Comparison of Scalp Microbiome According to the Severity of Androgenic Alopecia and Gender in a Korean Cohort

Yoo, Hye-Young[1+]; Jung, Da-Ryung[2+]; Jeong, Minsoo[2]; Kim, Min-Ji[2]; Jang, You-jin[1]; Park, Sung-Ha[1]; Park, Byoung-Jun[1*]; Shin, Jae-Ho[2*]

[1] Skin&Natural products Lab, Kolmar Korea Co., Ltd., Seoul, 06800, Republic of Korea;

[2] Department of Applied Biosciences, Kyungpook National University, Daegu, 41566, Republic of Korea

[+] These authors contributed equally.

* Park, Byoung-Jun: Kolmar Korea, 61, Heolleung-ro 8-gil, Seocho-gu, Seoul 06800, Korea, +82.2.3459.5570, A2001@kolmar.co.kr

* Shin, Jae-Ho: Kyungpook National University, 80, Daehak-ro, Buk-gu, Daegu 41566, Korea, +82.53.950.5716, jhshin@knu.ac.kr

Abstract

Background: The bacterial community on the scalp contributes to overall scalp and hair-related diseases. Thus, the purpose of this study is to identify the relationship between microorganisms and androgenetic alopecia (AGA) by analyzing the scalp microbiome according to the severity of AGA and gender.

Method: A total of 141 Korean women and men (aged from 20 to 65) participated, consisting of 46 normal subjects and 95 AGA subjects (stage 1 and 2). After measuring the clinical conditions of the scalp and hair, collected scalp microbial samples were identified by 16S rRNA sequencing, and further analysis was performed.

Results: There were differences in hair density and thickness, and scalp moisture level between each groups. Men had higher bacterial alpha diversity compared to women, and the AGA groups compared to the CON group. In the AGA groups, the ratio of total *Cutibacterium* and *Staphylococcus* decreased, and remaining bacteria commonly increased. We identified 7 and 9 taxa in women and men, respectively, using random forest. The network of the AGA group showed a more complex and unstable compared to the CON groups. The structural equation model provided potential evidence that the increase in scalp microbial diversity is aging-dependent, which indirectly affects AGA by affecting the decrease in resident bacterial abundance.

Conclusion: The differences of scalp microbiome were associated with gender and severity of AGA.

This is the first novel approach to the management of AGA by targeting the scalp microbiome, and can be expected as a scientific evidence for the cosmetic industry.

Keywords: Androgenetic alopecia; Scalp microbiome; 16S rRNA; Machine learning; Network; Structural equation modeling

Introduction

Androgenic alopecia (AGA) is one of the most common types of hair loss represented in both women and men. This is a phenomenon caused by androgen, androgens vary in influence depending on body site, and they appear in vertex, frontal, and occipital scalp. And short, thin, miniaturization of hair is a typical feature of AGA [1]. Clinically, thinning hair occurs as the anagen phase is shortened and the telogen is prolonged at the same time. When the rest period is extended in the hair cycle and the growth period is shortened, the hair is miniaturized by gradually replacing the terminal hair with short vellus hair [2].

AGA is actually a global problem that has been increasing every year for more than 2,000 years, and various factors such as aging, excessive mental stress, smoking, hormones, and living environment are expected to affect it [3, 4]. Because these various factors can affect complexly, hair loss treatment is difficult and takes a long time, and in this process, people with hair loss symptoms feel less confident and attractive to others' perceptions [5]. For these reasons, not only those who do not have much severe alopecia symptom but also healthy people are paying a lot of attention to the prevention and treatment of AGA. Genetic predisposition and sex hormones are also major factors in AGA development, but the skin's own metabolic system, especially microbiome, is emerging as another cause of hair loss because it forms specific clusters in the scalp to affect scalp ecosystem maintenance as well as differences in bacteria depending on hair follicle depth [6].

Human skin microbiome can be changed by various factors such as aging, residential location, ultraviolet radiation, cosmetics use, and disease, and the composed bacteria distributed vary depending on the location of the sites and its environment [7-11]. Although many studies have been found on intestinal microbiome, the studies on the skin or scalp have recently been promoted and the fields are also diverse. As skin microorganisms contribute to host immunity and inflammation activity, hair disorder related symptoms such as hair loss and miniaturization can be involved in [12, 13]. So, bacterial community of scalp can one of the affecting factor to alopecia. Previous studies have shown that people with AGA symptoms have shown scalp total imbalance (increase in *Cutibacterium* abundance and decrease in *Corynebacterium* abundance) compared to normal people, which also varies with age[14]. *Cutibacterium acnes* is correlated with miniaturized vertex hair in AGA and alopecia areata (AA) patients. And it is relatively abundance in dandruff and seborrhoeic dermatitis than healthy people [13, 15].

As such, studies on hair loss and scalp-related microbiomes have classified a test group simply with or without hair loss, or mostly dealt with scalp diseases for scalp condition relief or external

environmental factors through specific ingredients. However, since there is a difference in the incidence of AGA between men and women and people suffer a lot of psychological stress in areas that appear to be alopecia compared to normal people, a study on microbiome analysis according to gender and alopecia stage was needed.

Therefore, we are the first to divide the stage from preventable weak hair loss (the stage where psychological stress begins) to complete hair loss (the stage where treatment is needed) as well as the presence of hair loss and reveal the difference between men and women.

Materials and Methods

Subjects selection

Before start this study, it was approved by the Korean public institutional review board (IRB) of Korea national institute for bioethics policy (Approval number: P01-202002-33-001), and all participants submitted written informed consent after fully informing about the study process according to the guideline of IRB. A total of 141 Korean women and men (aged from 20 to 65) participated in the study, which was conducted at Kolmar Korea in Seoul.

Study design (Sample collection)

Through the visual evaluation of the researchers, the subjects were first divided into two groups based on the degree of scalp parting exposure by referring to the Basic and Specific (BASP) classification criteria: (1) 46 normal (CON) group consisting of 25 women and 21 men, and 95 AGA group consisting of 49 women and 46 men. Since then, the AGA group was divided into two groups again according to the severity of hair loss. The stage 1 of hair loss refers to a specific type (V1 or F1) having a slightly wider part than normal people (L, M0, C0). The stage 2 of hair loss refers to a specific type (V2-3 or F2-3) in which the part of the hair is wider than the stage 1 and the exposed scalp shape begins to change into a round shape. Participants with the following criteria were excluded: (1) is pregnant, (2) expose to any antibiotics within 1 month, (3) have an experience of a surgical procedure for AGA, (4) any infectious dermatitis etc., at scalp under study. The referenced BASP classification and participation restriction criteria were followed by the guidelines for cosmetic human application tests that help alleviate hair loss symptoms by the Ministry of Food and Drug Safety.

To standardize scalp conditions before sampling, subjects were required not to apply any hair care products and use shampoo for one day before. Sampling and clinical evaluation were performed in a maintaining room with constant conditions (temperature 24 ± 2 ℃, humidity 50 ± 5%) after 30 min acclimatization. The scalp microbial samples were collected with a sterile cotton swab for 3 minutes at the scalp and vertex and were stored at -80°C until further process.

Assessment of hair and scalp clinical condition

Total seven clinical conditions of scalp (moisturizing, sebum, desquamation, and temperature) and hair (thickness, density, and gloss) were measured. The level of scalp moisture, sebum and hair

gloss was respectively measured by Corneometer® CM825, sebumeter® SM815, glossymeter® GL200 (Courage & Khazaka, Cologne, Germany) and desquamation index value obtained with Corneofix® F20 (Courage & Khazaka) was visualized with Visioscan® VC98. Using portable Folliscope® (CCL-215, Sometech, Korea) and folliscope 2.8 software, we analyzed the thickness and density of hair within a specified range of captured scalp photos. Scalp temperature was measured by thermal imager [16-21].

DNA extraction and bacterial 16S rRNA amplicon sequencing

Scalp bacterial genomic DNA was extracted using DNeasy PowerSoil Pro Kit (Hilden, Germany), using modified methods to increase extraction efficiency. The concentration and quality of the extracted DNA were confirmed using a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. For making library for sequencing, bacterial genomic DNA was amplified for specific V4-V5 hypervariable region containing Illumina adapters and indexed sequences. All DNA sequencing libraries were multiplexed and paired-end sequenced by the Illumina MiSeq platform (San Diego, San Diego, USA) according to the manufacturer's instruments.

Bioinformatic and statistical analysis

The raw sequence data were analyzed using Quantitative Insights into Microbiological Ecology 2 (QIIME2) pipeline (version 2021.4). To identify the Amplicon Sequence Variant (ASV), the sequence was demultiplexed, trimmed, denoised using DADA2 (Q score > 30), and the ASV sequence was determined using a Silva v138 database with taxonomically 99% sequence identity.

All statistical analysis and visualization of our results in this study was performed with RStudio 1.4.1717 (https://www.rstudio.com/). Alpha diversity indices (Shannon and Simpson's index) of scalp microbiome were calculated using the Phyloseq and metagMisc R packages. Beta diversity was performed as the principal coordinates analysis (PCoA) based on the Bray-Curtis dissimilarity using vegan R package, and the distances between the samples within the groups were calculated in QIIME2. A random forest classification was used to identify the top discriminatory taxa for classifying the groups using randomForest R package. Co-occurrence analysis for network was performed using the igraph R package. The network topological statistic coefficients included density (ratio of the number of nodes and the number of possible edges) and transitivity (the probability that the adjacent nodes of a node are connected). Structural equation modeling (SEM) was used to examine the direct and indirect various

parameters on AGA. SEM was constructed and analyzed using the lavaan R package [22].

Results

Evaluation of physiological parameters of hair and scalp

141 Korean women and men participated in the study, and the average age of 74 women and 67 men was 44.8 years. The normal CON group and the AGA group are average 40.4 and 47.2 years old, respectively. In AGA groups, these are the 40.8 years old for the first stage and 57.2 years old for the second stage. Compared with normal people, it can be seen that the time when weak hair loss begins is in the early 40s.

When comparing the clinical results of hair according to the hair loss stage compared to normal people, the thickness and density of hair gradually decreased according to the severity for AGA stage 1 (AGA-S1) and AGA stage 2 (AGA-S2): thickness (0.0978, 0.0832, 0.0711) and density (137, 109, 91). On the other hand, in hair gloss, statistical significance between each group could not be confirmed (**Table 1**).

Temperature, moisture, sebum, and desquamation, known as clinical indicators representing typical scalp conditions, were classified and analyzed in detail according to AGA severity and gender. As a result, there was a significant difference in sebum and moisture. First, in the case of sebum, there was no significant difference between the CON and the AGA group, respectively, at 144 and 122. However, in particular, it was confirmed that the average value in women AGA-S1 and men AGA-S2 was higher than that of the CON. In addition, we confirmed that the moisture value gradually increased dependent on both women and men as hair loss progressed. Neither scalp temperature nor desquamation level showed any statistically significant differences according to the presence and severity of AGA and gender. (**Table 2**).

Table 1. The comparison of hair parameter measurement between control and AGA Stage group.

Gender	Group	Thickness (mm)	Density (N/cm^2)	Gloss DSC (A.U)
Women	CON	0.095 ± 0.013	113.8 ±18	2.45 ± 1.19
	AGA-S1	0.086 ± 0.01	105.7 ±14	2.47 ± 1.45
	AGA-S2	0.081 ± 0.014	83.5 ± 13	1.77 ± 0.82
Men	CON	0.1007 ± 0.012	140.8 ±17	2.14 ± 1.09
	AGA-S1	0.0804 ± 0.011	112.8 ± 15	2.29 ± 0.84
	AGA-S2	0.0613 ± 0.013	98.4 ± 22	2.12 ± 1.41

Data are mean ± SD.

167 **Table 2.** The comparison of scalp parameter measurement between control and AGA Stage group.

Gender	Group	Temperature (℃)	Hydration (A.U)	Sebum ($\mu g/cm^2$)	Desquamation index (%)
Women	CON	32 ± 1.6	6.08 ± 4.9	119 ± 122	24.78 ± 2.3
	AGA-S1	31.8 ± 1.7	9.68 ± 6	160 ± 145	23.96 ± 3.3
	AGA-S2	31.8 ± 1.4	15.22 ± 6.4	111 ± 97	24.68 ±1.9
Men	CON	30.2 ± 2	4.34 ± 3.1	108 ± 95	24.26 ± 1.4
	AGA-S1	31.3 ± 1.2	9.13 ± 6	93 ± 72	24.55 ± 2.6
	AGA-S2	31.7 ± 1.3	17.37 ± 16.5	125 ± 101	24.64 ± 1.9

168 Data are mean ± SD.

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Taxonomic composition of the scalp microbiome

At first, in order to compare whether there is a difference in scalp microbiome between genders regardless of the presence or absence of AGA, alpha diversity (Shannon and Simpson's index) and beta diversity were analyzed. Men had a significantly ($p < 0.05$, Wilcoxon rank sum test) higher alpha diversity than women, and the two groups showed a distinct differences ($p < 0.001$, Adonis) on PCoA plot of beta diversity based on Bray-Curtis distance (data not shown). Based on these results, we acknowledged the differences in the scalp microbiome of women and men, and conducted a following analysis by classifying the gender.

We investigated the relative abundance of the top 15 abundant genera in the scalp microbiome. Our results showed that *Cutibacterium* and *Staphylococcus* were the predominant genera in the scalp microbiome of the three groups (**Fig. 1A**). The top 2 abundant taxa slightly decreased in the AGA groups such as *Cutibacterium* (58.4% vs 52.4% vs 52.8%, women; 33.2% vs 30.4% vs 33.0%, men) and *Staphylococcus* (11.2% vs 10.0% vs 6.8%, women; 16.3% vs 5.5% vs 11.7%, men). Except for the two genera, the remaining genera generally increased in the AGA groups such as *Catenibacterium* (1.3% vs 3.5% vs 5.5%, women; 7.6% vs 12.9% vs 11.9%, men) and *Bifidobacterium* (0.7% vs 2.0% vs 4.5%, women; 7.2% vs 12.5% vs 9.9%, men). By comparing with core microbiome taxa, we found that each group uniquely had some taxa (**Fig. 1B and 1C**). In both women and men, the number of taxa only in the AGA-S1 and -S2 groups was higher than in the CON group.

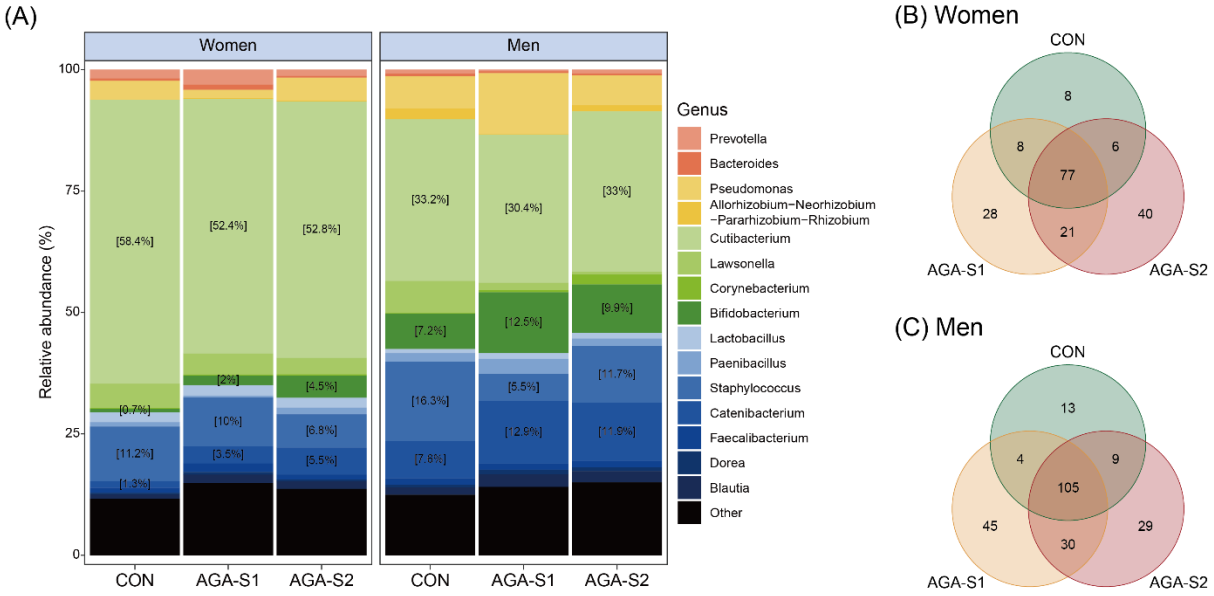


Figure 1. Taxonomic composition of the scalp microbiome. (A) Stacked bar chart showed the relative abundance (%) of predominant bacterial genera (top 15 genera). Venn diagram plot showed core microbiome with shared and unique genera between the three groups for (B) women and (C) men, respectively.

Diversity of the scalp microbiome

We compared the scalp bacterial structure of the three groups (CON vs AGA-S1 vs AGA-S2) using alpha diversity indices (Shannon and Simpson's index) (**Fig. 2A**). Although our results was not reached at statistical significance, both women and men showed a trend toward slightly increasing the alpha diversity as AGA progressed. To evaluate the dissimilarity of scalp bacterial composition, beta diversity was calculated based on the Bray-Curtis distance (**Fig. 2B and 2C**). On the principal coordinates analysis (PCoA) plot, no significant difference was observed between the centroid of the three groups, the distances between the samples within the each group were significantly different (**Fig. 2D and 2E**).

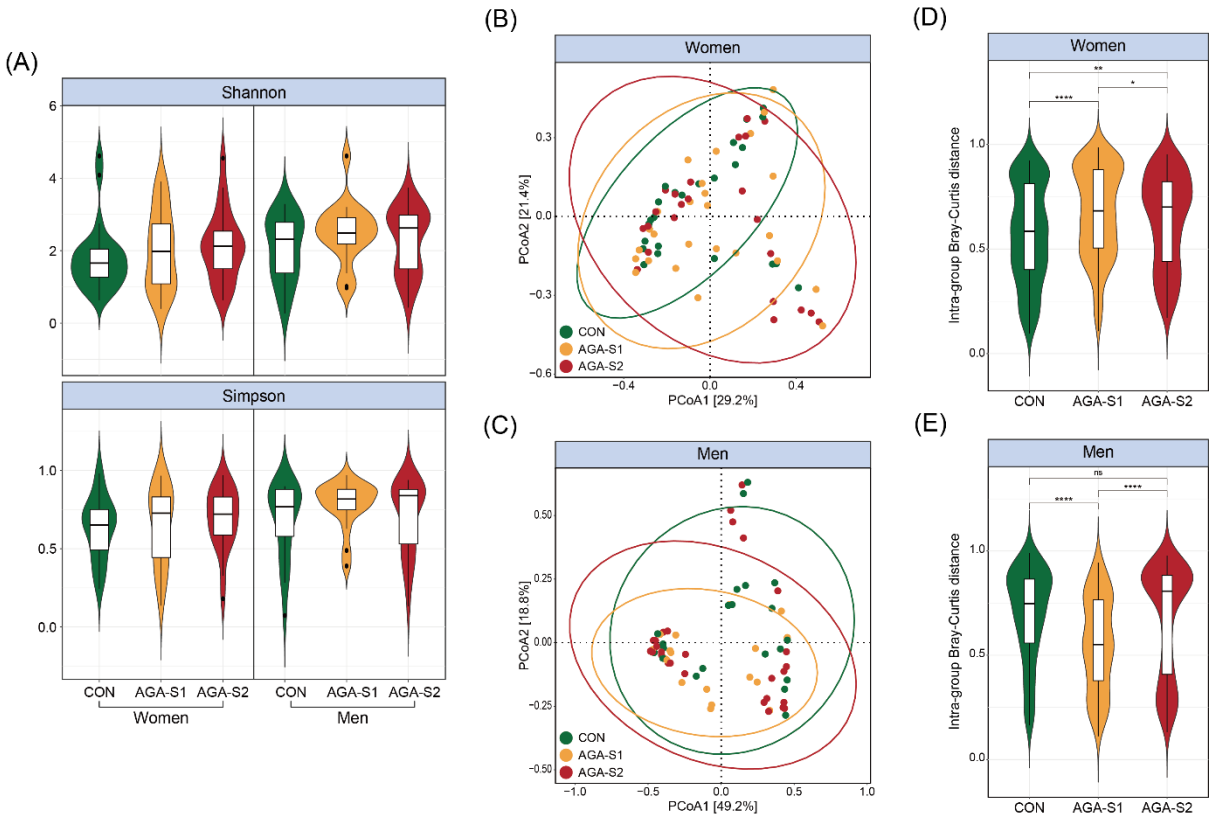


Figure 2. Comparison of diversity in scalp microbiome (A) Alpha diversity indices (Shannon and Simpson) were calculated compared for the three groups (CON vs AGA-S1 vs AGA-S2). The PCoA plot of the beta diversity were based on the Bray-Curtis distance matrices for (B) women and (C) men, respectively. Boxplots showed the average Bray-Curtis distances between samples within each group for (D) women and (E) men, respectively. Asterisks indicate *p*-values, where **p*-value < 0.05, ***p*-value < 0.01, and ****p*-value < 0.001.

Screening of differential scalp microbiome using random forest

The random forest analysis was performed to select the taxa that could discriminate between the CON and AGA (-S1 and -S2) groups as biomarkers. In the case of women, seven taxa, such as *Subdoligranulum*, *Bifidobacterium*, *Paracoccus*, *Pseudomonas*, *Blautia*, *Dorea*, and Unclassified Lachnospiraceae, were used as important variables for clustering the groups based on the mean decrease accuracy index (**Fig. 3A**). Also, nine taxa, such as *Lawsonella*, *Allisonella*, *Parasutterella*, Unclassified Lactobacillales, *Erysipelatoclostridium*, *Lachnospira*, *RF39*, *Phascolarctobacterium*, and *Negativibacillus*, were selected as important genera in men (**Fig. 3B**).

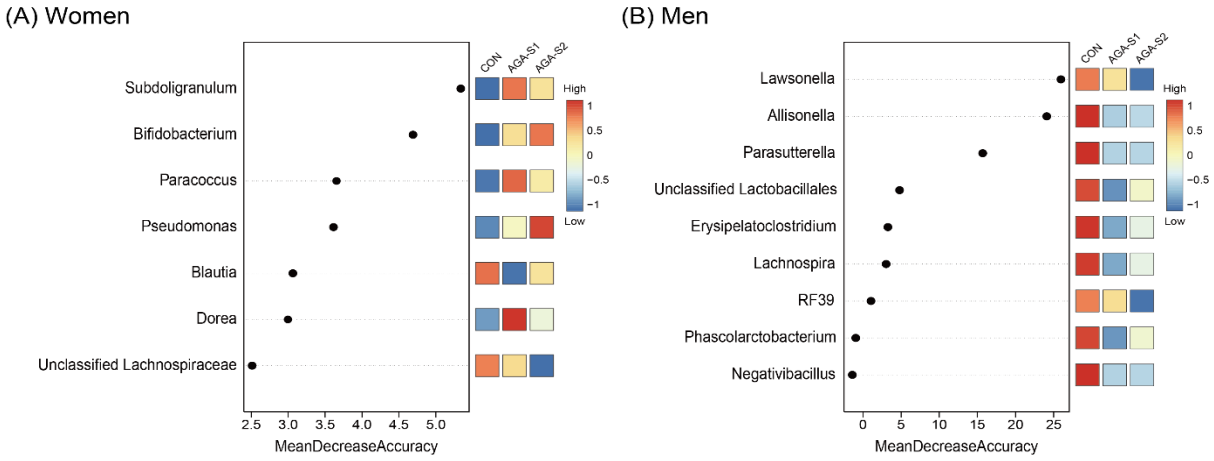


Figure 3. Significant taxa identified by random forest. A higher value of mean decrease accuracy indicated the importance of that taxa for group separation. The colored column on the right showed the group average value after log transformation.

Network of scalp bacterial community

To confirm the interaction of genera in the scalp microbiome, we performed co-occurrence analysis for each group (CON, AGA-S1 and -S2) in women and men (**Fig. 4A and 4B**). We used genera that were relatively abundant and present in more than 50% (women) and 70% (men) of samples in at least one group. Therefore, the network for women had 20, 21, and 24 nodes in the CON, AGA-S1, and -S2 groups, respectively. In the case of men, 14, 35, and 19 nodes were obtained in the CON, AGA-S1, and -S2 groups, respectively. Using Spearman's correlation, edges with p-value < 0.05 were used, and their thickness indicated the strength of the correlation between nodes. The network analysis showed that the transitivity (T) value was higher in AGA-S1 and -S2 (T = 0.86 and 0.61) than CON (T = 0.41) in women. Also, in men, the value of AGA-S1 (T = 0.86) was higher than that of CON (T = 0.75), but the value of AGA-S2 (T = 0.75) was the same as that of CON. In addition, similar to T value, the network density (D) value also showed higher in AGA-S1 and -S2 than CON in women, and only AGA-S1 in men was higher that CON.

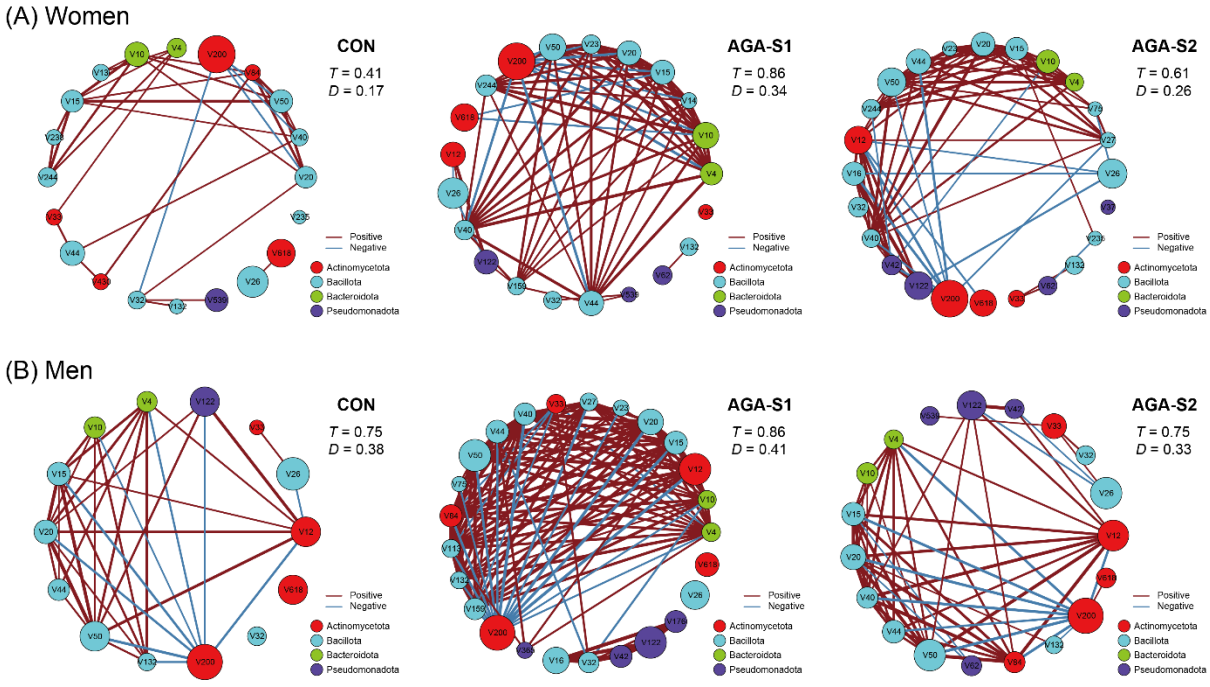
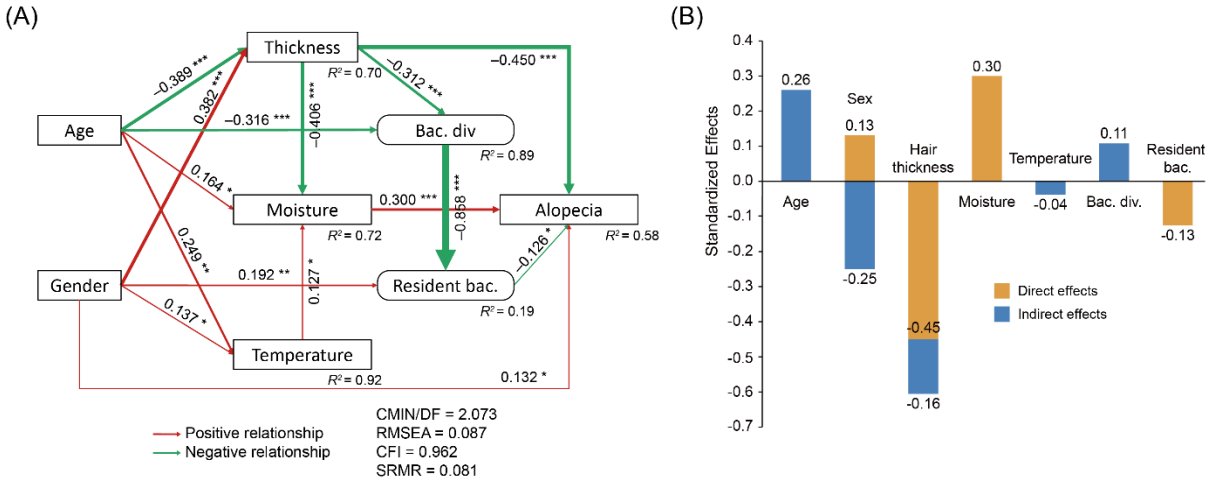


Figure 4. Network analysis of bacterial genera of the scalp microbiome. The size and color of each node was proportional relative abundance and phylum corresponding to that genus. Edge color (red or blue) represented positive or negative correlation, respectively. The T and D values represented the transitivity and density of the network.

240 Relationships between physiological parameters, resident bacteria, bacterial diversity, and AGA

241 We also conducted structural equation models (SEM) to characterize the differential effects of
242 the microbial properties and physiological parameters of hair and scalp on AGA (**Fig. 5**). Judging by
243 the standardized total effect, hair thickness and moisture had a great effect on AGA, and resident
244 bacteria affected AGA to a degree similar to gender. Furthermore, the resident bacterial community had
245 a significant negative direct effect on AGA ($\lambda = -0.126$, $p < 0.05$), and bacterial diversity indirectly
246 affected AGA through resident bacteria ($\lambda = 0.11$, $p < 0.05$).



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248 **Figure 5.** Structural equation models (SEM) indicating relationships between hair and scalp parameters and
249 bacterial parameters. (A) Direct and indirect effect of age, gender, temperature, moisturizing, hair thickness,
250 bacterial diversity, resident bacteria, and AGA (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001). (B) The
251 standardized effects on AGA (R^2) are the explained proportion of variance. CMIN/DF = 2.073, RMSEA = 0.087,
252 CFI = 0.962, SRMR = 0.081.

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Discussion

Microbiome for skin health is being studied as a new approach to understand the relationship between human skin and various microorganisms with recent advances in NGS technologies. For example, an imbalance between the skin microbial communities and the host can contribute to se such as alopecia, atopic dermatitis, and psoriasis [14, 23, 24]. Among the several skin diseases mentioned, the main causes of AGA are systemic androgens and genetic factors, but it is necessary to understand exogenous factors also influence the process as well [25]. Therefore, in this study, we suggest a preliminary possibility as the cause of AGA by revealing the association between AGA and the scalp microbiome.

The scalp provides a microenvironment for microorganisms through certain physiological properties such as sebum, moisture, pH, and temperature [26, 27]. Interestingly, the moisture content of the scalp increased with the severity of AGA. Harries et al. reported that the increased scalp sweating in a group of women with frontal fibrosing alopecia, and suggested a possible association between sweating and scalp inflammation [28].

The scalp microbiome of the subjects was mostly composed of *Cutibacterium* and *Staphylococcus*. These two genera, the most abundant, were easily found as the major bacterial colonizers on the skin surface [29, 30], and maintained skin health by regulating the immune response [31, 32]. As AGA progressed, the portion of total *Cutibacterium* and *Staphylococcus* decreased and the portion of remaining bacteria increased, which was associated with alpha diversity. Although not significant in our cohort, there was a sequentially higher alpha diversity in the AGA groups (**Fig. 2A**). In a previous study comparing the skin microbiome of older and younger groups, it was noted that the higher alpha diversity was due to an increased ratio of minor OTU/species, leading to changes in the overall structure [7]. Likewise, there was an increase in the number of unique and minor bacteria in the AGA groups in our cohort, and the Venn diagram of Fig. 1B also supported these findings. Also, a moister scalp in AGA subjects may induce colonization of unspecified microorganisms [33]. The low within-group variation in the CON groups compared to AGA groups indicated that the high relative abundance of skin commensal bacteria such as *Cutibacterium* and *Staphylococcus* was a conserved characteristic of this bacterial communities (**Fig. 2D and 2E**).

We identified differential taxa using random forest analysis, including the genus *Subdoligranulum* and *Bifidobacterium* enriched in women, and *Lawsonella*, *Parasutterella* and *Allisonella* enriched in men (**Fig. 3**). The taxa selected in this way can be used as bacterial biomarkers that can classify AGA, and has the potential to be treated as an important bacteria in further AGA studies.

In particular, *Lawonella*, which showed differences in men, had a negative correlation with moisture [34], suggesting that the moist scalp of AGA subjects was associated with a decrease of *Lawsonella*.

Our network analysis provided representative ecological relationships of bacterial communities (**Fig. 4**). Some theoretical studies suggested that ecological networks with weak correlations were more stable that those with strong correlations [35]. Although the network topology coefficients (T and D values) were not sequential according to the stages of AGA, it could be inferred that the CON group was more stable than the AGA groups due to weak correlations. Also, the AGA groups were more closely interdependent and may have been more susceptible to environmental interference. As mentioned above, the co-occurrence network of predominant bacteria was disrupted by an increase of minor bacteria in the AGA groups. This network disruption was similarly observed in the dandruff groups in Chinese cohort [36].

We conducted a structural equation model to investigate the direct and indirect effects of various parameters on AGA (**Fig. 5**). As in the previous studies, AGA increased as moisture increased, which was expected to be related to the destruction of the oil-water balance of the scalp [37]. SEM analysis results provide potential evidence that the increase in scalp microbial diversity is aging-dependent, which indirectly affects AGA greatly by affecting the decrease in resident bacterial abundance. Also, resident bacteria had a direct effect on AGA at a level similar to that of the gender, and it was expected that the reduction of the resident bacteria has a great effect on AGA as much as the heredity and sex hormones. Overall, we speculated that disruption of scalp microbiome homeostasis due to the reduction of resident bacteria significantly affects AGA.

Our study provides preliminary potential for an association between microorganisms and AGA by comparing the scalp microbiome between healthy controls and AGA subjects. Unlike previous studies that suggested this association, we identified patterns of the scalp microbiome according to the severity of AGA and gender, and analyzed them in depth using machine learning and structural equation model. However, for application, it is necessary to bacteria identification with species-level resolution, which requires shotgun metagenome sequencing. This will allow a functional evaluation of the specific bacteria that affect AGA on the scalp.

Finally, our research will not only lead to the development of cosmetics and therapeutics using microorganisms and metabolites contributing to AGA, but also provide a scientific evidence from a microbiological perspective in this process.

Conclusion

Taken together, there was a difference in the composition of microorganisms on the scalp of women and men, which was not only in the presence or absence of AGA but also in the severity stage. As AGA progressed, the proportion of total *Cutibacterium* and *Staphylococcus* decreased, and alpha diversity increased accordingly. Using random forest analysis, a type of machine learning, *Subdoligranulum* and *Bifidobacterium* in women and *Lawsonella* in men were selected as biomarkers that can classify AGA. The structural equation model provided potential evidence that the increase in scalp microbial diversity is aging-dependent, which indirectly affects AGA greatly by affecting the decrease in resident bacterial abundance. This finding is of far-reaching importance to understand microbiome as an exogenous factor in AGA.

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Conflict of Interest Statement

NONE

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