Accepted: 10 February 2018

DOI: 10.1111/jocd.12528

ORIGINAL CONTRIBUTION

WILEY JCD Journal of Cosmetic Dermatology

Prediction of skin anti-aging clinical benefits of an association of ingredients from marine and maritime origins: Ex vivo evaluation using a label-free quantitative proteomic and customized data processing approach

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Summary

Background: The application of ingredients from marine and maritime origins is increasingly common in skin care products, driven by consumer expectations for natural ingredients. However, these ingredients are typically studied for a few isolated in vitro activities.

Objectives: The purpose of this study was to carry out a comprehensive evaluation of the activity on the skin of an association of ingredients from marine and maritime origins using label-free quantitative proteomic analysis, in order to predict the clinical benefits if used in a skin care product.

Methods: An aqueous gel containing 6.1% of ingredients from marine and maritime origins (amino acid-enriched giant kelp extract, trace element-enriched seawater, dedifferentiated sea fennel cells) was topically applied on human skin explants. The skin explants' proteome was analyzed in a label-free manner by high-performance liquid nano-chromatography coupled with tandem mass spectrometry. A specific data processing pipeline (CORAVALID) providing an objective and comprehensive interpretation of the statistically relevant biological activities processed the results.

Results: Compared to untreated skin explants, 64 proteins were significantly regulated by the gel treatment (q-value ≤ 0.05). Computer data processing revealed an activity of the ingredients on the epidermis and the dermis. These significantly regulated proteins are involved in gene expression, cell survival and metabolism, inflammatory processes, dermal extracellular matrix synthesis, melanogenesis and keratinocyte proliferation, migration, and differentiation.

Conclusions: These results suggest that the tested ingredients could help to preserve a healthy epidermis and dermis, and possibly to prevent the visible signs of skin aging.

KEYWORDS
claim substantiation, clinical prediction, proteomics, seaweed, skin physiology

1 | INTRODUCTION

The marine environment is an exceptional source of both biological and chemical diversity, still largely unknown, although it has been exploited since ancient times. It has been estimated that 25% of the total number of species on Earth come from the marine environment.[1] The uniqueness of this hostile environment in terms of light, salinity, pressure, space, gave birth to original organisms and bioactive substances, many of which exhibit characteristics not found in the terrestrial environment. Numerous research teams across the world have investigated and continue to explore the application of marine ingredients in areas such as pharmaceuticals, human food, animal feed, biomaterials, or cosmetics. Furthermore, a specific science is dedicated to the study of marine compounds with pharmacological properties: marine pharmacology.[2] Many of these ingredients are secondary metabolites, not associated with primary functions such as development, growth, or species spreading. A recent review reported that researchers have isolated approximately 7000 marine natural compounds, 25% of which are derived from algae, 33% from sponges, 18% from coelenterates, and 24% from other invertebrate phyla.[3] Recent reviews recorded the identification of more than 28 000 marine compounds, of which at least 3000 exhibit bioactive properties, and hundreds of new compounds being discovered each year.[1,4] Few of them have been marketed or are under development, for various reasons including cost, time, and difficulties in harvesting the organism, low concentrations of natural compounds in producing organism, high toxicity of certain compounds, ecological issues, or insufficient investment by laboratories.[1] Notwithstanding these difficulties, marine drug discovery continues to develop thanks to technological developments facilitating structural elucidation and screening, and recent advances in understanding marine organisms. For several years now, researchers have investigated microorganisms[1,4] and, very recently, virus that lives in the world's oceans and plays a significant role in the population ecology of marine organisms and microorganisms.

Due to the rejection of animal-derived ingredients, algae are preferred by the cosmetic industry. Few speciality ingredients' suppliers sell to cosmetic manufacturers algae-derived ingredients of different natures (such as roughly or finely grinded algae powders, aqueous, or oily extracts that can be prepared by various different extraction processes) and from different species (*Laminaria digitata*, *Undaria pinnatifida*, *Palmaria palmata*, *Ulva lactuca*, *etc.*), which are from different geographical origins. Some of them were evaluated in vitro, and more rarely by clinical studies, to prove their beneficial activities on skin. The benefit claims are numerous: anti-aging, free radical scavenging, soothing, preventing redness, depigmenting, slimming, etc. However, assessment methods are less complex than those of marine pharmacology and mostly consist of isolated in vitro assays focusing on specific biological markers that are relevant for the cosmetic industry (such as collagen I or hyaluronic acid synthesis by human dermal fibroblasts). Very few algae derived ingredient suppliers carry out exhaustive in vitro studies to get a comprehensive view of the activity of ingredients on the skin. However, in recent years, exhaustive studies could finally be performed thanks to the rise of high-throughput technologies such as "omics."

These "omics" approaches share the same goal of assessing whole cell working and complexities using nonrestrictive means of observation. This enables to assess whole pathways and pathways interactions, regulation and functional networks, by choosing untargeted methods, analyzing a very large number of entities of the same type. They also enable to answer new questions after the initial analysis. On the other hand, these data have to be analyzed using specific tools and methods, while taking into account each analytic paradigm feature.

Most used "omics" paradigms included genomics (whole genome sequencing, SNPs), transcriptomics (gene expression and regulation), proteomics (protein expression and regulation), and metabolomics (metabolites). They allowed identification of biomarkers for exposition, effects or susceptibility, discovery of new knowledge in mechanistic studies, or creation of new predictive toxicology tools. Proteome is defined as the collection of proteins of a biological sample in given time and conditions (including the organism's responses to environment or internal signals such as hormonal or chronobiological ones). By definition, proteome is thus dynamic unlike cell genome, which remains relatively stable. Proteomics is a method of choice for assessing active compounds' effects, as they study proteins, which are some of the cell's final effectors, and thus closely related to phenotypes. This idea has proven to be relevant because multiple studies showed that proteomics results were often not correlated with transcriptomics.[5-9] Skin proteomics is taking increasing importance, with more than a hundred articles published each year for the last 3 years. It allows for various samples to be tested in vitro (primary fibroblasts or keratinocytes cultures), as well as ex vivo (skin explants and reconstituted epidermis) or in vivo, with skin swabs and D-Squames. Dermatology, therapeutics as well as cosmetology, has used it for studying the mechanisms of UV damage and "photo-aging," inflammation, differentiation, skin barrier, and it allowed to discover potential diagnostic markers,[10] to understand tegumentary structure and potential reinforcing treatment,[11] or active substances or pollution effects on skin.[12,13] It also has been shown recently that proteomics and transcriptomics may show discrepancies due to a variety of post-transcriptional regulations, making proteomics the shortest, straightest path to phenotype.[6,14-16] The activity on the skin of ingredients from algal origin and, more broadly, from marine origin is hardly ever evaluated with "omics" technologies. To our knowledge, only genomic analysis has been used for such purposes, and no cosmetic ingredients from marine or maritime origin have been evaluated using a proteomic approach.

Accordingly, we aimed to evaluate the activity on the skin of an association of ingredients from marine and maritime origins by a label-free quantitative proteomic and customized data processing approach. The tested blend is composed of carefully selected ingredients based on their molecular composition and their bioactivities substantiated by individual in vitro tests: a yeast-derived amino acid-enriched giant kelp (*Macrocystis pyrifera*) extract, a naturally trace

element-enriched seawater, and dedifferentiated sea fennel (*Crithmum maritimum*) cells. The three ingredients were combined in an aqueous gel, which was then topically applied on human skin explants. The skin explants' proteome was analyzed in a label-free manner by high-performance liquid nano-chromatography coupled with tandem mass spectrometry. A specific data processing pipeline providing an objective interpretation of the statistically relevant biological activities processed the results in order to get a comprehensive view of the effects of the association of marine and maritime ingredients and predict its clinical benefits if used in a skin care product.

2 | MATERIALS AND METHODS

2.1 | Tested product

In this study, we tested an association of the following specialty ingredients from marine and maritime origins: sterilized TONIKELP® (Gelyma, Marseille, France), which is an aqueous giant kelp *M. pyrifera* extract, sterilized EAU DE SOURCE MARINE® (Soliance France, Pomacle, France), which is a natural trace element-enriched seawater, and CELTOSOME™ CRITHMUM MARITIMUM ST (Seppic, Puteaux, France) which is a fine powder composed of dedifferentiated sea fennel *C. maritimum* cells. The selected giant kelp extract is enriched with yeast-derived amino acids. The activity on the skin of this nutrient-enriched algae extract was evaluated by a genomic analysis followed by some complementary in vitro assays, performed to confirm the results of the first study. These unusual in vitro studies have revealed the anti-aging potential of the algae extract, which could preserve skin cells against oxidative stress and enhance the dermal fibroblast proliferation and the synthesis of the extracellular matrix (P. L. Liliane Pellegrini & P. M. Max Pellegrini, unpublished data). The natural trace element-enriched seawater is a water drawn in Brittany from a natural underground reservoir more than 22 m in depth. As the seawater percolates down through tiny cracks and fissures before reaching the reservoir, it dissolves the sandy and then granitic bedrocks that characterize the area where the reservoir is located. As a consequence thereof, the seawater is enriched with silicon, manganese, and zinc compared with a standard seawater, while containing typical minerals such as sodium, potassium, calcium, and magnesium. These minerals and trace elements are recognized as essential for skin health.[17] In vitro studies have shown that the natural trace element-enriched seawater topically applied on reconstructed epidermis or skin explants improved the barrier function of the skin by stimulating the synthesis of epidermal lipids by keratinocytes and by strengthening cell adhesions. The dedifferentiated sea fennel cells are whole totipotent vegetal stem cells. They are derived from sea fennel *C. maritimum*, also called samphire or rock samphire, which is one among these unique plants able to adapt to hostile environments (high salt concentration, arid soil, and climate), by synthesizing large amounts of antioxidants to protect itself. The dedifferentiated sea fennel cells originate from plant stem cell technology, which is currently a new source of innovative cosmetic ingredients.[18] They differ from the differentiated plant cells by their richness in phenolic acids and more specifically in chlorogenic acid derivatives. Previous studies disclosed a positive influence of dedifferentiated sea fennel cells on epidermal regeneration, skin barrier recovery, and the inhibition of melanogenesis.[19,20] The three active ingredients were formulated in an aqueous gel as described in Table 1. In brief, glycerin and preservatives were mixed together and added to water. Then the addition of the synthetic gelling agent under stirring jellified the solution. Finally, the three active ingredients were successively added to the gel under stirring. All the steps were performed at room temperature.

2.2 | Human skin explants and treatment protocol

The effect of the association of ingredients from marine and maritime origins was examined in vitro using a skin explant model. This model was preferred as it is the closest to a real human skin, and as it is possible to evaluate the activity of the tested gel on the different compartments of the skin (epidermis and dermis) and its various cell types. Discarded skin tissue from a 50-year-old female who underwent surgery was obtained, then defatted and rinsed with a phosphate-buffered solution (PBS). Two batches of 8 mm diameter punched biopsies were prepared: 3 skin explants were topically treated with 10 μL of the tested gel and 3 more remained untreated as a control batch. Skin explants were incubated under classical cell culture conditions (37°C, 5% CO_2) in a culture medium specifically engineered for explants survival (DMEM/HAMF12 [50:50] supplemented with 2% of fetal bovine serum [FBS], 1% of penicillin/streptomycin, and 1% of Fungizone [amphotericin B]). Skin explants were maintained in survival for 48 hours, before being rinsed twice with PBS to remove gel residues and frozen at −80°C. Frozen skin explants were carefully shipped to Phylogene (Bernis, France) in dry ice to be analyzed.

2.3 | Protein extraction

Skin explants were minced and placed in 5-mL LoBind protein tubes (Eppendorf; Hamburg). Minced tissues were lysed by POLYTRON® PT 1200 E Manual Disperser (Kinematica; Luzern) in a 100 mmol/L

TABLE 1 Formulation of the marine and maritime ingredient-containing gel

Phase	Ingredients	% (w/w)
A	Purified Water	86.40 (qsp.)
	Glycerin	5.00
	Phenoxyethanol, Ethylhexylglycerin	0.70
B	Ammonium Acryloyldimethyltaurate /VP Copolymer	1.80
C	Maris aqua (seawater) (trade name: Sterilized EAU DE SOURCE MARINE®)	3.00
	Water, *Macrocystis pyrifera* extract, Hydrolyzed yeast extract (trade name: Sterilized TONIKELP®)	3.00
	Crithmum Maritimum Extract (trade name: CELTOSOME™ CRITHMUM MARITIMUM ST)	0.10

DTT—EDTA 5 mmol/L—Tris-HCl 100 mmol/L pH 8.5 PBS buffer (Sigma-Aldrich, St Louis, MO) with 1× HALT protease inhibitors, EDTA-free (Pierce; Thermo Scientific, Rockford, IL). After addition of 2% SDS (final concentration), homogenates were sequentially boiled (5 minutes 99°C) and sonicated with sonication probe on ice for 2 minutes (Sonorex; Bandelin electronic, Berlin). Protein content for each sample was estimated by 660 nm protein assay (Pierce; Thermo Scientific, Rockford, IL).

2.4 | Protein digestion and cleanup

Protein extracts were then digested. Amicon Ultra 0.5 10 kDa filters (Millipore, Billerica) were selected, employing 8 mol/L urea in 0.1 mol/L Tris (pH 8.5) to facilitate removal of protein-bound small molecules. Proteins (150 μg) were reduced with 10 mmol/L DTT (dithiothreitol) (Euromedex, Souffelweyersheim) for 60 minutes and alkylated with 50 mmol/L iodoacetamide (Sigma-Aldrich, St Louis, MO) for 30 minutes. Buffer exchange for ammonium bicarbonate 50 mmol/L (Sigma-Aldrich, St Louis, MO) was then performed by ultrafiltration. Cleaned proteins were digested overnight on filter with trypsin at a 1:50 ratio (Promega, Madison, WI), and resulting peptides were released from filter by centrifugation (14 000 × *g*, 20 minutes), followed by a first filter washing in 0.5 mol/L NaCl and second filter washing by 50% acetonitrile (both Sigma-Aldrich, St Louis, MO). Peptides were diluted in 5% acetonitrile and acidified by 0.1% trifluoroacetic acid 0.1% final concentration. Peptides were deposited on 500 mg SepPak tC18 sample extraction columns (Waters, Milford), then eluted with 1.2 mL of 70% ACN, dried, and diluted to a final volume of 100 μL with water, with sonication.

2.5 | Nano-liquid chromatography and tandem mass spectrometry

Peptide digest (250 ng per run) was loaded onto an Acclaim™ PepMap™ 100 C18, 300 μm × 5 mm (particle diameter 5 μm, pore size 100 fÅ) in trap and elute mode with Acclaim™ PepMap™ 100 C18, 75 μm × 500 mm (particle diameter 3 μm, pore size 100Å) (Both Thermo Scientific, Rockford, IL). Ultimate 3000 Nano RSLC (Thermo Scientific, Rockford, IL) nano-liquid chromatography system was used for run gradient from 3% to 35% buffer B (buffer A: 0.1% formic acid; buffer B: 80% acetonitrile, 0.1% formic acid) over a 60-minute period at a flow rate of 300 nL/min. This system was coupled to a Q-Exactive HF (Thermo Scientific, Rockford, IL) mass spectrometer interfaced to a Nanospray Flex source. Source parameters were set as follows: IS at 1500V, and IHT at 275°C. Acquisition parameters were as follows: for DDA mode one 60K resolution MS scan, to obtain MS/MS spectra for the 20 most abundant parent ions following each survey MS1 scan (with resolution 15K) for m/z 350-1800.

2.6 | MS data analysis

DDA spectra processing and database searching were performed with MaxQuant software (v1.5.3.30; Max Planck Institute of Biochemistry Research Group "Computational Systems Biochemistry," Martinsried) using the Andromeda algorithm. Search parameters were as follows: False discovery rate was set to 0.01 for proteins and peptides (minimum length of 7 amino acids) and was determined by searching a reverse database. Enzyme specificity was set as C-terminal to arginine and lysine, a maximum of two missed cleavages was allowed as well as Cys alkylation: iodoacetamide modification for database search. Peptide identification was performed with an allowed initial precursor mass deviation up to 7 p.p.m. and an allowed fragment mass deviation up to 20 p.p.m. Database was downloaded from UNIPROT (June 2014), filtering for reviewed human proteins only (20 194 entries). The resulting label-free quantification file was loaded into Perseus software (v1.5.3.2 Max Planck Institute of Biochemistry Research Group "Computational Systems Biochemistry," Martinsried). Statistics was performed using *t* test with Benjamini-Hochberg correction, we required *q*-value to be equal or inferior to 0.05 for statistical significance. We added two criteria for "technical significance." The first was a mass spectrometry quality criterion: Each protein is validated based on peptide signal. The second was technical significance thresholds on fold changes, which depend on apparel, sampling quality (number of biological samples and replicates, number of technical replicates, as well as on the nature of samples). For this study, we selected statistically significant proteins whose fold changes were equal or inferior to 0.5 or equal or superior to 2. These proteins are deemed as « significantly regulated ».

2.7 | Overrepresentation analysis and data integration

As CORAVALID analytic pipeline first step, we selected UNIPROT identifiers for proteins identified by LC-MS/MS and considered as significantly regulated in relative quantitation proteomics experiment. Relevant data were then extracted from various ontologies using overexpression analysis with Fisher's exact test[21-23] and Bonferroni multiple testing correction. Biological processes, molecular functions, and cellular components thus were collected from Gene Ontology (Gene Ontology, May 2016 release[24]), and metabolic and signaling pathways were retrieved from KEGG Pathways (KEGG Pathways, May 2015 release[25]) and WikiPathways (May 2015[26,27]).

In a second step, overrepresentation outputs were validated based on biological and experimental background, filtering out inappropriate terms. Enrichment was consolidated, both within and between ontologies, based on parent-child relationships as well as functional relationships. Available protein data were analyzed using natural language processing, semantic search, and data mining with tailored requests based on the experiment specificities to assess relationships between proteins, direct or indirect effects (through interactor proteins or generated mediators or metabolites) of protein regulations on specific processes, functions, components, or pathways. Tools were based on Python 3.5.1, December 6, 2015; NLTK 3.2, March 2016; Pandas 0.18.0, March 13, 2016; Numpy 1.11.0, March 30, 2016; SciPy 0.17.0, January 2, 2016. A proprietary

database including contextual information regarding metabolites, tissue specificities, and other protein-related information was used to complement analysis of results, as well as available literature (references as quoted in following text). This allowed to perform biological meaning assessment (from processes and functions to physiology), macroscopic activity assessment, and phenotype assessment.

In a third step, we used information about the assay and active substance, together with our observations and analysis results to substantiate claims about active substance cosmetic potential.

3 | RESULTS

3.1 | Skin explants' proteins identification and quantification

The whole data related to skin explants' proteins identification and quantification are provided as supplemental materials. The differences between chromatograms of each condition revealed an impact of the gel treatment on skin explants' proteomes (data not shown). Overlapping of each condition's 3 samples' chromatograms proved reliable intrasample reproducibility.

The total number and the average number of proteins and peptides, which were identified in treated and untreated skin explants, are reported in Tables 2 and 3, respectively. A reduction in the average and the total numbers of identified proteins was observed between the untreated skin explants and the gel-treated ones. As the quantity of processed proteins was almost the same for each condition (3.7-4.4 mg of proteins/mL), we therefore assumed that the gel treatment impacted skin explants' proteome dynamics: as some proteins became predominant following the gel treatment, they could have made minor proteins undetectable through a known masking phenomenon.

Different regulations were observed by comparing the gel-treated condition and the untreated condition. Among the regulated proteins, many of them were only observed in the gel-treated condition or in the untreated condition. They were considered as significantly and strongly downregulated (noted as $-\infty$) and upregulated (noted as $+\infty$) proteins according to the observed regulation. Among the identified proteins, 490 (55%) were unaffected by the gel treatment (q-value > 0.05), 50 (5.6%) were significantly and strongly downregulated (q-value < 0.05; fold change <0.5 or $-\infty$), 104 (12%) were significantly but moderately downregulated (q-value $< 0.,05$; $0.5 <$ fold change < 1), 236 (26%) were significantly but moderately upregulated (q-value < 0.05; $1 <$ fold change < 2), and 14 (1.6%) were significantly and strongly upregulated (q-value < 0.05; fold change > 2 or $+\infty$). Overall, 64 proteins were significantly and strongly regulated by the gel treatment compared with the untreated condition. As previously explained, only these 64 proteins were subjected to the CORAVALID analysis in order to determine the biological impact of the gel treatment on skin explants and its significance. A summary of the 64 significantly and strongly regulated proteins is provided in Table 4, and the enrichment analysis results from CORAVALID's first step, which was used as basis for further consolidation, information extraction and biological effect interpretation are provided as supplemental materials.

TABLE 2 Total number and average number of proteins identified in gel-treated and untreated skin explants

Experimental conditions	Samples			Total number	Average number
	1	2	3		
Untreated skin explants	1089	1072	1088	1292	1083
Gel-treated skin explants	999	983	996	1182	993
Both conditions				1400	1038

TABLE 3 Total number and average number of peptides identified in gel-treated and untreated skin explants

Experimental conditions	Samples			Total number	Average number
	1	2	3		
Untreated skin explants	6797	6843	6834	8527	6825
Gel-treated skin explants	5620	5602	5531	6943	5584
Both conditions				9130	6205

4 | DISCUSSION

Skin is the largest organ of the human body. It undergoes natural aging caused by intrinsic factors (cellular senescence) but also extrinsic ones as it is continuously exposed to harmful stresses and damages from environmental sources (sun exposure, dehydrating ambient air, pollution, and chemicals coming from human industrial and daily activities, etc.).[28] Skin also endures directly or indirectly some detrimental features of human modern life style (refined sugar-rich and fat-rich diet, skin's microbiota disruption by repeated use of antimicrobial cleansing, work-related stresses or societal pressure, sleep deprivation and circadian rhythm desynchrony, etc.).[28] As skin is the most external organ of the human body, aging-related changes deeply impact our social experience as the skin appearance has a significant psychological influence on our life quality.[29,30] Intrinsic and extrinsic skin aging have characteristic clinical features, but skin often undergoes a combination of both. Histological and ultrastructural studies have revealed deep modifications in skin structures and functions, as detailed below in the outcomes of this study.[31]

To help the readers, results are discussed according to the comparison between the gel-treated skin explants and the untreated skin explants. The regulated proteins are mainly named with their UNIPROT identifiers, and one can refer to the Table 4 to get their full names. Additionally, the upregulated proteins figure in green and the downregulated proteins figure in red.

TABLE 4 (a-d) Summary of the 64 proteins which were significantly and strongly regulated in skin explants, 48 h after being treated by the marine and maritime ingredient-containing gel compared to the untreated skin explants. Each table shows the full name, the UNIPROT name, a gene name, the fold change, and some relevant gene ontology (GO terms) related to the biological processes in which each protein is involved. (a) Upregulated proteins with an infinite fold change (+∞). (b) Upregulated proteins with a fold change ≥2 (q-value < 0.05). (c) Downregulated proteins with a fold change ≤0.5 (q-value < 0.05). (d) Downregulated proteins with an infinite fold change (−∞). The complete results of the CORAVALID analysis are provided as supplemental materials

Full name	UNIPROT name	Gene name	Fold change	Relevant gene ontology (biological process) terms
(a) Upregulated proteins with an infinite fold change (+∞)				
2-Oxoglutarate dehydrogenase, mitochondrial	ODO1_HUMAN	OGDH	+∞	Cellular metabolic process [GO:0044237]; Tricarboxylic acid cycle [GO:0006099]
4F2 cell surface antigen heavy chain	4F2_HUMAN	SLC3A2	+∞	Amino acid transport [GO:0006865]; Cell growth [GO:0016049]
EH domain-containing protein 3	EHD3_HUMAN	EHD3	+∞	Endocytic recycling [GO:0032456]; Protein targeting to plasma membrane [GO:0072661]
Interleukin-1 receptor antagonist	IL1RA_HUMAN	IL1RA	+∞	Inflammatory response to antigenic stimulus [GO:0002437]; Negative regulation of interleukin-1-mediated signaling pathway [GO:2000660]
Lymphocyte antigen 6 complex, locus D	LY6D_HUMAN	LY6D	+∞	Cell adhesion [GO:0007155]; Lymphocyte differentiation [GO:0030098]
Splicing factor 3B subunit 3	SF3B3_HUMAN	SF3B3	+∞	mRNA processing [GO:0006397]; mRNA splicing, via spliceosome [GO:0000398]
Splicing factor proline/ glutamine-rich	SFPQ_HUMAN	SFPQ	+∞	mRNA processing [GO:0006397]; Alternative mRNA splicing, via spliceosome [GO:0000380]
Prostacyclin synthase	PTGIS_HUMAN	PTGIS	+∞	Prostaglandin biosynthetic process [GO:0001516]; Negative regulation of inflammatory response [GO:0050728]
(b) Upregulated proteins with a fold change ≥2 (q-value < 0.05)				
60S Ribosomal protein L36	RL36_HUMAN	RPL36	2.04	Translational elongation [GO:0006414];
Collagen alpha-1(I) chain	CO1A1_HUMAN	COL1A1	2.09	Cellular response to amino acid stimulus [GO:0071230]; Cellular response to epidermal growth factor stimulus [GO:0071364]; Cellular response to fibroblast growth factor stimulus [GO:0044344]; Cellular response to retinoic acid [GO:0071300]; Cellular response to transforming growth factor beta-stimulus [GO:0071560]; Extracellular matrix organization [GO:0030198]; [GO:0030168]; Positive regulation of canonical Wnt signaling pathway [GO:0090263]; Positive regulation of cell migration [GO:0030335]; Skin morphogenesis [GO:0043589]
Collagen alpha-1(III) chain	CO3A1_HUMAN	COL3A1	2.38	Aging [GO:0007568]; Cell-matrix adhesion [GO:0007160]; Cellular response to amino acid stimulus [GO:0071230]; Extracellular matrix organization [GO:0030198]; Integrin-mediated signaling pathway [GO:0007229]; [GO:0001501]; Skin development [GO:0043588]; Transforming growth factor beta-receptor signaling pathway [GO:0007179]; Wound healing [GO:0042060]
Protein S100-A8 (calgranulin-A)	S10A8_HUMAN	S100A8	2.08	Cytokine production [GO:0001816]; Innate immune response [GO:0045087]; Leukocyte migration involved in inflammatory response [GO:0002523]; Positive regulation of inflammatory response [GO:0050729]
Protein S100-A9 (calgranulin-B)	S10A9_HUMAN	S100A9	2.02	Cytokine production [GO:0050832]; Innate immune response [GO:0045087]; Leukocyte migration involved in inflammatory response [GO:0002523]; Positive regulation of cell growth [GO:0030307]; Positive regulation of inflammatory response [GO:0050729]
Small proline-rich protein 2D	SPR2D_HUMAN	SPRR2D	2.33	Epidermis development [GO:0008544]; Keratinization [GO:0031424]; Keratinocyte differentiation [GO:0030216]; Peptide cross-linking [GO:0018149]

(Continues)

TABLE 4 (Continued)

Full name	UNIPROT name	Gene name	Fold change	Relevant gene ontology (biological process) terms
(c) Downregulated proteins with a fold change ≤0.5 (*q*-value)				
Calpain small subunit 1	CPNS1_HUMAN	CAPNS1	0.35	Extracellular matrix disassembly [GO:0022617]; Extracellular matrix organization [GO:0030198]; Positive regulation of cell proliferation [GO:0008284]
Cytoskeleton-associated protein 4	CKAP4_HUMAN	CKAP4	0.43	Cellular protein metabolic process [GO:0044267]
Pigment epithelium-derived factor (Serpin F1)	PEDF_HUMAN	SERPINF1	0.47	Aging [GO:0007568]; Cell proliferation [GO:0008283]; Cellular response to retinoic acid [GO:0071300]; Negative regulation of inflammatory response [GO:0050728]
Protein AHNAK2	AHNK2_HUMAN	AHNAK2	0.47	Ectoderm development [GO:0007398]; Epidermis development [GO:0008544];
Prothymosin alpha	PTMA_HUMAN	PTMA	0.37	Transcription, DNA-templated [GO:0006351]
Thymosin beta-4	TYB4_HUMAN	TMSB4X	0.19	Actin filament organization [GO:0007015]
Tropomyosin 1 (alpha)	TPM1_HUMAN	TPM1	0.41	Negative regulation of cell migration [GO:0030336]; Positive regulation of cell adhesion [GO:0045785]; Positive regulation of stress fiber assembly [GO:0051496]; Regulation of cell shape [GO:0008360]
Tropomyosin-2 (beta)	TPM2_HUMAN	TPM2	0.48	Regulation of ATPase activity [GO:0043462]
Tropomyosin-3	TPM3_HUMAN	TPM3	0.41	Movement of cell or subcellular component [GO:0006928]
Tropomyosin-4	TPM4_HUMAN	TPM4	0.48	Movement of cell or subcellular component [GO:0006928]
(d) Downregulated proteins with an infinite fold change (-∞)				
60S Ribosomal protein L26	RL26_HUMAN	RPL26	−∞	Translational elongation [GO:0006414]
60S Ribosomal protein L28	RL28_HUMAN	RPL28	−∞	Translational elongation [GO:0006414]
Acetyl-Coa acetyltransferase, mitochondrial	THIL_HUMAN	ACAT1	−∞	Cellular ketone body metabolic process [GO:0046950]
ATP synthase subunit delta, mitochondrial	ATPD_HUMAN	ATP5D	−∞	ATP biosynthetic process [GO:0006754]; Cellular metabolic process [GO:0044237];
Basal cell adhesion molecule (auberger B antigen)	BCAM_HUMAN	BCAM	−∞	Cell adhesion [GO:0007155]
Brain acid soluble protein 1	BASP1_HUMAN	BASP1	−∞	Negative regulation of transcription, DNA-templated [GO:0045892]
CD99 antigen	CD99_HUMAN	CD99	−∞	Cell adhesion [GO:0007155]
Claudin-1	CLD1_HUMAN	CLDN1	−∞	Bicellular tight junction assembly [GO:0070830]; Calcium-independent cell-cell adhesion via plasma membrane cell-adhesion molecules [GO:0016338]; Cell-cell junction organization [GO:0045216]; Establishment of skin barrier [GO:0061436];
Cytoplasmic dynein 1 intermediate chain 2	DC1I2_HUMAN	DYNC1I2	−∞	ER to Golgi vesicle-mediated transport [GO:0006888]; Microtubule-based movement [GO:0007018]; Mitotic cell cycle [GO:0000278]; Organelle organization [GO:0006996];
Elongation factor 1-delta	EF1D_HUMAN	EEF1D	−∞	Translational elongation [GO:0006414]
Enoyl-CoA hydratase, mitochondrial	ECHM_HUMAN	ECHS1	−∞	Fatty acid beta-oxidation [GO:0006635]
Eukaryotic translation initiation Factor 3 subunit C-like protein	EIFCL_HUMAN	EIF3CL	−∞	Translational initiation [GO:0006413]
Eukaryotic translation initiation factor 3 subunit F	EIF3F_HUMAN	EIF3F	−∞	Protein deubiquitination [GO:0016579]; Translational initiation [GO:0006413]

(Continues)

TABLE 4 (Continued)

Full name	UNIPROT name	Gene name	Fold change	Relevant gene ontology (biological process) terms
Far upstream element-binding protein 1	FUBP1_HUMAN	FUBP1	−∞	Positive regulation of gene expression [GO:0010628]; Transcription from RNA polymerase II promoter [GO:0006366]
Hepatoma-derived growth factor	HDGF_HUMAN	HDGF	−∞	Cell proliferation [GO:0008283]; Endoplasmic reticulum unfolded protein response [GO:0030968]; Negative regulation of transcription from RNA polymerase II promoter [GO:0000122]
Hypoxanthine-guanine phosphoribosyltransferase	HPRT_HUMAN	HPRT1	−∞	IMP metabolic process [GO:0046040]; Purine nucleotide biosynthetic process [GO:0006164]
Inositol monophosphatase 2	IMPA2_HUMAN	IMPA2	−∞	Inositol metabolic process [GO:0006020]; signal transduction [GO:0007165];
Isoleucine-tRNA ligase, cytoplasmic	SYIC_HUMAN	IARS	−∞	Isoleucyl-tRNA aminoacylation [GO:0006428]; tRNA aminoacylation for protein translation [GO:0006418]
Kallikrein-5	KLK5_HUMAN	KLK5	−∞	Epidermis development [GO:0008544]
Keratin, type I cytoskeletal 19 (cytokeratin-19)	K1C19_HUMAN	KRT19	−∞	Cell differentiation involved in embryonic placenta development [GO:0060706]; Notch signaling pathway [GO:0007219];
Keratin, type I cytoskeletal 23 (cytokeratin-23)	K1C23_HUMAN	KRT23	−∞	*No biological process described (only GO terms related to molecular functions)*
Keratin, type II cytoskeletal 6B (cytokeratin-6B)	K2C6B_HUMAN	KRT6B	−∞	Ectoderm development [GO:0007398]
Microtubule-associated protein 4	MAP4_HUMAN	MAP4	−∞	Cell division [GO:0051301]; Mitotic spindle organization [GO:0007052]
Myeloid-derived growth factor (interleukin-25)	MYDGF_HUMAN	MYDGF	−∞	Cellular protein metabolic process [GO:0044267]; Endoplasmic reticulum unfolded protein response [GO:0030968]; Positive regulation of MAPK cascade [GO:0043410]
Non-specific lipid-transfer protein	NLTP_HUMAN	SCP2	−∞	Cholesterol transport [GO:0030301]; Fatty acid beta-oxidation using acyl-CoA oxidase [GO:0033540]; Phospholipid transport [GO:0015914]; Steroid biosynthetic process [GO:0006694]; Unsaturated fatty acid metabolic process [GO:0033559]
Phosphoglucomutase-like protein 5 (aciculin)	PGM5_HUMAN	PGM5	−∞	Cell adhesion [GO:0007155]
Premelanosome protein	PMEL_HUMAN	PMEL	−∞	Melanin biosynthetic process [GO:0042438]; Melanosome organization [GO:0032438]
Proteasome subunit beta type-2	PSB2_HUMAN	PSMB2	−∞	Protein polyubiquitination [GO:0000209]; Regulation of cellular amino acid metabolic process [GO:0006521]
Protein FAM180B	F180B_HUMAN	FAM180B	−∞	*No biological process described (only GO terms related to molecular functions)*
Protein kinase C delta-binding protein (Cavin-3)	PRDBP_HUMAN	PRKCDBP	−∞	Positive regulation of ERK1 and ERK2 cascade [GO:0070374]
Prothrombin	THRB_HUMAN	F2	−∞	Acute-phase response [GO:0006953]; Positive regulation of cell growth [GO:0030307]; Positive regulation of cell proliferation [GO:0008284]; Positive regulation of collagen biosynthetic process [GO:0032967]
Rab GDP dissociation inhibitor alpha	GDIA_HUMAN	GDI1	−∞	Rab protein signal transduction [GO:0032482]; Regulation of small GTPase mediated signal transduction [GO:0051056]
Ribonuclease T2	RNT2_HUMAN	RNASET2	−∞	RNA catabolic process [GO:0006401]
Succinyl-Coa:3-ketoacid coenzyme A transferase 1, mitochondrial	SCOT1_HUMAN	OXCT1	−∞	Cellular ketone body metabolic process [GO:0046950]

(Continues)

TABLE 4 (Continued)

Full name	UNIPROT name	Gene name	Fold change	Relevant gene ontology (biological process) terms
Sushi repeat-containing protein SRPX	SRPX_HUMAN	SRPX	−∞	Cell adhesion [GO:0007155]; Negative regulation of cell proliferation involved in contact inhibition [GO:0060244]; Phagolysosome assembly [GO:0001845]; Positive regulation of extrinsic apoptotic signaling pathway in the absence of ligand [GO:2001241]; Response to endoplasmic reticulum stress [GO:0034976]
Translin	TSN_HUMAN	TSN	−∞	DNA recombination [GO:0006310]; Gene expression [GO:0010467]; Gene silencing by RNA [GO:0031047]
UDP-glucose 6-dehydrogenase	UGDH_HUMAN	UGDH	−∞	cellular glucuronidation [GO:0052695]; UDP-glucose metabolic process [GO:0006011]; UDP-glucuronate biosynthetic process [GO:0006065]
Vacuolar protein sorting-associated protein 4B	VPS4B_HUMAN	VPS4B	−∞	Endosomal transport [GO:0016197]; Membrane organization [GO:0061024]; Protein transport [GO:0015031];
Vesicle-associated membrane protein-associated protein A	VAPA_HUMAN	VAPA	−∞	ER to Golgi vesicle-mediated transport [GO:0006888]; Membrane fusion [GO:0061025]; sphingolipid metabolic process [GO:0006665]
Zinc finger protein 185	ZN185_HUMAN	ZNF185	−∞	*No biological process described (only GO terms related to molecular functions)*

4.1 | Effects of the gel treatment on the gene expression and the DNA-linked functions

According to the CORAVALID analysis, the treatment of the skin explants with the active gel impacted the regulation of gene expression and some DNA-linked functions of skin cells. We observed enrichment for mRNA processing toward a promotion of RNA splicing, with the splicing factor proline/glutamine-rich (SFPQ) and the splicing factor 3b, subunit 3, 130 kDa SF3B3. Both are related to the Notch pathway and potentially mediate its effects on the NF-kB pathway. They could be both involved in DNA repair, as SF3B3 is part of the STAGA (SPT3-TAF(II)31-GCN5L acetylase)-HAT (histone acetyltransferase) complex, and SFPQ may act with NONO factor to repair double-strand DNA breaks. It is also worth noting that while not being related to any enriched term, the ribonuclease T2 (RNT2) was underexpressed and could thus also influence mRNA processing and phosphate scavenging.[32] In addition, the hepatoma-derived growth factor (HDGF)[33] and the Translin (TSN) were also downregulated. The former is an RNA-binding protein interacting with the polyadenylate mRNA motifs, while the second normally activates RNA-induced silencing complex, which is responsible for RNA interference. Then we observed that multiple microRNA-related virtual pathways (from WikiPathways and TarBase pathways), which group proteins regulated by microRNA in a tissue/cell type-specific manner, were enriched following the gel treatment. MicroRNAs (miRNAs) are a highly conserved class of noncoding RNAs that regulate gene expression via translational repression. They dampen or silence gene expression by pairing with the at least partially complementary targeted messenger RNAs. Some miRNAs were identified in human skin in recent years, and it has been shown they are involved in the regulation of skin homeostasis[34] and in some skin diseases.[35] These suggest that some changes potentially occurred regarding isoforms, as well as mRNA turnover and interferences, all of which could change cellular protein equipment and changing functions.

Upstream, many protein regulations could eventually regulate gene expression and transcription. The far upstream element-binding protein 1 (FUBP1) regulates the c-myc oncogene promoter in undifferentiated cells, c-myc interacting further with PTMA, CPNS1, SFPQ, and CKAP4. Both splicing factors SFPQ and SF3B3 could regulate the chromatin relaxation, respectively, by interacting with the topoisomerase I and the STAGA transcription coactivator complex. SFPQ could also act as a nuclear hormone receptor transcriptional corepressor. In addition, the HDGF, a DNA-binding mitogenic growth factor, may be involved as a transcriptional corepressor involved in fibroblast proliferation and differentiation by the ERK pathway stimulation. The elongation factor 1-delta second isoform (EF1D) could also be involved in the heat-shock proteins (HSPs) promoter sequences regulation by direct binding, HSPs regulating many functions involved here. Such regulation could result in a decrease in heat-shock protein stress-induced expression, marine, and maritime active ingredients dampening such stresses. The zinc finger protein 185 (LIM Domain)(ZN185) is also involved in proliferation, differentiation, and apoptosis regulation by interacting with DNA.

The purine nucleotide synthesis appeared regulated by the treatment (hypoxanthine phosphoribosyltransferase 1 HPRT) and could influence the primary components of the DNA/RNA synthesis. HPRT catalyzes conversion of hypoxanthine to inosine monophosphate and plays a pivotal role in purine nucleotide synthesis. Its' downregulation could be linked to lesser need of DNA synthesis or repairing. It is worth noting that HPRT is commonly used as a reference in transcriptomic studies, as its expression is known to be stable. Its regulation by the gel treatment was thus surprising.

4.2 | Effects of the gel treatment on the protein translation

Various enrichments occurred regarding protein translation, especially with regard to ribosomes (with modification of 60S subunits RL26, RL28, RL36), translation factors (EIF3F, EF1D, EIFCL), and translation elongation (SYIC, EIF3F, RL28, RL26, RL36, EF1D) as well as for transfer RNA (EF1D), especially those associated with isoleucine (SYIC, THIL, ECHM and SCOT1).

Protein levels could also be regulated after their synthesis, as the proteasome (PSB2) and the ubiquitination (EIF3F) are involved in regulating protein turnover, related regulations potentially balancing each other. Also, the Myeloid-derived growth factor, which is related to the unfolded protein response, was regulated. Such change suggests that the stress associated with unfolded/immature proteins was alleviated by treatment, with a decrease in potentially destabilizing conditions such as glycation and oxidation reactions, and a greater availability of chaperones compared to the protein synthesis levels.

4.3 | Effects of the gel treatment on the signaling pathways

Such changes could highlight the fact that new cellular programs were at work in response to the active ingredients used for treatment. According to the CORAVALID analysis, the treatment of the skin explants with the gel impacted the regulation of different and closely interconnected signaling pathways. Some signaling pathways would be inhibited (Notch 1, PI3-AKT, c-myc, NF-κB, and TGF-β) while others would be activated (Src, cGMP-protein kinase G). For certain pathways, it is difficult to conclude to an activation or an inhibition (Wnt pathway which would be activated and thus favoring cell proliferation, ERK pathway).

In our study, the transforming growth factor beta (TGF-β) signaling pathway, which induces fibrosis, would be countered by PTGIS, inhibiting ERK pathway. It could thus not induce UGDH and SYIC, in accordance with their downregulation. The observed upregulation of the dermal type I and type III collagens (CO1A1 and CO3A1) would thus not be under the control of TGF-β like in wound healing and could contribute to an improvement of an aged dermis as detailed below.

The Notch 1 signaling pathway, required for the growth and differentiation of epidermal keratinocytes, could be inhibited by the treatment as many regulations point in this direction. Indeed we observed the absence of deubiquitinylation by EIF3F that could lead to the proteasomal degradation of Notch1 and SF3B3_HUMAN could lead to an inhibition of signal transduction. This could act on the inositol(Myo)-1(or 4)-monophosphatase 2 (IMPA2), which was shown to be underexpressed after an anti-Notch treatment, and lead to an inhibition of the PI3-AKT signaling pathway. Some protein regulations (SFPQ, CKAP4) could seem contradictory to Notch1 and PI3-AKT signaling pathway inhibitions, but we hypothesized they are signs of feedback loops.

The C-myc signaling pathway, whose promoter is regulated in undifferentiated cells by the far upstream element-binding protein 1 FUBP1, would affect in turn PTMA, CPNS1, SFPQ, and CKAP4. The C-myc pathway would be inhibited by the treatment, favoring cell survival in the absence of CPNS1.

The cGMP/protein kinase G pathway could be activated as it mediates a part of the signal related to PTGIS, as cGMP is linked to tropomyosins TPM3 and TPM4 and as nitric oxide is linked to 4F2_HUMAN. Then, the ERK pathway could be also regulated as it is typically activated by S10A8, S10A9, TYB4, PRDBP, and HDGF and inhibited by PTGIS.

The Notch 1, Wnt, PI3-AKT, Src, c-myc, and ERK signaling pathways are involved in cell proliferation, differentiation, and apoptosis, while PI3-AKT, c-myc, and ERK pathways regulate cell migration and cytoskeleton. The regulation of these signaling pathways could explain the structural effects observed in the epidermal and the dermal compartments of the skin explants treated with the active gel compared to the untreated explants.

4.4 | Effects of the gel treatment on skin cell activities and skin structures

4.4.1 | Effects on the epidermal keratinocyte proliferation, migration, and differentiation

Histological and ultrastructural studies have revealed deep modifications in structures and functions in aged skin, including thinning of the epidermis. The well-studied age-related thinning of the epidermis is linked to an impairment of the skin barrier function, leading to more pronounced skin dehydration and greater sensibility to environmental stressors. Consequently, an aged epidermis is visibly thinner, drier, coarser, and wrinkled.[31]

Our observations suggest that the events induced by the treatment with the marine and maritime ingredient-containing gel, in terms of gene and protein expressions and regulation of signaling pathways (as a consequence of the regulation of protein levels or through direct actions impacting them), led to modifications of cellular activity. The gel treatment would result in the regulation of cell proliferation, differentiation, senescence, apoptosis, and autophagy. The treatment of skin explants seemed to favor the proliferation and the migration of epidermal keratinocytes, along with an inhibition of the epidermal differentiation process.

More precisely, the analysis of enriched biological processes revealed various events linked to epidermal renewal (KLK5, SPR2D, K2C6B, AHNK2). Various structural events linked to the regulations of cell activity were observed, with an enrichment of cytoskeleton-linked cellular components and the downregulation of some keratins. Among the regulated proteins related to cytoskeleton-linked cellular components, the small proline-rich protein 2D (SPR2D) was upregulated by the treatment. SPR2D is produced by epidermal keratinocytes, where its transglutaminase-mediated reticulation to structural proteins results in the formation of an insoluble envelope located under the cellular membrane called the cornified envelope,

contributing at cellular level to the skin barrier function. With regard to keratins, we observed a downregulation of the keratin 19 type I (K1C19); keratin 6B, type II (K2C6B); and keratin 23, type I (K1C23). keratin 19 type I (K1C19) is expressed in growing tissues. Keratin 6B, type II (K2C6B) is involved in the regulation of the keratinocyte migration through the inhibition of the activity of Src, which would be activated in the present study. It is interesting to point out that the activation of Src was observed in neoplastic skins, leading to an increase in the keratinocytes migration.[36] Keratin 23, type I (K1C23) is expressed by keratinocytes in the course of their differentiation and the formation of the barrier function.[37] Our analysis about the keratins is based on scientific literature, as they are not supported by enriched terms in the structural category of the CORAVALID analysis. Their downregulation pairs well with an inhibition of the epidermal differentiation. The decrease in keratins may have an impact on skin texture. We also observed a strong downregulation of some cell adhesion proteins (phosphoglucomutase-like protein 5 PGM5) and cell anchoring structures such as desmosomal plaques (AHNAK Nucleoprotein 2 AHNK2), tight junctions (Claudin-1 CLD1, vesicle-associated membrane protein-associated protein A VAPA), and focal adhesions. These regulations may be viewed as potentially affecting epidermal cohesion and thus skin barrier function[38]; however, as damaging conditions might be alleviated or prevented and keratinocyte differentiation might decrease, this could be viewed as a consequence of the decrease in such aggression-related differentiation. In addition, it should be noted that cornification process cannot be totally impaired, as we observe the upregulation of SPR2D (see above).

It is interesting to note that the observed regulations were close to those induced by retinoids. Retinoids are the group of vitamin A derivatives such retinoic acid, tretinoin, isotretinoin, or retinol, which are well known in dermatology to improve acne and the appearance of aged skin by decreasing fine lines, wrinkles, and hyperpigmentation. However, their use in cosmetics is restricted due to side effects (erythema, scaling, dryness, and pruritus).[39] Topical treatment of skin with retinoids is known to induce a high stimulation of keratinocyte proliferation and migration and a downregulation of the epidermal differentiation process. Bernard et al[40] studied the transcriptional effect of retinoid (all-trans retinoic acid, 9-cis-retinoic acid, and all-trans-retinol) treatments in reconstituted human epidermis. In our study, we observed similar regulations of keratin 6B (K2C6B), interleukin-1 receptor antagonist protein (IL1RA), and calgranulins A and B (S10A8, S10A9). Then, as previously explained, in this study, we selected for the CORAVALID analysis statistically significant proteins whose fold changes were equal or inferior to 0.5 or equal or superior to 2. Besides these thresholds, we observed after the gel treatment the regulation of some typical retinoid-regulated proteins, while remaining within relevant fold change ranges such as 1.5-2 and 0.5-0.67, such as cellular retinoic acid-binding protein II (CRABP2; fold change $\times$1.95) and psoriasin also called S100 calcium-binding protein A7 (S10A7; fold change $\times$1.85) which were shown to be quickly induced in skin after a topical treatment with all-trans retinoic acid,[41,42] filaggrin (FILA; fold change $\times$0.61), filaggrin-2 (FILA2; fold change $\times$0.67), and loricrin (LORI; fold change $\times$0.72), which are all involved in the epidermal differentiation process. According to the above-mentioned regulations, it could be hypothesized that the association of ingredients from marine and maritime origins mimics the activity of retinoids. More precisely, it could be linked to the dedifferentiated sea fennel cells as the supplier previously found such retinoid-like activity (M. N. Nicole Mekideche, unpublished data).

4F2 cell-surface antigen heavy chain (4F2), also known as CD98c, encoded by the gene SLC3A2 (Solute Carrier Family 3 Member 2), is highly upregulated by the gel treatment, supporting the hypothesis of a stimulation of epidermal regeneration. CD98hc is one of the two chains composing the amino acid heterodimeric transporter LAT1. This transmembrane transporter preferentially transports large neutral amino acids such as valine, leucine, isoleucine, tryptophan, tyrosine, phenylalanine, and arginine. As skin ages, its expression and activity levels decrease, as well as the downstream integrin signaling.[43] The SLC3A2 gene deletion in mice impacted skin keratinocyte proliferation and migration, resulting in disturbed cutaneous homeostasis and the wound-healing process.[43] Boulter et al concluded that CD98hc could be a marker of the capacity of skin to regenerate itself. The upregulation of 4F2 by the gel treatment is thus very interesting in view of reducing the signs of skin aging and restoring proper structure and function to the epidermis.

Based on all the observed effects on the epidermis, one could presume that the association of the trace element-enriched seawater, the giant kelp extract, and the dedifferentiated sea fennel cells opposes the age-related thinning of the epidermis and the impairment of the skin barrier function, thereby contributing to the improvement of the skin hydration and texture, making the latter less coarse. Given the observed retinoid-like activity, one could also presume that the active ingredients help to reduce skin imperfections, fine lines, and wrinkles.

4.4.2 | Effects on the skin cell metabolism

Although the observed phenomena are complex to analyze and require further studies to be fully understood, some cell metabolism regulations were observed. The citric acid cycle completion was supported by the upregulation of the oxoglutarate dehydrogenase (ODO1) involved in the conversion of 2-oxoglutarate into succinyl-coenzyme A (succinyl-CoA) and carbon dioxide. The pentose and glucuronate interconversion pathways (UDP-glucose 6-dehydrogenase UGDH) and the synthesis and the degradation of ketone body pathways (acetyl-CoA acetyltransferase THIL, succinyl-CoA:3-ketoacid coenzyme A transferase 1 SCOT1) were also regulated by the treatment, as well as the phosphoglucomutase-like protein 5 (PGM5) contributing to the interconversion of glucose-1-phosphate and glucose-6-phosphate. These regulations could impact glycolysis by modifying the availability of its intermediary metabolites in order to optimize the pyruvate production and to favor the citric acid cycle and the succinyl-CoA production at the expense of the ketogenesis

and the use of sugars in others metabolic pathways. The propanoate and the butanoate metabolisms also appeared to be regulated toward the acetyl-CoA supply of the citric acid cycle, as well as the beta-alanine, ascorbate, and glyoxylate metabolisms, although enrichment for these pathways is somewhat less robust. Indeed, the raw enrichment analysis was significant (P-value $< .05$) but insignificant after multiple testing corrections. In this study, the latter was very stringent, and we considered these regulations to be relevant as they were consistent with other observed phenomena and involved, at least partly, in common enzymatic reactions.

The lipid metabolism, and more precisely, the fatty acid biosynthesis, mitochondrial elongation, and beta-oxidation were impacted by the treatment (THIL, ECHM, NLTP), suggesting a regulation of the fatty acid degradation and their recruitment as acetyl-coenzyme A in the citric acid cycle. The lipid transport would also be regulated (VAPA, which is involved in the sphingolipid, ceramide, and oxysterol metabolisms; NLTP, which deals with the transfer of all the common phospholipids and could be involved in the steroidogenesis), although there was no related enriched process. However, this hypothesis pairs well with the observed regulations of vesicular trafficking.

To sum up, cell metabolism would be oriented by the gel treatment toward glycolysis and the citric acid cycle, instead of lipid metabolism and ketogenesis. Such a phenomenon could be related to a Warburg effect, which is a characteristic metabolism of highly proliferative cancer cells or embryonic cells,[44] which, in our case, could be related to the epidermal stem cells. Considering that the treatment of the skin explants with the active gel seemed to favor the proliferation and the migration of the epidermal keratinocytes, it could be hypothesized that the cell metabolism regulation contributed to sustain a high-energetic requirement.

4.4.3 | Effects on the dermis and the extracellular matrix remodeling

An age-related loss of collagens, major structural proteins in the skin, and a reduced number of the dermal fibroblasts mainly characterize the aged dermis, which appears thinner than in young skin.[31,45,46] A disorganization of the dermal elastic materials and the glycosaminoglycan-rich extracellular matrix has also been observed in the aged dermis. These changes have been related to an increased fragility, a loss of firmness, and elasticity of the skin, leading to wrinkles and global sagging of the body volumes.[31]

The analysis of the enriched biological processes induced by the gel treatment revealed different events contributing to the growth of the dermis, as evidenced by the upregulation of the collagen alpha-1 (III) chain (CO3A1; fold change $\times 2.38$) and the collagen alpha-1 (I) chain (CO1A1; fold change $\times 2.09$), belonging, respectively, to the fibrillar collagens I and III. These collagens are structural macromolecules composing the dermal extracellular matrix and are of major importance when it comes to an antiwrinkle strategy. It was interesting to note that we also observed an upregulation of the Collagen alpha-2 (I) chain close to the threshold fold change value (CO1A2; fold change $\times 1.97$), supporting at least the hypothesis of a stimulation of the synthesis of the dermal collagen I. As previously explained, the upregulation of the dermal type I and type III collagens would not be under the control of TGF-β like in wound healing and could contribute to an improvement of the aged dermis. Furthermore, among the enriched molecular functions revealed in the CORAVALID analysis, we noted the platelet-derived growth factor (PDGF) binding function. It suggests that the PDGF could be involved in the upregulation of the dermal collagen synthesis.

At a cellular level, we observed enrichment of terms related to functions, cell components, and pathways linked to actin, the cytoskeleton, and its regulation. Among the proteins involved were the brain abundant, membrane attached signal protein 1 (BASP1), and the thrombin (THRB), the latter being implicated in wound healing by inducing the formation of actin stress fibers in different tissues. We also noted a downregulation of the thymosin beta 4, X-linked (TYB4), which regulates the polymerization of actin and influences cell proliferation, migration, and differentiation. Then we observed a downregulation of different actin-binding tropomyosins (TPMs): tropomyosin 1 (alpha) (TPM1), tropomyosin 2 (beta) (TPM2), tropomyosin 3 (TPM3), and tropomyosin 4 (TPM4). The tropomyosin family-related proteins stabilize the actin filaments, and they are key regulators of their specialization by determining the binding of molecules that control the actin filament organization.[47] The tropomyosins are involved in the regulation of the cell migration during wound healing and in the contractile function of the striated muscles, the smooth muscles and also in the nonmuscle cells. In our study, the downregulation of the tropomyosins could impact the intracellular fibril structures, including the cytoskeleton's contractile fibrils as we observed enrichment of terms related to the myofibrils and the contractile fibrils. These regulations could mean less differentiation of the dermal fibroblasts into myofibroblasts, thereby contributing to reduce a potential profibrotic behavior of these cells (which would be consistent with the above-mentioned inhibition of the TGF-β signaling pathway, a major inducer of skin fibrosis).[48]

The stimulation of the collagen synthesis is one of the main skin anti-aging strategies to oppose the age-related loss of collagen and thinning of the dermis. By stimulating the synthesis of collagen I and collagen III, it could be expected that the association of the trace elements-enriched seawater, the giant kelp extract, and the dedifferentiated sea fennel cells opposes the age-related thinning of the dermis, the formation of wrinkles, and contributes to improve the biomechanical properties of aged skin in order to reduce skin sagging.

4.4.4 | Effects on the inflammatory pathways

The active-containing gel had an influence on the inflammatory processes within the topically treated skin explants compared with the untreated ones, with the regulation of proteins involved in both anti-inflammatory and pro-inflammatory responses, although the former would be the more dominant.

The nuclear factor-kappa B (NF-κB) is a key transcriptional factor in the regulation of the inflammatory processes, which can influence cell behavior by modulating gene expression in response to different stresses such as free radicals, ultraviolet exposure, chemicals, or pollutants.[49,50] It was recently hypothesized that a lifelong chronic low-intensity stress exposure could continuously activate the NF-κB signaling pathway, achieving a chronic low-grade inflammation in the skin and thus contributing to premature aging.[49] In our study, the NF-κB signaling pathway would be inhibited by the gel treatment, as it is induced by the Notch signaling pathway (which would be also inhibited as previously discussed) and by the interleukin-1. The latter would be inhibited as well thanks to the upregulation of the interleukin 1 receptor antagonist (IL1RA), which inhibits the interleukins 1 alpha (IL1A) and beta (IL1B) by binding the interleukin 1 receptor 1 (IL1R1), thus preventing its linking to the coreceptor interleukin 1 receptor accessory protein (IL1AP) and the final induction of NF-κB. Then, if activated, the NF-κB signaling pathway would stimulate the expression of the thymosin beta 4, X-linked (TYB4), which was downregulated in our study, supporting an inhibition of the NF-κB signaling pathway.

We also observed the upregulation of the prostacyclin synthase (PTGIS) which catalyzes the isomerization of the prostaglandin H2 into prostacyclin, also known as prostaglandin I2. Prostacyclin is a strong vasodilator, inhibits the platelet aggregation, and prevents the TGF-beta-induced fibrosis by inhibiting the RAS/MEK/ERK signaling pathway.[51-53] In skin, it was described as part of an anti-inflammatory response.[54]

The gel treatment also impacted the level of the protein kinase C-delta (PRDBP), which is activated by the diacylglycerol and potentiates the immune functions. It is interesting to note that the PKC-delta mediates the epidermal growth factor signaling pathway, and is activated during wound healing.[55,56] We also noted a downregulation of the pro-inflammatory thrombin (THRB)[57,58] and of the prothymosin alpha (PTMA), the latter stimulating an immune response of the type TH1.[59,60]

Furthermore, we observed some pro-inflammatory regulations. Calgranulins A and B, also known respectively as the S100 calcium-binding proteins A8 and A9 (S10A8, S10A9), were upregulated by the treatment. Calgranulin A contributes to the antimicrobial defense, acts as a metal chelator and as an antioxidant, and regulates cytoskeleton, chemotaxis, cellular adhesion, and differentiation and cell cycle. Calgranulin A acts in the leukocytes both intracellularly and extracellularly, through the type 4 Toll-like receptor and the receptors to the advanced glycation end products, thereby activating the MAP-kinases/MEK/ERK, ITGAM/ITGB and NF-κB signaling pathways. Calgranulin B shares some activities within the Calgranulin A but within the neutrophils, favoring the phagocytosis. Calgranulin B also facilitates the production of cytokines and chemokines in leukocytes, as well as the metabolism of the arachidonic acid. However, the activities of such proteins could be mitigated by the prostacyclin synthase (PTGIS), converting the prostaglandins derived from the arachidonic acid into prostacyclin. Then, other regulated proteins were related to a pro-inflammatory response: the downregulation of the sushi-repeat containing protein, X-linked (SRPX), the potential decrease in the synthesis of anti-inflammatory terpenes due to the downregulation of the mitochondrial acetyl-CoA acetyltransferase (THIL) and the upregulation of the lymphocyte antigen 6 complex, locus D (LY6D), which may act as a specification marker at the earliest stage of the specification of the lymphocytes between B- and T-cell development. In order to put things into perspective, it is worth noting that both calgranulins are able to stimulate keratinocytes growth, and that hyperproliferative skin diseases such as psoriasis are also involving inflammatory processes. Hence, while these regulations may be related to inflammatory processes, they might be here not a cause of proliferation, but a normal part of the proliferative cell communication (in paracrine and autocrine manners), with other trigger for the initiation or increase in proliferation. One element that supports this idea is the potential regulation of Src as shown before, which shows that while we observe some elements related to hyperproliferative inflammatory diseases, the similarities are far from being absolute.

4.4.5 | Effects on the melanogenesis

Skin aging is also characterized by an uneven pigmentation, mainly driven by extrinsic factors such as daily ultraviolet and pollution exposures.[31] Uneven skin pigmentation has been shown to be a leading factor of the overall aged skin appearance.[61] It has its origin in vascular alterations and in melanocyte dysfunctions leading to the formation of localized abnormal brown hyperpigmentation.[31,61] The active ingredients contained in the gel could influence skin pigmentation. A downregulation of the premelanosome protein (PMEL) was observed. PMEL is a melanocyte-specific glycoprotein found in melanosomes, which are the intracellular organelles sheltering the melanin synthesis.[62] PMEL is involved in the formation of fibrillar sheets within the melanosomes that serve as a template upon which melanins polymerize as they are synthesized. PMEL is required for the biogenesis and the maturation of the melanosomes and characterizes the transition of the melanosomes from type I to type II. Its' downregulation by the gel treatment could thus contribute to limit the melanogenesis. It is interesting to note that it was recently shown that the apolipoprotein E (ApoE) regulates the formation of PMEL amyloid fibrils in endosomes and consequently melanosome maturation, within melanocytes.[63] ApoE was downregulated by the gel treatment, with a fold change close to the threshold value required for CORAVALID analysis (APOE; $\times 0.64$), supporting the hypothesis of an inhibition of melanosome formation and thus a downregulation of melanogenesis.

The gel treatment-induced restriction of the inflammatory processes could contribute to reduce unpleasant redness and to decrease the collateral induction of oxidative stress, which constitutes a melanogenic signal. The melanin production would be better controlled in order to prevent abnormal pigmentation linked to inflammation such as the solar lentigines and the postinflammatory hyperpigmentation.[64]

Moreover, the activation of the citric acid cycle (ODO1) could contribute to increase the level of NADH/NADPH, which is required for the recycling of glutathione. Glutathione is an important tripeptide involved in the maintenance of the cell redox homeostasis and in the quenching of reactive oxygen species, thereby contributing to decrease the oxidative stress. In addition, a high level of glutathione was also showed to favor the production of pheomelanin in the melanocytes, a sulfur-containing reddish-yellow melanin lighter than the brownish black eumelanin.[65] Indeed, glutathione can be processed by a glutamyl-transpeptidase to produce cysteine, a sulfur-containing amino acid required for the spontaneous conversion of dopaquinone leading to pheomelanin synthesis. This process is thermodynamically more favorable than the production of the dark eumelanin, which occurs only when the intracellular pool of cysteine is depleted. By potentially increasing the level of glutathione, the gel treatment could contribute to modulate the melanogenesis, by favoring the synthesis of pheomelanin, thus lightening the skin pigmentation.

Due to the stimulation of keratinocyte proliferation and migration, the restriction of the inflammatory processes and the inhibition, or at least the modulation, of melanogenesis by the gel treatment, an improvement of skin tone in terms of luminosity and homogeneity, and a reduction in age spots could be expected in a clinical study for the evaluation of a cosmetic formula containing the association of the trace elements-enriched seawater, the giant kelp extract and the dedifferentiated sea fennel cells.

5 | CONCLUSIONS

The purpose of this study was to carry out a comprehensive evaluation of the activity on skin of an association of ingredients from marine and maritime origins, composed of a trace element-rich seawater, a yeast-derived amino acid-enriched giant kelp extract and dedifferentiated sea fennel cells, formulated in a gel, using a label-free quantitative proteomic and customized data processing approach, in order to predict the clinical benefits if used in a skin care product.

Compared to untreated skin explants, 64 proteins were significantly regulated by the gel treatment (q-value ≤ 0.05). Computer data processing revealed activity of the ingredients on the epidermis and dermis. The significantly regulated proteins are involved in gene expression, cell survival and metabolism, inflammatory processes, dermal extracellular matrix synthesis, and melanogenesis. Several proteins involved in keratinocyte proliferation, migration, and differentiation were also regulated in a retinoid-like way. These results are consistent with what has been previously observed in unpublished in vitro assays on each ingredient. The present in vitro data suggest that our association of active ingredients could help to preserve a healthy epidermis and dermis, and possibly improve them in aged skin in order to prevent and reduce the visible signs of skin aging (rough skin texture, wrinkles, sagging, redness, and hyperpigmentation).

To our knowledge, only isolated in vitro assays focusing on specific biological markers or genomic analysis have been used for such purposes, and we are the first to assess the activity on skin with cosmetic ingredients from marine or maritime origin using a proteomic approach. This strategy offered a more comprehensive understanding of the mechanisms of action of the tested marine and maritime ingredients and some specific insights such as the retinoid-like activity impacting the epidermis. Nevertheless, as a label-free approach, our observations should be confirmed by more specific in vitro assays or clinical studies. Since the present study, cosmetic products containing the association of the trace element-rich seawater, the yeast-derived amino acid-enriched giant kelp extract, and the dedifferentiated sea fennel cells, at relevant percentages of incorporation, were tested in single-blind clinical studies under dermatological control and were proved to be objectively and subjectively effective in the reduction in the signs of aging in facial skin (H. S. Sebastien Hameury & P. D. Dominique Pradines, recent unpublished data).

ACKNOWLEDGMENTS

This study was fully funded by Laboratoires B.L.C. Thalgo Cosmetic S.A., Roquebrune-sur-Argens, France. S. Hameury and D. Pradines are employees of Laboratoires B.L.C. Thalgo Cosmetic S.A. We appreciate the expertise of Ephyscience (Nantes, France), a contract research organization, which were responsible for the collection and the preparation of the human skin explants used in this study. Phylogene S.A. was mandated by Laboratoires B.L.C. Thalgo Cosmetic S.A., to conduct the label-free quantitative proteomic analysis and the CORAVALID analysis and reports no conflict of interests.

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How to cite this article: Hameury S, Borderie L, Monneuse J-M, Skorski G, Pradines D. Prediction of skin anti-aging clinical benefits of an association of ingredients from marine and maritime origins: Ex vivo evaluation using a label-free quantitative proteomic and customized data processing approach. *J Cosmet Dermatol*. 2018;00:1–16. https://doi.org/10.1111/jocd.12528