Unlocking the Role of the Stratum Corneum Microenvironment in Shaping the Dynamics of Skin Microbiota

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Abstract
The stratum corneum (SC) of the skin serves as a platform for various barrier functions. As a biological barrier, the skin microbiota plays a crucial role interacting with skin diseases and physiology, making it a key target for cosmetics. While microbial communities vary between individuals, they remain stable on adult skin. To optimize the benefits from these microbes, understanding how the SC microenvironment influences the microbial balance is essential. Thus, this study aimed to clarify the relationship between SC and skin microbiota. Tape-stripped samples from control subjects (n=29) and atopic dermatitis (AD) patients (n=21) were analyzed by fluorescence-activated cell sorting, enzyme-linked immunosorbent assays, and 16S rRNA gene sequencing to evaluate vulnerable corneocytes: P2, soluble SC markers, and skin bacteria, respectively. The levels of P2 along with soluble SC markers, significantly increased in non-lesional and in lesional AD skin. P2 levels strongly correlated with *Staphylococcus aureus*, leading to dysbiosis, while soluble SC markers showed weak correlations with other bacteria. Furthermore, P2 levels in non-lesional areas positively correlated with local lesion severity, suggesting their potential contribution to exacerbation. Overall, our findings offer new possibilities for effectively caring for the SC microenvironment and nurturing its resident microbiota with cosmetics, thereby enhancing skin health.

Keywords: stratum corneum; biomarkers; skin microbiome; atopic dermatitis

Introduction

As the outermost layer of the skin, the stratum corneum (SC) constitutes a distinctive niche that orchestrates diverse barrier functions: physical, chemical, and biological systems. Among those, the skin microbiota has been increasingly recognized as crucial in the complex interplay with skin diseases and physiology, making it a key target for cosmetics. With significant variations between individuals, the skin microbiome interact with host skin cells and the immune system. Conversely, the skin provides a home and nutrients for the microbiome, which plays a role in maintaining skin homeostasis [1-3]. This bidirectional relationship highlights how imbalances can significantly affect skin conditions. In conditions such as atopic dermatitis (AD) and acne, there is an increase in *Staphylococcus aureus* and specific strains of *Cutibacterium acnes*, respectively, contributing to their pathogenesis [4, 5]. In healthy skin, the microbiota is linked to demographics, as well as lifestyle, environmental and local host factors, demonstrating the importance of a balanced microbiome for optimal skin function [6].

Given that background, various approaches involving the skin microbiota, such as prebiotics, probiotics, synbiotics and postbiotics, are emerging, although clinical guidelines do not currently exist [7]. On the other hand, longitudinal studies have shown that the microbial community generally remains stable on adult skin despite daily exposure to the external environment [8]. To optimize the benefits of our symbiotic allies, understanding how the SC microenvironment influences the microbial balance is essential. Previous studies have reported the relationship between the ceramide profile [9], proteins [10], and pH zones of the SC [11] with the microbiome. However, the relationship between the SC and the microbiome is much less studied than other skin parameters. Our goal here was to clarify the relationship between SC characteristics and the skin microbiota.

To this end, we first developed and validated markers involving the SC microenvironment. As SC markers, we used known soluble SC markers related to inflammation and new indicators related to vulnerable corneocytes: P2, then tested expression of those markers on the skin of healthy controls and of AD patients. Next, we performed correlation

analyses of the SC markers with *S. aureus* and other bacteria in these samples. Finally, we examined the relationship between local lesion severity and P2 in adjacent non-lesional areas in AD patients. Our work provides new insights into the SC microenvironment that correlates with microbial imbalances in AD skin, which should contribute to maximize the benefits of microbiome care and the management of skin diseases.

Materials and Methods

1. Study population

A comparative analysis of SC markers and skin microbiomes was conducted between healthy control subjects (CONT) and patients with AD. Patients were diagnosed as having AD by dermatologists according to the criteria of Hanifin and Rajka [12]. Mild to moderate AD patients, aged 18 years and older, were enrolled in this study from February to April 2023 at the Fukuda Dermatology Clinic. Age and sex-matched control subjects, without any history of inflammatory skin diseases, were enrolled in April 2023. The exclusion criteria were pregnancy and taking any aesthetic treatment. All subjects provided signed informed consent. This study followed the Declaration of Helsinki and was approved by the Ethics Committee of the FANCL Corporation (C2022-023).

2. SC sample collection

SC samples were obtained by a single tape-stripping using a skin tape (25 × 25 mm Horny Layer Checker, Asch Japan, Japan). The tape was placed on the skin, carefully pressed with a finger, and then removed. The tape-stripped SC up to the second layer in depth was used for the downstream analysis. For AD samples, SC was collected from faces with lesions and from the body, including both lesional and non-lesional skin under the supervision of dermatologists. More precisely, samples from lesional skin were taken based on the location of the lesion, and samples from non-lesional skin, were collected from adjacent areas. Topical treatment was discontinued on the examination day. For CONT samples, SC samples were collected from the

cheeks and lower backs of healthy subjects. The body was washed the day before, and the face was washed on the day of tape-stripping.

3. Assessments of the local severity of AD

The local severity scores of lesional AD skin were calculated based on assessments of redness, thickness, scratching and lichenification, each rated on a 4-point scale (0: none, 1: mild, 2: moderate, 3: severe). Clinical photographs, taken with a digital single-lens reflex camera (LUMIX GH5S, Panasonic, Japan) equipped with a LEICA DG VARIO-SUMMILUX 10-25 mm lens (Panasonic), were used for evaluation. The sum of these scores was used as the local severity score.

4. Measurement of the soluble SC markers

SC protein was extracted from tape-stripped SC as previously reported [13]. Briefly, the tape-stripped SC was homogenized with glass beads in T-PER Tissue Protein Extraction Reagent（Thermo Fisher Scientific, USA）at chilled conditions (Microsmash MS-100R, TOMY, Japan). The resulting extracts were directly subjected to enzyme-linked immunosorbent assay (ELISA) for the detection of SC markers: Galectin-7 (GAL-7), Heat shock protein-27 (HSP-27), Neutrophil gelatinase-associated lipocalin (NGAL) and DJ-1. These soluble SC markers were quantified using an R&D Systems sandwich ELISA kit (DuoSet, Bio-Techne, USA) following the manufacturer's instructions. Protein concentrations in the extracts were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Data are expressed as the nanogram of each marker protein content per microgram of the total protein content.

5. Analysis of corneocytes features

Tape-stripped SC was dissociated by boiling in dissociation buffer (2%SDS, 20 mM DTT, 5 mM EDTA, 0.1M Tris-HCl) [14] at 100°C for 10 min, then was sonicated in an ultrasound bath for 1 min. To wash SC samples, each suspension was centrifuged at 5,000 g for 5 min at room temperature. The supernatant was removed and replaced with fresh 0.1% phosphate-buffered saline with Tween 20 (0.1% PBST, Takara Bio, Japan). The SC samples were fixed with 4%

paraformaldehyde in phosphate buffer solution (FUJIFILM Wako Pure Chemical Corporation, Japan) at room temperature for 8 min, then washed 3 times. After filtration through tubes with a 35 µm nylon mesh (Corning, USA), samples were analyzed and sorted by fluorescence-activated cell sorting (FACS, FACS melody, Becton Dickinson and Company, USA).

6. SC lipid evaluation

The corneocytes were dispersed as described in section five, then stained with 0.03% Nile red for 10 min. After washing with 0.1% PBST, FACS analysis was performed on the filtered samples. For microscopic observations, the sorted corneocytes were stained with 0.03% Nile red for 10 min. After washing with PBS (-), corneocytes were observed using a BZ-X810 microscope (KEYENCE, Japan).

7. Surface observation of corneocytes

The corneocytes were sorted on glass slides as described in section five, then were observed using a VK-X3000 microscope (KEYENCE, Japan).

8. Skin microbiomes analysis

Skin microbiomes were analyzed by 16S rRNA sequencing, conducted by World Fusion Co., Ltd (Japan). Briefly, DNA samples were extracted from the tape-stripped SC from the outermost layer. The 16S rRNA V2, V3, V4, V6, V7, V8, and V9 region sequences were amplified by PCR using an Ion 16S Metagenomics Kit (Thermo Fisher Scientific) and sequenced with an ION S5 sequencer (Thermo Fisher Scientific). The reads that passed quality checks were subjected to a homology search against the NCBI 16S Microbial database (NCBI GenBank r228) using the BLAST program, and the species with the highest homology was assigned to each read.

9. Statistical analysis

Statistical differences were assessed using JMP 17.1 (SAS Institute Inc., USA) or R (R Core team, Austria) [15] and Rstudio (Rstudio team, USA) [16]. P values of < 0.05 were considered statistically significant.

Results

1. Verification of newly established SC features

An overview of the study design is shown in Figure 1a. We first developed a new method using FACS to understand the corneocytes features quantitatively. The FSC (forward scatter)-SSC (side scatter) plot revealed two distinct populations, P1 and P2, that were consistently present across all samples, with P2 accounting for approximately 7.3% to 55.2% of the total population (Fig. 1b, left).

Next, we elucidated the characteristics of P1 and P2 by performing Nile red staining on the respective SC samples. The mean intensity of SC lipids was lower in P2 compared to P1, a finding verified both by FACS and by fluorescence microscopy (Fig. 1b, right & 1c). Additionally, laser microscopy revealed that P2 exhibited a thinner profile and a finely villous surface structure compared to P1 (Fig. 1d).

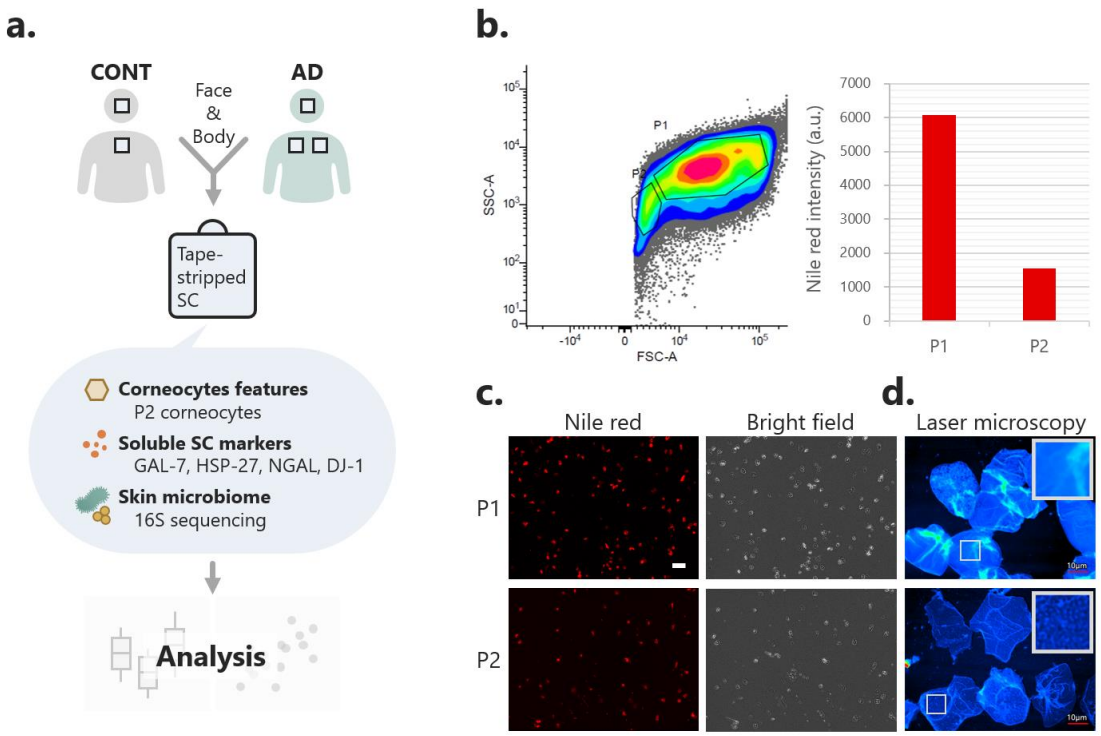

Figure 1 Corneocytes are divided into two groups by FACS analysis.
The overview of study design (a). The representative images of SSC/FSC plot of corneocytes (P1 and P2) and Nile red intensity of each population (b). Images of P1 & P2 corneocytes: Nile red staining, scale bar = 100 μm (c), and laser microscopy, scale bars = 10 μm (d).

To elucidate the association between skin inflammation and P2, we analyzed SC samples from AD patients. Our study enrolled 21 Asian subjects with AD, including 12 males and 9 females, with an average age of 38 years, and 29 Asian healthy controls, including 14 males and 15 females, with an average age of 45 years (Table I). Using body samples, we compared CONT with AD non-lesional and lesional skin. Among the four soluble SC markers tested, GAL-7, HSP-27, and NGAL levels significantly increased in the order of control, non-lesional, and lesional skin (Fig. 2a). P2 levels also significantly increased in the same pattern (Fig. 2b).

Table I Baseline characteristics of the patients and samples

Variable	Overall N=50	Control n=29	Atopic dermatitis n=21
Age (years)			
18≤29 (%)	12 (24.0)	6 (20.7)	6 (28.6)
30-39 (%)	11 (22.0)	6 (20.7)	5 (23.8)
40-59 (%)	11 (22.0)	6 (20.7)	5 (23.8)
50-59 (%)	12 (24.0)	7 (24.1)	5 (23.8)
60-69 (%)	1 (2.0)	1 (3.5)	0 (0)
≤90 (%)	3 (6.0)	3 (10.4)	0 (0)
Median (IQR)	42 (30-51)	45 (31-53)	38 (25-49)
Sex			
Male (%)	26 (52.0)	14 (51.7)	12 (57.1)
Female (%)	24 (48.0)	15 (48.3)	9 (42.9)
Sample location			
Face lesion	21	0	21
Face control	29	29	0
Body lesion	21	0	21
Body non-lesion	21	0	21
Body control	29	29	0

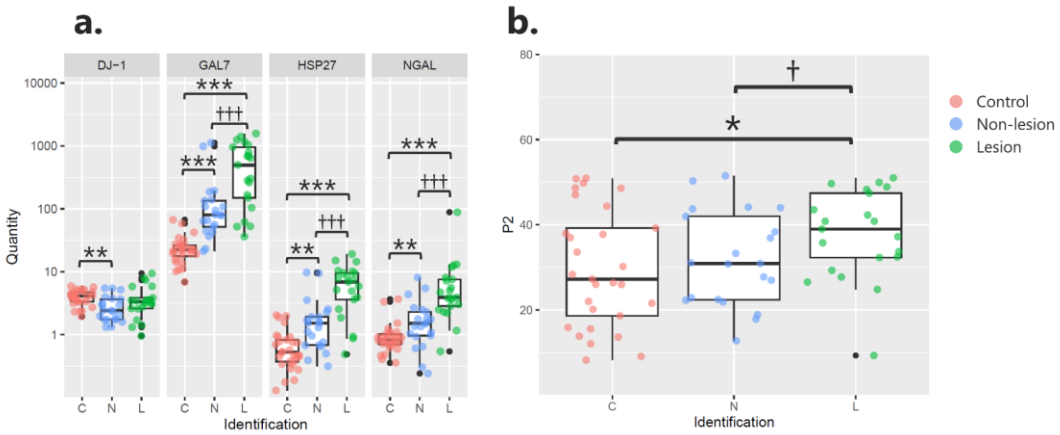

Figure 2 Soluble SC markers and P2 are increased in AD skin.
Expression levels of soluble SC markers: DJ-1, GAL-7, HSP-27, NGAL in healthy control and AD skin (a). Levels of P2 corneocytes in healthy control and AD skin (b). C: Control (Healthy control), N: non-lesion (AD), L: lesion (AD), Wilcoxon rank sum test: 0.05<*p**, 0.01<*p***, 0.001<*p****, Wilcoxon signed-rank test: 0.05<*p*†, 0.001<*p*†††

2. Integration analysis of skin microbiomes and SC markers

We then evaluated the relationship between the levels of P2 corneocytes, soluble SC markers and *S. aureus* using overall samples. In this dataset, AD skin showed a high colonization rate of *S. aureus* (Fig. 3a). In the correlation analysis of SC markers and bacteria with a relative abundance rate of 1% or more, P2 levels showed the strongest positive correlation with *S. aureus* (*p*=0.004) (Fig. 3b).

Additionally, exploratory correlation analysis was performed between SC markers and other bacterial species. Weak correlations were detected between soluble SC markers and several bacteria. i.e. DJ-1 protein and *Moraxella osloensis*. Different bacterial species exhibited correlations with P2 and SC biomarkers (Fig. 3c).

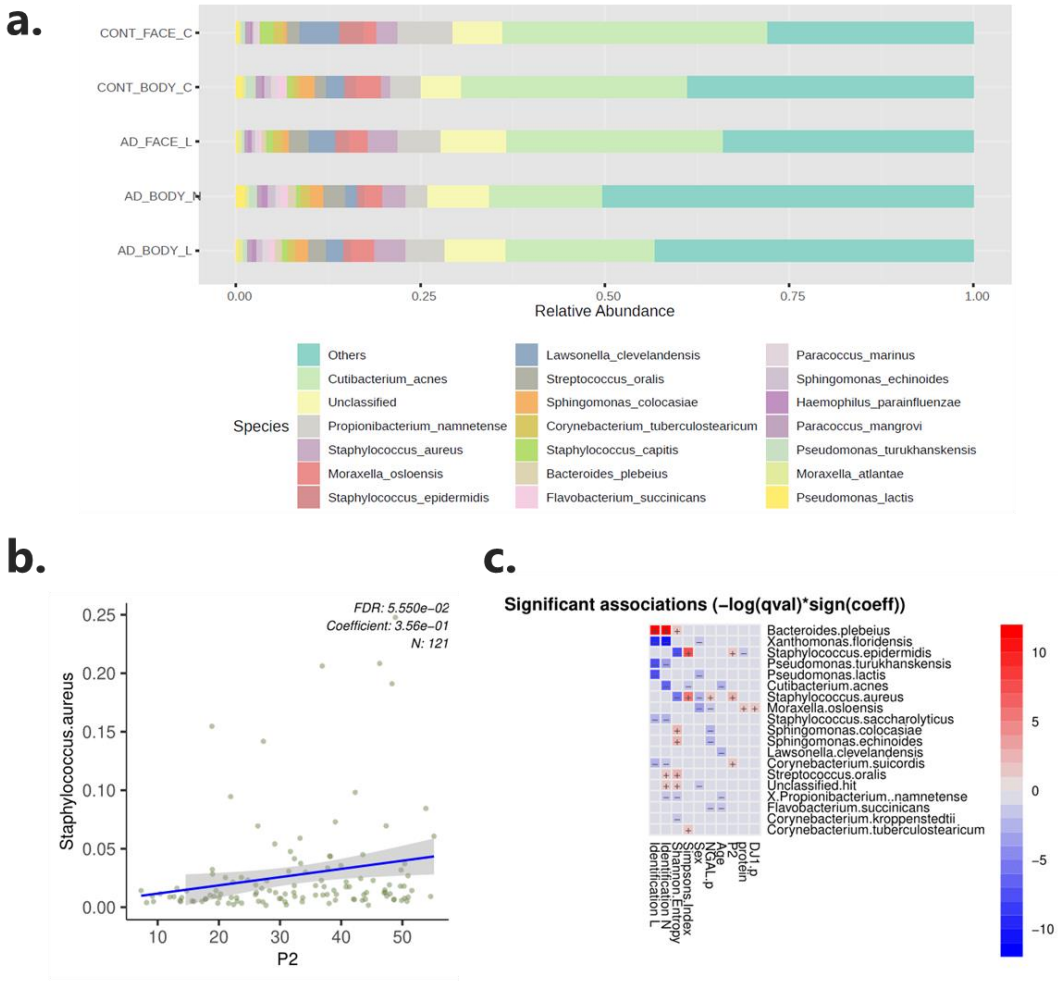

Figure 3 P2 and soluble SC markers are related to skin microbiome.
Occupancy rates of bacteria species by disease and site (a). Correlation analysis between P2 and *S. aureus* occupancy (b) and exploratory correlation analysis (c) in overall samples performed by R package MaAsLin2.

3. Relevance between P2 corneocytes and skin conditions

To gain further understanding of P2 and skin conditions, we conducted P2 analysis in lesional, and in adjacent non-lesional AD skin. P2 levels positively correlated with local severity scores (Fig. 4a, p=0.07) and non-lesional P2 levels (Fig. 4b, p=0.04). Furthermore, P2 levels in non-lesional skin showed a weak but positive correlation with local severity scores of adjacent lesions (Fig. 4c, p=0.16).

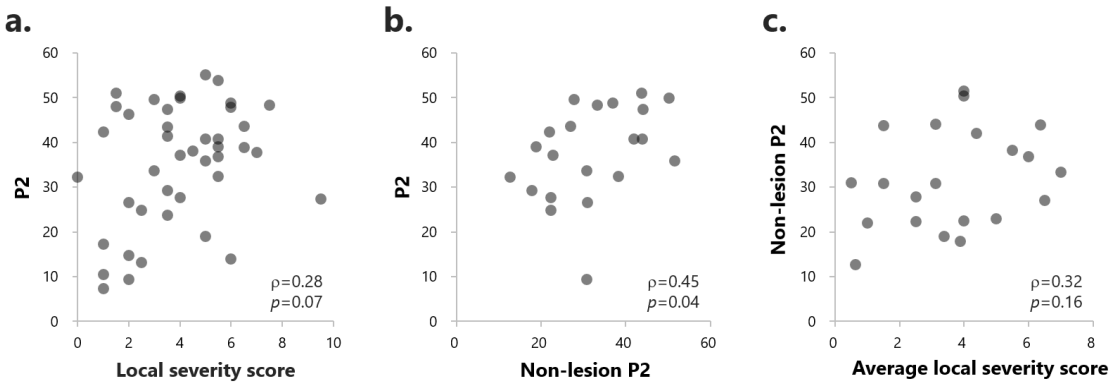

Figure 4 P2 levels are related to local severity of AD lesions and predisposition.
Correlation between P2 levels and the local severity score in overall AD lesion samples (a), P2 levels and non-lesion P2 levels in body samples (b), and non-lesion P2 levels and the average local severity score in body samples (c). All analyses were performed using Spearman's rank correlation coefficient.

Discussion

In this study, we explored the relationship between the SC microenvironment and skin microbiota in healthy controls and in patients with mild to moderate AD, focusing on *S. aureus*. Using FACS, we identified vulnerable corneocytes: P2. Not only soluble SC markers but also P2 corneocyte levels were significantly higher in AD skin. P2 was correlated positively with *S. aureus* colonization. Soluble SC markers showed weak correlations with bacteria. Moreover, P2 corneocyte levels in non-lesional AD skin showed a positive correlation with lesion severity. This study provides new insights into the significance of the SC microenvironment, particularly P2 corneocytes, in maintaining our skin microbiota.

Evaluating SC markers using tape-stripping is useful because it reflects the metabolism of the viable epidermis and allows for a non-invasive and detailed understanding of skin conditions. Targeting proteins and nucleic acids, applications have been expanding in the fields of cosmetics and medicine [17]. The assay using P2 corneocytes suggests that they represent vulnerable corneocytes, characterized by decreased lipid envelopes and coarse textures (Fig. 1). Previous studies reported that the SC from AD and sensitive skin has microvillus structures [18, 19], which are thought to be similar to P2 corneocytes. Additionally, this method enables the sorting of corneocytes, allowing the collected SC to be used in other assays. It has the

potential for wide applications, including screening cosmetic ingredients and selecting personalized skin care products based on individual SC conditions. We evaluated four soluble SC markers related to inflammation, as well as P2 corneocytes, using the SC from AD skin. Among these markers, GAL-7 and HSP-27 increased in non-lesional and lesional samples compared to CONT, which is consistent with previous studies [13, 20]. Furthermore, this research uniquely demonstrates the correlation between NGAL and P2 corneocytes with AD (Fig. 2). These findings suggest that both P2 corneocytes and soluble SC markers can serve as markers for AD.

Modern lifestyles and habits, along with global biodiversity losses and declining nature relatedness, are thought to trigger the dysbiosis and increase the risk of inflammatory diseases [21]. *S. aureus*, a prominent bacterium involved in the dysbiosis of AD, induces vicious cycles of inflammation and infection. This process affects the host skin barrier destruction and immunomodulation through the interaction of *S. aureus* virulence factors [22]. Remarkably, antimicrobial treatment correcting the dysbiosis can prevent the onset of dermatitis [23]. Clinical trials targeting *S. aureus* in AD patients using *Staphylococcus hominis A9*, which kills *S. aureus* and inhibits the production of toxins, are also currently underway [24]. These facts highlight the importance of controlling resident bacteria to maintain healthy skin in modern individuals. The initial step in *S. aureus* colonization involves adhesion to corneocytes. Towell *et al*. reported that fibronectin binding protein B and clumping factor B interact with host corneodesmosin, facilitating strong binding of *S. aureus* to corneocytes in AD skin. [25, 26]. Considering the lower lipid content in P2 and preliminary results showing distinctive protein expression (data not shown), P2 corneocytes may potentially modulate the relative abundance of resident bacteria through their surface environment. Interestingly, P2 corneocytes and soluble SC markers both showed association with the microbiome, but the associated bacterial species differed for each (Fig. 3), indicating distinct underlying mechanisms. Therefore, it is crucial to go beyond traditional approaches targeting bacteria and focus on improving the

metabolism of the SC and viable epidermis, which impacts the levels of P2 corneocytes and soluble SC markers. A combined analysis of these markers may be beneficial for a more detailed understanding of the microbiome and skin conditions.

Predispositions to AD involve a strong genetic background, epidermal dysfunction, and T-cell driven inflammation [27]. Based on our results (Fig. 4), maintaining low levels of P2 corneocytes in non-lesional areas may help prevent the worsening of lesions, suggesting that P2 corneocytes, which influence barrier function and inflammation, could serve as a predisposing factor for AD exacerbation. Resident microbiota are also deeply involved in the onset and remission of AD [10] [28], which marks the boundary with healthy skin and is closely related to skincare through cosmetics. Notably, individual data revealed the presence of high P2 levels even among healthy controls (Fig. 2b). Thus, P2 corneocytes could serve as important indicators influencing transient skin problems in healthy skin.

However, there might be some limitations to this study. While the data presented show correlations, verifying causation will be crucial for understanding the mutual influence between the microbiome and the SC microenvironment. Recent studies have shown that strain-level differences in *S. aureus* affect the responses of the viable epidermis [29, 30]. Although our study examined species-level differences, strain-level research is also essential. Further investigations will help confirm the applicability of our findings to healthy skin. By understanding how P2 corneocytes form, we can develop cosmetics that prevent their occurrence and enhance skin functions, driving innovation in microbiome-focused skincare.

Conclusion

In the present study, we provide novel insights into the relationship between SC characteristics and the skin microbiota: 1) Evaluation of the SC by FACS was associated with the presence or absence of AD rash, similar to soluble SC markers, indicating its potential as a useful marker. 2) The population of vulnerable P2 corneocytes showed a positive correlation with *S. aureus*

occupancy, suggesting its involvement in exacerbating AD lesions. Taken together, our findings unlock novel possibilities to effectively care for the SC microenvironment and nurture its resident microbiota with cosmetics, enhancing skin health. This might contribute to maximizing the benefits of microbiome care and the management of skin diseases.

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Conflict of Interest Statement
NONE.

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