CORYNEBACTERIUM CASSIRII sp.nov., A NEWLY DISCOVERED SKIN COMMENSAL WITH ANTI-AGING PROPERTIES

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Abstract

The skin microbiome is crucial to skin health. During aging, the skin surface composition changes and so does the microbiome. The objective of this study was to employ culturomics to investigate the evolution of the skin microbiome during aging and to isolate bacteria in order to gain insight into their characteristics and role on skin health.

We isolated skin bacteria from the foreheads of 47 women, young and old. We then analyzed the correlations between the bacterial carriage and clinical parameters as a function of age on 108 subjects. 3D skin models were inoculated with bacteria and monitored for bacterial growth, transcriptomic and metabolomic regulations. We isolated and discovered a new commensal, *Corynebacterium cassirii* sp. nov. (*C.cassirii*), that significantly decreased in the old group and was associated with young skin characteristics. When co-cultured with 3D skin models, *C.cassirii* increased the expression of genes and metabolites related to epidermal renewal while decreasing differentiation markers and terminal lipids such as ceramides and

lysophospholipids. Our data indicate that *C. cassirii* is a commensal bacterium with anti-aging properties. It is therefore important to restore its abundance on aged skin using specific prebiotics and to use it as a marker of young skin.

Keywords: microbiome; aging; culturomics; Corynebacterium; epidermis

Introduction.

The skin is an ecosystem inhabited by a plethora of microorganisms as diverse as bacteria, archaea, fungi and small arthropods [1]. Together, they make up the cutaneous microbiota. This microbiota is subject to numerous parameters of variation, both intrinsic and extrinsic. Recent studies have identified correlations between changes in the composition of commensal populations in the skin and the physiological state of this environment, influenced by factors such as ageing and/or the onset of dermatological pathologies [2-5]. With the recent advent of molecular biology and next-generation sequencing (NGS) as tools for microbiological identification, knowledge of the skin microbiome has increased exponentially. Metagenomics makes it possible to analyze a large number of cultivable and non-cultivable bacteria using current knowledge and microbial culture techniques. It has been used to demonstrate the decline in *Cutibacterium acnes*, one of the most abundant species in the skin microbiota, as we age [2,5]. However, this method only allows the characterization of abundant bacteria, which introduces biases into the analysis of the composition of the microbiota. Used on its own, metagenomics is therefore not sufficient to reflect the complexity and diversity of the skin microbiota.

Although the skin microbiota has been the subject of numerous studies, there is still scientific uncertainty about its composition and its role in skin health. It is a complex environment, made up of numerous species, particularly bacteria. However, certain bacterial species, which could

have an important function on the skin, are poorly understood because they are difficult to isolate and cultivate.

Culture methods therefore remain an essential tool for studying the characteristics of microorganisms *in vivo*. Only microorganisms that can be isolated and grown in the laboratory can be studied on their own or in contact with human cells or tissues in order to study their interactions.

In recent years, culturomics has been a method that has continued to improve and has become a pillar for the discovery of new organisms in relation to humans [6,7]. This technique is based on the multiplication of culture conditions for micro-organisms, allowing the growth of a greater diversity of bacteria, yeasts and fungi, some of which are considered non-cultivable. These new species are not necessarily in the minority in this habitat and their influence is largely unknown. One of the main difficulties in culturomics is determining the isolation and culture conditions that are favorable to the growth of certain unknown bacterial strains. Depending on the area of skin under consideration, there are differences in temperature, humidity and pH, which can influence the bacterial composition of the microbiota [1]. The major challenge is therefore to mimic these specific conditions, which favor the growth of certain bacterial species that are rare or difficult to cultivate [8]. Another limitation of culturomics is that we still don't know the conditions required to grow certain bacteria, which are therefore considered ‘non-cultivable’.

Based on this principle, a novel selective medium was developed and employed in this study for the isolation and selection of skin bacteria whose growth is masked by the predominant skin species. This study resulted in the isolation of a previously unidentified bacterial species. The commensal nature of this bacterium and its evolution in the context of skin ageing were investigated. Further investigations were conducted into its role and interaction with the skin via co-culture using 3D skin models and analysis of its metabolic profile.

Materials and Methods.

Culturomics - Isolation and identification of bacterial species

a- Subject recruitment and study design

The subject population was enrolled at L'Occitane's Natural Cosmetic Evaluation Centre in Marseille (CosNat, Marseille France) between August 2019 and December 2020. Written informed consent was obtained from all participants in accordance with the research ethics committee ID-RCB: 2019-A01508-49

Main population: 47 healthy French women, aged 18 to 30 or over 55 were recruited. They were not under antibiotic or antihistamine treatment for at least 1 month, did not washed their foreheads since the day before and did not apply any cosmetic product. For the young group, women were on contraceptive treatment, excluding high-dose pills. Women in the older group were postmenopausal, without hormone replacement therapy and showed signs of photoaging.

“Register” population included 62 women (33 from the young group and 19 from the old group).

b- Collection of skin microbiome samples

Samples were collected from a 10 cm² area in a Z-Stroked manner [9] using sterile swabs soaked in Culture Top® Transport Media (C-top Ae-Ana, Eurobio, France) [10]. Samples were immediately cultured using culturomics. From the complementary population, samples were collected using the same method but using a single Fecal Transwab®. After collection, these were immediately stored at -80°C before DNA extraction.

c- Direct seeding conditions for bacterial culture

Fresh samples were serially diluted in 0.1 M PBS (Dulbecco's Phosphate-Buffered Saline, ThermoFisher Scientific, Paisley, USA) to 10^{-5} and each dilution was plated onto a proprietary

medium (IHU méditerranée infection, France) or COS agar plates and incubated at 31°C under anaerobic and aerobic conditions.

d- Carriage analysis of *C.cassirii*

DNA from the “register” samples was extracted using the QIAamp 96 DNA QIAcube HT kit and qPCR was performed using the LightCycler® 480 Probes Master kit (Roche Life Science, Germany). *Corynebacterium cassirii* primers and probe were designed on the Ferredoxin gene F-GACGAACGGATGGTCGAAGT; R-CGAGGCGATCTTCTACGAGG; FAM-TCCACTCGTCCGGGGTGTCG-TAMRA

e- Bacterial identification

The isolated micro-organisms were deposited a MSP96 target and identified using MALDI-TOF (BrukerDaltonics, Germany) with the Flex Control™ software and MALDI BioTyper RTC identification software (Bruker Daltonics GmbH, Bremen, Germany). When identification failed using the MALDI-TOF technique, sequencing using Next Generation Sequencing methods was carried out using the IlluminaMiseq (Illumina Inc., San Diego, CA, USA) and the Oxford Nanopore method (Oxford Nanopore Technologies, Inc, Oxford, UK).

Genome analysis

Annotation was obtained using Prokka Galaxy Version 1.14.5+galaxy0 software. The closest type strain genomes were determined by comparison with all the type strain genomes available in the Type (Strain) Genome Server (TYGS) database using the MASH algorithm. The degree of genomic similarity with closely related species was estimated using the OrthoANI.

Association of C.cassirii sp.nov. with clinical parameters

a- Subject recruitment and study design

108 subjects, divided into 52 subjects aged 18-30 years and 56 subjects aged 55-70 were recruited between June and July 2022. Written informed consent was obtained from all participants in accordance with the research ethics committee ID-RCB: 2022-A00482-41. Visual scoring was performed by a dermatologist using a 5-point scale for complexion radiance and homogeneity based on High-resolution digital photographs of the face (CameraScan, Orion Concept, France). Crow's feet and sub-ocular wrinkles, and pigmented spots were determined using the visual scales of Bazin et al., (2007) [11]. Hydration (Corneometer®CM825, Courage & Khazaka electronic GmbH, Germany), sebum (Sebumeter® SM815, Courage & Khazaka electronic GmbH), Germany), Transepidermal water loss (TEWL, Tewametre® TM300) and biomechanical properties of face skin (Cutometer Dual MPA 580, Courage & Khazaka electronic GmbH, Germany) were measured.

b- Skin microbiome sampling and analysis

Skin microbiome was collected from a 25 cm² area on the forehead using a Transwab® as described earlier and immediately stored at -80°. After thawing, 20 µl of 20 mg/ml Proteinase K was added to each sample and heated for 1 hour at 50°C in a water bath, then concentrated in a SpeedVac before DNA extraction using the QIAamp® PowerFecal® Pro DNA kit (Qiagen). Quantitative PCR was then performed using *C.cassirii* specific PCR system described earlier, the TaqMan® Fast Advanced Master Mix (Applied Biosystems), using the QuantStudio™ 7 Pro (Applied Biosystems).

c- Statistical analysis

Analysis of clinical variations between the young and old groups was performed using the Wilcoxon signed-rank test, and p-values were adjusted using the Benjamini-Hochberg method on the R software. Potential outliers were looked for using the Z-score test. We then compared the qPCR results with the clinical variables (paired test) using the Wilcoxon test, adjusting the

p-values as before. For continuous variables, we looked for correlations between qPCR and the variable using Spearman's correlation on the overall population.

C.cassirii co-culture on 3D skin models

a- Preculture of *Corynebacterium cassirii* sp. Nov. (CC)

C.cassirii was grown on Tryptone salt agar (TSA)+ Tween 80 (T80) 1% plates at 32°C for 3 days. Colonies were suspended in peptone salt, inoculated in a tryptone-salt broth (TSB) + T80 1% liquid preculture at 32°C under agitation for 24 hours. Then 5% of the preculture was inoculated in fresh medium.

b- Bacterial inoculation and co-culture

Labskin reconstructed skin model from Innovenn (York, England) was cultivated with the supplier's culture medium for 24 hours at 37°C, 5% CO_2. The day after 10^5 CFU/cm² or 10^2 CFU/cm² of *C.cassirii* were inoculated and culture was prolonged for up to 5 days. Control models without treatment and models treated with the inoculation medium were also performed.

c- Evaluation of the bacterial growth on 3D skin

Bacteria were recovered from the skin models (n=3 per condition) surface with 1 mL of physiological water containing 0.1% T80 following the Williamson & Kligman method [12]. Bacterial quantification was assessed using colony counting method and PCR methods. For colony counting, 100 µL of the suspension was serially diluted and 100 µl were inoculated on TSA + T80 1% agar plates. For Q-PCR / PMA Q-PCR methods, 400 µL of the bacterial suspension was resuspended in PowerBead Solution (Extraction kit, Qiagen, Courtabœuf, France), and 400 µL were pre-treated with 50 µM PMAxx™ (Propidium monoazide PMAxx™, Biotium, Fremont, CA, USA). The sample was placed in the dark at 4°C for 5 minutes, exposed to the PhAST blue lamp for 2 minutes and centrifuged at 10,000g for 5 minutes. DNA was

extracted with the DNeasyR UltraCleanR Microbial Kit, (Qiagen). *C.cassirii* DNA was amplified by PCR as described earlier.

d- Transcriptomic analysis

The epidermis (n=4 per condition) was separated from the dermis with forceps, disaggregated and homogenized using the Omni tissue homogenizer in TRIzol® reagent (Invitrogen™,Walthlam, Massachusetts,USA). The upper phase was transferred to RNeasy spin columns and total RNA was extracted using Qiagen mini-RNeasy kit with the addition of the DNase. Total RNA was reverse transcribed, amplified, and labeled with cyanine 3 (Cy3) for the One-Color Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies, Les Ulis, France). Labeled cRNA was hybridized onto Agilent SurePrint G3 Human Gene Expression 8x60K v2 Microarray Kit. Slides were scanned with the Agilent SureScan microarray scanning system. One-color microarray images were extracted with Feature Extraction software (v12.0.0.7), for background subtraction and quality control. Microarrays raw were normalized, and flag filtered using GeneSpring GX13.0 software. Responsive genes analysis was performed with Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA). Statistical entry Moderated t-Test P<=0,05 FC>=1,5.

e- Metabolomic analysis

Samples (n=4 per condition) were extracted and split into equal parts for analysis on the LC/MS/MS and Polar LC platforms. Proprietary software was used to match ions to an in-house library of standards for metabolite identification and for metabolite quantitation by peak area integration. (Metabolon, Morrisville, USA).

Results.

Corynebacterium cassirii sp.nov. a newly discovered commensal of the skin

In this study, 128 species within 55 genera were isolated from the forehead of 47 women. The main genera were *Staphylococcus* (isolated from 100% of the subjects), *Cutibacterium* (76,60%) and *Streptococcus* (48,94%) with their main representant species being *Staphylococcus epidermidis*, *Cutibacterium acnes*, *Streptococcus mitis/ oralis*. 20 species, never described before, were discovered. After failed identification by MALDI-TOF, a full genome sequencing and comparison to the previously described species confirmed that they had never been described before. Their detection by PCR in the “register” population revealed that 7 were found on the skin of more than 20% of the subjects and were considered to be commensals, the others were considered to be transient. Among the commensals, *Corynebacterium cassirii* sp. nov. (CC) was isolated from the skin of a 60-year-old woman. In the register population, CC tended to decrease with age as it was detected on the skin of 27,3% of the subjects from the old group compared to 45% in the young group (Pearson's Chi-squared test, p value=0,1). The bacteria was deposited in the National Collection of Cultures of Microorganisms (CNCM) of the Pasteur Institute with the number CNCM-I-5678.

Corynebacterium cassirii sp.nov. is associated with young skin

To confirm this tendency, a new clinical study was conducted on 108 subjects with 52 women from 18 to 30 years old (young group) and 56 women from 55 to 70 years old (old group). A review of the clinical characteristics of the population exhibited significant differences for all variables, except for tewametry and corneometry values (Table I). Older subjects exhibited significantly reduced sebum production, more pigmentary spots, a duller and more uneven complexion, and more wrinkles under the eyes and around the crow's feet (Table I). Cutometer measurements revealed that the skin of older subjects exhibited significantly reduced firmness (as indicated by a higher R6 value, reflecting greater skin viscosity, which is commonly

associated with a loss of collagen) and reduced elasticity (as indicated by a higher R1 value, which reflects a poorer elastic return and lower R2, R5 and R7 values (elasticity parameters) (Table I). These data demonstrate the changes in skin parameters that occur during the process of skin ageing.

Table I : Evolution of the clinical parameters between the young and old groups. Variations are expressed as percentages for instrumental measures or as points for clinical scores (scores from 1 to 5) (Wilcoxon signed-rank test with adjustment for p-values using the Benjamini-Hochberg (BH) method). The significance threshold was set at 0.05 of the p-value. ****p<0,0001.

Clinical parameter	Difference in the old vs young group	Adjusted p-value (BH)	Statistical significance
Tewametry	-8,20%	0.11475	ns
Corneometry	5,20%	0.135	ns
Sebum	-17,20%	3.65E-06	****
Pigmented spots score	3	6.90E-16	****
Radiance score	-1	6.08E-16	****
Evenness of the complexion	-1	5.87E-10	****
Crow's feet wrinkles	3,2	3.18E-18	****
Sub-ocular wrinkles	2,6	1.56E-17	****
Cutometer R0	-12,6%	0.000372	****
Cutometer R1	-41,7%	3.17E-12	****
Cutometer R2	-38,1%	2.57E-15	****
Cutometer R3	-12,6%	0.000372	****
Cutometer R4	-41,7%	3.17E-12	****
Cutometer R5	-44,0%	1.45E-15	****
Cutometer R6	-47,3%	5.29E-13	****
Cutometer R7	-51,6%	2.08E-16	****
Cutometer R8	-47,3%	5.61E-13	****

The prevalence of *C. cassirii* was found to be significantly lower in the older group compared to the younger one. In the younger group, the new species was identified in 62.3% of subjects, while in the older group, it was found in 34.5% only (Fisher exact test, p=0.0067) (Figure 1).

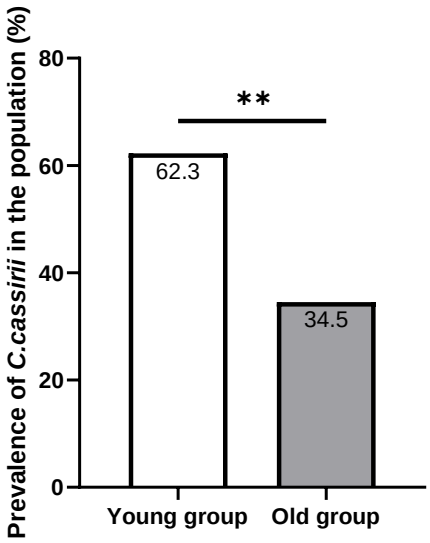


Figure 1: Prevalence of *C.cassirii* in the population. Percentage of positive subjects. Bacteria detected by Q-PCR. Fisher exact test ** p<0,01.

Furthermore, the quantity of *C. cassirii* detected by qPCR was found to be correlated with clinical parameters. The results presented in Table II demonstrate a significant inverse correlation between the quantity of *C. cassirii* and age, pigmented spot, crow's feet wrinkles, and sub-ocular wrinkles. Conversely, higher quantities of *C. cassirii* were associated with higher scores for skin radiance and even complexion. In terms of skin biomechanical parameters, *C. cassirii* was significantly associated with higher R2, R5 and R7 values, indicating more elastic skin. *C. cassirii* was not significantly associated with variations in hydration (corneometry), skin barrier (tewametry) or sebum levels.

Table II: Statistical associations between the quantity of *C. cassirii* sp. nov. and clinical parameters. Spearman's correlation coefficients are presented, along with their statistical significance for each parameter. ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Clinical parameter	*Correlation Coefficient (R)*	*p-value*	*Significance C.cassirii evolution with clinical parameter*
Age	-0,19	0,00038	**** Decrease
Sebum	0,14	0,15	NS
Corneometry	-0,1	0,3	NS
Tewametry	0,11	0,27	NS
Pigmented spots	-0,19	0,048	* Decrease
Radiance	0,29	0,0028	** Increase
Even complexion	0,17	0,072	NS None
Cow's feet wrinkles	-0,29	0,0028	** Decrease
Sub-ocular wrinkles	-0,28	0,003	** Decrease
Cutometer R0	0,2	0,041	* Increase
Cutometer R1	-0,17	0,081	NS
Cutometer R2	0,22	0,022	* Increase
Cutometer R3	0,2	0,041	* Increase
Cutometer R4	-0,17	0,081	NS
Cutometer R5	0,23	0,016	* Increase
Cutometer R6	-0,15	0,13	NS
Cutometer R7	0,23	0,014	* Increase
Cutometer R8	0,24	0,011	* Increase

Furthermore, the prevalence of two bacterial species, previously demonstrated to evolve during the ageing process, was investigated. These were *Cutibacterium acnes*, which is known to decrease in old skin, and *Corynebacterium kroppenstedtii*, which is known to increase. The results of our study indicate that *C. acnes* levels decrease significantly with age and in conjunction with a reduction in sebum production, an increase in wrinkles, pigmented spots and a loss of radiance and biomechanical properties of the skin. Conversely, *C. kroppenstedtii* exhibited a significant increase with age, which was associated with the development of

wrinkles and the loss of biomechanical properties of the skin, but not with the evolution of the skin's complexion (Table III).

Table III: Statistical associations between the quantity of *Cutibacterium acnes* and *Corynevbacterium kroppenstedtii* with age. Spearman's correlation coefficients are presented, along with their statistical significance for each parameter. ns = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Clinical parameter	*Correlation Coefficient with clinical parameter for C.acnes*	*p-value*	*Correlation Coefficient with clinical parameter for C.kroppenstedtii*	*p-value*
Age	-0,39 ****	3,5e-05	0,34 ***	0,00036
Sebum	0,55 ****	4,8e-10	0,027 NS	0,78
Corneometry	0,055 NS	0,57	0,17 NS	0,083
Tewametry	0,016 NS	0,87	-0,044 NS	0,65
Pigmented spots	-0,45 ****	1,1e-06	0,13 NS	0,2
Radiance	0,4 ****	1,4e-05	-0,16 NS	0,097
Even complexion	0,33 ****	0,00042	-0,086 NS	0,37
Cow's feet wrinkles	-0,34 ****	0,00031	0,26 **	0,0077
Sub-ocular wrinkles	-0,42 ****	4,6e-06	0,22 *	0,022
Cutometer R0	0,3 **	0,0018	-0,13 NS	0,19
Cutometer R1	-0,19 *	0,047	0,27 **	0,005
Cutometer R2	0,31 **	0,0013	-0,27 **	0,0056
Cutometer R3	0,3 **	0,0018	-0,13 NS	0,19
Cutometer R4	-0,19 *	0,047	0,27 **	0,005
Cutometer R5	0,33 ***	5e-04	-0,26 **	0,0074
Cutometer R6	-0,19 *	0,048	0,33 ****	4e-04
Cutometer R7	0,31 **	0,0011	-0,29 **	0,0026
Cutometer R8	0,36 ***	0,00011	-0,22 *	0,024

Corynebacterium cassirii sp.nov. has anti-aging properties.

The *C.cassirii* genome and metabolic pathways analysis suggested that this bacterial species utilizes lipids as nutrients, with the presence of genes coding for lipase and esterases. This was corroborated in culture, where the organism exhibited a markedly enhanced growth rate in TSB medium when Tween 80, a source of oleic acid, was included (data not shown).

In order to gain insight into the growth and interaction of *C. cassirii* with skin, the bacteria were inoculated at a density of 10^5 bacteria per cm² onto the surface of 3D skin models. The growth of the bacteria was then monitored over a period of 2- and 5-days post-inoculation. The following methods were employed: QPCR, PMA-XX QPCR and agar counting. The QPCR method enabled the detection of all bacteria present on the skin surface. In contrast, the PMA-XX PCR method, which prevents DNA amplification from dead bacteria, demonstrated the number of viable bacteria. Finally, the agar counting method enabled the detection of viable and cultivable bacteria. At day 0, immediately following inoculation, the three techniques yielded comparable results, with a total between 4.6. 10^4 and 9.6 10^4 CFU/cm² (Figure 2A). Following a two-day incubation period, a notable increase in bacterial growth was observed, accompanied by a higher total bacterial count (2.6 x 10^6 CFU/cm²) as determined by QPCR. However, the quantity of viable and cultivable bacteria remained relatively stable, with 4.3 x 10^5 CFU/cm² and 2.1 x 10^5 CFU/cm², respectively. After five days in culture, the number of total bacteria remained stable, while a decrease in cultivable bacteria was observed (2.10^3 CFU/cm²). At Day 5, the characteristic bacillus shape of C. cassirii could be observed on the epidermal surface by scanning electron microscopy (Figure 2B).

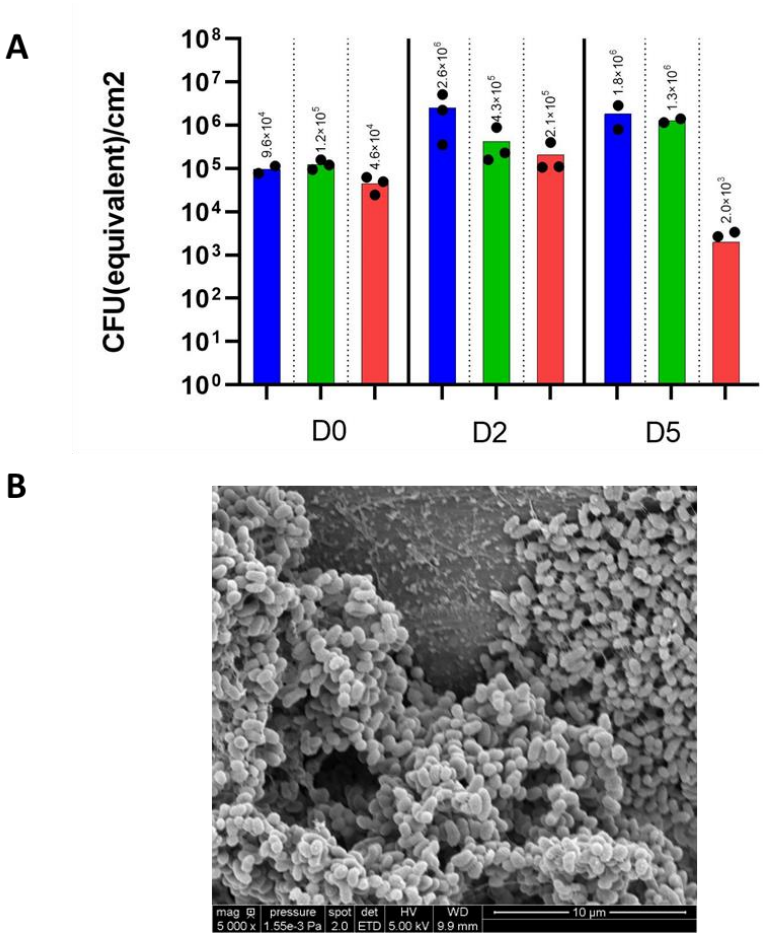


Figure 2: *C.cassirii* sp.nov. growth on 3D skin model. A- bacterial counting by QPCR (blue bar), PMA QPCR (green bar) and on agar plate (red bar) at day (D) 0, 2 and 5 after inoculation on the 3D skin. Each bar represents the mean of 2 or 3 samples (dots) per condition. B- Image of *C.cassirii* on epidermal surface by scanning electron microscopy at 5000x magnification.

We then investigated the regulation of gene expression in the 3D skin using DNA array. In this case, a low (10^2 UFC/cm²) and a high (10^5 UFC/cm²) inoculation densities were employed. Gene regulations were compared to that observed in the control 3D skin model which received the inoculation medium without bacteria. The initial analysis examined the impact of the inoculation medium on gene expression, comparing gene expression profiles between the control skin model and skin inoculated with the bacterial inoculation medium alone. The vast

majority of the 665 genes regulated by the medium were involved in the inflammatory response pathway, with a z-score activation of 4.43. In addition to the observed inflammatory response, genes involved in cellular remodeling were also upregulated, including metallopeptidase MMP9 and laminin LAMC2, which are involved in the skin healing pathway (data not shown). Among the 1403 genes regulated by *C.cassirii* at 10^5 CFU/cm², 314 genes were common with the inoculation medium condition and 1089 were bacteria-specific (Figure 3-A). The number of bacteria-specific genes was 364 for the 10^2 CFU/cm² condition (data not shown). Subsequently, our analysis was concentrated on genes that were specifically regulated by the bacteria at low or high bacterial concentrations. While 308 genes were regulated by both bacterial concentrations, the higher bacterial concentration led to a greater number of regulated genes, with 781 being specific to that concentration, while 56 were specifically regulated by the lower concentration (Figure 3-B). For instance, among the 29 genes encoding *stratum corneum* proteins that were regulated in the high concentration conditions, only five were also regulated in the lower concentration condition. Upon focusing on the high concentration condition that led to the most gene regulations, it was observed that the regulatory pathway of genes involved in epidermal differentiation, lipid biosynthesis, formation, and maintenance of the *stratum corneum* exhibited the most pronounced downregulation in the presence of bacteria in comparison to the medium control (Figure 3-C). For instance, the expression of genes encoding the Late Cornified Envelope 1 (LCE1), filaggrin, desmocollin or corneodesmosin was reduced, as was the expression of keratins 1 and 10, which are found in the supra basal layers of the epidermis, and loricrin, located at the last stages of keratinocyte differentiation. Conversely, several genes involved in cell proliferation were upregulated. For instance, the Ki-67 gene, a proliferation marker, Polo-Like Kinase 1 (Plk1) and Cell Division Cycle 25C (CD25C), which are responsible for cell division and proliferation, were upregulated.

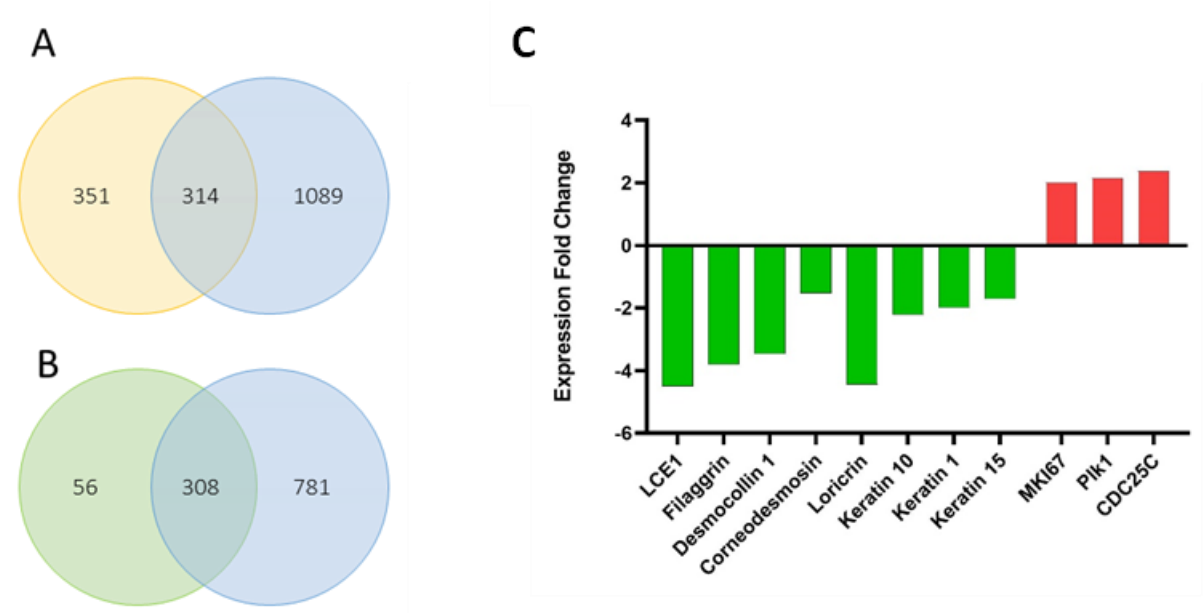
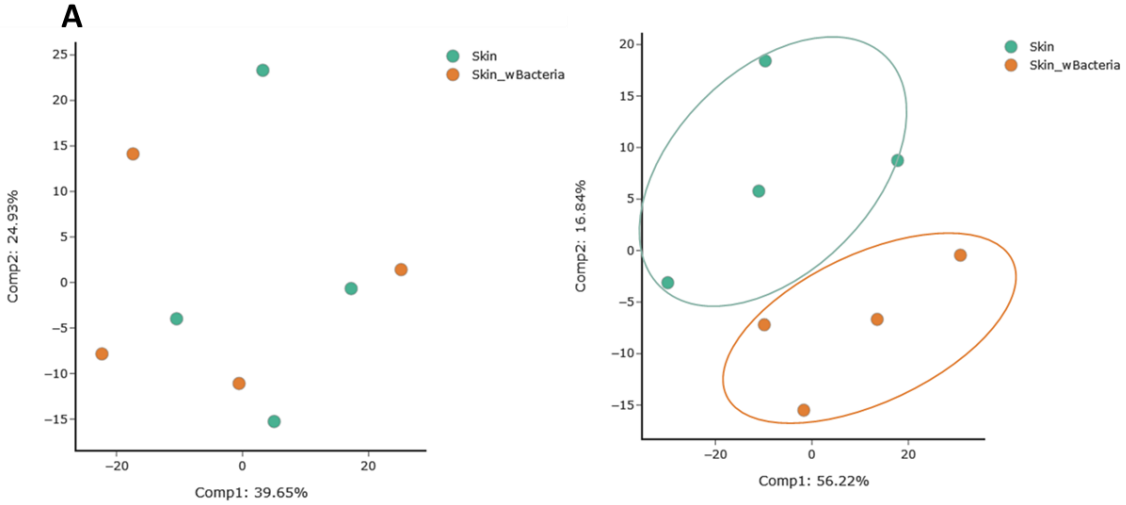


Figure 3: Transcriptomic analysis of the 3D skin models. A-Venn diagram representing the number of genes regulated by the bacterial culture medium (yellow) and by *C.cassirii* at 10^5 CFU/cm² in their culture medium (blue) compared to the control skin models without treatment (n=4). B- Venn diagram representing genes specifically regulated by *C.cassirii* at 10^2 CFU/cm² (green) or 10^5 CFU/cm² (blue). Genes regulated by both conditions appear at the intersection of the circles. C- Histogram representing significantly regulated genes related to epidermal differentiation (green) and proliferation (red). Moderated t-Test p<0,05.

At the metabolomic level, our investigation focused on the interaction between 3D skin and *C. cassirii* at the highest concentration (10^5 UFC/cm²). The metabolites produced by both the bacteria and the 3D skin were analyzed. Principal component analysis revealed minimal differences between control skin (inoculation medium without bacteria) and skin with bacteria at Day 2 (D2). At day 5 (D5), more differences emerged between the conditions, with a clustering of samples based on the presence or absence of bacteria at D5, indicating a different metabolite composition (Figure 4-A). Statistical comparisons revealed that the duration of the culture period was the primary driver of metabolic changes, with 179 significantly regulated and 66 approaching significance between days 2 and 5 in the control

model (CT). The metabolic pathways that exhibited significant changes were those of amino acids, peptides, sugars, nucleotides, cofactors and vitamins. Of particular interest was the lipid pathway, which was increased. This observation suggests that the epidermal differentiation occurring during the culture strongly modified skin metabolites, especially those related to the formation of the epidermal lipid barrier. This hypothesis was corroborated by histological examination of the 3D skin models, which demonstrated a thickened *stratum corneum* and thinned living layers, as expected in these models (Figure 5-C). Nevertheless, despite the pronounced effect of tissue evolution during culture, the presence of *C. cassirii* specifically regulated 42 metabolites at day 2 and 123 at day 5 (Figure 4-B).

A



B

Statistical comparisons				
ANOVA contrasts	**CT day5 /CT day2**	**CC day5 /CC day2**	**CC day2 /CT day2**	**CC day5 /CC day2**
Total biochemicals p≤0.05	179	104	21	72
Biochemicals ↑↓	151 | 28	89 | 15	6 | 15	11 | 61
Total biochemicals 0.05<p<0.10	66	47	21	51
Biochemicals ↑↓	59 | 7	38 | 9	5 | 16	2 | 49

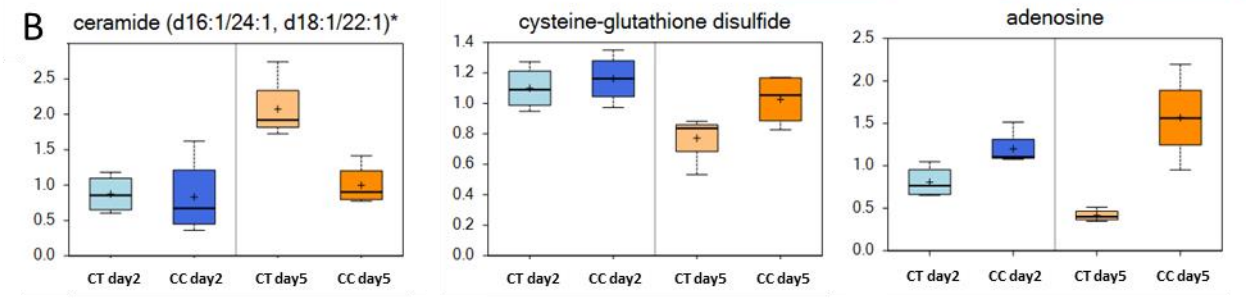
Figure 4: Metabolomic analysis of colonized and non-colonized 3D skin models. A- Two-dimensional principal component analysis plot for subgroup of samples colored by model type at day 2 (left) and day 5 (right). B- Number of metabolites regulated between day 2 and day 5 in the control (CT) and *C.cassirii* (CC)-colonized skin and between CC and CT at the two time points.

In the *C. cassirii*-colonized skin, the most notable changes were observed in metabolites associated with the lipid super-pathway. After five days of culture, the levels of lysophospholipids, ceramides, cholesterol, free fatty acids, monoacylglycerols, and diacylglycerols were found to be lower than in the control skin. The levels of these lipids were significantly elevated between days 2 and 5 in the control model, but remained stable in the colonized skin (Figure 5-A). Some metabolites in the glutathione metabolism related to

oxidative stress exhibited an increase over time in the skin inoculated with *C. cassirii*, while a decrease was observed in the control skin. Moreover, adenosine was identified as the most significantly increased molecule in this condition (Figure 5-B). Another study utilizing *C. cassirii* pellets derived from a bacterial culture also demonstrated that the bacteria were capable of producing adenosine (data not shown). In the colonized skin models, adenosine could thus be produced by *C. cassirii* alone or in conjunction with the skin model.

A

Super Pathway	Sub Pathway	Biochemical Name	CT day5 / CT day2	CC day5 / CC day2	CC day2 / CT day2	CC day5 / CT day5
Lipids	Lysophospholipids	2-myristoyl-GPC (14:0)*	2,87	1,44	0,95	0,48
		1-stearoyl-GPC (18:0)	1,80	1,29	0,81	0,58
		1-oleoyl-GPC (18:1)	2,83	1,27	0,84	0,38
		1-eicosenoyl-GPC (20:1)*	2,83	1,13	1,15	0,46
		1-dihomo-linolenoyl-GPC (20:3n3 or 6)*	2,40	1,24	0,97	0,50
		1-oleoyl-GPE (18:1)	1,85	1,11	0,96	0,58
		1-linoleoyl-GPG (18:2)*	1,95	1,00	0,86	0,44
	Ceramides	N-palmitoyl-sphingosine (d18:1/16:0)	1,95	1,20	0,84	0,52
		ceramide (d18:1/14:0, d16:1/16:0)*	1,97	1,31	0,90	0,60
		ceramide (d16:1/24:1, d18:1/22:1)*	2,38	1,20	0,95	0,48
	Dyhydroceramides	N-stearoyl-phytosphingosine (t18:0/18:0)*	2,26	1,03	0,93	0,42
	Sterols	cholesterol	1,83	1,35	0,87	0,64
Amino acids	Glutathione Metabolism	glutathione, reduced (GSH)	0,73	1,03	0,44	0,62
		glutathione, oxidized (GSSG)	0,49	1,23	0,75	1,88
		cysteine-glutathione disulfide	0,70	0,88	1,06	1,33
Nucleotides	Purine Metabolism, Adenine containing	adenosine	0,51	1,31	1,49	3,77



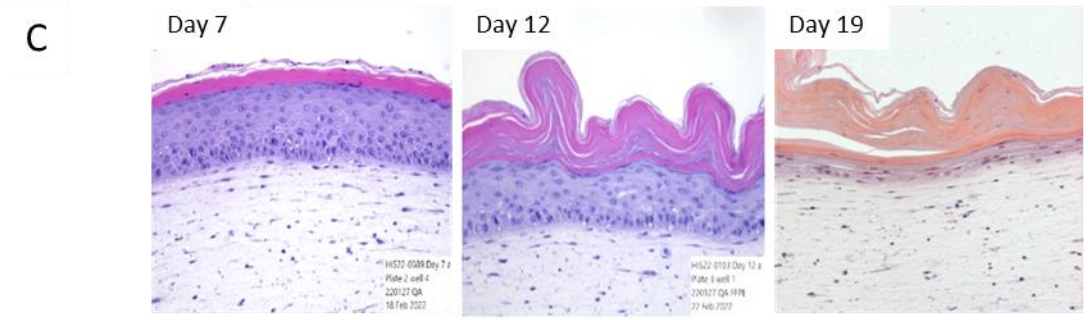


Figure 5: Regulation of metabolites with time and *C.cassirii* co-culture in 3D skin models, and illustration of epidermal differentiation with time. A- Examples of regulated biochemicals in different metabolic pathways expressed ad fold change. The red boxes represent significantly increased biochemicals ($p \leq 0.05$) while green and light green boxes represent decreased biochemicals ($p \leq 0.05$) or ($0.05 < p < 0.10$) respectively. Two-Way ANOVA Contrasts. B- Box plots of some regulated metabolites. CT: control skin, CC: *C.cassirii*-colonized skin, C- Images of hematoxylin and eosin staining of the control skin model at days 7, 12 and 19 of air/liquid culture. The bacterial strain inoculation was conducted on day 14.

Discussion.

The majority of studies investigating the evolution of the microbiome with age are based on metagenomic analysis, utilizing 16S gene sequencing and whole genome sequencing (WGS). These studies have enabled the description of an increase in microbiome diversity with age, as well as the specific evolution of some of the most abundant genera and, in some cases, species. For instance, the reduction in the prevalence of *C. acnes* with age is linked to a decline in sebum production by the skin. More recently, *Lactobacillus crispatus* has also been associated with young skin and has been observed to decrease significantly in the wrinkled areas of the face [4]. Conversely, several studies have indicated an increase in the prevalence of *Corynebacterium kroppenstedtii* with age [2,3,13,14]. However, if the genus Corynebacterium exhibited an overall increase, this was not observed for all species within the genus. Some species demonstrated an increase with age, while others remained stable or decreased. Furthermore, there is a shift from a lipophilic-dominated ecosystem of young skin to a less lipophilic and more varied metabolic profile in older skin. By means of culturomics, a new species of Corynebacterium was identified and its decline on old skin was demonstrated. Genome and metabolic pathway analysis revealed the organism's aerobic and lipophilic profile, which was confirmed in culture depending on the supplementation with Tween 80, a source of oleic acid, one of the most abundant unsaturated fatty acids in sebum. Tween 80 has previously been demonstrated to possess growth-promoting properties on several Corynebacteria species [17]. *C. cassirii* was not significantly correlated with sebum levels in our clinical study, but there was a tendency for it to decrease with decreasing sebum levels, with a p-value approaching significance. This suggests that other factors may be involved in the observed decrease during the ageing process.

The development of skin models is contingent on the division potential of keratinocytes, with a reduction in this potential with time, leading to a decline in epidermal renewal. Consequently, the thickness of the living layers is reduced and the *stratum corneum* becomes thickened, a

process that closely mimics the effects of ageing [18]. Our observations at the transcriptomic and metabolic levels are in accordance with these data, indicating an accumulation of terminal differentiation lipids and a decrease in proliferation. Notably, we observed that *C. cassirii* was capable of maintaining epidermal cell proliferation and slowing down differentiation. The production of adenosine by the bacteria in culture and the increase in adenosine levels in *C. cassirii*-colonized skin may in part explain the way in which *C. cassirii* favors epidermal renewal. It has been demonstrated that human epidermal keratinocytes express adenosine receptors. In normal human keratinocytes, physiological concentrations of adenosine activate A2B receptors, inducing cell cycle arrest. In contrast, the activation of A2A induces keratinocyte proliferation via p38–mitogen-activated protein kinase activation [19]. This aspect is of particular importance in the context of skin ageing, given that the epidermal turnover rate slows from 30% to 50% between the third and eighth decades of life [20]. Adenosine nucleoside is also a constituent of the adenosine triphosphate (ATP) molecule and plays an essential role in biochemical processes that provide energy to living cells. Furthermore, it is recruited by DNA polymerase to form new DNA. *In vitro* studies have demonstrated that adenosine nucleoside enhances protein synthesis in human skin fibroblasts. The activation of adenosine receptors by interacting with various skin layers may result in a reduction in the number of wrinkles, roughness, dryness, and laxity [23]. Reactive oxygen species (ROS) are byproducts of normal mitochondrial metabolism [24,25]. The observation that the signs of oxidative stress decreased in the control skin over time, while remaining stable in the *C. cassirii* colonized skin, may also indicate a higher mitochondrial activity of proliferating cells in the presence of the bacteria.

Given the anti-ageing benefits of *C. cassirii* on the skin, it is important to understand its metabolism and nutritional needs in order to provide the environment it needs to grow on the skin. This can be achieved either by stimulating skin cells to produce the appropriate nutrients, such as fatty acids, through the sebum, or by using these prebiotics in cosmetic formulations.

Conclusion.

A previously unidentified bacterial species, *Corynebacterium cassirii* sp. nov., was discovered to exhibit a higher prevalence on young skin and to correlate significantly with the characteristics of young skin. The anti-ageing properties of the bacterium were demonstrated by its ability to maintain epidermal renewal in 3D models of the human dermis. Consequently, it would be beneficial to maintain an abundance of this species to prevent skin aging.

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none.

Conflict of Interest Statement.

NONE.

References.

1. Grice EA, Segre JA (2011). The Skin Microbiome. Nature Reviews Microbiology 9, 244 253.
2. Shibagaki N, et al (2017) Aging-related changes in the diversity of women's skin microbiomes associated with oral bacteria. Sci Rep Sep 5;7(1):10567.
3. Jugé R et al (2018) Shift in skin microbiota of Western European women across aging. J Appl Microbiol Sep;125(3):907-916.
4. Garlet A et al (2024) Facial Skin Microbiome Composition and Functional Shift with Aging. Microorganisms May 18;12(5):1021.
5. Manos J (2022) The human microbiome in disease and pathology. APMIS Dec;130(12):690-705.
6. Lagier JC et al (2018) Culturing the Human Microbiota and Culturomics. Nature Reviews Microbiology 16, 540–550.
7. Fleming E et al (2021) Cultivation of Common Bacterial Species and Strains from Human Skin, Oral, and Gut Microbiota. BMC Microbiol 21, 278.
8. Wade W et al (2016) Uncultured Members of the Oral Microbiome. J Calif Dent Assoc 44, 447–456.
9. Rushing J (2007) Obtaining a wound culture specimen. Nursing 37(11):p 18, November

10. Cassir N et al (2021) Evaluation of Culture Top Transport Systems for Assessing the Bacterial Diversity of Microbiota by Culturomics as compared to a Routine Transport System. Journal of Medical Microbiology 2021, 70.
11. Bazin R, Doublet E (2007) Skin Aging Atlas. Vol. 1: Skin Aging Atlas: Caucasian Type. Editions MED'COM: Paris. ISBN 978-2-35403-001-8.
12. Williamson P, Kligman AM (1965) A new method for the quantitative investigation of cutaneous bacteria. J Invest Dermatol. Dec;45(6):498-503.
13. Zhou W et al (2023) Skin Microbiome Attributes Associate with Biophysical Skin Aging. Genomics. Exp Dermatol 32:1546–1556.
14. Li Z et al (2020) New Insights Into the Skin Microbial Communities and Skin Aging. Front Microbiol 11:565549
15. Dimitriu PA et al (2019) New Insights into the Intrinsic and Extrinsic Factors That Shape the Human Skin Microbiome. mBio 10:e00839-19.
16. Cao K et al (2022) Fatty Acid Profiling in Facial Sebum and Erythrocytes From Adult Patients With Moderate Acne. Front Physiol Jun 21:13:921866.
17. Menberu MA et al (2021) Tween 80 and its derivative oleic acid promote the growth of Corynebacterium accolens and inhibit Staphylococcus aureus clinical isolates. Int Forum Allergy Rhinol Apr;11(4):810-813.
18. Dos Santos M et al (2015) In vitro 3-D model based on extending time of culture for studying chronological epidermis aging. Matrix Biol Sep:47:85-97.
19. Andrés RM et al (2017) Adenosine A2A and A2B Receptors Differentially Modulate Keratinocyte Proliferation: Possible Deregulation in Psoriatic Epidermis. J Invest Dermatol Jan;137(1):123-131.
20. Baumann L (2007) Skin ageing and its treatment. J Pathol 2007;211:241–51.
21. Dobson JD (2002) Treatment of skin with adenosine or adenosine analog. United States patent US 6423327B1.
22. Dobson JG (2003) Treatment of skin with adenosine or adenosine analog. United States patent US 6645513B2.
23. Marucci G et al (2022) The possible role of the nucleoside adenosine in countering skin aging: A review. Biofactors Sep;48(5):1027-1035.
24. Dunn JD et al (2015) Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. Redox Biol Dec:6:472-485.
25. Murphy MP (2009) How mitochondria produce reactive oxygen species. Biochem J Jan 1; 417(Pt 1): 1–13.