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Biophysical and Microbial Conditions in Dandruff-afflicted Scalp Skin of Chinese Young Consumers, and the Shampoo Solutions to Address the Root Cause from Microbiota Perspective

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1. Introduction

Extensive research has been conducted on scalp health, particularly concerning conditions like dandruff. With advancements in sequencing technologies, recent studies have increasingly involved the microbial ecology of the scalp, shedding light on the relationship between microbiota and dandruff. However, most of them focus on visual dandruff assessments, or sometimes combined with amplicon sequencing techniques (e.g., 16S rRNA for bacteria and ITS for fungi). These sequencing methods are limited to taxonomic information that are often biased in abundance and left microbial functions unclear.

Hereby, we employed a more comprehensive approach for the study of dandruff situation in young chinese volunteers. Multiple aspects are considered in this study. For example, by utilizing metagenomic sequencing, we obtained species-level microbial data which significantly enhanced the breadth and depth of the analysis.

To specify, young volunteers from age 18 to 25 with healthy or dandruff-afflicted scalps were recruited. The basic conditions are detected with scalp samples collected before the dandruff volunteers were further divided into two groups and applied blank/active shampoo respectively. Same strategy was used to study the change of parameters before-after the shampoos application, in order to investigate the functions of both shampoos. By conducting microbial functional analysis, the mechanisms of action for both shampoos are revealed. The results suggest a fundamental difference on microbiota between healthy and dandruff-afflicted scalps in young volunteers. At the same time, the effect of shampoo base or antidandruff triplex proved crucial at altering the microbial functions.

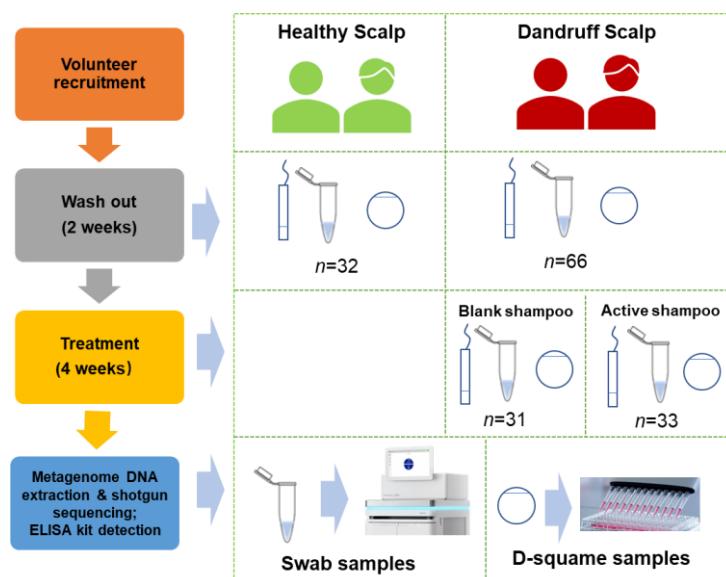


Figure 1. Illustration of the test flow: recruitment of the volunteers; after 2-weeks washout phase, the volunteers are separated as dandruff group(n=66) and healthy group(n=32) with biophysical parameters detected, scalp samples collected for subsequent analysis of microbiota and interleukins; after 4-weeks treatment for 2 dandruff groups by using blank and active shampoos respectively, all parameters are detected and scalp samples collected again.

2. Materials and Methods

Ethics approval and consent to participate:

The study was approved by the Scientific and Ethical Committee at Shanghai Jiao Tong University (approval ID: B20240204I).

Volunteer recruitment:

A total of 208 volunteers were recruited. Inclusion criteria were as follows: 1) aged 18–25 years (both males and females), 2) with hair length ≥ 5 cm, 3) the healthy group had an ASFS score ≤ 6 (scalp divided into 8 regions and scored using a 10-point scale, with scores summed); after a 2-week washout period, the ASFS score remained ≤ 6 , 4) the dandruff group had an ASFS score ≥ 10 with visible dandruff (scalp divided into 8 regions and scored similarly); after the 2-week washout period, the ASFS score remained ≥ 10 .

Sampling of the scalp microbiome and collection of scalp skin characteristics:

All the volunteers washed their hair every two days using a shampoo placebo with no active ingredients for two weeks, and 48 hours after their last wash, the first-run scalp sample collection was conducted. The sample collection of the scalp microbiome was performed on either the vertex region for healthy scalp, or the most severe dandruff site for dandruff scalp using swabs. The physiological parameters including TEWL, sebum, hydration and pH were detected at the adjacent location to the sampling site(Delfin Vapometer®, Sebumeter®SM-815, Dermalab®combo were used). The D-squame tapes were used to collect the samples for subsequent biomarker analysis (total protein, interleukins, and AMPs). For the AMPs samples, only a part of dandruff scalps was selected for collection.

After the first-run, 66 volunteers with dandruff were randomly divided into two groups that 32 of them used a blank shampoo without anti-dandruff agent and the other 34 used an active shampoo (which contains piroctone olamine, sorbitan caprylate and succinic acid) every two days. After 4 weeks of treatment, and 48 hours after the final wash, the sample collection and the physiological parameters detection were performed again following the same methods used in the first-run.

Metagenome DNA extraction and shotgun sequencing:

Total microbial genomic DNA samples were extracted using the OMEGA Mag-Bind Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's instructions, and stored at -20°C prior to further assessment. The extracted microbial DNA was processed to construct metagenome shotgun sequencing libraries with insert sizes of ~400 bp by using Illumina TruSeq Nano DNA LT Library Preparation Kit (Illumina, USA). Each library was sequenced by Illumina NovaSeq platform (Illumina, USA) with PE150 strategy at Personal Biotechnology Co., Ltd. (Shanghai, China).

Compositional and functional profiling:

MetaPhiAn4 (Blanco-Miguez et al., 2023) v.4.0.6 was used to generate taxonomic profiling data with database version mpa_vOct22_CHOCOPhiAnSGB_202212j. HUMAnN3 v3.7 was used to predict the gene families of each microorganism in each sample based on the taxonomic profile from MetaPhiAn4, and then summed their abundances according to the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations.

The significant differences in the relative abundance of GO annotations and KEGG pathways between the dandruff group and the healthy group were calculated using the LinDA package in R software, with sample coverage of functional terms >20%. The p-values were post-adjusted using the FDR method. Significant differences of the physiological parameters, interleukins, the relative abundance of microorganisms between the dandruff and healthy groups were analyzed using the Mann-Whitney test in GraphPad Prism software. The significant differences before and after treatment with shampoos were analyzed using the paired Wilcoxon test in GraphPad Prism software.

3. Results

We performed a 2-step study to investigate the relationship between dandruff and scalp microbiota functionality using metagenomic sequencing technology (Fig.1). First, we compared the biophysical properties, interleukins and compositions of the scalp microbiota between volunteers with dandruff ($n = 66$) and healthy scalps ($n = 32$). Second, the volunteers in the dandruff group were randomly divided into 2 subgroups, each using a different shampoo for 4 weeks. One group ($n= 31$) used a blank shampoo based on a simple composition with surfactants, conditioning agents and necessary preservatives. The other group ($n = 33$) used a shampoo with same composition as blank shampoo but containing additionally anti-dandruff triplex (piroctone olamine, sorbitan caprylate, and succinic acid).

3.1 Dermographics, scalp skin characteristics and microbiota difference between the dandruff and healthy Scalp

The dandruff rating in our study has medium-to-low severity, which reflect a more realistic condition in modern life. By comparing the dandruff with healthy scalps, they not only show elevated ASFS score, but also higher sebum, TEWL and pH level than healthy group, while its hydration level is significantly lower(Fig.2). In the dandruff group, the levels of IL-1 α and IL-1ra both decreased ($P<0.05$). The ratio of IL-1ra : IL-1 α increased in dandruff group as previously reported (but no sig. difference) [1].

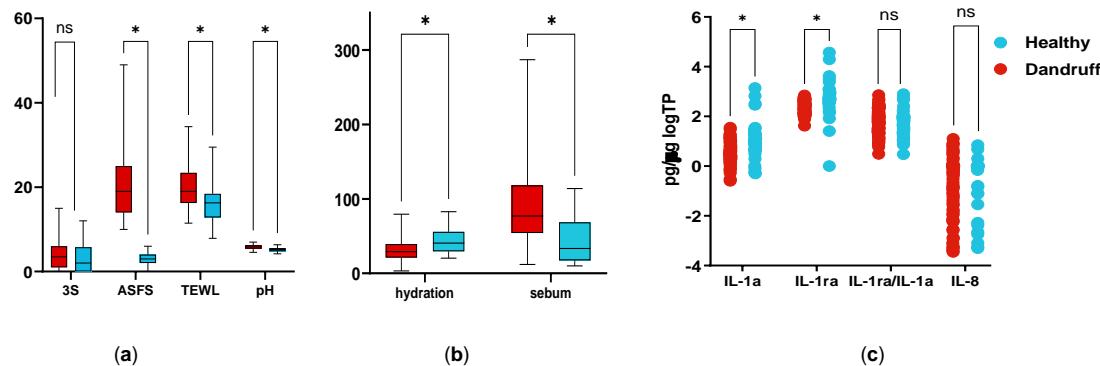


Figure 2. Comparison of self-evaluation, biophysical parameters and interleukins between dandruff and healthy scalp: (a) 3S score, ASFS score, TEWL and pH levels; (b) hydration and sebum levels; (c) Relative levels of interleukins IL-1 α , IL-1ra, IL-8 and the ratio of IL-1ra /IL1 α . (the differences with P<0.05 are labeled with *)

The dandruff group exhibited a total of 185 microbial species, whereas the healthy group harbored 133 species (data not shown). Both groups shared six microbial species with an average relative abundance exceeding 1%, namely *Cutibacterium acnes*, *Lawsonella clevelandensis*, *Malassezia restricta*, *Staphylococcus capitis*, *Cutibacterium namnetense*, and *Malassezia globosa* (Fig. 3). The first two species collectively accounted for over 50% of the total abundance. The prevalence of these high-abundance microbes on the scalp aligns with previous research[2].

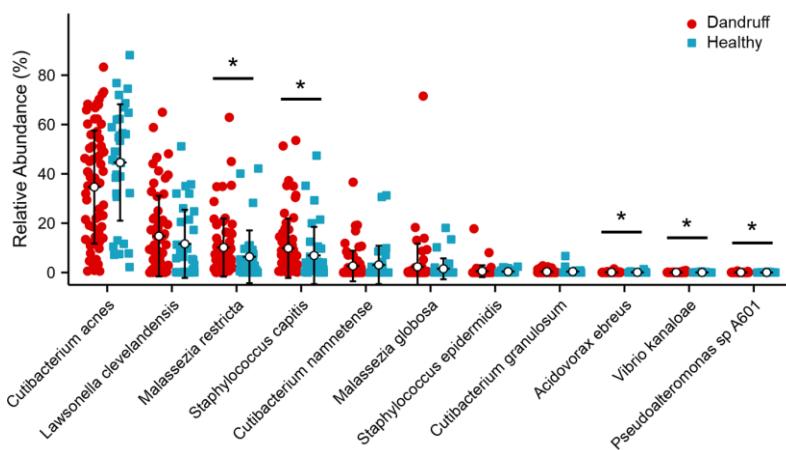


Figure 3. The relative abundance of dominant microbiota species on dandruff scalp and healthy scalp (the differences with P<0.05 are labeled with *).

C. acnes exhibited lower abundance in the dandruff group compared to the healthy group, although this difference did not reach statistical significance. *M. restricta* and *S. capitis* were significantly more abundant in the dandruff group. Furthermore, *C. namnetense* and *M. globosa* also showed increased abundance in the dandruff group ($P > 0.05$). Beyond these six species, several other bacteria, including *Acidovorax ebreus*, *Vibrio kanaloae*, *Pseudoalteromonas sp. A601* (Fig. 3), *Malassezia sympodialis*, *Escherichia coli*, *Pseudoalteromonas prydzensis*, and *Streptococcus varani* (data not shown), also displayed distinct differences between the two groups. Most of these species were significantly more abundant in the dandruff group than in the healthy group, with the exception of *Erythrobacter donghaensis* (data not

shown). These findings suggest that the dandruff scalp harbors a unique microbiota, characterized by higher microbial diversity and increased relative abundance for most species.

3.2 Interaction between microbiota, scalp skin characteristics and biomarkers

To further investigate the interplay between scalp physical conditions, microbiota, and biomarkers, a correlation analysis was performed on these parameters, as illustrated in Fig. 4. The severity of dandruff (ASFS) exhibits a positive correlation with biophysical attributes such as transepidermal water loss (TEWL), pH levels, and sebum production. This indicates that the condition is associated with barrier dysfunction or altered sebum secretion. Conversely, ASFS demonstrates a negative correlation with IL-1 α and IL-1ra ($P<0.01$). Although direct evidence linking ASFS to IL-1ra/IL-1 α is lacking, TEWL shows significant relevance to both IL-8 and IL-1ra/IL-1 α ; thus suggesting that these biomarkers may reflect underlying skin barrier issues.

By examining the relevance of the microbiome and other attributes more closely, it becomes evident that the top four species are significantly involved. *S. capititis* interacts with hydration, pH levels, and sebum production, contributing to a less healthy scalp condition. *C. acnes*, *L. clevelandensis*, *M. restricta*, and *S. capititis* exhibit strong associations with interleukin biomarkers. Notably, *M. restricta* and *S. capititis* show positive correlations with IL-8, suggesting their roles in elevating both inflammatory markers.

Regarding microbiota interactions, *C. acnes* serves as a key species that inhibits most other species except for *A. ebreus*. Additionally, *M. globosa* impedes the growth of *L.clevelandensis* and *C.namnetense*.The remaining major species appear to mutually support one another.

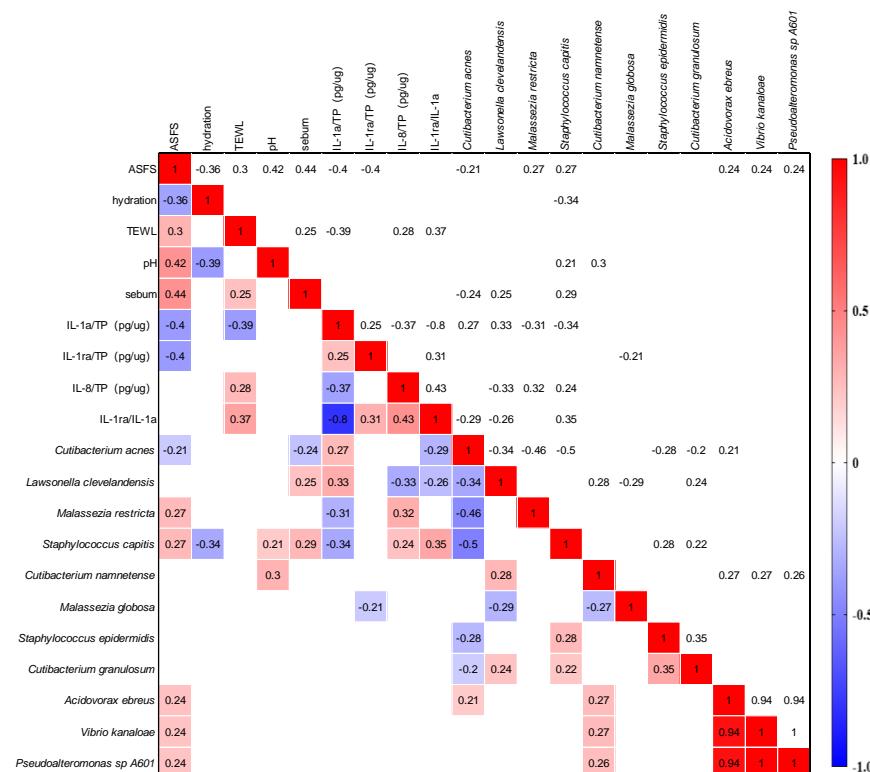


Figure 4. Correlation between dandruff severity measured by ASFS, biophysical parameters, interleukins and the top microbial species in all volunteers including both dandruff and healthy scalps. (The statistically significant correlation ($p<0.05$) was represented by the color of the square and the number.)

3.3 Intervention of dandruff by shampoos – the change of scalp skin characteristics and microbiota

Following four weeks of treatment, ASFS scores and all other biophysical parameters improved significantly for both groups. However, the group of active shampoo shows more significant improvement of dandruff score, skin barrier(TEWL) and hydration level(Fig. 5), along-side a significant decrease in the IL-1ra/IL-1 α ratio ($P < 0.05$, Fig. 6). Given its importance as a biomarker for dandruff and seborrheic dermatitis[3], the observed reduction in the IL-1ra/IL-1 α ratio suggests an alleviation of inflammation, likely attributable to the anti-dandruff triplex in the active shampoo. As a result, the overall performance of active shampoo wins over the placebo.

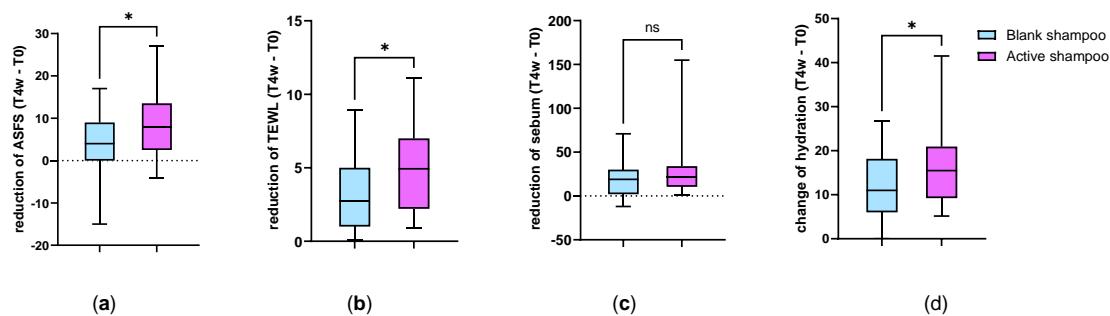


Figure 5. Pair comparison of biophysical parameters before/after the blank/active shampoo use for 4 weeks: (a) reduction of ASFS score; (b) reduction of TEWL; (c) reduction of sebum level; (d) change of hydration level (the differences with $P < 0.05$ are labeled with *).

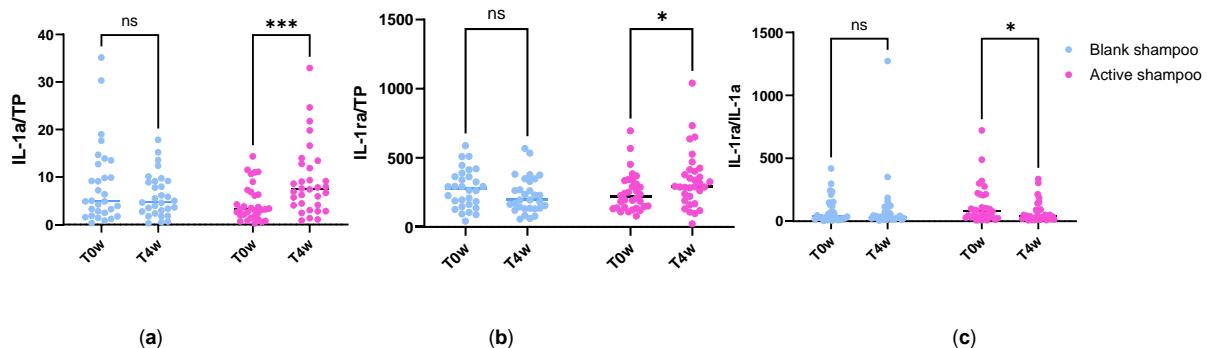


Figure 6. The change of interleukins level/ratio before/after the shampoo use: (a) ASFS score; (b) TEWL; (c) sebum level; (d) hydration level (the differences with $P < 0.05$ are labeled with *, $P < 0.001$ with ***).

Regarding the changes in microbial abundance, the blank shampoo significantly increased the abundance of *C. acnes* ($P < 0.05$), while having no significant impact on other dominant species(Fig. 7). Conversely, the active shampoo increased the abundance of *L. clevelandensis* ($P < 0.05$, Fig. 7) and reduced that of *S. capitis* ($P < 0.05$, Fig. 7). These findings suggest that both shampoos, through distinct mechanisms, partially altered the microbial composition associated with dandruff.

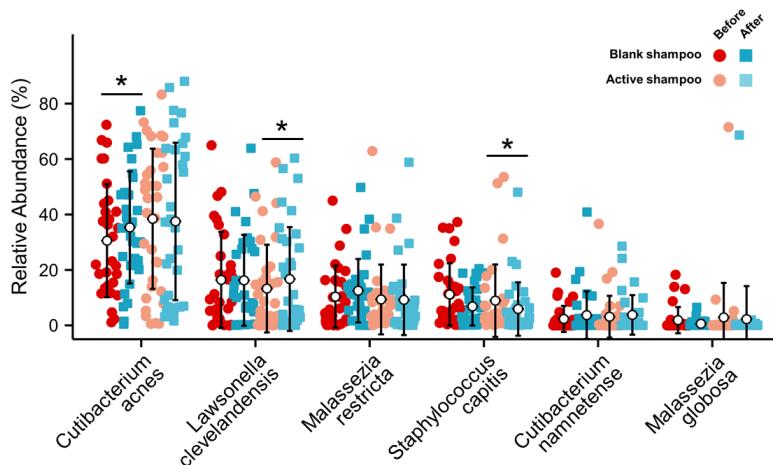


Figure 7. The change of relative abundance of dominant microbiota species before/after the shampoo use(the differences with $P<0.05$ are labeled with *).

3.4 Gene ontology and pathway analysis shows pathogenesis and amino acid transport as key biological process related to dandruff

In total, 653 significantly altered Gene Ontology annotations were identified, with 584 upregulated and 69 downregulated in the dandruff group. Among these, the most upregulated Gene Ontology annotations in dandruff group were biological process “pathogenesis”, and the most downregulated GOs were the BPs of alanine dehydrogenases, *L*-alanine catabolic process and amino acid transport (Fig. 8). And KEGG analysis also revealed the accumulation of amino acids due to their elevated biosynthesis (Fig. 9).

Following a 4 weeks treatment with shampoos, the GOs of [BP] pathogenesis showed reduction while [MF] alanine dehydrogenases and [BP] *L*-alanine catabolic process were upregulated by shampoo washing (Fig. 8), illustrating how shampoo washing can adequately remove the pathogenesis factor as well as improve the alanine transformation. Nevertheless, the anti-dandruff triplex in the active shampoo was more effective at changing alanine transformation and [BP] DNA replication initiation (Fig. 8). Alanine is a key component in the synthesis of peptidoglycans[4], and a reduction in its catabolism may promote peptidoglycan synthesis. In addition, alanine may enhance the synthesis of other amino acids(AA) such as threonine. All this manifest the unique actions of the antidandruff triplex intervening the microbial proliferation by limiting the cell wall and AA synthesis, as well as DNA replication.

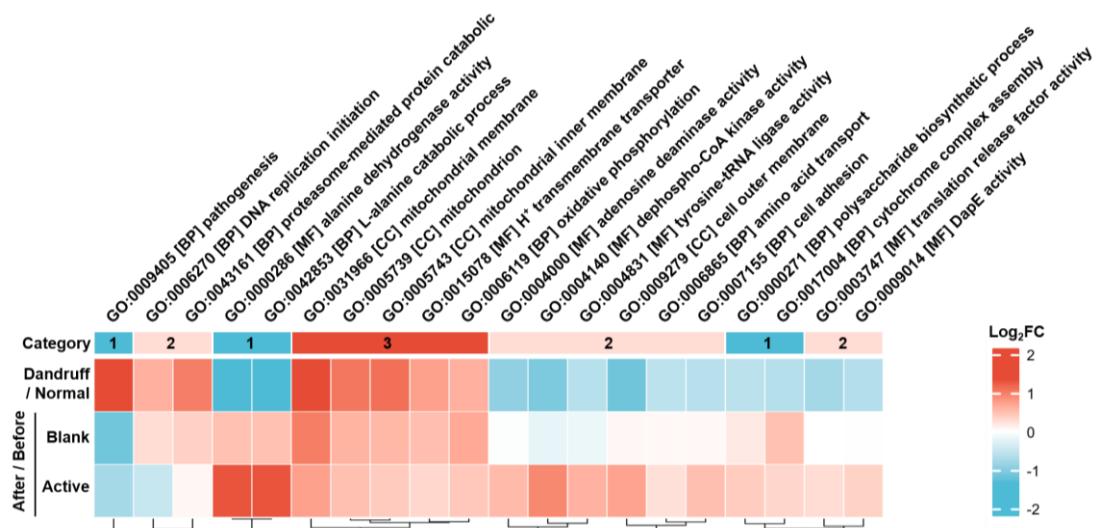


Figure 8. Heatmap of fold changes in the top 20 GO annotations to compare Dandruff/Healthy scalp, After/Before use of blank shampoo or active shampoo.

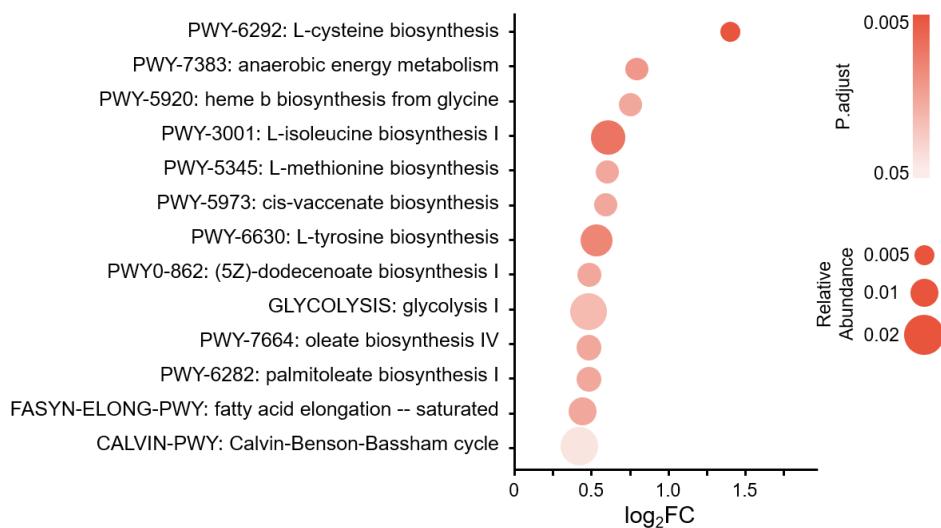


Figure 9. Abnormal KEGG pathway analysis for dandruff scalp microbiome.

4. Discussion

This study was conducted on a special cohort of young individuals presenting mild to moderate dandruff. The difference of ASFS scores, biophysical attributes are in line with previous studies, except the key biomarkers of IL-8 and IL-1ra: IL-1 α ratio. This can be attributed to the study participants, whose dandruff symptoms are not severe -- the inflammatory cascade remains at a low level. However, IL-1ra: IL-1 α ratio can still be tuned down greatly by employing active shampoo containing antidandruff triplex, suggesting the actives effective at regulating the inflammatory conditions.

In the microbiome analysis, we found 6 dominant microbial species concomitantly existing on both healthy and dandruff scalps. However, they are slightly different from the result reported by Hu et al. using metagenomic sequencing[2]. *L. clevelandensis* and *C. namnetense* is relatively more abundant in this study which may be relevant with the age of volunteers recruited or their residential location. Despite the non-severe dandruff symptoms, *S. capitis* and *M. restricta* can be discriminated between dandruff and healthy scalps among the key dominating species. Similar finding has been published although by using qPCR techniques[5].

After the shampoo treatment, the two groups exhibit different mode of microbiota change. The blank shampoo increased the abundance of *C. acnes* ($P<0.05$), while the active shampoo greatly reduced the abundance of *S. capitis* ($P<0.05$). In either groups, another key species- *M. restricta* was not affected after 4 weeks test. The triplex used in our study contains piroctone polamine (PO), sorbitan caprylate (SC) and succinic acid (SA). PO is a wellknown antidandruff active, proved to be effective when used in shampoo to shift the microbial composition(by reducing the abundance of *M. restricta*, *M. globosa*, and *S. capitis* [2]). SC works as a preservative booster usually[6]. SA has been applied in food industry to combat *Salmonella* or *E. Coli*[7] and is recognized as one kind of short-chain fatty acids (SCFAs) which can suppress the growth of *C. acnes*[8]. This might be the reason that the blank shampoo boost the abundance of *C.acnes* where as the active shampoo doesn't. Despite this, the antidandruff triplex together proved to be more effective on inhibiting *Malassezia spp.* and *Staphylococcus spp.* than PO alone in *in-vitro* model(data not shown). But the active shampoo didn't perform to decrease *M.*

restricta, which may be caused by the shampoo base - a slight increase of *M. restricta* was observed in the blank shampoo group(no significant difference).

By applying GO and KEGG functional analysis, we could tell the underlining causes of dandruff, lying particulary on 'pathogenesis' and 'amino acid transportation'. Using basic shampoo can remove most of the microbials so as to downreulate the above two processes to some extent. The active shampoo containing antidandruff triplex could enhance the effects and intervenes DNA replication in addition, resulting in better alleviation of dandruff.

5. Conclusion

We performed a study to investigate the difference between dandruff and healthy scalps, and the impact of using blank/active shampoos on the dandruff scalps, employing mutiple research approaches including microbiome metagenomic sequencing. Due to the relative moderate dandruff symptoms in our participants(closer to modern reality), the difference between dandruff and healthy scalps are not that significant as previously reported(e.g. interleukins). But the correlation analysis disclosed the relevance of such moderate dandruff situation and skin barrier condition, with biophysical parameters, interleukins and together the dominant microbiomes.

Combinning mcriobiota and functional analysis, we discovered the underlying mechism of action for base shampoo and the antidandruff triplex. Abnormal amino acid metabolism and elevated pathogenesis are the most critical processes related to dandruff. Simple washing can hardly change the microbiota conditions but is applicable to relieve the symptoms as well as alter the selective microbial functions. Addition of antidandruff triplex shows obvious advantages over basic washing by regulating the microbiome/functions more effectively.

As nowadays the hygiene condition in China improved greatly, the dandruff situation among general populations is no longer severe. Our study involving medium-low dandruff scalps, builds a fundamental basis to understand the realistic scalp issue from multiple points of view. This robust dataset also provides deeper insights into the mechanisms underlying the clinical observation, paving the way for more targeted solutions such as selective microbiota augmentation/inhibition.

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