A biomimetic approach inspired from resurrection plants to promote skin resilience

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Abstract

Background: Like plants, humans are exposed to harsh climatic conditions. These events affect human skin resilience properties and make cells more prone to damages causing detrimental effects. Inspired by resurrection plants, known for their strong resilience properties, we developed Galactinol Advanced (GA) a sustainable white biotechnology active ingredient able to improve skin resilience capacity. This work presents how GA preserves skin resilience and healthiness.

Methods: *In vitro* models of environmental stresses: Heat, Hydric, combination heat + hydric, UVA and UVB. Keratinocytes and fibroblasts morphologies by bright field pictures. HSP27 expression and quantification by immunofluorescence count. Clinical evaluations: wrinkles, firmness, elasticity and skin metaproteomics.

Results: GA protection against environmental stress was confirmed in the *in vitro* study model mimicking climatic changes. GA induces a stimulation of hyaluronic acid. After one month of use, skin benefits were demonstrated as visible reduction of wrinkles' volume, improvement of skin elasticity and firmness. GA wasn't harmful to skin microbiome, keeping its composition stable. Metaproteomics study identified 4785 proteins, among them, 343 proteins were significantly modulated (from human, fungi and bacterial origins) impacting positively skin healthiness: induction of antioxidant, proteostasis, metabolism, anti-inflammation and extra-cellular matrix pathways.

Conclusion: We demonstrated that "galactinol", a molecule described in plants as a "safeguarder" can bring a similar benefit to the skin modeling the resilience and healthiness observed in the nature. As in plants, GA activates defense mechanisms. Metaproteomics used for the first time on human skin clearly shows that modulating proteins expression at keratinocytes and microbiome levels leads to visible beneficial outcomes.

Key words: skin resilience, firmness, elasticity, wrinkles, HSP27, Metaproteomics

Introduction.

Due to climate evolution (warm weather, extreme cold, humid climates or air-dried state), such events may increase in intensity and frequency. Unfortunately, these events deeply affect skin integrity and resilience by profoundly altering skin defense capacity. High level of oxidative stress makes cells more prone to damage as protein alterations and DNA breakage, causing detrimental effects on cell and tissue functions [1][2]. This also leads to lower production or degradation of extracellular matrix elements resulting in disorganized skin structure. Inspired mainly by resurrection plants, and galactinol beneficial properties (against biotic and abiotic stresses) described in various other kind of plants [3], we developed Galactinol Advanced (GA) in order to help the skin adapting better to this climate changes. Indeed, as Galactinol is a rare and precious compound, it is not viable economically to isolate it from plants. Resurrection plants can survive over long periods, months and even years, of severe dehydration caused by drought stress. To endure severe dehydration, under very low cellular water content, these plants can reprogram their transcriptome, resulting in a profound alteration of metabolism toward alternative energy supply, hormone signal transduction, antioxidant, prevention of DNA/protein damage [4]. In addition, they produce also high levels of protective sugars as Raffinose Family Oligosaccharide's (raffinose, galactinol, stachyose, verbascose), trehalose and sucrose. After water exposure, they can quickly reactivate their metabolism, turning from a death-like state to an alive-looking plant. Moreover, in addition to its beneficial effects against environmental stress in plants [3], galactinol function as a signaling molecule for induction of plant disease resistance [5]. The aim of this work was to demonstrate that GA can mimic plants resilience strategy to face harsh environmental conditions. Moreover, work of the last decade on skin microbiota have shown that microorganisms localized at the skin surface are key players for the preservation of skin healthiness. Indeed, skin microbiota interacts with the host immune system to limit pathogen proliferation and maintain cutaneous homeostasis [6, 7]. Besides, the *in vitro* and biometrological skin analysis, we studied the effect of GA on skin microbiota by using the metaproteomics technology to demonstrate the beneficial properties of GA on skin microbiome. In contrast to metagenomics, the widely used technic to study skin microbiota, the metaproteomics provide valuable descriptive and functional insights [8, 9]. Metaproteomics study was used to demonstrate that skin resilience can be improved by GA through the induction of enzymes or proteins involved in detoxification process, protection of proteins such as HSPs or involved on cell metabolism expressed by skin microbiota and/ or human cells.

Materials and Methods.

GA is synthesized by white biocatalytic method from agricultural-food byproducts (from Cotton seeds and Rice bran). Biocatalysis is an environmentally friendly method as it avoids the use of toxic reagents and solvents.

Study of the protective properties of GA

In each condition tested (basal, UVA, UVB, heat stress, combined stresses hydric +heat), the protective effect of GA was followed by the observation of cell's morphology and by the quantification of HSP27 expression. Normal human epidermal keratinocytes (NHEK) from a 6-year-old child and Normal human dermal fibroblasts (NHDF) from a 47-year-old woman were used for the studies. The cells morphologies were observed under a bright field microscope (Zeiss Oberkoren Germany, Axiovert 40). After cells fixation (paraformaldehyde 4%), the staining was done with an anti-HSP27 antibody (Abcam, ab62339,) revealed with a goat anti-rabbit antibody conjugated with Alexa fluor® 568 (Thermofisher Waltham MA, A1101). Immunofluorescence pictures were taken with a Nikon microscope Eclipse Ti2 and quantified by Image J software.

Study of the protective properties of GA in human dermal skin cells under basal and UVA stress

Normal human dermal fibroblasts (NHDF) from a 47-year-old woman were cultured with DMEM medium (Dutscher Bernolsheim France, L0103-500), 10% SVF (Dutscher, S181H-100), 1% Non-Essential Amino Acid (Dutscher, X0557-100) and 1% Penicillin/Streptomycin (Duscher, L0022-100). Cells were seeded at 15000 cells/cm^2 then cultivated for 48 hours before treatment or not by GA 0.67% 24h at 37°C and 90% RH (basal condition). Cells were seeded at 15000 cells/cm^2 then cultivated for 48 hours before UVA stress (10J/cm^2) or not and treatment or not by GA 0.67% 24h at 37°C and 90% RH (UVA condition).

Study of the protective properties of GA on human epidermal skin cells exposed to various environmental stresses (figure 2)

Transitory heat stress

The choice of 44°C used to mimic a heat stress was based on the literature [10]. Cells were seeded at 15000 cells/cm^2, then after 6 hours the cells were put in contact with GA at 0.67% for 24h at 37°C and 90% relative humidity (RH). During this incubation time, in the transitory heat

stress condition, the cells were placed 6 hours before the end of the experiments at 44°C for 40 minutes. While in the control condition, the cells were maintained in an incubator at 37°C and 90% RH.

Hydric stress combined or not to a transitory heat stress

The rational of the design of these experimental conditions was based on several observations. The choice of 45% RH was taken as we observed that all around the globe our skin is daily exposed to fluctuation of RH from 45% to 90%. Therefore, in our experimental conditions, the cells were exposed in the same day at 45% RH and 90% RH to try to mimic realistic environmental conditions.

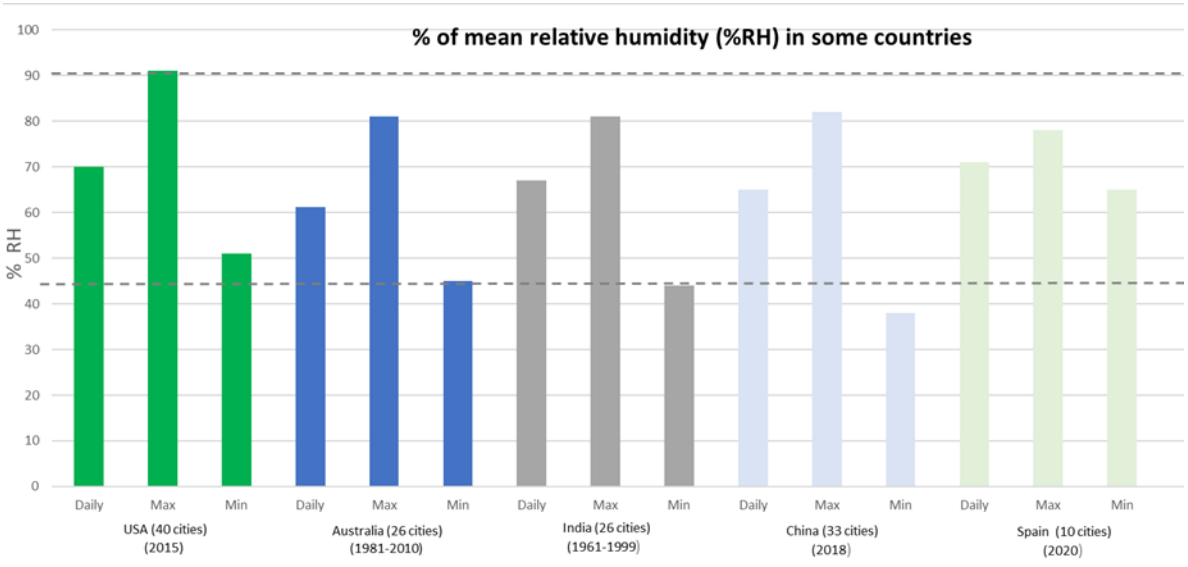

Figure 1: Daily RH% variation observed in some countries all around the world. The data were found on the following websites (https://www.currentresults.com/Weather/US/humidity-city-annual.php, https://sercc.com/climateinfo/historical/avgrh.html).

Cells were seeded at 15000 cells/cm^2, then after 6 hours the cells were put in contact with GA at 0.67% for 24h at 37°C and 45% RH. During this incubation time, the cells were placed 6 hours before the end of the experiments at 44°C for 40 minutes.

UVB stress

Cells were seeded at 15000 cells/cm^2, then cultivated for 48h at 37°C and 90% RH before exposure to UVB irradiation (30mJ/cm^2) then the cells were treated or not by GA. The cells were in contact with GA at 0.67% for 24 hours.

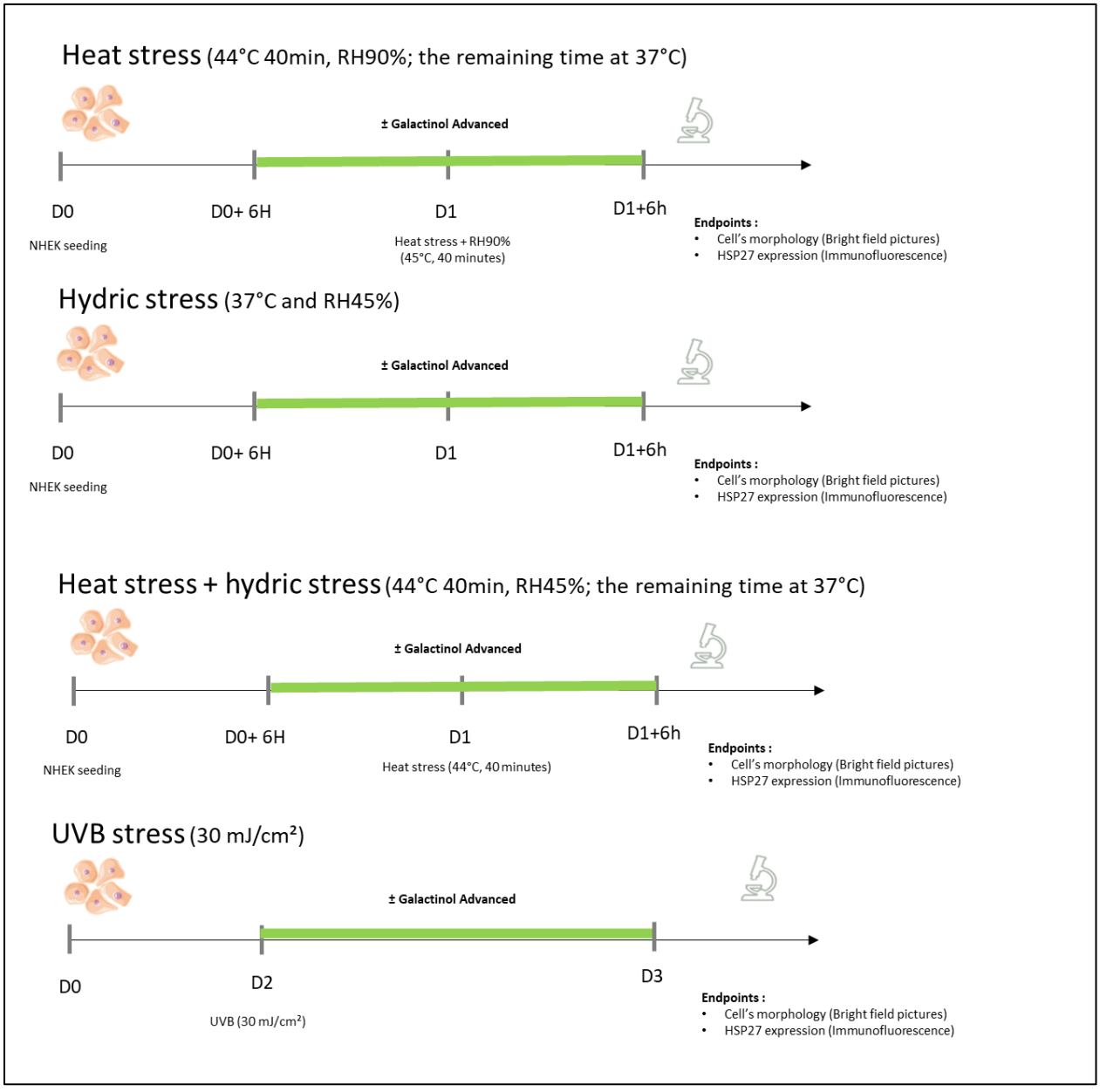

Figure 2: Study protocol related to the various environmental stresses studied.

Hyaluronic acid (HA) evaluation

NHEK were seeded in a 96 wells plate at 20000 cells/well and incubated at 37°C, 5% CO_2 for 24h. Cells were treated by GA (0.67% or 0.067%) or EGF (positive control) at 10^{-8}M and incubated at 37°C 5% CO_2 for 48h. The supernatant was collected from each well and centrifuged 10 min at 3000g on a 96 well plate (V-bottom). HA quantitation was realized using an ELISA Hyaluronan DHYALO (R&D system) assay kit.

Clinical investigation: skin biometrological analysis

Panel

A double blinded clinical trial was realized on 20 photo-exposed women (from 49 to 65 years old, mean age 54.6 y.o.). The peculiarity of this panel relies on their living habits, they spent most of the day "outside". The volunteers were asked to topically apply for 28 days on hemi-face, twice a day (morning & evening) a formula cream containing either GA at 2% or Placebo for 28 days. The same amount of glycerin is present on the two formulas (GA and Placebo one). Formula: water, ammonium acryloyldimethyltaurate/VP crossplymer, glycerin, dicarprylyl ether, citric acid and sodium citrate, phenoxyethanol, methylparaben, ethylparaben, fragrance, ± 2% Galactinol Advanced.

Evaluation of wrinkles, skin biomechanical properties and pictures

Analysis of the wrinkles of the crow's feet was made with AEVA-HE® device (Eotech). The Skin biomechanical properties (elasticity, firmness) were measured at the level of the maxillary area for each side of the face with the Cutometer® (Courage & Khazaka). Photographs of the faces were taken with the ColorFace® system (Newtone). The measurements were carried out at D0, D14 and D28.

Statistical analysis

Minitab software was used for *in vitro* and clinical biometrological study. As in most of the *in vitro* studies, we compared means of three or more independent groups we used the one-way analysis of variance (ANOVA) to determine whether there are any statistically significant differences between the means of groups. Then, if the one-way ANOVA shows a statistically significant result that means that there is at least two groups that are statistically significantly different from each other, post hoc tests need to be run according

to the assumption of homogeneity of variances. If the data met the assumption of homogeneity of variances, a Tukey's honestly significant difference (HSD) post hoc test, and if the data did not meet this assumption, a Games Howell post hoc test was considered. For gene expression study, the Statistical analysis was done with a student's t-test by BioRad CFX software.

Metaproteomics

Sampling, protein extraction, isobaric labeling

Skin swabs were taken from the cheeks from each side of the face at D0 and D28. Proteins were digested by LysC and Trypsin. Peptides were purified by SPE chromatography (SepPak tC18 3cc), dried and solubilized in HEPES 100 mM pH 8.5. Peptide concentration was determined using Pierce™ Quantitative Colorimetric Peptide Assay (Cat Number 15380517). For each sample, proteins digests were labelled by four sets of TMT pro™ 16plex Label Reagent Set (Thermo Scientific Cat Number A44520).

Mass spectrometry analysis

500 ng of peptides from each fraction of each multiplex sample were injected in triplicate. Chromatography was performed using Ultimate 3000 (Dionex) equipment using PepMap100 C18 (75 µm x 50 cm, 2 µm material) column applying a 2.5% to 35% acetonitrile 120 minutes gradient at a flow rate of 300 nl/min after a 3 min trapping step on precolumn. Data were acquired using a Q-Exactive Plus (Thermo) mass spectrometer. MS/MS scan was performed on the 10 most intense ions of each cycle.

Protein identification, taxonomic assignment, and functional analysis

Mass spectrometry results were analyzed by Proteome Discoverer 2.4. Proteins were identified using SEQUEST-HT algorithm against all bacteria, fungi and human reference proteomes mined from UniProt KB.

Taxonomic assignment: Peptides were assigned to the Lowest Common Ancestor (LCA) by submitting their sequences to Unipept tool. Each protein is assigned to the most precise LCA of its peptides. If one inconsistent peptide was detected, the protein was assigned to the majority LCA, and else, the previous taxonomic level was considered until the peptide assignment answered to the previously described standards. If no assignment or no unique peptide were

available, the protein was assigned to root. Identified taxa were gathered in 3 different taxonomic groups: host (all Metazoa), bacteria and fungi.

The functional analysis performed thanks to HolXplore (Phylogen proprietary tool). HolXplore analyzes functional involvement of proteins from various species. Indeed, as a large part of microbial proteins lacks functional annotation. To solve this issue, protein sequences (recovered from Uniprot and Uniparc databasis) were submitted to EggNOG mapper to associate each protein to its closest annotated ortholog. The functional annotation of orthologs was used for this analysis. Used functional terms were GO terms, COG category, COG and KOG terms and KEGG pathways, reactions and modules. To evaluate annotated terms enrichment, a reference dataset was built, using all identified terms in EggNOG and the associated proteins. This reference dataset was compared to differentially expressed proteins dataset by a modified Exact Fisher test. Terms were considered as enriched if they presented a fold enrichment ≥ 1.2, a minimal number of associated regulated proteins of 3 and a p-value ≤ 0.05. This analysis was performed for different taxonomic groups of proteins: all species, host only, bacteria only, and fungi only. For each taxonomic group, if the number of enriched terms was less than 20, the p-value cut-off was removed, and the 20 terms with lowest p-values were selected for further analysis

Results.

Study of the deleterious effect of various environmental stresses on normal human keratinocytes

Even though skin cells have intrinsic defense mechanisms allowing to protect themselves, we demonstrated that common environmental stresses such as heat, hydric stress or the combination of heat stress + hydric alter keratinocytes morphology. The cells appear thicker, elongated and less numerous (figure 3 B, C, D and E). In this experimental assay, we study the expression of HSP27 under these various stresses because, this heat shock protein has been describe to be involved in cellular protection against oxidative stress inducers [11][12]. UVB for example, is a strong trigger of oxidative stress. Moreover, a cause-effect relationship between the accumulation of HSP27 and thermotolerance has been established. Interestingly, we observed that the cells submitted to these various stresses are able to induce the expression of HSP27 (figure 3, red staining). These results suggest that the cells are trying to protect themselves by the upregulation of this beneficial heat shock protein. We clearly show that the protection provided by this intrinsic mechanism is not sufficient to avoid cell damages. Thus, if GA can increase the expression of HSP27 in the cells exposed to these various stresses, this might probably improve their intrinsic protection level.

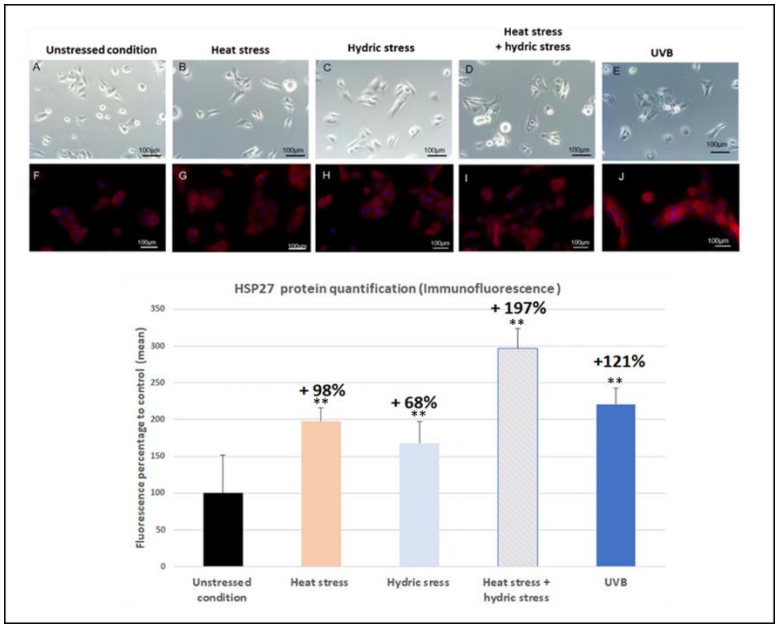

Figure 3: Bright field pictures of NHEK under basal condition (A) (37°C and 90% RH) and after various stresses heat stress (B) (44°C 40mn and 45% RH), hydric stress (C) (45% RH and 37°C), heat + hydric stresses (D) (44°C 40mn and 45% RH) and UVB stress (E) (30mJ/cm$^{2)}$. HSP27 immunostaining of NHEK under basal condition (F) (37°C and 90% RH), and after various stresses heat stress (G) (44°C 40mn and 45% RH), hydric stress (H) (45% RH and 37°C), heat + hydric stresses (I) (44°C 40mn and 45% RH) and UVB stress (J). HSP27 immunofluorescence intensity quantified by Image J software, Statistical analysis Minitab software; **p ≤0.01.

Study of the protective properties of Galactinol Advanced on human skin cells at basal level and when exposed to various environmental stresses

The results clearly demonstrate that GA by increasing the expression of HSP27 in the different stress conditions allows a better protection of skin cells. These results are consistent with literature data showing that in cell lines which constitutively overexpress various amounts of HSP27 or transiently express the protein after a selective induction revealed that cellular thermo-resistance correlates with the amount of total HSP27 present at the time of heat shock [13]. Our results might also be explained by the antioxidant and by the anti-inflammatory properties provided by this HSP27 thus ensuring a cellular protection under heat, hydric or UV irradiations [14].

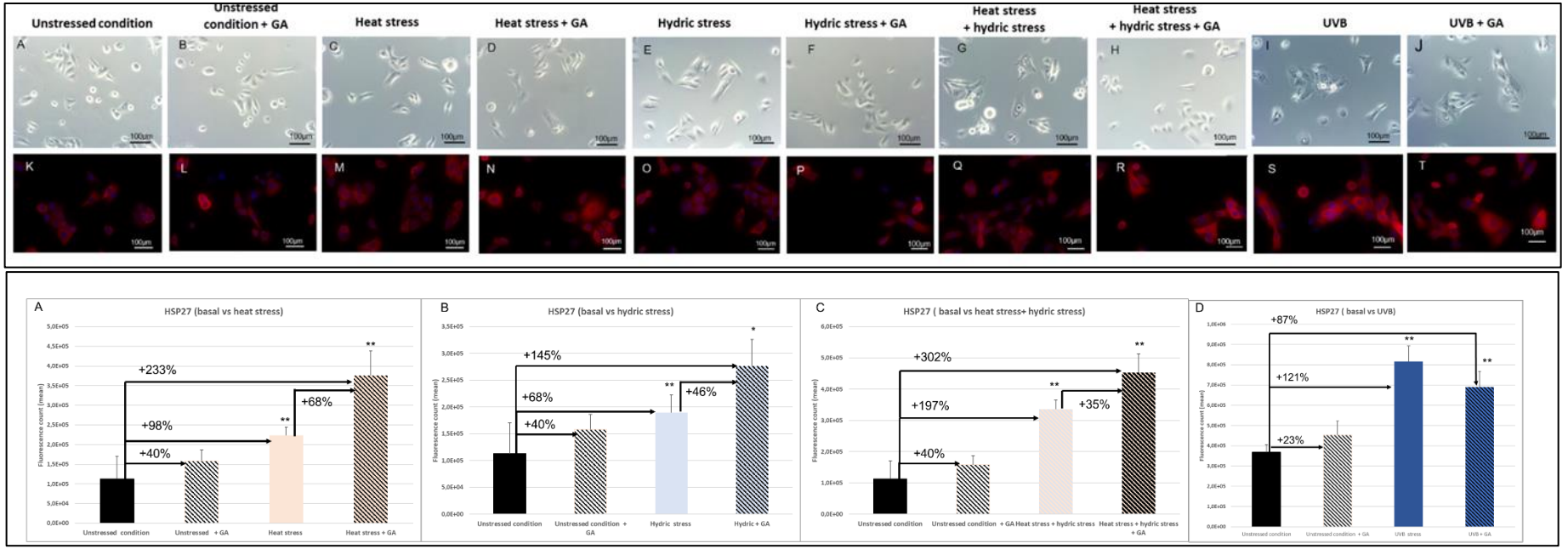

Figure 4: Bright field pictures of NHEK in basal condition i.e. 37°C and 90% RH (A: without GA, B: with GA), and after various stresses: heat stress (C: without GA, D: with GA), hydric stress (E: without GA, F: with GA), heat + hydric stresses (G: without GA, H: with GA) and UVB stress (I: without GA, J: with GA). HSP27 immunostaining of NHEK in basal condition (K: without GA, L: with GA) and after various stresses, heat stress (M: without GA, N: with GA), hydric stress (O: without GA, P: with GA), heat + hydric (Q: without GA, R: with GA) and UVB stress (S: without GA, T: with GA). HSP27 immunofluorescence intensity quantified by Image J software. Statistical analysis Minitab software; **p ≤0.01; *p ≤0.05.

Study of the protective properties of Galactinol Advanced in basal and stressed UVA condition: on normal human dermal fibroblasts

As expected, we demonstrated that UVA alter NHDF skin physiology (figure 5 A and 5C). The cells appear thicker and less numerous. Interestingly, when the cells are treated with galactinol the healthy cells morphology is maintained and the expression of HSP27 is increased. These results showed that the protective effect of GA is in part through HSP27 expression.

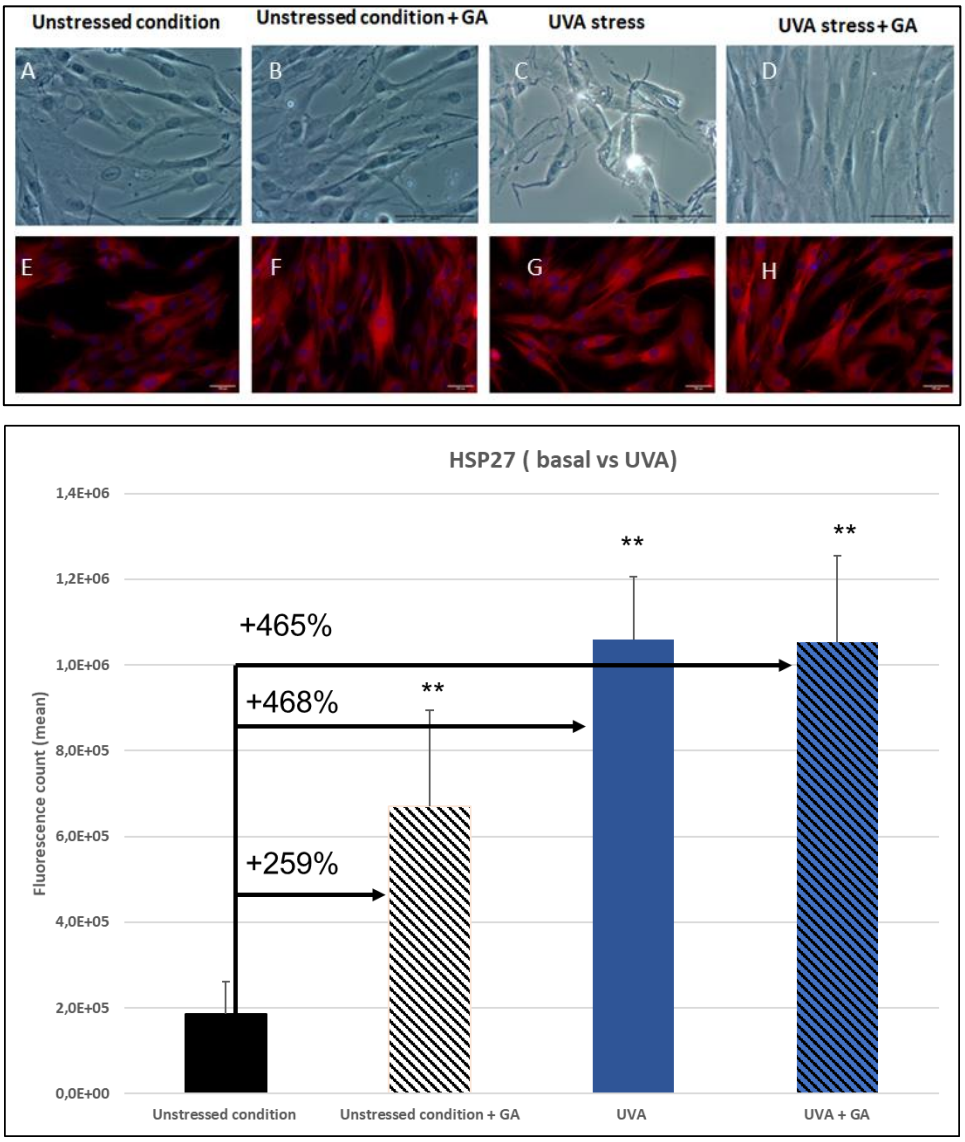

Figure 5: Bright field pictures of NHDF in basal condition i.e. 37°C and 90% RH (**A:** without GA, **B:** with GA) and after UVA stress i.e. 10J/cm^2 (**C**: without GA, **D**: with GA). HSP27 immunostaining of NHDF in basal condition i.e. 37°C and 90% RH (**E:** without GA, **F:** with GA), and after UVA stress i.e. 10J/cm^2 (**G**: without GA, **H**: with GA). HSP27 immunofluorescence intensity quantified by Image J software. Statistical analysis Minitab software; **p ≤0.01.

GA stimulates hyaluronic acid synthesis

Besides, the induction of HSP, the skin preserves its healthiness through other biological mechanism such as the stimulation of hyaluronic acid, a molecule known for its strong hydration properties. As shown in figure 6, GA stimulates in a dose dependent manner hyaluronic acid synthesis respectively by 40% and 78% when tested at 0.067% and 0.67%. As hyaluronic acid is known for its hydrating properties, this result suggests the potential hydration effect of GA. EGF was used as a positive control and validates the assay.

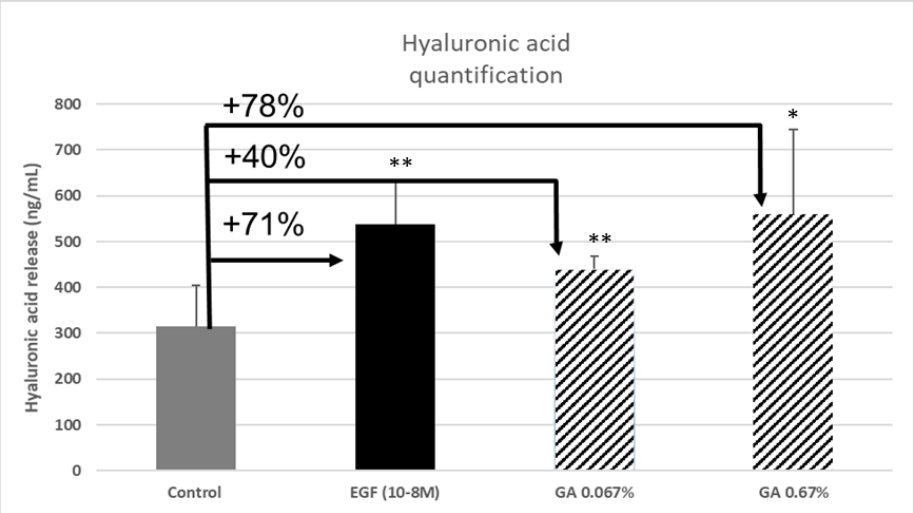

Figure 6: Hyaluronic acid quantification in NHEK treated with Galactinol Advanced at 0.67% or 0.067% for 48 hours. Statistical analysis Minitab software; **p ≤0.01 and *p ≤0.05.

Improvement of wrinkles, and skin biomechanical properties

After 28 days of topical application of GA treatment on the hemi-face, the wrinkles volume is significantly decrease by -18.9%. As expected in the placebo control group there is no improvement of wrinkles volume (Figure 8). Skin elasticity (Ue) and firmness (Ro) parameters were statistically significantly increased by GA treatment by +18.2% and +5.5% respectively after 28 days of twice daily use. A statistically significant difference was observed for both parameters between GA and the placebo control group. These results confirm that Galactinol Advanced improved efficiently skin biomechanical properties. These results might be explained by the significant upregulation of extracellular matrix constituents such elastin (data not shown) and hyaluronic acid. In the placebo group, a slight statistically significant increase of elasticity was observed. This result might be explained by the emollient properties of the formula used. In contrast, the placebo has no effect on skin firmness. The pictures clearly show a visible reduction of wrinkles after 28 days of use of 2% GA (grey arrows, Figure 7).

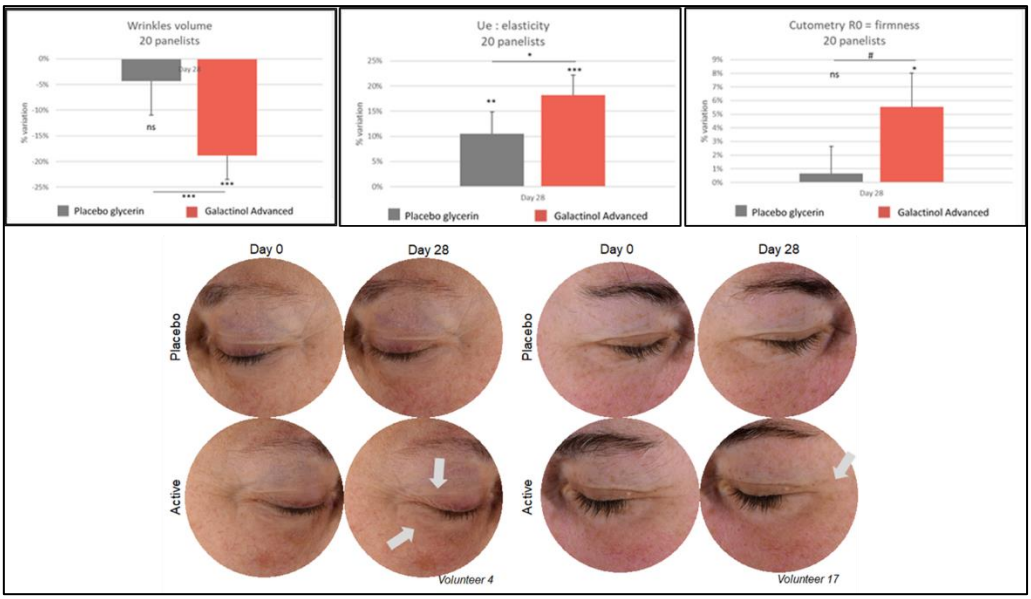

Figure 7: Wrinkles volume evaluated by the fringe projection technic with AEVA-HE® device, elasticity and firmness evaluated with a cutometer® before and after 28 days of 2% Galactinol Advanced treatment or Placebo. Illustrative pictures taking with ColorFace® device. Grey arrows highlight wrinkles improved by GA treatment. Statistical paired student t-test was realized with Minitab software. #=p<0.1.; *=p<0.05; **=p<0.01; ***=p<0.001.

Identification and determination of the number of proteins expressed at the skin surface according to their taxonomic assignment (human, bacteria, and fungi)

We studied the effect of GA on skin microbiota by using the metaproteomics technology as in contrast to metagenomics, this technic generates functional data relying on the study of proteins significantly regulated. We identified and assigned to the respective taxon 4785 proteins: human (2491 proteins), bacteria (1258 proteins) and fungi (656 proteins). Then, a ratio cut-off (ratio ≤ 0.833 or ≥ 1.2) was used to select only differentially expressed proteins between placebo and GA group. Only 343 proteins were significantly regulated in the full dataset by GA in comparison to placebo group. The Figure 8 presents proteins distribution in each taxonomic group and their regulations. Most of differentially expressed proteins were in bacteria (156 proteins) taxonomic group.

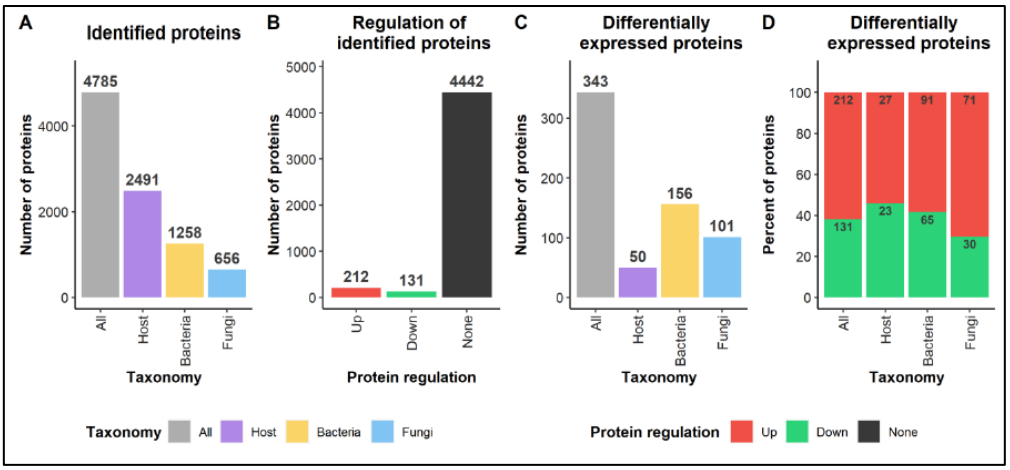

Figure 8: Number of identified proteins and their distribution in each taxonomic group.

Comparison of skin microbiota taxa abundances at the genus level before and after Galactinol Advanced or placebo treatment

The proteins identified at the genus level allow the determination of skin microbiota taxa abundances for each group studied (untreated skin D0, placebo and GA group (bar plots, figure 10). Most abundant genera are Cutibacterium for bacteria and Malassezia for fungi, a typical result for normal face skin, even if Staphylococcus is not a major genus in this dataset. The taxonomic analysis reveals no major difference in microbiome composition between sampling dates, highlighting that GA respects microorganism's balance. This result demonstrates that Galactinol Advanced can be considered as safe toward skin microbiota and can be quoted "microbiota friendly".

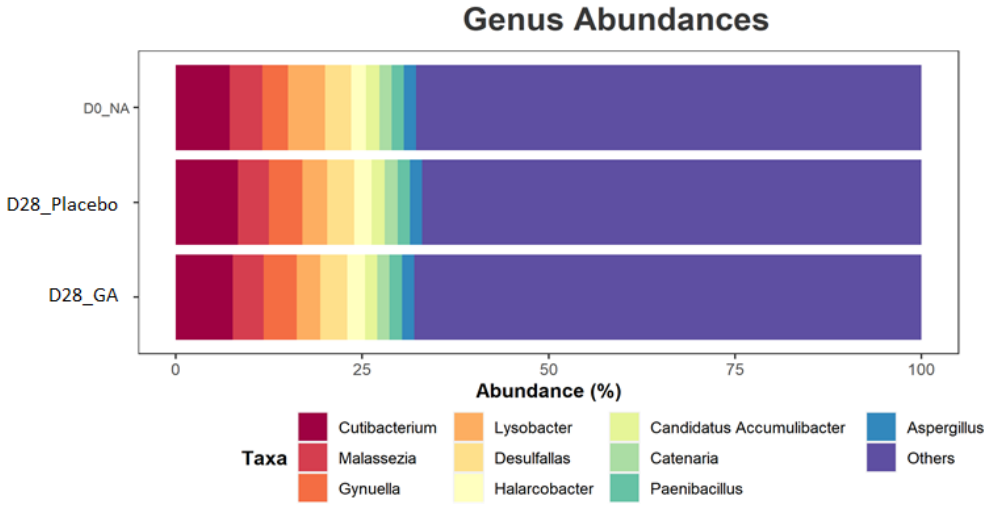

Figure 9: Major microbiota taxa abundances proportions at genus level for each group (Placebo or Galactinol Advanced GA).

Functional analysis of human and skin microbiota proteins regulated

The functional analysis revealed 25 terms statistically enriched with a p-value ≤ 0.05. The enriched terms show that GA treatment stimulates global cellular metabolism in human skin cells and in skin microbiota. Then, enriched terms have been gathered into major functional categories (table 2). Interestingly, we found a significant stimulation of antioxidant and detoxification enzymes such as superoxide dismutase and limonene 1.2 mono oxygenase. Moreover, we observed a common stimulation of the cell's metabolism in all taxa (human, fungi and bacteria). For example, the glyceraldehyde-3-phosphate deshydrogenase was upregulated in bacteria, human and fungi. While two other enzymes triosephosphate isomerase and enolase were upregulated in the human cells and in bacteria. In addition, GA regulates the proteostasis mechanism in human cells and in bacterial cells by stimulating the expression levels of proteins belonging to HSPs such as 60kda chaperonin, HSP70 binding protein 1 but also proteins involved in the translation process such as ribosomal protein 60S. Galactinol Advanced treatment stimulates the expression of UDP-glucuronic acid decarboxylase 1 a rate limiting enzyme involved in glycosaminoglycans synthesis pathway. Thus, the stimulation of this enzyme might explain the improvement of skin mechanical properties (elasticity and firmness). Moreover, the reinforcement of skin barrier function is also confirmed thanks to this metaproteomic study by the upregulation of Late cornified envelope proteins 1 B and 3B.

Origin	Function	Protein name	Fold change	P Value
Microorganisms	Antioxidant (ROS decrease/detoxification)	Super oxide dismutase	1.307	0.0009
		Limonene 1,2-monooxygenase	1.239	10^{-7}
Microorganisms/ human skin cells	Metabolism (energy increase/glycolysis)	Glyceraldehyde-3-phosphate dehydrogenase (bacteria)	1.241	0.00047
		Glyceraldehyde-3-phosphate dehydrogenase (fungi)	1.426	0.00001
		Glyceraldehyde-3-phosphate dehydrogenase (human)	1.125	0.00001
		Triosephosphate isomerase (bacteria)	1.236	0.003
		Triosephosphate isomerase (human)	1.085	0.014
		Enolase (bacteria)	1.39	0.00005
		Enolase (human)	1.132	0.000001
	Proteostasis (protein quality control /ribosome synthesis)	60 kDa chaperonin (bacterial)	2.677	10^{-15}
		HSP70 binding protein 1 (HSPB1) human	1.381	0.003
		Ribosomal protein (60S) bacteria	1.391	0.00005
		Ribosomal proteins (60S) human	1.260	0.001
Human skin cells	Anti-inflammatory	CD81	1.381	0.0001
		Complement 8B (lytic complex)	0.766	0.00003
	Skin barrier function (stratum corneum/ waterproofing)	Late cornified envelope protein 3B	1.349	0.007
		Late cornified envelope protein 1B	1.245	0.05
	Mechanical properties & hydration (GAG synthesis)	UDP-glucuronic acid decarboxylase 1	1.209	0.03

Table 1: Proteins from human and skin microbiota origins regulated by 2% Galactinol advanced

Discussion.

Skin is very sensitive to environmental stresses, such as UV irradiation and hydric fluctuations. For example, UV plays a major role in premature aging [15]. Moreover, it has been shown that when reconstructed human epidermis (RHE) are cultivated between 30–50% RH the properties of the epidermis at the structural, functional, and biochemical levels were profoundly altered in comparison to a control condition (90% RH). In particular, the *stratum corneum* of RHE become thicker, and TEWL decreased confirming a poorer barrier function [16]. We showed that when skin cells are exposed to these various stresses, the expression of HSP27 is increased. The results are consistent with literature data showing that cells respond to environmental stress by the preferential synthesis and the accumulation of a conserved family of proteins referred to as heat shock proteins (HSPs), and the acquisition of a dramatically increased capacity to survive subsequent hyperthermic stresses [17]. Heat Shock Proteins (HSPs) a large group of highly evolutionary conserved proteins, are main elements of the cellular proteoprotection system and are important for epidermis biology. For instance, HSP27 a stress-inducible chaperone is one of the most important elements of cellular proteostasis plays a role in the regulation of final steps of keratinization. HSP27 thanks to its holdase activity participates in transferring unfolded peptides to HSPA-HSPC-HSPH chaperone machinery supporting them to achieve native structures and preventing from unspecific aggregation [10, 18, 19].

In addition, to its role in epidermal keratinization, a specific interaction between HSP27 and filaggrin was described and confirm its crucial role in stratum corneum formation [20]. Moreover, an altered expression of HSP27 was observed in various skin ichthyosis pathological conditions such as bullous ichthyosiform erythroderma and annular epidermolytic ichthyosis and was not detected in keratinocytes derived from lesional skin of some patients with X-linked recessive ichthyosis [18]. For example, a cause-effect relationship between the accumulation of HSP27 and thermotolerance has been established. Moreover, it has been shown that HSP27 has an antioxidant property that generates a cellular protection against oxidative stress inducers [11][12]. The work of Sur *et al.* in 2008 [14], showed in HSP27 knockdown UVB irradiated keratinocytes an increase in pro-inflammatory cytokine IL-8. These works reveal the role of HSP27 in the regulation of inflammatory pathways in keratinocytes that might be trigger by UV irradiation or other environmental stresses. HSP27 has also been reported to attenuate protein oxidation, DNA damages, lipid peroxidation, and cytoskeleton architecture disruption [21, 22]. Thus, HSP27 overexpression induced by GA under basal and stress is consistent with a skin resilient powerful active. We demonstrate that in one hand, skin cells protect themselves

thanks to HSPs, but in the other hand, it's clearly established that our skin health rests mainly on its microbiome. Indeed, the skin is colonized by commensal beneficial microorganisms called microbiome. This complex ecosystem is a shield for our skin ensuring protective functions while educating our immune system. Therefore, studying epidermis and its microbiome with adapted methodologies to clinical sampling such as metaproteomics might help to gain valuable insight during the development of solutions dedicated to skin health such as cosmetic ingredients. Clinical results highlight that metaproteomics, used for the first time in the skin, is a powerful technology [8, 9]. We demonstrated that GA while preserving skin microbiota composition can stimulates human skin cells and skin microbiota to express beneficial proteins and enzymes involved in skin healthiness and resilience such as antioxidant, stimulation of metabolism, heat shock proteins, anti-inflammation, skin barrier function, and glycosaminoglycans synthesis.

Conclusions.

Thanks to a biomimetic approach, we developed a biotech based active ingredient efficient to promote skin resilience and healthiness. The *in vivo* investigations highlight the strong biological activities of GA to improve skin quality and healthiness when used topically at 2%. The following skin benefits were demonstrated wrinkles' reduction, improvement of skin elasticity and firmness. The skin metaproteomics results demonstrate that GA helps to maintain essential and various skin defense capacities beneficial against environmental stresses. These regulated proteins at keratinocytes and microbiome levels lead to visible outcomes: improvement of skin wrinkles, mechanical properties and skin healthiness.

Acknowledgments.

The authors thank Phylogen for their work on metaproteomics investigation.

References

1. Baek J, Lee M-G (2016) Oxidative stress and antioxidant strategies in dermatology. Redox Rep 21:164–169. https://doi.org/10.1179/1351000215Y.0000000015
2. Kammeyer A, Luiten RM (2015) Oxidation events and skin aging. Ageing Res Rev 21:16–29. https://doi.org/10.1016/j.arr.2015.01.001
3. dos Santos T. B. & Vieira L. G. E. (2020) Involvement of the galactinol synthase gene in abiotic and biotic stress responses: A review on current knowledge. Plant Gene 24:100258. https://doi.org/10.1016/j.plgene.2020.100258
4. Liu J, Moyankova D, Lin C-T et al. (2018) Transcriptome reprogramming during severe dehydration contributes to physiological and metabolic changes in the resurrection plant Haberlea rhodopensis. BMC Plant Biol 18:351. https://doi.org/10.1186/s12870-018-1566-0
5. Kim M. S., Cho S. M., Kang E. Y., Im Y. J., Hwangbo H., Kim Y. C., Ryu C.-M., Yang K. Y., Chung G. C. and Cho B. H. (2008) Galactinol Is a Signaling Component of the Induced Systemic Resistance Caused by Pseudomonas chlororaphis O6 Root Colonization 21:1643–1653
6. Baldwin H. E., Bhatia N. D., Friedman A., Eng R. M., Seité S. (2017) The role of cutaneous microbiota harmony in maintaining a functional skin barrier
7. Prescott S. L., Larcombe D.-L., Logan A. C., West C., Burks W., Caraballo L., Levin M., van Etten E., Horwitz P., Kozyrskyj A., Campbell D. E. (2017) The skin microbiome: impact of modern environments on skin ecology, barrier integrity, and systemic immune programming. World Allergy Organ J 10:29. https://doi.org/10.1186/s40413-017-0160-5
8. Karaduta O, Dvanajscak Z, Zybailov B (2021) Metaproteomics-An Advantageous Option in Studies of Host-Microbiota Interaction. Microorganisms 9(5)
9. Zhang X, Ning Z, Mayne J et al. (2016) MetaPro-IQ: a universal metaproteomic approach to studying human and mouse gut microbiota. Microbiome 4:31. https://doi.org/10.1186/s40168-016-0176-z
10. Echchgadda I, Roth CC, Cerna CZ et al. (2013) Temporal gene expression kinetics for human keratinocytes exposed to hyperthermic stress. Cells 2:224–243. https://doi.org/10.3390/cells2020224
11. Arrigo A.P. (2001) Hsp27: Novel Regulator of Intracellular Redox State
12. Merendino A. M., Paul C., Vignola A. M., Costa M. A., Melis M., Chiappara G., Izzo V., Bousquet J., Arrigo A.-P. (2002) Heat shock protein-27 protects human bronchial epithelial cells against oxidative stress–mediated apoptosis: possible implication in asthma
13. Lavoie J. N., Lambert H., Hickey E., Weber L. A., Landry J. (1995) Modulation of Cellular Thermoresistance and Actin Filament Stability Accompanies Phosphorylation-Induced Changes in the Oligomeric Structure of Heat Shock Protein 27
14. Sur R., Lyte P. A., Southall M. D. (2008) Hsp27 regulates pro-inflammatory mediator release in keratinocytes by modulating NF-kappaB signaling. J Invest Dermatol 128(5):1116–1122
15. Flament F, Bazin R, Laquieze S et al. Effect of the sun on visible clinical signs of aging in Caucasian skin. Clin Cosmet Investig Dermatol 6:221–232
16. Cau L, Pendaries V, Lhuillier E et al. (2017) Lowering relative humidity level increases epidermal protein deimination and drives human filaggrin breakdown. J Dermatol Sci 86(2):106–113
17. Landry J, Chrétien P, Lambert H et al. (1989) Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. J Cell Biol 109:7–15. https://doi.org/10.1083/jcb.109.1.7
18. Jonak C, Mildner M, Klosner G et al. (2011) The hsp27kD heat shock protein and p38-MAPK signaling are required for regular epidermal differentiation. j dermatol scie 61:32–37. https://doi.org/10.1016/j.jdermsci.2010.10.009

19. Scieglinska D., Krawczyk Z., Sojka D. R. & Gogler-Pigłowska A. (2019) Heat shock proteins in the physiology and pathophysiology of epidermal keratinocytes. Cell Stress Chaperones 24:1027–1044. https://doi.org/10.1007/s12192-019-01044-5
20. O'Shaughnessy RFL, Welti JC, Cooke JC et al. AKT-dependent HspB1 (Hsp27) activity in epidermal differentiation. J Biol Chem 282(23):17297–17305
21. Preville X., Gaestel M., Arrigo A.-P. (1998) Phosphorylation is not essential for protection of L929 cells by Hsp25 against H2O2-mediated disruption actin cytoskeleton, a protection which appears related to the redox change mediated by Hsp25.
22. Preville X., Salvemini F., Giraud S., Chaufour S., Paul C., Stepien G., Ursini M. V., Arrigo A.-P. (1999) Mammalian Small Stress Proteins Protect against Oxidative Stress through Their Ability to Increase Glucose-6-phosphate Dehydrogenase Activity and by Maintaining Optimal Cellular Detoxifying Machinery