Production and Quantification of Virulence Factors in *Malassezia* Species

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Abstract

A total of 77 strains of *Malassezia* were included in this study. Biofilm production and hydrolytic enzymes were studied by using specific solid media. Real-time Reverse Transcriptase qPCR method was applied to determine overexpression of genes encoding extracellular enzyme. All included *Malassezia* species produced biofilms. No statistical significant difference was observed between biofilm formation of the *Malassezia* species ($P = 0.567$). All *Malassezia* species produced lipase and 95% of *M. globosa* showed a strong enzymatic activity ($P_z = 0.55 \pm 0.02$). Statistical significant difference was observed between the mean keratinase indices of *M. slooffiae* and the others *Malassezia* species ($P = 0.005$). The overexpression of one or more genes was observed in 100% of strains isolated from patients with folliculitis, in 87.5% for pityriasis versicolor isolates and in 57.14% for the control group isolates. A statistical significant difference of the lipase gene expression ($P = 0.072$) was associated with the strains collected from patients with folliculitis vs group control. This investigation provides more information about the frequency of the production of the major enzymes considered to be virulence factors of *Malassezia* species. Interestingly, the overexpression of one or more genes was observed in strains isolated from patients with *Malassezia* disorder.

Introduction

Malassezia species, which are members of the human healthy skin, may cause a wide range of cutaneous and systemic diseases in predisposed individuals. Thus, they exist at the interface between commensal and pathogen. Their interaction with the human immune system is of great interest, despite being members of the indigenous cutaneous microbial population [1].

When the physical, chemical or immunological mechanisms of the skin are altered, this yeast can become pathogenic [2-6]. Classic skin diseases caused by *Malassezia* yeasts include pityriasis versicolor (PV), seborrheic dermatitis and *Malassezia* folliculitis.

Particular features of *Malassezia* organisms such as its non autoaggregation behavior and a very high lipolytic enzyme activity may play an important role in the pathogenicity of parenteral lipid emulsion *Malassezia*-related species [7].

Recently, the role of *Malassezia* lipases and phospholipases as pathogenicity mechanisms in seborrheic dermatitis was enhanced by the demonstration of significant increased expression of lipase genes [8]. This finding was also supported by the in vitro induction of phospholipase activity of *Malassezia* seborrheic dermatitis isolates by β -endorphin, another putative virulence factor increased in inflammatory skin conditions [9].

Among keratinophilic microorganisms, *Malassezia* for which the ability of digesting keratin constitutes a condition of their growth and development plays an important role.

A further health threat by these pathogens is their propensity to develop biofilm on skin and at the surface of indwelling medical devices. Biofilm represents a serious medical problem because it promotes resistance to antifungal drugs and acts as a reservoir for the seeding of infectious organisms at distant places [10]. Real-time PCR has become one of the most widely used methods of gene quantitation because it has a large dynamic range, boasts tremendous sensitivity, can be highly sequence-specific. The advent of real-time PCR and real-time reverse transcription PCR (real-time RT-PCR) has dramatically changed the field of measuring gene expression. Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity.

The aim of the present study was to quantify biofilm production and to investigate the extracellular production of lipase, phospholipase and keratinase for 6 *Malassezia* species by using microbiological and Real-time Reverse Transcriptase qPCR methods. In addition, we examined whether virulence factors inherent in the organisms themselves might predict clinical significance.

Materials And Methods

Isolates

A total of 77 strains of *Malassezia* were included in the study: 20 *M. globosa*, 10 *M. restricta*, 14 *M. furfur*, 7 *M. sympodialis*, 5 *M. slooffiae* and 21 *M. pachydermatis*. They were collected from patients with *Malassezia* folliculitis (12), from babies of neonatal intensive care unit (20), pityriasis versicolor (10) and control groups (35). These strains were identified by real time qPCR using a specific hydrolysis probe as previously described [11].

Evaluation of biofilm formation by crystal violet staining (CVS)

Biofilm formation of *Malassezia* species using crystal violet staining (CVS) was performed as reported previously with some modifications [12]. The strains were cultured on Dixon agar at 32 °C for 3 days. Thus, the culture was suspended in Dixon Liquid. The suspension (150 μ l) adjusted to match turbidity comparable to that of the 0.5 McFarland standard (10^6 CFU/ml) and 200 μ L of Dixon Liquid were placed into selected wells of 96-well polystyrene microtiter plates (Kartell, SPA, Italy). The plate was then incubated at 32 °C on a shaker at 120 rpm/min. At 24h, 200 μ L of sterile Dixon Liquid was added and incubated 48h under the same conditions. Subsequently the medium was removed and each well was washed with sterile ultra-pure water.

For fixation of the biofilm, 200 μ l of absolute methanol (15min) were added and removed. The polystyrene microtiter plates are kept at room temperature for drying and 200 μ l of crystal violet of CV (1% v/v) added to each well and incubated for 5 min. Then, multiple washing with ultra-pure water was done until complete elimination (CV) and finally 200 μ l of acetic acid were added.

A microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 620 nm was used for reading the absorbance in each well.

Evaluation of phospholipase production

The production of phospholipase was assessed using the egg-yolk plate method. Briefly, the isolates were incubated in Dixon agar. Thus, the culture was suspended in Dixon Liquid. Ten microliters of yeast suspension prepared from a fresh culture (10^6 cells/ml determined through densitometer) was spot inoculated in triplicate onto the medium and incubated at 32 °C for 10 days. The formation of precipitation zones surrounding the colonies was considered as an indicator for enzyme production. The diameter of the colony (*a*) and the diameter of the precipitation zone plus the diameter of the colony (*b*) were measured. Phospholipase index was designated as $Pz = a/b$, as described by Coutinho and Paula [13, 14]. According to this definition, low Pz values mean high enzymatic production and, inversely, high Pz values indicate low enzymatic production. The enzymatic activity was scored into four categories: a Pz of 1.0 indicated no enzymatic activity (nul); a Pz between 0.99 and 0.90 indicated weak enzymatic activity; Pz between 0.89 and 0.70 corresponded to high activity; and low Pz values ≤ 0.69 mean very high enzymatic activities.

Evaluation of lipase production

The strains were inoculated in triplicate on agar plates containing Tween 80 as substrate (bactopepton 10g; sodium chloride 5g; calcium chloride 0.1g; agar 20g; Tween 80 20% 50mL; water 950mL) and incubated for 7 days at 32°C. Precipitation halo and colony size were measured.

Degradation intensity was gauged by the ratio between colony diameter and precipitation halo (Pz). Pz coefficients were grouped into five classes: very low group: Pz between 0.9 and 1, low group: Pz between 0.89–0.8, high group: Pz between 0.79–0.70; and very high group: Pz less than 0.69 or null (Pz = 1).

Evaluation of keratinase production

The preparation of keratin substrate (KS) was extracted from white chicken feathers. Ten g of feathers was heated in 200 ml of DMSO (Dimethylsulfoxide) for 2 hours at 100 °C. Solution was precipitated for soluble keratin by using 2 volume of cold acetone for 1 volume of protein. Then, caseous precipitate of keratin protein was suspended in 0.1M of phosphate buffer. Afterwards, soluble keratin protein was added in the solid medium at a concentration of 0.06%. Solid medium was inoculated with micropipette, injecting 10 μ l of standard suspension into center of the petri plate and incubated at 32 °C. Control plates were containing medium without keratin substrate. Formation of precipitate zones around the colonies indicated keratinase enzyme production and zone was examined and measured. Pz coefficients were grouped into five classes as it was described for lipase activity.

Virulence gene expression

A RT-qPCR assay was newly developed to explore virulence gene expression of 20 *M. globosa* isolates. The levels of mRNA and DNA copies of the tested genes (Keratinase, Lipase, and Phospholipase) and the actin reference gene (ACT1) were measured.

The primers and probes were designed using Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>), verified by using sequence manipulation suite software (<http://bioinformatics.org/sms2/>) and were summarized in Table 1.

Cellular lysates were prepared from cells grown in culture in Dixon medium (reference) and lipase, keratinase and phospholipase medium to mid-log phase using proteinase K (Qiagen®).

RNA was extracted from cellular lysates using the RNeasy Mini Kit (Qiagen®, Germany) according to the manufacturer's instructions, and treated with DNase (Promega).

For cDNA synthesis, 2.5 μ l of total RNA was used as a template and subsequent reverse transcription was performed using the PrimeScript RT Reagent Kit (Perfect Real Time) from TaKaRa (Shiga, Japan), following the manufacturer's instructions.

The reaction mixture (20 μ l) for TaqMan assay contained 10 μ l TaqMan Universal PCR Master Mix (Applied Biosystems, UK), 20 pmol of forward and reverse primers, 7 pmol of hydrolysis probe and 1 μ l of template (extracted DNA or cDNA). The thermal conditions were as follows: initial holding stage at 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and a final step at 54 °C for 1 min. All reactions were performed in triplicate in 48-well reaction plates using a CFX 96 Real-Time System (BioRad).

Fold changes in target gene expression were then normalized to reference gene via the published comparative $2^{-\Delta\Delta Cq}$ method according to the formula: $RQ = 2^{-(Cq_{\text{target}} - Cq_{\text{reference}})}$ tested – $(Cq_{\text{target}} - Cq_{\text{reference}})$ control. Reference control was the isolate (TN371, KU597270) cultured in Dixon medium. A change of 2.5 times was considered as gene overexpression or an increase in gene copy number.

Statistical analysis

Statistical analyses were performed using IBM SPSS software (version 20.0; IBM SPSS Inc., New York, USA).

Results

Biofilm Formation

The formation of biofilm was positive for all *Malassezia* species (Figure 1). The amount of biofilm formed in each case was estimated in triplicate and an average was calculated for each isolate (Figure 1). The amounts of obtained biofilms were (Abs moy = 0.322 ± 0.01) for *M. globosa*, (Abs moy = 0.336 ± 0.01) for *M. furfur*, (Abs moy = 0.336 ± 0.02) for *M. restricta*, (Abs moy = 0.349 ± 0.02) for *M. pachydermatis*, (Abs moy = 0.256 ± 0.02) for *M. slooffiae* and (Abs moy = 0.235 ± 0.02) for *M. sympodialis* (Table 2).

No statistical significant difference was observed between biofilm formation of the *Malassezia* species ($P = 0.567$). Strains collected from neonates had the highest biofilm production (Abs moy 0.396 ± 0.02) followed by strains from folliculitis (Abs moy 0.342 ± 0.01) and pityriasis versicolor (Abs moy 0.313 ± 0.02). Strains isolated from control group had the lowest amounts of obtained biofilms (Abs moy 0.291 ± 0.02) (Table 2). However, there was no statistically significant association between biofilm-forming ability and the clinical origin of the isolates ($P=0.570$).

Lipase activity

The lipase activity was positive for all *Malassezia* species. A total of 19 isolates of *M. globosa* (95%) ($P_z = 0.55 \pm 0.02$) and 5 isolates of *M. sympodialis* (71.43%) ($P_z = 0.54 \pm 0.02$) showed a very high enzymatic activity (Table 3).

Strains isolated from pityriasis versicolor showed a very high activity with P_z values between (0.36 ± 0.02 , 0.28 ± 0.01) (Table 4). But, no statistical significant difference was observed between the mean lipase indices and the clinical origin of the isolates ($P=0.356$).

Phospholipase activity:

All isolates of *M. furfur*, *M. globosa*, *M. sympodialis*, *M. slooffiae* and *M. pachydermatis* included in this study demonstrated a high or a very high phospholipase activity (Figure 2). But, only 70% of the *M. restricta* isolates showed a high activity (Table 3). A high level of the activity with a P_z values (0.66 ± 0.02) was found in 95% of neonates samples (Table 4).

But, no statistical significant difference was observed between the mean phospholipase production and the clinical origin of isolates ($P= 0.65$).

Keratinase activity

The P_z values for keratinase activity of *Malassezia* isolates ranged from 0.33 to 0.91. All *M. slooffiae* strains showed a positive keratinase activity (Figure 2) and 40% were shown to be very high producers (Table 3).

A total of 16 isolates of *M. globosa* (80%) ($P_z = 0.65 \pm 0.02$), 4 isolates of *M. sympodialis* (57.15%) ($P_z = 0.62 \pm 0.03$), 7 isolates of *M. furfur* (50%) ($P_z = 0.67 \pm 0.02$), 2 isolates of *M. restricta* (20%) ($P_z = 0.69 \pm 0.02$) and 11 isolates *M. pachydermatis* (52.29%) ($P_z = 0.68 \pm 0.02$) showed a keratinase activity (Table 3).

Statistical significant difference was observed between the mean keratinase indices of *M. slooffiae* and the others *Malassezia* species ($P = 0.005$).

Correlation analysis results with Pearson's coefficient revealed a positive correlation between secretion of keratinase and phospholipase ($r = 0.31$, $P < 0.001$).

Expression of keratinase, Lipases and Phospholipases of *M. globosa*

We analyzed by RT-qPCR the quantitative expression and copy number of 3 genes (keratinase, lipase and phospholipase) responsible for the virulence of 20 *M. globosa* strains collected from patients with folliculitis (5), patients with pityriasis versicolor (8) and control group (7), comparing to the ACT1 gene and the isolate (TN371, KU597270) cultured in Dixon medium. The overexpression of one or more genes was observed in 5/5 (100%) of strains isolated from patients with folliculitis in 7/8 (87.5%) for pityriasis versicolor isolates and in 3/7 (57.14%) for control group isolates (Table 5). The level of expression of keratinase varied from 0.81 to 3.52 folds and the level of lipase varied from 1.84 to 12.99 in folliculitis strains. The phospholipase highest expression level was 12.4 folds shown in S3 isolate collected from folliculitis (Table 5). Upregulation was noted in strains collected from control group for lipase in only 1 isolate and for keratinase and phospholipase in 2 isolates. A statistical significant difference of the lipase gene expression ($P = 0.072$) was associated with the strains collected from patient with folliculitis vs group control. The overproduction of the 3 genes was observed in 5 strains (S2, S3, S7, S10 and S13). The overexpression of the keratinase, lipase and phospholipase genes was associated with an increased copy number of gene in 8 strains of *M. globosa* (Table 5).

In addition, correlation analysis results with Pearson's coefficient revealed that there was statistically significant positive association between the production of the enzymes and the expression of their genes: keratinase ($r = 0.881$, $P < 0.0001$), lipase ($r = 0.530$, $P = 0.016$) and phospholipase ($r = 0.754$, $P = 0.0001$).

Discussion

In the present study we examined multiple virulence factors such as extracellular secreted hydrolytic enzymes and biofilm development which contribute to the ability of *Malassezia* species to colonize host tissues, and cause disease. However, a few number of studies focused on virulence factors' expression and pathogenesis of the *Malassezia* species.

Our results demonstrated that all *Malassezia species* included in our study, like many other fungal and bacterial species [15] produced biofilms through a discrete sequence of events, including fungal surface adhesion, microcolony formation, and biofilm maturation.

A better knowledge of the mechanisms of antifungal drug resistance may lead to the development of novel therapies for biofilm-based diseases. Multiple mechanisms have been proposed for the biofilm resistance phenomenon [16]. Metabolic quiescence has been proposed as a mechanism of antimicrobial resistance in biofilm bacteria [17] and fungi [16]. However, our data revealed that CVS activity showed biofilm cells to be metabolically active in our *Malassezia species*, in line with data previously reported for *Candida albicans* and *C. parapsilosis* [18]. It is therefore unlikely that biofilm production is a major factor promoting antifungal resistance of *Malassezia species* [16].

Biofilm production was studied only for *M. furfur* [19, 20] and *M. pachydermatis* [21]. Angiolella et al, demonstrated that biofilm adherence and hydrophobicity are considered as virulence factors in *Malassezia furfur* species [20].

Also, these studies showed that *M. pachydermatis* strains from dogs with or without skin lesions are capable to form biofilm with variable quantity and structures which are likely to be strain-dependent [21].

In our study, we showed that all *Malassezia* species produced lipase and 95% of *M. globosa* showed a very high enzymatic activity. In fact, lipases catalyze the hydrolysis of ester bonds of triacylglycerols, resulting in the release of fatty acids. In almost all organisms, lipases play essential roles in lipid metabolism, including digestion, transport, and the processing of dietary lipids. Lipases also play important roles in the virulence of skin-associated lipophilic fungal pathogens of the *Malassezia* spp. The gene that encodes for lipases in *M. furfur*, *M. pachydermatis*, and *M. globosa* has been identified, and recent genome sequencing efforts have revealed at least 14 lipase-encoding genes in *M. globosa* [21].

Weerapong et al suggested that lipase may be a pathogenic factor in the skin disease associated with *Malassezia* and provide an explanation to why *M. globosa* is an important pathogenic species in several human skin diseases despite its slow rate of growth [12].

Interestingly, we showed a high lipase activity from *M. globosa* strains isolated from pityriasis versicolor. In previous study, *M. globosa*, one of the most frequently isolated *Malassezia* spp. from patients with dandruff and seborrheic dermatitis, displayed extracellular lipase activity [9, 22, 23].

Phospholipase activity is another virulence factor exhibited by pathogenic microorganisms which permits to hydrolyze one or more ester linkages in glycerophospholipids, resulting in the release of free fatty acids.

In this study, all strains had the ability to express phospholipase except for *M. restricta* with only 70% were phospholipase producers. Additionally, we noted a high phospholipase activity for *Malassezia* isolates recovered from Neonates group samples.

This virulence factor intrinsic to *Malassezia* yeasts has been discussed in association with the pathogenesis of seborrheic dermatitis. The increased level of production of phospholipase has been shown only for pathogenic *M. pachydermatis* strains [10].

Current information on phospholipases in *Malassezia* spp. is limited. Juntachai et al. detected extracellular phospholipase activities in *M. furfur*, *M. pachydermatis*, *M. slooffiae*, *M. sympodialis*, *M. globosa*, *M. restricta*, and *M. obtusa*. However, enzyme activity was higher only in *M. pachydermatis* [24].

The ability to express enzymes not only varies among different species of *Malassezia* but also differs among the strains of same species isolated from different clinical manifestations. Interestingly, we showed that strains isolated from different groups patients produced phospholipase. The production of *Malassezia* phospholipases on the skin could result in the removal of epidermal lipids, disruption of the epidermal barrier function, and the development of seborrheic dermatitis when sebum production is constitutionally decreased [10].

The keratinases are proteolytic enzymes in nature. They mainly attack the disulfide bond of the keratin substrate [25, 26].

As previously reported the dermatophytes and non-dermatophytes species were both keratinase producers and they are capable of damaging the keratinized structure of the skin [26-28]. In our study, all *M. slooffiae* isolates were keratinase producers. However, only the half of *M. furfur* has a positive enzymatic activity. Peyton et al found that *M. furfur* is capable of degrading keratin [26, 29]. But, Muhsin et al reported negative keratinase activity in *M. furfur* [26].

In our study we found a positive correlation between the secretion of keratinase and phospholipase. These virulence factors appear to act synergistically to contribute to the virulence of *Malassezia* strains.

The genome sequencing data revealed that *M. globosa* possesses a total of 14 lipases and 9 phospholipases. Among them, four lipases (MGL_3878, MGL_3507, MGL_0799, and MGL_0798) and two phospholipases (MGL_4252 and MGL_3326) were expressed on human scalps [30]. Few studies were carried out to analyze expression of lipases and phospholipases of the fungus [31]. However, to our knowledge no information is currently available for expression of keratinase. In our study, the RT-qPCR analysis of expression and copy number of the 3 genes responsible for the virulence of 20 *M. globosa*, showed the overexpression of one or more genes in 5/5 of folliculitis strains and 7/8 in pityriasis versicolor isolates. The phospholipase highest expression level was 12.4 folds shown in isolates collected from folliculitis. Moreover, we noted a statistical significant difference of the lipase gene expression ($P = 0.072$) was associated with the strains collected from patient with folliculitis vs group control. These data imply a possible role of lipase in the host environment to produce free fatty acids for the fungus. The concurrently overproduction of the three genes was observed in a five strains isolated from patients with folliculitis and pityriasis versicolor. However, overexpression was noted in only 2 cases of control group suggesting that these enzymes play an important role in the pathogenicity of *Malassezia* disorder inducing a transition from colonization to infection. Interestingly, further studies evaluated the expression of lipases and phospholipases of *Malassezia restricta* in patients with seborrheic dermatitis and suggested that majority of the patients displayed expression of these enzymes [32].

Moreover, Patino-Uzcategui et al. found a significant higher expression of MGL 0797, MGL 0798, and Mflp1 virulence genes in Seborrheic Dermatitis HIV patients. They concluded that lipases may be related to the development of Seborrheic Dermatitis and could be considered as virulence factors [8].

In the study of Gharehbolagh et al, the Real-time PCR was applied to investigate the contribution of the MGL_3741 gene to pathogenicity of *Malassezia globosa* in pityriasis versicolor. These authors revealed that this gene can be related to the pathogenicity of this yeast and it can be a candidate for new antifungal investigation with better action to treatment of pityriasis versicolor [33]. However, further studies are still needed to determine this role.

Conclusion

This investigation provides more information about the frequency of the production of the major enzymes considered to be virulence factors of *Malassezia* species. Interestingly, the overexpression of one or more genes was observed in strains isolated from patients with *Malassezia* disorder. These data emphasizes the role of lipase and phospholipase in the pathogenicity of this fungus and suggest that these enzymes act synergically for inducing *Malassezia* pathologies.

Declarations

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Tables

Table 1. The sequences of primers and probes used in RT-qPCR for gene expression of 20 *M. globosa* isolates

Enzymes	Names	Sequences
Keratinase	KerF	5'-ACGTCATGCTCAGATTGCAG-3'
	KerR	5'-GACTTCCGCGAAGAACAAAG-3'
	KerP	5'-FAM-CACAATTGCTCCCGATACCT-MGB
Lipase	LipF	5'-ACCCAACATTTGCTTCGTTC-3'
	LipR	5'-TCAATTATCAATGGTCGCGA-3'
	LipP	5'-FAM-CCTTGGTGGTCGTCAAAGTT-MGB
Phospholipase	PhosF	5'-AACTGGTGGATTTGCTGACC-3'
	PhosR	5'-CTTTACGGGTCCAAGGTGAA-3'
	PhosP	5'-FAM-CCGTGTGCCATTCTACATTG-MGB
Actin	ActF	5'-CTCTCCTTGCTACGCCTCTGG-3'
	ActR	5'-TTGACAAGATGCTCCGTCAG-3'
	ActP	5'-TET-GTATTGTGCTGGACTCGGGT-MGB

Table 2. Biofilm production for *Malassezia* species isolated from different clinical sites

groups	<i>M. globosa</i>			<i>M. restricta</i>			<i>M. furfur</i>			<i>M. sympodialis</i>			<i>M. slooffiae</i>			<i>M. pachydermatis</i>			To
	N	Mean Abs	Range	N	Mean Abs	Range	N	Mean Abs	Range	N	Mean Abs	Range	N	Mean Abs	Range	N	Mean Abs	Ra	
		± SD			± SD			± SD			± SD			± SD			± SD		
Folliculitis (N= 12)	5	0.321 ±0.01	0.155- 0.41	1	0.366 ±0.01	0.301- 0.398	6	0.339 ±0.01	0.317- 0.594	0			0			0			
Neonates group (N=20)	0			0			1	0.433 ±0.02		0			0			19	0.361 ±0.02	0.3 0.5	
Pityriasis versicolor (N=10)	8	0.317 ±0.01	0.16- 0.604	1	0.306 ±0.02		1	0.316 ±0.02		0			0			0			
Control group (N=35)	7	0.328 ±0.01	0.18- 0.51	8	0.338 ±0.02	0.12- 0.572	6	0.255 ±0.02	0.12- 0.311	7	0.235 ±0.02	0.137- 0.361	5	0.256 ±0.02	0.167- 0.331	2	0.336 ±0.02	0.2 0.4	
Total	20	0.322 ±0.01	0.155- 0.604	10	0.336 ±0.02	0.12- 0.572	14	0.336 ±0.02	0.12- 0.594	7	0.235 ±0.02	0.137- 0.361	5	0.256 ±0.02	0.167- 0.331	21	0.349 ±0.02	0.2 0.5	

N: number of tested isolates

SD: standard deviation

Table 3. Level of phospholipase, lipase and keratinase production by *Malassezia* species.

N: number of tested isolates

n: number of isolates with positive activity for the corresponding hydrolytic enzyme

SD: standard deviation

Table 4. Distribution of enzymatic activities of *Malassezia* species isolated from different clinical sites

	Phospholipase activity				Lipase activity			Keratinase activity			
	number of isolates (rate of isolates)				number of isolates (rate of isolates)			number of isolates (rate of isolates)			
	Mean (Pz) ± SD	very high n (%)	high n (%)	Nul n (%)	Mean (Pz) ± SD	very high n (%)	high n (%)	Mean (Pz) ± SD	very high	high n (%)	nul
<i>Malassezia globosa</i> (N=20)	0.64 ±0.03	9 (45)	11 (55)	0 (0)	0.55 ±0.02	19 (95)	1 (5)	0.65 ±0.02	10 (50)	6 (30)	4 (20)
<i>Malassezia restricta</i> (N=10)	0.76 ±0.02	0 (0)	7 (70)	3 (30)	0.36 ±0.03	10 (100)	0 (0)	0.69 ±0.02	0 (0)	2 (20)	8 (80)
<i>Malassezia furfur</i> (N=14)	0.64 ±0.02	2 (14.29)	12 (85.71)	0 (0)	0.45 ±0.03	14 (100)	0 (0)	0.67 ±0.02	1 (7.15)	6 (42.85)	7 (50)
<i>Malassezia sympodialis</i> (N=7)	0.67 ±0.02	1 (14.29)	6 (85.71)	0 (0)	0.54 ±0.02	5 (71.43)	2 (28.57)	0.62 ±0.03	1 (14.3)	3 (42.85)	3 (42.85)
<i>Malassezia slooffiae</i> (N=5)	0.64 ±0.02	2 (40)	3 (60)	0 (0)	0.4 ±0.04	5 (100)	0 (0)	0.67 ±0.02	2 (40)	3 (60)	0 (0)
<i>Malassezia pachydermatis</i> (N=21)	0.64 ±0.03	9 (42.86)	12 (57.14)	0 (0)	0.31 ±0.02	21 (100)	0 (0)	0.68 ±0.02	3 (14.29)	8 (38.5)	10 (47.66)
Total	0.66 ±0.02	23 (29.87)	51 (66.23)	3 (3.9)	0.48 ±0.02	74 (91.1)	3 (3.9)	0.66 ±0.02	17 (22.08)	28 (36.36)	32 (41.56)

		Folliculitis (N= 12)	Neonates group (N=20)	Pityriasis versicolor (N=10)	Control group (N=35)
Phospholipase activity Mean (Pz) ± SD	<i>M. globosa</i> (20)	0.69±0.01	-	0.74±0.02	0.73±0.02
		(5/12)	(0/20)	(8/10)	(7/35)
	<i>M. restricta</i> (10)	0.67±0.01	-	0.8±0.02	0.83±0.02
		(1/12)	(0/20)	(1/10)	(8/35)
	<i>M. furfur</i> (14)	0.74±0.01	0.70±0.02	0.69±0.02	0.71±0.01
		(6/12)	(1/20)	(1/10)	(6/35)
	<i>M. sympodialis</i> (7)	-	-	-	0.78±0.02
		(0/12)	(0/20)	(0/10)	(7/35)
	<i>M. slooffiae</i> (5)	0	-	-	0.77±0.02
		(0/12)	(0/20)	(0/10)	(5/35)
Lipase activity Mean (Pz) ± SD	<i>M. pachydermatis</i> (21)	0	0.66±0.02	-	0.79±0.01
		(0/12)	(19/20)	(0/10)	(2/35)
	Total	0.70±0.01	0.68±0.02	0.74±0.02	0.77±0.02
	<i>M. globosa</i> (20)	0.36±0.01	-	0.36±0.02	0.38±0.02
		(5/12)	(0/20)	(8/10)	(7/35)
	<i>M. restricta</i> (10)	0.37±0.01	-	0.32±0.02	0.36±0.02
		(1/12)	(0/20)	(1/10)	(8/35)
	<i>M. furfur</i> (14)	0.32±0.02	0.39±0.02	0.28±0.01	0.36±0.02
		(6/12)	(1/20)	(1/10)	(6/35)
Keratinase activity Mean (Pz) ± SD	<i>M. sympodialis</i> (7)	-	-	-	0.48±0.02
		(0/12)	(0/20)	(0/10)	(7/35)
	<i>M. slooffiae</i> (5)	-	-	-	0.4±0.01
		(0/12)	(0/20)	(0/10)	(5/35)
	<i>M. pachydermatis</i> (21)	-	0.31±0.01	-	0.38±0.02
		(0/12)	(19/20)	(0/10)	(2/35)
	Total	0.35±0.02	0.35±0.02	0.32±0.02	0.39±0.02
	<i>M. globosa</i> (20)	0.75±0.02	-	0.62±0.02	0.85±0.02
		(5/12)	(0/20)	(8/10)	(7/35)
Keratinase activity Mean (Pz) ± SD	<i>M. restricta</i> (10)	1	-	0.65±0.02	1
		(1/12)	(0/20)	(1/10)	(8/35)
	<i>M. furfur</i> (14)	0.82±0.02	0.53±0.02	0.65±0.02	0.96±0.02
		(6/12)	(1/20)	(1/10)	(6/35)
	<i>M. sympodialis</i> (7)	-	-	-	0.84±0.02
		(0/12)	(0/20)	(0/10)	(7/35)
	<i>M. slooffiae</i> (5)	-	-	-	0.8±0.02
		(0/12)	(0/20)	(0/10)	(5/35)
	<i>M. pachydermatis</i> (21)	-	0.85±0.02	-	0.79±0.02
		(0/12)	(19/20)	(0/10)	(2/35)
	Total	0.86±0.02	0.69±0.02	0.64±0.02	0.873±0.02

SD: standard deviation

Table 5. Relative quantification of gene expression and gene copy number of keratinase, lipase and phospholipase genes in 20 *M. globosa* strains

ID	clinical sites	RNA relative quantification			DNA relative quantification		
		Keratinase	Lipase	Phospholipase	Keratinase	Lipase	Phospholipase
S1	Folliculitis	2.11	1.84	2.59	4.43	7.22	3.06
S2	Folliculitis	2.52	7.51	7.57	1.36	2.04	1.08
S3	Folliculitis	3.52	12.99	12.40	1.50	2.12	3.36
S4	Folliculitis	0.81	3.20	3.91	0.32	12.98	0.69
S5	Folliculitis	3.40	5.55	1.48	6.58	9.57	6.36
S6	Pityriasis versicolor	0.49	0.31	0.78	0.29	0.21	0.02
S7	Pityriasis versicolor	2.65	3.52	3.17	1.35	1.46	1.10
S8	Pityriasis versicolor	2.82	2.35	2.54	2.79	7.43	1.34
S9	Pityriasis versicolor	1.25	3.10	2.53	0.77	1.60	0.24
S10	Pityriasis versicolor	2.57	2.55	2.52	1.96	1.56	3.33
S11	Pityriasis versicolor	2.84	2.75	1.32	0.97	1.74	1.03
S12	Pityriasis versicolor	0.96	2.86	3.37	0.46	0.86	2.56
S13	Pityriasis versicolor	2.55	2.86	3.38	1.50	2.58	1.88
S14	Control group	1.53	0.53	0.56	1.42	0.82	0.75
S15	Control group	1.04	1.19	2.55	0.92	1.05	0.86
S16	Control group	0.67	1.36	0.48	0.50	0.61	0.32
S17	Control group	0.94	2.37	2.43	0.50	1.06	2.33
S18	Control group	2.36	0.07	0.48	1.87	0.26	0.49
S19	Control group	0.24	2.52	2.80	0.56	1.31	1.39
S20	Control group	2.51	3.30	1.50	1.31	1.18	0.27

Figures

Biofilm production

Figure 1

Absorbance values of biofilm quantification using Crystal Violet staining (Abs CV) of different *Malassezia* species ($\lambda=620\text{nm}$). Error bars represent standard deviation.