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Skin Microbiome Compositional Changes in Atopic Dermatitis Patients Accompany Dead Sea Climatotherapy

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ABSTRACT

Dead Sea climatotherapy (DSC) is a well-established therapeutic modality for the treatment of several diseases, including atopic dermatitis. Skin microbiome studies have shown that skin microbiome diversity is anticorrelated with both atopic dermatitis severity and concurrent *S. aureus* overgrowth. This study aimed to determine whether DSC induces skin microbiome changes concurrent with clinical improvements in atopic dermatitis. We sampled thirty-five atopic dermatitis patients and ten healthy controls on both the antecubital and popliteal fossa. High-resolution microbial community profiling was attained by sequencing multiple regions of the 16S rRNA gene. Dysbiosis was observed in both lesional and nonlesional sites, which was partially attenuated following treatment. Severe AD skin underwent the most significant community shifts, and *S. epidermidis*, *S. mitis* and *M. luteus* relative abundance were significantly affected by Dead Sea climatotherapy. Our study highlights the temporal shifts of the AD skin microbiome induced by Dead Sea climatotherapy, and offers potential explanations for the success of climatotherapy on a variety of skin diseases, including AD.

Abbreviations:

AD – Atopic dermatitis

DSC – Dead Sea Climatotherapy

INTRODUCTION

Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disorder associated with a decreased ability of the skin to function as an efficient immunological barrier (1). The disease is now two to three times more prevalent in children than it was just four decades ago. It is manifested by eczematous skin lesions associated with severe itch, leading to a significant impairment in quality of life (2). Increased *S. aureus* skin colonization has long been documented on AD skin, which has been shown to contribute towards disease symptoms. Treatments aimed at reducing skin colonization of *S. aureus* have been moderately successful in improving AD and a *S. aureus* biofilm phenotype on AD skin has been postulated (3-5). Additionally, phototherapy has been shown to reduce S. aureus colonization in pediatric AD patients (6).

Human skin is home to a milieu of microorganisms that are located throughout the dermis, epidermis and skin surface (7, 8). The resident bacteria play a crucial role in immune training and show a degree of dysbiosis in various disease states, including psoriasis, AD, vitiligo and acne (9-13). Additionally, skin microbial communities have been shown to be shaped by biogeography, indicating site-specificity as an important factor in designing skin microbiome studies (14-16). Specifically, the antecubital fossa (anterior aspect of the elbow) and popliteal fossa (posterior aspect of the knee), both moist sites and clinically important for AD, are typically comprised of Proteobacteria, Firmicutes (mostly Staphylococcaceae), Bacteroidetes and some Actinobacteria (14).

Several culture-independent studies have explored the AD skin microbiome. Kong *et al*. showed that microbial diversity was anti-correlated with both disease severity and the overall proportion of *Staphylococcus* spp in childhood AD. This trend is reversed with treatment of either dilute bleach baths, topical steroids, emollients or systematic antibiotics (13). A subsequent study reaffirmed these conclusions for a cohort given topical corticosteroids and dilute bleach baths (17). The effect of other types of treatments for AD, including phototherapy, on the skin microbiome using culture-independent methods has not been reported. Further 16S profiling studies have shown significant differences between young children and teenager/adult cutaneous microbiota, whilst both age groups' skin microbiome is correlated with disease severity at lesional sites (18). Taken together, clinical evidence and previous microbiome studies point to the importance of the skin microbiota in the etiology of AD.

For decades, thousands of patients have undergone Dead Sea Climatotherapy (DSC), a therapeutic modality based primarily on sun exposure and Dead Sea bath, for a variety of skin diseases (19). Notably, DSC does not include application of Dead Sea mud or Dead Sea based cosmetics and skin care products. Natural harmful ultraviolet rays are attenuated in the Dead Sea basin due to its altitude, less-so in the therapeutic range (300-320 nm), enabling for a natural phototherapy facility (20). Several reports have been published attesting to the impressive clinical and quality of life improvements achieved by DSC in patients with psoriasis and AD (21-25). Finally, rare side effects, few reported contraindications, and cost-effectiveness add to the appeal of DSC over alternative

therapeutic modalities (26). We recently elucidated the temporal stability of the healthy human skin microbiome and mycobiome following DSC and showed that the microbiome remains stable, whilst the mycobiome experiences an outgrowth of *Malassezia* spp. following treatment (27). In this study, we hypothesized that DSC would induce skin microbiome changes concurrent with clinical improvements in disease state.

MATERIALS AND METHODS

Study design. Thirty-five dermatologist-diagnosed AD patients participated in the study. The patients underwent a full course of DSC (3 weeks) at the DMZ Medical Center in Ein Bokek, Israel between March and November 2015. Written informed consent was obtained by patients or their parents/guardians according to the approved protocols and procedures received from the Helsinki Committee of the Rabin Medical Center, Petah Tikva, Israel (approval number 0153-12-RMC). Figure S1 (Supporting Information) details the experimental design of this study. Additionally, ten healthy volunteers with no known skin diagnosis were enrolled. Genus-level skin microbial community analysis from the healthy volunteers has been previously published (27). Patients were sampled before beginning the therapeutic regimen, and again following a complete course of DSC. Exclusion criteria for both patients and healthy volunteers included pregnancy and the use of systemic or topical antibiotics over the past month.

Sample collection. Volunteers were instructed to avoid washing and emollient treatment in the 12 hours preceding sampling. A complete medical history and skin examination was carried out by a physician upon arrival. Additional important data was collected, including: date of birth, date of diagnosis, family history, previous hospitalizations, history of other illnesses, previous treatments, onset of disease, skin type, percent skin involvement, BMI and SCORAD, a scoring index which combines extent, severity and subjective symptoms (calculated before and after treatment) (28). Samples were taken from the antecubital fossa and popliteal fossa on lesional and contralateral unaffected sites. Samples were taken using a sterile rayon-tipped swab (COPAN, Brescia, Italy) soaked in sterile 0.15 M NaCl with 0.1% Tween 20 (J.T. Baker) from a 2x2 cm area of skin and was stored at -80° C until DNA extraction.

DNA extraction & sequencing. DNA extraction and library preparation were carried out as described previously (29). Briefly, total DNA was extracted using the MoBio Powersoil DNA extraction kit. PCR reaction mix consisted of 10 µL Kapa HiFi HotStart Readymix (Kapa Biosystems, Massachusetts, USA), .4 µL primer mix (10 µM of each primer), 7.6 µL Molecular Biology Water (Sigma, St. Louis, Missouri, USA), and 2 µL template DNA. The primer mix contained five different primer pairs, each targeting a different region of the 16S gene. PCR conditions followed the manufacturer's instructions, with the annealing temperature set to 61° C for 15 sec. DNA extraction and PCR setup were performed in a DNA/RNA UV Cleaner-Recirculator (Biosan, Latvia) located in a PCR-free facility. PCR reactions were set up in triplicates and pooled and cleaned using Agencourt AMPureXP kit (Beckman Coulter) following PCR. Barcodes and adapters were added in a second PCR reaction. Samples were pooled and cleaned using the Agencourt AMPure XP kit (Beckman Coulter). Sequencing was carried out at Hebrew University's Genomic Applications Laboratory, Ein Kerem on an Illumina MiSeq instrument, using the v2 2 x 150 kit.

Sequence analysis. Sequence reads were filtered and bacterial abundances were estimated using SMURF, an algorithm we developed to combine multiple sequence reads for high-resolution microbial community profiling, as previously described (30). Briefly, raw reads were filtered if either a) Phred score was less than 30 in more than 25% of nucleotides or b) more than three nucleotides had a Phred score of less than 10, or c) a read contained one or more ambiguous base calls (*e.g.,* 'N'). Unique reads with low counts were discarded. Reads were matched to k-mers per amplified region. Maximum likelihood bacterial abundances were estimated using the expectation-maximization algorithm. Taxonomy was assigned for each reconstructed amplicon based on Greengenes taxonomy (31). In cases when taxonomic information was missing or incomplete, Ribosomal Database Project (RDP) Sequence Match engine (32) was used to assign taxonomy.

Statistical analysis. All statistical tests were carried out in R (33) using the following packages: vegan (34), phyloseq (35), and DESeq2 (36). Clinical characteristics are presented at the mean $\pm$ Standard error of the mean (SEM). The Wilcoxon rank sum test was used to compare SCORAD scores before and after DSC, Shannon diversity measures, coefficient of variation values, relative abundances of specific bacteria and to determine statistical

significance. Permutational Multivariate Analysis of Variance Using Distance Matrices (PERMANOVA) was used to assess significance between groups on NMDS and hierarchical clustering (hclust in R) was used to assess the relationship between communities. The nonparametric Spearman's rank-order correlation was used to assess significant correlations.

RESULTS

Patients

Of the 35 AD patients, 21 were female (60%) with age range of 4 to 53: 18 were children (aged 4 to 12 years) 7 were teenagers (13 to 18 years) and 10 were adults (above 18 years). Family history of AD was recorded in 12 patients. Clinical data is shown in Fig. 1A. Average clinical SCORAD at baseline was 45 $\pm$ 4 and average clinical SCORAD following DSC was 7 $\pm$ 1 (Fig. 1B).

Summary of samples obtained for study

We prepared amplicon libraries for 309 samples, of which 265 were from AD patients, and 44 were from healthy volunteers. Following quality filtering, a total of 18,031,776 high-quality sequences were used as input for the SMURF algorithm (30). The resulting OTU table was input into R using the phyloseq package to obtain alpha and beta diversity metrics. Samples were rarified to 2300 sequences per sample.

All samples from healthy volunteers and AD patients were dominated by the phyla Firmicutes, Actinobacteria, and Proteobacteria and to a lesser extent Bacteroidetes (Fig. 2). These findings are consistent with previous reports of the skin microbiome (14). *Staphylococcus*, *Cutinibacterium*, *Streptococcus*, and *Acinetobacter* were the dominant genus' in all samples.

Skin microbiota of lesional sites on AD patients at baseline is reflective of disease severity

Following recent reports of the different skin microbiomes in pediatric versus adult AD (18), we sought to determine the degree of differentiation in the patients enrolled in our study. Shannon diversity, a community diversity measure which takes into account both species richness and evenness, was not significantly different between any age group (Fig. S2A). We

compared the microbiome of the lesional antecubital fossa and popliteal fossa separately between children, teenagers and adults. Individual species relative abundance was not significantly different between age groups, yet Bray-Curtis beta-diversity metrics of the entire antecubital fossa was significantly different between age groups ($P < 0.05$, Fig. S2B), but was not so for the popliteal fossa. Taken together, there is no significant difference in community diversity or individual taxa between children, teenagers and adults in our cohort, yet there is a site-dependent effect on beta-diversity.

Recent reports of the AD microbiome have demonstrated that the skin microbiota of AD patients differs from that of healthy volunteers. Additionally, several studies have shown a correlation between disease severity, as measured by SCORAD, and Shannon diversity. Our cohort consisted of fourteen individuals who presented with a baseline SCORAD below 30 (mAD) and twenty-one individuals who began treatment with a clinical SCORAD above 30 (sAD). We examined whether baseline disease severity affects community composition of the skin microbiome by comparing the healthy cohort's skin microbiome with that of mAD and sAD patients at baseline. Community diversity as measured by Shannon diversity of the elbows (Fig. 3A) indicated no significant difference between healthy individuals and mAD patients, yet significance was observed between healthy individuals and sAD patients ($P < 0.05$) as well as between mAD patients and sAD patients ($P < 0.05$). Shannon diversity of bacterial communities on the inner knee (Fig. 3D) indicated significance between healthy sites and sites from sAD patients ($P < 0.05$), yet not between healthy sites and mAD patients. PERMANOVA of non-metric multidimensional scaling (NMDS) indicated significant differences between groups on both inner elbow ($P < 0.001$, Fig. 3C) and inner knee ($P < 0.01$, Fig. 3F) communities. No significant clustering was associated with gender. We then assessed whether community structure, as assessed by hierarchical clustering of bacterial communities, is affected by disease severity (Fig. 3B, E). mAD skin microbiomes were closer to those of healthy skin than those of sAD skin on both antecubital fossa and popliteal fossa. Therefore, our analysis indicates a strong influence of disease severity on shaping the composition of the skin microbiome in our cohort.

Microbiome of unaffected skin of AD patients resembles that of lesional skin

We further sought to assess whether microbial communities on unaffected skin of AD patients were dysbiotic. We observed an insignificant increase in Shannon diversity of the unaffected elbow and knee microbiome in mAD patients as opposed to healthy patients, yet this trend did not exist on the unaffected areas of sAD patients (Fig. 3A, D). More so, we noted significantly decreased community diversity of sAD patients on unaffected sites when compared to unaffected sites on mAD patients ($P < 0.05$), similar to the difference in microbial diversity observed between lesional elbows of sAD and mAD patients. Of additional importance, no significance was observed between contralateral sites in either mAD or sAD patients on both elbows and knees. Therefore, the Shannon community diversity of unaffected skin of AD patients resembles that of lesional skin. We further examined this relationship through hierarchical clustering of bacterial communities which indicated that lesional and unaffected skin of mAD patients more closely resembled each other than any other group (Fig. 3B, E). Similarly, lesional and unaffected skin of sAD patients more closely resembled each other than any other group examined. Additionally, both lesional and unaffected sites of mAD patients more closely resembled healthy volunteers than sAD patients.

Effect of DSC on the AD Skin Microbiome

We then assessed skin microbiome compositional changes following DSC. We first measured the coefficient of variance (CV) of Shannon diversity values, a measure of community stability in each of the groups. Significantly higher CV measures were observed between both lesional and unaffected sites on sAD patients and healthy volunteers ($P<0.05$, Fig. 4A, C). No significance was observed between sites on mAD patients and either healthy volunteers or sAD patients. Therefore, both lesional and nonlesional skin on sAD patients undergoes a higher degree of change than healthy volunteers following DSC. Hierarchical clustering of mean taxonomic relative abundances of the most abundant species indicated that of the three groups examined (Healthy, mAD and sAD), mAD lesional sites were the only ones that experienced a significant shift, ultimately bringing them closer to healthy skin (Fig. 4B, D). We further identified three bacterial genera which were significantly affected by DSC. *S. epidermidis* experiences a dramatic decrease in relative abundance following DSC on the popliteal fossa (Fig. 5D, $P<0.01$), yet not so on the antecubital fossa (although an

insignificant trend is noted, Fig. 5A). *Micrococcus luteus* relative abundance is reduced following DSC on the antecubital fossa (Fig. 5B, $P<0.05$), yet not so on the popliteal fossa (although an insignificant trend is noted. Fig. 5E). *Streptococcus mitis* relative abundance increased following DSC on both the antecubital fossa (Fig. 5C, $p<0.01$) and popliteal fossa (Fig. 5F, $p<0.001$).

S. aureus and AD

Previous reports have shown that SCORAD is anticorrelated with Shannon diversity and positively correlated with the relative abundance of *S. aureus*. The negative correlation between relative abundance of *S. aureus* and Shannon diversity is present in our dataset for lesional and nonlesional antecubital and popliteal fossa (Fig. 6A, B, C, D). Additionally, the correlation between the percent relative abundance of S. aureus and SCORAD values is significant on both lesional and nonlesional antecubital fossas (Fig. 6E, F), yet not so for lesional or nonlesional popliteal fossas (Fig. 6G, H).

DISCUSSION

Over the past few years, a plethora of microbiome studies have been published elucidating different aspects of the skin microbiome in AD. The microbiome of pediatric AD skin is different than that of adult AD skin (18), yet microbial community diversity has been anticorrelated with disease state in pediatric patients (13), and subsequently in adult AD skin (37). Our cohort, consisting of both pediatric and adult AD patients, exhibited the same traits (Fig. 3A). Furthermore, severe AD skin is dysbiotic (Fig. 3C, D), primarily due to significant overrepresentation of *S. aureus* (Fig. 1B, E), consistent with previous reports (13, 18, 38). We previously leveraged the high-resolution microbial community profiling enabled by the SMURF method (30) and identified specific sAD associated *S. aureus* 16S sequences (39).

DSC is a proven modality for the treatment of a variety of skin diseases, including AD (19). Whereas the therapeutic mechanism has been documented for DSC on psoriatic skin (25), such a report is lacking for AD. Given the consistent successful clinical results achieved by DSC in AD patients (Fig. 1B, (24)) and the concurrent reports that various therapeutic modalities affect the composition of the skin microbiome on AD skin (17, 40), we sought to

determine whether DSC-induced AD remission would be accompanied by significant microbiome compositional changes. Of note, we previously reported that prolonged UV exposure in the form of DSC, does not alter the composition of the skin microbiome in healthy individuals, yet contributed to changes in skin fungal communities (29). Significant temporal changes were observed on lesional and nonlesional sAD skin, yet not so on mAD and healthy skin (Fig. 4A, C), thereby indicating that DSC induces compositional changes in the sAD skin microbiome. However, post-DSC microbial communities remained closer to their baseline state than to mAD or healthy skin (Fig. 4B, D), thereby indicating that despite the DSC-induced microbiome compositional changes, the microbial community of sAD skin does not shift to mirror that of healthy skin. The post-DSC skin microbiome still retains the microbial footprint of sAD.

A recent report of ours highlighted the temporal stability of the healthy skin microbiome following DSC (29). In the current study, we observed three bacterial species which were responsive to DSC, including *S. epidermidis* and *M. luteus* whose numbers decreased, and *S. mitis* which increase in relative abundance following DSC. These changes were observed on healthy, mAD and sAD skin on both lesional and nonlesional sites. The latter two species are commonly found in the skin microbiota, yet their contribution to skin health and homeostasis is poorly understood. In contrast, *S. epidermidis*, another important member of the skin microbiome, is often thought to increase skin health, primarily by colonization resistance and immune training against *S. aureus (41)*. However, *S. epidermidis* has been documented to be present at greater numbers on moderate AD skin (42). In our study, the diminishment of *S. epidermidis* following therapy is most pronounced on moderate AD skin (Fig. 5A, D), consistent with the aforementioned observations.

Interestingly, *S. aureus* overrepresentation is not significantly mitigated following DSC, despite the substantial clinical improvement observed. These findings may dovetail with the supposition that *S. aureus* overrepresentation on AD skin is part of the secondary events resulting from AD, as opposed to primary events, including immune dysregulation, which cause AD (2). This would explain how our cohort experienced sweeping SCORAD improvements without diminished *S. aureus* colonization. This supposition is supported by

the fact that many diseases which lack a clear microbial etiological factor, e.g. psoriasis, vitiligo, arthritis, and heart conditions, are treated successfully at the Dead Sea.

CONCLUSION

DSC-induced clinical improvement in AD severity occurs independent of significant reductions in *S. aureus* skin colonization. Additionally, DSC induces significant changes in the percent relative abundance of *S. epidermidis*, *M. luteus* and *S. mitis*. DSC is an effective therapeutic modality for a variety of skin diseases, and may also prove an effective modality for inducing compositional shifts in the diseased skin microbiome.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Figure S1. Study design. Eleven healthy volunteers and 35 AD patients were sampled on their lesional antecubital fossa and popliteal fossa and contralateral nonlesional sites were sampled as well. Samples were taken before beginning the Dead Sea Climatotherapy therapeutic regime and following 21 days of treatment.

Figure S2. Effect of age on the AD skin microbiome**.** (A) Shannon diversity index score on elbows for children, teenagers and adults. Line in box represents median, boxes represent 25^{th} and 75^{th} percentile, and whiskers represent minimum and maximum values. (B) Non-metric multidimensional scaling of Bray-Curtis dissimilarities contrasting children (green), teenagers (blue) and adults (red) microbiomes.

Figure S3. Bacterial community composition differs in moderate and severe AD on unaffected skin. (A) Non-metric multidimensional scaling of Bray-Curtis dissimilarities contrasting moderate (peach) and severe (aqua) AD on unaffected elbows and (B) knees. Individual taxonomic relative abundance values of the nine 16s groups present in greatest abundance sorted by SCORAD values of (C) unaffected elbows and (D) knees.

Figure S4. Unaffected and lesional skin microbiomes are not significantly different. NMDS of Bray-Curtis dissimilarities shows no significant clustering between lesional and unaffected AD skin.

AUTHOR CONTRIBUTIONS

MB, MH, ZB, NS & SM designed the study. MB, GF, AI, FS & MH performed research. MB, GF, AS, EH, DS, ZB, NS & SM analyzed data. MB wrote the paper. All authors read and approved the final manuscript.

DATA AVAILABILITY

Sequence data and clinical metadata per subject can be obtained through direct communication with the corresponding author.

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Figure Legends:

Figure 1. Summary of clinical characteristics of study participants. (A) Clinical data recorded at baseline (B) SCORAD (Scoring atopic dermatitis) values for patients enrolled in this study before and after undergoing treatment at the Dead Sea ($P < 0.0001$). Line represent the mean.

Figure 2. Healthy and AD microbiome is dominated by four phyla. (A) Relative abundance of the four major phyla Bacteroidetes, Actinobacteria, Proteobacteria and Firmicutes on elbows and (B) knees for healthy, AD lesional and AD nonlesional sites at baseline and following DSC.

Figure 3. Dysbiosis is pronounced in Severe AD, but not Moderate AD. (A) Shannon diversity index score on elbows and (D) knees for healthy, moderate AD and severe AD on unaffected (blue) and lesional (red) sites. Line in box represents median, boxes represent 25^{th} and 75^{th} percentile, and whiskers represent minimum and maximum values. (B) Mean taxonomic relative abundance of the nine 16s groups present in greatest abundance sorted by hierarchical clustering of elbows and (E) knees. (C) Non-metric multidimensional scaling of Bray-Curtis dissimilarities contrasting moderate (peach) and severe (aqua) AD on lesional elbows and (F) knees.

Figure 4. Temporal changes in the AD microbiome following DSC**.** (A) Coefficient of variation between samples taken at baseline and following DSC on elbows and (C) knees indicated significant temporal changes in the severe AD cohort compared to the healthy cohort. (B) Mean taxonomic relative abundance of the nine species present in greatest abundance on lesional sites sorted by hierarchical clustering of elbows and (D) knees. Moderate AD samples taken from the knee shift away from the AD microbiome and towards the healthy microbiome.

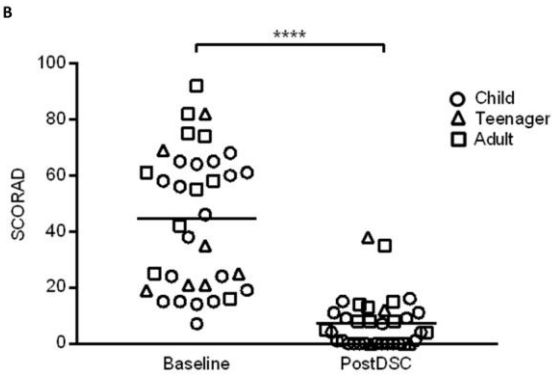
Figure 5. Bacterial species affected by DSC**.** (A) Percent relative abundance of individual taxa at baseline (circle) and following DSC (square) on unaffected (blue) and lesional (red) sites of antecubital fossa (A,B,C) and popliteal fossa (D,E,F). *S. epidermidis* and *M. luteus* decrease in relative abundance following DSC, while *S. mitis* increases.

Figure 6. *Staphylococcus aureus* and AD. (A-D) Correlation between relative abundance of *S. aureus* and the Shannon diversity index on lesional (A,C) and unaffected (B,D) elbows (A,B) and knees (C,D). (E-F) Correlation between relative abundance of *S. aureus* and clinical SCORAD on lesional (E,G) and unaffected (F,H) elbows (E,F) and knees (G,H).

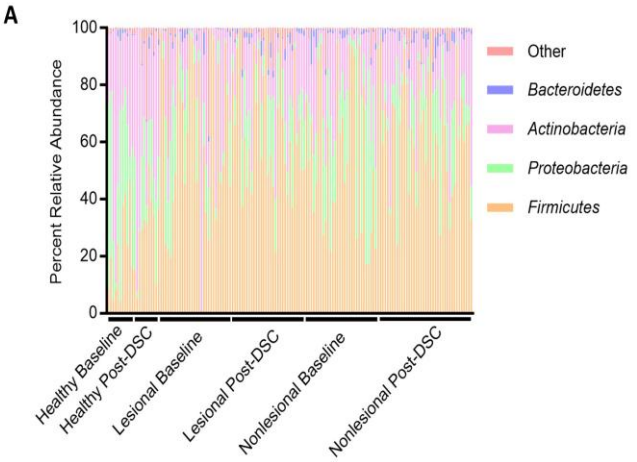
A

Summary of samples obtained for study	Age Range: 4 -53
	Children (4-12): 18 Teenagers (13-17): 7 Adults (18+): 10
	SCORAD: 45 ± 4
	Moderate AD (<30): 14 Severe AD (>30): 21
	Females: 21 Males: 14

B



Antecubital fossa



Popliteal fossa