C or above, with a maximum temperature identified at ∼40–41°C (Gaitanis et al., 2012).

Oxygen

For microorganisms, a low oxygen concentration can be a potent inhibitor of growth, but again, this might not represent a strong challenge for *Malassezia* strains. The level of oxygen is not constant along the intestine but follows a decreasing gradient from the mouth to the rectum (Espey, 2013); thus, *Malassezia* might find parts of the gut with suitable conditions for development. As stated above, the observation of growth in follicles and sebaceous glands supports a certain flexibility in terms of the levels of oxygen needed for *Malassezia*.

Reports on the growth capacity of *Malassezia* strains in anaerobic conditions are sparse and do not give a general or specific overview for each species. The most often cited work is a publication in 1981 in *Sabouraudia* from two Sweden researchers, Faergemann and Bernander, describing the microaerophilic and anaerobic growth of *Pityrosporum* species (Faergemann and Bernander, 1981). *Pityrosporum* and *Malassezia* were two names given to the same fungi before *Malassezia* was chosen in 1996 (Dolenc-Voljč, 2017). In this study (Faergemann and Bernander, 1981), the authors tested only *M. furfur*, *M. sympodialis,* and *M. pachydermatis* in different media and with different levels of oxygen. They concluded that the strains were able to grow in microaerophilic and anaerobic conditions but that anaerobic growth was much weaker with slow growth and very small colonies. However, a recent study reported that *M. pachydermatis* is unable to grow under anaerobic conditions, which contradicted the data from the Swedish team suggesting that *M. pachydermatis* might have the capacity to grow under these conditions but that the growth is clearly dependent on many environmental factors that we do not master and understand yet (Tylicki et al., 2008) and can also simply depends on strains variability. It is well-known that it is difficult to grow *Malassezia* strains *in vitro* under laboratory conditions. In addition, as *Malassezia* form aggregates and grow better when they are not isolated on a plate, the evaluation of the CFU as well as using counting cells under the microscope is almost impossible. Altogether, this probably explains the lack of specific and quantitative information about growth under anaerobic conditions.

Youngchim et al., while studying the hyphae formation of *M. furfur*, used microaerophilic conditions (anaerobic jar) for the induction of morphogenesis, but no data are available on the growth rate under these conditions (Youngchim et al., 2013). However, it seems that low levels of oxygen induce *Malassezia* morphogenesis, which is an interesting feature that can participate in the colonization of this strain in the human epithelium. Indeed, germ tubes and hyphae are known to be penetrating structures that can help *Malassezia* cross epithelial barriers (Tati et al., 2016). Consequently, *Malassezia* hyphae might help fungal cells reach areas rich in nutrients over the highly keratinized top layers of the skin (Brand, 2012).

Altogether, these data suggest that although *Malassezia* mainly localizes with the skin microbiota, this fungal microorganism has the capacity to at least survive within the gut. However, the resistance to acidic pH, for example, has not been clearly documented, so thorough characterization of each *Malassezia* species is still needed.

FUNGAL GUT MICROBIOTA

In the past few years, the gut microbiota has become a key player in human health studies. When the gut microbiota is described, it tends to be reduced to its bacterial population only. Indeed, bacteria represent ∼99.1% of the population established in the colon (Qin et al., 2010). However, technological advances, such as NGS, have allowed deeper investigation of the microbial population and have revealed that the microbiota is also populated by archaea, viruses, protozoans and 0.01–0.1% fungi (Qin et al., 2010; Huffnagle and Noverr, 2013; Nash et al., 2017). In addition, changes in the composition of fungal gut microbiota, the mycobiota, have been associated with several gut-related diseases, such as inflammatory bowel disease (IBD) (Chehoud et al., 2015; Liguori et al., 2016; Sokol et al., 2017), irritable bowel syndrome (Botschuijver et al., 2017), colorectal cancer (Gao et al., 2017), and alcoholic liver disease (Yang et al., 2017).

Fungal Gut Colonization

The current theory is that most fungi transit through the digestive tract without being able to implant. As with bacteria, fungi can primo-colonize the intestine at birth (Bliss et al., 2008; Nagata et al., 2012) and during breastfeeding (Nagata et al., 2012; Boix-Amorós et al., 2017); then, they can simply be brought by food, the respiratory tracts or the contacts between the mouth and the skin (Schulze and Sonnenborn, 2009; Koh, 2013). Indeed, on the skin, fungi represent 5–10% (Byrd et al., 2018) of the microbial population, consequently representing a large reservoir for colonization. Additionally, food is an important source of fungi, which play an important role in the transformation processes during food preparation and can be ingested in large quantities. There is no doubt, however, that there are huge differences between continents or even countries depending on their food cultural habits. Nevertheless, it has been shown that some species can survive in this specific environment and manage to adhere to human epithelial cells more effectively and thus persist in the intestine, which is a characteristic of the *Candida* genus, such as *C. albicans* (Raimondi et al., 2019). The fact that a strain can persist or only transit in the body does not account for its capacity to influence host health. A good example is the positive effect of *Saccharomyces boulardii* CNCM I-745 on antibiotic associated diarrhea and acute gastroenteritis, since *S. boulardii* is known to be unable to settle in the human gut but is cleared in 2 to 5 days (Buts and De Keyser, 2006).

The mycobiota appears to be relatively stable along the digestive tract: from 10^3 (Darabi et al., 2009) in the stomach to up to 10^{5-6} (Gaitanis et al., 2008; Magiatis et al., 2013) microorganisms per gram of content in the colon (Slmon and Gorbach, 1984). The bacterial microbiota abundance increased dramatically from the stomach to the colon, from 10^2 (Gupta et al., 2004) in the stomach to up to 10^{12} (Richard and Sokol, 2019) microorganisms per gram of content in the colon (Slmon and Gorbach, 1984). As such, the fungal to bacterial cell abundance ratio is variable from the stomach to the colon and can be much more favorable to fungi in the upper digestive tract. Further investigations are thus needed in these compartments where fungi may have a stronger influence on host health.

Composition and Diversity of the Mycobiota

The composition of the mycobiota, as the rest of the gut microorganisms, varies according to the environment (Suhr et al., 2016), diet (Hoffmann et al., 2013; David et al., 2014; Hallen-Adams and Suhr, 2017; Heisel et al., 2017; Yang et al., 2017), sex (Markle et al., 2013; Strati et al., 2016; Borges et al., 2018), and health of the host (Richard et al., 2015; Nash et al., 2017; Sokol et al., 2017). There are two major approaches to investigate the gut mycobiota: culture-dependent (Gouba et al., 2013; Becker et al., 2014; Borges et al., 2018) or culture-independent methods (Qin et al., 2010; Donovan et al., 2018). Culture-dependent methods have the strong advantage of resulting in a microorganism that can be used directly either in interactions with cells or for metabolite production, for instance. However, this method allows only the isolation of a very low percentage of living organisms in samples for simple technical reasons: the media, pH, oxygenation, or temperature may not be optimized. On the other hand, culture-independent methods are much more efficient for the identification of a large percentage of the microorganisms present with fewer technical constraints. To analyze the fungal population with a culture-independent method, the major targets are the internal transcribed sequences (ITSs) ITS1 and ITS2 (Huffnagle and Noverr, 2013; Tang J. et al., 2015; Wang et al., 2015; Donovan et al., 2018; Yang et al., 2018). These regions are highly divergent between fungi and can even allow identification to the fungal species level; for further information, refer to Richard and Sokol (2019). However, some strains have been identified by culture-dependent methods and not by culture-independent methods (Hamad et al., 2017; Richard and Sokol, 2019), revealing that both methods show advantages and limitations (Richard and Sokol, 2019) and that they remain complementary for the exhaustive identification of the intestinal mycobiota.

From these diverse techniques, we have concluded so far that the major phyla of the intestinal mycobiota are Ascomycota and Basidiomycota (Suhr et al., 2016; Nash et al., 2017; Borges et al., 2018; Raimondi et al., 2019), with a much lower abundance of Zygomycetes (Borges et al., 2018). The diversity of the mycobiota is relatively low, since 10 genera and 20 different species are usually identified within a healthy individual: *Candida*, *Saccharomyces, Malassezia*, *Penicillium*, *Aspergillus, Debaryomyces, Trichosporon, Galactomyces, Cryptococcus,* and *Cladosporium* (Strati et al., 2016; Suhr et al., 2016; Nash et al., 2017; Auchtung et al., 2018; Raimondi et al., 2019; Richard and Sokol, 2019). With culture media methods from feces, mostly *Candida* (Strati et al., 2016; Borges et al., 2018; Raimondi et al., 2019) and *Saccharomyces* (Strati et al., 2016; Borges et al., 2018; Raimondi et al., 2019) as well as *Debaryomyces* (Raimondi et al., 2019), *Penicillium* (Strati et al., 2016; Borges et al., 2018), *Malassezia,* and *Aspergillus* (Strati et al., 2016; Borges et al., 2018; Raimondi et al., 2019) have been identified.

Role of Fungal Gut Microbiota in Diseases

As previously stated, the increase in the number of studies on the mycobiota revealed changes in the mycobiota composition associated with intestinal diseases. The causality is still to be proven in most of the studies, but it is difficult to consider that this association is simple coincidence. It is probably a sum of different modifications that trigger and enhance the disease with a vicious circle effect.

In the IBD context, mycobiota in the feces and associated with the mucosa have been studied in various cohort patients, showing a clear modification of the fungal community during intestinal inflammation. *In vivo* studies in mouse models have also reinforced the hypothesis that fungi are directly or indirectly involved in IBD symptoms (Tang C. et al., 2015; Wang et al., 2016). From the various studies published to date, in IBD patients, fungal load was increased during flare (Liguori et al., 2016), showing a modification of the equilibrium between Ascomycota and Basidiomycota with a decrease in Ascomycota and an increase in Basidiomycota (Sokol et al., 2017). At the genus and species levels, *Candida* (Chehoud et al., 2015; Liguori et al., 2016) (particularly *C. glabrata* Liguori et al., 2016, *C. tropicalis* (Hoarau et al., 2016), *C. albicans* Standaert-Vitse et al., 2009, *C. utilis* Chehoud et al., 2015, and *C. parapsilosis*

Chehoud et al., 2015) was the genus showing an increase in the vast majority of the studies, and *Saccharomyces* was decreased (Hoarau et al., 2016; Liguori et al., 2016; Sokol et al., 2017). Some studies also identified variations in the relative abundance of *Malassezia* with an increase in *M. globosa* (Liguori et al., 2016) and a decrease in *M. sympodialis* (Sokol et al., 2017) (see next section).

In irritable bowel syndrome (IBS), to date, only one team has specifically made a link between the fungal gut community and IBS symptoms. Botschuijver et al. compared 3 groups of 19+/−1 subjects of healthy controls, hypersensitive IBS and normosensitive IBS patients (Botschuijver et al., 2017) and showed a decrease in diversity in both IBS groups; the *Saccharomyces* and *Candida* genera represented two-thirds or more of the mycobiota in these patients, and they represented ∼57% in the healthy controls. Additionally, an increase in *Kazachstania turicensis* was one marker of IBS in these specific cohorts. Further analyses and clinical trials are needed to confirm this hypothesis.

In alcoholic liver diseases, the mycobiota is very strongly modified with a drop in diversity in patients, in which *Candida* seems to replace all other fungal genera within the intestinal content. Interestingly, a previous study demonstrated *in vivo* in mice that the use of antifungal drugs can improve alcohol-induced liver injury (Yang et al., 2017). Again, even if this does not prove causality, the authors made an interesting link between the increase in alcohol in the gut, triggering at the same time an increase in gut permeability and fungal burden: the direct consequence was an increase in circulating **ß**-glucans in the blood reaching the liver and triggering liver injuries via IL-1**ß** and Kupffer cells.

Finally, recently, there has been increasingly more data indicating a potential role of the fungal microbiota during the course of colorectal cancer (CRC) or colitis-associated cancer (CAC). Two reports were published in 2019 on the role of Card9 in the regulation of the fungal burden, myeloid-derived suppressor cell expansion and inflammasome activation, allowing the restriction of CRC or CAC development (Malik et al., 2018; Wang et al., 2018). CARD9 indeed participates in the recognition of microorganisms, especially fungi, through several receptors, such as Mincle, NOD2, and Dectin, and thus orchestrates an important part of the host response against fungi from simple overgrowth to deep infection (Richard et al., 2015).

Additionally, in 2019, chitooligosaccharides were shown to prevent the development of CAC through their effect on the balance between bacterial and fungal microbiota (Wu et al., 2019). In addition to these results, several other publications, which are presented in the next chapter, described the potential role of *Malassezia* strains in cancer development.

MALASSEZIA INFLUENCES GUT HEALTH

As highlighted earlier in this review, although a well-described resident of the skin, *Malassezia*, appears to be a prominent component of the gut mycobiota, numerous studies have identified *Malassezia* in fecal samples through culture-dependent and culture-independent methods (Chen et al., 2011; Hamad et al., 2012; Gouba et al., 2013; Suhr et al., 2016; Hallen-Adams and Suhr, 2017). *Malassezia* has been reported in healthy volunteers as a major genus, reaching up to 4% of the total abundance (Gouba et al., 2013; Suhr et al., 2016; Raimondi et al., 2019). *M. restricta* (Hamad et al., 2012; Gouba et al., 2013; Suhr et al., 2016; Nash et al., 2017; Auchtung et al., 2018), *M. globosa* (Hamad et al., 2012; Gouba et al., 2013; Nash et al., 2017), *M. pachydermatis* (Chen et al., 2011; Hamad et al., 2012; Gouba et al., 2013), and *M. sympodialis* (Nash et al., 2017) are the main species that can be found in the gastrointestinal tract. However, these previous studies made very few cases of this presence, mostly considering it transient and with no effect on the host. It is only very recently that *Malassezia* strains have been specifically identified in association with gut diseases and possibly other types of diseases (**Figure 1**).

The Impact of Malassezia on IBD

The *Malassezia* genus has only very recently been associated with IBD, both in patients and in mouse models. IBD is composed of two types of disease: Crohn's disease (CD) and ulcerative colitis (UC). Both are characterized by inflammation of the wall of the digestive tract, from the mouth to the rectum for CD and only for the colon for UC (Seyedian et al., 2019).

Two studies highlighted the potential role of *Malassezia* in the development of IBD in the last 3 years: (i) our study while characterizing the global modifications of the bacterial and fungal microbiota for UC and CD patients (Sokol et al., 2017); (ii) and Limon and coworkers in a specific study focused on CD patients (Limon et al., 2019).

In a work where we compared a cohort of 235 IBD patients (UC, CD, flare, or remission) to 38 healthy subjects, our team found that the relative abundance (percentage of reads) of *Malassezia* increased globally during IBD flare. Interestingly, we also showed that *Malassezia* had a negative correlation with many bacteria, especially in UC patients, something that we did not see in other types of disease or during CD. Finally, *M. sympodialis* was negatively correlated with the Dectin1 SNP associated with medically refractory UC (rs2078178, "T" allele12). Altogether, these data from human samples suggest a potential role of *Malassezia* in IBD with possible opposite effects between species.

Using human samples from CD patients (no data on UC) and *in vivo* experiments with a mouse model of colitis, Underhill's team was able to highlight a possible relationship between gut inflammation and the presence of *M. restricta*. Through amplicon-based analysis of the mycobiota of CD patients compared to healthy subjects, *M. restricta* was found to be enriched in the mycobiota-associated mucosa of CD patients (Limon et al., 2019). In addition, in a model of dextran sodium sulfate (DSS)-induced colitis, germ-free or wild-type mice colonized by *M. restricta* showed a worse disease activity index with shorter colons, suggesting that the addition of *M. restricta* alone exacerbated the severity of colitis. However, in their model, *C. albicans*, a well-known pro-inflammatory fungus did not trigger stronger inflammation than the non-treated mice, underlying the complexity of these effects, probably due to the composition of the basal mouse microbiota. The authors also

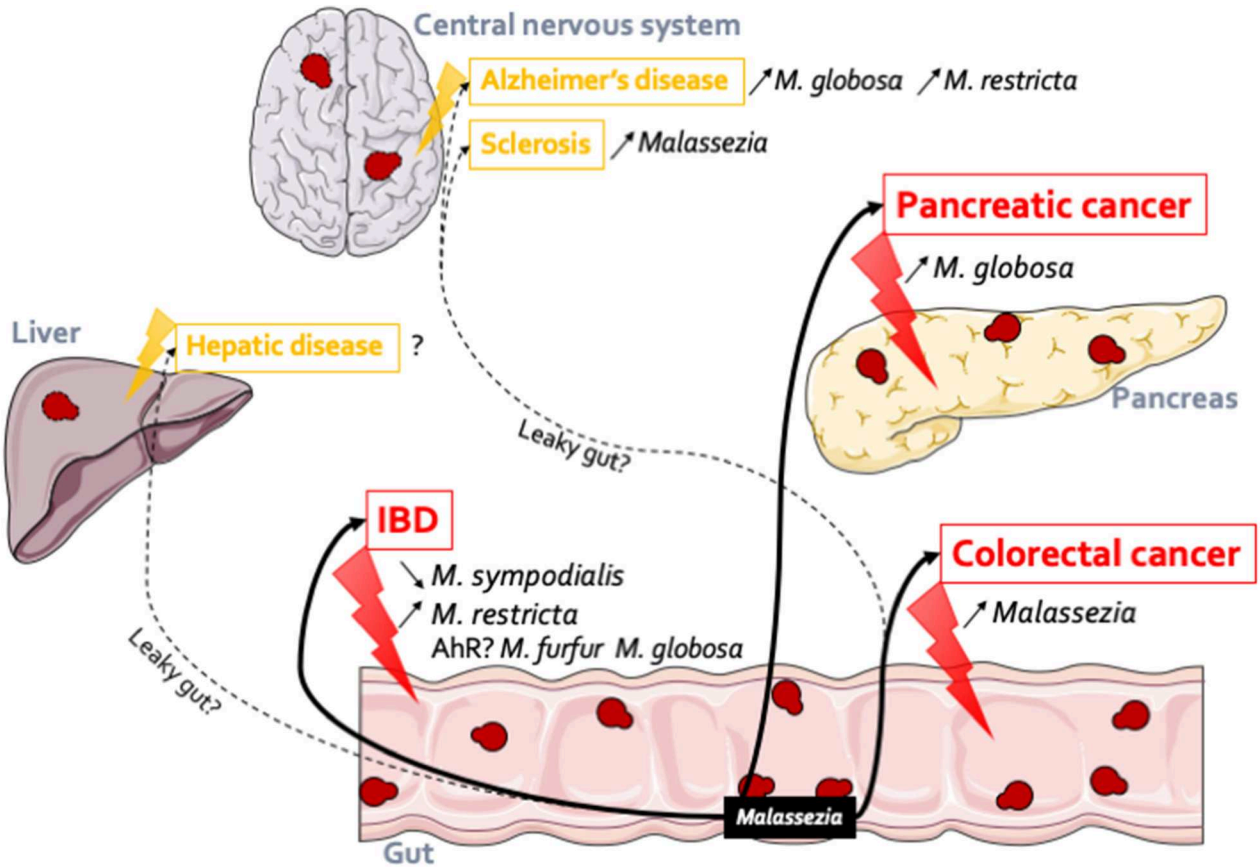

FIGURE 1 | *Malassezia* in the gut: association with several diseases. *Malassezia* strains have an action within the gut. In IBD, *M. sympodialis* has been identified both in flare (Sokol et al., 2017) (decreased) and in remission (Liguori et al., 2016) (increased) of Crohn's disease patients; *M. restricta* (Limon et al., 2019) participates to the production of inflammatory factors and can so exacerbates severe colitis. These observations might be linked to the gene expression regulations through AhR. *Malassezia* genus is more abundant in patients with polyp and colorectal cancer (Gao et al., 2017; Coker et al., 2019). In pancreatic cancer (Aykut et al., 2019), in both patients and mice model, *Malassezia* was increased. *Malassezia* fungi can be implicated in other diseases related or not to the gut. Indeed, their DNA were identified in the central nervous system of Alzheimer's patients (Alonso et al., 2018b) (*M. globosa* and *M. restricta*) and in both multiple sclerosis (Alonso et al., 2018a) and amyotrophic lateral sclerosis patients (Alonso et al., 2017). In the same line we can hypothesis that *Malassezia* could be implicated in hepatic disease due to the liver-gut axis but to date no data confirm this hypothesis.

showed a link between *M. restricta* and Card9 signaling: the Card9-S12N polymorphism in CD patients which was strongly linked to the presence of *Malassezia* spp. Using CARD9KO mice and colonization with *M. restricta*, the authors suggested that the pro-inflammatory effects (cytokine production, colitis symptoms) due to *M. restricta* were dependent on Card9.

A possible mechanism explaining the effect of *Malassezia* strains on IBD development can be the link between these fungi and the aryl hydrocarbon receptor (AhR). Indeed, the majority of *Malassezia* strains, especially *M. furfur* (Gaitanis et al., 2008) and *M. globosa* (Magiatis et al., 2013), are capable of synthesizing indole ligands that can act on AhR (Gaitanis et al., 2008; Magiatis et al., 2013; Furue et al., 2014; Wheeler et al., 2017). AhR is a cytoplasmic transcriptional regulator found not only in epithelial cells such as skin cells but also in many other cell types throughout the body (Lamas et al., 2018), and AhR has numerous endogenous ligands with opposite effects on cell functions, generating a very complex network of regulation that has not been completely elucidated. AhR is involved in many functions, including the regulation of the expression of enzymes involved in xenobiotic metabolism, participation in cutaneous homeostasis and the modulation of ultraviolet-induced damage (Furue et al., 2014). Some studies have linked the impact of *Malassezia* on skin diseases to its capacity to produce AhR ligands (Gaitanis et al., 2008; Magiatis et al., 2013). AhR ligands produced by *Malassezia* can possibly regulate the production of inflammatory mediators (Swanson, 2004) and/or change the function of keratinocytes (Vlachos et al., 2012). However, studies also highlighted the role of AhR ligands in host immunity (Lamas et al., 2018) on a more global view and on other types of cells; for example, AhR ligands are directly made by the gut microbiota from tryptophan transformation and have been connected recently to intestinal diseases such as IBD (Lamas et al., 2016; Agus et al., 2018). Consequently, the hypothesis of

the involvement of *Malassezia* in gut pathologies via the AhR receptor should be investigated further.

The Impact of Malassezia on Cancer

As mentioned above, there is an increasing number of clues suggesting that fungi can be implicated in the development of some cancers or at least that the mycobiota of CRC or CAC patients is modified. However, to date, very few specific fungi have been identified as central to these phenomena. Surprisingly, in the last 2 years, *Malassezia* strains have been independently identified as potentially key to cancer development in several publications.

Initial interest was obviously directed to gut-related cancer (CRC or CAC), and 2 recent works showed that the development of carcinoma was concurrent with the enrichment of *Malassezia* strains (Gao et al., 2017; Coker et al., 2019), suggesting a potential deleterious effect of this genus. In the first study, Gao and coworkers analyzed the mycobiota of colon polyps and CRC. Colorectal cancer is a malignant tumor in the colon and rectum, beginning with colon polyps that eventually evolve into carcinoma. There is strong evidence now that the gut bacteria composition plays a role in cancer development with a decrease in bacterial diversity and bacterial dysbiosis in favor of detrimental bacteria such as *Fusobacterium* spp. (Wong and Yu, 2019). Analyzing the mycobiota diversity of stool samples of patients with colon polyps, patients with colorectal cancer and healthy volunteers did not show any difference. However, the composition of the gut mycobiota revealed that the *Malassezia* genus was more abundant in patients with polyp and colorectal cancer. From these datasets, we can speculate that *Malassezia* strains can play a role in the genesis of colorectal cancer development, as they are present at the same level in precancerous lesions and in cancer.

In a recent work, Coker and collaborators performed a very interesting analysis on 3 large cohorts, 184 patients with CRC, 197 patients with adenoma and 204 control subjects, and showed that the mycobiota associated with CRC was specifically altered and might be responsible for triggering or amplifying colon adenoma (Coker et al., 2019). Using these data, they also showed that *Malassezia* was significantly increased in CRC, while *Saccharomycetes* was decreased, allowing the definition of potential efficient diagnostic markers for CRC prediction.

Finally, a study highlighted the role of mycobiota in the pathogenesis of pancreatic cancer (Aykut et al., 2019). The analysis of mycobiota in pancreatic patients showed an increased abundance of intrapancreatic fungi compared to healthy individuals, as well as in an associated mouse model. In pancreatic tumor tissue, the *Malassezia* genus was increased in both patients and mice. Furthermore, by administering GFP-labeled *S. cerevisiae* to mice, the authors demonstrated that large numbers of fungi migrate from the intestinal lumen to the pancreas. The authors highlighted the specific implication of *Malassezia* strains in the development of pancreatic lesions by ablating the mycobiota in mice using amphotericin B treatment and repopulating the gut with *M. globosa*; the genus accelerated the growth of pancreatic ductal adenocarcinoma tumors.

The gut-liver axis and the potential impact of microorganisms of the intestine on liver pathologies, such as non-alcoholic and alcoholic steatohepatitis, cirrhosis and hepatocellular carcinoma, could also be investigated, as the liver and intestine have a physical pathway via the portal vein and the bile duct (Alvarez-Silva et al., 2019).

CONCLUSION

Malassezia can be associated with human gut-related disease. These fungi have been found in abundance in fecal samples, both in healthy and pathology contexts. Future studies designed to increase our understanding of *Malassezia* within intestinal dysbiosis as well as in other organs that can be connected to the gut may lead to novel therapeutic approaches that target this specific genus.

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MS and MR constructed and wrote together the mini review.

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Analysis of *Malassezia* Lipidome Disclosed Differences Among the Species and Reveals Presence of Unusual Yeast Lipids

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Malassezia yeasts are lipid dependent and part of the human and animal skin microbiome. However, they are also associated with a variety of dermatological conditions and even cause systemic infections. How these yeasts can live as commensals on the skin and switch to a pathogenic stage has long been a matter of debate. Lipids are important cellular molecules, and understanding the lipid metabolism and composition of *Malassezia* species is crucial to comprehending their biology and host–microbe interaction. Here, we investigated the lipid composition of *Malassezia* strains grown to the stationary phase in a complex Dixon medium broth. In this study, we perform a lipidomic analysis of a subset of species; in addition, we conducted a gene prediction analysis for the detection of lipid metabolic proteins. We identified 18 lipid classes and 428 lipidic compounds. The most commonly found lipids were triglycerides (TAG), sterol (CH), diglycerides (DG), fatty acids (FAs), phosphatidylcholine (PC), phosphatidylethanolamine (PE), ceramides, cholesteryl ester (CE), sphingomyelin (SM), acylcarnitine, and lysophospholipids. Particularly, we found a low content of CEs in *Malassezia furfur*, atypical *M. furfur*, and *Malassezia pachydermatis* and undetectable traces of these components in *Malassezia globosa*, *Malassezia restricta*, and *Malassezia sympodialis*. Remarkably, uncommon lipids in yeast, like diacylglyceryltrimethylhomoserine and FA esters of hydroxyl FAs, were found in a variable concentration in these *Malassezia* species. The latter are bioactive lipids recently reported to have antidiabetic and anti-inflammatory properties. The results obtained can be used to discriminate different *Malassezia* species and offer a new overview of the lipid

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composition of these yeasts. We could confirm the presence and the absence of certain lipid-biosynthesis genes in specific species. Further analyses are necessary to continue disclosing the complex lipidome of *Malassezia* species and the impact of the lipid metabolism in connection with the host interaction.

Keywords: lipidomic, ultra-high-pressure liquid chromatography/mass spectrometry, *Malassezia*, partial least squares discriminant analysis, fatty acids esters of hydroxyl fatty acids, diacylglyceryltrimethylhomoserine

INTRODUCTION

Lipid-dependent *Malassezia* species belong to the phylum Basidiomycota and are the most important constituent of the human skin mycobiota. *Malassezia* species have been associated with dermatological conditions, such as dandruff/seborrheic dermatitis, pityriasis versicolor, and atopic dermatitis, and with more severe conditions, like systemic infections and pancreatic cancer (Grice and Dawson, 2017; Theelen et al., 2018; Aykut et al., 2019).

The absence of *de novo* synthesis of fatty acids (FAs) in *Malassezia* species is determined by the absence of genes that encode for FA synthase in their genomes (Triana et al., 2015; Wu et al., 2015; Lorch et al., 2018). This characteristic is related to the requirement to exploit lipid sources contained in the human sebum [triglycerides (TAG), FAs, wax esters, sterol esters, cholesterol, cholesterol esters, and squalene] (Ro and Dawson, 2005). For this reason, *Malassezia* species secrete several enzymes, such as esterases, lipases, lipoxygenases, and proteases, in order to supply their lipid requirements (Mayser and Gaitanis, 2010; Park et al., 2017).

This yeast can metabolize or modify FAs to carry out a variety of important biological processes, such as membrane biogenesis, energy homeostasis, energy storage, and metabolism, to carry out signal transduction and to contribute to fungal pathogenicity (Ro and Dawson, 2005; Celis Ramirez et al., 2017). Thus, changes in the external FA composition represent a challenge for *Malassezia* metabolism; however, not much is known about the lipid composition and adaptation of species of this genus (Shifrine and Marr, 1963; Porro et al., 1976; Huang et al., 1993; Mayser et al., 1998).

Considering the relevance and the complexity of lipid metabolism and potential applications of lipids, the emerging field into the omics known as lipidomics (as a subset of metabolomics) has been developed, and its application (Ibáñez et al., 2017), sensitivity, and reliability have increased in recent years along with the rapid advancement in mass spectrometry (MS) techniques (Wenk, 2005; Han, 2016; Yang and Han, 2016). The lipidome of the yeast *Saccharomyces cerevisiae* is well-characterized, and it has been widely used as an experimental system for studying lipid-related processes (Ejsing et al., 2009; Klose et al., 2012). Furthermore, lipidomic analyses have been carried out in various fungal pathogens, such as *Cryptococcus* species, *Candida* species, and *Paracoccidioides brasiliensis*, to investigate aspects related to virulence, antifungal resistance, and new antifungal targets (Hein and Hayen, 2012; Longo et al., 2013; Singh et al., 2017; Zamith-Miranda et al., 2019). Factors that affect lipid composition have also been studied in oleoginoseous yeasts in order to efficiently obtain biofuels (Beopoulos et al., 2011; Pomraning et al., 2015). Recently, differences in lipid profiles were detected via Raman spectroscopy and used to differentiate three *Malassezia* species (Petrokilidou et al., 2019); nonetheless, to date, no lipid profiles of *Malassezia* species based on MS have been reported.

The present study implemented lipidomics in combination with an *in silico* genomic analysis to investigate the lipid composition and synthesis of *Malassezia furfur*, atypical *M. furfur*, *Malassezia pachydermatis*, *Malassezia globosa*, *Malassezia restricta*, and *Malassezia sympodialis* after growth in complex Dixon medium broth.

MATERIALS AND METHODS

Strains and Culture Conditions

The reference *Malassezia* strains—*M. furfur* CBS 1878, *M. globosa* CBS 7986, *M. pachydermatis* CBS 1879, *M. restricta* CBS 7877, and *M. sympodialis* CBS 7222 (Westerdijk Institute, Utrecht, The Netherlands)—and a previously reported isolate of *M. furfur* with atypical assimilation of Tween 80 (hereafter referred to as atypical *M. furfur*) (González et al., 2009) were precultured at 33°C using modified Dixon agar [mDixon agar; 36 g L^{-1} mycosel agar [BD, Franklin Lakes, NJ, USA], 20 g L^{-1} Ox bile, 36 g L^{-1} malt extract [Oxoid, Basingstoke, UK], 2 ml L^{-1} glycerol, 2 ml L^{-1} oleic acid, and 10 ml L^{-1} Tween 40] (Guého-Kellermann et al., 2010).

After 5 days of growth on mDixon agar, the yeast cells were suspended in 3 ml of distilled water with 0.1% Tween 80 used to inoculate 27-ml mDixon broth [20 g L^{-1} Ox bile, 6 g L^{-1} peptone [BD], 36 g L^{-1} malt extract [Oxoid, Basingstoke, UK], 2 ml L^{-1} glycerol, 2 ml L^{-1} oleic acid, and 10 ml L^{-1} Tween 40 and 500 mg L^{-1} chloramphenicol] that was grown at 33°C with 180 rpm to reach the stationary phase (Guého-Kellermann et al., 2010).

Lipidomic Analysis

Aliquots of *Malassezia* species in the stationary phase were washed three times with phosphate-buffered saline with intermediate centrifugation at 1,248 g for 10 min. Cells were resuspended in 10 ml of phosphate-buffered saline and disrupted with a sonicator (Sonic Vibra Cell, Newtown, CT, USA) at 40% amplitude, performing the following procedure 10 times: 1 min sonication and 30 s of cooling on ice. The disrupted cells were centrifuged at 4°C at 5,000 g for 10 min, and the pellet was washed with deionized water and centrifuged using the same conditions. Each sample was freeze-dried and weighted before analysis.

The lipidomic analysis was performed at the West Coast Metabolomic Center (University of California at Davis). Lipid extraction was conducted according to the center's workflow involving sample extraction based on the "Matyash" method (Matyash et al., 2008) with some modifications, as described. Extraction was carried out using a biphasic solvent system of cold methanol, methyl-tert-butyl ether (MTBE), and water. The extraction was followed by ultra-high-pressure liquid chromatography (UHPLC), and the chromatographic analysis was performed as reported by Cardona Jaramillo et al. (2019). The separation was carried out in a Waters charged surface hybrid (CSH^{TM}) column, UHPLC CHS C18 (100 mm × 2.1 mm × 1.7 μm; Waters Corporation, MA, USA). The lipid detection was achieved with an Agilent 6530 quadrupole time-of-flight (QTOF) mass spectrometer with resolution $R = 10{,}000$ for positively charged lipids and with an Agilent 6550 QTOF mass spectrometer with resolution $R = 20{,}000$ for negatively charged lipids. The mobile phase A was a 90:10 mixture of isopropyl alcohol and acetonitrile with 10-mM ammonium formate and 0.1% formic acid. The mobile phase B was a 60:40 mixture of acetonitrile and water with 10 mM of ammonium formate and 0.1% formic acid. The elution gradient was 0 min 15% (A), 0–2 min 30% (A), 2–2.5 min 48% (A), 2.5–11 min 82% (A), 11–11.5 min 99% (A), 11.5–12 min 99% (A), 12–12.1 min 15% (A), and 12.1–15 min 15% (A). Raw data were processed qualitatively by Agilent's MassHunter software. Peak alignment was performed using MassProfiler Professional. MS/MS information and the LipidBlast library were used to identify the lipid compounds. A unique ID was given to each lipid, based on its retention time and mass–charge ratio, and an additional manual verification was made. Lipids were identified based on MS/MS fragmentation patterns using in-house Lipidblast software (Kind et al., 2013). Lipid peak heights were normalized using the yeast biomass weight, and the concentration of each metabolite in terms of %mol was calculated using the internal standard method when possible [ceramide C17 for ceramides; CE [22:1] for CE; DG [12:0/12:0/0:0] for DG; LPC [17:0] for lysophosphatidyl esters; MG [17.0/0:0/0:0] for monoglycerides; PC [25:0] for PC; PE [17:0/17:0] for PE; TAG [17:0/17:1/17:0] for TAG] (**Supplementary Table 1**). Lipidomic analyses resulting from six biological replicates were analyzed.

Statistical Analysis

To test whether *Malassezia* strains can be discriminated based on (a) the whole chromatographic profiles and (b) the sole basis of the FAHFA lipids, we built partial least squares models coupled with discriminant analyses (PLS-DA). Briefly, the relative amount of lipid compounds was used as a predictor of a categorical variable, the strain. The use of DA alone would lead to spurious predictive models for two main reasons. First, groups of lipid compounds tend to co-occur throughout the analyzed samples, which leads to significant correlation between them and, thereby, to multicollinearity, an undesirable property for a set of predictors. Second, the number of predictors was very large (above 400 compounds for the whole lipidome and 77 for the FAHFA lipids) and far larger than the number of analyzed samples (36, six strains each repeated six times). The PLS model reduces the number of predictors to a lower number of uncorrelated (i.e., orthogonal) variables, which are then used to discriminate (DA) among categories (strains). PLS is related to principal component analysis (PCA). Whereas, PCA reduces the dimensionality of predictors based on the sole covariation between them, PLS further considers the ability of the new components to predict the output variables.

To estimate the lowest number of components that suffice to discriminate among strains, we first fitted complete PLS-DA models and then assessed the classification performance of our models. We used unscaled maximal multivariate distances because all descriptors (compound concentrations) were expressed in the same units. The performance curve was used to visualize the contribution of each additional principal component (PC) to reduce the error rate of classifications to the point that adding new components represented negligible (i.e., statistically non-significant) contributions. As expected, both overall error rate and balanced error rate in classification decreased with the number of components and then stabilized after the fifth (all lipids) or eighth (FAHFA lipids) PC. Therefore, we used five and eight PCs, respectively, in the following analyses.

Because PLS-DA deals with a high ratio of variables to samples, it may eventually lead to good classifications by chance (Gromski et al., 2015). To reduce the risk of overfitting (tendency to overfit), we refrained from reporting both the PLS-DA score plots obtained from the training data (Szymańska et al., 2011; Brereton and Lloyd, 2014; Gromski et al., 2015) and the R^2, Q^2, and DQ^2 statistics (Szymańska et al., 2011; Gromski et al., 2015). Instead, we report here the number of misclassified cases (NMC) and the area under the receiver operating characteristic (AUROC) (Szymańska et al., 2011). Finally, we fitted two sparse versions of the model (sPLS-DA), which were intended to identify the subset of uncorrelated lipid compounds that best discriminated the strains and to eliminate the uninformative lipids. The number and identity of the lipids represented by each component was selected by cross-validation with 5-folds (groups) and 200 repetitions each, which rendered 1,000 permutations. M-fold values between 5 and 10 have been empirically shown to estimate relatively unbiased and stable error rates (Rohart et al., 2017). We then plotted the decrease in classification performance (error rate) with regard to the number of selected descriptors for each PC and checked both the load of compounds to the PCs as well as the stability of PC classification performance after adding compounds. All analyses were conducted on the package mixOmics (Rohart et al., 2017), as implemented in R[1] (details of the code are given in **S4 File**).

Gene Prediction and Annotation

Among the species considered, only those of *M. restricta* and *M. pachydermatis* had their associated proteome sequences uploaded to RefSeq as of June 2019, with 3,742 and 2,960 sequences, respectively (Park et al., 2017; Triana et al., 2017). These sequences were downloaded from the National Center for Biotechnology Information (NCBI) in June 2019, using their

[1]http://www.R-project.org/

TABLE 1 | Predicted *Malassezia* homologs of enzymes involved in lipid biosynthesis.

Gene	EC number	Function	MF	AMF	MP	MS	MG	MR
Lipid synthesis								
FAS1	EC 3.1.2.14	Fatty acid synthase (β subunit)	X	X	X	X	X	X
FAA1, FAA2, FAA3, FAA4, FAT1	EC 6.2.1.3	Long-chain fatty acyl-CoA synthetase						
GPT2 (GAT1)	EC 2.3.1.15	Glycerol-3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferase						
SCT1 (GAT2)	EC 2.3.1.42	Glycerol-3-phosphate/dihydroxyacetone phosphate sn-1 acyltransferase						
AYR1	EC 1.1.1.101	1-Acyl-DHAP reductase						
ALE1/SLC1/ SLC4	EC 2.3.1.51/ EC 2.3.1.23	1-acyl G-3-P acyltransferase (lyso-phospholipid acyltransferase)						
CDS1	EC 2.7.7.41	Phosphatidate cytidylyltransferase						
PAH1	EC 3.1.3.4	Phosphatidate phosphatase						
CHO1	EC 2.7.8.8	CDP-diacylglycerol–serine O-phosphatidyltransferase						
PSD1	EC 4.1.1.65	Phosphatidylserine decarboxylase						
PSD2	EC 4.1.1.65	Phosphatidylserine decarboxylase						
CHO2	EC 2.1.1.17	Phosphatidylethanolamine N-methyltransferase				X	X	
OPI3	EC 2.1.1.71	Phosphatidyl-N-methylethanolamine N-methyltransferase				X		
EKI1	EC 2.7.1.82	Ethanolamine kinase	X	X	X	X	X	X
CKI1	EC 2.7.1.32	Choline kinase	X	X		X	X	X
PCT1	EC 2.7.7.15	Cholinephosphate cytidylyltransferase						
CPT1	EC 2.7.8.2	Cholinephosphotransferase						
VPT29	EC 2.7.1.137	Phosphatidylinositol 3-kinase						
PIS1	EC 2.7.8.11	Phosphatidylinositol synthase				X		
LSB6	EC 2.7.1.67	1-phosphatidylinositol 4-kinase						
PIK1	EC 2.7.1.67	1-phosphatidylinositol 4-kinase						
STT4	EC 2.7.1.67	1-phosphatidylinositol 4-kinase						
MSS4	EC 2.7.1.68	1-phosphatidylinositol-4-phosphate 5-kinase						
FAB1	EC 2.7.1.150	1-phosphatidylinositol-3-phosphate 5-kinase						
PGS1	EC 2.7.8.5	Phosphatidylglycerolphosphate synthase						
GEP4	EC 3.1.3.27	Phosphatidylglycerophosphatase			X			
CRD1	EC 2.7.8.41	Cardiolipin synthase						
PLB1	EC 3.1.1.5	PC/PE specific phospholipase B						
SPO14	EC 3.1.4.4	Phospholipase D						
PLC1	EC 3.1.4.11	Phospholipase C				X		
DGK1	EC 2.7.1.107	Diacylglycerol kinase	X	X	X	X	X	X
DGA1	EC 2.3.1.15 EC 2.3.1.42	Diacylglycerol acyltransferase						
LRO1	EC 2.3.1.158	Phospholipid:DAG acyltransferase				X		
ARE1	EC 2.3.1.26	Acyl-CoA:cholesterol acyltransferase					X	
ARE2	EC 2.3.1.26	Acyl-CoA:cholesterol acyltransferase					X	
ELO1, ELO2, ELO3	EC 2.3.1.199	Elongases						
IIFA38	EC 1.1.1.330	β-keto acyl-CoA reductase/very-long-chain 3-oxoacyl-CoA reductase						
PHS1	EC 4.2.1.134	3-Hydroxy acyl-CoA dehydratase						
TSC3	EC 1.3.1.93	Enoyl-CoA reductase						
INO1	EC 5.5.1.4	Inositol 3-P synthase						

(Continued)

TABLE 1 | Continued

Gene	EC number	Function	MF	AMF	MP	MS	MG	MR
INM1	EC 3.1.3.25	Inositol-phosphate phosphatase						
OLE1	EC 1.14.19.2	Δ9-desaturase					X	X
ECI1	EC 5.3.3.8	$\Delta^{3,2}$-enoyl-CoA isomerase	X	X	X	X	X	X
SPS19	EC 1.3.1.34	2,4-dienoyl-CoA reductase						
DCI1	EC 5.3.3	Delta(3,5)-delta(2,4)-dienoyl-CoA isomerase						
FAD2/3	EC 1.3.1.35	Δ12-desaturase/ω3-desaturase						
YJU3	EC 3.1.1.23	Acylglycerol lipase						
TGL3	EC 3.1.1.3	Bifunctional triacylglycerol lipase and LPE acyltransferase						
TGL4	EC 3.1.1.3	Multifunctional lipase/hydrolase/phospholipase						
TGL5	EC 3.1.1.3	Bifunctional triacylglycerol lipase and LPA acyltransferase						
TGL1	EC 3.1.1.13	Steryl ester hydrolase						
YEH1	EC 3.1.1.13	Steryl ester hydrolase						
YEH2	EC 3.1.1.13	Steryl ester hydrolase						
DPP1	EC 3.1.3.81	Diacylglycerol pyrophosphate phosphatase 1			X	X		
APP1	EC 3.1.3.4	Phosphatidate phosphatase		X	X	X	X	X
LPP1	EC 3.1.1.-	Lipid phosphate phosphatase 1		X	X	X	X	X
Glycosphingolipid synthesis								
LCB1/2	EC 2.3.1.50	Serine palmitoyltransferase						
TSC10	EC 1.1.1.102	3-ketosphinganine reductase						
LAC1/LAG1	EC 2.3.1.24	Sphingosine N-acyltransferase						
YDC1	EC 3.5.1	Alkaline dihydroceramidase						
SUR2	EC 1	Sphinganine C4-hydroxylase						
YPC1	EC 3.5.1	Alkaline ceramidase						
SCS7	EC 1.14.18.-	Sphingolipid alpha-hydroxylase						
LCB4	EC 2.7.1.91	Sphingoid long-chain base kinase						
LCB3	EC 3.1.3	Long-chain base-1-phosphate phosphatase						
YSR3	EC 3.1.3	Dihydrosphingosine 1-phosphate phosphatase			X	X	X	X
DPL1	EC 4.1.2.27	Dihydrosphingosine phosphate lyase						
AUR1	EC 2	Phosphatidylinositol:ceramide phosphoinositol transferase						
CSH1/CSG1	EC 2.4	Mannosylinositol phosphorylceramide (MIPC) synthase catalytic subunit						
IPT1	EC 2	Inositolphosphotransferase						
Sterol synthesis enzymes								
ERG10	EC 2.3.1.9	Acetyl-CoA C-acetyltransferase						
ERG13	EC 2.3.3.10	3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase						
HMG1/2	EC 1.1.1.34	HMG-CoA reductase						
ERG12	EC 2.7.1.36	Mevalonate kinase						
ERG8	EC 2.7.4.2	Phosphomevalonate kinase						
ERG19	EC 4.1.1.33	Mevalonate pyrophosphate decarboxylase						
IDI1	EC 5.3.3.2	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase						
ERG20	EC 2.5.1.10	Farnesyl pyrophosphate synthetase						
ERG9	EC 2.5.1.21	Farnesyl-diphosphate farnesyl transferase (squalene synthase)						
ERG1	EC 1.14.13.132	Squalene epoxidase						
ERG7	EC 5.4.99.7	Lanosterol synthase						

(Continued)

TABLE 1 | Continued

Gene	EC number	Function	MF	AMF	MP	MS	MG	MR
ERG11	EC 1.14.13.70	Sterol 14 α-demethylases						
ERG24	EC 1.3.1.70	Sterol C-14 reductases						
ERG25	EC 1.14.13.72	Sterol C-4 methyl oxidases						
ERG26	EC 1.1.1.170	Sterol C-4 decarboxylases			X	X	X	X
ERG27	EC 1.1.1.270	Sterol C-3 dehydrogenase				X		
ERG6	EC 2.1.1.41	Sterol C-24 methyltransferases						
ERG2	EC 5.3.3.5	Sterol C-8 isomerases				X		
ERG3	EC 1.14.19.20	Sterol C-5 desaturases				X		
ERG4	EC 1.3.1.71	Sterol C-24 reductases						
ERG5	EC 1.14.19.41	Sterol C-22 desaturases						
DHCR7	EC1.3.1.21	7-dehydrocholesterol reductase						
Acetylation/deacetylation (Sterol acetates formation)								
ATF2	EC 2.3.1.84	Alcohol O-acetyltransferase 1	X	X	X	X	X	X
SAY1	EC 3.1.1	Steryl acetyl hydrolase 1						

Corresponding gene in: MF, Malassezia furfur CBS1878; AMF, atypical Malassezia furfur; MP, Malassezia pachydermatis CBS1879; MS, Malassezia sympodialis CBS7222; MG, Malassezia globosa CBS7986; MR, Malassezia restricta CBS7877; EC number, enzyme commission number. X indicate absence.

respective protein name with the basic query "(((scientific name) NOT partial) NOT hypothetical)," where "scientific name" took the values *Malassezia restricta* or *Malassezia pachydermatis*. For *M. sympodialis*, *M. globosa*, *M. furfur*, and atypical *M. furfur*, the previously annotated and experimentally validated proteins from Xu et al. (2007), Gioti et al. (2013) and Triana et al. (2017) were used.

For detection of lipid metabolism proteins, associated sequences for each lipid metabolism gene were downloaded from NCBI in June 2019. The search was conducted in three stages: first, only using RefSeq entries and hits from Basidiomycetes (taxID:5204). If this stage yielded less than four sequences, the possible hits were expanded to those coming from all fungi (taxID:4751). If this approach yielded <10 sequences, then all NCBI databases were considered, and duplicates were removed manually.

A custom BLAST protein database with the downloaded sequences was created using BLAST V.2.6.0+ (Camacho et al., 2009). For hidden Markov model (HMM), sequences associated with a given protein were aligned with Muscle V3.81 (Edgar, 2004) and then used to create an HMM with HMMER V.3.1 (Eddy, 1998). Lipid metabolism proteins and their respective ECs are presented in **Table 1**.

The protein sequences of the six *Malassezia* species were subjected to three analyses. First, they were blasted against the aforementioned BLAST database. Next, they were scanned for HMMs against the HMM database. Finally, both the *Malassezia* sequences and all downloaded, annotated proteins retrieved from NCBI were subjected to an orthologous group analysis using OrthoFinder V.2.3.3 (Emms and Kelly, 2015). In that context, a *Malassezia* proteome was suspected to harbor one of the lipid metabolism proteins if it fulfilled one of three conditions: (1) It had a BLAST hit against one of the sequences associated with the corresponding protein, with an average nucleotide identity score of 60% or higher and an e-value of 1e-5 or less. (2) It was grouped by OrthoFinder in an orthologous cluster associated with the same protein. Here, an orthologous cluster was considered to be associated with a given protein if at least 60% of the downloaded sequences from that protein were present and if they constituted at least 40% of the total number of sequences in the cluster. Note that the orthologous clusters were created *de novo* with OrthoFinder using the lipid metabolism and *Malassezia* sequences. (3) It had a HMM that can hit against the HMM associated with that protein, with a coverage of at least 60% of the protein and an e-value of 1e-5 or less. All *Malassezia* sequences that fulfilled these criteria were subjected to a final verification in which their domains and motifs were identified with InterProScan (Jones et al., 2014) and compared with those harbored by the proteins that were downloaded from NCBI. Note that the conditions in question could be fulfilled by separate proteins from the same genome, in which case both were further analyzed with InterPro.

InterProScan verification was the definitive criterion for asserting that a given lipid metabolism protein was present in a genome. Given the variations in the length of some proteins from *Malassezia* species as compared with the downloaded and annotated lipid metabolism genes, in some cases, the HMM criterion was relaxed to 40% coverage if no criteria were fulfilled by any sequence in a given species with the original thresholds. Any hits obtained with the new 40% coverage criterion were then verified with InterPro as described previously.

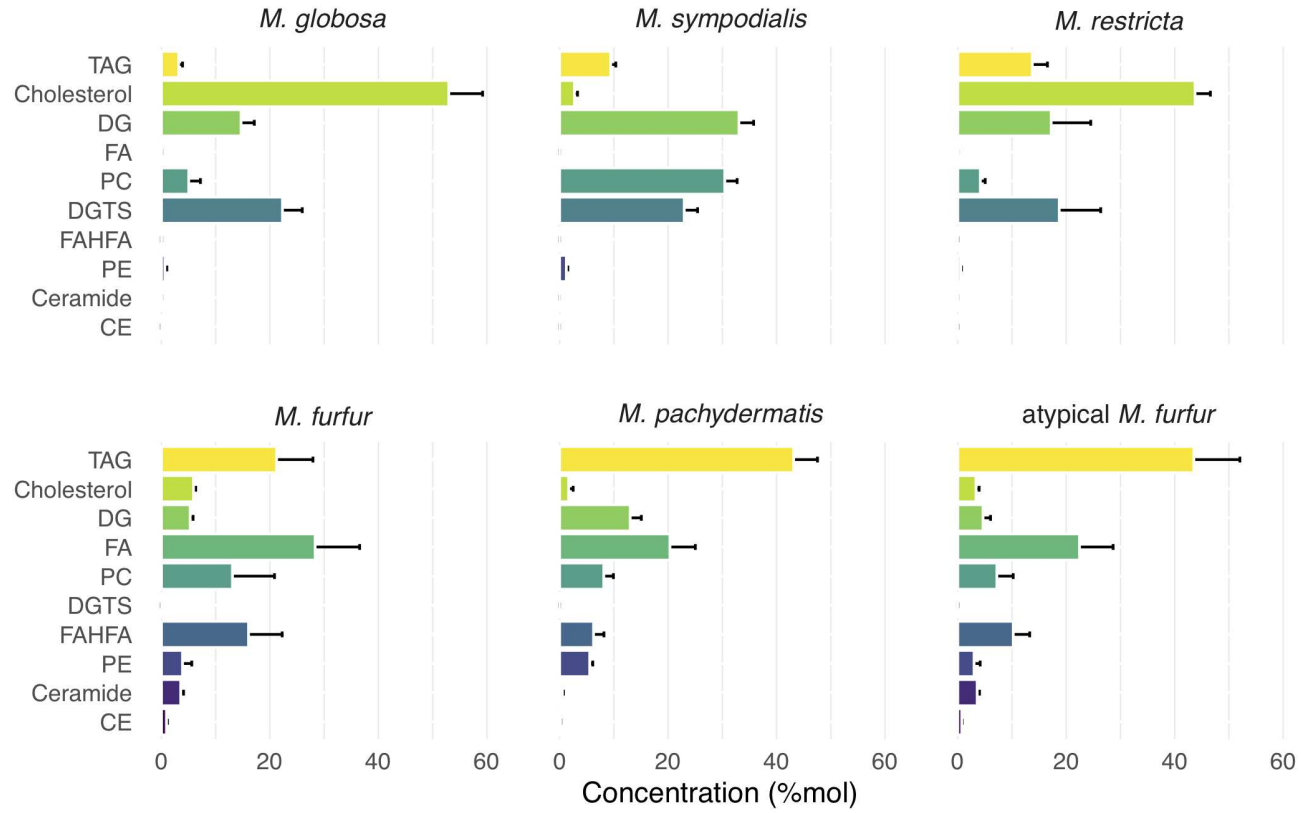

FIGURE 1 | Relative concentration of major lipid classes in five species of *Malassezia* and a putative *M. furfur* (atypical *M. furfur*) strain. Lipid classes are organized top-down according to the average concentration throughout all species. Species are organized from left to right according to the decreasing concentration of triacylglycerols (TAG) lipids. Each lipid class is expressed in %mol by their relative molar contribution to total lipids. Cholesterol should be read as: cholesterol and closely related sterols that could currently not be resolved by the technique used. Bars and lines denote mean and standard deviation (SD), respectively. TAG, triglycerides; DG, diglycerides; FA, fatty acids; PC, phosphatidylcholine; DGTS, diacylgyceryltrimethylhomoserine; FAHFA, fatty acids hydroxyl fatty acids; PE, phosphatidylethanolamine; CE, cholesteryl ester.

RESULTS

Lipid Profiles of *Malassezia* Strains

Using ultra-high-performance liquid chromatography combined with quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS) (Köfeler et al., 2012), we identified 18 lipid classes and 428 lipidic compounds. Lipid identification was performed by combining the retention time, the exact precursor mass, and the product ion spectra. The lipid classes identified are as follows: TAG, sterols (indicated in **Figure 1** as cholesterol), diglycerides (DG), FAs, phosphatidylcholine (PC), diacylglyceryltrimethylhomoserine (DGTS), fatty acids esters of hydroxyl fatty acids (FAHFAs), phosphatidylethanolamine (PE), ceramides, cholesteryl ester (CE), and ceramide or sphingomyelin (SM); the relative abundance (%mol) of these species differed between the six species analyzed (**Figure 1**). Remarkably, we observed an unusually high relative amount of the neutral lipids' sterols and TAG, and *M. furfur*, atypical *M. furfur*, and *M. pachydermatis* that contained more TAG and *M. globosa*, *M. sympodialis*, and *M. restricta* that contained more sterols. The latter three contained more sterol as compared with TAG. We detected sterols, but we could not distinguish among the different sterol species in fungi based on the currently used analysis (Weete et al., 2010). TAG was the most abundant among *M. furfur*, atypical *M. furfur*, and *M. pachydermatis*, followed by FA, FAHFAs, PE, ceramides, and CE. In contrast, *M. globosa*, *M. restricta*, and *M. sympodialis* were characterized by the presence of sterols, DG, PC, and DGTS. A low concentration of CE was found in *M. furfur*, atypical *M. furfur*, and *M. pachydermatis*, and these were undetectable in the lipidome of *M. globosa*, *M. restricta*, and *M. sympodialis*. In *M. sympodialis,* a low concentration of sterols was detected similarly in *M. furfur*, atypical *M. furfur*, and *M. pachydermatis* (**Figure 1**). Phosphatidylinositol (PI) were not identified in the current analysis.

The FAs detected had chain lengths of 11–28, including even-chain and odd-chain FAs. Odd chain FAs found would be the α-oxidation products (Řezanka and Sigler, 2009). Precursors of eicosanoid FAs, such as longer PUFAs, including arachidonic (20:4) and docosahexaenoic acid DHA (22:6), were also detected (**Supplementary Table 1**). Glycerophospholipids were characterized by the presence of PA, PC, PE, and PG (**Figure 1**; **Supplementary Table 2**). Particularly, we found a

higher concentration of PC in *M. sympodialis*, *M. furfur*, *M. pachydermatis*, and atypical *M. furfur* in comparison with that in *M. globosa* and *M. restricta*. *M. pachydermatis*, *M. furfur*, and atypical *M. furfur* had higher PE levels compared with *M. sympodialis*, *M. globosa*, and *M. restricta*. Besides PS, phosphatidylglycerol (PG) and cardiolipin were also detected but in a low concentration (data not shown). We also detected lysophosphatidylcholine, lysophosphatidylethanolamine, and lysophosphatidylinositol (**Supplementary Table 2**).

A subset of the FAs detected were those that are linked via a hydroxyl group to a second FA (FAHFA), of which we detected 77 different molecular species (**Supplementary Table 2**). Our analysis showed the presence of a wide variety of FAHFA in variable concentrations in *Malassezia* strains (**Figure 1**; **Supplementary Table 3**). In *M. furfur*, atypical *M. furfur*, and *M. pachydermatis*, FAHFA species were found in higher amounts. In contrast, for *M. globosa*, *M. restricta*, and *M. sympodialis*, approximately a 100-fold lower amount was detected when compared with the other three *Malassezia* species (**Supplementary Table 1**).

FA moieties of FAHFAs were mainly represented by palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, and arachidonic acid, and less frequently by saturated FAs like capric acid, undecylic acid, mysristic acid, lauric acid, and PUFAs such as dihomo-γ-linolenic acid and docosahexaenoic acid (DHA) (**Supplementary Table 2**). We could not determine the fragments corresponding to the hydroxylation position of hydroxy FA because this cannot be determined precisely via the MS/MS data obtained (Zhu et al., 2018). Based on previous investigations by Wilde and Steward, however, a hydroxyl group at the 9th position can be expected since 9-hydroxypalmitic acid was shown to be the major product of metabolism of *Pityrosporum ovale* and 9-hydroxystearic to a lower extent (Wilde and Stewart, 1968). Although low amounts of DGTS (34:1), (34:2), (34:3), and (36:3) were detected, these compounds were characteristically present in *M. globosa*, *M. restricta*, and *M. sympodialis* and absent in *M. furfur*, atypical *M. furfur*, and *M. pachydermatis*.

We did not perform a lipidomic analysis of mDixon broth only to address the possibility that some of the lipids in the medium associated to cells. However, the lipidome profiles showed clear differences among the species analyzed, indicating that similar amounts of lipids are not simply carried over to all strains. Further analysis is necessary to rule out specific association of certain lipid species in this media to the *Malassezia* species.

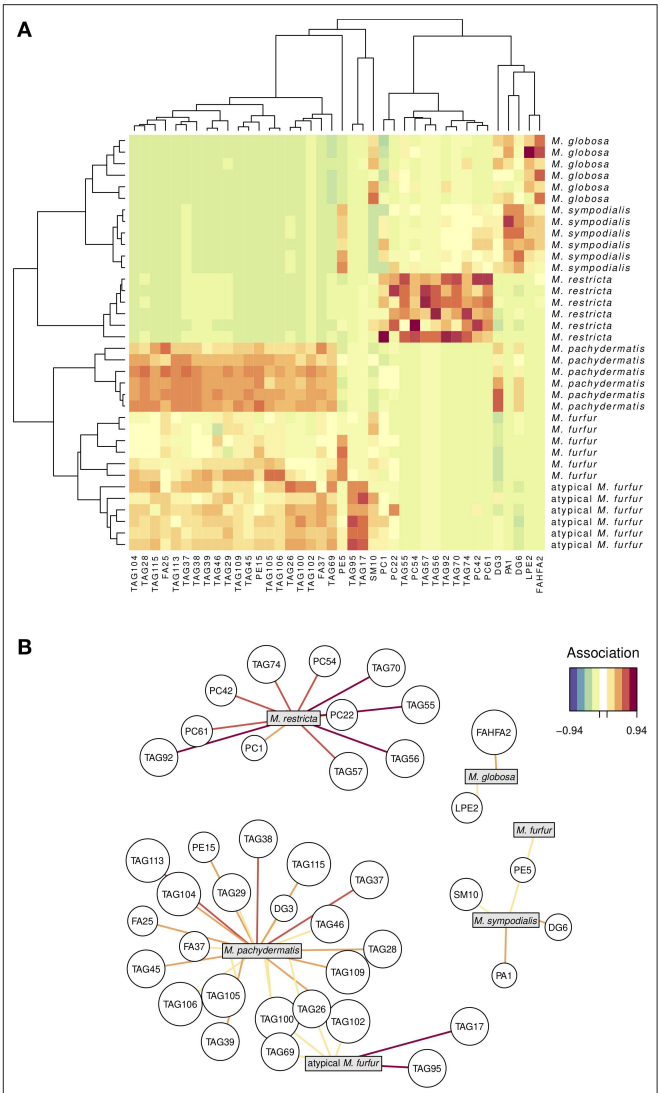

FIGURE 2 | Lipidomic signature in five species of *Malassezia* and a putative *M. furfur* (atypical *M. furfur*) strain, six biological replicates were analyzed. A sparse least partial square analysis coupled to a discriminant analysis (sPLS-DA) reduced the dimensionality of 428 to 40 compounds, which successfully discriminated among the studied lineages. Above **(A)**, both species and compound clustering according to the similarity in lipid profiles; the heat map denotes higher (red) to lower (green-blue) concentrations of each compound. Below **(B)**, network visualization of the correlation between each species and the main compounds that characterize it; both positive and negative correlations with magnitude below 0.52 are not included for clarity. The color of the line denotes the magnitude of the correlation coefficient.

Discrimination of *Malassezia* Strains

We next investigated whether we could discriminate the six *Malassezia* species examined in this study using the lipid profiles in which we identified 428 molecular species (**Supplementary Tables 1**, **2**). For example, DG1 represents the molecular species of diacylglycerol, with two FA species represented as 32:0. Using the 428 molecular species as the lipid profiles, we performed an sPLS-DA and reduced the dimensionality of 428 original molecular species to 40 relevant compounds, represented by five orthogonal PCs (**Figure 2**). These compounds successfully discriminated the six *Malassezia* strains used in this study, with an error rate of 0.017 and no misclassified cases (NMC = 0) (**Figure 2A**). Hence, the AUROC was 1.0 ($p = 0.0013$) when classifying each species against all others. The hierarchical clustering resulting from the sPLS-DA (**Figure 2A**) showed two evident species clusters based on lipid profiles. The first joined *M. globosa* with *M. sympodialis* and then both to *M. restricta*. The second included *M. pachydermatis* and

the two strains of *M. furfur* (**Figure 2A**). The clustering approach distinguished particularly well among species based on lipids, like TAG, cholesterol, FA, PC, DGTS, FAHFAs, PE, ceramides, and CE (**Figure 2A**).

We used the molecular species data set (**Supplementary Table 1**) and the network graphic representation of the sPLS-DA (**Figure 2B**) to perform a more detailed exploration of which molecular species appear associated with certain *Malassezia* species. Four out of the six *Malassezia* species revealed positive associations with a unique compound. The other two were both associated with four TAG (69, 100, 26, and 102) compounds, but the atypical *M. furfur* was strongly and uniquely associated with TAG17 and TAG95, whereas *M. pachydermatis* was linked with a large number of TAG, FA, PE, and DG compounds (**Figure 2B**; **Supplementary Table 2**).

Regarding the FAHFA lipid compounds, the sPLS-DA reduced the dimensionality of the original 77 to only 74 relevant compounds, represented in turn by eight PCs. Although they successfully discriminated three of the six *Malassezia* species (*M. globosa*, *M. restricta*, and *M. sympodialis*), there were seven misclassifications (NMC = 7) in the other *Malassezia* species (**Figure 3A**) and an error rate of 0.069. As expected, the AUROC values were 1.0 ($p = 0.0013$) when classifying the three former species but between 0.98 and 0.99 for the other ones. Interestingly, *M. globosa* revealed an idiosyncratic lipid profile of about 35 FAHFA compounds that were not associated with all other strains (**Figure 3B**). These FAHFAs belonged to previously undescribed families (**Supplementary Table 3**).

In silico Genomic Analysis Suggested the Presence of Genes Associated With the Lipid-Biosynthesis Pathway

We found the key genes involved in lipid synthesis (**Table 1**; **Figure 4**). The bioinformatic analysis suggested the presence of enzymes involved in sterol biosynthesis. Although sterols were detected, we could not differentiate among cholesterol, ergosterol, or other fungal sterols (**Figure 1**). *DHCR7* (7-dehydrocholesterol reductase), related to cholesterol synthesis, was present. *ERG8* (phosphomevalonate kinase), *ERG27* (sterol C-3 dehydrogenase), *ERG2* (sterol C-8 isomerases), and *ERG3* (sterol C-5 desaturases) are apparently missing in *M. sympodialis*. *ERG26* (sterol C-4 decarboxylases) homolog was present only in *M. furfur* and atypical *M. furfur* (Weete et al., 2010; Kristan and RiŽner, 2012).

TAG and CE synthesis genes were detected in the genome of all the strains (**Table 1**; **Figure 4**). *DGA1* and *LRO1* are associated with the acylation of DG to TAG and were present in almost all *Malassezia* strains. In *M. sympodialis*, *LRO1* is absent. Homologs of the yeast *ARE1* and *ARE2* genes that encode acyl-CoA: cholesterol acyltransferase (EC 2.3.1.26) were present in all the strains, but interestingly, these were absent in *M. globosa*. These transferases are predicted to catalyze the acylation of ergosterol, and the absence of CE in the lipidome of *M. globosa* (**Figure 1**) can be explained by the absence of these two genes. The phosphatidate phosphatase gene *PAH1* and the acylation gene *DGA1*, which contribute to the synthesis of TAG, were also present in all strains (**Table 1**; **Figure 4**). In contrast, the additional phosphatase genes *DPP1* (not present in *M. pachydermatis* or *M. sympodialis*), *APP1*, and *LPP1* were present in *M. furfur* (Pascual and Carman, 2013). Dgk1 (*DGK1*), a kinase related to the regulation of levels of PA, seems to be missing in all strains (Klug and Daum, 2014). The absence of this gene and the impact on *Malassezia*'s lipid metabolism require further investigation. In addition, a homolog of the alcohol O-acetyltransferase 1 gene *ATF2* was absent. This gene encodes an enzyme that, together with *SAY1* (present in all *Malassezia* strains), is part of a sterol acetylation/deacetylation cycle.

In general, homologs for enzymes involved in the glycerophospholipid synthesis were found, and an overview of the differences between the strains are summarized in **Table 1** and schematically represented in **Figure 4**. A subset of genes involved in lipid metabolism was found to be absent in specific *Malassezia* species. In *M. sympodialis*, neither *PIS1* (phosphatidylinositol synthase), involved in the formation of PI (Klug and Daum, 2014), nor *CHO2* (phosphatidylethanolamine N-methyltransferase) or *OPI3* (CDP-diacylglycerol-serine O-phosphatidyltransferase), which catalyze the reactions to the formation of PC, were found. This latter gene was also missing in *M. globosa*. *PLC1* encoding phospholipase C was missing in *M. sympodialis*. Homologs for enzymes related to the synthesis of PE via the Kennedy pathway, such as *EKI1* (ethanolamine kinase), were apparently absent in all strains. The choline kinase gene *CKI1*, required for PC synthesis, was present only in *M. pachydermatis* (Klug and Daum, 2014). An important enzyme encoded by (*GEP4*) involved in the dephosphorylation of PGP to PG was apparently not present in *M. pachydermatis* (**Figure 4**) (Henry et al., 2012).

The glycosphingolipid synthesis genes were present, except *YSR3* (dihydrosphingosine 1-phosphate phosphatase), which was present only in *M. furfur* and atypical *M. furfur*; however, a paralog *LCB3* was present in all strains (**Table 1**; **Figure 4**).

Lastly, homologous genes for enzymes required to degrade unsaturated FAs, such as *ECI1*, were absent in all *Malassezia* strains (**Figure 4**), as was reported previously (Gordon James et al., 2013). In contrast, *SPS19* (2,4-dienoyl-CoA reductase) and *DCI1* (delta (3,5)-delta (2,4)-dienoyl-CoA isomerase) were detected in our analysis. *FAD2/3* Δ12-desaturase/ω3-desaturase, involved in the synthesis of PUFAs such as linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3), was present (**Table 1**; **Figure 4**) (Leonard et al., 2004).

DISCUSSION

Malassezia yeast lacks *de novo* FA synthesis, and it is therefore lipophilic. Limited information is present about the lipid metabolism in these species as well as the lipidic components produced. Here, we present the results of lipidomic and *in silico* genomic analysis of six strains of *Malassezia* (*M. globosa*, *M. sympodialis*, *M. restricta*, *M. furfur*, *M. pachydermatis*, and atypical *M. furfur*). The data reveal novel insights and are of importance to understand lipid metabolism in this yeast.

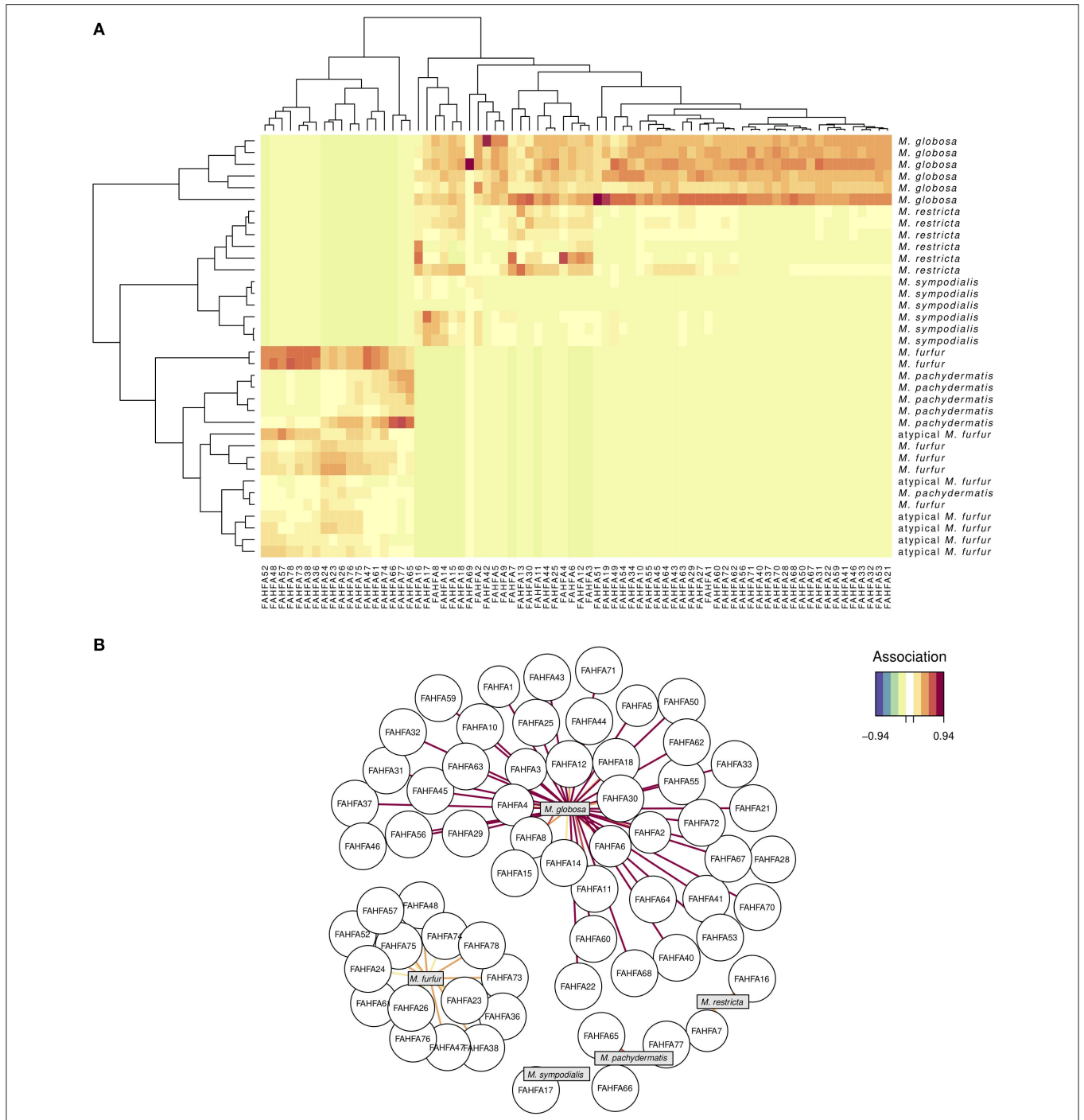

FIGURE 3 | FAHFA lipid signature in five species of *Malassezia* and a putative *M. furfur* (atypical *M. furfur*) strain, six biological replicates were analyzed. A sPLS-DA (see **Figure 2**) barely reduced the dimensionality of 77–74 FAHFA lipids, which successfully discriminated among *M. globosa, M. restricta*, and *M. sympodialis* but not among the other studied lineages. See **Figure 2** for explanation of the corresponding visualizations: the clustering heat map (above, **A**), and the correlation network (below, **B**).

Lipid content based on the currently described analysis allowed us to discriminate the *Malassezia* strains because the phylogenetic relations were maintained among the species during the examination of their lipid composition. In this study, we determined that the most common lipids of these strains are TAG, sterols, diglycerides, FAs, phosphatidylcholine, phosphatidylethanolamine, ceramides, sphingomyelin, acylcarnitine, and lysophospholipids. The lipid composition was

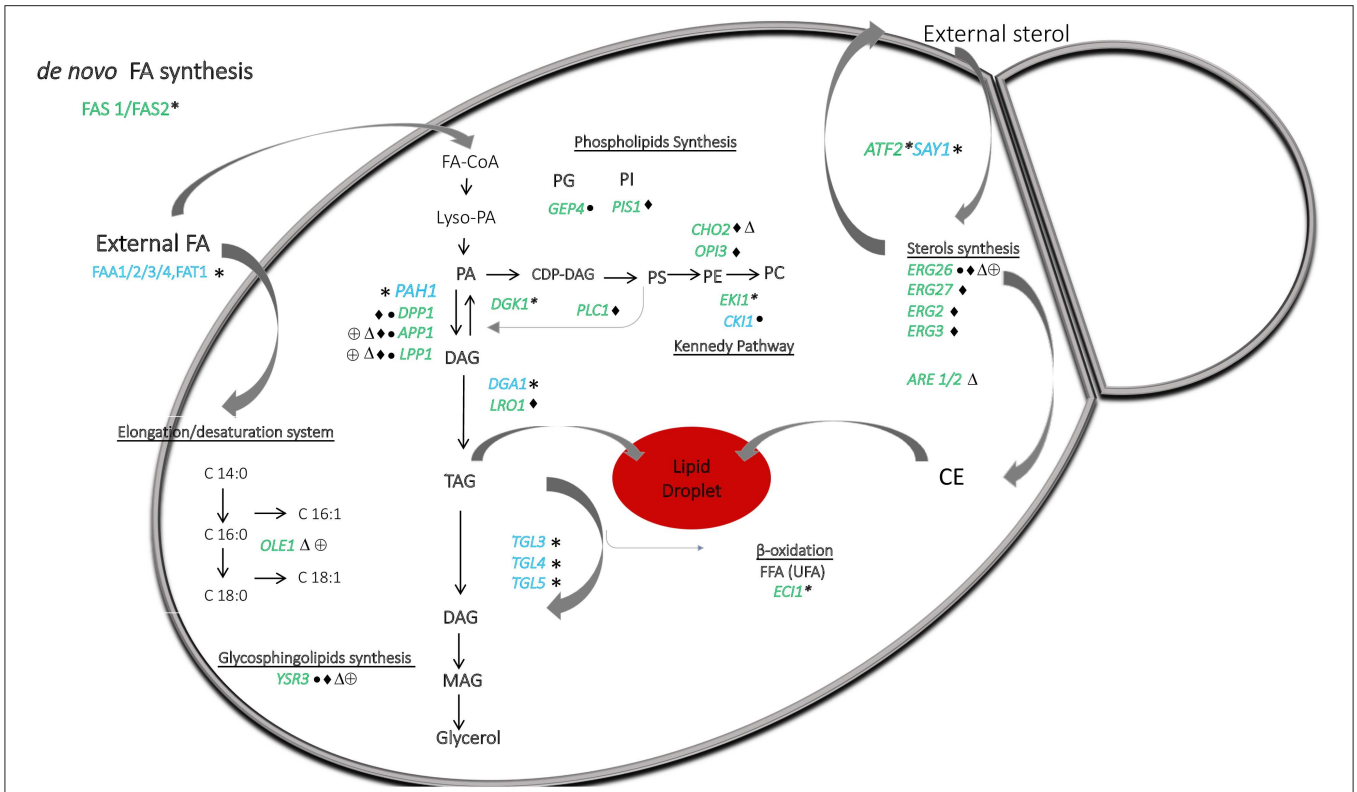

FIGURE 4 | A general overview of the lipid metabolism in *Malassezia,* based on lipidomic and *in silico* genomic analysis presented in **Figure 1** and **Table 1**. *Malassezia* species are lipid dependent due to the lack of fatty acid synthase (*FAS1/FAS2*). External sources of free fatty acids are taken up and activated by the acyl-CoA synthases *FAA1/2/3/4*. Δ9-desaturase (*OLE1*) catalyzing the conversion of saturated to unsaturated fatty acids was absent in *M. globosa* and *M. restricta.* The presence of phosphatidate phosphatase *PAH1* can replace the lack of *DPP1, LPP1*, and *APP1* to allow production of DAG from PA and to form TAG in lipid droplets. PA can normally be resynthesized from DAG by *DGK1*, but this gene was absent in all strains. Genes implicating in phospholipids synthesis involved CDP-DAG as a precursor were present in all strains. Importantly, in *M. sympodialis*, *PIS1* involved in PI synthesis was absent as well as *CHO2* and *OPI3*, which both are participating in the phosphatidylethanolamine N-methyltransferase pathway to form PC. *GEP4* involved in PG synthesis was absent in *M. pachydermatis*, whereas this strain did contain *CKI1* involved in PC synthesis but was absent in the other strains. *ECI1* required to degrade unsaturated FAs was absent in all strains. Genes involved in sterol and sphingolipids synthesis were present in all strains, except *ERG26* and YSR3, respectively, in *M. pachydermatis, M. sympodialis, M. globosa,* and *M. restricta*. Genes *ERG27*, *ERG2*, and *ERG3* were only absent in *M. sympodialis*. *ARE1/2* predicted to catalyze the acylation of ergosterol was absent in *M. globosa*. Lastly, sterol acetylation catalyzed by *ATF2* was absent in all strains. Blue letters represent the presence and in green the absence of genes denoted by *All *Malassezia* strains, ● *M. pachydermatis,* ♦ *M. sympodialis,* △ *M. globosa,* and ⊕ *M. restricta.* CE represents cholesteryl ester the species.

similar to that of *S. cerevisiae* (Kohlwein, 2017). Furthermore, we cannot exclude the possibility that other lipid classes are present but were left undetected due to the currently used methods of extraction on stationary grown cells (e.g., PI would be subject to future research). Lipidomic analysis of mDixon medium itself is also necessary to clarify whether specific association of lipids from the medium does occur.

Importantly, we could not differentiate among sterols, and further sterol profiling is required to determine which species are actually produced. Sterols could also be taken up as indicated in **Figure 4** and used in the production of CE stored in lipid droplets. We did, however, detect the apparent presence of all genes encoding sterol synthesis enzymes. It would be important to differentiate among sterol species due to the fact that sterols others than ergosterol, regarded as the "fungal sterol," have been described. Ergosterol was considered the unique fungal sterol. However, other sterol species, such as lanosterol, brassicasterol, 24-ethyl cholesterol, 24-methyl cholesterol, and cholesterol, are characteristic for some fungal groups included in the phyla Chytridiomycota and Mucoromycota. These sterols may also be present in the Dikarya (Weete et al., 2010). Particularly, the homolog of *DHCR7* (7-dehydrocholesterol reductase) that is related to cholesterol synthesis was found in the *Malassezia* genomes. *Pneumocystis jirovecii*, an important human pathogen, is characterized by the presence of cholesterol, and previous studies have suggested that it contributes to the flexibility of the membrane in the trophic form of these fungi (Ma et al., 2016). Among the phylum Basidiomycota, rust fungi contain intermediates in the formation of 24-ethyl cholesterol (Weete et al., 2010). Further analyses are required to corroborate the presence of the sterol species in *Malassezia* species.

TAG and CE are neutral lipids produced from FA and sterols, respectively, to avoid possible toxicity due to an excess of these compounds in the cell (Klug and Daum, 2014). A relatively

high amount of neutral lipids was detected in some of the *Malassezia* species. In biological membranes, the phospholipid-to-cholesterol ratio cannot exceed 2:1; we believe that the ratio of phospholipids is higher and that the relatively high amount of neutral lipids might be due to the currently used methods of analysis. In the future, different methods of extraction are required to clarify this issue. It should, however, be mentioned that *Malassezia* species were reported to have a lipid-rich cell wall (Hechemy and Vanderwyk, 1968; Thompson and Colvin, 1970) and even a lipid-like capsular layer (Mittag, 1995) that modulates immune responses (Thomas et al., 2008), and the possibility that some of the neutral lipids are derived from this source requires further investigation. TAG were present in different concentrations among the strains with a wide range of molecular species, and some of them showed a positive association with *M. pachydermatis* that enables the differentiation of this strain from the others. We identified genes related to TAG synthesis. Particularly, *LRO1*, which is associated with the acylation of DG to TAG, seemed to be absent in *M. sympodialis* (Klug and Daum, 2014). However, *DGA1*, which has the same function, was present in this strain; thus, the possible absence of *LRO1* might not affect the TAG synthesis (Klug and Daum, 2014).

We found a low content of CEs in *M. furfur*, atypical *M. furfur*, and *M. pachydermatis* and undetectable traces of these components in *M. globosa*, *M. restricta*, and *M. sympodialis*. On the other hand, *ARE1* and *ARE2* genes are transferases predicted to catalyze the acylation of ergosterol to CE and were present in all strains except *M. globosa*. The absence of these genes in *M. globosa* may explain why CE was undetected in this strain, but it cannot explain the absence in *M. sympodialis* and *M. restricta* unless these genes are not expressed. Expression analysis is required to corroborate differences in the functionality of *ARE1/ARE2* in *Malassezia* strains. These differences could also be due to possible inhibition of CE synthesis by oleic acid due to competitive inhibition of *Are2p* by free oleate, as has been described for *S. cerevisiae*; however, all strains were grown in the same medium (Connerth et al., 2010; Grillitsch et al., 2011).

The mechanism used by the cell to avoid toxic effects due to excess of sterols involves HMG-CoA reductase (HMGR), a conserved enzyme in eukaryotes, and the acetylation/deacetylation cycle performed by *ATF2/SAY1* (Burg and Espenshade, 2011; Klug and Daum, 2014). Here, we did not detect homologs to *ATF2*, but a homolog of *SAY1* was shown to be present in all strains. The absence of *ATF2* would imply that this detoxifying mechanism is not operative in *Malassezia* or that these strains contain a different enzyme. The presence of organelles such as lipid droplets may contribute to the detoxification mechanisms (Celis Ramírez, 2017). Why high levels of sterols are tolerated in some of the species remains to be determined.

Phospholipids are structural components of membranes and play many essential roles in cell biology, like membrane trafficking, membrane identity, and anchoring of membrane proteins, and also serve as signaling molecules and as precursors of signaling molecules. We found differences in the content of PC and PE, which were detected in higher concentrations than PS, phosphatidylglycerol (PG), and cardiolipin. CDP-DAG and Kennedy pathways can synthesize PE and PC (Klug and Daum, 2014). Genes associated with CDP-DAG were present in all strains, but important genes in the Kennedy pathways were not (*EKI1* was absent in all strains, and *CKI1* was present only in *M. pachydermatis).* This probably means that *Malassezia* strains can synthesize PE and PC only using CDP-DAG and that *M. pachydermatis* can synthesize PC also via the Kennedy pathway. *PLC1* encodes phospholipase C, forming DG, inositol, and G3P (glycerol-3-phosphate), which can serve again as precursors for phospholipid synthesis (Henry et al., 2012). This is missing in *M. sympodialis* and may be related to differences observed in the metabolism of this species. However, further analyses are required to confirm this. GEP4p is involved in the dephosphorylation of PGP to PG and is apparently not present in *M. pachydermatis* (Henry et al., 2012), and it may be the reason why this lipid species was in fact not detected in our analysis.

Homologs to several genes (among others, *ERG26*, *ERG27*, *ERG2*, *ERG3*, *PIS1*, *CHO2*, *OPI3*, *PLC1*, *CKI1*, *EKI1*, and *GEP4*; see **Table 1** for a complete overview) were not identified in some strains, and *M. sympodialis* and *M. globose*, in particular, were missing most homologs. However, we cannot exclude the presence of genes with similar functions that might have escaped detection due to very limited similarity.

PUFAs are important structural components that confer membrane fluidity and selective permeability (Leonard et al., 2004). Arachidonic acid (ARA; 20:4) and docosahexaenoic acid (DHA; 22:6) were detected in our analysis. These FAs are not produced by *S. cerevisiae*, which mainly produces saturated and monounsaturated FAs of 16- and 18-carbon compounds because it contains only one FA desaturase, a Δ9-desaturase (OLE1) (Uemura, 2012). However, bifunctional D12/D15-FADs can desaturate D9-UFAs to PUFAs, and these have been detected in different species belonging to Basidiomycota and Ascomycota (Buček et al., 2014). A previous study predicted the metabolism of ARA in atypical *M. furfur*, suggesting its role as a precursor of eicosanoids (Triana et al., 2017). We were able to detect the enzymes involved in the biosynthesis of these FAs.

The recently discovered class of FAHFA lipids (Yore et al., 2014) was detected in *Malassezia* species with, additionally, a high variety in the composition of their acyl chains. It is noteworthy that some of these lipids have not been reported previously (Liberati-Cizmek et al., 2019). These lipids might provide anti-inflammatory properties, but further studies should provide insights into their role in fungal biology and host–pathogen interactions (Zhu et al., 2018).

Betaine lipid diacylglycerol-trimethyl-homoserine (DGTS) is an analog of phosphatidylcholine (PtdCho), which is synthesized by many soil bacteria, green plants, chromophytes, fungi, and amoebae (Sohlenkamp and Geiger, 2016). In the human fungal pathogens, *Candida albicans* and *Cryptococcus neoformans* phosphate starvation induces the replacement of phosphatidylcholine with betaine lipid, and this event was related to fungal virulence during host interaction (Naik et al., 2017; Lev et al., 2019). DGTS was also detected in *M. globosa*, *M. sympodialis*, and *M. restricta*. Some evidence suggests reciprocity between PC and DGTS content, meaning that, in most cases, in which PC is a major lipid, no DGTS is detected and vice versa.

However, this was not the case in this study. In some algae, PC and DGTS accumulate at the same time (Sohlenkamp and Geiger, 2016). The role of DGTS in *Malassezia* species requires further analysis.

The lipidomic analysis of the *Malassezia* species described in this paper is in the context of growth to the stationary phase in mDixon broth. This is a rich medium that contains the lipid-containing components Ox bile, peptone, malt extract, oleic acid (>78% pure), and Tween 40 (>90% pure). Ox bile is a complex lipid mixture containing bile salts and bilirubin cholesterol, FAs, and lecithins, which are a mixture of different phospholipids, glycolipids, or TAG (Hall and Guyton, 2011). Peptone is a protein hydrolysate that contains small amounts (∼0.6%) of lipids (Klompong et al., 2009). Malt extract is prepared by extracting the soluble products from sprouted grain and might contain lipids, but to our knowledge, a detailed analysis has not been presented.

There is a possibility that some of the lipidic components present in the mDixon associate with yeast cells and were detected in our analysis. The relative amount of the different lipid species we detected in the six different *Malassezia* species (**Figure 1**) do, however, vary considerably between these species. These rules out the possibility that similar amounts of the medium lipids were simply carried over with these six species because they all were cultured in the same medium. Whether differential association of specific lipid species to cells occurs remains to be determined, but it would be very remarkable and might suggest that different *Malassezia* species have a preference for accumulating certain lipid sources from the medium. A lipidomic analysis of the medium is required to confirm that preferential association does occur. To eliminate the problem of carryover of lipids, synthetic media with pure lipids as a source should be used, but such a medium has not yet been developed. Furthermore, more work is required to link the current lipid analysis to lipidomic analysis of *Malassezia* species grown with culturing methods that resemble the *in vivo* conditions, which remains a major challenge. In addition, the role of gene products and their importance in the biosynthetic pathways require further studies, such as knockouts, mutant genes as well as studies with stable isotope labeled FAs coupled with lipidomic analysis to unravel these routes.

Taken together, our data provide a general overview of the lipid composition and metabolism in six *Malassezia* strains. This study contributes to the knowledge in this genus and provides fundamental information with which future studies can advance comprehensive knowledge of the role of lipids in the life cycle of *Malassezia* yeast as commensal and pathogen.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AC, AA, AB, and SR contributed to the design of the work. AC performed the experiments. AC, AA, JC, LM-C, JA-M, and HC wrote the manuscript. AC, HC, SR, and AB made revisions. All authors were involved in the analysis and interpretation of data. All authors approved the version to be published and agreed to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00338/full#supplementary-material

Supplementary Table 1 | Raw data with concentration (%mol) of lipid species detected in *Malassezia* strains.

Supplementary Table 2 | Class of lipid species detected by UHPLC/MS.

Supplementary Table 3 | FAHFA species detected by UHPLC/MS.
*Abbreviations were adapted from: Kolar et al. (2019), Liberati-Cizmek et al. (2019), Balas et al. (2018), Zhu et al. (2018), Hu et al. (2018), and Kuda et al. (2016).

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BRIEF RESEARCH REPORT
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Expression of a *Malassezia* Codon Optimized mCherry Fluorescent Protein in a Bicistronic Vector

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The use of fluorescent proteins allows a multitude of approaches from live imaging and fixed cells to labeling of whole organisms, making it a foundation of diverse experiments. Tagging a protein of interest or specific cell type allows visualization and studies of cell localization, cellular dynamics, physiology, and structural characteristics. In specific instances fluorescent fusion proteins may not be properly functional as a result of structural changes that hinder protein function, or when overexpressed may be cytotoxic and disrupt normal biological processes. In our study, we describe application of a bicistronic vector incorporating a Picornavirus 2A peptide sequence between a *NAT* antibiotic selection marker and mCherry. This allows expression of multiple genes from a single open reading frame and production of discrete protein products through a cleavage event within the 2A peptide. We demonstrate integration of this bicistronic vector into a model *Malassezia* species, the haploid strain *M. furfur* CBS 14141, with both active selection, high fluorescence, and proven proteolytic cleavage. Potential applications of this technology can include protein functional studies, *Malassezia* cellular localization, and co-expression of genes required for targeted mutagenesis.

Keywords: *Malassezia*, fluorescent, multicistronic, bicistronic, transformation, tagging, 2A

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INTRODUCTION

Malassezia, comprising 18 currently recognized species, are a unique group of lipophilic basidiomycetes that evolved independently from related plant pathogen lineages (Xu et al., 2007), and represent a ubiquitous and dominant eukaryotic microbial community on human skin (Grice and Segre, 2011; Findley et al., 2013; Grice and Dawson, 2017). In adaption to life on mammalian skin, *Malassezia* genomes have been reshaped to secrete an armory of proteases, lipases, and phospholipases, amongst other enzymes to support their growth. These skin-dwelling yeasts usually maintain a symbiotic relationship with their human host but can quickly shift into opportunistic pathogens, usually induced by environmental alterations such as breaches in skin barrier integrity, dysfunctional immune response, or age related changes that affect skin, such as aging, puberty, or menopause (Grice and Segre, 2011). *Malassezia* are the causative agents of dandruff and seborrheic dermatitis, and are associated with myriad clinical conditions such as atopic dermatitis, pityriasis versicolor, and folliculitis (Gaitanis et al., 2012; Theelen et al., 2018). Beyond superficial cutaneous disorders, *Malassezia* are also responsible for catheter-associated

infections and invasive septic fungemia (Barber et al., 1993; Gaitanis et al., 2012; Kaneko et al., 2012; Iatta et al., 2014). Recent reports illustrate pathogenic roles for *Malassezia* in Crohn's Disease and pancreatic ductal adenocarcinoma through an elevated inflammatory response linked to CARD-9 and the complement cascade (Aykut et al., 2019; Limon et al., 2019). Relatively little is known about the specific mechanisms of *Malassezia* pathogenesis, despite decades of investigation and their broad significance, meaning much remains to be explored about this important group of yeasts. Research efforts on *Malassezia* are gaining traction as more species are being identified in human gut microflora, animal skin, and even in Antarctic and marine environments, making them amongst the most ubiquitous of fungi (Amend, 2014; Theelen et al., 2018). These new findings have rapidly increased interest, and are driving advances in diverse species identification, definition of axenic culture conditions, and development and application of tools to dissect *Malassezia* genomic complexity (Dawson, 2019).

Malassezia were widely accepted as highly recalcitrant to conventional transformation techniques including biolistic, electroporation, and lithium acetate methods. Recent developments in genetic modifications with the use of *Agrobacterium* have not only established gene transfer, but also provided tremendous headway in studies of previously uncharacterized *Malassezia* gene function (Ianiri et al., 2016, 2019; Sankaranarayanan et al., 2020). Earlier studies have also applied the use of fluorescence protein tagging (Celis et al., 2017; Sankaranarayanan et al., 2020). Additionally, the simultaneous co-expression of multiple genes and fluorescent proteins has applications ranging from monitoring gene expression (Rasala et al., 2012; Lewis et al., 2015), protein tagging, to live cell or whole organism labeling and imaging (Provost et al., 2007; Kim et al., 2011; Ahier and Jarriault, 2014), enabling this technique as a cornerstone in biomedical research.

One of the most common approaches in collective gene expression exploits the incorporation of the 2A oligopeptide, first identified in viral genome of foot-and-mouth disease virus (F2A). Subsequently, other 2A sequences were discovered in porcine teschovirus-1 (P2A), equine rhinitis A (E2A), and *Thosea asigna* virus (F2A). 2A peptides are usually between 18 and 22 residues and reports have suggested 2A sequences encode a single open reading frame (ORF), and impedes the formation of a peptide bond between glycine and proline residues, allowing the generation of discrete protein products (Trichas et al., 2008; Lewis et al., 2015). The use of 2A sequence overcomes the need of bidirectional or multiple promoters and skips use of numerous co-transfection plasmids, offering greater efficiency and simplicity in construct designs and transformation.

In this report, we demonstrated use of a bicistronic expression system to simultaneously produce active fluorescent mCherry and dominant nourseothricin resistance (*NAT*) non-fusion proteins, delivered via *Agrobacterium tumefaciens*-mediated transformation (ATMT) in *Malassezia furfur*. This is achieved through incorporating viral P2A sequence between gene ORFs, with mCherry upstream of *NAT*, designed to ensure expression of mCherry if *NAT* protein is expressed. *Agrobacterium* Transfer DNA (T-DNA) expression vector was designed and constructed with unique restriction enzyme sites to allow straightforward modification to any of the genetic elements to support broad experimental use and complement alternative experimental needs.

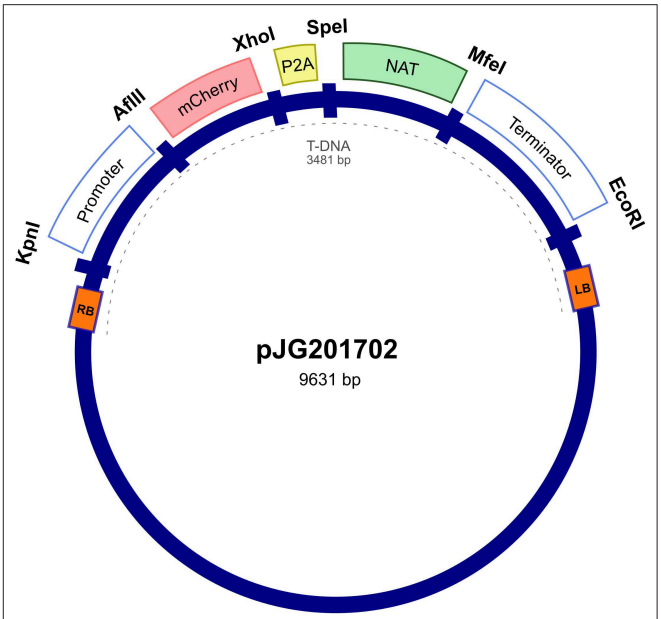

FIGURE 1 | Random integration bicistronic vector. Generation of bicistronic construct comprised use of promoter and terminator sequences from *Malassezia sympodialis* ATCC 42132 in *Agrobacterium tumefaciens* backbone vector. Actin encoding regulatory elements govern the sequential expression of mCherry and nourseothricin sulfate (*NAT*) antibiotic resistance marker, separated by porcine teschovirus-1 2A (P2A) pseudo-autolytic cleavage sequence. pJG201702 vector was created with multiple restriction cut sites between each genetic element for future modification.

RESULTS

Random Insertional Mutagenesis in *M. furfur*

The expression vector was first constructed in *E. coli* pUC57 cloning vector by insertion between the actin *ACT1* promoter and terminator of *Malassezia sympodialis* ATCC 42132, a codon optimized mCherry gene and a P2A viral sequence (**Supplementary Table 1** and **Supplementary Figure 1**) followed by the *NAT* selection marker gene, forming a single ORF. The expression cassette was digested from purified pUC57 and cloned into an *Agrobacterium* tumor-inducing (Ti) backbone binary vector, generating the resulting plasmid, pJG201702 (**Figure 1**). The bicistronic expression plasmid pJG201702 was verified by PCR and Sanger sequencing (data not shown) prior to electroporation into competent *Agrobacterium tumefaciens*.

Transformations were carried out as previously described (Ianiri et al., 2016; Celis et al., 2017), and randomly selected *NAT*-resistant colonies were analyzed by PCR to detect the presence of *NAT* and mCherry ORFs. Predicted amplicons of 576 and 708 bp for *NAT* and mCherry respectively, were observed in 4 out of 23 transformants but absent in wild type

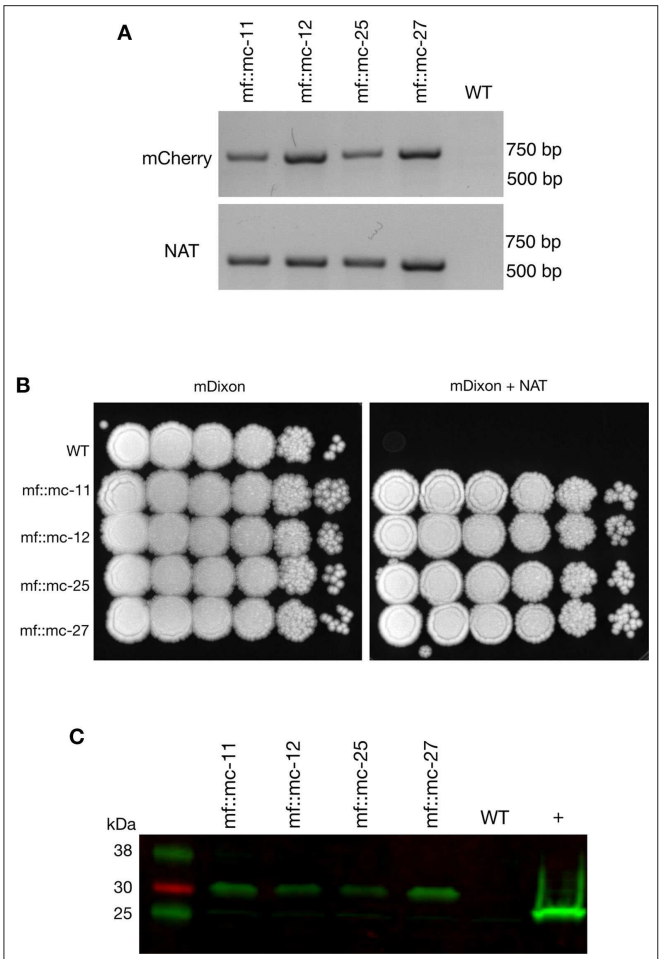

FIGURE 2 | Characterization and analysis of transformants. **(A)** PCR screening of transformants and wild-type cells were analyzed with mCherry and *NAT* ORF spanning primers, indicating the appropriate sized product in transformants and not wild-type cells. **(B)** Transformants and *M. furfur* CBS 14141 wild-type clones were ten-fold serially diluted in PBS and 3 uL of cell suspensions were spotted on mDixon growth medium and selection media-supplemented with 100 ug/mL *NAT*, indicating *NAT* resistance in selected clones. **(C)** Immunoblot was performed with whole cell lysates, probed with anti-RFP primary antibodies. Transformants expressing mCherry contained additional amino acid residues from viral P2A protein tag, produced expected protein bands with an upward shift of molecular weight at 29 kDa. In comparison, 26.7 kDa native molecular weight band was detected in positive control, TurboRFP expressing keratinocyte cell lysate.

(**Figure 2A**). The recovery of 17 false positive *NAT*-resistant colonies are likely to be attributed to spontaneous mutations and this chemically induced resistance to *NAT* was also observed in previous work (Ianiri et al., 2016). The 4 mCherry- and *NAT*-positive transformants and wild type were serially diluted in PBS and spotted on mDixon and selective medium-supplemented with *NAT*. Wild type CBS14141 was not able to proliferate in the presence of *NAT* while engineered *M. furfur* strains displayed growth similar to wild type on mDixon (**Figure 2B**). These results suggest in the selected transformants, the *NAT* resistance gene was incorporated into the genome, expressed, and translated into a functional protein without affecting fitness.

P2A Cleavage Efficiency

To determine whether P2A peptide allows proper and efficient cleavage and release of the mCherry and *NAT* proteins, total protein lysates were prepared and analyzed from wild type and transformants. Immunoblot analysis using a red fluorescent protein antibody (RFP, mCherry) revealed the expected single band of 29 kDa with no observable band detected in wild type lysate (**Figure 2C**).

In vivo Fluorescence Assessment

M. furfur CBS 14141 wild type and three selected genetically engineered strains, mf::mc-12, −25, and −27 were fluorescently imaged. Live cell imaging revealed transformants exhibit a higher fluorescence signal compared to wild type (**Figure 3A**). Wild type displayed classical autofluorescence observed in *Malassezia* species that localizes mainly in the yeast cell wall, while all selected transformants displayed a strong mCherry cytoplasmic signal, with mf::mc-27 demonstrating the strongest fluorescence. In an effort to reduce the autofluorescence background, mf::mc-27 and wildtype were cultured in liquid media and cells were collected at mid-log phase to avoid the accumulation of fluorescent metabolites, dead or stationary cells. Further, cells were washed in PBS to remove residual culture media before imaging but detectable autofluorescence was still observed in wildtype. Regardless, mf::mc-27 transformant demonstrated higher fluorescence, an indicator that the mCherry protein was expressed and functional (**Figure 3B**).

Location of the Inserted Genetic Cassette

Transformant mf::mc-27 was subjected to Illumina sequencing (Novogene) to determine the site of the T-DNA insertion. Sequenced DNA fragments were mapped to *M. furfur* CBS 14141 reference genome assembly (Sankaranarayanan et al., 2020) and RNAseq data (TLD lab, unpublished data). Bioinformatic analysis located the insertion of the exogenous DNA cassette in an intergenic region on chromosome 2 (**Figure 4**). Sanger sequencing of T-DNA through to upstream chromosomal DNA confirmed the presence of a complete ORF of a putative *CDC25*-related phosphatase gene. PCR amplifications of the downstream chromosomal DNA from the insertional point were unsuccessful. However, Illumina sequencing reads were able to detect both intact transformation DNA cassette and predicted ORF of the adjoining adiponectin receptor.

DISCUSSION

Malassezia are indispensable members of a healthy human skin microbiome, found on almost all warm-blooded animals, and can even be traced in a marine ecosystem (Amend, 2014; Theelen et al., 2018). This diverse group of lipophilic yeasts is often implicated in various cutaneous diseases and recent studies have identified *Malassezia* as playing pivotal roles in the progression of Crohn's Disease and exocrine pancreatic cancer (Ashbee and Evans, 2002; Aykut et al., 2019; Limon et al., 2019). Yet much of the specific mechanisms and disease pathogenesis remain elusive due to the lack of capability to perform gene studies, hampering the advance of research developments. Today, two

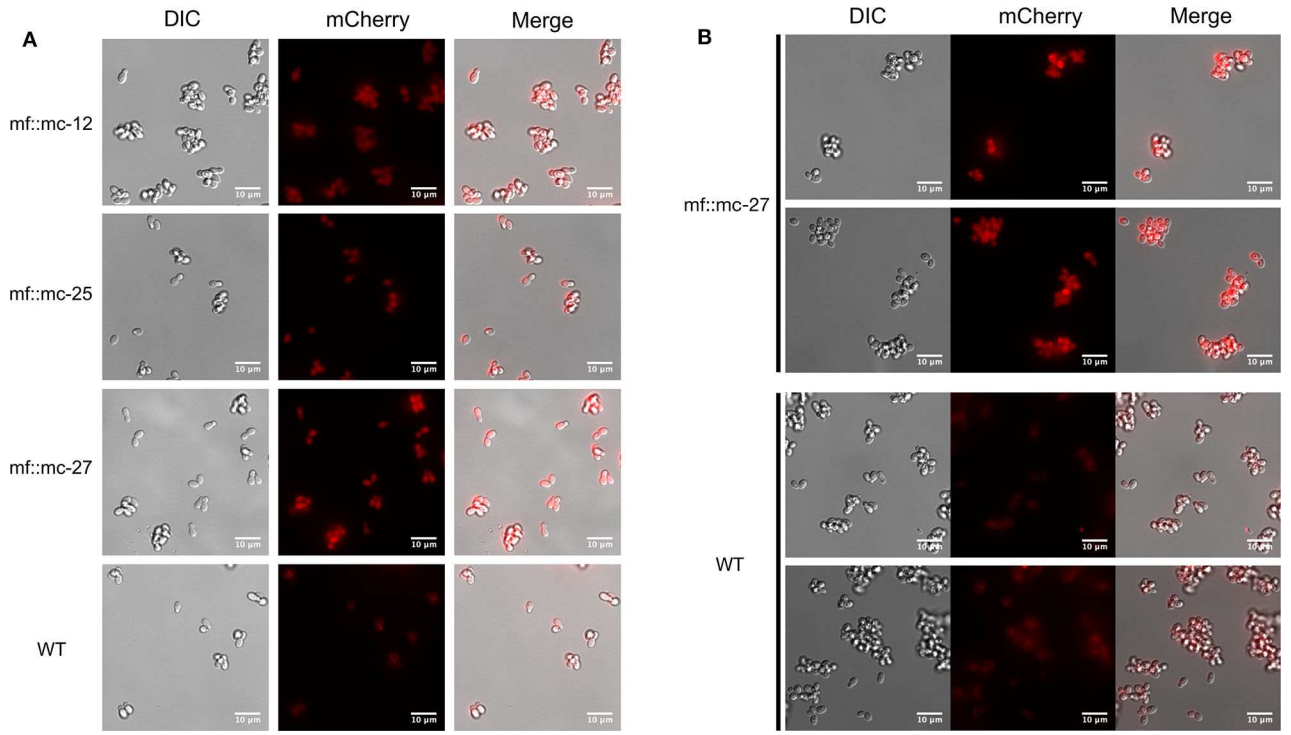

FIGURE 3 | Live fluorescent Imaging. **(A)** Transformants and wild-type cells were collected from mDixon agar and imaged under TRITC channel and differential interference contrast (DIC). Wild-type cells displayed localized autofluorescence in cell wall, in contrast to transformants exhibiting stronger fluorescence distributed uniformly throughout the cells. **(B)** Cells in exponential growth phase were collected to minimize accretion of dead or stationery cells and aggregation of fluorescence metabolites. Transformant mf::mc-27 presented higher fluorescence intensity in comparison to *M. furfur* CBS 14141 wild type cells which displayed persistent low levels of fluorescence background.

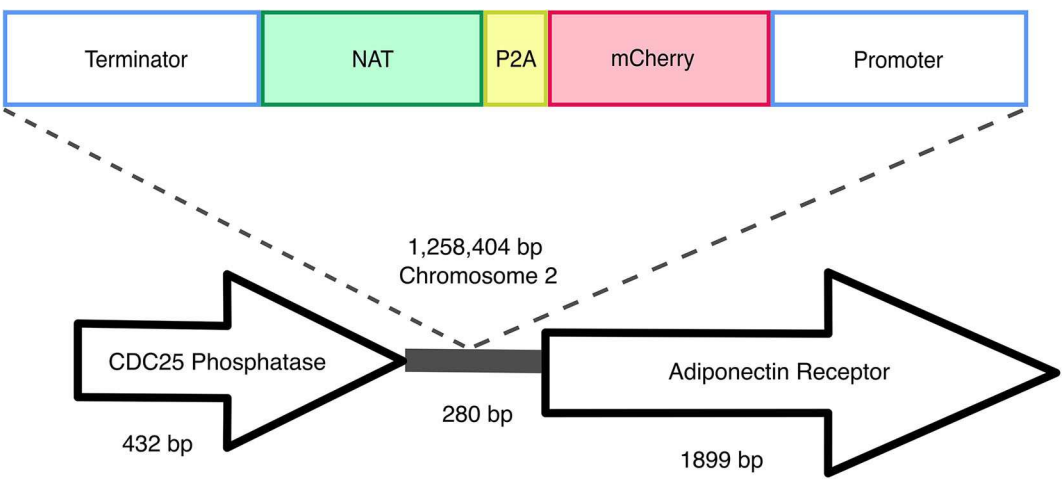

FIGURE 4 | Random insertional mutagenesis. Using Illumina and Sanger sequencing, exogenous T-DNA was identified in chromosome 2 of mf::mc-27 mutant. Sanger sequencing further validates the presence of *CDC25* phosphatase gene, adjacent to exogenous DNA of actin terminator and *NAT*. Illumina reads were able to detect both complete flanking ORFs of the hypothetical adiponectin rector protein, and exogenous T-DNA containing *Malassezia* actin promoter, terminator, and mCherry-P2A-*NAT* cassette.

research groups have demonstrated the use of a soil bacterium, *Agrobacterium tumefaciens* to genetically modify *Malassezia*, paving a new avenue to investigate functional genomics (Ianiri et al., 2016; Celis et al., 2017). In this study, we leveraged the application of *Agrobacterium*-mediated transformation to introduce a bicistronic expression vector to co-express red

fluorescence protein, mCherry, and *NAT* resistance marker in *Malassezia furfur*.

The usage of inter-kingdom *Agrobacterium*-mediated transformation in delivering exogenous DNA is a convenient and versatile tool applied in diverse engineered eukaryotic species. This pathogenic bacterium effectively housebreaks into the host genome and influences host cellular processes to its benefit. It also provides an inexpensive and flexible approach in designing and utilizing expression vectors. ATMT approach also has its set of limitations including the unpredictable efficiency of transgene expression due to position effects, leading to varying transgenes expression levels among the same pool of transformants. In our study, *Agrobacterium tumefaciens* Transfer DNA (T-DNA) does not contain homologous DNA sequences to *Malassezia furfur* genome, in a deliberate attempt to assess random integration and gene disruption.

The construction of the bicistronic vector, pJG201702 included the use of actin encoding promoter and terminator to regulate the expression of mCherry and *NAT* proteins. The arrangement of the transcribed proteins was specifically designed to direct the obligatory expression of mCherry when *NAT* protein is simultaneously expressed. It was engineered to avoid the possibility of selective gene expression as *Malassezia* acquired the potential to undergo genomic rearrangements, excising non-essential genes in maintaining a condensed genome (Xu et al., 2007; Sankaranarayanan et al., 2020).

Generation of non-fusion proteins was facilitated through the insertion of P2A viral sequence between mCherry and *NAT*. Ribosomal skipping of the peptide bond formation between glycine and proline residues within P2A sequence results in a pseudo-cleavage event to take place, separating the two protein products (Kim et al., 2011; Szymczak-Workman et al., 2012; Liu et al., 2017). In pJG201702, each genetic component is flanked by restriction enzyme sites, designed for the ease of future modification including the development of multi-cistronic vectors through incorporating multiple 2A sequences to generate varied gene products. Additionally, the mCherry and P2A sequences were codon optimized to improve protein expression in *Malassezia*.

To assess the functionality of P2A sequence in the generation of discrete mCherry and *NAT* protein products, a monoclonal antibody specific for red fluorescent protein was probed against whole cell lysates extracted from wild type and transformants. We detected the predicted protein band at 29 kDa in all transformants. The pseudo autolytic-cleavage occurs near the end of P2A sequence resulting in the retention of a 2A tag (21 amino acid residues) at the end of mCherry C-terminus, explaining the significant shift of the cleaved mCherry protein from its native weight of 26.7 kDa. This suggests adequate self-cleaving efficiency with the inclusion of a Gly-Ser-Gly (GSG) linker in P2A residues which promotes cleavage efficiency (Kim et al., 2011; Wang et al., 2015). Correct functionality of the generated vector was demonstrated by strong mCherry expression of *NAT* resistant transformants compared to wild type, which displayed autofluorescence background probably due to the presence of lipids within the growth media (Croce and Bottiroli, 2014) and the production of naturally occurring intracellular metabolites (Mayser et al., 2002; Maslanka et al., 2018).

We sequenced the genome of one fluorescent transformant and confirmed correct T-DNA integration in *M. furfur* genome. The T-DNA was inserted between two genes, the gene upstream of T-DNA encodes a putative *CDC25*-related phosphatase, an yeast ortholog of a Ras guanyl-nucleotide exchange factor. This gene is thought to be involved in Ras protein signal transduction and cell cycle regulation, traversing the start control point of the mitotic cell cycle (Chen et al., 2000). Downstream of the T-DNA insertion encodes for an adiponectin receptor gene that is predicted to be associated with zinc ion homeostasis (Lyons et al., 2004). Based on the orientation of the genes, it is likely that the T-DNA integrated in the terminator region of *CDC25*-related phosphatase and the promoter region of the predicted adjoining adiponectin-encoding gene. Intergenic insertions of T-DNA are very common and represent a disadvantage of ATMT in fungi, and they are probably due to a preference for the T-DNA to insert in low-transcribed regions (Michielse et al., 2005; Idnurm et al., 2017). Intergenic insertions were also reported in two previous studies in *Malassezia*, suggesting that additional mechanisms that favor insertion in a ORF-free regions may exist (Ianiri et al., 2016, 2019); this is surprising if we consider that *Malassezia* genomes are consistently small and compact (Wu et al., 2015). This intergenic insertion may explain the lack of phenotypic abnormalities or variations in fitness in mc::mf-17 transformant. Intergenic insertions can also be turned into an advantage by using a conditional promoter that drives the expression of the gene downstream the T-DNA insertion (Kilaru et al., 2015; Ianiri et al., 2017). Another advantage could be the use of non-protein coding region as a safe haven for future genetic engineering in *Malassezia*, especially useful for the reintroduction of genes as complementation without compromising cell viability or disruption of neighboring genes as reported in *C. neoformans* (Arras et al., 2015; Upadhya et al., 2017).

We demonstrated the effective use of a bicistronic vector in *Malassezia*, to generate a codon optimized RFP in a *NAT*-resistant strain. The application of a bicistronic system can potentially be expanded to a multi-cistronic construct with the aid of multiple cloning sites, to include the expression of assorted genes according to the users' preference. Most of current research models in understanding skin health include the use of transgenic mice expressing green fluorescence protein (GFP), mammalian cell culture, reconstructed skin epidermis (RHE) or even *ex-vivo* skin models, which can be complemented with these fluorescently labeled *Malassezia*. In addition, the competence to fluorescently label yeast cells, overexpression of genes, and targeted gene replacement can be applied to deepen our understanding of *Malassezia* gene functions and host-microbes interactions.

METHODS

Strains and Culture Conditions

Haploid strain *M. furfur* CBS 14141 was obtained from Westerdijk Fungal Biodiversity Institute. Cells were cultured

at 32°C, with mDixon medium (36 g/L malt extract, 20 g/L desiccated oxbile, 6 g/L peptone, 2 ml/L glycerol, 2 ml/L oleic acid, 10 ml/L Tween 40, pH 6). Genetically transformed cells were maintained on mDixon supplemented with 100 μg/ml nourseothricin sulfate (*NAT*).

Plasmid and Constructs

pJG201701 was first constructed with *M. sympodialis* ATCC 42132 actin promoter and terminator sequences, encoding for codon-optimized fluorescent mCherry and porcine teschovirus-1 2A (P2A) sequences (as detailed in **Supplementary Table 1**), followed by *NAT* antibiotic resistance marker (GenScript) in pUC57 cloning vector. To enable the translation of both proteins, the stop codon of mCherry was removed and retaining a single stop codon in the second protein, *NAT*. Both actin regulatory and *NAT* sequences were taken from pAIM2 (Ianiri et al., 2016). Each genetic elements are adjoined with restriction cut sites to allow efficient modification of the construct. pJG201701 was digested with EcoRI and KpnI, and gene fragments were cloned in pPZP201-BK (Covert et al., 2001), which contains *Agrobacterium tumefaciens* backbone vector using T4 DNA ligase (New England Biolabs). Resultant plasmid, pJG201702 was transferred to *A. tumefaciens* EHA105 strain via electroporation and verified by digestion pattern and PCR.

A. tumefaciens-Mediated Transformation

Transformation of *M. furfur* were performed using previously established protocol (Ianiri et al., 2016; Celis et al., 2017). *A. tumefaciens* harboring pJG201702 was grown overnight in Luria-Bertani medium supplemented with 50 μg/ml kanamycin at 30°C and 250 rpm in a shaking incubator. Aliquot of cells were used as inoculum, resuspended in induction medium (IM) containing 100 μM acetosyringone (Sigma) and cultured for additional 6 h to OD_{600} of 1. *M. furfur* CBS 14141 cells were collected at mid-log phase and used at OD_{600} of 1. Proportional volumes of bacterial and yeast cells were mixed and filtered through 0.45 μm mixed cellulose membrane (Merck, Millipore) before transferring onto IM agar supplemented with 200 μM acetosyringone. Co-culture plates were incubated at 25°C for 5 days, after which cells were washed in 20 mL sterile PBS and plated on selection medium—mDixon agar containing 100 μg/ml *NAT* to select for transformants, and 200 μg/ml cefotaxime with 10 μg/ml tetracycline to halt the growth of *A. tumefaciens*.

Molecular Analysis

Genomic DNA of wild-type and engineered strains of *M. furfur* CBS 14141 were isolated with MasterPure yeast DNA kit (Epicenter) as per manufacturer's protocol with additional step of homogenizing at 6 m/s for 50 s (MP Biomedicals). PCR amplification of mCherry and *NAT* genes with mCherry-forward (5'-ATGGTGTCGAAGGGCGAG-3') and mCherry-reverse (5'-CTTGTAGAGCTCGTCCATGC-3'), and *NAT*-F (5'-ATGGCGGCCGCCACTCTTGAC-3') and *NAT*-R (5'-TTATGGACAAGGCATACTCATATAAAG-3') primers respectively to screen for positively transformed *Malassezia*.

Total Protein Extraction and Immunoblot

Washed transformant and wild type yeast cells were collected by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5) with added protease inhibitor cocktail (Nacalai tesque). Cells were mechanically lysed with mixture of ceramic and glass beads (Lysing Matrix E, MP Biomedicals) for 50 s and repeated twice to achieve complete cell breakage. Cell debris were separated by centrifugation at 12,000 rpm at 4°C for 5 min and supernatants were transferred to new tubes for repeated centrifugations. Prepared protein samples were separated using 4–20% Tris-glycine SDS-PAGE gradient gel and blotted to PVDF membrane. The blot was blocked in Intercept PBS blocking buffer (Li-cor) and subsequently incubated with RFP monoclonal antibody (ThermoFisher, MA5-15257) and probed with fluorescent anti-mouse secondary antibody. Protein bands were visualized with Odyssey CLx digital imaging system (Li-cor).

Genome Sequencing

Mf::mc-27 isolate was selected for whole genome analysis and nucleic acid was extracted using method described previously. Whole genome sequencing was performed with Illumina Novoseq 6,000 at 100x coverage, from 150 bp short insert paired-end reads (NovogeneAIT). CDS prediction using Augustus gene prediction tool identified putative *CDC25* Phosphatase and Adiponectin Protein Receptor proteins (**Supplementary Table 2**).

Fluorescence Microscopy

Live cell imaging was obtained on inverted wide-field microscope (Olympus IX-83). Fluorescence and differential interference contrast (DIC) images were achieved with 60 × 1.2 oil objective (plan-Apochromat). Exposure time was kept at 700 ms, and cells were held at 37°C with applied CO_2 incubator chamber during image acquisition. Images were processed and analyzed with FIJI (ImageJ).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/; PRJNA638523 https://www.ncbi.nlm.nih.gov/genbank/; MT559569-MT559575.

AUTHOR CONTRIBUTIONS

JG, GI, JH, and TD designed the experiments. JG performed the experiments and data analysis. JG and TD wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00367/full#supplementary-material

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REVIEW
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Malassezia spp. Yeasts of Emerging Concern in Fungemia

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Malassezia spp. are lipid-dependent yeasts, inhabiting the skin and mucosa of humans and animals. They are involved in a variety of skin disorders in humans and animals and may cause bloodstream infections in severely immunocompromised patients. Despite a tremendous increase in scientific knowledge of these yeasts during the last two decades, the epidemiology of *Malassezia* spp. related to fungemia remains largely underestimated most likely due to the difficulty in the isolation of these yeasts species due to their lipid-dependence. This review summarizes and discusses the most recent literature on *Malassezia* spp. infection and fungemia, its occurrence, pathogenicity mechanisms, diagnostic methods, *in vitro* susceptibility testing and therapeutic approaches.

Keywords: *Malassezia* spp., epidemiology, pathogenesis, fungemia, diagnosis, therapy, antifungal profile

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INTRODUCTION

Malassezia are lipid-dependent yeasts inhabiting the skin of healthy humans and other warm blooded animals. However, these yeasts may also act as opportunistic pathogens, causing dermatitis and otitis in animals, and dermatitis with (i.e., atopic dermatitis, folliculitis, and psoriasis) and without inflammation (*Pityriasis versicolor*) in humans (Guillot and Bond, 2020; Saunte et al., 2020). Besides their involvement in skin diseases, *Malassezia* spp. have increasingly been reported to cause severe systemic infections, especially among premature neonates and immunocompromised patients receiving parenteral nutrition (Miceli et al., 2011; Iatta et al., 2014a, 2018; Ilahi et al., 2017; Pedrosa et al., 2018). While fungemia caused by *Candida* species has been recognized as a cause of morbidity and mortality in hospitalized patients worldwide (Mellinghoff et al., 2018), the epidemiology of *Malassezia*-related fungemia remains largely underestimated most likely due to the difficulties in the isolation of these yeasts species due to their lipid-dependent growth (Iatta et al., 2018). Currently, the genus comprises 18 lipid-dependent species with a variable distribution on different hosts and pathologies (reviewed in Lorch et al., 2018; Guillot and Bond, 2020). Additionally, by using both fingerprinting methods and multigene sequence analysis, different *Malassezia* genotypes were identified as strictly related to the host, geographical origin, and/or clinical manifestations (Cafarchia et al., 2008, 2011b; Theelen et al., 2018). Several hypotheses have been proposed to explain the pathogenic mechanisms of these fungi, but the role of single species and genotypes in clinical manifestations remains to be elucidated (Theelen et al., 2018). In addition, scientific data suggest that *Malassezia* antifungal susceptibility profiles against

azoles, amphotericin B (AmB) and terbinafine (TER) largely vary between *Malassezia* species or genotypes, thus influencing clinical management of patients (Theelen et al., 2018). This review summarizes and discusses the most recent literature on *Malassezia* fungemia, its occurrence, pathogenic mechanisms of the involved species, diagnostic methods, *in vitro* susceptibility testing and therapeutic approaches.

THE *MALASSEZIA* GENUS AND ETIOLOGY OF *MALASSEZIA* FUNGEMIA

Since the designation of the genus *Malassezia* by Baillon in 1889, the taxonomy has been updated and currently the genus comprises 18 lipid-dependent species with different genotypes showing variable pathology and distribution on different hosts (reviewed in Lorch et al., 2018; Guillot and Bond, 2020). All *Malassezia* species are lipid-dependent and, with the exception of *Malassezia pachydermatis*, do not grow on Sabouraud Dextrose Agar (SDA), which is most commonly used for culturing fungi in clinical labs. The genus *Malassezia* occurs on the skin of humans and animals but some species have only been observed on either humans or animals (reviewed in Guillot and Bond, 2020). Interestingly, *Malassezia* yeasts are the major component of the healthy human skin mycobiome (Findley et al., 2013; Oh et al., 2014; Wu et al., 2015). A recent phylogenetic study evaluating six genes suggested that the genus is deeply rooted in the Ustilaginomycotina and has a sister relationship with the Ustilaginomycetes and Exobasidiomycetes, assigning the genus its own class, Malasseziomycetes (Wang et al., 2014). Based on a phylogenomics study using 164 core eukaryotic genes, three main clusters were identified: Cluster A consisting of *M. furfur, M. japonica, M. obtusa, and M. yamatoensis*; Sub-cluster B1, with the most abundant human skin inhabitants *M. globosa* and *M. restricta*; Sub-cluster B2 consisting *of M. sympodialis*, *M. dermatis*, *M. caprae, M. equina, M. nana*, and *M. pachydermatis*; and Cluster C forming a basal lineage with *M. cuniculi and M. slooffiae* (Wu et al., 2015). Four more species have been described since then: *M. brasiliensis* and *M. psittaci* from parrot (Cabañes et al., 2016), *M. arunalokei* from human scalp (Honnavar et al., 2017), and *M. vespertilionis* from bat (Lorch et al., 2018). Multiple genotypes of a species can colonize the same patient (Cafarchia et al., 2008; Machado et al., 2010; Ilahi et al., 2017) but some genetic types might be linked to a particular body site or pathology thus indicating an affiliation of *Malassezia* genotypes with host, geographical origin and/or clinical manifestations (Cafarchia et al., 2008, 2011a,b; Ilahi et al., 2017). In particular, amplified fragment length polymorphism (AFLP) patterns of *M. furfur* skin isolates from Ontario, Canada clustered separately from mainly European references from other body sites, suggesting geographical or ecological/clinical variability in the species (Gupta et al., 2004). Sequence analysis of the intergenic spacer (IGS1) distinguished specific *M. globosa*, *M. restricta*, and *M. pachydermatis* variants in seborrheic dermatitis, atopic eczema, and on healthy skin of humans and animals (Sugita et al., 2003, 2004; Kobayashi et al., 2011). Moreover, sequence analyses of the LSU rDNA showed distinct *Malassezia* spp. subtypes on different host species (Gaitanis et al., 2012). Multilocus sequence analysis that included the D1/D2 domains of LSU rDNA, the chs2 gene, and the ITS1 region grouped *M. pachydermatis* strains from skin of healthy dogs and from skin lesions in three main genotypes (A, B, and C) with eight ITS1 subtypes. Genotype B included isolates from dogs of European origin and appeared to be present on healthy dog skin, without producing phospholipase. The A and C genotypes and their subtypes seemed to be predominantly associated with skin lesions and their isolates showed high phospholipase production (Cafarchia et al., 2008; Machado et al., 2010). Similarly, IGS1 subtypes 3C and 3D displayed high phospholipase production and were more frequently isolated from skin lesions of dogs with atopic dermatitis (Kobayashi et al., 2011). Only three *Malassezia* species have been described to cause bloodstream infections: *M. furfur*, *M. pachydermatis*, and *M. sympodialis*. Interestingly, AFLP analysis or ITS sequences showed that only one main *M. furfur* or *M. pachydermatis* genotype seems to be involved in blood stream infections in immunocompromised hosts (Theelen et al., 2001; Kaneko et al., 2012; Ilahi et al., 2017). All these *Malassezia* genotypes might also colonize the skin of patients or of hospital staff which might represent the driver for these systemic infections (Theelen et al., 2001; Gupta et al., 2004; Kaneko et al., 2012). With respect to the assessment of antifungal microbiological profiles, a reference method has not yet been developed for these yeasts and the culture media, inoculum sizes, incubation times, and the criteria used to determine MIC endpoints differ among studies. However, evidence suggested that *Malassezia* antifungal susceptibility profiles against azoles, AmB and TER vary according to the *Malassezia* species, regardless of culture medium or other conditions employed. *M. sympodialis* and *M. pachydermatis* are the most susceptible and *M. furfur* and *M. globosa* the least susceptible species to azoles, AmB and TER (Rojas et al., 2014; Cafarchia et al., 2015; Pedrosa et al., 2019b). Itraconazole (ITZ) and ketoconazole (KTZ) were the most active drugs for all *Malassezia* species, and fluconazole (FLZ), voriconazole (VOR) and AmB the least active (Rojas et al., 2014; Cafarchia et al., 2015; Pedrosa et al., 2019b). Interestingly, antifungal profiles may also vary in relation to genotype (Sugita et al., 2005; Cafarchia et al., 2012). In particular, isolates derived from animal skin lesions and belonging to a unique genotype, showed reduced susceptibility to azoles when compared to genotypes associated with healthy skin (Cafarchia et al., 2008).

EPIDEMIOLOGY OF *MALASSEZIA* YEASTS AND FUNGEMIA

The epidemiology of *Malassezia* fungemia has not been well-investigated until now due to the scant surveillance studies with this focus (see **Table 1**). *Malassezia furfur*, *M. sympodialis*, and *M. pachydermatis* are the only *Malassezia* species isolated from bloodstream infections to date (**Table 1**). Some authors propose that the fungal density on the skin as well the host immunological competence might be driving factors influencing their pathogenic role (Wheeler et al., 2017; partially reviewed in Theelen et al.,

TABLE 1 | *Malassezia* yeasts fungemia: yeast species, risk factors, diagnosis, treatment, and outcome.

References	Yeast species	Hosts and number	Cause of admission	Risk factors	Diagnosis	Treatment protocol	Length of treatment	Outcome
Redline and Dahms (1981)	*M. furfur*	Preterm neonate/1	Respiratory distress syndrome	CVC, Preterm neonates, TPN	Histology/cytology	AmB + Flucytosine	NR	Dead
Hassall et al. (1983)	*M. furfur*	Children/1	Fever, vomiting	CVC, bowel syndrome, urokinase therapy	Counter current immune-electrophoresis	CVC removal, AmB	42 days	Alive
Powell et al. (1984)	*M. furfur*	Preterm neonate/4 Children/1	Fever, SEPSIS interstitial pneumonia, elevated neutrophil band counts, and thrombocytopenia	CVC, TPN, preterm neonates, prolonged hospitalization, pneumonia	CCVC tip culture on lipid media	CVC removal	NR	Alive
Redline et al. (1985)	*M. furfur*	Preterm neonate/3 Children/2 Adult/2	Fever, thrombocytopenia in neonates, respiratory distress	CVC, TPN, preterm neonates, severe gastrointestinal disease and immunosuppression	Histology/cytology/ blood and CVC tip cultures on lipid media	Discontinuation of the lipid administration, AmB (0.5–1 mg/kg/d), or AmB (40 mg/day), Flucytosine every 6–8 h	30–35 days	Dead (preterm neonates) Alive (children and adults)
Alpert et al. (1987)	*M. furfur*	Preterm neonate/6 Children/1	Fever, respiratory distress asymptomatic (2 infants)	CVC, TPN, multiple potential causes for immunosuppression	Blood culture on lipid media	CVC, AmB alone or with fluorocytocine	5–42 days	2 Dead due to unrelated causes and 5 alive
Aschner et al. (1987)	*M. furfur*	Children/2	Fever, pulmonary diseases, thrombocytopenia	Apnea, bradycardia, pulmonary deterioration, thrombocytopenia	CVC tip on lipid media	CVC removal	NR	1 Dead 1 Alive
Dankner et al. (1987)	*M. furfur*	Preterm neonate/5 Adult/2	Fever, thrombocytopenia, hyperbilirubinemia	CVC, Premature neonates, prolonged hospitalization, tpn, broad-spectrum antibiotics, multiple potential causes for immunosuppression	Blood culture bottles on lipid media	AmB alone or AmB and flucytosine	4 days−6 weeks	Alive
Garcia et al. (1987)	*M. furfur*	Adult/2	Fever and leukocytosis	CVC, TPN, Broad-spectrum antibiotics, multiple potential causes for immunosuppression	Blood culture on lipid media	Discontinuation of the lipid administration AmB (total dose 665 mg)	28 days	Dead
Wurtz and Knospe (1988)	*M.furfur*	Adult /1	Fever	Multiple potential causes for immunosuppression	Blood culture on lipid media	AmB followed by cephalexin and KTZ	18–25 days	Alive
Shek et al. (1989)	*M. furfur*	Preterm neonates/3	NR	NR	Histology	NR	NR	Dead due to *Malassezia* septicemia
Surmont et al. (1989)	*M. furfur*	Preterm neonates/6	Fever, pulmonary diseases, leucocytosis, thrombocytopenia	CVC, total parenteral nutrition, preterm neonates, broad-spectrum antibiotics, multiple potential causes for immunosuppression	CVC culture on lipid media	CVC removal, discontinuation of the lipid administration miconazole (13–30 mg/kg per d) alone or in combination with or cloxacillin and netilmicin	10–15 days	1 Dead 5 Alive
Masure et al. (1991)	*M. furfur*	Preterm neonates/1	Leukemia	CVC	Blood culture on lipid media	NR	NR	NR
Weiss et al. (1991)	*M. furfur*	Preterm neonates/1	Preterm neonates	CVC, total parenteral nutrition, preterm neonates	Blood culture on lipid media	Discontinuation of the lipid emulsion without CVC Removal	–	Alive

(Continued)

TABLE 1 | Continued

References	Yeast species	Hosts and number	Cause of admission	Risk factors	Diagnosis	Treatment protocol	Length of treatment	Outcome
Welbel et al. (1994)	*M. pachydermatis*	Preterm neonates /5	Thrombocytopenia	CVC, TPN, interavenous lipid, preterm neonates (interveuticular hemmorhage, sepsis, respiratory distress syndrome), prolonged hospitalization	Blood culture	NR	NR	NR
Barber et al. (1993)	*M. furfur*	Adults /4 children/3	Fever, leukocytosis, cough, apnea, myalgia, nausea/vomiting	CVC, Multiple potential causes for immunocompromise	CVC culture on lipid media	AmB (1 mg/kg/d)	NR	2 Dead due to unrelated causes 5 Alive
Shparago et al. (1995)	*M. furfur*	Adult/1	Fever, chills, dyspnea, pleuritic chest pain, and multiple bilateral pulmonary nodular infiltrates	CVC, TPN	Lysis-centrifugation fungal blood cultures on lipid media	CVC removal discontinuation of the lipid emulsion, and AmB (300-mg total cumulative dose)	>2 weeks	Alive
Schoepfer et al. (1995)	*M. furfur*	children/3	T cell lymphoma. meningeal relapse bone marrow transplantation.	CVC, TPN with lipids, broad-spectrum antibiotic and corticosteroid treatment,	Cytology	AmB, flucytosine FLZ	NR	Alive
Chang et al. (1998)	*M. pachydermatis*	Preterm neonates/8	Fever intubation or reintubation and tachycardia	CVC, TPN with Lipids,	Blood cultures	AmB for at least 10 days	NR	1 dead 7 alive
Morrison and Weisdorf (2000)	*M. furfur*	Children/1 Adult/1	Fever, Hurler's allogeneica trasplant	CVC, TPN with Lipids,	Histology, dupont isolator system on lipid media	CVC removal discontinuation of the lipid emulsion	NR	1 dead due to unrelated causes 1 alive
Schleman et al. (2000)	*M. pachydermatis*	Adult /1	Fever	CVC, TPN with lipids, multiple abdominal surgeries	Blood culture bottles	CVC Removal AmB	NR	Alive
Chryssanthou et al. (2001)	*M. pachydermatis*	Preterm neonates/8	Fever, respiratory distress syndrome	Preterm neonates, TPN	BACTEC blood culture bottles	L-AmB (1 mg/Kg/d), Flucytosine (8 mg/Kg/d PO or EV), FLZ (50–150 mg kg/d)	21–35 days	Alive
Kikuchi et al. (2001)	*M. sympodialis*	Adult/1	Gastric cancer	Fever elevated leukocyte counts and C-reactive protein	NR	NR	NR	NR
Chu and Lai (2002)	*M. furfur*	Adult/1	Fever	Broad-spectrum antibiotic corticosteroid treatment,	Blood culture bottles	AmB (0.7 mg/kg/d for 10 days), FLZ (200 mg daily for 14 days)	24 days	Alive
Rosales et al. (2004)	*M. furfur*	Preterm neonate/1	Hypotension, Thrombocytopenia, High level C-reactive protein apnea, temperature instability, Bilious residual	CVC, Parenteral nutrition, preterm neonate (low birth-weight), chronic lung disease, necrotizing, enterocolitis intraventricular	Histology, radiographically/ photomicrograp	AmB	26 days and 2 days	Died
Giusiano et al. (2006)	*M. furfur* *M. sympodialis*	Adult/2	Oncology	NR	CVC tips cultures	Nr	NR	NR
Oliveri et al. (2011)	*M. furfur*	Preterm neonate/1	Fever, gastrointestinal disturbs, cyanosis	CVC, Parenteral nutrition, prolonged hospitalization, antibiotic treatment, ileostomy surgery	Lysis-centrifugation blood culture on Dixon	CVC removal L-AmB (4 mg/Kg/d)	45 days	Alive

(Continued)

TABLE 1 | Continued

References	Yeast species	Hosts and number	Cause of admission	Risk factors	Diagnosis	Treatment protocol	Length of treatment	Outcome
Iatta et al. (2014a)	*M. furfur*	Preterm neonates/6 (3 with FLZ prophylaxis) and children/2	Fever, apnea, high level c reactive protein, abdominal distension, thrombocytopenia	CVC, Parenteral nutrition, Preterm neonates, Prolonged hospitalization, Neonatal asphyxia, Abdominal surgery (infants)	Lysis-centrifugation blood culture on Dixon	CVC removal L-AmB (2.5–5 mg/Kg)	6–20 days	Alive
Al-Sweih et al. (2014)	*M. pachydermatis*	Preterm neonate/1	Fever, respiratory distress syndrome	CVC, Preterm neonates TPN, necrotizing enterocolitis	BACTEC blood culture bottles	L-AmB	7 days	Alive
Choudhury and Marte (2014)	*M. pachydermatis*	Adult with posaconazole prophylaxis/1	Fever	CVC, acute myeloid leukemia, chemotherapy	BACTEC blood culture bottles	CVC removal L-AmB (1 mg/Kg/d)	NR	Alive
Aguirre et al. (2015)	*M. sympodialis*	Children /1	Fever, respiratory distress with hypoxemia	CVC, History of prolonged hospitalization, broad-spectrum antibiotic treatment Abdominal surgeries, viral infections	Lysis-centrifugation blood culture on Dixon	CVC removal, L-AmB (1 mg/Kg/d)	21 days	Alive
Roman et al. (2016)	*M. pachydermatis* + mycobacteria	Adult/1	Fever, pneumonia, dyspnea	CVC, Bacteremia, Leprosis	BACTEC blood culture bottles	CVC removal, AmB (5 mg/Kg/d) and nafcillin (400 mg IV d)	7 days	Alive
Iatta et al. (2018)	*M. furfur*	Preterm neonates/9	Fever, respiratory distress, elevated or depressed leukocyte count, increased C-reactive protein levels, thrombocytopenia	CVC, Preterm neonates TPN	CVC tips cultures on lipid media	NR	NR	1 Died and 8 Alive
Pedrosa et al. (2018)	*M. furfur*	Preterm neonates/1 and Adult /2 with fluconazole prophylaxis	Fever	CVC, Broad-spectrum antibiotic and glucocorticoid therapies, Auto-immune diseases, Carcinoma	BACTEC blood culture bottles and (MALDI-TOF-MS)	CVC removal, FLZ (10 mg/kg/d) and/or L-AmB (3 mg/kg/d) or (5 mg/kg/d) for Adults	50 days	2 Dead (adults) 1 Alive
Chen et al. (2019)	*M. furfur*	Preterm neonates with fluconazole prophylaxis/1	Fever, apnea, bradycardia, thrombocytopenia	CVC, TPN with Lipids Premature infant (Low birth-weight).	Blood culture in media with lipid	CVC removal, AmB (1 mg/kg/d)	NR	Alive
Lee et al. (2019)	*M. pachydermatis*	Adult/1	Intraabdominal abscess	CVC, TPN with Lipids, Multiple potential causes for immunocompromised, Gastric cancer, Chemotherapy	Blood culture on 5% on Sabouraud dextrose agar.	AmB	2 days	Dead due to unrelated causes
Huang et al. (2020)	*M. pachydermatis*	Preterm neonates with fluconazole prophylaxis/4	Respiratory distress syndrome, low Apgar scores	CVC, broad-spectrum antibiotics, TPN with Lipids, low birth weight	BACTEC blood culture bottles	CVC removal (2/4), AmB	14–21 days	Alive
Chow et al. (2020)	*M. pachydermatis*	Preterm neonates with fluconazole/3	CLABSI, necrotizing enterocolitis, peritonitis, infected urinoma	CVC, Broad-spectrum antibiotics, TPN with Lipids, Preterm neonate (low birth-weight)	CVC culture	NR	NR	Alive

NR, not reported; CVC, central venous catheter; TPN, total parenteral nutrition; AmB, Amphotericin b; L-AmB, Liposomal Amphotericin b; FLZ, fluconazole; CLABSI, central line-associated bloodstream infection.

2018). *Malassezia* bloodstream infections have been described in adult and child immunocompromised patients, and neonates (**Table 1**). According to general knowledge, the main ecological niche of *Malassezia* yeasts is human and animal skin, and they represent 50 to 80% of the total human skin mycobiome (Nagata et al., 2012; Findley et al., 2013; Gupta et al., 2014). Therefore, it would make sense to consider the skin mycobiome as a potential reservoir and point d'entrée for bloodstream infections. More is currently known about the adult human skin mycobiome but little information is available about the child and neonatal skin mycobiome. Even if *Malassezia* skin colonization may be the result of both maternal and environmental sources, data suggests that colonization of human skin begins immediately after birth and *Malassezia* species distribution varies with age (Jo et al., 2016; Ward et al., 2018). The limited data currently available about the cutaneous mycobiomes in preterm and term neonates shows that *Malassezia* species distribution on skin of neonates and children varies between studies, but *M. globosa*, *M. furfur*, *M. sympodialis,* and *M. restricta* seem to be the most prevalent species described (Bernier et al., 2002; Zomorodain et al., 2008; Jang et al., 2009; Gupta et al., 2014; Prohic et al., 2014; Jo et al., 2016; Paul et al., 2019). Variation between studies could be the result of methodological differences such as number of subjects studied, DNA extraction, target region selection and PCR-conditions, and data processing and interpretation; but also geographical and host-specific differences may explain study discrepancies. The study of Paul et al. determined *M. restricta* to be the most abundant *Malassezia* species in both term and preterm neonates ($n = 30$), similar to adult skin (Paul et al., 2019). Interestingly, this species has thus far not been linked to bloodstream infections. *Malassezia* colonization increases quickly after birth but a very important shift, making *Malassezia* the most predominant genus of the human skin mycobiome, takes place during adolescence and has been linked to lipid composition shifts of the skin (Grice and Segre, 2011; Findley et al., 2013; Jo et al., 2016). In adults, *M. restricta* and *M. globosa* seem to be the dominating *Malassezia* species on the skin, followed by *M. sympodialis*, depending on body site (Findley et al., 2013; Wu et al., 2015). Geographical factors may influence *Malassezia* species distribution on healthy human skin (Leong et al., 2019; Saunte et al., 2020). In addition, the proportion of *Malassezia* species isolated from the skin varies considerably among different medical conditions (Saunte et al., 2020). As the two most abundant *Malassezia* species on healthy human adult skin have not been linked to *Malassezia* bloodstream infections, studies focusing on genetic and functional differences between cells of *Malassezia* species involved in bloodstream infections and ones that are not, in addition to potentially selective host parameters, could help explain this. Recent molecular studies highlighted the genetic variability among single species implicated in both skin and systemic infections (Theelen et al., 2001; Kaneko et al., 2012; Gupta et al., 2014), suggesting pathogenicity variation at species and sub-species levels. *Malassezia pachydermatis* is mainly known from animals and is not commonly associated with human skin colonization yet in multiple cases it was linked to *Malassezia* bloodstream infections (**Table 1**).

A study evaluating cats and dogs (107 healthy and 123 with chronic otitis externa), showed that occurrence and population size of *M. pachydermatis* increased according to the presence in skin lesions, not only in affected areas of the skin but also at other sites without detectable skin lesions, suggesting that the yeasts could be easily transmitted from site to site because of scratching induced by pruritus (Cafarchia et al., 2005). This finding is also relevant from a zoonotic point of view since the yeasts may be mechanically transmitted from dogs to their owners (Morris et al., 2005) and subsequently can cause health problems as previously reported from an intensive care nursery (Chang et al., 1998). A similar transmission route—animal to human or human to human—from health care worker or family member for *M. furfur* or *M. sympodialis* may also occur and should be further explored in future studies. Even when these species are part of the stable mycobiomes of *Malassezia* bloodstream infection patients, isolates causing the infection could belong to different genotypes, transmitted from external sources. The first case of *Malassezia* spp. as a pathogen in bloodstream infection and sepsis was reported in 1981 by Redline and colleagues (**Table 1**). Until now, a total of 118 cases were published, but only three surveillance studies (**Table 1**). *Malassezia furfur* was the most encountered species with 82 cases, followed by *M. pachydermatis* (33 cases), and only three cases of *M. sympodialis* fungemia have been described. To date, *Malassezia* fungemia cases were observed with highest incidence in neonates (82 cases), followed by 23 in adults and 17 in children. Numerous cases were reported in the past two decades, particularly in neonates and infants receiving intravenous lipids. In our 1-year survey on yeast fungemia involving 290 neonatal and 17 pediatric patients with intravascular catheters, lipid parenteral nutrition, prolonged ward stay, and surgery, were evaluated. This diagnostic survey on bloodstream infections (BSIs) resulted in a higher prevalence of *M. furfur* (2.1%) than *Candida* spp. (1.4%) and suggested that *Malassezia* BSIs might be underestimated, due to improper diagnosis (Iatta et al., 2014a). The surveillance study was repeated 4 years later, and a total of 202 neonatal patients with intravascular catheters were enrolled (Iatta et al., 2017). A total of 10 cases of BSIs were registered, thus suggesting the relevance of these yeasts in catheter mediated fungemia (Iatta et al., 2018). Incubator, sheets and the skin of patient or hospital staff may represent potential sources of *Malassezia* infection. Recently, lipid infusion type, phototherapy light sources, central venous catheter placement, and prophylactic fluconazole were proposed as risk factors affecting *Malassezia* colonization and /or fungemia more than candidemia (Chen et al., 2019).

PATHOGENESIS

Almost all-available research to date focuses on *Malassezia* virulence factors linked to skin and pathogenesis for *Malassezia* BSIs has hardly been studied, likely due to the relatively low number of cases when compared to skin diseases. With an increasing number of cases in recent years and the likely underestimation of *Malassezia* BSIs as a result of the use of standard culture media without lipid supplementation in

the clinic, future studies will hopefully address some of the missing knowledge. Here, we focus on known virulence factors of the three fungemia causing *Malassezia* species (i.e., *M. pachydermatis, M. furfur,* and *M. sympodialis*) and discuss findings, even if they were derived from skin. Virulence factors, to some extent, may be of a more general nature and could potentially also be relevant for bloodstream infections. Several hypotheses have been proposed to explain the pathogenic behavior of these fungi. The relationship between host and *Malassezia* metabolism seems to be key for the understanding the pathogenesis of infection (Cafarchia and Otranto, 2004; Velegraki et al., 2015; Theelen et al., 2018).

Various *Malassezia* cell characteristics may be involved in BSI pathogenesis. *Malassezia* spp. have cell walls with a very thick multi-layered structure that may protect them from different environmental stresses and have been described to help evade phagocytosis (Celis et al., 2017a). Biofilm formation has been linked to increased drug resistance and virulence. *M. furfur*, *M. sympodialis*, and *M. pachydermatis* are able to form biofilms, a process that seems to be strain dependent (Figueredo et al., 2013; Angiolella et al., 2017; Pedrosa et al., 2019a). In particular, *M. pachydermatis* strains isolated from dogs with and without skin lesions were able to form biofilms with variable extracellular matrix (ECM) quantity and structure depending on the sources (Figueredo et al., 2013). Accordingly, a structural heterogeneity of biofilm was found between those formed by *M. furfur* and *M. sympodialis* isolates, with both species exhibiting yeast aggregates in multilayer clusters but with a denser entrapment by a more gelatinous ECM in case of *M. furfur* biofilms (Pedrosa et al., 2019a). Biofilm formation and the extracellular matrix generation were responsible for the emergence of antifungal resistance (Figueredo et al., 2013; Angiolella et al., 2017). The biofilm formation was well-correlated with hydrophobicity, adherence, and phospholipase production of pathogenic *M. pachydermatis* and *M. furfur* cells, which may help explaining the change from a commensal to a pathogenic phenotype of these organisms (Figueredo et al., 2013; Angiolella et al., 2017). *Malassezia furfur* colonization of central venous catheters was already observed in 1994 (Sizun et al., 1994) and the ability *M. pachydermatis* to form biofilms on catheter surfaces in the laboratory was confirmed in another study (Cannizzo et al., 2007). Though not observed frequently, *Malassezia* yeasts are able to produce mycelium, a feature first suggested from an observation of hyphae in the scales of PV. Later, researchers managed to also obtain a mycelial phase for *M. furfur in vitro* (Saadatzadeh et al., 2001). In a recent review, the importance of morphological switching for fungal pathogens was highlighted: shape-shifting between different morphologies allows fungi to adapt to different host environments. The authors suggest that to understand pathogenesis mechanisms, it is crucial to establish how fungal morphology impacts virulence strategies (Min et al., 2020). It would be useful to further investigate *Malassezia* morphology switching and its potential relevance in bloodstream infections.

It has been shown that μ-opioid receptors (MORs) are present on *M. pachydermatis* cell membranes, having a role in modulating the phospholipase activities (Cafarchia et al., 2010). In animals without lesion MORs were expressed as dimers with other opioids thus resulting in an inactive form (Cafarchia et al., 2010). The high concentration of beta-endorphin normally present on lesioned skin of hosts (Pan, 2005; Honnavar et al., 2017) influenced the expression of MOR in their active form, thus favoring phospholipase production (Cafarchia et al., 2010). Increased phospholipase activity may be linked to the appearance of skin lesions and in some cases septicaemia (Cafarchia and Otranto, 2004; Vlachos et al., 2013).

Malassezia yeasts also produce esterases, lipases, and proteases, of which the latter have a crucial role in interactions with the host and microbial community such as *Staphylococcus aureus* thus making it unfavorable for colonization (Chen and Hill, 2005; Li et al., 2018; Tee et al., 2019). Additionally, *M. furfur* secretes aspartyl proteases, capable of degrading a wide range of human skin associated extracellular matrix (ECM) protein, and might also be able to modify the skin environment potentially interfering with wound re-epithelization (Poh et al., 2020). These enzymes could on one hand contribute to pathogenesis but on the other hand have a protective function, leading to a potentially very complex role for *Malassezia* on and in the human host. Lipases particularly have been considered virulence factors of *Malassezia* yeasts since they may be involved in the invasion, colonization, persistence and proliferation within host tissues (Petrokilidou et al., 2019). The first *Malassezia* gene encoding an extracellular lipase was identified in *M. furfur* and was designated as LIP1. Subsequently, orthologs were identified in *M. pachydermatis*, *M. globosa,* and *M. restricta*. *Malassezia* species possess multiple genes encoding putative lipases (from 9 to 14 depending on the species) that are differently involved in various skin disorders (Park et al., 2017; Tee et al., 2019). Interestingly, the lipase and phospholipase activities of *Malassezia* yeasts vary according to the species and they are implicated in both skin diseases and fungemia. In particular, *M. furfur* strains causing fungemia showed very high lipolytic enzyme activity thus suggesting that parenteral lipid emulsions may play an important role in modulating the growth and pathogenicity of *Malassezia*-yeasts in sepsis (Kaneko et al., 2012). *Malassezia* strains from skin disorders produce metabolites, such as indirubin and indolo[3,2-b] carbazole (ICZ) which are associated with carcinogenesis, immune regulation and mediation of ultraviolet radiation (UVR) damage (Gaitanis et al., 2011; Theelen et al., 2018). Recently, it has been shown that both *M. furfur* and *M. sympodialis* are able to produce nanovesicles enriched with allergens and/or proteins that interact with keratinocytes and monocytes, thus causing and maintaining the inflammation (Johansson et al., 2018; Zhang et al., 2019). In particular, nanovesicles produced by *M. sympodialis* (MalaEx) are also able to activate human keratinocytes causing an enhanced intercellular adhesion molecule-1 (ICAM-1) expression, which can cause an attraction of immune competent cells, thus causing host cutaneous defense to *M. sympodialis* (Vallhov et al., 2020). So far, the function of these extracellular vesicles for *Malassezia* spp. has only been studied in relation to skin but various studies in other fields related to similar kinds of nanovesicles show that their function in cell-to-cell communication may be much broader than that. For example, a study on human pathogen *Cryptococcus gattii*, showed that vesicles were taken

up by macrophages of the infected host, allowing long distance pathogen-to-pathogen communication resulting in virulence enhancement (Bielska et al., 2018). It would be interesting to explore whether extracellular vesicles might also play a role in *Malassezia* BSIs.

In order to gain better understanding of *Malassezia* pathogenesis in general, and in BSIs in particular, it is important to assess known *Malassezia* virulence factors, as well as investigate potentially unknown factors, and perform comparative studies between BSI-derived isolates and skin isolates. In recent years, many useful new tools have been developed for this purpose. Although to date, no model systems for studying systemic *Malassezia* infections have been described, recent advances for other *Malassezia*-affected areas of the human body may offer useful insights for future application to studying host-pathogen interactions in BSIs. Sparber et al., reported of a murine epicutaneous infection model that allowed studying the interaction of *Malassezia* with mammalian skin *in vivo* (Sparber and LeibundGut-Landmann, 2019; Sparber et al., 2019) and in the same year the association of *Malassezia* with Crohn's disease was reported using mouse models (Limon et al., 2019). Performing *Malassezia* host-pathogen interaction studies in model systems such as the mouse may sometimes be difficult, but with the recent establishment of an *in vivo* infection model using *Galleria mellonella*, host-pathogen interaction studies are becoming more accessible. The *G. mellonella* model has multiple advantages, such as the absence of ethical hurdles, low cost, ease of use, yet the immune response has similarities with the human system (Torres et al., 2020). In addition, recently developed *Malassezia* transformation systems can aid in studying the role of specific genes and virulence factors in *Malassezia* pathogenicity. *Agrobacterium tumefaciens* mediated transformation systems for the BSI-relevant *Malassezia* species were developed, allowing direct gene manipulation to better understand gene function (Ianiri et al., 2016; Celis et al., 2017b). With the recent improvements to the CRISPR/Cas9 strategy, this gene editing system has already contributed to various fungal virulence studies (Malavia et al., 2020), and also its first application for research in functional *Malassezia* genetics has recently been reported (Ianiri et al., 2019).

DIAGNOSIS

Since multiple *Malassezia* species and/or genotypes with varying antifungal susceptibility profiles may cause unique or similar pathologies, serious concern about the diagnostic procedures and antifungal treatment has been raised. Isolation and enumeration of *Malassezia* cells from clinical specimens remain a challenge because of their lipid dependency. Although microscopy of swab specimens is useful for diagnosing animal and human dermatitis, a more accurate etiological diagnosis is needed in high-risk patients (Iatta et al., 2018). Clinical features, laboratory markers, strategies of patient management, and outcomes of *Candida* and *Malassezia* fungemia do not differ. In some studies, *M. furfur* fungemia appeared earlier than candidaemia (average day 26 vs. day 42), most likely due to its exogenous origin. In addition, duration of *Malassezia* fungemia is longer than candidaemia, most likely due to the late removal of the central venous catheters (CVC) and also as a result of the lower efficacy of the antifungal therapy (Iatta et al., 2014a, 2018). The first signs causing suspicion of *Malassezia* fungemia usually are bloodstream infections manifested in fever of unknown origin in hospitalized and severely immunocompromised patients with CVC (i.e., preterm neonates or cancer patients) or in patients with pulmonary distress (**Table 1**) (Morrison and Weisdorf, 2000; Iatta et al., 2015, 2018). *Malassezia* fungemia should however be confirmed with the laboratory isolation of the responsible agent and identification by molecular means (Morrison and Weisdorf, 2000; Iatta et al., 2015, 2018; Pedrosa et al., 2018). In literature *Malassezia* yeasts were mainly isolated by culturing blood or CVC-tip directly on specific media and mainly by using the Isolator system (**Table 1**). It has been shown that the automated blood culture system BacT/Alert is not suitable for detecting *M. furfur* fungemia (Campigotto et al., 2016; Iatta et al., 2018). Out of 9 *M. furfur* fungemia cases reported by using BacT/Alert bottles only 1 case of *M. furfur* fungemia was detected whereas all the *M. pachydermatis* cases were easily detected using the above mentioned method, since this species grows on the media included in the bottles (Iatta et al., 2018, **Table 1**). Interestingly, it has been shown that human blood has a toxic effect on yeast growth and the addition of 3% of palmitic acid in the bottle might be able to overcome the inhibitory effect of both small (0.5 ml) and larger (3 ml) volumes of blood, thus favoring *Malassezia* growth (Nelson et al., 1995). Finally, although molecular tools have been used to detect *Malassezia* yeasts from biological samples no studies were performed to molecularly diagnose *Malassezia* directly from blood, thus culture remains the gold standard to isolate and identify the yeasts. This method is also suitable for yeast quantification, viability assessment, and genotyping and eventually to test the antifungal susceptibility profile of the isolated species (Peker et al., 2018). Generally, clinics use standard culture media without lipid supplementation, likely leading to underdiagnosis of both *M. furfur* and *M. sympodialis* in BSIs. It is recommended to carefully assess *Malassezia* BSI risk factors and apply the use of lipid-rich culture media when *Malassezia* spp. may be the causative agent.

THERAPY, ANTIFUNGAL PROFILE, AND PROBABLE RESISTANCE PHENOMENA

For treatment of *Malassezia*-related infections, azoles, and the polyene AmB are frequently employed, both in humans and animals. Topical antifungal agents (mainly azoles) are adequate for the management of localized skin lesions, while systemic ITZ or FLZ for severe skin diseases (Bond et al., 2020; Saunte et al., 2020). For catheter-related *Malassezia* infections, there are only recommendations, and patients were usually treated with catheter removal, discontinuation of lipid infusion and administration of antifungal drugs such as FLZ, AmB, and or VOR (Arendrup et al., 2014; **Table 1**). Amphotericin B is

effective in the treatment of *Malassezia* systemic infections, both in preterm infants and adults, but both FLZ and posaconazole (POS) fail to prevent *Malassezia* fungemia (**Table 1**). Usually, about 24 days of AmB treatment might be useful for a positive outcome of *Malassezia* fungemia but the length of the treatment might be different depending on the *Malassezia* species (**Table 1**). *M. pachydermatis* fungemia resolve more quickly than *M. furfur* fungemia (about 14 vs. 24 days, **Table 1**).

Despite attempts to treat these fungal infections, a trend toward recurrence is often observed in humans and animals with dermatitis (Negre et al., 2009; Bond et al., 2010, 2020; Saunte et al., 2020). Moreover, the induction of *in vitro* FLZ resistance in *M. pachydermatis* as well as the clinical evidence of treatment failure with TER in patients with *pityriasis versicolor* and, with KTZ in dogs with otitis (Kim and Pandya, 1998; Gupta et al., 2014; Kim et al., 2018) or with FLZ or POS in preventing *M. furfur* fungemia in humans, suggested the occurrence of drug resistance phenomena in these yeast species (Choudhury and Marte, 2014; Iatta et al., 2014a; Pedrosa et al., 2018; Chen et al., 2019). Antifungal susceptibility test methods have not yet been standardized, neither by the Clinical and Laboratory Standards Institute (CLSI) nor by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Arendrup et al., 2014), resulting in the absence of clinical breakpoints for these yeasts species. A recent study showed that drug efflux pumps (EPMs) are involved as defense mechanisms to azole drugs in *Malassezia* yeasts (Iatta et al., 2017). By using a broth microdilution chequerboard analysis, the *in vitro* efficacy of azoles in combination with EPMs (i.e., haloperidol-HAL, promethazine-PTZ, and cyclosporine) was evaluated. The MICs of FLZ and VOR of *Malassezia* spp. decreased in presence of sub-inhibitory concentrations of HAL and/or PTZ, and a synergistic effect was observed only in strains with FLZ MIC $\geq$ 128 μg/mL for *M. furfur*, FLZ MIC $\geq$ 64 μg/mL for *M. pachydermatis,* and VOR MIC $\geq$ 4 μg/mL in both *Malassezi*a spp., suggesting that the above FLZ and VOR MIC values might be considered the cut-off to discriminate susceptible and resistant strains (Iatta et al., 2017). Finally, the *in vitro* susceptibility of *Malassezia* for echinocandins suggests that this genus is intrinsically resistant to these drugs. Indeed, MIC $>$ 32 μg/mL were usually recorded for *M. pachydermatis* and *M. furfur* regardless of the employed CLSI protocol for testing drug efficacy (Prado et al., 2008; Yurayart et al., 2013; Al-Sweih et al., 2014; Leong et al., 2017).

DISCUSSION

Our knowledge on *Malassezia* yeasts has increased tremendously during the last two decades. Many questions remain ambiguous however, such as their role in pathology, diagnostic procedures, azole susceptibility profiles and clinical implications. *Malassezia* is the major component of the healthy human skin mycobiome but data on the constitution of the skin mycobiome and the abundance of *Malassezia* yeasts directly after birth and during the first years of life are highly inconclusive. Additional studies are needed as a better understanding of early (skin) mycobiome development may aid in understanding transmission routes, and may subsequently aid in infection containment. For example, in cases where *M. pachydermatis* was involved, transmission from pets via healthcare workers to patients was suggested which warrants better hygiene measures for hospital staff (Chang et al., 1998; Morris et al., 2005). Interpreting available literature on this topic should be done with caution as variation between studies may arise from methodological differences for factors such as study group size, identification methods and geography.

Concerning the causative species of bloodstream infection, epidemiological surveys suggest that *M. furfur* is the most common species involved. Although further studies are needed to understand the exact mechanisms of this finding; the colonization of skin of patients, as well as the higher virulence of *Malassezia* strains involved in fungemia, might be the driving factors influencing BSI epidemiology.

The occurrence of these infections seems lower among adult patients, and higher among neonatal patients, yet a slanted view exists as cases are likely underestimated, given the special lipid requirement of these yeast species (Iatta et al., 2018). Interestingly neonatal patients seem more frequently colonized with *M. furfur* strains than adults, but more data is needed to support this trend. Although lipid infusion and/or total parenteral nutrition seems to be one of the major risk factors in causing *Malassezia* fungemia due to the lipolytic properties of *Malassezia* yeasts, cases unrelated to intravenous nutrition were also observed (**Table 1**; Pedrosa et al., 2018; Chen et al., 2019). Interestingly, these infections are usually confined to severely immunocompromised hosts with CVCs, thus confirming the importance of this portal of entry (Iatta et al., 2018; Pedrosa et al., 2018; Chen et al., 2019). Several potential virulence factors have been described and may be involved in *Malassezia* pathogenesis of BSIs. In particular, the ability to produce enzymes such as lipases and/or phospholipases, production of various indolic compounds, the ability to form biofilms, and allergen enriched nanovesicle production were described for *M. furfur* and *M. sympodialis* which are frequently related to fungemia. The properties of these microorganisms to adhere to the skin and to medical indwelling devices by forming biofilms influence the antifungal profile of cells, which might represent another virulence factor in fungemia of severely immunocompromised hosts (Figueredo et al., 2013; Pedrosa et al., 2019a). Interestingly the structure of *Malassezia* biofilms is strain dependent and those of *M. furfur* and *M. pachydermatis* from skin lesions are composed of a more gelatinous extracellular matrix (Figueredo et al., 2013; Angiolella et al., 2018; Pedrosa et al., 2019a). Since the extracellular matrix is directly linked to the virulence of these yeasts, *M. furfur* and *M.pachydermatis* should be more virulent than *M. sympodialis*, which may explain the lower observed incidence of *M. sympodialis* fungemia (Figueredo et al., 2013; Angiolella et al., 2018; Pedrosa et al., 2019a). In recent years, useful new tools for studying virulence and host-pathogen interactions have been developed. *Galleria mellonella* has proven to be a promising *in vivo* infection model (Torres et al., 2020) and two studies showed the potential of a *Agrobacterium tumefaciens* mediated transformation system for studying the role of specific virulence related genes (Ianiri et al., 2016; Celis et al., 2017b). As the clinical outcome of *Malassezia* fungemia

does not differ from that of *Candida* yeasts (Iatta et al., 2014a), clinical guidelines need to be organized for an early diagnosis of these yeasts infections. In particular, fever of unknown origin and very high values of C-reactive protein (CRP) should alert the clinicians to suspect *Malassezia* related systemic infections. Diagnostic procedures to recover *Malassezia* organisms from blood are not routinely available, and the most common system used in many laboratories for the detection of bacterial and fungal pathogens (i.e., BacT/Alert system), is ineffective for diagnosing *M. furfur* fungemia (Iatta et al., 2018). Recently, CVC culturing on lipid-supplemented media, has been proposed as a routine procedure to diagnose *M. furfur* fungemia in severely immunocompromised patients (Iatta et al., 2018).

Guidelines for the treatment of *Malassezia* spp. skin disorders of pet animals and humans have been assessed, but those related to systemic infections still need to be addressed. Clinical evidence indicated efficacy of azole drugs for the control of skin infections and of AmB for systemic ones (**Table 1**). However, common recurrences of skin disorders (Negre et al., 2009; Theelen et al., 2018; Bond et al., 2020) as well as the clinical evidence of treatment failure with TER in patients with *pityriasis versicolor,* with KTZ in dogs with otitis (Kim and Pandya, 1998; Gupta et al., 2014) or with FLZ or POS in preventing *M. furfur* fungemia in humans, suggested the occurrence of drug resistance phenomena in these yeast species.

Additionally, the high level of inter- and intraspecies differences of *Malassezia* antifungal profiles might explain the differences in mycological cure rates when an antifungal agent is used to treat what appears to be clinically the same disease state. Different *Malassezia* species might be involved in the same clinical diseases, and/or different genetic types with different antifungal profiles might colonize the same host (Prohic et al., 2015; Velegraki et al., 2015). However, even if the MIC data of *Malassezia* species vary according to the protocol used for susceptibility testing, there are evidences of a very low susceptibility of these yeasts to FLZ, VOR and echinocandins (reviewed in Theelen et al., 2018; Bond et al., 2020). Although *Malassezia* species show differences in their antifungal susceptibility *in vitr*o, the *in vivo* efficacy of antifungal agents needs to be further evaluated by comparing *in vitro* MICs and clinical outcomes. Correlation of high azole MIC values for *Malassezia* spp. with unsuccessful treatment has been reported in some studies (Velegraki et al., 2005; Iatta et al., 2014a; Rojas et al., 2014). The above results need to be further validated with multicentre studies in order to develop therapeutic guidelines. For the moment, it is important to be aware that the genus *Malassezia* comprises a heterogeneous group of species and genotypes that may cause the same pathology, but may vary in their susceptibility to different antifungal agents. Species identification is of paramount importance not only for epidemiological surveillance and outbreak investigation, but also when therapy failure is registered. Interestingly, AmB is among the preferred therapeutic options for the first-line approach, mainly in patients under FLZ prophylaxis (Iatta et al., 2014b). Liposomal-AmB seems to be the most active drug due to the lipophilic nature of these yeasts even if *in vitro* resistance phenomena were registered for L–AmB. The favorable outcome of patients after therapy with micafungin and L-AmB followed by FLZ, might suggest *in vivo* synergism between these drugs as has been previously reported for *Candida* spp. (Rosato et al., 2012; Iatta et al., 2015). Although the observed frequency of systemic *Malassezia* infections is not very high, this may in part be due to underdiagnosis. Use of FLZ prophylaxis with reduced susceptibility for this drug, and the increase of immunocompromised patients, may lead to a higher number of observed *Malassezia* BSIs in the future. Regardless, emerging infections need to be timely diagnosed to aid clinicians in better patient management. The persistence of *Malassezia* yeasts on incubator surfaces and on the hands of health care workers or parents suggested the need for punctilious hygienic measures (Chang et al., 1998; Iatta et al., 2014a,b). The role of lipid infusion in aiding the spread of different *Malassezia* species infections should be also explored. The lack of sufficient literature showing the prevalence of *Malassezia* fungemia, low specificity and sensitivity of different blood culture systems used for the diagnosis of this fungal sepsis, the lack of standardized methods for *in vitro* antifungal susceptibility testing, as well as the lack of studies investigating drug resistance phenomena in these yeasts, call for further studies drafting guidelines for the diagnosis and correct management of *Malassezia* related diseases. Until specific guidelines for diagnosis and treatment of *Malassezia* bloodstream infections are available, clinicians must be aware of the patient population at risk for these infections and they must communicate to the laboratory the need to include special procedures to recover the organisms. Importantly, commonly used culture media in the clinic do not include lipid supplementation, which is needed for *Malassezia* yeasts to grow due to their lipid dependence. Finally, the very low susceptibility of some of these yeasts to azole drugs (i.e., FLZ and VOR) and echinocandins should be considered when a long term or prophylactic therapy is expected to be used. As stated before, *Malassezia* are the major fungal component of the human skin microbiome but for a long time the vast majority of scientific research focused on the role of bacterial microbiota in health and disease. In recent years, the role of fungi attracted more attention regarding their interplay with the human host, but also with other members of the microbiome. Any alteration in either host or microbiota can result in infections and some recent studies emphasized a role for *Malassezia* spp. in other parts of the body, and linked to other diseases known until then (Kong and Segre, 2020). One study reported a role for *M. restricta* in Crohn's disease, observing higher relative abundances of intestinal *Malassezia* compared with healthy controls, evoking inflammatory responses through CARD9 signaling (Limon et al., 2019). Though a few recent studies reported dysbiosis signatures of mycobiota in colorectal cancer (CRC) with enrichment of *Malassezia* in CRC compared with controls (Gao et al., 2017; Coker et al., 2019); a new study, for the first time, showed direct proof for the involvement of *Malassezia* in the pathogenesis of cancer. A much increased fungal community in pancreatic ductal adenocarcinomas (PDAs) was significantly enriched for *Malassezia*. Removal of the mycobiome reduced tumor growth, and only repopulation with *Malassezia* accelerated oncogenesis. PDA progression was dependent on mannose-binding lectin

(MBL), which binds to glycans of the fungal cell wall to activate a part of the immune system called the complement cascade (Aykut et al., 2019). The relationship between *Malassezia* and the host is complex and more research is needed to understand the various roles that *Malassezia* may express in/on the human host, and the conditions that trigger them. However, the above mentioned recent advancements have paved the way with new tools and insights that may also benefit a better understanding of *Malassezia* bloodstream infections.

AUTHOR CONTRIBUTIONS

CC planned, wrote, and contributed to the critical review of the manuscript. WR and BT performed an initial electronic search and drafted and edited the manuscript. CC, BT, TB, and DO performed data cleaning and reviewed the manuscript. CC and BT approved the manuscript for submission. All authors contributed to the article and approved the submitted version.

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REVIEW
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Approaches for Genetic Discoveries in the Skin Commensal and Pathogenic *Malassezia* Yeasts

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Malassezia includes yeasts belong to the subphylum Ustilaginomycotina within the Basidiomycota. *Malassezia* yeasts are commonly found as commensals on human and animal skin. Nevertheless, *Malassezia* species are also associated with several skin disorders, such as dandruff/seborrheic dermatitis, atopic eczema, pityriasis versicolor, and folliculitis. More recently, associations of *Malassezia* with Crohn's disease, pancreatic ductal adenocarcinoma, and cystic fibrosis pulmonary exacerbation have been reported. The increasing availability of genomic and molecular tools have played a crucial role in understanding the genetic basis of *Malassezia* commensalism and pathogenicity. In the present review we report genomics advances in *Malassezia* highlighting unique features that potentially impacted *Malassezia* biology and host adaptation. Furthermore, we describe the recently developed protocols for *Agrobacterium tumefaciens*-mediated transformation in *Malassezia*, and their applications for random insertional mutagenesis or targeted gene replacement strategies.

Keywords: *Malassezia*, genomics, *Agrobacterium tumefaciens*-mediated transformation (AMT), insertional mutagenesis, targeted gene replacement

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MALASSEZIA YEASTS AS COMMENSALS AND PATHOGENS

Malassezia includes a monophyletic genus of yeasts that are the main fungal species resident on human skin and hair, representing more than 90% of the eukaryotic components of the skin microbiome (Findley et al., 2013). To date, 18 species of *Malassezia* have been identified (Theelen et al., 2018). The limited number of species isolated so far most likely reflects the difficulties in cultivating *Malassezia* under laboratory conditions, given their ability to grow *in vitro* only in the presence of exogenous lipids, and at a narrow range of temperatures. As commensal organisms living on the skin, *Malassezia globosa*, *Malassezia restricta*, and *Malassezia sympodialis* are the most common species found in humans, followed by *Malassezia furfur, Malassezia yamatoensis, Malassezia dermatis, Malassezia obtusa, Malassezia japonica*, and *Malassezia arunalokei*. *Malassezia pachydermatis* is mainly found in dogs and cats, *Malassezia slooffiae* in pigs and cats, *Malassezia nana* in cats and horses, *Malassezia caprae* in goats, *Malassezia equina* in horses, *Malassezia cuniculi* in rabbits, *Malassezia brasiliensis* and *Malassezia psittaci* in parrots, and *Malassezia vespertiliones* in hibernating bats (Theelen et al., 2018; Guillot and Bond, 2020). Aside from their commensal lifestyle, *Malassezia* yeasts are associated with a number of skin disorders, the most common of which are dandruff/seborrheic dermatitis, atopic eczema, pityriasis versicolor, and folliculitis. Occasionally, in immunocompromized hosts or patients receiving total parenteral nutrition, *M. furfur, M. sympodialis*, and *M. pachydermatis* can also cause systemic disease (Gaitanis et al., 2012; Saunders et al., 2012; Velegraki et al., 2015; Theelen et al., 2018; Guillot and Bond, 2020). Moreover, novel

studies have linked *Malassezia* yeasts with Crohn's disease in patients with an S12N polymorphism in the gene encoding CARD9, a signaling adaptor critical for innate antifungal immunity (Limon et al., 2019), with pathogenesis of pancreatic ductal adenocarcinoma through activation of the MBL pathway (Aykut et al., 2019), and with cystic fibrosis pulmonary exacerbation (Soret et al., 2020).

EVOLUTIONARY TRAJECTORY OF *MALASSEZIA* GENOMES CORRELATES WITH PATHOGENICITY AND NICHE ADAPTATION

In the last decade several groups contributed to generate genomics data for the majority of species within the *Malassezia* genus. A GenBank search (last accessed on March 7th, 2020) finds 45 genome assemblies that include 15 known *Malassezia* species (Xu et al., 2007; Gioti et al., 2013; Triana et al., 2015; Wu et al., 2015; Park et al., 2017; Zhu et al., 2017; Lorch et al., 2018; Cho et al., 2019; Morand et al., 2019; Sankaranarayanan et al., 2020). Analysis of the genomes available contributed to resolve *Malassezia* taxonomy, and shed light on the evolutionary trajectory of pathogenesis and niche adaptation of this unusual fungal genus.

Taxonomically *Malassezia* are included in the subdivision Ustilaginomycotina within the Basidiomycota phylum, which also includes human and plant pathogens (Wang et al., 2014; Wu et al., 2015). Surprisingly, from a phylogenetic viewpoint *Malassezia* fungi are more closely related to the basidiomycete plant pathogen *Ustilago maydis* than the human pathogen *Cryptococcus neoformans*, and are very divergent from other fungi that are found on the skin, such as the dermatophytes and *Candida albicans* (Xu et al., 2007; Saunders et al., 2012; Wu et al., 2015). Within the *Malassezia* genus we found three clades that include two sister clades, clade A and clade B, with clade A including subclades A1 and A2, and clade C that includes early-divergent species (**Figure 1A**). Phylogenetic relationships of the tree of **Figure 1A** based on D1D2 domains of LSU rDNA agree with the previous phylogenomics data (Wu et al., 2015; de Hoog et al., 2017; Lorch et al., 2018; Theelen et al., 2018).

All haploid *Malassezia* species have small and compact genomes compared to other phylogenetically related fungi (7–9 Mb compared to ∼20 Mb) (**Figure 1B**), with genes being arranged very close to each other, and containing very short introns. At the karyotype level, haploid *Malassezia* species have from 6 to 9 chromosomes, based on pulsed-field gel electrophoresis (PFGE) and telomere-to-telomere genome assemblies generated with PacBio long-read sequencing technology (Boekhout and Bosboom, 1994; Boekhout et al., 1998; Sankaranarayanan et al., 2020). Using a combination of genomics, biochemical, cell biology, and molecular genetics techniques (described later in the text), Sankaranarayanan and colleagues elucidated the mechanisms of karyotype evolution within the *Malassezia* genus. In particular, the authors proposed an ancestral state of 9 chromosomes and two distinct mechanisms of chromosome number reduction that involve newly-identified AT-rich, fragile, centromeres: a chromosome breakage followed by loss of centromere that gave rise to 8 chromosomes in *M. sympodialis* and closely related species; and centromere inactivation accompanied by changes in DNA sequence following chromosome-chromosome fusion that gave rise to 7 chromosomes in *M. furfur* (Sankaranarayanan et al., 2020). It is intriguing to note that species with 9 chromosomes, such as *M. globosa* and *M. restricta*, are difficult to isolate and replicate in axenic conditions, while *M. sympodialis* and *M. furfur* are more readily cultivated.

At the gene level, comparative genomics revealed extensive turnover events, with significant gene loss and gene gain. Some *Malassezia* species have lost nearly 800 genes and have <4,000 predicted genes. All species have lost genes for lipid metabolism, including fatty acid synthase, Δ9-desaturase, and $\Delta^{2,3}$ -enoyl-CoA isomerase, hence explaining *Malassezia* lipid dependency (**Figure 2**); *M. pachydermatis* has also lost the genes for lipid metabolism but is the only known *Malassezia* species that is able to grow *in vitro* without the addition of exogenous lipids (**Figure 2**); however, a recent study identified some *M. pachydermatis* isolates that are unable to grow in synthetic medium without lipids (Puig et al., 2017). Other major groups of lost genes include those encoding glycosyl hydrolases and enzymes involved in carbohydrate metabolism, concordant with the evolution of a skin-adapted fungus that uses lipids as carbon sources. Moreover, the *Malassezia* genomes have a low density of transposable elements, and they lack core genes of the RNA interference (RNAi) pathway, such as dicer, argonaute, and RNA-dependent RNA polymerase.

Because the lack of the RNAi pathway in other fungi such as *Saccharomyces cerevisiae* and *U. maydis* is associated with the presence of dsRNA viruses (Drinnenberg et al., 2011), it was hypothesized that *Malassezia* species could also harbor mycoviruses. Corroborating this hypothesis, dsRNA mycoviruses of the Totiviridae family were found in *M. sympodialis, M. globosa, M. obtusa, M. pachydermatis, M. yamatoensis*, and *M. restricta* (Clancey et al., 2019; Park et al., 2019). In *M. sympodialis,* the viral genome includes two dsRNA elements, one of 4.6 kb that encodes an RNA-dependent RNA polymerase and a capsid protein, and one of 1.4 kb that encodes a novel unknown protein predicted to be secreted from the fungal cells and involved in host-pathogen and/or microbial interactions. Fungal cells can be cured of the mycovirus upon exposure to high temperature. Transcriptomic analysis of infected and cured strain pairs revealed that the presence of the mycovirus strongly enhances the expression of ribosomal genes, suggesting that the virus conscripts the *Malassezia* transcription and protein synthesis machineries. Lastly, the presence of the *Malassezia* mycovirus correlated with higher pathogenicity in *ex vivo* models (Clancey et al., 2019; Park et al., 2019).

With respect to gene gain, several unique events found in *Malassezia* genomes warrant consideration. First, a set of 44 *Malassezia*-specific gene clusters was identified, but unfortunately most of them have unknown functions that could not be predicted through bioinformatics analyses (Wu et al., 2015). One gene gain event that Wu and colleagues described regarded a gene with a PF06742 domain of unknown function.

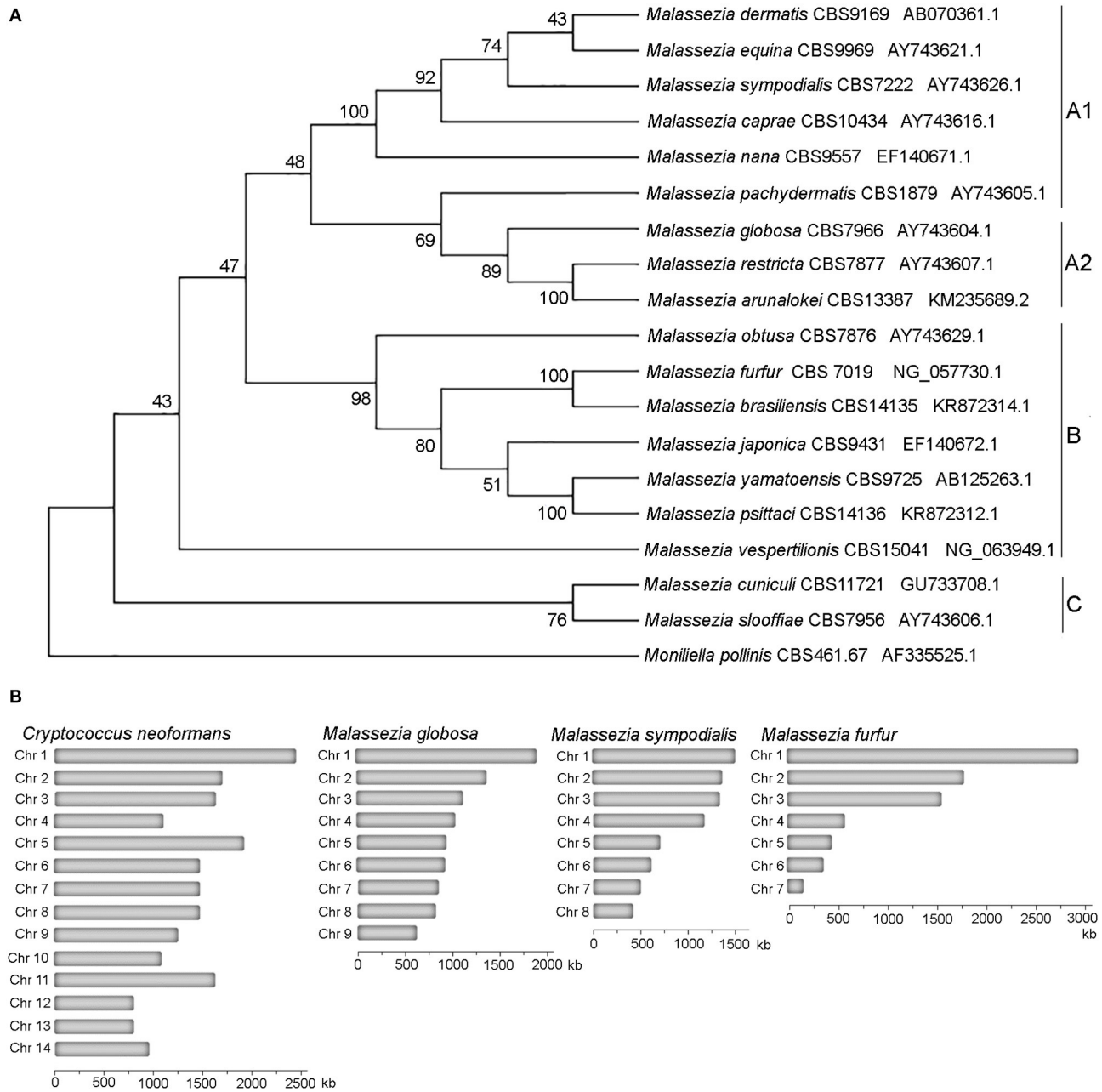

FIGURE 1 | Phylogeny of *Malassezia* yeasts. **(A)** Topology of the type strains of the eighteen recognized *Malassezia* species. The evolutionary history using sequences of the D1D2 domains of the LSU rRNA gene was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (−3126.28) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.1690)]. The rate variation model allowed for some sites to be evolutionarily invariable [(+I), 27.23% sites]. The analysis involved 19 nucleotide sequences. There were a total of 718 positions in the final dataset. **(B)** Karyotypes of representative *Malassezia* species with 9, 8, and 7 chromosomes compared to the Basidiomycete human pathogen *C. neoformans*.

This gene is conserved in all *Malassezia* species and is absent in all Basidiomycota, suggesting its acquisition by a *Malassezia* ancestor and an important role in *Malassezia* evolution (Wu et al., 2015).

Second, *Malassezia* genomes are characterized by a significant expansion of lipase, phospholipase, peptidase, and protease gene family-encoding products predicted to break down lipids and proteins for growth, and to play roles in host and microbial

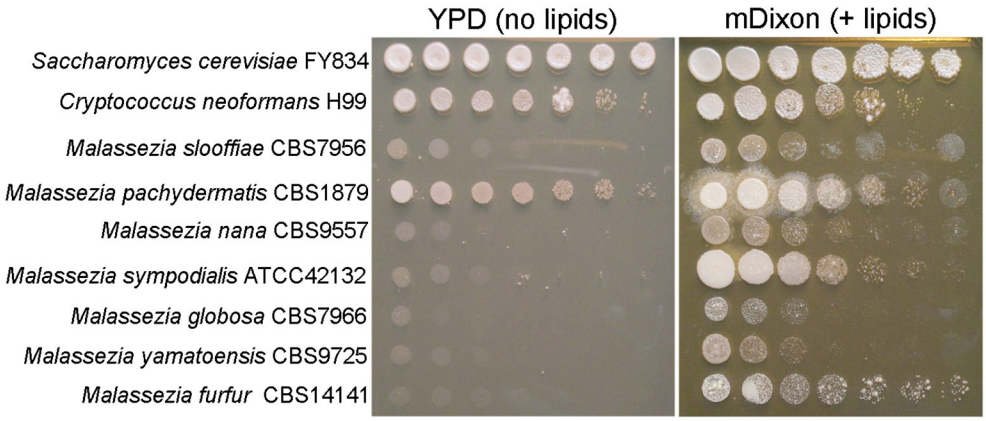

FIGURE 2 | *Malassezia* yeasts are lipid dependent. Ten-fold serial dilution of representative *Malassezia* species on medium without exogenous lipids (YPD, yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L, agar 20 g/L), and on lipid-rich medium mDixon (36 g/L malt extract, 10 g/L desiccated ox-bile, 10 g/L mycological peptone, 2 ml/L glycerol, 10 ml/L Tween 60, agar 20 g/L).

interactions (Wu et al., 2015). Intriguingly, a similar set of enzymes is found in the genome of *C. albicans*, a phylogenetically distant fungus that also lives on the skin, suggesting an important role in skin colonization and niche adaptation. Moreover, analysis of the *M. sympodialis* and *M. globosa* genomes identified 89 and 169 predicted secreted proteins, most of them without any domain (Schuster et al., 2018). These predicted secreted proteins include several MalaS allergens, such as MalaS1, a β-propeller-folded protein that has fungal orthologs/homologs in some basidiomycetes and ascomycetes (Vilhelmsson et al., 2007; Gioti et al., 2013), MalaS12 that is similar to other fungal GMC oxidoreductases (Zargari et al., 2007) that play diverse roles in fungi, such as mycotoxin biosynthesis in species of *Aspergillus* and *Penicillium* (Tannous et al., 2017), and MalaS7 (in 3 copies) and MalaS8, both of which are *Malassezia*-specific and have unknown predicted roles (Gioti et al., 2013). Besides these, *M. sympodialis* has genes encoding six additional MalaS allergens that are conserved proteins that share high similarity with the corresponding mammalian homologs, and hence can potentially cross-react with T cells and induce skin inflammation (Glatz et al., 2015).

Another characteristic of *Malassezia* genomes is the presence of bacterial genes acquired through horizontal gene transfer (HGT) events. While the number of these events is usually limited, in *Malassezia* more than 30 HGT have been identified (Wu et al., 2015; Ianiri et al., 2020). HGT candidates found in the majority of the *Malassezia* species include genes involved in broad stress resistance, such as flavohemoglobin, catalase, and oxidoreductases, found in some cases in multiple copies. An interesting HGT candidate is the gene encoding a septicolysin-like protein, which is known as a pore-forming bacterial toxin that might play a role as virulence factor (Beceiro et al., 2013; Mosqueda et al., 2014). This gene is absent in all *Malassezia* species phylogenetically related to *M. sympodialis*, and is present in five copies in *M. globosa*. Other acquired genes encode a variety of proteins with different functions, such as hydrolysis, protein transport and folding, and detoxification of xenobiotics (Ianiri et al., 2020).

Using molecular techniques described in the section *Agrobacterium tumefaciens*-Mediated Transformation Enables Insertional Mutagenesis and Targeted Gene Deletion in *Malassezia*, we demonstrated that the HGT of the bacterial flavohemoglobin in *Malassezia* resulted in a gain of function critical for resistance to nitrosative stress and nitric oxide (NO) detoxification (Ianiri et al., 2020). Analysis of the available *Malassezia* genomes revealed additional HGT of another flavohemoglobin-encoding gene that originated from different donor bacteria. Endogenous accumulation of NO in the flavohemoglobin mutant results in downregulation of the allergen-encoding genes, and accordingly, we found that flavohemoglobin has a dispensable role for *Malassezia* pathogenesis. This study represents the first functional analysis of an HGT-acquired gene in *Malassezia*, and the first evaluation of a *Malassezia* mutant in a novel murine skin model (Sparber and LeibundGut-Landmann, 2019; Sparber et al., 2019) to assess the involvement of a *Malassezia* gene in pathogenesis.

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION ENABLES INSERTIONAL MUTAGENESIS AND TARGETED GENE DELETION IN *MALASSEZIA*

Although the availability of sequenced genomes revealed insights about *Malassezia* evolution, adaptation, and gene turnover, the function of specific genes could not be studied because of the lack of transformation systems. Genetic transformation in fungi can be carried out through the combined use of lithium acetate (LiAc) and polyethylene glycol (PEG), biolistic

bombardment, electroporation of intact cells or protoplasts, or *A. tumefaciens*-mediated transformation (AMT). We tested the effectiveness of these four techniques to successfully transform *Malassezia*, but despite several attempts, AMT was the only technique that allowed the generation of stable transformants of *M. furfur, M. sympodialis*, and *M. pachydermatis* (Ianiri et al., 2016; Celis et al., 2017).

A. tumefaciens is a soil-borne bacterium that has the ability to infect plants to cause a crown gall disease. The infective process is unique and relies on the natural ability of *A. tumefaciens* to genetically engineer host plants by introducing a short DNA fragment into their genome. The DNA fragment is called T-DNA (transfer DNA) and its excision is enabled by virulence proteins induced by acetosyringone, a chemical compound that is produced by wounded plant roots and that attracts *A. tumefaciens.* The T-DNA contains genes that encode products that mimic plant hormones, and once integrated in the host genome, causes an undifferentiated growth of the plant tissues forming a tumor or gall. Researchers have exploited this natural genetic ability of *A. tumefaciens* to transfer a desired DNA molecule, usually a gene marker, into a variety of eukaryotic organisms, such as plants, animal cells, oomycetes, and fungi.

The most common use of AMT in fungal research is based on a binary vector system: one *A. tumefaciens* plasmid contains *vir* genes required for virulence (i.e., transfer of DNA into the host), and another plasmid, the Ti (tumor inducing) plasmid—usually it is a binary vector and is the most commonly manipulated by researchers—contains the marker gene between two 25-bp direct repeats (right and left borders, RB and LB, respectively) that define the T-DNA. The *vir* proteins are induced by acetosyringone and act on the T-DNA borders enabling the production of single-stranded DNA. The T-DNA is coated with proteins forming the T-complex, which is transferred into the fungal cell. The T-complex is then disassembled, and nuclear localization signals drive the translocation of the T-DNA within the fungal nucleus where integration into the genome occurs (Michielse et al., 2005) (**Figure 3A**). Compared to other transformation methods, AMT requires basic reagents that are common in most microbiology laboratories, and therefore it has been largely utilized for transformation of yeasts and fungi since its first use in *S. cerevisiae* in 1995 (Bundock et al., 1995). For more information about the method and its use in fungal biology research, there are several reviews available (Michielse et al., 2005; Frandsen, 2011; Idnurm et al., 2017).

In general, the method is straightforward: after growing the *A. tumefaciens* with the binary vector of interest and the fungal strain to be transformed, these two organisms are co-cultured on induction medium (IM) for a few days depending on the growth of the fungus, and subsequently transferred to a selective medium that differs based on the gene marker used (usually a dominant gene that confers resistance to an antifungal drug). A key role in the transformation process is played by the induction medium (IM), which contains acetosyringone to induce the *vir* genes, and it physically supports the *A. tumefaciens*-fungus co-culture ensuring the tight contact between the cells, which is a critical requirement for the success of the trans-kingdom conjugation process (Michielse et al., 2005).

While AMT is relatively simple in the majority of fungi, its use in *Malassezia* turned out to be more difficult because of the unique biology of this fungus. The first successful application of AMT was carried out in *M. furfur*, one of the species that displays more robust growth compared to others within the *Malassezia* genus (Ianiri et al., 2016). The method employed followed a previously published protocol (Ianiri et al., 2011), with the only difference being the use of a modified IM (mIM) that also included exogenous lipids (i.e., Tween and ox-bile) to favor growth of *Malassezia*. Stable *Malassezia* transformants could be generated for the first time, although the efficiency of the ATM was very low (<5 transformants per transformation plate). The AMT method was then improved using a higher density of *Malassezia* cells, a longer co-incubation period of up to 6 days, and by performing the co-incubation step on slightly concave spots generated on nylon membranes placed on the modified IM. The latter modification was critical to facilitate cell-to-cell contact between bacterial and *Malassezia* cells, which was otherwise hindered by the presence of Tween in the modified IM. Subsequently, the AMT protocol was further optimized by Celis et al. (2017) and Ianiri et al. (2019), as illustrated in detail in the flow charts of **Figures 3B–D**. Examples of representative steps of the AMT of *Malassezia* are shown in **Figure 3E**, and representative NAT-resistant *M. furfur* transformant are shown in **Figure 3F**. Lastly, we could never obtain transformants for *M. globosa*, a species characterized by very slow growth at a limited range of temperatures (30–34°C) (unpublished data).

Several binary vectors proved to be effective for *Malassezia* transformation. Plasmids pAIM2 and pAIM6 were generated through fusing the *ACT1* promoter and terminator of *M. sympodialis* with the *NAT* and *NEO* genes to confer resistance to nourseothricin (NAT) and neomycin sulfate G418 (NEO), respectively (**Figure 4A**) (Ianiri et al., 2016). Celis and colleagues successfully employed plasmid pBHg that includes the *Escherichia coli hpt* gene under the control of the *Agaricus bisporus* promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene to confer resistance to hygromycin B (HYG) (**Figure 4B**). Vector pBH-GFP-ActsPT further includes the *eGFP* gene from *Aequorea victoria* under the control of the *ACT1* promoter and terminator of *A. bisporus* (**Figure 4C**). Another vector that encodes a fluorescent marker includes a *Malassezia* codon-optimized mCherry gene fused with the *NAT* marker, as in plasmid pAIM2, through a P2A sequence (Goh et al., 2020); P2A is a 2A self-cleaving peptide derived from the porcine teschovirus-1 that was used to guarantee high expression of both the *NAT* and mCherry genes. These gene markers were also modified and reassembled to perform protein localization and chromatin immunoprecipitation (ChIP) through the generation of both N-terminal and C-terminal GFP fusion proteins, and 3xFLAG-tagged proteins (Ianiri et al., 2020; Sankaranarayanan et al., 2020).

One of the greatest advantages of AMT for *Malassezia* is its efficacy for approaches of both random insertional mutagenesis and targeted mutagenesis, which is not common for Basidiomycota fungi such as *C. neoformans* (McClelland et al., 2005). Insertional mutagenesis is carried out through AMT of a *Malassezia* species with one of the binary vectors

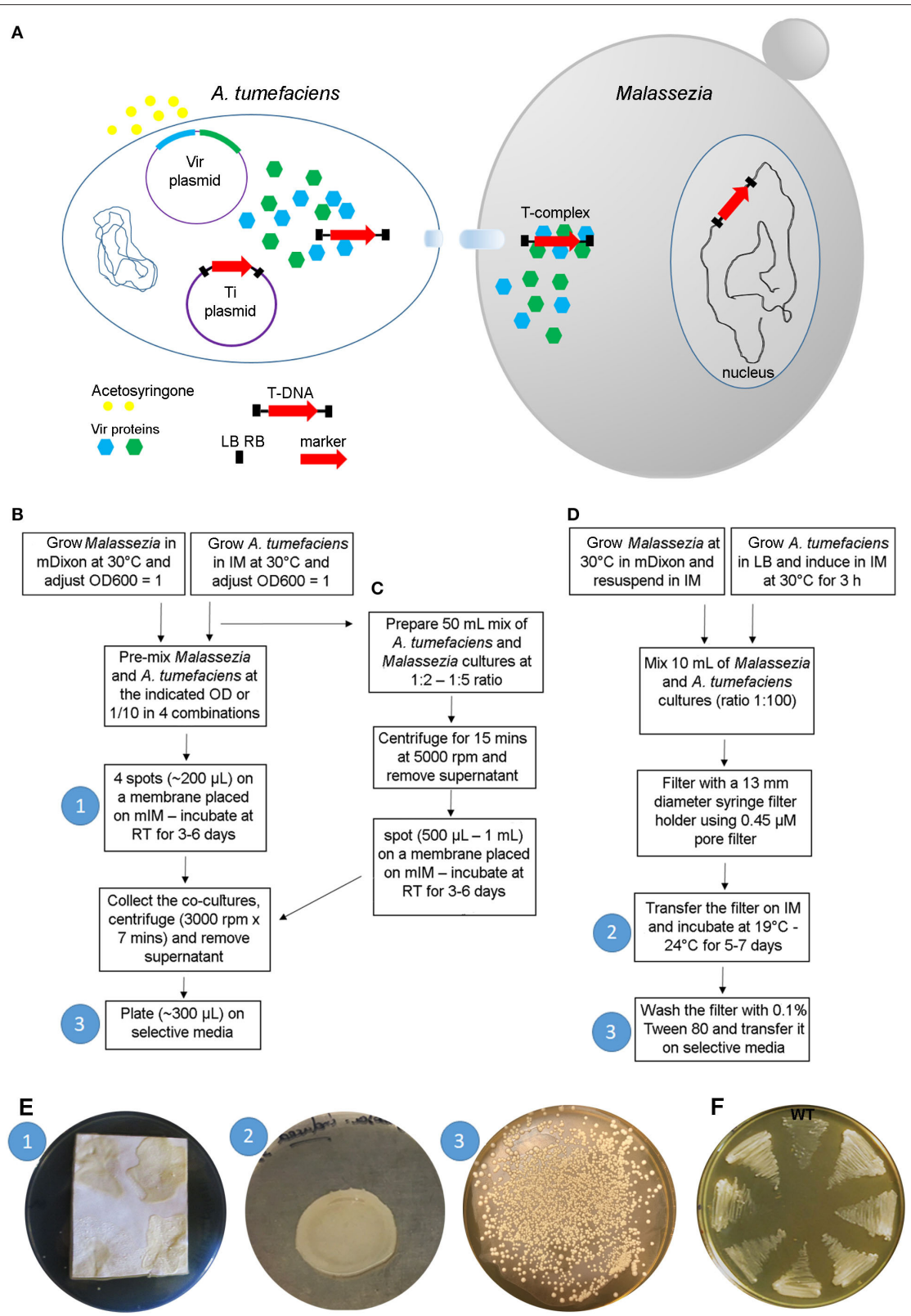

FIGURE 3 | *A. tumefaciens*-mediated transformation of *Malassezia*. **(A)** Schematic overview of the transformation process; see text for details. **(B–D)** Main steps of the protocols available for AMT of *Malassezia* according to Ianiri et al. (2016) **(B)**, Ianiri et al. (2019) **(C)**, and Celis et al. (2017) **(D)**; the white numbers in the blue circle

(Continued)

FIGURE 3 | reflect the corresponding step shown in images in **(E)**. **(E)** Representative pictures of the *Malassezia-A. tumefaciens* co-incubation step (1) as described in Ianiri et al. (2016), (note the non-homogenous spots due to the presence of Tween that altered the physical proprieties of the IM agar) and (2) in Celis et al. (2017), and example of a highly efficient AMT of *M. furfur* with selection on NAT (3); the white numbers in the blue circles reflect the corresponding steps displayed in the charts shown in **(B–D)**. **(F)** Growth of 7 representatives NAT-resistant M. furfur transformants on NAT selective media compared to the *M. furfur* WT strain.

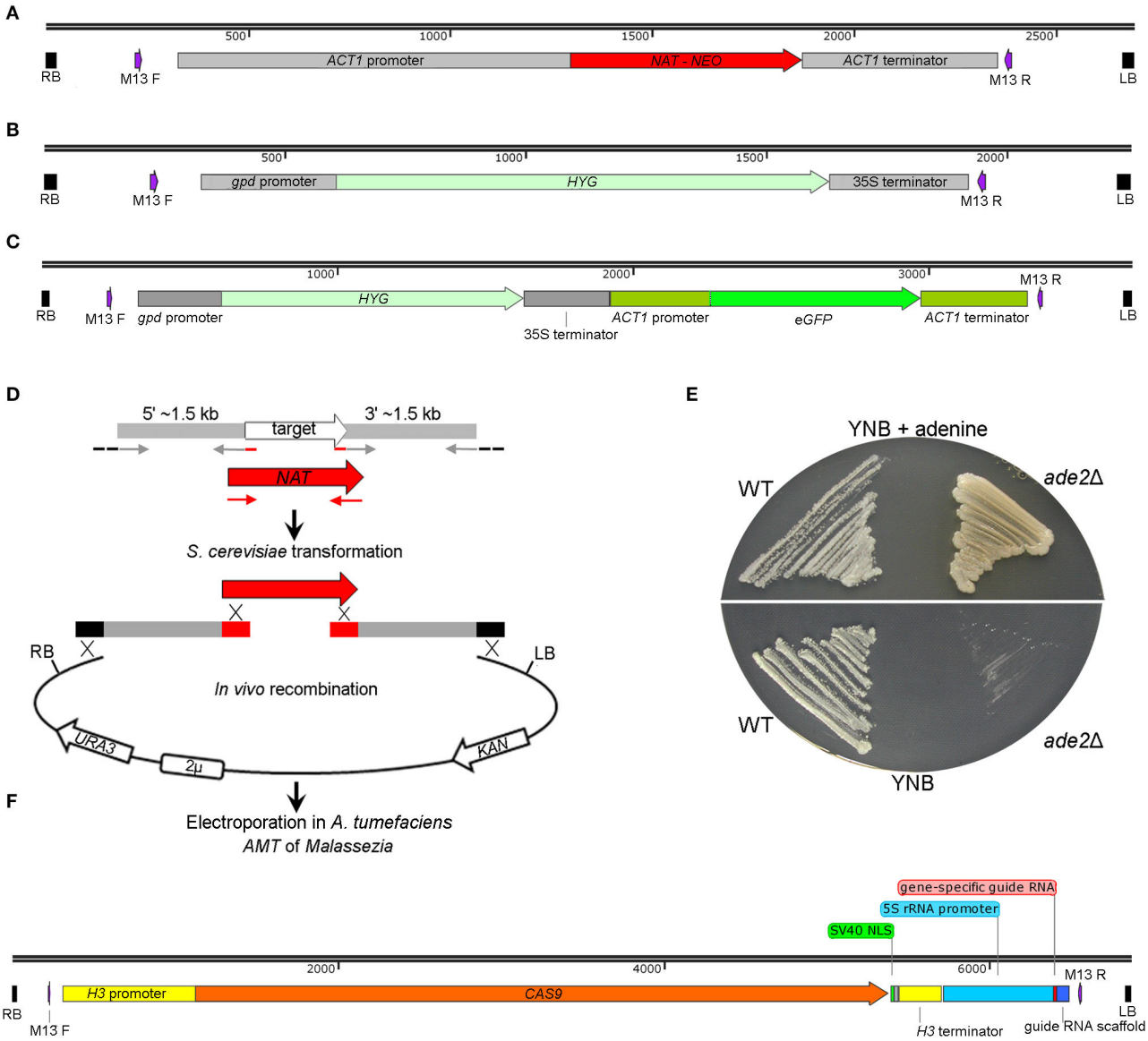

FIGURE 4 | Plasmids available for *A. tumefaciens*-mediated trasformation of *Malassezia.* **(A)** T-DNA of the plasmids pAIM2 and pAIM6 conferring resistance to NAT and NEO, respectively, as reported by Ianiri et al. (2016). **(B)** T-DNA of plasmid pBHg conferring resistance to HYG, and in **(C)** the same T-DNA including also a e*GFP*-expression cassette, as reported by Celis et al. (2017). **(D)** Strategy for *in vivo* recombination in *S. cerevisiae* developed to generate plasmids for targeted gene replacement in *Malassezia;* the schematic representation is adapted from Ianiri et al. (2016). **(E)** *M. furfur ade2*Δ mutants generated through AMT in Ianiri et al. (2016); note the different growth pigmentation of the *ade2*Δ mutant compared to the WT strain on mYNB supplemented or not with adenine (mYNB stands for "modified YNB", which includes Tween 60, Tween 20, and ox-bile). **(F)** T-DNA of the plasmid pGI40 used by Ianiri et al. (2019) for transient CRISPR/Cas9-mediated targeted gene replacement in *M. furfur*.

described above, selection of stable drug-resistant transformants to be screened for a phenotype of interest, and identification of the genes that bear the random T-DNA insertion within the *Malassezia* genome; PCR-based techniques (inverse PCR and/or Splinkerette PCR) or whole-genome sequencing can be used to identify the site of insertion of the T-DNA (Idnurm

et al., 2004; Ianiri et al., 2011; Ianiri and Idnurm, 2015). The random insertional mutagenesis approach was applied mainly in *M. furfur* and allowed the identification of (i) transformants unable to grow on minimal medium with T-DNA insertions in the *TYR1* and *ARG1* genes, (ii) a temperature-sensitive transformant with a T-DNA insertion in the promoter region of the *JEN1* gene, (iii) a UV-sensitive transformant with a T-DNA insertion in the *CDC55* gene, and (iv) several other transformants sensitive to the antifungal drug fluconazole, heavy metals, and cell wall stressing compounds (Ianiri et al., 2016, 2019). Insertional mutagenesis has the advantage that it can be used to discover novel genes and phenotypes; conversely, it has the disadvantage that transformants selected might have irregular and/or multiple T-DNA insertions and chromosomal rearrangements, factors that hinder the correct association between the mutated genes and the observed phenotypes. For such situations, in *Malassezia* the mutant phenotype can be confirmed through the *de novo* generation of a targeted deletion mutant for the identified gene, as we recently described (Ianiri et al., 2019).

Gene disruption mutagenesis involves the generation of a specific targeted mutant for a defined gene via homologous recombination. The first step is the generation of a gene deletion construct that includes ∼ 1–1.5 kb of sequence homologous to the regions flanking the gene of interest fused with a gene marker; when recipient organisms are transformed with this allele, homology with the flanking regions allows homologous recombination and the replacement of the target gene with the gene marker. For the use of AMT for targeted gene replacement, the gene deletion allele has to be assembled and cloned within the T-DNA of a binary vector. Although this can be achieved using several approaches, for gene deletion in *Malassezia* we developed a high-throughput strategy based on *in vivo* recombination in *S. cerevisiae* to simultaneously assemble and clone the gene replacement cassette within the T-DNA of a shuffle plasmid (Ianiri et al., 2017b). Briefly, three PCR fragments that include the gene marker gene and the 1.5 kb upstream (5′) and downstream (3′) regions flanking the target genes, and the KpnI-BamHI digested pGI3 plasmid, are transformed in *S. cerevisiae* wherein endogenous recombination is enabled by homologous regions between the PCR fragments and the digested plasmid (Ianiri et al., 2016), (**Figure 4D**).

In our first attempt, we tested the feasibility of AMT to generate *M. furfur* targeted mutants for the *ADE2* gene, which was chosen because mutations in this gene result in a differential pigmentation compared to the WT hence allowing rapid evaluation of the results. We obtained several *M. furfur ade2*Δ mutants that displayed adenine auxotrophy and a pigmentation that varied from light pink on rich media to yellow on minimal medium supplemented with adenine (**Figure 4E**), which is different from other yeasts such as *S. cerevisiae* (Zonneveld and van der Zanden, 1995). Subsequently, we applied this approach to study the function of the *M. furfur* laccase-encoding gene *LAC1* expected to play a role in pathogenesis (Ianiri et al., 2016), to elucidate the mechanisms of resistance of *M. sympodialis* to calcineurin inhibitors through mutations of the *FKB1* and *MSH2* genes (Ianiri et al., 2017a), and to demonstrate that the HGT-mediated acquisition of the flavohemoglobin gene *YHB1* in *M. sympodialis* resulted in a gain of function as described above (Ianiri et al., 2020). Other mutated genes under investigation were the allergen-encoding gene MalaS8 in *M. sympodialis*, and the Rim101-alkaline pathway genes *RIM101* and *RRA1* in *M. sympodialis* and in *M. furfur* (unpublished data).

During the generation of these deletion mutants, we observed a lower rate of homologous recombination (HR) in *M. furfur*, about ∼50%, compared to *M. sympodialis*, which had homologous recombination rates ranging between 90 and 100%. While the mechanisms that control the rate of HR in these fungi are unknown and worthly of further investigation, in some cases we were unable to generate targeted mutants in *M. furfur*, especially for large genes. For these reasons, a novel CRISPR/Cas9 system to increase the rate of HR and efficiently generate targeted mutants in *M. furfur* was developed. The system is based on co-transformation of *M. furfur* mediated by two *A. tumefaciens* strains to deliver both a CAS9-gRNA construct that induces double-strand DNA breaks, and a gene replacement allele that serves as a homology-directed repair template. The binary vector for Cas9 expression, pGI40, consists of the *CAS9* gene fused with the histone *H3* promoter and terminator of *M. sympodialis*, followed by the *M. sympodialis* 5S rRNA promoter fused with a gene-specific guide RNA, and a guide RNA scaffold (Ianiri et al., 2019), (**Figure 4F**). Using our AMT protocol, targeted deletion mutants for the *M. furfur* genes *CDC55* and *PDR10* were readily obtained with a HR rate of 100 and 83%, respectively; note that *PDR10* is large gene (∼5 kb) and such a high rate of HR was achieved using shorter flanking regions of 800 bp.

CONCLUDING REMARKS

Malassezia yeasts are attracting the interest of both basic and applied scientists because of their unique biological features, and importance in clinical and cosmetic settings. The availability of genome assemblies and robust tools for genetic manipulation allows both insertional mutagenesis and targeted gene replacement to be conducted. Results from these experiments can be combined with the increasing availability of transcriptomic data, with the possibility to focus further studies on novel key genes that characterize the *Malassezia* fungi. Moreover, from a more clinical perspective, tools for genetic manipulation can be combined with the use of host-pathogen interaction models, such as the easy-to-use wax moth larvae of *Galleria mellonella* (Torres et al., 2020), or a more complex murine skin model (Sparber and LeibundGut-Landmann, 2019; Sparber et al., 2019), enabling the characterization of both the fungal components that trigger skin damage and inflammation, and the inflammatory and antifungal response of the host to prevent fungal infection through immunological and molecular analyses of experimentally infected tissue.

AUTHOR CONTRIBUTIONS

GI and JH planned the review, read, and approved the final version. GI wrote the initial draft. All authors contributed to the article and approved the submitted version.

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Host Responses in an *Ex Vivo* Human Skin Model Challenged With *Malassezia sympodialis*

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Malassezia species are a major part of the normal mycobiota and colonize mainly sebum-rich skin regions of the body. This group of fungi cause a variety of infections such as pityriasis versicolor, folliculitis, and fungaemia. In particular, *Malassezia sympodialis* and its allergens have been associated with non-infective inflammatory diseases such as seborrheic dermatitis and atopic eczema. The aim of this study was to investigate the host response to *M. sympodialis* on oily skin (supplemented with oleic acid) and non-oily skin using an *ex vivo* human skin model. Host-pathogen interactions were analyzed by SEM, histology, gene expression, immunoassays and dual species proteomics. The skin response to *M. sympodialis* was characterized by increased expression of the genes encoding β-defensin 3 and RNase7, and by high levels of S100 proteins in tissue. Supplementation of oleic acid onto skin was associated with direct contact of yeasts with keratinocytes and epidermal damage. In oily conditions, there was increased expression of *IL18* but no expression of antimicrobial peptide genes in the skin's response to *M. sympodialis*. In supernatants from inoculated skin plus oleic acid, TNFα, IL-6, and IL1-β levels were decreased and IL-18 levels were significantly increased.

Keywords: *Malassezia sympodialis*, skin model, immune response, cytokines, antimicrobial peptides

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INTRODUCTION

The genus *Malassezia*, previously known as *Pityrosporum*, is a group of lipophilic yeasts. *Malassezia* species are part of the normal mycobiota and colonize several regions of the body, mainly sebum-rich skin areas such as the scalp and thorax (Marcon and Powell, 1992). To date, 17 *Malassezia* species have been proposed (Sparber and LeibundGut-Landmann, 2017). *Malassezia* spp. are the most abundant genus on the skin of individuals with psoriasis and atopic eczema (Paulino et al., 2006; Paulino et al., 2008; Zhang et al., 2011) and are highly increased in seborrheic dermatitis and dandruff (DeAngelis et al., 2005; Park et al., 2012).

M. globosa, *M. furfur*, *M. restricta*, and *M. sympodialis* are the most frequent *Malassezia* species on both healthy (Findley et al., 2013) and diseased skin, such as pityriasis versicolor and seborrheic dermatitis (Prohic and Ozegovic, 2007; Prohic, 2010; Amado et al., 2013; Lyakhovitsky et al., 2013; Lian et al., 2014; Rodoplu et al., 2014). *M. sympodialis* is one of the most frequent colonizers of healthy skin (Findley et al., 2013), but is found less frequently in *Malassezia* related-diseases, such as seborrheic dermatitis and pityriasis versicolor (Prohic and Ozegovic, 2007; Prohic, 2010; Amado et al., 2013).

Most *Malassezia* species are unable to synthesize fatty acids and degrade carbohydrates and are dependent upon the acquisition of exogenous fatty acids. *Malassezia* species have a large repertoire of lipolytic enzymes such as lipases, phospholipases and esterases (Saunders et al., 2012; Sparber and LeibundGut-Landmann, 2017). Lipases hydrolyze sebum triglycerides from the host skin to release fatty acids (oleic acid and arachidonic acid). Lipase activity is significantly higher in *M. globosa* and *M. pachydermatis* than in *M. sympodialis* and *M. slooffiae*, although normal phospholipase activity is found in these species (Juntachai et al., 2009). The fatty acids oleic acid (OA) and arachidonic acid are released by phospholipase and lipase activity and have an irritating/inflammatory effect on skin (Ashbee and Evans, 2002). OA is also increased in dandruff scalps compared with non-dandruff scalps (Jourdain et al., 2016) and increases the severity of flaking (or *stratum corneum* desquamation) facilitating better penetration of *M. sympodialis* so that the fungus directly interacts with cells of the inner skin layers (DeAngelis et al., 2005).

Exogenously acquired fatty acids contribute to the formation of a thick cell wall in *Malassezia* sp. characterized by a unique lipid-rich outer layer, which contributes to triggering the immune response against this group of fungi (Thomas et al., 2008; Gioti et al., 2013). Human studies have shown that cytokine levels (IL-1α, IL-1β, IL-2, IL-4, IFN-γ, IL-10, and IL-12) in the skin of individuals with seborrheic dermatitis and *Malassezia* folliculitis were higher than levels in the skin of healthy volunteers (Faergemann et al., 2001). However, this pattern of cytokine induction varied depending on the fungal cell wall structure. Higher levels of IL-8 and lower levels of IL-10 were produced by keratinocytes *in vitro* when they were stimulated with *M. sympodialis* lacking the lipid-rich outer layer compared to the same yeasts with the outer layer (Thomas et al., 2008).

Antimicrobial peptides (AMPs) are key in the innate immune response to *Malassezia* spp. Individuals with pityriasis versicolor have significantly higher AMP levels (β-defensin 2, β-defensin 3, S100A7, and RNase7) in their skin (Brasch et al., 2014). The β-defensins are specifically increased in the *stratum corneum*, RNase7 in the *stratum granulosum*, and S100A7 in the *stratum corneum, granulosum* and *spinosum* (Brasch et al., 2014). *Malassezia* sp. also play a role in NLRP3 inflammasome activation when yeast cells are sensed by dendritic cells but not by keratinocytes (Kistowska et al., 2014). Activation of NLRP3 depends on Dectin-1 and leads to high expression of caspase-1-dependent IL-1β in dendritic cells of patients with seborrheic dermatitis (Kistowska et al., 2014). The adaptive immune response against *Malassezia* is characterized by the production of specific IgG and IgM antibodies in healthy individuals and specific IgE antibodies in atopic eczema (Glatz et al., 2015).

Atopic eczema (AE) is a chronic inflammatory disease affecting up to 20% of children and 3% of adults (Nutten, 2015). Multiple factors have been associated with AE, such as impairment of skin barrier function due to physical (scratching, skin dryness) or chemical damage (pH changes due to soap), genetic factors (mutations in *FLG* and *SPINK5* genes encoding filaggrin and serine protease inhibitor Kazal-type 5 protein, respectively), and environmental factors (cold climate, no breastfeeding, pollution) (Sääf et al., 2008; Nutten, 2015). *Malassezia* sp. has been linked with AE pathogenesis as *Malassezia* sp. allergens induce specific IgE antibodies and autoreactive T-cells that can cross-react with skin cells (Zargari et al., 2001; Glatz et al., 2015).

The *M. sympodialis* genome contains 13 allergen genes, Mala S1, Mala S5 to S13 and three orthologs of *M. furfur* allergens (Mala F2, 3, and 4) (Andersson et al., 2003; Gioti et al., 2013). Some of these allergens are highly similar to human proteins (Mala S11, Mala S13) and have been specifically linked to cross-reactive immune responses (Schmid-Grendelmeier et al., 2005; Gioti et al., 2013; Roesner et al., 2019).

The aim of this study was to investigate the host skin response to *Malassezia sympodialis.* An *ex vivo* human skin model was used either directly for non-oily skin or supplemented with oleic acid to represent oily skin, and the skin surface was scratched to represent a disrupted skin barrier. Host-pathogen interactions were analyzed by SEM, histology, gene expression, immunoassays, and proteomics.

The skin response to *M. sympodialis* was characterized by increased expression of the genes encoding β-defensin 3, and RNase7 and high levels of S100 proteins. Supplementation of the skin with oleic acid resulted in epidermal damage, direct contact of yeasts with keratinocytes, and AMP gene expression was not detected. IL-18 levels were significantly increased in supernatants from inoculated skin plus oleic acid and *IL18* gene expression was increased in tissue. TNFα, IL-6, and IL-1β levels were decreased in the same supernatants.

MATERIALS AND METHODS

Fungal Strains and Culture Conditions

Modified Dixon (mDixon) broth and agar (3.6% w/v malt extract, 2% w/v desiccated ox-bile, 0.6% w/v bacto tryptone, 0.2% v/v oleic acid, 1% v/v Tween 40, 0.2% v/v glycerol, 2% w/v bacto agar for agar plates) was used to grow *Malassezia sympodialis* (ATCC 42132) for skin experiments. Yeast cells were grown on mDixon agar at 35°C for 4–5 days in a static incubator, one colony was selected from an agar plate and inoculated into 10 ml of mDixon broth for 4 days at 37°C in a shaking incubator at 200 rpm. Yeast cells were recovered and a final inoculum of $1x10^6$ yeasts in 10 µl of PBS ($1x10^8$/ml) was prepared and applied to the skin surface.

An *Ex Vivo* Human Skin Model

The *ex vivo* human skin model was set up as previously described with modifications (Corzo-León et al., 2019). Human skin tissue (no stretch marks), without an adipose layer (adipose layer removed by the surgeon during surgery), from abdominal or breast surgeries was supplied by Tissue Solutions® Ltd. (Glasgow, UK). Human tissue from four different donors was obtained according to the legal and ethical requirements of the country of collection, with ethical approval and anonymous

consent of the donor or nearest relative. Tissue Solutions® comply with the UK Human Tissue Authority (HTA) on the importation of tissues. Explants were transported at 4°C with cooling packs and maintained at 4°C until processed, which occurred within 36 h of surgery.

Skin was washed with DMEM (supplemented with 1% v/v antibiotics (penicillin and streptomycin) and 10% heat inactivated FBS, Thermo-Fisher Scientific, Loughborough, UK) and kept moist in a Petri dish in the same medium. The explant was cut into 1 cm^2 pieces. The surface of each piece was gently wounded using a needle, without penetrating the entire skin thickness, by pricking with a needle several times (6-10) to a depth of approximately 2–3 mm.

After wounding, each piece of skin was placed into an individual well of a 6-well plate. An air-liquid interphase was maintained by adding 1 ml of supplemented DMEM. A well containing only DMEM growth medium was added as a negative control and served as a contamination control. The medium was changed every 24 h and the spent medium was stored in 2 ml tubes for subsequent analysis at the same time points as the rest of the samples. Recovered medium was stored at -80°C until analyzed. In a previous study (Corzo-León et al., 2019), the skin samples were demonstrated to remain viable for 14 days under non-oily conditions using the TUNEL system (Promega, Southampton, UK). This same assay was used to evaluate cell viability and apoptosis in 6 μm skin tissue sections from the frozen OCT blocks in this study. Apoptosis levels were measured in the four groups of skin samples in three biological replicates which were analyzed in duplicate. Apoptosis levels were averaged, statistical analysis was done using one-way ANOVA test. For post-hoc analysis, Tukey's test was used. Prism 8 software (GraphPad, La Jolla, CA, USA).

Skin explants were inoculated by applying 10 μl of fungal suspension (1 x 10^6 yeasts) directly on to the epidermis. Yeast suspension was prepared as described below and resuspended in PBS. Uninfected/non-inoculated skin controls were included in all experiments. Two additional experimental conditions were added: 1) uninfected skin with 10 μl 100% oleic acid (Oleic Acid, Extra Pure, catalogue number: O/0200/15, Thermo-Fisher Scientific) applied to the surface of the skin explant and 2) infected skin with same *M. sympodialis* inoculum, followed by application of 10 μl 100% oleic acid on to the surface of the skin explant.

Skin samples were incubated for 6 days at 37°C and 5% CO_2 before being recovered in a Petri dish. Prior to processing, the macroscopic appearance of explants was evaluated by eye and images captured with a Stemi 2000-c Stereo Microscope (Carl Zeiss, Oberkochen, Germany). Samples were then processed for further analyses.

Tissue samples for histology were placed into molds, embedded in OCT compound (Cellpath Ltd. Newtown, UK) and flash-frozen with dry ice and isopentane. These samples were stored at -20°C for immediate analysis or at -80°C for longer term storage. For scanning electron microscopy (SEM), tissue samples were fixed in glutaraldehyde buffer (2.5% glutaraldehyde in 0.1 M cacodylate) overnight at 4°C and sent to the Microscopy and Histology technology hub, University of Aberdeen, for further sample preparation. Tissue samples for RNA extraction were cut into smaller pieces and placed in a microcentrifuge tube with RNAlater® (Sigma, Dorset UK) for subsequent RNA extraction. These samples were stored at -20°C for immediate analysis or at -80° C for longer term storage. Experiments were replicated at least three times using skin from different human donors.

Scanning Electron Microscopy and Histopathology

Several microscopy analyses were performed on recovered skin tissue for histological confirmation of fungal infection. Sections (6 μm) were cut from the frozen OCT blocks for histological analysis and stained with fluorescent dyes (1 μg/ml calcofluor white (CFW), and propidium iodide (1 μg/ml). Tissue sections were also stained with Periodic acid solution (1%), Schiff reagent and counterstained with Hematoxylin solution (Sigma). Fluorescent images were captured with a DeltaVision™ confocal microscope (GE Healthcare, Buckinghamshire UK). PAS histological sections were imaged by light microscopy using a Zeiss™ Axioskop microscope. SEM samples were observed using a Zeiss™ EVO MA10 Scanning Electron Microscope. Images were generated by detection of secondary electrons (SE1) and backscatter electron detection (NTS BSD) and captured at 10 kV resolution and at different magnifications.

TUNEL Analysis to Measure Cell Viability

The percentage of apoptotic cells was determined by counting the apoptotic cells (green cells stained by fluorescein-12-dUTP) and expressing them as a percentage of the total number of cells (apoptotic cells and non-apoptotic cells stained red with propidium iodide). Three biological replicates were analyzed for each experimental condition with duplicate slides prepared for each replicate. Three fields were analyzed per slide with a total of at least 600 cells counted for each biological replicate.

Gene Expression and Proteomics Analyses

Tissue samples for RNA extraction were thawed and the RNAlater® discarded before processing the samples. Samples were washed twice with PBS. RNA and proteins were extracted from the same recovered samples in a single 2-day sequential process based on previously published methods (Chomczynski and Sacchi, 1987; Berglund et al., 2007; Corzo-León et al., 2019).

The RNA yield and purity were evaluated by Nanodrop™ spectrophotometry (Thermo-Fisher Scientific) and samples were stored at -80°C until further use. All samples had an initial yield between 200 and 800 ng/μl and a 260/280 ratio between 1.8 and 2.0. To produce cDNA, RNA samples (1 μg) were treated with DNase I (1 U/μl per 1 μg of RNA sample) (Thermo-Fisher Scientific), then reverse transcription carried out using the SuperScript™ IV first-strand synthesis system (Thermo-Fisher Scientific) with Oligo dT primer, following the manufacturer's instructions.

Intron spanning primers and qRT-PCR assays were designed using Roche's Universal Probe Library Assay Design Centre (lifescience.roche.com/en_gb/brands/universal-probe-library.html#assay-design-center) for different target genes known to be expressed in skin. Genes, accession numbers, Roche probes

paired with target primers and primer sequences are shown in **Table 1**. The Roche probes were hydrolysis probes labelled at the 5′ end with fluorescein (FAM) and at the 3′ end with a dark quencher dye. A reference gene (*B2M* encoding β2-microglobulin) primer pair and probe was also designed (Lossos et al., 2003) using the Eurogentec web tool (secure.eurogentec.com/life-science.html) (**Table 1**). The probe was modified at the 5′ end with Cy5 and at the 3′ end with quencher QXL 670.

qRT-PCR reactions (10 µl) were set up in Light cycler 480 plates using the LightCycler 480 probe master mix (Roche, Welwyn Garden City UK) according to the manufacturer's instructions (**Table 2**). Dual hydrolysis probe assays were analyzed in the same well, FAM probe was used for target gene primer pairs and Cy5 probe for the reference gene. For each cDNA, assays were performed in triplicate.

Reactions were run in a LightCycler 480 (Roche). Following manufacturer's recommendations, reaction settings were as follows: one cycle at 95°C for 10 min (ramp rate 4.8°C/s), 55 cycles of amplification phase with denaturation at 95°C for 10 s (ramp rate 4.8°C/s), annealing at 60°C for 30 s (ramp rate 2.5°C/s), extension 72°C for 1 s (ramp rate 4.8°C/s); and, finally, one cycle of cooling phase at 40°C for 30 s (ramp rate 2.5°C/s). Results obtained for each target gene were normalized against β2-microglobulin gene expression levels. The corresponding uninfected/non-inoculated skin samples (with or without OA) were used as negative controls for infection and to measure baseline gene expression levels. Results were analyzed using the 2-ΔΔCT method (Livak and Schmittgen, 2001). Statistical analysis was performed using the Student's t-test or Mann-Whitney test depending on the data distribution using Prism 8 software (GraphPad, La Jolla, CA, USA).

TABLE 2 | qRT-PCR reactions for skin samples.

Reagent	Volume (µl)	Final concentration (nM)
Master mix (Roche)	5.0	
10 µM Target primer forward	0.25	250
10 µM Target primer reverse	0.25	250
1 µM Target probe (FAM)	0.5	50
10 µM Reference primer forward	0.25	250
10 µM Reference primer reverse	0.25	250
1 µM Reference probe (Cy5)**	0.25	50
Water	1.25	–
cDNA	2.0	150–200 ng/µl
Total volume	**10 µl**	

For skin samples, dual hydrolysis probe assays were analyzed in the same well, with a FAM probe used for target genes primer pairs and a Cy5 probe for the reference gene.

Protein concentration was determined by Coomassie G-250 Bradford protein assay kit following manufacturer's instructions (Thermo-Fisher Scientific). Protein samples were sent for trypsin digestion and LC-MS/MS analysis (Aberdeen Proteomics Core Facility (www.abdn.ac.uk/ims/facilities/proteomics/). Four biological replicates were analyzed per condition (infected and uninfected skin). Only proteins having two or more identified peptides and two or more Peptide Spectrum Matches (PSM)

TABLE 1 | Primers targeting human genes.

Target Gene/Accession number	Primer	Sequence (5′-3′)
TGFB1	Primer F	TGGACATCAACGGGTTCAC
NM_000660.6	Primer R	GGCCATGAGAAGCAGGAA
	Roche hydrolysis Probe++	#49
IL18	Primer F	CAACAAACTATTTGTCGCAGGA
NM_001243211.1	Primer R	CAAAGTAATCTGATTCCAGGTTTTC
	Roche hydrolysis Probe++	#66
RNASE7	Primer F	CAGGAGTCACAGCACGAAGA
NM_032572.3	Primer R	CAGCAGAAGCAGCAGAAGG
	Roche hydrolysis Probe++	#15
S100A8/	Primer F	CAGCTGTCTTTCAGAAGACCTG
NM_001319196.1	Primer R	TGTGGTAGACGTCGATGATAGAG
	Roche hydrolysis Probe++	#78
S100A7	Primer F	CCAAACACACACATCTCACTCA
NM_002963.3	Primer R	TCAGCTTGAGTGTTGCTCATC
	Roche hydrolysis Probe++	#33
S100A9	Primer F	GTGCGAAAAGATCTGCAAAA
NM_002965.3	Primer R	TCAGCTGCTTGTCTGCATTT
	Roche hydrolysis Probe++	#85
DEFB4A	Primer F	TCAGCCATGAGGGTCTTGTA
NM_004942.3	Primer R	AGGATCGCCTATACCACCAA
	Roche hydrolysis Probe++	#35
DEFB103B	Primer F	TTCTGTTTGCTTTGCTCTTCC
NM_018661.4	Primer R	CGCCTCTGACTCTGCAATAA
	Roche hydrolysis Probe++	#85
	REFERENCE GENE	**Sequence**
β2-microglobulin	β2M Primer F*	TGACTTTGTCACAGCCCAAGATA
NM_004048.2	β2M Primer R*	CGGCATCTTCAAACCTCCA
	Probe**	ACATGTCTCGATCCCAC

*++Primers and matching probes were selected using Universal Probe Library Assay Design center. All Roche probes were hydrolysis probes labelled at the 5′ end with fluorescein (FAM) and at the 3′ end with a dark quencher dye. *Primers were designed to amplify the β2-microglobulin gene, which was used as the reference gene **Modified probe at the 3′ end with QXL 670, and at the 5′ end with Cy5.*

were selected. Finally, proteins found in at least two out of four analyzed samples per condition were included for further Gene Ontology (GO) analysis using the GO consortium online tool (geneontology.org). Area under the curve (AUC) values for each protein were averaged and compared between conditions, inoculated and non-inoculated skin, non-inoculated skin with and without oleic acid and, finally analyzed using the Student's t-test or Mann-Whitney test depending on the distribution of the data, with a value of $p<0.05$ considered statistically significant (Prism 8 software). Proteins identified as significant were compared to the CRAPome database (www.crapome.org/). The CRAPome web tool is a Contaminant Repository for Affinity Purification and contains lists of proteins identified in negative control samples, collected using affinity purification followed by mass spectrometry (AP-MS). Proteins found in the CRAPome database in >10% of the cases were not considered significant as they are probably the result of carryover contamination during mass spectrometry experiments.

Immunoassays

Recovered supernatants from inoculated skin and non-inoculated skin controls were analyzed for different cytokines (TGFβ1, TNFα, IL-1β, IL-6, IL-8, IL-18, IFNγ, and TNFRI) at 2–3 days of incubation. Four biological experiments were analyzed in duplicate. Multiplex immunoassays were carried out following the manufacturer's instructions (Milliplex ® Map kits. EMD Millipore Corporation, Livingston UK). Data were analyzed using either one-way ANOVA or Kruskal-Wallis test depending on the homogeneity of variance (tested by Bartlett's test). A p value <0.05 was considered statistically significant (Bonferroni correction) and post-hoc analysis done by Dunnett's or Dunn's test (Graphpad Prism 8).

RESULTS

M. sympodialis Invaded *Ex Vivo* Skin Supplemented With Oleic Acid and Interacted Directly With the Inner Epidermal Layer

Host-pathogen interactions between human skin and *M. sympodialis* were investigated using an explant human skin model with skin collected from four healthy donors undergoing cosmetic surgeries (Corzo-León et al., 2019). All skin samples were gently wounded by scratching the surface with a needle. Four different skin conditions were used: untreated skin left uninfected or inoculated with *M. sympodialis* (see below) or skin supplemented with 10 µl of 100% oleic acid (OA) and uninfected or inoculated with *M. sympodialis* (MS). Inoculated skin samples were inoculated with $1x10^6$ *M. sympodialis* yeast cells in 10 µl applied onto the skin surface.

Skin inoculated with yeasts without OA supplement did not show macroscopic differences when compared to the non-inoculated skin control after 6 days. The inoculated skin did not show any macroscopic changes and will be referred to as inoculated skin instead of infected skin. Meanwhile, skin inoculated with yeasts and supplemented with OA had visible yeast growth (**Figure 1**).

When analyzed microscopically by SEM, only the *M. sympodialis*-inoculated skin had fungal structures on the epidermis. The fungal structures differed between the skin supplemented with OA and skin that did not receive OA. Skin without OA (MS or *Malassezia* only) had yeast cells on the top of the skin, while skin receiving both yeasts and OA (MSOA) had not only more abundant yeasts but also elongated fungal structures (**Figure 2**).

Tissue sections were stained with propidium iodide to stain nuclei in the epidermis and calcofluor white (CFW) to stain chitin in the yeast cell walls. In parallel, sections were stained with PAS. The sections were examined to investigate the integrity of keratinocytes and presence of fungal elements. The epidermis in different samples had differences in structure. Sections of MSOA skin had detached keratinocyte layers and had yeast cells in direct contact with the inner epidermal layers. In contrast, MS skin had completely intact epidermis and yeast cells were trapped in the outer *stratum corneum* layer. Intact epidermis was also seen in the uninfected skin control without oleic acid. In uninfected skin receiving OA, thinner epidermis and damaged keratinocytes were observed, but no detachment of epidermal layers was seen (**Figure 1**).

When cell viability was evaluated by the TUNEL system, no differences were found between the uninfected skin conditions and their corresponding infected counterparts (**Figure 3**). Cell viability was affected by OA as skin samples treated with OA (either infected or uninfected) had higher numbers of cells showing apoptosis compared to untreated samples (**Figure 3**).

Local Skin Response to *M. sympodialis* Is Characterized by Higher Expression of Genes Encoding β-Defensin 3, Ribonuclease 7, and Higher Levels of S100 Proteins

RNA was extracted from skin tissue samples for gene expression analysis, at day 6 post-inoculation under the four experimental conditions described above. Due to the importance of AMPs and cytokines in the innate immune response, the expression of eight human genes encoding different AMPs and cytokines was analyzed by qRT-PCR. AMP genes included *S100A7* (psoriasin), *S100A8*, *S100A9*, *DEFB4A* (β-defensin 2), *DEFB103A* (β-defensin 3), *RNASE7* (ribonuclease 7, RNase7), *IL18*, and *TGFB1* (transforming growth factor β1). Expression of AMP genes was found only in uninfected skin and MS skin (inoculated skin without OA). No expression of AMP genes was found in samples receiving OA. We can confirm that reference genes were amplified and expressed in samples receiving OA verifying that the lack of expression of AMP genes was not due to a technical problem. In MS skin, expression of *DEFB103A* ($p \leq 0.03$) and *RNASE7* ($p \leq 0.03$) was increased 193 (IQR 139 to 843) and 7 (IQR 2 to 343) times, respectively, compared to non-inoculated, negative control skin (**Figure 4**). Meanwhile, *S100A9* was significantly ($p \leq 0.03$) decreased -10.2 (IQR -15 to -4) fold.

The four different experimental conditions (described above) were also analyzed by proteomics at 6 days post-infection to gain a better understanding of the host response to oleic acid and *M. sympodialis* and to investigate the *M. sympodialis* proteome when it was interacting with human skin. Trypsin digestion

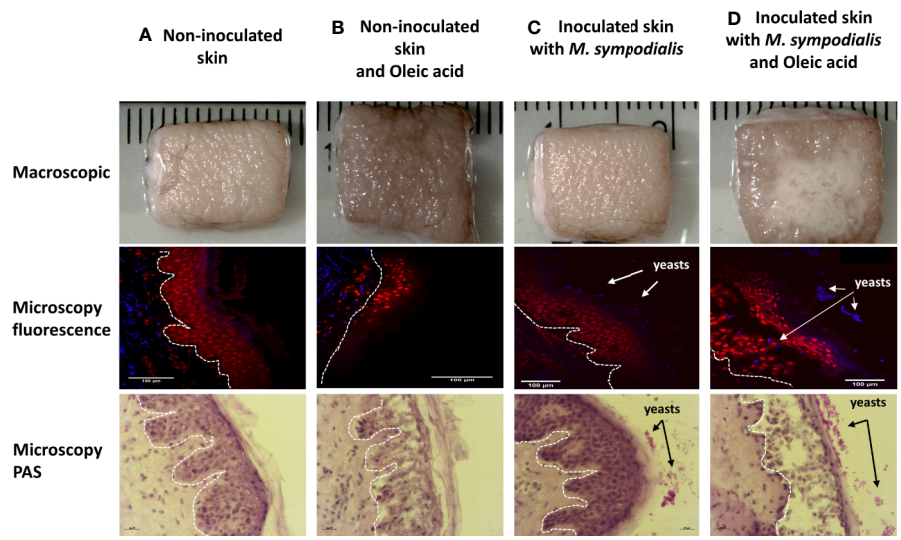

FIGURE 1 | Macroscopic and histologic appearance of skin in four different conditions. Human skin explant samples were untreated **(A, C)** or supplemented with oleic acid **(B, D)**. *M. sympodialis* (1 x 10^6 yeasts) was inoculated in **(C, D)**, with **(A, B)** uninoculated. All samples were incubated at 37°C in 5% CO_2 for 6 days. Scale is indicated by the ruler, with the space between each bar = 1 mm. Fluorescence microscopy images show propidium iodide (PI) (red) indicating keratinocytes in epidermis and CFW (blue) staining *M. sympodialis* yeast cells after 6 days incubation. Channels used were DAPI (358 nm/461 nm) for CFW, and Rh-TRITC (543 nm/569 nm) for PI. Scale bars represent 100 µm. PAS staining images are shown on the bottom row. Scale bars represent 20 µm. In all histological samples, the white line separates the dermis from epidermis. Epidermis in all images are facing right side. Yeasts are seen on the external side of the skin. Images are representative of four biological replicates per condition.

and protein identification by LC-MS/MS was performed by the Aberdeen Proteomics Facility (data in **Supplementary Material S1**, **S2**). Database searches were conducted with the Mascot server v 2.5 using *Homo sapiens* and *M. sympodialis* protein sequences (Swiss-Prot database). Data are available *via* ProteomeXchange with identifier PXD018404.

A total of 1488 proteins were found in skin tissue of the four biological experiments at the 6-day time point. After screening for proteins having ≥2 PSM and ≥2 peptides, the total number of proteins included in the analysis was 1048. Non-inoculated skin conditions (with and without OA) were compared. Most of the proteins (98%) found in the non-inoculated skin receiving OA had significantly decreased levels (-2 fold change) when compared to proteins in the non-OA non-inoculated skin control. Fifteen proteins were significantly decreased ($p \leq 0.05$) in non-inoculated skin receiving OA compared with the non-OA control from which five are involved in cornification, keratinocyte differentiation and wound healing (KRT5, KRT6A, KRT6B, KRT6C, KRT84). (**Supplementary Material S3**).

Compared to non-inoculated skin, 267/1488 (18%) proteins were greatly increased in the MS condition, while 376/1488 (25%) proteins were greatly increased in the MSOA samples compared to non-inoculated skin that received oleic acid only. Proteins with higher levels compared to their corresponding negative control (± OA) were analyzed by gene ontology. The most significantly enhanced biological processes in MS skin were cornification (fold change 27, FDR <0.0001), antimicrobial immune response (fold change 16.5, FDR <0.0001), and defense response to fungus (fold change 14, FDR 0.009). Meanwhile, for MSOA, chylomicron remodeling (fold change 38, FDR <0.0001) and removal of superoxide radicals (fold change 32, FDR <0.0001) were the most significantly increased processes (**Figure 5**). Keratins were significantly more abundant in MSOA, compared to the plus OA uninfected control and included the five keratins that were less abundant in the plus OA non-inoculated control when compared to non-inoculated control without oleic acid, mentioned above (**Figure 5**, **Supplementary Material S3**).

AMP levels were analyzed separately and compared to non-inoculated control by Kruskal-Wallis test and Dunn's test, as well as using the CRAPome online tool. RNase7 (fold change 2, IQR 1–3, CRAPome 0%, p=0.05), S100B (fold change 5, IQR 3-9, CRAPome 0, p=0.03), S100A4 (fold change 5, IQR 4-5, CRAPome 2%, p=0.01), and S100A2 (fold change 14, IQR 7–24, CRAPome 1%, p=0.05) were significantly increased in the MS skin but not in MSOA skin, where they were either decreased or undetectable (**Figure 6**). Along with these AMPs, CD99/MIC2, a glycosylated transmembrane protein (fold change 2, IQR 1.7–3.6, p=0.05, CRAPome 1%) was also found at higher levels in the MS skin (**Figure 6**).

Secreted Cytokine Responses of Human Skin Tissue Exposed to *M. sympodialis* Is Characterized by High Levels of TGFB1 and IL-18

After 2 days of incubation supernatants were recovered from the four experimental conditions and analyzed for seven different cytokines (IL1-β, IL-6, IL-8, TNFR1, TNFα, TGF1β, IL-18). Post-hoc analysis indicated no differences in IL-8 and TNFR1 levels, while IL1-β levels were significantly increased in skin treated with OA compared to MSOA skin (p=0.04) (**Figure 7**). IL-6 were significantly higher (p=0.03) in OA supernatants

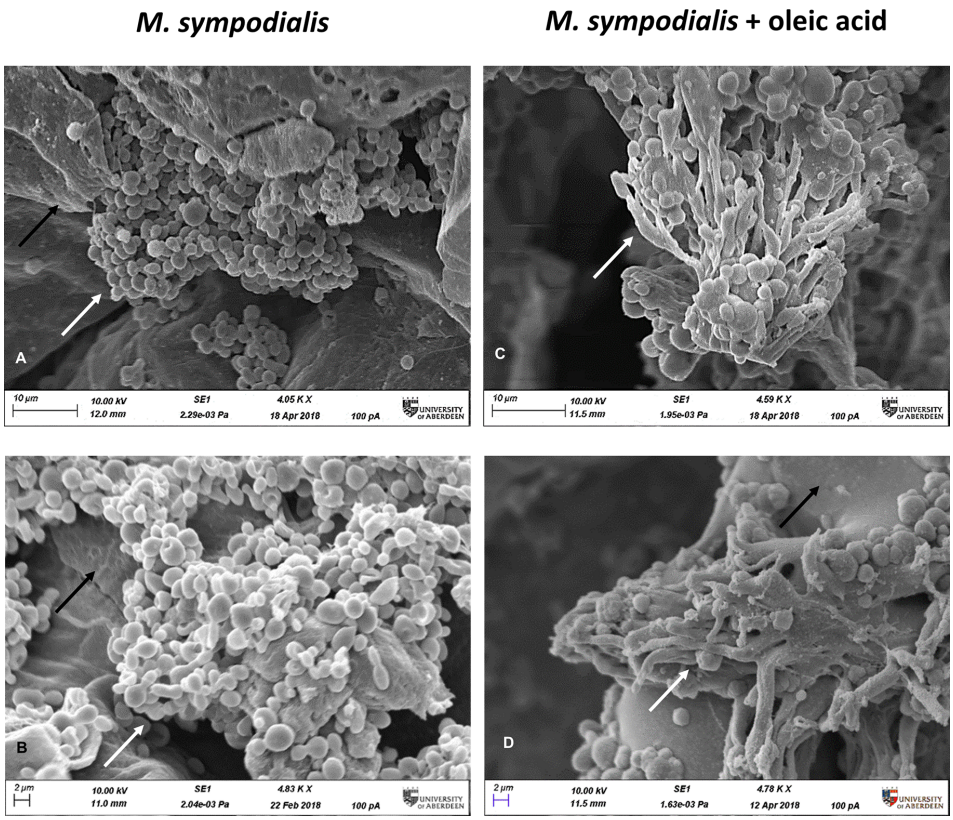

FIGURE 2 | Microscopic appearance of skin inoculated with *M. sympodialis*. *M. sympodialis* yeast cells (1 x 10^6) were inoculated onto the human skin explants, with or without OA supplementation. All samples were incubated at 37ºC in 5% CO_2 for 6 days. **(A, B)** SEM images *M. sympodialis*-inoculated skin, **(C, D)** skin co-inoculated with *M. sympodialis* and oleic acid. Black arrows indicate corneocytes (skin), white arrows indicate fungal structures. Scale bars represent 10 µm **(A, C)** or 2 µm **(B, D)**. Images are representative of four biological replicates.

compared to MSOA supernatants, but neither levels were significantly different to the non-inoculated control. TNFα levels were not different between non-inoculated control and MS skin but were significantly decreased in MSOA skin compared to OA skin (p=0.005) (**Figure 7**).

To further explore the role of IL-18 and TGF β1 a more detailed analysis of these two cytokines in tissue and supernatant samples was performed. Gene expression of *TGFB1* and *IL18* in tissue was measured by qPCR and expressed as fold changes compared to uninfected control. *TGFB1* and *IL18* were both increased in MSOA skin compared to oleic acid uninfected skin levels ($p \leq 0.01$). *TGFB1* had a fold change of 5.2 x 10^3, (IQR 1.6 x 10^3 to 1 x 10^4) in MSOA versus a fold change of -9.8 x 10^5, (IQR -4.9 x 10^6 to -1.5 x 10^2) in oleic acid uninfected skin. *IL18* had a fold change of 2.8 x 10^6, (IQR 2 x 10^6 to 4.2 x 10^6) in MSOA versus a fold change of 1.9 x 10^2 (IQR -4.0 x 10^2 to -56.6) in oleic acid uninfected skin levels. No differences were found between MS skin and its corresponding uninfected control (**Figure 8**). Immunoassays were performed to examine levels of TGFβ1 and IL-18 cytokines in supernatants. IL-18 levels were increased in supernatant samples from MSOA skin compared to the oleic acid uninfected control (73.9 pg/ml, IQR 25.1 to 135.1, vs. 8.5 pg/ml, IQR 5.8 to 11.4; p=0.03) (**Figure 8**). There were no differences in levels of TGFβ1 and IL-18 in supernatants from MS skin and uninfected control skin (**Figure 8**). TGFβ1 levels did not significantly change in supernatant samples, which contradicts what was observed in the gene expression analysis. This may be due to post-transcriptional regulation of TGFβ1. Addition of OA to uninoculated skin resulted in no significant changes in IL-18 and TGFβ1 levels as measured by RNA or protein levels.

Nine Allergens of *M. sympodialis* Were Identified in Inoculated Skin

We next examined protein levels of *M. sympodialis* allergens when the yeast was inoculated onto the skin explant model and incubated for 6 days. A total of 202 *M. sympodialis* proteins were detected by mass spectrometry and identified by matching peptide fingerprints to *M. sympodialis* (reference strain ATCC 42132) protein sequences (Swiss-Prot database). However, after filtering to include proteins found only in *M. sympodialis* inoculated skin in ≥2 biological experiments, only 48 fungal proteins were included in the final analysis.

Most of the proteins were found only in MSOA skin (32/48, 66%); 12 (25%) were uncharacterized proteins, nine (19%) were allergens, and only one lipolytic enzyme, Lipase 3, was identified in MSOA skin. The allergens found in infected skin were Mala

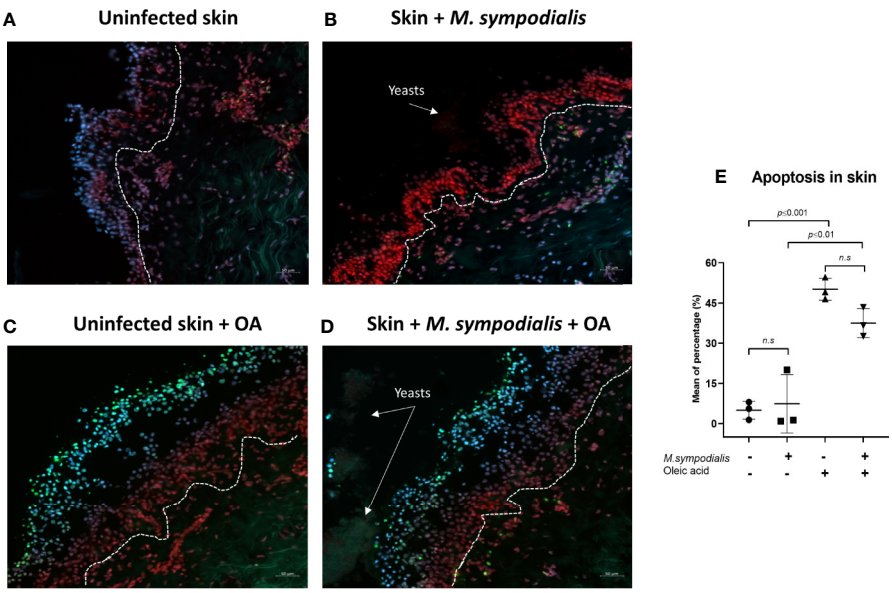

FIGURE 3 | Apoptosis in skin. Apoptosis levels were measured in the four groups of skin samples after 6 days of incubation (images **A–D**). Skin sections were stained with TUNEL system, DAPI and propidium iodide. Apoptotic cells were green, non-apoptotic cells were red/blue/purple (merging of DAPI and Propidium iodide). Channels used were FITC (495 nm/519 nm), DAPI (358 nm/461 nm), Rh-TRITC (543 nm/569 nm). **(E)** Mean apoptosis levels and SD. One-way ANOVA test was applied for statistical analysis ($p \leq 0.0001$), post-hoc analysis used the Tukey's test with *p* values shown in figure, n= 3 biological replicates, with each sample analyzed in duplicate. Scale bars are 50 μm.

S1, Mala S4, Mala S5, Mala S6, Mala S8, Mala S9, Mala S11, Mala S12, Mala S13. Mala S11, and Mala S13 were the only two allergens found in both MSOA and MS skin (**Supplementary Material S2**).

DISCUSSION

We have characterized the host-pathogen interactions of *M. sympodialis* with human skin at the molecular level using a human skin explant model. The model consisted of inoculating skin explants with *M. sympodialis* yeasts and incubating them for 6 days. The influence of added exogenous oleic acid, mimicking lipid-rich skin niches, was also investigated. First, the presence of yeasts was confirmed by fluorescence microscopy and SEM in the inoculated samples. Then, host responses were evaluated by gene expression, proteomics and immunoassays.

In this model, increased mRNA and protein expression of β-defensin 3 and RNase 7 was detected in MS skin, similar to previous reports in skin of individuals with pityriasis versicolor and atopic dermatitis (Gambichler et al., 2008; Brasch et al., 2014). However, AMP gene expression was not detected when oleic acid was applied to the skin. Oleic acid was added to this model to allow evaluation of host response to *M. sympodialis* on oily skin. Previous studies found fatty acids (oleic acid, palmitic acid and lauric acid) to be protective, resulting in the upregulation of β-defensin 2 expression by sebocytes in cell culture (Nakatsuji et al., 2010). This is contrary to what was observed with this model in the non-inoculated and MSOA skin and may be due to the high concentration of OA used in the current report. Skin damage by OA has been documented previously with concentrations as low as 5% and is macroscopically evident as dermatitis in healthy volunteers and histological damage in reconstructed epidermis (Boelsma et al., 1996). Therefore, the lack of AMP expression in OA or MSOA skin may be due to epidermal damage as a result of OA supplementation, which requires further validation. The direct effect of OA on skin was analyzed here by proteomics and the majority of proteins (including keratins involved in cornification, keratinocyte differentiation and wound healing responses) were detected at lower levels in OA skin compared to untreated skin. Only a handful of proteins had increased levels in OA skin but the differences in fold change were not statistically significant. It is known that OA can damage and produce desquamation of the *stratum corneum* (DeAngelis et al., 2005), which was also observed in histological sections in our model. In addition, OA-induced damage facilitates penetration of *M. sympodialis* so that it contacts and damages keratinocytes in deeper skin layers. These two consequences could result in the absence of AMPs produced by the epidermis in MSOA skin.

The lack of AMP protein expression in MSOA skin in this model is similar to what has been reported in skin lesions of AE individuals, where previous studies have documented decreased or no expression of β-defensin 2 and LL37 in acute and chronic skin lesions of AE individuals (Ong et al., 2002; Clausen et al., 2018). Both AMP genes and protein expression in the epidermis have been reported to be lower in atopic dermatitis patients compared to psoriasis patients (de Jongh et al., 2005).

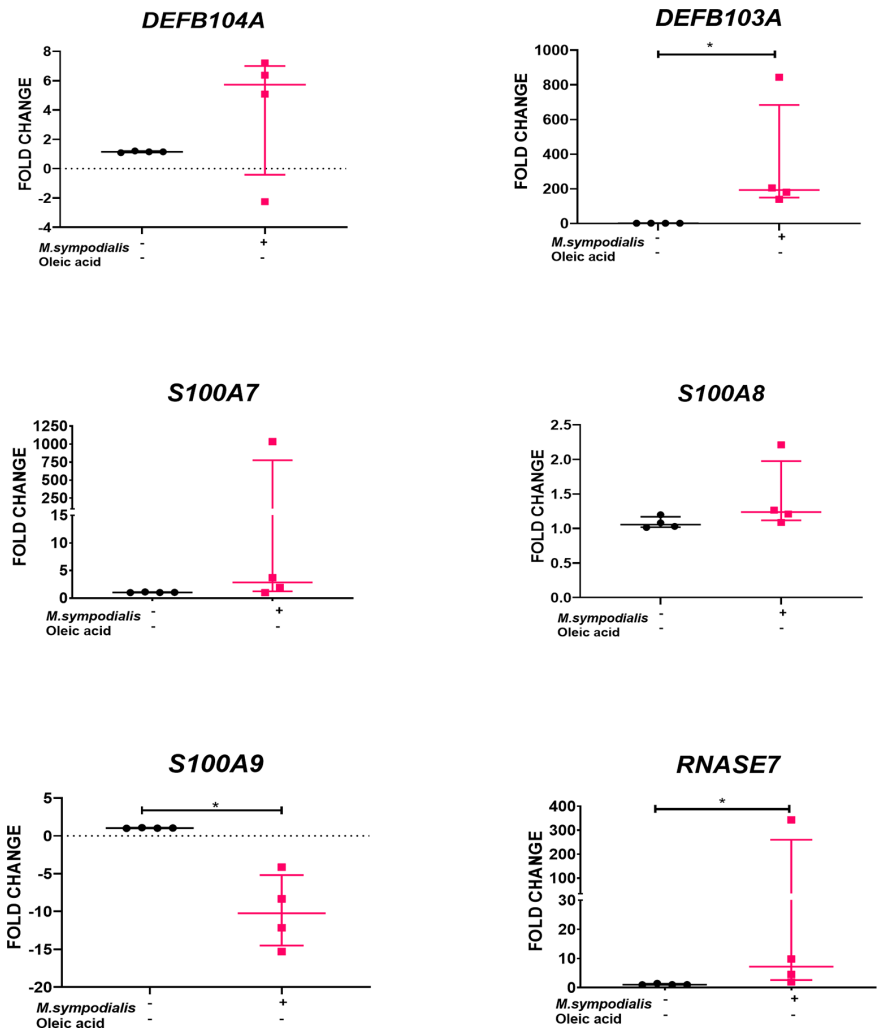

FIGURE 4 | AMP gene expression in *M. sympodialis* infected human skin without OA. RNA was extracted from skin samples after 6 days incubation for qRT-PCR analysis. Results obtained for each target gene were normalized against β2-microglobulin gene expression levels and expressed relative to uninfected skin negative controls. Data is shown as median and IQR and compared by Mann-Whitney U test. n=4 biological replicates, each analyzed in triplicate. *p value = ≤0.05.

High levels of certain S100 proteins were found in MS skin (S100A2, S100A4, and S100B). These have not been reported to have a role in *Malassezia* infections or allergic reactions. However, these three S100 proteins have been reported to have a role in macrophage migration, cell proliferation and migration, and as apoptosis inhibitors and regulators of p53 protein (Donato et al., 2013). The increase of these S100 proteins should be confirmed with different techniques, such as gene expression, immunoassays, or immunofluorescence as LC-MS/MS could misidentify these peptides with other similar proteins sharing S100 domains such as filaggrin (Bunick et al., 2015). This differentiation will be important as filaggrin is essential for epidermal barrier formation (Hänel et al., 2013) and the loss of its function is already recognized as a causative factor for AE (Nutten, 2015). Further study of the role of filaggrin in the response to *M. sympodialis* is required. In addition, the lower expression of *S100A9* gene found in skin inoculated with *M. sympodialis* has not been reported before. The expression of *S100A9* gene was expected to be increased or unchanged as seen with the rest of S100 proteins. In order to investigate whether decreased expression of *S100A9* is a feature of *M. sympodialis* skin response, a follow up of the dynamics of *S100A9* gene and protein expression in earlier and later stages of *M. sympodialis* infection would be required.

Uninfected OA skin had significantly higher levels of TNFα, IL1-β, and IL-6 compared to MSOA. These higher cytokine levels may reflect an inflammatory effect of OA on skin, which as mentioned above is associated with skin damage.

The effect of *Malassezia* species on cytokine production can vary depending on the clinical and experimental context (Faergemann et al., 2001; Watanabe et al., 2001; Pedrosa et al., 2019). High levels of IL-1β, IL-6, IL-8, and TNFα have been found in supernatants of human keratinocyte cell cultures after 3–6 h of co-incubation (Watanabe et al., 2001). However, the

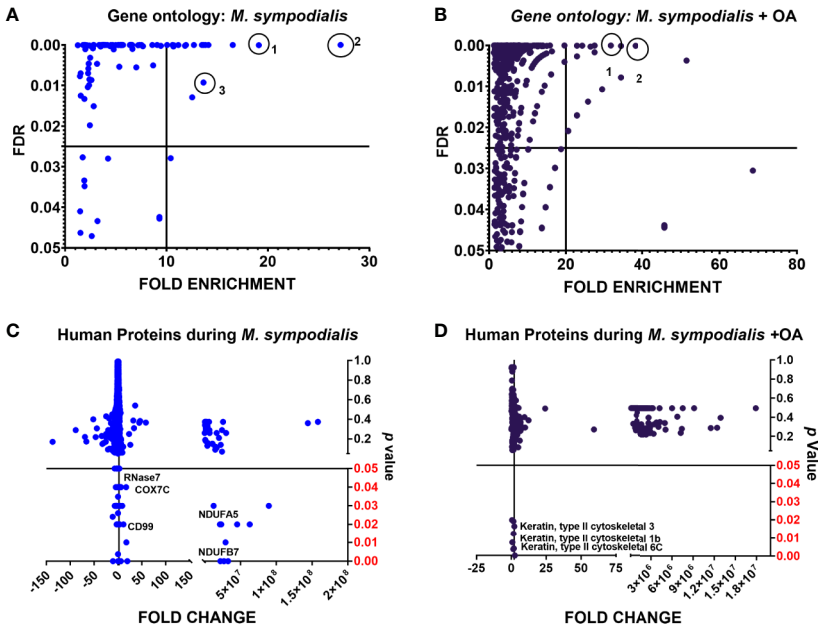

FIGURE 5 | Analysis of human proteins in skin tissue inoculated with *M. sympodialis*. Human proteins with higher levels in tissue at day 6 post-infection (≥2 fold change compared to their corresponding non-inoculated control: non-OA, OA) were analyzed by gene ontology. Statistical analysis was done by Fisher's test with FDR correction (*p* value). **(A)** biological processes in *M. sympodialis*-inoculated skin, circles are 1) antimicrobial response, 2) cornification 3) defense response to fungus. **(B)** processes in skin co-inoculated with *M. sympodialis* and oleic acid, circles are 1) removal of superoxide radicals, 2) chylomicron remodeling. **(C, D)** show proteins with significantly increased levels in each condition. Fold change was estimated relative to protein level in non-inoculated skin without OA.

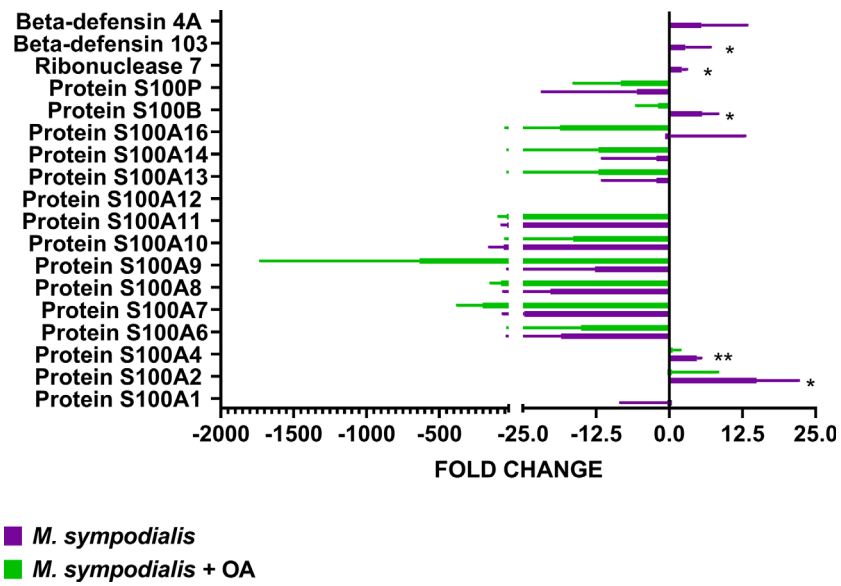

FIGURE 6 | Proteomic analysis of AMP response in skin inoculated with *M. sympodialis*. Single proteins were analyzed separately by Kruskal-Wallis test. If *p* value <0.05 after Kruskal-Wallis then post-hoc analysis by Dunn's test was performed and indicated in the graphic as $^{*}p \leq 0.05$, $^{**}p \leq 0.001$. Data are presented as median and IQR, n=4 biological replicates.

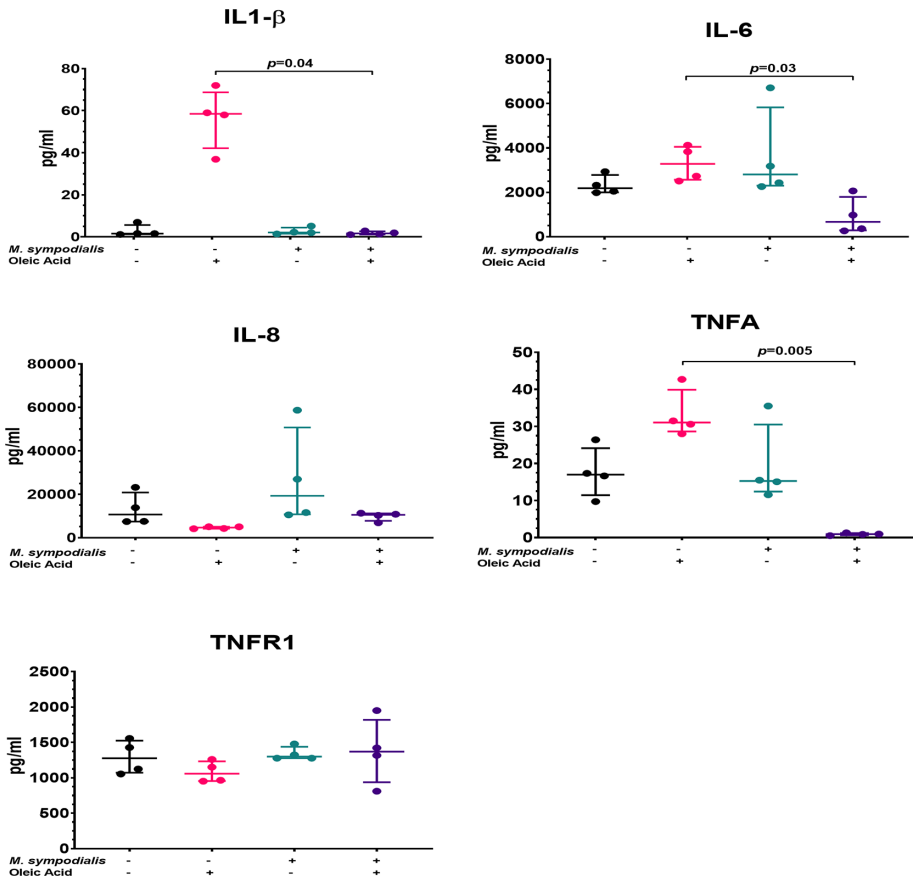

FIGURE 7 | Supernatant cytokine levels from *M. sympodialis*-inoculated skin at 2 days incubation. Cytokine levels were measured by immunoassay. Fold change was calculated by comparing levels to negative control, non-inoculated skin. Data is shown as median and IQR, and analyzed by Kruskal-Wallis test. If p value <0.05 after Kruskal-Wallis test then post-hoc analysis by Dunn's test was performed and indicated in the graphic. n=4 biological replicates, each analyzed in triplicate.

cytokine response to *Malassezia* sp. varies depending on the species; *M. pachydermatis* induced the highest levels of all these cytokines and *M. furfur* induced almost no response (Watanabe et al., 2001). In our study, none of these cytokines had high levels when MS skin was compared to the corresponding OA non-inoculated controls and TNFα, IL1-β, and IL-6 levels were significantly decreased in MSOA skin. This finding can be explained in three ways. Firstly, the time point for cytokine analysis differs. In a recent study using reconstructed epidermis, gene expression of *IL1B, IGFB1*, and *TNFA* in tissue was increased after 6 h incubation with *M. sympodialis*, but these same genes were downregulated after 48 h of co-incubation (Pedrosa et al., 2019). Secondly, models to study host response to fungal infection can differ from the response in real human infections. Faergemann et al. (2001) reported no difference in skin TNFα levels in individuals with *Malassezia* folliculitis and seborrheic dermatitis when compared to healthy volunteers, similar to the current *ex vivo* skin model and contrary to what has been reported from monolayer keratinocyte culture. In addition, lower TNFα serum levels were found in individuals with chronic AE, along with low serum levels of IL-10, β-defensin 3 and high levels of β-defensin 2 (Kanda and Watanabe, 2012). Finally, the lack of AMP expression seen in the MSOA skin can explain the low levels of some cytokines as these AMPs (especially, β-defensins, S100 proteins and cathelicidin) are key for inducing cytokine responses (Niyonsaba et al., 2017).

Thomas et al. (2008) demonstrated that lack of the external lipid layer in *Malassezia* species increased the inflammatory response by keratinocytes, characterized by high levels of IL-6, IL-8, and IL-α. Similarly, Kesavan et al. (2000) reported that *Malassezia* cells with lipid-depleted surfaces triggered higher levels of inflammatory cytokines such as IL-1β, TNFα and IL-6. In our model of *M. sympodialis* infected skin, supplementation with oleic acid had the opposite effect, with reduced levels of acute inflammatory cytokines. The direct effect of oleic acid on *M. sympodialis* cells and the cell wall was not evaluated in this study, hence the impact of OA on cytokine responses to *M. sympodialis* cannot be considered; however, it is a factor currently under investigation by our group.

IL18 and *TGFB1* were highly expressed in MSOA skin, whilst only IL-18 levels were significantly higher in the analyzed supernatants. IL-18 belongs to the IL-1 cytokine family, along with IL-1α and IL-1β, and is cleaved by caspase-1 after being activated by 3NLRP inflammasome (Fenini et al., 2017). This

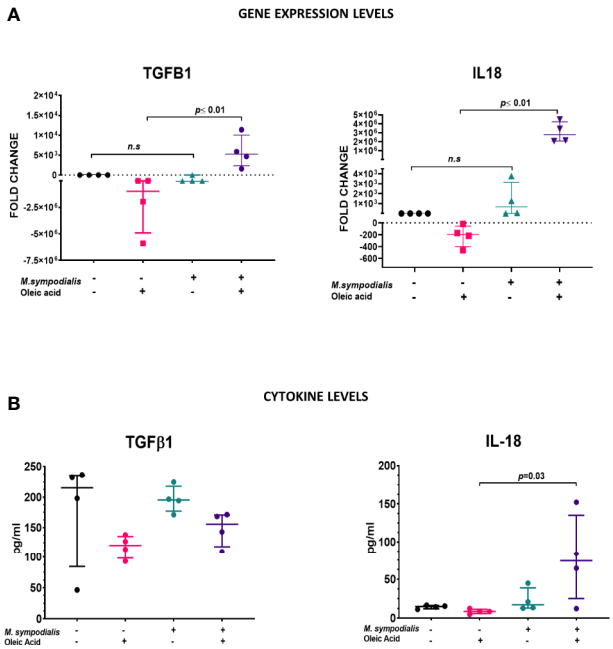

FIGURE 8 | Comparison of TGFB1 and IL-18 expression in tissue and supernatants. **(A)** For gene expression, relative quantification was performed by qRT-PCR using RNA extracted from samples at 6 days post inoculation. Results obtained for each target gene were normalized against β2-microglobulin gene expression levels. Fold change was estimated relative to expression in non-inoculated skin. **(B)** Protein levels in supernatant at 2 days post inoculation were measured by immunoassay and expressed as pg/ml. Data is shown as median and IQR and statistical analysis was done with Kruskal-Wallis test. If p value <0.05 after Kruskal-Wallis test, then post-hoc analysis by Dunn's test was performed and this value it is indicated in the graphic. n=4 biological replicates, each analyzed in triplicate.

inflammasome pathway is crucial for inducing Th1/Th2 responses. IL-18 induces the formation of high serum levels of IgM, IgG_1, IgG_{2a}, and very high levels of IgE antibodies (Enoksson et al., 2011). The production of these antibodies depends on $CD4^+$ T cell-derived IL-4 and the auto-reactivity of these antibodies is regulated and depends on NK T cells (Enoksson et al., 2011). The production of high levels of IL-18, along with high levels of IL-4 (Th2 biased response), has been associated with worse prognosis in other infections such as leishmaniasis (Gurung et al., 2015). High serum levels of IL-18, IL12/p40 and IgE antibodies have been found in individuals with atopic eczema and their serum levels correlate proportionally with clinical severity of AE skin lesions (Zedan et al., 2015).

As mentioned previously, *M. sympodialis* allergens play a crucial role in the pathogenesis of atopic dermatitis (Gioti et al., 2013). In this study, higher numbers of allergens were identified in the MSOA skin compared to MS skin. This finding could be explained by the higher number of yeasts present on the surface of MSOA skin. Due to the skin damage caused by OA, it is possible that these allergens were in contact with inner epidermal cells and contributed to the host response seen in MSOA skin. The role of allergens in *M. sympodialis* pathogenicity is a future avenue of investigation with this *ex vivo* human skin model.

In conclusion, the local host response to *M. sympodialis* can be characterized using this *ex vivo* human skin model. Such host response can vary as previously described, depending on the *Malassezia* species, host intrinsic and extrinsic factors, time of clinical evolution, and type of infection. Most of these conditions can potentially be mimicked in this *ex vivo* skin model. Comparison of responses between different skin conditions has already been shown to be possible in this study. In non-oily and intact skin, AMPs and S100 proteins are key in the response to *M. sympodialis*, but in oily and damaged skin, allergens and yeasts are in direct contact with keratinocytes, and inflammasome responses seem to lead to increased IL-18, which can promote chronic inflammation, auto-reactivity in skin and continuing local damage.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DC, DM, and CM conceptualized and designed the study. DC performed the experiments and data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.561382/full#supplementary-material

S1 | Results **Supplementary Material S1** proteomics human database OA.

S2 | Results **Supplementary Material S2** *Malassezia sympodialis* proteins.

S3 | Results **Supplementary Material S3** Analysis of uninfected skin samples with and without OA.

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Cutaneous *Malassezia:* Commensal, Pathogen, or Protector?

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The skin microbial community is a multifunctional ecosystem aiding prevention of infections from transient pathogens, maintenance of host immune homeostasis, and skin health. A better understanding of the complex milieu of microbe-microbe and host-microbe interactions will be required to define the ecosystem's optimal function and enable rational design of microbiome targeted interventions. *Malassezia*, a fungal genus currently comprising 18 species and numerous functionally distinct strains, are lipid-dependent basidiomycetous yeasts and integral components of the skin microbiome. The high proportion of *Malassezia* in the skin microbiome makes understanding their role in healthy and diseased skin crucial to development of functional skin health knowledge and understanding of normal, healthy skin homeostasis. Over the last decade, new tools for *Malassezia* culture, detection, and genetic manipulation have revealed not only the ubiquity of *Malassezia* on skin but new pathogenic roles in seborrheic dermatitis, psoriasis, Crohn's disease, and pancreatic ductal carcinoma. Application of these tools continues to peel back the layers of *Malassezia*/skin interactions, including clear examples of pathogenicity, commensalism, and potential protective or beneficial activities creating mutualism. Our increased understanding of host- and microbe-specific interactions should lead to identification of key factors that maintain skin in a state of healthy mutualism or, in turn, initiate pathogenic changes. These approaches are leading toward development of new therapeutic targets and treatment options. This review discusses recent developments that have expanded our understanding of *Malassezia*'s role in the skin microbiome, with a focus on its multiple roles in health and disease as commensal, pathogen, and protector.

Keywords: *Malassezia*, commensal, pathogen, mutualist, multikingdom, immunity, skin, host and disease

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MALASSEZIA: A MAJOR COMPONENT OF THE SKIN MICROBIOME

Human skin serves as our protective physical barrier, but also consists of a complex micro-environmental ecosystem. The skin surface micro-environment is colonized by a wide range of microorganisms, including bacteria, archaea, viruses, and fungi, collectively referred to as the skin microbiome. While initially considered as a 2 m^2 flat surface representing a smaller and less influential

niche than gut or lung, when one considers skin's 3-dimensional topography it becomes an estimated 30 m^2, similar in surface area to gut or lung. This, coupled with access to viable epidermis in deeper invaginations (such as the follicle infundibulum), the skin becomes an important and relevant microbial niche (Gallo, 2017). The complex ecosystem in any individual is governed by the skin's divergent niches, ranging from dry (heel, volar forearm) to moist (antecubital fossa, axilla) to dry and oily (face, upper back) to moist and oily (scalp), driving high microbial variability between niches. While there remains microbial variability between individuals, it is relatively low compared to that between different body site niches (Grice et al., 2009; Oh et al., 2014). Multikingdom DNA sequencing has revealed the skin microbiome has notably higher viral and fungal representation when compared to gut (Arumugam et al., 2011; Oh et al., 2014). The skin microbiome is also temporally stable over long periods regardless of the environmental perturbations experienced in daily life (Oh et al., 2016). Factors such as short term washing with common (non-antimicrobial) hygiene products does not disrupt the skin commensal microbial diversity, but can help displace opportunistic pathogenic colonizers (Two et al., 2016). The eukaryotic component of skin microbiome is dominated by *Malassezia* (Findley et al., 2013; Oh et al., 2014; Jo et al., 2016; Byrd et al., 2018), which are found in highest abundance on sebaceous sites including scalp, face, chest, and upper back, and in lower abundance on trunk and arms. Feet are the exception with lower *Malassezia* content and higher overall fungal diversity (**Table 1**) (Findley et al., 2013). Interestingly, the diversity of commensal skin fungi can also vary with geography and possibly ethnicity in healthy individuals (Leong et al., 2019).

Metagenomics defines the genetic information from a single microbe as one functional unit, a set of genes, and is used as such to determine the presence of individual microorganisms and predict abundance. However, the relative number of genomes provides an estimate of abundance independent of the cellular size, or interactive biomass, which differs considerably between organisms. For metagenomic ecological analyses, it is important to consider the potential biomass of each microbial community member, as biomass is how bacteria, fungi, or viruses interact with the host as a functional unit. Using conservative estimates of cell size, *Malassezia* have 200–500 times the cellular biomass per genome relative to *Staphylococcus epidermidis*. Hence it would be reasonable and likely meaningful to consider biomass availability during interaction with the host (Ramasamy et al., 2019). Doing so promotes the fungal biomass component of sebaceous skin to at least an equal footing with bacteria.

Molecular phylogenetic and genomic studies have shown *Malassezia* belong to Basidiomycota, *Ustilagomycotina* and class *Malasseziomycetes*. *Malassezia* have undergone an unfortunate and complex series of nomenclature changes which have clouded their research history. Originally discovered by Malassez and Sabouraud in the late 19th century, their resistance to cultivation led to the conclusion that there was one species, named *Malassezia* (Malassez, 1874; Sabouraud, 1897). In the 1950's there were three identified species, renamed as *Pityrosporum* (which is still found in some textbooks): *P. ovale*, named for their oval shape; *P. orbiculare*, for their round shape; and *P. pachydermatis*, as the species found on animal as opposed to human skin (Rhoda, 1939). Other nomenclature included *M. furfur* serovars (A, B or C) now re-classified as A-*M. furfur*, B-*M. globosa* and C-*M. restricta* (Cunningham et al., 1990; Ashbee et al., 1993; Batra et al., 2005). Today the *Malassezia* genus is diverse and comprises 18 species, with numerous functionally distinct strains (**Figure 1**) (Theelen et al., 2019). *Malassezia* have haploid genomes of 8–9 Mb, among the smallest for free-living fungi. They have evolved genetic content enriched for genes specific to their environment, encoding lipases, phospholipases and acid sphingomyelinases for utilization of lipids, and proteases for utilization of proteins (Poh

TABLE 1 | Summary of currently described *Malassezia* species present in human body sites associated with health and/or disease.

S/N	*Malassezia* Species	Group (Figure 1)	Body site† (Human)	Health/ Disease‡	References
1	*M. furfur*	A	D, S, SC, Sy	AD, D, SD, PV, SI, F	(Simmons and Gueho, 1990; Boekhout T et al., 2010; Cabanes, 2014; Iatta et al., 2014)
2	*M. obtusa*	A	D, S	AD, D, SD	(Boekhout T et al., 2010; Cabanes, 2014; Prohic et al., 2016)
3	*M. japonica*	A	D, S	AD	(Sugita et al., 2003)
4	*M. yamatoensis*	A	D, S	AD, SD	(Sugita et al., 2004; Boekhout T et al., 2010)
5	*M. sympodialis*	B	D, M, S	D, SD, AD, PV, F	(Gupta et al., 2004; Boekhout T et al., 2010; Aguirre et al., 2015; Patron, 2016)
6	*M. dermatis*	B	D, S	AD	(Sugita et al., 2002; Boekhout T et al., 2010; Guého-Kellermann and Begerow, 2010)
7	*M. restricta* (all humans)	B	D, M, S, F	D, SD, AD, P	(Nakabayashi et al., 2000; Batra et al., 2005; Boekhout T et al., 2010; Guého-Kellermann and Begerow, 2010)
8	*M. globosa* (all humans)	B	D, M, S, F	D, SD, AD, PV, P	(Gupta et al., 2004; Boekhout T et al., 2010; Cabanes, 2014)
9	*M. pachydermatis* (normally animal, not human)	B	Sy	SI	(Boekhout T et al., 2010; Chow et al., 2020)
10	*M. arunalokei*	B	S, ear	D, SD	(Honnavar et al., 2016)
11	*M. slooffiae* (rare)	C	D, S	SD	(Boekhout T et al., 2010)

Non-human associated species: M. brasiliensis (Parrot-Group A); M. psittaci (Parrot-Group A); M. equina (Horse, Cow); M. nana (Cat, cow, Dog-Group B); M. caprae (Goat, Horse-Group B); M. vespertilionis (Bat-Group C); M. cuniculi (Rabbit-Group C).

†Body Site: SC-Scalp; F-foot; Sy- systemic (blood, urine); S-sebaceous; M-moist; D-dry.

‡Health/Disease: AD-Atopic dermatitis, PV-Pityriasis Versicolor; D-Dandruff; SD-seborrheic dermatitis; P-psoriasis; F-folliculitis; SI-systemic infections.

et al., 2020). They lack a fatty acid synthase and δ-9 desaturase, likely due to their habitation on oil rich sebaceous skin, and hence have an evolutionary inability to synthesize lipids as part of their adaptation to life on skin (Saunders et al., 2012; White et al., 2014; Wu et al., 2015). The most closely related and well-established fungus is the plant pathogen *Ustilago maydis*, which also targets their plant host for degradation, but *via* secretion of enzymes to break down proteins, pectin, and wax common to plant surfaces. Interestingly, while closely phylogenetically related to *Ustilago*, *Malassezia* secrete an enzyme armada much more similar to the distantly related *Candida albicans*, an example of niche-specific evolution (Xu et al., 2007).

The *Malassezia* clade can be subdivided into three major groups, as seen in **Figure 1** and **Table 1**. Group A are represented as *M. furfur*-like, are more robust in culture, less frequent inhabitants of human skin and more often linked to skin or septic disease. Group B are more common on healthy human skin, with *M. restricta* and *M. globosa* by far the most common and found on the skin of all humans, followed by *M. sympodialis*, then distantly by the other Group B members. The Group B exception is *M. pachydermatis*, which can cause human septic infections but is only normally found on animal skin. **Figure 1** also reveals pathways of chromosomal rearrangements resulting from centromere loss of function (Sankaranarayanan et al., 2020). A nine-chromosome ancestral lineage is hypothesized, which carried through to most of Group B. One centromere was lost to generate the eight-chromosome *M. sympodialis* group, and then another to generate the seven-chromosome Group C. *M. pachydermatis* and *M. obtusa* remain six-chromosome outliers. A series of horizontal gene transfers have been defined and further cloud the phylogeny (Ianiri et al., 2020). For example, another recent LSU tree, while very similar implies different grouping and relationships (Ianiri and Heitman, 2020). These findings highlight that while much has been learned about *Malassezia* phylogeny there is still a long way to go to understanding this unusual clade. Unfortunately, many *Malassezia* species do not have complete genomes, so further long read sequencing and assembly of chromosome-level genomes will be necessary to further refine *Malassezia* evolution. The lack of capability to cultivate many *Malassezia* species and strains has been an impediment to genome sequencing and characterization of gene function. Developing new culture conditions to allow cultivation of new species and continuation of the consortium/repository based exchange of genome information will advance understanding about *Malassezia*. The genomic relatedness of known and sequenced *Malassezia* spp. is useful for future species genome assembly and gene assignment, but unfortunately a large number of *Malassezia* genes fall into the category of unknown function and exist as families of similar gene structure. The reasons for duplication or multiplication of genes in *Malassezia* in the perspective of evolution could be addressed by genome evolution studies (Sankaranarayanan et al., 2020). The availability of more complete *Malassezia* genomes will further the understanding of unknown gene function, identify

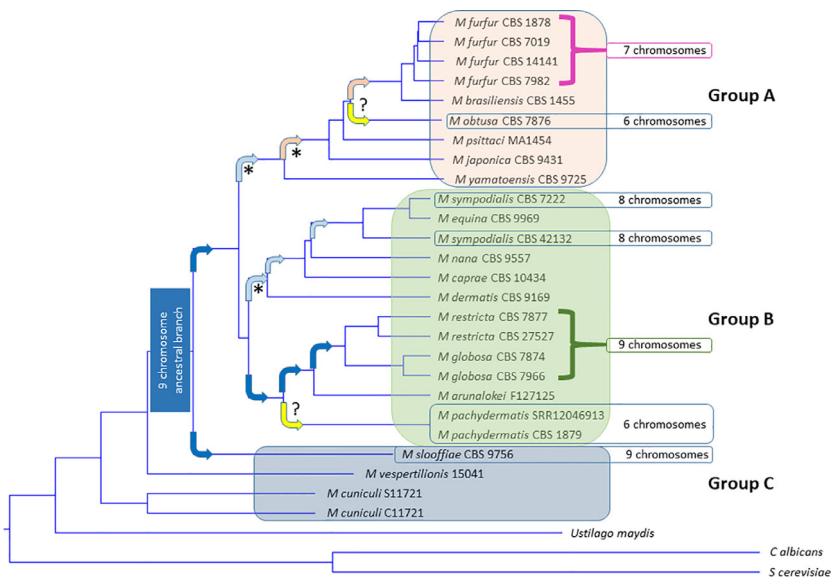

FIGURE 1 | Phylogenetic tree of all 18 currently accepted *Malassezia* species. *Malassezia* can be subdivided into subgroups A, B, and C based on Wu et al. PLoS Genetics 2015. Tree constructed from most current NCBI LSU data (**Table 1**) using the MAFFT method to generate multi-align sequence (MSA), UPGMA (unweighted pair group method with arithmetic mean) for clustering, phylogenetic tree viewed using Newick viewer. MAFFT v6.864 is a multiple alignment program for amino acid or nucleotide sequences (Katoh et al., 2005). (http://www.trex.uqam.ca/index.php?action=mafft and http://mafft.cbrc.jp/alignment/software/). Known species of inhabitation and activities can be found in **Table 1**. Arrows indicate hypothesized chromosome losses associated with development of new species (Sankaranarayanan et al., 2020). Dark blue box = nine chromosome ancestral lineage. Dark blue arrows, nine chromosome lineages. Light blue arrow predicted centromere loss to eight chromosomes. Pink arrow predicted centromere loss to seven chromosomes. Orange arrow unknown chromosome loss to six chromosomes. Yellow unknown event ()? resulting in loss to six chromosomes. (* = documented by centromere loss) (*M. pachydermatis* chromosome number postulated from complete genome assemblies, *M. obtusa* hypothesized based on PGFE karyotype) (Kiuchi et al., 1992; Boekhout et al., 1998; Kim et al., 2018).

potential pathways to harness unidentified roles in host-commensal or host-pathogen interactions.

Historically, Malassezia genetic engineering has been extremely challenging. However, it has now been accomplished *via* agrobacterium-mediated transformation (Ianiri et al., 2016; Celis et al., 2017; Ianiri and Heitman, 2020). For example, gene deletion of a bacterially derived flavohemoglobin gene found in all *Malassezia* spp. detoxifies skin generated nitric oxide and is involved in *Malassezia*/Host interaction (Ianiri et al., 2020). Other gene deletion studies have shown that the multidrug transported PDR10 is involved in Malassezia furfur antimicrobial resistance (Ianiri et al., 2019). Finally, insertion of marker and tracking genes into Malassezia for use in *in vitro*, *ex vivo*, and *in vivo* models should assist in more detailed investigation of *Malassezia*/Host interactions (Goh et al., 2020). Improved gene and genome information will assist with further identification of novel proteins that trigger skin disease, inflammation, antifungal response, immunological response of host and mechanistic functions in clinical perspective.

Microbial community and host interactions have multiple effects: broadly classified as commensal, pathogenic, or mutualistic (Schommer and Gallo, 2013). Commensalism is an active relationship between individuals of two species in which one species obtains benefits from the other without benefiting the latter. In a commensal paradigm *Malassezia* obtains the benefit of a food source while causing no effect to the human host. Pathogenicity is a relationship where one member is harmed, in this situation with *Malassezia* activity resulting in direct host damage through specific secreted virulence factors or toxins that negatively affect the host; or indirectly through induction of a damaging host response. In fungal infection, it is necessary to have a functional definition of virulence and pathogenicity, termed a "Damage Response Framework (DRF). In the DRF, a "causal" microorganism may manifest disease directly through products or antigens, or indirectly *via* induction of a harmful host response. In the DRF a pathogen is defined as eliciting a functional change from a commensal to a pathogenic state (Casadevall and Pirofski, 1999; Casadevall and Pirofski, 2003; Casadevall and Pirofski, 2018). Mutualism is classed as an active relationship where both species benefit. For *Malassezia*, they may not only survive on our skin but also may provide protection from contextually pathogenic microbes such as *S. aureus* (Li et al., 2018). Many acute skin infections have underlying microbial contributions which are improved by antimicrobial treatment (Golan, 2019). However, due to the frequency of unknown individual susceptibilities, it is often challenging to successfully satisfy Koch's postulates to prove causality by individual microbial components (Koch, 1893; Grice and Dawson, 2017). It is therefore important to delineate the context of microbial interactions with skin disease outcomes. Functional interactions can be scenarios where (i) the skin microbiota is a direct cause, (ii) the skin microbiota is altered by changes in the skin and hence generate a deleterious host response, exacerbating the situation, or (iii) where the microbiota is uninvolved. Differentiating these functional interactions is complex and for *Malassezia* a still developing research area.

MALASSEZIA INTERACTIONS WITH SKIN AND THEIR ROLE AS A COMMENSAL

Most metagenomic datasets reveal that microbial communities in different skin ecosystems are determined by topography and driven by water (sweat), oil (sebum), or other temporally stable attributes (Findley et al., 2013; Oh et al., 2016). *Malassezia* are enriched in sebaceous zones, particularly breast, back, and head, due to the abundance of the lipid nutrient source (Findley et al., 2013; Jo et al., 2017). *Malassezia* density is associated with the maintenance of skin health (Ashbee and Evans, 2002; Prohic et al., 2016), and *Malassezia* are the most abundant fungi identified at eleven core body sites, all except those on the foot. *M. restricta* and *M. globosa* are by far the most abundant on human skin, with other species occurring at much lower frequency (Findley et al., 2013).

In addition to body site, age plays a role in shaping the skin mycobiome. During gestation, the fetus is exposed to microbes from the placenta, fetal membranes, amniotic fluid, and umbilical cord (Pelzer et al., 2017). Immediately after birth, early skin colonization is influenced by vernix caseosa, a multi-component defense system (anti-microbial sebum) composed of cellular contents, water, lipids, and proteins produced by fetal sebaceous glands during the third trimester (Pickens et al., 2000; Tollin et al., 2005; Michalski et al., 2017; Szabo et al., 2017). Neonates born through vaginal delivery acquire microbial communities from the birth canal and vagina, resembling their mother's vaginal microbiota, where neonates born *via* cesarean section have skin microbial communities similar to the mother's skin surface (Dominguez-Bello et al., 2010; Oh et al., 2010; Aagaard et al., 2014; Dunn et al., 2017; Georgountzou and Papadopoulos, 2017). *Malassezia* colonization occurs immediately after birth, when neonatal sebaceous glands are active, being driven by maternal hormones which cross the placental barrier with a progression over the first few months of life to more closely resemble the adult microbiome assemblage (Ashbee and Evans, 2002; Bernier et al., 2002; Ayhan et al., 2007; Nagata et al., 2012). The succession of mycobiome during birth within individual infants is variable either due to mothers vaginal mycobiome or vertical transmission depending on mode of delivery and environmental impact such as caregivers and other sources (Ashbee and Evans, 2002; Bernier et al., 2002; Ward et al., 2018). In contrast to the mother's skin *Malassezia* colonization, infant skin only contains two percent relative abundance shown by either sequencing, PCR, or culture-based approaches. Despite the higher abundance in mother's skin, cesarean compared to vaginally born infants have lower *Malassezia* abundance. The reasons are currently unknown but could be biological, environmentally influenced, or a reported technical bias of Internal Transcribed Spacer (ITS) 2 region amplification (Anna and Bazzicalupoa; Bellemain et al., 2010).

As *Malassezia* are lipid dependent colonizers, their skin abundance would be hypothesized to follow the level of sebaceous gland activity and hence lipid level on skin (Ro and Dawson, 2005). Soon after birth (3-6 months), the sebaceous glands become dormant, and *Malassezia* revert to a low abundance. With the onset of puberty, increased lipid levels in

sebaceous regions result in a concomitant increase in *Malassezia* abundance (Ro and Dawson, 2005; Prohic et al., 2016). Comparison of skin fungal communities between healthy children and adults showed that *Malassezia* predominates on adults while in children (age < 14) *Malassezia* were present but at lower abundance, and with a more diverse fungal community including Eurotiomycetes and common dermatophytes (Jo et al., 2016). This observation of fungal ecological dynamics may partly be responsible for the prevalence or severity of common skin disorders seen more frequently in children. Together this points to a key protective role of *Malassezia* when fully occupying the skin niche.

IMMUNE EDUCATION, HOST TOLERANCE, AND THE RESPONSE TO *MALASSEZIA*

The skin is reliant on commensal microbiota to "train" the immune system and develop appropriate tolerance, host defense mechanisms, and immunity against invading pathogens. Dynamic signals from commensals during early development are used by the immune system to provide heterologous defense mechanisms (Naik et al., 2015). These host interactions are skin-specific, and a resident immune cell population is recruited for establishing innate and adaptive responses in the periphery. One of the foremost factors for skin-microbiota coexistence is host immune tolerance, as shown in **Figure 2**. Tolerance is established after birth, maintained throughout life, and is defined as the capability of the host to suppress the active immune response against itself and certain microbes. The microbes in turn also have mechanisms to evade skin antimicrobial defenses and co-exist with skin (Zhang and Gallo, 2016). There is a significant body of literature, reviewed in brief here, regarding the role of the skin microbiome on normal immune development. However, little is known about the specific role of *Malassezia*. This will be an important area for future research.

Fetal immune development begins as early as nine to fifteen weeks with formation and maturation of multiple cell types including B and T lymphocytes (Hayward, 1983). The fetus

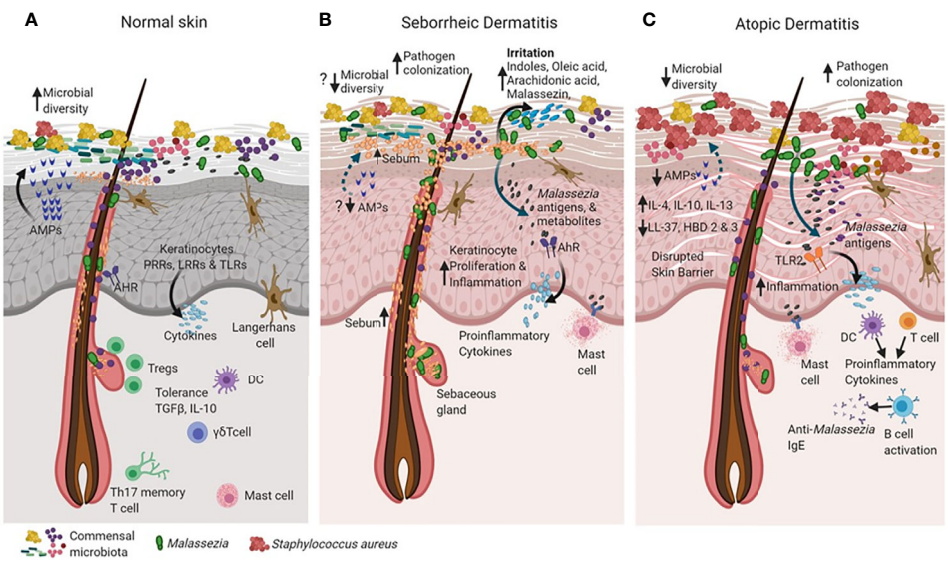

FIGURE 2 | Cross talk between skin and microbiota in healthy and diseased skin states (such as seborrheic dermatitis and atopic dermatitis). The skin and the immune system evolve together with resident microorganisms to establish commensal microbial relationships (for example, *Malassezia* in green). In the healthy state **(A)**, skin maintains high microbial diversity when compared to active disease states in seborrheic dermatitis (SD) and atopic dermatitis (AD) as shown in **(B, C)**. Keratinocytes sense microbial population through recognition of microbial pathogen-associated molecular patterns (PAMPs) motifs *via* their pattern recognition receptors (PRR's), Leucine rich repeat (LRR's) containing receptors and Toll-like receptors (TLR's) as shown in **(A)**. The binding of PAMPs to PRRs, LRRs and TLRs triggers innate immune responses, resulting in the secretion of antimicrobial peptides that can rapidly inactivate a diverse range of pathogenic microorganisms, including fungi, bacteria and parasites. The Langerhans cells interact with microbial antigens in the epidermis to detect barrier breach and maintain homeostasis **(A)**. The skin tolerance is dependent on regulatory T cells, a subset of lymphocytes infiltrate skin, concomitant with hair follicle and skin microbial colonization in based on cytokines TGF-Beta and IL-10 **(A)**. In SD, alterations in sebum content creates favorable conditions for expansion of *Malassezia* as the dominant species that may cause the disease **(B)**. Increased *Malassezia* colonization initiates specific utilization of stratum corneum fatty acids that are converted into by-products such as oleic acid, arachidonic acid which irritates and causes inflammation in the skin. Irritants such as indole and Malassezin could increase keratinocyte (KC) proliferation and induce inflammation **(B)**. Accumulation of histamine in the SD lesions is suggestive of mast cell degranulation **(B)**. In AD there is increased pathogen colonization that causes probable decreased commensal microbial diversity and a defective skin barrier**(C)**. *Malasszeia* and other pathogens could stimulate sub epidermal layers and releases antigens which are recognized by TLR2 receptors feedback to DC or T cell population which stimulates immune response The disrupted skin barrier allows microbial entry in to skin which probably increases cytokines IL-4, IL-10 and IL-13 levels. Circulating anti- *Malassezia* IgE has been reported in AD **(C)**. The figure was created using Biorender.com.

maintains a high Th2-biased immune system to prevent proinflammatory Th1-type alloimmune responses to maternal tissues (Philbin and Levy, 2009), but acquires the ability to produce IgG and IgM antibodies at 10 weeks gestation with IgG levels increasing till 22 weeks. The neonates undergo extreme physical and physiological changes at birth, with the skin surface experiencing a drastic shift from aqueous and sterile to dry with a high load of microbial antigens (Hoeger and Enzmann, 2002). The high Th2 dependent-IL6 cytokine levels, formed in the prenatal stage, shield against microbial infections, and these antigens are cleared at birth (Georgountzou and Papadopoulos, 2017).

Dynamic signals from commensals during early development are used by the immune system to provide heterologous defense mechanisms. The evolutionary interaction between cutaneous commensal microbiota and the skin immune system involves changing antigen signals to calibrate immunity against pathogens (Naik et al., 2015; Quaresma, 2019). From the time of birth, the skin microbiota develops as a highly diverse and dynamic ecology which undergoes remodeling due to host and environmental factors and aids in immune education (Grice et al., 2009; Kong and Segre, 2012). However, host tolerance is established in the early phases of immunity development immediately after birth. During this period skin regulatory T cells (Tregs) establish immune tolerance to commensal microbes, preserve homeostasis with skin microbiota, and protect against pathogens (Belkaid et al., 2002). In neonates, there is a steep influx of Tregs into skin within the first two weeks, to mediate tolerance specific to the commensal microbiota (Scharschmidt et al., 2015; Ali and Rosenblum, 2017). As *Malassezia* are among the major commensal fungi in neonates, it can be hypothesized that they may also induce and establish specific tolerance pathways in the skin involving Tregs (**Figure 2**).

In healthy skin, *Malassezia* exist as a commensal and benignly interact with keratinocytes and the immune system, as they reside mainly on the outer skin surface and the follicular infundibulum (Sanmiguel and Grice, 2015; Mittermann et al., 2016). *Malassezia* are detected by the host immune system through keratinocytes and various immune cell populations. The *Malassezia* cell wall components β-(1,6)-glucans, glycolipids, and glycoproteins are recognized by proline rich region (PRR) motifs present in Dectin-2 and Macrophage inducible Ca2+-dependent lectin receptor (Mincle) host cell membrane bound CLR (C-type Lectin) receptors in multiple immune cell types (Ishikawa et al., 2013; Dambuza and Brown, 2015; Underhill and Pearlman, 2015; Sparber and Leibundgut-Landmann, 2017). Langerin, a PRR expressed on epidermal Langerhans cells and a subset of dermal DCs, can recognize beta-glucans expressed on the *Malassezia* cell wall (De Jong et al., 2010; Tateno et al., 2010)(de Jong Mol Immunol 2010; Tateno J Biol Chem 2010). However, what roles these (and other PRRs) play in *Malassezia*-induced commensalism, inflammation, and adaptive immunity is currently not well understood.

Host innate immune activity to *Malassezia* has been well documented with various *in vitro* studies in human keratinocytes based on secretion of proinflammatory cytokines, chemokines and AMPs. *M. furfur, M. globosa and M. restricta* induced increase in expression of Toll-like receptor 2 (TLR-2), IL-8, Human beta-defensin 2 (HBD-2), HBD-3 suggests their role in skin protection. (Baroni et al., 2006; Donnarumma et al., 2014; Georgountzou and Papadopoulos, 2017). These cytokines and chemokines recruit immune cells to skin sites with minimal or compromised barrier, such as the follicle infundibulum, where *Malassezia* may be directly exposed to keratinocytes, tissue resident dendritic cells (DCs), macrophages, myeloid cells and γδ-T cells (Sparber and Leibundgut-Landmann, 2017). *Malassezia* can inhibit phagocyte responses to Toll-like receptor (TLR) stimulation and contribute to cutaneous invariant γδT cell homeostasis through specific indole metabolites and AhR receptor signaling in the skin (Kadow et al., 2011; Vlachos et al., 2012). A murine skin infection model indicates *Malassezia* can induce Th17 immunity (IL-23/17 axis). In healthy human skin, *Malassezia* are also known to modulate the inflammatory cytokine response of $CCR6^+$ Th17 memory T cells (Sparber et al., 2019). It is not clear how *Malassezia* mediates an immune response for either a commensal or inflammatory state in human skin.

Innate immune activation in skin enhances the adaptive immune response (Holt and Jones, 2000). Generally, adaptive immune responses are stronger in *Malassezia*-associated diseases, but their status during commensalism is less clear. Emerging evidence also indicates that innate lymphoid cells (ILCs) respond directly to skin fungal populations by producing IL-17 cytokine (Gladiator and Leibundgut-Landmann, 2013; Gladiator et al., 2013). To substantiate the immune response, *Malassezia*-specific immunoglobulins IgG, IgM, IgE, and IgA have also been identified in healthy human sweat and shown to coat *Malassezia* on the skin surface (Page and Remington, 1967; Forstrom et al., 1975; Metze et al., 1991; Cunningham et al., 1992). In summary, although there are studies beginning to describe the immunological response to *Malassezia* as commensals, further studies are required to elucidate precise mechanisms.

MALASSEZIA AND SKIN DISEASE

Malassezia have now been associated with numerous skin diseases (Gaitanis et al., 2013; Harada et al., 2015; Prohic et al., 2016; Limon et al., 2017; Saunte et al., 2020). These skin conditions are either caused, or exacerbated by, alterations by *Malassezia* in a changing skin environment. *Malassezia* normally exist as a skin commensal without inflicting disease, suggestive of contextual pathogenesis. One possible mechanism of *Malassezia* mediated skin disease is host genetic susceptibility (Deangelis et al., 2005). Skin barrier defects, for example, may change microbiota composition and/or behavior, leading to a corresponding immune inflammatory response. There are two modes by which *Malassezia* interact with the skin. One is direct, where specific *Malassezia* metabolites introduce physiological

changes such as irritation. The second is indirect, where immune or allergic pathways are activated and manifest as inflammation (Grice and Dawson, 2017). One emergent challenge is to decipher the role of *Malassezia* as a cause or consequence in its multifaceted interaction with the skin. *Malassezia* can cause hypo- or hyper-pigmented non-inflammatory lesions through interaction with melanocytes, and with mild barrier defects can cause pityriasis versicolor, a common skin infection. Often *Malassezia* metabolites trigger a scalp inflammatory response causing dandruff and in severe situations seborrheic dermatitis, and can invade and inflame hair follicles to cause folliculitis (Theelen et al., 2018). In other inflammatory skin conditions, such as atopic dermatitis and psoriasis, there is increasing evidence about the role of *Malassezia*. As host susceptibility is often a prerequisite for fungal pathogenicity, it remains important for mechanistic investigational studies to be performed longitudinally in susceptible individuals, as important pathogenic mechanisms may well be present in non-susceptible individuals and confound parallel group studies by not inducing the disease phenotype.

Malassezia and Childhood Skin Infections

New-born fungal infections are usually topical with mild symptoms. However, in immune compromised or prematurely born infants', skin resident *Malassezia* may spread into to blood circulation and disseminate as sepsis, with serious and often lethal consequence. The initial *Malassezia* colonization may cause hypersensitivity reactions in neonatal face, scalp, and neck skin with non-follicular papulopustular eruptions referred to as neonatal acne or sebaceous miliria. It is not clear how these eruptions spontaneously resolve, but they usually do so within weeks. Neonatal and infantile seborrheic dermatitis associated with *M. furfur* shows a scaling scalp, 'cradle cap' phenotype and is treated by applying ketoconazole shampoo or petrolatum gently on scalp skin or affected area (Wananukul et al., 2005; Zuniga and Nguyen, 2013). Low birth weight infants are also susceptible to *Malassezia* skin infections and these infants are reported to have high fungal load (Speer et al., 1976; Sperling et al., 1988; Ng, 1994; Sohn et al., 2001; Kaufman and Fairchild, 2004). Proposed mechanisms for preterm neonatal skin and systemic infections involves factors such as the developing fragile skin structure having an incomplete barrier, an under-developed immune system, and microbial transmission from caregivers and hospital sources. Other clinical manifestations of infants of premature births are invasive *Malassezia* infections through catheters in neonatal intensive care units (NICU) (Shek et al., 1989; Pedrosa et al., 2018; Chow et al., 2020). At the onset of puberty there is increased activity of sebaceous glands and lipid content predominantly in facial, scalp and trunk skin. This favors the growth of specific lipophilic microbial populations such as *C. acnes* and *Malassezia.* However, it is not clear which biological factors, including changes in skin microbiota, are causative for acne vulgaris during adolescence (Common et al., 2019; Ramasamy et al., 2019).

A Role for *Malassezia* in Pityriasis Versicolor

Pityriasis versicolor (PV), also called Tinea versicolor, is a mild, chronic, superficial fungal infection and frequently occurs in children and adolescents when sebaceous activity is maximum. The lesions appear as hyper- or hypo-pigmented (discolored patches), mostly around the trunk and shoulders. Common clinical presentations are mild itch and scaling in affected areas (Gordon, 1951; Gaitanis et al., 2013). This mild cosmetic disease is usually more active in hot and humid weather conditions than temperate climates. *Malassezia* are known to cause PV (Gupta et al., 2002; Crespo-Erchiga and Florencio, 2006). To date *M. furfur*, *M. globosa* and *M. sympodialis* are the most commonly identified species in PV (Saadatzadeh et al., 2001), but due to the numerous changes in *Malassezia* nomenclature and the recent identification of many new species it remains unclear if any specific species is causal for PV. *Malassezia* usually exist as individual spherical yeast, however in PV they become mycelial with profuse hyphal growth and abnormal expansion in the affected site. Interestingly, histopathological staining from skin biopsies shows milder signs of skin barrier defects and no sign of inflammation despite the heavy fungal load. It is possible that *Malassezia* take advantage of a compromised skin barrier and the mycelial form can go in search of a nutrient rich skin layer (Saadatzadeh et al., 2001). It is not clear how and why there is minimal inflammation despite the increased mycelial form and fungal load. Humoral specific IgG response toward *M. furfur* antigens has been reported for PV (Silva et al., 1997). Although PV could be treated by specific antifungal treatments there is high risk of relapse at up to 80%, which severely impacts the patients quality of life (Theelen et al., 2018).

Direct Pathogenesis: *Malassezia* in Seborrheic Dermatitis and Dandruff

Seborrheic dermatitis and Dandruff (D) are skin conditions found in sebaceous areas with hair. Dandruff is restricted to the scalp and involves itchy, flaking skin without visible inflammation, and is considered a mild non-inflammatory form of SD (Priestley and Savin, 1976; Danby et al., 1993; Warner et al., 2001). SD is a common chronic relapsing inflammatory skin disorder characterized by greasy scales with erythematous skin and exofoliative scaling (oily-yellow desquamation) on the scalp, which may extend to face, ears and upper chest associated with pruritus (Borda and Wikramanayake, 2015). Triggering factors include stress and cold, dry weather (Gary, 2013; Borda and Wikramanayake, 2015). The prevalence is higher in men than women, potentially due to hormonal influence by androgens (Islamoglu, 2019). However, this may also be a result of differences in grooming practices between genders. *Malassezia* have been identified and correlated to SD and D phenotypes, with *M. globosa, M. restricta, M. dermatis* and *M. furfur* associated with these conditions (Nakabayashi et al., 2000; Gupta et al., 2001; Gemmer et al., 2002; Sugita et al., 2002; Kim, 2009). There are three basic etiologic factors for SD and D;

Malassezia, sebaceous activity, and individual or host susceptibility (Deangelis et al., 2005; Ro and Dawson, 2005). Intrinsic host factors, such as composition of sebum and defective epidermal barrier, likely have an effect on *Malassezia* activity (density, lipase expression and nutrient utilization, immune stimulatory metabolites) that elicits the host inflammatory response. Metabolites such as oleic acid, arachidonic acid, malassezin, and indole-3-carbaldehyde act as skin irritants and are implicated in keratinocyte proliferation and inflammation as shown in **Figure 2**. The causative role of *Malassezia* in SD and D may be assessed through Koch's postulates (Koch, 1893). As *Malassezia* are found on all humans, Koch's first postulate cannot be fulfilled (Mcginley et al., 1975). However, it remains unclear whether there are *Malassezia* strain level differences between healthy and D or SD skin that manifest the disease. It is also not known whether the same *Malassezia* strain(s) that exist as commensals in healthy skin contextually become pathogenic due to unknown host environment and susceptibility factors. However, removal of *Malassezia* using antifungals improves D and SD, while removal of bacteria does not, and removal of both bacteria and fungi provides a similar benefit to the removal of fungi alone (Vanderwyk, 1964; Vanderwyk, 1967; Leyden et al., 1976). Furthermore, reintroduction of resistant "*P. ovale*" (likely *M. globosa*) during application of an antifungal (nystatin) are able to induce D and SD flaking (Gosse, 1969). Finally, a specific *Malassezia* metabolite, oleic acid, induces a D like desquamation when applied to scalp free from *Malassezia* and flaking (in individuals previously determined to be susceptible to D and SD) (Ro and Dawson, 2005). These observations fulfill three of four Koch's postulates and clearly establish the pathogenic role of *Malassezia* in causing D and SD (Gran et al., 2020). The mechanisms of individual susceptibility to D and SD remains unclear, but host genetics implicate immune response (ACT1, C5, IKBKG/NEMO, STK4) and epidermal differentiation (ZNF750). However, it is still not known how disruption to these genetic factors is related to the clinical presentation of D and SD (Jacobs and Miller, 1972; Evans et al., 1977; Mancini et al., 2008; Abdollahpour et al., 2012; Crequer et al., 2012; Nehme et al., 2012; Boisson et al., 2013; Halacli et al., 2015; Karakadze et al., 2018).

Malassezia in Atopic Dermatitis: Pathogenesis or Mutualism?

Atopic dermatitis (AD) and psoriasis are characterized by chronic skin inflammation due to multiple genetic, immune and environmental factors (Bjerre et al., 2017; Weidinger et al., 2018; Nowicka and Nawrot, 2019; Langan et al., 2020). The majority of AD patients have skin barrier dysfunction either due to mutations in genes such as filaggrin and tight junctions or from a more generalized disruption from the presence of Th2 cytokines (Palmer et al., 2006; Sandilands et al., 2009; Jungersted et al., 2010; Rerknimitr et al., 2017; Drislane and Irvine, 2020). The disruption is similar in both instances with a compromised barrier as demonstrated by increased trans-epidermal water loss, high pH, reduced stratum corneum hydration, and altered microbiota (Yang et al., 2020). Increased percutaneous sensitization from microbial products or allergens then produces a vicious cycle stimulating host immunity with resulting dryness, itching and erythema that often progresses into lesional flares and infections (De Benedetto et al., 2012; Lunjani et al., 2018). The incidence of AD is approximately 15% to 20% of children and up to 10% of adults with an age specific disease pattern (Eichenfield et al., 2014; Nutten, 2015). The skin microbiome is strongly associated in pathogenesis of AD with an over growth of *Staphylococcus aureus* at the infected lesions and a distinct microbial configuration in non-lesional skin including alterations in *Malassezia* species (Leyden et al., 1974; Kong et al., 2012; Kobayashi et al., 2015; Chng et al., 2016).

The role of *Malassezia* in AD is supported by both antifungal treatments reducing the severity of symptoms and that application of *Malassezia* extracts or recombinant *Malassezia* antigens on AD subjects exacerbates the phenotype (Zargari et al., 2001; Johansson et al., 2003; Brodska et al., 2014; Glatz et al., 2015; Prohic et al., 2016) (**Figure 2**). *Malassezia* are frequently isolated from and associated with AD, with *M. globosa* and *M. restricta* found more frequently followed by *M. sympodialis* and *M. furfur* (Sugita et al., 2001; Amaya et al., 2007; Kaga et al., 2011). However, one study reported that AD patients yielded exclusively *M. sympodialis* isolates (Sandstrom Falk et al., 2005; Jagielski et al., 2014). One possible explanation why *Malassezia* may be in lower abundance on AD skin is the reduced lipid content associated with the dry skin phenotype (Pilgram et al., 2001; Chng et al., 2016; Theelen et al., 2018). In AD the susceptibility of host to *Malassezia* infection has also been associated with a cytokine gene polymorphism and clinical outcome (Jain et al., 2017).

Malassezia are even more strongly implicated in development and persistence of a specific subset of adult head and neck eczema, proposed to be caused by *Malassezia* allergens (Saunte et al., 2020). This subset of patients responds to oral or topical antifungal therapy and have circulating antibodies to multiple *Malassezia* antigens (Scheynius et al., 2002). While the subgroup and antigens were initially found with *M. sympodialis* antigens (Mala S1-9), antigens to other species have also come to light (Andersson et al., 2003). *Malassezia* antigens MGL 1304 (*M. globosa*), Mala s 8, Mala s 13 (*M. sympodialis*), and Mala r 8 (*M. restricta*) have now been shown to be released through *Malassezia* nanovesicles due to the increased skin pH in AD (Selander et al., 2006; Gehrmann et al., 2011; Kohsaka et al., 2018). The antigenic protein MGL-1304 is involved in histamine release and implicated as a component of the AD pathogenesis (Kohsaka et al., 2018). These antigenic proteins are found in sweat, and can cause sweat allergy and an IgE specific AD skin immune response (Hiragun et al., 2013). It is also known that the IgE sensitization profile to skin commensal *M. sympodialis* and specific allergens from the bacterial pathogen *S. aureus* differ between moderate and severe AD patients (Mittermann et al., 2016). One other AD subject sub-group has a $CD4^+$ T cell

population specifically reacting to *Malassezia* thioredoxin antigen (Mala s 13). Mala S 13 is a homolog of human thioredoxin, and the $CD4^+$ T cells cross react with human thioredoxin, leading to AD skin inflammation. A similar triggering mechanism is attributed to Mala S 11, manganese dependent superoxide dismutase (Vilhelmsson et al., 2007; Balaji et al., 2011).

The disrupted skin barrier can provide a constant source for *Malassezia* and its allergens to enter the skin and interact with TLR2 receptors on DCs and keratinocytes. Specific pro-inflammatory cytokines and *Malassezia* specific IgE antibodies are produced through T cell response and B cell activation. The mast cells and DCs also contribute to skin inflammation sustained by cross reacting auto reactive T cells. In AD patients the increased levels of cytokines IL-4, IL-10 and IL-13 suggest inflammatory response and these cytokines are known to reduce the levels of antimicrobial peptides such as Cathelicidin (LL-37) and Human beta-defensins 2 and 3, as well as and skin barrier proteins filaggrin, loricrin and involucrin produced by keratinocytes (**Figure 2**; (Mcgirt and Beck, 2006; Howell et al., 2007; Kim et al., 2008). The growth of pathogens such as *Staphylococcus aureus* and their biofilms in AD skin is probably favored due to lack of abundance of *Malassezia* and reduction of human defensins (Glatz et al., 2015; Chng et al., 2016). *Malassezia* is also known to inhibit the growth of *S. aureus* biofilms through secretion of specific proteases, suggesting its possible role in healthy and AD skin (Li et al., 2018). *Malassezia* antigens are also responsible for activation of NLRP3 inflammasome in DCs and can trigger production of IL-1β, IL-4, IL-5, IL-13 and IL-18 cytokines *in vitro* (Kistowska et al., 2014). A number of these cytokines contribute to inflammation in AD skin as well as other allergic diseases, however, it is not clear whether *Malassezia* antigens are specifically expressed in AD skin or healthy subjects. Additionally, a specific subset of Th17 memory T cells is elicited to *Malassezia* in AD patients aggravating inflammation directly dependent on IL-17 (Sparber et al., 2019). Taken together, there is mounting evidence that *Malassezia* have a role in AD. The specific *Malassezia* contribution to the AD phenotype is not clear and interactions could occur *via* cell wall, cell membrane or lipid metabolite components that stimulate an immune response. However, what has been shown is that antifungals are effective in reducing AD severity, and this should be further explored (Kolmer et al., 1996; Nikkels and Pierard, 2003).

Malassezia in Psoriasis: Evolving to a Pathogenic Relationship in Susceptible Individuals?

Psoriasis affects skin, nails, and joints and is characterized by epidermal hyperproliferation and hyperkeratinisation (Perera et al., 2012; Hugh and Weinberg, 2018). Psoriasis is a T cell mediated autoimmune disease and primarily the result of a combination of genetic and environmental factors (Barker, 1998). Psoriasis (as with AD) has a strong microbial component that could drive or exacerbate the disease phenotype (Fyhrquist et al., 2019; Hurabielle et al., 2020). Certain *Malassezia* species have been associated with particular subtypes of psoriasis such as *M. japonica* and *M. furfur* with psoriasis vulgaris and *Malassezia* yeasts with guttate and scalp psoriasis (Aydogan et al., 2013; Gomez-Moyano et al., 2014; Honnavar et al., 2015). *Malassezia globosa* is the predominant yeast found in scalp psoriatic lesions, followed by *M. furfur* and *M. sympodialis*, but as these are also the same species found on normal scalp there is little evidence they are causal in pathogenesis. However, serum analysis from psoriatic individuals has indicated antibodies against *Malassezia* and its antigens (Squiquera et al., 1994; Gemmer et al., 2002; Jagielski et al., 2014). In Psoriasis, interleukin 23 (IL-23)/Th17 immune axis has been identified as a major pathway (Blauvelt, 2008; Girolomoni et al., 2017; Li et al., 2020). *Malassezia* can also induce Th1-related cytokines in peripheral blood mononuclear cells *in vitro* (Kanda et al., 2002; Valli et al., 2010) and induce keratinocyte proliferation and proinflammatory cytokine production which could potentially enhance inflammation (Baroni et al., 2004). Topical and systemic antifungal treatments show marked efficacy of improvement in psoriatic lesions (Rosenberg and Belew, 1982; Farr et al., 1985; Amichai, 2004; Armstrong et al., 2016; Beck et al., 2018). In an epicutaneous psoriasis mouse model involving pre-exposure to *Malassezia* followed by Imiquimod (IMQ) there is induction of skin inflammation in a Th17-dependent manner with a transcriptome similar in profile to human psoriatic lesions. Taken together *Malassezia* is likely an exacerbating factor in psoriasis where antifungal treatment can lead to symptomatic improvement but is not the initiating event (Hurabielle et al., 2020). This necessitates attempting more controlled antifungal treatment strategies in psoriasis (Stehlikova et al., 2019). Further work is required to fully understand the role of *Malassezia* in psoriasis.

The strongest evidence that *Malassezia* can directly cause skin disease remains their role in D and SD, where it is clear they play a causal role (Grice and Dawson, 2017; Theelen et al., 2018). They are also very likely to be the causative agent in several less common disorders, including PV and *Malassezia* folliculitis (Gupta et al., 2004). It still remains less clear as to what specific role *Malassezia* may have in inflammatory skin disease pathogenesis. To fully elucidate the role of *Malassezia* in inflammatory skin disease more longitudinal treatment-based studies are needed to elucidate the protective or pathogenic role in susceptible individuals. It is likely that strain level analysis will also aid in defining the molecular mechanisms by which *Malassezia* contextually interact with our skin and how to strategize fungal targeted therapy toward improving clinical outcomes.

MALASSEZIA AS A PROTECTOR AGAINST SKIN PATHOGENS: MUTUALISM?

Skin barrier homeostasis is maintained in part due to multi-kingdom microbial communities' protective roles against pathogens (Byrd et al., 2018). A number of bacterial species have now been shown to provide a protective and synergistic

relationship that maintains skin homeostasis (Nakatsuji et al., 2017). It has been hypothesized that the skin fungal mycobiome, primarily *Malassezia*, can protect human skin through its large biomass and spatial-temporal expansion. *Malassezia* metabolize sebum and produce short chain fatty acids (SCFA), such as azelaic acid, which are known to have dual antibacterial and anti-mycotic properties (Brasch and Christophers, 1993). Moreover, *Malassezia* mediated esterification of medium-chain fatty acids generate ethyl ester derivatives with *in vitro* antimycotic activity (Mayser, 2015). *M. globosa* secreted aspartyl protease 1 (MgSAP1) has been shown *in vitro* to disrupt biofilm formation of *Staphylococcus aureus via* hydrolysis of *S. aureus* protein-A (Li et al., 2018). In AD associated microbiomes, there is significant reduction of *Malassezia* as a genus and specifically *M. globosa* resulting in a potentially decreased protective function which could otherwise be restricting *S. aureus* pathogenicity (Chng et al., 2016).

CONCLUSION

Malassezia are now known to cause skin and scalp disorders, but there remain numerous gaps in the mechanistic understanding of how body site microenvironments affect multi-kingdom skin microbiota composition and function in both healthy homeostasis and disease states. To reach this end goal of defining the role of *Malassezia* as commensal, pathogen, or mutualist we still need to rationalize differences between detection methodologies. Although there is ongoing, rapid advancement in sequencing technologies and metagenomics analysis, it is not yet clear why there is such a large discrepancy between results obtained with cultures, ITS, and metagenomics. It is imperative that future microbiome studies, including any *Malassezia* mediated skin or systemic disease, needs appropriately controlled, robust, and reproducible detection methods which consider and balance both new, cutting edge sequencing techniques and established, well vetted technologies. Also, *Malassezia* have had multiple confusing changes in nomenclature and continuing expansion of the known species, making tracking of the primary literature challenging and assignment of pre-1998 activities to current strains nearly impossible (Theelen et al., 2018). As recent work has shown divergent function between even closely related species and strains, care must be taken in strain identification and assignment of function (Chng et al., 2016; Li et al., 2018). Additionally, many current studies investigating the relationships between microbe and host are limited to parallel group studies of specific pathways in target diseases, limiting understanding of molecular mechanisms that initiate or exacerbate disease progression. Mode of disease onset, function of the skin microbiota, and their role in healthy homeostasis will need to be investigated by implicit longitudinal study design, with appropriate comparison of severity and treatment stages (Grice and Dawson, 2017). These multiple factors have limited our current understanding of the role of mycobiota in healthy skin homeostasis.

In conclusion, the relationship between *Malassezia* and their human host is complex, varies with body site, age, and host susceptibility, and can be in any given circumstance a commensal, pathogenic, or mutualistic relationship. It is important for future clinical studies to account for intrapersonal anatomical variations in the skin microbiota, individual susceptibility, gender, age, seasonality, and ethnicity. Detailed information should also be included to capture the various stressors and perception of skin health or disease, which may promote endocrine and metabolic host changes within the cutaneous microenvironments. Full analysis of these variations will help to delineate the direct influence of microbial alterations in homeostasis of healthy skin and to develop understanding of solid causal relationships.

An improved understanding of the host-*Malassezia* relationship offers tremendous potential for development of treatments to improve skin health outcomes. There are opportunities to develop mycobiome targeted solutions using prebiotic or post-biotic metabolites with the potential to restore healthy skin microbiome and functional attributes such as barrier, dryness, inflammation, and reverse dysbiosis. While most studies to date describe bacterial interactions, it is crucial for future endeavors to address the mechanistic processes between fungal-fungal, inter-kingdom communities, and microbe-host for skin health and disease (Arvanitis and Mylonakis, 2015; Tipton et al., 2018; Zhang et al., 2018). Although there are now an increasing number of detailed studies that demonstrate the mycobiota's role in commensal and disease states, a substantial knowledge gap remains in understanding fungal virulence determinants and requires further attention.

AUTHOR CONTRIBUTIONS

All authors planned the review, read, and approved the final version. SHVC and RS wrote the initial draft. All authors contributed to the article and approved the submitted version.

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