ORIGINAL RESEARCH

A Pilot Study: Composition and Diversity of 16S rRNA Based Skin Bacterial Microbiome in Indonesian Atopic Dermatitis Population

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Background: Atopic dermatitis (AD) interferes with quality of life and is influenced by important factors like skin microbiome. The results of the skin microbiome composition and diversity in AD varied in some studies.

Purpose: This study aims to determine the composition and diversity of the skin microbiome in Indonesian AD patients.

Patients and Methods: Genomic deoxyribonucleic acid (DNA) preparations were obtained from skin swabs of the cubital fossa of 16 subjects, nine of which were having mild AD, three moderate AD, and four healthy individuals. DNA extraction and sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene using next-generation sequencing and bioinformatics analysis were further performed.

Results: *Firmicutes* (p), *Bacilli* (c), *Bacillales* (o), *Staphylococcaceae* (f), and *Staphylococcus* (g) were dominant in moderate AD. On the contrary, *Proteobacteria* (p), *Gammaproteobacteria* (c), *Pseudomonadales* (o), *Moraxellaceae* (f), and *Acinetobacter* (g) were dominant in mild AD. *Staphylococcus aureus* was found in the highest number in individuals with moderate AD. Interestingly, *Ensifer adhaerens* was found in mild AD. Microbial diversity was decreased in moderate AD.

Conclusion: Metagenomic analysis in this study identified microbes in moderate and mild AD and showed a low diversity of skin microbiomes in moderate AD. Interestingly, this is the first time that the bacteria *Ensifer adhaerens* was detected on the human skin.

Keywords: atopic dermatitis, microbiome, skin microbiome

Introduction

Atopic dermatitis (AD) is the most common chronic, and recurrent inflammatory skin disease. The prevalence of AD varies throughout the world and still increasing. In developing countries, 10–20% of children and 1–3% of adults are affected by AD. This disease is characterized by significant itching.[1,2] Intense itching causes trauma on the skin due to scratching and disturbed sleep patterns. Individuals with AD lesions can also face social stigma due to the visibility of the lesions. Additionally, chronic conditions causes individuals with AD to require ongoing treatment, in the form of daily moisturizers, repeated topical and oral therapy, and repeated consultations with doctors. These may affect the individuals, and to some extent, their family, financially.[2,3]

Atopic dermatitis is caused by a complex interaction between impaired skin barrier function, dysregulation of immunity, and skin microbiome.[2,4] Impaired skin

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barrier function and immune dysregulation affect skin microbiome, which leads to an increase in *Staphylococcus aureus* (*S. aureus*) colonization.[5] This condition could decrease bacterial diversity on the skin due to decreased colonization of other bacteria such as *Streptococcus, Propionibacterium, Acinetobacter, Corynebacterium*, and *Granulicatella*.[5–7] The change in the microbial composition that differs from normal conditions is called skin microbiome dysbiosis.[6,7]

A study by Kong et al revealed a decrease of microbial diversity in skin lesions of acute exacerbation of AD due to an excessive increase in *S. aureus*.[5] One study also reported an increase in *S. aureus* colonization in individuals with AD lesions that had not been treated with moisturizer compared to healthy individuals.[7] However, the most predominant microbes in individuals with AD *Stenotrophomonas maltophilia*, which differs from that of healthy individuals.[8] Kim et al found that *Staphylococcus, Pseudomonas*, and *Streptococcus* are dominant in AD skin.[9]

Identifying specific bacteria in individuals with AD is difficult. Therefore, it is necessary to conduct further microbial profile studies in individual AD populations. Molecular techniques help detect various microbes that have not yet been identified in human organs.[8] Deoxyribonucleic acid (DNA) sequencing method against 16S ribosomal ribonucleic acid gene (16S rRNA gene) in bacteria produces a more outstanding picture of skin microbiome diversity compared to the culture method,[6] as this method can detect bacteria that are difficult to culture or isolate.[8] This study aims to determine the composition and diversity of the skin microbiome in Indonesian AD patients.

Materials and Methods
Study Population and Inclusion Criteria
The study was approved by the Institutional Review Board of Health Research Ethics Committee in Dr. Hasan Sadikin Hospital, Bandung, West Java, Indonesia and conducted in accordance with the latest version of the Declaration of Helsinki. Each subject and their parents gave written informed consent and consent to being photographed prior to enrolment.

The subjects were 12 AD patients that had an onset or exacerbation and four normal individuals. Dermatologists clinically diagnosed the AD patients, then evaluated the severity of their condition using the Scoring Atopic Dermatitis (SCORAD) index, a complex measures that assess a weighted average of the severity of AD signs and extent of lesions.[10] The inclusion criteria were AD patients and their parents gave their consent to participate in the study, The exclusion criteria were subjects who had another skin disease, received topical or oral antibiotics or corticosteroids within the previous seven days, applied emollient routinely within the last seven days, or have bathed within the last 12 hours. For normal individuals agree to participate in the study and the exclusion criteria were have previous skin diseases, including AD, allergic or significant underlying diseases, and history of taking medications, including corticosteroid, antibiotic, and emollient.

Data Collection (Sampling/Collection of Skin Swab)
A cross-sectional descriptive study with consecutive sampling was performed from January 13 to July 13, 2020. For all subjects, the following data were collected: sex, age, education, occupation, AD duration, the onset of AD symptoms, medication history, and disease severity as assessed using the SCORAD index. Microbial skin samples were collected using skin swab taken with single-use sterile cotton-tipped swabs. Swabs were pre-moistened with a sterile buffer solution and rubbed firmly for 20 seconds over affected skin at the volar region of the forearm (cubital fossa). The cotton-tip samples were then stored in a tube filled with a sterile buffer solution and shipped to the Genetika Science Laboratory for processing.

DNA Extraction, Polymerase Chain Reaction (PCR) Amplification, Next Generation Sequencing (NGS), and NGS Analyses
Genomic DNA was extracted from each swab using the Qiagen Gel Extraction Kit (Qiagen, Germany). The 16S rRNA genes of distinct regions (16SV3-V4) were amplified using a specific primer of variable regions 3 through 4. Primers 341F: CCTAYGGGRBGCASCAG and 806R: GGACTACNNGGGTATCTAAT were used for the amplification of regions. The following PCR conditions were: 95°C for 1 minute followed by 35 cycles of 95°C for 15 seconds, 52°C for 15 seconds, and 72°C for 10 seconds,Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the

barcode and primer sequences. Paired-end reads were merged using FLASH, a fast and accurate analysis tool designed to merge paired-end reads when at least some reads overlap the read generated from the opposite end of the same DNA fragment.[11] The splicing sequences were called raw tags. Quality filtering on the raw tags was performed under specific conditions to obtain the high-quality clean tags according to the QIIME quality-controlled process.[12,13] The tags were then compared with the reference database using UCHIME algorithm to detect chimera sequences. The chimera sequences were removed, and the Effective Tags were finally obtained.[14,15]

Sequence analysis was performed by Uparse software using all the effective tags. Sequences with ≥97% similarity were assigned to the same Operational Taxonomic Unit (OTU).[16] A representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was used to compare against the small subunit ribosomal ribonucleic acid (SSU rRNA) database of SILVA Database for species annotation at each taxonomic rank (Threshold: 0.8~1) (kingdom, phylum, class, order, family, genus, species).[17,18] To obtain the phylogenetic relationship of all OTUs representative sequences, the MUSCLE (Version 3.8.31) was used to compare multiple sequences rapidly.[19] OTUs abundance information was normalized using a standard sequence number corresponding to the sample with the least sequences.

Alpha diversity is applied in analyzing the complexity of biodiversity for a sample through 6 indices, including Shannon and Simpson. All the indices in our samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Subsequent analysis of alpha diversity was all performed based on this output normalized data.

Results

Demographics of Study Participants

The majority of the study participants with AD were women (58.3%), aged >18 years (58.3%), with a bachelor degree (75%), and were housewives (33.4%). All subjects had <6 months duration of AD (100%), with most of them having their AD exacerbation at >18 years and starting in early life (50%). Mild AD was the most frequently found, based on the SCORAD assessment (75%). All participants in the current study had a history of previous treatment (Table 1). While the normal group consisted of 2 females and 2 males, it consisted of 1 participant <10 years old and 3 other participants over 18 years old. These four normal subjects' occupations and education were a kindergarten pupil, a gardener with high school graduated, a medical doctor, and a houseworker high school graduated.

Overview of Skin Microbiomes in Study Participants with Atopic Dermatitis

Skin microbiome composition in patients with AD was investigated using 16S rRNA next-generation sequencing. The most abundant phylum in moderate AD was the *Firmicutes* phylum, followed by the *Proteobacteria* and *Actinobacteria* phyla (Figure 1A). In contrast, the skin microbiome of mild AD patients and healthy individuals were predominated by *Proteobacteria*, followed by *Firmicutes* and *Actinobacteria* (Figure 1A). At the class level, the predominant bacteria in moderate AD were of the *Bacilli* class, was followed by *Alphaproteobacteria* and *Gammaproteobacteria*. *Gammaproteobacteria* were more frequent in mild AD, followed by *Alphaproteobacteria* and *Bacilli*. Furthermore, *Bacilli, Unidentified Actinobacteria*, and *Gammaproteobacteria* were dominant in healthy individuals as shown in Figure 1B.

The most abundant order in moderate AD were *Bacillales*, followed by *Rhizobiales* and *Micrococcales*. Moreover, mild AD was predominated by *Pseudomonadales*, followed by *Bacillales, Micrococcales*, and *Rhizobiales*. The most predominant order in healthy individuals was *Bacilles*, followed by *Micrococcales* and *Pseudomonadales*, as shown in Figure 1C. Furthermore, *Staphylococcaceae, Rhizobiaceae*, and *Bacillaceae* were the dominant family in moderate AD. In contrast, *Moraxellaceae, Rhodobacteraceae, Staphylococcaceae*, and *Rhizobiaceae* made up the most prominent family in mild AD, whereas *Staphylococcaceae, Intrasporangiaceae*, and *Moraxellaceae* were abundant in healthy individuals (Figure 1D). The most observed genus in moderate AD was *Staphylococcus*, followed by *Ensifer* and *Bacillus*. Furthermore, *Acinetobacter* was the most abundant genus in mild AD, followed by *Paracoccus, Staphylococcus*, and *Ensifer*. In contrast, *Staphylococcus, Knoellia*, and *Acinetobacter* were dominant in healthy individuals (Figure 1E).

Table 1 Demographics of Study Participants with Atopic Dermatitis

Demographic Characteristics	n=12	
	n	%
Gender		
Male	5	41.7
Female	7	58.3
Age (Year)		
≤10	4	33.4
11–18	1	8.3
>18	7	58.3
Education		
Primary school	0	0
Junior high school	0	0
Senior high school	3	25
Diploma 1	0	0
Diploma 3	0	0
Bachelor	9	75
Occupation		
Do not have job:		
-Student	3	25
-College student	2	16.7
-Housewife	4	33.4
Civil servants	0	0
General employees	0	0
Entrepreneur	1	8.3
Administration employees	1	8.3
Online driver	1	8.3
AD duration		
<6 months	12	100
>6 months	0	0
AD onset or exacerbation		
Age <2 years	1	8.3
Age 2–18 years	5	41.7
Age >18 years	6	50
Severity (SCORAD index)		
Mild (0–25)	9	75
Moderate (26–49)	3	25
Severe (≥50)	0	0
History of medication		
Yes	12	100
No	0	0

Abbreviations: AD, atopic dermatitis; SCORAD, Scoring Atopic Dermatitis.

In mild AD, *Ensifer adhaerens* (12.8%) accounted for the highest proportion of species, followed by *S. aureus* (10.17%) and *Pseudomonas stutzeri* (6.53%). On the other hand, in moderate AD, the most prominent species found was *S. aureus* (49.32%), followed by *Ensifer adhaerens*

(29.8%) and *B. aerophilus* (9.55%). Bacterial diversity was also analyzed using 16S rRNA next-generation sequencing, and the highest diversity of skin microbiomes was seen in mild AD. Moderate AD patients had lower microbial diversity as measured by Shannon and Simpson indices, as shown in Figure 2.

Discussion

The skin is the most exposed organ of the body to environmental changes and stress. It has a dynamic and complex microbial ecosystem. The bacteria that live on the skin are closely related in type and density to those found in skin glands or hair follicles in different areas. For example, *Staphylococcus* is dominant in the sebaceous and the moist regions, while *Propionibacterium* dominates only in the sebaceous and *Corynebacterium* only in the moist areas.[20] A complex microbial community termed "the microbiome" colonizes the skin.[21] There are links between intestinal microbiota and autoimmune diseases (arthritis, psoriasis, diabetes, and others) that target different tissues (joints, skin, and others), not only those that target the intestine. The most common immune-mediated skin diseases are atopic dermatitis, psoriasis, vitiligo, scleroderma, pemphigus vulgaris, bullous pemphigoid, lichen planus, erythema multiforme, and systemic lupus erythematosus (SLE).[20]

Fahlen et al showed that the level of *Actinobacteria* was significantly increased in the skin from healthy individuals compared with that of psoriasis patients, while *Proteobacteria* dominated in lesional skin.[22] Additionally, they observed a higher ratio of *Streptococcus/Proteobacteria* in the skin of these patients. A study reported by Ganju et al analyzed the cutaneous microbiota in patients with vitiligo. They observed a reduction of microbiota diversity in lesional sites compared with non-lesional sites; *Actinobacteria* represented the dominant taxa in non-lesional skin while *Firmicutes* and *Proteobacteria* dominated in the lesional areas.[23] Only a few studies have shown the presence of dysbiosis in patients with SLE. Specifically, a lower ratio of *Firmicutes/Bacteroides* was identified in SLE patients in Spain and southern China. The increase in *Actinobacteria* was only significant in patients from the latter region.[20]

Metagenomic analysis using 16S rRNA next-generation sequencing can detect bacteria in greater numbers and in more variations compared to conventional methods such as bacterial culture.[6,8] This culture-independent method makes it possible to identify and
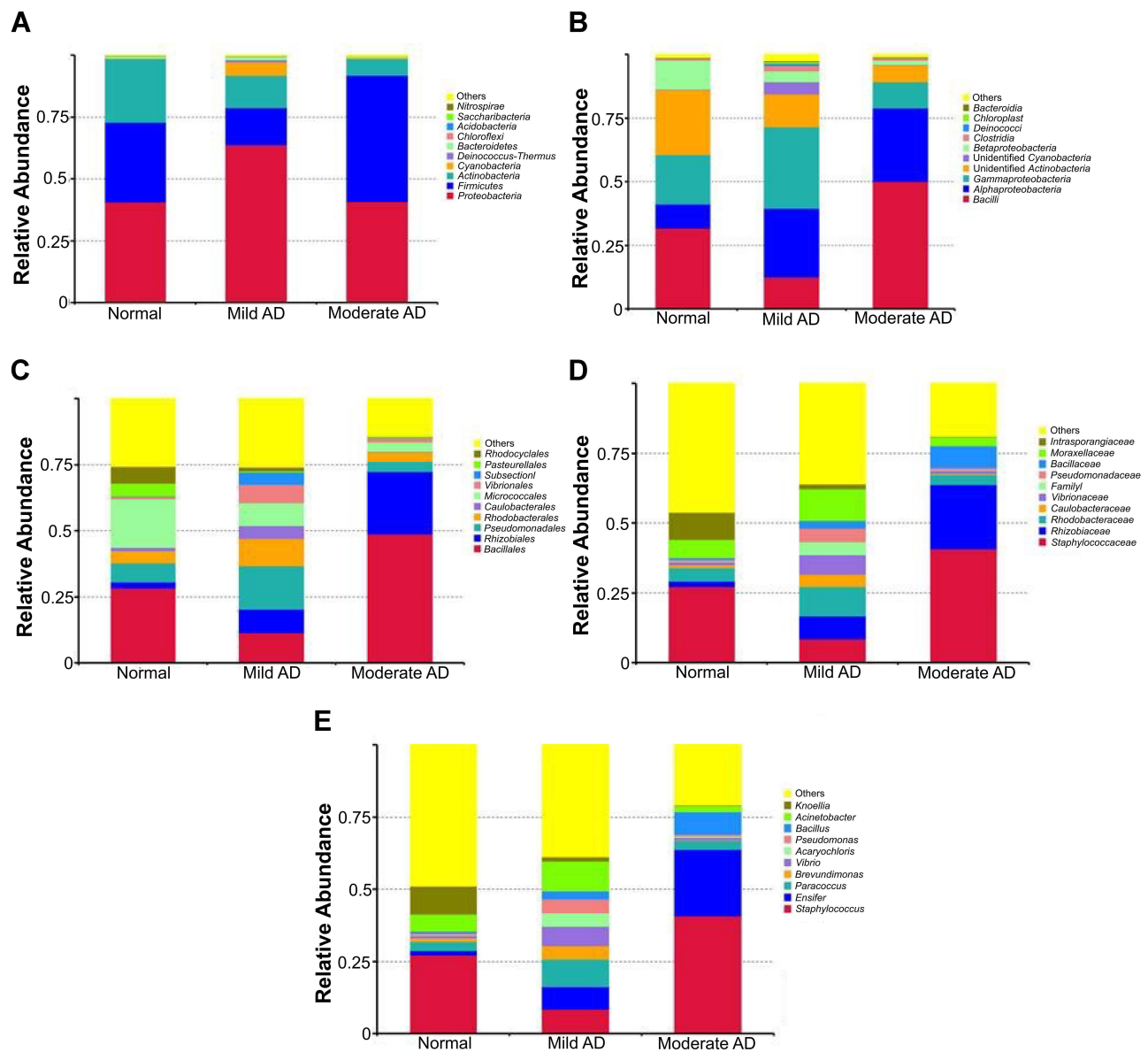
Figure 1 The most abundant phylum (**A**) classes (**B**) order (**C**) family (**D**) and genus (**E**) on the skin of AD patients.

count the relative abundance of all species present. The results of this study support the results of previous studies for moderate AD, although the results also vary in several studies. The skin microbiome composition found in moderate AD in this study was similar to those of Kim et al and Flores et al with moderate AD participants. Kim et al reported the most genera detected in patients with moderate AD in Korea was *Staphylococcus*.[9] Other genera found in abundance include *Pseudomonas, Streptococcus*, and *Acinetobacter. Lactobacillus, Xanthomonadaceae, Leuconostoc, Alcaligenaceae, Lactococcus, Rhizobiales*, and *Methylobacterium*, although their composition was relatively small. In addition to genus level, most of the

bacteria detected in AD patients were of the *Staphylococcaceae, Pseudomonadaceae*, and *Streptococcaceae* families. At the order level, the majority of bacteria came from the *Bacillales* and *Pseudomonadales* orders, while at the class level, most of the bacteria came from the *Bacilli* class. At the phylum level, the majority of bacteria were from the *Firmicutes* phylum. In healthy individuals, the most dominant bacteria detected at the family level were *Alcaligenaceae*, followed by *Sediminibacterium* and *Lactococcus*; at the order level, *Burkholderiales* was found to be most abundant; while at the class level it was *Betaproteobacteria* and at the phylum level it was *Proteobacteria*.[9]
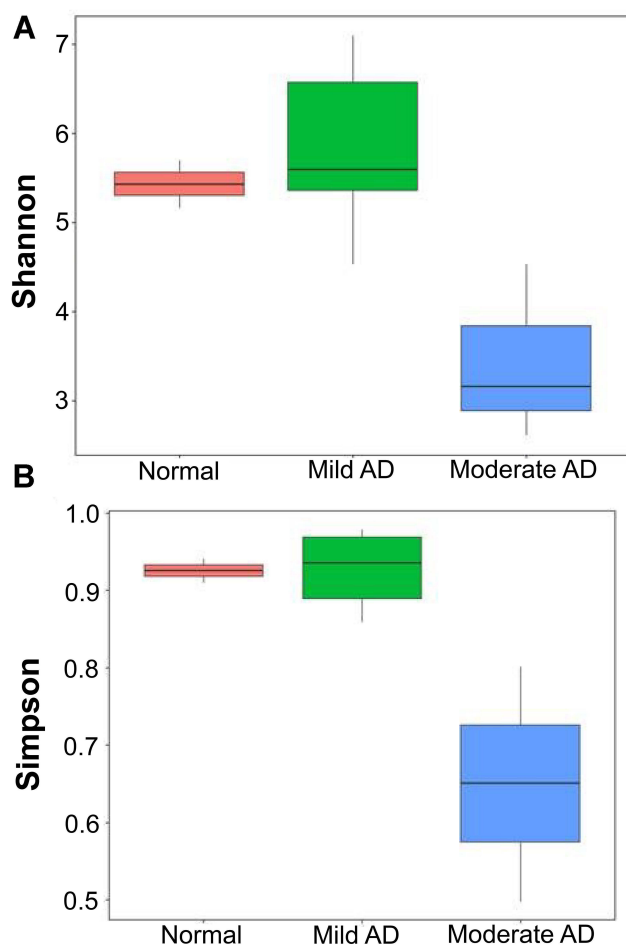
Figure 2 The skin microbiome diversity as assessed using the Shannon index (**A**) and Simpson index (**B**).

A study of skin microbiomes from the United States by Flores et al on moderate AD patients before and after using moisturizers showed that *Staphylococcus* was the most frequent genus on the skin of patients with AD before moisturizing. Other genera found in patients with AD were *Propionibacterium, Streptococcus, Alicyclobacillus, Corynebacterium, Stenotrophomonas, Streptococcaceae, Acinetobacter*, and *Prevotella*.[7]

The composition of the skin microbiome of healthy individuals in this study was similar to the results of several studies. Gao et al from the United States reported the molecular analysis of healthy individual skin bacteria from the volar surface of the forearm.[24] This study showed that there were three phyla found, *Actinobacteria, Firmicutes*, and *Proteobacteria*. There were four genera detected, namely *Propionibacteria, Corynebacteria, Staphylococcus*, and *Streptococcus*.[24]

One study from the United States reported that the sequencing results of five samples taken from the volar

forearm of healthy individuals with no history of skin disease were the *Proteobacteria* group with *Pseudomonas* being the most frequent genus, followed by *Janthinobacterium*.[25] Other identified genera were *Serratia, Halomonas, Stenotrophomonas, Delftia*, and *Comamonas*. The *Actinobacteria* group consisted of the genera *Corynebacterium, Kocuria, Propionibacterium, Microbacterium*, and *Micrococcus. Firmicutes* were the third most-frequently identified, with the genus *Staphylococcus* and *Clostridium* being the most abundant. Furthermore, the fourth phylum detected was *Bacteroidetes*, with the predominant genus being *Sphingobacterium* or *Chryseobacterium*.[25] Another study analyzed the skin microbiota in healthy individuals from 10 study participants. The study detected 16 phyla with the most dominant phyla being *Actinobacteria* (51.8%), *Firmicutes* (24.4%), *Proteobacteria* (16.5%), and *Bacteroidetes* (6.3%). Of the 12 genera detected, the most dominant ones were *Corynebacteria* (22.8%), *Propionibacteria* (23.0%), and *Staphylococci* (16.8%).[26] Washing hands might increase the *Staphylococcaceae, Streptococcaceae*, and *Lactobacillaceae* on the skin of the hands. However, it did not change the composition of the majority of bacteria on the skin.[27]

In our study, the highest number of species detected in individuals with moderate AD was *S. aureus*. It was similar to Kim et al's study that found *S. aureus* as the most frequently found species in AD patients, followed by *S. epidermidis* and *S. haemolyticus*.[9] Flores et al also reported that *S. epidermidis, S. aureus*, and *S. haemolyticus* as the most abundant bacteria found in AD patients.[7] Dekio et al reported that the skin microbiota in AD patients mostly consists of *Staphylococcus* sp. This study also discovered a new species that have never been reported before in the skin of AD patients. The new species or phylotype detected in patients with AD was *Stenotrophomonas maltophilia*. While *D. maris*, also a never-before-seen species, was found in normal individuals.[8] The burden of *S. aureus* on lesional and non-lesional skin correlates with the severity of the disease. *S. aureus* directly influences pathogenesis, and several factors produced by the bacterium increase inflammation and exacerbate AD symptoms, including *staphylococcal* superantigen B and delta-toxin.[28]

From 102 AD patients in Kong et al's study, the most detected bacteria came from the genus *Staphylococcus*, including *S. epidermidis* and *S. aureus*, followed by bacteria from the *Streptococcus, Corynebacterium*, and *Propionibacterium* genera.[5] Grice et al reported that *Propionibacteria* and

Staphylococci were the most numerous species in the seba-
ceous regions. In the moist skin areas, such as nostrils, armpits,
volar arms, between fingers, hips, buttocks, behind the knees,
and umbilicus, most species found were *Corynebacteria*,
although *Staphylococci* also been identified.[26]

Flores et al reported that the diversity of microbiome in AD
lesions was reduced and, there was a significant decrease in the
genus *Staphylococcus* and a significant increase in the variety
of skin microbiome (p = 0.01) post-administration of
moisturizers.[7] Other study using dupilumab as treatment sig-
nificantly changed the skin microbiome and decreased
S. aureus.[29] We also studied the skin microbiomes of
mild AD patients. In this study, the highest diversity of skin
microbiome was seen in samples of individuals with mild AD,
and the lowest diversity was seen in individuals with
moderate AD. Kong et al found that treatment changed the
bacterial skin diversity in AD and suggested that treatments
diversify skin bacteria preceding improvements in disease
activity. Dramatic reductions in the skin microbial diversity
occurred during AD flares, restored with standard AD thera-
pies. In AD, the proportion of *Staphylococcus* sequences,
particularly *S. aureus*, was more significant during disease
flares than at baseline or post-treatment and correlated with
worsened disease severity.[5] Higher *S. aureus* abundances in
patients with AD relative to healthy subjects, in lesional sites,
compared to non-lesional sites, and in patients with higher vs
lower SCORAD also revealed in Khadka et al's study.[30]

Furthermore, the most abundant species found in
mild AD was *Ensifer adhaerens*, a bacterial species of
the genus *Ensifer*. The bacteria was first isolated in 1982
and 1983 and located in the soil.[31] No study had pre-
viously detected this bacteria on the human skin. The
microbiota is generally conceived of as two groups, resi-
dent and transient microbes.[5] In this study, *Ensifer adhae-
rens* could be as transient microbes that do not establish
themselves permanently on the skin's surface but rather
arise from the environment and persist for hours to days.
Furthermore, people with soil-related occupation or daily
activities could be the source of inoculation of the soil
bacteria, and soil is the habitat of the *Ensifer adhaerens*.[31]
Additionally, Indonesia has high humidity conditions,
about 43–98%, making the bacteria grow high.[32]

Conclusion

Metagenomic analysis in this study succeeded in identify-
ing microbes in moderate and mild AD and showed a low
diversity of skin microbiome in moderate AD. The *Ensifer*

adhaerens detected in mild AD is a newly detected bac-
teria on human skin.

Disclosure
The authors report no conflicts of interest in this work.

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