Utility of a quantitative approach to microbial dysbiosis using machine learning in an African American cohort with self-reported hair loss

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Abstract

Microbial dysbiosis has been identified as a therapeutic target for several dermatologic conditions. However, the concept of dysbiosis is poorly defined, limiting reproducibility. We developed a reproducible metric for dysbiosis that can be applied to research within dermatology. Thirty-six individuals from an African-American cohort with self-reported hair loss without formal clinical diagnoses provided scalp swabs from both afflicted (hair loss) and normal (no hair loss) sites. The scalp microbiome was characterized via 16S rRNA gene sequencing. A dysbiosis score that considers the proportion of all taxa within the samples was calculated. Further, we identified the taxa most associated with dysbiosis using both a machine learning random forest classifier and a negative binomial mixed effects model to control for participant age. Sites of hair loss exhibited an increase in microbial diversity, in particular for individuals older than 40 years. We identified a core set of OTUs assigned to 7 genera that were significant contributors to increased scalp dysbiosis. This work demonstrates the utility of applying a quantitative approach to dysbiosis and provides a framework that can be applied to other microbiome-associated conditions.

Introduction. Recent research has revealed that changes in the microbiome are associated with several dermatologic conditions including different types of alopecia [1], dandruff, seborrheic dermatitis [2], atopic dermatitis [3], scalp psoriasis, and folliculitis decalvans [4]. The term “dysbiosis” is often invoked when describing microbial associations with disease. However, in most studies the definition of “dysbiosis” lacks clarity; its use ranges from a broad shift in the microbial community composition to a change in a specific taxon [5]. Dysbiosis is readily applied to the gut microbiome and therefore is frequently equated with decreased microbial diversity [5]. This conflation with decreased diversity may lead to confusion, as increased diversity is associated with aging and disease in dermatologic conditions [6, 7]. With treatments for skin disorders targeting microbial “dysbiosis” on the rise [8-10], there is a need for a more quantitative approach for the concept of dysbiosis.

We built a reproducible microbial dysbiosis score and identified the taxa differentiating dysbiotic from non-dysbiotic scalp sites, without *a priori* selection of the organisms associated with dysbiosis. This work involved a cohort of thirty-six African-American individuals with self-reported hair loss. We posited that skin sites exhibiting hair loss would show significant shifts in microbiome composition, indicative of dysbiosis development. Individuals provided both afflicted (hair loss) and normal (no hair loss) scalp swabs and the bacterial 16S rRNA gene was sequenced.

Methods

Recruiting and Sample Collection. Adults with self-reported hair loss were recruited for the study without restrictions in ethnic background. Exclusion criteria included participants with complete hair loss, the presence of open sores, drainage and/or injury to the scalp, and cancer or chemotherapy treatment. Participants were instructed to use a sterile swab (Puritan catalog # BD 25-806 10WC) to collect two samples: one from an afflicted (hair-loss) site and one from a normal site, swabbing the areas for 10 seconds each to collect microbial biomass. All qualifying participants completed a hair density scale; a questionnaire where participants rated their degree of hair loss using an anchored line scale from 1 to 10. An index score of 1 represented a participant whose scalp was readily viewable, and 10 represented a scalp completely covered by hair fibers [11, 12]. Participants also completed a hair stress scale, which contained anchored line scales where they indicated their level of stress on a scale from 1 – 10 [13]. Study protocols were approved and overseen by The University of Chicago Biological Sciences Division institutional review board, Chicago, Illinois (protocol number IRB16-1541). A signed consent was obtained from all participants prior to sampling and questionnaire completion.

Library preparation and sequencing. Swabbed samples were placed inside of a sterile Whirl Pak® bag and mailed to the testing facility. Upon arrival, samples were stored in a -80°C freezer until DNA extraction. Genomic DNA was extracted using the recommended protocols of the Earth Microbiome Project (EMP) and American Gut Project [14, 15]. The V4 region of the 16S rRNA gene (515F-806R) was amplified. Barcoded primer sets were adapted for the Illumina MiSeq System as described by [16].

Statistical analysis. All statistical analyses were performed using R v4.4.2 and visualized with the package 'ggplot2' v 3.5.1 [17]. Sequences were quality-filtered using QIIME v1.9.1 [18]. Operational taxonomic units (OTUs) were defined and assigned taxonomy by clustering sequences against 97% OTU reference data set from the Greengenes database [19, 20]. Alpha diversity measures were generated using 'microbiome' package v1.28.0 [21]. Pearson correlations were calculated to determine the linear relationship between age and alpha diversity. Age groups were further split into 20-year intervals (< 40, 40-59, 60+) and a Wilcoxon rank-sum test was conducted to evaluate differences in alpha diversity between age groups.

Dysbiosis score. Dysbiosis scores were calculated using the R package 'dysbiosisR' v1.0.4 (https://github.com/microsud/dysbiosisR). Our dysbiosis score is a modification of the score reported in [22]. The microbial dysbiosis score is defined as the difference between (the Aitchison distance between the test sample and the healthy class centroid) and (the Atichison distance between the test sample and the diseased class centroid). In other words, the dysbiosis score for each sample measures its closeness to the group (hair-loss afflicted or normal) mean. The dysbiosis score **D** of the test sample **z**, is defined as

$$D(z;\ \mu C_N, \mu C_A) = d_A(z,\ \mu C_N) - d_A(z,\ \mu C_A)$$

where μC_N and μC_A are the centroids (mean composition) of the normal and afflicted samples, respectively and d_A is the Aitchison distance either between the test sample and the μC_N or μC_A. A dysbiosis score of 0 indicates that the test sample is at equal distance from the center of both class (afflicted and normal) centroid, and higher scores indicate more deviation from normal. Calculated dysbiosis scores were classified into two groups of either dysbiosis (score > 0) or normobiosis (score < 0). Code used to calculate dysbiosis scores along with all microbial analyses conducted is publicly available (https://github.com/jud-m/Defining-microbial-dysbiosis).

Determination of taxa contributing to dysbiosis. To determine the bacterial taxa that differentiated dysbiotic hair sites from the normal hair sites, a random forest (RF) machine learning classifier was used to determine which data features (OTUs) were most important for classifying dysbiotic versus normobiosis samples using the R package 'randomForest' v4.7-1.2. An out-of-bag random forest model was used to assess the importance of each microbial OTU by removing each OTU in turn and observing its effect on the classification accuracy. The100 most important OTUs (out of 6,009 total OTUs) for classification were considered further. In addition, because age can be a confounding variable in determining the microbial taxa associated with dysbiosis, a negative binomial mixed-effect model (NBMM) was used to model the microbiome count data using the R package 'NBZIMM' v1.0 [23]. The NBMM identified differentially abundant taxa in the afflicted hair sites relative to normal sites while controlling for the effect of age. As a final check, a Wilcoxon rank-sum test was conducted on the log10 abundance of the OTUs that were flagged in both the NBMM and the random forest classifier. OTUs that were not differentially abundant according to the Wilcoxon rank-sum test were excluded.

Results

Cohort characteristics. The majority of recruited participants were African American (36 of 38) and the remaining two participants were Caucasian. Caucasian participants were excluded from further analyses as microbiome differences are associated with ethnicity [24], and there were not enough Caucasian participants to control for this confounding variable. The average age of the participants was 51.6 years (sd +/- 13.5). The cohort included twenty-nine female and seven male participants. The state of the hair among participants included: natural (n = 20), relaxed (n = 8), color (n = 5), keratin treatment (n = 1), and other (n = 2). None of the participants wore hair prostheses. Both scalp density (Pearson, p = 0.0019, R^2 = -0.51) and scalp stress (Pearson, p = 0.073, R^2 = -0.31) decreased with age but only the correlation with scalp density was statistically significant. The frequency of scalp cleansing reported by participants included monthly (n = 7), biweekly (n = 16), weekly (n = 12), and daily (n = 1).

Microbial diversity. Following quality filtering,1,720,130 microbial sequences comprising 6,009 OTUs were observed for 67 samples (35 afflicted, 32 normal). Firmicutes (50.6%), Actinobacteria (33%) and Proteobacteria (11.6%) were the most abundant phyla. *Staphylococcus, Corynebacterium*, and *Propionibacterium* were the most abundant genera (**Figure S1**). Shannon alpha diversity, a measure of richness and evenness of species within a sample, was significantly higher in the afflicted hair sites (p = 0.004). Shannon alpha diversity was positively correlated with age in both afflicted and normal hair sites (R^2 = 0.62 and 0.4, respectively; **Fig. 1A**). When categorized by 20-year intervals, participants above 40 years of age have significantly higher Shannon diversity values in the afflicted sites (**Fig. 1B)**. Neither hair state (including treatments) nor cleansing frequency were significantly associated with alpha diversity of the afflicted hair sites.

Dysbiosis. Individual samples were assigned a dysbiosis score **(Fig. 2)**. Calculated dysbiosis scores were classified into groups of either dysbiosis (score > 0) or normobiosis (score < 0). It was determined that the majority, but not all, sites of hair loss are dysbiotic (**Fig. 2**). Based on the threshold set, 24 of 35 afflicted samples were classified as dysbiotic (68.5%) and 30 of 32 samples normal samples as normobiosis (93.75%).

Core taxa contributing to dysbiosis. A RF machine learning classifier was used to determine the most important OTUs for distinguishing the dysbiotic from normobiosis samples. The overall error rate for the RF was 20.9%. The model was better at classifying normobiosis sites; dysbiotic sites were classified to an accuracy of 57.6%, while the normobiosis sites were correctly classified in 92.6% of samples. Twenty-seven unique genera were assigned to the 100 most important OTUs for classification (**Fig. S2**). The mean decrease accuracy (MDA), a measure that indicates the percentage decrease in the random forest model accuracy when a specific OTU is excluded, was >10% for OTUs assigned to the genera *Streptococcus* (66%), *Corynebacterium* (34.2%), *Anaerococcus* (15.8%), *Haemophilus* (14.3%), *Peptoniphilus* (12.5%), *Staphylococcus* (11.9%), *Actinomyces* (11.4%), and *Rothia* (10.5%) (**Fig. S2**).

To control for the potential confounding effect of age, a NBMM was generated each of the 6009 OTUs were independently tested for differential abundance. A total of 100 OTUs belonging to 29 unique genera were found to be differentially abundant in the afflicted sites compared to the normal sites, the majority of which (83%) were more abundant in the afflicted sites (**Fig S3)**. OTUs associated with dysbiosis from the RF were compared to the differentially abundant genera from the NBMM (**Fig. S4**). Taxa that were not identified as differentially abundant by the NBMM were excluded. After conducting a Wilcoxon-rank-sum test to confirm differential abundance, 20 OTUs assigned to 7 genera were determined to be significantly associated with dysbiosis while controlling for age (**Fig 3**).

Discussion

We have developed a microbial dysbiosis score as a proxy for scalp health. Sparse areas on the scalp are associated with dysbiosis in individuals experiencing self-reported hair loss. In addition, we determined a core set OTUs belonging to 7 genera that are significantly more abundant in dysbiotic sites.

Dysbiosis has been broadly defined as a condition differing from the healthy or normal state [25]. The taxonomic composition of the skin microbiome is a result of a multitude of factors including host physiology, environment, lifestyle, and underlying pathobiology [26, 27]. In addition, the microbiome is affected by factors such as skin thickness, folds, and the density/type of hair follicles and glands [24, 27, 28]. Therefore, “normal” is subject to interindividual variation, rendering an informal definition of dysbiosis ineffective. Here, we have defined dysbiosis quantitatively. This approach allows for a gradient approach to characterizing dysbiosis so researchers can quantify the degree to which a particular sample differs from normal and set different thresholds depending on study goals.

Bacterial genera commonly identified on healthy human scalp are *Propionibacterium (P. acnes), Staphylococcus (S. epidermidis),* and *Corynebacterium*. Relative changes in the abundance of these bacteria have been implicated in dysbiosis, though there are inconsistencies between studies (Table 1). Discrepancies may reflect an environmental, disease state, or ethnicity-specific response; most reported studies, including this one, focus on a particular population of subjects (Table 1). In addition, studies often aggregate data at the genus level, though different species within a genus can be either beneficial or antagonistic to human health. Therefore, considering the abundance of the entire genus may be too coarse of a metric. Here, we evaluated abundance differences at the OTU level, which allowed for the differentiation of OTUs within a genus that expressed different patterns of association. In this study, taxa of the genera *Corynebacterium* and *Staphylococcus* were less abundant in hair loss sites when evaluated in aggregate (**Fig S1**), however, some OTUs within these genera were more abundant in hair loss sites (**Fig S3)**. *Corynebacterium* increases and decreases have been associated with scalp-related conditions (Table 1); here, only a few *Corynebacterium* OTUs contributed to dysbiosis (**Fig 3**), potentially accounting for the differences between studies. Therefore, we recommend that future work evaluate taxonomic abundances without aggregating sequence data at the genus level.

Our results indicate that an increase in less abundant residents of the scalp, in addition to an increase of certain OTUs within the genus *Corynebacterium,* is associated with the symptom of hair loss. Taxa within the genera *Acinetobacter, Anaerococcus, Corynebacterium, Finegoldia, Gamella, Haemophilus, Rothia,* and *Streptococcus* were identified as the most significantly associated with dysbiosis (**Fig. 3**). Several of these genera can be highly pathogenic though the pathogenicity is species-dependent. The genera *Acinetobacter* and *Anaerococcus* have previously been identified as more abundant in the scalp dermis of patients with Alopecia Areata [30]. *Streptococcus,* which is implicated in many infectious diseases including cellulitis and toxic shock syndrome [34], contributed most to dysbiosis (**Fig. S2; Fig 3**).

While this work does not include formal clinical diagnoses for our subjects, our findings show that the symptom of hair loss can serve as an indicator of scalp health. Hair shafts promote the growth of gram-negative biofilms while inhibiting the growth of gram-positive *Staphylococcus* species [28]. We observed a decrease in *S. epidermidis* OTUs in the afflicted sites, which could have allowed colonization of other, potentially harmful species. In addition, antimicrobial proteins (lysozyme and histones) have been extracted from human hair [35]. Therefore, the loss of hair in the afflicted sites may have opened the door to enhanced colonization and thus increased diversity due to the loss of protective biofilms and/or antimicrobial properties of the hair. Microbial diversity increased significantly at the afflicted sites for those older than 40 years (**Fig. 1**), which may indicate some degree of protection from colonization of harmful bacteria in younger individuals. Alternatively, the older individuals in the cohort may have had differing underlying scalp disorders. For example, androgenetic alopecia is more common in older individuals, and this condition may elicit a different microbial response compared to traction alopecia, which is the predominant alopecia type in younger individuals of African descent [36]. Future work should compare hair loss sites from more individuals with clinical diagnoses.

CONCLUSIONS

Here, we have corroborated previous work that scalp-related disorders are associated with increased microbial diversity and have shown that this diversity is spatially significant on the scalp within individuals and driven in several taxa including *Streptococcus*. We have shown that within a genus, taxa can be more or less abundant depending on the species, and we recommend against aggregating 16S sequence data at the genus level for differential abundance analyses. Importantly, we have developed a reproducible dysbiosis score that can be used to inform dermatological diagnoses and compare research findings meaningfully. The limited research into treatment modalities that target the scalp microbiome has shown promise [7, 37] and future work should explore targeting the reduction of dysbiosis-associated taxa.

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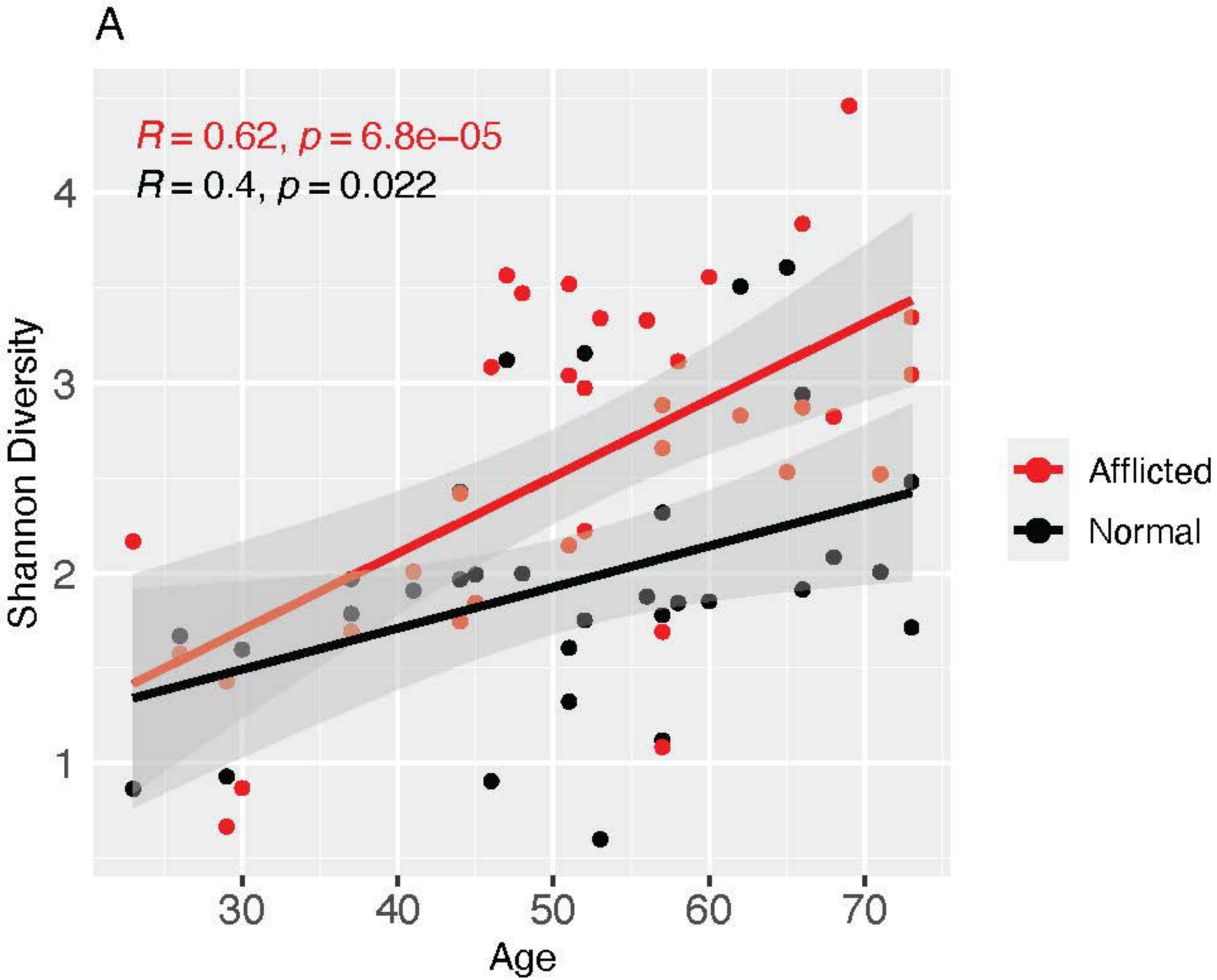
Table 1. Summary of findings from studies on the association between microbial dysbiosis and scalp-related dermatological conditions.

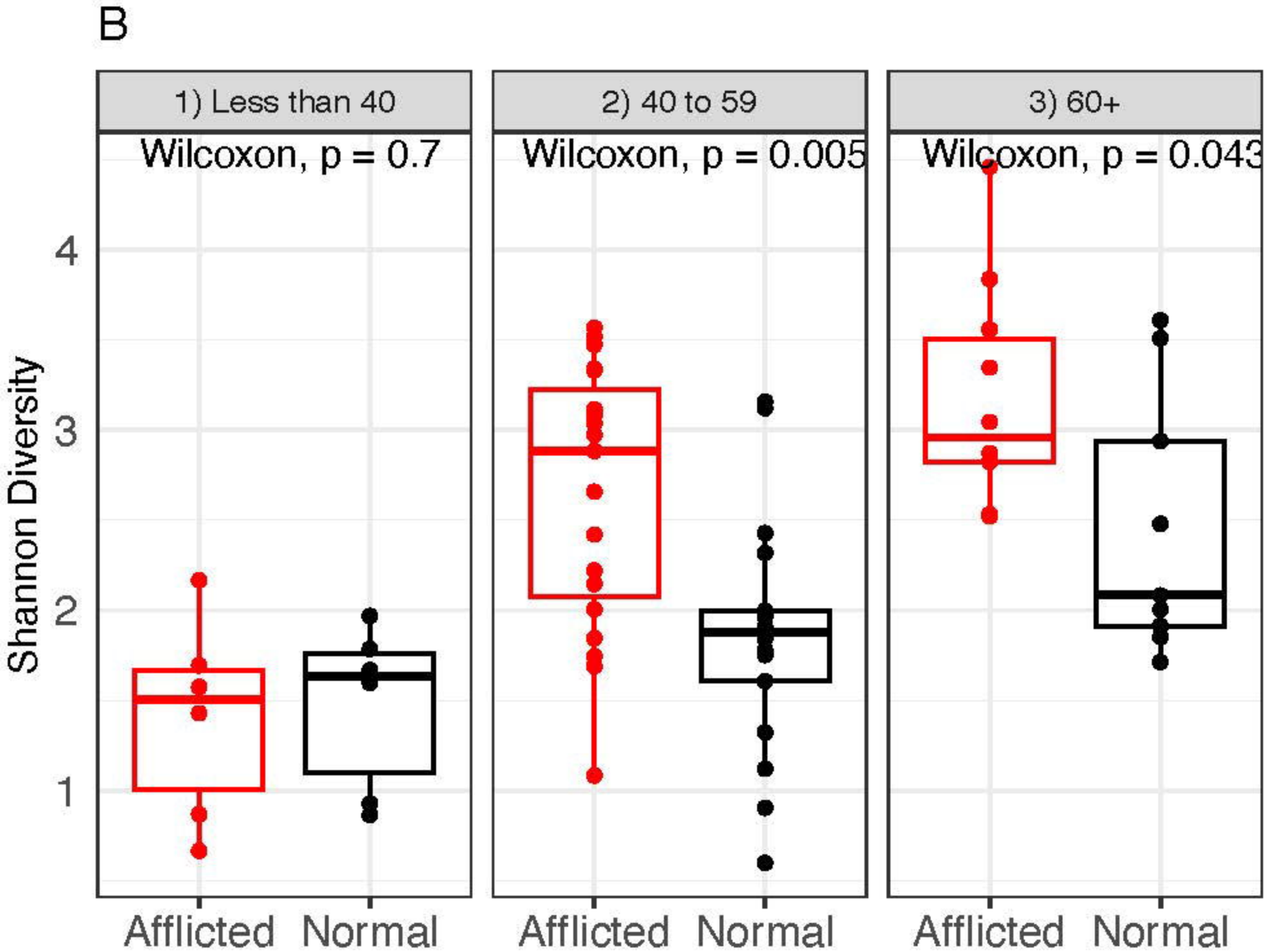
Ref	Population	Condition	Diversity	*Staphylococcus*	*Corynebacterium*	*Propionibacterium / Cutibacterium*
[7]	Indian women	Dandruff	Increase	Increase in *S. epidermidis*	No change	Decrease in *P. acnes*
[30]	Italian men	Alopecia Areata	Increase	Decrease in Genus Decrease in *S. epidermidis*	No change	Increase in Genus Increase in *P. acnes*
[1]	Japanese men	Androgenetic Alopecia	No change	Increase	Decrease	Increase
[31]	Patients recruited from hospital in Korea	Alopecia Areata	Increase	Decrease in Genus Decrease in *S. carprae*	Increase	Increase in severe cases
[32]	Korean women and men	Androgenetic Alopecia	Increase	Decrease in men only	Increase in men only	No change
[33]	Black women	Central Centrifugal Cicatricial Alopecia	No Change	No change	Increase	No change
This study	African American men and women	Hair loss	Increase	Increase in several OTUs Decrease in *S. epidermidis*	Increase in several OTUs	Decrease in *P. acnes*

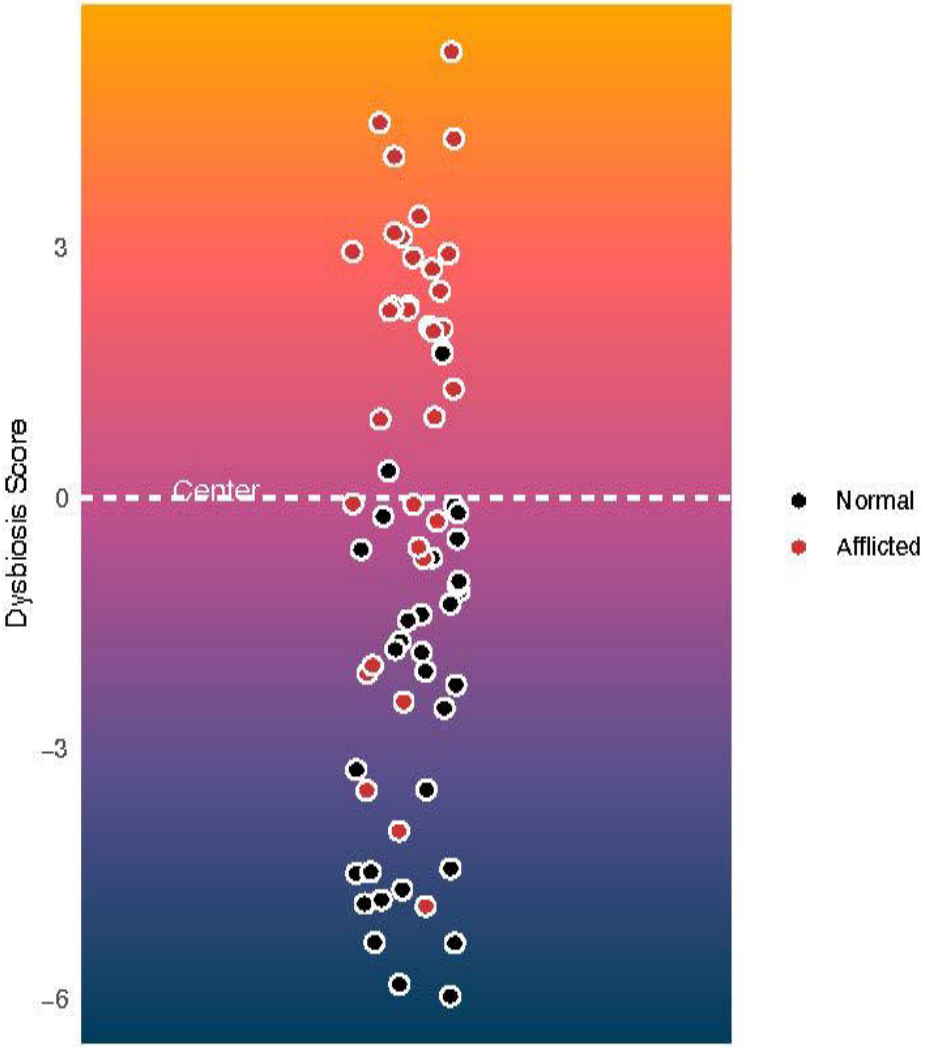
Figure 1. A) Linear regression of age of participants with Shannon Alpha diversity of the scalp microbiome. Red points are the afflicted sites, black points are the normal sites. B) Shannon alpha diversity of the afflicted and normal hair site. Each panel represents an age group 1) Less than 40, 2) 40-49 and 3) 60+.

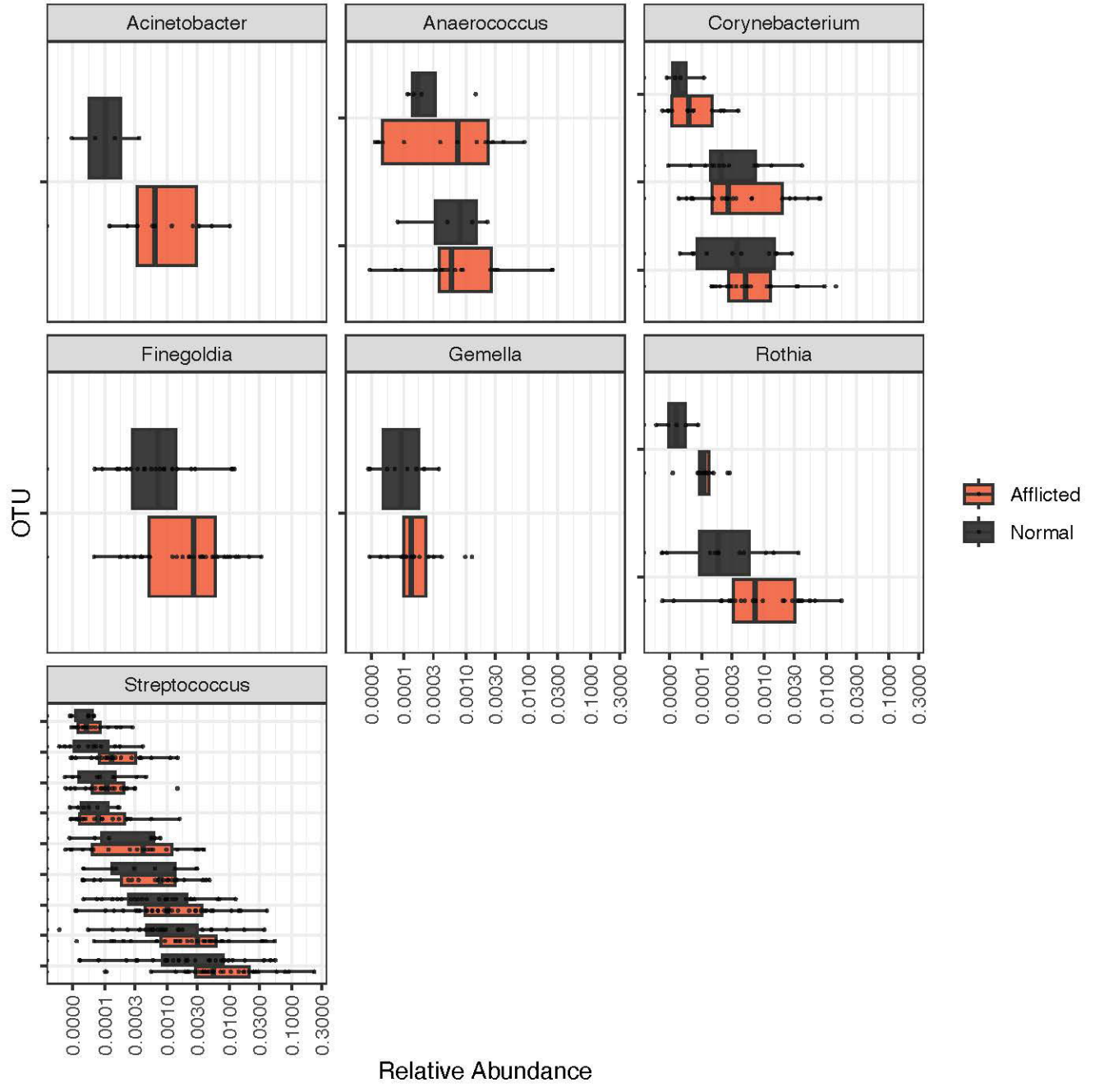
Figure 2. Dysbiosis scores plotted with threshold (white dotted line). A dysbiosis score of 0 indicates that the test sample is at equal distance from the center of both class (afflicted and normal) centroid, and higher scores indicate more deviation from normal.

Figure 3. Relative abundances of core taxa differentiating dysbiotic from normobiosis hair sites while controlling for age differences. Within each genus, only OTUs that are significantly associated with dysbiosis are displayed.


A
R = 0.62, p = 6.8e−05
R = 0.4, p = 0.022
Shannon Diversity
4
3
2
1
30
40
50
60
70
Age
Afflicted
Normal


B
1) Less than 40
2) 40 to 59
3) 60+
Wilcoxon, p = 0.7
Wilcoxon, p = 0.005
Wilcoxon, p = 0.043
Shannon Diversity
4
3
2
1
Afflicted
Normal
Afflicted
Normal
Afflicted
Normal


Dysbiosis Score
3
0
−3
−6
Center
Normal
Afflicted


Acinetobacter
Anaerococcus
Corynebacterium
Finegoldia
Gemella
Rothia
Streptococcus
OTU
Afflicted
Normal
0.0000
0.0001
0.0003
0.0010
0.0030
0.0100
0.0300
0.1000
0.3000
Relative Abundance