Caenorhabditis elegans and *Tenebrio molitor -* New tools to investigate *Malassezia* species

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Abstract

Malassezia species are part of human commensal microbiota and is also related to diseases. Little is known about the interaction of these microorganisms with their host. Here we established two standard culture conditions for *Malassezia* sp. to perform infection assays using *C. elegans* and *T. molitor*. Invertebrate hosts infected by *Malassezia* sp. cultured in M9M resulted in higher death rate on survival assays when compared to yeasts cultured in the standard Dixon medium, indicating that M9M cultured *Malassezia* species have increased virulence. The culture and infection conditions established in this work, using invertebrate models, are valuable tools to understand Malassezia-host interaction.

Introduction

Malassezia genus comprises the more abundant lipophilic and/or lipodependent yeasts in the human resident microbiota, establishing a commensal relationship with their hosts [1; 2; 3]. However, when immune system homeostasis is impaired Malassezia can act as pathogens. In this context, some Malassezia cellular characteristics takes place at pathological processes acting as virulence factors: cell wall structure, lipases, morphological transition and indole compounds [3; 4]. Despite many years of Malassezia genus studies, the knowledge about species interaction with human host is not completely understood [3; 5; 6; 7].

Invertebrate host models to study microorganism-host interactions is becoming very popular to understand many aspects of microbial virulence [8; 9]. The nematode *Caenorhabditis elegans*, an invertebrate model used since the 1960s in several areas of scientific research, and more recently the coleopteran *Tenebrio molitor*, represent excellent tools to study fungal diseases [8; 10; 11; 12; 13; 14; 15; 16].

We evaluated these alternative hosts as models to study infection and diseases associated with Malassezia species using two distinct and standardized culture conditions and we showed that nutrients starved *Malassezia* sp. significantly reduces *C. elegans* end *T. molitor* survival, and thus promotes increased virulence of these pathogenic yeasts.

Materials and Methods

Malassezia sp. culture

Malassezia species used in this study are clinical samples from the yeast library of the Biotechnology and Ecology of Yeasts Laboratory (Biological Sciences Institute - Federal University of Minas Gerais). Three clinical isolates, previously identified by PCR and sequencing (unpublished data), were selected: *M. furfur* (MIE), *M. sympodialis* (A88) and *M. yamatoensis* (MZ2).

Cultivation in standard Dixon medium

M. furfur, *M. sympodialis* e *M. yamatoensis* were grown and maintained in Dixon medium [6% (w/v) malt extract, 2% (w/v) ox-bile, 1% (v/v) Tween40, 0.25% (v/v) glycerol mono oleate, supplemented or not with 2% (w/v) agar] [17] for 3 days at 30°C (in a rotatory shaker for liquid cultures), except when adapted to alternative culture in M9M, as described below.

Cultivation and adaptation to alternative medium M9M

The M9 buffer, commonly used for *C. elegans* manipulation [18], supplemented or not with agar [2% (w/v)], was established as an alternative culture medium for *Malassezia* species and named in this work as M9M (M9 Medium). When necessary selected species where grown in liquid Dixon medium and inoculated in M9M for at least 3 days at 30°C (in a rotatory shaker for liquid cultures).

Malassezia species were adapted to M9M through at least three successive passages before infection assays.

Caenorhabditis elegans culture and synchronization

C. elegans N2 Bristol (wild-type strain kindly provided by CGC) were maintained in Petri dishes containing NGM media and a layer of *Escherichia coli* OP50 [18]. Plates were maintained at 16°C until the experimental procedures were performed. In order to obtain worms at the same larval stage *C. elegans* where subjected to synchronization as described by Porta de la Riva et al[19].

Caenorhabditis elegans survival assays

For the survival tests, one isolated colony of *Malassezia* sp., picked from Dixon or M9M agar, was inoculated into a sterile Falcon tube containing 8 mL of the selected culture medium (Dixon or M9M) and maintained at 30°C for 3 days. On the experiment day these cultures were centrifuged (4000 rpm during 10 minutes in conical tubes), washed with M9 buffer and cell density was measured (OD_{660}). Cell suspensions were diluted in order to obtain a final concentration of 1.25×10^6 UFC/mL [20] and 10 µL were placed at the top M9M agar supplement with streptomycin (50 µg/mL - Sigma) and 5-fluoro-2'-deoxyuridine (FUDR - 80 µg/mL- Sigma) in a 24-well plate. To survival determination, 10-15 young adults (L4) *C. elegans* were added to the pathogenic yeast lawn and incubated at 25°C. Worms viability was examined at 24h intervals and those not responding to mechanical stimulus with a platinum wire were considered dead [15].

Tenebrio molitor survival assays

T. molitor beetle larvae were commercially obtained and maintained at our laboratory. To perform survival analyses, larvae presenting 0.1 - 0.2g without signs of contamination, spots and exoskeleton changes, were selected. Each larva, previously cleaned with 70% ethanol, were infected directly into the hemocele using a Hamilton syringe (701N, caliber 26, 10µL capacity). *T. molitor* was injected with 5µL of yeasts suspension (2.65×10^7 cells), cultured in Dixon or M9M. Experimental group were composed of 10 larvae each, belonging to three experimental groups: 1- Dixon-cultured Malassezia, 2- M9M-cultured Malassezia and 3- negative control. The control group was inoculated with 5 µl of Phosphate Buffered Saline (PBS). Infected larvae and negative control were incubated in Petri dishes at 37ºC and viability was examined at 24h intervals for ten days, being considered dead those larvae not responding to mechanical stimulus.

Statistical analyzes

C. elegans and *T. molitor* survival curves were plotted, and estimation of differences (log-rank and Wilcoxon tests) in survival analyzed with the Kaplan-Meier method performed using GraphPad Prism software 5.01 (GraphPad Software). A p-value of 0.05 was considered significant.

Results

Malassezia sp. cultured in M9M maintain viability and undergoes filamentous growth

In this work we cultured and adapted Malassezia species in M9M, as previously described, to prevent an observed overgrowth of these yeasts in the standard *C elegans* culture medium NGM. Also, we tried to perform *C. elegans* assays using the Malassezia standard medium Dixon, that showed to be toxic to nematodes. When *Malassezia* sp. were subjected to successive passages through liquid M9M cells remained viable, as determined by MTT assays (data not shown) and plating in Dixon agar, but presented reduced growth when compared to those cultivated in Dixon (data showed for *M. furfur* - Figure 1). In addition, we observed that yeasts are subjected to morphological changes in M9M, presenting filamentous growth (Figure 2).
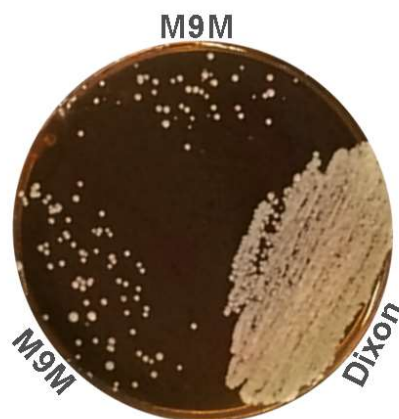

Figure 1 - *Malassezia furfur* growth in Dixon agar after adaptation to M9M. M9M adapted yeasts are viable but present reduced growth after M9M passages (representative image for all tested yeasts).
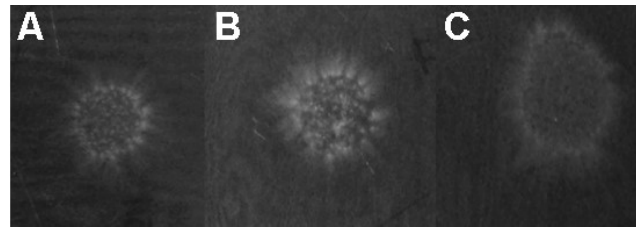
Figure 2 – *Malassezia* sp. filamentous growth in M9M. Growth aspect of *M. furfur* (A), *M. yamatoensis* (B) and *M. sympodialis* (C) after three passages in liquid M9M and spotted in M9M agar.

Malassezia *species* adapted to M9M reduce *C. elegans* survival.

Survival assays using *C. elegans* showed significant differences when *Malassezia* spp. were cultivated in M9M. Nematodes infected with yeasts grown in Dixon medium has LT50 (time required to kill 50% of the nematodes) of 6 days for *M. furfur* (figure 3A), *M. yamatoensis* (Figure 3B) and *M. sympodialis* (Figure 3C). Differently, when the same yeasts species were previously cultured and adapted to M9M medium we noticed a significant reduction of LT50, from 6 to 4 days in worms infected with *M. yamatoensis* (Figure 3B, p=0,0092), *M. sympodialis,* (Figure 3C, p=0,004) and LT50 from 6 to 2 days in worms infected with *M. furfur* (Figure 3A, p=0,0001).
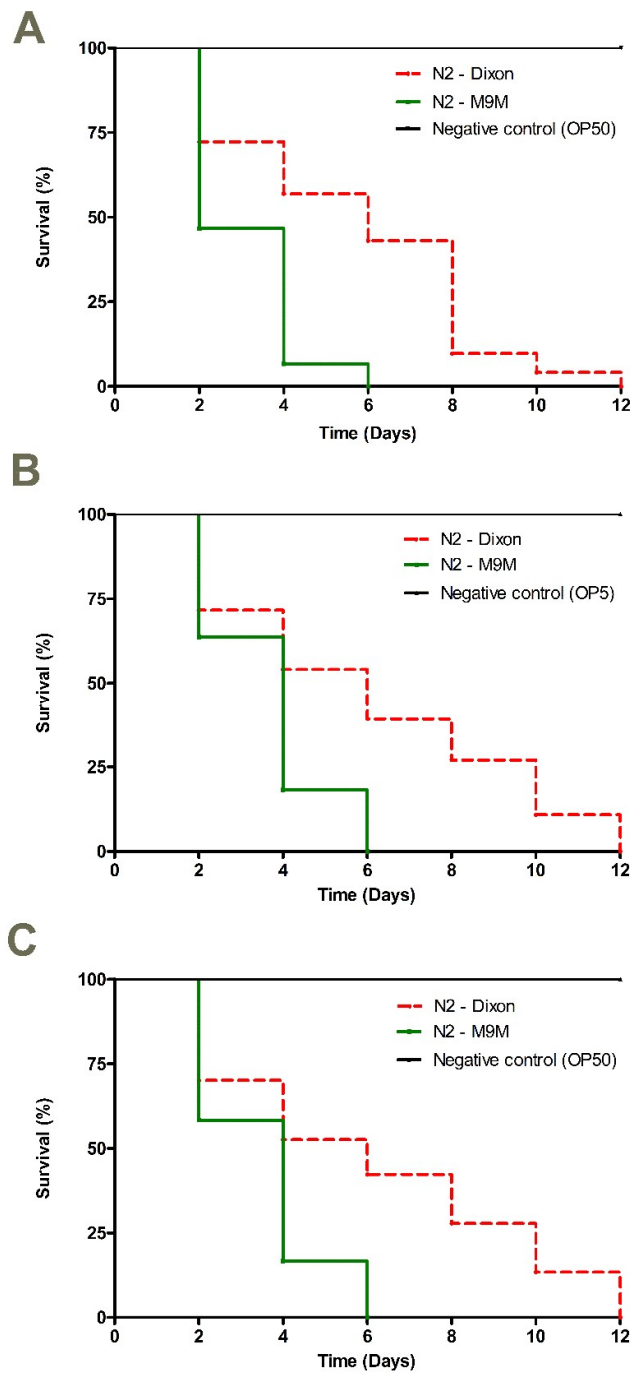
Figure 3 - *Malassezia* sp. adapted to M9M reduces *C. elegans* survival. *C. elegans* infected with *Malassezia* sp. grown in M9M presented significant reduction of LT50 and survival when compared to worms infected with yeasts

maintained in Dixon. *M. furfur* (A, p=0,0001), *M. yamatoensis* (B, p=0,0092), *M. sympodialis* (C, p=0,004).

Malassezia furfur adapted to M9M reduces *Tenebrio molitor* larvae survival

T. molitor can be a useful model to study fungal infections and we selected *M. furfur* for the survival assays since it was shown to be more virulent to *C. elegans* in both tested culture conditions (Figure 3A). Using this inverterbrate, we showed that *M. furfur* adapted to M9M is more virulent to *T. molitor* larvae than yeasts grown in Dixon. After 24h, 100% of larvae infected with *M. furfur* from M9M died, whereas only approximately 25% of larvae infected with yeasts grown in Dixon medium died at the same period, maintaining this number until the end of the experiment (Figure. 4).
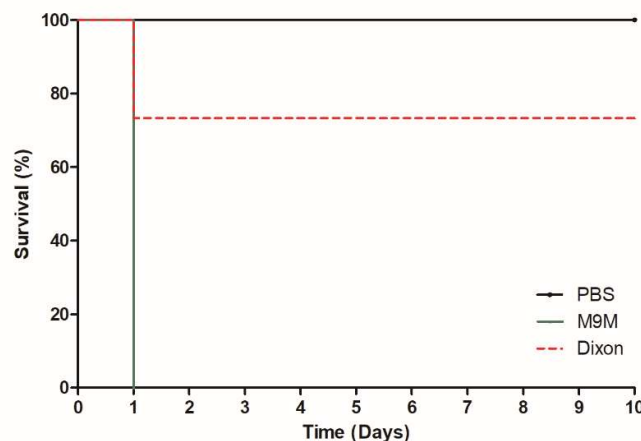

Figure 4 - *Malassezia furfur* adapted to M9M decreases *T. molitor* larvae survival. Survival curves of *T. molitor* larvae (n=10) infected with *Malassezia*

furfur grown in Dixon medium and liquid M9M. Control group (PBS - black line). p<0,0001.

Discussion

Malassezia species are described in literature as lipophilic or lipid-dependent yeasts according to its colonization site and availability of lipidic components [5; 21]. At the laboratory these yeasts only grow in media containing external lipid sources. In this work we showed that *M. furfur*, *M. sympodialis*, *M. yamatoensis* can grow in M9 (named here as M9M), a *C. elegans* physiological buffer, as well as are viable in this culture condition. External lipid source is absent in M9M and the observed growth of *Malassezia sp.* can be explained by stable storage of lipdis. *Malassezia* spp. can store lipids in cytoplasmic droplets as occur with *Saccharomyces cerevisae*, and these sources could be used when yeasts are growing in M9M as other conditions when lipids are scarce [22].

Microbial cells have extremely adaptable metabolism in order to survive to diverse conditions, for example, when they are in a hostile environment as in the case of infection of host tissues, and even during commensalism. Nutritional stress is a very common example of metabolism dynamics, in which microbial cells became more vulnerable and hence can adapt, sometimes influencing its morphology and virulence [23, 24, 25]. Stress inducing morphogenesis and virulence changes can be clearly observed when microorganisms are submitted to culture conditions that limit nutrients and were demonstrated by several works. Weerasekera et al. evaluated how culture conditions influence growth, adhesion and biofilm formation in *C. albicans* and *C. tropicalis* [24]. They found that maximal growth of *C. albicans* and *C. tropicalis* was favored in Sabouraud

Dextrose medium, but *C. tropicalis* presented optimal growth in the Yeast Nitrogen Base medium. In paralell, they observed a facilitated adherence and biofilm formation in RPMI 1640 medium in both species. Differently, O'Connor and cols. showed that *C. dubliniensis* cultivation in nutrient limited conditions can promote yeast transition to filamentous growth [25]. Rodríguez-Gomes et al. [26] observed *Beauveria bassiana* virulence changes using different culture conditions and found that Sabouraud-dextrose agar increased virulence for *T. molitor* when compared to fungal cells that were grown in other culture media. As occur with other yeasts, here we show that *Malassezia* sp. have optimal growth in Dixon medium, but when adapted and cultured in M9M can form hyphae (Figure 2), and thus can be potentially more invasive and virulent. Corroborating our growth data, Malassezia species adapted to M9M (liquid or agar) showed a significant increase in virulence to invertebrate hosts, evidenced by the rapid death of *C. elegans* and T. *molitor* when compared to Dixon cultured cells (Figures 3 and 4).

There are few studies about the mechanisms involved in altering *Malassezia* spp. commensal state to pathogenic life [3; 27]. Existing studies are mostly restricted to in vitro tests using cell cultures or in vivo using murine models [3]. Brilhante et al. evaluated the virulence of *M. pachydermatis* using *C. elegans* as invertebrate model and describe it as a valuable model to investigate this yeast [28]. In this work we could define a standard culture condition that increase *Malassezia* sp. virulence by simply removing nutrients and lipid exogenous sources. In addition, we established *C. elagans* and *T. molitor* as new models to study this pathogenic genus thus contributing to future works to investigate malasseziosis pathogenesis and treatment, reinforcing the excellence of

invertebrate models to expand the knowledge in pathogen-host interaction research field [8; 10; 11; 12; 13; 14].

Conclusion

Here we established two alternative invertebrate model hosts to investigate *Malassezia* sp. infections, adding one more tool to understand its virulence and pathogenesis. Moreover, this is the first work demonstrating *Malassezia* sp. culture in the absence of external lipid sources that potentiates its virulence to *C. elegans* and *T. molitor*.

Author contributions

A.P.R.S., A.D.V., S.J. and V.S.A. conceived and designed the experiments; A.P.R.S., A.D.V., R.O.V., A.C.S.R. performed the experiments; A.P.R.S., A.D.V. and V.S.A analyzed the data; A.D.V. and V.S.A. wrote the paper. All the authors revised and approved the manuscript.

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Interest conflicts

No conflicts of interest.

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