In vitro studies of skin microbiota-host interactions based on 3D skin models

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Abstract

Background: The human skin is host to trillions of microorganisms including bacteria, viruses, and fungi. This natural flora which lives on our skin is called the skin microbiome**.** Hence, new skincare products are now evolving to better preserve or enhance this natural ecosystem and suitable experimental models are then required for research on the skin microbiome. Currently, Human Reconstructed Epidermis (RHE) models are widely accepted as a valuable tool in dermatological research. On its MicroBIOS Platform, StratiCELL combines skin microbiota key components and 3D RHE, to study the efficacy of dermo-cosmetic ingredients on microbial homeostasis and disorders linked with dysbiosis.

Methods: StratiCELL is studying commensal and opportunistic strains of the skin including *Staphylococcus epidermidis, Staphylococcus aureus, Cutibacterium acnes* and *Malassezia furfur*. The RHEs are colonized with these microbiological strains and a two-tiered approach is developed to objectivate the influence of dermo-cosmetic actives. The first is the monitoring of the microorganism's adhesion and growth on the stratum corneum. The second is the study of the epidermal response to bacteria, yeasts and fungi.

Results: For each bacterial or fungal species selected to infect RHE, a systematic methodology was applied. According to their characteristics and their metabolism, the protocols of culture, infection, harvesting and counting of CFU were optimized. The tissue response to infection was studied after a period of growth of the microorganism on the top of the RHE. For each species, we established a transcriptomic signature following infection. These expression profiles allowed us to identify multiple response genes shared for all micro-organisms and some specific to each of them. These genes are most often related to the antimicrobial response, inflammation and skin barrier. The relevance and reliability of these infection biomarkers was then validated by qPCR. For some of them, a quantification by ELISA was performed to confirm that the changes in mRNA expression also affect protein abundance.

Conclusion: The infection of *in vitro* reconstituted epidermis by microorganisms constitutes an essential tool to approach the conditions of *in vivo* infection, allowing among other things the inoculation of strains in a topical way. The technical challenge is the adaptation of the different models to the specificities of the microorganisms of interest.

Keywords: Reconstructed human Epidermis*;* Skin microbiome*;* dysbiosis

Introduction.

The human skin is host to trillions of microorganisms including bacteria, viruses, and fungi. This natural flora which lives on our skin is called the skin microbiome**.** Its impact and influence on skin homeostasis is booming. Hence, new skincare products are now evolving to better preserve or enhance this natural ecosystem and suitable experimental models are then required for research on the skin microbiome.

Few *in vitro* models are available to facilitate the preclinical phase of research and objectification of new microbial homeostasis. Microbiological tests and 2D cellular systems exist, but with poor similarity to the real microenvironment. Animal models also exist, but their access is limited, or even forbidden in the context of the development of active ingredients for cosmetic purposes. This is why access to 3D *in vitro* skin models replicating the cutaneous biology is becoming a priority to accelerate the preclinical phases of development of new solutions. In order to fill this gap, StratiCELL develop new models to study the interactions between the skin and its flora, by colonizing reconstructed human epidermis (RHE) with microorganisms of interest. On its MicroBIOS Platform, StratiCELL combines skin microbiota key components and 3D RHE to study the efficacy of dermo-cosmetic ingredients on microbial homeostasis and disorders linked with dysbiosis.

Materials and Methods.

StratiCELL is studying commensal and opportunistic strains of the skin including *Staphylococcus epidermidis, Staphylococcus aureus, Cutibacterium acnes* and *Malassezia furfur*. The RHE are colonized with these microbiological strains and a two-tiered approach is developed to objectivate the influence of dermo-cosmetic actives. The first is the monitoring of the microorganism's adhesion and growth on the stratum corneum of the RHE through CFU counting upon harvest with a swab. The second is the study of the epidermal response to bacteria and yeasts with a transcriptomic tool based on the TaqMan Low Density Array (TLDA) technology. StratiCELL develops a proprietary TLDA that we have named "Skin Response to Microorganisms" to evaluate the expression of 93 key genes selected from full-transcriptome analysis and involved in the skin response to micro-organisms. We selected these 93 genes for their key role in three processes related to bacterial infection, namely inflammation, innate immunity response, and skin barrier structure and homeostasis. This TLDA can be used to evaluate the effect of an ingredient to interfere on bacteria-host interactions. This powerful transcriptomic tool is therefore an important step in understanding the mechanism of action of innovative compounds that target the skin microflora **(Figure 1)**. We also focus on specific biomarkers expression (RT-qPCR) and mediators release in the culture media (ELISA).

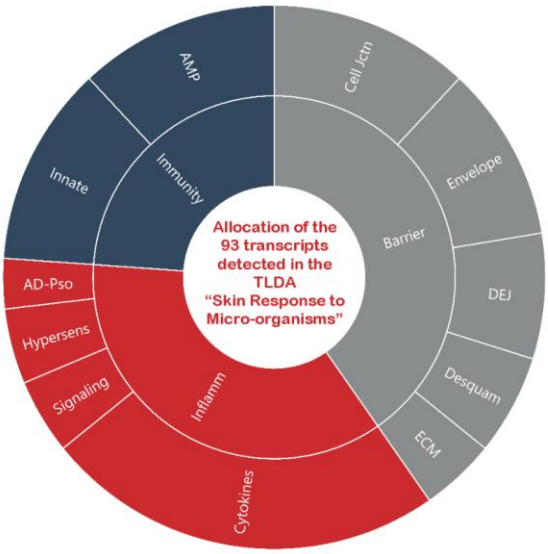

Figure 1 : *StratiCELL "Skin Response to Microorganisms" TLDA to evaluate the expression of 93 key genes selected from full-transcriptome analysis and involved in the skin response to micro-organisms.*

For each bacterial species selected to infect RHE, a systematic methodology was applied. According to their characteristics and their metabolism, the protocols of culture, infection, harvesting and counting of CFU were optimized. The tissue response to infection was studied after a period of growth of the microorganism on the top of the RHE. For each species, we established a transcriptomic signature following infection. These expression profiles allowed us to identify multiple response genes shared for all micro-organisms and some specific to each of them. These genes are most often related to the antimicrobial response, inflammation and skin barrier (e.g. *CXCL8*, *DEFB4*, *IL1*, *HBEGF*, *S100A7*, *CLDN17*, etc.). The relevance and reliability of these infection biomarkers was then validated by qPCR. For some of them, a quantification by ELISA was performed to confirm that the changes in mRNA expression also affect protein abundance (e.g. IL-8, TNF-α, HßD2).

Nevertheless, it should be noted that the development of each model was subject to adaptation of culture protocols, CFU enumeration, infection conditions of RHE, kinetics, recovery using swabs, *etc*. The data related to the four different infection models will be presented into the Results section.

Results.

Staphylococcus epidermidis

S. epidermidis is one of the most abundant bacterial species in the skin microbiota. It is a Gram-positive, facultative anaerobe that constitutes up to 90% of the resident aerobic flora. Some strains have been associated with healthy skin[1,2]. Indeed, it has been shown that S. epidermidis can benefit the skin in different ways. On one hand, it can produce lantibiotics, which are AMPs (Antimicrobial peptides) containing lanthionine, also called bacteriocins[3] that directly inhibit pathogenic microorganisms[4]. On the other hand, the recognition of *S. epidermidis* lipoteichoic acids modulate inflammation via a cross-communication between TLR2 and TLR3[5]. Activation of TLR2 stimulates tight junction expression and limits the production of pro-inflammatory cytokines via TLR3 in cultured keratinocytes[5,6]. This induces a better detection and a more efficient response to pathogens by the keratinocytes [7]. Microbiome-Friendly skin care products intended to preserve the natural flora of the skin, including the commensal *Staphylococcus epidermidis*. The safetyness of skin care product can be asses by measuring its impact on the growth of *S. epidermidis* in the real context of an *in vitro* 3D reconstructed epidermis.

Colonization of RHE with S. epidermidis

RHE were reconstruced from primary normal human keratinocytes (NHEK). At the end of their reconstruction, we laided on the *stratum corneum* a quantity of 10^3 colony forming units (CFU) /RHE of an *S. epidermidis* strain (ATCC 12228). As a growth control, bacteria were applied in the absence or presence of a molecule known for its activating effect on this bacterium. After 24h of culture, all bacteria were harvested and the bacterial growth was evaluated by counting CFU (colony forming units) on agar media. This count allowed us to observe a bacterial growth up to 10^7 CFU in the absence of activator, against a bacterial growth up to 10^8 CFU with the activator (**Figure 2A**). These results confirm the survival and growth of the *S. epidermidis* strain on the RHE, and the possibility to activate its growth with a specific activator.

From a histological point of view, we did not notice any profound change in the morphology of the epidermis colonized by the bacteria (**Figure 2B**).

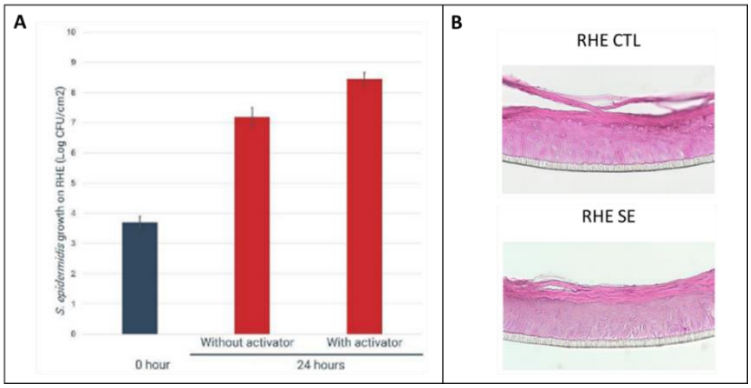

Figure 2: *Colonization of RHE with S. epidermidis.* ***A)*** *Growth of S. epidermidis on RHE by C.F.U.(Colony Forming Unit) counting. Positive control: S. epidermidis growth activator.* ***B)*** *Morphological analysis of RHE± S. epidermidis (SE) after Hemalun/Eosin staining*

RHE response to S. epidermidis

To evaluate the RHE response to *S. epidermidis*, RNA were extracted from RHE colonized or not by *S. epidermidis*. After retro-transcription the cDNA were amplified with *StratiCELL "Skin Response to Microorganisms" TLDA* **(Figure 3)**.

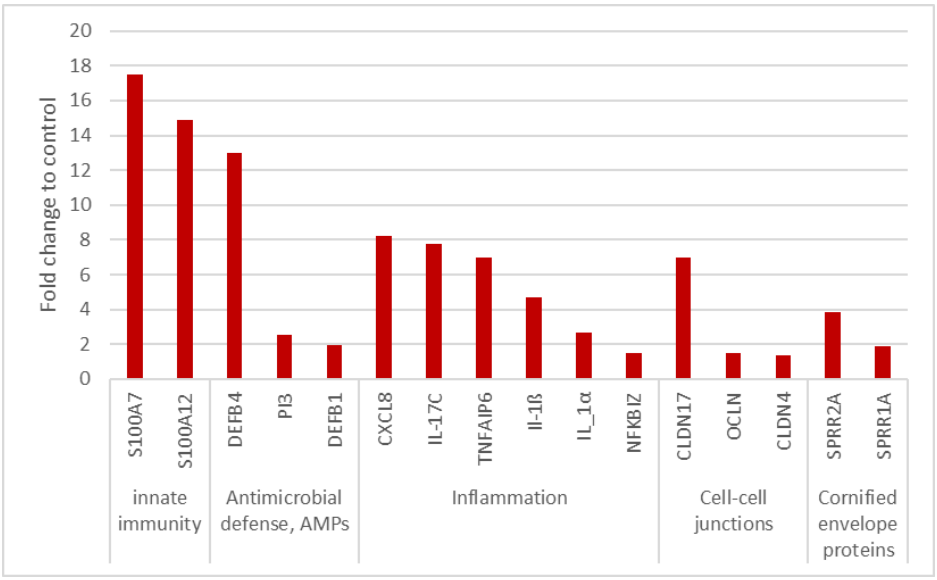

Figure 3: *Analysis of 93 genes selected for their key role in three processes related to bacterial infection (inflammation, innate immunity response, and skin barrier structure and homeostasis) with StratiCELL proprietary TLDA "Skin Response to Microorganisms". These 93 genes expresions were evaluated in human reconstructed epidermis colonized by S. epidermidis during 24h. Change in gene expresion are expressed as fold change to uncolonized control reconstructed epidermis.*

The epidermis response to the infection by *Staphylococcus epidermidis* is mainly characterized by a strong increase in the expression of AMPs such as S100A7, S100A12, DEFB4 (encoding HBD2), DEFB1, as well as other proteins known to carry such activity (SPRR2A, HBEGF, SPRR1A, PI3/SKALP). AMPs are part of the innate immune response allowing rapid tissue reaction. AMPs are powerful broad-spectrum natural substances that are able to destabilize and permeabilize the membranes of bacteria. They can also enhance cellular immunity by acting as immunomodulators.

As described in the literature, several genes encoding for pro-inflammatory mediators (IL-8 (CXCL8), IL-1α, IL-1β, IL-17C) are upregulated following *Staphylococcus epidermidis* infection[8–12]. Genes involved in inflammatory pathways are also overexpressed such as NFKBIZ coding for IκBζ, an NFκB modulator, activated in response to stimulation of TLRs by *S. epidermidis* [12]. TNFAIP6 (TSG-6) is also overexpressed. TSG-6 induces the transition of macrophages from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype following an infection [13]. TNIP1 overexpression in response to bacterial infection and subsequent stimulation of TLRs, according to an activation-dependent regulation of the NFκB pathway and p38 kinase. It seems that TNIP1 plays a role in the regulation of innate immunity and homeostasis of the epidermis, by attenuating the signaling pathways and the inflammatory reaction dependent on TLRs, as demonstrated on *Cutibacterium acnes* [14].

Finally, an increase in the expression of genes coding for constituents of cell-cell junctions, such as claudins 17 and 4 and occludin, is detected. These overexpression suggest a reinforcement of the epithelial barrier promoted by *S.epidermidis*.

In summary, infection of RHE by *S.epidermidis* induces an expected and well-documented response. These results confirm the relevance of the reconstructed epidermis as study model to objectivate active agents acting on the cutaneous microbiota.

Staphylococcus aureus

Staphylococcus aureus is a gram-positive bacteria that cause a wide variety of clinical diseases. *S. aureus* is rarely detected in healthy skin, it is an opportunistic strain. Skin diseases that imbalanced immune system signalling and impaired skin barrier often enhanced *Staphylococcus aureus* skin colonization. In atopic dematitis, skin bacterial communities are characterized by increasing abundance of *S. aureus*, leading to reduced diversity compared with the bacterial communities on healthy skin. This dysbiosis is knwon to increase the disease severity [15–17].

Colonization of RHE with S. aureus

1.10^3 CFU/RHE of an *S.aureus* strain were laided on the stratum corneum of reconstructed epidermis for 24h. As a growth control, bacteria were applied in the absence or presence of a molecule known for its inhibitory effect on this bacterium. After 24h of culture, all bacteria were harvested and the bacterial growth was evaluated by counting CFU on agar media. This count allowed us to observe a bacterial growth up to 10^8 CFU/cm^2 in the absence of inhibitor, against a total absence of bacteria in the presence of the inhibitor molecule (**Figure 4A**). These results confirm the survival and growth of the *S.aureus* strain on the RHE, and the possibility of inhibiting this growth with a specific inhibitor.

From a histological point of view, we did not notice any profound change in the morphology of the epidermis colonized by the bacteria. Indeed, after Hemalun/Eosin staining of paraffin-embedded tissues, we observed that the presence of bacteria is limited to the *stratum corneum*, without impact on the morphology of the epidermal layers (**Figure 4B**).

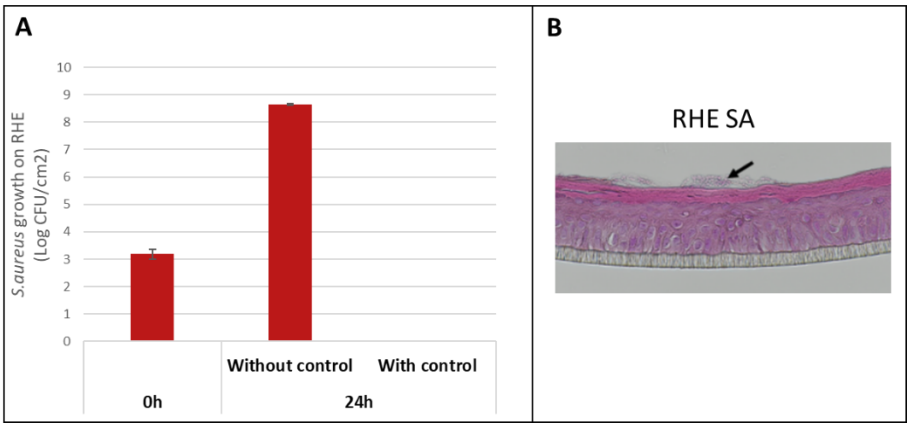

Figure 4: *Colonization of RHE with S. aureus* ***A)*** *Growth of S. aureus on RHE by C.F.U.(Colony Forming Unit) counting. Positive control: S. aureus growth inhibitor.* ***B)*** *Morphological analysis of RHE-SA after Hemalun/Eosin stain*

RHE response to S. aureus

To evaluate the RHE response to *S. aureus,* RNA were extracted from RHE colonized or not by *S. aureus*. After retro-transcription the cDNA were amplified with *StratiCELL "Skin Response to Microorganisms" TLDA* **(Figure 5)**. After statistical analysis of the results obtained with this technology, the most upregulated genes are genes involved in the antmicrobial defences (RNASE7, DEFB4A, CAMP, PI3). We also observed an upregulation of several genes involved in inflammation (CXCL20, IL-17C, IL-1α, IL-1ß and TSLP) and overexpression of AMPs such as S100A7 and S100A12. Upregulation of genes coding for cornified envelop proteins confirm the response of the tissue to the presence of a *S. aureus*.

Finaly, tow genes were sligthy dowregulated, NELL2, biomarker of Atopic Dermatitis and MMP10 involved in the extracelular matrix remodeling.

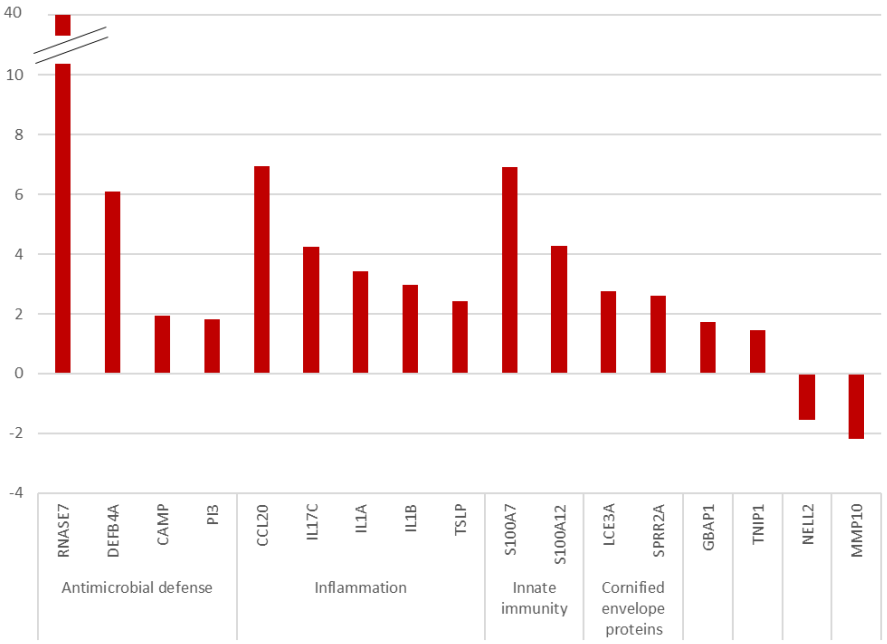

Figure 5: *Analysis of 93 genes selected for their key role in three processes related to bacterial infection (inflammation, innate immunity response, and skin barrier structure and homeostasis) with StratiCELL proprietary TLDA "Skin Response to Microorganisms". These 93 genes expresions were evaluated in human reconstructed epidermis colonized by S. aureus during 24h. Change in gene expresion are expressed as fold change to uncolonized control reconstructed epidermis.*

Cutibacterium acnes

While it was long thought that acne was due to the presence of a bacterium named at this time *Propionibacterium acnes*, it is now admitted that the development of acne spots is related to an imbalance in the phylotypes (phylogenetically related individuals) of this bacterium now renamed *Cutibacterium acnes*. The pathophysiology of acne is indeed the result of an increase in the IA1 phylotype of *C. acnes*, in sebum-rich areas [18]. The hyperseborrhea combined to the hyperkeratinization form a lipid-rich anaerobic environment that favours the growth of *C. acnes*[19]. Additionally, acne-prone skins also display an overexpression of genes encoding pro-inflammatory molecules such as interleukin 8 (IL-8, encoded by the CXCL-8 gene) and interleukin 1 beta (IL-1β, encoded by the IL1B gene), as well as antimicrobial peptides like the human β-defensin 2 (hBD2, encoded by the DEFB4 gene)[19,20]. All these factors lead to the appearance of uncomfortable acne breakouts in susceptible individuals. The population most affected by acne is the prepubertal population at 90%, even though acne is also found in 30% of adults [21]. The prolonged wearing of masks

during the pandemic further increases these percentages with the emergence of the new phenomenon of maskne, a contraction of "mask" and "acne".

Assuming that the cause was bacterial, several antibiotic treatments exist on the market to cure acne. However, in addition to the problem of bacterial resistance to antibiotics, it is now known that antibiotics will also impact the growth of commensal bacteria, thus slowing down the restoration of a balanced microflora. Retinoid derivatives such as isotretinoin or benzoyl peroxide have also been prescribed to reduce acne spots, but their adverse side effects reduce their use and possible treatments [21,22]. Therefore, it appears important to find new topical acne treatment solutions that can restore a balanced skin flora without undesired effects. Here, is presented the model of RHE colonized with a IA1 phylotype strain of *C. acnes*.

Colonization of RHE with C. acnes

For the first step in the development of this new model, we reconstructed epidermis from primary normal human keratinocytes (NHEK). At the end of their reconstruction, we laid on the *stratum corneum* a quantity of 100.000 colony forming units (CFU) per cm^2 of an acneic phylotype IA1 *C. acnes* reference strain in a lipid mixture representative of the ecological niche of this lipophilic bacterium. As a growth control, bacteria were applied in the absence or presence of a molecule known for its inhibitory effect on this bacterium. After 72h of culture, all bacteria were harvested and the bacterial growth was evaluated by counting CFU on agar media under anaerobic culture conditions. This count allowed us to observe a bacterial growth up to 10^7 CFU/cm^2 in the absence of inhibitor, against a total absence of bacteria in the presence of the inhibitor molecule (**Figure 6A**). These results confirm the survival and growth of the *C. acnes* strain on the RHE, and the possibility of inhibiting this growth with a specific inhibitor.

As demonstrated by colony counting, the bacteria showed 2-logs positive growth on the *stratum corneum* of RHE in three days. The addition of a growth inhibitor confirmed these observations, allowing the inhibitor to be used as a reference to compare the performance of potential anti-acne molecules.

From a histological point of view, we did not notice any profound change in the morphology of the epidermis colonized by the bacteria. Indeed, after Hemalun/Eosin staining of paraffin-embedded tissues, we observed that the presence of bacteria is limited to the *stratum corneum*, without impact on the morphology of the epidermal layers (**Figure 6B**).

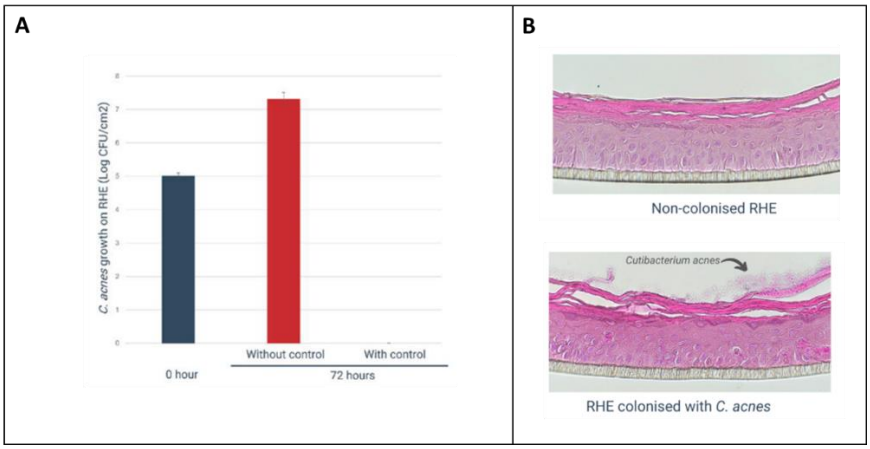

Figure 6: *Colonization of RHE with C. acnes.* ***A)*** *Growth of C. acnes on RHE by C.F.U.(Colony Forming Unit) counting. Positive control: C. acnes growth inhibitor.* ***B)*** *Morphological analysis of RHE-C.acnes after Hemalun/Eosin staining.*

RHE response to C. acnes

In order to demonstrate that the presence of bacterial elements on reconstructed epidermis induces a response similar to that described in the literature [19,20]. The monitoring of three biomarkers CXCL-8, IL-1β and DEFB4 has been assesd and revealed a significant increase in the expression of these genes in the presence of *C. acnes* (**Figure 7**).

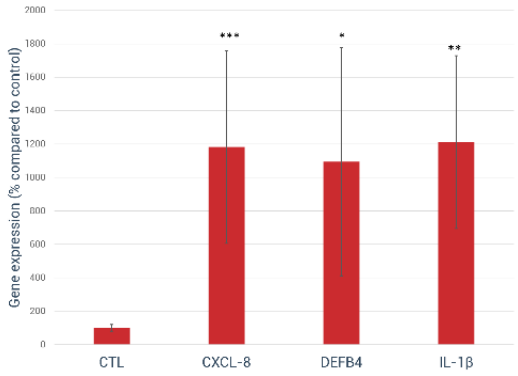

Figure 7: *Gene expression analysis by RT-qPCR. Histogram showing the percentage of CXCL-8, IL-1β and DEFB4 gene expression in reconstructed human epidermis colonised with Cutibacterium acnes compared to non-colonised control reconstructed epidermis (CTL).*

To measure and compare the simultaneous expression of these 93 genes, we amplified retro-transcripted RNA extracted from RHE colonized or not by *C. acnes* using a microplate-based quantitative PCR system known as “TaqMan Low Density Array” (TLDA). After statistical analysis of the results obtained with this technology, we observed an upregulation of several genes involved in inflammation and immune defences. In parallel, we also observed changes

in the expression of some genes involved in the structure and homeostasis of the skin barrier (**Figure 8**), confirming a response of the tissue to the presence of a *C. acnes* phylotype IA1 strain.

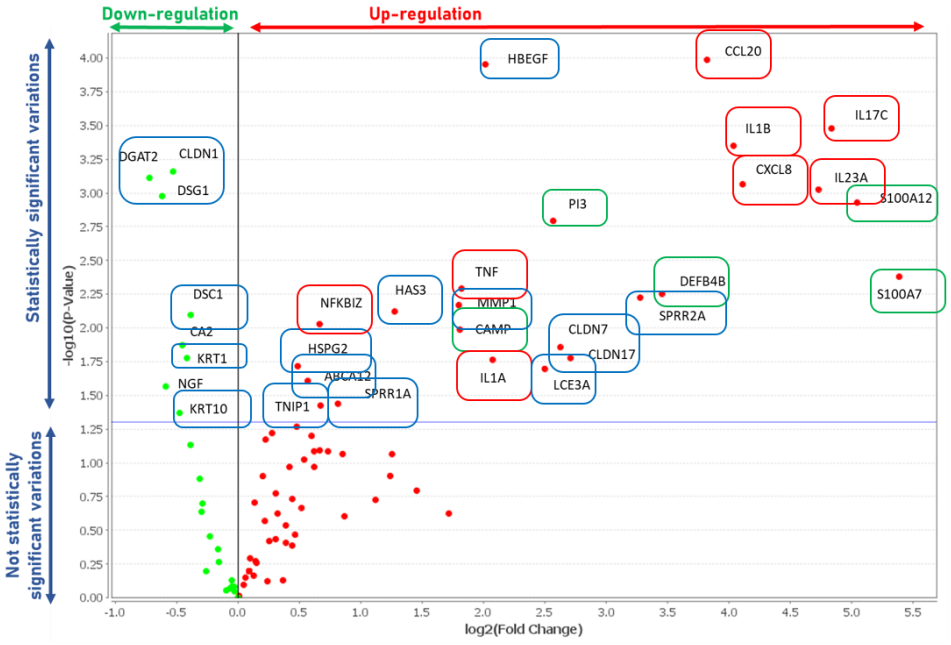

Figure 8: *Gene expression analysis by TaqMan Low Density Array (TLDA) technology. Volcano Plot obtained by comparison of 93 gene expression in non-colonised versus Cutibacterium acnes-colonised reconstructed human epidermis, after total RNA extraction, reverse transcription and amplification using StratiCELL's TLDA referred as "Skin Response to Microorganisms". Y axis: statistically significant gene expression variations defined as Log10 of the p-value set at 0.05. X axis: gene expression regulation defined as the Log2 of fold change expression set at 1. Genes involved in the skin inflammation are framed in red. Genes involved in the innate immunity are framed in green. Genes involved in the skin barrier are framed in blue.*

Malassezia furfur

Malassezia furfur is a lipid-dependent yeast naturally living on the skin. Despite its tolerance by the immune system under healthy conditions, *M. furfur* overgrowth is associated with skin disorders such as seborrheic dermatitis (SD), an inflammatory condition affecting oily rich areas and leading to dandruff and pruritic lesions. In dandruffs, the quantity of *Malassezia* can increase up to 1.5 to 2 times its normal rate [23]. This huge spread of yeast highly invades the epidermis, with detrimental consequences on the skin barrier function. It is known that disruption of the epidermal barrier increases microbial invasion and triggers both innate and acquired immune response [24]. Keratinocytes are playing a central role in this response by

secreting key signaling molecules like defensins and antimicrobial peptides to overcome microbial 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 innate defensins and pro-inflammatory cytokines in response to infection [25–27].

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 its capacity of multiplication. Recently, KTZ has demonstrated its efficacy to reduce *Malassezia* species in SD, while restoring the skin microbial communities [28].

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.

Colonization of RHE with M. furfur

For the development of this new 3D model, human epidermis were infected with *M. furfur* in a lipid mixture representative of the ecological niche of this lipophilic yeast. As a growth control, the yeast was applied in the absence or presence of Ketoconazole (KTZ). After 3 days of culture, yeast were harvested and plated on an adapted solid microbiological media for the counting of colonies forming units (CFU). A 2-fold growth was counted in the absence of antimycotic agent. However, in the presence of KTZ, the growth was reduced to a 1.5-fold level (**Figure 9**). These results confirm the survival and growth of *M. furfur* on the RHE, and the efficacy of KTZ to effectively reduce this multiplication.

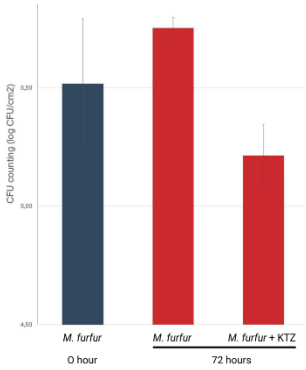

Figure 9: *Growth of M. furfur on RHE by C.F.U.(Colony Forming Unit) counting. Positive control: C. Ketoconazole (KTZ).*

From a histological point of view, a Periodic Acid Schiff (PAS) staining of paraffin-embedded RHE demonstrated that after 3 days, the invasion of *M. furfur* passes the *stratum corneum* of the RHE to reach the first epidermal layers and induce a disruption of the barrier (**Figure 10A**). In order to evaluate the barrier disruption, a transepidermal Lucifer Yellow (LY) dye diffusion assay was performed on colonized RHE. Resulting fluorescent images (**Figure 10B**) as well as quantification of LY dye in the culture media (**Figure 10C**) confirmed a disruption of the outside/inside epidermal barrier by *M. furfur*. Treatment with KTZ however, could reduce the quantity of LY in the culture media, confirming its efficacy to limit the spread of the yeast thoughout the epidermal layers.

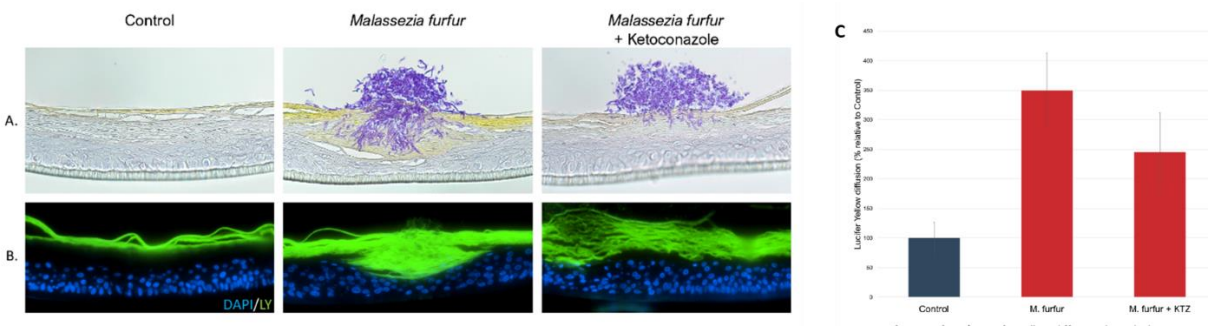

Figure 10: *Representative images of reconstructed human epidermis colonized by Malassezia furfur or not (Control), in the presence of Ketoconazole (KTZ) or not.* ***A)*** *Periodic Acid Schiff (PAS) staining of paraffin-embedded epidermis.* ***B)*** *Lucifer Yellow (LY) fluorescence after out/in epidermal barrier diffusion assay.* ***C)*** *Quantification of out/in Lucifer Yellow diffusion through the stratum corneum of reconstructed human epidermis colonized by Malassezia furfur or not (Control), in the presence of Ketoconazole (KTZ) or not.*

RHE response to M. furfur

The observation of the disrupted barrier leads 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 11**) 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 matrix metalloproteins MMP-1 and -9 involved in the turnover of those structure components were upregulated. Regarding the expression of inflammatory genes, we observed that the expression of the interleukins IL-1-alpha, IL-1-beta, IL-8, and IL-23A genes were upregulated around 200-fold time. Same, the expression fold change of defensin genes like the Human Defensin DEFB4A gene, or the antimicrobial psoriasin S100A7 gene were around

200 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.

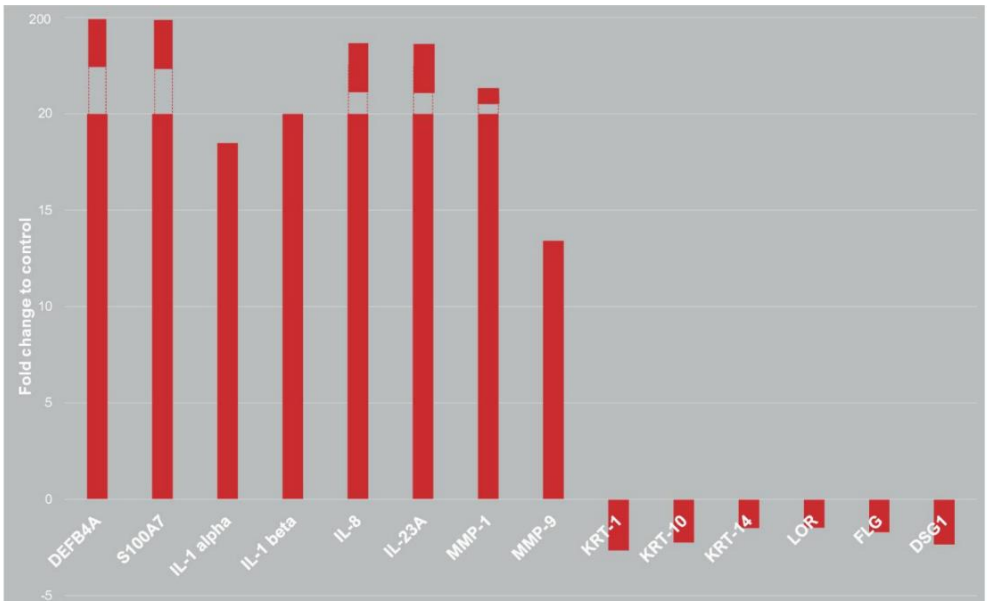

Figure 11: *Analysis of inflammatory, innate immunity and epidermal genes differentially expressed in human reconstructed epidermis colonized by Malassezia furfur during 3 days. Changes in gene expression are expressed as fold to uncolonized control reconstructed epidermis. DEFB4A: Human Beta Defensin 4A - DSG1: Desmoglein 1 - FLG: Filaggrin - IL's: Interleukins - KRT's: Keratins - LOR: Loricrin - MMP's: Matrix Metallo Proteinases - S100A7: Psoriasin.*

In order to support those transcriptomic data, we decided to quantify the release of some of the expressed immune proteins. Two specific ELISA assays measuring the Human Beta Defensin HBD2 (encoded by the gene DEFB4A) and the IL-8 cytokines were used to define the respective level of those proteins in the culture media of RHE colonized with *M. furfur*, in the presence or absence of KTZ. In agreement with the previously obtained RT-qPCR results, ELISA assays demonstrated that *M. furfur* significantly induces the secretion of HBD2 and IL-8 proteins in the culture media, in a KTZ-limiting way (**Figure 12**). These observations confirmed that data obtained at the gene expression level are consistent with protein secretions by reconstructed epidermis.

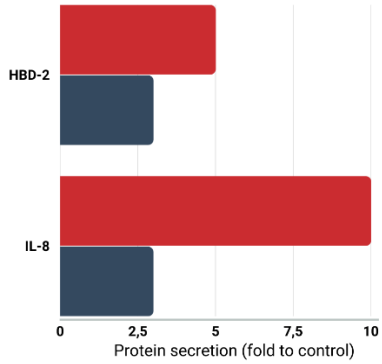

Figure 12: *Quantification by ELISA of proteins differentially secreted in the cell culture media of reconstructed epidermis colonized by Malassezia furfur during 3 days, in the presence (blue bars) or absence (red bars) of Ketoconazole. Changes in protein secretion is expressed as fold to uncolonized control reconstructed epidermis. HBD-2: Human Beta Defensin 2 (encoded by the DEFB4A gene) - IL-8: Interleukin 8.*

Discussion and Conclusion.

In this paper, we described the completion of four *in vitro* 3D models within our *MicroBIOS Platform*. These models allow to study the impact of living bacterial strains (*Staphylococcus aureus, Staphylococcus epidermidis, Cutibacterium acnes)* and yeast (*Malassezia furfur*) on RHE.

These four models allow to study both the survival of a microorganism on the surface of the epidermis, and the responses of the skin to the presence of this microorganism. In a context of dermo-cosmetic compounds targeting the skin flora exploration, this double approach enables to investigate the potential effects on the microorganism itself, as well as the response of the tissue to this infection. Those different available *in vitro* 3D models and associated tests offer a promising tool to speed up the research, understanding and objectivation of innovative dermo-cosmetic actives that target the skin flora.

In conclusion, the infection of *in vitro* reconstituted epidermis by microorganisms constitutes an essential tool to approach the conditions of *in vivo* infection, allowing among other things the inoculation of strains in a topical way. The technical challenge is the adaptation of the different models to the specificities of the microorganisms of interest.

Acknowledgments.

This work is financed by Service Public Wallonie (SPW) through grant *Microsbios-8000*

Conflict of Interest Statement. NONE.

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