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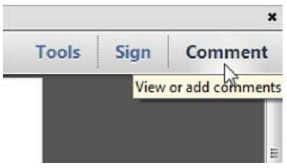

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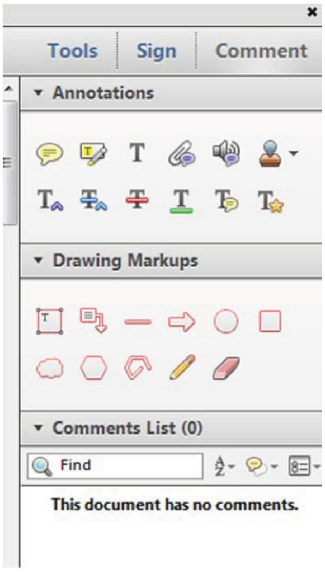

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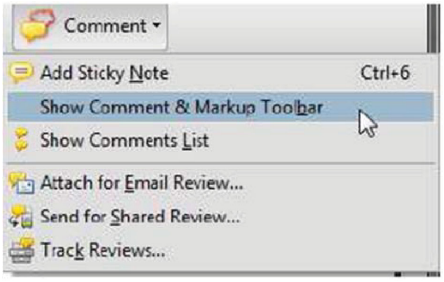

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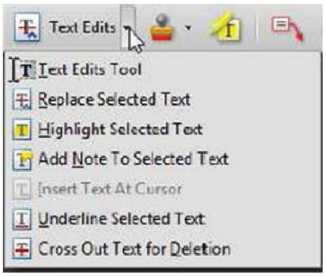

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Original Article

Original Article

Biofilm formation, adherence, and hydrophobicity of *M. sympodialis, M. globosa,* and *M. slooffiae* from clinical isolates and normal skin

Virulence factors of *M. sympodialis, M. globosa* and *M. slooffiae*

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Abstract

The genus *Malassezia* comprises a heterogeneous group of species that cause similar pathologies. *Malassezia* yeasts were considered as the most abundant skin eukaryotes of the total skin mycobiome. The ability of this fungus to colonize or infect is determined by complex interactions between the fungal cell and its virulence factors. This study aims to evaluate *in vitro* the hydrophobicity levels, the adherence capacity on a polystyrene surface and the ability to form biofilm of 19 isolates, including *M. sympodialis*, *M. globosa,* and *M. slooffiae*, from healthy subjects and from dermatological disorders. Cellular surface hydrophobicity levels were determined by two-phase system. The biofilm formation was determined by tetrazolium salt (XTT) reduction assay and by Scanning Electron Microscopy (SEM). Strain dependence was observed in all virulence factors studied. All isolates of *M. sympodialis*, *M. globosa,* and *M. slooffiae* demonstrated their ability to form biofilm at variable capacities. SEM observations confirmed a variable extracellular matrix after 48 hours of biofilm formation. All isolates of *M. globosa* were highly adherent and/or hydrophobic as well as biofilm producers. In contrast, *M. slooffiae* was the least biofilm producer. No significant differences between virulence factors were demonstrated for *M. sympodialis*, either as clinical isolate or as inhabitant of human microbiota. Results of this work together with the previous *M. furfur* research confirm that the most frequently *Malassezia* species isolated from normal subject's skin and patients with dermatosis, form biofilm with different capacities. The study of these virulence factors is important to highlight differences between *Malassezia* species and to determine their involvement in pathological processes.

Key words: hydrophobicity, adherence, biofilm, SEM, *Malassezia sympodialis*, *Malassezia globosa*, *Malassezia slooffiae*.

Introduction

The genus *Malassezia* comprises a heterogeneous group of species that cause similar pathologies.[1] Although the relative abundance of fungi on human skin was found to be lower compared to bacteria, *Malassezia* yeasts are the most abundant eukaryotes representing about 50%–80% of the total skin mycobiome.[2] *Malassezia* species are also involved in the pathogenesis of different skin disorders, including pityriasis versicolor (PV), seborrheic dermatitis (SD), and atopic dermatitis (AD).[3,4] *Malassezia* yeasts seem to have an opportunistic nature and have been considered an emergent pathogen.[5,6]

M. sympodialis is the third most abundant species on healthy human skin, appearing with significantly lower frequency compared to *M. globosa* and *M. restricta*.[7] It was also isolated from PV, SD, AD, skin in patients with human immunodeficiency virus (HIV)/AIDS and various warm-blooded animals.[1,8]

Occasionally, *M. sympodialis* was also found to be responsible for systemic infections.[9] *M. globosa* is the most frequent species linked to various dermatological conditions, including PV, SD and AD in humans and it also causes otitis in animals.[10] *M. slooffiae*, commonly isolated from animals, is also associated with very low frequency on healthy human skin and several dermatosis.[4]

The pathogenic role of *Malassezia* in PV is well established, being mainly related to *M. globosa*, followed in frequency by *M. sympodialis* and *M. furfur*.[4,11,12] AD is a chronic multifactorial inflammatory disease of the skin with increasing prevalence over the last decades. Symptoms of AD are obvious and easy to recognize, and a high proportion of AD patients have a positive reaction to *Malassezia* allergens.[13] Additionally, *Malassezia* is associated with SD, where *M. globosa*, *M. sympodialis*, and *M. furfur* have been the most frequently isolated agents from the skin affected with this dermatosis.[3,4,14]

It is known that microbial adhesion to host surfaces is an essential step for infection and also promotes the biofilm formation on implanted biomaterials like prosthetics and catheters.[15] The hydrophobic properties of microbial surfaces are conducive for adhesion. Depending on the type of surface, hydrophobicity of cells can increase the propensity of microorganisms to adhesion and to form biofilm. This microbial structure is another medical problem; it is a community of cells affording drug resistance and increased virulence.[16,17] Cellular surface hydrophobicity (CSH) also precludes the pathogens from phagocytosis by host immune cells.[18]

In a previous work, we demonstrated the ability of *M. furfur* clinical isolates to form biofilm related to CSH and adherence.[19] Nowadays, culture-independent studies showed *M. furfur* with a low frequency and abundance as inhabitant of healthy skin; moreover, other lipid-dependent *Malassezia* species are more frequently related to several dermatosis than *M. furfur*.[1,20]

The aim of this work was to study the hydrophobicity levels, adherence capacity, and biofilm formation of the lipid-dependent *M. sympodialis*, *M. globosa,* and *M. slooffiae*, isolated from skin of healthy subjects and individuals suffering dermatological disorders such as PV, DS, and DA.

Methods

Microorganisms and growth conditions

Seven clinical *M. sympodialis* isolates from PV lesions, five from skin of healthy subjects (HS), and the *M. sympodialis* CBS 7222 strain; two clinical *M. globosa* isolates from PV, one from SD and one from HS; in addition, two *M. slooffiae* isolates from SD and one from atopic dermatitis (AD) lesions were studied (Table 1). No clinical isolates came from immunocompromised subjects. All isolates were deposited in the culture collection (IMR-M) of the Micology Department, Instituto de Medicina Regional, Universidad Nacional del Nordeste, Argentina. Using a polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP), all isolates deposited were previously identified.[20]

Table 1. Isolates of *Malassezia* spp.

Strains	Origin
M. sympodialis	
IMR- MM 11	Pitiriasis versicolor
IMR- MM 156	Pitiriasis versicolor
IMR- MM 305	Pitiriasis versicolor
IMR- MM 331	Pitiriasis versicolor
IMR- MM 381	Pitiriasis versicolor
IMR- MM 455	Pitiriasis versicolor
IMR- MM 849	Pitiriasis versicolor
IMR- MM 851	Healthy skin
IMR- MM 852	Healthy skin
IMR- MM 854	Healthy skin
IMR- MM 855	Healthy skin
IMR- MM 857	Healthy skin
CBS 7222	Laboratory strain (healthy skin)
M. globosa	
IMR- MM 130	Pitiriasis versicolor
IMR- MM 351	Pitiriasis versicolor
IMR- MM 371	Healthy skin
IMR- MM 737	Seborrheic dermatitis
M. slooffiae	
IMR- MM 139	Seborrheic dermatitis
IMR- MM 147	Atopic dermatitis

Prior to studying their virulence factors, all yeasts were grown in Leeming-Notman modified broth at 32°C for 72 hours.

Hydrophobicity assay

The cell-surface hydrophobicity was determined by two-phase system.[21] A yeast cell suspension was washed with sterile saline buffer 0.5% Tween 20, then resuspended in 0.05 M phosphate-buffered saline (PBS) (pH 7.2) at a final concentration of 2×10^6 cells/ml. OD600 of each suspension was recorded (OD600 control). Furthermore, the suspension was transferred to a glass tube containing 500 μl octane (Sigma Aldrich) and shaken for 1 minute using a vortex mixer. The aqueous phase was measured at OD600 (OD600 after octane overlay). Relative CSH was calculated as: [(OD600 control - OD600 after octane overlay)/OD600 control] × 100.

Value for each strain was the average of three independent biological replicates.

Adherence assay

The adhesion on polystyrene surface was measured as we previously reported.[19] Yeast cells were washed twice with sterile PBS and then resuspended at 37°C in Roswell Park Memorial Institute (RPMI) 1640 modified for *Malassezia*, plus 10% FBS at 7.5×10^2 cells/ml. Incubation was performed in six-well

polystyrene plates (Corning Incorporated, Corning, NY, USA) for 24 hours at 37°C. By aspiration of the medium, nonadherent cells were removed; thereafter, fresh Leeming-Notman modified agar medium was added into each well and let solidify. After incubation for 72 hours at 37°C, colonies were counted. The inoculum size was 7.5×10^2 cells/ml. As a control, each cell suspension was cultured directly in plates with the same Leeming-Notman agar medium modified. Results were expressed as a percentage of the inoculum size.

Biofilm formation

The *in vitro* biofilm formation assay was carried out as described Ramage et al.[22] A suspension of *Malassezia* cells (1.0×10^7 cells/ml) was incubated for 24 and 48 hours at 37°C in 96-well microtiter plates (Corning, NY, USA). After incubation, the medium was aspirated and nonadherent cells were removed by washing with sterile PBS. Biofilms were measured using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay, a reaction catalyzed by mitochondrial dehydrogenases.[22] Briefly, the washed biofilm cells were incubated for 2 hours at 37°C with a solution of 0.5 mg/ml of XTT and 1 µM of menadione in PBS. From each well, 500 µl was transferred into a fresh 12-well plate. The colorimetric change resulting from the XTT reduction was measured at 490 nm.

Scanning electron microscopy (SEM) of biofilm

Under the same conditions, reading at 24 hours and 48 hours, all isolates of *Malassezia* spp. were grown on polyurethane material to observe the biofilm formation. Afterward, cells were fixed in 2.5% (v/v) glutaraldehyde in 0.01 M cacodylate buffer (pH 7.4) containing 2% (w/v) sucrose for 20 minutes at room temperature and then post-fixed with 1% (w/v) OsO4 for 1 hour. Ethanol gradient and critical point dried in CO2 were used to dehydrate. Samples were examined under a Joel 5800 LV (Tokyo, Japan) scanning electron microscope at the Electronic Microscopy Service of Universidad Nacional del Nordeste, Argentina.

Statistical analysis

All tests were performed in triplicate and the values presented are the mean with standard deviation.

Associations between disease and virulence factors were evaluated using Fisher exact test. In addition, Pearson coefficient was applied to analyze linear correlation between those variables.

Student *t* test was used to compare between 24- and 48-hour biofilm formation. *P* value $< .05$ was considered to be statistically significant.

INFOSTAT statistical software was used to perform data analysis.[23]

Table 2. Adherence and hydrobobicity of *Malassezia* spp.

Strains	% adherence	% hydrophobicity
M. sympodialis		
IMR- MM 11	28.4 ± 7.07	29.4 ± 6,11
IMR- MM 156	15 ± 2.8	42.6 ± 16.6
IMR- MM 305	43.48 ± 16.51	9.8 ± 2.1
IMR- MM 331	24.8 ± 3,5	11.6 ± 22.6
IMR- MM 381	54.5 ± 7.7	63.3 ± 14.8
IMR- MM 455	33.8 ± 6.36	31 ± 3.7
IMR- MM 849	36.0 ± 11.3	31.1 ± 6.9
IMR- MM 851	59.73 ± 9.19	62.5 ± 16.8
IMR- MM 852	50.6 ± 26.1	32.3 ± 3.2
IMR- MM 854	98.7 ± 3.2	49.2 ± 2.0
IMR- MM 855	43,14 ± 8.4	70 ± 3.5
IMR- MM 857	17.72 ± 3.5	32.47 ± 8.0
CBS 7222	51.4 ± 3.5	41.2 ± 9.5
M. globosa		
IMR- MM 130	45.6 ± 3.5	55.15 ± 13.0
IMR- MM 351	67.2 ± 11.3	33.0 ± 6.8
IMR- MM 371	56.4 ± 15.5	87.7 ± 15.2
IMR- MM 737	64.3 ± 9.5	40.6 ± 5.4
M. slooffiae		
IMR- MM 139	72.3 ± 14.6	36.17 ± 10,0
IMR- MM 147	48.4 ± 11,3	79.86 ± 4.9

Percentage of plastic adherent cells and cell surface hydrophobicity values measured with a two-phase system. Mean values of % hydrophobicity and adherence ± SD of three independent experiments.

Results

Hydrophobicity and adherence

Table 2 shows the hydrophobicity levels and adherence values obtained for the CBS 7222 *M. sympodialis* and *Malassezia* isolates (IMR-M) tested. All strains were hydrophobic and adherent on polystyrene surface. The values in percentage were variables and ranged from 9.8 ± 2.1% to 87.7 ± 15.2% and from 15.0 ± 2.8% to 98.7 ± 3.2% for hydrophobicity and adherence, respectively.

In particular, only one isolate of *M. sympodialis* (IMR-M 855) was highly hydrophobic (70 ± 3.5%), five isolates (IMR-M 156, 381, 851, 854, and CBS 7222) showed medium CHS with values among 41.2 ± 9.5% to 63.3 ± 14.8% and seven (IMR-M 11, 305, 331, 455, 849, 852 and 857) showed low hydrophobicity values ranging from 9.8 ± 2.1 to 32,47 ± 8.0. One isolate of *M. globosa* (IMR-M 371) showed high hydrophobicity (87.7 ± 15.2%), two isolates (IMR-M 130 and 737) showed medium CHS with values 55.15 ± 13.0 and 40.6 ± 5.4%, respectively, and one isolate (IMR-R 351) was low hydrophobic. One *M. slooffiae* clinical isolate (IMR-M 147) showed high, while other (IMR-M 139) showed low CSH.

M. sympodialis, *M. globosa,* and *M. slooffiae* did not showed significant differences in hydrophobicity; about 50% of the strains were high or medium hydrophobic.

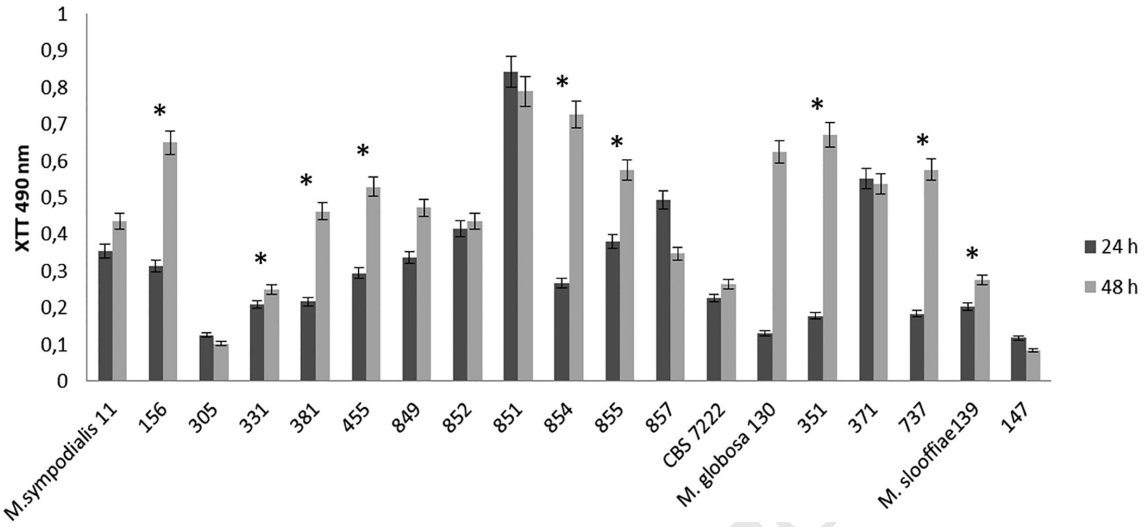

Figure 1. Histograms of biofilm formation of *Malassezia* spp. isolates (IMR) and the reference strain CBS 7222 *M. sympodialis* after 24 and 48 h measured by XTT.

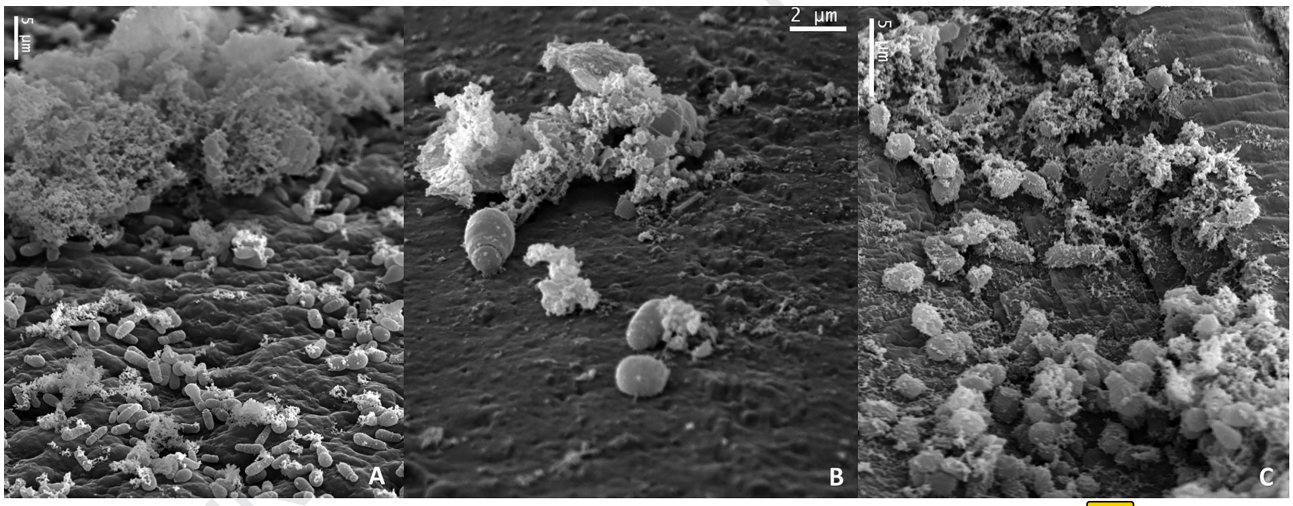

Figure 2. Scanning electron microscopy of *Malassezia* cells on polyurethane catheter after 48 h (a) *M. globosa* (IMR-M 371) (×2000) (b) *M. slooffiae* (IMR-M 147) (×5000) (c) *M. sympodialis* (CBS 7222) (×3000).

Adherence values presented in Table 2 also displayed variability. One clinical isolate (IMR-M 854) of *M. sympodialis* was highly adherent (98.7 ± 3.2%), six isolates (IMR-M 305, 381, 851, 852, 855) and the CBS 7222 *M. sympodialis* strain showed values among 43.14 ± 8.4% to 59.73 ± 9.19% and the other isolates (IMR-M 11, 156, 331, 455, 849, 857) were low adherent. About 50% of *M. globosa* and *M. slooffiae* isolates showed medium (IMR-M 130, 371, 147) and high adherence (IMR-M 351, 737, 139).

Biofilm formation

Using XTT assay and SEM observations, the biofilm formation capacity of all strains after 24 hours and 48 hours were analyzed (Fig. 1). Significant differences between XTT assay at 24 h and 48 h ($P < .05$) for only 9 strains (MM-M 156, 331, 381, 455, 854, 855, 351, 737, 139) were obtained (*).

All isolates and the CBS 7222 *M. sympodialis* strain were able to form biofilm with different capacities. Indeed, the variability of biofilm formation after 48 hours ranged from 0.102 to 0.789 at OD 490 nm for *M. sympodialis*, from 0.538 to 0.67 for *M. globosa* and from 0.085 to 0.275 for *M. slooffiae*.

Results obtained by XTT assays were also confirmed by SEM observations. Figure 2 shows a) *M. globosa*, b) *M. slooffiae*, and c) *M. sympodialis* cells on polyurethane catheter after 48 hours. The biofilm formation of *M. sympodialis* was characterized by a monolayer structure covering the cells. The evident biofilm formation of *M. globosa* presented an abundant extracellular matrix, organized in multistructures, while the biofilm produced by *M. slooffiae* was low and an aggregation of adherent cells was observed.

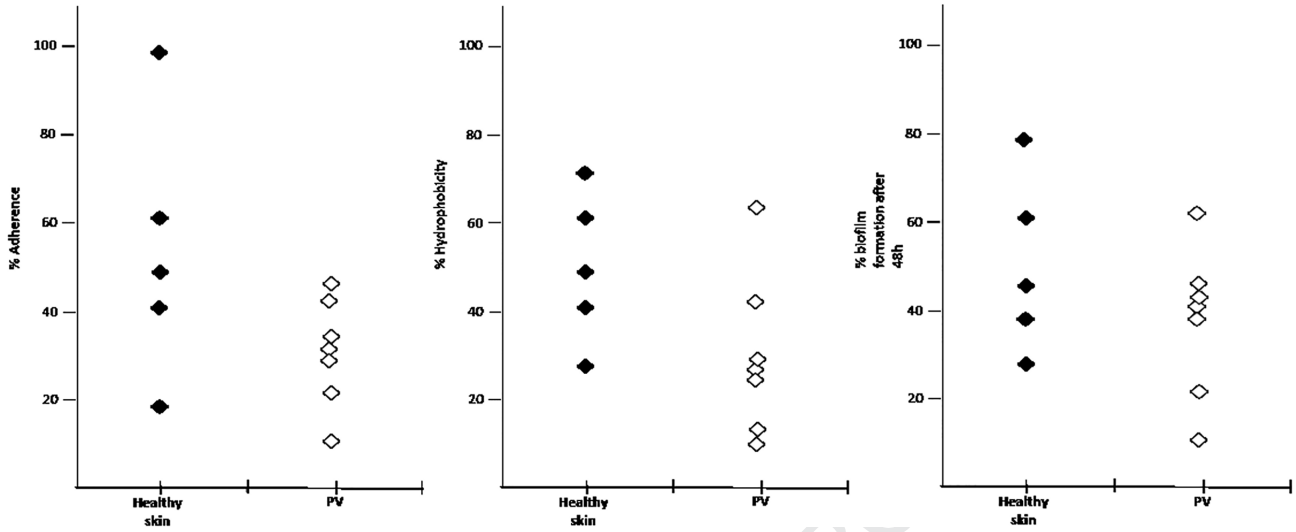

Figure 3. Adherence, hydrophobicity and biofilm formation of *M. sympodialis* isolates from normal skin and PV.

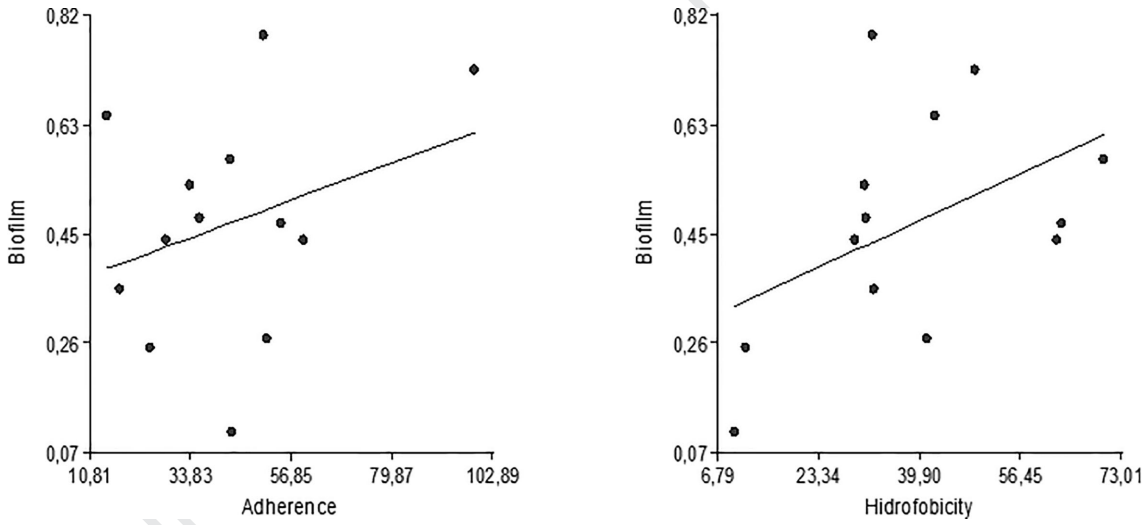

Figure 4. Dispersion diagrams between biofilm production as main pattern associated to adherence and hydrophobicity.

Virulence factors of *M. sympodialis* isolates from pitiriasis versicolor and healthy subjects

Results of virulence factors studied in *M. sympodialis* isolates of both groups of subjects are shown in Figure 3. Both from isolates of PV and from healthy skin, the ability to form biofilms of *M. sympodialis* associated with hydrophobicity and adherence, showed no statistical significance with the Fisher's exact test. Dispersion diagrams are shown in Figure 4. Pearson correlation coefficients obtained between biofilm production and adherence and hydrophobicity were 0.2945 and 0.1070, respectively.

Discussion

Human microbiome studies mainly focused on prokaryotic inhabitants, nowadays, fungi have received more attention. To avoid misunderstandings, first, it is important to emphasize that microbiota refers to microorganisms on the skin, and the microbiome represents the total genomic component, measured by DNA analysis. Genomes do not interact with the host, however, the biomass associated with each genome does. Presently, it was observed that microorganisms of the human microbiota play a crucial protective role in the maintenance of health as well as in development of diseases.[24] Microbial cells are highly variable in size, consequently, the biomass per genome fluctuates widely. Furthermore, cell size *Malassezia* species was reported to have 200–500 times the biomass per genome relative to bacteria, it strengthens the fungi relevance to skin.[24] Even though, the role of the skin microbiome has been deeply studied in recent years, some aspects are yet to be elucidated. Although *M. sympodialis* and *M. globosa* were included within the most abundant *Malassezia* species present in skin mycobiome of healthy humans and also were involved in several dermatological disorders

and systemic infections,[1,9] the knowledge of the expression of virulence factors of these species is scarce.

At varying frequencies, *M. sympodialis, M. globosa,* and *M. slooffiae* were related as members of healthy skin in different body sites. Investigations showed *M. sympodialis* as the third most abundant commensal species, but it was also recovered from a great number of individuals with PV, DS, AD, and systemic infections. *M. globosa* and *M. slooffiae* were found essentially in skin lesions; *M. globosa* in PV, DS, and AD and *M. slooffiae* found only in DS with constant low frequency.[1,25,26] The species distribution is affected by geographical location while depending on other factors such as, the skin disease, the body site of the lesion(s) and the age of the individual.[27,28] In particular, *M. globosa* was reported as an agent with about 60% of PV in Europa, while *M. sympodialis* has higher isolation rates in Latin America.[1] Other factors influencing *Malassezia* species distribution are the difficulties involved in the isolation from clinical samples, cultivation and survival in culture mediums.[4] Although *M. restricta* is considered as one of the most frequent inhabitant of the healthy skin and diseased skin such as SD and AD, it is one of the most difficult species to grow in culture, even under the most favorable conditions. Hence, less data on the epidemiology of this species are available.[4] All of these important factors reflect the reason for the different number of isolates included in this work.

Adhesion is a determinant step for the microbial infections pathogenesis and it is favored by the hydrophobic interactions between cells and surfaces, whether biological or not.[29,30] The best adherence performance of hydrophobic *Candida albicans* isolates has been demonstrated instead of hydrophilic cells on different host tissues.[31] In a previous study, we have demonstrated that about 60% of *M. furfur* isolates displayed similar range values in both hydrophobicity and adherence.[19] Less than 40% followed this trend in the present work. In particular, *M. sympodialis* isolates showed variable adherence; about 50% were medium or low adherent to abiotic surface. No significant differences were demonstrated, either as clinical isolate or as inhabitant of human microbiota. Probably it is a characteristic of *M. sympodialis*.

Considering the scarce number of *M. globosa* and *M. slooffiae* isolates studied it is difficult to conclude on their behavior, but medium or high adherence was observed in all isolates. All strains of *M. globosa* were also highly adherent and/or highly hydrophobic.

Currently, the knowledge about the biological properties and pathogenicity of the lipophilic *Malassezia* yeasts is scarce. In a previous study, we demonstrated that about 83% of *M. furfur* isolates tested were hydrophobic and 60% displayed similar range values in both hydrophobicity and adherence on an abiotic surface. In that study, all *M. furfur* isolates were related to dermatological conditions such as PV and DS and no isolates from healthy skin were tested.[19] The current study included other lipid-dependent *Malassezia* species even more frequently isolated from dermatological disorders than *M. furfur,* such as *M. sympodialis* and *M. globosa*. Our results show a similar behavior of *M. sympodialis, M. globosa,* and *M. slooffiae* isolates demonstrating hydrophobicity and adherence values on an abiotic surface with a high variability strain dependence. *M. furfur* is a diverse group and the divergence with the previous data may be related to the physiological and genomic diversity.

Other virulence factors secreted by *Malassezia* spp. could affect our results. *Malassezia* genome encode enzymes (e.g., lipases, phospholipases, aspartyl proteases, and acid sphingomyelinases) to mediate proteins, lipid utilization, and also, pathogenesis. The specific roles of *M. globosa* proteases secreted have been recognized as virulence factors correlated with the hydrolysis of host proteins to supply nutrients, degradation of host tissues, modification of host cells to facilitate adhesion or to alter the immune response by secretion of proinflammatory cytokines.[1,32] Other data suggest that hydrophobicity drives the systemic distribution of lipid-conjugated siRNAs via lipid transport pathways.[33] Since these virulence factors influencing both hydrophobicity and adherence have not yet been studied for all *Malassezia* species, differences in our results could be traced to them.

In a previous study we have demonstrated the high ability of clinical *M. furfur* isolates to form biofilm.[19] Also, Figueredo et al.[34] observed this capacity in *M. pachydermatis*. In this work, 12 clinical isolates showed a linear increase in metabolic activity over time; three (IMR-M 331, 852, and CBS 7222) showed the same metabolic activity and four (IMR-M 305, 851, 371, 147) showed a low reduction in development. It is interesting to note that about 76% of *M. sympodialis* and all *M. globosa* were the highest biofilm producers, while *M. slooffiae* was the specie with the lowest production. Considering only isolates with significant difference ($P < .05$) in the biofilm formation, about 78% were isolated from skin lesions. SEM observations showed an abundant extracellular matrix, after 48 hours. *M. sympodialis* and *M. slooffiae* demonstrated the ability to form biofilm but with variable capacity depending on the strain. All isolates of *M. globosa* were high producers of biofilm with an abundant extracellular matrix; in contrast, *M. slooffiae* was the lowest biofilm producer.

Biofilm formation is enhanced by the pathogen's CSH and capacity of adherence. Although nonlinear correlation was obtained between those variables with the ability to form biofilms, dispersion diagrams showed a tendency to increase biofilm production when CHS and adherence increased. More effort, including study of a greater number of isolates, is required to both find the best fit and interpret the role of the independent variables.

Analysis of human mycobioma showed *Malassezia* yeasts as the most prevalent fungus in the skin and other body sites.[35] Multiple complex interactions occur between bacterial species, bacterial and fungal skin inhabitants, and the human host. Any of these interactions can lead to commensality or pathogenesis.[24] The change of habitual inhabitant to pathogen status has been

associated with changes in the normal physical, chemical, or immunological processes of the skin, which may enhance or downregulate the molecular production of yeast virulence factors.[27] It is important to highlight the ability of *M. sympodialis*, *M. globosa*, and *M. slooffiae* to form biofilm, since this characteristic makes them potential pathogens of systemic infections after catheter colonization and proliferation. Our group published a clinical case of fungemia and interstitial lung compromise caused by *M. sympodialis* in a pediatric patient.[9] In addition, the extracellular matrix generation observed by SEM might be responsible for a low antifungal susceptibility to common clinical drugs.

Results of this work together with the previous ones in *M. furfur* [19] confirm that the most frequent *Malassezia* species isolated from a normal subject's skin and also from patients with dermatosis are able to form biofilm with different capacities. Strain dependence was observed in all virulence factors studied. The study of these virulence factors is important to highlight differences between *Malassezia* species and to determine their involvement in the pathological process.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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