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From Skin Commensals to Bloodstream Pathogens: In Vitro Virulence Traits and In Vivo Pathogenicity of *Malassezia* Isolates

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ABSTRACT

Background: The lipid-dependent yeast genus *Malassezia* is composed of skin commensals that can become pathogenic. This genus can cause bloodstream infection in low-weight preterm neonates and immunocompromised adults. However, little is known about the virulence mechanism involved in the transition from commensal to pathogenic.

Objective: This study aimed to evaluate and compare the in vitro and in vivo virulence of *M. furfur*, *M. pachydermatis* and *M. sympodialis* strains from fungemia cases and healthy skin or superficial clinical entities.

Methods: Twenty-five isolates, along with three reference strains of the three evaluated species, were assessed in vitro for virulence traits, including phospholipase and hemolytic activity, thermotolerance, oxidative stress resistance and biofilm production. Those isolates that presented the highest or lowest in vitro virulence based on the evaluated traits were evaluated in vivo in the *Galleria mellonella* infection model.

Results: Fungemia isolates of the three species showed higher phospholipase activity, biofilm formation, thermotolerance and oxidative stress resistance. Only for *M. furfur*, a high hemolytic activity was registered for the fungemia isolate. In vivo evaluation supported the previous in vitro results, in which larvae inoculated with isolates from fungemia and with high in vitro virulence were demonstrated to induce higher mortality and tissue burden, contrasting with isolates from healthy skin or skin clinical entities.

Conclusion: The evaluated isolates exhibited heterogeneous in vitro phenotypes and experimental pathogenicity. The findings support further investigation of relationships between these traits and infection outcomes. Finally, *M. furfur* showed a higher capacity to survive in the host, correlating with the *Malassezia*-fungemia epidemiology.

1 | Introduction

The genus *Malassezia* comprises 20 legitimately described species [1–3] and 4 proposed new species by metagenomic [4] and phylogenetic [5] analyses, primarily found as part of the skin microbiota of humans and animals. This genus is characterized by lipid dependence due to the lack of the fatty acid synthase (FAS) complex, which is required for the de novo synthesis of fatty acids [6, 7]. Although most of the species of this genus behave as commensals, they have been associated with different clinical entities such as seborrheic dermatitis (SD) [3] which has shown a high incidence in HIV patients [8], pityriasis versicolor

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(PV) [3], atopic dermatitis (AD) [3], psoriasis, chronic diseases like colorectal and pancreatic cancer [9, 10] and bloodstream infection (BSI) [11, 12].

Regarding BSI, *Malassezia furfur*, *Malassezia pachydermatis* and *Malassezia sympodialis* are the three species linked to this infection [11]. Among them, *M. furfur* is the most associated species [13, 14]. *M. pachydermatis*, typically isolated from animals, is the second most frequent cause of *Malassezia* fungemia, followed by *M. sympodialis*. Fungemia caused by *Malassezia* primarily affects low birth-weight neonates with prolonged hospitalization, parenteral lipid nutrition and central venous catheters (CVC) [11, 15]. Furthermore, immunosuppressed adults with CVC can also develop fungemia caused by this species [15].

Despite the clinical importance, the mechanisms that drive the transition of *Malassezia* species from commensal to opportunistic pathogens remain poorly understood. Increasing evidence suggests that isolates from the mycobiota of healthy skin and clinical entities differ in enzyme activity, biofilm production and antifungal resistance to fluconazole and amphotericin B [16, 17]. However, most studies focus on virulence mechanisms involved in superficial infections rather than in fungemia. One of the most important reasons for the lack of information on the pathogenesis of systemic infection is the misdiagnosis of fungemia caused by *Malassezia*. This is largely due to the lipid dependence of these yeasts, which complicates their isolation from clinical samples [18, 19]. However, screening studies conducted in neonatal intensive care units (NICU) have suggested that the prevalence of BSI caused by *Malassezia* could surpass that of *Candida* spp. [18–20].

Lipases and phospholipases that enable *Malassezia* to acquire lipids from the environment are among the main virulence mechanisms. These enzymes may contribute to the ability of *M. furfur*, *M. pachydermatis* and *M. sympodialis* to thrive in lipid-rich environments, including parenteral nutrition systems used in intensive care units [3, 11]. Furthermore, this genus has been shown to produce biofilms that may allow it to colonize medical devices [21], evade the host immune response and resist antifungal activity, as has also been shown for other pathogenic fungi such as *Cryptococcus* spp. [22]. Species such as *M. furfur*, *M. pachydermatis* and *M. sympodialis* have been reported to have a high capacity for adherence and biofilm formation on abiotic surfaces, which explains their involvement in nosocomial infections, particularly in intensive care units [21].

In addition to their ability to adhere and form biofilms, *Malassezia* spp. have developed mechanisms to evade the host immune response [3, 23, 24]. Also, *Malassezia* spp. have been shown to possess efficient antioxidant systems, including catalase and superoxide dismutase, which enable them to neutralize oxidative stress and persist in host tissues [3, 25]. Likewise, azelaic acid and melanin could also contribute to this oxidative stress tolerance [26]. Moreover, these yeasts can modulate the host immune response [11, 24]. Thermotolerance is another critical factor contributing to *Malassezia* survival during systemic infection, as it has been reported for fungal pathogens [27, 28]. Furthermore, it has been reported that fungal pathogens like *Candida* spp., *Cryptococcus* spp. and *Aspergillus* spp. rely on stress responses, such as thermotolerance and oxidative stress tolerance, to survive during systemic infections [27–29].

This study aims to compare thermotolerance, oxidative stress tolerance, biofilm formation, phospholipase and hemolytic activity among *M. furfur*, *M. pachydermatis* and *M. sympodialis* isolates from fungemia and non-bloodstream sources. Isolates demonstrating the lowest and highest in vitro virulence were evaluated in vivo for virulence using the previously standardized systemic infection model for *Malassezia* spp. in *Galleria mellonella* larvae [13].

2 | Materials and Methods

2.1 | Fungal Strains and Isolates

Three reference strains were included in this study: *M. furfur* CBS 1878, *M. pachydermatis* CBS 1879 and *M. sympodialis* CBS 7222. These reference strains were obtained from the culture collection of the Westerdijk Fungal Biodiversity Institute (WI-KNAW), Utrecht, The Netherlands. In addition, 25 clinical and commensal isolates were assessed: 19 *M. furfur*, 4 *M. pachydermatis* and 2 *M. sympodialis* isolates from the microorganism's collection of Grupo de Investigación Celular y Molecular de Microorganismos Patógenos (CeMoP group), Universidad de Los Andes, Colombia and kindly provided by the Mycology Department of Instituto de Medicina Regional (IMR-M), Universidad Nacional del Nordeste, Argentina, as shown in Table 1, were used in this study. These isolates were obtained from the skin of non-HIV patients with SD [31], AD [33], PV [34] and fungemia [35, 36], as well as from patients without lesions [31, 33]. Skin samples from HIV-positive and HIV-negative individuals without *Malassezia*-associated skin disorders were denominated, in this study, as isolates from 'non-lesioned skin (NL)'. Finally, two of the *M. pachydermatis* isolates were obtained from canines. The isolates were previously identified and reported by earlier studies [31, 33–36] (Table 1). In this study, isolates stored in 10% skim milk [Oxoid, UK] liquid medium at −80°C were reactivated by plating on modified Dixon (m-Dixon) agar, which contained 36 g/L mycosel agar [BD, USA], 20 g/L Oxgall [BD, USA], 36 g/L malt extract [Oxoid, UK], 2 mL/L glycerol [Sigma Aldrich, USA], 2 mL/L oleic acid [Sigma Aldrich, USA] and 10 mL/L Tween 40 [Sigma Aldrich, USA] [30]. Plates were incubated at 33°C for 72 h. For inoculum preparation, colonies were subcultured onto fresh m-Dixon agar plates and incubated for 48–72 h at 33°C [30]. The isolates were phenotypically characterized by evaluating beta-glucosidase and catalase activity, assimilation of Tweens (20, 40, 60 and 80) [Sigma Aldrich, USA] and Cremophor EL [Sigma Aldrich, USA], and growth on Sabouraud agar. The results agree with those reported [8] for the three evaluated species.

2.2 | Inoculum Preparation for Stress, Biofilm and In Vivo Assays

To conduct the stress droplet and biofilm production assays, the exponential growth phase of isolates and reference strains of *M.*

TABLE 1 | Origin of Isolates and reference strains.

Species	Name	Origin	Microorganisms' confirmation method/GenBank accession number, collection and reference
M. furfur	MF CBS 1878	Reference strain SD	[30]
M. furfur	MF 103.76 NL	Non-lesioned skin	5.8S rDNA-ITS2/JQ088202, CeMoP, [31]
M. furfur	MF 110.80 NL	Non-lesioned skin	5.8S rDNA-ITS2/JQ088274, CeMoP, [31]
M. furfur	MF 58.42 NL	Non-lesioned skin	5.8S rDNA-ITS2/JQ088203, CeMoP, [31]
M. furfur	MF 46 NL	Non-lesioned skin	RPB2/LR794350, CeMoP, [32]
M. furfur	MF 115.84 DS	SD	5.8S rDNA-ITS2/JQ088269, CeMoP, [31]
M. furfur	MF 57.41 DS	SD	5.8S rDNA-ITS2/JQ088204, CeMoP, [31]
M. furfur	MF 99.72 HIV	HIV patient Non-lesioned skin	5.8S rDNA-ITS2/JQ088215 CeMoP, [31]
M. furfur	MF 117.86 HIV	HIV patient Non-lesioned skin	5.8S rDNA-ITS2/JQ088218, CeMoP, [31]
M. furfur	MF 24.14 DSHIV	HIV patient with SD	5.8S rDNA-ITS2/HQ710825.1 CeMoP, [31]
M. furfur	MF 26.16 DSHIV	HIV patient with SD	5.8S rDNA-ITS2/HQ710828.1 CeMoP, [31]
M. furfur	MF 29.18 DSHIV	HIV patient with SD	5.8S rDNA-ITS2/HQ710824.1, CeMoP, [31]
M. furfur	MF 37.24B DSHIV	HIV patient with SD	5.8S rDNA-ITS2/JQ088205, CeMoP, [31]
M. furfur	MF 36 DA	AD	RPB2/LR794344 CeMoP, [31, 32]
M. furfur	MF 3 DA	AD	RPB2/LR794362, CeMoP, [32, 33]
M. furfur	MF 1828 PV	PV	RPB2/LR794356, CeMoP, [32, 33]
M. furfur	MF 1 PV	PV	RPB2/LR794367, CeMoP, [32, 34]
M. furfur	MF IMR-MM 829	Fungemia	Polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP), IMR-M, [35, 36]
M. furfur	MF IMR-MM 848	Fungemia	Polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP), IMR-M, [35, 36]
M. furfur	MF IMR-MM 959	Fungemia	Polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP), IMR-M, [37]
M. pachydermatis	MP CBS 1879	Reference strain Dog externa otitis	[38]
M. pachydermatis	MP 131	Canine isolate	26S rDNA/KU757222, CeMoP, [39]
M. pachydermatis	MP 80	Canine isolate	26S rDNA/KU757203, CeMoP, [39]
M. pachydermatis	MP 041 H	Non-lesioned skin	5.8S rDNA-ITS2 and 26S rDNA/PQ643978 and PQ571773 CeMoP, [40]
M. pachydermatis	MP IMR-MM 954	Fungemia	Polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP), IMR-M, [35, 36]
M. sympodialis	MS CBS 7222	Reference strain Non-lesioned skin	[41]
M. sympodialis	MS 1DA	AD	5.8S rDNA-ITS2 and 26S rDNA/PQ647265 and PQ571752, CeMoP, [42]
M. sympodialis	MS 5S	Fungemia	5.8S rDNA-ITS2/MT648822, CeMoP, [43]

furfur, *M. pachydermatis* and *M. sympodialis* was induced by transferring a loop of the growth lawn into 3 mL of 0.5% Tween 80 [Sigma Aldrich, USA] and adjusting the turbidity to McFarland Standard 2. This suspension was then added to 27 mL of m-Dixon broth (6 g/L peptone [Oxoid, UK], 20 g/L Oxgall [BD, USA], 36 g/L malt extract [Oxoid, UK], 2 mL/L glycerol [Sigma Aldrich, USA], 2 mL/L oleic acid [Sigma Aldrich, USA] and 10 mL/L Tween 40 [Sigma Aldrich, USA]) in a 125 mL Erlenmeyer flask. The flasks were incubated at 33°C with shaking at 180 RPM for 72 h. Following this activation period, 0.3 mL of the liquid culture was transferred into 29.7 mL of fresh m-Dixon broth and incubated overnight at 33°C with continuous shaking at 180 RPM; at this point, the culture reached the logarithmic (log) phase [44].

The inoculum preparation for the stress droplet assay was performed as described in a previous study [29] with slight modifications. Briefly, metabolically active yeasts from the log-phase liquid cultures of the three species isolates and reference strains were washed for the stress droplet assay. The liquid cultures were transferred to 50 mL conical tubes and centrifuged at 1248 ***g*** for 10 min. The supernatant was discarded, and 20 mL of 1X phosphate-buffered saline (PBS) (pH 7.4) [GibcoTM, Thermo Scientific, USA] was added. The pellet was resuspended, and the mixture was subjected to a second centrifugation at 1248 ***g*** for 10 min. After discarding the PBS, 20 mL of m-Dixon broth was added, and yeasts were counted in a Neubauer chamber to adjust the inoculum to 1.0×10^7 colony-forming units (CFU)/mL. This inoculum was then serially diluted from 1.0×10^7 CFU/mL to 1.0×10^4 CFU/mL [29, 45].

The inoculum was adjusted to 1.0×10^6 CFU/mL for biofilm production evaluation by following the same procedure used for the stress droplet assay inoculum preparation, starting from the log-phase liquid cultures [29]. For the virulence in vivo assay, the reference strain and the *M. furfur* 57.41 DS and *M. furfur* IMR-MM 959 isolates were plated on m-Dixon agar and incubated for 48–72 h. The resulting culture lawn was then diluted with 0.5% Tween 80 to adjust the inoculum to 1.5×10^9 CFU/mL, as previously described [13]. Since the *Malassezia* genus tends to aggregate, in all assays, inocula were prepared in 0.5% Tween 80, which is a detergent, to facilitate *Malassezia* homogenization.

2.3 | Phospholipase Activity Assay

Phospholipase activity was assessed according to Triana et al. [46] with minor modifications. Briefly, isolates were inoculated on egg-yolk agar supplemented with Tween 80 (8% egg yolk, 2.5 g/L yeast extract [Difco, USA], 0.5 g/L ammonium sulphate [Merck, Germany], 0.5 g/L calcium chloride, [Sigma-Aldrich, USA], 0.1 g/L Dibasic Potassium Phosphate, 18 g/L agar-agar [Oxoid, UK], 2% Tween 80 [Sigma Aldrich, USA]) [24, 25] and incubated at 33°C for 21 days. Then, the phospholipase activity (PHz) was calculated as the ratio of the colony growth diameter to the colony growth area plus the zone of precipitation [46].

Phospholipase activity was classified according to the following PHz values:

- No phospholipase activity: PHz = 1.
- Low phospholipase activity: $0.8 \leq$ PHz ≤ 0.9.
- Moderate phospholipase activity: $0.64 \leq$ PHz ≤ 0.79.
- High phospholipase activity: PHz < 0.64.

2.4 | Hemolytic Activity

Hemolytic activity was determined using the method described by Chebil et al. [47] with slight modifications. Sabouraud dextrose agar [Oxoid, UK] was prepared, supplemented with 0.1% Tween 80, 0.5% chloramphenicol and cycloheximide [Sigma-Aldrich, USA] and 5% sheep blood.

Inocula of the isolates were prepared in a 0.5% (v/v) Tween solution in sterile distilled water and adjusted to a concentration equivalent to the McFarland standard 5. Then, 10 µL of each inoculum was seeded in triplicate and incubated at 33°C for 21 days. The presence or absence of hemolysis was recorded daily. At the end of the incubation period, hemolysis halos were measured, and the hemolysis coefficient (Hz) was calculated as the ratio between the colony diameter and the diameter of the hemolysis zone plus the colony growth area. Hemolytic activity (Hz) ranges were defined as follows [47]:

- No hemolytic activity: Hz = 1.
- Low hemolytic activity: $0.7 \leq$ Hz ≤ 0.99.
- Moderate hemolytic activity: $0.4 \leq$ Hz ≤ 0.69.
- High hemolytic activity: Hz < 0.4.

2.5 | Stress Droplet Assays

Oxidative stress and thermotolerance assays were performed for all *Malassezia* strains and isolates to compare interspecies and intraspecies variation. To evaluate oxidative stress and capacity to grow at 37°C (thermotolerance), two concentrations of menadione (0.2 mM and 0.4 mM) and incubation at two temperatures (the optimal growth temperature at 33°C [26, 48], which is also the surface human temperature, and the internal human body temperature of 37°C) were used [27, 28]. m-Dixon agar plates were supplemented with either of these menadione concentrations. Then, 3 µL of each inoculum, adjusted to concentrations ranging from 1.0×10^7 CFU/mL to 1.0×10^4 CFU/mL, were sequentially spotted onto the agar. The plates were incubated at 33°C and 37°C for 72 h. Following incubation, oxidative stress and thermotolerance were assessed by measuring growth inhibition. To quantify growth inhibition, the Background Grey Value (BGV) of each spot was determined using the ImageJ software [49]. Briefly, each plate was imaged using a Bio-Rad ChemiDoc MP Imaging System with Universal Hood III at Universidad de Los Andes, Bogotá, Colombia and the images were processed in ImageJ by subtracting the average local BGV from the spot images [49]. Since the most distinct differences among treatments were observed in spots inoculated with 1.0×10^5 CFU/mL, these spots were selected for analysis because of the clear difference in cell growth.

2.6 | Indirect Biofilm Production Evaluation

Biofilm production was evaluated following previously published protocols with slight modifications [50]. Briefly, biofilm production was indirectly quantified in polystyrene flat-bottom 96-well culture plates through a metabolic assessment [Corning, USA]. 200 μL of inoculum, adjusted to 1.0×10^6 CFU/mL, from each of the 25 evaluated isolates and the reference strains was added to each well in triplicate. Three additional wells containing 200 μL of m-Dixon broth served as negative controls. The plates were incubated at 37°C with continuous shaking at 75 RPM for 24 h. To remove non-adherent cells, the wells were carefully washed three times with 200 μL of 1X PBS (pH 7.4). The plates were then re-incubated at 37°C with continuous shaking at 75 RPM for 96 h, with daily medium changes.

Biofilm production was indirectly quantified using the XTT reduction assay [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] [Sigma Aldrich, USA] [21]. After 96 h, the wells were washed three times with 1X PBS (pH 7.4) to remove any remaining non-adherent cells. Then, 1 μL of 10 mM menadione [Sigma Aldrich, USA] in acetone was added to 10 mL of a 0.5 g/L XTT stock solution. A 100 μL volume of the XTT menadione mixture was added to each well, and the plates were covered with aluminium foil and incubated at 37°C for 2 h. After incubation, 80 μL of the solution from each well was transferred to a new plate, and absorbance was measured at 490 nm [51] using a Bio-Rad iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 | Virulence In Vivo Assays

Before the assay, larvae weighing between 200 and 300 mg and showing no visible markings were selected [13]. The larvae were then decontaminated by immersing them for 30 s in a 0.1% sodium hypochlorite solution, followed by two consecutive rinses in sterile distilled water for 30 s and 10 s, respectively [13]. The larvae were inoculated with the previously prepared inoculums (1.5×10^9 CFU/mL) of *M. furfur* CBS 1878, *M. furfur* 57.41 DS, *M. furfur* IMR-MM 959, *M. pachydermatis* CBS 1879, *M. pachydermatis* 131, *M. pachydermatis* IMR-MM 954, *M. sympodialis* CBS 7222, *M. sympodialis* 1DA, *M. sympodialis* 5S suspended in 0.5% Tween 80. Using a BD Ultra-Fine needle (31G) insulin syringe (0.5 mL), 20 μL of either the inoculum or 0.5% Tween 80 (negative control) was injected into the last left proleg of 15 larvae per treatment [13]. The chosen isolates were those that showed the highest and lowest in vitro virulence, respectively. The inoculated larvae were incubated in separate Petri dishes, each corresponding to a specific treatment, at 37°C for 15 days. Daily evaluations of larval mortality and melanization were conducted throughout the incubation period [13].

2.8 | Melanization Evaluation

After larval inoculation, the larvae were monitored daily for 5 days to assess melanization on the cuticle surface. The melanization of each larva was scored on a scale of 0 to 4, where 0 indicated full melanization and 4 indicated no melanization, as described by Torres et al. [13]. However, the melanization scores were plotted from day 0 to 5 in a cumulative form, with a maximum value of 24 for the control larvae.

2.9 | Larval Fungal Burden

For reference strains, the mean lethal time to 50% mortality (LT_{50}), which is the time needed to reach 50% larval mortality, was determined; three larvae per treatment were euthanized by incubating at −20°C for 10 min [13]. Then, larvae were homogenized in 1 mL of 0.5% Tween 80 using a micro-pestle at 1500 RPM for 1 min. Homogenized tissue in 0.5% Tween 80 was serially diluted to 1.0×10^{-3} [13]. Each dilution was plated on m-Dixon agar and incubated at 33°C for 15 days. CFUs were counted daily [13].

2.10 | Statistical Analysis

Each assay was performed in triplicate. GraphPad Prism 8 (version 8.2.0) was used for data analysis, with a p-value < 0.05 considered statistically significant. Oxidative stress assay, heat tolerance assay, and melanization evaluation were analysed through a Two-way ANOVA test followed by a Tukey test. Biofilm production evaluation, plasmocyte count and fungal burden data were analysed using the Kruskal–Wallis test, followed by a Dunn's multiple comparisons test for multiple comparisons. For larval survival data, Kaplan–Meier curves were constructed, and then the curves were compared using the Log-Rank (Mantel-Cox) test.

3 | Results

3.1 | Phospholipase Activity Varies Among *Malassezia* Isolates

According to the results, 3/4 (75%) of *M. pachydermatis* isolates exhibited moderate phospholipase activity, in contrast to *M. furfur* isolates. In this study, only (6/19) 31.6% of the *M. furfur* isolates exhibited moderate phospholipase activity. For *M. sympodialis* isolates, only 1/2 (50%) isolates showed moderate phospholipase activity. Regarding the reference strains, *M. furfur* CBS 1878 exhibited the lowest phospholipase activity among the three strains evaluated. Finally, there was no evidence of phospholipase activity in five of the *M. furfur* isolates. For *M. furfur*, isolates recovered from non-lesioned skin showed no or low phospholipase activity. Concerning *M. furfur* isolates that come from any of the skin disorders, phospholipase activity ranges from low to moderate; likewise, this is observed in isolates from fungemia, where *M. furfur* IMR-MM 829 and *M. furfur* IMR-MM 959 showed moderate phospholipase activity. For *M. pachydermatis*, only one isolate, which came from a canine (*M. pachydermatis* 131), showed low phospholipase activity. Regarding *M. sympodialis*, the isolate with moderate phospholipase activity was recovered from a patient with fungemia (*M. sympodialis* 5S). Remarkably, the three isolates (from the three evaluated species) that were later found to show higher in vitro virulence and then evaluated in vivo were the ones with higher phospholipase activity (Figure 1). To summarize, a higher phospholipase activity was observed in isolates derived from fungemia than in those from healthy skin or skin clinical entities.

3.2 | Higher Hemolytic Activity for *M. furfur* Isolates

Hemolytic activity was markedly higher in *M. furfur* isolates compared to *M. pachydermatis* and *M. sympodialis*. 4/19 (21%) of *M. furfur* isolates did show high hemolytic activity. *M. pachydermatis* and *M. sympodialis* isolates presented only low and moderate hemolytic activity. High hemolytic activity was particularly evident in *M. furfur* IMR-MM 829 and *M. furfur* IMR-MM 959 isolates. This suggests that hemolysis may be a relevant virulence mechanism for *M. furfur* in bloodstream infections. In contrast, fungemia isolates from *M. pachydermatis* and *M. sympodialis* species showed low hemolytic activity but moderate phospholipase activity. However, two *M. furfur* isolates from patients without skin lesions presented high hemolytic activity. Only one *M. pachydermatis* isolate had moderate hemolytic activity (Figure 1).

3.3 | *M. furfur* Isolates Showed a Lower Tolerance to Oxidative Stress and Temperature Increase

To assess their potential to cause systemic infection, isolates were evaluated for thermotolerance, oxidative stress tolerance and biofilm production. In vitro evaluations were conducted under three specific conditions. The first condition tested thermotolerance, based on the premise that a microorganism must withstand human body temperature (37°C) to become a successful pathogen [24]. The second condition focused on oxidative stress induced by the immune system. Phagocytic cells, acting as the first line of defence against pathogenic fungi, release reactive oxygen species (ROS) during immune responses [52]. The third condition assessed was biofilm production, an essential factor for colonizing medical device surfaces [11].

For the thermotolerance evaluation, *Malassezia* species isolates were cultured at either 33°C or 37°C; all *M. furfur* isolates grew at both temperatures, with no significant differences between isolates (p-value = 0.3271, 2-way ANOVA) or growth temperatures (p-value = 0.1543, 2-way ANOVA) (Figure 1). However, the reference strain exhibited significantly higher growth at 37°C than several isolates, including *M. furfur* 46 NL (p-value = 0.026, 2-way ANOVA), 115.84 DS (p-value = 0.0034, 2-way ANOVA), 57.41 DS (p-value = 0.0075, 2-way ANOVA), 3 DA (p-value = 0.0153, 2-way ANOVA), 1828 PV (p-value = 0.0362, 2-way ANOVA) and IMR-MM 829 (p-value = 0.0346, 2-way ANOVA) (Figure 1). Notably, (13/25) 52% of the isolates exhibited better growth at 37°C than at 33°C. These isolates were recovered from healthy skin (5 isolates), SD from HIV patients (4 isolates), AD (1 isolate), PV (1 isolate) and fungemia (2 isolates). At a concentration of 0.2 mM menadione, significant differences were observed between isolates (p-value < 0.0001, 2-way ANOVA) and growth temperatures (p-value = 0.0007, 2-way ANOVA). At this concentration, four isolates failed to grow at 37°C, including one from an HIV patient with SD, one from PV and two from fungemia (Figure 1). The effect of temperature at 0.4 mM menadione also resulted in significant differences among isolates (p-value < 0.0001, 2-way ANOVA). Three of the four isolates that grew at 0.4 mM

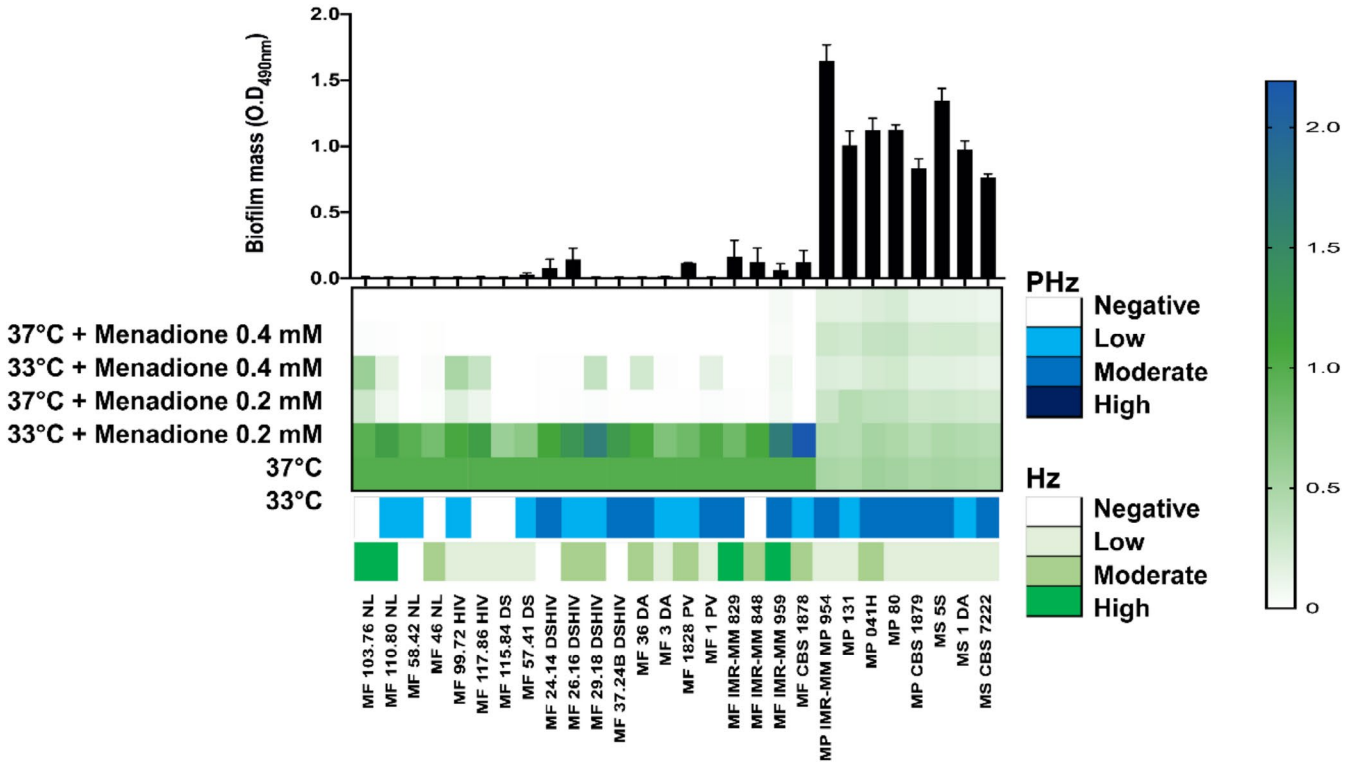

FIGURE 1 | In vitro evaluation of the virulence factor of *M. furfur*, *M. pachydermatis* and *M. sympodialis*. Metabolic activity associated with biofilm production was significantly higher for *M. pachydermatis* and *M. sympodialis* isolates. Moreover, for the three species, biofilm formation was higher for the fungemia isolates. Likewise, oxidative stress tolerance and thermotolerance were significantly higher for *M. pachydermatis* and *M. sympodialis* isolates. Concerning phospholipase activity (PHz), it was not evident or was low in isolates from healthy skin but more pronounced in isolates from fungemia. In these isolates, this activity was high. *M. furfur* isolates from fungemia showed high hemolytic activity (Hz).

menadione failed to grow at 37°C. All these isolates were from healthy skin samples (Figure 1). For *M. pachydermatis* and *M. sympodialis*, growth at either temperature was similar and higher than that of *M. furfur* (Figure 1).

Regarding oxidative stress, only one *M. furfur* isolate from healthy skin and two from SD samples failed to grow in the presence of any of the menadione concentrations. A significant difference was observed between treatments with and without menadione, and across the two concentrations, at both 33°C (p-value <0.0001, 2-way ANOVA) and 37°C (p-value <0.0001, 2-way ANOVA). At 37°C, menadione treatment significantly differed between isolates (p-value $=0.0013$, 2-way ANOVA). Only one isolate grew at 37°C with 0.4 mM menadione. At 33°C, only four isolates (three from healthy skin and one from fungemia) grew at 0.4 mM menadione; this number dropped to just one isolate at 37°C (*M. furfur* IMR-MM 959), which showed a slight increase in growth at this temperature. However, most *M. furfur* isolates could not grow under the effect of menadione. This contrasted with *M. pachydermatis* and *M. sympodialis* isolates, which could grow at either of the two menadione concentrations and at the evaluated temperatures. However, the thermotolerance and oxidative stress tolerance were lower for *M. sympodialis* isolates and the reference strain.

3.4 | Biofilm Production Is Higher for *M. pachydermatis* and *M. sympodialis* Isolates

To assess the capacity of the *Malassezia* species isolates to adhere to medical devices and evade immune responses, a metabolic activity associated with biofilm production assay was performed. In general, *M. furfur* isolates and the reference strain showed lower biofilm production. For the three species, a significant difference in biofilm production was observed between isolates (p-value <0.0001, Kruskal–Wallis test). Isolates derived from healthy skin and atopic dermatitis (AD) samples exhibited the lowest biofilm production. Additionally, four isolates from seborrheic dermatitis (SD) lesions and one isolate from pityriasis versicolor (PV) demonstrated low biofilm production (Figure 1). In contrast, all fungemia isolates exhibited high biofilm production. Notably, one isolate from PV and two isolates from HIV patients with SD also produced high biofilm (Figure 1). As found in *M. furfur* isolates from fungemia, *M. pachydermatis* and *M. sympodialis* isolates from fungemia showed high biofilm production. Importantly, biofilm production is higher in isolates from fungemia of the three evaluated species (Figure 1).

3.5 | Isolates With Higher In Vitro Virulence Are Also Highly Virulent In Vivo

In vivo virulence was assessed using survival and melanization assays in *G. mellonella* larvae. *G. mellonella* survival significantly varied across *Malassezia* isolates. For *M. furfur*, the fungemia isolate (IMR-MM 959) and the reference strain (CBS 1878) were the most virulent, both reaching lethal time (LT_{50}) at day 11, whereas isolate 57.41DS showed significantly higher survival, LT_{50} at day 15 (p-value <0.0057, log-rank [Mantel-Cox] test). Notably, while the reference strain showed a bimodal mortality pattern, the fungemia isolate exhibited a continuous decline (Figure 2A).

Regarding *M. pachydermatis*, all strains showed a continuous survival decrease (p-value <0.0001, log-rank [Mantel-Cox] test), unlike the bimodal pattern of *M. furfur*. Isolate IMR-MM

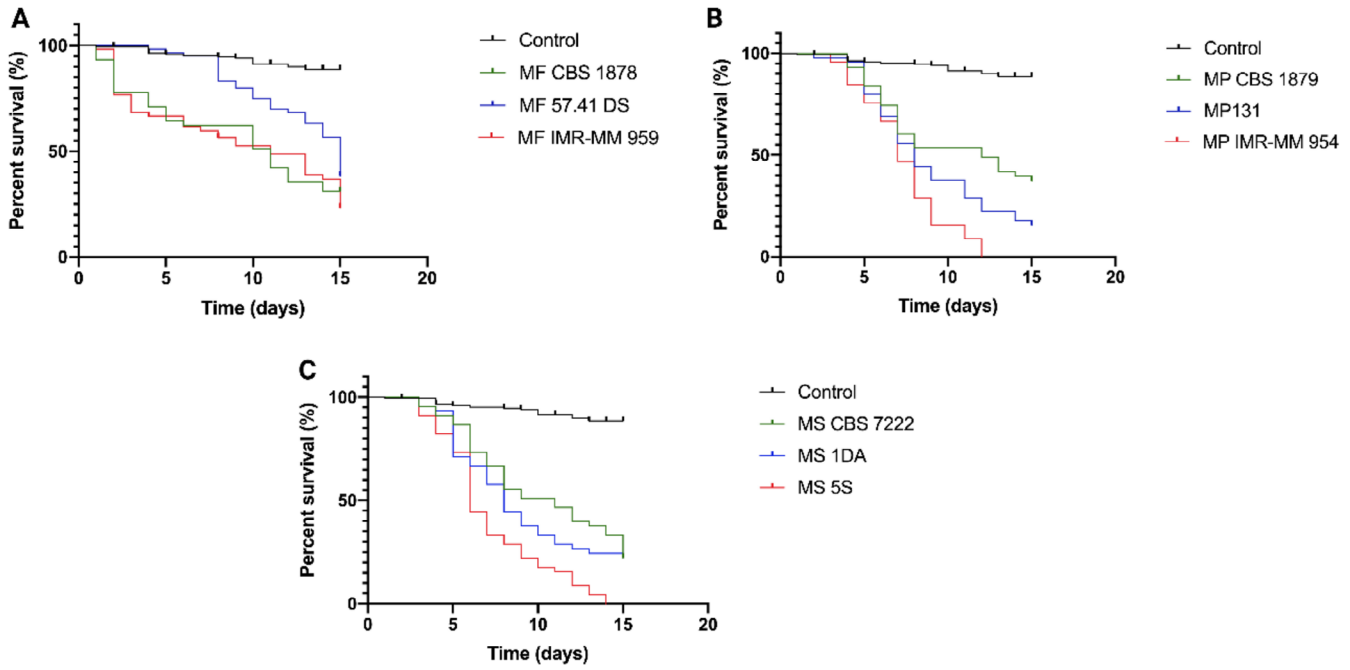

FIGURE 2 | Survival curves of *G. mellonella* larvae inoculated with *M. furfur, M. pachydermatis* and *M. sympodialis reference* strains and isolates. (A) Larvae inoculated with *M. furfur*. (B) Larvae inoculated with *M. pachydermatis* and (C) larvae inoculated with *M. sympodialis*. Isolates from fungemia showed higher increase in larval mortality rate.

954 was the one that induced a faster larval survival decrease (LT_{50} at day 7, 0% survival by day 12), followed by isolate 131 (LT_{50} at day 8) and the reference strain CBS 1879 (LT_{50} at day 13, 40% survival at the endpoint) (Figure 2B). For *M. sympodialis* (p-value <0.0001, log-rank [Mantel-Cox] test), isolate 5S showed the fastest decline (LT_{50} at day 6, 0% survival by day 14), while the reference strain and isolate 1DA showed a similar behaviour with LT_{50} at days 11 and 8, respectively (Figure 2C).

3.6 | *M. furfur* Induces a Stronger Immune Response

Melanization assays revealed distinct immune activation patterns (p-value = 0.05, two-way ANOVA). Larvae inoculated with *M. furfur* isolates presented the highest overall melanization scores, with the fungemia isolate inducing a distinct brownish coloration after day 5 (Figure 3A). Among *M. pachydermatis*, larvae inoculated with the *M. pachydermatis* IMR-MM 954 isolate triggered the strongest response (score around 3 from day 2), while the reference strain elicited the lowest levels. For *M. sympodialis*, isolate MS 1DA induced higher melanization than MS 5S.

Finally, plasmocyte counts significantly increased in all inoculated larvae with any of the *Malassezia* species (p-value = 0.0007, Kruskal–Wallis test), particularly in those infected with *M. furfur*. This trend correlates with the higher melanization scores observed, suggesting that *M. furfur* isolates trigger a more robust cellular and humoral immune response in *G. mellonella* compared to the other species (Figure 3B).

3.7 | Yeasts of Isolates of *Malassezia* From Fungemia Patients Were the Most Recovered From Inoculated Larvae

Fungal burden was used as a quantitative parameter to assess fungal virulence, reflecting the ability of *Malassezia* isolates to invade host tissues and evade immune responses [53, 54]. A significant difference in fungal burden was observed among isolates (p-value <0.0027, Kruskal–Wallis test) (Figure 4). Overall, fungemia isolates showed a tendency toward higher larval recovery rates than other strains. However, no significant differences were found between fungemia and reference strains for *M. furfur* (p-value >0.9999, Dunn's multiple comparisons test), *M. pachydermatis* (p-value >0.9999, Dunn's multiple comparisons test) or *M. sympodialis* (p-value >0.9999, Dunn's multiple comparisons test).

3.8 | Isolates That Showed Lower Virulence Presented Higher Melanin Accumulation

Histological assessment enables evaluation of immune response, tissue invasion and tissue integrity [55]. In all inoculated larval tissue, tissue integrity is lost with cuticle detachment and degradation of fat body tissue (Figure 5). About melanin nodule formation, higher melanin deposition was observed in larval tissue of larvae inoculated with isolates that showed lower in vitro and in vivo virulence (Figure 5C,D,I,J,O,P). Also, a higher number of yeast cells was observed inside nodules in larvae inoculated with isolates with higher in vitro and in vivo virulence for the three evaluated species of *Malassezia* (Figure 5E,F,K,L,Q,R).

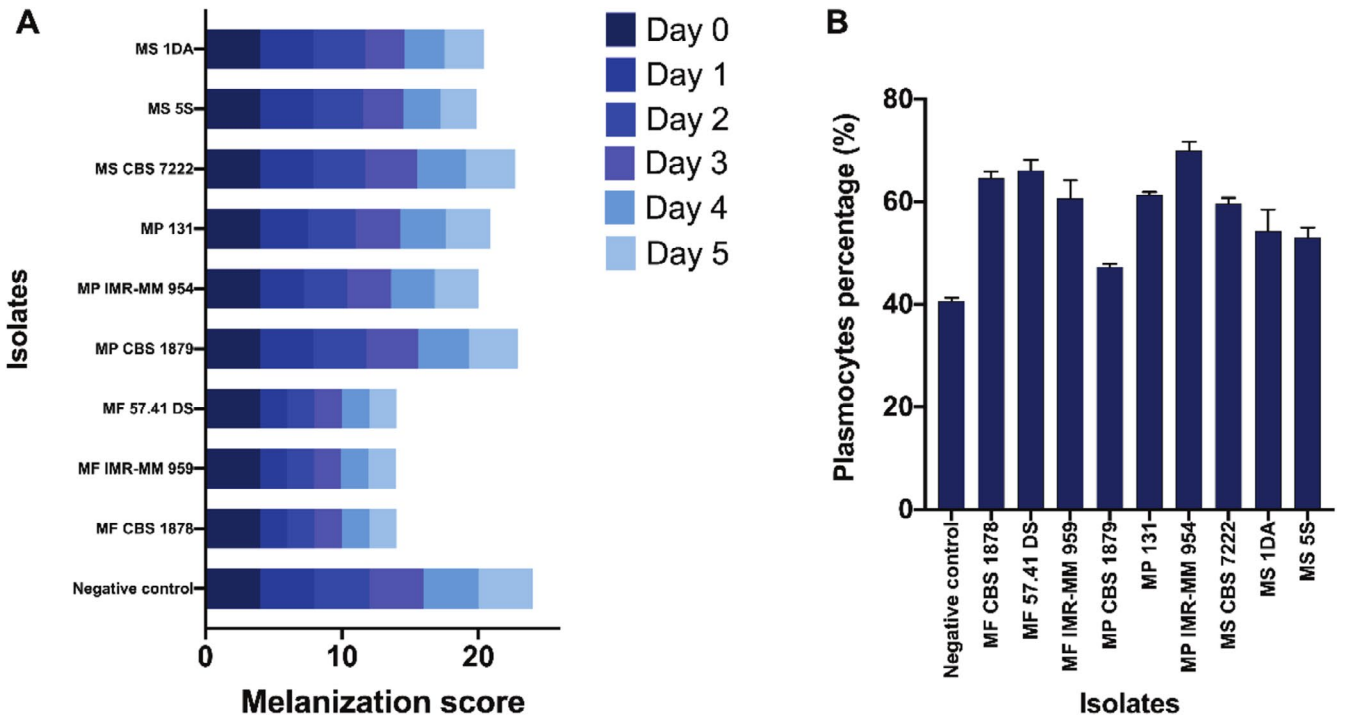

FIGURE 3 | Immune response indirect evaluation. (A) Melanization scores assessment with higher melanization in *M. furfur* larvae. (B) Plasmocytes Percentage showing a significant increase in inoculated larvae with any of the three evaluated species compared to the negative control larvae. However, the melanization scores were plotted from day 0 to 5 in a cumulative form, with a maximum value of 24 for the control larvae.

4 | Discussion

The genus *Malassezia* has been mostly known for being a skin commensal, but this genus can also be recognized as capable of causing life-threatening diseases such as fungemia in immunosuppressed adults and neonates in intensive care units [11]. *M. furfur* is the primary causative agent of *Malassezia*-fungemia, followed by *M. pachydermatis* and *M. sympodialis*. However, the mechanisms associated with the systemic invasion remain poorly understood. This study provides insights into the virulence profiles of these three species of *Malassezia*, comparing their in vitro and in vivo virulence. Highlighting how hemolytic activity, phospholipase activity, thermotolerance, oxidative stress resistance and biofilm formation correlate with their pathogenic potential.

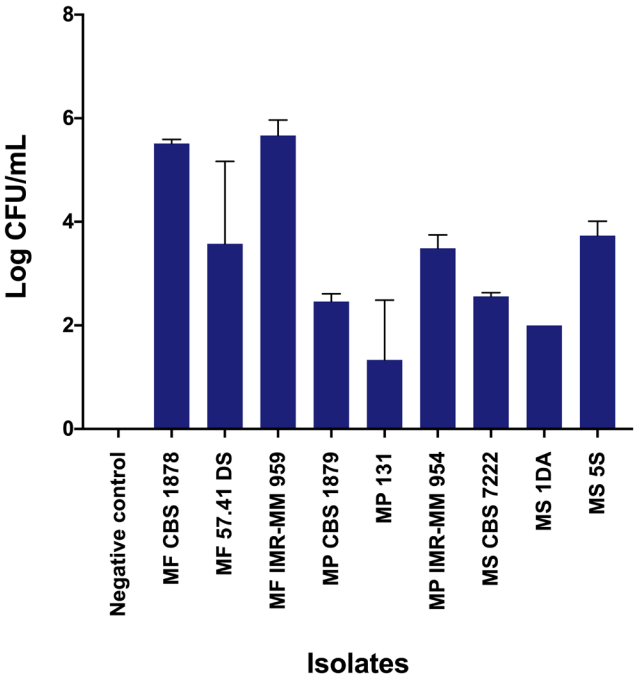

FIGURE 4 | Fungal burden. Larvae inoculated with *M. furfur* showed higher fungal burden than larvae inoculated with *M. pachydermatis* and *M. sympodialis*. Isolates from fungemia patients were recovered most often in all cases. MF: *M. furfur*; MP: *M. pachydermatis*; MS: *M. sympodialis*.

M. furfur is a skin-dwelling microorganism [56], where the surface temperature typically ranges from 33°C to 36°C. Consequently, the optimal growth temperature for the *Malassezia* genus is approximately 34°C [26]. Therefore, all *M. furfur* isolates in this study exhibited satisfactory growth at 33°C; however, 52% showed increased growth at 37°C, the typical human internal body temperature. Previous studies have demonstrated that growth at 37°C is essential for establishing fungal systemic infection [27, 28, 57]. This 52% of isolates that grew better at 37°C came from healthy skin, DS, PV and fungemia, and one isolate from AD. This thermotolerance in non-fungemia isolates poses a latent risk, as the origin of infection has been traced to the mycobiota of healthcare personnel and the mothers of neonates [11]. Regarding oxidative stress, menadione, a quinone that induces reactive oxygen species (ROS) production in mitochondria [58], was used to assess isolates' tolerance to ROS generated by the immune system. Eight isolates tolerated low concentrations of menadione at both 33°C and 37°C. At 33°C, only 4/19 (21%) of isolates could grow at high menadione concentrations, with three of these from healthy skin. At 37°C, only the *M. furfur* IMR-MM 959 isolate grew, and the higher temperature appeared to enhance tolerance to oxidative stress. This observation aligns with previous studies indicating that temperature can trigger oxidative stress responses, such as enzyme synthesis [27–29,

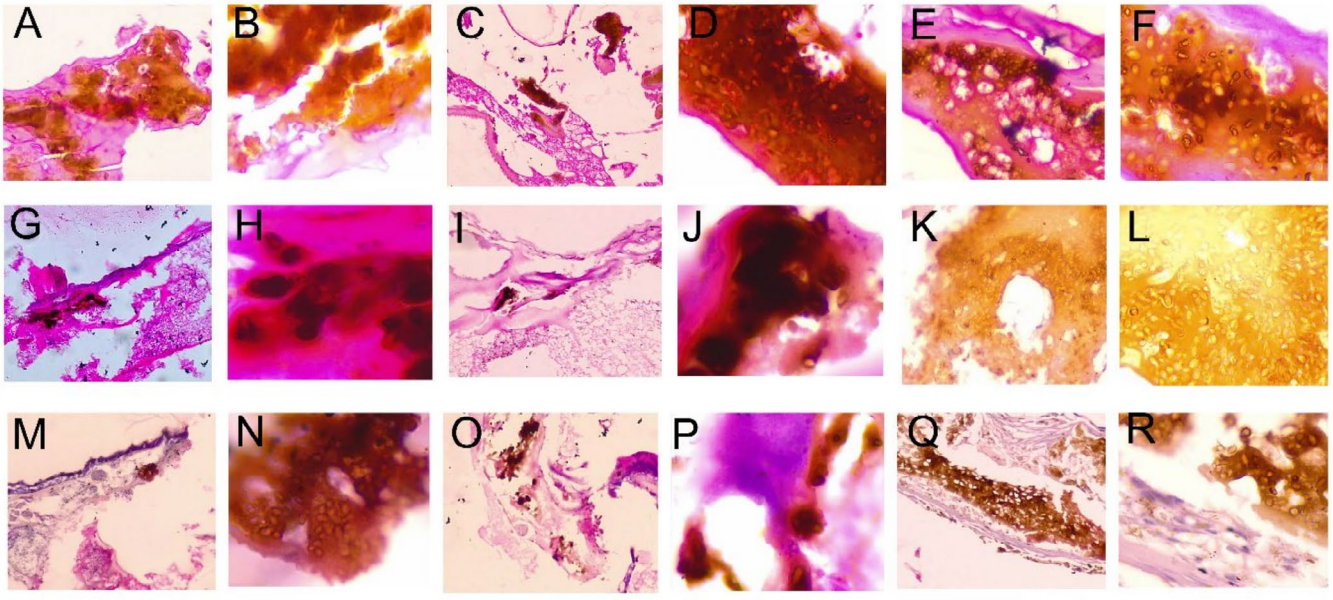

FIGURE 5 | Histological evaluation. (A, B) *M. furfur* CBS 1878 at 40X and 100X, (C, D) *M. furfur* 57.41 DS at 10X and 100X, (E, F) *M. furfur* IMR-MM 959 at 10X and 100X, (G, H) *M. pachydermatis* CBS 1879 at 10X and 100X, (I, J) *M. pachydermatis* 131 at 10X and 100X, (K, L) *M. pachydermatis* IMR-MM 954 at 10X and 100X, (M, N) *M. sympodialis* CBS 7222 at 10X and 100X, (O, P) *M. sympodialis* 1DA at 10X and 100X and (Q, R) *M. sympodialis* 5S at 10X and 100X. Higher melanization in nodules from larvae that showed a lower virulence.

57, 59]. Moreover, the mechanisms that activate heat tolerance often overlap with those required for oxidative stress responses [57]. The thermotolerance and oxidative stress resistance in the healthy skin and skin disorder isolates denote the opportunistic character of this genus. Instead of being obligate pathogens, skin commensals harbour these latent virulence traits. In this way, human skin and indeed healthcare personnel's skin, act as a reservoir for these microorganisms. This could mean that when they are introduced into the bloodstream via CVC, the pre-existing traits enable them to transition from commensals to invasive systemic pathogens.

Concerning *M. pachydermatis*, this is primarily recognized as a commensal of canine skin and mucosae, but is increasingly implicated as an opportunistic pathogen in humans, especially in neonates with indwelling catheters and immunocompromised individuals [11]. In this study, *M. pachydermatis* exhibited thermotolerance, with all isolates sustaining growth at 37°C. This was observed even under oxidative stress conditions. This aligns with previous reports in which a successful adaptation to host body temperature represents a key virulence trait in fungemia [28, 29]. *M. pachydermatis* IMR-MM 954 displayed the capacity to survive under menadione-induced stress. However, all isolates of this species were shown to grow under oxidative stress conditions. Moreover, two isolates that were not associated with any medical entity did not change in growth pattern when exposed to oxidative stress or at 37°C. For this species, oxidative stress did not depend on thermotolerance, which may indicate that the capacity of *M. pachydermatis* to survive this kind of host condition relies on another heat-independent mechanism different from that of *M. furfur*, like catalase or superoxide dismutase (SOD) [60]. Interestingly, all isolates exhibited variable oxidative stress resistance, reinforcing the notion that virulence traits in this species are isolate-dependent rather than species-dependent.

M. sympodialis is generally considered a skin commensal with strong associations to atopic dermatitis (AD), seborrheic dermatitis and other superficial skin conditions, rather than invasive infections. Nonetheless, cases of fungemia attributed to *M. sympodialis* have been increasingly reported, particularly in neonates, although at lower frequencies than those of *M. furfur* and *M. pachydermatis* [11]. In this study, *M. sympodialis* isolates exhibited moderate thermotolerance, with only a subset demonstrating high growth at 37°C. The oxidative stress tolerance of *M. sympodialis* isolates was lower than that of the *M. pachydermatis* and *M. sympodialis* fungemia isolates. At 33°C, most isolates displayed sensitivity to high concentrations of menadione, and growth recovery at 37°C was lower. This pattern suggests that *M. sympodialis* may rely more heavily on skin-associated microenvironments with lower oxidative pressures, reflecting its specialization as a superficial commensal [61]. The limited oxidative stress resistance may also contribute to a reduced capacity to persist in systemic circulation, where phagocytic responses and ROS generation play central roles in fungal clearance [62, 63]. This may result in its lower reported prevalence with respect to *M. furfur* and *M. pachydermatis* [11].

Biofilm formation in *Malassezia* species has emerged as a critical virulence attribute, especially in *M. pachydermatis* and *M. sympodialis*, where fungemia isolates have demonstrated enhanced biofilm-forming ability compared to reference strains. Likewise, this high biofilm formation in fungemia isolates was also observed for *M. furfur*, but to a lower proportion. This agrees with previous reports in which biofilm formation was high in isolates that were associated with any clinical entity [21, 64, 65]. These biofilms are characterized by dense cellular communities embedded in a self-produced extracellular matrix composed of polysaccharides, proteins and lipids, which confer protection against environmental stress and host immune responses [21]. Although less is known than for *Candida* spp., recent findings have confirmed that *Malassezia* can adhere to inanimate surfaces and form structured biofilms under in vitro conditions, likely facilitating persistence on medical devices and skin surfaces [21]. Taking this into account, these structures may help the three *Malassezia* species to thrive and gain access to the bloodstream.

Biofilm production is isolate-dependent, as has been shown for the genus *M. furfur* in a previous study [21]. This isolate-dependence was also observed in this study for thermotolerance and oxidative stress responses. Furthermore, biofilm production has been linked to the ability of *M. furfur* to colonize and establish infections on the skin surface [65]. In the context of fungemia, biofilm production is generally crucial for thriving within catheters [50, 66], evasion of phagocytosis [67] and resistance to antifungal treatments [68]. In this study, all isolates of the three species obtained from cases of fungemia exhibited a higher capacity for biofilm production. However, compared to *M. furfur*, *M. pachydermatis* and *M. sympodialis* demonstrated greater biofilm production. This pattern of isolates from fungemia patients is consistent with previous studies showing that bloodstream isolates exhibited enhanced biofilm capacity [17, 64].

Even though the ability to form biofilms has contributed to the capacity of these species to cause systemic infection, it is not a decisive factor in establishing infection. Indeed, the phospholipase activity was higher in isolates from patients with fungemia for the three evaluated species of *Malassezia*, contrasting with previous reports [17]. For hemolytic activity, it was found to be higher in isolates of *M. furfur* that came from fungemia and lower in isolates of *M. pachydermatis* and *M. sympodialis*. Concerning the high hemolytic activity of *M. furfur* isolates from fungemia, this also contrasted with a previous report [17]. However, it has been reported that hemolytic activity is an important virulence factor that facilitates fungal invasion, as it helps to acquire iron and to develop filaments in *Candida* species [69, 70], contributing to their virulence. Adding to this, it has been reported for *C. tropicalis* that high phospholipase activity can induce filamentation [71], which may facilitate invasion. Since *M. furfur* is only one of these species capable of inducing mycelial transformation, and this has been associated with skin disorder severity [72, 73], and, as mentioned, facilitates invasive infections by *Candida* species, this may explain the lower prevalence of *M. pachydermatis* and *M. sympodialis* as causative agents of fungemia and the importance of hemolytic and phospholipase activity, mainly in the former.

In vivo assays further showed that the in vivo virulence correlated with the results obtained from the in vitro evaluated traits. In that way, isolates exhibiting higher thermotolerance, oxidative

stress resistance, high biofilm formation, phospholipase activity and hemolytic activity were associated with lower larval survival (shorter LT_{50} values). As expected, the in vitro virulence of the two evaluated *M. furfur* isolates correlates with their in vivo virulence. Isolate *M. furfur* 57.41 DS exhibited lower virulence, with an LT_{50} on day 15, compared to the *M. furfur* IMR-MM 959 isolate, which had an LT_{50} on day 11. For *M. pachydermatis* and *M. sympodialis*, the fungemia isolates also reached the L_{50} earlier than isolates from healthy skin or skin disorders associated with the *Malassezia* genus. Following the larval survival evaluations, the virulence of *M. furfur* isolates was further assessed by examining their ability to withstand the host immune response. Regarding the initial inoculum burden, yeast recovery was generally lower, particularly in larvae inoculated with *M. furfur* 57.41 DS, *M. pachydermatis* 131 and *M. sympodialis* 1DA. This reinforces the idea of low virulence of these isolates, which contrasts with the fungemia isolates also in terms of thermotolerance, oxidative stress tolerance and biofilm production. Comparing the three evaluated species, the fungal burden demonstrated the higher capacity of *M. furfur* isolates to survive the host immune response and to proliferate, which, together with the in vitro and in vivo virulence results, agrees with the high number of reports of this species associated with fungemia [11].

On the other hand, the strong immune response observed in infected larvae was reflected in the melanization index measured over time [74]. Melanization is one of the key mechanisms of the innate immune system in invertebrates, such as *G. mellonella* and is activated as a direct response to the detection of exogenous microbial structures (PAMPs), including glucans, mannans and membrane lipids, components broadly conserved across fungal pathogens [75, 76], including yeasts such as *Malassezia* [23, 77]. This process is mediated by the activation of the phenoloxidase cascade, an enzymatic pathway that depends on the enzyme pro-phenoloxidase, which generates melanin as an end product [76, 78]. The melanin generated not only acts as a visible pigment but also forms part of a strategy for encapsulating and containing the pathogen [78]. It is deposited in the infected tissues or around the invader, promoting its physical isolation and the local generation of reactive oxygen species (ROS), which increases oxidative stress and contributes to cellular damage of the microorganism [78]. Therefore, intense melanization is interpreted as a robust immunological marker of threat perception and host defence activation [76]. Melanization was higher in larvae inoculated with *M. furfur* isolates, suggesting that immune response induction in these cases may be higher. Considering that the melanization cascade system is analogous to vertebrates' complement activation [76], this high immune response induction may contribute to *M. furfur* virulence by leading to sepsis and organ damage [79]. Previously, it has been reported that pseudo-hyphal induction in *C. albicans* in the *G. mellonella* model could be a melanin evasion mechanism and that the development of blastoconidia from those pseudo-hyphae may lead to a stronger secondary melanization response [78]. This may explain the stronger melanization induction in larvae inoculated with *M. furfur*, which, as mentioned before, is capable of inducing the mycelial transformation. This may add to virulence during bloodstream infection, as filamentation helps to evade the host immune response associated with ROS release.

Overall, these results highlight key differences in the pathogenic potential of *M. furfur*, *M. pachydermatis* and *M. sympodialis*. When comparing the three species, distinct virulence profiles emerge that correspond to their epidemiological distributions. *M. furfur* remains the leading cause of *Malassezia* fungemia in neonates and immunosuppressed adults, likely due to the combination of its thermotolerance, capacity to persist in bloodstream conditions such as oxidative stress, phospholipase activity, hemolytic activity and strong biofilm formation on medical devices [11]. *M. pachydermatis*, although traditionally considered a veterinary pathogen, shows an alarming potential for human invasive infections through zoonotic transmission, highlighting its relevance within a One Health framework at the human–animal interface [80]. Its ability to form robust biofilms and withstand oxidative stress underscores its clinical significance, making it a secondary cause of *Malassezia* fungemia. In contrast, *M. sympodialis* appears to be a less aggressive pathogen, with virulence traits optimized for superficial colonization rather than systemic invasion, explaining the low prevalence in bloodstream infections [11, 43]. This highlights its opportunistic nature under favourable host conditions. However, further studies are needed to address this.

Even though 28 isolates and reference strains of three different species were evaluated, the samples of *M. pachydermatis* and *M. sympodialis* were relatively fewer. Further studies with a higher number of isolates from different origins of these two species are needed to confirm the observed pattern. Moreover, although the *Malassezia* genus comprises about 20 described species, in this study only 3 of them were studied, owing to the capacity of these species to cause fungemia, which was our objective. However, it could be interesting, in the future, to equally evaluate other species under the same conditions to find out if other species could be associated with this kind of clinical entity. It is important to take into account the limitations of the in vitro and in vivo assays conducted in this study. The assays conducted in vitro simplify the human body conditions that may contribute to favour or disfavour thermotolerance, oxidative stress tolerance, phospholipase and hemolytic activity, as well as biofilm formation. Moreover, even though the infection model has many advantages, such as its ease of handling and obtaining results, this model does not fully reflect the complexity of vertebrates with their immune systems. Taking this all together, it is clear that results cannot be directly extrapolated to clinical outcomes in affected patients. This is why further validation is required in a vertebrate model.

In summary, these findings underscore the heterogeneity of virulence within the *Malassezia* genus. Likewise, it showed that the evaluated in vitro parameters could be good predictors of *Malassezia* virulence in vivo, and that this relates to the isolates' origin. Moreover, these results emphasized the higher in vitro and in vivo virulence as a result of the combination of different virulence traits of *M. furfur* as a cause of fungemia, which, according to reports, is the main cause of *Malassezia* fungemia. The capacity of *M. pachydermatis* to bridge zoonotic and nosocomial contexts represents a particular concern for neonatal intensive care units, where contaminated environments and healthcare personnel may act as reservoirs [11]. Meanwhile, *M. sympodialis* demonstrates that even when this species shows low virulence, it can emerge as an opportunistic pathogen in immunocompromised hosts. Further genomic and proteomic analyses

are warranted to clarify the molecular mechanisms involved in these differential virulence traits and to identify targets for antifungal alternatives.

Author Contributions

Maritza Torres: conceptualization, investigation, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, data curation. **María Alejandra Gómez-Rivera:** conceptualization, investigation, writing – original draft, methodology, validation, visualization, writing – review and editing, formal analysis, data curation. **Jineth Alejandra Silva Triana:** investigation, methodology, validation. **Adriana Marcela Celis-Ramírez:** conceptualization, formal analysis, validation, funding acquisition, methodology, writing – review and editing, project administration, supervision, resources.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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