Malassezia interaction with a reconstructed human epidermis: imaging studies

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ABSTRACT

Background: Biofilm formation represents a major microbial virulence attribute especially at epithelial surfaces such as the skin. *Malassezia* biofilm formation at the skin surface has not yet been addressed.

Objective: The present study aimed to evaluate *Malassezia* interaction with a reconstructed human epidermis (RhE) model.

Methods: *Malassezia* clinical isolates were previously isolated from volunteers with pityriasis versicolor and seborrheic dermatitis. Yeasts of two strains of *M. furfur* and *M. sympodialis* were inoculated onto the SkinEthic™ RHE. The tissues were processed for light microscopy, wide-field fluorescence microscopy and scanning-electron microscopy.

Results: Colonization of the RhE surface with aggregates of *Malassezia* yeasts entrapped in a multilayer sheet with variable amount of extracellular matrix was unveiled by imaging techniques following 24, 48, 72 and 96 hours of incubation. Whenever yeasts were suspended in RPMI medium supplemented with lipids, the biofilm substantially increased with a dense extracellular matrix in which the yeast cells were embedded (not seen in control samples). Slight differences were found in the biofilm architectural structure between the two tested species.

Conclusion: Skin isolates of *M. furfur* and *M. sympodialis* were capable of forming biofilm *in vitro* at the epidermal surface simulating *in vivo* conditions. Following 24 hours of incubation, without added lipids, rudimental matrix was barely visible, conversely to the reported at plastic surfaces. The amount of biofilm apparently increased progressively from 48 to 96 hours. A structural heterogeneity of biofilm between species was found with higher entrapment by a denser and more gelatinous extracellular matrix in *M. furfur* biofilm.

INTRODUCTION

Malassezia yeasts are lipophilic organisms, members of the cutaneous microbiome, [1] despite being often involved in numerous skin diseases, such as pityriasis versicolor and seborrheic dermatitis.**[2]**

Most microorganisms live and survive in special communities called biofilms, especially at epithelial surfaces including the skin. Such biofilm accounts as a major microbial virulence attribute.[3] Biofilms consist in sessile microbial cells encased in an extracellular matrix (ECM) displaying a well-developed communication system that enables regulation of microbial growth and metabolism, which also confers promoted resistance to antimicrobials, to host inflammatory cells and to immune response. These communities attach to biotic and abiotic surfaces and exhibit altered phenotypes with promoted growth features comparing to planktonic counterparts.[3, 4] The composition of fungal biofilm ECM is complex and usually comprises polysaccharides, proteins, extracellular DNA and even host produced factors[5, 6] conferring structural support and protection from host response through informative, redox-active and nutritive roles.[5, 7]

Malassezia biofilm formation has been demonstrated *in vitro* for some species at abiotic surfaces; *M. pachydermatis* isolates from healthy dogs and dogs with seborrheic dermatitis were able to form biofilm at 48, 72 and 96 hours in plastic material.[8] Mature *M. pachydermatis* biofilms were however rudimental consisting of unipolar budding yeasts with collarets and ECM without hyphae.[9] More recently, Angiolella *et al*[10] described biofilm formation by *M. furfur* clinical isolates at the surface of polyurethane material following 24 and 48 hours with no evidence of hyphae formation. To date, there is no description of *Malassezia* biofilm formation at the surface of a reconstructed human epidermis (RhE) model simulating *in vivo* conditions. The aim of

the present study includes the analysis, by imaging techniques, of biofilm formation by *Malassezia* clinical isolates at the surface of a RhE.

MATERIALS AND METHODS

Yeast isolation and identification

Malassezia clinical isolates previously retrieved from the skin of patients with pityriasis versicolor and seborrheic dermatitis at the Dermatovenereology Department of the University Hospital *Centro Hospitalar Universitário de São João EPE*, Porto, Portugal during a prospective clinical study [11] were selected. The study was approved by the Institutional Board, namely the Ethics Committee for Health of *Centro Hospitalar Universitário de São João EPE*, Porto, Portugal. Oral explanation about the study and time for reading the detailed information provided were given to each participant. All participants signed an informed consent. The identity of all isolates was confirmed by PCR-based sequencing using specific primers for amplification of the *ITS-1*, *ITS-2* and *IGS-1* regions.[12, 13]

Growth conditions

The selected isolates were firstly cultured on CHROMagar Malassezia® (CHROMagar, Paris, France) at 32ºC for 2 to 7 days, and then subcultured on Leeming-Notman Agar (LNA) at 34ºC for 72 hours. After an overnight culture on Leeming-Notman broth, yeast cells were harvested by centrifugation and washed thrice with phosphate buffered saline (PBS; pH7). A yeast suspension was prepared both in PBS and in modified RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with lipids; a densitometer (DEN-1, Grant Instruments, Cambridge, UK) was used to obtain a final optical density (OD) of 0.5 McFarland. Suspensions were plated onto LNA to assess the number of colony-forming units.

Biofilm assessment on RhE

Two clinical strains, one of *M. furfur* (from seborrheic dermatitis face lesions of a 65-year old male patient) and the other of *M. sympodialis* (from pityriasis versicolor torso lesions of a 37-year old female patient) were selected to be inoculated onto a RhE in order to mimic *in vivo* conditions. Both patients denied topical and/or systemic antifungal administration during the previous year. The species were selected based on the most frequent isolate involved in bloodstream infections for *M. furfur* [14], and the most common isolate retrieved from the skin during a previous prospective clinical study carried out at the Dermatovenereology Department of the University Hospital *Centro Hospitalar Universitário de São João EPE*, Porto, Portugal for *M. sympodialis*.[11] Moreover, *M. furfur* had been previously studied for biofilm formation at an abiotic surface [10] which might be used for comparison with our results at the surface of a RhE. The models used reproduce epidermal morphology and have been fully characterized.[15] Despite being apparently similar, the two types of RhE display differences; EpiSkin™ shows a larger surface area (1.07 cm^2) *versus* 0.5 cm^2 of SkinEthic™ RHE small; it is cultured on a collagen matrix at the air/ liquid interface with serum in the culture media, while SkinEthic™ RHE model is cultured on an inert polycarbonate filter at the air-liquid interface, in a chemically defined medium without serum. Upon arrival, RhE cells were managed according to the manufacturer's instructions. The EpiSkin™ and SkinEthic™ RHE small inserts were removed from the agarose nutrient solution under sterile airflow and rapidly transferred to 12-well and 6-well plates, respectively, previously filled with the provided growth medium without antifungals and incubated at 37°C in a 5% CO2 atmosphere with saturated humidity. In a pilot study, *Malassezia* yeasts (approximately $1x10^4$ cells) suspended in PBS, in a total volume of 50 µL, were inoculated onto the RhE inserts. Control samples were

inoculated with 50 µL of PBS without *Malassezia*. Following 24 and 48 hours of incubation at 37ºC, 5% CO_2, the samples were processed for image acquisition, namely light microscopy stained with hematoxylin and eosin (H&E), wide-field fluorescence microscopy (WFFM) and scanning electron microscopy (SEM). Following this pilot assay, some parameters were optimized, namely the timepoints of incubation which were extended to 48, 72 and 96 hours; the inoculum volume was increased to 100 µL to allow an easier uniform cover of the surface area of the SkinEthic RHE small (0.5 cm^2); the number of yeast cells was increased to obtain a standardized suspension of about $1x10^6$ cells/mL (spectrophotometer OD 0.5 McFarland). Two distinct approaches were used for SEM samples: one with yeasts suspended in PBS and the other suspended in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with Tween 60 and oleic acid as recommended by Leong *et al*.[16] For WFFM and light microscopy (H&E) the yeasts were suspended in the abovementioned modified RPMI medium. For each timepoint, control samples were used, namely 100 µL of PBS or of modified RPMI medium without yeasts.

Histological imaging by light microscopy

Samples for histological analysis were initially fixed in 10% (v/v) neutral buffered formalin (Leica Biosystems, Newcastle-upon-Tyne, UK) for 48 hours. Tissues were dehydrated through an increasing alcohol concentration series and then included in paraffin wax. Sections (20 µm) were placed on microscope slides and de-waxed in xylene, with subsequent rehydration using water, and afterwards stained with H&E.

Wide-field fluorescence microscopy analysis

Samples were fixed with room temperature methanol for 30 minutes and afterwards stained, separately for 5 minutes, with 10% calcofluor white (Sigma-Aldrich, St. Louis, MO), a compound that binds to cellulose and chitin in fungal cell walls

followed by 50 µg/mL fluorescein isothiocyanate-concanavalin A (FITC-ConA; Sigma-Aldrich, St. Louis, MO), which has affinity for polysaccharides, specifically for α-D-mannosyl and α-D-glucosyl residues found in the ECM of biofilms. These two fluorochromes exhibit distinct spectral properties and quantum yield potentially enabling the simultaneous staining of the yeasts (cell wall rich in chitin) and of the ECM (rich in carbohydrates). After washing with PBS, the RhE inserts were mounted on glass slides with 20 mM Tris pH 8, 0.5 n-propyl-gallate, 90% glycerol, as previously reported.[17] Image acquisition (0.24 µm thick z-stacks) was performed with a Zeiss AxioObserver Z1 wide-field microscope equipped with a planapochromatic (1.46 NA 100x; 1.4 NA 60x) DIC objective and a cooled CCD (Hamamatsu Orca R2). All images show maximum intensity projections. Image analysis was performed in Fiji (ImageJ) and Adobe Photoshop CS4 was used for histogram adjustments and panel assembly.

Scanning electron microscopy (SEM) imaging

Samples (in duplicate) were fixed with glutaraldehyde 1.2% in sodium cacodylate 0.1 M for 1 hour, washed thrice with sodium cacodylate 0.1 M for 10 minutes and postfixed with osmium tetroxide 1% in sodium cacodylate 0.1 M for 1 hour. After removing the osmium tetroxide solution and rinsing twice with bidistilled water, the samples were gradually dehydrated by means of an ethanol series and dried overnight. For analysis, samples were coated with gold/palladium (40%/60%) by sputtering using SPI Module Sputter Coater and observed in a scanning electron microscope (Quanta 400FEG ESEM/ EDAX Genesis X4M) in high vacuum mode.

RESULTS

Pilot study

Initially, a pilot study was conducted with two types of RhE, EpiSkin™ and SkinEthic™ RHE small (Episkin, Lyon, France), in order to assess the best substrate to carry on subsequent studies.

The preliminary results unveiled that *Malassezia* colonization at the surface of the RhE (EpiSkin™ and SkinEthic™ RHE small, aged 17 days) involved aggregates of yeasts and an incipient ECM following 24 hours of incubation; SEM showed yeast adherence to the RhE surface and attachment to each other, embedded within an ECM which is compatible with biofilm formation at the RhE surface, by both *Malassezia* species. At 48 hours a slight increase in the number of microorganisms was noticeable with expansion of the communities of surface-associated cells enclosed in a thicker matrix.

The ability of both *M. furfur* and *M. sympodialis* clinical isolates to colonize the RhE surface forming biofilm was demonstrated in this pilot study. Its three-dimensional structure was evident by SEM; incipient ECM formation was found for both species, but more notable in case of *M. furfur*, soon after 24 hours of incubation (Fig. 1).

Following these preliminary results, SkinEthic™ RHE small (aged 12 days) was selected to proceed with the experiments to confirm and expand the results of the pilot study, since growth and adherence of *Malassezia* clinical isolates were higher in SkinEthic™ comparing to EpiSkin.™

Biofilm imaging

Colonization of the RhE surface with aggregates of *Malassezia* cells entrapped and embedded in a multilayer sheet with variable amounts of ECM was unveiled by light microscopy, WFFM and SEM following 48, 72 and 96 hours of incubation confirming biofilm formation at the surface of SkinEthic RHE® small. At earlier stages of incubation, yeasts exhibiting a tendency to aggregate in clusters were visible at the

surface of the stratum corneum by light microscopy (Fig.2); wide-field images demonstrated the extracellular polysaccharides surrounding the budding yeast aggregates (Fig.3); SEM unveiled a very rudimental ECM formation in the samples with yeasts suspended in PBS compared to a thicker and more elaborated ECM in case of yeast cells suspended in modified RPMI medium (Fig.4). Over time a notable increase of yeast aggregates and ECM density was apparent, especially whenever yeasts were suspended in modified RPMI medium (Fig.5). The elemental analysis of the ECM, performed by the scanning electron microscope functionality, revealed more calcium and less sodium compared to the yeast surface, no nitrogen was present in this amorphous structure (Fig.6), which was not present in the control samples. Interestingly, there was an apparent slight difference in the biofilm architectural structure between the two tested species, with *M. furfur* exhibiting an apparently more intricate and gelatinous ECM visible by SEM (Fig.5). No hyphae formation was ever detected with the three imaging techniques used.

DISCUSSION

Skin biofilms have been associated with several dermatological diseases such as acne, rosacea and atopic dermatitis.[3] To the best of our knowledge, and although *Malassezia* organisms have been implicated in numerous skin diseases, its ability to form biofilm at the skin surface has not yet been demonstrated.

There have been very few previous studies examining the ability of *Malassezia* yeasts to form biofilms *in vitro* at abiotic surfaces.[8, 10, 18] The present study unveils that skin isolates of *M. furfur* and *M. sympodialis* were capable of forming biofilm at the surface of a RhE model simulating *in vivo* conditions. During the pilot study, we have demonstrated the ability of both *M. furfur* and *M. sympodialis* to colonize and

form biofilm at the RhE surface. A difference in the biofilm architectural structure between the two tested species showed that *M. furfur* exhibited a higher entrapment by a denser and more viscous extracellular matrix.

The amount of *M. furfur* and *M. sympodialis* biofilm apparently increased progressively from 48 to 96 hours, in contrast to what was previously reported about *M. pachydermatis* whose ability to form biofilm on polystyrene material was maximal at 72 hours, with the amount decreasing at 96 hours.[8] Figueredo *et al* [18] found that *M. pachydermatis* strains isolated from dogs with and without skin lesions were able to form biofilm with variable ECM quantity and structure at 72 hours and the authors hypothesized that this likely to be strain-dependent. Notably, our results regarded *M. furfur* and *M. sympodialis* human skin isolates being both lipid-dependent species, conversely to *M. pachydermatis* [19], which might influence the results of biofilm formation and establishment. A structural heterogeneity of biofilm between *M. furfur* and *M. sympodialis* isolates was found , with both species exhibiting yeast aggregates in multi-layer clusters but with a denser entrapment by a more gelatinous ECM in case of *M. furfur* biofilm. Conversely to our pilot study findings on a RhE substrate, *M. furfur* biofilm formation on plastic material was reported to produce no ECM during the first 24 hours, but with an abundant ECM after 48 hours of incubation. [10] In fact, at the surface of the epidermal model, we have found rudimental ECM following 24 hours of incubation, meaning a possible faster formation of biofilm at the skin surface *versus* an abiotic surface. This finding might be related to the particular features of the biological substrate used, SkinEthic^{TM} RHE small, which contains fatty acids [20], most probably supporting an easier and faster biofilm formation, as may occur *in vivo*.

Hyphae produced by *Malassezia* are sometimes observed *in vivo* in individuals with hyperactive sebaceous gland activity, since excessive sebum seems to induce

hyphae formation.[21, 22] Although one study reported hyphae induction *in vitro* using a specific culture medium with glycine,[23] we did not observe hyphae structure with the three employed imaging techniques, even though the modified RPMI 1640 medium contains glycine and other nutrients that promote yeast growth. Considering that the used epidermal model mimics *in vivo* conditions with stratum corneum constitutive lipids,[20] one possible explanation for the absence of hyphae formation might be related to the amount and type of lipids added to the RPMI 1640 medium not perfectly reproducing the human sebaceous gland produced lipids.[24]

The limitations of the present study included the use of only two clinical isolates from two distinct species to test possible biofilm formation at the surface of the RhE. For the abovementioned reasons, it seemed reasonable that these two species were tested in a first study, but in the future other species and more clinical isolates should be included in further investigations. Another limitation was related to the absence of a biofilm quantification assessment which should be addressed in future studies. Nevertheless, this study remains pioneer in illustrating by imaging the biofilm formation of *M. furfur* and *M. sympodialis* clinical isolates at the surface of a RhE in qualitative terms.

Malassezia-related skin diseases, particularly pityriasis versicolor and seborrheic dermatitis represent an important clinical and economic burden with relevant impact in patients' quality of life and self-esteem.[25] The recurrent character of these skin diseases despite long-term use of topical and systemic antifungals [26] stresses the need to better understand the mechanisms of pathogenicity of these yeasts, also inhabitants of the skin microbiome.[27] The inappropriate use of antifungals, in particular at sub-minimal inhibitory concentrations, may enhance biofilm formation and may promote hypothetical emergence of antifungal resistance. Notably, our findings might prompt a

shift towards a more targeted therapeutic approach in case of *Malassezia*-related skin diseases based on the understanding of biofilm structure and organization, which might be more complex than previously reported for other non-lipid dependent species.[9] Although SkinEthic™ RHE small contains different epidermal classes of lipids comprising ceramides,[20] *M. furfur* and *M. sympodialis* biofilm highly increased in amount whenever lipids were added to the yeast suspension, which supports that clinical approaches to reduce seborrhea[28] might affect the development of biofilm.

In summary, results from this study represent an essential approach to the formation of *M. furfur* and *M. sympodialis* biofilm as a virulence factor. A better elucidation of the virulence attributes exhibited by *Malassezia* species, namely the characterization of its biofilm particularities and its susceptibility profile to antifungals might further contribute to the disclosure of clinical associations and therapeutic targets.

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FIGURES

Figure 1: Pilot study; SEM images following 24 hours of incubation. (A) *Malassezia sympodialis* and (B) *Malassezia furfur* yeast aggregates at the surface of the SkinEthic™ RHE exhibiting rudimental extracellular matrix formation.

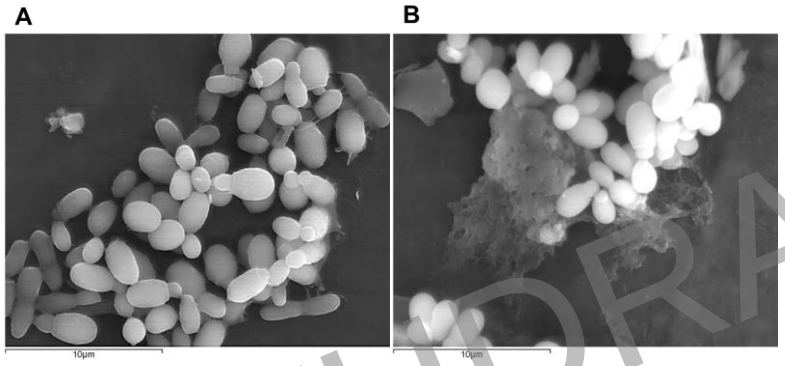

Figure 2: Light microscopy (H&E) images of the SkinEthic™ RHE colonized with (A) *M. sympodialis* at 48 hours and (B) *M. furfur* at 96 hours of incubation; the latter showing yeast aggregates on the top of the stratum corneum clustered in an eosinophilic amorphous structure.

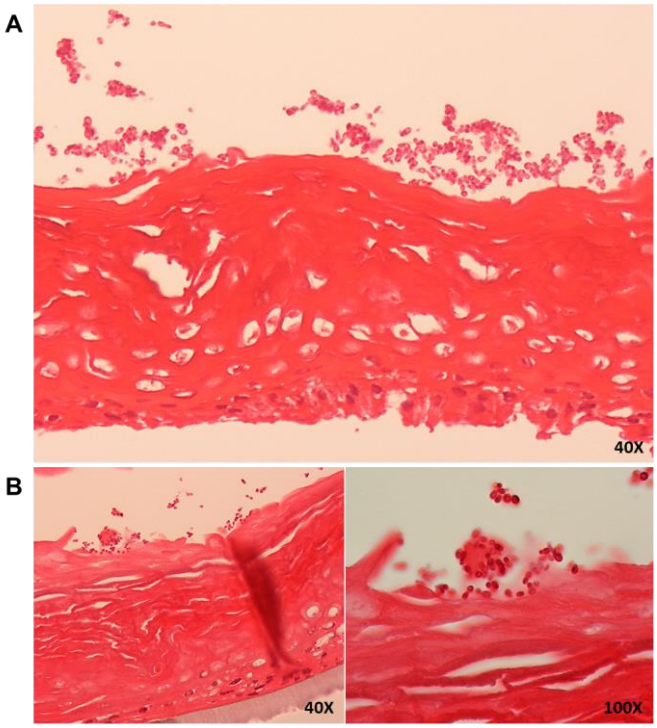

Figure 3: Representative images obtained following 48, 72 and 96 hours incubation of SkinEthic™ RHE with *M. furfur* and *M. sympodialis* on wide-field fluorescence microscopy. Calcofluor white staining is represented in blue and FITC-ConA staining in red. Scale bar: 20µm.

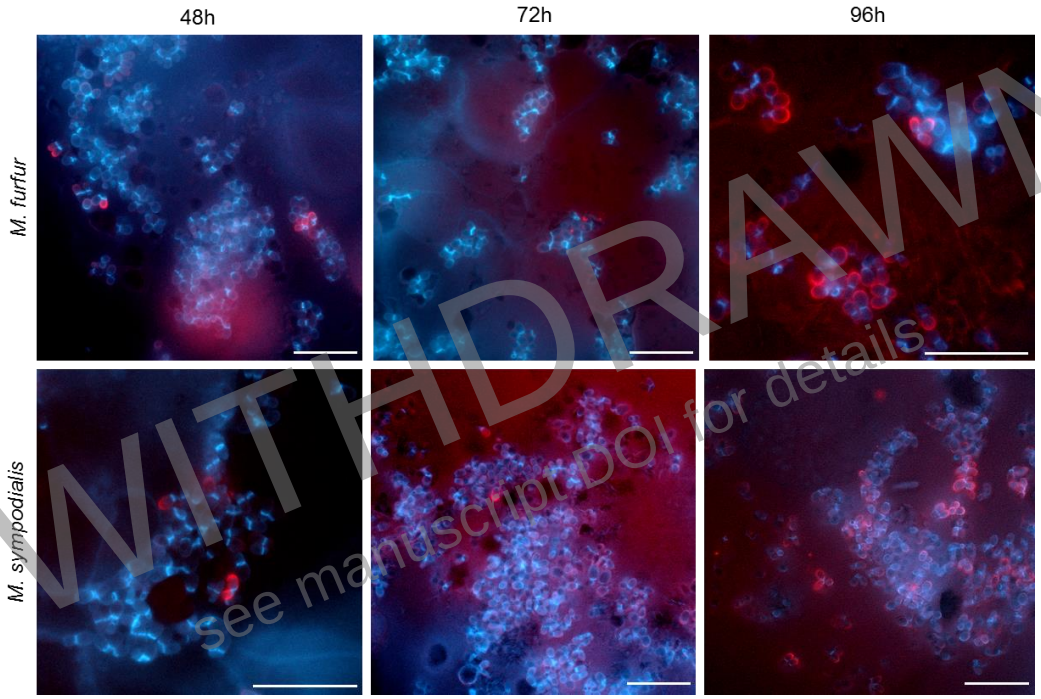

Figure 4: Representative SEM images of (A) *M. furfur* and (B) *M. sympodialis* biofilm at 48 hours of incubation (yeast suspension in PBS); (C,D) *M. sympodialis* biofilm at 72 hours of incubation (yeast suspension in modified RPMI medium).

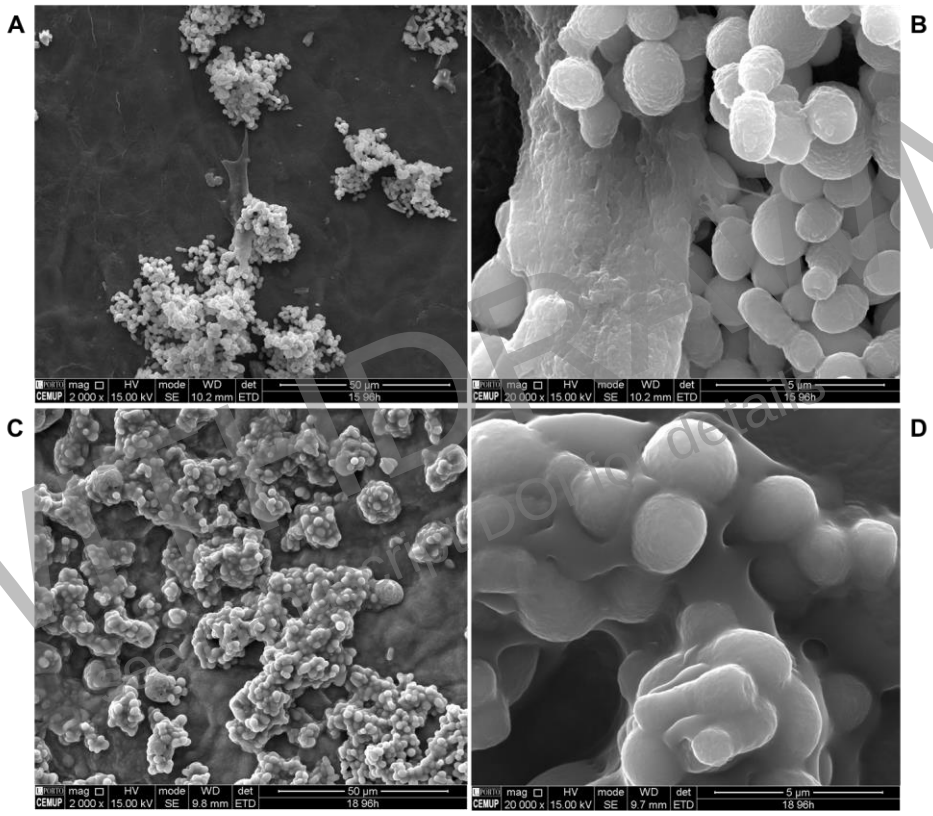

Figure 5: Representative SEM images of *Malassezia furfur* biofilm at 96 hours of incubation; yeast suspension in PBS (A,B) and in modified RPMI medium (C,D).

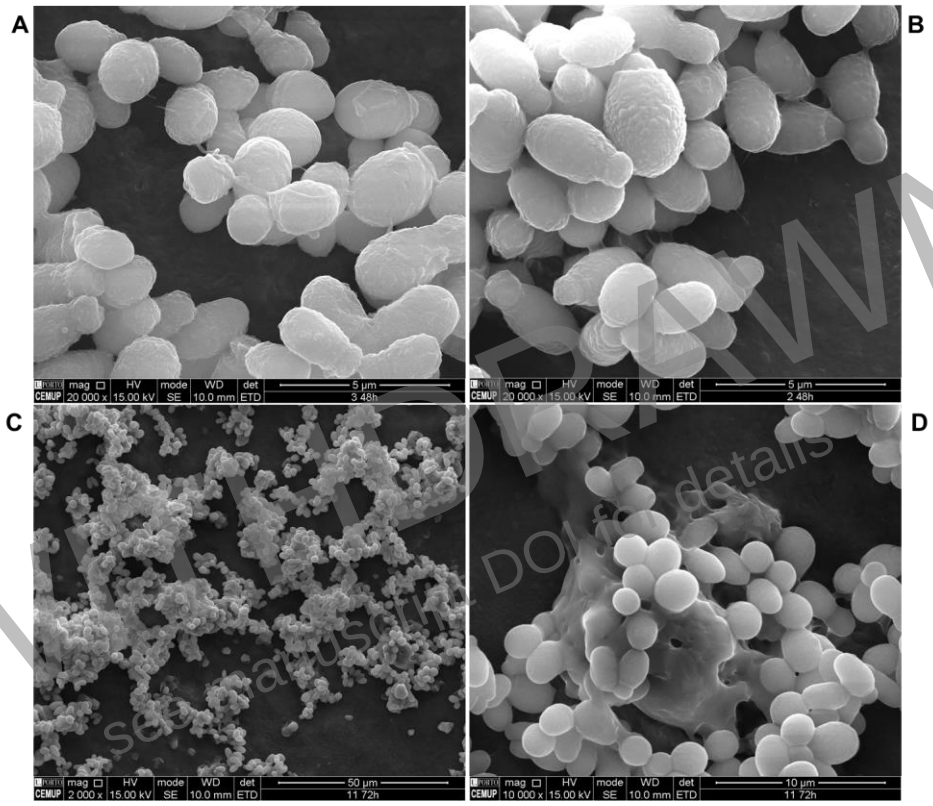

Figure 6: Elemental analysis of the (A) extracellular matrix and of the (B) yeast surface applied to SEM images. C: carbon; N: nitrogen; O: oxygen; Na: sodium; Os: osmium tetroxide; S: sulphur; Cl: chlorine; Ca: calcium.