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Identification and characterization of lipid droplets in Malassezia furfur

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Objective: *Malassezia furfur* is a lipid-dependent yeast that is part of the human skin microbiota. Mechanisms underlying growth on skin including its lipid metabolism are largely unknown. Lipid droplets (LDs), also known as lipid particles, lipid bodies, oil bodies, or oleosomes, are highly dynamic organelles that can be produced in nearly all cells. The presence of LDs in *Malassezia* (*Pityrosporum orbiculare)* have been reported, but their role has not been studied. LDs contribute to a variety of processes including lipid storage, cell signaling, temporary protein storage, cell lipid homeostasis, prevention of lipotoxicity, biosynthesis and secretion of inflammatory mediators such as prostaglandins and leukotriene's, interferon responses, and antigen cross presentation. Lipid droplets are expected to play an important role in *Malassezia* spp during their growth and/or adaptation on the skin yet this has not been investigated. The purpose of this study is to characterize LD of *M. furfur* in complex medium (mDixon) and minimal medium supplemented with sources of palmitic acid (PA; C16:0) and/or oleic acid (OA; C18:1). We addressed the presence of LD during starvation for fatty acids, the composition of the lipid droplets using lipidomics and we used genomic analysis to identify the pathways involved in neutral lipid synthesis in this yeast.

Methods: Lipid droplets (LDs) were identified in *M. furfur* growing in mDixon broth or MM with or without PA and/or OA through confocal microscopy using nile red staining. LD dynamics and cell viability was monitored upon transferring *M. furfur* cells that had been pre-grown in MM with PA and / or OA to MM without fatty acids. Lipidomic analyses were performed on chloroform/methanol extracted lipids from LD isolated via density gradient centrifugation.

Results: CFUs strongly decreased upon lipid starvation irrespective of the type(s) of fatty acid present in the pre-culture and no CFUs were found after 160 h. Interestingly, cells observed in the 3 cultures after 160 h still contained LDs with distribution and morphology indistinguishable form those at the 72 h time point. Yet, they were less abundant as compared to those observed at t = 0 h. Lipidomic analyses revealed the presence of phospholipids as well as triglycerides (TGs) in LDs of *M. furfur* but sterol esters (SEs) were not detected. TG species 52:4, 54:3, 54:4, and 54:5 were enriched in LDs after growth in the presence of Tween 80 or oleic acid (both donors of C18:1), while TG species 50:1 and 52:2 were enriched after growth in the presence of Tween 40 (donor of C16:0). All enzymes involved in TG and SE synthesis were detected in the genome of *M. furfur*.

Conclusion: Remarkably, LD remain present in *furfur* cells even when starved for 160 min in MM lacking fatty acids.

Lipidomic analyses of LD of *Malassezia furfur* indicated the presence of triglycerides but absence of SEs.

Genomic analyses showed the presence of genes encoding proteins for esterification of ergosterol, indicating that regulation prevents SE storage and confirmed the presence of all genes required for the formation of the main precursor for synthesis of triglycerides.

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Evaluation of pathogenicity of Fusarium spp. isolates from humans, animals and plants in Galleria mellonella as experimental host.

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Objective: *Fusarium* species are widely recognized as phytopathogens devastating crops worldwide. However, they are not limited to the agricultural sector, they are emerging pathogens in immunocompetent and immunocompromised patients, and also produce superficial lesions and mycotoxicosis in animals. Taking into account their potential to cause trans-kingdom infections, it is crucial to develop cross-pathogenicity tests in different host models, particularly animal hosts. Therefore, the aim of our work was to evaluate the pathogenicity of *Fusarium* spp. isolates from humans, animals and plants in *Galleria mellonella*, as an experimental host which circumvents the constraints of murine host models for pathogenesis research.

Methods: The first stage of the project focused on the determination of conidial inoculum volume and concentration to inject in last instar *G. mellonella* larvae. Subsequently, we performed the pathogenicity tests assessing the larvae response to twelve *Fusarium* spp. isolates from plants, animal superficial mycoses, and human superficial and systemic mycoses. The response variables evaluated during a 15-day time course after inoculation were melanization, survival and emergence of fungal mycelium.

Results: The injection of 5 μL of a 1×10^6 conidia/mL suspension was determined as the experimental inoculum for pathogenicity tests in *G. mellonella* larvae. The pathogenicity tests demonstrated the infective capacity of *Fusarium* spp. in *G. mellonella* larvae as experimental host. Melanization of the larvae was observed parallelly to their death, indicating that *Fusarium* spp. isolates are able to overcome *G. mellonella* immunological barriers. Most of the larvae cadavers showed the emergence of fungal mycelium, which confirms that larvae death was actually caused by *Fusarium* spp. isolates inoculation comparing to negative control. This result also reveals that the whole pathogenicity cycle is fulfilled.

Conclusion: Our results corroborate the utility of *G. mellonella* as an invertebrate host to evaluate *Fusarium* spp. pathogenicity, as well they contribute to the understanding of *Fusarium* spp. potential as trans-kingdom pathogens, since all evaluated isolates were virulent to *G. mellonella* larvae regardless their origin or species.

Picture 1: https://www.eventure-online.com/parthen-uploads/89/8ISH/add_1_424872_332f0c24-850c-401c-bfca-e3b63790af67.png

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Comparison of a Histoplasma/Blastomyces Lateral Flow Assay to Enzyme Immunoassay Using Dog and Cat Sera

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Objective: Quantitative enzyme immunoassays (EIAs) for *Histoplasma* and *Blastomyces* antigen are highly sensitive for the detection of fungal antigen to aid in the diagnosis of histoplasmosis and blastomycosis in dogs and cats. These assays show nearly complete cross reactivity; therefore, both infections may be detected by a single antigen test. A serum lateral flow assay (LFA) was developed for human histoplasmosis, and this might be used in dogs and cats. Use of the LFA as a point-of-care assay in veterinary clinics would allow for more rapid diagnosis and earlier treatment of histoplasmosis and blastomycosis. The objective of this study was to compare agreement between the *Histoplasma* antigen LFA and the quantitative EIAs for *Histoplasma* and *Blastomyces*, using canine and feline sera.

Methods: Serum samples previously tested in either EIA were stored frozen until batch evaluation in the LFA. Serum was treated with EDTA and extracted supernatant was loaded onto the LFA device. LFA devices were interpreted visually by three qualified human evaluators, as well as two different automated readers. A consensus RANN score (ranging from 0–10 based on intensity of the test line compared to scorecard standards) was obtained by using two evaluator agreement. The samples were randomized and evaluators were blinded to EIA results. All samples were analyzed in duplicate. A total of 11 EIA-negative serum samples (22 LFA devices) and 21 EIA-positive serum samples (42 devices) were evaluated, including 12 feline samples and 20 canine samples. The EIA-positive samples ranged from 0.2 ng/mL to 19 ng/mL.

Results: Of the EIA-negative group, 19/22 devices had a consensus RANN score of 0. The three devices with positive RANN scores included two devices that were also positive with both automated readers, and one device positive with only one automated reader. Of the EIA-positive group, 41/42 devices had a positive consensus RANN score; however, only 40/42 and 38/42 devices had positive results by automated readers 1 and 2. Kappa agreement score compared to EIA was 0.858 (visual assessment), 0.861 (automated reader 1) and 0.760 (automated reader 2).

Conclusion: The LFA showed overall good qualitative agreement with the EIA tests for canine and feline specimens. The occasional discordant results did not appear to be caused by evaluator error, as they were also detected by the automated readers. Causes of discordant results will need further investigation. Unexpectedly, visual assessment of the positive samples outperformed the automated readers and could detect as low as 0.2 ng/mL. Further studies will assess quantitative agreement between RANN score and antigen concentration. The LFA will potentially be useful for in-clinic screening for histoplasmosis and/or blastomycosis.

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Identification, characterization and gene expression of novel adhesins in trichosporon Asahii

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Objective: *Trichosporon asahii* is an emergent opportunistic yeast pathogen, responsible for invasive infections with mortality rates up to 80%, and it is known as a high biofilm producer. Thus, this work aimed to identify and characterize putative genes in *T. asahii* genomes encoding adhesins that may participate in biofilm formation and adhesion on host cells.

Methods: We searched *T. asahii* genomes deposited in the NCBI database for hypothetical adhesin genes using as query 23 adhesin sequences from *Candida albicans* and *Cryptococcus neoformans* by tBLASTn-NCBI tool. In addition, we used *FungalRV* and *Faapred* adhesin prediction programs to analyze the complete proteome of the *T. asahii* CBS2479. Predictions of the presence of signal peptide sites, secretion by classical secretory pathway, transmembrane helices, glycosylations, mannosylations, and acetylations were performed in the Center Biological Sequence Analysis (CBS prediction) server. Adhesin conserved domains were searched by BLASTp-NCBI tool. Gene expression analysis by RT-PCR were performed using RNA from planktonic cells of two low biofilm producers: isolates CBS2479 (from superficial mycosis) and L773 (blood culture), and one high biofilm producer, L2585 (blood culture). Actin gene was used as internal control.

Results: Comparisons by tBLASTn analysis revealed only one protein with 30% identity and 85% similarity to the recently described adhesin CFL1p of *C. neoformans*. Through *FungalRV* analysis, 22 proteins with putative adhesin function were found, lacking CFL1p-like. Of the 23 proteins predicted as adhesins, 17 were confirmed by *Faapred* analysis. After applying inclusion criteria such as: being present in at least two adhesin prediction analyzes, protein size ($\geq$300 amino acids), presence of signal peptide, secretion by classical secretory pathway and absence of transmembrane helices, four putative adhesin sequences were finally selected. The first *T. asahii* sequence was named CFL1p-like and contains 325 amino acids, predictions of *O*-glycosylation in four serine/threonine residues (ser/thr) and *C*-mannosylation in 14 tryptophan residues, without known adhesion conserved domains. The second, BETAp-like contains 1383 amino acids with 463 putative *O*-glycosylation and 21 *N*-glycosylation sites, and the conserved Herpes_*BLLF1* adhesion domain. The third protein was RESTIN-like which contains 507 amino acids, had predictions for 101 ser/thr *O*-glycosylation sites, one *C*-mannosylation residue and two conserved domains: Von Willebrand factor type A (vWA) superfamily and Mucin-like superfamily. The last protein was named MAR-*like* and contains 406 amino acids, predictions of 65 *O*-glycosylation and 12 *N*-glycosylation residues, and the Herpes_*BLLF1* and Endomucin superfamily conserved domains. The RT-PCR analysis showed that *BETA-like*, *RESTIN-like* and *MAR-like* genes were significantly highly expressed in the CBS2479 strain from superficial mycosis ($p \leq 0.05$). Interestingly, *CFL1*-like was more expressed in the blood culture isolate L773 ($p \leq 0.05$), which exhibited arthroconidial morphology, as oppose to the other isolates that grew only as long hyphae.

Conclusion: We were able to demonstrate that all four genes were expressed in planktonic cells and the isolation site and morphology may influence their differential expression. This work also contributed to the genomic annotation of the four sequences which are no longer "hypothetical" by identifying and characterizing genes that may encode novel adhesins of the Basidiomycota phylum using as model the emerging pathogen *T. asahii*.

Picture 1: https://www.eventure-online.com/parthen-uploads/89/8ISH/add_1_425247_d8f6ae43-5a6e-4c5c-b99f-99be56e1d446.jpg

Caption 1: Expression levels of four adhesin genes from T.asahii isolates obtained from superficial mycosis (CBS2479) and blood cultures (L773 and L2585).* $P < 0.05$

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Histopathologic score for granulomatous injuries experimentally induced by Fusarium spp.

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Objective: The study of pathogenicity mechanisms of emerging pathogens, which cause systemic mycoses, involves a rigorous analysis of infected tissues. The qualitative and semiquantitative data, obtained from histological evaluation, can be categorized to generate a scoring system that allows to characterize and compare the host response to different pathogen isolates; therefore, leading to deepen understanding of virulence mechanisms of emerging pathogens, such as *Fusarium* spp. Considering the potential of Fusaria to cause trans-kingdom infections, we aimed to propose a histopathologic score for tissues injured by *Fusarium* spp., in experimentally induced infections in immunosupressed BALB/c mice.

Methods: Female and male BALB/c mice weighing 20 – 25 g were immunosuppressed by a single intraperitoneal injection of cyclophosphamide (200 mg/kg dose) 48 h pre-inoculation. Afterwards, mice were injected into the tail lateral vein with 0.1 ml of fungal inoculum (1×10^8 conidia/ml). *Fusarium* spp. isolates came from human superficial and systemic mycoses, animal superficial mycoses and necrotic vascular tissues of plants. Mice were housed in micro-isolation cages with HEPA filtration, under controlled environmental conditions for 7 days. Liver, spleen and lungs from died and euthanized mice were sampled for microbiological and histopathological (Hematoxylin-Eosin and Grocott stains) analysis.

Results: Histopathological analyzes of liver, spleen and lungs led to classifying tissue injuries into four categories with different scores. a) Degree of injury, inflammatory granulomatous lesion: score 1 (mild), 2 (moderate), 3 (severe). b) Distribution of injury: score 1 (single injury defined as focal tissue lesion), 2 (multiple injury defined as multifocal tissue lesion). c) Tissue necrosis associated with granulomatous injury: score 0 (absence), 1 (mild), 2 (moderate), 3 (severe). d) Detection of fungal structures by Grocott stain: score 0 (negative), 1 (positive). The sum of each category scores for each organ tissue yielded a final score that was categorized into three grades. Grade 1 (mild) - total score: 1 - 4. Grade 2 (moderate) - total score: 5 - 6. Grade 3 (severe) - total score: 7 - 9.

The evaluation showed that all *Fusarium* spp. isolates disseminated and colonized liver, spleen and lung tissues, despite their origin or species. The most injured organ was the liver (grade 3), other organs were scored at grades 2 and 3. *Fusarium* spp. capacity for dissemination and colonization was confirmed microbiologically by fungus re-isolation of the pathogen in at least one of the organs analyzed.

Conclusion: Dissemination, colonization and tissue injury produced by *Fusarium* spp. isolates, from humans, animals and plants, in the different organs evaluated is key to study the virulence of *Fusarium* spp. as a model for trans-kingdom pathogenesis. The histopathological score established in our study could be used to evaluate the virulence of other emerging pathogens which cause invasive mycosis.

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Infection of chick chorioallantoic membrane (CAM) As a model for the pathogenesis of Cryptococcus Gatti

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Objective: The use of embryonated egg as an alternative in the study of the pathogenesis of fungi is evolving, although murine models are "gold standard" however, these models are used to screen determinants of virulence among fungi species. This study was aimed at the evaluating the virulence potential of Cryptococcus gattii strains R265, R272, EJB18 and *Malassezia sympodialis* via Chorio-allantioc membrane (CAM) in embryonated egg.

Methods: Three strains of *C.gattii* (R265, R272, EJB18) with well characterized murine model were used and a strain of *Malassezia sympodialis*, the study was conducted in duplicates. Twenty(20) embryonated eggs were used for each isolate. After inoculation of test isolates, the eggs were incubated for 8days. Embryo viability was determined and survival recorded. Fungal burden was also determined. Inoculation of isolates were made into the Chorio-allantoic membrane following standard procedures.

Results: At a concentration of 10^7 cfu/ml, C .gattii R272 was more virulent than R265 in the egg model while EJB18 had a low virulence *M. sympodialis* however, showed indisguishable mortality when compared with the control group. The CAM model supported the growth of Malassezia sympodialis strain and induced the formation of hyphae. The formation of lesions by the organism and re-isolation of organism from CAM suggests that the model can be used for evaluating the virulence of C.gattii. Histopathology of CAM from both strains revealed massive disruption of CAM

Conclusion: This study suggests that embryonated eggs is a useful alternative tool to pre-screen *Cryptococcus gattii* strains to select strains for subsequent testing in murine models and could be a potential medium for studying the hyphal growth in *Malassezia* species.