Skin Microbiota and Claims Substantiation

The evaluation of the effect of dermocosmetics or active products on the skin microbiota is evolving into more and more complex models. And yet, these never reach the sophistication of the ecosystem of the bacterial biofilm of the skin. In addition, it is now known that the skin microbiome is subject to intra-individual variations depending on the body areas and inter-individual according to genetic, intrinsic, and environmental factors.

A complex ecosystem deeply linked to the skin homeostasis

Healthy skin promotes a skilled balance of the different bacteria present and vice-versa, skin homeostasis involves a diverse and highly controlled microbiome. Sometimes the simple decrease of one species can benefit others, which then become potentially pathogenic. 2D or 3D models including the inoculation of one or more microorganisms, living, or inactivated, allow to progress on specific problems, such as those associated with dysbiosis such as acne, atopic dermatitis, psoriasis, or scalp disorders… These approaches, which can sometimes be considered "reductionist" as the bacterial ecosystem of the skin is complex, remain unavoidable and contribute to the advancement of knowledge in the fields of skin biology and microbiology. The **intra and inter variability in skin microbiota** characteristics is large and it is difficult to say how a good microbiota should be. The diversity of microbiota species (number and distribution) that is influenced by urban way of life and excessive hygiene, plays a major role in **the healthy skin conditions.**

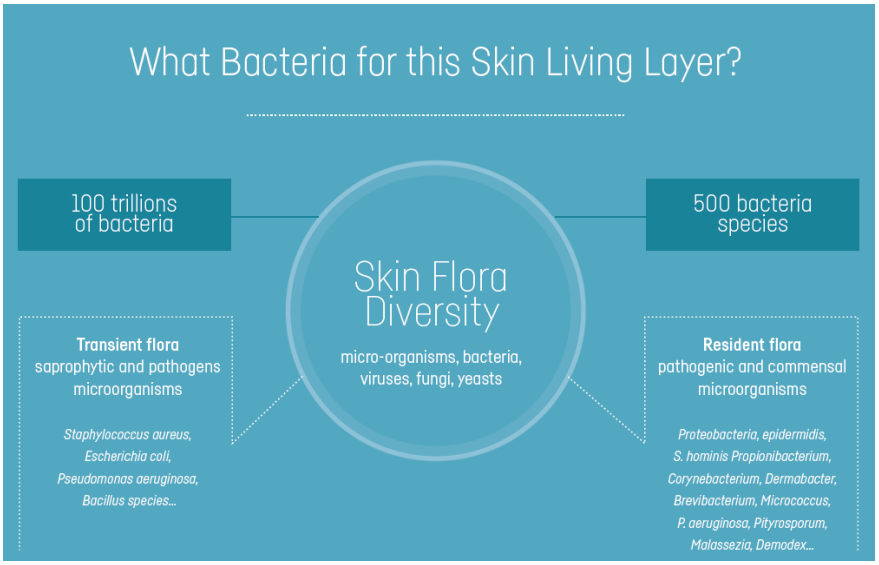
Microorganisms and hosts skin cells are in a continuous relationship to insure a healthy skin balance. Various scientific research demonstrates that the biofilm of the skin, constitutes by microorganisms and host cells, maintains skin immunity and supports the good skin barrier (i.e. Atopic Dermatitis). Cosmetics should **preserve this bacterial balance of the skin** as varied as it may be and allow fragile and injured skin to regain a natural biofilm.

Since the discovery of the intestinal microbiota, the question of the skin microbiota (micro-organisms, bacteria, viruses, fungi, yeasts), is under every lip. **100 trillion of bacteria** are living in our bodies and everybody wants to know more about the impact of the cosmetics use on the skin bacteria ecosystem and how these phenomena can be measured?

The microflora is usually subdivided in 2 groups:

- **The transient flora**, saprophytic and pathogens microorganisms with *Staphylococcus, aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus species…*
- **The resident flora,** pathogenic and commensal microorganisms with *Proteobacteria, epidermidis, S. hominis Propionibacterium, Corynebacterium, Dermabacter, Brevibacterium, Micrococcus, P. aeruginosa, Pityrosporum, Malassezia, Demodex…*

The balance of cutaneous microflora (500 bacteria species) is dependent of the several conditions of its ecosystem: temperature, pH, hormones, light, UV, lipids, proteins, water… It is mainly influenced by the genetic, the lifestyle and the diet. Each person has their own skin flora composition, distributed from the epidermis until the dermis, which is lifelong qualitatively stable, like a personal microbial footprint. This skin microflora is fundamental for the skin homeostasis and participates to the immune and barrier functions.



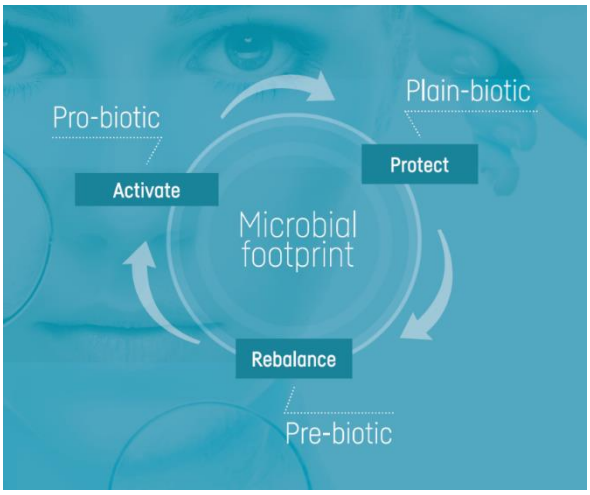
A paradoxical interest of the beauty consumers for the cutaneous microbiome

The Benchmarking company published in 2021 the results of a study regarding how **American customers** increasingly care about the skincare that they purchase in relation with the good conditions of their own skin microbiota. 21% of them are aware of microbiome specific skincare but 93% have not yet purchased microbiome beauty products. The consumers know for 87% what the microbiome is gathering of microbes (good and bad bacteria) that live on body, skin & hair.

Finally, these beauty consumers believe that the **main benefits of probiotic skincare are** balances skin pH, it kills bacteria on skin that causes acne/blemishes, contains live

bacteria that fight 'bad' bacteria on the skin, keeps microbiome balanced and creates protective barrier on skin surface. While the regulation for probiotic skin care integrating live or non-viable bacteria is not well defined, formulate such products remains a tricky challenge for cosmetics chemists. **The "Microbiome-friendly" claim** should ensure that the cosmetics, free from contamination, respect the microbiota diversity and does not impact the balance of the skin

Microbiome claims, the era of a new revolution for the cosmetics?



Currently, the approach of supporting this activity of cosmetics is still in its early stages. Many testing laboratories are studying these new claims looking in the direction of the metagenomic field. The studies of the cutaneous flora are complex, and it is not always easy to understand its **functionalities and interactions with the skin metabolism.** The first way is to analyze the genome of the bacteria of the skin flora. It is **a living layer** of the skin to be discover like a new continent of the body.

First, we must consider that the skin microbiota does not belong to the epidermis layer of the skin.
It is a "foreign" substance of our body:

- acting as a resident of the skin
- forming a biofilm at the epidermis surface
- maintaining the good conditions of the skin
- regulating inflammation,
- shielding the body from aggressive environmental conditions
- protecting from various internal stresses.

Various bacteria disorders might be considered as a source of cutaneous dysfunctions like **acne, eczema or atopic dermatitis** modifying this precious balance. The cosmetics search for 3 main benefits:

- the rebalancing, pro-biotic, pre-biotic
- the probiotic-like
- the anti-microbial effects.

In a regulatory approach, the first thing is that the personal care targeting the skin microbiota must be safe following the EC 1223/2009. They must include in their formula only prebiotics, probiotics and postbiotics ingredients that are not listed on the Annex II of the prohibited substances. Moreover, each brand must assess that the microorganism involved in the formula do not produce any toxin.
The claims of "skin microbiota" care must be not misleading, must give sufficient grounds to the consumers. Cosmetics are allowed only to keep the skin healthy and are not allowed to make it healthy or to modify the physico-chemical processes of the epidermis.

Personal care, toiletries or cosmetics claiming that they support and protect the skin microbiota are allowed using claims such as supports or protects the microbiota, microbiota friendly. But these products cannot claim that they stimulate, boost, reduce, or improve the microbiota or its diversity even it is admitted that **higher diversity is linked with higher hydration, and less infected skin.**

The beneficial **anti-ageing effects** of probiotics are now often reported with significant anti-ageing effect by increasing the skin water content, the skin elasticity, the skin gloss and decreasing transepidermal water loss as well as the wrinkle depth.

How to evaluate cosmetics activity on the skin microbiota?

As the important **intra-individual diversity** of the cutaneous flora is also associated with a high level of **inter-individual variability**, the protocols will compare the **skin swabbing** of the treated zone and of the non-treated or placebo zone, before and after treatment. To get the best results implementing these protocols, it is necessary to strengthen the **inclusion of the volunteers** with specific criteria of age, gender, lifestyles…

The skin swabbing method represents a **classical and non-invasive skin sampling**, easy and fast that can be implement on every subject. Thus, the DNA is extracted from each skin sampling, specific regions of bacteria are then amplified by 16S-rRNA. Finally, the selected segments are sequenced and analysed.

Analysing these skin samples, it is possible to evaluate the changes balance. To go deeply in this objectivation, is it also possible to study the microbiota and the skin simultaneously and investigate what are the functions and the metabolic pathways impacted. Analysis of the skin microbiota and the performance of personal care can be directly evaluate analysing the quantity and the quality of the species collected on the skin or scalp samples.

For the microbiota diversity, well known as an **indicator of healthy skin conditions**, several indexes are used. This index information varied giving indication of the taxonomic richness of the bacteria community present at the skin surface and in some cases, they inform about the relative abundance of species (taxon) and their distribution.

Main evaluation opportunities:

1- **In vivo identification of the Propionibacterium acnes bacteria on the skin surface**
The Visiopor developed by Courage & Khazaka immediately analyses the number of fluorescing areas as well as the intensity of the fluorescence.

2- **Identification of the skin microbiota at molecular level:** PCRs, rRNA, S16 rDNA + ITS sequencing: Mass Spectrometry: High-resolution Nano LC-MS/MS.

- **Measurement of the bacteria number**
Quantitative PCRs and follow-up of major species or genus. It gives answers such as*: «The product has/does not have an effect on tested genus».*
- **Identification of the bacteria composition**
Bacterial Metagenomics comparative study of microbiome by sequencing 16S ribosomal RNA (rRNA) genes. It gives answers in a report as: *«The product/treatment does or does not impact the quantitative composition and microbiome diversity».*
- **Relative quantification of functions and interactions**
Metaproteomic comparative study by relative quantification LC-MS/MS « shotgun proteomics » and bioinformatics/biostatistics. The assays enable the taxonomic analysis of the bacteria, identifying what are the bacteria that can be found. Then the genome of the bacteria to better know their action. Now a major part of the skin bacteria is known. It gives answers relative to the *effects of the cosmetics on functions and interactions of host and microbiome simultaneously.*

However, to interpret the raw data of the metagenomics analysis and translate them into an easy understandable skin claim for evaluation managers, the marketing team and the consumers it requires a minimum of **3 skills** from the testing laboratories:

1. a solid experience of data treatment,
2. a proven expertise of the dedicated bioinformatic database
3. an essential and thorough knowledge of the skin biology.

4. Counting method at cell level,

The cosmetics approach of microbiota

A lot has happened since she first wrote about the Microbiome, back in 2012. Small brands have rushed in, large ones, not so much, but there is a lot of activity on the dermo brands front, and the premium consumer side is slowly starting. So why are premium brands so timid in using the Microbiome argument?

The Skin Microbiome is young. It is a discipline that is still in its infancy. Especially in Skin Care. Sampling is less complicate than it has been. Lifestyle impacts the Microbiome as much as genetics does. The liminal nature of the "organ" complicates matters a great deal. So, yes, there are a lot of claims, many of them implying things that we just cannot be sure of. Yet. What we need, before we speak with the consumer, is industry-wide guidelines and consensus. If understanding what is happening on the skin with the Microbiome remains complex despite the presence of excellent testing labs in the space, one thing would help a great deal: **understanding the impact of the products before they are formulated.** Let 's make it routine to test the raw materials for their Microbiome impact before the regulator makes us.

Skin is a rather hostile environment, salty, dry, and poor in nutrients. Certain parts remain however moist and lipid rich propitious to bacteria blooming. As the skin matures with age, notably during puberty where hormones are kicking-in and triggering a cascade of physiological and physical transformations, the skin microbiome is constantly evolving until adulthood. On average, a person has around 1,000 species of bacteria on their skin offering a variety of distinct ecosystems, which create conditions that promote different subsets of organisms.

Researchers have uncovered **extensive communication between bacteria, skin cells and immune cells.** These interactions have been described in plethora of physiological functions as in skin barrier reparation, limitation of trans-epithelial water loss and defences against infections. Deciphering the role of skin bacteria functions is a tremendous work and this domain remains however poorly understood.

Functional analysis and deciphering mechanisms of actions of bacteria /microbes are a prerequisite to better understand their role and activities. Skin is an accessible and untapped reservoir to dissect and improve the comprehension of host- microbiome interactions. Furthermore, an extensive analysis of the skin and its microbiome via specific protocols using mass spectrometry proteomics and dedicated **bioinformatics pipelines are keys to both decipher functional correlations and mechanisms of actions between skin and its microbiome.**

For optimum beauty results, every cosmetic should follow the approach of fostering the natural homeostasis of skin and hair instead of imposing additional stress with aggressive chemicals. This is achieved best by exclusively high-quality ingredients that enhance the microbes dwelling on the skin. The skin's microbiome differs significantly between the

various body parts. To make sure a product does not harm the microbiome, some testing laboratories have developed different standards: face and body; scalp; infant skin; private parts; foot….

Also, cosmetics claims that are attributed to microbiome skin or scalp care can be easily assessed by using classical biometrological methods objectivating pH balance, Hydration, Barrier fonction, Trans Epidermal Water Loss, blemishes, irritation, inflammation, sebum and lipids, Skin turn-over and desquamation, sensitivity, or dandruff. The Skinobs Clinical Testing platform allow you, for free, to easily find all methods selecting a specific claim.

The future of skin microbiota evaluation

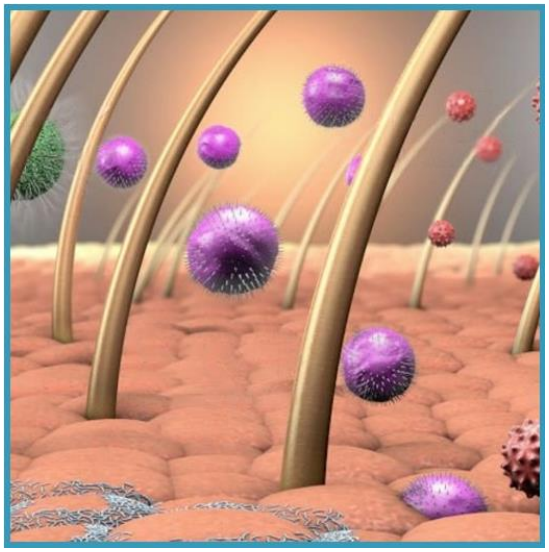
In conclusion, there is no ideal composition of the skin microbiota as people are living in different ecosystems and have various lifestyles. Scientists agree that among the huge inter- and intra-individual variation, a wide variety of microbiota species assure a good health. This bacteria ecosystem synthetizes a myriad of elements which have an important metabolic activity for our skin health. It could be necessary to protect, to rebalance and activate it on the cosmetics side. Simply said the aim for personal care could be to reduce the "bad" bacteria and protect the "good" ones! But the notion of "bad" or "good" is relative depending on the physiological state of the skin. Now things are not so simple!

The microbiota will play a key role in the cosmetics of tomorrow. We move towards personalized and preventive cosmetics. Claim substantiation will evolve with the regulation and the products development. We must keep in mind the diversity and the balance of the skin flora in the future developments of actives and personal cares. May be one day we should measure their impact of on the microbiota before launching the products on the market.

Anne Charpentier |CEO Skinobs

Our partners have the floor

We are glad to introduce the several topics presented by testing experts



Healthy Skin Microbiote of the Human Face by Complife

Vincenzo Nobile - R&D Manager and Cosmetics Market Manager

INTRODUCTION

The microbiota is the sum of all bacteria, fungi and arthropods that live in and on everything that composes an ecosystem, as the **human being, for example.**

The set of the genetic patrimony that constitutes the microbiota is known as microbiome. Analysing the microbiome allows to know the structure of the microbiota in order to evaluate its state, its composition, and how it can be affected by its environment (including the use of cosmetic products).

The role of the human microbiome in health and disease has been recognized for many years, nevertheless still relatively little information is available on the standard **composition of the healthy microbiome of the human face.**

In this article we report the results of the re-elaboration of the basal data collected during various studies designed by the Complife team to **assess the impact of the use of cosmetic products on the skin microbiome**.
The variables analysed were sex, age, season, and living area (urban or rural).

THE STUDY

COMPLIFE offers safety and efficacy testing both *in vivo* and *in vitro* to the cosmetic industry and everyday more of **our clients are interested in developing products that respect or improves the skin microbiome.**

Thanks to the many test requests, the team collected a lot of microbiome samples and ended up with **254 samples total**, 183 from women and 71 from men.

ANALYSIS PIPELINE

After sample preparation, the bacterial DNA was extracted and purified, then the study proceeded with the PCR amplification.

Since working with skin microbiota, the decision was to use primers targeting the V1-V3 hypervariable regions of the 16S rRNA gene.

Then, samples were pooled together, the library prepared, and the mix loaded on the sequencer.

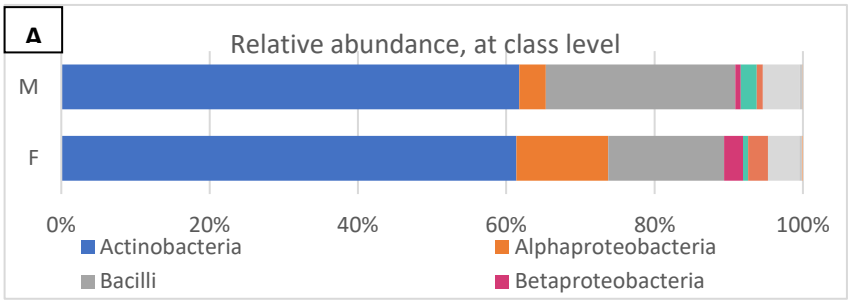
Data were analysed using the MicrobAT (SmartSeq) and MicrobiomeAnalyst (www.microbiomeanalyst.ca) software. Analysis was done based on 2 endogenous factors (sex and age) and 2 exogenous factors (season and living area).

GENERAL CONSIDERATIONS

Primary analysis at the **Phylum level** is in accordance with the literature on skin microbiota:

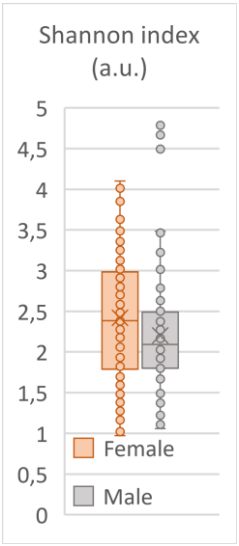
- 3 major phyla were identified (Actinobacteria, Proteobacteria and Firmicutes) with Actinobacteria accounting for more than 60% of all bacteria.
- very low percentage of other bacteria: a median percentage could be calculated only for Bacteroidetes and Cyanobacteria.

Comparison of abundances at various taxonomic levels indicates that men and women do not host the same type of bacteria (A+C). Secondary analysis of the results allowed to estimate the biodiversity either at sample (alpha-diversity) or group (beta-diversity) level. With p-values clearly lower than our 0.05 threshold, the calculations demonstrated statistical differences in bacterial population between men and women, in terms of **both taxa population and community structure**.



α-diversity (Shannon):
p-value = 2.42E-04
β-diversity (PERMANOVA):
p-value <0.001

B



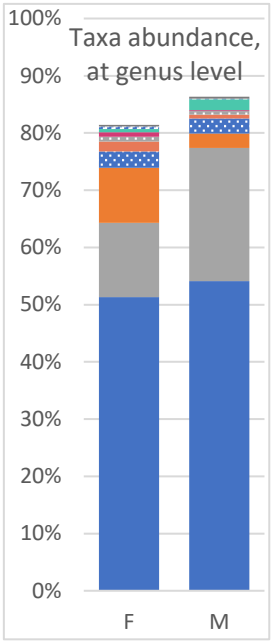
In terms of biodiversity (B), **women** within our cohort showed a **higher bacterial diversity**, which is **in line with traditionally lower sebum production and higher hydration levels**.

Frequent use of cosmetics could also play a role in modulating the microbiota, either by frequent depletion/recolonization of the skin or by microenvironment modifications.

With higher standard deviation and higher maximum and minimum biodiversity**, men appear to have higher interpersonal variations.**

At the genus level, we focused on the **10 most abundant genera (C and table)**. The analysis demonstrated that most are statistically different between the sexes, except three: *Propionibacterium*, *Corynebacterium*, and *Anaerobacillus.*

GENUS	F	M	*p (LefSe analysis)*
Propionibacterium	*51.30%*	*54.11%*	*1.21E-01*
Staphylococcus	12.99%	23.30%	2.62E-09
Sphingomonas	9.61%	2.52%	9.58E-22
Corynebacterium	*2.88%*	*2.63%*	*2.77E-01*
Pseudomonas	1.75%	0.65%	6.87E-08
Streptococcus	0.80%	0.59%	1.20E-03
Pelomonas	0.78%	0.18%	4.97E-20
Anaerococcus	0.57%	1.92%	4.06E-06
Microbacterium	0.44%	0.10%	2.95E-18
Anaerobacillus	*0.30%*	*0.36%*	*7.77E-01*



The statistical differences between men and women, in terms of both taxa population and community structure, prompted us to analyse the **influence of the other factors separately** for men and women, to evaluate if the **same factor effect the same variations** in both sexes.

Age does not seem to impact men since both α- and β-diversity p-values are over 0.05, while microbiome composition in women varies over time.

The same can be said for the effect of **seasonality** on the microbiome: although it shows fluctuations in both sexes, microbiota composition is statistically unchanged in men but varies in women. In any case, the higher diversity in summer for both sexes could be linked to higher skin humidity.

The **living area** on the other hand affect significantly both sexes.

Interestingly, the median biodiversity follows the same trend in men and women depending on their geographic location.

CONCLUSIONS

Conclusions can be summarized as follows:

1) **The cheek hosts more Proteobacteria and Firmicutes** than the neighboring sites (alar crease, nares, glabella).
   So this means that performing whole-face studies could help to further elucidate local population variations.
2) **Men have a lower median biodiversity and less statistically relevant factor-induced microbiome variations**.
   This could be due to the more frequent use of cosmetics by women, but it could also be the translation of a higher interpersonal variability in men.

3) The general observation is that the major taxa are not necessarily impacted by endogenous and exogenous factors - suggesting that **factor-induced microbiome variations could be driven by minor taxa populations**.

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Contact: Vincenzo Nobile, vincenzo.nobile@complife.com

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Personalized beauty? Start paying attention to your skin microbiome

by Mérieux NutriSciences

Valentina Abbondandolo - Product Manager

Human skin is a complex ecosystem with various micro-environmental conditions and microbial communities, and it is the home to millions of bacteria, fungi and viruses which compose its microbiota. It is extremely important to the skin health, and it plays an essential role in protecting it against external infectious or toxic substances and allergens. Skin microbial communities are site-, individual-, and ethno-specific, and they are largely stable over time, despite skin's exposure to different external environments. Various factors, including the use of antibiotics, cosmetics, soaps, personal care products, and living conditions such as lifestyles and nutrition habits can influence the skin microbiome.

Various factors can **disrupt the balance of microbiota and cause skin dysbiosis**. Described as an imbalance between the microbiota and its host, dysbiosis can be considered a form of impaired homeostasis in which the microbiota is shifted towards a less complex, less varied pathological spectrum.

Researchers have suggested that the fragile balance of the skin microbiota may have a strong influence on the functional differences between healthy skin and diseased or damaged skin, and that strong treatments can deeply alter the microbiota and compromise the health of the skin.It becomes important to **maintain the microbiota equilibrium to preserve the best skin physiology, also choosing biome-friendly cosmetic products.**

The skin microbiota *in-vitro* approach: our outstanding expertise on disease models.

POINT 1. Be safe - Check the formula composition

- Skin cytotoxicity evaluation: the potential cytotoxicity of the formulation assessed through a skin *in vitro* model.
- Skin irritation evaluation: the potential irritation of the formulation assessed through OECD test N. 439 - *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method.

POINT 2. Respect your microbes - Check the formula skin compatibility

- Bacteriostatic or bactericidal activity of the cosmetic formulation: to investigate if the formulation at its highest non-irritant concentration negatively affects bacteria growth, its bacteriostatic and bactericidal activity will be assessed following a standard MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) assays.
- Formulation impact on bacteria growth kinetic: the impact of the formulation on skin bacteria growth is evaluated by combining a broth culture and a spread plate seeding approach.

POINT 3. Evidence-based effect - Prove the real effect of the product

- Skin microbes' competition for active substances in the formulation: depending on the effect of cosmetic formulation on bacterial strains growth kinetics, it is evaluated the potential competition between different skin microbe strains for active substances as energy sources.
- 3D *in vitro* skin model for skin microbes' colonization: it is evaluated the bacterial strains colonization potential and growth kinetic on *in vitro* skin model specifically designed for each application. Skin colonization and growth of different bacteria, as single strains or in combination, will be assessed both in the presence and in the absence of the cosmetic formulation.

The skin microbiota approach on human skin: our outstanding expertise on normal and sensitive skin.

Our experts dedicated to skin microbiota projects have developed a sound strategy to provide a complete service that **prove the real effect of beauty products that claim for microbiota or microbiome**, through the design of specific protocol that fits customized needs.

STEP 1. The selection of the right panel
The right panel of volunteers is screened and selected according to skin bacterial communities. Screening results to select the right panel for specific efficacy test services are based on the total bacterial count through a direct collection of skin microbiota on volunteers.

STEP 2. Test your product effect on a selected panel
Thanks to a customizable evaluation on skin microbiota before and after product application, we can prove the product effect on selected volunteers with an integrated and multi-tool approach. Our integrated & multi-tool approach exploits the synergies of different techniques to investigate:

- how a product works on skin microbiota
- the clinical proof of your product effect on the selected panel

Conclusions

Innovation is the engine of cosmetics. The continuous research and the development of technology have always been part of the DNA of cosmetics. New claims, new production processes, new ingredients, new actives, new formulations, new products are continuously designed and developed to meet the needs and demands of the consumers of today and tomorrow. Efficacy tests give real data to support all these innovations.

Contact: Thibaud Pichard, thibaud.pichard@mxns.com

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Microbiota testing services: Through co-culture to microbiota-skin cells adhesion studies by GLYcoDiag

Dr Benoit Roubinet, Dr Mateja Senicar - R&D Managers

Microbiota co-culture models for cosmetics actives and formulation testing Skin microbiota constitutes the first biological barrier in association with innate immunity. The cutaneous microbiota is composed of various populations of microorganisms (bacteria, archaea, fungi, viruses, and mites) that are distributed in well-defined balance which depends on the different environments and localizations on the skin.

Topographical studies of the cutaneous microbiota have revealed three main areas:
- **the sebaceous environment**, essentially the face area
- **dry environment** mainly volar forearm, hypothenar palm and buttocks
- **moist environment** mainly are antecubital fossa, inguinal fold and foot

Most bacterial species identified in *skin microbiota are Corynebacterium, Propionibacterium and Staphylococcus*. Therefore, for more than five years, GLYcoDiag has developed simple **co-culture models** which allow to study the effect of a cosmetic product on the growth and/or the decrease of each strain **growing together in the same medium and environmental conditions**.

All of the co-culture models developed by GLYcoDiag are based on the following criteria:

- Referenced strains either from well-known collections or isolated and characterized wild-type strains from GLYcoDiag's own cell bank.
- Calibrated inoculum of each strain mixed (four to five strains) in proportion mimicking eubiosis equilibrium or dysbiosis related to specific cutaneous diseases.
- A common culture medium based on minimum nutrients, allowing to keep the growth of each strain in at wanted proportions during the study (usually 24 to 72h).
- Respective pH and temperature according to the environment targeted.
- Miniaturized, accurate and reliable.

Standardized co-culture models available:

1. **The common environment model** performed on the following strains selection: *S. aureus, S. epidermidis, S. hominis and C. acnes.*
2. **The dry environment model** performed on the following strains selection: **C. xerosis, M. luteus, P. aeruginosa, and C. acnes**.
3. **The moist environment model** performed on the following strains selection: **S. aureus, S. epidermidis, S. hominis, C. Xerosis.**
4. **The sebaceous environment** model performed on the following strains selection: *S. aureus, S. epidermidis, C. acnes, C. xerosis.*

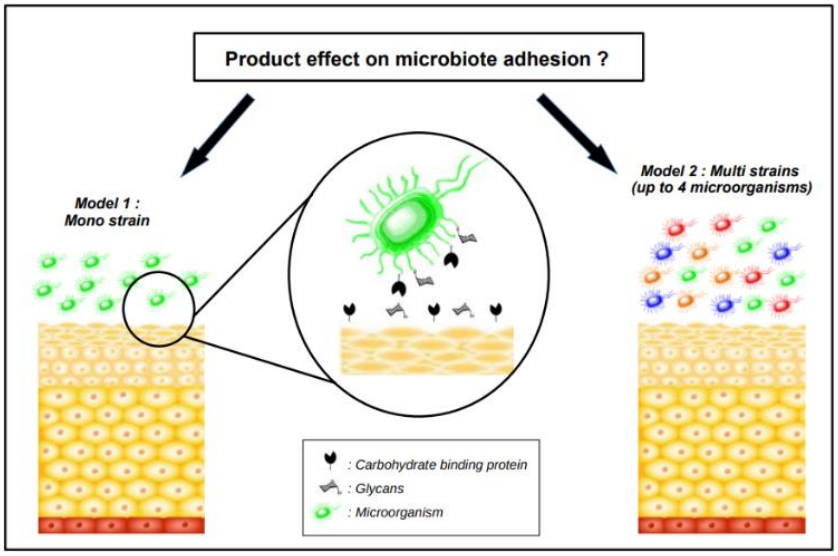
Moreover, customized co-culture mix (strains, proportions) according to your needs can be specifically developed with wild type human skin strains. These strains have a broad interest to mimic more precisely the reality of the skin microbiota and can be used in our standard miniaturized methods, and/or for the development of a wild type of experimental models according to your needs.

Glyco-microbiota studies by GLYcoDiag: Microbiota-corneocytes/keratinocytes adhesion model.

On the other hand, **there is no cutaneous microbiome without cutaneous cells.** Hence, the symbiosis of micro-organisms with skin cells (mainly corneocytes and keratinocytes) is the key to ensuring functionality of biological skin barrier preventing pathogen colonisation, microbiota equilibrium, appropriate immune response, and a healthy skin. **The symbiosis depends on number of interactions/ communications between cells among which glycobiological interactions** play the main role, notably in micro-organisms adhesion, biofilm formation, communication with skin cells and innate immunity through Langerhans and dendritic cells.

Taking into account these important considerations and beside the co-culture models described above, GLYcoDiag manages to further develop its services that are intended to study the microbiota-corneocytes (or microbiota-keratinocyte) adhesion.

The testing services intended to study **the effect of a product on the adhesion of microorganisms on skin cells** (corneocytes and/or keratinocytes) are *in vitro* models useful for screening of new actives and/or to confirm the effect of your products (actives or final products) on **microbiota-skin adhesion.** We have recently upgraded this method **through the monitoring of adhesion of up to 4 differents strains simultaneously.** The upgraded model is now adapted to study the effect of products on the adhesion of different mix of up to four strains on corneocytes, **giving a complementary and totally adapted adhesion models to our co-culture models** (proportions, identity and number of species can be modulated according to the targeted skin environment).



Contact: Dr Benoit Roubinet, Dr Mateja Senicar, contact@glycodiag.com

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In-vitro Evaluation of Microbiotic Strains on Sebocyte Function

by Phenocell

Helene Leménager | R&D Scientist

The healthy human skin is populated with a characteristic microbiome (Grice et al., 2011). As a major commensal species of the skin microbiome, *Cutibacterium acnes* (*C. acnes*) participates to skin protection against chronic inflammatory diseases (Dréno et al., 2020). However, ***C. acnes*** and its interplay with the skin innate immune system is instrumental in acne development. **Deregulation of the balance between its various phylotypes** (or dysbiosis), participates to increased sebogenesis, sebum production and inflammatory response with up-regulation of innate immune markers.

C. acnes is mainly present in the sebocyte gland

Using in-vitro models allows mimicking of the harmful effects of any exogenous or endogenous disrupter of homeostasis if they accurately reproduce human pathophysiology. Unfortunately, immortalized cell lines do not always answer this requirement, on responses to pro-seborrheic agents such as testosterone. Cell lines also present genomic abnormalities that might impair their behaviour. Sebocyte extraction from skin samples is time-consuming and stressing for the cells.

Phenocell has successfully set-up production lines for **human sebocytes** (PCi-SEB) **derived from human induced pluripotent stem cells** (iPSC) of donors with varying sebum production abilities linked to their genetic background (African, Caucasian, and Asian). PCi-SEB have proven to be a reliable tool to study biological pathways involved in

- sebogenesis dysregulation,
- inflammatory states
- oxidative stress

induced by atmospheric pollutants, chemical agents, blue light, or microbiota components.

The **iPSC technology presents several advantages,** among which large-scale production of somatic cells, highly reproducible batches, control of genetic background and clinical status of donors, and absence of genetic modifications. PCi-SEB follow the normal maturation process, followed by specific cell death and holocrine secretion of sebum. PCi-SEB are amenable to bioprinting, to 3D-organogenesis or to co-culture with other skin cell types. PCi-SEB therefore are highly relevant tools to evaluate active ingredients in hyper- or hypo-seborrhea, stress and anti- or pro-inflammation settings.

The panel of bioassays developed at Phenocell allows to assess actives **potential use to regulate acne, aging, or pathological conditions** such as psoriasis, atopic or seborrheic dermatitis and rosacea. Given their unlimited availability, they also allow going deeper into the mechanisms of action of compounds and gain insight into their potential intracellular targets.

LPS triggers the secretion of pro-inflammatory cytokins by PCi-SEB.

Lipopolysaccharide (LPS) is part of the outer membrane of gram-negative bacteria, including *E. coli*, Pseudomonas aeruginosa, Serratia marcescens, Klebsiella and Proteus species. LPS acts on the skin innate immune system through pattern-recognition receptors (PRRs) that are present on sebocytes. Upon stimulation with LPS, we demonstrated that PCi-SEB significantly increase their release of IL-8, TNF-a and Interferon-a after 36h of exposure (Fig. 1). The effect was stable on 3 different batches of cells and observed with Caucasian and Asian PCi-SEB.

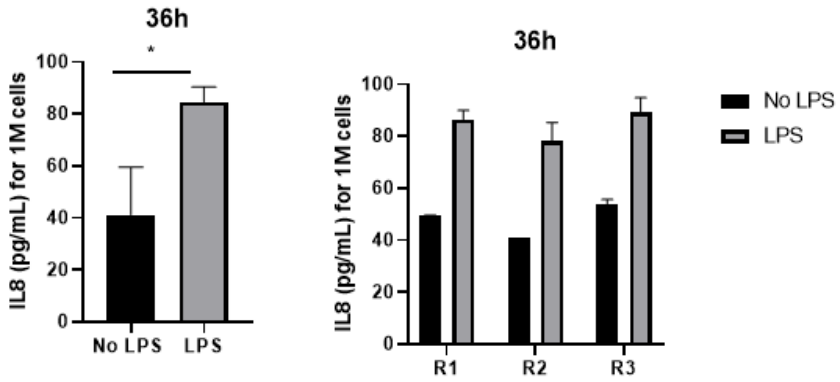

Figure 1. Release of the pro-inflammatory cytokine IL-8 by PCi-SEB exposed to LPS.

***C. acnes* strains**

The toxicity of several strains of *C. acnes* has been analysed on PCi-SEB. As for LPS, PCi-SEB respond to *C. acnes* Type IA1 pre-conditioned supernatant or direct contact by strong upregulation of sebogenesis. Treatment also elicited strong activation of IL-8 and IL-6 genes and protein release in the supernatant. TNFa or MCP-1 genes were also modulated, showing strains differences in pro-inflammatory effects mediated by sebocytes. As expected, the effects of *C. acnes* were potentialized by arachidonic acid induction of sebogenesis in PCi-SEB.

These analyses are particularly relevant to the development of solutions for acne-prone skin.

Contact: Helene Leménager, Helene.lemenager@phenocell.com

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BIOFILM CONTROL
EXPERT IN MICROBIOTA & BIOFILM

New Relevant Acne Models for Objectivation of Microbiota Claims by Biofilm Control

Thierry Bernardi - President and Sophie Cole - Engineer

These last years, consumers have increasingly opted for more conscious and sustainable purchases, and "microbiome/microbiota-friendly" care products benefit from this trend.

Amongst the skin microorganisms, *Cutibacterium acnes* (*C. acnes*) has a key role in the skin ecosystem, participating in skin homeostasis**. This bacterium adapts to changing skin microenvironments and can shift to being opportunistic pathogens, forming biofilms,** and thus are involved in common skin dysbiosis, such as acne vulgaris.

In acne sufferers, sebaceous glands produce excess sebum that mixes with dead skin cells and forms a plug that clogs the pores. This creates a suitable environment for *C. acnes* proliferation, considered as an aerotolerant anaerobe, allowing it to be sustained also on the surface of the skin.
Many studies have identified several virulence factors involved in the pathogenicity of *C. acnes*:

- attachment to target cells, p
- olysaccharide-based biofilm synthesis,
- molecular structures mediating inflammation (reaction of the immune system),
- imbalance with the skin microbiota (dysbiosis) and
- enzymatic degradation of host tissues (scarring).

Paying attention to the skin care, the Cosmetic industry is looking for emerging claims, about the maintenance, protection and restoration of microbiota diversity and equilibrium, as well as prevention of skin dysbiosis. The principal challenges of such demonstrations are evaluation studies able to be assessed by the regulatory authorities.

To answer this need, BioFilm Control has developed **5 relevant "standardized" study models to investigate step-by-step**, with increasing level of significance, the biofilm behavior of C. acnes and to characterize the activity of Cosmetic products. Thus, 1) isolated strains (reference strains and wild types) are tested alone for their ability to form a first layer of biofilm on surface (with the BioFilm Ring Test® (BRT, Chavant et al., 2007)). With this information, 2) Cosmetic products are tested for their **ability to prevent this first layer of biofilm** (with the BRT), 3) for their ability to prevent the biomass made of secreted exopolysaccharides by the biofilm (with Crystal violet staining method), 4) for their ability to treat a 24h mature biofilm (with Crystal violet staining method), and 5) and for their ability to prevent biofilm on Restricted Human Epidermis (by enumeration).

First, a panel of *C. acnes* strains was selected to be more representative of the strains present on the skin. Clinical isolates were isolated from "healthy" patients without signs of acne as well as from "acne" patients. A total of **40 strains, with various phylotypes** (IA1, IA2, IB, II et III), were characterized with 4 reference strains (ATCC 6919, CIP

110516, CIP 110519 and CIP A.179), 16 strains isolated from acne patients and 20 strains isolated from healthy volunteers (Dermatology service, Nantes).

The first layer of biofilm forming *C. acnes* strains is detected with the proprietary BioFilm Ring Test® method, clinically validated (Sotto *et al*., 2021), here used for the benefit of the Cosmetic industry.

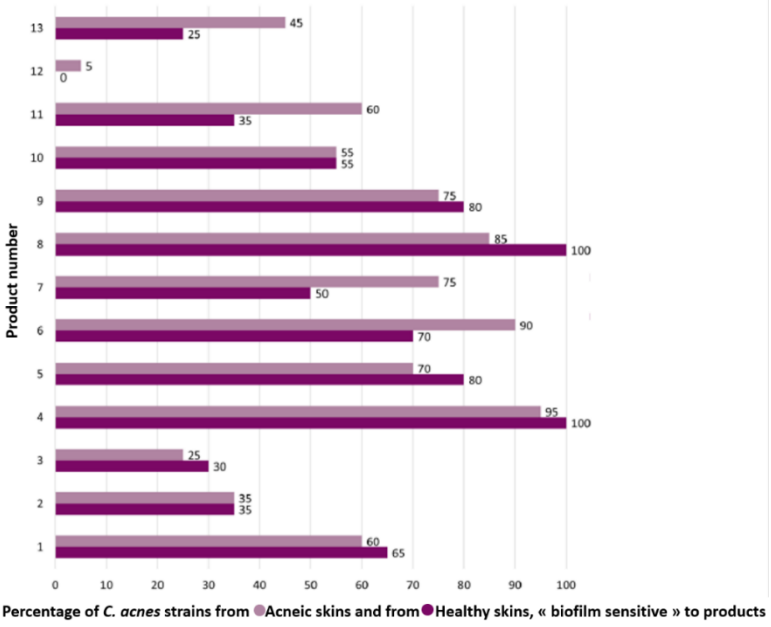
The principle of BRT is based on the use of magnetizable microbeads which are blocked by the cell bodies or the matrix of the early-stage biofilm.

Interestingly, 85% of the 40 *C. acnes* strains showed a low adhesion strength, meaning thin and fragile biofilms, needing at least 24h for adhesion, with no difference between the healthy volunteer's *vs* acneics.

Next, BioFilm Control tested **13 dermo-cosmetic products** already on the market with claims "active against acne or on the *C. acnes* germ" on these 40 strains.

On the second range of tests for their preventive effect on early-stage adhesion, the BRT allowed to discriminate **4 cosmetic products** that showed significant activity on *C. acnes* strains, and one product was clearly a cut above the rest.

On the third rang of test, for their preventive effect on biomass (Crystal violet staining), the results discriminate products combining efficiency with the BRT and/or the CV. Combining these results, some products showed a profile of activity **"anti-biofilm installation"** more efficient on the acne skin strains than on the healthy skin strains. That kind of data could help as for R&D qualification than for the validation of claims on a product on shelf.



In addition to the preventive models, a 4th range of **"curative" studies** were conducted on biofilms already formed by *C. acnes* (24h) using Crystal violet staining.
Some products showed activity to destroy biofilms **after 2h and after 6h of contact**, effective products showed a % reduction of biomass between 70 and 100% for the strains of the 2 groups.
To further investigate the products on *C. acnes*, the 5th range of tests on 2 strains in the study. showed an inhibition of *C. acnes* adhesion on RHE by almost 80%.

Today, Biofilm Control proposes **the most extensive and complete *in-vitro* "*C. acnes* testing"** offer with step-by-step progress in complexity, from the testing on isolated bacteria culture to the testing of these bacteria on Reconstructed Human Epidermis, that pay attention to the whole bacterial life cycle on skin, planktonic and biofilm.

Contact: Hervé Molinero, contact@biofilmcontrol.com

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Pre-clinical models for the study of host/microbiome interaction by InnovSkin

Rachida Nachat-Kappes, PhD - Doctor of Biology and Scientific Consultant

The skin, like other organs, has its own microbiome. Diverse and variable from one individual and one anatomical region to another, the microbiome is a key player in skin homeostasis. As the host's first line of defence, its characterisation and understanding of its imbalance or dysbiosis, observed in various skin disorders, are the subject of much research and require the development of pre-clinical study models.

Preserving and/or restoring the microbiome is now a major challenge for many sectors, including the pharmaceutical and cosmetics industries. In 2007, the launch of the **"Human Microbiome Project" by the National Institutes of Health** (NIH) made it possible to identify "healthy" microbiomes at the intestinal, vaginal, nasal, oral, and cutaneous levels [(1)]. The second phase of the project (HMP2) studies microbiomes in pathological situations [(2)].

Thanks to the advent of multi-omics approaches, HMP2 has been designed to explore the host-microbiome interaction in its entirety, including immunity and metabolism.

The skin is a complex ecosystem, colonised by about 1,000 species. Today, one of the **main limitations** of the study of skin-microbiome interactions is the **absence of pre-clinical models capable of reproducing the complexity of the cutaneous bacterial community**.

Both reconstructed epidermis (RHE) and explants have advantages and disadvantages. For example, in the case of RHE, although they give more reproducible results, they are less favourable for studying aerotolerant anaerobic bacteria such as *C. acnes* than explant models.

The latter also undergo sanitising treatments that can interfere with and disrupt the inoculated microorganisms [(3)]. Recently, a **synthetic microbiome including 119 species** of bacteria naturally present in the human body has been created and inoculated *in vivo*, demonstrating the advances in this field of research [(4)].

References:

[(1)] Turnbaugh, P. J. *et al.* Nature 449, 804-810 (2007).
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[(3)] Larson PJ, *et al.* J Invest Dermatol. 2021 Jan;141(1):228-231.e4.
[(4)] Cheng, A., G. *et al.* Cell. 2022 Sep 15;185(19):3617-3636.e19.

Contact : Rachida Nachat-Kappes, rachida@innovskin.com

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