SCALP MICROBIOME, A NEW PLAYGROUND FOR COSMETIC INNOVATION

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Abstract

Background: The human skin including the scalp surface, serves as the body's first line of defense as well as a host to a myriad of microorganisms, which includes both bacteria and fungi. The application of high-throughput next-generation sequencing, and robust computational analysis has led to an in-depth understanding of the scalp microbiome in the recent years, providing novel clues on the scalp-related disorders and scalp health. Global studies have revealed that the scalp microbiome is characterized by a rather low bacterial diversity, as compared to the other body sites, and is dominated by *Cutibacterium spp*., *Staphylococcus spp*. and *Malassezia spp*. The scalp microbiome acts as guardians of hair and scalp quality. Various environmental and intrinsic factors are reported to be linked to the scalp barrier, sebum composition, scalp microbiome disequilibrium, thus it is important to propose adapted scalp cosmetic products. For this purpose, we developed a specific formula designed to act on scalp and its ecosystem.

Methods: The formula is a serum containing vitamin CG, polysorbate 21 and a complex of 7 pre- and probiotics fractions including *Bifidobacterium longum*, *Saccharomyces cerevisiae*, two *Lactobacillus* extracts and two long polysaccharides and one short sugar. The benefits of this new scalp care were evaluated on two cohorts of 74 healthy adult men and women with different hair and scalp types aged 18-65y old.

First study: open-label, single center study in 41 subjects with sensitive scalp. Evaluations were performed immediately after 1st application and at D21, assessing the cosmetic acceptability and efficacy of the product. Transepidermal water loss (TEWL), squalene and

squalene peroxide (SQOOH) were assessed at baseline and D21. Tolerance was assessed during the whole study.
Second study: open-label, single center study in 33 subjects (9 Males, 24 Females) during 2 weeks of daily application of the serum. Microbiome evaluations were performed before and after scalp surface aggression and along the time. Cutibacterium spp., Staphylococcus spp. and Malassezia spp (quantitative) were evaluated by QPCR (region V1-V3 16S rRNA KAPA Biosystems kit with SYBER Green) and amplicon sequencing analysis of bacterial and fungal communities (qualitative) was conducted using Next Generation Sequencing (Amplification V1-V3 on Illumina MiSeq platform).

Results: Scalp discomfort score and scratching frequency significantly ($p<0.0001$) improved immediately after first serum application and lasted until the end of the study (D21) compared to baseline. There was a significant reduction of SQOOH content (-15%) between baseline and D21. Subject satisfaction was high. Tolerance was good.
The scalp surface aggression decreased the bacterial and fungal load and diversity of the scalp microbiota. Application of the serum do not change the recovery of the quantity but promoted a faster recovery of scalp diversity compared to bare scalp after 15 days of application. Indeed, very interestingly, based on the beta diversity, after 15 days of serum application, the scalp microbiome showed a significant faster and total recovery relative to bare scalp.

Conclusion: These findings show that for the first time a specific designed cosmetic formula containing vitamin CG, polysorbate 21 and a combination of 7 pre- and probiotics fractions can bring a quicker and complete recovery of scalp microbiota after aggression and reduce global discomfort and symptoms in subjects with sensitive scalp, and as well as markers of oxidative stress.
These results bring a key milestone in scalp knowledge and paving the way for new products for healthy scalp bringing hair quality.

Keywords: microbiome; prebiotic; probiotic fractions; sensitive scalp; discomfort

Introduction.

Skin/scalp microbiota has a strong impact on skin/scalp quality link to its power on the regulation of inflammation, on the protection against invaders and oxidants and on helping the barrier function recovery. [1, 2, 3, 4]

The disequilibrium of skin microbiota may contribute to facilitate skin disorders. Many factors such as environmental pollution, solar exposures, psychologic stress or harsh skin care routine are reported to impact the skin microbiota homeostasis. [5, 6, 7, 8, 9, 10] The human skin including the scalp surface, serves as the body's first line of defense as well as a host to a myriad of microorganisms, which includes both bacteria and fungi. The application of high-throughput next-generation sequencing, and robust computational analysis has led to an in-depth understanding of the scalp microbiome in the recent years, providing novel clues on the scalp-related disorders and scalp health.

Global studies have revealed that the scalp microbiome is characterized by a rather low bacterial diversity, as compared to the other body sites, and is dominated by *Cutibacterium spp., Staphylococcus spp*. and *Malassezia spp*. The scalp microbiome acts as guardians of hair and scalp quality. [11, 12, 13] Various environmental and intrinsic factors are reported to be linked to the scalp barrier, sebum composition, scalp microbiome disequilibrium, thus it is important to propose adapted scalp cosmetic products. For this purpose, we developed a specific formula designed to act on scalp and its ecosystem.

Materials and Methods.

1. Clinical studies and clinical evaluation

These open-label, single center, non-invasive and prospective studies were conducted between September 2020 and February 2021 at one investigational site in France according to the guidelines for the conduct of clinical trials in alignment with local and international regulations. All subjects provided written informed consent prior to their participation.

41 Subjects from first study were included with sensitive scalp at baseline meaning with global scalp discomfort score of ≥8 (0-27), defined as the sum of individual symptom scores (0-9) for itching, stinging and warming sensations, each rated on a 10-point self-grading scale from 0=none to 9=very much. Subjects had to apply the serum every evening on the full scalp

for 21 days and they were asked to wash their hair 2 to 3 times per week with their usual non-treating shampoo. Discomfort sensations and frequency of scratching were assessed at the investigational site at D0T0 (before application of serum), at D0Timm (immediately after 1st application), at D21T0 and at D21Timm and every evening at home between D1 and D20.
Cosmetic acceptability and efficacy of the serum were assessed at D0Timm and D21 according to Likert scale from “totally agree” to “neither agree”.
Transepidermal water loss (TEWL) using a Vapometer® SWL4001 (Delfin®) was evaluated at D0T0 and D21T0 on shaved scalp mini area of 2,25 cm^2.
Squalene (SQ) and squalene peroxide (SQOOH) were sampled with swab method by rubbing the tip of the swab over the entire area of interest at D0T0 and D21T0 from a defined area of the scalp closed to shaved mini area. Samplings were stored at -20°C then extracted. SQ and SQOOH were quantified by liquid chromatography (Ultimate 3000 HPLC system) combined with a single quadrupole spectrometer MSQ (Thermo) (LC-MS). Total proteins were also analyzed with Microplate reader Spark (Tecan) to normalize SQ and SQOOH results to limit the potential sampling difference influence. [14, 15]
Scalp tolerance was assessed during the whole study.

33 subjects (9 Males, 24 Females) from the second study were included with normal scalp, no dandruff condition, and a high heterogeneity according to hair and scalp types, hair curliness and scalp sensitivity. (Fig.-1)

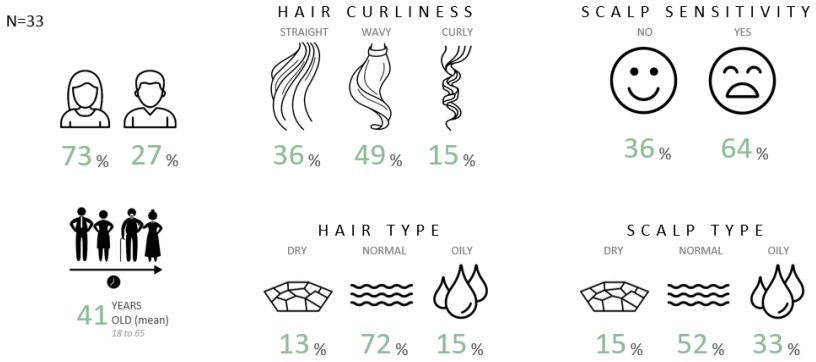


Fig.-1: Demography of the second clinical study on 33 subjects focused on scalp and hair demographic data

Subjects had to apply the serum every evening on the full scalp for 2 weeks. Microbiome evaluations were performed at D0 (baseline before serum application), D7 (after 1st application of serum) and D26 (after 2 weeks of daily serum application). For each condition, microbiome sampling was done before (D0T0, D7T0 & D26T0) and at different timepoints after scalp surface aggression with shampoo: immediately after (D0Timm, D7Timm and D26Timm) and after 6h (D0T6h, D7T6h and D26T6h), 24h (D1, D7 and D27) and 48h (D2, D8 and D28).

2. Formula tested

The formula is a scalp serum containing vitamin CG, polysorbate 21 and a complex of 7 pre- and probiotics fractions including *Bifidobacterium longum extract*, *Saccharomyces cerevisiae extract*, two *Lactobacillus* extracts and two long polysaccharides and one short sugar. [16-17]

3. Microbiome evaluation

Cutibacterium spp., *Staphylococcus spp*. and *Malassezia spp* (quantitative) were evaluated by QPCR (region V1-V3 16S rRNA KAPA Biosystems kit with SYBER Green) and amplicon sequencing analysis of bacterial and fungal communities (qualitative) was conducted using Next Generation Sequencing (NGS) (Amplification V1-V3 on Illumina MiSeq platform).

i) Sampling method

A sterile cotton swab soaked in a solution containing collection solution (0.15M NaCl and 0.1% Tween 20) was rubbed onto the scalp surface to cover a total surface of 4 cm^2 in a non-overlapping manner. At the end of the sampling in an Eppendorf sterile tube and store at -80°C until DNA extraction, qPCR and NGS analysis. A sterile cotton swabs were kept as negative controls each day of sampling.

ii) Bacterial DNA extractions

Genomic DNA was extracted by using the PowerSoil DNA Isolation kit (MOBIO Laboratorie Inc., Carlsbad, USA) according to the manufacturer's instructions 1. DNA

concentration was measured using Qubit ds DNA HS kit on Qubit 2.0 fluorometer (Life technologies, Carlsbard, CA, USA).

iii) DNA sequencing and processing

To prepare 16S amplicon libraries, 2,5 µl of DNA was used for a first PCR amplification step of 25 cycles with specific bacterial 16S rRNA (V1-27S and V3-535R) hypervariable regions oligos using the KAPA HiFi HotStart ReadyMix PCR kit (Roche Diagnostics, Laval, Québec, Canada). After purification with Agencourt AMPure XP beads (Beckman Coutler, Missisauga, Ontario, Canada), a second PCR amplification step was performed to incorporate specific index adaptors for multiplexing. This step was followed by a purification step using Agencourt AMPure XP beads. The quality of final libraries was examined with a DNA screentape D1000 on a TapeStation 2200 (Agilent Technologies, Santa Clara, CA, USA) and the quantification was done on the QBit 3.0 fluorometer and/or by Quant-iT dsDNA Assay (ThermoFisher Scientific, Canada). Subsequently, 16S libraries with unique index were pooled together in equimolar ratio (366 samples/pool) and both pools were sequenced twice on Illumina Miseq system for 300pb paired-end sequencing at the Next-Generation Sequencing Platform, Genomics Center, CHU de Québec-Université Laval Research Center, Québec City, Canada.

iv) Sequence processing and bioinformatics

The raw sequence data was subjected to quality trimming and ambiguity filtering using FASTQC v0.11.2 4 and multiQC v1.3 5. Quality filtration of fastq reads and adaptor removal was carried out using Trimmomatic 6 with the following options: ILLUMINACLIP: 2:30:10, TRAILING:20 and MINLEN:36. The paired-end reads were assembled for each amplicon sequence using PANDASeq 7. Clustering was carried out using closed-reference OTU picking and de novo OTU picking protocol of QIIME v1.9.1 8 at ≥97% identities. The Silva database v119 10 was used as a reference for bacterial taxonomic assignment. For the taxonomic assignment of de novo OTUs, sequence was clustering against their respective databases with the QIIME's assign_taxonomy.py script v1.9.1 8 using the UCLUST algorithm 11. Beta diversity was analyzed by measuring the UniFrac distances for the bacterial population.

v) Quantitative PCR analysis

The quantification of total bacterial DNA was performed with a fluorescent based Realtime PCR system using the LightCycler 480 (Roche Diagnostics). The PCR reaction was performed using the reagent KAPA SYBR FAST qPCR kit optimized for LightCycler 480 (KapaBiosystems).

4. Statistical analysis

All statistical analyses for the first study were performed at a 5% significance using 2-sided tests, except normality testing at 1% threshold (Shapiro-Wilk test). Evolution over time was investigated by using Student or Wilcoxon test for paired series (according to the normality of the data distribution). The count and percentage of subject responding to questionnaires were provided for each timepoint.

To show the formula-efficacy in the second study, a time effect analysis was performed on the whole study population using the Linear Mixed Model with: each study parameter as dependent variable; time as fixed and repeated factor: overall time effect and comparisons Dx versus D0 with adjustment for multiple comparison according to the Bonferroni procedure. Subject as random variable; Unstructured model as covariance matrix; Checking of residuals distribution normality assumption using normal QQ plot.

A gap compared to residuals distribution normality was found for raw data, a log10 transformation of data, was therefore applied to obtain normally distributed residuals for each QPCR variable.

For sequencing data analysis, R software was used (vegan: Community Ecology Package. R package version 2.4-5.14. Pairwise analysis of Similarities (ANOSIM 15) was applied to assess differences based on treatment and time point. ANOSIM global R value ranges from 1 to −1 ($R \sim 0$ indicates the same level of variation within and between groups). ANOSIM test was performed using $\alpha = 0.05$ for statistical significance. Alpha diversity (Shannon) was analyzed following the same method as for clinical parameters (Linear Mixed Model, see above). Benjamini–Hochberg procedure was used for multiple comparisons adjustment.

Results and discussion

1. <u>Global scalp discomfort</u>

Global scalp discomfort score and scratching frequency significantly ($p<0.0001$) improved immediately after first serum application (D0Timm) (respectively -52% and -67%) and lasted until the end of the study (D21Timm) compared to baseline. (Fig.-2)

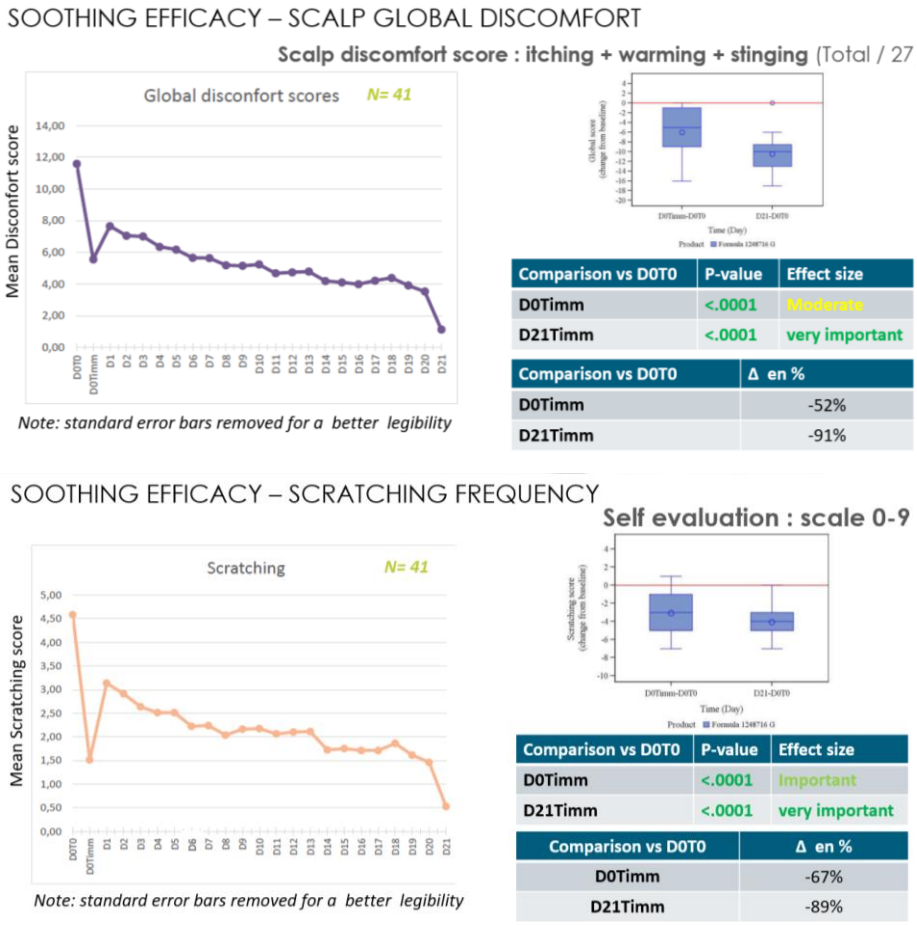
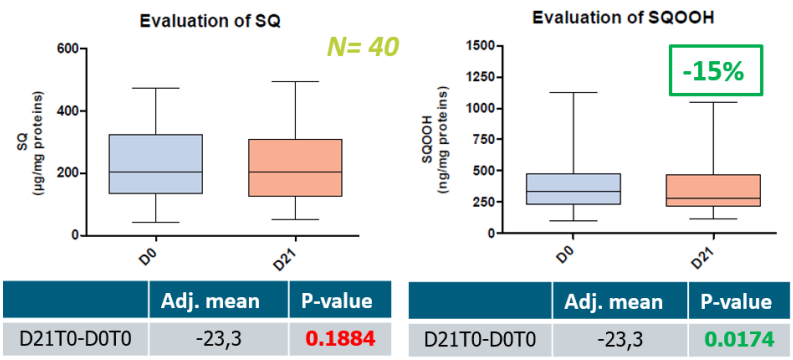


Fig.-2: Evolution of the mean discomfort score and the mean scratching score during the soothing clinical study on n=41 volunteers; boxplots of change from baseline for global discomfort score and scratching score and statistical comparisons versus baseline (D0T0)

2. <u>SQ and SQOOH biomarkers results</u>

No significant difference ($p=0,1884$) on SQ content was observed between D0T0 and D21T0, no seboregulation activity was observed in this study.

The SQOOH content is significantly reduced (p = 0,0174) between D0T0 and D21T0 (-15%) highlighting a reduction of oxidative stress of Squalene. (Fig.-3)



	Adj. mean	P-value
D21T0-D0T0	-23,3	0.1884

	Adj. mean	P-value
D21T0-D0T0	-23,3	0.0174

Fig.-3: Boxplot's representation of the quantity of squalene (expressed in µg/mg total proteins) and squalene peroxide (expressed in ng/mg total proteins)

3. <u>Self-assessment of serum efficacy and acceptability</u>

Immediately after the first application, 100% of the subjects stated that the serum provided a cooling sensation, 74% that their scalp felt hydrated, 26% that hair and scalp were better protected from external aggression and 33% that the serum left the hair shiny. In total, 55% stated that itching sensations had reduced, 57% for stinging sensations, 50% for tightness sensations and 69% for warming sensations attenuation. (Fig.-4)

After 21 days of daily treatment subjects highly appreciated the efficacy and product properties and the acceptability was good on scalp.

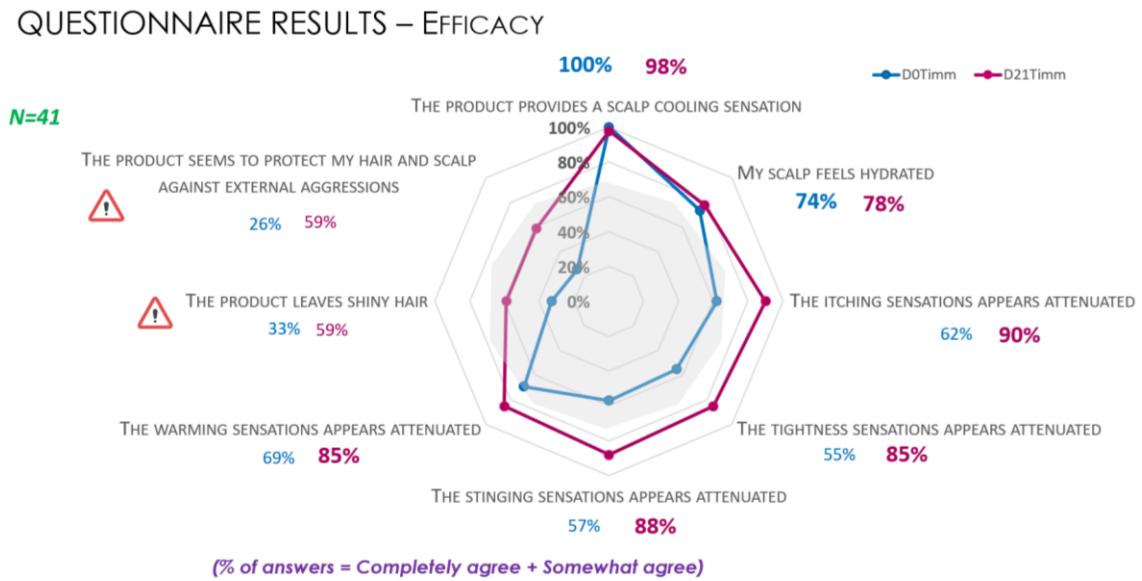


Fig.-4: Spider graphs representation of self-assessment efficacy TOP2BOX results by 41 subjects at D0Timm and D21Timm. TOP2BOX represents the % of subjects that answer “completely agree and somewhat agree.

4. Microbiome results

The scalp surface aggression decreased the bacterial and fungal load and diversity of the scalp microbiota. (Fig.-5)

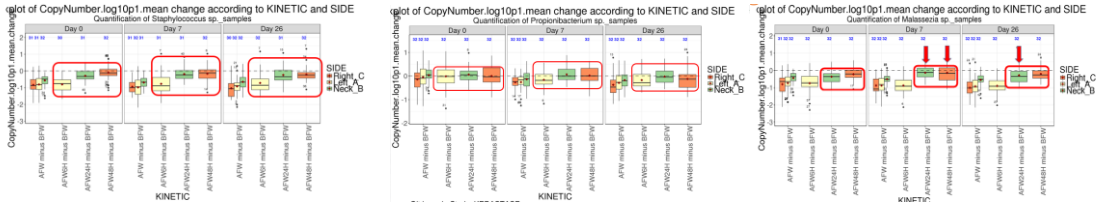


Fig.-5: Copy number log 10, kinetic of evolution of total bacterial load, qPCR, in bare scalp (D0), immediately after 1st serum application (D7) and after 2 weeks daily application of serum (D26).

The relative abundance for fungi and bacteria at the genus taxonomic level at D0, D7 and D26 shows that fungi is dominate by Malassezia at >98% so the harsh shampoo shows no difference in fungi quality, in opposite an important diversity in bacteria was shown. (Fig.-6)

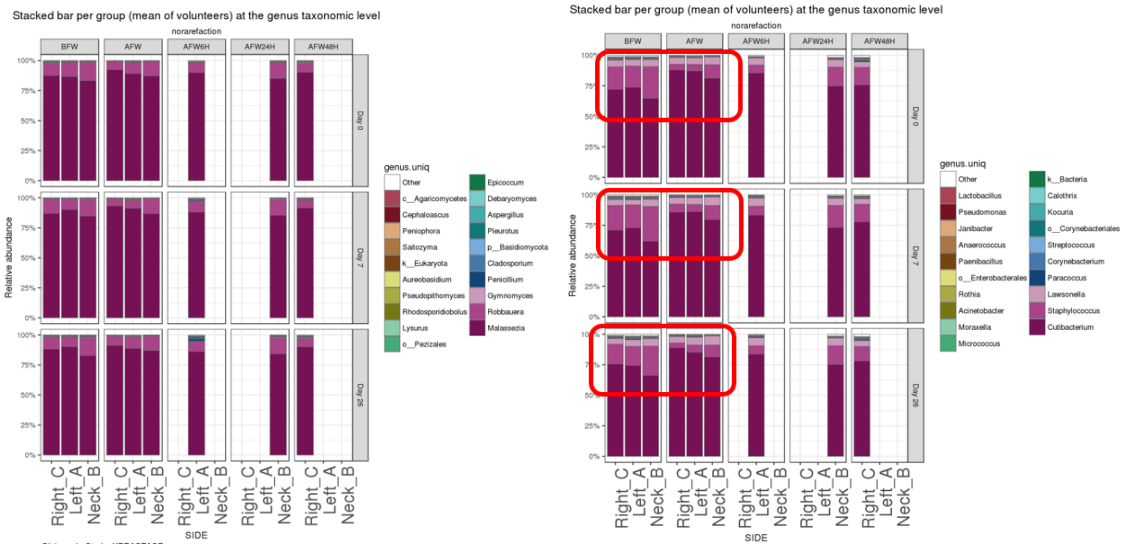


Fig.-6: Relative abundance Stacked bar per group (mean of volunteers) for fungi and bacteria at the genus taxonomic level and with no rarefaction, kinetic of evolution, at D0, D7 and D26.

Application of the serum doesn't change the recovery of the quantity but promoted a faster recovery of scalp diversity compared to bare scalp after 15 days of application. (Fig.-7) Indeed, very interestingly, based on the beta diversity, after 15 days of serum application, the scalp microbiome showed a significant faster and total recovery relative to bare scalp.

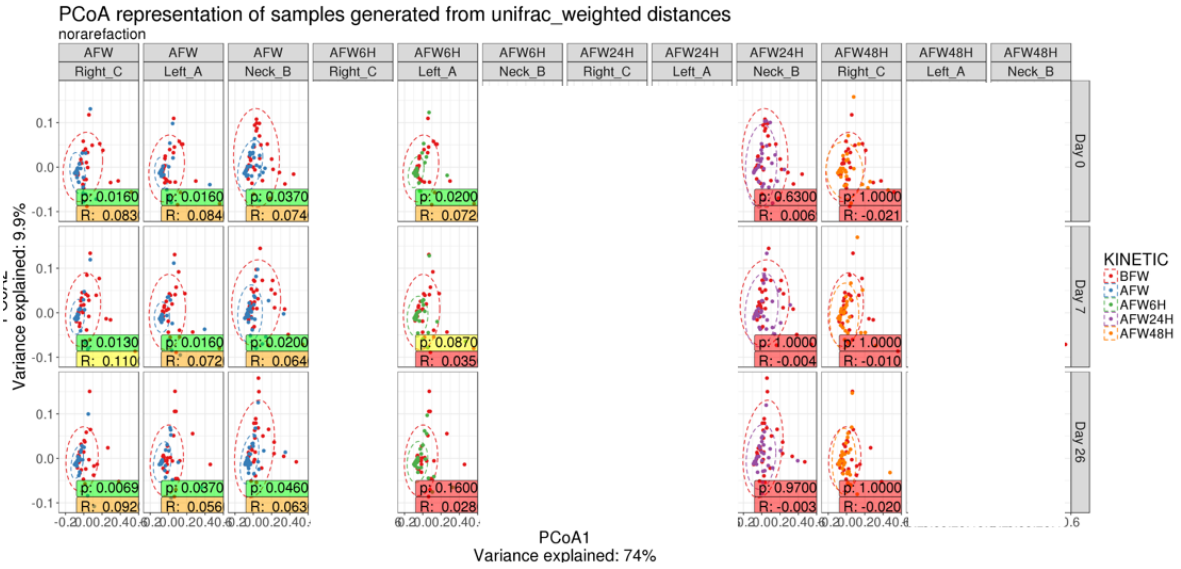


Fig.-7: Beta diversity, unifrac distance / no rarefaction / weighted distance showing kinetic effect at D0, D7 and D26.

Conclusion

These findings show that for the first time a specific designed cosmetic formula containing vitamin CG, polysorbate 21 and a combination of 7 pre- and probiotics fractions can bring a quicker and complete recovery of scalp microbiota after aggression and reduce global discomfort and symptoms in subjects with sensitive scalp, and as well as markers of oxidative stress.

These results bring a key milestone in scalp knowledge and paving the way for new products for healthy scalp bringing hair quality.

Acknowledgments

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Conflict of Interest Statement

NONE

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