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Use of protection of alga to skin stressed by exposome

KEYWORDS: Exposome, microbiota, reactive skin, alga.

ABSTRACT

The treatment of sensitive skin represents excellent target for ingredients in cosmetics. The involvement of microbial communities in sensitive pathophysiology is unknown.
We performed a study to examine 1) the effects of Halymenia durvillei (Hd) active ingredient, a red alga, using an ex vivo and in vivo model on inflammation and neurosensory discomfort 2) the characterization of the skin microbiota before and after 15 days of Hd active ingredient application.
Hd active ingredient calms feelings of discomfort and redness, and controls the microvascularization. Our data demonstrate that specific bacterial genus and species are associated with sensitive and reactive skin. Hd active ingredient decreases the proportion of Corynebacterium and particularly Corynebacterium kroppenstedtii and increases the Chryseobacterium genus, showing that Hd active ingredient promoted the development of specific bacteria.

INTRODUCTION

The skin is constantly exposed to various endogenous, exogenous and life style factors that may affect the skin barrier function at the physical, mechanical and microbial levels. The resulting impact can potentially lead to inflammatory skin conditions involving sensitive or irritated skin as well as chronic inflammatory skin diseases, allergies or autoimmune diseases. These endogenous and exogenous factors are included in the exposome concept, which was proposed by Wild (1) to encompass "the totality of human environmental exposures from conception onwards, complementing the genome". More recently, Miller and Jones (2) proposed an alternative definition of the exposome, which explicitly incorporates behavioural risk factors, the body's response to environmental influences, and the endogenous metabolic processes that could alter or process the chemicals to which humans are exposed. Increasing attention is being paid to the exposome of human skin. Mirroring the cutaneous responses to environmental stress (3), the major environmental factors that contribute to skin alterations have been recently regrouped in the so- called "skin aging exposome" which includes (i) sun radiations, i.e. ultraviolet, visible light and infrared wavelengths, (ii) air pollution, (iii) tobacco smoke, (iv) nutrition, and (v) other factors such as temperature, stress and lack of sleep that can alter skin conditions.

It has become evident that the exposome needs to be considered in relation with sensitive skin. The etiology of sensitive skin is multifactorial, involving an underlying genetic susceptibility combined with exogenous and endogenous factors that can trigger or aggravate the clinical expression of the condition (4). Potential mechanisms of sensitive skin involved skin neurosensory dysfunction, neurogenic inflammation, epidermal barrier disruption, immune cells activity (transient receptor potential channels), and hyperreaction of the skin blood vessels (5). Sensitive skin is also defined by the self-reported presence of different sensory perceptions, including tightness, stinging, burning, tingling, pain and pruritus in response to stimuli that normally should not provoke such sensations (6).

Despite the physiological similarities between sensitive skin subjects and normal skin subjects, lower concentrations of pyrrolidone carboxylic acid (PCA) and transglutaminase (TG) activities together with a greater number of smaller and immature corneocytes indicated an altered stratum corneum (SC) maturation in the sensitive subjects were found (7). Recently, the role of cutaneous microbiota in skin sensitivity has been hypothesized (8). It appears in fact a direct effect of substance P and CGRP, which are neuropeptides released by nerve ending in the skin, on the skin microbiota in relation to the modulation of bacteria virulence (9).

Sensitive skin shows high incidence in France (10). In response to this prevalence, the effective treatment of sensitive skin represent excellent target for active ingredients in cosmetics. Various naturally derived complex mixtures such as botanical extracts have been used for long time. The application of algae in cosmetic products have recently received more attention in the treatment of skin problems. Algae are rich-sources of structurally novel and biologically active metabolites, with great industrial potential and accessibility. A wide range of metabolites such as alginates, polysaccharides, carotenoids with biological activities like antioxidants have been investigated for cosmeceutical preparations. Algae are therefore a source of raw materials for one of the most promising and profitable sectors of the biotechnology industry (11). Among them, *Halymenia durvillei (Hd)* is a red alga belonging to the Rhodophyceae family, abundant in a vast area of the Indian Ocean. Red algae are often small and can live in the deepest known as depths for organisms containing chlorophyll. *Halymenia durvillei* contains phycocolloids, which are the constituent polysaccharides of cell membranes. Today, the use of this alga for its phycocolloids is one of the most important industrial opportunities and a source of innovation and valorisation. The current interest in these

polysaccharides is due to their known bioactivities, conferred by their anti-allergic, neuroprotective, cytotoxic, anti-nociceptive, and immunomodulatory properties, making them promising bioactive products and biomaterials.

Based on these data, the first aim of this study was to evaluate the effect of *Hd* active ingredient on reactive skin, a skin which is aggressed by exposome. We therefore investigated the effects of *Hd* on inflammation (TNF-α) and neurosensory discomfort (TRPV-1, NKR-1) using an *ex vivo* model. An *in vivo* study was also performed in a panel of 25 volunteers using clinical and instrumental evaluations. The second objective of this study was to characterize the microbiome before and after 15 days of *Hd* application

EXPERIMENTAL SECTION

Ex vivo study

Twenty-one skin explants were prepared from an arm plasty of a woman of the Negroid type aged of 31 years (reference P1822-BN31). The explants were kept alive in BEM (BIO-EC's Explants Medium) at 37°C in a humid atmosphere, enriched with 5% CO2. The explants were divided into 4 batches as follows: Product, Placebo, Control plasty, and Control (T0). At Day 0, Day 2 and Day 5, Placebo and *Hd* active ingredient were applied to the surface of the explant at the rate of 2 µL.cm^{-2} and spread with a spatula. The control group received no treatment, except for the renewal of the medium (2 mL) on Day 0 and Day 2. At D0, the explants of batch T0 were removed and cut in half. One half was fixed in buffered formalin and one half was frozen at -80°C. On Day 2 and Day 6, 3 explants of each lot were taken and treated in the same way as on Day 0. Culture media from all lots on D2 (2mL) and D6 (2mL) were removed and frozen at -80°C for assays.

We first evaluated the effect of *Hd* active ingredient (5%) on TNF-α secretion. The assay was performed with the human Elisa TNF-α kit (ref 589201, Cayman). The culture medium and the TNF-α standard were incubated with acetylcholinesterase (AChE): TNF-α-binding Fab'conjugate in the wells containing the immobilized TNF-α anti-body, overnight at 4°C. Absorbance at 412 nm was measured with the Tecan M200Pro microplate reader and Magellan7 software.

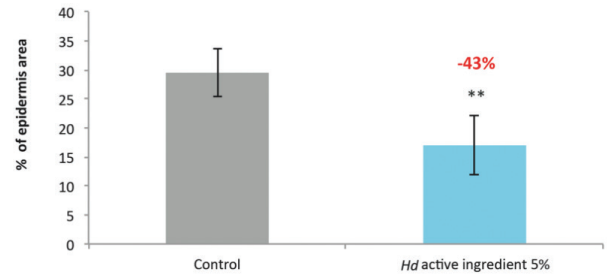

Figure 1. Percentage of area occupied by the substance P receptor in the epidermis at Day 6. ** p< 0.01 vs control.

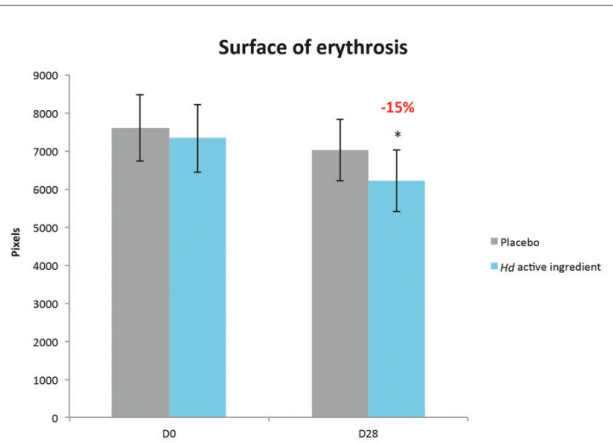

Figure 2. Effect of Hd active ingredient on erythrosis. * p<0.05 D0 versus D28.

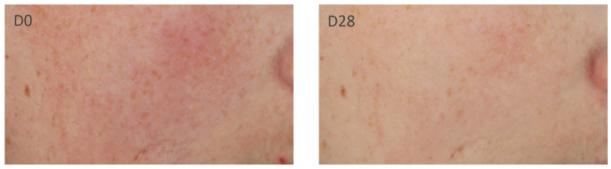

Figure 3. Reduction of redness induced by 28 days of *Hd* active ingredient application.

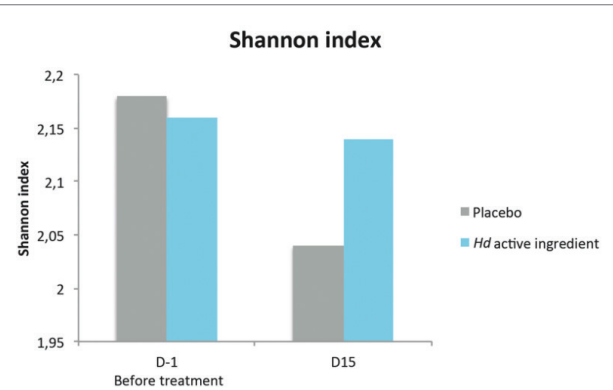

Figure 4. Average of Shannon index in the samples.

Then, we evaluated the effect of *Hd* active ingredient on TRPV-1. During the protocol explained above, histological treatments were realized. After 24h in the buffered formalin, the samples were dehydrated and impregnated in paraffin using a Leica PEARL dehydration automaton. They were packaged using a Leica EG 1160 coating station. Sections of 5 µm were made using a Minot microtome, Leica RM2125 and mounted on Superfrost® histological glass slides. TRPV-1 is highlighted thanks to a specific anti-body (Abcam, ref. ab3487).

Thirdly, NK1-R was scored on paraffin sections with anti-NK1-R polyclonal anti-body (Santa-Cruz, ref sc-365091), diluted 1/50 in PBS-BSA 0.3% - Tween20 0.05% for 1h at room temperature with a Universal VECTASTain RTU VECTOR streptavidin/peroxidase system and revealed in VIP, a purple peroxidase substrate (Vector, SK-4600). Immunostaining was performed using an immunostaining automaton (Dako, AutostainerPlus) and evaluated by microscopic observation and image analysis. Finally, VEGF was scored on paraffin sections with anti-VEGF polyclonal anti-body (Santa-Cruz, ref sc-7269).

In vivo study

Twenty-five female volunteers aged 41 ± 10 years old with skin phototypes I-IV and dry and sensitive skin participated in this study after having given their written informed consent. The study was performed by comparison before and after hemi-face application of *Hd* active ingredient 3% vs placebo. All of them presented reactive skin as defined from the stinging test (total score ≥ 3) and also presented erythrosis as defined from clinical score.

Exclusion criteria were the presence of any skin-related pathologies and abnormalities (eczema, psoriasis, etc.), allergies, hypersensitivity to the tested product, acute and/or chronic inflammation or infection of facial skin, an exposure of sunlight or artificial UV rays within 15 days, pregnancy, and nursing. Subjects were advised to avoid the application of other similar product during the whole study.

Evaluations were performed at baseline (D0) and 28 days (D28) after twice-daily application. Subjects were evaluated in the standard skin situation (last face washing the night before visit, without any cosmetic, water, and makeup application until the measures). Clinical scoring of erythrosis (Measurement and analysis of blood micro-circulation by videocapillaroscopy : Moritex MS500 with a x50 magnification: exploration area = 13.6 mm^2), assessment of functional signs using the Sensitive Scale (12) and soothing effect were evaluated. We also took pictures, using a VISIA from CANFIELD® imaging system. Women were also asked to report their overall opinion about the product, with 3 sentences maximum for remarks after the 28 days of application.

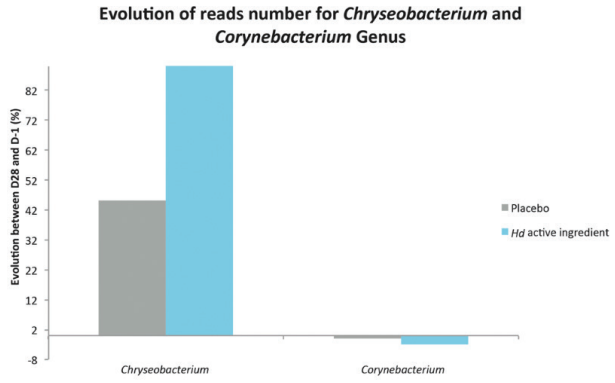

Figure 5. Evolution of Corynebacterium and Chryseobacterium genus after 15 days of Hd active ingredient use.

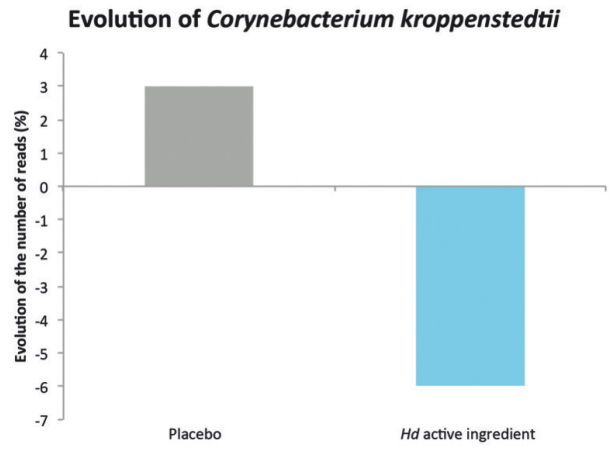

Figure 6. Evolution of Corynebacterium kroppenstedtii after the use of Hd active ingredient.

Microbiota

At D0 2 samples were collected from each volunteer (one on each cheek: D0-No Treatment and D0-Treatment). Similarly, 2 samplings were performed after 15 (D15-NT and D15-T) days of treatment. Microbiota composition analysis of samples was performed by amplifying the hypervariable regions V1-V3 of the 16S RNA gene (primers 24F-533R which target the conserved regions of this gene common to all bacteria). The products of this amplification were sequenced by MiSeq Illumina technology. Pretreatment of sequences was performed by an informatic pipeline developed by our provider operated under "Mothur program". High quality sequences were grouped into Operational Taxonomic Units (OTUs) with a sequence identity threshold of 100%, and taxonomy was assigned by interrogating the Greengenes database with the high- quality sequences. The Shannon diversity index was calculated and used as a measure of diversity.

Statistical Analyses

The analyses were carried out with SPSS 21. Descriptive statistics are provided for each parameter. Statistical comparison was carried out with the Student-t Test or Wilcoxon test (depending on the normality of the distribution). *P* value < 0.05 was considered statistically significant.

RESULTS

Anti-inflammatory properties of *Hd* active ingredient

Hd active ingredient induces a non significant decrease of TNF-α (-21%) compared to control after 6 days of treatment. At the same time, the expression of TRPV-1 in the epidermis significantly decreases. A significant decrease of NK1-R (-43%, p< 0.01: Figure 1) and a decrease of VEGF (-16%) were also reported when compared to control.

***Hd* active ingredient improves sensitive skin symptoms**

Significant decreases of skin sensitivity, irritability, heat sensations, pain, flushes, and redness were reported using the SS-10 scale (12) after 28 days of repeated applications of the *Hd active ingredient* (Table 1). A significant decrease (-15%: p< 0.05) of the surface of erythrosis was also observed with our extract (Figure 2 and 3).

Finally, 90% of the women declared that the product had a soothing effect when applied after the stinging test.

Hd* active ingredient reduces *Corynebacterium kroppenstedtii

We observed that three phyla: *Proteobacteria*, *Firmicutes*, and *Actinobacteria* dominate the skin microbial communities with no differences between before and after *Hd active ingredient* treatment.

Regarding Shannon index, we observed stable values at D-1. At D15, this index decreased only for areas treated by the placebo and remained stable for cheeks treated with active product, suggesting a protecting effect of the active molecule (Figure 4).

The average taxonomic composition of skin microbial communities with *Hd* active ingredient treatment during 15 days significantly decreased the level of *Corynebacterium* and increased the *Chryseobacterium* genus (Figure 5). Finally, we observed a significant proportion of *Corynebacterium kroppenstedtii*, a specie particularly observed in the case of redness, the main criteria for volunteer's inclusion. *Hd* active ingredient decreased the proportion of this specie (Figure 6).

DISCUSSION

The concept of the human skin exposome was introduced more than a decade ago and refers to the constellation of external exposures, inducing cutaneous aging and sensitive and reactive skin (13). The treatment of reactive skin is challenging and generally based on continuous and

topical application of antisensitive moisturizing tolerance extreme product that improves skin features associated with itching, stinging, dryness, tightness, burning, or pain. To maintain a healthy skin, it is recommended to hydrate and protect it. In this study, we investigated whether *Halymenia durvillei (Hd)* active ingredient, riched in polysaccharides and particularly in Galactose, could calm feelings of discomfort, redness.

	D28 vs D0
Skin sensitivity	-19% *
Skin irritability	-34% *
Heating sensations	-40%*
Pain	-33%*
Flushes	-37%*
Redness	-24%***

Table 1. Evolution of the skin irritability, sensitivity, heating, pain, flushes and redness state from D0 to D28 using the SS-10 scale (12). * $p<0.05$, *** $p<0.001$ vs D0.

Neurosensory dysfunction in the skin might represent one of the pathomechanisms of sensitive skin. Another mechanism through which the cutaneous nervous system could contribute to sensitive skin might be by functional hyperreactivity of cutaneous nerves. Our results are in line with the pathophysiology of sensitive skin, *Hd* active ingredient inducing decrease of TRPV-1 and NK1-R expression, as well as reducing VEGF and TNF-α.

The Sensitive Scale (12) enables to measure the severity of skin sensitivity was also used in a clinical study including twenty-five females. Significant decreases of skin sensitivity, irritability, heat sensations, pain, flushes, and redness were reported after 28 days of repeated applications of *Hd* active ingredient (Table 1). Erythrosis takes the form of diffuse, persistent red patches on the face. It appears as the result of exposome, such as shaving or temperature fluctuations. It occurs mainly around the nose, chin, forehead and cheeks, and is associated with a sensitive skin. We also note that *Hd* active ingredient decreases erythrosis after 28 days of application (Figure 3).

Four main phyla have been characterized in healthy skin: *Actinobacteria, Firmicutes, Proteobacteria, Bacteroides* with three predominant genus: *Corynebacterium, Propionibacterium* and *Staphylococcus* (14). *Hd* active ingredient didn't modify the proportion of each phylum. It has been shown that several skin disorders are characterized by shifts in the skin microbiome, most notably, loss of protective bacteria and outgrowth of pathogenic bacteria (15), increased severity correlating with decreased microbial diversity. Figure 5 shows that *Hd* active ingredient application during 15 days maintained a high microbial diversity, suggesting a protecting effect of the active molecule.

A decrease of *Corynebacterium* genus was reported, *Corynebacteria* being identified as a dominant mediator of skin immunity and inflammation, and recognized as pathogens, particularly among immunocompromised hosts (16). *Hd* active ingredient also fights against red skin conditions by reducing *Corynebacterium kroppendenstedtii levels*, a liphophilic bacteria, which increased levels are associated with age and skin redness (17) (Figure 6).

A dramatic increase of the *Chryseobacterim* genus was evidenced after *Hd* active ingredient application during 15 days. *Chryseobacteruim* genus belongs to *Bacteriodetes* phylum. *Chryseobacterium* have documented the significance of flexirubin as a biocontrol agent, antioxidant, sulfobacin A, protease producer (18). This pigment is used in the treatment of chronic skin disease, eczema etc. and may serve as a chemotaxonomic marker (19).

CONCLUSION

This study demonstrates that our alga extract may provide protection for sensitive and reactive skin by daily environmental insults, named exposome. Our data demonstrated that specific bacterial genus and species are associated with sensitive and reactive skin. This active ingredient is able to modulate skin microbiota and promote specific bacteria metabolism and development.

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