IFSCC 2025 full paper

"Corynebacterium in Skin Aging: Species Distribution and Anti-Aging Potential"

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1. Introduction

Corynebacterium is a bacterial genus that is part of the normal skin microbiome with a growing concern about its role in skin health and aging. Interestingly, it was previously reported a decrease in *Cutibacterium* [1-3] but an increase in *Corynebacterium* [1, 2, 4] on forehead and/or cheeks from older donors. Moreover in a study conducted by 16S ribosomal ribonucleic acid (16S RNA) on 495 North American subjects, *C. kroppenstedtii* was shown to be increased, while others staying constant or decreased [4].

The dominance of *C. kroppenstedtii* on older skin can be particularly driven by the metabolism and production of free fatty acids, which have been found on older skin [5]. Although considered as a skin commensal, there is evidence that this specie can act as an opportunistic pathogen [6]. It was among the most abundant bacteria on skin of patients suffering from rosacea and redness compared to healthy skin [7, 8].

In this study, our goal was to analyze the content and distribution of *Corynebacteria* in skin samples from young and old donors focusing particularly on the wrinkle area, as well as from individuals with sensitive skin. We then used droplet-based microfluidic technology (DBMT) to extract and assess after sequencing some isolates for their potential skin benefits.

2. Materials and Methods

The study was conducted in accordance with the Declaration of Helsinki (statement of ethical principles applicable to medical research involving human beings, including research on human biological material and identifiable data) and informed consent was obtained from all subjects involved in the study.

Sensitive skin study

We conducted a thorough bacterial analysis utilizing 16S RNA sequencing alongside droplet-based microfluidics (Biomillenia Romainville, France) for the extraction and cultivation of a wide range of clinical bacterial isolates from individuals with Normal Skin (NS) and Sensitive Skin (SS) [9]. We recruited male and females, aged from 18 to 77 years-old, and having a skin phototype ranging from 1 to 5. Among the 73 volunteers on which the analysis was completed, 30 reported having a thin and SS, and were tested for their hypersensitivity to heat before sampling. More precisely, panelists from the SS cohort showed a 155% increase in

electrodermal response on the cheeks after heating stimulation (conductance measured using a Galvanic Skin Response electrode).

DNA was extracted using the kit Zymo BIOMICS™ DNA miniprep (D4300, Zymo Research, Irvine, CA, USA) following manufacturer recommendations. The primers used to amplify the full-length 16S RNA gene for the PacBio library preparation were composed of the specific regions 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') combined to asymmetrical barcodes. PCR samples were then purified using AMPure PB beads (PacBio, SanDiego, CA, USA).

For bioinformatic analysis, samples were analyzed with the bioinformatics pipeline DADA2, Taxonomic assignment was achieved with the database Silva 138.1 and if the algorithm did not manage to provide a classification at the species level, the species annotation was built with the corresponding genus combined to the mention spp (species plural). To make the data manipulation easier and to exclude potential sequencing artifacts, ASVs having less than 10 reads in the overall study or present in a single sample were also discarded. A cutoff of 5,000 reads was selected to avoid a loss of rare species. Total Sum Scaling (TSS) was achieved with the function transform_sample_counts and rarefied counts, with and without replacement, were achieved with the rarefy_even_depth function from the Phyloseq package. Phyloseq is a tool to import, store, analyze, and graphically display complex phylogenetic sequencing data that has already been clustered into Operational Taxonomic Units (OTUs) (GitHub, San Francisco, CA, USA).

Aging study

We conducted a study to discover differences in microbiota structure between an old group and a young group, particularly focusing on the wrinkled area of the skin [10]. To this end, we used whole-genome sequencing (WGS) to gain deeper insight into the composition and to gain access to the microbial structure at a species level as well as microbial functional potential. A total of 100 healthy female Caucasian volunteers aged from 18-85 years old were recruited. A total of 95 panelists completed this study: 49 in the older group (subjects aged above 55 years old with crow's feet wrinkles of grade 5-6) and 46 in the younger group (18-35 years old with crow's feet wrinkles of grade 0-1). The microbial samples were collected as follows: inside the crow's feet wrinkles (wrinkle), around the crow's feet/under eye area and on the cheek adjacent to earlobe area (control). DNA extraction and whole-metagenome sequencing libraries were prepared using the Epicentre MasterPure kit, NEBNext Ultra II FS DNA Library Prep kit (Epicentre, Madison, WI, USA) and Ampure XP beads (Beckman Coulter, Indianapolis, USA) following the guidelines provided by the manufacturers. Libraries of more than 2 nM were submitted to paired-end (2x100 base pairs) sequencing on the HiSeq 3000 (San Diego, CA, USA).

Bacterial sampling

More than 3,500 isolates were retrieved by Droplet-Based Microfluidic Technology (DBMT, Biomillenia, Romainville, France). Briefly bacteria from skin swabs coming from 6 subjects (35-70 years old - upper cheek) were processed using the high-throughput microfluidic platform. The isolated strains were analyzed via 16S sequencing (1600 bp on specific regions) and phylogenetic analysis was done for close clades as explained Figure 1. Among the 3,500 isolates, 500 strains were sequenced and analyzed by BLAST; 8 genera were identified and 36 species isolated.

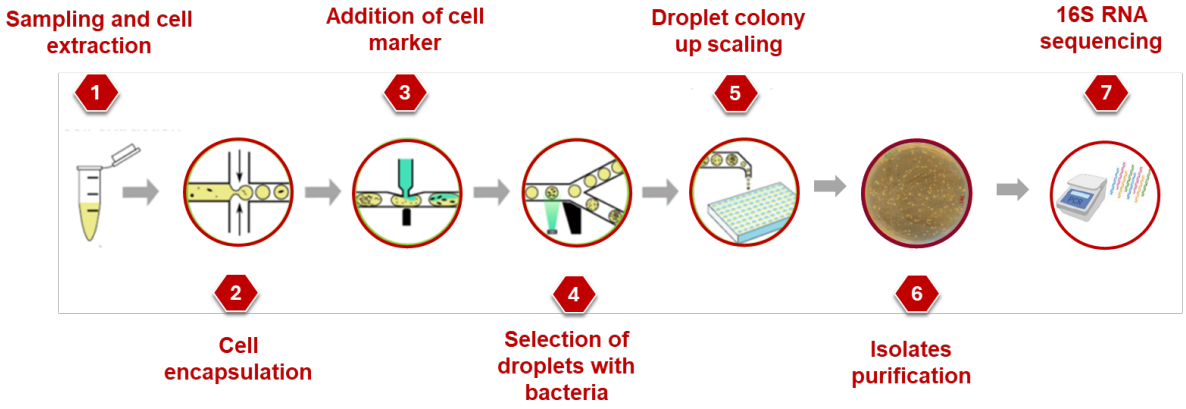

Figure 1. Bacterial collection isolation by Droplet-Based Microfluidic Technology followed by identification by 16S RNA sequencing.

Analysis of bacterial effects on antiaging targets

We selected Corynebacteria species to be studied thanks to the results of previous experiments and thus explored C. accolens, C. amycolatum and C. kroppenstedtii potential for skin benefits.

We first evaluated their antioxidant potential using DPPH assay. Briefly, bacteria were cultivated during 72h and adjusted to 10^7 CFU/mL. Then we centrifugated these bacterial susspensions to recover the supernatant tested using DPPH assay (1-diphenyl-2-picrylhydrazyl, 0.09 mM). The absorbance at 530 nm was determined, and the percent scavenging activity was calculated. *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus* aureus ATCC 35556 were used as controls and and vitamin C (1mM) as positive control. Statistical analysis performed using Student t-test *versus* untreated control.

We also measured the *Corynebacteria* indirect effect on collagen type I synthesis by normal human fibroblasts (Delfia immunoassay) compared to the same ATCC strains of *S. epidermidis*, S. *aureus* plus *Cutibacterium acnes ATCC* 6919 (Figure 2). Bacteria were cultivated during 72h and adjusted to 10^5 CFU/mL and then were placed in a transwell insert above normal human fibroblasts grown on the bottom of a culture well. After 48h of treatment with the bacteria or vitamin C (50 µM), deposited collagen type I content was measured in the fibroblasts monolayers using Delfia method with primary antibody anti collagen I (OriGene R1038). Results are expressed *versus* untreated control. Statistical analysis performed using Student t-test *versus* untreated control.

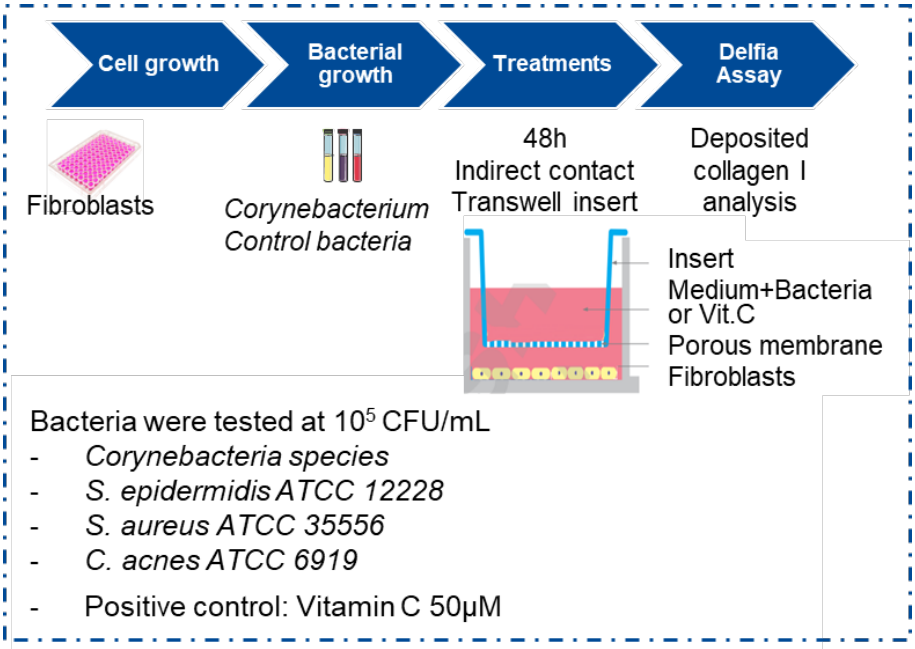

Figure 2. Protocol of collagen modulation evaluated by Delfia after fibroblasts treatment by some *Corynebacteria* versus *C. acnes*, *S. epidermidis* and *S. aureus* and positive control (vitamin C).

The effect of *Corynebacteria* was evaluated on fibrillin-1 produced by fibroblasts. Bacteria were cultivated during 72h, adjusted to 10^6 CFU/mL and then were placed in a transwell insert above normal human fibroblasts grown on the bottom of a culture well. After 72h of treatment with the bacteria or TGF beta (10 ng/mL), fibroblasts were fixed and stained to visualize fibrillin-1 thanks to a primary antibody anti-Fibrillin-1 (Novus NBP1-847220) and nuclei were stained with dapi. Observation was performed with a confocal microscope (Zeiss LSM700).

The effect of *Corynebacteria* was evaluated on the synthesis of Filaggrin and Claudin-1 by normal human keratinocytes cultivated up to confluence into Lab-tek II coated with 3T3 fibroblasts. At confluence, fresh medium containing bacterial supernatant was added for 3 days for Claudin-1 and 5 days for Filaggrin. Medium alone and supplemented by $CaCl_2$ at 0.5mM served as controls. The synthesis of Filaggrin and Claudin-1 was evaluated after fixation using primary antibody anti-Claudin-1 (Thermo Fisher 71-7800) or anti-Filaggrin (Abcam ab218395) and counterstaining by Evans blue. Observation was performed with a confocal microscope (Leica SPE).

Cosmetic ingredients will be finally tested on *C. accolens* growth. Briefly *Corynebacteria* will be grown in Tryptic Soy Broth medium supplemented in Tween, then incubated for 48h in diluted medium with and without ingredients. OD will be measured at 600nm. Advantageously, some selected prebiotic hits will be tested on collagen I assay (Delfia).

3. Results

Sensitive skin study

The prevalence of the species was analyzed by determining the absence or presence of different bacterial genera on NS or SS. Among the 3 major skin genera, we observed that the genus *Cutibacterium* was found in all the volunteers regardless of cohort. The genus

Staphylococcus was also found in all NS volunteers, but the prevalence slightly decreased by 3% in SS cohort. **T**he prevalence of *Corynebacterium* was on the contrary increased by 7% in the SS skin cohort (Figure 3). When looking more in detail, in SS, the most represented *Corynebacterium* species was *C. kroppenstedtii* and this species was 1.6 times more present in SS than in NS. Less represented, but maybe important in the biology of sensitive skin, *C. coyleae C. tuberculostearicum,* and *C. imitans* were increased by 16.5, 3.8, 7.9-fold respectively in SS. Conversely, the relative abundance of *C. accolens* decreased by 4-fold in SS subjects**.**

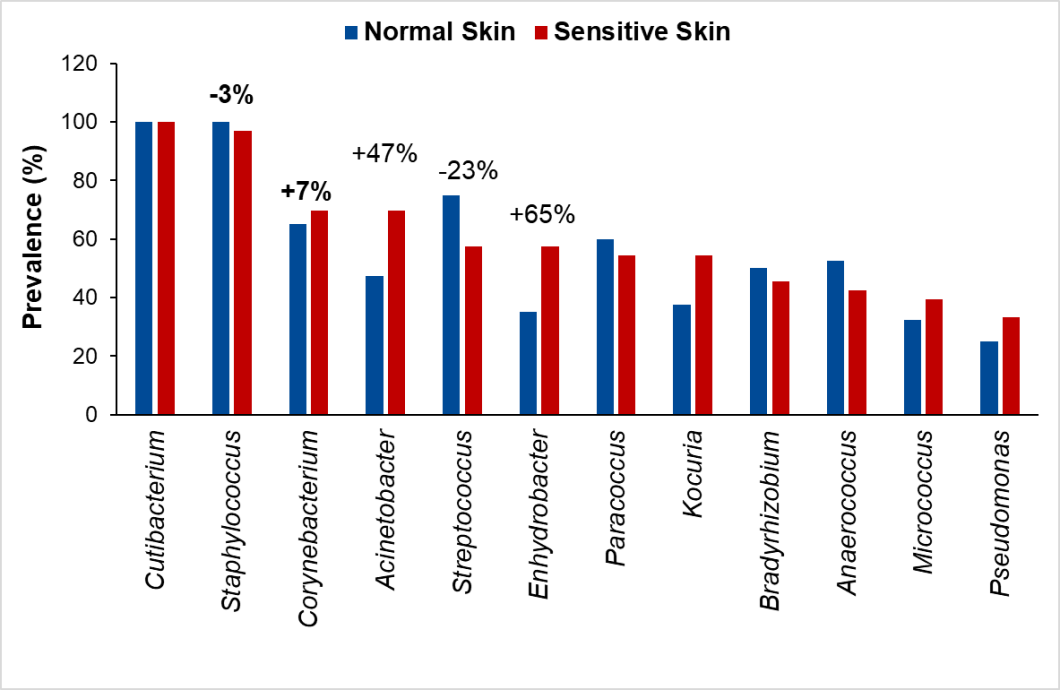

Figure 3. Sequencing analysis between normal and sensitive skin: illustration of 12 selected genera ranked from more to less prevalent in sensitive skin (ex of percentage of negative or positive variation in SS versus NS).

Aging study

Here, we more precisely analyzed the Corynebacteriaceae repartition among different zones (into the wrinkle, crow's feet area and control area near the ear). Whatever the area, we observed that *C. pseudogenitalium*, *tuberculostearicum*, *kroppenstedtii and matruchotii* were the 4 more prevalent species. C. *Kroppenstedtii and matruchotii prevalences* were always increased in the older group, with *Kroppenstedtii* reaching +47% in the wrinkle area. On the contrary, among the more prevalent, *C. accolens* was always decreased with the age (up to -43% in the contol zone). To a lesser extend *C. amycolatum* prevalence was also decreased in some areas (Figure 4). For *C. kroppenstedtii,* whatever the area, we also noted a significant 6-fold increase of the mean relative abundance per cohort (in %) in the older compared to the younger group.

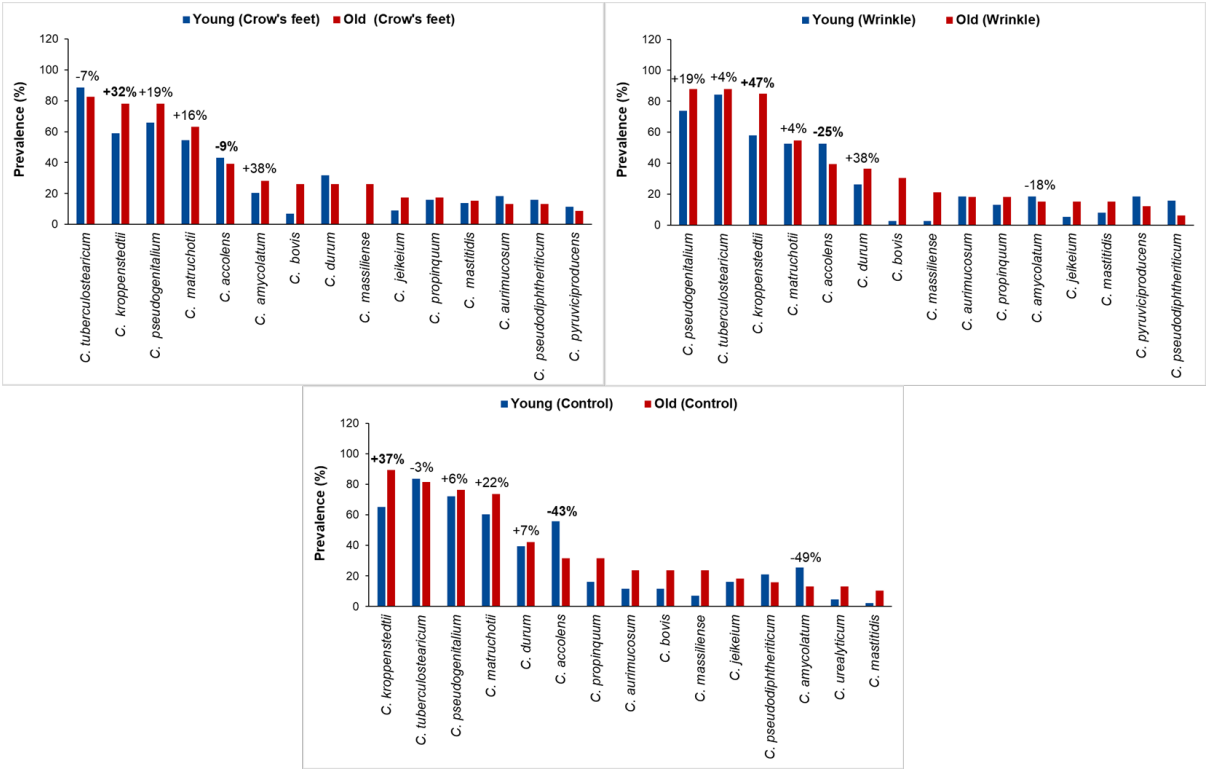

Figure 4. *Sequencing analysis between young or old donors: illustration of 15 selected genera ranked from more to less prevalent in older skin (ex of percentage of negative or positive variation in old versus young).*

When it comes to bacterial extraction using DBMT, we succeeded to isolate and grow 13 *Corynebacterium* strains that were sequenced for identification. Based on the results obtained with the aging study and SS skin study, we decided to run free radical scavenging assay, collagen and epidermal differentiation evaluations with *C. kroppenstedtii, accolens* and *amycolatum* in parralel to control bacteria.

Using DPPH assay, we evidenced that all *Corynebacteria* secretomes had a significant antioxidant effect whereas *S. epidermidis*, and *S. aureus* used as control had not. The 3 *Corynebacteria* strains also significantly boosted collagen content when *S. epidermidis, S. aureus* and *C. acnes* showed inhibition. However, *C. accolens* outperformed C. *kroppenstedtii* and *amycolatum*, achieving a 2-fold increase in collagen induction *versus* untreated (Figure 5). We also found that *C. accolens* and *amycolatum* promoted fibrillin-1, filaggrin and claudin-1 (data not shown). Finally the screening of prebiotic ingredients for *C. accolens* is ongoing.

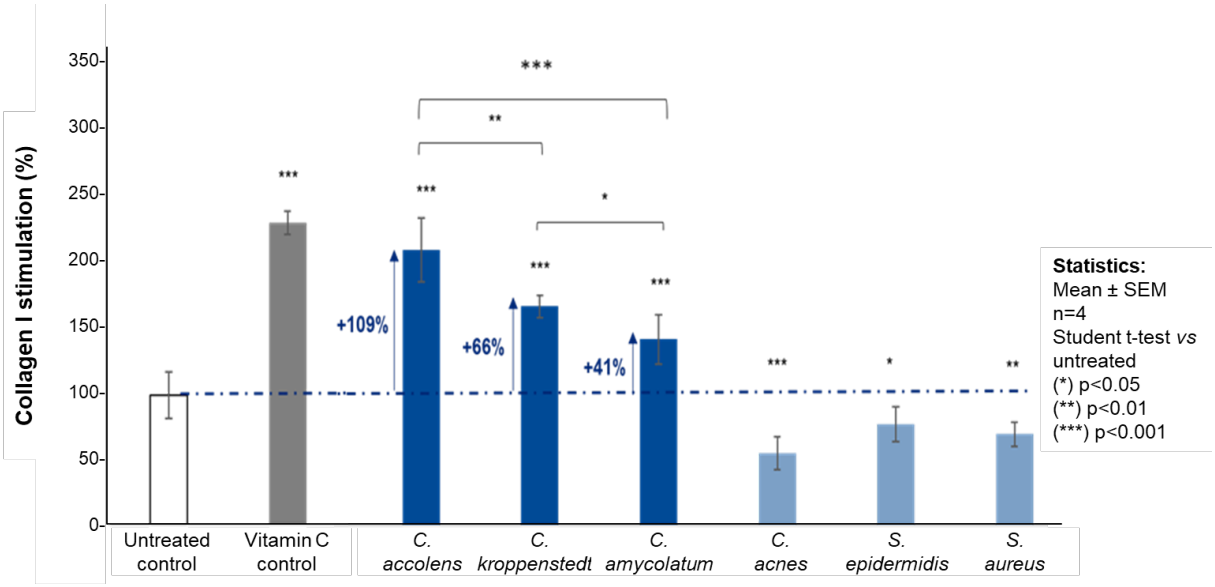

Figure 5. Collagen modulation evaluated by Delfia after fibroblasts treatment by some Corynebacteria *versus C. acnes*, *S. epidermidis* and *S. aureus* (positive control vitamin C).

4. Discussion

The results of this study confirmed that *Corynebacterium* prevalence and abundance changed in skin condition such as sensitive skin or skin aging. In SS compared to NS, we first observed a 3% decrease in *Staphylococcus* and a 7% increase in *Corynebacterium* prevalence [10]. This confirmed Hillion *et al*. study [11] who observed an increase in *Corynebacterium* and a decrease in *Staphylococcus* but partially aligned with the findings of Bai's study [12], which indicated a trend towards an increase in *Corynebacterium* and a decrease in *Staphylococcus,* or Jarrin's study [13] who reported a significant increase in *Corynebacterium* but not significant decrease in *Staphylococcus*.

Within the *Corynebacterium* genus, most species exhibited an increase in abundance in SS, with *C. kroppenstedtii* being particularly more abundant in SS compared to NS (x1.6). On the contrary, we observed a decrease for 2 species *C. accolens* or *mucifaciens*. Little is known about the *Corynebacteria* genus members contribution in SS, however an increase in *C. kroppenstedtii* was previously correlated to skin redness [14].

As reported previously on forehead and/or cheeks, we observed that in older facial wrinkled skin, there is a decrease in *Cutibacterium* and an increase in *Corynebacterium* [1-4]. In our aging study, we also detected a strong change regarding *Corynebacterium*, particularly *C. kroppenstedtii* [9]. Its prevalence in older skin increased whatever the area analyzed (crow's feet, wrinkles and control area). Moreover, we also noticed a significant increase in its abundance whatever the area. Furthermore, the old group is characterized by a higher prevalence of *C. pseudogenitalium* and *tuberculostearicum*, or *C. matruchotii, bovis* and *massiliense* when *C. accolens* and to a lesser extent *C. amycolatum* seemed decreased.

Through whole genome sequencing (WGS), we found that the shift in *Corynebacteria* was more intricate than what was previously described [1-4, 15]. To our knowledge, the presence of *C. massiliense,* as a member of the skin microbiome, has never been previously reported.

Out of the fact that *C. kroppenstedtii* could take advantage of metabolism and production of free fatty acids found on older skin [5] and that it is among the most abundant bacteria on skin of patients suffering from rosacea and redness [7, 8], little is known about *Corynebacteria* impact on skin cells.

For that reason, we used DBMT, recognized to help the extraction of germs difficult to isolate and grow, to extract as many *Corynebacteria* species we could. Notably, we found that the 3 *Corynebacteria* tested exhibited free radical scavenging activities and may play a role in enhancing skin density by promoting collagen deposition in the extracellular matrix. We particularly identify *C. accolens* and C. *amycolatum* as good bacteria to target as they are the more efficient to promote collagen and increase epidermal differentiation and cohesion. However these positive effects may be reduced while aging as they seemed decreased.

Advantageously, they could therefore be promoted by ingredients improving their growth and metabolic pattern to help the skin to recover a younger skin profile.

5. Conclusion

In the skin, resident microbiota protect the skin from invading, pathogenic, or opportunistic microbes through the process of colonization resistance. However, since few years, a new trend emerged showing some additional skin protection or antiaging benefits that could be provided by skin microorganisms. For example *C. acnes* could help protecting the skin from free radicals through the production of an antioxidant protein named RoxP which production could be increased by cosmetic ingredients [16, 17]. *Lactobacilli* are also interesting skin residential microogranisms as some species, and were shown to not only provide protection against pathogens [18] but also to promote important extracellular matrix proteins like collagen or elastin [19].

The findings of this new study suggest that *Corynebacteria* are also now intriguing and promising candidates for modulation through ingredients aimed at providing anti-aging benefits for the skin.

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