LAYMAN'S SUMMARY

Atopic dermatitis (AD, eczema) is an itchy inflammation of the skin, and a chronic condition that tends to flare periodically and then subside, and may be accompanied by asthma or hay fever [1-3]. AD is associated with significant social and financial burden, and affects adults and children with worldwide prevalence rates of up to 20% [4-6]. The cause of AD is unknown, but has been associated with changes in microbial flora, skin barrier defects and dysregulation of innate and adaptive immunity [7-9]. The funded proposal was a "proof-of-principle pilot study" of the skin bacterial microbiome ("bacteriome") and fungal microbiome ("mycobiome") at affected and unaffected sites in AD patients and in affected and unaffected sites in patients with allergic contact dermatitis to identify changes in the microbiota associated with dermatitis. Our analysis showed that AD is associated with distinct changes in the bacterial and fungal microbiota, and identified significant correlations among both bacteria and fungi in the setting of AD disease. The specific aims of the funded grant were:

Aim 1. Use Next-Gen deep-sequencing approach to determine the identity of bacterial and fungal microbiota in AD patients and their relationship with the disease.

Aim 2. Use robust bioinformatics algorithms to identify correlation between skin microbiota and AD.

PROGRESS ACHIEVED DURING THE FUNDING PERIOD

In the funded period, we obtained swabs of affected and unaffected skin in post-pubertal AD patients and contact dermatitis (CD) patients after informed consent, following an IRB-approved protocol. Patients using antibacterial/antifungal within the previous 4 weeks, those undergoing topical treatment on the day of examination, and patients on an ultraviolet treatment scheme were excluded from the study. AD was defined as a history of chronic dermatitis including childhood flexural dermatitis, and severity was assessed using physician global assessment. Allergic contact dermatitis was defined as a positive patch test to an allergen with relevance to exposure at the affected site. Several patients had both atopic and allergic contact dermatitis and were stratified accordingly, creating three groups of subjects. Skin swabs were collected as described previously by our group [10].

The collected swab samples were processed and their DNA was extracted. Next, we initiated amplification of fungal and bacterial DNA in these samples using polymerase chain reaction (PCR). All tested swabs yielded a robust amount of DNA, and all amplified samples generated positive reactions for fungal and bacterial DNA, indicating the presence of fungi and bacteria in the skin swabs collected. The amplified DNA samples were purified, converted to tagged libraries, and proessed through the Ion Torrent PGM sequencer. The resulting DNA sequences were analyzed using data analytics and biostatistics algorithms (using packages based on R programming software) to gnereate the profile of bacterial and fungal microbiota in particpants with AD as well as non-AD individuals. Our results provide insight into the diversity, abundance and frequency of bacteria and fungi present in the skin, and reveal whether changes in the skin microbiome are correlated with AD.

TECHNICAL SUMMARY

Subject Demographics

Skin swab samples were collected from 13 patients after informed consent, following an IRB-approved protocol. We collected six swab samples from areas of affected and unaffected skin of subjects with a clinical diagnosis of AD. Potential areas of swabbing included the neck, anterior or posterior axillary line, axillary vault, antecubital fossa, volar wrist, and retroauricular fold. Swab samples were also collected from the nares of each subject. Skin swab samples in the same locations were also collected from the affected and unaffected skin of six subjects with allergic contact dermatitis (CD) (Table 1). These subjects were selected based on a positive patch test result to a relevant substance. Swabs were not collected from patients within four weeks of taking oral antibiotics or swimming in a chlorinated pool. Eight of the thirteen subjects swabbed were managing their dermatitis using a prescribed topical corticosteroid. Additionally, two of the thirteen subjects were using antidandruff treatments containing the active ingredient zinc pyrithione, known to have anti-fungal effects. Swabs were stored at -80 °C until analyzed.

Table 1. Summary demographics of study participants

Variable		AD*	CD*	Both AD and CD	Total
Age	≤ 55 years	1	3	3	**7**
	> 55 years	1	3	2	**6**
Gender	Male	0	6	3	**9**
	Female	2	0	2	**4**
Affected Sites	Neck (N)	1	3	3	**7**
	Axillary line (AL)	0	3	0	**3**
	Axillary vault (AV)	0	2	1	**3**
	Volar Wrist (VW)	0	2	3	**5**
	Antecubital fossa (AF)	0	4	2	**6**
	Retroauricular fold (RF)	2	2	1	**5**

*AD: Atopic Dermatitis; CD: allergic Contact Dermatitis

Extraction of DNA from skin swabs: DNA was extracted from the collected swabs using the QIAamp DNA Mini kit (Qiagen) according to manufacturer's instructions. Briefly, samples were thawed at room temperature, sonicated for 10 min and placed in Fast Prep-24 device for vortexing for 3 rounds. After vortexing, each tube was heated and then centrifuged. Supernatant from each sample was aspirated to a new tube containing 200

μL 100% ethanol, pulse-vortexed and transferred to QIAmp mini spin column. DNA from each column was eluted using elution buffer. DNA was quantitated using NanoDrop 2000 spectrophotometer. Our results showed robust DNA yield from the tested skin swab samples (range: 21.5 ng/μL to 36.0 ng/μL).

Amplification of DNA: For PCR amplification, 2x Phusion High-Fidelity PCR Master Mix with GC Buffer, and ITS1/ITS2 primers were used for fungal DNA. For bacterial DNA 2X Dream Taq Master Mix and 16S forward/reverse primers were used. PCR products were validated using 1.5% agarose gel and ethidium bromide. Positive amplification was noted for both fungal and bacterial DNA, as shown in Figures 1 and 2.

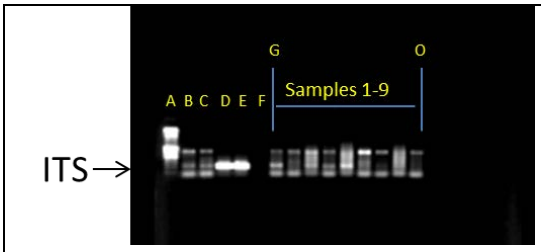

Figure. 1. Gel picture showing PCR amplified fungal DNA: (A) Mid Range DNA Ladder (Marker), (B) Sample 10, (C) Sample 7, (D and E) Positive Control, (F) Negative control, (G-O) Samples 1- 9, PCR amplified DNA showing signals for fungal specific ITS1 and ITS2 primers.

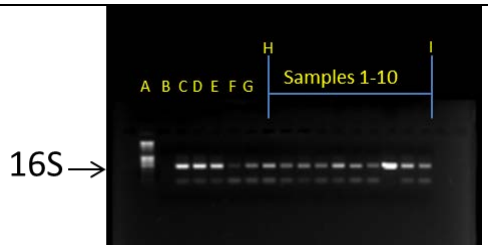

Figure. 2. Gel picture showing PCR amplified bacterial DNA: (A) Mid range DNA ladder, (B) Blank, (C-E, G) Positive Control, (F) Negative Control, (H-I) Samples 1-10, PCR amplified DNA showing signals for bacterial specific 16S primers.

Sequencing Analyses

The amplified DNA was subjected to amplicon library preparation, followed by ligating and barcoding of library, size-selection and enrichment of targeted amplicons, and sequencing on the Ion Torrent PGM sequencer. The resulting sequencing data was cleaned, denoised, and analyzed on the QIIME platform to generate tagged HTML files and operational taxonomic unit (OTU) datasets. These OTU datasets were then analyzed using bioinformatics algorithms (based on R and PARTEK software packages) to obtain summarized results on the richness, diversity, abundance and frequency profiles of the bacteriome and mycobiome, and their correlations with disease status. A summary of the sequencing results is presented in Table 2, which show that roughly equivalent number of raw sequences were obtained for both the bacterial and fungal DNA (1.59 and 1.77 million, respectively), with the mean length being 537 bp for bacteria and 985 bp for fungi. Of note, the strong fidelity of the sequencing data can be noted by the low number of “No Blast” hits.

Table 2. Summary of sequencing output		
Sequencing Summary	**Bacterial Microbiome (Bacteriome)**	**Fungal Microbiome (Mycobiome)**
Raw input sequences	1.59M	1.77M
Length (bp): min/max/avg	185/537/311	185/985/415
Sample count: min/max/mean (n=86)	22 / 333922 / 13772	335 / 55094 / 6770
High Quality Sequences	1145423	582244
OTU's Generated	630568	580592
Number of “No Blast” Hits	13072	493939
Unculturable/Unclassified Sequences	368646	43231
Classified Sequences Used in Analysis	761546	44074

Cluster Analysis

Next, we performed cluster analysis by principal components (PCA) to assess the overall variance at the genus level in the collected data, grouped by affected or unaffected skin samples in AD, AD_CD (both AD and CD), and CD patients. We found that in AD patients, the bacteriome data clustered along the PC2 axis for affected skin samples, while the unaffected group clustered further apart along the PC2 axis (Fig. 3A). Overall, 81.6% of the variance was represented by the three principal components. Analyses of the mycobiome data in the same set of samples in AD patients revealed that the affected group clusters similar to the distribution of the bacteriome, while the unaffected samples cluster along the PC1 axis; 62.1% of the variance was represented by the three principal components (Fig 3B).

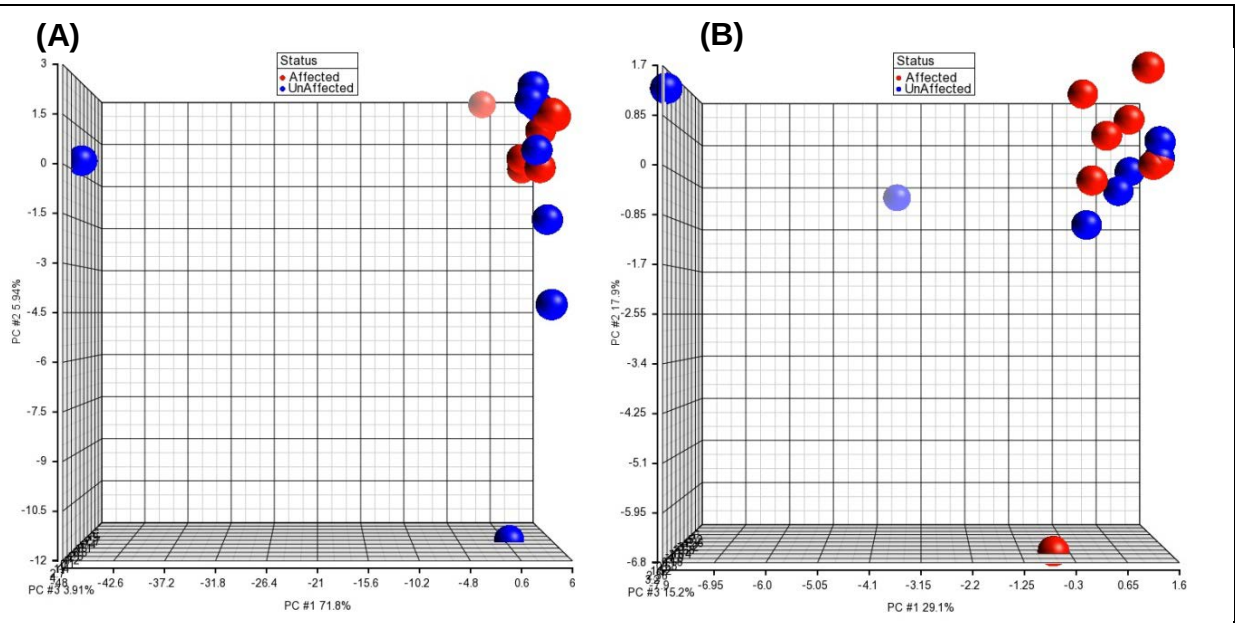

Figure 3. Principal components analysis (PCA) of variance in the affected and unaffected skin samples of AD patients. (A) Bacterial microbiome (bacteriome); (B) fungal microbiome (mycobiome). Red: affected sites; Blue: unaffected sites.

Next, PCA analyses of affected and unaffected skin swabs obtained from patients with both AD and CD revealed that in the bacteriome, both the affected and unaffected sites showed diffuse clustering, and one patient in each group was an outlier (Fig. 4A). The three PCs represented 55.6% of the variance. In contrast, analysis of mycobiota revealed that the affected sites exhibited stronger clustering compared to the unaffected sites, which demonstrated diffuse distribution along the PC1 axis (Fig. 4B). Moreover, 63.8% of the variance was represented by the three principal components.

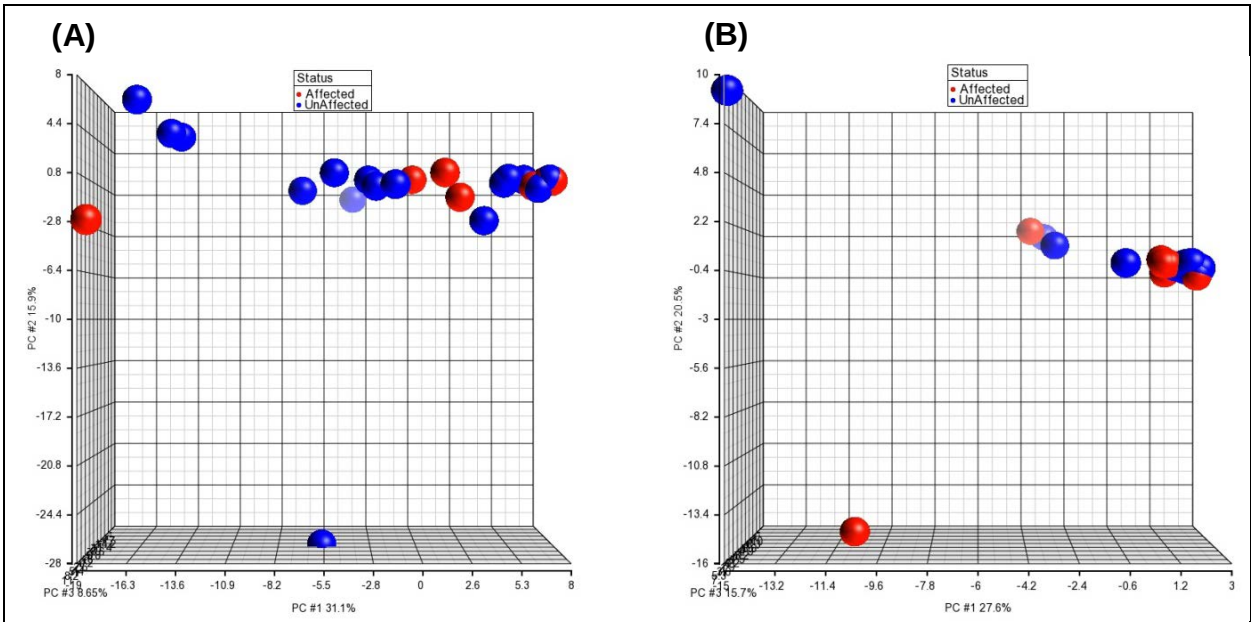

Figure 4. Principal components analysis (PCA) of variance in the affected and unaffected skin samples in patients with both AD and CD. (A) Bacterial microbiome (bacteriome); (B) fungal microbiome (mycobiome). Red: affected sites; Blue: unaffected sites.

Finally, PCA analyses of CD patients revealed that in the bacteriome profile, the affected sites clustered along the PC1 axis, while unaffected sites demonstrate diffusion along the three axes (Fig. 5A). Overall, 47.7% of the variance was represented by the three principal components. In comparison, the mycobiome showed tightly clustered communities in both affected and unaffected sites, with 53.2% of the variance being represented by the three principal components (Fig. 5B).

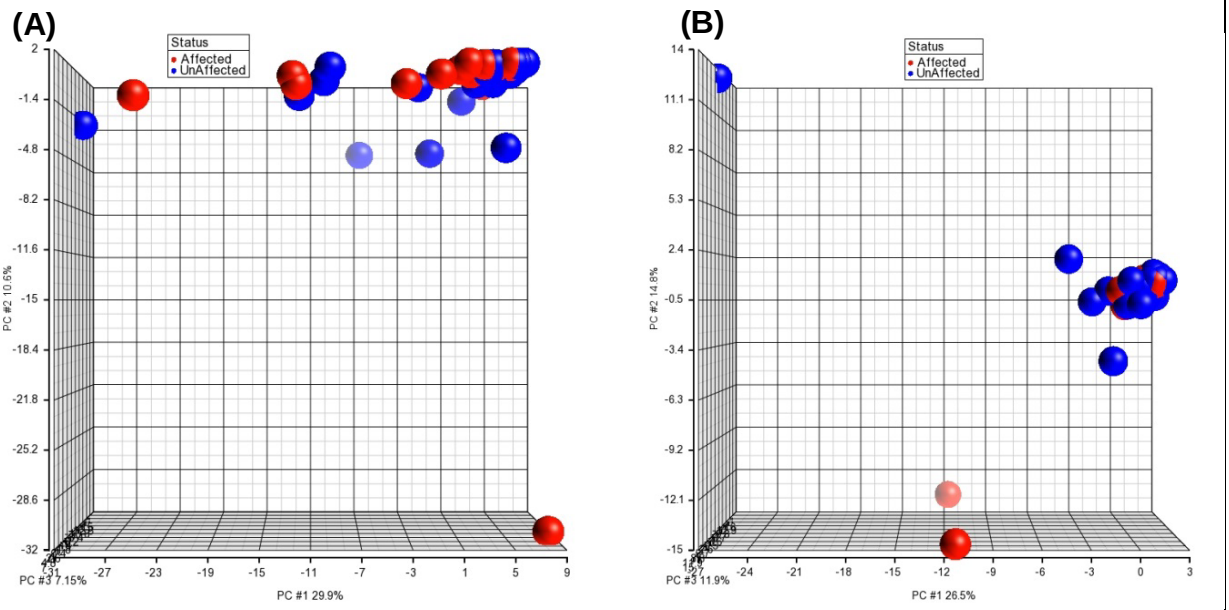

Figure 5. Principal components analysis (PCA) of variance in the affected and unaffected skin samples in patients with CD. (A) Bacterial microbiome (bacteriome); (B) fungal microbiome (mycobiome). Red: affected sites; Blue: unaffected sites.

Taken together, these PCA analyses revealed that the bacteriome and mycobiome cluster differently between affected and unaffected sites, and that this pattern of clustering differed greatly between AD and CD patients (interestingly, patients with both AD and CD exhibited intermediate variability patterns).

Community Richness and Diversity

A key set of metrics commonly used to describe microbial communities at a global level involves evaluation of their richness and diversity (Shannon) indices. We performed analysis of community richness and diversity to determine whether the skin microbiota differs between unaffected and affected sites in AD, and whether such differences are also seen in CD or AD_CD settings. As shown in Table 3, the richness of both bacteriome and mycobiome was higher in unaffected sites (324 and 65, respectively) compared to affected sites (294 and 42, respectively). Similar patterns were noted for AD-CD and CD patients. Diversity analysis showed that the Shannon diversity index (SDI) of bacteriome and mycobiome was higher in unaffected skin (2.73 and 2.63, respectively) than in affected skin (2.37 and 2.26, respectively), suggesting that AD was associated with a decrease in microbiome diversity. In contrast, the link between diversity and disease was not observed for patients with CD or both AD and CD (AD_CD). Interestingly, previous studies have associated reduced microbial diversity with diseases including inflammatory bowel disease, obesity, atopy, and susceptibility to infection [11,12]. Our results reveal similar association between diversity of the microbiome and AD (Fig. 6). Moreover, site-wise analysis of diversity showed that in three sites (axillary line, axillary vault and retroauricular fold), the diversity of microbiome was lower in affected skin compared to unaffected skin in the same anatomical sites (Fig. 7).

Table 3. Distribution of genus richness and diversity

Biome Type	Variable	AD		AD_CD		CD	
		Affected	Unaffected	Affected	Unaffected	Affected	Unaffected
Bacteriome	Sample Size (N)	8	8	10	17	18	25
	Richness	294	324	275	336	321	334
	Diversity Index	2.37	2.73	3.07	3.00	3.16	2.70
Mycobiome	Sample Size (N)	8	8	10	17	18	25
	Richness	42	65	86	99	112	130
	Diversity Index	2.26	2.63	2.17	1.71	3.10	2.23

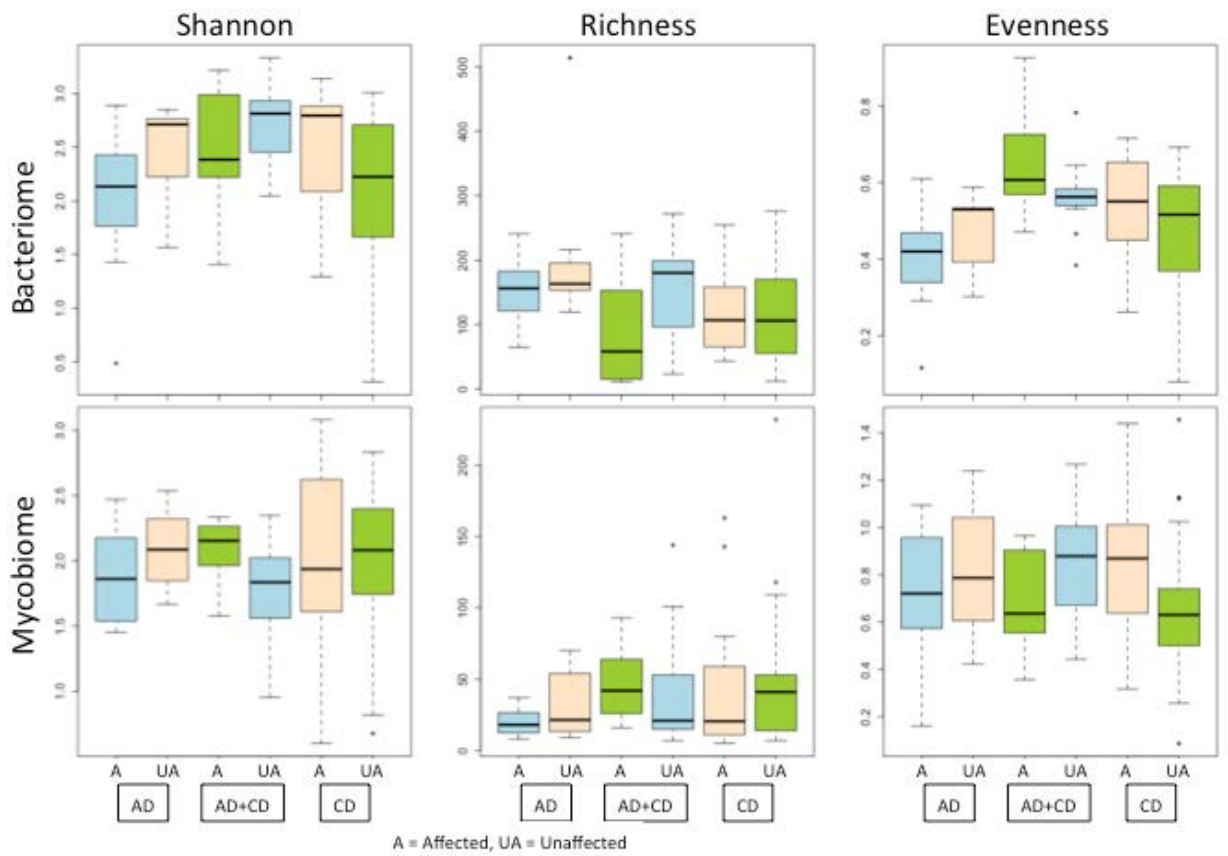

Figure 6. Community distribution indices in bacteriome and mycobiome of enrolled individuals.

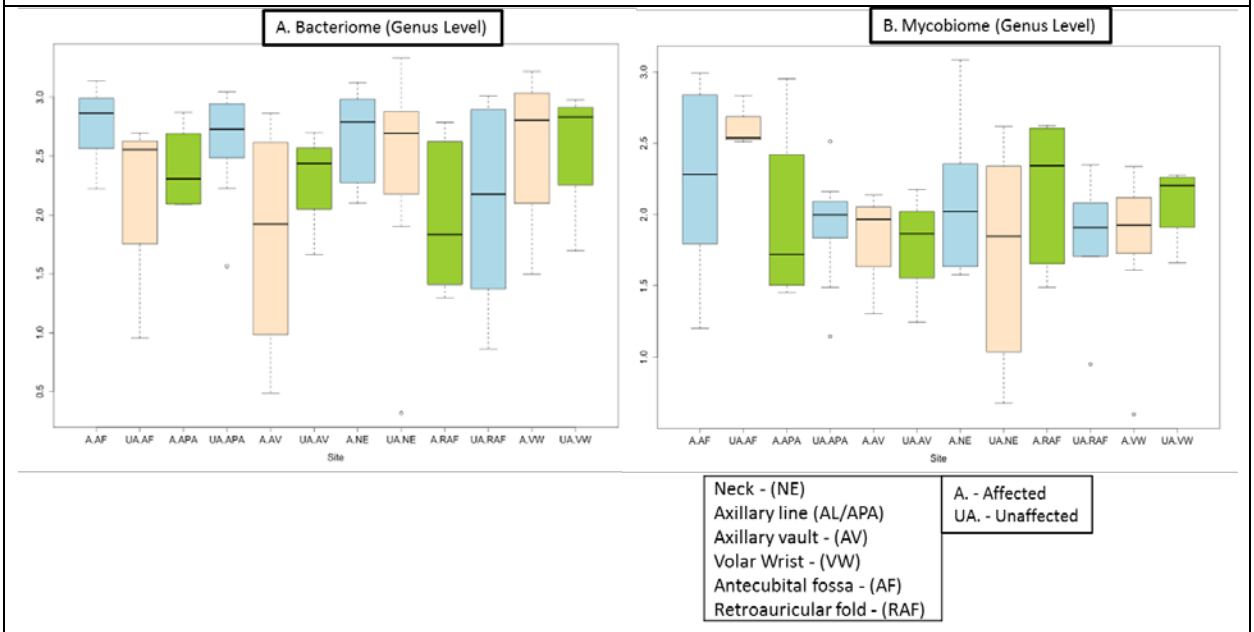

Figure. 7. Distribution of microbial diversity at different sites. (A) Bacteriome, (B) Mycobiome

Distribution of Bacterial and Fungal Abundance

Next, we determined whether the abundance of different bacterial and fungal genera differs between affected and unaffected sites in the setting of AD, compared to similar distribution in CD and AD_CD patients. We found that patients with AD had more unique bacteria and fungi compared to AD_CD and CD patients. Interestingly, the abundance profile of bacteriome and mycobiome in AD_CD was more similar to that of CD patients (Fig. 8), while distinct clustering was observed between the three cohorts (Fig. 9).

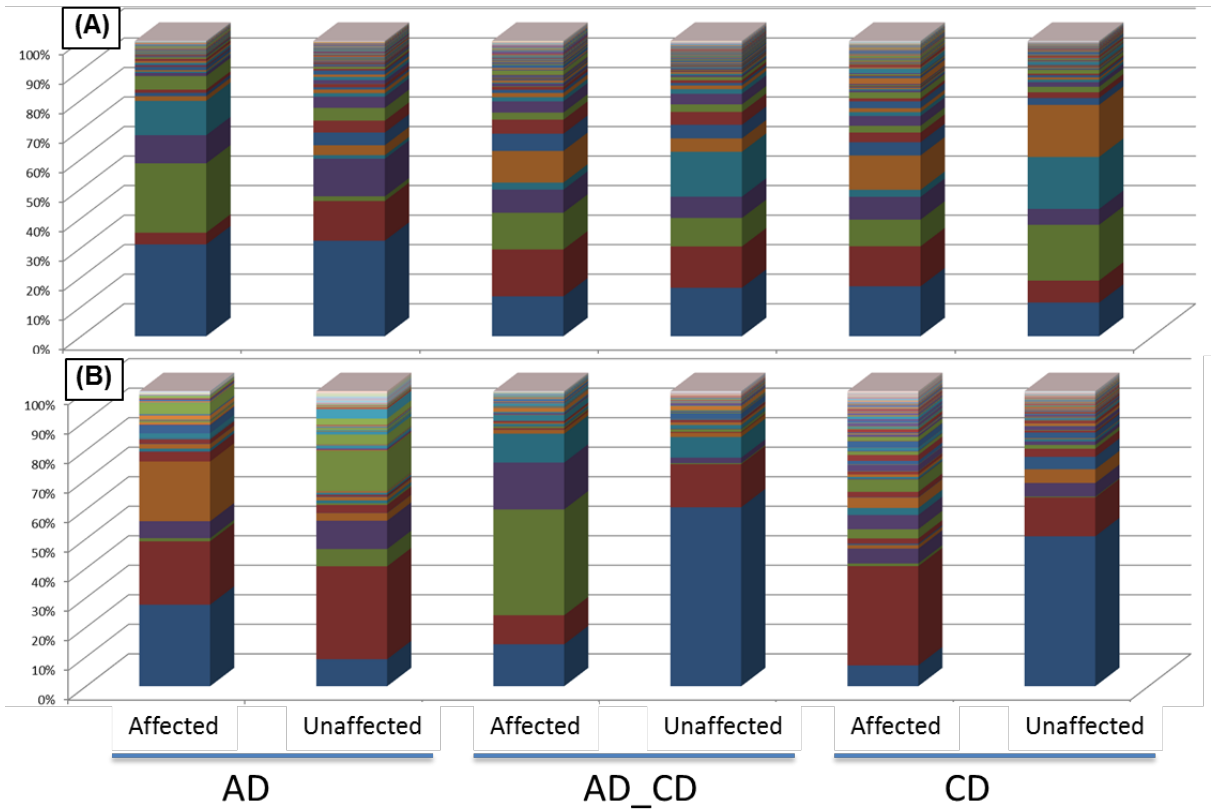

Figure 8. Abundance of different genera in the affected and unaffected sites in AD, AD_CD and CD patients. (A) Bacteriome; (B) Mycobiome.

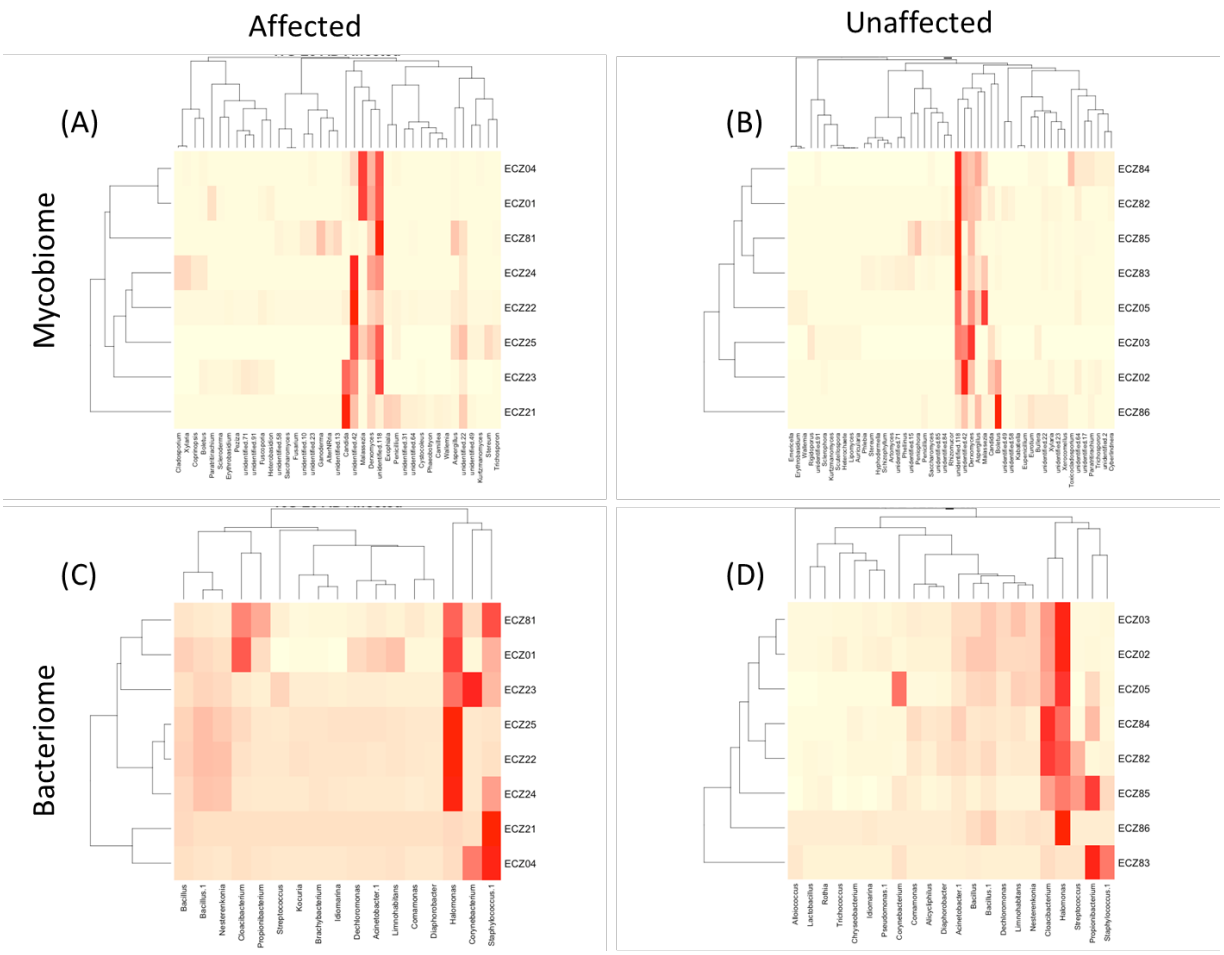

Figure 9. Heatmap showing clustering of bacterial and fungal abundnace in samples from AD patients.

Furthermore, analysis of frequency distribution of microbial genera in the affected and unaffected skin sites of AD patients showed the presence of 221 bacterial genera and 77 fungal genera (Fig. 10). Among the bacteriome, a majority (213/221) were presented in both affected and unaffected sites, while one genus (*Sphingobacetrium*) was uniquely present in affected sites and seven were unique to unaffected sites (Fig. 10A). Frequency distribution analysis of mycobiome revealed the presence of 12 genera unique to affected sites, 35 unique genera in unaffected sites, and 30 genera that were present in both affected and unaffected sites (Fig. 10B). These results demonstrated that the bacteriome and mycobiome of AD patients are distinct from those of AD_CD and CD patients.

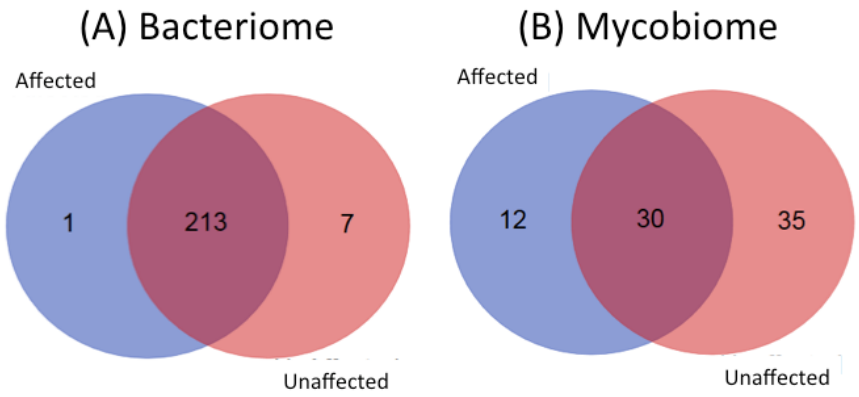

Figure 10. Frequency distribution of microbial genera in affected and unaffected skin sites of AD patients. (A) Bacteriome; (B) Mycobiome.

Correlation analysis revealed significant associations among fungi and bacteria (Fig. 11-12), and also revealed that *Staphylococcus* and *Alternaria* were correlated positively with each other (Fig. 13).

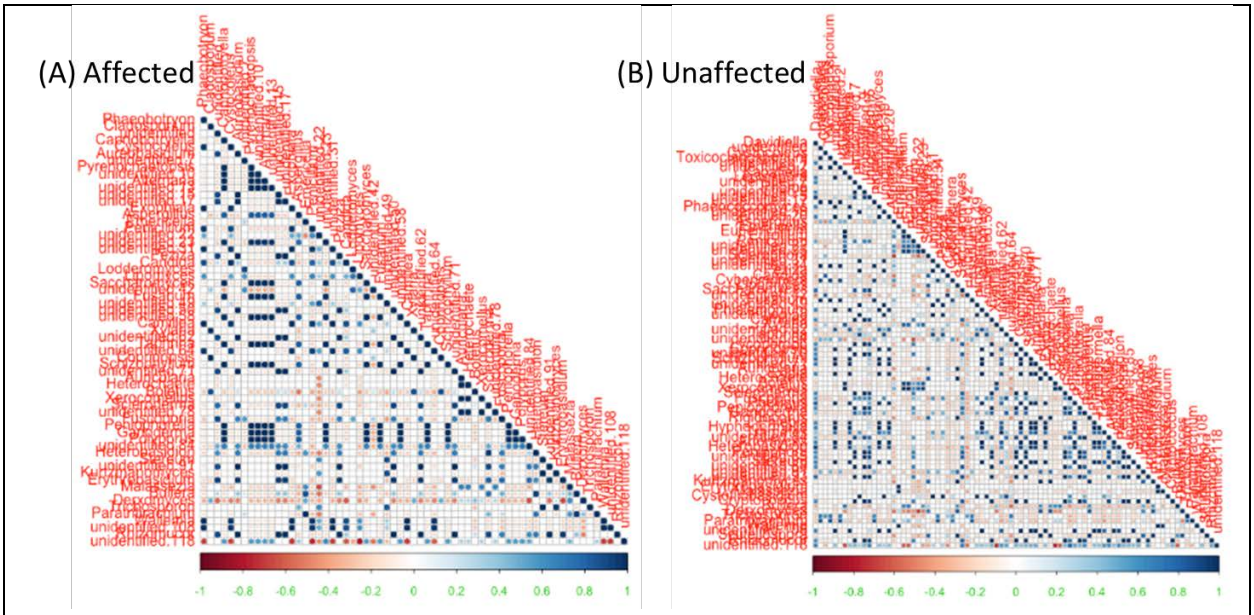

Figure 11. Correlation among fungal genera in (A) affected and (B) unaffected samples of AD patients

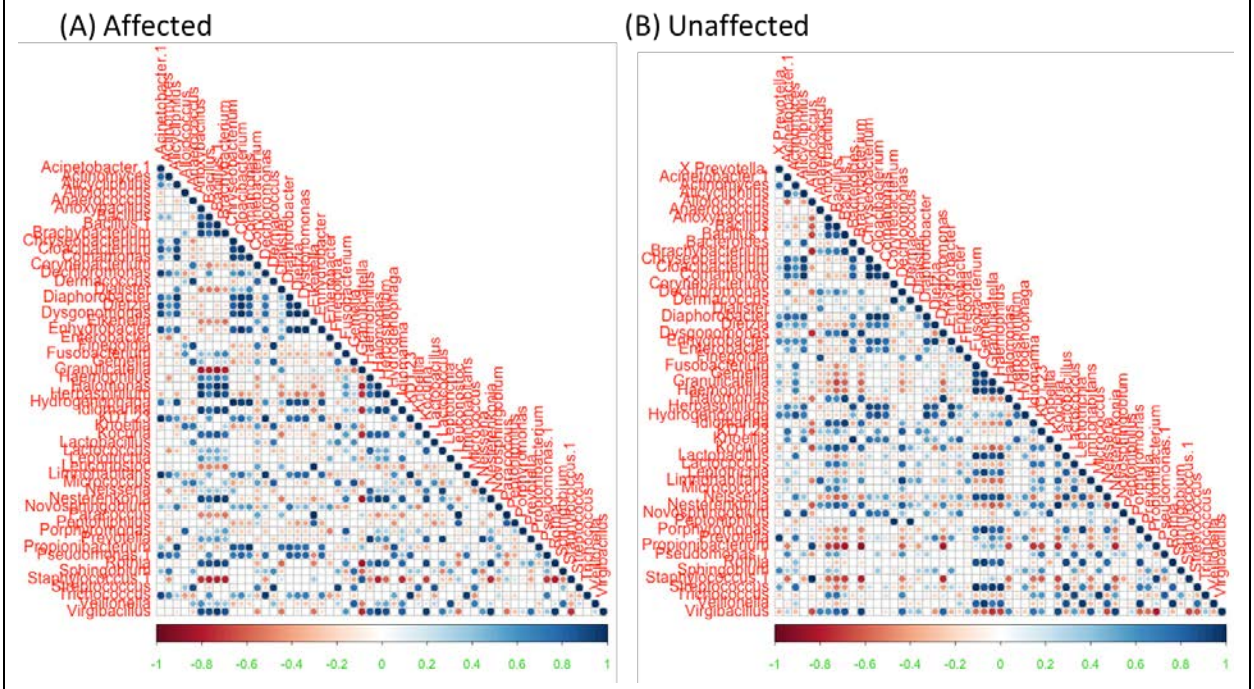

Figure 12. Correlation among bacterial genera in (A) affected and (B) unaffected samples of AD patients.

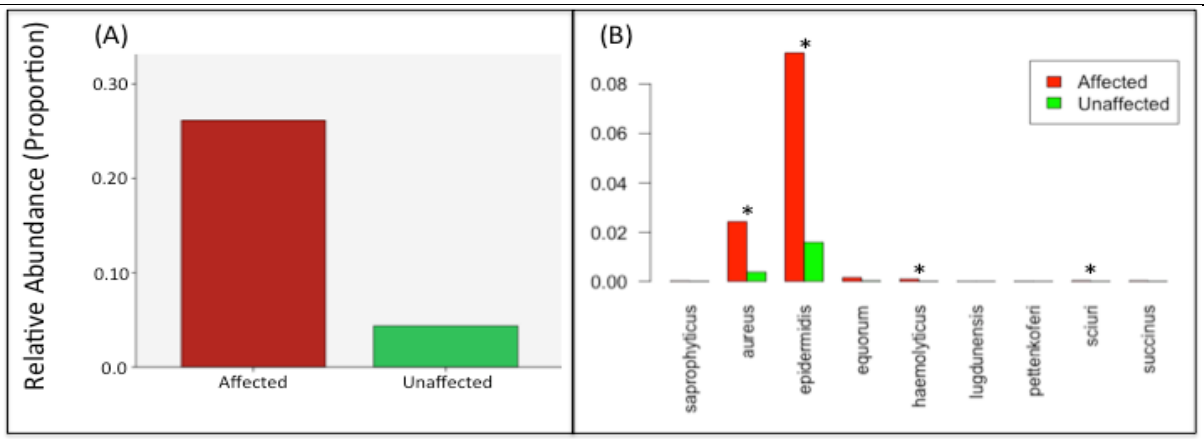

Figure 13. Level of *Staphylococcus* in affected and unaffected sites in AD patients. Panels show distribution of (A) Genus *Staphylococcus* and (B) different species of *Staphylocccus*.

Conclusions

Our study was the first to preform detailed analysis of the bacteriome and mycobiome of the same skin swab samples obtained from AD patients, compared to AD_CD and CD patients. We demonstrate that community richness and diversity exhibit association with disease status, and identified bacterial and fungal genera that are differentially distributed between the affected and unaffected sites in the three disease conditions.

Future Plans

We are currently preforming further analyses based on specific anatomic sites. For example, we know that atopic dermatitis in the axillary line and neck in adults improves with azole antifungals that inhibit yeast. We expect that there will be less mycobiome diversity in the affected axillary line/neck in AD than in the same affected areas in CD patients. AD patients are known to have innate immune deficits that interfere with containment of *Staphylococcus* infection and therefore may exhibit less diversity of bacteria in affected than unaffected sites prone to impetiginization such as the volar wrist and post-auricular fold. We are preparing to submit a manuscript describing our results. We also intend to submit an NIH R01 grant application targeted to investigate the mechanism by which the identified interactions between bacteriome and mycobiome, and with the host immune system, modulate the severity and onset of AD.

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