Postbiotics for hair and scalp microbiome balance

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Recent research has discovered the deep connection between microbiota and human skin. The skin is an ecosystem where about 1,000 microbe species live in symbiosis with each other and with the host. Symbiotic microbes support skin barrier function, modulate immune response protecting the skin against pathogens, allergens and so on.

Since the skin is a finely organized ecosystem, a perfect balance between host and microbe communities exists to preserve homeostasis and wellness. Disruption of this delicate balance can induce perturbations in skin barrier function, onset of dermatological and scalp disorders. Skin microbiota interacts with keratinocytes and the immune system, thus inducing beneficial responses in the host.

The scalp microbiota shows some similarities with the skin microbiota, but possesses unique characteristics. Indeed, the scalp surface provides specific environmental conditions to the microorganisms, depending by moisture, pH, sebum content and other typical physiological conditions of the host.[1,2]

The hair follicles (HFs), directly connected to the sebaceous glands through the dermis, represent unique hydrophobic niches which selectively allow the growth of specific microorganisms.[3]

The exchanges between the scalp surface and the microbiota allow biofilm formation in commensal or pathogenic form,[4] i.e. a dysbiosis in the scalp microbiota was observed in the case of dandruff, similarly as reported for skin microbiota in the case of seborrheic and atopic dermatitis.[5-8]

A microbial dysbiosis has been also identified on the scalp of subject affected by hair growth disorders.[9] Dandruff is generally recognized as a mild type of seborrheic dermatitis affecting the scalp. Unbalance of the scalp microbiota, with an increase in *Staphylococcus spp.* and a reduction in *Cutibacterium spp.*, has been identified as a potential trigger in dandruff formation.

Inflammation and microbiome role in hair disorders

Androgenetic alopecia (AGA) is a hair growth disorder featuring the miniaturisation of hair and shortening of the anagen phase, caused by an increased androgen activity. The miniaturisation process is accompanied by micro-inflammation in the HF, as suggested by lymphocyte infiltration, presence of activated

T-cells and degranulation of mast cells. Micro-inflammation can be triggered by UV radiations, micro-organisms biofilm, IL-6 and androgen activity.[11]

Micro-inflammation takes place mainly in the upper part of HF, where many microorganisms are present. Many patients affected by androgenetic alopecia, show also seborrheic scalp dermatitis, associated with inflammatory processes, dysbiosis featuring increased growth of *S. aureus* and decreased colonisation by commensals such as *Cutibacterium* and *Corynebacterium*. Proteases, antigens and toxins produced by *S. aureus* amplify inflammation and induce skin barrier function alterations.

Inflammation is also involved in ageing of the hair. The scalp, as the skin, is subjected to intrinsic and extrinsic ageing. Oxidative stress plays a major role in hair ageing. Outcomes in hair ageing are reduction in melanocyte function, associated with hair greying, and decrease in hair regeneration, causing alopecia.

Free radicals may damage melanocytes in hair bulb inducing hair graying and induce apoptosis in hair follicle cells, thus accelerating the onset of the catagen phase, followed by hair loss.

Keratinocytes react to oxidative stress by releasing IL-1α, a proinflammatory cytokine able to inhibit the growth of isolated HFs *in vitro*. IL-1 stimulate adjacent keratinocytes to express TNF-α, IL-1α and the chemokines IL-8 and monocyte chemoattractant protein-1 (MCP-1) and -3 (MCP-3), which allow the recruitment of neutrophils and macrophages in the follicle, thus enhancing the inflammatory response.

Prevention of oxidative stress and inflammation could therefore represent an innovative approach to counteract hair graying, hair weakness and alopecia. Scalp microbiota interacts with host keratinocytes and innate immune system producing beneficial effects on HFs and hair mediated by the activity of antimicrobial peptides (bacteriocins), biosurfactants and free fatty acids.

The use of topically applied probiotics could therefore represents a targeted approach to hair and scalp disorders. Many studies reported indeed the health-promoting efficacy of probiotics on skin health.

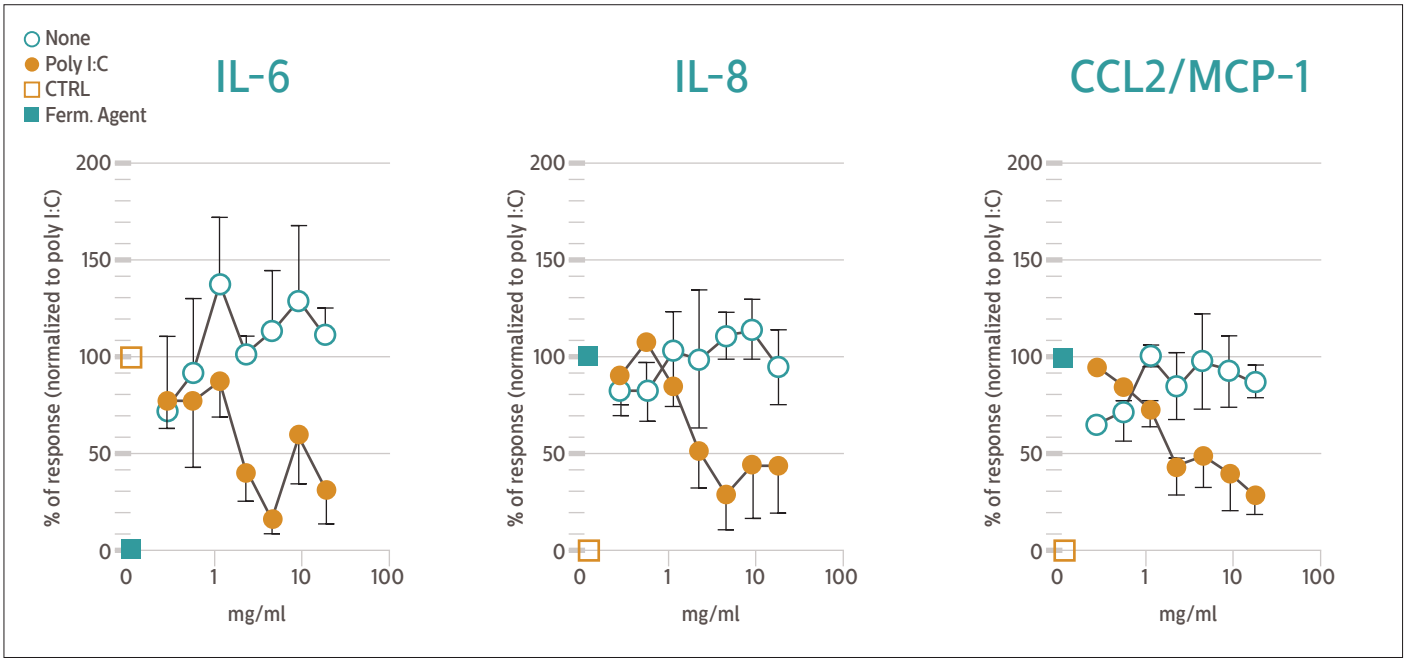

Figure 1: Modulatory action on IL-6, IL-8 and CCL2/MCP-1

Introducing postbiotics

However, the use of living bacteria on scalp or skin poses several challenges, such as skin safety, the compatibility with the preservatives and the ability of the microorganisms to grow and inhabit the host. The innovative approach we propose is represented by the use of postbiotics. The term refers to molecules – not living, nor inactivated microorganisms – but released by beneficial bacteria that mediate the beneficial effects of probiotics itself.

Postbiotics include bioactive peptides enzymes, short-chain fatty acids (SCFAs), biosurfactants, vitamins and more. It has been hypothesised that these substances are responsible for probiotic efficacy. Compared to probiotics, postbiotics can interact in a more targeted and direct way with local microbiota and host cells. Recent research highlighted the main properties of postbiotics including antioxidant, antimicrobial and immunomodulatory activities.[12]

Postbiotics can therefore represent a novel frontier in hair care to prevent ageing of hair, hair growth disorders, e.g. alopecia, and scalp issues such as dandruff, by directly counteracting oxidative stress and inflammation in HFs, due to their high specifity and selectivity of action on resident microbiota and host cells.

An *in vivo* study showed the efficacy of a cosmetic gel containing postbiotics for the treatment of alopecia areata. A significant improvement in hair regrowth was observed in the subjects treated with the active product in comparison to placebo.[13]

We here demonstrate the ability of Kalibiome postbiotics – a pull of postbiotic molecules obtained from L. paracasei through an original and unique biofermentation – in reducing the expression of inflammatory mediators, decreasing oxidative stress by reducing the production of free radical species, and inhibiting S.aureus biofilm formation, thus suppressing the main causes involved in scalp disorders previously discussed.

Kalibiome postbiotics

Solutions aimed to preserve the skin and hair scalp microbiota balance have become one of the main cosmetic industry priorities. In the past years, living probiotics, tyndallised bacteria, lysates or prebiotics use has expanded. However, the production of stable, reproducible and safe formulations containing most of these ingredients remains a topic of discussion.

In light of the previous scientific literature background, it can be asserted that bacteria interact with each other and skin and hair scalp cells, using complex network of molecules, the postbiotics, which represent a new class of bacteria-derived factors, helpful in regularising the microbiota balance, reinforcing skin, hair and scalp defense and other beneficial effects.

Through a patented biotech fermentation process (PB Tech), Kalibiome postbiotics for cosmetic applications were designed and produced starting from *L. Paracasei*, a species known for its beneficial skin effects.

Kalibiome postbiotics are produced naturally and released by living microbes and extracted by a technique evading bacterial fragments or toxins presence enabling to obtain highly reproducible output, avoid interferences with cosmetics preservatives and formula stability issues, while optimising the safety profile and increase the efficiency of the microbiota regulation process on skin and hair.

Kalibiome postbiotics are pure substances that do not contain any residue of bacterial fragments nor culture broth media residues or bacteria toxins. Due to their reproducible composition, organoleptic features, long shelf life, safety, handling and efficacy, the use of postbiotics is an elegant and efficient approach to directly provide the skin with microbial actives.

In addition, Kalibiome postbiotics come in hydro-soluble powdery form, they have a preservative-free composition and a significant applicative versatility, as they can be included in several cosmetics forms while avoiding interferences with cosmetics preservatives and formula stability issues.

Kalibiome: biological targets to counteract hair disorders and stimulate hair follicle

Kalibiome postbiotics were developed with the aim of obtaining specific metabolites able to modulate the expression of:

- **Interleukin 6** (IL-6), cytokine acting as mediator of the inflammatory process leading to the miniaturisation of the hair follicle and shortening of the anagen phase
- **Interleukin 8** (IL-8) and Monocyte chemoattractant protein-1 (MCP-1), chemokines enhancing the inflammatory response on the hair bulb subject to UV rays and exposed to increased risk of catagen phase onset acceleration and hair greying
- **Thymic Stromal Lymphopoietin** (TSLP), cytokine demonstrated to be significantly increased during the anagen phase of the hair cycle and able to promote hair growth in response to injury.14 TSLP protein is found in two variants: Long-form TSLP (linked to type 2 immune responses, highly induced in pathological conditions such as allergic diseases) and Short-form TSLP (sfTSLP) constitutively expressed in healthy human epidermis. sfTSLP was found to be upregulated throughout the hair cycle, reaching peak expression during mid-anagen, when hair follicles extend deeper into the tissue, and then downregulated to lowest expression during catagen. Scientific evidence suggests that TSLP functions locally in the hair follicle microenvironments to promote tissue regeneration

Furthermore, Kalibiome proved to act at additional functional values, by:

- **Reducing free radicals induced by UV radiations**: the oxidative stress is among the most relevant factors of risk triggering the onset

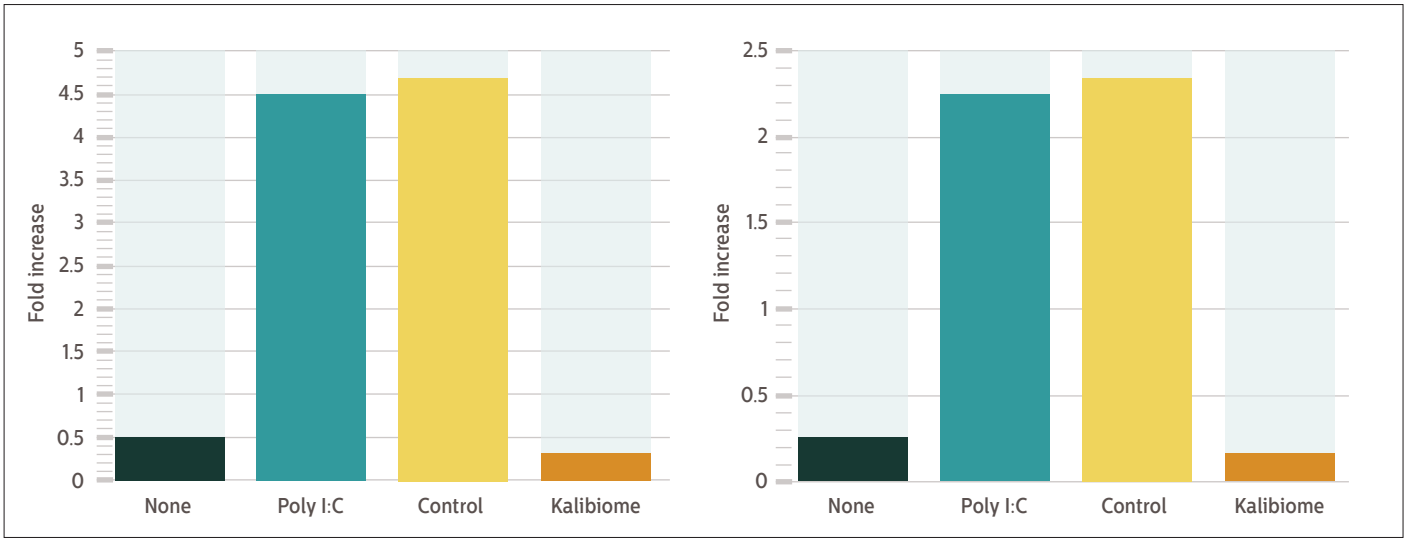

Figure 2: Modulatory action on long/short TSLP

of the involution of the hair follicle (given their induced exacerbation of local inflammations), and shortening of the anagen phase

■ **Promoting interference on pathogens biofilm formation**: hair scalp microbiota alterations and pathogens hyper-colonization contribute to the premature termination of the anagen phase and miniaturisation of the hair follicle. The maintenance of a heterogenous balance of the microbiota eco-system is one of the main targets where the cosmetics industry has focused the attention in the past years. Kalibiome acts at this level through its bio-surfactants, which create a physical barrier inhibiting pathogens quorum sensing, hence preventing over-expressions of single species

Material and methods

In vitro tests were carried out normal human epidermal keratinocytes (NHEKs) cultured in NEHK growth medium supplemented with 1% growth supplement (NHEK-GS) and 50µg/ml Gentamycin. Cells were stimulated with 1µg/ml of Polyinosinic:polycytidylic acid and treated with different concentration (10mg/ml and following serial dilutions 1/3) of Kalibiome (fermented agent) and its related control (containing non-fermented substrate). The inflammatory mediators IL-6, IL-8 and CCL2/MCP-1 were evaluated by ELISA.

Then, gene expression of the two forms of TSLP, *longTSLP* and *shortTSLP*, were evaluated by qPCR. Real time PCR (qPCR) was performed with Fast SYBR Green PCR kit on Applied Biosystems 7Flex Fast Real Time PCR System (Applied Biosystems) with 20 ng of cDNA and specific primers for the two forms of TSLP. The relative mRNA quantification was calculated by ΔΔCt method.

In order to quantify the free radical scavenging properties, a superoxide evaluation through a MitoSOX tracker on keratinocytes cell line was carried out. Mitomycin-C massively produces superoxide and hydroxyl radicals in the cells. Following the Mitomycin treatment, the cells were washed and treated with the antioxidant actives object of the analysis (blank solution as control, Glutathione, Kalibiome).

The treatment foresaw an exposure of the cells to the active for 18 hours. At the end of such exposure, a measurement of the production of superoxide was performed. The molecules with the best *in vivo* antioxidant features are those that determine lower formation of superoxide inside the cells.

The inhibition of bacterial biofilm was assessed with the following procedure: pathogen bacteria (*Staphylococcus aureus*) were taken from a frozen stock at -80°C in 25% glycerol before each experiment. The cryovials were placed on ice to avoid thawing of the sample. Then, bacterial strains were streaked onto a TB Agar plate in a zig-zag pattern and incubated at 37°C overnight.

Bacterial suspension at the desired optical density is injected into rectangular straight microchannels followed by a period (30 minutes) of rest in which the cells will have time to adhere to the surface. Different Kalibiome solutions in fresh culture medium and control were driven in the channels through syringes at 1.2µL/min for 17 hours. A fully automated image acquisition routine records the position of bacteria on the surface for several hours at different locations along the same channel, for each of the channels.

For the free radical scavenging assessment, a cell line of human Keratinocytes (HaCat) was treated for one hour with Mitomycin-C, in order to replicate a stressing condition to the cell and to check how antioxidants react in front of factors inducing oxidative stress on the skin.

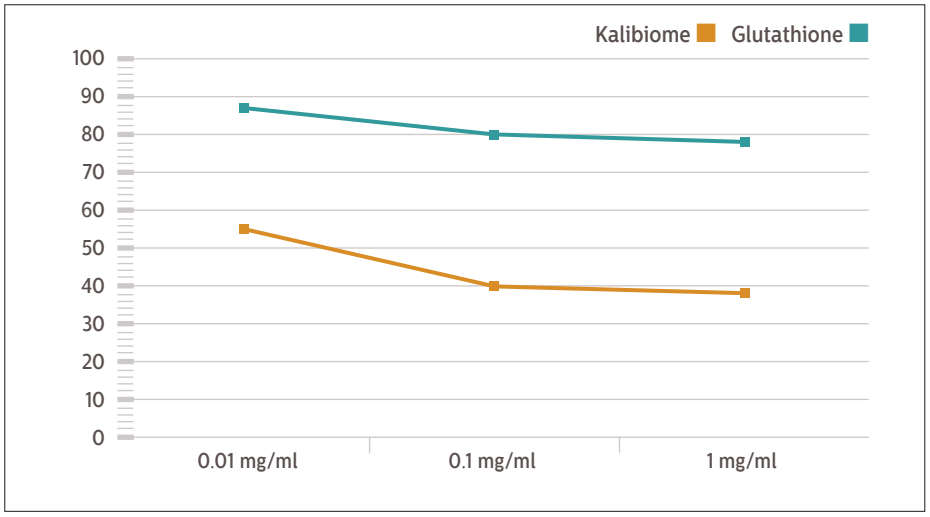

Figure 3: Percentage of production of Mit-c dependent superoxide

Results

As shown in Figure 1, Kalibiome postbiotics were proven to show a dose-dependent immunomodulatory action, with a refined reduction of the pro-inflammatory IL-6, IL-8, MCP-1. The regulation of these cytokines could represent a key mechanism to normalize the anagen phase of the hair follicle and retarding the onset of catagen phase; such a mechanism may be exploited both for anti-hair loss treatments as well as for daily routine treatments to keep under control acute and chronic local inflammations on the scalp.

In Figure 2, qPCR results show the activity of Kalibiome in reducing eightfold the expression of the pathogenic Long TSLP (cytokine responsible of itching, pathologically occurring

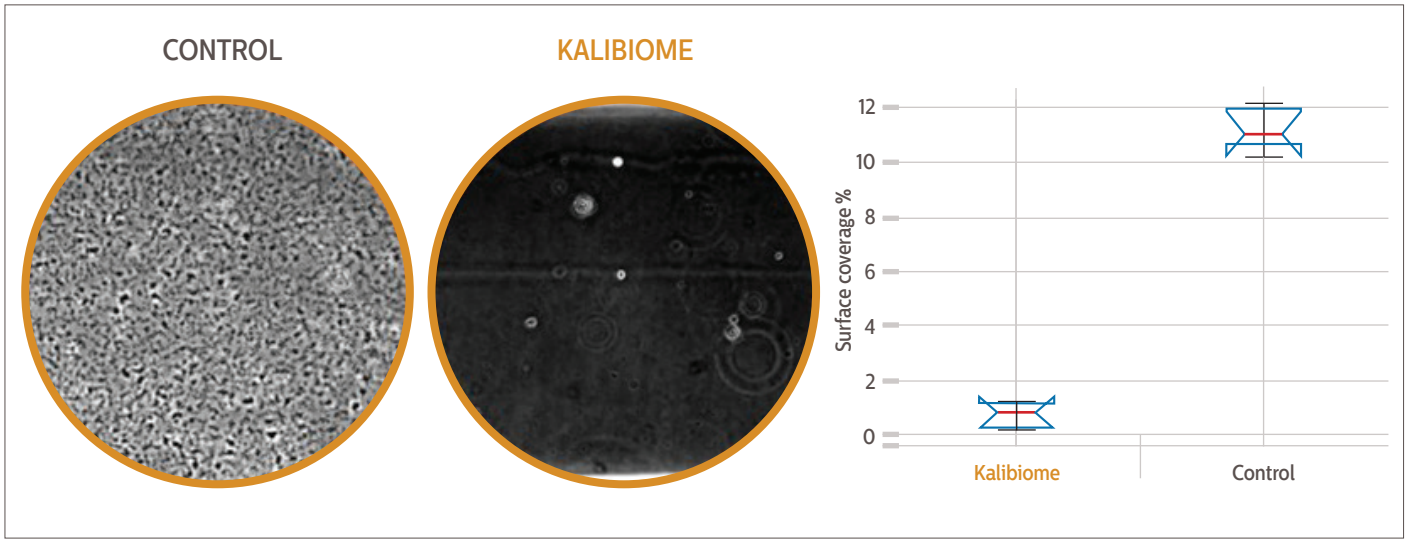

Figure 4: Surface coverage of S.aureus after 280 minutes of continuous infusion of medium and Kalibiome, and control

during allergic and acute inflammations), while increasing by 300% versus control the expression of the physiological short TSLP, cytokine physiologically expressed during the mid-anagen phase and promoting hair follicle tissues regeneration.

Not only does the activity on TSLP represent a biological mechanism enabling to balance the anagen/telogen phases throughout the hair life cycle, it also supports the process of hair regeneration following traumas or wounds.

As for the free radical scavenging action, the tests analyzed the mitochondrial ROS (O_2 superoxide) reduction in living cells; Kalibiome postbiotics reduced more effectively than glutathione the superoxide radical found in the cells subject to oxidation, outperforming it at every dosage analyzed (Figure 3).

The oxidative stress decrease contributes to the immune system modulation activity, as it downregulates the activation of pathways involving the induction of lipid peroxidation, membrane disruption, protein denaturation that leads to nucleic acids damages, mitochondrial damage and oxidative explosion. Proper protection against free radicals represents one of the main mechanisms of last generation products for anti-hair loss and hair anti-ageing.

The interference with pathogens biofilm formation is shown in Figure 4. Kalibiome postbiotics inhibit the formation of *S.aureus* biofilm, germ involved in inflammation diseases and frequently occurring in cases of *androgenetic alopecia*, whose proteases, antigens and toxins amplify inflammation and induce skin barrier function alterations. Kalibiome showed significant bacteriostatic action by totally inhibiting the growth of *Staphyloccocus aureus* following 280 minutes according to the previously mentioned assessment procedure.

Conclusion

Kalibiome postbiotics are a complex of biotech actives developed through a patented fermentation technique that enables to obtain with standardised reproducibility bacterial active metabolites with proven safety and with patented composition.

Their combined immune system modulation, refined cytokine and chemokines regulation - specifically those involved in the regularisation of the hair follicle anagen/telogen phases equilibrium - together with their bacteriostatic action on pathogens, microbiome balancing and free-radical scavenging properties can be key mechanisms to support daily routine scalp microbiome treatments, as well as anti-hair loss and hair anti-ageing applications, with potential expansion to anti-greying and dandruff support treatment.

Kalibiome postbiotics represent the ideal ingredients in all formulations developed to strengthen the hair and make it healthier and more beautiful. Their powdery and preservative-free nature, their hydro-solubility and lack of interaction with cosmetics preservatives, makes them suitable for several applications, including glycolic and hydro-alcoholic lotions, shampoos (traditional and dry), mousse, conditioner, masks, sprays and gels. **PC**

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