Exploring Changes in Facial Microbiota of Maskne Patients and Healthy Individuals Before and After Mask Wearing Using 16S rRNA Analysis

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Abstract

Whether in the field of medical care or in people's daily life and health protection, increased emphasis has been put on the significance of masks. Acne is the most common complication after wearing masks, which is also called as Maskne and has been successfully introduced into the common language, and been a common topic of dermatologist consultations. This study aims to study the microflora changes of Maskne patients and normal people before and after wearing masks.

In the summer 2023, we collected a total of 50 samples from 15 Maskne patients and 10 normal people before and after wearing masks for a long time. 16S ribosomal DNA sequencing and identification technology with V3-V4 variable region were adopted to explore the microbiome changes caused by mask-wearing, analyzing the changes in microbial diversity, and making interaction network. LDA effect size analysis and ANOVA t-test were used to find changed microorganisms.

After Maskne patients wore masks, the changing degree of their microbiomes was significantly more than those of normal people, with both $\alpha$ diversity and $\beta$ diversity lower than those of Maskne patients before wearing masks and those of normal people after wearing masks. Co-occurrence network analysis showed that compared with other groups, the network of Maskne patients after wearing masks for a long time had the lowest connectivity and complexity, but the highest clustering property, while the normal people group was the opposite. Many microbes that are potentially beneficial to the skin decreased significantly after wearing the mask. There was almost no difference in normal people before and after wearing masks.

1. Introduction

Acne is a chronic inflammatory disorder of the pilosebaceous unit. It ranks as the eighth most common disease worldwide, with a prevalence of 9.4%[1]. Approximately 85% of individuals aged 12-24, 8% of adults aged 25-34, and 3% of adults aged 35-44 are afflicted by acne globally[2, 3]. Besides causing severe physical burdens such as painful abscesses and unsightly scars, it can also lead to psychological issues including depression[4], contribute to economic burdens, and increase unemployment rates[4]. Since the beginning of the 21st century, the significance of masks has permeated various aspects of daily life, following several global respiratory infectious disease outbreaks. Whether in healthcare settings or for personal health protection, the importance of mask usage has become increasingly emphasized. However, the prolonged use of masks has brought about facial skin issues. The most frequent adverse skin reaction associated with mask-wearing, as observed in previous studies, is acne, followed by itching symptoms and rashes [5, 6].

Maskne, a newly coined term deriving from mask-related acne, is a form of mechanical acne resulting from continuous textile–skin adherence and friction.[7] Maskne has become a common topic of discussion among dermatologists and has successfully integrated into everyday language. On social media platforms, there are over 2000000 hashtags related to maskne[7], The diagnostic criteria for maskne include:
I. Appearance of acne within six weeks of mask-use or aggravation of pre-existing acne in the mask area [8, 9];

II. Elementary lesions as papules, pustules, and comedones;

III. Localization in the area of mask;

IV. Temporal relationship with mask use: aggravation/development of acne with prolonged usage (>4–6 h/day ) and improvement when not worn for a long period[10];

V. Exclusion of other dermatoses such as perioral dermatitis, rosacea, seborrheic dermatitis, irritant contact dermatitis, allergic contact dermatitis[8];

Maskne is essentially acne issues related to wearing masks. It has been observed that acne patients exhibit noticeable facial microbiota imbalance and reduced microbial diversity [11]. The underlying pathology of Maskne is thought to arise from the microclimate formed beneath the mask-covered skin. This microclimate results in elevated temperature, increased humidity, reduced air circulation, toxin accumulation, and excessive sebum secretion, leading to changes in skin microbiota and acne development.

However, there is currently no specific research regarding alterations in skin microbiota before and after mask wearing. The specific changes in skin microbiota diversity and the compositional differences in microflora from the phylum to genus levels between Maskne patients and healthy individuals before and after mask usage remain unclear. Therefore, this study aims to utilize 16S rRNA sequencing technology to uncover the alterations in skin microbiota of Maskne patients and healthy individuals before and after mask wearing. By exploring the changes in facial microflora, the study seeks to elucidate the potential reasons behind acne caused by prolonged mask wearing in Maskne patients. This study helps address concerns of health care workers and others who need to wear masks for long periods of time about mask complications, such as Maskne, and facilitates research on facial microbiome balances.

2. Method and Materials

2.1 Volunteers recruitment

The study aimed to enroll Maskne patients and healthy individuals (HCs) aged 16-25 residing in Hunan Province, China, during the summer months from June 2023 to September 2023. The average low and high temperatures during the study period range approximately between 23-26°C and 33-38°C, respectively, with a relative humidity of around 70% to 85%. Participants who had been living in Hunan for several years, not undergone antibiotic treatment in the three months prior to sampling, and willing to avoid any other medications during the testing period were recruited. Participants were requested to choose a testing time point that did not overlap with their menstrual time. Written informed consent was obtained from each participant before registration. All procedures involving human participants in this study adhered to the principles of the Helsinki Declaration. The study had been approved by the
Institutional Review Board of the Third Xiangya Hospital, Central South University (Protocol Number: [21141]).

**Inclusion Criteria:**

1. Residing in Hunan Province, China.

2. **Patients:**
   
   Individuals diagnosed with Maskne who had completed a three-month washout period under medical supervision, during which they did not wear any facial masks and did not use any skincare or medications that influence the skin. Patients were classified as moderate or severe according to the Global Acne Grading System (GAGS) during the period of illness, regardless of their current skin condition.

3. **HCs:**
   
   Individuals without acne or any other skin diseases, such as atopic dermatitis, allergic dermatitis, eczema, etc.

4. **Age between 16 and 25 years.**

**Exclusion Criteria:**

- Participation in other clinical studies in the past three months; pregnancy or lactating women; presence of any skin disease (e.g., atopic dermatitis, psoriasis, bruises, eczema, urticaria, rosacea, etc.); deformities or tattoos in the area of study or open wounds; use of medications within the past three months, including antibiotics, hormonal medications, retinoids, adapalene, alpha hydroxy acids, beta hydroxy acids, salicylic acid, tretinoin, or other skincare products with known efficacy in treating acne or skin were excluded.

2.2 **Samples collection:**

Within one week, participants should avoid swimming in chlorinated pools, as well as using hot water, saunas, or ultraviolet. Considering the time required for microbiota to regain balance after facial cleaning patients refrained from any facial cleansing for 24 hours before the study beginning. For 12 hours before the study beginning, participants should avoid touching their faces with hands, tissues, towels, or other items. Volunteers were not allowed to wear any type of facial mask during the washout period three months before the study.

Using a sterile skin swab moistened with physiological saline, random samples were collected from the cheek area within the mask-covered region. The swab sample covered an area of 2.5 x 2.5 cm². During collection, the skin should be swabbed alternately in a cross pattern about 25 times. Samples should be collected before and after wearing the mask for 4 hours. Swabs were placed in phosphate-buffered saline and immediately stored at -20°C before DNA extraction.
In order to express conveniently, we abbreviate normal people before and after wearing masks to NB and NA, and abbreviate Maskne patients before and after wearing masks to B and A in the following paper.

2.3 DNA extraction, PCR amplification, and sequencing

F medial primer 5’-TTCCCTACACGACGCTCTTCCGATCT-barcodeF1Specific primers-3’

F Lateral primers 5’-AATGATACGGCGACCACCGAGATCTACAC-

TCTTTCCCTACACGACGCTC-3’

R medial primer 5’-GAGTTCTCTTGGCACCACCGAGATTCCA- barcodeR1Specific primers-3’

R Lateral primers 5’-CAAGCAGAAGACGGCATACGAGAT- barcodeR2 -

GTGACTGGAGTTCTTGGCACCACCGAGA-3’

16S V3-V4 specific segment :

357F 5’-ACTCCTACGGRAGGCAGCAG-3’

806R 5’-GGACTACHVGGGTWTCTAAT-3’

The PCR amplification products were add to 2% agarose gel for electrophoresis and subsequently excised and collected from the gel. AxyPrepDNA Gel Collection Kit from AXYGEN was used for the recovery process. Following recovery, Illumina high-throughput sequencing and bioinformatics analysis were carried out.

2.4 Data processing

The sequencing work was conducted by Tinygene Biotech Co., Ltd. (Shanghai, China). The obtained paired-end reads (PEreads) were initially sorted based on barcodes for each sample. Subsequently, sequences quality control and filtering were performed. The sequences were then merged based on their overlap relationships, then the quality control and filtering were performed again. The specific steps are outlined below:

Sequence quality control using Trimmomatic (version: 0.38): If the average quality score within the window dropped below 20, the bases starting from the window were trimmed off the end, and reads below 50 bp after filtering were discarded. A sliding window approach was employed, using a window size of 50 base pairs (bp).

Adapter and primer trimming using cutadapt (version: 1.16): cutadapt software was used to process sequencing adapters and primers.
Sequence Merging using FLASH (version: 1.2.11): PE reads were merged into a single sequence using FLASH, taking into account the overlap relationship between PErads. The minimum overlap length was set to 10 bp, and the maximum allowed mismatch rate in the overlap region was 0.2. Additional parameters were set as follows: maxambig=0, maxhomop=8, minlength=200, maxlength=485. Merged sequences that didn't meet the criteria were filtered out.

The resulting optimized sequences were subjected to Operational Taxonomic Units (OTUs) clustering analysis and taxonomic classification. Using USEARCH (version: 8.1.1861) software to cluster the assembled sequences into OTU, the main steps were as follows:

Utilizing UPARSE, clustering was carried out at a 97% sequence similarity level, resulting in representative sequences for each OTU. UCHIME and the existing chimera database, golddatabase (v20110519), were used for sequence alignment to remove chimeric sequences generated during PCR amplification from the representative sequences of OTUs. The usearch_global method was employed to align all sequences back to the representative sequences of OTUs, generating abundance tables for every OTU in each sample. Following the acquisition of representative OTU sequences, mothur (classify.seqs, version: 1.39.5) software was used to annotate these sequences with species information by aligning them against a reference database. The confidence threshold of 0.6 was set for the annotations. The databases used for alignment were Silva128 (bacteria) and Silva119 (archaea). OTUs without annotation results or annotated to species not relevant to the analysis project were filtered out. For example, if the analysis project focused on bacterial 16S samples, OTUs annotated as archaea were removed. Software used for this step included USEARCH (version: 8.1.1861) and mothur (version: 1.39.5).

### 2.5 Statistical analysis

All statistical analyses were performed using the R V3.6.0 environment. With no special instructions, the statistical results were visualized using the 'ggplot2' package. Alpha diversity was measured using the function 'diversity' in the package "Vegan" based on a flat taxonomy table. Gini-Simpson diversity index was obtained by subtracting the value of the classical Simpson index from 1. Beta diversity was compared using principal coordinate analysis (PCoA) based on Bray-Curtis distances. Redundancy analysis (RDA) was also conducted using Vegan. Beta diversity across sample groups was compared by PERMANOVA with permutations of 999. The ANOSIM test was selected to assess significance between groups following the criteria of Wang et al., where R > 0 and p < 0.05 were considered statistically significant. Relative abundance data at the genus level (excluding unclassified) were divided by group to construct separate network graphs. The 'cor.test' function from R version 3.6.3 was employed to compute Spearman correlation coefficients, with p-values corrected using the Benjamini-Hochberg (BH) method. Species pairs with correlation coefficients above 0.8 and p-values below 0.05 were selected to establish correlations. The 'ggraph version 2.0.3' package was used for network graph visualization, and the 'group_components' function determined Modules within the network. Nodes and edges within the same Module were assigned the same color. The 'centrality_degree' calculated degrees, the 'triangles' calculated local_triangles, and the 'group_edge_betweenness' computed Cluster attributes. Visualization
involved the use of ‘ggplot2 version 3.4.2’ and ‘ggsignif version 0.6.0’ to create box plots, with the wilcox.test method assessing inter-group differences. The ‘centrality_hub’ computed hub_score attributes to select the Module with the highest hub_score value as the hub_network to visualization.

In addition, biomarkers of sample groups were discovered by Linear Discriminant Analysis (LDA) Effect Size (LEfSe)3. The strategy for multi-class analysis was set one-against-all, and the threshold on the logarithmic LDA score for discriminative features was set to 3.0.

It needs to be emphasized that A and B, NA and NB, are paired sample groups

3. Results

1.1 Background characteristics of study cohort

This study included 15 patients with maskne and 10 HCs, whose background characteristics are shown in Table.1. The participants ranged in age from 18 to 25 years old; the average age was 19.4 and 19.8 years old in the Maskne and HC groups, respectively. In the patients group, there had been 9 cases of moderate acne, 8 of severe acne respectively during their Maskne period. A total of 50 skin microbiome samples were collected, distributed as follows: 15 samples obtained from Maskne patients before wearing masks, 15 samples obtained from Maskne patients after wearing masks, 10 samples obtained from HCs before wearing masks, 10 samples obtained from HCs after wearing masks. All skin samples met the standard for analysis. No participants withdrew from the study, and there were no missing data.

Table.1 Background characteristics of patients with acne and healthy controls

<table>
<thead>
<tr>
<th>Factors</th>
<th>Normal</th>
<th>Acne</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F/M)</td>
<td>10(5/5)</td>
<td>15(7/8)</td>
<td></td>
</tr>
<tr>
<td>Age, years, mean±SD</td>
<td>19.8±2.4</td>
<td>19.4±2.1</td>
<td>0.97</td>
</tr>
</tbody>
</table>

3.2 Quality of 16sRNA sequencing

Sequencing of 16S rRNA genes with variable regions V3-V4 of the skin samples was performed using Illumina MiSeq platforms. A total of 3287779 clean reads were obtained, with an average of 53190 reads per sample after removing unqualified sequences. Shannon index analysis showed that the depth of sequencing covered rare new phylotypes and most microbial diversity (Online Resource Fig.S1a). The species accumulation curve exhibited a sharp initial rise followed by a gradual ascent, indicating adequate sampling. As the sampling size increases, the rate of species addition diminished. This trend suggested that the number of samples from the system was sufficient to represent the species composition of the system (Online Resource Fig.S1b).

3.3 Changes in facial microbiome diversity before and after wearing masks
Alterations in facial microbiome diversities before and after mask-wearing were examined among Maskne patients. As depicted in Fig.1a, it was evident that the α-diversities of the microbiome after mask-wearing among Maskne patients were notably lower compared to those of normal individuals. It includes observed diversity (P=0.0096, α=0.05), Chao diversity (P=0.0192, α=0.05), Ace diversity (P=0.0357, α=0.05), Shannon diversity (P=0.0036, α=0.005), and Gini-Simpson diversity (P=0.0096, α=0.005). There were also substantial alterations in α-diversity among Maskne patients before and after mask-wearing, particularly with a significant reduction in Shannon diversity (P=0.0036, α=0.005) and Gini-Simpson diversity (P=0.0096, α=0.005) after mask-wearing. In contrast, there was no significant difference in the microbial diversity before and after mask-wearing among HCs (α=0.05). However, when comparing HCs with Maskne patients before mask-wearing, only Shannon diversity exhibited significant differences (P=0.041, α=0.05), consistent with previous literature reports. Compared by principal coordinate analysis (PCoA), and the ANOSIM test using Bray-Curtis distance (R=0.07, p<0.05), Beta diversities demonstrated significant differences among groups (Fig.1b). From Fig.1c and Fig.1d, it could be seen that the NA and NB groups almost coincided, but the A and NA groups were slightly different as shown by ANOISM test (P<0.1).

As depicted in Fig.2a, at the phylum level, among the top 10 abundant bacterial phyla in Maskne patients, the microbial communities showed significant reductions in Bacteroidetes (p=0.0125, α=0.05) and Fusobacteria (p=0.026, α=0.05) after mask-wearing, while Chloroflexi exhibited a significant reduction after mask-wearing (P=0.002, α=0.05) among the top 10 abundant phyla in HCs. However, no significant difference was observed at the phylum level between Maskne patients and HCs after wearing masks. As illustrated in Fig.2b, at the genus level, among the top 30 abundant bacterial genera in Maskne patients, statistically significant decreases were noted after mask-wearing in the genera Corynebacterium (P=0.021, α=0.05), Enhydrobacter (P=0.005, α=0.05), Acinetobacter (P=0.033, α=0.05), Rothia (P=0.035, α=0.05), Veillonella (P=0.012, α=0.05), Brevundimonas (P=0.011, α=0.05), Leptotrichia (P=0.028, α=0.05), and Paracoccus (P=0.038, α=0.05). No statistically significant change was observed in the top 30 abundant genera among HCs after mask-wearing. However, when comparing Maskne patients and HCs after wearing masks, significant decreases were observed in the genera Streptococcus (P=0.023, α=0.05), Actinomyces (P=0.036, α=0.05), Pseudarthrobacter (P=0.039, α=0.05), Acinetobacter (P=0.020, α=0.05), and Pseudomonas (P=0.046, α=0.05) (Fig.2c).

3.4 Microbial network analysis of maskne patients and normal individuals before and after wearing masks

We performed a network co-occurrence analysis to unravel the relationships among microorganisms. With the same network construction parameters, the network of Maskne group after wearing masks (Fig.3a) had 246 nodes and 563 edges, the group of Maskne (Fig.3b) had 217 nodes and 619 edges before wearing. There were 333 nodes and 1602 edges in normal group network after using mask (Fig.3c), with 373 nodes and 2131 edges before (Fig.3d). In addition, A group and B groups were clustered into 45 and 42 modules respectively, while NA and NB groups were 39 and 41 (Online Resource Tab.S1). The results suggested that microbial networks were composed of tightly connected nodes and formed a
kind of ‘small-world’ topology (Online Resource Tab.S1; Online Resource Fig.S2). Correspondingly, we also analyzed the network properties for each group of networks. The average degree of the A and NA groups were 4.577 and 9.622, while the B and NB groups were 5.705 and 11.335, which were higher than their paired groups respectively (Fig.3e), but only the difference between NA and NB groups had statistic significance (P<0.05). The difference between A and NA groups was also significant (P<0.001). Forming triangles in B and NB groups were also higher than their paired groups respectively (Figure.3e), but only the difference between A and B groups had statistic significance (P<0.05), and the forming triangles between A and NA groups was also significant (P<0.001). These suggested that total connectivity and complexities about facial skin microbiomes were higher in the B and NA groups than in the A group. NB group was also higher than its paired NA group. The network Clusters of the A and NA groups were also higher than those of the paired B and NB groups respectively (Fig.3e), but there was no statistic significance yet, while the property of Clusters of A group was significantly higher than that of NA statistically (P<0.0001). These results manifested that the average “clustering property” of the whole networks within facial skin microbiomes in the A and NA groups were higher than the paired B and NB groups respectively, as well as the difference between A and NA groups.

To understand each network in four groups deeply, we extracted the microbial hub network. Among hub networks of four groups, Crinalium had the highest abundance in the A group (Online Resource Fig.S3a), Hansschlegella was the most abundant in the B group (Online Resource Fig.S3b), and in the NA group hub network (Online Resource Fig.S3c), Methylotenera was the highest abundant genus while Crossiella is the highest in NB group. (Online Resource Fig.S3d). Overall, the NB group hub-network had the highest number of nodes and the highest agglomeration. The above findings can be concluded that there were differences in the facial microorganism interaction networks among the A, B, NA, NB groups. Compared with other groups, the A group network had the lowest connectivity and complexity and the highest clustering property.

3.5 LEfSe analysis of the four groups

As indicated by the above results, the changes on facial microbiomes of the Maskne group before and after wearing mask were significantly greater than those of normal people, and microfloras between A and NA groups were also significantly different. Thus, LEfSe analysis was performed to explain which bacteria had significant changes in their relative abundances from phylum to genus. The threshold of adjusted P and LDA score were 0.05 and 3. The findings demonstrated that the phyla Bacteroidetes and Fusobacteria were the most significantly altered ones, and at the genus level, Acinetobacter, Campylobacter, Leptotrichia, Porphyromonas, Finegoldia, Dialister had significant decreases in their relative abundances in the A group compared to their paired B group (Online Resource Fig.S4a). As for A and NA groups, Bacteroidetes Fusobacteria Deinococcus_Thermus were significant in the NA group with increased relative abundances at phylum level. (Online Resource Fig.S4b). Meanwhile, Sphingomonas Rothia Corynebacterium Acinetobacter Pseudarthrobacter Actinomyces Veillonella Kocuria Pseudomonas Chryseobacterium Caulobacter genus were significantly associated with the NA group, and Methylobacterium was significantly associated with the A group at the genera level. For groups
NA and NB, there was no change at the phylum and genera level when the LDA score is 3. The relative abundances of the genera Acinetobacter related to the B and NA groups dropped almost to 0% in the A group (Online Resource Tab. S2). In terms of relative abundance, Acinetobacter Rothia, Pseudarthrobacter Veillonella Actinomyces were the most 5 altered genera in A and NA groups. The cladogram showed the most relevant clades among groups, which was in accordance with the above-mentioned results (Fig. 4a; Fig. 4b).

4 Discussion

4.1 Acne and related microbiota

The clinical characteristics of acne include excessive sebum secretion, non-inflammatory lesions (open or closed comedones), inflammatory lesions (papules and pustules), and varying degrees of scarring. Acne lesions are most commonly found in areas with a high density of pilosebaceous units, such as the face, neck, upper chest, shoulders, and back. In some cases, nodules and cysts can lead to severe nodulocystic acne [12]. The main factors contributing to the development of acne are as follows: Excessive sebum secretion leads to the accumulation of excess oil on the skin. Abnormal follicular keratinization results in the disordered shedding of epithelial cells, causing blockage of the sebaceous ducts. Excessive inflammatory response and secretion of inflammatory factors. Altered distribution of bacteria. Bacteria associated with acne mainly include Propionibacterium acnes, Staphylococcus epidermidis, Malassezia spp., and Staphylococcus aureus [13]. According to Shi et al.'s study, acne patients exhibit lower α-diversity in their facial microbiome, indicating that they have fewer species within their skin microbiome and less evenness compared to healthy individuals [11].

Lachnospiraceae, Clostridiales, Moraxellaceae, Prevotella, and Lactococcus garvieae were the top 5 most abundant species found in patients with acne using 16sRNA sequencing, but were not present in HCs. Achromobacter, Stenotrophomonas, Porphyromonas, Prevotella, and Pseudomonas were the top 5 most abundant species in HCs but were not found in patients with acne. [11] Furthermore, there is a correlation between the integrity indicators of the epidermal barrier and the skin microbiota in acne patients [14]. In comparison to HCs, acne patients often exhibit increased trans-epidermal water loss (TEWL) and reduced diversity in the microbiota [14]. The quantitative analysis of skin microbiota diversity using Shannon and Simpson diversity indices reveals a negative correlation with the severity of acne and TEWL [14]. These findings highlight the close involvement of the microbial community in the pathological changes observed in acne patients.

4.2 Determination of some experimental conditions and parameters in the present study

For patients already suffering from acne, it has been reported that wearing masks for extended periods (> 4 hours) exacerbates acne symptoms, intensifying facial discomfort such as itching, stinging, redness, and swelling [15]. Prolonged mask wearing (> 4 hours), mask type, frequency of mask reuse, and wearing
frequency have been demonstrated to be significantly associated with acne exacerbation[15, 16]. Besides surgical masks are the most widely used and common mask type globally [5]. Therefore, in this study, the collection of facial swabs was conducted with volunteers wearing medical surgical masks for a duration of 4 hours.

4.3 Comparison with the previous researches

Based on the findings of this study, the α-diversity and β-diversity of Maskne patients significantly decreased after wearing masks compared to their pre-mask-wearing state and to the HCs after mask-wearing. This is contrary to the previous study by Wongtada et al., where their results indicated no significant impact of mask-wearing on the skin microbiota in patients with mild acne vulgaris[17]. We hypothesize that this notable discrepancy from prior research is attributed to differences in the severity of acne selected for the present analysis. Specifically, we focused on patients with moderate to severe acne, while previous studies examined individuals with mild acne. Additionally, they used different facial regions for their microbial controls, while we performed before-and-after comparisons all on the mask-covered regions. Prior to the beginning of our experiment, we strictly enforced a mask-free period to establish baseline conditions. Furthermore, the effects of mask-wearing on the facial microbiota of HCs were found to be negligible, aligning with the findings of Hillebrand et al., who concluded that mask-wearing did not induce changes in the facial microbiota of HCs [18]. Moreover, the bacteria collected from facial swabs in our study were predominantly Cutibacterium acnes, Corynebacterium, and Staphylococcus, similar to previous research where the bacteria present in masks after wearing were primarily Cutibacterium acnes and Staphylococcus.

4.4 Possible roles of some microorganisms that varied at the phylum and genus levels

T-test and LefSe were used to search for bacteria that have changed in Maskne patients and normal people after wearing mask for a long time.

We observed that in HCs, there was a decrease only in the phylum Chloroflexi after wearing masks. Although there hasn't been sufficient exploration regarding Chloroflexi [19], it's worth noting that in probiotic-based skin therapies, an increase in its abundance has been associated with improved skin conditions[20]. Moreover, the phylum Chloroflexi and the genera Brevundimonas have been identified as significant microbial components in some spring waters known to promote skin regeneration[21].

After mask-wearing, Maskne patients exhibited significant decreases in the Corynebacterium, Enhydrobacter, Acinetobacter, Rothia, Veillonella, Brevundimonas, Leptotrichia, Paracoccus, Campylobacter, Porphyromonas, and Finegoldia at the genus level. Staphylococcus is a major member of the skin microbiota and has been found to be inversely correlated with skin UV spots[22]. The process of immune regulation is highly complex, and Staphylococcus promotes a substantial increase in the number and activation of γδ T cell subsets. This effect is persistent, independent of other microorganisms, and partially mediated by interleukin-23. Under steady-state conditions, the impact of Staphylococcus is
Genus Enhydrobacter shows a positive correlation with various skin physicochemical parameters such as stratum corneum hydration [24]. Veillonella, Leptotrichia, and Campylobacter have poor researches related with skin. Some species of Paracoccus exhibited increased abundance after facial cleansing [25] and produced potent antioxidant astaxanthin [26]. In facial microbiota of acne and pyoderma patients, Porphyromonas is nearly absent. Finegoldia is enriched in healthy controls compared to rosacea patients. Interleukin-2 receptor displays a negative relationship with Dialister, which serve as predictors of psoriasis activity [27].

Compared to HCs after wearing masks, Maskne patients exhibited significant reductions in the abundances of Bacteroidetes and Fusobacteria at the phylum level after mask-wearing. Bacteroidetes has been found to significantly decrease in adverse skin conditions [28], and in atopic dermatitis, the abundances of Bacteroidetes and Fusobacteria decreased significantly with increasing disease severity [29]. At the genus level, there were notable decreases in Streptococcus, Actinomyces, Pseudarthrobacter, Acinetobacter, Pseudomonas, Sphingomonas, Kocuria, Chryseobacterium, Caulobacter, Veillonella, and Rothia. Certain species of Streptococcus produce bacteriocin-like inhibitory substances (BLIS) that inhibit Propionibacterium acnes growth [30] and secrete ceramides promoting improvement in atopic dermatitis [31], preventing skin aging [32]. There seems to be an antagonistic relationship between Actinomyces and Propionibacterium acnes, as Actinomyces viscosus supematant demonstrating lytic activity against Propionibacterium acnes growth cells [33]. Some strains of Pseudomonas possess immunomodulatory and anti-biofilm capabilities [34, 35], with a potential role in skin repair [36, 37]. An extract from Sphingomonas could mitigate the negative effects of senescence in human skin [38]. Kocuria has skin-protective effects [39] and is significantly reduced in inflammatory lesions [40]. Chryseobacterium can produce a unique bacterial pigment called Flexirubins, used in treating chronic skin diseases like eczema [40]. Research related to Pseudarthrobacter, Caulobacter, Veillonella, and Pseudomonas in dermatologic field remains limited.

In Maskne patients after wearing masks, the quantities of Acinetobacter and Rothia were significantly lower compared to both pre-mask-wearing patients and normal individuals. Certain species of Acinetobacter in the skin microbiota have been found to protect against allergic sensitization and inflammation [41]. Additionally, research has shown that certain types of Acinetobacter on mucosal surfaces increased after surgery and were associated with improved quality of life in chronic rhinosinusitis [42]. Topical ozone therapy has been found to restore microbiome diversity in atopic dermatitis, leading to an increased abundance of Acinetobacter [43]. Rothia has been proved to prevent skin aging and possesses certain anti-inflammatory properties, possibly attributed to its short-chain fatty acids [44, 45].

Methylobacterium has been found to significantly increase in Maskne patients compared to HCs after mask-wearing. Methylobacterium is a highly lipophilic opportunistic pathogen that primarily exists in soil and plants. It possesses strong adhesive and biofilm-forming characteristics, as well as the ability to withstand high temperatures (50–60°C) [46]. However, there is currently no research linking Methylobacterium to skin diseases. Some studies have found significant associations between
Methylobacterium and antibiotic-induced dysbiosis[47], as well as its involvement in the progression of various cancers and higher mortality rates [48]. It can also serve as a prognostic indicator for malignant pleural effusion [49]. Therefore, Methylobacterium, as a newly emerging genus, warrants further investigation into the interaction between itself and acne or skin conditions.

4.5 Discussion on Network Analysis

Nowadays, a wealth of evidence suggests that changes in the microbiome are related to acnes. Dysbiosis of the facial microbiome is reflected not only at the changes in the abundances of microbiome components but also in the altered relationships of microbial interactions. Many studies point out that there are widespread competitions between bacteria in addition to cooperation in networks [50]. Our network analysis showed that the state before HCs wearing masks had the highest network connectivity and complexity and lower aggregation, while the A group had significantly sparser network connectivity. Pathogenic bacteria breaching the skin protective barrier may make contributions to the decreased aggregation in the A and B group, thereby promoting a decrease in the degree of microbial interaction in the facial skin.

4.6 Limitations

It's worth mentioning that we found no significant changes in Propionibacterium acnes in this study. The proliferation of Propionibacterium acnes has historically been closely associated with the occurrence and development of acne. However, recent research has shown that there is no difference in the abundance of Propionibacterium acnes between acne patients and healthy individuals[51], and its abundance is not correlated with the severity of the disease either [51]. The severity of acne is characterized by specific strains of C. acnes [52]. A study identified the top 10 genotypes (RT1-RT10) of Propionibacterium acnes with the highest abundance in acne patients and HCs, and found that the top 3 genotypes, RT1, RT2, and RT3, were evenly distributed in both acne and normal follicular sebaceous units [53]. However, the RT4 and RT5 were significantly enriched in up to 40% of acne individuals but rarely found in healthy individuals. In contrast, RT6 was enriched in 99% of healthy skin individuals[53]. RT4 and RT5 are classified as IA-2 type clades, possessing certain virulence and closely associated with acne inflammation [52]. However, current 16S rRNA technology cannot accurately classify specific phylotypes of Propionibacterium acnes, which is a limitation of our study. In the future, we will conduct research on the relationship between changes in the abundance of different Propionibacterium acnes phylotypes and mask-wearing.

In addition, this study also has other shortcomings. The sample size was relatively small, therefore while there were numerical changes in many bacteria that are closely related to acne, these changes did not reach statistical significance, hindering meaningful conclusions. Moreover, the experimental period primarily took place during the summer season, which could have been influenced by factors like temperature and humidity. Furthermore, the study was conducted in a limited population from central China, so it's anticipated that future research will explore a larger and more diverse cohort to provide broader insights.
5 Conclusion

In conclusion, our research has provided evidence for the imbalance in facial microbiome on Maskne patients, clarified the differences in facial microbiome among NA, NB, A and B groups, elucidated the changes of facial microbiome on Maskne patients before and after wearing masks with A exhibiting significant dysbiosis of the microbiota and the lowest microflora diversities. Network analysis showed different network connectivity, complexity and aggregation degree, while the A group had significantly sparser network connectivity and the lowest network connectivity and complexity. T-test and LefSe were used to search for bacteria that have changed in Maskne patients and normal people after wearing masks for a long time. The changes in phylotypes of Propionibacterium acnes are still worth exploring in Maskne patients after wearing masks. This study has identified specific microbial changes in acne, the most common complication caused by prolonged mask wearing, which will help researchers and factories further improve mask manufacturing.

Abbreviations

Healthy individuals (HCs) Global Acne Grading System (GAGS) Paired-end reads (PEreads); Operational Taxonomic Units (OTUs); Principal coordinate analysis (PCoA); Redundancy analysis (RDA); Linear Discriminant Analysis (LDA) Effect Size (LEfSe); Trans-epidermal water loss (TEWL); Bacteriocin-like inhibitory substances (BLIS); Genotypes RTs;

Declarations

Statements and Declarations

Acknowledgments: There are no people or institutions to acknowledge in this article.

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Ethics approval: Written informed consent was obtained from each participant before registration. All procedures involving human participants in this study adhered to the principles of the Helsinki Declaration. The study had been approved by the Institutional Review Board of the Third Xiangya Hospital, Central South University (Protocol Number: [21141]).
Consent to participate: Informed consent was obtained from all individual participants included in the study or their legal guardians.

Consent to publish: Patients signed informed consent regarding publishing their facial microbiome data.

Data availability statements: Data is stored in https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1017432 and the project is PRJNA1017432

References


Figures
Changes of microbial diversity (a) $\alpha$ diversities include observed diversity, Chao diversity, Ace diversity, Shannon diversity, and Gini-Simpson diversity. Paired sample groups: A and B, NA and NB were analyzed with the paired ANOVA t-Test. Non-paired sample groups: A and NA, B and NB were analyzed with ANOVA t-Test. *p<0.05; **p<0.01; ***p<0.001. (b) ANOISM demonstrated significant differences among A-B-NA-NB groups using Bray-Curtis distance. (c) Principal coordinate analysis (PCoA) of NA and NB groups.
using Bray-Curtis distance. (d) ANOISM demonstrated statistical differences among A-NA groups using Bray-Curtis distance (p<0.1).

**Figure 2**

Composition of bacterial communities at the phylum and genus levels (a) Composition of the microbiomes of the four groups at the phylum level; (b) Comparison of bacterial community composition at the genus level between groups A and B (c) Comparison of bacterial community composition at the genus level between groups A and NA. A: Maskne patients after wearing masks for a long time. B: Maskne patients before wearing masks for a long time. NA: Healthy individuals after wearing masks for a long time. NB: Healthy individuals before wearing masks for a long time.
Figure 3

Co-occurrence network in four groups (a) Network of Maskne patients after wearing mask (A), (b) Network of Maskne patients before wearing mask (B), (c) Network of healthy controls after wearing mask (NA) and (d) Network of healthy controls before wearing mask (NB). (e) Comparison of network topology properties among groups, weighted degree, triangles, and cluster. Wilcoxon test, *p < 0.05; **p < 0.01; ***p < 0.001.
LEfSe analysis of taxonomy with significant differences in abundance among groups. Evolutionary branching diagram: the circles radiating from the inside to the outside represent taxonomic levels from the phylum to the genus. Each small circle at different taxonomic levels represents a taxon at that level, and the diameter size of the small circles is proportional to the relative abundance size. (a) Cladogram between A and B. Species without significant differences are uniformly hided or colored in chartreuse, red
nodes indicate microbial taxa that play an important role in the B group. The names of the species indicated by letters in the figure are shown in the legend on the right. (b) Cladogram between A and NA. Species without significant differences are uniformly hided or colored in chartreuse, red nodes indicate microbial taxa that play an important role in the A group, green nodes indicate microbial taxa that play an important role in the NA group. The names of the species indicated by letters in the figure are shown in the legend on the right. A: Maskne patients after wearing masks for a long time. B: Maskne patients before wearing masks for a long time. NA: Healthy individuals after wearing masks for a long time. NB: Healthy individuals before wearing masks for a long time.

**Supplementary Files**

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