Combined metagenomic- and culture-based approaches to investigate bacterial strain–level associations with medication-controlled mild-moderate atopic dermatitis

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Background: The skin microbiome is disrupted in atopic dermatitis (AD). Existing research focuses on moderate to severe, unmedicated disease.
Objective: We sought to investigate metagenomic- and culture-based bacterial strain–level differences in mild, medicated AD and the effects these have on human keratinocytes (HKs).
Methods: Skin swabs from anterior forearms were collected from 20 pediatric participants (11 participants with AD sampled at lesional and nonlesional sites and 9 age- and sex-matched controls). Participants had primarily mild to moderate AD and maintained medication use. Samples were processed for microbial metagenomic sequencing and bacterial isolation. Isolates identified as *Staphylococcus aureus* were tested for enterotoxin production. HK cultures were treated with cell-free conditioned media from representative *Staphylococcus* species to measure barrier effects.
Results: Metagenomic sequencing identified significant differences in microbiome composition between AD and control groups. Differences were seen at the species and strain levels for Staphylococci, with *S aureus* found only in participants with AD and differences in *Staphylococcus epidermidis* strains between control and AD swabs. These strains showed differences in toxin gene presence, which was confirmed *in vitro* for *S aureus* enterotoxins. The strain from the participant with the most severe AD produced enterotoxin B levels more than 100-fold higher than the other strains ($P < .001$). Strains also displayed differential effects on HK metabolism and barrier function.
Conclusions: Strain-level differences in toxin genes from *Staphylococcus* strains may explain varying effects on HK, with *S aureus* and non–*aureus* strains negatively affecting viability and barrier function. These differences are likely important in AD pathogenesis. (J Allergy Clin Immunol Global 2024;3:100259.)

Key words: *Atopic dermatitis, skin microbiome,* Staphylococcus aureus*,* Staphylococcus epidermidis*, metagenomics, bacterial isolation, human keratinocytes*

Atopic dermatitis (AD) is the most common inflammatory skin disease in children, with recent estimates suggesting that as many as 1 in 5 children experience AD, with 80% exhibiting symptoms within the first 6 years of life.[1-3] AD is characterized by recurrent eczematous lesional skin sites. During flares, these erythematous patches are associated with pruritus, exudation, blistering, and lichenification, which can cause loss of sleep, poor mental health, and decrease in school/work performance.[1,4,5] It has been proposed that disruptions to the skin barrier during AD flares promote AD pathogenesis.[6,7] Numerous causes have been proposed for this disruption of the skin barrier, including genetic mutations (eg, in the filaggrin gene),[8] immune dysregulation (classically characterized by T_H2 and IgE predominance),[4,6,7] environmental triggers (such as detergents and epicutaneous protease exposure),[9-11] and alterations in the skin microbiome (particularly overgrowth of *Staphylococcus aureus*).[12,13]

The microbiota plays a critical role in the maturation and function of the skin barrier,[14] including pH balance,[15-17] water retention,[18] keratinocyte differentiation,[19,20] wound healing,[21-24] innate[25,26] and adaptive[16,27,28] immune responses, and pathogen competition.[29-31] Commensal skin bacteria, such as *Staphylococcus epidermidis*, produce antimicrobial peptides (AMPs) that are bactericidal and increase keratinocyte production of host AMPs.[29,32,33] These bacterial AMPs are active against *S aureus* and are depleted on AD skin.[29] In contrast, *S aureus* is important in AD pathogenesis. Multiple studies have shown an increased prevalence of *S aureus* on the skin of patients with AD, particularly on lesional sites.[34,35] *S aureus* is known to produce a number of toxins, with staphylococcal enterotoxin B (SEB) in particular associated with more severe AD.[36,37] However, existing studies, including those associating increased *S aureus* with lesional skin, have largely focused on moderate to severe participants while holding AD medications and have focused on the microbiome at the

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Abbreviations used
AD: Atopic dermatitis
AMP: Antimicrobial peptides
CFU: Colony-forming unit
DM: Differentiation medium
HK: Human keratinocyte
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD_{600}: Optical density at 600 nm
P_{adj}: Adjusted *P* value (using Bonferroni)
SEB: Staphylococcal enterotoxin B
ST: Strain type
TEER: Transepithelial electrical resistance

genus and species levels. Whether *S aureus* plays a similarly important role in less severe or well-controlled disease remains less well understood. In addition, variability at the strain level in AD progression is only beginning to be addressed, with early studies suggesting that strains from subjects with severe AD more negatively affect the skin in a murine model.[35,38]

METHODS

Overall approach

Skin swabs were collected from 20 participants aged 0.5 to 14 years for metagenomic analysis of the microbiome. When present, lesional samples were collected in addition to matched nonlesional samples. In contrast to previous studies, participants with AD primarily had mild to moderate AD and continued use of topical therapies to control disease, mimicking real-world clinical scenarios. Shotgun metagenomic- and culture-based approaches were used to evaluate the microbial communities associated with these samples. To determine the effects of strain variation on keratinocyte viability and barrier integrity, keratinocyte cultures were exposed to conditioned media containing secreted metabolites from select *Staphylococcus* isolates derived from participants with a range of clinical presentations (see Fig E1 in this article's Online Repository at www.jaci-global.org).

Patient recruitment and classification

Recruitment occurred at the University of Wisconsin—Madison's Pediatric Allergy-Immunology and Dermatology clinics. Consent for the study and publication of results was obtained from participants' legal representatives. Approval was granted by the University of Wisconsin School of Medicine and Public Health's Institutional Review Board. Participants were assigned nonidentifying subject IDs for the study. AD was defined as board-certified pediatric allergist or pediatric dermatologist diagnosis with typical signs and symptoms of disease (eczematous dermatitis with typical morphology and distribution) using the Hanifin and Rajka criteria.[39] AD severity was determined as previously described using guidelines-based criteria.[40] Age- and sex-matched controls were recruited if they had no current eczematous rash, no history of AD, and were not using any topical medication. See the Online Repository at www.jaci-global.org for criteria for other allergic disease classification.

Sample collection

Skin swabs were collected from 20 pediatric participants using standard Copan swabs for metagenomic analysis and eSwabs (Copan, Murrieta, Calif) to obtain live isolates. Lesional (if present) and nonlesional swabs were collected from all participants. See the Online Repository for full details.

Metagenomic library preparation and analysis

DNA extraction was done as previously described.[41] Samples were sent to the University of Minnesota Genomics Center for library preparation and metagenomic sequencing. A total of 27 skin samples and 1 extraction negative control were sequenced. The average read depth was 7.6 million paired-end reads per sample. Resulting FASTQ files were processed using quality filtering, adapter removal, human decontamination, and tandem repeat removal as described previously.[41] Taxonomic classification and abundance estimation were performed using Kraken2 (v2.0.8-beta)[42] and Bracken (v2.5).[43] Reads assigned as *Homo sapiens* or that which were unclassified at the genus level were filtered out. Decontam (v1.16.0) was then used to identify and remove contaminant reads. See the Online Repository for additional details and Fig E2 (in the Online Repository available at www.jaci-global.org) for read counts at each step. StrainGST (v1.3.3)[44] was used to infer the presence and relative abundance of specific *Staphylococcus* strains, using a set of 230 representative genomes from across the genus.[45] PubMLST was used to determine strain types (STs) for *S epidermidis*.[46]

Bacterial isolation and identification

Single isolates were obtained through selective culturing on multiple media types until single colonies were obtained. Sanger sequencing on the full-length 16S rRNA gene was used for taxonomic classification. See the Online Repository for full details.

Whole-genome sequencing

Bacteria identified as *Staphylococcus* were sent for whole-genome sequencing at SeqCenter (Pittsburgh, Pa) using the Illumina DNA Prep kit (San Diego, Calif) and IDT (Coralville, Iowa) 10 bp unique dual indices, with sequencing performed on an Illumina NextSeq 2000. Demultiplexing, quality control, and adapter trimming were done with bcl-convert (v3.9.3). These genomes were then used to generate a phylogenetic tree with autoMLST in the *de novo* mode and concatenated alignment functions.[47] The resulting tree was visualized using iTOL (Interactive Tree of Life).[48] Gene calling and standard annotation were performed using PROKKA (v1.13).[49] Toxin genes were identified through DIAMOND BLASTp alignment to the full Virulence Finder Database[50] download in January 2023, retaining the best hit per query on the basis of bitscore with additional filters to retain only those alignments that exhibited a maximum *e* value of 1×10^{-5}, a minimum percent identity of 50%, and minimum query and subject coverages of 70%.

Generation of staphylococcal cell-free supernatants

Staphylococcus isolates were grown from stock on trypticase soy agar overnight at 37°C. Single colonies were then grown in trypticase soy broth overnight at 37°C with shaking. The following morning, liquid cultures were used to inoculate

50 mL supplemented keratinocyte growth media (see the Online Repository for recipe) to a starting OD_{600} of 0.1, followed by incubation at 37°C with shaking. OD_{600} was read every 2 hours until an OD_{600} of 0.7 was reached (range, 0.693-1.156; average, 0.774; typically 3-7 hours). The cultures were centrifuged at 3220*g* for 10 minutes. The supernatants were collected and passed through a Watman cellulose grade 1 filter paper (GE Healthcare Life Sciences, Chicago, Ill) and then through a 0.2-μm pore filter. Supernatants were stored at −20°C until use. Cultures were serially diluted for colony-forming unit (CFU) plating immediately following inoculation and immediately preceding collection to confirm similar CFUs across strains (average of 2.64×10^9 CFUs/mL).

In vitro enterotoxin assay

Staphylococcus isolates from stock were grown on brain heart infusion agar overnight at 37°C. Single colonies were grown in 3 mL brain heart infusion liquid culture overnight at 37°C with shaking. The next day, the cultures were centrifuged at 3500*g* for 5 minutes. The supernatants were collected and passed through a pore filter (0.2 μm). Enterotoxin levels were then measured using the BioPharm Ridascreen kit according to the manufacturer's instructions.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Effects of staphylococcal cell-free supernatants on neonatal human keratinocyte (HK; American Type Culture Collection, Manassas, Va) cell viability were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Roche Diagnostics, Mannheim, Germany) as per the manufacturer's instructions. See the Online Repository for a full description.

Transepithelial electrical resistance assay

HKs were also used for the transepithelial electrical resistance (TEER) assay. The cells were cultured in HK media on a semipermeable filter insert in a 5% CO_2 atmosphere at 37°C for 24 hours until they reached confluence. They were then switched to a differentiation medium (DM; Dulbecco modified Eagle medium supplemented with 1.8 mM calcium ion and 4 mM glutamine [ThermoFisher Scientific, Waltham, Mass]) alone (control) or DM containing one of the *Staphylococcus* supernatants diluted to 1×10^6 CFUs/mL. The integrity of the HK monolayer was verified by measuring the TEER assay after 2 days of treatment. Three independent replicates were used to calculate the results. The electrical resistance, measured in ohms, of an empty insert was subtracted from that of the insert with cells to yield the resistance of the cells. The electrical resistance was then multiplied by the area of the insert to determine the TEER. Results are expressed as the percentage change in TEER, which reflects the change in resistance after 2 days of treatment normalized to the TEER value of the control (DM alone).

Statistical analysis

The R software (version 4.2.0) was used to do all analyses and figure generation. The Kruskal-Wallis test was used to compare select taxonomic levels and the Shannon diversity index. Permutational multivariate ANOVA testing was used with Bray-Curtis dissimilarity estimates. These calculations were done on relative abundance set to 100% for microbes, bacteria, or *Staphylococcus*, where appropriate. A *t* test was used to compare enterotoxin levels. Following a significant ANOVA, pairwise *t* tests were used to compare the MTT and TEER effects of the *Staphylococcus* isolates to media controls, with a Bonferroni correction used to account for multiple testing.

RESULTS

Clinical characteristics and sample collection

Twenty pediatric participants, aged 5 months to 14 years, were recruited from the University of Wisconsin Pediatric Allergy-Immunology and Dermatology clinics (Table I). One control participant was omitted because of having a parent-reported history of AD but no physician diagnosis and no symptoms of disease. The remaining participants consisted of 8 healthy controls and 11 with diagnosed AD, primarily mild to moderate in severity. Participants with AD were not asked to halt usage of topical treatments (see Table E1 in this article's Online Repository at www.jaci-global.org). Skin swabs collected from participants were classified as control, lesional, or nonlesional and were processed for DNA extraction and metagenomic analysis and untargeted bacterial isolation.

Skin metagenomes from well-controlled participants with AD resemble controls

Metagenomic analysis of skin swabs was completed to provide a comprehensive view of the microbiome across participants. After filtering and quality control, most of the reads (>97%) were identified as Bacteria (Fig 1, *A*). The remaining 3% of reads mapped to either the Eukaryota or the Viral database. The dominant bacterial genera were consistent with previous reports of pediatric skin—*Streptococcus*, *Cutibacterium*, *Micrococcus*, and *Staphylococcus* (Fig 1, *B*). Of note, the Eukaryota were dominated by *Malassezia restricta*, but there were no significant differences across genera (see Fig E3, *A*, in this article's Online Repository at www.jaci-global.org). Interestingly, viral relative abundance was elevated in AD (adjusted *P* value [using Bonferroni], $P_{adj} = .010$). This was likely driven by the presence of *Escherichia virus T4*, which was identified only in participants with AD ($P = .004$) (Fig E3, *B*). Molluscum contagiosum virus, which is associated with skin infection, was also present on the skin of several participants, but was not significantly different among the groups (Fig E3, *B*).

Alpha (within-sample) diversity of the bacteria, as measured by the Shannon diversity index, did not significantly differ between the 3 groups (Fig 2, *A*) nor when considering combined AD (pooled lesional and nonlesional samples) versus control samples. However, a trend emerged showing lower diversity but greater evenness in the lesional group compared with control (see Fig E4 in this article's Online Repository at www.jaci-global.org). A similar trend was noted with the combined AD group for evenness ($P = .07$). Similarly, beta (between-sample) diversity, measured by Bray-Curtis dissimilarity, showed weak grouping of controls versus combined AD ($P = .056$).

TABLE I. Demographic data for the participants

Characteristic	Total (N = 19)	Control (n = 8)	AD (n = 11)
Sex, female, n (%)	8 (42.11)	4 (50)	4 (36)
Age (y)			
Range	0.46-14.02	0.46-15	0.667-13.167
Median (IQR)	3.07 (1.74-6.82)	5.5 (2.20-9.50)	5.0 (2.83-6.33)
Race, n (%)			
Asian	2 (10.53)	0	2
Black or African American	2 (10.53)	1	1
Other	1 (5.26)	0	1
White	14 (73.68)	7	7
AD severity, n (%)			
None	8 (42.11)	8	0
Mild	7 (36.84)	0	7 (36.84)
Moderate	2 (10.53)	0	2 (10.53)
Severe	2 (10.53)	0	2 (10.53)

IQR, Interquartile range.

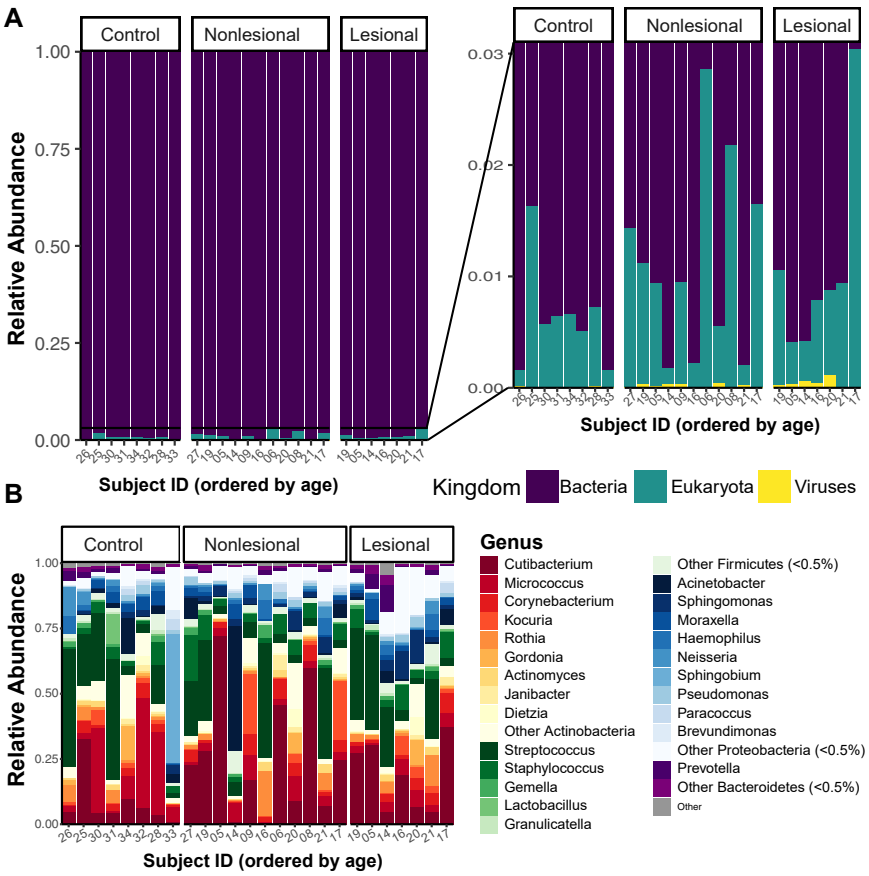

FIG 1. Metagenomic analysis of skin swabs from AD and control groups. **A,** Bar graphs of the relative abundances of kingdom-level data, with an inset to show differences in Eukaryota and Viruses. **B,** Relative abundance plots of bacterial genera, with hue corresponding to phylum, are ordered by phylum abundance. For both, each swab was normalized to 100% relative abundance of all included taxa along the y-axis and bars are grouped by AD status and then ordered by age along the x-axis.

Staphylococcal strain variation by AD status

Staphylococcus species are among the most dominant taxa on human skin, and blooms of *S aureus* have been associated with AD flares. Focusing on the *Staphylococcus* species within the metagenomes showed large intraindividual variability (Fig 3, *A*). Although the proportion of *Staphylococcus* species present in the 3 groups did not significantly differ, the total number of different *Staphylococcus* species present in participants with AD trended higher (Fig 3, *B*; $P = .15$). Notably, we did find a decrease in the Shannon diversity index when comparing combined AD with control ($P = .048$; Fig 3, *C*).

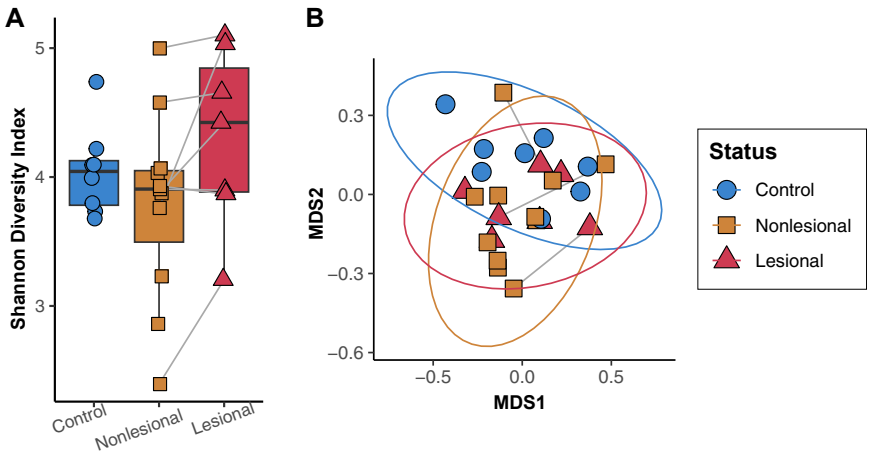

FIG 2. A and **B,** Diversity metrics did not differ significantly by AD status. The Shannon diversity index (Fig 2, *A*) and Bray-Curtis dissimilarity (Fig 2, *B*) were calculated for all metagenomes and then grouped by AD status. *MDS1/2*, Multidimensional scaling.

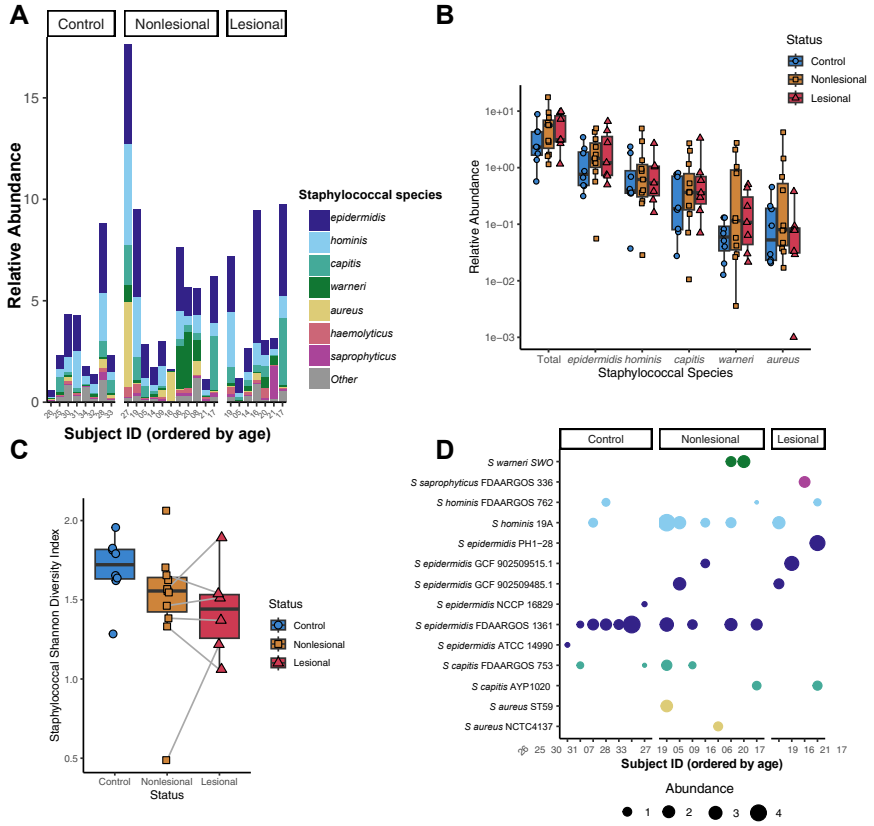

FIG 3. *Staphylococcus* species and strain-level variation by AD status. **A,** Bar chart showing relative abundances of *Staphylococcus* species detected in each metagenome, grouped by AD status and ordered by age across the x-axis. **B,** Bar chart showing the relative abundances of the top 5 *Staphylococcus* species (as called by Kraken/Braken), grouped by AD status. **C,** Shannon diversity index of all *Staphylococcus* species (as called by Kraken/Braken), grouped by AD status. **D,** *Staphylococcus* strains (as called by StrainGST), grouped by AD status and ordered by age, highlight strain differences.

To determine whether specific strains of Staphylococci vary by AD status, we classified the *Staphylococcus* strains in metagenomes using StrainGST.[44] This analysis showed a striking lack of *S epidermidis FDAARGOS_1361* (ST 153) in lesional samples, but was present in 5 of 7 control samples and 4 of 8 nonlesional samples (Fig 3, *D*). Conversely, 3 other strains of *S epidermidis*

(ST 5, 89, and 387) were present only in AD swabs. These data suggest that the association of *S epidermidis* with disease activity may be strain-dependent. Using StrainGST, we observed *S aureus* strains only in AD samples, specifically in the nonlesional swabs. Strains of *S warneri* and *S saprophyticus*, skin commensals that may act opportunistically,[51,52] were also observed only in AD samples (Fig 3, *D*).

Isolation and identification of live bacterial isolates

To determine the role different strains may be playing in AD pathogenesis, we performed untargeted bacterial isolation. Using 4 different media to capture greater microbial diversity, 601 isolates from 26 swabs were obtained. Full-length 16S rRNA gene sequencing was used to taxonomically classify each isolate, resulting in the classification of 22 genera—13 among the 305 isolates from control swabs, 10 from the 84 lesional isolates, and 14 from the 210 nonlesional isolates (see Table E2 in this article's Online Repository at www.jaci-global.org). Of those, 93% were genera represented in the metagenomes. Actinobacteria were the most abundantly represented phylum, followed by Firmicutes and then Proteobacteria in both the metagenomic- and culture-based approaches. Of note, control swabs had more than 50% more isolates collected than from nonlesional swabs, which, in turn, had twice the number of isolates as lesional swabs. *Micrococcus* was the most abundantly cultured genera from control samples, whereas *Staphylococcus* species (specifically *aureus*, *capitis*, *epidermidis*, *hominis*, *saprophyticus*, *succinus*, and *warneri*) were the most abundantly isolated in lesional and nonlesional swabs (Table E2). Combined, these 2 genera accounted for more than 40% of all isolates. Strikingly, the proportion of isolates that are *Staphylococcus* species rose greatly in the AD samples, particularly those from nonlesional sites.

Toxins are elevated in AD-associated Staphylococci

To better understand the role of Staphylococci strain-level differences in AD, we used the isolate library described earlier. We used bacterial strains representing all of the Staphylococci species isolated across participants and performed whole-genome sequencing of each strain chosen. From this, a phylogenetic tree was generated showing relatedness of the selected strains (Fig 4, *A*). Of note, all *S aureus* isolates were collected from participants with moderate and severe AD. Conversely, all *S epidermidis* and *S hominis* isolates were from participants with mild AD or control participants. The *S capitis* strains were split into 2 clusters, of which one comprised participants with moderate and severe AD and the other was associated with a single mild participant. Genes encoding toxin production in each genome were predicted using the Virulence Factor Database. We noted 3 patterns for genes encoding toxins—those universally present (not shown), those present in a specific species, and those present in only select isolates. All isolates, even coagulase-negative Staphylococci, contained at least 5 toxin-associated genes. As expected, *S aureus* strains contained increased enterotoxin, hemolysin, and leukocidin genes (Fig 4, *B*). Only 1 of 3 *S aureus* strains isolated encoded for *seb*. This *S aureus* strain, designated LK1493, was isolated from a participant recorded as having severe disease. To confirm the genomic predictions, the production of enterotoxins in each *S aureus* isolate was tested *in vitro*, confirming high levels of SEB production by LK1493 ($P < .001$) (Fig 4, *C*).

Effects of *Staphylococcus* supernatants on keratinocytes *in vitro*

To test the *in vitro* effects of these differences on keratinocytes, cell-free conditioned media derived from all of the *Staphylococcus* isolates were incubated with keratinocytes to investigate cell viability and barrier integrity using MTT and TEER assays, respectively. Isolates from AD swabs significantly decreased cell viability (Fig 5, *A*) and barrier function (Fig 5, *B*). However, there was wide variability within each group.

To investigate the variability within each group, we first compared among species (Fig 5, *C* and *D*). *S hominis* species isolated from control swabs had no effect on cell viability, whereas *S hominis* species from AD lesional swabs were detrimental. Indeed, many *Staphylococcus* species isolated from lesional swabs significantly impaired TEER ($P_{adj} < .05$). We next examined each isolate (Fig 5, *E* and *F*). We found intriguing differences driven by the strain of Staphylococcus tested. For example, *S hominis* LK2134, isolated from a control participant, showed a trend toward improved barrier function ($P_{adj} = .12$; $P = .005$), whereas *S hominis* LK2024, isolated from a lesional swab, showed the most detrimental effect on barrier function ($P_{adj} < 1 \times 10^{-10}$). *S warneri* LK1434 significantly decreased MTT ($P_{adj} = .006$) but not TEER, whereas *S warneri* LK1908 and LK2055 significantly decreased TEER ($P_{adj} < 1 \times 10^{-4}$) but not MTT. None of the *S epidermidis* isolates significantly affected MTT, although 2 decreased TEER ($P_{adj} < .001$). All isolates of *S capitis* significantly decreased TEER ($P_{adj} < .005$), but only 1 trended toward a decrease in MTT ($P_{adj} = .060$). Similarly, 2 strains of *S aureus* significantly decreased TEER ($P_{adj} < 1 \times 10^{-4}$), whereas the third trended toward a decrease in MTT ($P_{adj} = .14$).

DISCUSSION

The skin microbiome is critical in maintaining proper functioning of the skin barrier, and previous research has focused on the role of *S aureus* in moderate to severe AD while medications are held. In this setting, the relative abundance of *S aureus* has been shown to increase during acute disease flares.[12,13] In addition, *S aureus* has been shown to produce toxins, particularly SEB, which are associated with more severe disease.[36,37] However, there is limited research into medication-controlled AD, which is more reflective of the clinical setting.

In our medicated cohort, we did not observe the emergence of *S aureus* blooms reported in previous studies enrolling unmedicated AD, even in lesional samples. When *S aureus* was detected, it was primarily identified from nonlesional AD swabs. This is similar to a previous study that found mitigation of *S aureus* overgrowth when taking medication.[13] How medications interfere or alter the microbiome is not well described, but topical corticosteroids have been shown to decrease *S aureus* bioburden.[53] Our data, together with the previous work, suggest that the interventions to manage AD are decreasing *S aureus* overgrowth, despite disease flares.

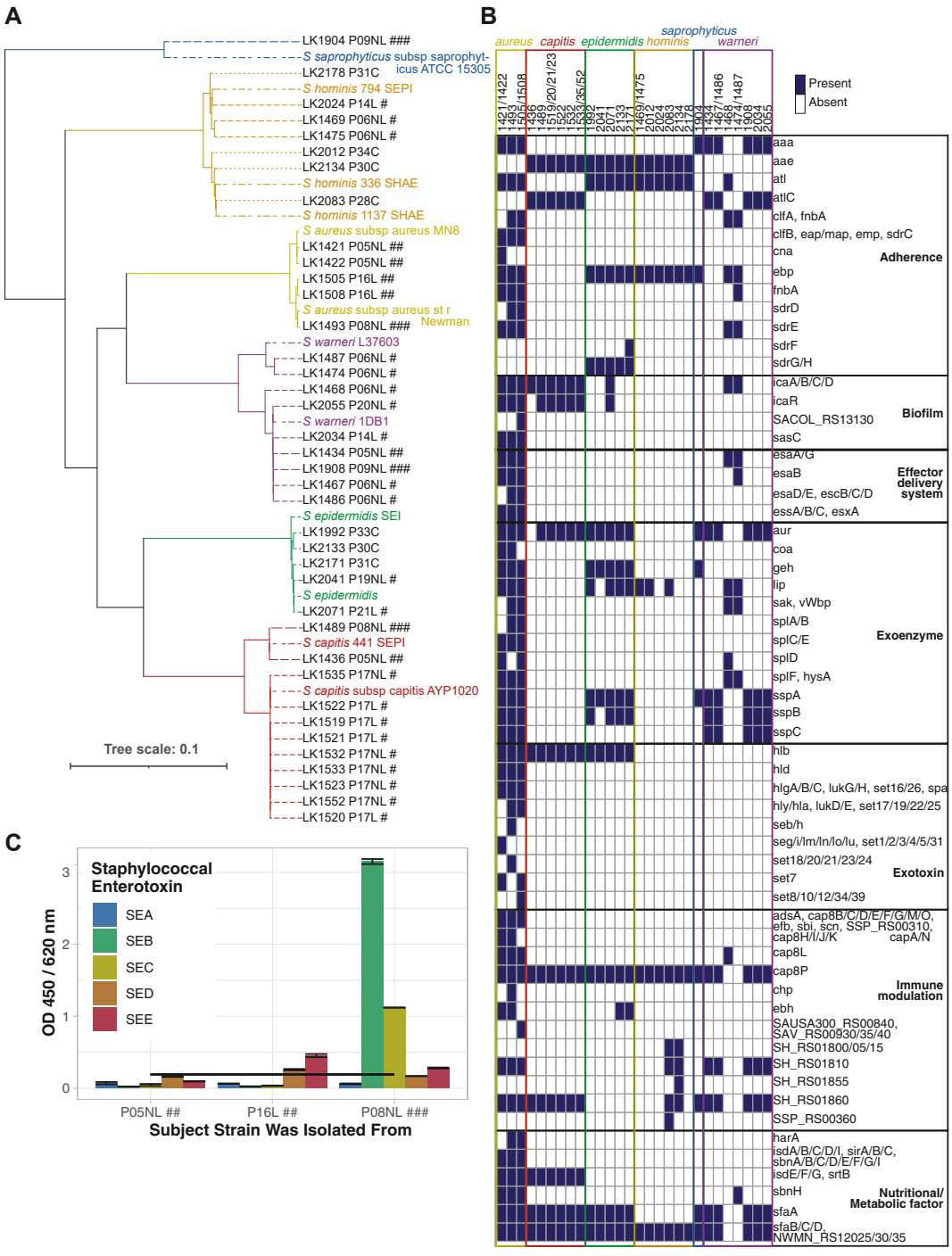

FIG 4. Toxins are elevated in AD-associated Staphylococci. **A,** Phylogenetic tree of select cultured Staphylococcus strains. Sequenced whole genomes were uploaded to autoMLST using *de novo* mode and concatenated alignment function. The resulting tree was annotated with iTOL (Interactive Tree of Life). Type strains are colored by species. Cultured strains are in *black,* with the "#" symbol indicating subject's AD severity (none: control; #: mild AD; ##: moderate AD; ###: severe AD). **B,** Heat map indicating presence of toxin genes observed in the whole-genome sequences of those same selected Staphylococcus strains, as called by PROKKA. **C,** *In vitro* enterotoxin levels in the 3 putatively unique *S aureus* strains show strain-level differences, with SE levels increasing with severity. Bar graph depicts mean and SE; subject label color denotes AD severity; and line represents positivity threshold.

Differences in *Staphylococcus* strains were present, including in coagulase-negative Staphylococci. A strain of *S epidermidis* (ST 153) was primarily associated with healthy participants. This strain was also associated with healthy volunteers in a study of catheter-related bacteremia.[54] Conversely, metagenomes from AD lesions contained a number of other *S epidermidis* strains. Although previous studies in AD have suggested potential protective effects of *S epidermidis*, it can behave as an opportunistic

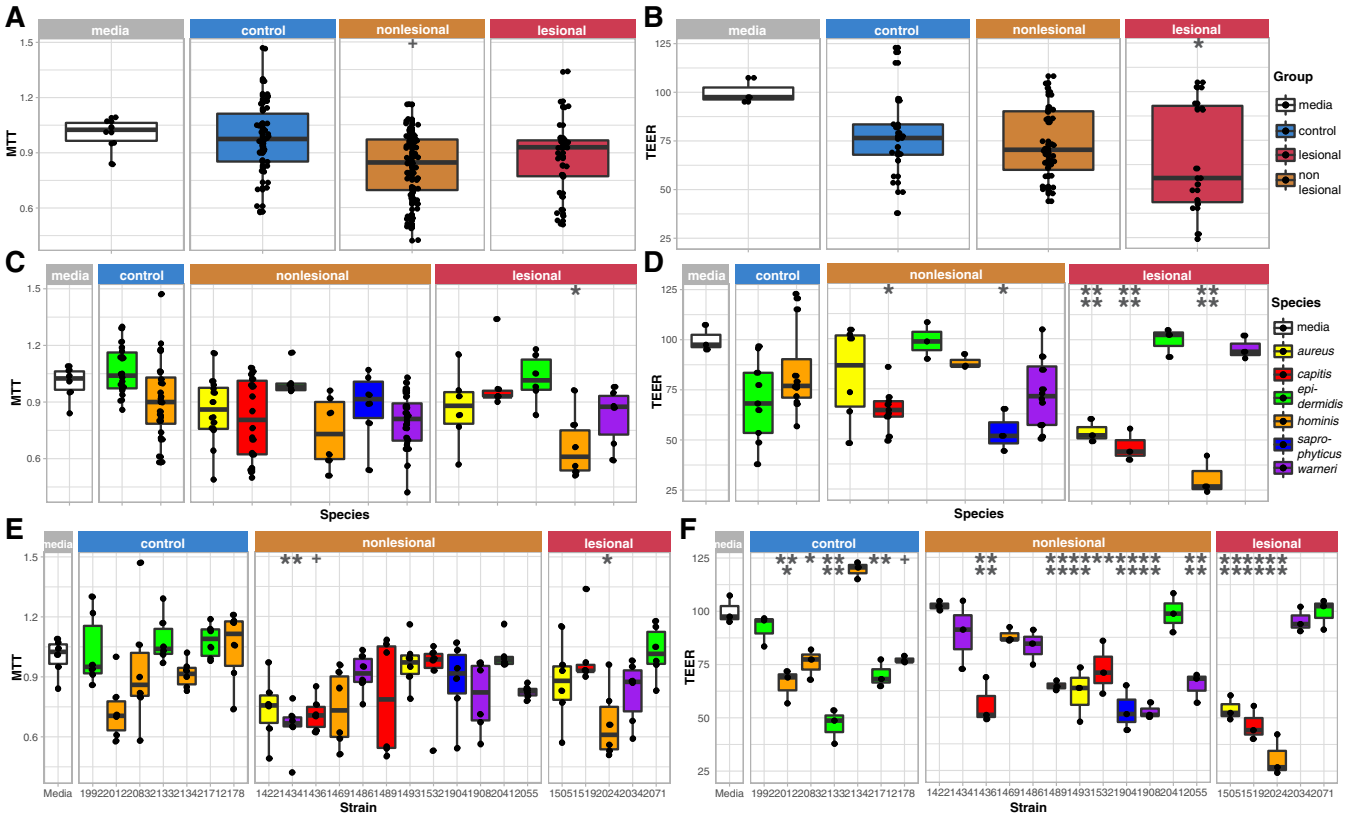

FIG 5. *Staphylococcus* cell-free media differentially affects keratinocyte function. **A-F,** Box plots showing effects of *Staphylococcus* cell-free conditioned media on MTT (Fig 5, *A, C, E*) and TEER (Fig 5, *B, D, F*) assays, faceted by groups and colored by species. The MTT (Fig 5, *A*) and TEER (Fig 5, *B*) data at the group level (ie, all isolates in the control group are pooled, as are all in the lesional and nonlesional groups). The MTT (Fig 5, *C*) and TEER (Fig 5, *D*) at species-level resolution. The MTT (Fig 5, *E*) and TEER (Fig 5, *F*) with each individual strain. Bonferroni adjusted *P* value symbols: +, .15; •, .1; *.05; **.01; ***.001; ****.0001.

pathogen. As demonstrated by the TEER data, strains of *S epidermidis* from intact skin can disrupt keratinocyte barrier function. The pathogenicity of *S epidermidis* seems to vary greatly by strain, with certain strains being associated with health and others with disease.[35,55,56] On human skin in the native microbiome, these strains exist in balance with a diversity of other species. Thus, these data suggest the importance of strain-level differences in both *S aureus* and non–aureus *Staphylococcus* species in AD disease expression and severity.

In addition, we observed marked differences in toxin gene presence across the Staphylococcal isolates. All 3 *S aureus* strains, isolated from participants with moderate and severe disease, encoded for more toxin genes than other strains. One *S aureus* strain (isolated from a severe patient) contained the *seb* gene and expressed this protein at high levels *in vitro*. Previous studies have suggested that SEB is associated with more severe AD,[36,37] likely because of its cytotoxic effects that disrupt barrier function.[57]

Importantly, our data evaluating keratinocyte metabolism and barrier function after exposure to secreted factors from diverse Staphylococci species demonstrate that many Staphylococcal strains are capable of disrupting keratinocyte function, even in the absence of enterotoxin production, similar to other work.[58-60] In addition, keratinocyte dysfunction occurred across several species and was strain-dependent. In general, *S aureus* and *S capitis* were associated with deleterious effects on barrier function, whereas *S epidermidis* and *S warneri* had strain-level varied effects as measured by TEER. Finally, different strains of *S hominis* induced opposing effects on keratinocyte integrity as measured by MTT. Ongoing research will attempt to clarify the drivers of these differential effects.

This study was designed to demonstrate feasibility of the approach and to determine whether strain-level differences are important to clinical disease. Therefore, the small sample size is a limitation, because we are underpowered to detect metagenomic differences comparing control participants and those with AD. Other limitations include that we did not capture what medications (if any) were being applied to nonlesional skin. We attempted to collects swabs from the upper extremity when available. However, we did not capture the exact body site for each swab. The study was cross-sectional, and it is unclear whether alterations in skin barrier facilitate *S aureus* colonization or whether colonization induces changes to the skin barrier. Prospective data, perhaps from a birth cohort, will help address these issues. Regardless, the TEER and MTT data suggest that the microbiome may affect the skin barrier. In addition, MTT and TEER assays are both limited in that they are unable to accurately capture the complexity of the 3-dimemsional skin environment. Finally, we examined each strain in isolation, and this does not allow us to measure the interactions between members of the bacterial communities. The issues of the influence of live bacterial isolates (and not supernatants) and the role of bacterial

interactions will be investigated in future work with a larger sample size. We also found differences in the virome between AD and control participants. This will be investigated further in future work with a larger sample size.

Taken together, our data underscore the importance of bacterial strain in AD disease expression and pathogenesis and suggest that topical steroids can be effective in controlling *S aureus* overgrowth. It is worth noting that this was a small cross-sectional study, with participants covering a range of developmental stages. The skin microbiome can change significantly from infancy through puberty,[61] and controls were age- and sex-matched to help address this issue. In addition, medications used varied somewhat among participants, which may have differential effects on the skin microbiome. Thus, future research will add additional participants to clarify these effects, and to collect additional strains of *Staphylococcus*, as well as expanding to genera beyond *Staphylococcus*.

The data presented demonstrate the critical role of strain-level differences in AD pathogenicity. Strains differed with regard to presence and amount of toxins expressed, effect on keratinocyte viability, and barrier function. In addition, deleterious effects were not exclusive to *S aureus*. Thus, both interspecies and intraspecies differences affect AD pathogenesis, suggesting that strain-level differences are important considerations in AD pathogenesis and disease expression.

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Key messages

- **Staphylococcal strain effects, more so than species effects, affect keratinocyte barrier function and metabolism, suggesting that strain-level differences, and not species-level differences, may be critical in AD pathogenesis.**
- **The microbiome from mild, medicated patients with AD harbor *Staphylococcus* strains with detrimental effects on skin barrier and may not only be mediated by *S aureus*.**

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