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TITLE:

Infecting Mice with *Malassezia* spp. to Study the Fungus-Host Interaction

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SUMMARY:

This protocol outlines the establishment of a mouse model for studying *Malassezia*-host interactions in the skin. It describes the cultivation of *Malassezia* in vitro, the infection of the murine skin with *Malassezia*, and the subsequent analysis of the inflammation and the fungal burden in the skin tissue.

ABSTRACT:

Animal models are crucial for infectious disease research. They provide an important basis for analyzing the full spectrum of interactions that occur between microbes and their host in vivo in a tissue-specific manner. Pathogenic fungi are increasingly recognized as a serious threat for humans and exploiting such infection models have greatly improved our understanding of fungal pathogenicity. Species of the genus *Malassezia* are the most abundant fungi of the human skin microbiota and they are also associated with the development of severe inflammatory skin disorders such as seborrheic dermatitis and atopic dermatitis. However, a causative link between *Malassezia* and disease pathogenesis remains unknown, a fact that can be attributed to the poor knowledge of the complex crosstalk of *Malassezia* with the skin immune system. This protocol describes the establishment of an experimental mouse model that allows studying the interaction of *Malassezia* with the mammalian skin in vivo. It outlines the method for cultivating *Malassezia* spp. under laboratory conditions, how to infect the murine skin with *Malassezia* spp. and how to assess the outcome of infection by means of the skin inflammation and fungal burden analyses. The model described here works in fully immunocompetent animals and does not rely on immune suppressive or antibiotic pretreatment of the animals. It is furthermore adaptable to virtually all genetically modified mouse strains and can be combined with other skin disease models. These features make this infection model a very powerful tool for studying in detail the innate and adaptive immune response of the host against *Malassezia* in the skin in vivo.

INTRODUCTION:

The skin is populated by many different microbes. The constant exposure of the skin to the microbiota contributes to shaping and educating the immune system of the host. Fungi are increasingly recognized as a vital part of the microbiota and they fulfill an important role for the host physiology and immunity, similar to bacteria and viruses[1]. Species of the genus *Malassezia* are by far the most abundant fungi colonizing the skin of warm-blooded vertebrates and they make up for more than 90% of the human skin mycobiome[2,3]. Eighteen different species of *Malassezia* have so far been identified from the human and animal skin[4].

Various pathologies of the skin are thought to arise, at least partially, as a result of a dysbalanced microbiota composition. Dysbiosis may lead to the overgrowth of species with pathogenic potential resulting in opportunistic infections and disease[5]. Consistently, there is an increasing evidence that *Malassezia*, besides its commensal lifestyle, contributes to the development of various skin pathologies, ranging from dandruff and pityriarsis versicolor to more severe inflammatory disorders such as seborrheic dermatitis and atopic dermatitis[4,6]. While a causative link between *Malassezia* and pityriarsis versicolor has been established, the pathophysiological role of the fungus in more severe skin pathologies remains largely unknown.

Determining the role of *Malassezia* in skin homeostasis and disease calls for more in-depth knowledge about the interaction of the fungus with the skin and the cutaneous immune system. Of note, research on *Malassezia* is, compared to other human fungal pathogens (e.g., *Candida albicans* or *Aspergillus fumigatus*), still in the fledgling stage. This can be attributed to the difficulty in the cultivation of *Malassezia* under laboratory conditions and the lack of appropriate experimental models for studying the fungus in contact with the host in vivo. Previous experiments with isolated cells in culture indicated a broad range of direct and indirect interactions between *Malassezia* and various immune and non-immune cells[7]. However, these in vitro experiments only partially recapitulate the situation of the complex skin environment in vivo where numerous cellular and molecular events occur concomitantly between the fungus and various cell types.

Herein, we outline the protocol for an experimental model of *Malassezia* skin infection in mice, which we recently established, to study the fungus-host interaction in vivo[7]. This includes procedures for (1) the successful cultivation of *Malassezia* in vitro, (2) the epicutaneous application of *Malassezia* onto the murine ear skin, and (3) the technical details of how to analyze *Malassezia*-induced skin inflammation and the fungal burden of infected skin. Importantly, this model does not rely on immunosuppression (e.g., by corticosteroids) or antibiotic treatment of mice prior to infection, as it is practiced in other mouse models of fungal infection[8,9]. In turn, it allows studying the full spectrum of the innate and adaptive immune response against *Malassezia* in the normal skin. Of note, inbred wild type mice kept under specific pathogen-free (SPF) conditions are not naturally colonized with *Malassezia* and, therefore, their exposure to the fungus does not result in persistent colonization but is cleared from the host within approximately 1.5 weeks. However, the model allows for studying the mechanisms of antifungal host response initiation and regulation which, in turn, is the basis of how immune memory is generated. The model is versatile in that it can easily be applied to a wide variety of genetically modified mouse strains and it can be combined with other existing skin disease models, such as

models of barrier deficiency, to study the impact of *Malassezia* under pathological and inflammatory skin conditions[7]. Therefore, the described model of experimental *Malassezia* skin infection in mice provides a high degree of flexibility to investigate the interaction of the fungus with the skin immune system in the context of homeostasis and disease.

This protocol describes the experimental skin infection of mice with *Malassezia* spp. Due to its pathogenic potential, *Malassezia* spp. are classified as BSL2 pathogens in some countries, including Switzerland. Please check the local guidelines and follow the regulations by the local authorities. BSL2-classified organisms should be handled by trained personnel under a BSL2-certified biosafety cabinet (BSC). Biological waste contaminated with BSL2-classified organisms, as well as, carcasses from mice infected with such organisms should be autoclaved prior to disposal. For experiments with mice, all efforts should be made to minimize suffering and ensure the highest ethical and humane standards according to the 3R principles (replace, refine, reduce)[10]. The experiments described in this protocol were carried out with *M. pachydermatis* (ATCC 14522), *M. furfur* (ATCC 14521) and *M. sympodialis* (ATCC 42132)[7].

PROTOCOL:

All procedures described in this protocol were carried out in accordance with the ordinance on handling organisms in contained systems of the Federal Office for the Environment, Switzerland (www.bafu.admin.ch). The mouse experiments were conducted in strict accordance with the guidelines of the Swiss Animals Protection Law and were performed under the protocols approved by the Veterinary Office of the Canton Zurich, Switzerland (license number 168/2018).

1. Cultivation of *Malassezia* under laboratory conditions

NOTE: Store all the reagents and media used for this protocol at room temperature (RT, 20 – 25 °C) unless stated otherwise, as the lower temperature can inhibit fungal growth.

1.1. Prepare the liquid modified Dixon (mDixon) medium for *Malassezia* growth. To prepare 500 mL of liquid mDixon medium, dissolve 18 g Malt extract, 10 g desiccated Ox-bile, 5 mL Tween-40, 3 g Peptone, 1 mL Glycerol and 1 mL Oleic Acid in 500 mL distilled H_2O (dH_2O). Adjust the medium to pH 6 with HCl and autoclave. Store the medium at RT.

1.2. Prepare mDixon agar plates by adding 7.5 g agar to 500 mL mDixon medium prior to autoclaving. Slowly cool down the mDixon agar after autoclaving using a steering bar and a magnetic heating plate to avoid partial solidification of the medium while cooling down.

1.3. Once the agar has cooled down to 50 – 60 °C, dispense the liquid into Petri dishes in a laminar flow hood and let them dry at RT overnight.

NOTE: The agar plates can be stored at 4 °C for several weeks when wrapped and kept upside down to avoid evaporation.

1.4. Obtain *Malassezia* isolates and revive lyophilized stocks of *Malassezia* according to the

instructions obtained by the provider.

1.5. Inoculate 10 mL of liquid mDixon medium in a sterile 100 mL Erlenmeyer flask with the revived *Malassezia* suspension according to the instructions obtained by the provider. Incubate the culture in a shaking incubator at 30 °C and 180 rpm.

1.6. Inspect the growth of the *Malassezia* culture regularly by checking for the appearance of cream color and turbidity. Growth kinetics depend on the species and strain of *Malassezia* and may be particularly slow when *Malassezia* is freshly revived from a lyophilized stock. (**Figure 1A**).

1.7. Prepare glycerol stocks by mixing 3 parts of the densely grown *Malassezia* culture in mDixon medium with 1 part of sterile 99% glycerol. Aliquot the *Malassezia*/glycerol mixture into sterile screw-cap tubes and store at -80 °C.

1.8. For in vitro propagation, plate *Malassezia* from the liquid culture in mDixon or from the frozen glycerol stock onto a mDixon agar plate (brought to RT from 4 ˚C) using an inoculation loop.

NOTE: Transfer mDixon agar plates to RT from 4 °C prior to the use, since cold mDixon agar inhibits fungal growth.

1.9. Incubate the agar plate(s) with *Malassezia* upside down in an (non-shaking) incubator at 30 °C. Inspect the growth of *Malassezia* colonies regularly.

NOTE: *Malassezia* colonies on mDixon agar plates appear within 3 - 5 days and are cream-colored dull, smooth with convex elevation (**Figure 1B**).

1.10. Store *Malassezia* colonies on mDixon agar plates at RT for ~ 2 weeks. Thereafter, prepare a new mDixon agar plate by streaking *Malassezia* from the frozen glycerol stock as described in 1.7.

2. Preparation of the inoculum for experimental *Malassezia* infection of mice

2.1. Inoculate 10 mL of liquid mDixon medium in a sterile 100 mL Erlenmeyer flask with 3 - 5 individual *Malassezia* colonies from a mDixon agar plate (see step 1, **Figure 1B**).

2.2. Incubate the *Malassezia* culture for ~ 48 to 96 h at 30 °C and 180 rpm until the culture is cream-colored and turbid (**Figure 1A**).

NOTE: The time necessary for *Malassezia* growth depends on the *Malassezia* species and strain and the amount of fungus used for inoculation.

2.3. Transfer 2 mL of the *Malassezia* culture into a sterile 2 mL microcentrifuge tube and

centrifuge for 1 min at 10, 000 *x g.*

2.4. Discard the supernatant and wash the pellet by suspending it in 1 mL of phosphate-buffered salt solution (PBS). Centrifuge again for 1 min at 10, 000 *x g*.

2.5. After the washing, suspend the pellet in 1 mL of PBS by vigorous pipetting and measure the optical density of the solution at 600 nm (OD_{A600}) using a spectrometer. Dilute the *Malassezia* suspension 20 – 50 x with PBS for the OD measurement to assure that the reading is between 0.1 and 1.

NOTE: The density of a 3-day culture of *Malassezia* generally varies between 15 and 30 OD_{A600}, depending on the *Malassezia* species and strain and on the number of yeast cells used for inoculation of the culture (step 2.1). *Malassezia* tends to form aggregates, therefore, vigorous pipetting is necessary to ensure homogeneity of the suspension.

2.6. Aliquot a volume of the *Malassezia* suspension in PBS that corresponds to a density of 4 OD_{A600} into a sterile 2 mL tube. Prepare 1 tube per animal to be infected.

2.7. Centrifuge the tubes containing *Malassezia* for 1 min at 10, 000 x g.

2.8. Discard the supernatant and suspend the *Malassezia* pellet in 200 µL of native olive oil (corresponding to 2 OD_{A600} yeast cells/100 µl olive oil)**.**

NOTE: Olive oil was found to be a good vehicle for epicutaneous infection with *Malassezia*, as *Malassezia* is a lipophilic and lipid-dependent yeast. Olive oil is better absorbed by the skin than PBS. However, be aware that it is not easy to suspend *Malassezia* in olive oil. Improve the *Malassezia*/olive oil suspension by vortexing. Keep the suspension at RT until it is used for infection.

2.9. Prepare tubes with olive oil alone for mock infection of control animals.

3. Infecting mice with *Malassezia*

3.1 Order female C57BL/6 mice at an age of 6 - 8 weeks and allow them to acclimatize in the experimental animal facility for at least one week. Calculate for 3 - 5 mice per group, including an uninfected control group.

3.2 Prepare sterile anesthetic cocktail containing 1.3 mg/mL Xylazine and 6.5 mg/mL Ketamine in PBS. 5 mL of the anesthetic cocktail is enough to anesthetize 20 animals. Adjust the volume of the cocktail according to the number of animals to be anesthetized.

3.2.1 Anesthetize animals by injecting 10 µL/g bodyweight of anesthetic cocktail intraperitoneally (corresponding to 65 mg Ketamine and 13 mg Xylazine per kg body weight) and place the anesthetized animals onto a heating pad at 37 °C.

NOTE: At the indicated dose, animals usually remain anesthetized for ~ 30 - 60 min.

3.3 Check the reflexes by pinching the rear foot with forceps to assure that the animals are fully anesthetized.

3.4 Apply an eye cream onto the eyes to prevent dehydration during anesthesia.

3.5 Optionally, measure the ear thickness of both ears using a caliper (0 - 5 mm range). Measure two different areas of each ear and calculate the average ear thickness per ear.

NOTE: Measuring ear thickness is optional and depends on the research question. However, if the ear thickness is used as a readout for skin inflammation, it is necessary to measure the baseline ear thickness prior to infection. (see Step 4).

3.6 Optionally, disrupt the epidermal barrier of the dorsal ear skin by mild tape stripping: manually apply a small piece of tape to the skin and remove it again. Repeat for 5 consecutive rounds using a fresh piece of tape for each round.

NOTE: *Malassezia* induces a more pronounced skin inflammation in barrier-disrupted skin compared to unperturbed skin (**Figure 2A**) [7].

3.7 Topically apply 100 µL (2 OD_{A600}) of the *Malassezia*/olive oil suspension onto the dorsal side of each ear using a sterile pipette. Include a control group of animals that are treated with olive oil only (vehicle-treated control group).

NOTE: Vortex the *Malassezia*/olive oil suspension vigorously to ensure a homogenous *Malassezia* suspension immediately prior to the application.

3.8 Leave the anesthetized animals on the heating pad to avoid hypothermia until they show signs of recovery (whisker movement, increased breathing rate, etc.).

3.9 Inject 200 µL of sterile and pre-warmed 2% glucose solution subcutaneously into the nuchal fold to support their metabolism and rehydration.

NOTE: To prepare a sterile 2% glucose solution, dissolve 1 mg glucose in 50 mL PBS and filter it using a 0.2 µm filter. The solution can be stored at 4 °C.

3.10 Transfer the animals back to their cage.

4. Analysis of *Malassezia*-induced skin inflammation

NOTE: This procedure describes the analysis of *Malassezia*-induced ear swelling during infection which serves as a parameter of skin inflammation. A prerequisite for analyzing the fungus-

induced ear swelling is to measure the baseline ear thickness prior to tape-stripping and/or infection (Step 3.5).

4.1 Prepare isoflurane chamber for short term anesthesia of *Malassezia*-infected and control animals.

4.2 Transfer one animal at a time to the chamber and wait for the animal to be fully anesthetized.

NOTE: Signs of proper anesthesia include complete body relaxation as well as slow and heavy (flank) breathing. Carefully monitor anesthesia as extended exposure to isoflurane can be fatal.

4.3 Remove the animal from the chamber and place it onto a tissue.

4.4 Measure the thickness of the ear(s) using a caliper (range 0 - 5 mm). Measure two different areas of each ear and calculate the average thickness per ear (see step 3.5).

4.5 Transfer the animal back to the cage.

NOTE: Isoflurane anesthesia is very short-lived, and the animals recover within ~ 30 s after removal from the isoflurane chamber.

4.6 Calculate the increase in ear thickness by subtracting the average baseline ear thickness, measured prior to the tape stripping and/or infection, from the average ear thickness measured at each time point after infection.

4.7 Plot the calculated values as the increase in ear thickness or, alternatively, as the total ear thickness over time for each animal or group of animals (**Figure 2B**).

5. Analysis of fungal burden in the infected skin

5.1 Prepare a sterile 2 mL microcentrifuge tube for each ear to be harvested, containing 0.5 mL of sterile 0.05% NP40 in dH_2O and an autoclaved steel ball (5 mm diameter).

5.2 Weigh the tubes using a precision balance and write down the precise weight.

5.3 Euthanize the mice by CO_2 asphyxiation.

5.4 Remove the ear(s) at the base and transfer into the tube containing 0.5 mL of sterile 0.05% NP40 in dH_2O, as described in steps 5.1 - 5.2.

5.5 Weigh the tube containing the ear tissue and calculate the actual weight of each sample by subtracting the weight of the tube without the organ from the weight of the tube with the

organ.

5.6 Homogenize the ear tissue for 6 min at 25 Hz using a tissue homogenizer. Ensure that the tissue is well homogenized.

5.7 Plate 100 µL of each sample (corresponding to 1/5 of each homogenate, dilution factor = 5) onto mDixon agar plates and incubate the plates upside down in a 30°C incubator.

NOTE: The amount of homogenate plated should be adjusted according to the fungal load to be expected. Make sure to plate sufficient homogenate to obtain at least 10 and no more than 250 colonies per plate to allow easy enumeration. Optionally, plate multiple plates per sample with different dilutions of homogenate.

5.8 Inspect the growth of *Malassezia* colonies regularly.

NOTE: Colonies usually become visible after 2 - 3 days. The time necessary for *Malassezia* colonies to grow depends on the species and strain of *Malassezia*.

5.9 Count the colonies per plate.

5.10 Calculate the number of CFU/g tissue by using the following formula:
CFU/g tissue = (number of colonies/plate) x (dilution factor) / (weight of the skin sample in g).

NOTE: The approximate minimal detection limit can be assessed using the following formula: minimal detection limit = (1 colony/plate) x (dilution factor) / (average weight of all skin samples in g).

5.11 Fungal loads are usually plotted on a logarithmic scale (**Figure 2C**).

REPRESENTATIVE RESULTS:

In vitro* cultivation of *Malassezia

Compared to other more commonly used fungal model pathogens such as *C. albicans* or *A. fumigatus*, *Malassezia* is more difficult to culture in vitro. This can be attributed to the fact that *Malassezia* relies on exogenous lipid sources for its nutritive requirements, due to its inability to synthesize fatty acids[11]. The mDixon medium is suitable for culturing several *Malassezia* species including *M. pachydermatis*, *M. furfur*, *M. sympodialis*, *M. slooffiae, M. globosa* and *M. yamatoensis* in vitro[7,12]. **Figure 1** shows representative images for the growth of *M. sympodialis* in liquid mDixon medium and on mDixon agar as described in step 1 and step 2.

Analysis of skin inflammation and fungal burden of *Malassezia* skin inflammation

Exposure of *Malassezia* to the mouse ear skin that was barrier disrupted by tape-stripping prior to infection results in exacerbated inflammation of the skin, characterized by epidermal and dermal hyperplasia and development of edema[7]. Step 4 and 5 outline the methods for analyzing

Malassezia-induced ear swelling and the fungal burden of the skin. Both parameters represent key readouts for monitoring the course of the infection. **Figure 2A** illustrates the increase in ear thickness that can be observed after *M. furfur* exposure to skin that was barrier-disrupted compared to unperturbed skin of WT C57BL/6 mice. **Figure 2B** shows a representative summary graph of the increase in ear thickness over time. **Figure 2C** displays the fungal burden in the ear skin on day 2 after infection with *M. pachydermatis*.

FIGURE LEGENDS:

Figure 1: In vitro cultivation of *Malassezia.* (**A**) *M. sympodialis* strain ATCC 42132 grown for 3 days at 30 °C and 180 rpm in liquid mDixon medium (left) next to a control Erlenmeyer flask containing mDixon medium that was not inoculated (right). (**B**) Colonies of *M. sympodialis* strain ATCC 42132 on mDixon agar after 5 days of incubation at 30 °C.

Figure 2: Analysis of *Malassezia* skin infection on the basis of ear thickness and fungal burden. (**A**) Histology of ear sections obtained from C57BL/6 mice that were treated with olive oil (vehicle, left) or infected with *M. furfur* strain JPLK23 for 5 days (middle and right). On the right, the ear skin was tape-stripped prior to infection. Sections were stained with hematoxylin and eosin (H&E). (**B**) Summary graphs showing the increase in ear thickness over time for C57BL/6 mice that were exposed to *Malassezia* or left uninfected as controls. The absolute thickness of *M. pachydermatis* strain ATCC 14522-exposed or vehicle-treated ear skin at each time point is displayed on the left; the increase in ear thickness at the indicated time points relative to the baseline on day 0 is shown on the right. (**C**) Fungal burden in the skin of C57BL/6 mice that were infected with *M. pachydermatis* strain ATCC 14522 or treated with olive oil as a control (vehicle). In both cases, the skin was tape-stripped. Each symbol in the summary graphs **B** and **C** represents one animal. The statistical significance of the differences between groups was calculated using one-way ANOVA (B) or Student's t-test (C). ***p <0.001, ****p<0.0001, D.L.: Detection Limit

DISCUSSION:

This protocol describes the infection of the skin of the commonly used inbred mouse strain C57BL/6 by *Malassezia* spp. Adapting this protocol to other mouse strains with a different genetic background (e.g., Balb/c) or to genetically modified mouse strains may need adjustment of the infection dose, the time point(s) of analysis, etc. To ensure reproducibility, groups of mice should always be of the same age and sex. The source of mice should be kept stable, as even slight changes in the genetic background and differences in the microbiota, which exist between vendors and may exist even between different units of a single breeding facility, can have an unpredictable impact on the course of infection. When setting up the *Malassezia* infection model described in this protocol, it is advised to perform a pilot study to carefully monitor the course of infection, including the extent of colonization, the kinetics of fungal clearance and the degree of inflammation and pathology that might be induced (e.g., if the ear skin is barrier-disrupted prior to infection) to determine the optimal assay conditions.

To ensure reproducibility and to reliably detect differences between experimental groups, the number of animals used per group must be calculated based on the statistical analysis. The

sample size is calculated based on effect size, error rate and power, which consider biological and experimental variations (e.g., due to variation in the immune system). For ethical reasons avoid using unnecessarily high numbers of animals. Regarding *Malassezia* skin infection, treating only one ear with the fungus and using the other ear as a control within the same mouse, is not advised because mice may spread the fungus to both ears when grooming. However, using ½ ear for different methodological read outs such as determination of fungal burden, isolation of immune cells or histological analysis is often enough and results in a significant reduction in animal numbers used for experiments.

18 different species of *Malassezia* have been described up to date. Inter- and intraspecies variations within the genus *Malassezia* can affect the interaction with the host, as we have also learned from studies on other human pathogenic fungi[13]. Different *Malassezia* species and strains differ in their origin (e.g., *M. pachydermatis* is the most frequent species isolated from animals, while *M. restricta*, *M. globosa* and *M. sympodialis* are the most prominent members of the fungal skin microbiome in humans with variable distribution of these species between different skin areas). Some species have been associated with commensalism, while others are thought to be more pathogenic, although detailed evidence remains relatively weak. Importantly, some species and strains are inherently more difficult to grow than others. Thus, the decision of which species/strain to use for the infection must be based on the research question.

Experimental infection of the murine skin with some microbial organisms such as *Candida albicans* or *Staphylococcus aureus* require the disruption of the epidermal barrier prior to infection, e.g., with sand paper[14-16]. In contrast, the model of *Malassezia* infection described here is equally efficient with and without barrier disruption[7]. The degree of inflammation induced by the fungus is massively enhanced if the skin is tape stripped prior to infection[7]. Therefore, whether the skin should be manipulated before the application of *Malassezia* depends on the research question. Various models of chronic and acute skin inflammation (e.g., models for delayed type hypersensitivity (DTH) and contact hypersensitivity (CHS)) and models of barrier deficiency exist that may be of interest for investigating the contribution of commensal yeast to skin pathologies.

Inbred mice maintained under specific pathogen free (SPF) conditions are (to our knowledge) not naturally colonized with *Malassezia*. Therefore, the experimental application of *Malassezia* to the mouse ear skin represents a primary exposure to the fungus that induces an acute response in the host, which in turn leads to fungal clearance within 1 - 2 weeks [7]. While the model described in this protocol therefore only partially reflects the situation in immunocompetent humans or other host organisms that are permanently colonized with *Malassezia*, the experimental infection allows an ample window of opportunity to study antifungal immunity and the cellular and molecular mechanisms that underlie this response. It also allows investigating variations in the response to different *Malassezia* species and strains under different experimental conditions (e.g., with and without barrier disruption of the skin).

The study of *Malassezia* - host interactions have been limited in the past to in vitro experiments with isolated cell types in cultures (e.g., keratinocyte cell lines, PBMCs). Although these studies have shed some light on fungal and host determinants that shape the interplay between *Malassezia* and the host[17], they do not allow to gain a comprehensive understanding of the fungus - host interaction in the complex environment of the skin, which involves multiple cell types that are in constant communication, such as keratinocytes, fibroblasts and tissue-resident immune cells, but also leukocyte populations that infiltrate the tissue only upon microbial encounter of the skin. This multicellular network cannot be fully reproduced in the in vitro models, even with most advanced organoid systems. Thus, the experimental infection of mice still represents the gold standard in immunology and infectious disease research, and the availability of the model described here represents a breakthrough in the field of *Malassezia* research. Importantly, this model relies on the epicutaneous application of *Malassezia* on the otherwise unperturbed mouse ear skin, and it does not implicate inoculation of the fungus by injection into the tissue, e.g., subcutaneously or intraperitoneally, as previous studies reported[18], both of which are more distant from the situation in naturally colonized hosts.

The possibility to combine the model of *Malassezia* infection described in this protocol with other available mouse models greatly increases the scope and flexibility of the application. The latter include various models of specific skin disorders, such as the model of barrier deficiency that mimics important features of atopic dermatitis, a disease associated with *Malassezia* in both humans and dogs. Moreover, epicutaneous infection of the skin with *Malassezia* can easily be applied to mice with genetic defects in host genes of interest, or mice in which a cell type of interest are genetically deleted or can be pharmacologically depleted (e.g., by means of diphtheria toxin administration in diphtheria toxin receptor-expressing mice). Such models represent an inevitable tool for dissecting the host response to commensal and pathogenic microbes, including *Malassezia*, and to assess the role of these genes and cell type in the fungus-host interaction. The analyses of the *Malassezia*-host skin interaction can be expanded far beyond of what is described in this protocol. These include analyses by histology (e.g., to determine the degree of skin pathology or the epidermal thickening induced by the fungus), by immunohistochemistry or immunofluorescent staining of tissue sections using antibodies directed against cell type specific markers or other molecules of interest. It may also involve the isolation of cells (e.g., tissue resident or tissue-infiltrating leukocyte subsets) from the infected skin tissue to study the polarization, regulation, and dynamics of the immune response to *Malassezia* in great depth.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1:

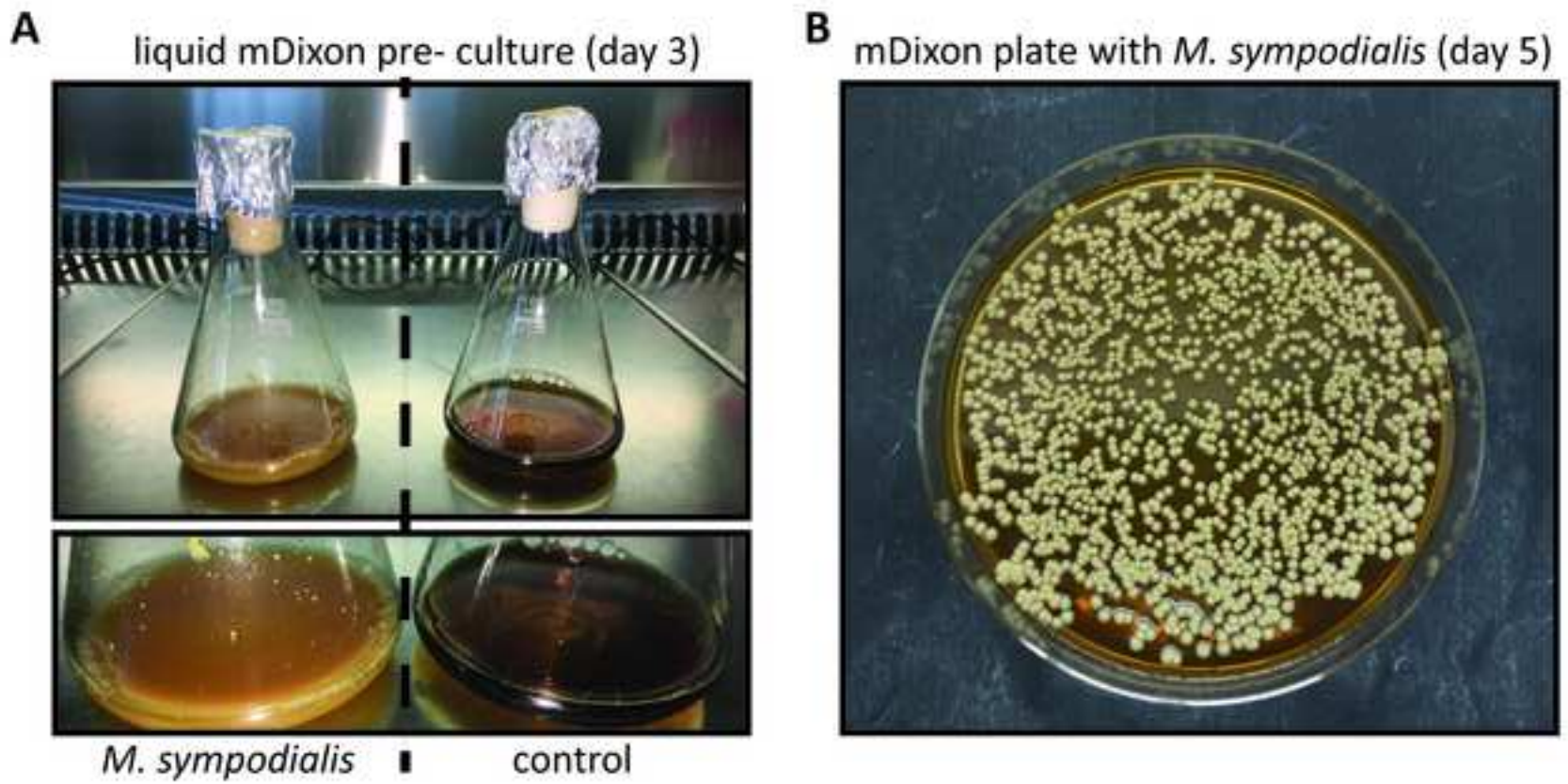
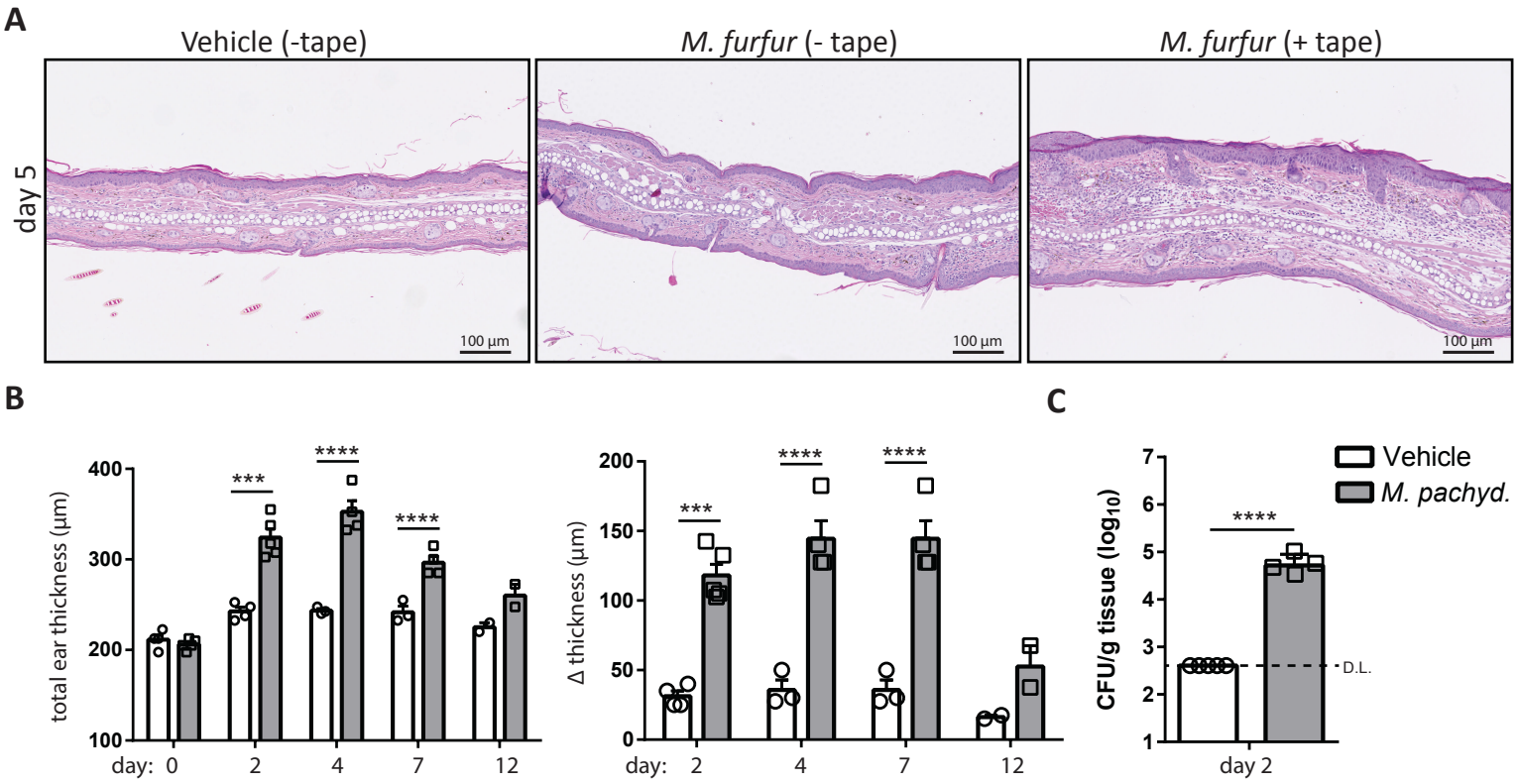


Figure 2:



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Agar	Sigma-Aldrich	A1296-1KG	
Attane Isoflurane	Piramal Healthcare	-	
Biosaftey cabinet (BSC) Faster Ultra Safe	DASIT GROUP	TEC 5594	BSL2 certified
Centrifuge	Eppendorf	5415D	
C57BL/6 mouse	Janvier	-	
Dessicated Ox-bile	Sigma-Aldrich	70168-100G	
Eppendorf Tubes (2 ml)	Eppendorf	0030 120.094	Safelock
Glucose	Sigma-Aldrich	49159-5KG	commerc. available
Gylcerol (99 %)	Honeywell	10314830	
Heating pad	Eickenmeyer	648048	
Incubator Hereaus B20	Heraeus	412047753	
Ketasol (100 mg)	Graeub AG	6680416	
Malassezia spp.	ATCC	14522, 14521, 42132	
Magentic heating plate MR Hei-Standard	Heidolph Instruments	442-1355	
Malt extract	Sigma-Aldrich	70167-500G	
Multiply Biosphere Tubes (200 µl)	Sarstedt AG	7084211	BSL2 certified
Native olive oil	e. g. SPAR	-	
Nonidet P40	Axon Lab	A1694,0250	
Oditest measurment devise	Kroeplin	S0247	compatible with 2ml Eppendorf tubes
Oleic Acid	Sigma-Aldrich	75090-5ML	
Phosphat buffered salt solution (PBS, 1x)	Amimed/Bioconcept	3-05F39	
Petri dishes	Sarstedt AG	82.1473	
Peptone	Oxoid	LP0037	
Rompun (2 %)	Bayer	KP0BFHR	
Shaking incubator Infors Minitron	Infors	-	BSL2 certified
Spectrometer	Jenway	20308	
Spectrometer Cuvettes	Greiner Bio-One	613101	
Stainless Steel balls (5mm)	ABF	KU.5G80 1.3541	
Syringes 1 ml Sub-Q	BD Bioscience	305501	range 0-5 mm

Tissue Lyzer II	Quiagen	85300	optical density measurement at 600nm
Transpore Hypoallergic Tape	3M	1527-1	
Tween 40	Sigma-Aldrich	P1504-100ML	
Vitamin A eye Cream	BAUSCH & LOMB		commerc. available

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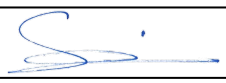
Signature:	
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Point-to-point reply to the editorial comments:

Line 1, The title is reworded to reflect the protocol. Please check.

→ *We thank the editor for the suggestion and we acknowledge the title as suggested.*

Line 91, We cannot have paragraph of text in the protocol section. Hence this section is moved here. Please check.

→ *We acknowledge the change.*

Line 100, Please move this part to the table of materials.

→ *We added the information about the mice in the table of materials.*

Line 111: This is redundant and can be deleted.

→ *We deleted the sentence as adviced.*

Line 133, What volume? OR do you inoculate with a single colony? Please be specific.

→ *For the establishment of the first Malassezia culture we strictly followed the instruction from the datasheet of the vendor (in our case ATCC, see documentation/data sheet of M. furfur ATCC 14521 under www.lgcstandards-atcc.org), which suggested to use "several drops" of the revived Malassezia suspension for the inoculation of the medium. The amount of Malassezia used to establish the first culture most likely varies, depending on the source/vendor. Thus, no accurate information about a particular volume or density can be provided. We rephrased this sentence for further clarity (line 138).*

Line 159, So you do not plate from the already growing culture?

→ *As described in the section 2, for the establishment of an infection inoculum we use colonies from the plate, which we store at RT for approximately two weeks. After the two weeks we prepare a new Dixon agar plate by streaking from frozen glycerol stock.*

Line 163, Again this is redundant and can be removed.

→ *We removed the paragraph as adviced.*

Line 164, Do you inoculate single colony in a tube? This needs clarity. If not then how many colony per flask? Also how many flasks are inoculated per mouse/ per infection?

→ *We are not sure what the editor means with "single colony in a tube". For the establishment of an infection inoculum, 10ml of liquid Malassezia culture needs to be set up in an 100ml Erlenmeyer flask, as indicated (point 2.1). We rephrased the sentence for more clarity and added the approximate number of colonies ("3-5 colonies") used for the inoculation of the 10 ml of liquid mDixon medium (line 172).*

Line 170, It will also depend on number of colonies inoculated. Do you check the O.D at this stage?

→ *We thank the editor for the suggestion and rephrased the sentence for more clarity. We do check the OD of the liquid culture after 48 – 96 hours of incubation, as outlined in point 2.3 – 2.5.*

Line 177, Is this correct?

→ *Yes, 10, 000 x g is correct.*

Line 179, How much ?

→ *The amount of the volume of the aliquot used for OD measurements depends on the particular device and should be used accordingly. We rephrased the sentence accordingly.*

Line 184: So you do the OD measurement after 3 days. Please include this in step 2.3 to bring out clarity.

→ *We measure OD after 48 to 96 hours of incubation of the liquid culture, as indicated in point 2.2). The NOTE under 2.5 should give the reader an approximation of what OD can be expected after 3 days of Malassezia culture (with the Malassezia strains and species indicated in the protocol).*

Line 197, Why is this done? Why can't the infection be performed in PBS? Please discuss this in the discussion section.

→ *We extended the NOTE under 2.8 by one sentence explaining why olive oil is suitable for topical infection with Malassezia (line 205).*

Line: 206, Again, this is redundant and can be removed.

→ *We deleted this sentence as adviced.*

Line 207, Please provide age and sex of the mice used for the study.

→ *We added the specification of sex and age of the C57BL/6 mice we used for this particular protocol in last paragraph of the introduction (line 102). However, the age and the sex of the animals can vary depending on the particular research question and thus we did not give any specification in point 3.1. We also mention in the discussion (line 408) that animals should be age and sex matched to ensure reproducibility of the experiments.*

Line 211, We cannot have commercial term in the manuscript. Please move the commercial term to the table of materials and use generic term instead.

→ *We changed the terminus to "Ketamine".*

Line 211, Please specify what is meant by according to need. Do you have a specific volume/concentration for injection?

→ *We rephrased the sentence about the adjustment accordingly (Line 227). We also provide details about the final concentration of each compound in the anesthesia cocktail (point 3.2) and the amount of anesthesia cocktail to be injected per g body weight (3.4). The final volume of anesthesia cocktail to be injected depends on the animal's weight.*

Line 221, Point 3.4, After anesthesia, do you check the depth of anesthesia before proceeding?

→ *Injecting the suggested amount of narcotics should result in an appropriate depth of anesthesia of the animals. However, we added a NOTE to point 3.4., which explains how to check the depth of anesthesia (Line 233/234).*

Line 226, We cannot have commercial terms in our manuscript. Please use generic term instead.

→ *We adjusted the sentence accordingly.*

Line 229, Step 3.5 states this is optional however the note states this is necessary, since this is a parameter of skin inflammation. Please clarify.

→ *Malassezia induces ear swelling when applied onto tape stripped skin and measuring the ear swelling over time is a frequently used read out for skin inflammation. Whether ear swelling is measured during the course of the infection (or not) is up to the experimenter and the research question and thus optional (point 3.6). However, if the research question requires the measurement of the ear thickness, it is necessary to measure baseline ear thickness prior to infection (as indicated in the NOTE). We adjusted the text for further clarification.*

Line 233: Please move the commercial term to the table of materials.

→ *We moved the commercial term to the Material table.*

Line 241: Would the right vs left ear would be a better control in this case? Please explain why or why not?

→ *No, treatment of just one ear is not advised because the animals might spread the fungus when grooming. Treating animals with just the vehicle should be used as a negative control. We mention this in the discussion (line 405).*

Line 292, Again this is redundant as all these are mentioned in the steps below.

→ *We deleted this paragraph.*

Line 301, Reworded to bring out clarity.

→ *We thank the reviewer for the suggestion and rephrased the wording.*

Line 307, Please move this to the table of materials. We cannot have commercial terms in the manuscript.

→ *We moved the commercial term to the Material table.*

Line 334, Did you perform any biomarker studies in the host- any mRNA, protein studies?

→ *We carried out several measurements of immunological parameters at the mRNA and/or protein level. They are all included in our recent publication (Sparber et al. Cell Host & Microbe, 2019).*

Also how did you confirm which fungal species was growing on the plate?

→ *Our SPF mice are Malassezia-free as we did not recover any fungal colonies from sham-infected animals. Microscopic analysis confirmed that the recovered yeast cells growing on mDixon agar was Malassezia.*

Line 367, How many per group?

→ *Each symbol in the summary graphs in Figure 2B and 2C represents one animal. We added this sentence to the figure legend for more clarity (Line 395).*

Line 377, The subheadings can be removed.

→ *We removed the subheadings.*