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"Microbial Dynamics in Dandruff: Insights from Co-occurrence Network Analysis"

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1. Introduction

Seborrheic dermatitis and dandruff (SD/D) are prevalent dermatological conditions that primarily affect seborrheic areas of the body [1]. These disorders are common both in the United States and worldwide [1]. The incidence of SD/D peaks during three key life stages: in the first three months of life, during puberty, and in adulthood, with the highest prevalence observed between the ages of 40 and 60 years [2–4]. In infants up to three months old, the incidence can reach as high as 42% [4–6]. In adolescents and adults, SD/D typically manifests on the scalp and other seborrheic regions, including the face, upper chest, axillae, and inguinal folds [4,7,8]. The incidence among the general adult population is estimated to be between 1% and 3% [3,9]. Notably, men are affected more frequently than women across all age groups (3.0% vs. 2.6%), suggesting a potential association with sex hormones, such as androgens [3,8]. Furthermore, no significant differences in SD/D incidence have been observed among various ethnic groups [3].

Research has established links between skin disorders and skin microbiota for several conditions [10,11], including SD/D [12,13]. However, discrepancies among studies may arise from individual heterogeneity and variations in study populations, sampling methods, and sequencing techniques [14]. These inconsistencies underscore the necessity for our own study to better elucidate the relationships between SD/D and scalp microbiota, as well as to investigate the effects of active ingredients on both scalp microbiota and SD/D.

Despite these discrepancies, common findings have emerged from previous studies, such as the involvement of fungal species like *Malassezia restricta*, which may produce cytotoxic compounds that contribute to dandruff exacerbation [15,16]. Additionally, an increase in *Staphylococcus* populations has been reported in cases of dandruff [13].

Microorganisms form a complex community characterized by extensive interactions that influence the overall structure of the community and, consequently, scalp health. While examining individual microorganisms is valuable, understanding their interactions as a collective is essential. This ecological perspective extends beyond merely identifying the microorganisms present; it encompasses how these microorganisms interact, which significantly impacts microbial community dynamics and stability, as well as the unique characteristics that emerge from these communities [17].

However, inferring networks across multiple kingdoms of life, such as bacteria and fungi, presents challenges, particularly when dealing with sequencing data due to its compositional and zero-inflated nature [18]. To address these issues, we opted to study the absolute abundance of microorganisms using quantitative PCR (qPCR) [18]. This approach enables the exploration of correlations between microbial abundances and scalp features, such as dandruff area. A limitation of qPCR is that it provides access to a restricted number of microorganisms compared to next-generation sequencing (NGS) data. This restriction might reveal numerous indirect connections [19]. To avoid potential misinterpretations arising from these indirect connections, we focused solely on the global parameters of the inferred networks, rather than the individual links observed between taxa.

Based on this methodology, we first analyzed the relationships between scalp features and the absolute abundances of microorganisms. Next, we compared the community structures of healthy and dandruff-affected scalps. Finally, we evaluated the impact of a plant extract on scalp health and microbiota structure.

2. Materials and Methods

2.1. Panel description

The aim of this study was to evaluate on a panel of 20 volunteers on the reduction of dandruff and related erythema of an active product versus a placebo formulated in shampoo by biometrological measurements for 14 days of home application.
Scalp microbiota was collected to assess the impact of product on Interabiome which include the microflora composition and their interconnectivity. An healty scalp group of 10 volunteers was added exclusivily for doing comparative analysis at microflora level. The panel was divided in two equal group including Caucasian females and male volunteers, having between 18 and 70 years old, having a phototype between I and IV and suffering from itching sensation (itching score > 2 scale from 0 to 5) with sensitive and irritated scalp, were included in the study.
After 15 days of wash out period volunteers started to applied tested products with one groupe used active shampoo containing 3% of *Crocus sativus* flower extract, and the other one used placebo shampoo (same formula without active) for 14 days. The product was applied in normal conditions of use, *i.e.* 3 times a week with one shampoo every 2 days. Dandruff, erythema and scalp microflora were studied at D0 and D14.

2.2. Tested formula – INCI

Placebo and active shampoo:
AQUA/WATER, SODIUM LAURETH SULFATE, COCAMIDOPROPYLBETAINE,± **BETAINE (AND) POTASSIUM LACTATE (AND) LACTIC ACID (AND) *CROCUS SATIVUS* FLOWER EXTRACT**, SODIUM BENZOATE, SODIUM CHLORIDE, HYDROXYPROPYL GUAR HYDROXYPROPYLTRIMONIUM CHLORIDE, CITRIC ACID, FRAGRANCE

2.3. Clinical measurement of dandruff by Dandruffemeter®

Dandruff sample analysis: the analysis is made with DandruffMeter DA20 just after the collect. The system consists of a device in which the collected dandruff is inserted. A circularly arranged LED light source illuminates the sample homogeneously on a dark background. The high-resolution camera above the sample takes the image and the software detects all dandruff, categorizes it in 9 different size classes and gives total area covered with green spots in mm^2.

2.4. Clinical measurement of erythema by C-cube®

Dermatoscope C-Cube® (PIXIENCE) allows realizing high resolution skin pictures. The capture will be taken on the hair line. Standardized photographs of the scalp with C-Cube®, were performed at D0 andD14. In this study, erythema index was analyzed. From the standard CIE L* a* b* measurements given by the C-Cube® for every pixel, we define a specific severity parameter (based on a*) that can be used to grade erythema. This index can be displayed with blue/red color map to localize and illustrate the erythema. Drawing ROI on images, it is possible to monitor changes across time or to analyze differences of evolution between zones.

2.5. Scalp microflora analysis

2.5.1. Microbiota sampling and storage

Samples of microbiota were collected from the scalp of volunteers, by a non-invasive swabbing method, using sterile swabs moistened with a sterile solution of 0.15 M NaCl. Swabs were transferred at -20°C and kept frozen until DNA extraction. Sampling was done before treatment (D0), and after 14 days (D14) of treatment, using a standardized procedure. For healthy volunteers, sampling was done using the same procedure without treatment (D0).

2.5.2. DNA extraction

DNA extraction was performed, using the DNeasy PowerLyzer® PowerSoil® DNA Isolation Kit with Qiacube device (Qiagen, Hilden, Germany), with the following modifications. The tip of each swab was detached and transferred into 750 µL of Bead Solution. The sampled biomass was suspended by stirring and pipetting, and then transferred to a bead beating tube. The remaining steps were performed according to the manufacturer instructions. DNA concentration was determined using the QuBit dsDNA HS fluorometric quantitation kit (Invitrogen, ThermoFisher Scientific, Courtaboeuf, France) according to the manufacturer instructions. DNA was then frozen and send to SEQUENTIAL SKIN LTD, in Singapore, for quantitative PCR processing, using highly specific probes, designed, and validated for key bacteria and fungi.

2.5.3. quantitative PCR (qPCR)

The scalp microbiome composition was monitored through quantitative PCR focusing on 20 microbial targets (8 genera ans 12 species – Table 1):

Table 1. List of the 20 microbial targets followed during the study. In total 3 yeast species; 9 bacterial species 1 fungal genus and 7 bacterial genera were targeted. Spp. stands for *Species pluralis*, and means all species within the specified genus (spp. stands for “species plural", referring to multiple species within a given genus).

Species		Genus	
Cutibacterium acnes	*Staphylococcus capitis*	*Cutibacterium* spp.	*Malassezia* spp.
Cutibacterium modestum	*Malassezia restricta*	*Streptococcus* spp.	*Staphylococcus* spp.
Cutibacterium granulosum	*Malassezia globosa*	*Enterococcus* spp.	*Prevotella* spp.
Staphylococcus epidermidis	*Malassezia sympodialis*	*Corynebacterium* spp.	*Lactobacillus* spp.
Staphylococcus aureus	*Lawsonella clevelandensis*		
Staphylococcus caprae	*Pseudomonas aeruginosa*		

2.5.4. Microbial network analysis

The construction, analysis, and comparison of networks were conducted using the R package NetCoMi [20], developed by Peschel and colleagues in 2021. The input data consisted of quantifications obtained via qPCR (measured in genome copies per µL). The Spearman correlation coefficient was employed to evaluate the relationships between different microbial targets (measure = "spearman"). Data were normalized to stabilize variance (normMethod = "VST"), and no method for zero replacement was applied (zeroMethod = "none"), as such transformations are necessary in cases of data sparsity, such as with sequencing data. For network representation, absolute values of the Spearman correlation coefficient below 0.5 are not considered.

For network comparisons, permutation tests were conducted (permTest = TRUE) using 1,500 permutations (nPerm = 1500).

When quantifications for both genus and species were available (specifically for *Cutibacterium*, *Staphylococcus*, and *Malassezia*), only species quantifications were retained.

2.5.5. Statistical analysis

For all studies in vivo, a Shapiro Wilk test was used to verify whether the raw data followed the Gaussian Law. In case of Normally-distributed data, the mean values were compared using either an unpaired or paired t student test. In case of non-Normally-distributed data, a Wilcoxon (paired) or Kruskal-Wallis test followed by a Mann-Whitney U (unpaired) test were used for paired data or unpaired data respectively. Regardless the test, it was considered as a significant results; $p<0.1$ with #, $p<0.05$ with *, $p<0.01$ with ** and $p<0.001$ with ***.

For the interabiome analysis, Graphs and statistical comparisons were conducted using R version 4.4.0 [21], along with the ggplot2 package [22] and the rstatix package [23]. Non-parametric tests were employed for all comparisons: dependent data were analysed in the context of the treatment impact study, while independent data were used for the comparison between dandruff and healthy scalps.

3. Results

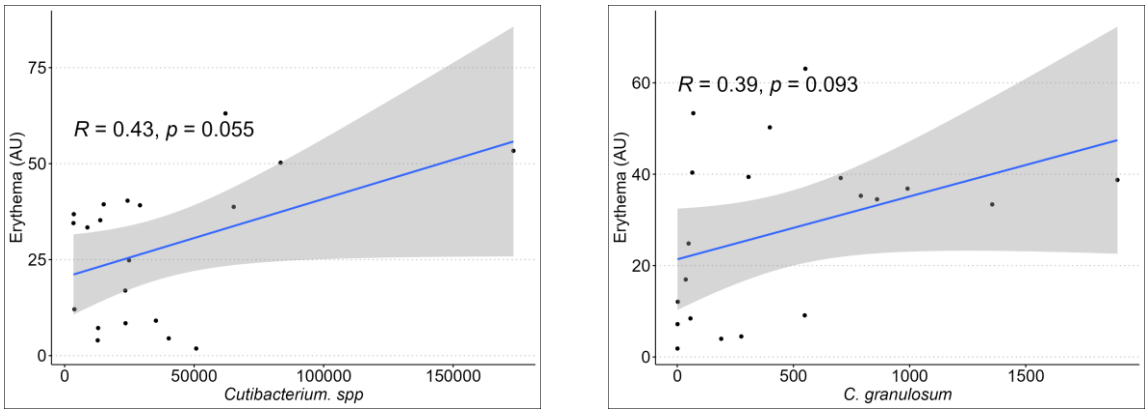
3.1. Relationship between scalp disorders and microbial abundance

3.1.1. Erythema

Among the 20 microbial targets quantified from healthy scalps (10 volunteers) and dandruff-affected scalps (10 volunteers), one bacterial genus and one bacterial species exhibited significant correlations with erythema intensity (Figure 1).

3.1.2. Dandruff area

Among the 20 microbial targets quantified from healthy scalps (10 volunteers) and dandruff-affected scalps (10 volunteers), two genera (one bacterial, one fungal) and three species (2 bacterial and one fungal) showed significant correlations with dandruff area (Figure 2). On Figure 2, the relationships with the genus *Staphylococcus* was not depicted, but the correlation was also significant (R = 0.41, p-value = 0.075). All correlations were positively correlated with dandruff area, except for Staphylococcus capitis, which was less abundant on scalps with larger dandruffs area.



(a) (b)

Figure 1. Significant correlation between microbial abundance (genome copy number) and Erythema intensity (arbitrary unit): (a) Correlation with *Cutibacterium* genus abundance; (b) Correlation with *Cutibacterium granulosum* species abundance.

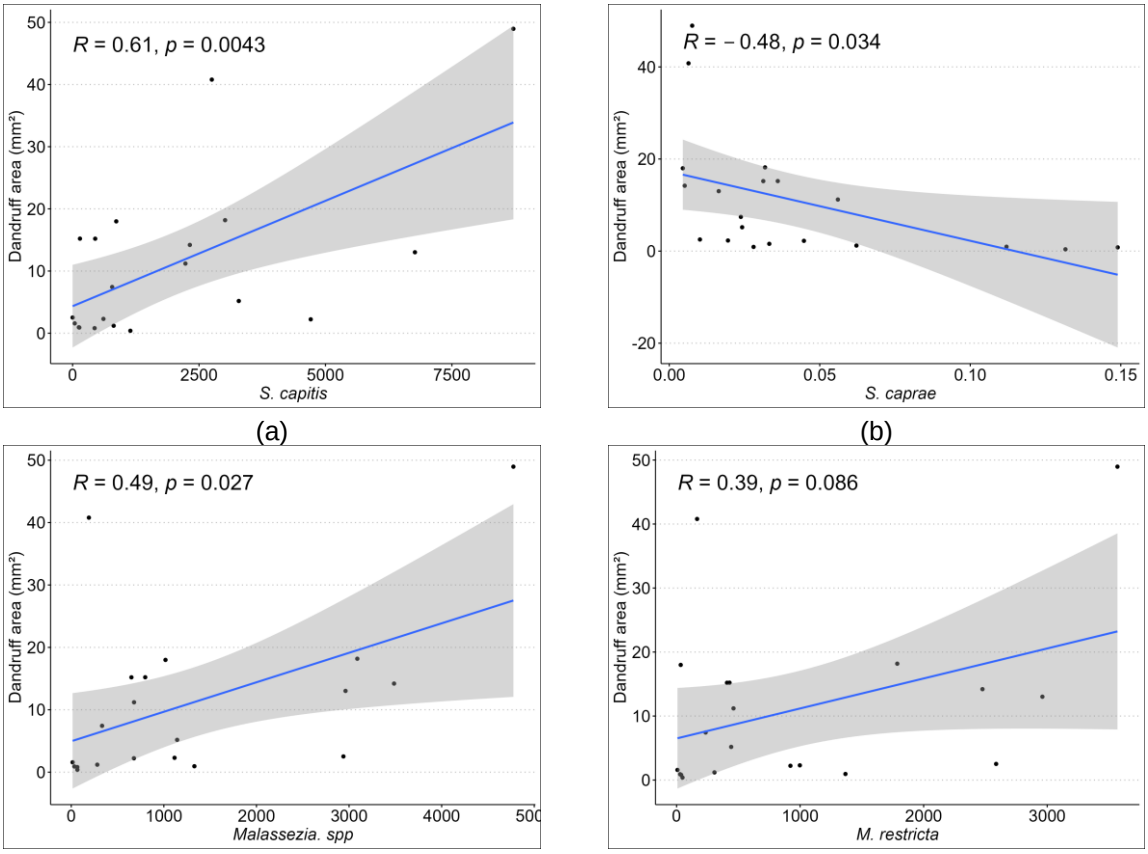


Figure 2. Significant correlation between microbial abundance (genome copy number) and Dandruff area (mm²): (a) Correlation with *Staphylococcus capitis* species abundance; (b) Correlation with *Staphylococcus caprea* species abundance; (c) Correlation with *Malassezia* genus abundance; (d) Correlation with *Malassezia restricta* species abundance.

3.2. Healthy *versus* dandruff-affected scalps microbial community structures

As explained in the introduction, the connections between microbes highlighted in Figure 3 will not be discussed or interpreted. Instead, we will focus on the significant differences in the overall structure of the community, emphasizing the metrics with significant differences (Table 2) when comparing microbial networks from healthy and dandruff-affected scalps.

Table 2. Network (Figure 3) topological metrics showing significant differences comparing healthy and dandruff-affected scalps, representative of 10 volunteers for each condition. Significance levels are indicated as follows: # = p-value < 0.1 ; * = p-value < 0.05 ; ** = p-value < 0.01.

metric	*Healthy scalp*	*Dandruff scalp*	*Difference*	*p-value*	*significance*
Clustering coefficient	25.2	74.4	(+)49.2	0.071	#
Positive edge percentage	76.47	84.55	(+)8.08	0.028	*

The clustering coefficient of a microbial network serves as a proxy for estimating the interdependence among the microorganisms within that network. A low to moderate level of interdependence among microorganisms enables the microbial community to be less sensitive to disturbances affecting only a single species; such communities are considered stable. In contrast, high interdependence reflects a community with low stability [24].

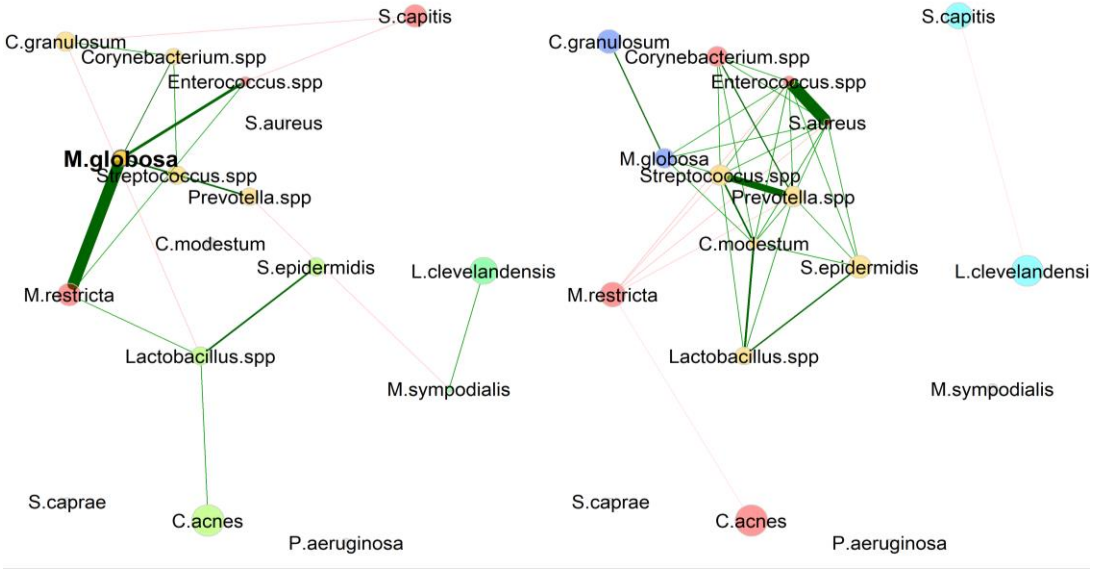


Figure 3. Representation of microbial networks based on Spearman correlation of the absolute abundances of bacteria and fungi targeted by qPCR. (a) Microbial network representative of a healthy scalp of 10 volunteers, without any treatment; (b) Microbial network representative of a dandruff-affected scalp of 10 volunteers, without any treatment.

The positive edge percentage reflects the overall relationships among microorganisms within the community. Positive links are interpreted as collaboration, while negative links indicate competition. A community characterized by a predominance of competitive relationships is described as more stable [19,25]; thus, a stable community is defined by a low to moderate proportion of collaborative interactions, represented by a low to moderate positive edge percentage. For these reasons we made the choice to describe Positive edge percentage as forced collaborations.

In Table 2, we observed that both the clustering coefficient and positive edge percentage are significantly higher in the case of dandruff-affected scalps. This indicates that interdependence and forced collaboration are greater in dandruff-affected scalps community compared to healthy scalps community, suggesting that microbial communities are more stable on healthy scalps.

3.3. Treatments effect

3.3.1. Impact on Erythema and Dandruff area

The utilization of a shampoo containing 3% Crocus sativus flower extract over 14 days resulted in a significant decrease in both erythema and dandruff area (Figure 4). This effect was significantly different compared to the vehicle treatment (Figure 4), which did not have a significant impact on either erythema or dandruff area.

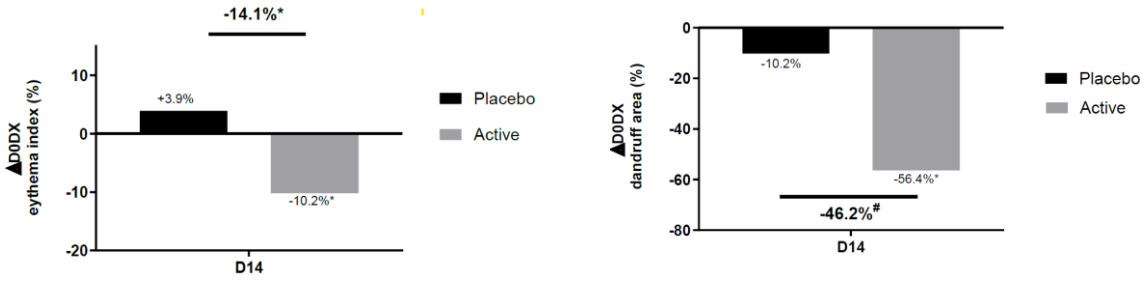


Figure 4. Treatment comparisons between the placebo (vehicle) and the active formulation (vehicle containing 3% Crocus sativus flower extract). (a) Evolution of erythema measured by C-Cube® in volunteers after 14 days of product application; (b) Evolution of dandruff area measured by Dandruffmeter® in volunteers after 14 days of product application.

3.3.2. Impact on absolute abundances

No significant changes (p-value > 0.1) in bacterial absolute abundances were observed at both the species and genus levels after 14 days of treatment, regardless of the treatment applied. On the other hand, significant reductions in *Malassezia* (both at the genus and species levels) were observed after 14 days of treatment with the active formulation (Figure 5b), while no significant impact was noted with the vehicle (placebo – Figure 5a). In case of *M. sympodalis*, the low mean abundance (1.86 genome copy numbers) combined with high variability (standard deviation = 5.69), may account for the lack of effect observed with the extract.

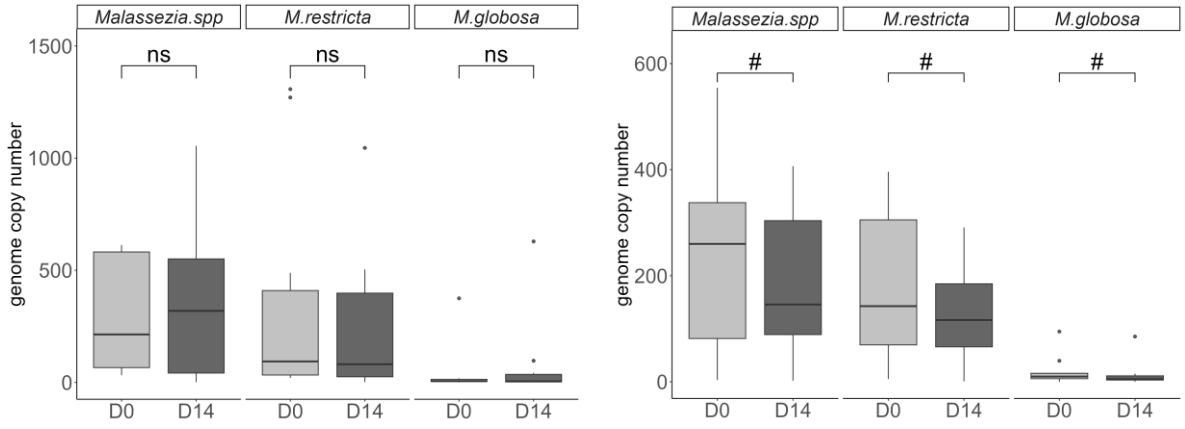
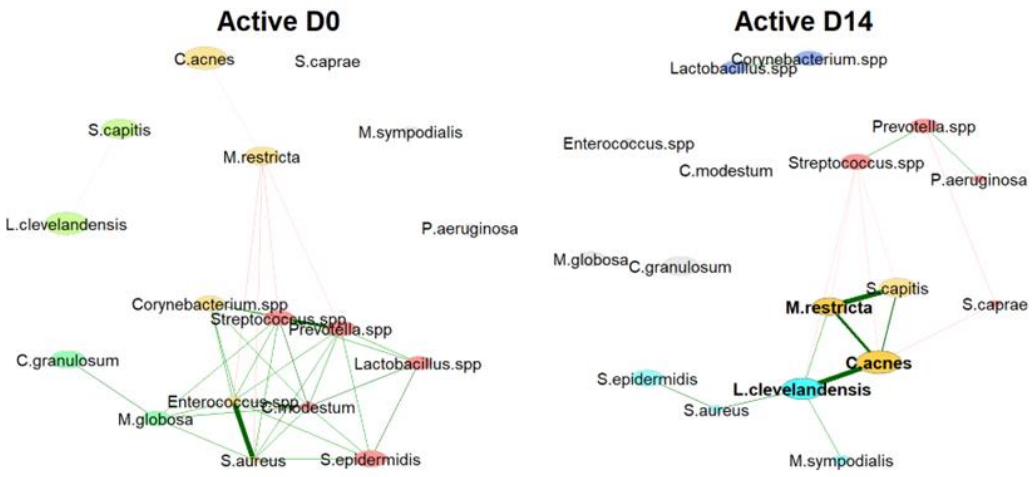


Figure 5. Dispertion of the abundances of the genus *Malassezia* and the two species *Malassezia restricta* and *Malassezia globosa* . (a) Abundance before and after 14 days of treatment with the vehicle; (b) Abundance before and after 14 days of application of the vehicule containing *Crocus sativus* flower extract.

3.3.3. Impact on the structuration of microbial community

After 14 days of treatment with Crocus sativus flower extract (Active), the inferred co-occurrence networks (Figure 6) revealed distinct structural changes in the scalp microbiota.



(a) (b)

Figure 6. Representation of microbial networks based on Spearman correlation of the absolute abundances of bacteria and fungi targeted by qPCR. (a) Microbial network observed before treatment with *Crocus sativus* flower extract; (b) Microbial network observed after 14 days of treatment with *Crocus sativus* flower extract.

Results depicted in Table 3 highlight the lack of significant effects after 14 days of treatment with the vehicle alone. In contrast, treatment with the active formulation (Table 4) resulted in a significant decrease in both the clustering coefficient and the percentage of positive edges.

Table 3. Network topological metrics comparing community structures before and after treatment with the vehicle. Significance levels are indicated as follows: ns = p-value > 0.1; # = p-value < 0.1; ** = p-value < 0.01.

metric	*Vehicle D0*	*Vehicle D14*	*Difference*	*p-value*	*significance*
Clustering coefficient	63.8	64.2	(+)0.4	0.777	ns
Positive edge percentage	66.7	63.5	(-)3.67	0.921	ns

Table 4. Network (Figure 5) topological metrics comparing community structures before and after treatment with the active formulation. Significance levels are indicated as follows: ns = p-value > 0.1; # = p-value < 0.1; ** = p-value < 0.01.

metric	*Active D0*	*Active D14*	*Difference*	*p-value*	*significance*
Clustering coefficient	74.4	54.7	(-)28.7	0.089	#
Positive edge percentage	84.55	52.17	(-)32.31	0.008	**

4. Discussion

Given the potential cytotoxic effects of *Malassezia restricta* [15], we anticipated a positive correlation between its abundance and erythema; however, no significant association was observed. In contrast, a significant positive correlation was found with the abundance of *Cutibacterium* spp. and specifically *Cutibacterium granulosum* (Figure 1). This finding was unexpected, as *Cutibacterium* spp. has been reported to contribute to maintaining a healthy scalp environment [26] and is often depleted in lesional sites of SD/D [14]. For *Cutibacterium granulosum*, it has been suggested that this species may play a role in preserving the normal skin barrier [27], making a positive correlation with erythema unexpected in this context. Nevertheless, as previously mentioned, discrepancies among studies are frequently

observed when examining specific genera or species [14]. To our knowledge, this is the first investigation into the relationship between scalp erythema and scalp microbiota, highlighting the need for further exploration, potentially down to the strain level, to gain a deeper understanding of the relationship between erythema and *Cutibacterium*.

Regarding the dandruff area (Figure 2), to our knowledge, the abundances of both *Staphylococcus capitis* and *Staphylococcus caprae* have not been previously studied in relation to SD/D. However, *S. capitis* has been reported to have a strong presence in atopic dermatitis and is the most abundant staphylococcal species identified in ichthyotic skin [28]. This suggests that a positive correlation between this bacterium and dandruff area may be plausible. Conversely, *S. caprae* has been associated with healthy scalps when compared to those of patients with severe alopecia areata [29]. Our observation of a negative correlation between *S. caprae* and dandruff area supports the notion that this bacterium may serve as a marker for scalp health.
In the case of *Malassezia* spp. and specifically *Malassezia restricta*, our findings of higher abundances associated with larger dandruff areas align with observations reported in most studies [13,14], thereby confirming the deleterious effect of *M. restricta* on scalp health.
When comparing the microbiota structures of healthy scalps and dandruff-affected scalps (Figure 3), we observed distinct organizational patterns. However, it is more informative to focus on the overall statistics of the networks rather than the connections between individual microorganisms, due to the potential influence of unreported factors, such as undetected microbial species and abiotic drivers, which can create indirect edges [19].

The statistics presented in Table 2 reveal significant differences in the microbiota structure between healthy and dandruff-affected scalps. Notably, dandruff-affected scalps exhibited higher clustering coefficients and positive edge percentages, indicating lower stability within the microbial community. A high clustering coefficient suggests a tightly-knit network that, while efficient, may be less stable due to its vulnerability to node loss. Increased efficiency can imply lower error tolerance, making these networks more susceptible to extinction events [24]. Furthermore, elevated clustering coefficients have been associated with greater levels of network degradation, which further diminishes stability [24]. To finish, positive correlations suggest cooperation among species, which can destabilize the community by fostering dependencies and positive feedback loops. Conversely, negative correlations reflect competition among microorganisms, contributing to a more stable microbiota structure [12,25]. Wang and colleagues [30] also observed that the microbiota of dandruff-affected scalps may be more susceptible to environmental interference, resulting in decreased stability.

After 14 days of treatment (Figures 4 to 6, Tables 3 and 4), we observed an improvement in scalp conditions with the use of *Crocus sativus* flower extract, evidenced by smaller dandruff flakes and reduced erythema. It is surprising that such improvement occurred despite the extract primarily only impacted *Malassezia* growth at the microbiota level. Given our previous

findings linking dandruff severity and erythema to various Cutibacterium and Staphylococcus species, changes in the abundance of these microorganisms could be anticipated. This discrepancy suggests that examining individual microorganisms alone is insufficient to capture the complexity of interactions between the microbiota and the host. When considering the microbiota as a whole through inferred networks, we found that *Crocus sativus* flower extract stabilized the structure of the scalp microbiota by lowering both the clustering coefficient and the positive edge percentage. The observed improvement in skin condition likely results from a synergistic effect between the extract's impact on the microbiota and its influence on other skin factors. This synergistic effect, along with the stabilization of the microbial community, may contribute to a long-lasting benefit, as the scalps of volunteers remained healthy even 14 days after discontinuing treatment with the extract.

5. Conclusion

This study offers novel insights into the intricate relationships between scalp microbiota and scalp health, particularly in relation to erythema and dandruff. While examining bacteria and fungi individually yields valuable information, it often leads to discrepancies across studies. Our research emphasizes the importance of viewing microorganisms as an ecological community, utilizing co-occurrence networks inferred from quantitative PCR data. Through this approach, we demonstrated that the skin microbiota on dandruff-affected scalps forms a less stable community compared to that on healthy scalps.

Interestingly, the improvement in scalp conditions observed following treatment with *Crocus sativus* flower extract did not correspond to the anticipated changes in the abundances of microorganisms associated with erythema and dandruff. However, when assessing the microbial community as a whole, we noted a significant stabilization post-treatment with the extract.

Furthermore, the beneficial effects of *Crocus sativus* flower extract on scalp conditions highlight the necessity of considering synergistic interactions within the microbiota and other skin factors. These findings underscore the need for further research, particularly at the strain level, to enhance our understanding of the scalp microbiome's role in health and disease.

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