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Cutaneous fungal microbiome: *Malassezia* yeasts in seborrheic dermatitis scalp in a randomized, comparative and therapeutic trial

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ABSTRACT

Malassezia spp in skin microbiome scalp has been implicated in seborrheic dermatitis pathogenesis. Thus, treatment based in antifungal combined to topical keratolitic agents have been indicated as well as oral isotretinoin as it reduces the sebum production, glandular's size and possesses anti-inflammatory properties. This randomized, comparative and therapeutic trial aimed toper form the genotypic identification of *Malassezia* species before and after low-dose oral isotretinoin or topical antifungal treatments for moderate to severe seborrhea and/or seborrheic dermatitis on scalp. Scales and sebum of the scalp were seeded in the middle of modified Dixon and incubated at 32°C. For genotypic identification polymerase chain reaction primers for the ITS and D1/D2 ribossomal DNA were used and followed by samples sequencing. The procedure was conducted before and after therapeutic and randomized intervention for moderate to severe seborrhea/seborrheic dermatitis on the scalp, including oral isotretinoin, 10 mg, every other day and anti-seborrheic shampoo (piroctone olamine), over six months. The *M. globosa* and *M. restricta* were the most frequent species isolated on the scalp before and after both treatments. Other non-*Malassezia* species were also identified. The *Malassezia* spp. were maintained in the scalp after both treatments that were equally effective for the control of seborrhea/seborrheic dermatitis clinical signs.

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Introduction

Seborrheic dermatitis (SD) is a chronic and inflammatory dermatosis with recurrent character and its pathogenesis remains unclear.[1,2] The prevalence of SD is estimated between 2.35% and 11.30% in the general population, according to the geographic region.[2,3] Some American studies referred to its occurrence in 30% to 50% of the general population when they included dandruff which is restricted to the scalp, and involves itchy, flaking skin without visible inflammation.[4] Several intrinsic and environmental factors, such as sebaceous secretion, increase in triglycerides and cholesterol and decrease in squalene and free fatty acids, skin surface fungal colonization such as *Malassezia* yeasts, host factor susceptibility, and interactions between these factors, all contribute to the pathogenesis.[5,6] The *Malassezia* spp. are lipophilic yeasts and the major fungi colonizing the human scalp and of the most relevant represent yeast of skin fungal microbiome. *M. restricta* and *M. globosa* represents the most relevant species of the ten known isolated species of *Malassezia* on human skin according to some studies.[7,8]

Althought, the role of *Malassezia spp.* in seborrheic dermatitis pathogenesis is sustained on observation that removal of the yeasts by antifungal agent may lead to remission, this yeast may not be considered the etiologic agent of seborrheic dermatitis.[9,10]

Some authors pointed out its association with seborrhoea and although it affects areas with greater density of sebaceous glands, that is not being well established association between higher sebaceous secretion and seborrheic dermatitis.[5,11] Likewise, it has been described that women with seborrheic dermatitis may even have a lower sebum flow than individuals without seborrheic dermatitis and cases of extreme oiliness without signs of seborrheic dermatitis were mentioned. Moreover, seborrheic dermatitis therapy is traditionally obtained through the use of several classes of topical keratolytic, corticosteroid and

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antifungal products and the options for systemic treatment consist in the antifungals such as imidazoles (itraconazole, fluconazole and ketoconazole) and terbinafine with variable results and there is no gold standard among them.[1] In the worldwide, oral isotretinoin is considered the drug of choice in the treatment of severe acne vulgaris and nevertheless being off-label use, this is a more effective option since it reduces the synthesis of sebum and modulates toll-like receptors 2 and 4.[12-14]

Considering the *Malassezia spp.* as a lipophilic yeast that needs an adequate environment to its development, it has been hypothesized that the low-dose oral isotretinoin treatment may have a possible benefit in the erradication of its colonization in the scalp of individuals with moderate to severe seborrheic dermatitis and / or seborrhea.

Patients and methods

This therapeutic interventional, randomized, and comparative study with parallel groups was approved by the Institutional Review Board of the Federal University of Sao Paulo (protocol no. 0126/10) and registered at ClinicalTrials.gov (NCT01139749). Subjects were recruited at the outpatient dermatology clinic of a public hospital. All subjects signed a consent form prior to enrollment. The inclusion criteria were: age from 18 to 40, moderate to severe seborrhea and/or seborrheic dermatitis on the scalp and/or face, with a clinical severity score of $\geq$4, according to evaluation of six clinical parameters (oiliness, erythema, and scaling on the face and scalp) and a 4-point numeric scale (total score corresponding to the sum of the values ranged from 4 to 18 at baseline and 0 to 18 atend of the study). The exclusion criteria for all subjects were the presence of chronic and inflammatory dermatoses on the scalp and face, and paraben hypersensitivity. Additional exclusion criteria for subjects to be treated with isotretinoin were: previous treatment with oral retinoids, tetracyclines and derivatives, vitamin A and polyvitamins, chemotherapy, carbamazepine and phenytoin; autoimmune, bone, muscle, renal andhepatic-diseases; alterations in laboratorytests; a positive serumpregnancytest, lactation, andthe non-use ofbarrierand non-barriercontraceptionmethods in women. After randomization two groups were created: ISO, patients treated with 10 mg oral isotretinoin every other day and SHAMPOO (SH), patients using only topical treatment anti-seborrheic shampoo to clean the scalp and hair three times a week. The shampoo composition included 0.1% lipo hydroxy acid (LHA), 1.3% salicylic acid, 0.2% glycacil, 1% piroctone olamine, and 2% lipo amino acid. The efficacy variables were assessed at baseline and 6 months by the same investigator. The sebum secretion at the midline of the scalp was assessed using a Sebumeter (Courage &Khazaka Electronic GmbH, Cologne, Germany). This assessment was conducted in a room maintained at relative humidity of 40–44% and temperature of 22–24°C.

Molecular identification of Malassezia species

simultaneously to phenotypic identification, *Malassezia* yeast strains were identified at species level by sequencing of ITS and D1/D2-28S of rDNA. Previously, the clinical samples were cultivated in modified Dixon agar[15] for 7–14 days at 32°C. After yeast growth, culture samples were transferred to microtubes containing 1 mL of phosphate buffered saline (PBS), centrifuged three times at 16,000 × *g* for 3 min. The final pellet was resuspended in 100 μL of PrepMan^{TM} reagent (Applied Biosystems, USA) for yeast DNA extraction, according to the manufacturer's instructions.The polymerase chain reaction (PCR) was performed for amplification of ITS and D1/D2-28S of rDNA using the PCR Master Mix (Promega, USA) and universal primers for panfungal identification. The forward V9G (5′-TTACGTCCCTGCCC TTTGTA-3′) and reverse LS266 (5′-GCATTCC-CAAACAACTCGACTC-3′) primers were employed for ITS amplification,[16] and the forward NL1 (5′-GC ATATCAATAAGCGGAGGAAAAG-3′) and reverse NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers for D1/D2-28S rDNA amplification.[15,16] A total volume of 25 μL was used for each reaction and PCR was run on a *Proflex* PCR System (Applied Biosystems, Inc., Foster City, CA, USA).DNA sequencing was performed by using the dideoxynucleotide chain termination method with a Big Dye Terminator Reaction kit v3.1 (Applied Biosystems, USA), following the protocol previously described.[16] For ITS sequencing, the forward primers V9G and ITS5 (5′-GGAAG-TAAAAGTCGTAACAAGG-3′) and the reverse primers LS266 and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) were used.[17,18] The same primers used for D1/D2-28S PCR were applied for DNA sequencing.

Samples were run on an automated ABI 3130 genetic analyzer (Applied Biosystems, Inc., Foster City, CA, USA). Sequencher® version 4.1.4 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI USA, http://www.genecodes.com) was used for consensus sequence assembly and edition, and a phred score ≥ 40 was considered to obtain high-quality data. The consensus sequences were aligned and compared with sequences deposited in public genomic databases: GenBank (NCBI, USA, http://blast.ncbi.nlm.nih.gov/) and CBS database (the Netherlands, http://www.cbs.knaw.nl/). For accurate *Malassezia* species identification, a maximum identity $\geq$ 98% and an e-value $< 10^{-5}$ were considered.

The randomization was performed in blocks of four at baseline and generated by computer. For statistical analysis, the intention-to-treat (ITT) population was used. Categorical data were compared using the chi-squared test, Mann–Whitney U-test or Wilcoxon's test. Analyses were conducted in IBM SPSS Statistics for Windows Version 19.0 (IBM Corp., Armonk, NY, USA). The level of significance was established at $P < 0.05$.

Results

At the baseline and six months after therapy, the total of 87 scalp samples were collected from 46 and 41 subjects and forty three and thirty nine of them had positive cultures for *Malassezia* species respectively (Table 1). The most frequent species isolated on the scalp were M. *globosa*, M. *restricta* and M. *sympodialis* at baseline (Table 2). There was a reduction in M. *globosa* and an increase of M.*restricta* after treatment. Similarly, in individuals with clinical diagnosis of seborrheic dermatitis, M. *globosa* followed by M. *restricta* (Table 3). There was no difference between M. *globosa* and M. *restricta* frequency and age, gender, clinical diagnosis, clinical severity, treatment group and sebum secretion rate variables (Table 4). The identification of *Malassezia* spp. strains obtained from scalp subjects before and after treatment was demonstrated in Table 5.

Some different *Malassezia* species were isolated cohabiting the scalp before and after both treatment, such as *M. globosa-M. restricta, M. globosa-M. sympodialis and M. restricta-M. sympodialis*were the most registered peer of scalp colonization.

Table 1. Fungal species identified on scalp samples population study overall (n = 87).

Isolated species	N (%)
M. globosa	40 (45.8)
M. restricta	26 (29.8)
M. sympodialis[§]	7 (8.0)
M. dermatis[§]	3 (3.4)
M. furfur	2 (2.3)
M. japonica	2 (2.3)
M. slooffiae	1 (1,1)
Non- *Malassezia* species	5 (5.7)

Non-Malassezia species: T. asteroids, T.faecales, Rhodotorula spp., C. haemulonis var. vulnera and C. parapsilosis.
One without growth.
[§]ITS-rDNA region identification.

Table 2. Distribution of yeasts of *Malassezia spp*. at the baseline and six month after treatment including all subjects (n = 81 samples).

Species (isolates)	Baseline N(%)	Month 6 N(%)
M. dermatis[§]	1 (2.3)	2 (5.6)
M. furfur	1 (2.3)	1(1.8)
M. globosa	24 (55.8)	16(44.4)
M. japonica	1 (2.3)	1 (2.8)
M. restricta	10 (23.3)	14 (38.9)
M. sloofiae	0 (0)	1(2.8)
M. sympodialis[§]	6 (14)	1 (2.8)

[§]ITS-rDNA region identification.

Table 3. Distribution of yeasts of *Malassezia spp*. at the baseline and six month after treatment in seborrheic dermatitis subjects (n = 81 samples).

Species (isolates)	Baseline N(%)	Month 6 N(%)
M. globosa	24 (29.6)	16(19.7)
M. restricta	10 (11.3)	14 (17.3)

Table 4. Baseline characteristics in subjects according to yeasts of *Malassezia* genus.

Variables	*M. globosa* (n = 24)	*M. restricta* (n = 10)	p
Demographic			
Age, years, mean ± SD	28.5 ± 5.6	28.3 ± 7.2	0,985
Female, n (%)	17 (70.8)	7(70)	1,000
Clinical diagnosis			
Seborrheic dermatitis, n (%)	20 (83.3)	6 (60.0)	0,309
Seborrhoea, n(%)	4 (16.7)	4 (40.0)	
Clinical severity			
Moderate level, n (%)	22(91.7)	10 (100)	0.888
Severe level, n(%)	2 (8.3)	0 (0.0)	
Treatment group			
ISO group, n (%)	13(54.2)	7 (70.0)	0,637
SH group, n(%)	11(45.8)	3(30.0)	
Laboratorial			
Sebum secretion rate, ug/cm2, mean ± SD	127.8 ± 48	121.5±31.6	0.615

SD: standard deviation.
ISO: low-dose oral isotretinoin therapy.
SH: topical therapy with anti-seborrheic shampoo.

Table 5. Identification of *Malassezia* spp. strains obtained from patients before and after treatment based on molecular methods (internal transcribed spacer region, ITS-rDNA and D1/D2-28S-rDNA sequencing).

	Molecular Identification			
Strain number	Yeast ID	Maximum ID (%)	Yeast ID	Maximum ID (%)
1	*M. globosa*	99%	*M. globosa*	99%
1F	*M. japonica*	99%	*M. japonica*	100%
2	*M. globosa*	98%	*M. globosa*	99%
2F	*M. globosa*	99%	*M. globosa*	99%
3	*M. globosa*	99%	*M. globosa*	98%
3F	*M. restricta*	99%	*M. restricta*	99%
4	*M. restricta*	99%	*M. restricta*	98%
4F	*M. restricta*	99%	*M. restricta*	99%
5	*M. restricta*	100%	*M. restricta*	99%
5F	*M. globosa*	99%	*M. globosa*	100%
6F	*M. restricta*	99%	*M. restricta*	99%
7	*M. restricta*	99%	*M. restricta*	99%
7F	*M. restricta*	99%	*M. restricta*	99%
8	*M. sympodialis*	99%	*M. sympodialis / M. dermatis*	99%
9	*M. furfur*	99%	*M. furfur*	99%
10	*M. globosa*	99%	*M. globosa*	99%
10F	*M. globosa*	99%	*M. globosa*	99%
11	*M. restricta*	99%	*M. restricta*	99%
11F	*M. restricta*	99%	*M. restricta*	99%
12	*M. globosa*	99%	*M. globosa*	99%
12F	*M. restricta*	99%	*M. restricta*	99%
13	*M. japonica*	99%	*M. japonica*	100%
13F	*M. globosa*	98%	*M. globosa*	99%
15	*M. globosa*	98%	*M. globosa*	99%
15F	*M. slooffiae*	99%	*M. slooffiae*	100%
16	*M. globosa*	99%	*M. globosa*	100%
16F	*M. globosa*	99%	*M. globosa*	98%
17	*M. globosa*	99%	*M. globosa*	100%
17F	*M. globosa*	98%	*M. globosa*	99%
19	*M. globosa*	98%	*M. globosa*	99%
19F	*M. restricta*	99%	*M. restricta*	99%
20	*M. sympodialis*	99%	*M. sympodialis*	100%
20F	*M. globosa*	99%	*M. globosa*	99%
21	*M. restricta*	99%	*M. restricta*	99%
21F	*M. furfur*	99%	*M. furfur*	100%
23	*M. globosa*	99%	*M. globosa*	100%
23F	*M. restricta*	99%	*M. restricta*	99%
24	*M. sympodialis*	99%	*M. sympodialis*	100%
24F	*M. globosa*	99%	*M. globosa*	99%
25	*M. restricta*	99%	*M. restricta*	99%
	Identificação molecular – região ITS-rDNA		Identificação molecular – D1/D2 da região 28S-rDNA	
N° Paciente	Yeast ID	Maximum ID (%)	Yeast ID	Maximum ID (%)
25F	*M. restricta*	99%	*M. restricta*	99%
27	*M. globosa*	98%	*M. globosa*	98%
28	*M. restricta*	99%	*M. restricta*	99%
29	*M. globosa*	99%	*M. globosa*	99%
29F	*M. dermatis*	99%	*M. dermatis/M. sympodialis*	98%
30	*M. globosa*	99%	*M. globosa*	99%
30F	*M. globosa*	100%	*M. globosa*	99%
31	*M. globosa*	99%	*M. globosa*	99%
31F	*M. globosa*	99%	*M. globosa*	99%
32	*M. globosa*	99%	*M. globosa*	100%
32F	*M. sympodialis*	100%	*M. sympodialis*	100%
33	*M. restricta*	99%	*M. restricta*	99%
33F	*M. restricta*	99%	*M. restricta*	99%
35	*M. globosa*	99%	*M. globosa*	99%
36	*M. globosa*	98%	*M. globosa*	99%
37	*M. globosa*	98%	*M. globosa*	100%
37F	*M. globosa*	98%	*M. globosa*	99%
38	*M. globosa*	98%	*M. globosa*	99%
38F	*M. restricta*	99%	*M. restricta*	100%
39	*M. sympodialis*	99%	*M. sympodialis*	99%
39F	*M. restricta*	99%	*M. restricta*	99%

(*Continued on next page*)

Table 5. (*Continued*)

	Identificação molecular – região ITS-rDNA		Identificação molecular – D1/D2 da região 28S-rDNA	
N° Paciente	Yeast ID	Maximum ID (%)	Yeast ID	Maximum ID (%)
40	*M. globosa*	99%	*M. globosa*	100%
40F	*M. restricta*	99%	*M. restricta*	99%
42	*M. restricta*	97%	*M. restricta*	99%
43	*M. globosa*	99%	*M. globosa*	99%
43F	*M. globosa*	99%	*M. globosa*	99%
44	*M. globosa*	98%	*M. globosa*	99%
44F	*M. globosa*	99%	*M. globosa*	99%
45	*M. globosa*	99%	*M. globosa*	99%
45F	*M. globosa*	99%	*M. globosa*	99%
46F	*M. restricta*	99%	*M. restricta*	99%
47	*M. sympodialis*	100%	*M. sympodialis*	100%
47F	*M. restricta*	<90%	*M. restricta*	99%
48	*M. restricta*	100%	*M. restricta*	100%
48F	*M. globosa*	99%	*M. globosa*	99%
51	*M. sympodialis*	100%	*M. sympodialis/ M.dermatis*	99%
51F	*M. restricta*	<90%	*M. restricta*	99%
52	*M. globosa*	98%	*M. globosa*	100%
52F	*M. globosa*	98%	*M. globosa*	99%
54	*M. dermatis*	99%	*M. dermatis/M. sympodialis*	99%
54F	*M. dermatis*	99%	*M. dermatis/M. sympodialis*	99%

ID =Identity (chance of similarity between sequences with those of gene bank); E *value*= Probability of alignment occurred by chance; 6= *T. asteroides*; 14=*T. faecales*; 14F= *Rhodotorula spp.*, 9F= *C. haemulonis var. vulnera*, 27F=without growth; 46F = *C. parapsilosis*; 8F, 28F, 35F, 36F, 42F= dropout; 40Do= dorsal region (*M. sympodialis*); 40Lo= lombar region (*M. globosa*).

Discussion

Malassezia spp. constitute a resident of the healthy skin microbiome as well as atopic dermatitis, seborrheic dermatitis and probably psoriasis lesioned skin.[10,19-21] Its role in the pathophysiology of seborrheic dermatitis has not yet been fully elucidated. It is believed that *Malassezia* species contribute as a triggering factor of the inflammatory process of the innate immunity of the skin mediated by complex interactions between the fungal cell and its virulence factors, just as, similarly *P.acnes* acts in acne.[22] Its importance is based on the fact that when there is a quantitative reduction of the fungal load after specific topical and / or systemic antifungal treatment the signs and symptoms improve significantly.[1,23-31] Topical immunomodulators was also reported as therapeutic option.[32] There are about fourteen identified species of which ten were isolated on human skin with clinical importance (*M. globosa, M. restricta, M sympodialis, M. dermatis, M. japonica, M. obtusa, M. sloofiae, M. furfur, M. pachydermatis, M. nana*).[7,8] Published epidemiological data suggest geographical variations in the rate of the isolated species, andmolecular typing methods have been developed to evaluate the distribution of different *Malassezia* subtypes.[7,9,15]

This study presented unprecedentedly the prevalence of *Malassezia* yeasts isolated on the scalp with seborrheic dermatitis before and after treatment in a randomized, comparative and therapeutic trial. Initially, *M. globosa* and *M. restricta* were the predominant species isolated both in SD and seborrhea lesions on the scalp skin microbiome corroborating findings previously described.[33] It is noteworthy that *M. sloofiae* was identified only after treatment and *M. sympodialis* did not show growth in any participant at the end of the study. Such findings corroborate those already described that *M. globosa* and *M. restricta* represented the most frequent species both in DS lesions and in healthy skin through the real-time PCR method. The same study showed that *M.sympodialis, M. dermatis and M. sloofiae* were found in skin lesions in 25.8-35.5% and in rates of 14.8-22.2% in healthy skin.[33]

In some subjects, the *Malassezia spp.* identified after treatment were not the same as those of the baseline. Moreover, in seborrheic dermatitis subjects more than one *Malassezia spp.* was found in culture concluding that probably there is a coexistence of different species on the scalp as described by some authors.[9,21] Study conducted in Greece demonstrated that individuals with pityriasis versicolor and seborrheic dermatitis presented *M.globosa* as the most commonly isolated species (33.3%) or in combination with *M.sympodialis* or *M.restricta* (13.3%) or together with *M. sympodialis* or *M. restricta* in one individual (2.2%) in cases of SD.[9]

In a Canadian study, *M.globosa* was the predominant species (45%) isolated from DS skin lesions followed by *M.sympodialis* (30.8%) and *M.sloofiae* (10%). The trunk was the most colonized region with 82.1% of the individuals.[34] In contrast, studies in Japan showed that *M.globosa, M.furfur* and *M.sympodialis* corresponded to the species identified in 20.8%, 20.8% and 6.3% of the samples respectively.[19] Interestingly, *M.restricta* has not been described in either of the two previous studies.

In this context, genotypic identification through PCR followed by sequencing are essential methods. The ITS region of rDNA is not specific for *Malassezia* spp. Because the same region is employed as the universal target for molecular identification of *Candida* spp[16] as well. Initially, the ITS region of the rDNA was selected as the only target for the molecular identification of *Malassezia* spp. However, the literature also describes the use of the D1-D2 domains of the 28S rDNA region, which are the most variable portions of that region, in the identification of isolates of *Malassezia* spp.[35] Thus, sequencing of the ITS region as well as the D1-D2 domains of rDNA is recommended for accurate identification at the species level for isolates of unknown identity. In the present study, it was chosen to perform the methods that identify two molecular targets to guarantee the reliability in the species-level differentiation. We observed the agreement between the molecular identification results using the two targets, with percentages of identity between the sequences greater than or equal to 98%, confirming the utility of the ITS region and D1 / D-28S region sequencing of the rDNA as a laboratory tool to differentiate the Species of the genus *Malassezia*.[10,36] Strains of the genus *Trichosporon* identified in two isolates do not represent contamination. It was reported that occasionally in humans some species of the genus *Trichosporon* may be part of the microbiome in the gastrointestinal and respiratory tract, oral and vaginal mucosa and transiently in the skin.[37] It was described that such fungus can lead to infection of the armpit, pubic and perianal hairs in man.[15] We believe that *Rhodotorula* present in our study represented a contaminating fungus on the skin of the scalp.

A systematic review concluded that there is no standard treatment for seborrhea and DS which are chronic conditions.[1] We compared the two therapies most reported in the literature – topical antiseborrheic shampoo[23,25,26-28,31] and low-dose oral isotretinoin, as this option have also been reported for moderate acne treatment.[12,13,38-42] Its use for seborrhea and DS is not approved as well as for other dermatosis reported in the literature.[43] Despite clinical improvement the reduced sebum secretion on scalp environment was not sufficient to eliminate *Malassezia* yeasts significantly in patients treated with oral isotretinoin. It is possible that most adapted *Malassezia* yeasts remained in the scalp despite the treatment. Based in our findings it was not possible to corroborate with the hypothesis that *Malassezia* spp are etiological factor. However they just acts as triggering factor in etiopathogenesis of seborrheic dermatitis. New mechanisms such as oxidative stress have been discussed as responsible for seborrheic dermatitis activity.[44]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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