Skin barrier defects and inflammation related to leaky skin can be prevented by potent natural active compounds

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Abstract (Maximum of 200 words)
As microbiota participates to barrier function, dysbiosis may also contribute to leaky skin. Two natural actives, from bacterial (B) and marine (M) origins respectively, were applied on a leaky skin *ex-vivo* model in order to assess their ability to prevent skin barrier defects and induced inflammation. Strong *in situ* penetration of the fluorescent dye showed that skin explants became permeable after application of *S. aureus* proteases, inducer of leaky skin damages. Claudin-1 and Desmoglein-1 protein expressions were significantly down-regulated in stressed conditions confirming that skin barrier integrity was compromised. Expression of TSLP, TNFα and Interleukin-31, specific markers of atopic and/or itchy skin, were up-regulated in same conditions. Results obtained with selected natural ingredients showed that both B and M significantly counteracted the penetration of the dye. Active B was also able to prevent the down-regulation of Claudin-1 and Desmoglein-1 expression induced by *S. aureus* proteases. Moreover, active M inhibits protein expression of inflammation markers TSLP, TNFα and IL-31. Reinforcing skin physical barrier might be the key to prevent inflammatory diseases, treating not only the symptoms but the cause: a leaky epithelium. Here we developed two natural actives that may be applied *in vivo* to prevent leaky skin manifestations.

Keywords: leaky skin; barrier; inflammation; atopy; natural solutions

Introduction.

Under healthy conditions, the intestinal barrier prevents ingested toxins, allergens and harmful elements from entering the blood circulation. However, different factors including stress, unhealthy diet, drugs or excessive alcohol consumption can compromise the composition of the gut microbiota and its balance, leading to intestinal barrier dysfunction and thus increased epithelial permeability. This phenomenon, named leaky gut syndrome, can allow the entry of harmful agents through the junctions of the intestinal epithelium, which pass into the bloodstream and can affect various organs [1].

Both colonized epitheliums acting as barriers to protect the body from external stressors, gut and skin are closely associated [2]. For instance, it has been shown that atopic dermatitis (AD) and rosacea are both linked to changes in the gut barrier and intestinal microbiota [3]. A link between imbalances of the intestinal microbial communities and development of skin psoriasis

has also been documented in mice and patients [4]. Given this close relationship between skin and gut, it seems logical that leaky gut and skin, two essential barriers that became porous, may be also connected and/or rely on similar biological mechanisms.

Skin is the first barrier of our organism to external toxins, allergens and bacteria. As microbiota participates to barrier function homeostasis, dysbiosis may also contribute to leaky skin [2]. When barrier function is less efficient, external harmful compounds may cross over the epithelial barrier triggering systemic immune response, leading to various skin diseases as ichthyosis, acne, psoriasis or atopic dermatitis [5].

Natural solutions able to prevent leaky skin harmful impacts, especially skin inflammation and barrier defects, are necessary. That is why this study has been conducted on an ex vivo model mimicking leaky skin triggered by application of proteases from *Staphyloccocus aureus*. Indeed, *S. aureus* secretes proteases that lead to endogenous epidermal proteolysis promoting *in fine* inflammation [6]. Induction of leaky skin state has been assessed thanks to the study of a fluorescent dye penetration as well as immunostainings of barrier function proteins such as Claudin-1 (CLDN1) and Desmoglein-1 (DSG1) and global inflammation marker Tumor Necrosis Factor alpha (TNFα), a potent modulator of cutaneous immune function [7], on skin explants stressed with *S. aureus* proteases vs unstressed control.
CLDN1 is a protein marker of tight junctions, adhesion molecules crucial for skin epidermal barrier [8]. DSG1 is a cadherin specifically expressed in desmosomes, intercellular junctions that confer mechanical strength to the skin [9].
As colonization of the skin by *S. aureus* is also associated with exacerbation of atopic dermatitis [10], inflammation markers more specific to that skin disease have been explored to extend leaky skin assessment. Expression of Thymic Stromal Lymphopoietin (TSLP) and Interleukin-31 (IL31) have thus been measured thanks to immunostainings. TSLP acts as a master switch that triggers both the initiation and maintenance of atopic dermatitis and, broadly speaking, the atopic march as well as itch [11]. Similarly, IL31 has been recognized as the “itchy” cytokine, main driver of pruritus in various inflammatory and allergic skin diseases [12].
After checking the stress induced-modulation on that different parameters to validate the leaky skin *ex vivo* model, two actives, respectively from marine (M) or bacterial (B) origins, have been assessed on explants stressed with *S. aureus* proteases. Active M, previously identified for its soothing action, has been tested on inflammation markers whereas active B has been challenged on barrier function proteins CLDN1 and DSG1 modulation. Both were evaluated on fluorescent dye (Lucifer Yellow) penetration to assess their ability to prevent skin barrier defects induced by proteases.

Materials and Methods.

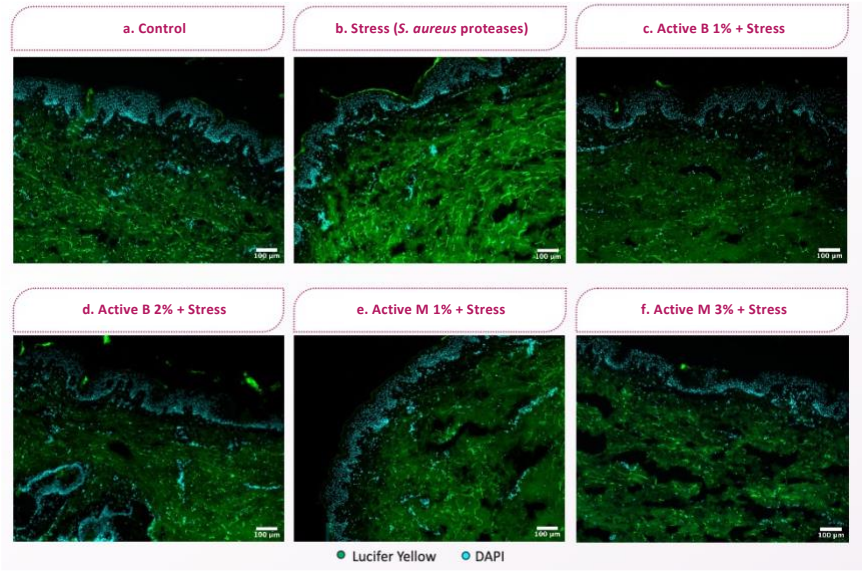
To establish a model of leaky skin, skin explants were stressed with application of proteases secreted by *Staphylococcus aureus* to mimic permeable damaged skin.
Twenty-four explants were obtained with the informed consent of a 41-year-old female Caucasian donor. They were kept alive by culturing on metal grids into standard 12-well plates at 37°C in a humid atmosphere, enriched with 5% CO_2. They were topically stressed (or not, control condition) for 48h with *S. aureus* proteases only, or in solution with active B at 1% or 2% or active M at 1% or 3%. The culturing medium was renewed every 24 hours. Twenty-four hours after the last application, the skin explants were sampled, transferred in OCT for cryopreservation, snap-frozen in liquid nitrogen and conserved at -80°C until analyses. Skin cryosection of 5 µm thickness were fixed with a solution containing 95% ethanol, and 5% acetic acid for 10 min.

Previously Lucifer Yellow (LY) treated and cryofixed sections were stained with DAPI (4',6-diamidino-2-phenylindole) for nuclear detection. The analysis of the skin barrier integrity was achieved evaluating the specific fluorescent signal intensity (Ex. 428nm / Em. 536 nm) at 250 µm from the explant surface.
For immunodetection, a saturating step of the non-specific sites was carried out with a solution of PBS (Phosphate Buffer Saline) containing 3% BSA (Bovine serum albumin). Skin sections were incubated with diluted specific primary antibodies targeting CLDN1, DSG1, TNFα, TSLP or IL31 in a PBS-BSA solution. The excess of primary antibodies was eliminated with washing steps using a solution of PBS 0.1% Tween (PBS-T), then skin sections were incubated for 1 hour with the secondary antibody coupled to a fluorophore in PBS-BSA. The nuclei were labelled with diluted DAPI in PBS. Finally, the antibody and DAPI excess were removed with a sequence of washing steps with PBS-T. Fluorescent images were collected with an epi-fluorescent microscope (ThermoFisher, EVOS M5000 Imaging System) and analyzed with ImageJ software. The intensity of targeted biomarker levels was obtained by the integration of the specific fluorescence signal over threshold normalized by the evaluated area. The quantification of biomarkers was normalized in relation to the control (considered at 100%), to finally obtaining a mean value and a standard deviation. Statistical analyses were carried out using the "GraphPad" software (La Jolla, California, USA) by using one-way ANOVA and Dunnett's post-hoc test for multi-comparisons analyses vs Stress group (confidence interval of 95%). An efficacy value (%) was obtained for the experimental groups using, as references, the control group, considered at maximum efficiency (100%), and the stress group, at minimum efficiency (0%).

Results.

Skin explants stressed with *S. aureus* proteases were first evaluated vs unstressed explants to validate leaky skin induction. Strong increase of LY fluorescent signal (+44%) has been observed in stressed explants vs unstressed control (Figure 1A a vs b). This *in situ* penetration of the fluorescent dye showed that skin explants became permeable after application of appropriate dose of *S. aureus* proteases.

1A



1B

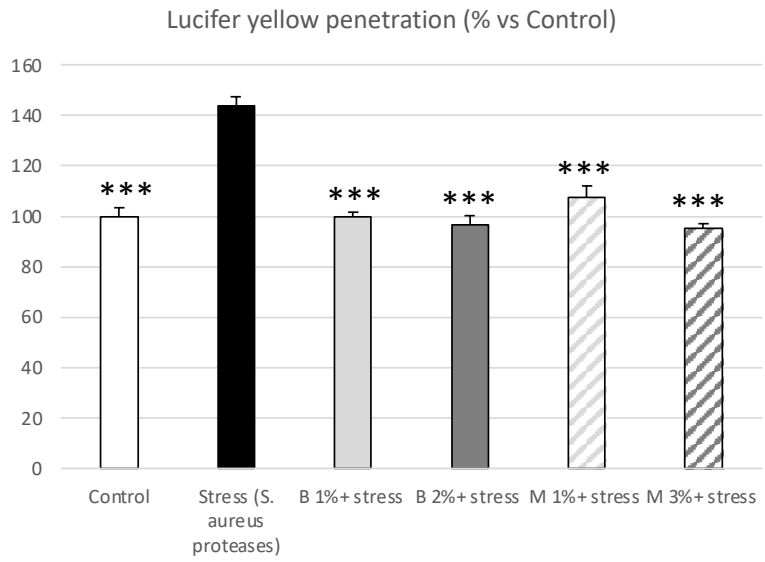
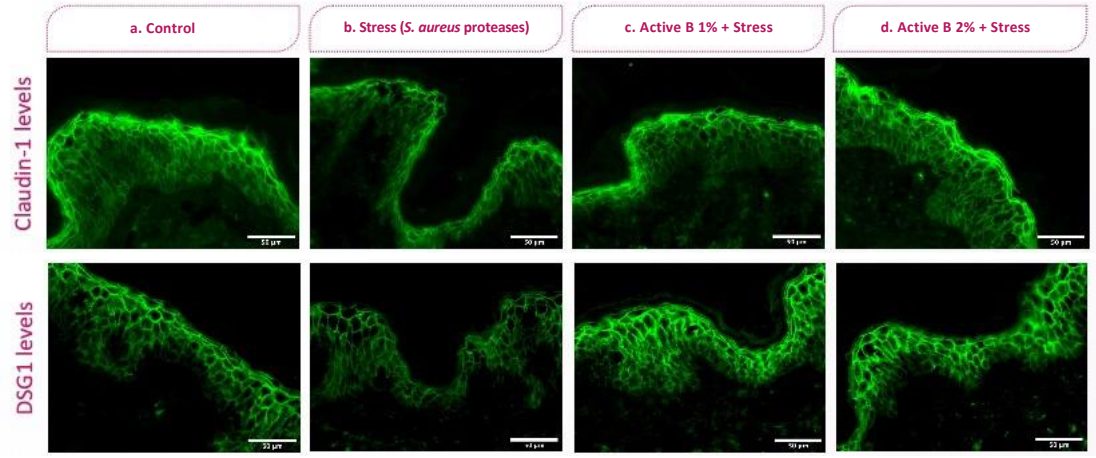


Figure 1A - In situ visualization of fluorescent dye permeability for skin barrier integrity evaluation. Representative images were obtained for Lucifer Yellow penetration on skin explant surface (10x objective). The specific signal of Lucifer Yellow (green) is shown superposed to the nuclei staining (DAPI, in cyan). Scale bar, 100 µm. a-f: Unstressed control (a), untreated stressed (b) and Active B (c-d) or Active M (e-f)-treated stressed explants.

Figure 1B – Quantification of fluorescent dye permeability. The intensity of the specific fluorescent signal of Lucifer Yellow at 250 µm from the explant surface is reported as mean ± SD per experimental group. – one-way ANOVA and Dunnett's post-hoc test for multi-comparisons vs Stress group. ***p<0.001

CLDN1 and DSG1 protein expression were significantly down-regulated in untreated stressed conditions (Figure 2A a vs b) confirming that skin barrier integrity was compromised. Inflammation was then assessed to check if its induction by skin dysbiosis and/or barrier function alteration observed in atopic dermatitis, has been mimicked successfully ex vivo. Expression of Thymic Stromal Lymphopoietin (TSLP, +17%), Tumor Necrosis Factor alpha (TNFα, +40%) and Interleukin-31 (IL-31, +47%), specific markers of atopic and/or itchy skin, were significantly up-regulated in stressed conditions (Figure 3A a vs b) demonstrating that skin permeability defects induced inflammation. *S. aureus* proteases induce thus both skin barrier defects and inflammation.

2A



2B

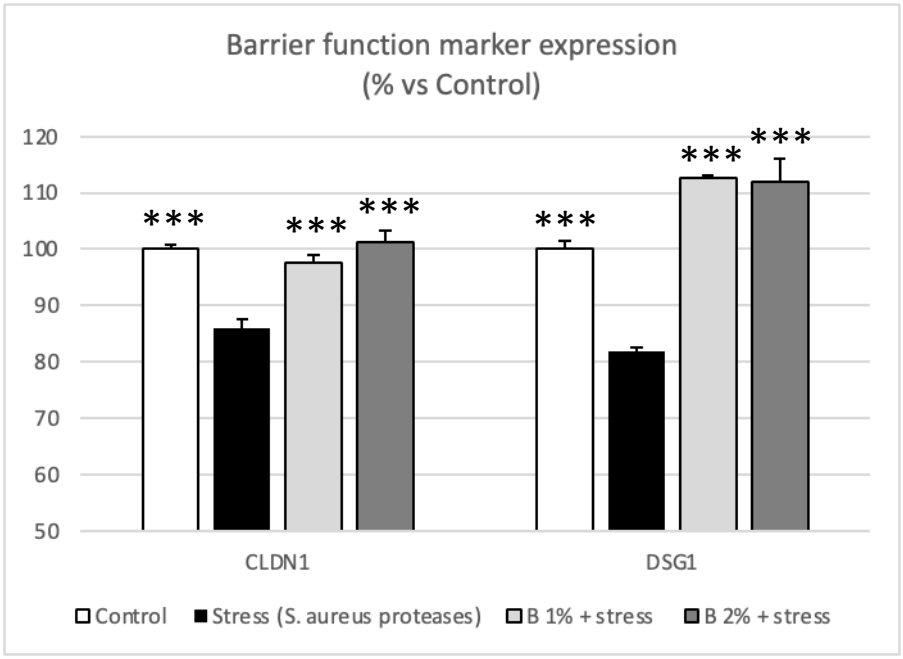
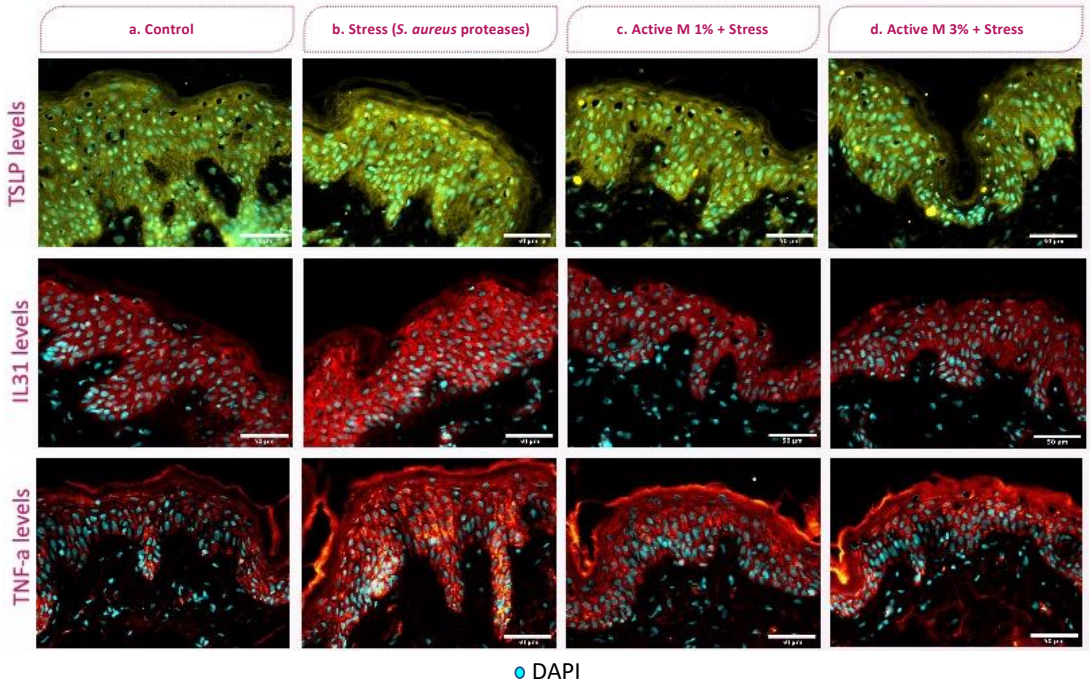


Figure 2A - *In situ* visualization of specific signal of Claudin-1 (green, upper part) and DSG1 (green, lower part) levels by epifluorescence microscopy. Scale bar, 50 µm. a-f: Unstressed control (a), untreated stressed (b) and Active B (c-d)-treated stressed explants.

Figure 2B - Quantification of CLDN1 and DSG1 levels. The levels of each barrier function marker of each experimental group are expressed as relative values (% vs Control) and shown as mean +/- S.D. one-way ANOVA and Dunnett's post-hoc test for multi-comparisons vs Stress group. ***p<0.001.

3A



3B

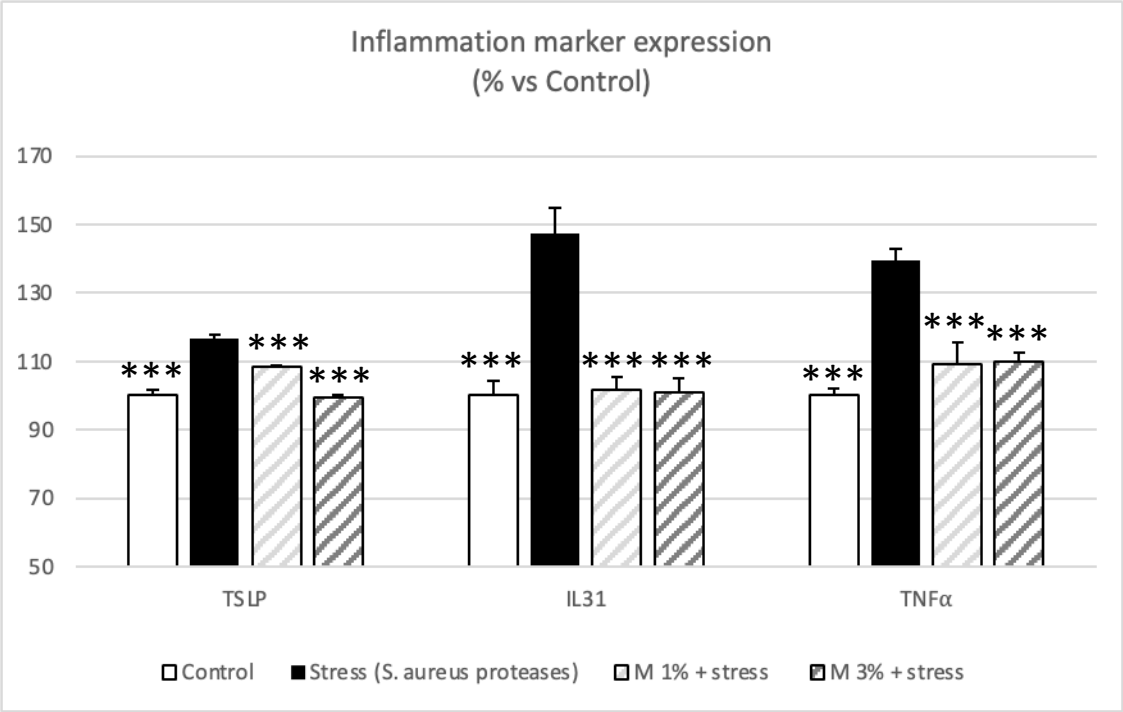


Figure 3A - *In situ* visualization of specific signal of TSLP (yellow, upper part), IL31 (red, middle part) and TNFα (red range, upper part) levels by epifluorescence microscopy. Scale bar, 50 µm. a-f: Unstressed control (a), untreated stressed (b) and Active M (c-d)-treated stressed explants.

Figure 3B - Quantification of TSLP, IL31 and TNF-a levels. The levels of each inflammation marker of each experimental group are expressed as relative values (% vs Control) and shown as mean +/- S.D. one-way ANOVA and Dunnett's post-hoc test for multi-comparisons vs Stress group. ***p<0.001.

Results obtained with selected natural ingredients showed that both B and M significantly counteracted the penetration of the LY dye (Figure 1). Active B allows complete protection at both concentrations whereas as well as Active M at 3%. Active B was also able to prevent the down-regulation of CLDN1 (98% for treated condition vs 86% for untreated stressed, vs control normalized at 100%) and DSG1 expression induced by *S. aureus* proteases treatment (Figure 2), reinforcing skin barrier. Moreover, results showed that active M inhibits protein expression of inflammation markers TSLP, TNFα and IL-31 (Figure 3).
Actives B and M allow thus preservation of skin integrity as well as, respectively, protection of skin barrier function proteins (specific to desmosomes and tight junctions) and prevention of associated inflammatory reaction.

Discussion.

Ex vivo model using application of *S. aureus* proteases mimics faithfully leaky skin syndrome as induced dysbiosis leads to porous skin showing barrier defects and inflammation. Thus, *S. aureus* proteases lead to skin leakage, confirmed by LY penetration, via its deleterious action on tight junction and desmosome markers. This alteration of skin barrier function induces an inflammatory reaction, confirmed with increase of TNFα levels. Atopic-prone or atopic skin inflammation is also reproduced here as protein levels of TSLP and IL31, specific to atopic dermatitis and pruritus, increase significantly after application of S*. aureus* proteases.

Natural cosmetic solutions able to bring relief to atopic skins have been identified here as Active B is able to reinforce skin barrier proteins whereas Active M has a strong soothing activity in case of dysbiosis and/or leaky skin defects. Both are able to preserve skin integrity by preventing penetration of LY.
As claudin-1 ([13] Xia et al., 2022) and desmoglein-1 ([14] Sherrill et al., 2013) are also expressed in gastro-intestinal tract epithelial barrier, Active B may also be relevant to prevent leaky gut syndrome. Similarly, TNFα ([15] Ruder et al., 2019), TSLP ([16] Blázquez et al., 2010) and IL31 ([17] Dambacher et al., 2007) are also linked to intestinal, allergic or not, inflammation. Active M could also act to prevent gut induced-inflammatory reaction.

Conclusion.
In conclusion, this *ex vivo* model can mimic harmful impacts of *S. aureus* proteases and thus be used to screen compounds dedicated to leaky skin prevention. More specifically, natural ingredient B, previously demonstrated as skin barrier and immunity booster in healthy skin, reinforces protein junctions responsible of epidermis cohesiveness. On the other hand, active M, revealed *in vivo* as sensitive skin reliever, decreases also inflammation triggered by leaky skin.
Using cosmetics as emollients can delay or even prevent eczema and food allergies. To reinforce skin (and gut!) physical barrier might thus be the key to prevent inflammatory diseases, treating not only the symptoms but, more importantly, the root of the problem: a leaky epithelium. Here we bring a deeper understanding of the underlying biological mechanism of leaky skin as well as natural cosmetic solutions that may be applied *in vivo* to prevent it. These actives allow the balance of skin microbiota necessary to maintain optimum interactions with the cutaneous structures.

Acknowledgments.
We thank OxiProteomics for conducting this *ex vivo* study and Dr Lionel Breton for his assistance to design it on leaky skin model.

Conflict of Interest Statement.
Lauriane IMBERT-ROUX, Carine BOUTOT, Assia DREUX-ZIGHA and Jean-Yves BERTHON are employees of GREENTECH SA.

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