First-time use of a droplet-based microfluidic method to highlight specificities of microbiota communities from sensitive skin

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Abstract

Background:

Despite the importance of the skin microbiome for skin health, only a few studies in the literature have compared the skin microbiome of normal skin (NS) and sensitive skin (SS) [1-3]. They showed no variations in bacterial diversity indices but shifts in abundance of specific genera were noted in SS.

Here, we compared the microbiome from individuals with/without SS at strain level resolution and isolated representative strains in culture to evaluate ingredients.

Methods:

Face skin swabs from individuals with normal (40) or sensitive skin (33) were analyzed using 16S rRNA sequencing to study strain-level bacterial diversity, prevalence and abundance. We used droplet-based microfluidic technology (DBMT) to create a bacterial collection from individuals of each cohort and evaluated the effects of active ingredients on the growth of selected representative strains.

Results:

Here we confirmed the observations from previous studies of SS that there are increases of abundance in *Corynebacterium*, *Kocuria*, *Micrococcus* and *Lactococcus* and decreases in Staphylococci. We additionally observed an increase in *S. aureus*, *C. kroppenstedtii* and *Pseudomonas*, and a decrease in *Lactobacilli*.

Using DBMT, we isolated several hundred strains and selected ~50 representatives of SS and NS. Some ingredients showed a "microbiome-friendly" profile while others showed the potential to reestablish a microbial profile resembling NS.

Conclusion:
A better understanding of shifts in microbial communities in individuals with SS and subsequent ingredient testing on a clinically representative set of bacterial strains is of real interest to select effective microbial rebalancing and skin soothing solutions for consumers with SS.

Keywords: droplet-based microfluidic; sensitive skin; bacterial collection; ingredient profiling

Introduction

Up to 70% of women and 60% of men report having sensitive skin [4]. Sensitive syndrome is a complex clinical phenomenon defined by the self-reported presence of different sensory perceptions, including tightness, stinging, burning, tingling, pain and pruritus, even though objectively measurable signs of irritation are not always present.

Although the skin microbiome is considered important in skin health, the relationship between the skin microbiome and skin sensitivity has received little attention. A first comparison was performed on several body zones of 3 men and 3 women (20-35yo) suffering from sensitive skin (SS) or not (NS). In this limited cohort, decreased percentage of *Staphylococci* and absence of *Acinetobacter* or *Proteus* were shown after culture of skin bacteria under aerobic conditions. However, there was no clear skin microbiome signature of the sensitive skin phenotype [1]. A later study on 42 Korean women (22-52yo, cheek) showed equivalent diversity of skin bacteria in SS, but a significant increase of Lactobacillus and *Mucor racemosus* and a decrease in *Malassezia restricta* [2]. Finally, a larger study on Caucasian women (SS 20-50yo and NS 23-50yo, cheeks) likewise did not show differences in diversity. However, a signature of discriminant genera was proposed with significant decreases in *Cutibacterium*, *Lawsonella*, *Bacillus* in SS (with a tendency for *Staphylococcus*), and increases in *Corynebacerium*, *Snodgrassela*, *Kocuria*, *Micrococcus*, *Lactobacillus* and *Lactococcus* (tendency for Chryseobacterium and Roseomonas) [3].

In our study, we compared the composition of the skin microbiota of individuals with normal and sensitive skin. To characterize the composition of the skin microbiota, we sequenced the entire 16S rRNA gene using the PacBio technology to obtain unprecedented taxonomic resolution of the microorganisms present. We used droplet-based microfluidic technology

(DBMT) for the first time to create a collection of specific bacterial strains isolated from individuals with sensitive and non-sensitive skin. The growth of representative strains was then tested in the presence of active ingredients to identify neutral ingredient or those ingredients with the potential to rebalance sensitive skin microbiota.

Materials and methods

Recruitment of participants

78 volunteers were enrolled in this study and assessed by a dermatologist with healthy skin on the studied anatomic unit (free of eczema, psoriasis, wounds, inflammatory scar….). We recruited male and females, aged from 18 to 77 years old, and having the skin phototype ranging from 1 to 5. Among the 73 volunteers on which the analysis was completed, 30 reported having a thin and sensitive skin and were tested for their hypersensitivity to heat before sampling. The study was conducted in accordance with the Declaration of Helsinki and informed consent was obtained from all subjects involved in the study.

Sampling protocol

The skin site investigated in this study was the face. Participants were asked not to wash their skin within 24 hours prior to sample collection, and not to apply any cosmetics during this time frame. A cotton swab immersed in 1,5mL of skin sampling buffer (10% glycerol, 0.1% Tween80 in 1xPBS) was used for sampling. An area of 4 cm^2 was vigorously rubbed with the cotton swab for 30 seconds. Swabs were then vortexed for 30s at maximum speed in their container to resuspend biological matter in solution, and they were subsequently squeezed to maximize the volume of SSB in the container. Samples were concentrated in 400µL of 1x PBS and stored at -80°C until DNA extraction.

DNA extraction

The DNA was extracted using the kit Zymo BIOMICSTM DNA miniprep (D4300) following manufacturer recommendation including a mechanical lysis step. Blank extractions were also performed to check for potential contamination. The final DNA was eluted in 50µL of nuclease-free water. DNA samples were kept at -80°C until bacterial quantification.

DNA quantification

A Taqman probe-based qPCR assay was used to measure the bacterial concentration of the samples. A culture of E. coli was grown overnight at 37°C in BHI, which was followed by a DNA extraction. The eluted DNA was quantified using Qubit dsDNA HS assay kit. 5µL of E. coli DNA solution was used for quantification. The DNA solution was serially diluted in nuclease-free water to reach a final dilution factor of 10^6. The six dilution steps were used as standard for the qPCR assay.

20µL reactions were performed in triplicate will the following reactants: 10µL PrimeTime Gene Expression Master Mix, 0,8µL forward primer, 0,8µL reverse primer, 0,4µL of probe, 6µL of nuclease free water and 2µL of DNA template. A reaction was performed with a blank extraction as DNA template, and another was performed with nuclease-free water as DNA template. The reactions were performed in 96-well plates, low profile and white, and they were sealed with thermoresistant optical films. The qPCR was performed in Agilent Aria Mx machine with the following cycling conditions: initial denaturation 95°C – 3 min – 1 cycle; denaturation 5seconds – 95°C; Annealing/extension: 60°C -40 cycles; final extension: 60°C - 5min- 1 cycle.

PacBio library preparation

The primers used to amplify the full-length 16S rRNA gene were composed of the specific regions 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') combined to asymmetrical barcodes. PCRs were performed with the following solution mix: 10µL Q5 reaction buffer, 1µL 10mM dNTPs, 2µL 10µM forward primer, 2µL 10µM reverse primer, 2µL 10-2ng/µL DNA template, 0.5µL Q5 high-fidelity DNA polymerase, 32.5µL nuclease-free water. The cycling conditions were as follows: initial denaturation: 95°C – 30s – 1 cycle; denaturation: 5s – 95°C – 30 cycles; Annealing – 59°C – 30 cycles; extension – 45s – 72°C; final extension: 72°C - 2min- 1 cycle.

Amplifications were checked on 1% v/v agarose gel, 30min migration using 100mV. A band was expected around 1500bp. Samples were then purified using AMPure PB beads. The entire PCR volume was mixed in equal ratio with AMPure XP beads, and two washes were performed with 80% ethanol. Finally, DNA was eluted in 25µL of nuclease-free water.

DNA concentration was measured using Qubit dsDNA HS assay kit. 3µL of eluted DNA was used for quantification. Samples were pooled in equal amount to reach a final DNA mass of 1000ng. In total, 3 DNA pools were generated to ensure sufficient sample coverage. Pools were then kept at -20°C until completion of library preparation.

Pools were sent to Maryland Genomics to perform a PacBio short-insert library preparation, which included DNA damage repair, end repair/A-tail, ligation and AMPure PB bead purifications. Each pool was sequenced in a PacBio Sequel II 8M run, 30h movie, to generate Circular Consensus Sequences (CCS). Samples were demultiplexed and exported in fastq format.

Mock community preparation

To assure the quality of the sequencing and bioinformatic workflows, as a positive control a mock community composed of 14 wild strains previously isolated from the skin was built. The community includes the following strains: *Cutibacterium acnes*, *Cutibacterium avidum*, *Cutibacterium namnetense*, *Cutibacterium granulosum*, *Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus aureus*, *Staphylococcus hominis*, *Corynebacterium tuberculostearicum, Corynebacterium kroppenstedtii, Micrococcus luteus*, *Kocuria palustris*, *Kocuria rhizophila*, and *Bacillus thuringiensis*. Strains were plated in BHI agar supplemented with 0.1% Tween80 at 37°C in aerobic or anaerobic conditions depending on the species requirements. A single colony was picked from each plate and grown in liquid medium. 50µL of each culture was pooled in a single tube. The bacterial density of each culture was assessed as colony forming units per volume unit (CFU/mL) after growth in agar medium under appropriate conditions. The pool of cultures was subjected to two DNA extractions using ZymoBIOMICSTM DNA miniprep. These two DNA solutions from the same mock community were prepared for sequencing using the same protocol as for the skin samples. Two amplification replicates were performed for each DNA solution.

Bioinformatic analysis

Samples were analyzed with the pipeline dada2 [5]. A metadata sheet was used to gather all information specific to samples such as participant characteristics (age, sex, skin phenotype), skin sites and the corresponding sequencing run. Sequences were filtered based on size, quality, and the presence of primers. The error rate was learnt by gathering samples sequenced in the same run and the subsequent denoising was performed with the pseudo-pooling method. Taxonomic assignment was achieved with the database Silva 138.1 at the species level. If the algorithm didn't manage to provide a classification at the species level, the species annotation was built with the corresponding genus combined to the mention spp. (e.g. *Staphylococcus spp*.). Amplicon sequence variants (ASVs) that were not assigned to any genera were excluded from the analysis. ASVs classified as mitochondria and chloroplast at the family and class level,

respectively, were also discarded from the analysis. In order to make the data manipulation easier and to exclude potential sequencing artefacts, ASVs having less than 10 reads in the overall study and that were present in a single sample were also discarded. Samples with less than 5000 reads were removed from this study. Rarefaction curves were generated with the Vegan package to ensure sufficient sequencing depth.

Normalization of the sequencing data

Sequencing data were treated according to four different workflows to check the impact of normalization methods. Total Sum Scaling (TSS) was achieved with the function transform_sample_counts and rarefied counts with and without replacement were achieved with rarefy_even_depth. Both functions are from the phyloseq package. The Cumulative Sum Scaling was produced by exporting data in the appropriate format using phyloseq_to_metagenomeSeq from the phyloseq package and counts were normalized with MRcounts from metagenomeSeq package. TSS was used as default method for the subsequent analysis.

Creation of a bacterial collection from the normal and sensitive skin subjects

More than 4,000 isolates were retrieved and added to the normal and sensitive skin collection. Amplification of the 16S rRNA gene was performed with the high-fidelity polymerase (Q5 polymerase) and sequenced using PacBio Sequel II 8M.
16S rRNA gene sequences allowed for isolate identification (genus, species, ASVs level). The normal and sensitive skin microbiota was constituted by coupling the information from the bioinformatic analysis regarding prevalence and abundance.

Active ingredient profiling on the bacterial collection from individuals with normal and sensitive skin

31 strains were isolated from normal skin: 10 Cutibacterium, 17 Staphylococcus, 1 Micrococcus and 3 Corynebacterium. 31 strains were isolated from sensitive skin: 8 Cutibacterium, 12 Staphylococcus, 1 Micrococcus and 3 Corynebacterium, 2 Kocuria 2 Streptococcus, 1 Roseomonas, 1 Enhydrobacter and 1 Bacillus strains. A classical microtiter plate method was performed. Briefly, the strains were grown in 96-well culture plates with triplicates of the ingredients at 4 concentrations or without ingredient in 200µl of suboptimal proprietary medium. Plates were incubated at 37°C in a shaking or non-shacking incubator

depending on the strains. Growth was assessed after 24h, 48h and 72h by optical density measurement D0600 nm (Spark Tecan).

Results

I- Sequencing analysis between normal and sensitive skin

The analysis was completed for the73 volunteers (40 subjects in the NS and 33 subjects in the SS cohort). The diversity was analyzed using the Shannon index and highlighted no significant differences between the two cohorts (Figure 1).

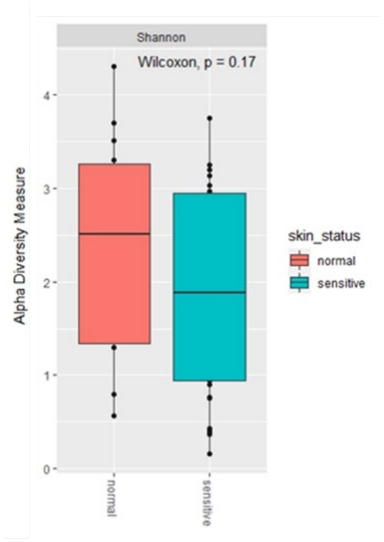

Figure 1: Diversity analysis between normal skin and sensitive skin

We then analyzed the prevalence of the strains. This means that we observed the absence or presence of different bacterial genera on normal or sensitive skin. Figure 2 represents the selected 20 genera having a prevalence of above 30% for both cohorts. The genus *Cutibacterium* was found in 100% of the volunteers in each cohort. The genus *Staphylococcus* was also found in each volunteer in NS but the prevalence slightly decreased by 3% in SS. Interestingly, this analysis showed a 23% decrease in the prevalence of the *Streptococcus* genus in SS and an increase of the prevalence of *Acinetobacter* by 47%. The prevalence of the *Corynebacterium* genus increased by 7% in SS.

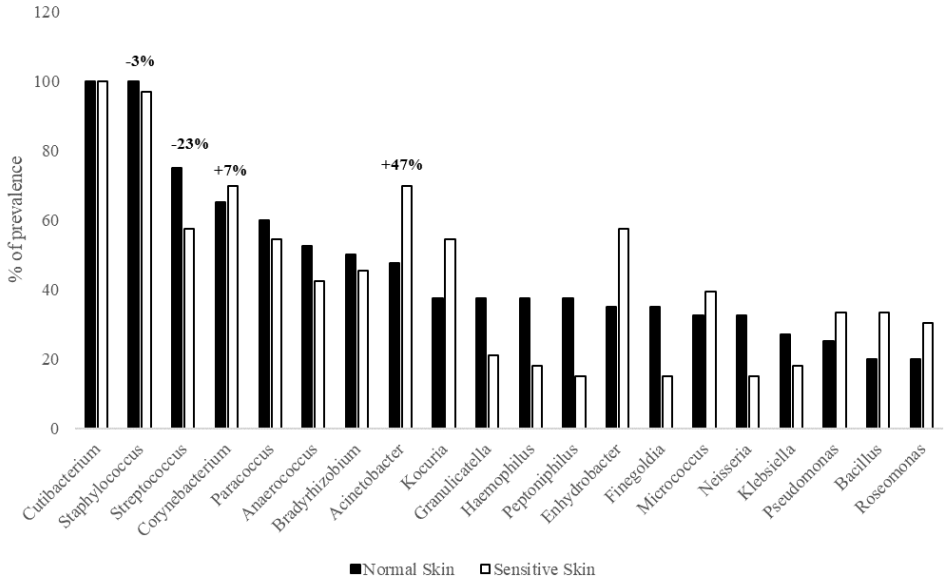

Figure 2: Illustration of selected 20 genera having above 30% prevalence in both cohorts (percentage of negative or positive variation are given as example for some stains).

Next, we compared the two cohorts to examine the presence of common genera and to determine variations in abundance (Figure 3). *Cutibacterium* and *Staphylococcus* are the two most abundant genera in both cohorts, but the proportion is different. NS showed 47% of *Cutibacterium* abondance and 38% of *Staphylococcus* compared to the SS with 60% of Cutibacterium and only 22 % of *Staphylococcus*. 3 genera *Ruminococcus*, *Anaerococcus* and *Romboutsia* are present in the top 10 most abundant genera in NS but are absent from this ranking in SS. 5 genera, *Bacillus*, *Acinetobacter*, *Actinomyces Kocuria* and *Micrococcus* were abundant in SS and decreased in NS.

However, the analysis of abundances that notably vary between the two cohorts seems very interesting to us. But further investigation of strain differences within genera with no strong variation between cohorts revealed new subtleties. Bacterial genera that vary little between the two cohorts can still provide important information about the changes in skin flora between the two cohorts.

Further examination of species-level diversity (Figure 3) showed that in NS subjects, the most prevalent and abundant members of the *Cutibacterium* are 4 species *C. acnes*, *C. granulosum*, *C namnetense* and *C. avidum*. The respective abundances are 46%, 2%, 0.4% and 0.1% (data not shown). In SS subjects, only 3 strains of *Cutibacterium* were found, with *C. avidum* no longer

being detected. Moreover, *C. acnes* was increased in SS with an abundance of 61 % while the abundance of *C. granulosum* was decreased 3 folds.

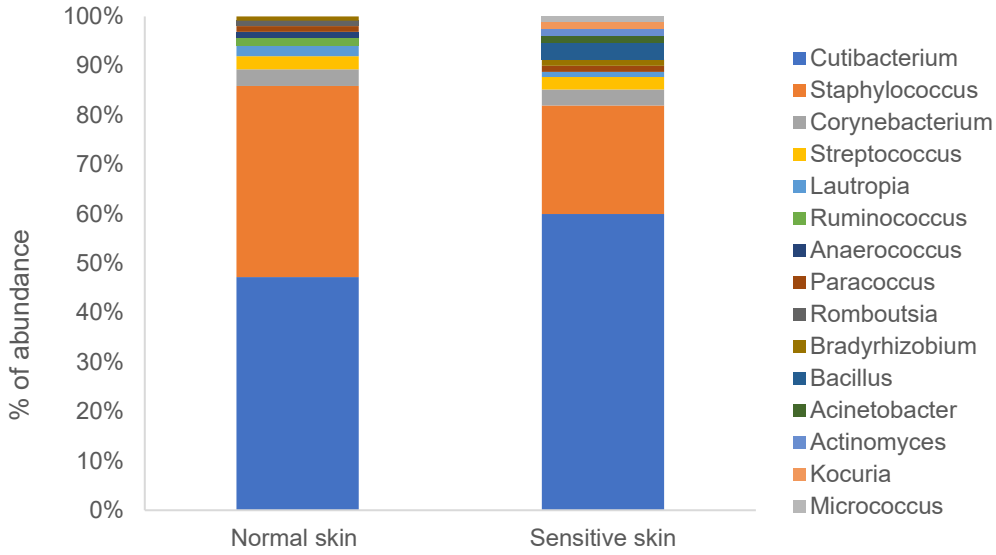

Figure 3: Illustration of abundance of the 10 most genera present in each cohort

On the contrary, as shown in Figure 4, the abundance of the genus *Staphylococcus* decreased in SS. Among that genus, *S. epidermidis* and *S. capitis*, the most abundant *Staphylococcus* species in NS decreased ~ 1.6 fold in SS. In the SS subjects, we observed a 2.8-fold increase of *S. aureus* abundance compared to the NS subjects.

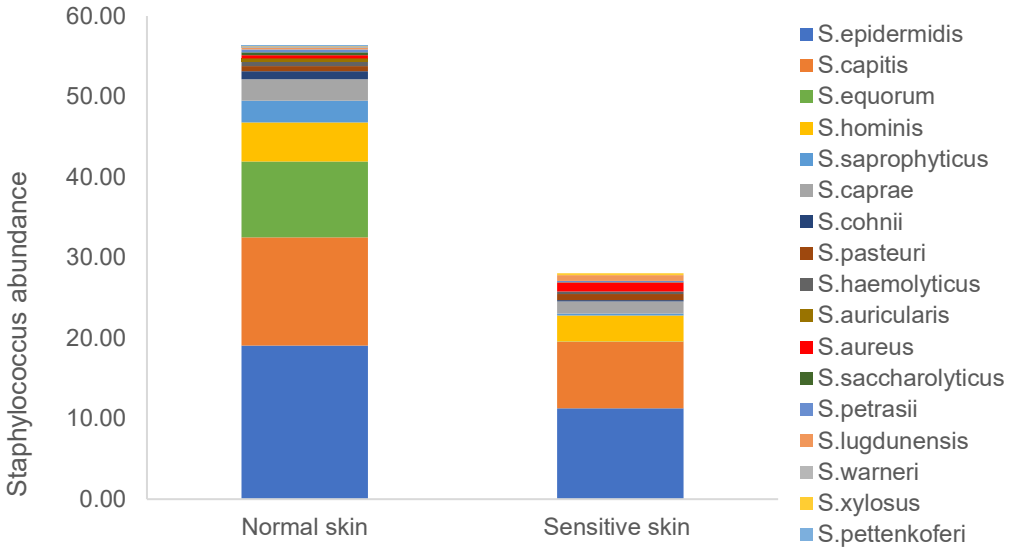

Figure 4: Staphylococcus species abundance relative to the global abundance bacterial species between normal and sensitive skin

The genus *Corynebacterium* is another good example of the interest of an analysis at the strain level. At the genus level, the variation of abundance between the two cohorts was not significant but if we look at the strain level, we can see lot of variations between NS and SS (Figure 5). Firstly, the most represented *Corynebacterium* species is the *C. kroppenstedtii*. This strain is 1.6 times more present in SS than NS. Less represented but maybe important in the biology of sensitive skin, we observed an increase of *C. tuberculostearicum* by 3.8 folds in SS. Conversely, the abundance of *C. accolens* decreased by 4 folds in SS subjects.

Interestingly we found *C. pseudogenitalium C. jeikeium C.amycolatum C. bovis* only in NS but we observed a lack in other *Corynebacterium* (for example *C. casei*, *C. lipophiloflavum, C. resistens* and *C. mastitidis*).

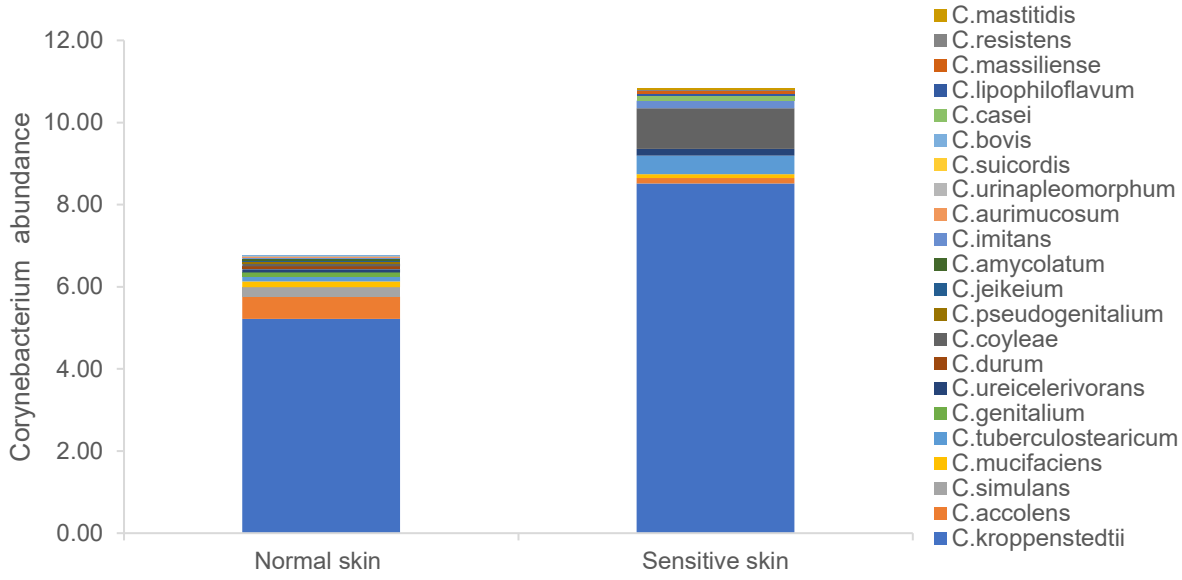

Figure 5: Corynebacterium species abundance relative to the global abundance bacterial species between normal and sensitive skin

II – Creation of bacterial collection from normal and sensitive skin to screen active ingredients

Using droplet-based microfluidic technology, we isolated several hundred strains and built our bacterial collection from which we selected the strains representative of sensitive and normal skin conditions. For some bacteria, we decided to select multiple species from a single genus.

For the NS we isolated and selected 10 *Cutibacterium*, 17 *Staphylococcus*, 1 *Micrococcus* and 3 *Corynebacterium* species. Then 31 strains were isolated and selected from SS with 8 *Cutibacteria*, 12 *Staphylococci*, 1 *Micrococci* and 3 *Corynebacteria*, 2 *Kocuria* 2 *Streptococci*, 1 *Roseomonas*, 1 *Enhydrobacter* and 1 *Bacillus*.

5 preselected active ingredients were then tested on the growth of these strains to evaluate if one would provide a good performance to help to recover a microbial profile closer to the normal skin. The results are presented in the Figure 6. The results show different profiles of active ingredients.

One of them has an appreciable impact on bacterial growth of strains isolated from NS (Figure 6A). We can observe for instance variations in the growth for some concentrations, at different times and for several strains of *Staphylococcus* and *Corynebacterium*. On the other hand, some ingredients could have slight effect on the growth of strains (Figure 6B). This type of ingredient could fit to the "microbiome-friendly" trend, *ie* an ingredient that would have no or the least damaging impact on the normal skin flora to preserve its protective function of the skin.

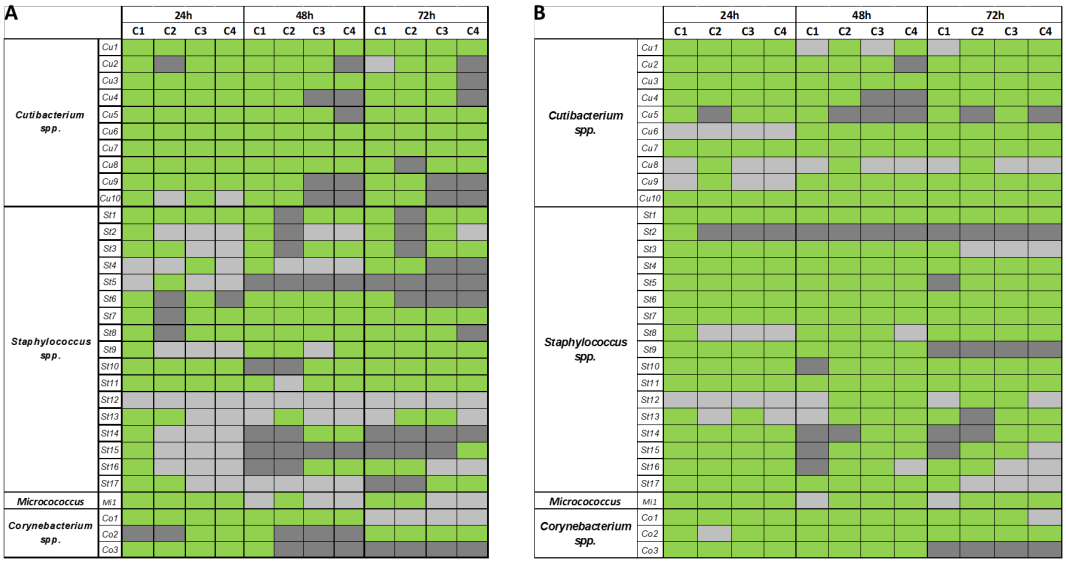

Figure 6: Growth results of ingredients on the bacterial collection from normal skin.

The growth was evaluated with adequate medium and environments with 4 concentrations of active ingredient during 24h, 48h or 72h. A: example of an active ingredient with some effects on bacterial growth B: example of an active ingredient with a "microbiome-friendly" profile. Results from n=4 and compared to the no treated. No significative difference in green, growth decrease in light gray and increase in dark gray (Dunnett's multiple comparison test * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Discussion

Sensitive skin is defined by the self-reported presence of different sensory perceptions, including tightness, tingling and pain in response to stimuli that normally should not provoke such sensations [6]. Sensitive skin may occur in individuals with normal skin, or with skin barrier disturbance. The aim of our study is to compare the composition then to isolate in culture the skin microbiota of individuals with sensitive and normal skin to better understand this skin condition.

In congruence with the existing literature, we confirmed that there are no significant differences of microbial diversity between the two cohorts [1-3]. Therefore, it may be necessary to further analyze the balance of the microbiota between sensitive and normal skin at the genus and species levels to define a putative bacterial signature for sensitive skin. This may help in better understanding and improving this skin condition.

Unlike Keum's study [2] that shows no changes in *Cutibacterium* and *Staphylococcus* genus levels, we showed an increase of *Cutibacterium* and a decrease of *Staphylococcus* genus abundance between cohorts. We are more in accordance with Filaire *et al.* who showed in sensitive skin an increase of *Actinobacteria* (phylum composed essentially by *Cutibacterium* and *Corynebacterium* genus) and a decrease of Firmicutes (phylum composed by the Staphylococcus genus) [7].

To complete the analysis, as Jarrin et al. [3], we observed in SS subjects an increase in abundance of *Corynebacterium* and also of *Kocuria*, *Micrococcus* and *Lactococcus* genera (data not shown) and a decrease of *Staphylococcus*. However, we observed a contrasting result for *Cutibacterium* whose abundance was higher in our study. We obtained also contrasting results for *Bacillus* and *Lawsonella* that were found to increase in abundance in SS subjects, and *Lactobacillus* that we found to decrease (data not shown).

Our results are also in accordance in part with Hillion *et al*. [1] as we showed in SS an increase in the abundance of *Corynebacterium* and *Brevibacterium* (data not shown) and a decrease of *Staphylococcus*. We also observed an increase in *Micrococcus* that they observed only in male sensitive skin. However, in our case, we rather observed an increase in *Pseudomonas* in the SS cohort.

Altogether, our results and previous results seem to demonstrate a core group of genera that were changed in SS: those increased as *Corynebacterium, Kocuria, Micrococcus, Lactococcus*

and *Brevibacterium*, and one decreased, the *Staphylococcus*. For the other bacteria, a more precise analysis of the data or new studies would certainly help to define a clear status in SS.

Going deeper to the strain level definitely provides an additional insight as it more precisely highlights the changes within a genus. For example, in *Corynebacteria*, we particularly observed a 1.6 fold increase of *C. kroppenstedtii,* the already most abundant in NS. Interestingly, an increase of *C. kroppenstedtii* was previously correlated to skin redness showing that this bacterium could be particularly involved in the redness and the sensitivity of the skin [7]. Ridaura et al. also showed that *Corynebacteria* promote a dramatic increase in the number and activation of a defined subset of γδ T-cells. This effect is long-lasting, occurs independently of other microbes, and is partly mediated by interleukin (IL-23). Under steady-state conditions, the impact of *Corynebacterium* is discrete and non-inflammatory. However, under specific conditions, *Corynebacterium* alone promotes inflammation [8]. *Corynebacterium* and particularly *C. kroppenstedtii* could thus be important to take into consideration in cosmetic care for sensitive skin.

Recent research indicates that the bacterial diversity and the relative abundance of different microbes present on and in the skin may contribute to skin barrier dysfunction [9-10]. In this study we have particularly showed that if *Staphylococcus* genus level was decreased in the SS, but a 2.8 increase of *S. aureus* was observed with a correlated decrease of *S. epidermidis* and S. *hominis*. As *S. aureus* secretes a lot of virulence factors known to contribute to skin barrier dysfunction [11] it is also important to pay attention to it for sensitive skin care and to restore the equilibrium with its counterparts *S. epidermidis* and S. *hominis* known to synthesize antimicrobial peptides that could selectively kill *S. aureus* [12].

After the analysis of the metagenome between the two cohorts, we have used droplet-based microfluidic technology to isolate in culture bacterial strains from NS and SS and to create a bacterial collection from these clinical isolates. We have previously demonstrated that this technology is particularly suitable for isolation of bacteria known to be difficult to isolate and grow. Here we got hundreds of strains from each cohort and selected 31 strains as representative of each one. On these 62 strains, we evaluated 5 preselected ingredients on the growth profile of the selected strains from both collections. As bacteria do not growth at the same speed and

are not sensitive at the same level to active ingredients, we checked the growth over 3 days and with several concentrations of ingredients. On one hand, we could learn how the ingredient will act on the clinical strains from the NS. This could be interesting as a strategy for identifying some prebiotic ingredients for specific strains of interest. For example, some strains have been shown to be decreased in aging and could thus be promoted to restore an expression closer to a younger skin one. Additionally, this system can be used to identify ingredients with neutral effects, i.e. those that can be said to be "microbiome-friendly". On the other hand, using the clinical strains from the SS cohort will be of real interest to identify ingredients that could help to compensate the shifts in abundances of various strains observed between the two cohorts.

Conclusion

Our preliminary analysis showed interesting changes in the microbiome of individuals with sensitive skin not in terms of diversity but rather in terms of the abundance of genera and bacterial species. DBMT allowed the successful isolation in culture of the bacterial strains to build both our "normal" and "sensitive" skin bacterial libraries, including some strains recognized as difficult to culture.

Analyzing in more depth the changes in microbial communities in people with sensitive skin associated with the selection of ingredients on a clinically representative microbial collection is of real interest to select effective solutions for microbial rebalancing and skin soothing for these specific consumers. Moreover, having this collection coming from clinical isolates could be used to evaluate *in vitro* bacterial interactions between 2 strains, up to community of strains, with or without variation of their environment, to better understand clinical shifts observed in sensitive skin.

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