# 3D modelling of *Malassezia furfur* skin interaction

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*Malassezia furfur* is a lipid-dependent yeast naturally living on the skin. Despite its tolerance by the immune system under healthy condition, *M. furfur* overgrowth is associated with skin disorders such as dandruff.

Dandruff and its more severe form seborrheic dermatitis (SD), are common chronic inflammatory conditions characterized by an abnormal shedding of the skin in seborrheic areas of the body. While dandruff is restricted to the scalp, SD also affects oily rich areas of the face, causing itching, pruritic lesions and erythema.

Environmental and internal stress factors, dysregulation of the immune system and fungal colonization are amongst the main contributors to the development of SD and dandruff.[1] It has been described that in dandruff, the quantity of *Malassezia* can increase up to 1.5 to 2 times its normal level.[2]

This huge spread of yeast highly invades the epidermis, with detrimental consequences on the skin barrier function. Because the epidermis is the principal barrier against the penetration of chemicals and pathogens, any disruption increases microbial invasion and triggers both innate and acquired immune response.[3]

Keratinocytes are playing a central role in this response by secreting key signaling molecules like antimicrobial peptides to overcome pathogenic infections, as well as various cytokines and interleukins to recruit inflammatory cells. In the case of *Malassezia* overgrowth, previous studies have reported the expression of some defensins and pro-inflammatory cytokines in response to infection.[4,5,6]

SD and dandruff are highly disconformable scalp disorders. Above the itchy feeling, flaking skin is socially embarrassing, forcing patients to expanse large amount of money on treatments. Given the association of *M. furfur* with SD and dandruff, one treatment option is to prevent its expansion using broad spectrum antimycotic agents like Ketoconazole (KTZ).

KTZ increases the fungal membrane fluidity, therefore limiting the yeast multiplication. Recently, KTZ has demonstrated its efficacy to reduce the quantity of *Malassezia* in SD while restoring the skin microbial communities.[7,8]

Nowadays, new antifungal solutions are entering the market, with a need to demonstrate their efficacy to reduce the growth of *M. furfur* and restore the skin barrier. Organotypic 3D skin models have become essential tools to understand the biological activity of compounds on the skin.

In order to accelerate the knowledge about *Malassezia*-host interactions, we decided to develop a 3D skin model that replicates *Malassezia* infection. Here, we present the successful colonization of reconstructed human epidermis (RHE) with a living strain of *M. furfur*.

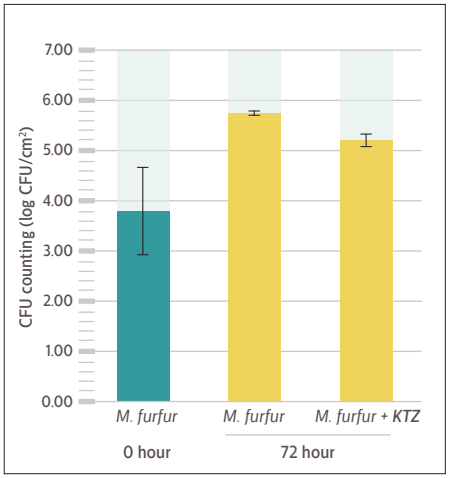


**Figure 1:** *Malassezia* furfur growth on the *stratum corneum* of reconstructed human epidermis in the presence of Ketoconazole (*M. furfur* + KTZ) or not (*M. furfur*). After 72 hours, yeasts were harvested and plated on an adapted solid microbiological media for the counting of colony-forming units (CFU)

After confirming the effective growth of the yeast on the *stratum corneum* of the RHE, the response of the tissue to this colonization has been studied by monitoring key biomarkers.

## Colonization of RHE with *M. furfur* and barrier disruption

For the development of this new 3D model, human epidermis were reconstructed from primary normal human epidermal keratinocytes (NHEK). At the end of the reconstruction, *M. furfur* was laid on the *stratum corneum* in a lipid mixture representative of the ecological niche of this lipophilic yeast.

As growth control conditions, the yeast was applied in the absence or presence of KTZ. After three days, yeasts were harvested from the top layer of RHE, and plated on an adapted solid microbiological media for the counting of colony-forming units (CFU). A sevenfold growth was counted in the absence of KTZ (Figure 1).

However, in the presence of this antimycotic agent, the growth was reduced fourfold compared to the initial yeast inoculum. These results confirmed the survival and growth of *M. furfur* on the RHE, and the efficacy of KTZ to effectively reduce this multiplication.

From a histological point of view, the yeast-specific periodic acid-Schiff (PAS) staining of paraffin-embedded RHE demonstrated that after three days of growth, *M. furfur* passed the *stratum corneum* of the RHE to clearly invade

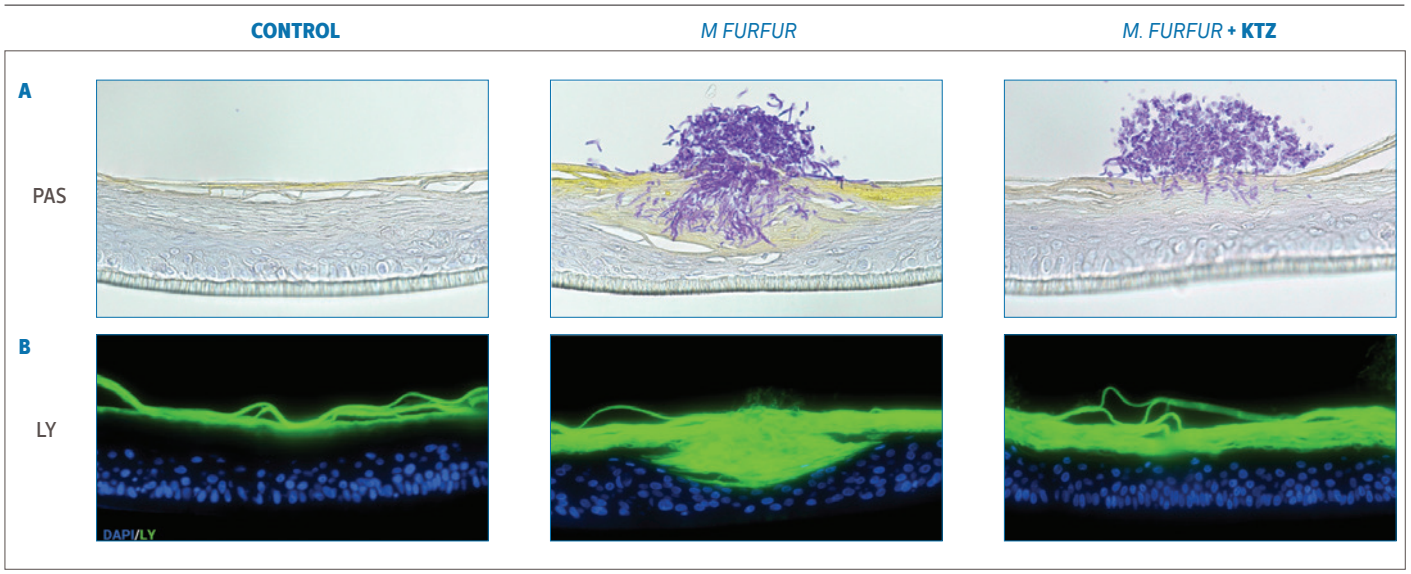


**Figure 2:** Representative images of reconstructed human epidermis uncolonized (Control) or colonized by *Malassezia furfur* in the presence of Ketoconazole (*M. furfur* + KTZ) or not (*M. furfur*). A: Periodic acid-Schiff staining of paraffin-embedded epidermis. B: Lucifer yellow fluorescence after out/in epidermal barrier diffusion assay

the epidermal barrier (Figure 2A).

A slight thickening of the epidermis was also observed in the presence of the yeast. Additionally, as expected, addition of KTZ weakened the multiplication of *M. furfur*, therefore reducing the invasion of the epidermal barrier.

In order to evaluate this barrier disruption, we performed a transepidermal Lucifer yellow (LY) diffusion assay on colonized RHE. LY is essentially a fluorescent dye able to diffuse from the outside to the inside layers of a tissue if the intercellular spaces are enlarged, typically like in disrupted RHE.

Both the quantification of fluorescence in the culture media after diffusion of the dye throughout the RHE and the quantification of remaining fluorescence in paraffin-embedded RHE, reflect the permeability of the RHE. In the case of *M. furfur*-colonized RHE, LY applied on the *stratum corneum* was able to diffuse to the basal layer, as observed by fluorescent images (Figure 2B) and by quantification in the culture media (Figure 3).

In contrast, treatment with KTZ reduced *M. furfur* intrusion to allow an accumulation of green dye in the top layer of the RHE (Figure 2B) and a resulting lower quantity of fluorescence in the culture media (Figure 3). Altogether, those data confirmed that the invasive yeast clearly declined the outside/inside epidermal barrier of the RHE, in a KTZ-limiting way.

## Impact on gene expression

The observation of the disrupted barrier led us to investigate deeper the impact of *M. furfur* on the response of the tissue to this invasive stress. To this end, we conducted a RT-qPCR gene expression analysis (Figure 4) and observed that the expression of genes playing role in the structure of the barrier such as loricrin, filaggrin, desmoglein, or keratin-1, -10 and -14 was statistically downregulated.

Additionally, the expression of genes coding for the matrix metalloproteinases MMP-1 and -9 involved in the turnover of extracellular matrix components were upregulated. Regarding the expression of inflammatory genes, we observed that the expression of the interleukins IL-1-

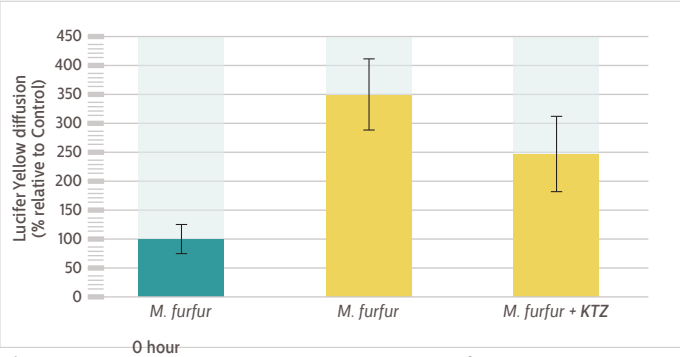


**Figure 3:** Quantification of out/in Lucifer Yellow diffusion through the *stratum corneum* of reconstructed human epidermis uncolonized (Control) or colonized by *Malassezia furfur* in the presence of Ketoconazole (*M. furfur* + KTZ) or not (*M. furfur*).

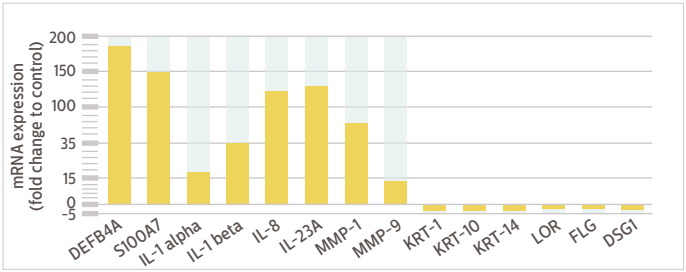


**Figure 4:** Analysis of inflammatory, innate immunity and epidermal genes differentially expressed in reconstructed human epidermis colonized by *Malassezia furfur* during three days. Changes in gene expression are expressed as fold to control (uncolonized reconstructed human epidermis). DEFB4A: Human Beta Defensin 4A – DSG1: Desmoglein 1 – FLG: Filaggrin – IL's: Interleukins – KRT's: Keratins – LOR: Loricrin – MMP's: Matrix Metalloproteinases – S100A7: Psoriasin

alpha, IL-1-beta, IL-8, and IL-23A genes were upregulated, up to 140-fold time for the IL-8 gene.

Similarly, the expression fold change of defensin genes like the Human Defensin DEFB4A gene or the antimicrobial psoriasin S100A7 gene were around 150 time overexpressed. This extended transcriptomic analysis confirmed the effective activation of the immune response following *M. furfur* colonization, as well as an impairment of the epidermal structure, at the gene level.

## Secretion of protein biomarkers

In order to support those transcriptomic data, we aimed to quantify the release of some proinflammatory mediators. As described earlier, the RT-qPCR analysis revealed a particularly significant overexpression of IL-8 and DEFB4A genes.

Given the major involvement of these two biomarkers in the response to infection, we selected two specific ELISA assays measuring the Human Beta Defensin 2 (HBD-2, encoded by the gene DEFB4A) and the IL-8 cytokines to define the respective levels of those proteins in the culture media of RHE colonized with *M. furfur*, in the presence or absence of KTZ.

In harmony with the previously obtained RT-qPCR results, ELISA assays demonstrated that *M. furfur* significantly induces the secretion of HBD-2 and IL-8 proteins in the culture media, by a respective fivefold and tenfold change compared to uncolonized RHE control (Figure 5, red bars).

Additionally, in the presence of KTZ, the overproduction of the two biomarkers was reduced to only a threefold secretion level (Figure 5, red bars), supporting again the use of this antimycotic molecule to reduce the influence of *M. furfur* on the tissue responses.

## Conclusion

Organotypic 3D models are nowadays a prerequisite for accelerating our comprehension of skin interactions with its microbiome. In order to accelerate our knowledge on *Malassezia* overgrowth and provide the community with a tool to evaluate new antimycotic compounds, we presented here the development of a new 3D RHE model mimicking the colonization by *M. furfur*.

Using microbiological, histological, transcriptomic and immunological assays, we clearly demonstrated that the condition of colonization allows the living yeast to grow and interfere with the epidermal tissue.

We illustrated that in our 3D model, *M. furfur* invades the upper layers of the reconstructed epidermis with deleterious impacts on the barrier function, inducing an effective immune and inflammatory response, at both gene and protein levels.

Indeed, the transcriptomic analysis revealed that, compared to uncolonized controls, colonized tissues displayed a down-regulation of genes involved in the skin barrier homeostasis, as well as an over-expression of genes playing key roles in the immune response.

Moreover, to further attest to the responsiveness of RHE to *M. furfur* colonization, we demonstrated that gene expression was consistent with the corresponding protein secretion, as reported by the quantification of IL-8 and HBD-2 proteins by ELISA assays. The fact that KTZ treatment moderated the observed impacts of *M. furfur* on RHE, supports the use of KTZ as an effective antimycotic agent in this model.

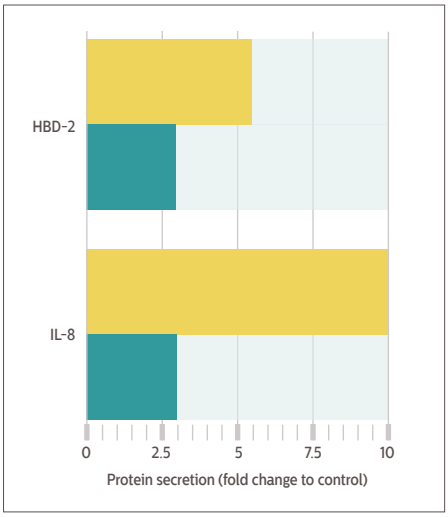


**Figure 5:** Quantification of proteins differentially secreted in the cell culture media of reconstructed human epidermis colonized by *Malassezia furfur* during three days, in the presence (blue bars) or absence (red bars) of Ketoconazole. Changes in protein secretion are expressed as fold to control (uncolonized reconstructed human epidermis). HBD-2: Human Beta Defensin 2 – IL-8: Interleukin 8

Altogether, this new 3D microbial skin model and its associated assays are fully adapted to objectivate the efficacy of innovative dermo-cosmetic compounds by two complementary approaches, namely their interaction with the skin in response to infection, as well as their interference with the yeast overgrowth.

Efficacy in comparison to KTZ used as a positive reference is possible in the model presented here. This new infectious 3D model and associated efficacy tests are therefore a promising tool to evaluate and promote the entry of new SD and dandruff treatment solutions on the market. **PC**

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