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"Ideal controlling of androgenetic alopecia clinical characteristics with rebalancing scalp microbiome dysbiosis via droplet digital PCR"

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1. Introduction

In our previous studies, the bacterial flora differences between sex according to the severity of androgenetic alopecia (AGA) was revealed. Interestingly, the scalp microbiome (SM) of male and female was totally different, and scalp or hair conditions affect the AGA stage and are correlated with the microbiome. As AGA progressed, *Cutibacterium* and *Staphylococcus* proportion (%) decrease and alpha diversity increase accordingly. Thus, it indicates that AGA is more affected by multi-bacteria than single, because of dysbiosis in the SM [1]. SM dysbiosis can be considered to be an extrinsic factor as important as genetic or hormones in hair loss and rebalancing can be a solution [2, 3]. These findings support our ongoing research into modulating the SM as a potential approach to mitigate AGA-related changes. However, so far, no study has accurately assessed the absolute microbial abundance of SM to determine whether controlling SM imbalance can alleviate the clinical characteristics of AGA.

Among the predominant taxa in the SM, *Actinobacteriota, Firmicutes, Proteobacteria*, and *Bacteroidota* were the most abundant, with *Actinobacteriota* being more prevalent in healthy individuals and *Firmicutes* being more prevalent in individuals with hair loss [1, 4]. Therefore, within the *Firmicutes*, *Staphylococcus aureus*, which thrives in high-pH environments and contributes to persistent negative scalp conditions, was identified as a target bacterium for alleviating AGA [5]. Using essential oils known for their antimicrobial properties and hair loss prevention effects, we aimed to regulate the SM imbalance using them. A newly formulated blended essential oil (BEO), combining lemongrass [6], ylang-ylang [7], and lavender [8] as targeted ingredients to control AGA-associated bacteria, was incorporated into products to evaluate its ability to restore the microbial balance in the AGA group to resemble that of the healthy control (HC) group.

This study aims to demonstrate for the first time whether modulating AGA microbiome imbalance has the potential to change clinical symptoms. In addition, to determine and verify the recovery of the SM, it is essential to accurately understand the abundance of the SM. Therefore, we aim to overcome the limitations of relative microbiome profiling (RMP) by utilizing quantitative microbiome profiling (QMP) to improve accuracy. Through this study, we propose clinical changes, ideal validation, and application methods by AGA SM imbalance control.

2. Materials and Methods

Study design (Clinical parameters and microbiome sample collection)

This study scheme was approved by Institutional Review Board (N01 2022022 001 HR), and a total of 60 subjects (male: 28, female: 32) submitted written informed consent after being fully informed about the study process. Through the visual evaluation of the researchers, the subjects were first divided into two groups based on the degree of hair loss by referring to the Basic and Specific classification criteria; 30 HC, and 30 AGA group consisting each 28 male and 32 female. All selected participants were restricted to using only the shampoo provided by the institution for a total of 5 weeks. They visited three times (baseline, after 2 weeks, and after 5 weeks), and clinical measurements and microbial sample collection were conducted at each visit. The shampoo (TEST or CON) was provided in a blinded, and participants were instructed to use the shampoo once daily, applying it for 2-3 minutes before rinsing. Except for the inclusion of 10,000 ppm BEO (TEST), all other formulations were identical between CON and TEST. A total of eight clinical conditions of scalp (moisturizing, sebum, desquamation, redness, and temperature) and hair (thickness, density, and gloss) were measured prior to microbiome sample collection at each visit. The scalp microbial samples were collected with a sterile cotton swab for 3 minutes and were stored at -80°C until further processing.

16S rRNA gene amplicon sequencing and downstream analysis

The quality and quantity of the extracted DNA was evaluated using the NanoDrop One Microvolume UV-Vis Spectrophotometer and the Qubit Flex Fluorometer (Thermo Fisher, USA), respectively. The V3-V4 hypervariable region of the 16S rRNA gene was targeted for PCR amplification with in-house primers designed to improve coverage when assigning bacterial taxonomic information using SILVA database. Amplified sequencing libraries were purified and sequenced on the Illumina MiSeq platform (Illumina, USA) at the KNU NGS core facility (South Korea). Raw sequencing data were quality-filtered and processed using the Quantitative Insights into Microbial Ecology 2 (QIIME2) pipeline [9]. Amplicon Sequence Variants (ASVs) were identified, and taxonomy was assigned referencing the SILVA database.

Quantitative microbiome profiling (QMP)

Bacterial quantification was performed using droplet digital PCR (ddPCR) to target the 16S rRNA gene, measuring gene copies per 1.5 mL of skin swab preservative. For microbiome quantification, the total 16S rRNA gene copies per 1.5 mL were converted to bacterial cell counts per 1.5 mL. Initially, the average 16S rRNA gene copy number for each sample was obtained from the rrnDB35 database [10] by dividing total sequencing reads by the average 16S rRNA gene copies. The ddPCR-derived total 16S rRNA gene copies per 1.5 mL were then divided by this sample-specific average to estimate bacterial cells. Subsequently, samples were normalized to a uniform sampling depth, determined by the corrected 16S rRNA gene copy number and microbial load, represented as the average bacterial cell count per 1.5 mL of skin swab preservative.

Shotgun metagenome sequencing and downstream analysis

High-quality DNA was utilized for sequencing library construction using the DNBSEQ-G400RS High-Throughput Sequencing FCL PE100 Kit (MGI Tech, China). Shotgun metagenome sequencing was carried out on the DNBSEQ-G400RS platform (MGI Tech, China) at the KNU NGS core facility (South Korea). Adapter sequences were removed from raw sequencing data, and low-quality reads were filtered out using SOAPnuke v2.1.7 [11]. Reads were then mapped to the GRCh38 human reference genome with Bowtie2 v2.5.0 [12], and aligned reads were excluded from downstream analysis. Quality-filtered reads were used for taxonomic profiling

with Kraken2 v2.1.2, employing a bacterial genome from the NCBI. Microbial abundance was quantified at various taxonomic levels using Bracken v2.8 based on Kraken2 results [13].

Statistical analysis

All statistical analyses and data visualizations were conducted using R software version 4.2.2. Beta-diversity was assessed using both principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS), each based on Bray-Curtis dissimilarity matrices using vegan v.2.6-8 and phyloseq v1.48.0 R packages. Group-wise differences in microbial community composition were statistically evaluated using permutational multivariate analysis of variance (PERMANOVA). Additionally, taxon-specific abundance patterns across different microbiome profiling methods were visualized in three-dimensional plots using the scatterplot3d v0.3-44 R package to facilitate integrated comparisons between groups.

3. Results

3.1. 16S amplicon microbiome (HC vs AGA in 0 week)

Beta diversity analysis using PCoA based on Bray-Curtis dissimilarity revealed a significant difference in scalp microbial community structure between HC and AGA in the total cohort ($p = 0.033$) (Figure 1). Stratified analysis showed that this difference was significant in females ($p = 0.003$), but not in males ($p = 0.261$).

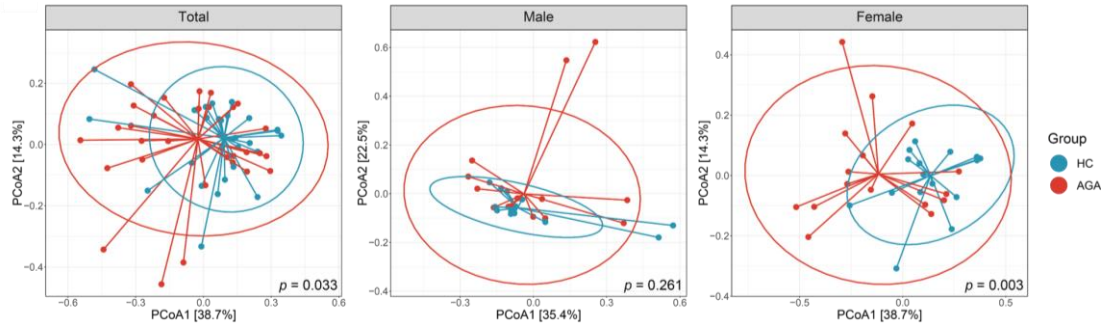

Figure 1. Comparison of SM beta diversity between HC and AGA. PCoA plots based on Bray-Curtis dissimilarity showing in the distribution of microbial communities in HC (blue) and AGA (red) groups. Plots are shown for the total population, males, and females. Each point represents an individual, and ellipses indicate 95% confidence intervals.

Alpha diversity, assessed by Chao1 and Shannon indices, was significantly higher in the AGA group compared to the HC group in the overall population ($p < 0.01$ for all indices) (Figure 2a). This trend was primarily observed in females, where all indices were significantly increased in the AGA group ($p < 0.01$). No significant differences were detected in males for either index (Figure 2b).

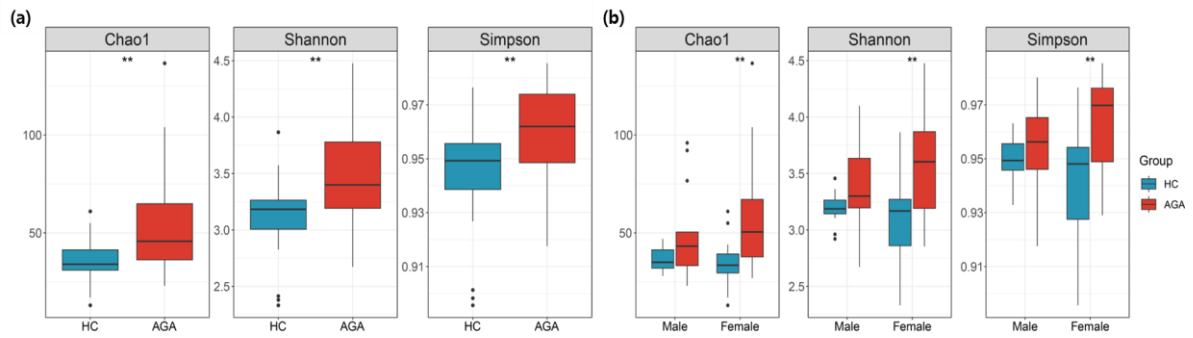

Figure 2. Comparison of SM alpha diversity between HC and AGA. Boxplots of diversity metrics (Chao1, Shannon, and Simpson indices) for the (a) total population and (b) sex-stratified groups of males and females.

3.2. RMP vs QMP (0 week)

To investigate taxonomic differences between HC and AGA, we analyzed SM using both RMP based on 16S rRNA gene sequencing (Figure 3a and b) and QMP integrating 16S data with absolute bacterial load measured by ddPCR (Figure 4a and b). Also, we compared the relative and absolute abundances of three dominant scalp bacterial genera—*Cutibacterium, Staphylococcus*, and *Lawsonella*—between between HC and AGA, stratified by sex.

In the RMP, the relative abundance of *Cutibacterium* was increased in AGA females compared to HC females ($p < 0.5$), while *Staphylococcus* showed reduced relative abundance in AGA females ($p < 0.01$). Also, *Lawsonella* was significantly increased in HC males compared to AGA males ($p < 0.05$).

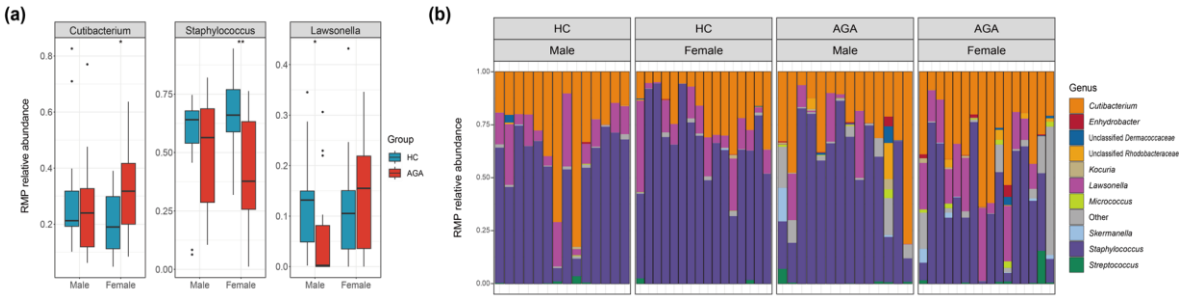

Figure 3. Comparison of scalp microbial composition between HC and AGA using RMP. The relative (a) abundance of three major bacterial genera (*Cutibacterium, Staphylococcus, and Lawsonella*), stratified by sex and AGA. Stacked bar plots showing the RMP (b) in top 10 genera across individual subjects, grouped by sex and AGA.

In the QMP, which incorporate total bacterial load from ddPCR, *Cutibacterium* was markedly reduced in AGA males ($p < 0.05$), and *Staphylococcus* showed decreased absolute abundance in AGA females ($p < 0.05$). *Lawsonella* was significantly higher in HC males compared to AGA males ($p < 0.01$), but the distinction in males was more evident compared to RMP data.

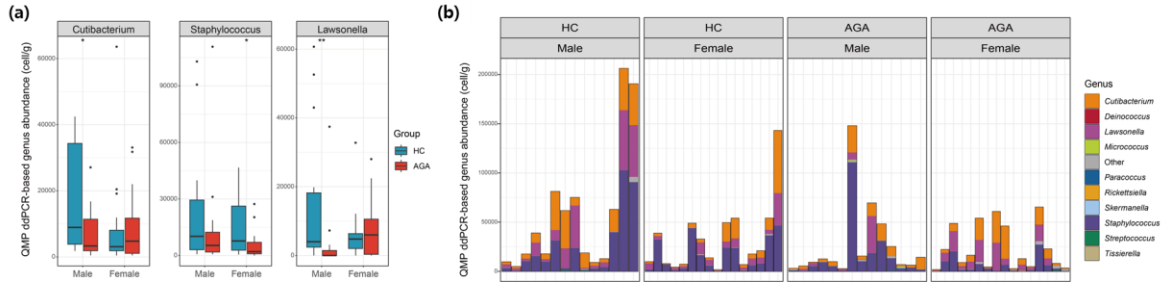

Figure 4. Comparison of scalp microbial composition between HC and AGA using QMP. The absolute (a) abundance of three major bacterial genera (*Cutibacterium, Staphylococcus, and Lawsonella*), stratified by sex and AGA. Stacked bar plots showing the QMP (b) in top 10 genera across individual subjects, grouped by sex and AGA. Asterisks indicate statistical significance *(*p < 0.05, **p < 0.01*).

These findings highlight discrepancies between relative and absolute abundance data, emphasizing the added resolution provided by QMP. While RMP captures compositional shifts, QMP allows for detection of true microbial loss or enrichment by accounting for total bacterial load. Notably, the increase in *Lawsonella* in HC males appears more prominent in absolute abundance than in relative terms.

3.3. Scalp microbiome rebalancing (0w vs 2w vs 5w in QMP)

To assess the effect of BEO treatment on SM, we performed longitudinal profiling at 0, 2, and 5 weeks in HC and AGA, with and without treatment (CON vs TEST). NMDS plots showed that the microbial community structure exhibited greater dispersion over time in AGA compared to HC, and within AGA, greater dispersion was observed in CON than in TEST (Figure 5a and b). In other words, the HC group exhibited a relatively more cohesive structure compared to the AGA group. Although not statistically significant, it was observed that in the AGA-TEST group, particularly among females, the bacterial flora became more cohesive over time, resembling the HC group due to BEO treatment.

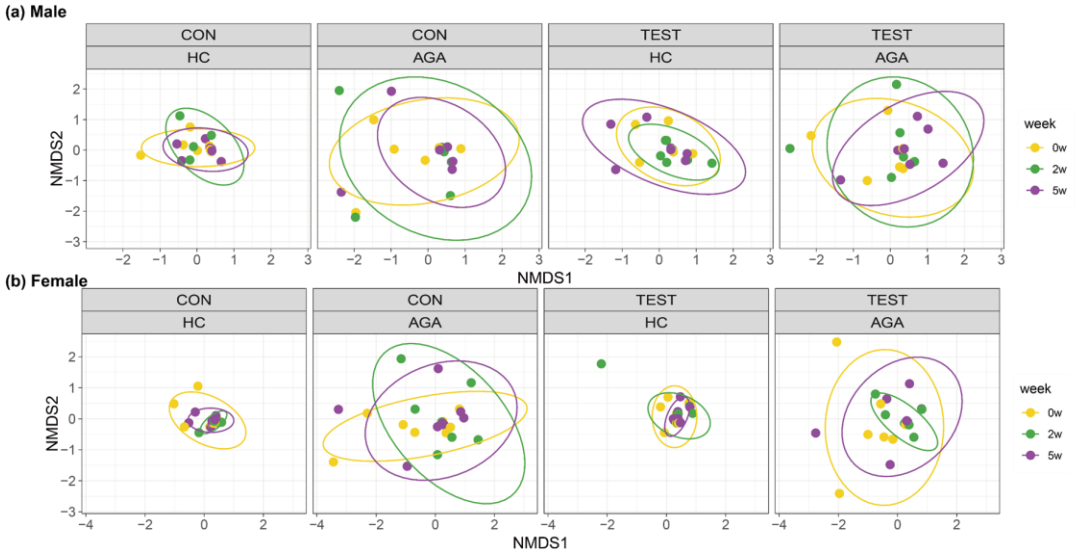

Figure 5. Temporal dynamics of SM in HC and AGA, with and without BEO treatment. NMDS plots based on Bray-Curtis dissimilarity in male (a) and female (b). Each point represents a sample collected at week 0 (yellow), week 2 (green), and week 5 (purple). Ellipses represent 95% confidence intervals.

In males, upon BEO treatment, the absolute abundance of three major genera (*Cutibacterium, Staphylococcus,* and *Lawsonella*) increased further in the AGA of TEST group at week 2 and remained enhanced through week 5 (Figure 6a). In females, although a baseline difference in *Staphylococcus* abundance was observed between HC and AGA at week 0, no significant change was detected in response to BEO treatment over time (Figure 6b).

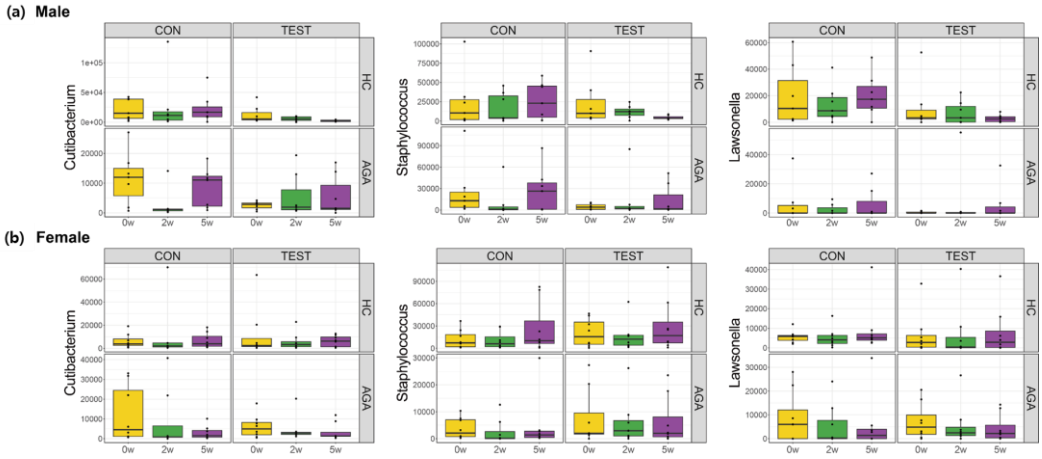

Figure 6. Temporal dynamics of SM in HC and AGA, with and without BEO treatment. Absolute abundances of three major genera across sampling points (week 0, 2, and 5) in male (a) and female (b).

3.4. Metagenome vs RMP vs QMP (in 0 week)

To assess baseline differences in SM between HC and AGA, metagenomic profiling on samples collected at week 0, stratified by sex. PCoA based on Bray-Curtis dissimilarity showed a clear separation of microbial community structures between HC and AGA in females ($p = 0.002$), whereas no significant difference was observed in males ($p = 0.999$) (Figure 7a).
Taxonomic composition analysis at the genus level revealed that both *Cutibacterium* and *Staphylococcus* dominated the SM in HC and AGA groups, yet their relative proportions varied between phenotypes (Figure 7b).

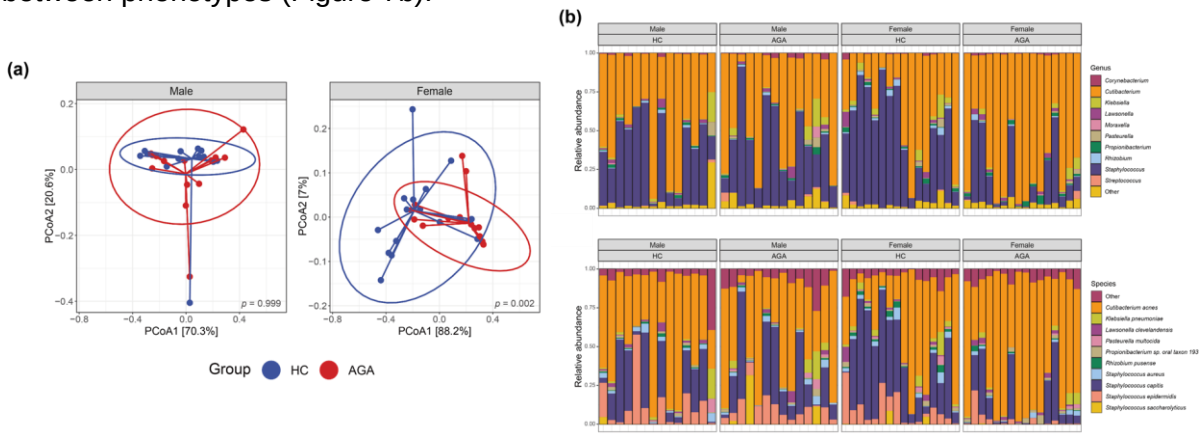

Figure 7. Metagenome (0 week). (a) PCoA plots based on Bray–Curtis dissimilarity of metagenomic data, comparing microbial community structure between HC (blue) and AGA (red) in males and females. Stacked bar plots showing genus-level and species-level (b) relative abundances derived from metagenomic sequencing.

In AGA of females, a notable increase in *Cutibacterium* and a decrease in *Staphylococcus* were observed compared to HC of females. Male subjects exhibited higher inter-individual variability, and the genus-level shifts between HC and AGA were less pronounced. At the

species level, similar trends were observed. In females, *Cutibacterium acnes* was more dominant in the AGA group, while other taxa such as *S.aureus, Staphylococcus epidermidis* and *Staphylococcus capitis* appeared in lower proportions. In males, although *C. acnes* remained the dominant species in both groups, the relative abundance of secondary species varied more widely across individuals, with no consistent disease-associated pattern. Overall, metagenomic analysis at baseline revealed sex-specific microbial signatures associated with AGA. The differences were more apparent in females, with distinct alterations in community structure and relative abundance of key taxa, whereas the male scalp microbiota showed less divergence between HC and AGA groups.

To assess the agreement between different microbiome profiling methods, three-dimensional scatter plots were constructed using genus-level abundance data from metagenomic sequencing, 16S rRNA gene sequencing, and 16S-based QMP with ddPCR (Figure 8). Each point represents a genus, plotted by its abundance in metagenome (x-axis), RMP (y-axis), and QMP (z-axis). While metagenome and 16S data showed moderate concordance, QMP values often diverged, reflecting differences in total microbial load. Notably, QMP enabled more accurate quantification of dominant taxa and revealed that several low-abundance genera in relative methods had high absolute abundance, underscoring the limitations of RMP compositional data alone.

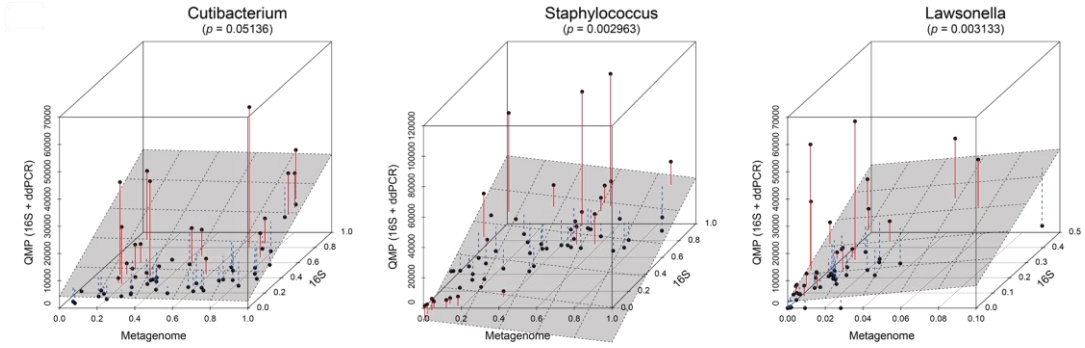

Figure 8. Metagenome vs 16S vs 16S + ddPCR (0 week). Three-dimensional plots comparing relative abundances of major genera across three data modalities: 16S rRNA gene amplicon sequencing, QMP using 16S data with ddPCR, and whole metagenome sequencing.

3.5. Correlation between scalp microbiome and hair/scalp characteristics

To explore the relationship between SM and subject clinical conditions, Spearman correlation analysis was performed between the abundance of major bacterial taxa and various hair and scalp characteristics, including age, hair thickness, density, gloss, moisture, sebum, desquamation, temperature, and redness. Microbial abundance was evaluated at the genus level using QMP (16S + ddPCR) and at the species level using metagenomic data, with results analyzed separately for males and females.

In the QMP dataset (Figure 9a), all three major taxa in males showed positive correlations with hair thickness, with *Lawsonella* additionally displaying negative correlations with moisture and redness of scalp. Over time, *Lawsonell*a also showed a decreasing association with desquamation following BEO treatment. In females, *Staphylococcus* exhibited strong and more frequent correlations, positively associated with hair thickness, density, and gloss, and negatively associated with moisture, desquamation, and redness.

In the metagenomic data (Figure 9b), species-level resolution revealed more detailed associations. In males, *S. epidermidis* showed a positive correlation with sebum levels, while *Lawsonella clevelandensis* was negatively correlated with scalp moisture. In females, multiple significant associations were observed. *C.acnes* showed a negative correlation with gloss and a

positive correlation with sebum. Most *Staphylococcus spp*. showed positive correlations with hair thickness, density, and gloss. In contrast, *L. clevelandensis* did not exhibit any significant associations with clinical hair or scalp characteristics.

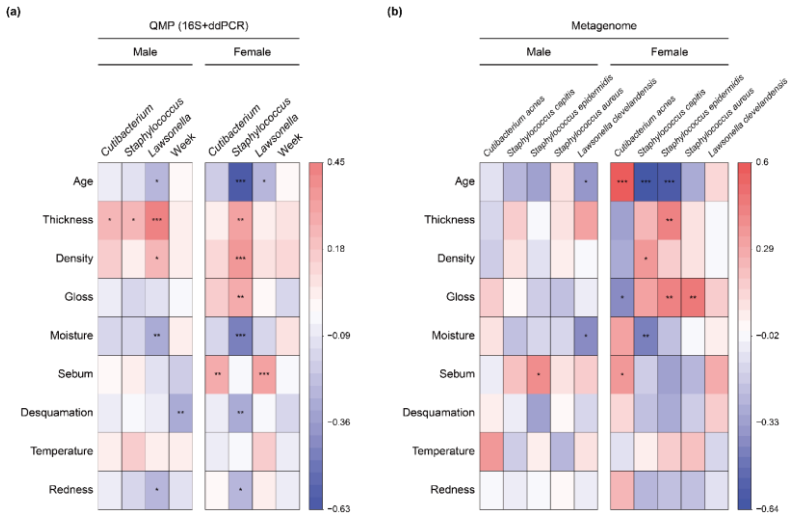

Figure 9. Correlation between scalp microbiota and hair/scalp characteristics based on QMP and metagenomic profiling. Heatmaps of Spearman correlation coefficients between bacterial abundance and clinical hair/scalp characteristics in males and females. Genus-level abundance was obtained from QMP (16S + ddPCR) (a), and species-level abundance was derived from metagenomic data (b). Positive correlations are shown in red, negative correlations in blue. Asterisks indicate statistical significance *(*p < 0.05, **p < 0.01, ***p < 0.001*).

4. Discussion

AGA, induced by androgens, is the most common type of hair loss. Recent studies have revealed that the occurrence of AGA is associated with microbial imbalances in the SM [14]. However, the patterns of AGA occurrence differ between males and females, it is necessary to classify and compare them from a microbiome perspective [15]. In the preliminary results of this study, it was revealed that there are differences in microbiome diversity between Korean male and female regardless of hair loss status, and that there are distinct differences in microbial communities depending on the presence, absence, and severity of hair loss. In the AGA group, imbalances occurred due to changes in dominant genera such as *Cutibacterium* and *Staphylococcus*, leading to the emergence of minor or unique AGA-specific taxa. Therefore, to alleviate hair loss or maintain a healthy scalp environment through rebalancing, it is crucial to accurately understand the balanced composition based on the presence or absence of AGA. However, no studies have compared sequencing methods for accurate bacterial validation, and most rely on RMP, which limits the accurate assessment of absolute microbial differences in the SM. Thus, QMP using ddPCR was applied to overcome the limitations of RMP by assessing the absolute microbial abundance.

As expected, significant differences in diversity between AGA and HC were observed in both sexes, with higher diversity in AGA due to imbalances, which were more prominently observed in females. Notably, NMDS results showed that the changes in the SM by regulating the diversity (imbalance) of AGA subjects were achieved through the use of BEO shampoo for 5 weeks, resulting in cohesion and rebalance. However, no correlation was observed with the three major genera. In AGA-TEST, the changes in the three genera were not identical to those in CON-HC, but the increase and decrease of these genera likely had a significant impact on other minor bacterial taxa, leading to rebalancing. Further analysis is required to confirm this. We

focused on the similarity of bacterial populations between HC and AGA using metagenome, 16S, and QMP to identify the main bacteria affecting the scalp. Notably, the major genera frequently mentioned in other studies—*C. acnes, S. aureus, and S. epidermidis*—were confirmed through metagenomic species analysis, consistent with the results of 16S with real-time PCR in alopecia areata [16]. In terms of clinical correlations, the *Staphylococcus* cluster showed an impact on clinical condition improvement in both QMP and metagenomic analyses. *S. epidermidis* is known to strengthen the skin barrier and provide protective effects against external pathogens. On the other hand, this bacterium was significantly increased in the metagenomic results of males when sebum levels were elevated, suggesting that inflammatory *S. epidermidis* biofilms may have formed communities in the sweat glands [17].

The absolute microbial analysis of skin conditions (dryness, moisture, and sebum) has shown that *Streptococcus* is more abundant in dry and oily part, while *Staphylococcus* and *Corynebacterium* are more concentrated in moisturized skin. In our QMP results, *Cutibacterium* and *Lawsonella* (females) were influenced by scalp sebum, while *Staphylococcus* (females) and *Lawsonella* (males) showed negative correlations with moisture, contrary to other findings. However, these results also differ from our study in that the microbial composition of the skin and scalp varies by location and that the quantification was performed using real-time PCR [18]. In other words, our comparative validation data suggest that using more sensitive PCR technology (ddPCR) allows us to gain important insights into potential mechanisms of AGA that may have been difficult to detect with traditional qPCR approaches. This method can also be used to validate or investigate specific bacterial species identified through unbiased sequencing and explore their potential roles in AGA development and related clinical features. We demonstrated that the observed differences in alpha and beta diversity across sex and AGA were consistent using both profiling methods. In this study, the correlation between 16S and metagenome was the highest, while the correlation between metagenome and QMP was present but relatively weaker. Nevertheless, in low biomass samples such as the scalp (skin), the total microbial load itself can be a critical factor for interpretation, making a quantitative-based strategy like QMP a more suitable choice depending on the situation. Therefore, while 16S-based profiling is time- and cost-efficient, whole metagenome analysis, despite its lower sensitivity, may be ideal for studies where distinguishing specific species within genera is essential. Consequently, the choice of sequencing and quantification methods can depend on whether the goal is to explore the entire microbial community or to target the abundance of specific species. These insights enable more effective use of limited resources by reducing sequencing costs and minimizing bias in results.

5. Conclusion

Building on our previous research, we propose that improving the SM imbalance phenomenon may represent a novel approach and therapeutic factor for AGA. Additionally, using QMP to compare absolute abundances prevents data distortions that can occur with RMP, yielding more reliable and accurate SM results for AGA. Consequently, quantitative or qualitative changes in SM can positively influence human scalp conditions.

Reference

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