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ORIGINAL ARTICLE

Affinity of *Malassezia* and Other Yeasts for Pulmonary Lipids

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Abstract Pulmonary surfactant, the primary substance lining the epithelium of the human Lower Respiratory Tract (LRT), is rich in lipids, with dipalmitoyl-phosphatidylcholine (DPPC) being the most abundant. Although surfactants are known to have antifungal activity against some yeast species, the significant presence of species like *Malassezia restricta* in the lung mycobiome suggests that these yeasts may exhibit some level of lipo-tolerance or even lipo-affinity for pulmonary lipids. This study explored the affinity and tolerance of yeasts, identified as significant members of the lung microbiome, to pulmonary lipids through culture-based methods. Eleven species from the genera *Malassezia, Candida* (including the new genera *Nakaseomyces* and *Meyerozyma*), and *Cryptococcus* were tested for their growth on media containing pulmonary lipids such as DPPC and commercial porcine surfactant and in other culture medium that contain non-pulmonary lipids such as glycerol monostearate and tweens. The yeasts' lipo-affinity or lipo-tolerance was assessed based on their growth on these lipids compared to standard media, specifically Modified Leeming Notman Agar (MLNA) for *Malassezia* species and Sabouraud Dextrose Agar (SDA) for the other genera. The addition of DPPC or surfactant to the media enhanced the growth of most *Malassezia* yeasts and some *Cryptococcus* species. *C. parapsilosis, Meyerozyma guilliermondii and Cryptococcus neoformans s.s.* showed similar growth to that on the standard media, while the other yeasts primarily demonstrated lipo-tolerance without lipo-affinity for these compounds. To our knowledge, this is the first report on the influence of pulmonary lipids on the in vitro growth of *Malassezia* spp. and other yeast members of the lung mycobiome. Some yeasts, such as *Malassezia restricta*, commonly found in the lower respiratory tract (LRT), exhibit specific affinity for lung lipids like DPPC and commercial porcine surfactant. This finding suggests that lung lipids may play a significant role in shaping the LRT mycobiome.

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Introduction

Research on the lung microbiome has primarily focused on bacteria, with fungi often overlooked due to their lower abundance and the lack of widely accepted standards in both experimental and analytical methods [1, 2]. However, fungi, or the mycobiome, also contribute to maintaining homeostasis and immunomodulation in the respiratory tract [1, 3]. Compared to gut microbiome studies, the pulmonary microbiome has been less explored due to its technical complexities and constant environmental interactions [2].

Over the past six years, our group has been studying the mycobiome of the Lower Respiratory Tract (LRT) in patients with non-infectious lung diseases [4, 5]. Our findings, along with others in the literature [6–11], indicate that the core mycobiome mainly consists of yeast species. The core, or nuclear microbiome, is defined as taxa with an abundance greater than 0.1% in most samples (85–95%, depending on the authors) [4, 5, 12, 13]. Among the most prevalent species are *Malassezia restricta, Candida parapsilosis, Cryptococcus neoformans* s.l., *Naganishia albida*, and *Rhodotorula mucilaginosa*. *Malassezia restricta* is the most frequently detected species, with the highest number of sequences in Broncho-Alveolar Lavage (BAL) samples [4, 5, 14, 15].

Given the reported antifungal properties of surfactant and the need to assess the role of the pulmonary mycobiome, it was crucial to demonstrate the viability of the species detected by molecular methods. We conducted BAL cultures on Sabouraud media parallel to molecular studies and successfully cultivated some species identified as part of the nuclear mycobiome [4, 5]. However, *Malassezia* species were not isolated in culture, primarily due to their lipodependence.

The genus *Malassezia* belongs to the class Malasseziomycetes, within the subphylum Ustilaginomycotina (phylum Basidiomycota). It comprises 18 species of lipophilic yeasts commonly found as commensals in the cutaneous microbiota of mammals [15, 16]. *Malassezia furfur, M. restricta, M. globosa, M. pachydermatis* and *M. sympodialis* stand out for their presence in humans. *M. pachydermatis* typically found in animals and occasionally in humans [17]. Genomic studies of this genus reveal a loss of genes for the fatty acid synthase complex and an increase in genes related to lipid degradation and carbohydrate metabolism, indicating an adaptation to life in skin and other lipid-rich tissues [18, 19]. This genomic reorganization prevents them from synthesizing their own lipids, making them dependent on external sources. The Sabouraud Dextrose Agar (SDA) medium, a general fungal medium, does not support the growth of lipo-dependent *Malassezia* species due to its lack of sufficient lipids [20]. The exception is *M. pachydermatis*, which can grow using the small lipid portion in the peptone [17]. In addition to their lipid dependence, *Malassezia* yeasts can consume L-DOPA and produce melanin, a virulence mechanism also found in species like *Cryptococcus neoformans* and *C. gattii* species complexes [21].

The genus *Candida*, belonging to phylum Ascomycota, includes species that are common fungal pathogens in humans, colonizing the skin, gastrointestinal tract, oral cavity, upper airways, and genitourinary tract of women [22]. Over 200 *Candida* species have been identified, but only a few are part of the human microbiota capable of causing infections. In the absence of carbohydrates, *Candida* species can metabolize amino acids and lipids as supplementation for metabolic adaptation [23]. Recent studies emphasize the role of lipids in fungal pathogenicity, including drug resistance, biofilm formation, and the release of extracellular vesicles [23].

The lung surface, primarily composed of alveoli, is lined with pulmonary surfactant, a substance that reduces surface tension at the air–liquid interface within the alveoli, preventing their collapse. Produced by type-II pneumocytes from the 24th week of gestation [24], surfactant consists of about 80% phospholipids, 10% cholesterol, and 2–5% surfactant proteins (SP-A, SP-B, SP-C, and SP-D). The most abundant phospholipid, dipalmitoyl-phosphatidylcholine (DPPC), is exclusive to pulmonary surfactant [24, 25]. The more hydrophilic components, SP-A and SP-D, participate in pulmonary defense and immune response modulation. This lipid-rich environment may influence the LRT microbiota and the types of opportunistic mycoses that enter through the lungs.

This study aims to investigate the tolerance/affinity of yeasts species described as common members of the pulmonary mycobiome for lipids such as the components of pulmonary surfactant. This may lead to a better understanding of the lung physiology related to its mycobiome, and the pathogenesis of some opportunistic fungal infections.

Materials and Methods

Design

This experimental study was conducted at the Medical Mycology Laboratory, University Miguel Hernández (UMH), Alicante, Spain.

Yeast Isolates

Eleven yeast species previously described as members of the Lower Respiratory Tract were used: *Candida albicans, Candida parapsilosis, Nakaseomyces glabrata* (formerly *Candida glabrata*), *Meyerozyma guilliermondii* (formerly *Candida guilliermondii*), *Cryptococcus neoformans, Cryptococcus deneoformans, Cryptococcus deuterogattii*, and four *Malassezia* strains: *Malassezia arunalokei, Malassezia globosa, Malassezia pachydermatis*, and *Malassezia restricta*. *M. restricta* and *M. globosa* were obtained from the CBS-KNAW Culture Collection (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands), and the others were from our own collection, isolated from clinical samples including human BAL samples (*C. parapsilosis* and *M. guilliermondii*), human oral cavity (*Nakaseomyces glabrata, and C. albicans*), human blood (*C. deneoformans*), human cerebrospinal fluid (*C. neoformans* and *C. deuterogattii*), human ear canal (*M. arunalokei*) and dog ear (*M. pachydermatis*). The origin of the isolates is listed in Table 1.

The strains were stored in skimmed milk at -80ºC, except for *Malassezia* species, which were kept at 32 ºC following the instructions of CBS-KNAW curator. Fresh cultures were grown on SDA (for *Candida, Nakaseomyces, Meyerozyma,* and *Cryptococcus*) and MLNA (for *Malassezia* species), considered as "standard media". All isolates were incubated at 30-32ºC (optimal temperature for *M. restricta* and *M. globosa*) for 24 to 48 h, up to 21 days for *M. restricta*. After growth, the macroscopic and microscopic morphology was verified. Catalase tests were conducted to confirm the presence of *M. restricta* (catalase-negative) and the avoidance of contamination from the other *Malassezia* species (catalase-positive). The colony appearance on SDA and MLNA, and the colony size (diameter in mm) were used to assess growth on other lipidic media.

In vitro Growth Assays on Different Lipidic Sources

Inoculum Preparation

The inoculum was prepared from fresh cultured cells suspended in sterile distilled water, adjusted to a concentration of 10^6 CFU/mL using the

Table 1 Yeast strains used in the study: Abbreviation, name of the species, identification code and origin of the isolates. CLA: Collection of yeasts of Alicante (Spain); CCA: Collection of *Cryptococcus* of Alicante (Spain); CBS-KNAW: VI-KNAW Culture Collection. Utrecht (The Netherlands). *Name of species according to Hagen F et al. [26], and Kidd SE, et al. [27]

Abbreviation	Species	ID CODE	SOURCE
N.g	*Nakaseomyces gtabrata**	CLA44	Human dental prothesis
Co	*Candida albicans*	CLA45	Human oral cavity
Cp	*Candida parapsitosis*	CLA46	Human bronchoalveolar lavage
M.g	*Meyerozyma guilliermondU**	CLA47	Human bronchoalveolar lavage
Cn	*Cryptococcus neoformans s.s.* *	CCA494	Human cerebrospinal fluid
Cdn	*Cryptococcus deneoformans**	CCA420	Human blood culture
Cdeu	*Cryptococcus deuterogattii**	CCA56	Human cerebrospinal fluid
M.a	*Malassezia arunalokei*	CLA42	Human ear canal
M.glo	*Malassezia globosa*	CBS7966	CBS-KNAW Collection
M.p	*Malassezia pachydermatis*	CLA43	Dog ear canal
M.r	*Malassezia restricta*	CBS7877	CBS-KNAW Collection

McFarland scale. Incubation was at 32 ºC in an air incubator (Heidolph Inkubator 1000).

Culture Media Used

Culture media with different lipidic content were assayed, including the standard media for lipophilic yeasts (Modified Leeming and Notman agar medium -MLNA) [28]. From this one, eight other media were designed, modifying the lipidic composition of the MLNA. All of them had a common nutrient base (to which different lipids were added separately: Glycerol Monostearate (GME), 4 Tween components (T20, T40, T60 and T80), purified DPPC (Sigma-Aldrich®) and pig surfactant extract (Curosurf®, Chiesi Laboratories). Table 2 shows the detailed composition of these media. SDA was used also as a culture medium, as mentioned above, as standard medium for non-lipophilic yeasts.

Culture Methods

Two different culture methods were tested, each performed in triplicate: growth on the surface of solid medium and growth in depth, around dug wells in the same solid media.

Mean values of the diameter of the growth area of yeast inoculated with 5 μL droplets of cells suspension on different media after 48 h incubation.

Friedman's test was performed to assess whether there were differences between the growth of strains in lipid media compared to standard media (SDA and MLNA).

Mean values of the radius of the growth area of yeast inoculated with 5 μL droplets of cells suspension on different media after 48 h incubation (values at different time of 48 h incubation have not been considered for mean calculation).

Growth on Surface of Solid Medium

Plates prepared with the described media were divided into numbered sections. Each isolate was inoculated in its section with a drop of inoculum of 5 μL, 10 and 50 μL respectively in three different experiments. The plates were left to stand and incubated at 30 ºC, with growth checked at 24 h, 5 days, and every 3 days until 21 days.

Growth in Depth in Wells in Solid Medium

Another set of plates was prepared and divided into numbered sections. Wells (3–5 mm deep) were made

Table 2 Detailed composition of the culture media used for testing the ability of yeast to grow on different lipidic sources

Composition of Media										
components	MLNA	NMl	Tw20	Tw40	Tw60	Tw80	GME	DPPC	SURF	SDA
Peptone	Igr	Igr	Igr	Igr	Igr	Igr	Igr	Igr	Igr	Igr
Bile salt	0.8 gr	0.8 gr	0.8 gr	0.8 gr	0.8 gr	0.8 gr	0.8 gr	0.8 gr	0.8 gr	
Glycerol	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL	
Glucose	Igr	Igr	Igr	Igr	Igr	Igr	Igr	Igr	Igr	Igr
Yextract	200 mg	200 mg	200 mg	200 mg	200 mg	200 mg	200 mg	200 mg	200 mg	200 mg
Olive oil	2 mL	2 mL								
Glycerol monostearate	50 mg						50 mg			
Tween 20			1 mL							
Tween 40				1 mL						
Tween 60					500 liL					
Tween 80						500 u ±				
DPPC		16,7 mg						16,7 mg		
Surfactant*									627 ul	
Destiled water	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL	100 mL
Agar–agar	1,5 gr	1,5 gr	1,5 gr	1,5 gr	1,5 gr	1,5 gr	1,5 gr	1,5 gr	1,5 gr	1,5 gr

*Commercial surfactant (Curosurf). Concentration of surfactant in the vial 80mg/mL with a sterile punch (5 mm diameter) in each section, inoculated with 50 µL of yeast cell suspensions.

They were left to stand and incubated at 30 ºC and the results were read at 24 h, 5 days, and every 3 days until 21.

Growth in Liquid Media (Microtiter Plates)

Eight liquid media were prepared as described for Tw20, Tw40, Tw60, Tw80, GME, DPPC, SURF, and SDB (SDA excluding agar–agar). The media were placed in the columns of three 96-well microtiter plates, each well filled with 150 µL of medium. Rows A to I were inoculated with 50 µL of yeast cell suspension, and row H was kept sterile as a control. Three sets of plates were used to test all strains. Growth was assessed by serial readings, starting at 24 h and every 3 days until 21 days. Growth was indicated by a color change from blue to red-pink using alamar blue solution (Resazurin, Sigma-Aldrich®) [29].

Results

Growth on Surface Experiments

Most tested yeasts developed on lipid-enriched media. For non-pulmonary lipids like tweens and GME, yeasts mainly showed similar growth to the standard medium except for *M. pachydermatis* and *M. restricta* that showed scarce growth or negative cultures on these media (Table 3). For pulmonary-lipid-enriched media (DPPC and surfactant), *C. albicans, N. glabrata, C. neoformans,M. guilliermondii* showed scarce growth or did not develop, *Malassezia* species, *C. parapsilosis* and *C. deneoformans* showed better or similar growth than on the standard (Figs. 1 and 2 and Table 3). However, no significant differences were found ($p=0{,}135$) using Friedman's test.

In particular for *Malassezia* species, the area of growth on the pulmonary lipid media and on the MLNA showed that, except for *M. globosa*, for all other species the surfactant gives the largest colonies. In DPPC colonies appeared similar or smaller in size to the standard except for *M. restricta* which is always larger in lung lipid than in standard (Fig. 2, Table 3).

For *M. restricta* its better development with lung lipids was confirmed using NM1 medium in which GME was replaced by DPPC (Fig. 3).

Growth in Depth Experiments

The growth in depth in lung-lipid-enriched media is shown in Fig. 4. All isolates showed some growth around the wells, moderate even for those inoculated with *M. restricta* in the DPPC medium. *M. pachydermatis* showed abundant growth in both media, as did *C. glabrata* and *C. deuterogattii* in the surfactant medium. For non-pulmonary lipids, most of the results with tween compounds were similar to the surface experiments, although in the case of *Malassezia* species it was only reproducible for two of them.

Table 3 Mean values of the diameter growth of yeast inoculated with 5 µL droplets of cells suspension on different media after 48 h incubation (values at different time of 48 h incubation have not been considered for mean calculation)

Species	Standard		Tween compounds				GME	Pulmonary lipids	
	SDA	MLNA	T20	T40	T60	T80		DPPC	SURF
N. glabrata	11		10	12	11	9	10.5	9	9,5
C. albicans	11		11	12	9	9	10	9	9
C. parapsilosis	10		11	12	10	8,5	9	10,5	9,5
M. guilliermondii	9,5		12	12	11,5	10	10	9	10
C. *neoformanss.s*	10		12	11	10	9	7,5	10	10
C. deneoformans	8,5		10	9,5	9	9	10	9	10,5
C. deuterogattii	10		10	12	10	9	11	14	10,5
M. arunalokei		15					0	14	16
M. globosa		17						14	16
M. pachydermatis		12	12	9	12,5	20	25*	13,5	16
M. restricta		12,5					0	16	17

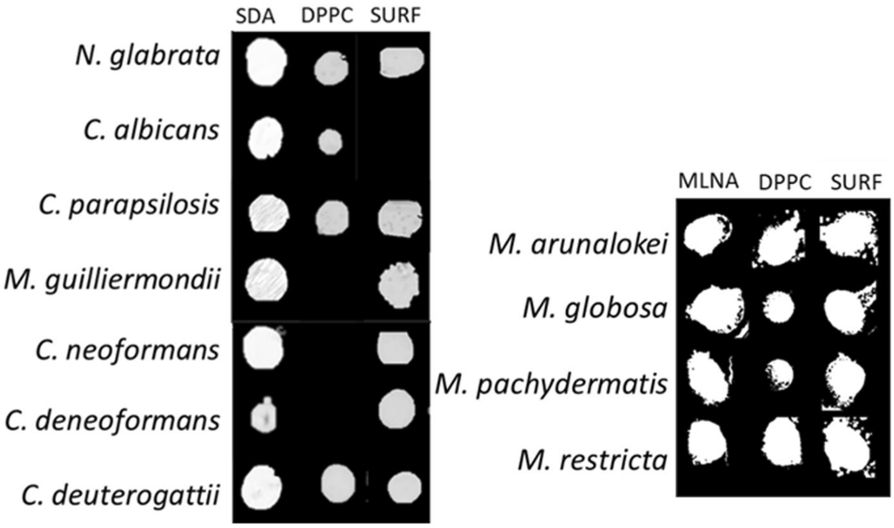

Fig. 1 Growth of all isolates tested on the standard medium (SDA for *non-Malassezia* species and MLNA for *Malassezia* species) and on media containing pulmonary lipids (DPPC and Surfactant—SURF). The background colour of the colonies has been removed and replaced with a black square using the PowerPoint "remove background" tool

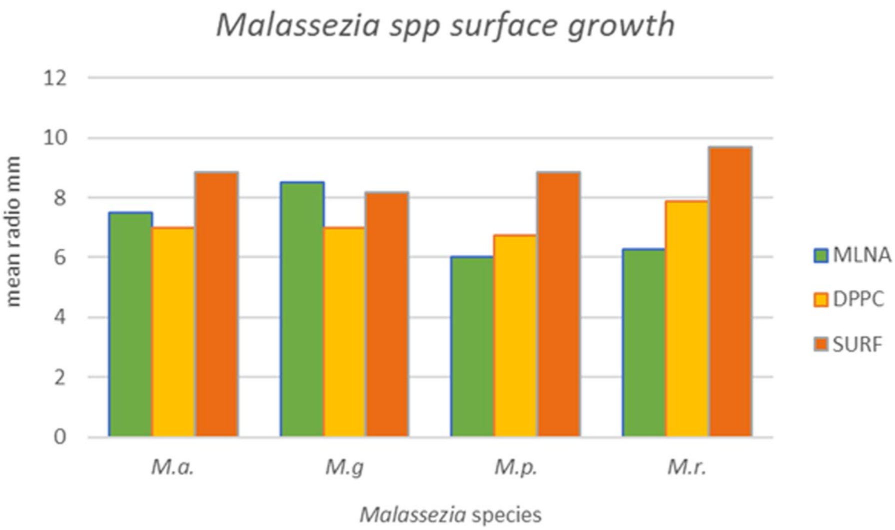

Fig. 2 Representation of the mean values of the growth radius of *Malassezia* spp inoculated in 5µL droplets on the surface of different media with and without lung lipids. MLNA: Modified Leeming Notman Agar. DPPC: Dipalmytoyl phosphatidylcholine; SURF: commercial porcine surfactant (Curosurf®)

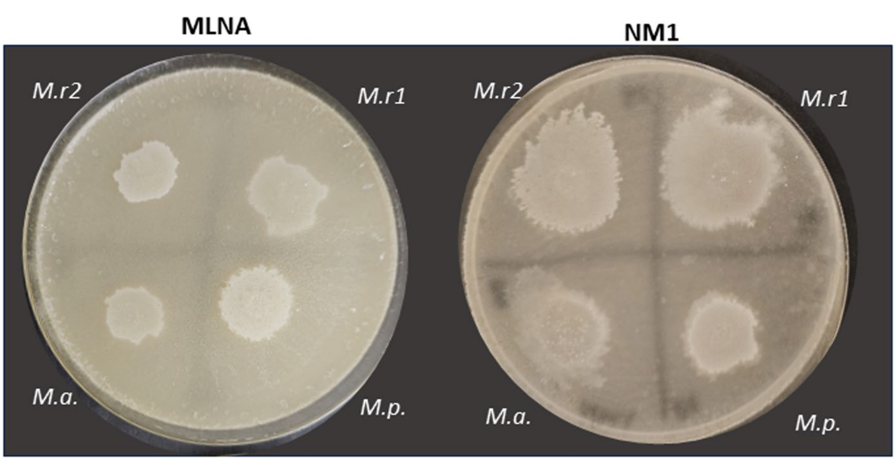

Fig. 3 Growth of *Malassezia* species on MLNA and NM1 (MLNA in which GME has been substituted by DPPC). *M.r.*: *M. restricta* (two replicates *M.r.*1 and *M.r.*2), *M.a.*: M *arunalokei* and *M.p.*: *M. pachydermatis*

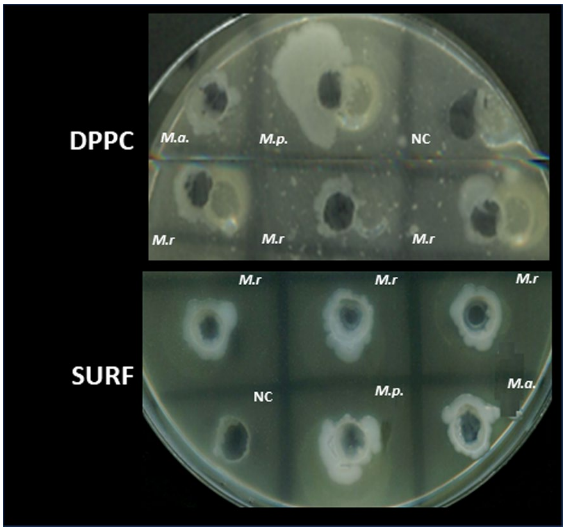

Fig. 4 Growth of *Malassezia* isolates inoculated in wells on media containing pulmonary lipids. Upper plate: medium with DPPC and lower plate: medium with commercial surfactant (SURF). *M.r*: *Malassezia restricta* (triplicated); *M.p.: M. pachydermatis; M.a.: M. arunalokei*; NC: Negative Control

Growth in wells on GME was variable for all strains tested. In general, reading these results was somewhat difficult due to the frequent precipitates appearing around the wells, which were misleading to interpret as growth (Figure S1).

Experiments in Liquid (Microtiter Plates)

These experiments showed relatively good growth of *C. parapsilosis* in all media tested, while *C. albicans* and *M. guilliermondii* had limited growth, especially in pulmonary lipids compared to Sabouraud Dextrose Broth (SDB). *Malassezia* isolates, did not grow in liquid and for *Cryptococcus* species results were inconsistent due to, color changes from resazurin reduction that were sometimes outside the normal scale, making reading inaccurate. Only results consistent across three assays were considered, such as plates with *Candida* and related species (Supplementary material Figure S2).

Global results of all isolates in all media tested are summarized in Fig. 5 and provided in supplementary material (Tables S1 and S2).

Comparing growth on non-pulmonary lipids with pulmonary lipids, some yeasts can be inhibited by surfactant, while others are stimulated. Our findings from surface experiments (the most reproducible) indicate that *C. deneoformans and C. deuterogatii* developed better on surfactant, as did *M. restricta* and *M. pachydermatis*. *Malassezia* species developed irregularly on DPPC and M. globosa showed less development on lung lipids than on standard (Fig. 5).

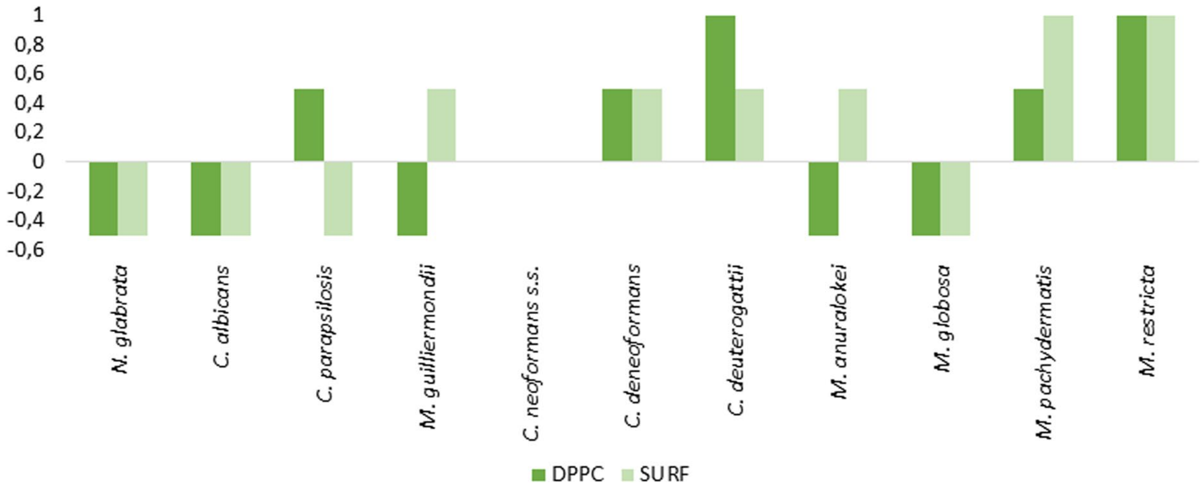

Fig. 5 Results of yeast growth capacity with the tested lipids compared to the standard medium in the three types of experiments carried out. The result is expressed as a semi-quantitative value, with a value of 0 for growth similar to the standard and negative or positive values depending on whether it is better (0.5), much better (1), worse (− 0.5) or no growth (− 1). DPP: Dipalmytoil phosphatidylcholine; SURF: commercial pig surfactant (Curosurf®)

Discussion

This study provides a preliminary understanding of how pulmonary lipids may influence the composition of the LRT mycobiome. The key finding is the demonstrated growth of yeast species, identified as important members of the LRT, on pulmonary lipids such as DPPC and surfactant.

Our experiments showed that some these species, especially *Malassezia restricta*, form larger colonies on media with DPPC and surfactant than on MLNA. This, coupled with their high prevalence in lung mycobiome studies, suggests that pulmonary lipids may be a significant factor in determining the LRT mycobiome composition. The high prevalence of *M. restricta* in the lungs, as shown by mycobiome studies, suggests that the lower respiratory tract may be its ecological niche, similar to how the skin and external auditory canal are considered niches for *Malassezia furfur* and *M. globosa*, and the auditory canal of dogs for *M. pachydermatis*. Other *Malassezia* species tested (*M. arunalokei*, *M. globosa* and *M. pachydermatis*), even though they also showed a good growth rate on pulmonary lipids, have only been occasionally detected in samples of the LRT by molecular methods [4]. Although, no significant differences were found using Friedman's test between, the *Malassezia* group shows a tendency towards greater growth in media with DPPC and Curosurf®, respect to the rest of the strains.

The significant presence of *M. restricta* in the respiratory tract warrants further investigation, as this species has recently been linked to the development of carcinomas in the digestive system, particularly pancreatic cancer [30]. This fungus is also implicated in the development of carcinoma of the uterine cervix and has been proposed to play a role in cutaneous carcinogenesis [31, 32]. Some studies even explore a potential link between the mycobiome and non-small cell lung cancer, although the evidence is currently limited [13, 30, 33–35].

For the other species tested, *Candida parapsilosis* demonstrated equal or better growth on media containing pulmonary lipids compared to SDA, consistent with its description as part of the nuclear mycobiome of BAL samples [4]. Conversely, species such as *Candida albicans,* and *Nakaseomyces glabrata*, important members of the oral mycobiota, showed less ability to grow on pulmonary lipids, with some displaying minimal growth. Notably, these yeasts are not considered permanent members of the pulmonary mycobiome and have only been sporadically detected in the LRT [4–7].

For the genus *Cryptococcus*, the so-called *C. neoformans* s.s. generally exhibited limited growth on lipids compared to SDA. However, *C. deneoformans* and *C. deuterogatii* showed good growth on surfactant and DPPC. Since 2015, seven species of *Cryptococcus* are recognized within the *C. neoformans* and *C. gattii* species complexes: *C. neoformans* sensu stricto*, C. deneoformans,* and *C. gattii* sensu stricto*, C. deuterogattii, C. tetragattii, C. bacillisporus,* and *C. decagattii* [26]. While *C. neoformans* has been reported as a member of the lung mycobiome, it is unclear which specific members of these complexes are being referred to, as mycobiome studies often rely on the ITS region sequence, which does not differentiate between these complex members. The differing behaviors observed in our experiments warrant further study to explore potential correlations between lipo-tolerance or lipo-affinity and yeast pathogenicity. Although species within these complexes are known to cause invasive pulmonary infections in humans, members of the *C. gattii* complex are more frequently associated with lung invasions, while the *C. neoformans* complex is more commonly linked to central nervous system infections. Differences in the pathogenesis of cryptococcosis between these complexes could relate to their varying tolerance or affinity for different lipidic compounds. In this context, recent findings by Rossi et al. using an in vitro 2D organoid model of minilung are noteworthy, as they demonstrate that *C. neoformans s.s.* induces pneumocytes to secrete surfactant, using it as an opsonization method to facilitate cell entry [36]. This behavior is likely linked to lipo-tolerance rather than using surfactant as a nutrient, and further study is needed, particularly among the pathogenic *Cryptococcus* complexes.

The methods employed in this study were standardized to favor the growth of *Malassezia restricta*, potentially limiting the optimal growth conditions for other species. Consequently, the growth results for some species may not reflect their best development under the media used. Surface growth generally provided the most stable and reproducible results, while growth in liquid media posed challenges, likely due to lipids acting as a barrier and limiting oxygen availability. Culturing *Malassezia restricta* remains challenging, with long incubation periods required for growth in specific lipidic media, and success not always guaranteed [37].

Most yeasts studied showed lipo-tolerance or even lipophilicity, particularly for pulmonary lipids. However, aside from the genus *Malassezia*, none are currently classified as lipophilic. To our knowledge, there are no major database studies on lipophilicity for human lipids in these yeasts. This new finding could significantly impact future microbiome studies and have implications for understanding the pathogenic potential of these yeasts in respiratory processes and how they might use the lung as a gateway for distant infections.

Conclusions

This study indicates that specific pulmonary surfactant lipids support the optimal development of *Malassezia restricta*, the most prevalent yeast in the lower airways according to previous mycobiome studies. This species may have a natural niche in the LRT of humans. Pulmonary lipids may be a major determinant of the LRT mycobiome, as many species considered part of the nuclear mycobiome in this site improve their growth when surfactant or DPPC is available, even though they are not traditionally considered lipophilic yeasts.

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Declarations

Conflict of interest None.

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