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ORIGINAL RESEARCH

Analysis of the Bacterial Flora of Sensitive Facial Skin Among Women in Guangzhou

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Background: Sensitive skin (SS) is easily irritated by various environmental stimuli, and epidemiological surveys surprisingly find that self-perceived SS is widespread worldwide.

Objective: To investigate whether SS is linked to changes in the skin bacterial population using 16S rRNA sequencing and bioinformatic analysis.

Patients and Methods: According to both the Huaxi SS Questionnaire and Lactic Acid Stimulation Test, 60 female volunteers in Guangzhou were classified into normal skin (NS) and SS groups. Skin barrier parameters were assessed by the CK skin tester. The DNA of the bacterial flora on the facial skin surface was extracted and was subjected to 16S rRNA sequencing.

Results: The skin hydration was significantly lower in the SS group compared to the NS group (P =0.032). Based on 16S rRNA sequencing and bioinformatic analysis, the number of operational taxonomic units (OTUs) significantly decreased in the SS group (P =0.0235, SS vs NS). The relative abundance of *Neisseriaceae* in SS group decreased significantly (P <0.05, SS vs NS), while that of *Neisseria* (within the *Neisseriaceae* family) increased significantly (P <0.05, SS vs NS).

Conclusion: SS is accompanied by a decrease in species diversity and richness, which may be relevant to the weakening of the microbial barrier (due to the increase of *Neisseria* or the decrease of *Neisseriaceae*). Thus, corresponding treatment for *Neisseriaceae* may be a new idea in the treatment of SS.

Keywords: sensitive skin, 16S rRNA sequencing, bacterial, skin microbiome, skin barrier

Introduction

It is becoming more widely accepted that the present increase in allergies and (chronic) inflammatory diseases are a result of our modern lifestyle, and they are also related to changes in the human microbiome.[1] Disturbances in the homeostasis between the host and its microbiota may drive many inflammatory skin diseases, such as atopic dermatitis (AD),[2] psoriasis,[3] and acne.[4] Sensitive skin (SS) is generally characterized by subjective discomfort, no obvious signs of irritation, with symptoms such as itching, dryness, redness, and swelling.[5,6] Although initially believed to be an unusual reaction to common products, epidemiological surveys surprisingly find that self-perceived SS is widespread worldwide.[7] The global prevalence of self-declared SS (to varying degrees) was 71% in the adult population, and the prevalence of highly or moderately SS was 40%.[8] Subjects with SS can experience inappropriate reactions, such as itching, dryness, redness, and swelling, after exposure to dry and cold climates due to damage of the skin barrier function. In short, changes in the skin barrier, microbes, and consequent cutaneous sensitization plays crucial roles in these inflammatory skin diseases.

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Every person's skin carries a unique microbial population, which may help to protect skin or increase its vulnerability.[9] The microbiota present on the skin (which consists of both commensal and pathogenic microbes) can be transient or resident,[10,11] and these microbes affect the skin barrier and epithelial innate immune responses.[12,13] When the barrier is disturbed or the balance between commensal microbiota and pathogens is disrupted, skin disease or even systemic disease may occur.[14,15]

The skin microbiome plays important roles in preventing invading pathogens, educating the host immune system, and breaking down natural products, similar to the gut microbiomes.[15] However, the research on the skin microbiota is not as far along as research on gut microbes, because the comparatively highly open skin microenvironment leads to large differences in the flora between individuals. In recent years, due to the development of 16S rRNA gene sequencing technology and bioinformatic analysis, understanding of the composition of skin microbiomes has increased.[16,17] Many human skin disorders and diseases are related to changes in the composition or functionality of skin microbiota, termed dysbiosis.[18] In patients with AD, there is increased colonization by *Staphylococcus aureus*.[19,20] In psoriatic lesions, *Streptococcus spp*. is significantly more frequent.[21] *Cutibacterium acnes* is the main resident of the skin that is considered to contribute to acne.[22] In the past few decades, the prevalence of allergic diseases such as cutaneous allergy has risen sharply. Recent studies have pointed to the core role of the microbiome, which is highly influenced by a variety of environmental and dietary factors.[23] However, it is still unclear whether these changes are the cause or the result of the underlying disease. Nevertheless, such changes may have diagnostic, preventive, and potential therapeutic implications.[18]

Therefore, given the associations between the microbiome and skin conditions, we aimed to assess whether the microbiome in SS differs from that of normal skin (NS) by bacterial 16S rRNA V3-V4 gene Illumina sequencing. SS and NS were defined using the Huaxi SS questionnaire (SSQ) and Lactic Acid Stimulation Test (LAST). Skin barrier parameters, including hydration and transdermal water loss (TEWL), were assessed by the CK skin tester. A key objective was to identify biological targets associated with SS and NS by 16S rRNA gene Illumina sequencing. Comparing the microbiomes of SS and NS could help us find the cause of sensitivity and advance our understanding of the mechanisms that underlie the pathophysiology of SS.

Materials and Methods

Study Design

This was a single-center observational study designed to investigate the association between the skin microbiome composition and SS, using 16S rRNA gene Illumina sequencing and skin test system. Our study protocol was reviewed and approved by the Hospital Institutional Ethics Review Committee of Jinan University (Guangzhou, China). Informed consent was obtained from all subjects before they participated in this study. All procedures and protocols used in this study were carried out following the principles of the Declaration of Helsinki.

Using self-assessment questionnaires to identify SS is a useful approach.[6] According to previous research,[24] the self-assessed Huaxi SSQ (which is a newly developed tool designed by the Department of Dermatology, West China Hospital, Sichuan University) has superior reliability and validity in Chinese individuals to the Bauman SSQ, and it is more suitable for screening of SS in China.

Based on Huaxi SSQ and LAST scores, 60 female volunteers in Guangzhou were classified into the NS group (Huaxi SSQ score = 12–17, LAST score = 0) and the SS group (Huaxi SSQ score = 18–32, score $\geq$ 3) (Figure 1). Sterile cotton swabs were used to sample the flora on each subject's cheek (C) and jaw (J). Thereafter, DNA was extracted for amplification and sequencing of the 16S rRNA gene. We also assessed the differences in facial skin barrier parameters (including skin hydration level and TEWL) between the NS and SS groups.

Subjects

We classified the 60 female volunteers in Guangzhou into the NS and SS groups based on the Huaxi SSQ and LAST scores. Eligible subjects were: Chinese females aged 20–40 years (mean 28.67±11.33 years); the history of sensitivity to general cosmetics or other skin products (such as moisturizers); diagnosed with chronic skin allergies; and known hypersensitivity to cosmetic products. Exclusion criteria were: under the care of a doctor; taking drugs that may mask or conflict with the test results; any form of skin cancer or any other disease that could conflict with test results, such as mental illness, serious diseases of heart, liver and kidney, autoimmune diseases, or severe herpes simplex infection; chemical peeling treatment within the previous 6 months; skin treatment within past

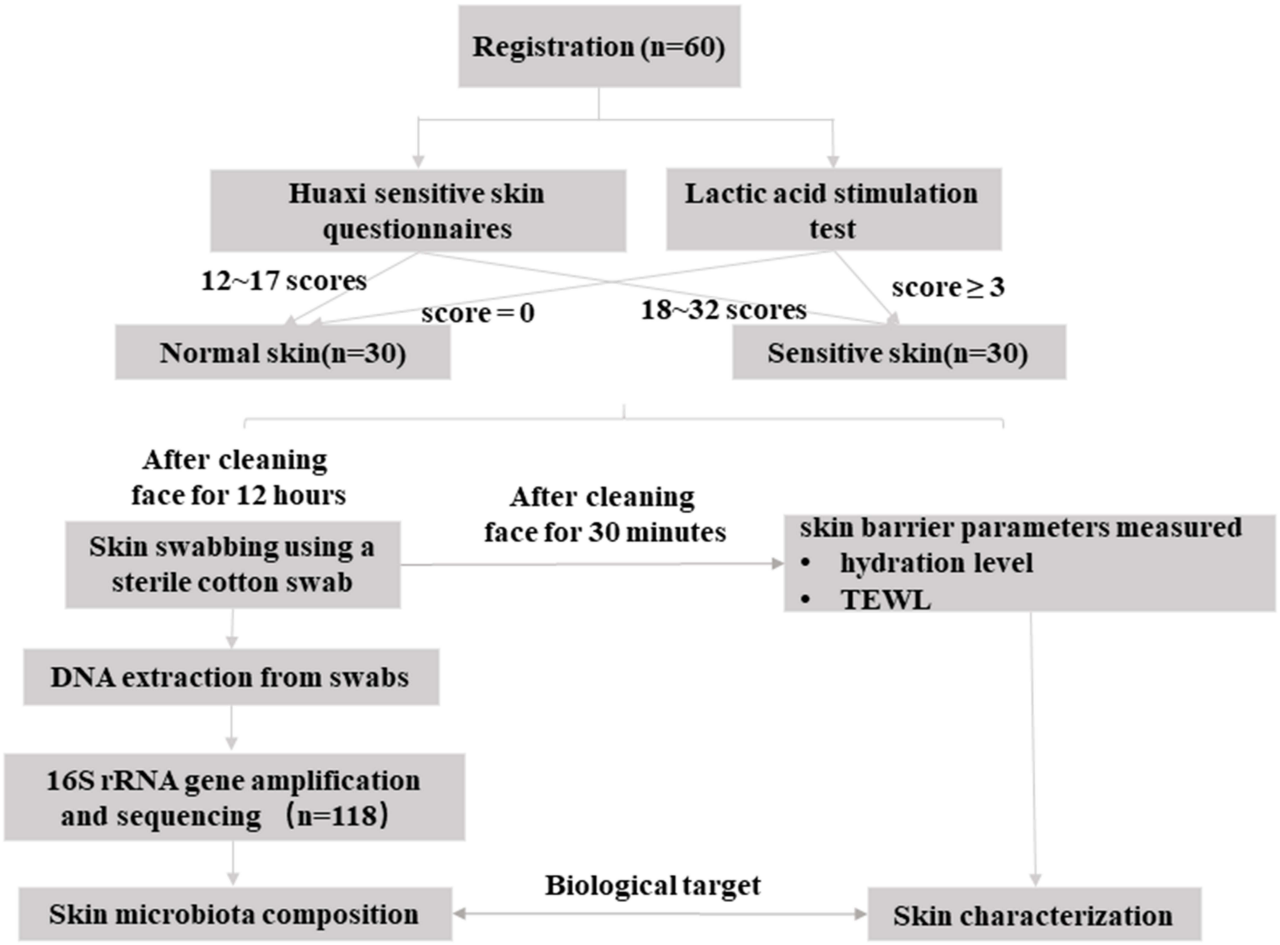

Figure 1 Consort flow chart demonstrating the progress of subjects in the study.
Abbreviation: TEWL, transepidermal water loss.

3 months; any type of laser or intense pulsed light within the previous 6 months; pregnant or nursing an infant; and refusing to give informed consent.

Lactic Acid Stimulation Test (LAST)

To conduct the LAST, 5% lactic acid solution was rubbed with a cotton swab onto the test site (which produces a moderate to severe stinging sensation a few minutes later in the "stingers group"), and the inert control (phosphate-buffered saline [PBS]) was rubbed onto the contralateral test site. Thereafter, the subjects reported the types of discomfort (pain, burning sensation, itch, or crawly feeling) and severity score (0 to 3 points in increments of 1) at three time points (30 s, 2.5 min, and 5 min). The LAST score was defined at each time point as the difference between the lactic acid- and PBS-treated skin regarding the severity score with the maximum value (at the lactic acid-treated site).[25] Finally, if the sum of 2.5 min and 5 min LAST scores ≥3, the subject was classified as LAST-positive.

Sample Collection and DNA Extraction

Sample Collection

The choice of sampling method, anatomical location, and sequencing approaches are critical factors in any skin microbiome study.[26] Widespread methods for sampling the skin microbiome include non-invasive (swabbing, scraping, and tape stripping [D-squame])[27] and invasive (punch biopsies) methods.[28] We selected swabbing skin with a sterile cotton swab, which is the most practical method for large-scale skin sampling. It is fast and simple but can certainly allow resident microbiomes to be obtained from the stratum corneum.

After cleansing their skin, the subjects did not touch their face or put any products on their face, and cotton swabs were used to collect samples after 12 h. The

operator wore sterile gloves, and used a medical sterile cotton swab dipped in sterile PBS, wiping 50 times (rotating cotton swabs) on the cheek (C) or jaw (J). The tip of each cotton swab was cut off into a sterile 2 mL centrifuge tube containing 1 mL DNA protection agent, and the tube was then covered and shake by hand for 30 s. Finally, it is sealed with parafilm and store at −80 °C. It was necessary to ensure aseptic operation during sampling to avoid contamination.

DNA Extraction from the Swab

Samples in DNA protection agent were left in a 37 °C water bath for 2h. Thereafter, the DNA was extracted using the MicroElute Genomic DNA Kit (Omega, USA) following the manufacturer's instructions.

16S rRNA Gene Amplification and Sequencing

To obtain an overview of each subject's skin microbiome, we generated amplicons from the V3-V4 region, which represents a region among the nine hypervariable regions (V1–V9) of the bacterial 16S rRNA gene. The PCR primers were 341F (5′ CCTAYGGGRBGCASCAG 3′) and 806R (5′ GGACTACNNGGGTATCTAAT 3′). They span over 1 of the 9 hypervariable regions of the bacterial 16S rRNA, which were chosen as most of the sequences in databases were used for variable regions V3–V4.[29]

The forward and reverse primers were labeled by Illumina adapter, pad, and linker sequences. PCR was performed using a 50 μL reaction mixture including 30 ng template, primers, and PCR master mix. The PCR products were eluted using elution buffer. The purity, integrity, and concentration of the libraries were examined using an Agilent 2100 bioanalyzer (Agilent, USA). They were sequenced on the Illumina MiSeq platform by Chi Biotech (Shenzhen, China) according to the Illumina standard pipelines, producing 2×300 bp paired-end reads.

Skin Tests

After obtaining samples of each subjects ' facial flora, the subject cleaned their face and then equilibrated for 30 minutes in a closed environment (20°C~22°C and 40% ~60% relative humidity). Skin parameters, including TEWL and hydration of the skin surface, were assessed using a multi-probe adapter system–Cutometer® dual MPA 580 (Courage + Khazaka Electronic GmbH, Cologne, Germany).

Statistical Analysis

The statistical analysis was carried out in GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Data are reported as mean ± standard deviation (SD). To evaluate the statistical significance of the between-group differences, the groups were compared by one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Analysis of Biophysical Parameters of NS and SS Groups

In this study, we assessed SS in Chinese individuals (n=1321) using the Huaxi SSQ and found that about 80% of men and about 90% of women have SS, and most of them are aged 15–34 years (Supplementary Table 1). Based on overall Huaxi SSQ scores of 18–23, 24–32, 33–42, and 12–17, subjects can be classified as having mild SS, moderate SS, severe SS, and NS, respectively.

The Huaxi SSQ scores in the NS and SS groups were approximately 16 and 28, respectively, with significant differences ($P < 0.05$) (Table 1). The LAST scores for all subjects in the NS and SS groups were 0 and ≥ 3, respectively, which corresponded to the classification based on the Huaxi SSQ scores. Based on the skin barrier parameters measured by the Courage + Khazaka multiprobe adapter system, the hydration levels in the NS and SS groups were approximately 63 and 58, respectively, with a significant difference ($P < 0.05$). The SS group had a non-significantly higher TEWL than the NS group (P=0.0572) (Table 1). Thus, SS was more sensitive to lactic acid stimulation and had significantly decreased hydration compared to NS, but there was no significant difference in TEWL.

Skin Microbiomes in the NS and SS Groups

To explore the difference in the skin microbiomes between the various NS and SS subgroups, we used bacterial 16S rRNA V3-V4 gene sequencing to determine the composition of the bacterial flora (Figure 2A–F). Notably, in NS group, the number of operational taxonomic units (OTUs) was significantly higher in the NS-C subgroup (3764.07 ±1057.10) than the NS-J subgroup (3137.10±884.79) ($P <$ 0.05). However, there was no significant difference between the SS-C (3081.93±943.42) and SS-J (2710.83

Table 1 Skin Characteristics from Different Facial Sites of Normal and Sensitive Skin

	Normal Skin (n = 30)		Sensitive Skin (n = 29)		*P*-value
Demographics					
Age, years	30±7 years		28±8 years		-
Sex	Female				-
Huaxi SSQ	16.37±1.37		28.17±3.39		<0.001
LAST	0		2.36±1.74		-
	Cheek	Jaw	Cheek	Jaw	
Skin test					
TEWL	11.57±5.07		14.69±6.30		0.0572
Hydration level	63.40±8.25		58.33±9.99		<0.05
Alpha diversity estimates					
OTU Count	3764.07±1057.10	3137.10±884.79	3081.93±943.42	2710.83±695.09	-
No. of Genera	14,546	12,457	11,933	10,016	-
Shannon Index	6.52±0.86	5.89±1.07	6.56±0.72	6.27±0.55	-
Chao Index	4532±1172.08	3828±987.19	3824.6±1034.67	3380±822.85	-

Abbreviations: Huaxi SSQ, Huaxi sensitive skin questionnaires; LAST, Lactic acid stimulation test; TEWL, transepidermal water loss.

±695.09) subgroups. The number of OTUs was significantly higher in the NS-C group than the SS-C group ($P < 0.05$), but there was no significant difference in the number of OTUs between the NS-J and SS-J subgroups (Figure 2A). In addition, there were 6349 and 4550 genera in the NS-C and SS-C subgroups, respectively, of which 3547 were shared. There were 5343 and 3948 genera in the NS-J and SS-J subgroups, respectively, of which 2944 genera were shared (Figure 2B). In short, the NS group had more No. of genera and OTUs than the SS group. However, the quality control process indicated that the quality of a sample in the SS group was unqualified, so all the data of this group of samples were removed.

To further analyze the microbial community diversity within each subgroup, we conducted an α diversity analysis using Qiime software, including species richness and community diversity, represented by the Chao1 index and Shannon index, respectively. The trends in the Chao1 index (representing species richness) was the same as for the number of OTUs (Figure 2C), with a significant increase in the NS-C subgroup compared to the SS-C subgroup, and in the NS-C subgroup compared to the NS-J subgroup ($P <0.05$). However, there was not a significant difference in the Shannon index (representing community diversity) between the NS-C and SS-C subgroups ($P > 0.05$) (Figure 2D).

Based on the species annotation results, the relative abundance at phylum (Figure 2E) and genus (Figure 2F) level were assessed. The microbiotas in the SS and NS groups were mainly composed of three bacterial phylum (*Proteobacteria, Firmicutes*, and *Actinobacteria*) with no significant differences between the SS and NS groups for either the cheeks or jaws ($P > 0.05$) (Figure 2E). This indicated site-specific distribution of flora at the phylum level. Interestingly, *Neisseriaceae* family were dominant, and there were significant differences between the NS and SS groups, particularly in the relative abundance of the *Neisseriaceae* family and the *Neisseria* genus (Figure 2F). The relative abundance of *Neisseria* genus significantly increased in the SS group compared to the NS group ($P <0.05$), while that of *Neisseriaceae* family significantly decreased ($P <0.05$).

Differences in Microbial Communities Among the NS and SS Groups

To compare the microbial community composition among the four subgroups, we performed β diversity analysis based on principal component analysis (PCA) and found

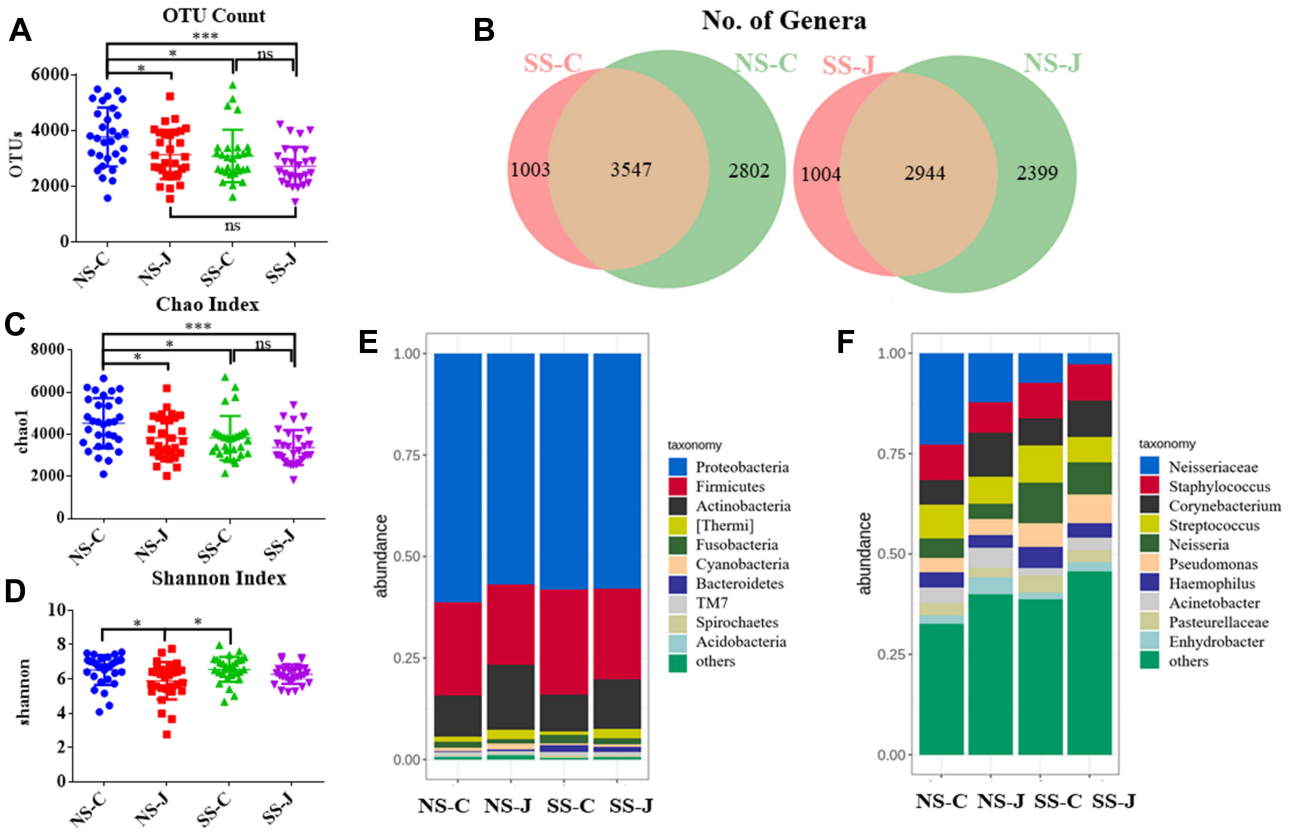

Figure 2 Microbiota characteristics (alpha diversity) from different facial sites of normal skin (NS) and sensitive skin (SS). (**A**) The operational taxonomic units (OTUs) represents the total bacterial load. The line represents the median. *P*= 0.0414, NS-C vs NS-J; *P*= 0.0235, NS-C vs SS-C; *P*=0.0001, NS-C vs SS-J. (**B**) Venn diagram of No. of Genera. (**C**) Chao index. *P*= 0.0454, NS-C vs NS-J; *P*= 0.0465, NS-C vs SS-C; *P*=0.0002, NS-C vs SS-J. (**D**) Shannon index. *P*= 0.0226, NS-C vs NS-J; *P*= 0.0142, NS-C vs SS-J. (**E** and **F**) Histogram of bacterial structures for all groups at the phylum (**E**) and genus (**F**) level, just phylum or genus with abundance higher than 1% were pointed. **P* < 0.05; ****P* < 0.001; ns means no significant differences.

Abbreviations: NS, normal skin; SS, sensitive skin; C, cheek; J, jaw.

no significant differences among the four subgroups ($P > 0.05$) (Figure 3). Nevertheless, the microbial community composition of the NS group was more diverse, as was the microbial community composition of the cheek samples compared to the jaw samples.

We used linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify taxa with significant differences in abundance between subgroups. *Neisseria* genus and *Neisseriaceae* family exhibited significant differences between the SS-C and NS-C subgroups (Figure 4A), with *Neisseria* genus significantly increasing in the SS-C group (P<0.05) and *Neisseriaceae* family significantly decreasing (P<0.05). *Intrasporangiaceae* exhibited significant differences between the SS-J and NS-J subgroups (Figure 4B), with a significant decrease in the SS-J group (P<0.05).

Discussion

Compared to NS, SS is more sensitive to lactic acid stimulation and had significantly decreased hydration, which is also similar to the results of previous studies.[30–32] Studies have shown that the major reason for SS in women might be barrier dysfunction induced by water loss.[30] Impaired skin barrier function facilitates the course of the disease.[33] However, there is no significant difference in TEWL (*P*=0.0572), which is similar to previous results.[32] Although TEWL was not significantly different between the NS and SS groups, the *P* value was close to 0.05, and the lack of a significant difference may have been due to insufficient sample size or lack of damage to the stratum corneum. The stratum corneum or horny layer in the epidermal barrier acts in the control of TEWL.[34] It indicates that the itching of SS may be related to nerve sensitivity and dry skin, rather than damage to the stratum corneum.

The three dominant bacteria phylum on the skin in both the SS and NS groups were *Actinobacteria, Firmicutes*, and *Proteobacteria*. Nevertheless, depending on the skin topography and distinct chemical makeup of the site and

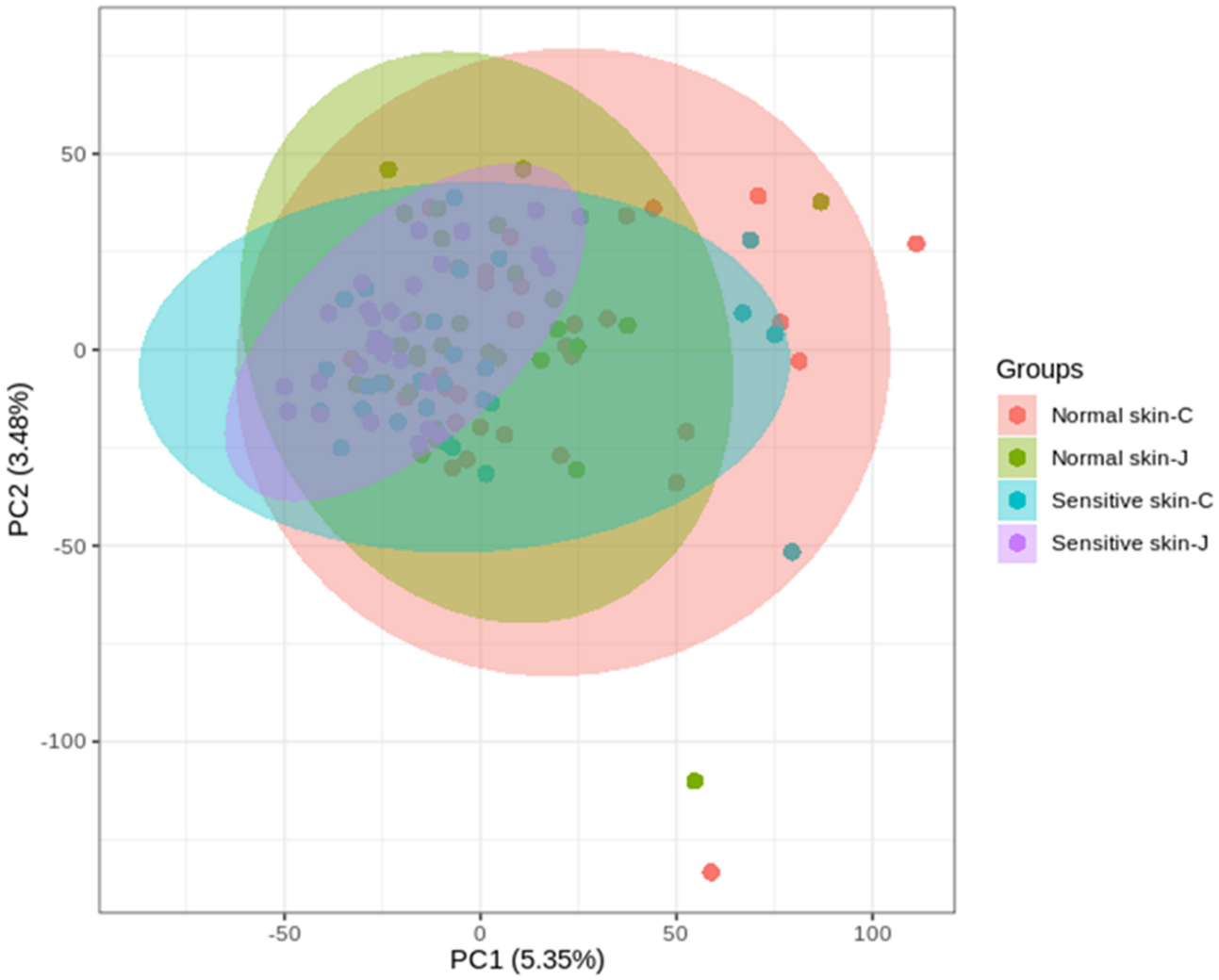

Figure 3 Principal component analysis (PCA) of species-level operational taxonomic unit (OTU) relatedness of 118 skin samples from two different facial sites of 59 subjects. The axes are labeled with the percent of the distinction defined by various principal components. The circle describes all cheek (C) or jaw (J) skin samples, which cluster closely together.

Abbreviations: NS, normal skin; SS, sensitive skin; C, cheek; J, jaw.

the individual, the structure and variety of the resident microbiomes may vary significantly. A previous study found that personality and skin topography influenced the microbiome composition.[21] The microbiome of NS was previously found to have a high diversity and high interpersonal variation. The microbiota on diseased skin lesions has been shown to have distinct differences compared to the microbiota on NS.[10] The occurrence of AD has been proved to be the result of the skin microbiome interactions with host immunity.[35,36] SS may also be an immune protection mechanism that encounters microbiomes.

In addition, the number of OTUs was significantly higher in the NS-C subgroup than the SS-C subgroup. Additionally, the Chao1 index (representing species richness) was significantly higher in the NS-C subgroup than the SS-C subgroup, but there was no significant difference in the Shannon index (representing community diversity) between the NS and SS groups. In short, the SS is accompanied by a decrease in species diversity. These findings differ from previous findings,[32] which may be related to regional differences, inclusion criteria, and different companies selected for sequencing. Based on PCA, there were no significant difference in the microbial community composition among the four subgroups. However, the microbial community composition of the NS group was more diverse, which high diversity and high inter-individual variation. These findings are consistent with previous research observations.[37]

In SS group, the relevant abundance of *Neisseria* significantly increased, while the relevant abundance of

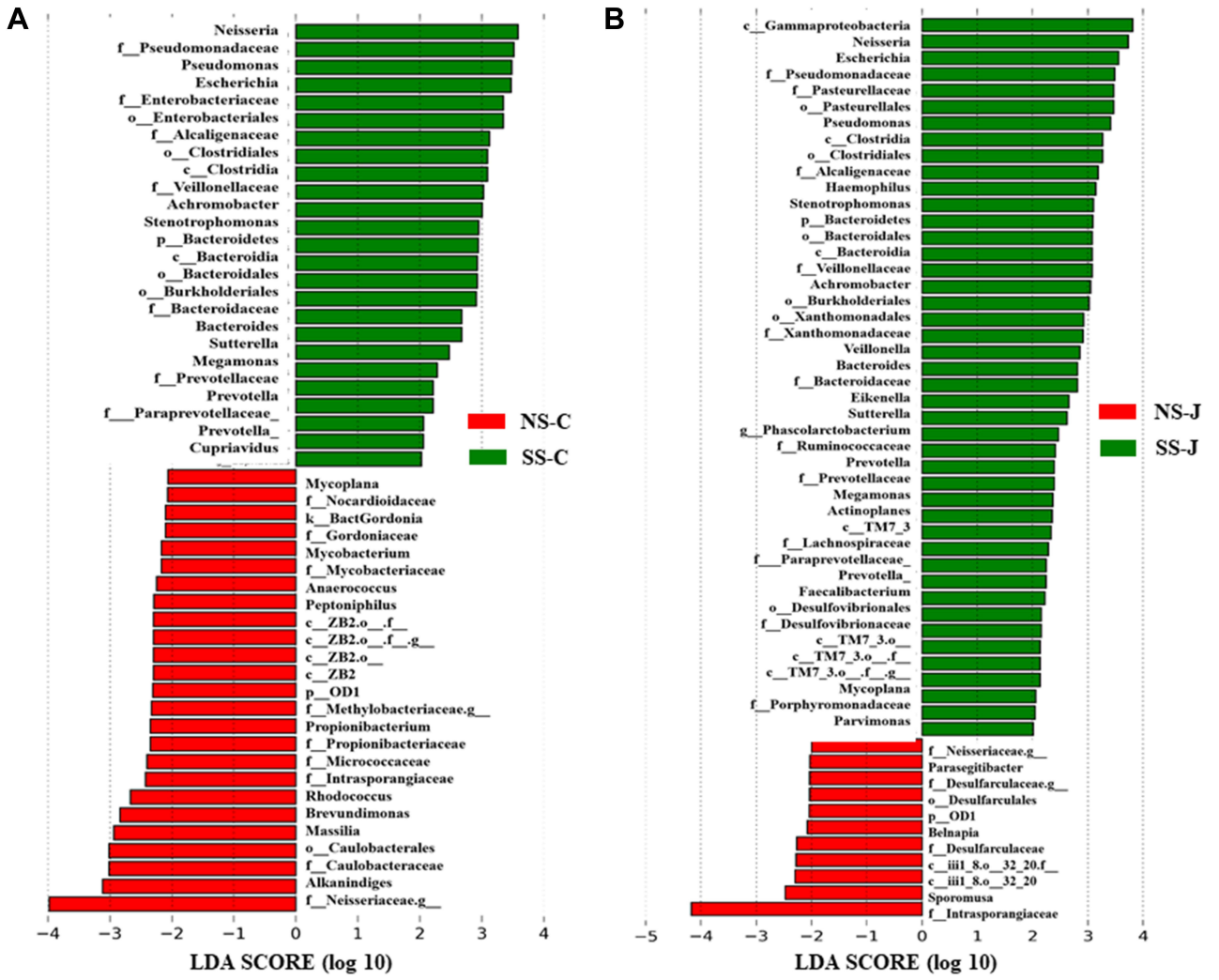

Figure 4 Skin microbiome differences between normal skin (NS) and sensitive skin (SS). (**A** and **B**) A histogram of the distribution of the linear discriminant analysis (LDA) effect size (LEfSe) shows a significant difference in the relative abundances of the biomarkers with LDA scores of 2.0 or more between the two groups.
Abbreviations: p, Phylum; c, Class; o, Order; f, Family; NS, normal skin; SS, sensitive skin; C, cheek; J, jaw.

Neisseriaceae significantly decreased (more significantly in the samples with larger physiological differences) (Supplementary Figure 1). We speculated that the occurrence of SS may be relevant to the weakening of the microbial barrier related to a significant increase in *Neisseria* or decrease in *Neisseriaceae*. The genus *Neisseria* includes a variety of commensal bacteria that typically colonizes the mucosal surfaces of humans and other animals.[38] Kim et al[39] described an intriguing mechanism of colonization resistance that commensal *Neisseria* kill pathogen *Neisseria gonorrhoeae* by releasing DNA into the environment. There may be a similar mechanism related to the microbes on the skin surface, but this remains to be verified. Regarding *Intrasporangiaceae* (within the phylum Actinobacteria), which was significantly decreased in the SS-J subgroup compared to the NS-J subgroup, there are relatively few descriptions in the literature. Ouyang et al[40] proved that *Intrasporangiaceae* assimilate sulfamethoxazole in soil, accelerating the degradation of antibiotics. Similarly, we can hypothesize that the presence of *Intrasporangiaceae* accelerates the degradation of antibiotics on the skin surface and maintains the species diversity of the NS surface. Finally, we found no difference in the relative abundance of *Staphylococcus epidermidis* and *Staphylococcus* (Supplementary Figure 2), which conflicts with previous research.[32] This requires further verification.

The selection of the V3-V4 region in this study was based on research involving mock microbial populations[29] and the recommendations of sequencing companies,

without considering the differences between different organs. Meisel et al.[41,42] showed that sequencing of the V1–3 regions of the 16S rRNA gene more accurately recapitulates skin microbial communities, while sequencing of the V4 region poorly captures the skin commensal microbiota, especially severely underrepresented *Staphylococcus epidermidis* and *Propionibacterium acnes* and overrepresented *Staphylococcus aureus*. Therefore, the V3-V4 area maybe not the most suitable target for studying skin microbial communities. Nevertheless, we identified differences in bacterial flora that have not been previously reported, and they may be potential biomarkers for SS. Furthermore, the main reason why our results differ from those of other studies may be the choice of primers, but the mistake can actually be overcome, and sufficient literature research is very important.

SS indicates a general susceptibility to exogenous factors. To develop effective approaches to improve SS, it is important to fully understand the mechanism underlying SS. It remains unclear why some individuals experience subjective symptoms such as itching, burning or tingling. The increased permeability of the stratum corneum and the acceleration of neural responses in the skin are thought to be related to SS.[7] To determine the relationship between SS, skin barrier, and skin microbiota, more research is necessary. Our findings will not only contribute to the development of skincare products but also provide evidence for developing diagnostic and treatment strategies of SS syndrome.

Conclusions

In summary, our results underscore that subjects with SS are more sensitive to lactic acid stimulation and have significantly reduced skin hydration, but there was no significant difference in TEWL. SS was also accompanied by a decrease in species diversity. The relative abundance of Neisseriaceae in the SS group significantly decreased, while that of *Neisseria*, a genus in the *Neisseriaceae* family, significantly increased. PCA showed that there was no significant difference in β diversity. We speculate that the SS is accompanied by a decrease in the species diversity and richness (based on the number of OTUs and Chao1 index), which may be relevant to the weakening of the microbial barrier (due to the decrease in *Neisseriaceae* or increase in *Neisseria*). Corresponding treatment for *Neisseriaceae* may be a new idea in the treatment of SS.

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Disclosure

The authors report no conflicts of interest in this study.

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