World's first human *ex-vivo* acne sebaceous gland model colonized with virulent phylotype of *C. acnes*

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Abstract

***Background*:** Acne-prone skin is associated with dysbiosis involving among *Cutibacterium acnes* (*C. acnes*) and *Staphylococcus epidermidis* (*S. epidermidis*) causing increased seborrhea in Sebaceous Glands (SG) and inflammation.

***Methods**:* Human primary sebocytes were cultivated with 1.10^6 UFC/ml *C. acnes* Type IA (facial acne, ATCC6919) and/or 1.10^5 UFC/ml *S. epidermidis* (unknown origin, ATCC12228) for 48h in our SEB4GLN optimized media without antibiotics. Bacteria and sebocytes were enumerated and assessed for viability. Lipid production was imaged and quantified with Nile Red staining. SG with hair follicles were microdissected from healthy skin and cultured with 1.10^5 UFC/ml *C. acnes* Type 1A and/or 1.10^4 UFC/ml *S. epidermidis* (wild facial skin strain) prior fixation and immunostaining for MC5R, *C. acnes* and nuclei (DAPI) with Z-stack confocal microscopy bioimaging (Leica SP5X & FIJI software)

Results: *C. acnes* growth was not impacted when co-cultivated with sebocytes (2D) or SG (3D) models. Phylotype IA stimulated sebocyte lipid production with no impact on viability. *S. epidermidis* reference strain overproliferated inducing sebocytes mortality. For 3D SG model, culture conditions were optimized by using a facial skin wild type strain lower concentration, 1:10 ratio to *C. acnes,* reduced contact time, sequential inoculation and a rinsing step. Bioimaging revealed strong *C. acnes* labelling in the active areas of the

pilosebaceous unit. *S. epidermidis* formed biofilm distributed across the SG with non-specific fluorescence imaging.

Conclusion: We have developed an innovative model of sebaceous gland that mimics acne-prone skin with lipid overproduction and virulent phylotype IA *C. acnes* inoculation.

Keywords: Acne; Sebaceous; Bacteria; Sebum; Microbiota.

1.Introduction

Sebum is produced by the sebaceous glands, holocrine glands within the pilosebaceous unit composed of sebocytes. Undifferentiated sebocytes located at the periphery of the gland proliferate and, regularly, some of them start to differentiate and produce more and more lipid droplets while migrating towards the center of the gland. The final stage of sebocyte differentiation is cell death which allows the release of the cell contents into the hair follicle duct to which the gland is usually connected. This constitutes sebum, which is mainly composed of neutral lipids, and the amount of sebum produced depends on both the proliferation of sebocytes and the stimulation of lipid metabolism [1].

Acne is a common chronic inflammatory disease estimated to affect 70% of teenagers with discomfort, sometimes subsequent skin scarring and often psychological distress and emotional burden. Although what triggers acne remains not fully understood, androgen-induced hypersecretion of sebum in the pilosebaceous unit is linked to recurrent inflammation, keratinization and bacterial colonization of *Cutibacterium acnes* (*C. acnes*) causing skin microbiota dysbiosis with commensal bacteria such as *Staphylococcus epidermidis* (*S. epidermidis)* [2] [3]. Due to the cosmetic strategies in association with the microbial component, we described here our new accurate 3D *ex vivo* human sebaceous gland model colonized with *C. acnes* and/or *S. epidermidis*. The objectives of the study were: in a first step to validate co-culture conditions of human sebocytes grown in 2D monolayers in presence of *C. acnes* and / or *S. epidermidis*, prior to a second step for the development and the validation a robust reproducible 3D *ex vivo* human sebaceous gland model colonized with normal and dysbiotic microbiota.

2.Materials and Methods.

2.1 Human biological specimen collection

All skin samples were obtained from human donor surgery following the provision of informed consent in accordance with applicable ethical guidelines and regulations in France. CTIBIOTECH has a double accreditation from the French Ministry in charge of research for the preparation (AC-2018-3243 and DC-2018-3242) and conservation of elements derived from the human body with a view to their transfer for scientific purposes. The operation was carried out according to the strictest hygiene rules. The sample was maintained in aseptic conditions throughout its journey.

2.2 Primary human sebocytes isolation and 2D monolayer cell culture.

Primary human sebocytes were isolated from micro-dissected sebaceous glands on freshly resected *ex vivo* human skin biopsies as previously described [1]. Epidermal and dermal tissue around the sebaceous gland were progressively removed with scalpels under stereomicroscope in aseptic conditions. Sebaceous glands were then transferred in fibronectin-coated 6-well plates for "air-liquid" *ex vivo* culture for sebocytes amplification in monolayers in CTIBIOTECH's proprietary cell culture SEB4GLN.

2.3 Optimization of experimental conditions for primary human sebocytes co-culture with C. acnes and S. epidermidis.

2.3.1. Bacterial strains selection and amplification

Two commercial reference strains were selected and amplified (Eurofins BactUp, St Priest, France) DSM 1897 (ATCC6919) a phylotype IA strain of *C. acnes* isolated from acne-prone facial skin and CIP28.61 (ATCC12228) a strain of *S. epidermidis of* unknown origin (reference strain for FDA quality control tests). Strains were quality controlled for growth charts under optimal conditions as below:

Strain	Environment	Growth condition	Temperature / Time
Staphylococcus epidermidis CIP28.61	TSA agar	Aerobic	37°C for 24/48*h
Cutibacterium acnes DSM 1897 - Type IA	COS agar	Anaerobic	37°C for 48/72*h

* For optimal enumeration.

Strain	Number of cells	Enumeration Point	Incubation
Cutibacterium acnes	1,00E+07	2h / 6h / 24h / 48h	37°C + 5% CO2
Staphylococcus epidermidis	1,00E+07	2h / 6h / 24h / 48h	

Table 1: Growth conditions and stability kinetics of the strains

Suspensions of OD_{600} 0.5 to 1 were made and counted on solid culture medium. From these data, correlations between OD_{600} and Colony Forming Units (CFU) were used to determine the parameters for preparing the inoculum.

Several types of media were used to enumerate germs:

- TSA medium - Tryptone Soy Agar: a classic isolation medium used for enumeration/isolation of undemanding germs.

- COS medium - Columbia Blood Agar: a classic isolation medium used for enumeration/isolation of more demanding germs and a classic medium used for anaerobic germs.

Both media were used in all pharmaceutical and cosmetic controls. Based on 48 h pre-culture on agar medium of the different microorganisms, different inoculum were tested to evaluate the survival rate of the microorganisms in the sebocyte growth medium SEB4GLN in one duplicate per strain.

After inoculation, counts were made at T2h, T6h, T24h and T48h of incubation at 37°C +5% CO2. Enumerations were performed on the media and under the specific conditions for each strain: on TSA agar for *S. epidermidis* and on COS agar for C. *acnes* (see Table 1).

2.3.2. Primary human sebocytes co-culture with bacterial strains

Human primary sebocytes from facial skin were seeded at 30,000 cells per well in 6 well plates coated with fibronectin (Sigma-Aldrich, St Quentin Fallavier, France) and grown in SEB4GLN medium supplemented with linoleic acid (CTIBIOTECH, Meyzieu, France). **The medium did not contain any antibiotics** so as to not impede on the bacterial growth. After 5 days *C. acnes* and *S. epidermidis* were inoculated at concentrations of $2.46.10^5$ and $3.35.10^6$ respectively into wells containing approximately 60,000 sebocytes per well according to the experimental conditions described below:

- Sebocytes
- Sebocytes + *C.acnes*
- Sebocytes + *S. epidermidis*
- Sebocytes + *S.epidermidis* + *C.acnes*
- *C.acnes*
- *S.epidermidis*
- *S.epidermidis* + *C.acnes*

These values were based on previous studies using a ratio of 1:10 between *S. epidermidis* and *C. acnes* due to the predominant effect of S. epidermidis on *C. acnes* [4] and an optimised ratio of 1:100 between SZ95 sebocyte cell line and *C. acnes* [5].

2.3.3. Analysis of the impact of sebocytes on the survival of bacteria

48h after inoculation of the bacteria (D7), supernatants were collected, and the bacteria counted according to the conditions described in Table 1. The counts of the culture supernatants are performed on both COS agar and TSA agar. Wells inoculated with only *S. epidermidis* are plated only on TSA aerobically, wells containing only *C. acnes* are plated only on COS anaerobically. Wells inoculated with both strains simultaneously are plated on COS and TSA. On TSA agar, only the *S. epidermidis* strain could be counted. On COS agar, both strains are countable but morphotypically different to differentiate between *C. acnes* and *S. epidermidis* strains.

The supernatants are diluted in saline solution (Peptone Salt Solution) and 100 µl of each dilution is spread in duplicate on 2 agars using a single-use rake. The concentration results for each strain indicate the concentration of the bacteria in the wells for each plate.

2.3.3. Analysis of the impact of bacteria on the survival of sebocytes

At day 5 (D5, just prior to inoculation of the bacteria) and day 7 (D7, end of culture), the culture supernatant was removed and replaced with culture medium containing 10% Alamar Blue (Fisher Scientific, Illkirch, France #DAL1025). After incubation for 1h at 37°C, the medium containing Alamar Blue was collected, and the cells were rinsed with Phosphate Buffered Saline solution (Fisher Scientific) before being returned to the culture medium and inoculated (D5) or fixed for further analysis (D7).

For each condition, the fluorescence intensity (FI) emitted by the solution containing Alamar Blue was measured using a fluorometer TECAN Spark®) at a rate of 3 measurements per well and with the following wavelengths: excitation at 570nm and emission at 585nm.

2.3.4. Analysis of the impact of bacteria on lipid production by sebocytes

At the end of the experiment (D7), the sebocytes were fixed with 4% w/v formaldehyde (Sigma-Aldrich, France). Then, nuclei and cell lipids were labelled with 50µg/mL Hoechst solution and 7µg/mL Nile Red solution respectively. The fluorescence intensity emitted by the cells was then measured in each well using a fluorometer (TECAN Spark®) with the following wavelengths:

- Core (Hoechst): excitation 356nm - emission 465nm
- Neutral lipids (Nile Red): excitation 475nm - emission 530nm
- Total lipids (Nile Red): excitation 520nm - emission 625nm

The raw values obtained were collected with the iControl software and exported to Excel for analysis.

Microscopic analysis was also performed using a fluorescence microscope (Eclipse Ti, Nikon) and processed with NIS software. For each condition, photos were taken in bright field and with different excitation and emission filters to reveal nuclei in blue ("DAPI"), neutral lipids in green ("FITC") and total lipids in red ("TRITC"). A superposition of the 3 photos taken with the 3 types of filters revealed the lipid droplets in yellow.

2.4 Optimization of experimental conditions for 3D *ex vivo* sebaceous glands with a with *C. acnes* and *S. epidermidis*.

2.4.1. Sebaceous Gland extraction, culture and viability assessment.

24 sebaceous glands were isolated from a 46-year-old female under aseptic as previously described (CTISebogland, CTIBIOTECH, Meyzieu, France) [1].

Sebaceous glands were obtained by microdissection under the assistance of a stereomicroscope (Leica, France) under aseptic conditions in a laminar flow hood from the skin sample. The sebaceous glands were then transferred to 48-well plates for *ex vivo* air-liquid survival culture in 300µL antibiotic-free SEB4GLN media on a fibronectin-coated well (Fisher Scientific) at the "air-liquid" interface [1].

Figure 1: Example of an ex vivo sebaceous gland micro-dissected from human skin.

Metabolic activity was assessed overnight, the day before inoculation of the bacteria into the model and 48 hours afterwards. The culture supernatant was removed and replaced with culture medium containing 10% Alamar Blue. After 12 hours of incubation, for each duplicate condition, the fluorescence intensity (FI) emitted by the solution containing Alamar Blue was measured using a fluorometer (TECAN Spark®).

2.4.2. Inoculation of bacteria on the 3D sebaceous gland model

Bacterial strains were selected, validated, and amplified (Eurofins BactUp) DSM 1897 (ATCC6919) a phylotype IA strain of *C. acnes* isolated from acne-prone facial skin and *S. epidermidis* derived from human facial sebaceous gland. The change from CIP28.61 (ATCC12228) strain of *S. epidermidis* of unknown origin for a wild type of facial *S. epidermidis* was justified by the overproliferation characteristics oberserved in the 2D monolayer sebocyte model (cf. section 3.1, **fig 2).**

3 days after extraction of the sebaceous glands, *S. epidermidis* and *C. acnes* were inoculated at concentrations of 1.10^4 and 1.10^5 respectively into wells each containing a single

sebaceous gland. These values were established according to results from 2D monolayer co-culture and literature showing the predominance of *S. epidermidis* over *C. acnes* in our experimental conditions.

8 different experimental condition were tested as below:

Conditions tested with 48 hours of culture:

- Sebaceous gland (negative control)
- *S. epidermidis* (1.10^4 CFU/ml)
- *C. acnes* (1.10^5 CFU/ml)
- *S. epidermidis* (1.10^4 CFU/ml) + *C. acnes* (1.10^5 CFU/ml)

Rinsing of contamination after 2 hours of contact - Enumeration after a total of 48 hours of contact :

- *S. epidermidis* (1.10^4 CFU/ml)
- *S. epidermidis* (1.10^4 CFU/ml) then after rinsing - *C. acnes* (1.10^5 CFU/ml) for 46 hours

Rinsing of contamination after 6 hours of contact - Enumeration after a total of 48 hours of contact:

- *S. epidermidis* (1.10^4 CFU/ml)
- *S. epidermidis* (1.10^4 CFU/ml) then after rinsing - *C. acnes* (1.10^5 CFU/ml) for 42 hours

After incubation for 48 hours at 37°C + 5% CO_2 the supernatants were recovered for enumeration. Depending on the conditions, the counts of the culture supernatants were performed on one agar (COS or TSA) or simultaneously on both types of agars as described in section 2.3.1.

2.4.3. Bio-imaging of microbiota on sebaceous glands

2.4.3.1 Immunostaining

Following Alamar Blue viability assay, the sebaceous glands were gently rinsed three times with PBS and then fixed with 4% w/v formaldehyde (Sigma-Aldrich, St Quentin Fallavier, France) for 1 hour and then maintained in PBS. After rinsing, the samples underwent a permeabilization step necessary to allow the penetration of the antibodies.

Indirect immunostaining was then carried out to highlight the morphology of the sebaceous gland (MC5R, Abcam, Cambridge, UK) and its colonization by the microbiota (*C. acnes*, MBL International, Woburn, USA).

Sebaceous glands were incubated 66 hours prior to rinsing and incubation with secondary antibodies (Alexa Fluor 488 goat antirabbit IgG or Alexa Fluor 546 - goat anti mouse IgG1, Fisher Scientific). Cells nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole), a fluorescent molecule capable of binding strongly to DNA (Sigma-Aldrich).

2.4.3.2 Confocal microscopy bioimaging

Prior to confocal acquisition, the samples were placed in a 35 mm glass-bottomed petri dish covered with a coverslip. Image acquisition was performed with Leica SP 5 X confocal microscope (Leica, Germany) with oil immersion objectives (0.5mm magnification). Cells nuclei labelled blue (UV), MC5R green (AlexaFluor 488) and finally *C. acnes* red (AlexaFluor 546). Acquisition parameters (gain, laser power, etc.) were defined during the analysis and then applied uniformly to all the conditions of the same magnification to allow for image comparison. The optical focusing allows acquisition on a very precise focal plane to make a series of images at different depths in the sample called "Z-Stack" (every 2 to 3 µm). 3-dimensional reconstruction of Z-stacks, data processing and analysis were performed using ImageJ software and the FIJI module (NIH, Bethesda, USA).

*3.**Results**. It must describe the main findings of the research.*

3.1 *S. epidermidis* inoculum must be adjusted to prevent over proliferation in presence of *C. acnes* and sebocytes.

Following culture with and without sebocytes, TSA agar permitted sole enumeration of *S. epidermidis* as C. *acnes* does not multiply under those conditions. On COS agar, both strains

could be counted and were morphotypically different, which allowed the differentiation between *C. acnes* and sole enumeration *S. epidermidis* strains.

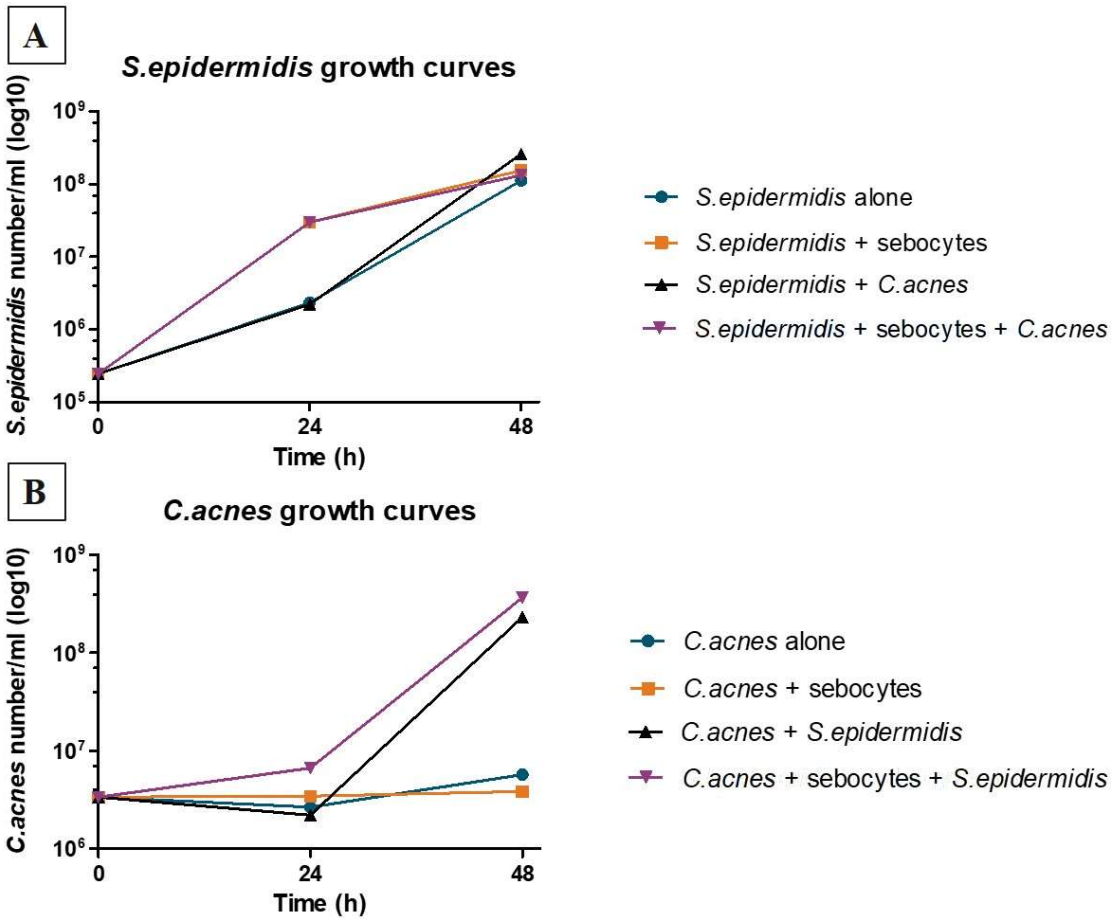


Figure 2: Evolution of the counts C. *acne s*and *S. epidermidis* in the presence or absence of sebocytes.

C. acnes was maintained for 48h, without overgrowth, whether it was grown alone or in the presence of sebocytes, confirming that sebocytes culture conditions were suitable (without antibiotics) for the assay. Similar results were obtained in the presence of *S. epidermidis* during the first 24 h of culture after inoculation. Stimulation of *C. acnes* proliferation was generated by *S. epidermidis* between 24 and 48 h following inoculation. Significant growth of *S. epidermidis* occurred during the 48 h of culture and this was observed in the presence

or absence of *C. acnes* and/or sebocytes, indicating the absence of a significant effect of *C. acnes* and sebocytes on the growth of *S. epidermidis*. Since the selected *S. epidermidis* strain did not appear appropriate for the establishment of the desired model, an endogenous strain isolated from a human sebocyte gland was selected for the 3D sebaceous gland *ex vivo* assay (fig. 2).

3.2 Human sebocytes viability and metabolism are negatively affected by overproliferation of *S. epidermidis* but not by *C. acnes*.

Sebocyte metabolism and viability were comparatively assessed prior to bacteria inoculation and 48 hours post inoculation with the Alamar Blue test.

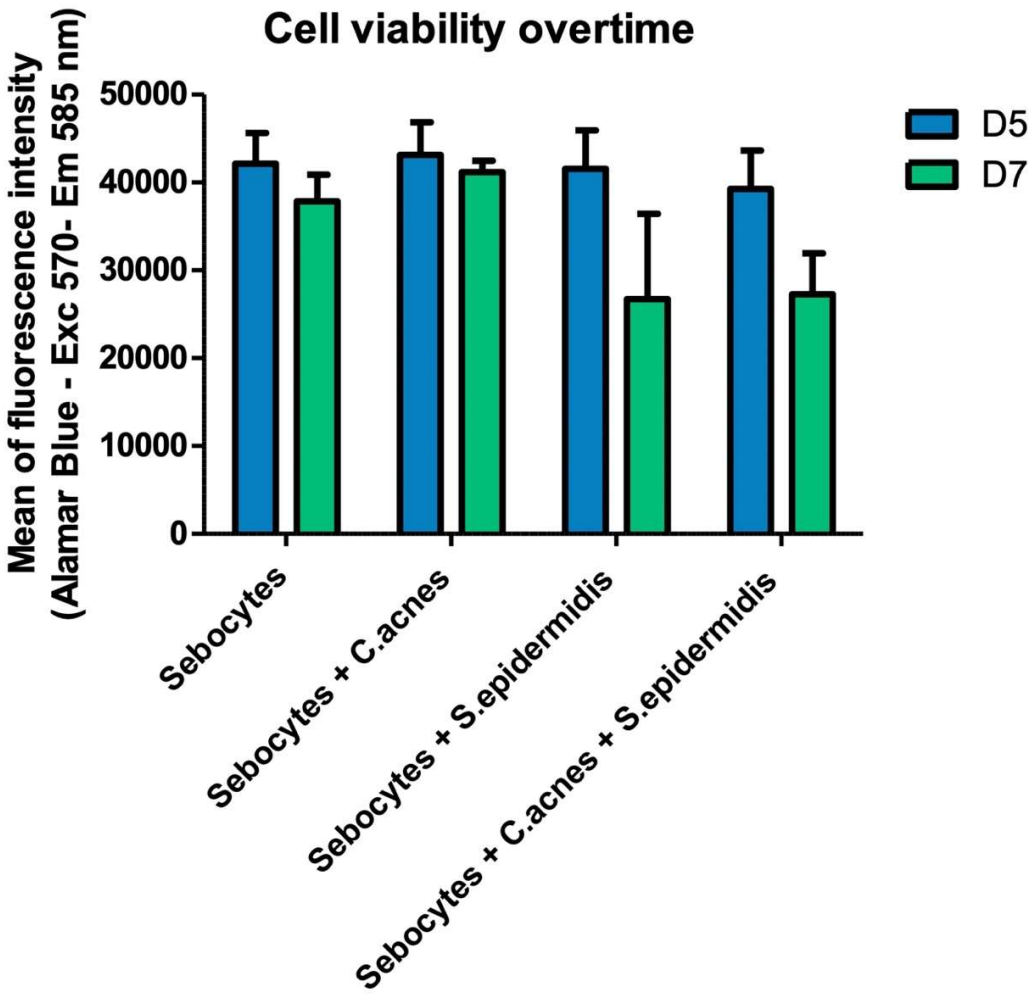


Figure 3: Comparison of cell viability under different culture or co-culture conditions

of bacteria and sebocytes just before inoculation with bacteria (D5-day 5) or 48h after inoculation (D7-day 7).

A good viability homogeneity in sebocyte numbers between all replicates and cell culture wells was observed at Day 5 **(fig.3)**, estimated at 60,000 sebocytes per well. 48h after inoculation of the bacteria, no significant variation of this viability was observed in the control condition (sebocytes without bacterial inoculation) or after inoculation of *C. acnes*. Furthermore, no signal was detected in the condition where *C. acnes* was inoculated in the absence of sebocytes (**fig.3**) and almost no bacteria were visible at the bottom of the culture plate (**fig.4**). *C. acnes* therefore had no positive or negative effect on sebocytes proliferation or survival, as it was therefore selected for subsequent evolution of the model on 3D sebaceous glands.

Sebocyte metabolic activity and viability was significantly decreased in the presence of *S. epidermidis*, whether *C. acnes* was present or not, and a relatively strong signal was detected in wells containing *S. epidermidis* alone without sebocytes (**fig. 3**). Microscopic observations also showed that the sebocytes were much less numerous under these conditions and tended to detach, while many bacteria adhered to the bottom of the plate and onto the sebocytes (**fig. 4**). The inoculated *S. epidermidis* strain therefore significantly decreased the viability of sebocytes and confirmed the need to select a skin specific strain for progression to the 3D sebaceous gland model.

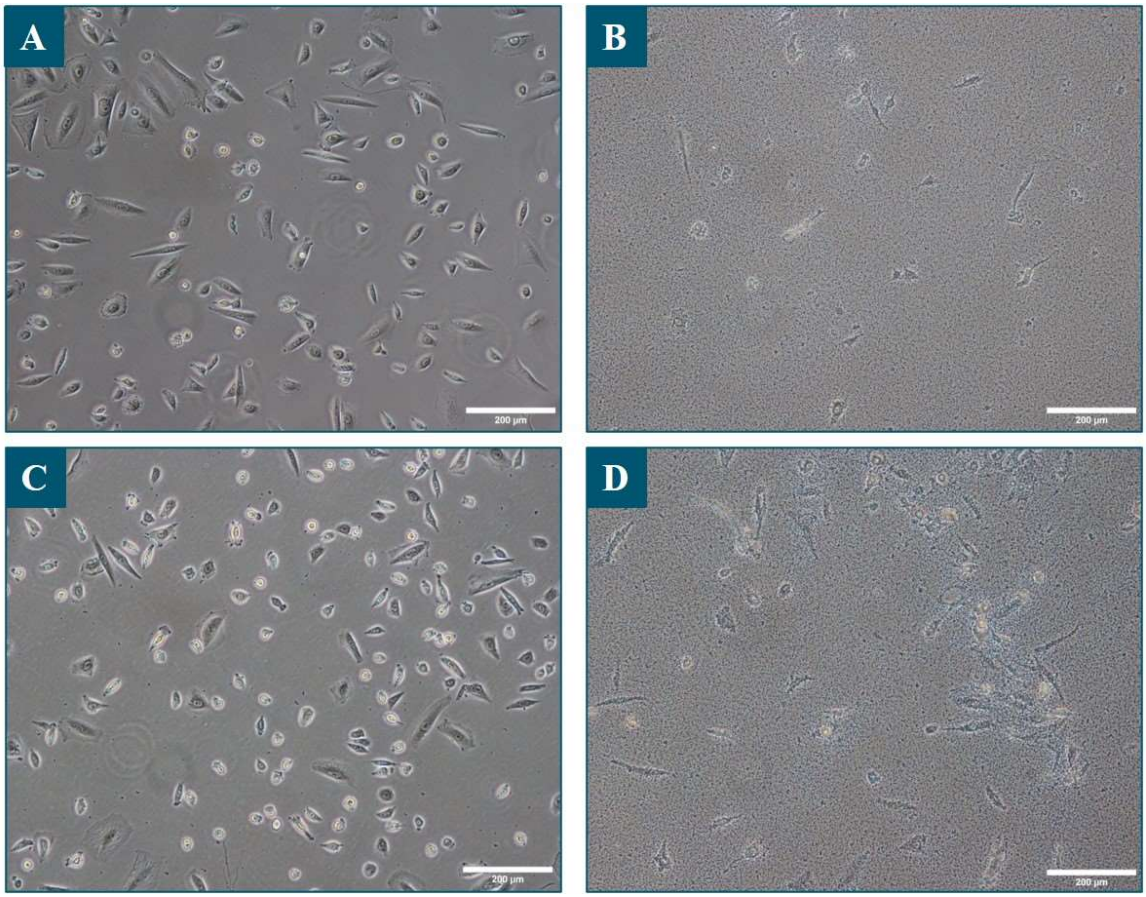


Figure 4: Bright field microscopy of sebocytes grown under the different conditions.

The pictures were taken 48h after inoculation of the bacteria, just after removing the culture supernatant containing the bacteria and just before performing the Alamar Blue test. A: sebocytes only; B: sebocytes + *S. epidermidis*; C: sebocytes + *C. acnes*; D: sebocytes + *C. acnes* + *S. epidermidis*. Scale bar: 200µm.

3.2 *C. acnes* induced total and neutral lipid production by human sebocytes.

Nile Red labels lipids and, depending on the excitation and emission wavelengths used, it reveals more neutral lipids (mainly constituting lipid droplets containing sebum), polar lipids (mainly constituting membranes) or total lipids (all lipids). Furthermore, Hoechst labels nuclei. The ratio between the fluorescence intensity of Nile Red and Hoechst therefore allows the quantity of lipids per cell to be estimated.

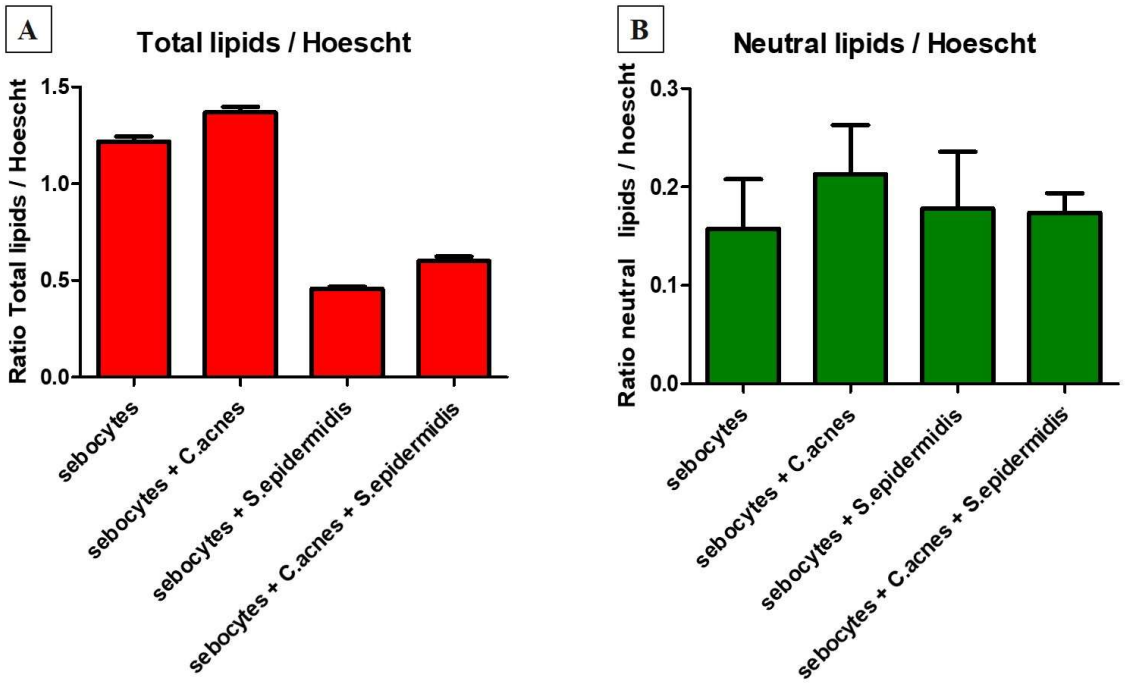


Figure 5: Comparison of fluorescence intensity ratios between Nile Red and Hoechst signals obtained using excitation and emission wavelengths of 520 and 625nm for total lipids and 475 and 530nm for neutral lipids, and 356 and 465nm for nuclei respectively CA: C. *acnes*; SE: *S. epidermidis*

C. acnes inoculation of human sebocytes induced an increase in total and neutral lipid (constitutive of sebum) both quantitatively and qualitatively (**fig.5 & 6**) in the absence of *S. epidermidis*. *S. epidermidis* appeared to decrease total lipid production which can be correlated with the deleterious effect it caused on sebocytes viability. Interestingly, when combined to *C. acnes, S. epidermidis* maintained the decrease in neutral lipid secretion and increased slightly total lipid production when compared to the condition inoculated with *S. epidermidis* alone.These results were confirmed by fluorescence bioimaging (**fig.5 & 6**).

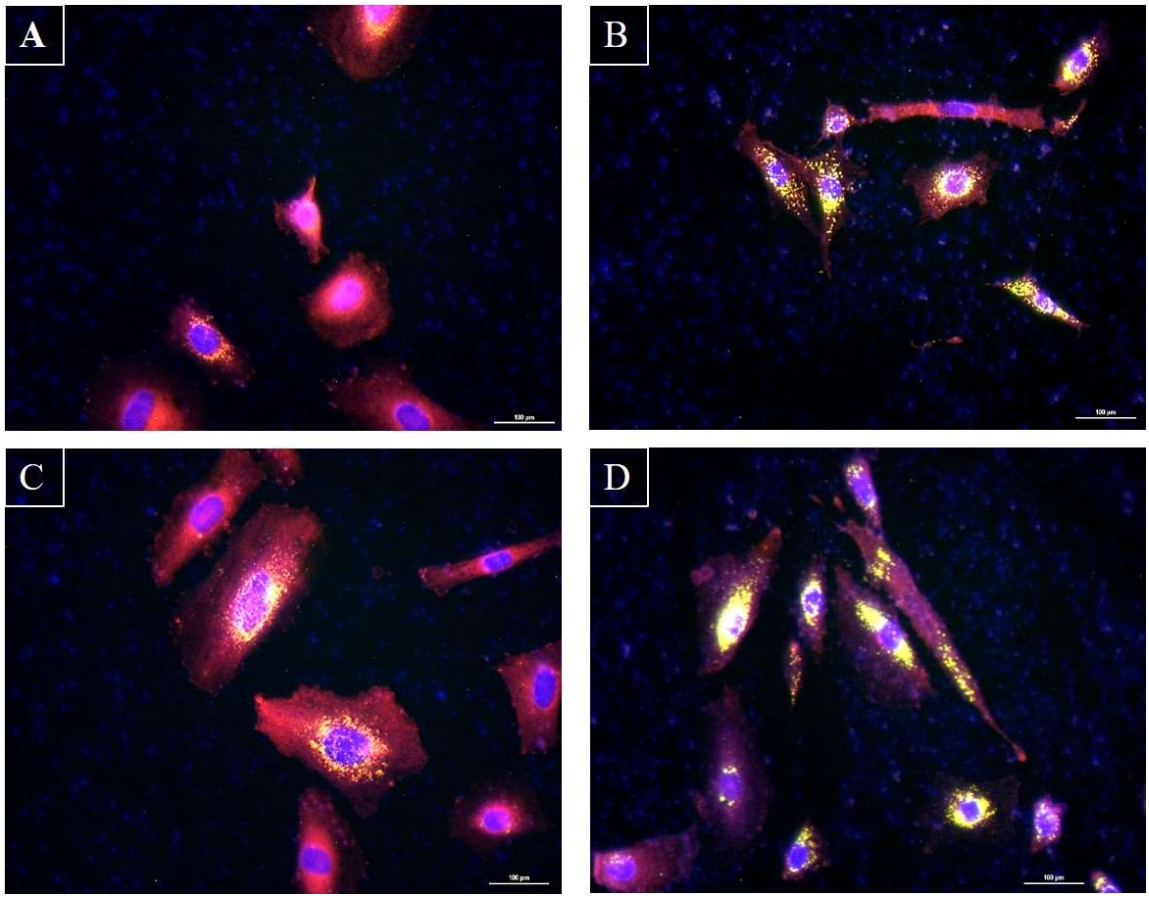


Figure 6: Microscopic observation of sebocytes cultured under the different conditions after fixation and labelling with Nile Red and Hoechst.

Pictures were taken with an epifluorescence microscope. Hoechst marks the nuclei in blue while Nile Red reveals neutral lipids in green and total lipids in red. Superimposing the two highlights the lipid droplets in yellow. . A: sebocytes only; B: sebocytes + *S. epidermidis*; C: sebocytes + *C. acnes*; D: sebocytes + *C. acnes* + *S. epidermidis*. Scale bar: 100µm.

3.3 *C. acnes* and *S. epidermidis* strains and inoculation ratio can be optimized for 3D sebaceous gland *ex vivo* culture.

Following observations made for 2D sebocytes culture, a facial skin-specific strain of *S. epidermidis* (isolated from sebaceous gland) was isolated and amplified for the project to replace the CIP28.61 (ATCC12228) strain of *S. epidermidis* of unknown origin with high proliferation characteristics.

Condition	TSA (UFC/ml)	Average TSA	COS (UFC/ml)	Average COS
S.epidermidis + C.acnes 1E5	3,20E+05	**1,87E+08**	5,50E+05	**4,37E+08**
	1,90E+08		6,20E+08	
	3,70E+08		6,90E+08	
S.epidermidis + rinse at 2h	1,99E+08	**1,46E+08**		
	2,03E+08			
	3,70E+07			
S.epidermidis + rinse at 2h + *C.acnes 1E5* after rinse	2,06E+08	**1,72E+08**	3,70E+08	**2,71E+08**
	2,59E+08		3,90E+08	
	5,10E+07		6,90E+08	
S.epidermidis + rinse at 6h	5,90E+08	**4,57E+08**		
	5,40E+08			
	2,41E+08			
S.epidermidis + rinse at 6h + *C.acnes* 1E5 after rinse	2,30E+08	**7,76E+07**	5,30E+08	**1,79E+08**
	1,10E+06		3,50E+06	
	1,78E+06		2,60E+06	
S.epidermidis 1E4	8,50E+08	**7,10E+08**		
	5,40E+08			
	7,40E+08			
C.acnes 1E5			1,30E+09	**1,09E+09**
			1,38E+09	
			5,80E+08	
No IC	> 3,00E+04	**> 3,00E+04**	> 3,00E+04	**> 3,00E+04**
	> 3,00E+04		> 3,00E+04	
	> 3,00E+04		> 3,00E+04	

Table 2: Concentration of micro-organisms under different 3D ex vivo sebaceous gland culture conditions.

The bacterial counts summarized in Table 2 highlight that when *C. acnes* and *S. epidermidis* (facial skin strain) that the 10:1 ratio in the inoculum was suitable to observe bacterial proliferation in the 3D *ex vivo* sebaceous gland model. The counts further confirmed that the specific sebaceous gland culture conditions classically (fibronectin coating, SEB4GLN, 37°C, 5% CO2) are favourable to a sebaceous gland/bacteria (*C. acnes* / *S. epidermidis*) co-culture with a balanced ratio of inoculum "*S. epidermidis* 1E4 + *C. acnes* 1E5". However, the final concentrations measured were found to be lower than the concentrations obtained from the conditions inoculating the two strains individually (*S. epidermidis* 1E4 or *C. acnes* 1E5).

The concentrations obtained after rinsing the wells at 2 hours and 6 hours (*S. epidermidis* + rinsing at 2 hours and *S. epidermidis* + rinsing at 6 hours) confirmed the interest of this method to limit the potential overproliferation of S. *epidermidis* in the model. The final concentration measured after 2 hours (1.46E+08) is lower than the concentration after 6 hours of contact. The concentration with rinsing at 6 hours (4.57E+08) is also lower than the concentration measured after 48 hours (7.10E+08).

The inoculation of *C. acnes* after rinsing also shows a certain proportionality between the final concentration measured and the contact time, which validates the rinsing method:

- *C. acnes*: 1.09E+09
- *S. epidermidis* + rinse at 2h + *C. acnes* 1E5 after rinse: 2.71E+08
- *S. epidermidis* + rinse at 6h + *C. acnes* 1E5 after rinse: 1.79E+08

Interestingly, this ratio of inoculation, regardless of any rinsing method, did not cause any significant negative effect on the sebaceous gland overall metabolism, and viability as highlighted by Alamar blue fluorescence intensity with a rather homogeneous signal measured by spectrophotometry. Very strong increase in fluorescence intensity in all conditions tested compared to the values detected before inoculation. All the values obtained can be associated with the presence of the bacteria and not with the presence of the gland alone **(fig .7).**

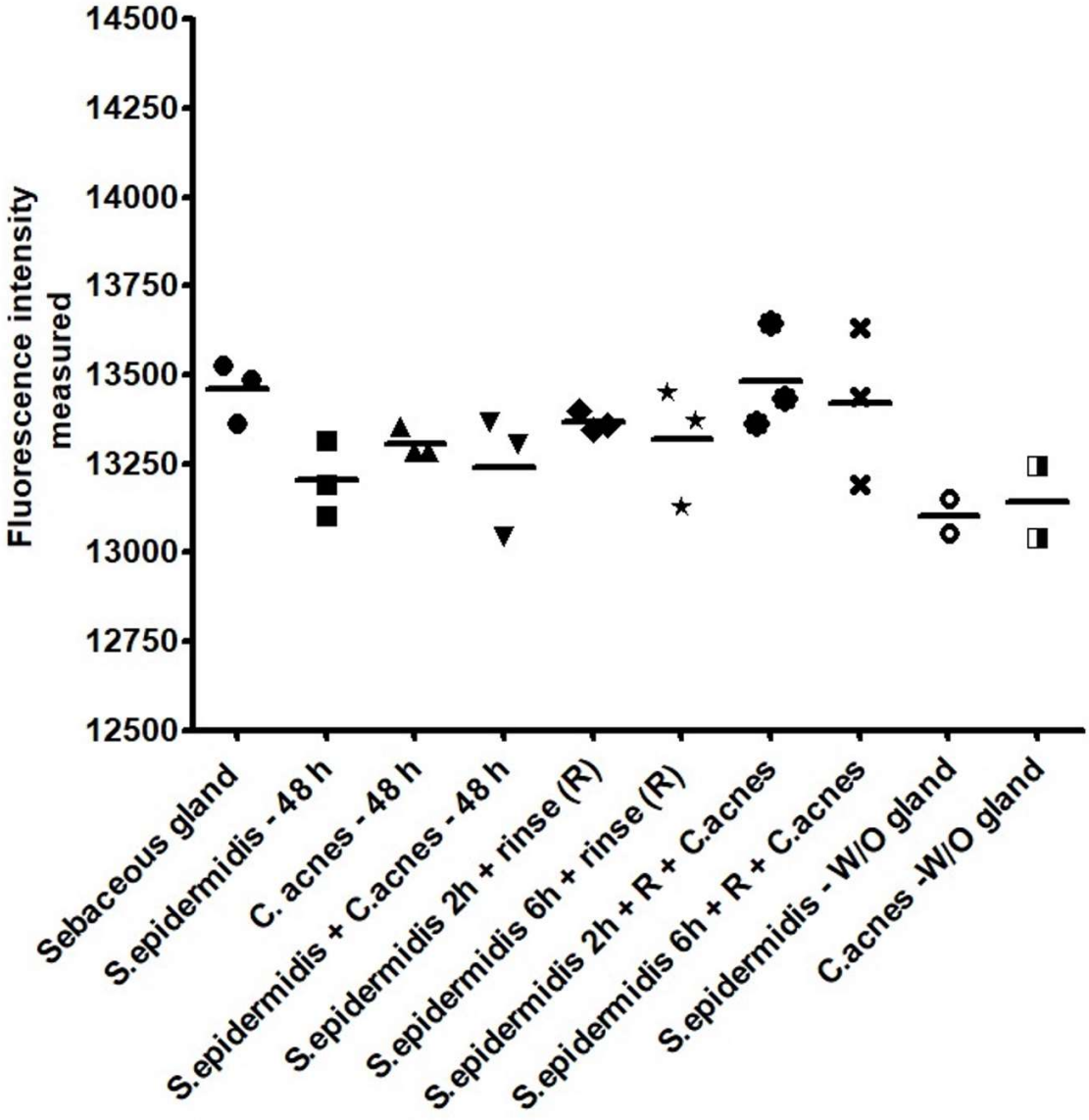
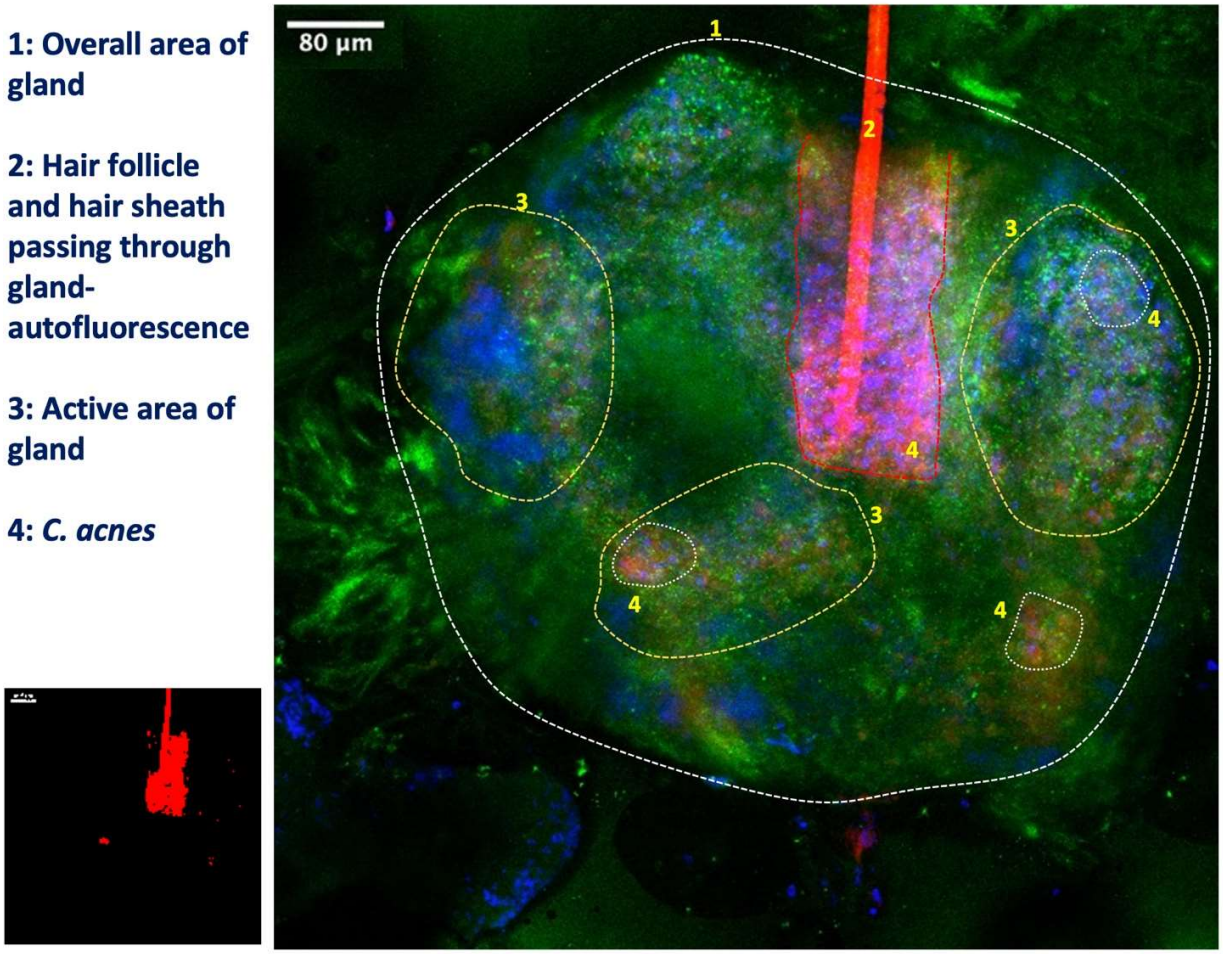


Figure 7: Alamar blue fluorescence intensity measured 48 hours after inoculation of the bacteria confirmed a preserved viability/metabolism of the sebaceous glands.

3.4 C. acnes bacterial load can be imaged by 3D confocal microscopy of sebaceous glands.

The negative control without inoculum, did not show any *C. acnes* labelling but that the morphology of the gland (in green, MC5R labelling) as well as the nuclei could be clearly identified (in blue). The positive control, with inoculation of *C. acnes* alone showed positive red staining localized at the center of the sebaceous gland. *C. acnes* was further detected with the same pattern in the active areas of both sebaceous glands and the hair follicles, with a higher concentration in the central areas of glands. *S. epidermidis* was imaged by non-specific fluorescence and located more globally across the entire gland (to be presented at conference). The condition "*S. epidermidis 2h* + rinse + *C. acnes*" provided the most interesting bioimaging of C. acnes labelling in the active part of the sebaceous gland **(fig. 8).** Rinsing after 2 hours or 6 hours is effective since we observe a decrease in non-specific labelling.



(Orientation corrected to the skin surface view)

Figure 8: Representative 3D bioimaging of C. acnes on ex vivo sebaceous gland by confocal microscopy. Blue represents cell nuclei, Green labels MC5R positive sebocytes, Red stains for C. acnes bacteria.

4. Discussion.

The involvement of microbiota on skin homeostasis is gaining much interest and is a wealth of opportunities for innovation in dermocosmetics. We had previously reported 2D sebocyte monolayer *in vitro* methods combined to 3D *ex vivo* culture systems for sebaceous gland tissue engineering as a robust approach for screening and development of innovative active ingredients, formulated products, and medical/aesthetic devices for skincare [1]. This study investigated optimization of culture conditions to integrate two specific bacterial strains involved in acne dysbiosis *S. epidermidis* and *C. acnes*, and particularly phylotype IA known to be particularly virulent [7] [8].

The challenge for any predictive *in vitro* / *ex vivo* human tissue bioassay is to be sufficiently predictive to mimic what happens *in vivo* in humans whilst providing robust measurable biological signals to understand the effect of products on skin systems.

This study adopted a two-step approach with a progression from 2D primary human sebocytes co-culture with bacteria to a 3D *ex vivo* sebaceous gland system for further investigation of microbiota on seborrhea.

Both the 2D and 3D approaches showed that human sebocytes and sebaceous glands could be successfully cultured with C. acnes and/or S. epidermidis. The origin of the strain choice is further important, particularly for S. epidermidis. Although we had initially hypothesized that the FDA reference CIP28.61 (ATCC 12228) of *S. epidermidis* (unknown origin) would be a good candidate for the model, its growth profile was rather intense, and adopting a facial skin-derived *S. epidermidis* strain was more suited for the 3D *ex vivo* sebaceous gland model. The sebocyte-friendly culture conditions enable inoculation of both bacteria types in 2D and 3D whilst enabling lipid production measurement, viability assessment and bioimaging. Total and neutral (sebum) lipid neo synthesis induction by *C. acnes* could be measured efficiently with and without presence of *S. epidermidis.* For a long time, *C. acnes* (particularly phylotype IA) was implicated in inflammatory acne, with elevated seborrhea, hyperkeratinization, and induced hypoxia with the pilosebaceous gland unit and its epidermal

pathophysiology was previously reported in reconstructed human epidermis models [10]. However, no difference in *C. acnes* load can be detected from healthy and acne-affected skin *[7].* The dysbiosis observed acne skin microbiota can lead to the selection of specific C. acnes lineages, (phylotypes IA1/IA2) and a recent study confirmed that this could lead to production of Christie–Atkins–Munch–Petersen factor (CAMP)1 by *C. acnes* lineages with strong toll like receptor (TLR)-2 binding activity to induce inflammation [10].

The dose and relative ratio of the inoculum between *C. acnes* versus *S. epidermidis* (10:1) was paramount to maintain the cultures over 48 hours to reduce the risk of *S. epidermidis* taking over the artificial microbiota of the gland. From the results obtained and our observations, *C. acnes* is detected in the 3D *ex vivo* sebaceous gland model during a 48-hour culture (*C. acnes* alone and *S. epidermidis 2h* + rinse + *C. acnes).* The 3D model confirmed that when co-inoculated, *S. epidermidis* should be inoculated first, and rinsed within 2 hours prior to inoculation with *C. acnes*. Over and beyond the growth characteristics observed this was confirmed with the bioimaging as *S. epidermidis* which induced unspecific autofluorescence signal, probably due to the known pattern of this bacteria to rapidly form biofilm [5] [6]. Previous studies further observed that *C. acnes* could stimulate biofilm formation by S. epidermidis [7] which could be observed in the 3D *ex vivo* sebaceous gland model described here. Taken together, the results presented here confirmed the interest of using microbiota strain in co-culture with sebocytes to have a relevant model to investigate dermocosmetics products efficacy on acne-like models.

5. Conclusion.

We have developed innovative models with 2D *in vitro* primary sebocytes monolayer and 3D *ex vivo* sebaceous gland that mimics acne-prone skin with possibility to measure lipid overproduction, metabolic activity and evaluate 3D bioimaging of virulent phylotype *C. acnes* inoculation. The specific localization of *C. acnes* to the active regions of the sebaceous glands and hair follicles is new and of great importance to allow for accurate research. This model will allow us to develop actives in skin dysbiosis conditions, but also investigate skin microbiota, which is of increasing importance in cosmetics strategies.

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Conflict of Interest Statement. NONE

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