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Original Research

Preliminary characterization of human skin microbiome in healthy Egyptian individuals

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Abstract: Human skin is a large, complex ecosystem that harbors diverse microbial communities. The rapid advances in molecular techniques facilitate the exploration of skin associated bacterial populations. The objective of this study was to perform a preliminary characterization of skin associated bacterial populations in Egyptian individuals. Samples were collected from five healthy subjects from two skin sites; Antecubital Fossa (AF) and Popliteal Fossa (PF). Genomic DNA was extracted and used to amplify bacterial 16S rRNA genes which were sequenced on Illumina MiSeq platform. The two sites showed distinct diversity where PF was more diverse than AF. Taxonomic analysis of sequences revealed four main phyla *Proteobacteria*, *Firmicutes, Actinobacteria* and *Deinococcus–Thermus*, with *Proteobacteria* presenting the highest diversity. *Klebsiella, Bacillus, Pseudomonas* and *Escherichia* were the most predominant genera. Our data suggest that environmental factors can shape the composition of the skin microbiome in certain geographical regions. This study presents a new insight for subsequent analyses of human microbiome in Egypt.

Key words: Antecubital fossa, Egypt, Skin Microbiome, Popliteal fossa.

Introduction

Human skin doesn't only protect the body against harmful conditions but also provides favorable medium for various bacterial communities (1). Topography and biogeography of human skin represented in different sites and niches with various climates, nutrition and pH, determine and control the structure and abundance of residents (2-5). With these features, human skin is considered a unique ecosystem (6). As they live together, bacterial communities interact with skin in various ways which vary from friendship extending to hostel relations (7). Thus, configure the immunological makeup of a given location (8). Skin physiology is also thought to be directly or indirectly influenced (9). Disturbance in the relations between skin and its inhabitants has a direct or indirect role in the emergence of skin diseases (10-13).

The development of human skin microbiome doesn't start from birth but recently it has been proven that fetus contacted with maternal microbiome (14-16). Skin microbiome is characterized by highly dynamic interpersonal diversity, in other words, as each site or person has his/ her own bacterial signature (3, 17). Studying human skin associated bacterial communities is recognized as crucial for understanding human diseases, disease progression and its role in maintaining human health (18). Studying intersubject variation in bacterial population composition provides a smart tool to understand the pathogen site associated diseases. For example AF and PF are considered as moist sites and their importance stems from implication in dermatological disorders such as atopic dermatitis (AD) (19-21). Community members were closely assumed to have clinical significance through their influence on the healthy state of the skin as well as participation in many dermatological disorders, for instance severity of AD is indirectly proportional to bacterial diversity at the site of disease (21). In addition, the composition of bacterial communities exists with high inter-individual variability which can be utilized as a tool in forensic identification (3, 22).

Traditional culture based techniques are unable to meet the demands of scientists to explore new world of bacterial communities. These techniques are restricted to very limited range 0.01-1% of bacteria which only survive in standard laboratory conditions excluding uncultivable bacteria (23-26). In comparison with culture independent approaches, characterizing bacterial diversity of human skin with high and precise resolution including 99-99.9% of bacteria is possible (27-30). The aim of this study is to characterize skin associated bacterial communities in AF and PF samples taken from Egyptian individuals.

Materials and Methods

Subject enrollment and sample collection

This study was approved by scientific research ethics committee at the faculty of pharmacy, Suez Canal University, Egypt (reference number of 20156H1). Subjects provided written informed consent for screening, enrollment and specimen collection. Before sampling, at screening time, subjects were excluded if they had any of the following; topical corticosteroid or antibiotic use in the previous seven days, presence of multiple blisters, boils, ulcers or boils at or within 4cm from sampling site, and oral or systemic antibiotic intake 2 months prior to sampling time, as stated by human microbiome project manual of procedures (31). From five healthy volunteers who passed screening for required criteria (Supplementary data S1), biological samples were collected from two moisten sites; AF and PF by rubbing the selected site with sterile cotton tipped swabs pre-

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moisten with a sterile solution of (0.15 M NaCl with 0.1%Tween 20) (11) with rolling movement in all directions. A negative control, sterile pre-moisten swab without rubbing to skin, was used in parallel to check the presence of contaminants in cotton swabs. After collection, the cotton tips of the swabs were broken off directly into a 1.5 ml micro- centrifuge tube containing 1ml of lysis buffer (Mo Bio) with vortex at low speed to disperse the trapped bacteria.

DNA extraction

Genomic DNA was extracted from the clinical specimens and the negative control using Mo Bio power soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA cat. No 12888-50) with some modifications. The steps briefly were; the cotton tips were removed and the solution transferred to bead tubes to which 60 µL of solution C1 had been added. Tubes were incubated at 65 °C for 10 min and then shaken horizontally at maximum speed for 2 min using the MO Bio vortex adapter. Then instructions were followed as directed by the manufacturer except eluting DNA with 30 µL instead of 100 µL of PCR DNAase free water.

PCR amplification and sequencing of *16S rRNA* gene

Immediately after DNA extraction, PCR reactions were conducted to amplify hyper variable regions V3-V4 of 16S rRNA gene using the 16S metagenomic sequencing library protocol (Illumina). Initially the DNA was amplified with primers which also incorporated the Illumina overhang adaptor (underlined), Forward Primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG Reverse Primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. PCR reactions were carried out in 25µl reactions with 0.8µL for each forward and reverse primer (10µM, Metabion, Germany), 3 µL of template DNA for specimens or 3 µL of elution solution for negative control, and 12.5 µL of 1×of Hot Master Mix (Genedirex PCR supermix). Thermal cycling consisted of initial denaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 5 min at 72 °C. The PCR products were visualized using agarose (1%) gel electrophoresis. PCR products were purified using the Agencourt XP Ampure Beads (Beckam Coulter, USA). The quality of the final products were assessed using a Bioanalyzer 2100 (Agilent Technologies, USA) and after quantification with a Qubit (Invitrogen, USA), the samples were pooled in equal proportions and sequenced paired-end in an Illumina MiSeq (Illumina, USA) with 600 cycles (300 cycles for each paired read and 12 cycles for the barcode sequence) at IGA Technology Services (Udine, Italy). To prevent focusing and phasing problems due to the sequencing of "low diversity" libraries such as 16S amplicons, 30% PhiX genome were spiked in the pooled library.

Sequence processing and analyses

Sequences analysis was performed using the metagenomics workflow of MiSeq Reporter v2.3 (Illumina). Briefly, sequences were demultiplexed based on index sequences. FASTQ files were generated with Quality Score Trim sample-sheet set, in order to make trimming. Classification step was performed using Classify Reads, a proprietary Illumina algorithm that provides species-level classification for paired-end reads. The process involves matching short subsequences of the reads (words) to a set of 16S reference sequences. The accumulated word matches for each read were used to assign reads to a particular taxonomic classification. The taxonomy database for the metagenomics workflow was an Illumina-curated version of the Greengenes database (greengenes.secondgenome.com/downloads/database/13_5)

All previous preprocessing steps were essentially repeated using MOTHUR (v.1.35.0) software package (32). Assembly of forward and reverse reads was done using (command make.contigs).Quality filtering included rejecting reads <440 nt and >490nt, excluding homopolymer runs >8nt, and 0 ambiguous bases, and requiring minimum average Phred quality ≥25 using (command trim.seqs). Aligning of unique sequences was performed using reference database, SILVA 119 (arb silva.de/documentation/release-119/) (33). Chimeras sequences were removed using the MOTHUR implementation of UCHIME algorithm (34). Taxonomic classification and operational taxonomic units (OTUs) were determined with (command classify.seqs) using naïve Bayesian method (35) at 97% of similarity against SILVA database.

Diversity analysis

Analyses of diversity and statistical significance were performed using random resampling of contigs with average 40,000 sequences per sample. In this study, two approaches were used to analyze sequences from two points of view; the first approach was to deal with each sample as a separate community and the second approach was to combine samples from each sampling site to form groups representing the two sites.

Phylotypes based analysis

In these analyses, operational taxonomic units (OTUs) were clustered for richness and diversity estimation using (command dist.seqs) for calculation of uncorrected pairwise distances between aligned sequences, then the resulted distance matrix was analyzed with (command cluster) to calculate OTUs using the average-neighbor algorithm and a similarity cutoff of 97%.

Alpha diversity

Ecological diversity indices; Chao1 richness estimators, rarefaction curves, the inverse Simpson's diversity index and Shannon diversity index were calculated with bacterial communities diversity calculator using (command sub.sample) with subsampling cutoff of 40,000 sequence/sample, while community evenness was calculated with Shannon equitability index using Microsoft Excel spread sheet.

Beta diversity

Interpersonal and intrapersonal diversity were determined using OTUs based analysis using two measures, Jaccard index for shared community membership and Theta index for shared community structure, were cal-

culated with calc parameter thetayc and jclass (32, 36).

Beta diversity was measured by phylogenetic based analysis using UniFrac based principle component analysis (PCA). It was calculated to visualize high dimensional data. For factor analysis only taxa with relative abundance consistently >0.03 in all samples were chosen.

Relationships between samples were determined using principle coordinate analysis (PCoA) based on both weighted and unweighted UniFrac distances (37, 38).

Statistical analysis

To assess the significant differences between samples as well as sites, *P* values were calculated on the basis of the Mann-Whitney U test (MW) *U* test to determine the significant difference between two samples or sites, while Kruskal-Wallis (KW) sum rank test was used to determine species with significant difference between more than two samples using (command lefse) (39). Spearman correlation coefficient (SpCC) was used to assess the correlation between OTUs using (command otu.association) with cutoff value 0.001.

R programming language version 3.2.0 2015 (40) was used to assess all statistics. Bacterial composition at genus level was used to calculate herarical dendrogram based on the Bray-Curtis distances (41) using Vegan package (v.1.15–1) in the R statistical framework (42). All charts and plots were produced using *ggplot2* package R (43).

Data access

The sequence data from this study had been submitted to NCBI BioProject (http://www.ncbi.nlm.nih.gov/bioproject) under the accession number; PRJNA285083.

Patient and sample metadata had been submitted to db biosample (http://www.ncbi.nlm.nih.gov/biosample) under the accession numbers; SAMN03998657, SAMN03854518, SAMN03838213, SAMN03998829, SAMN04002327. Representative sequences of *Klebsiella* species were submitted to Gene bank under the accession numbers; KT291801-KT301936.

Results

Sequences analysis and classification

After assembly of forward and reverse reads, a total of 11,957,880 sequences were generated from 10 samples. Over 99.98% of sequences were bacterial and the non-bacterial sequences were removed from the analysis. After trimming, deduplication and removal of sequences<420 nt and clustering at 97% similarity level, there were about 1,679,567 reads as representative sequences (read counts for each sample are listed in Supplementary data S2).

Bacterial community composition

Relative abundance of bacterial taxa was determined at different taxonomic levels to assess if there were any significant shifts in the composition of the bacterial communities according to the body site. A total of 29 phyla were detected across all samples. The distribution of the major phyla revealed that *Proteobacteria* was the most predominant phyla in all samples with (57% for AF and 49% for PF), followed by *Firmicutes, Actinobacteria* and *Deinococcus–Thermus* members with (36% for AF and 38% for PF, 4% for AF and 7% for PF, 0.42% for AF and 0.27% for PF, respectively) (Fig. 1).

At family level, the bacterial community composition was found highly diverse. There were 238 families, and the predominating families were *Enterobacteriaceae, Bacillacae, Pseudomonadaceae* and *Thermaceae*.

Core microbiome

At genu level, there were 711 genera; the most predominant genera were *Klebsiella, Bacillus, Pseudomonas* and *Escherichia* in PF, while AF had a different order where *Meiothermus* was in the fourth level (Fig. 2). There were 56 genera that detected in all samples, their relative abundance was found to be equal or more than 0.1%, these called core genera. While the genera that were detected in only one sample and represented more than 5 % of total genera, may considered as unique genera for that sample.

Moreover, our results showed unique presence of

Figure 1. Relative abundance of the most predominant phyla in all samples. X-axis represents the relative abundance of predominant phyla. Samples codes represented on Y-axis.

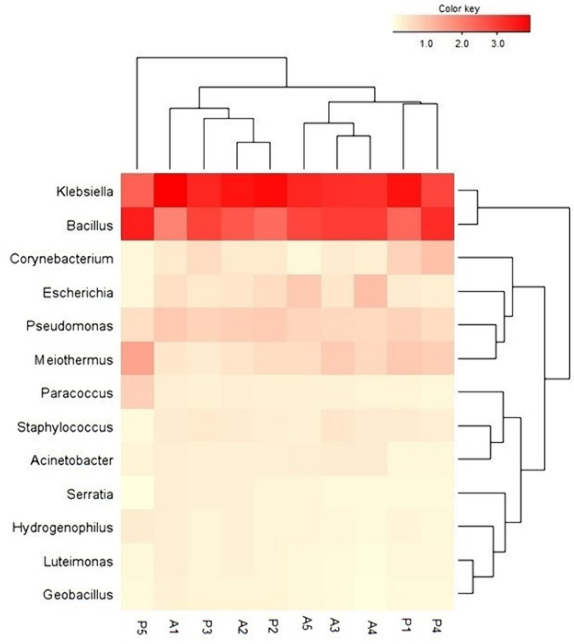

Figure 2. Heat map with dendrogram clustering of samples. The most predominant 13 genera were used in hierarchical clustering to assess the relationships between samples using weighted pair clustering based on Bary-Cruits measurements. The dark red color indicated the genus with more predominance.

opportunistic as well as potential pathogen residents in the skin of our individuals. Depending on their clinical significance, this study focused on these three genera *Klebsiella*, *Staphylococcus* and *Bacillus* with more details. *Klebsiella* genus was present with the highest proportions in all samples ranged from 20- 50.48% of reads without significant difference between the two sites (KW p=0.45). Sequences that clustered as *Klebsiella* genera were classified at species level using USEARCH algorithm (34). Three *Klebsiella* species were detected; *Klebsiella variicola, Klebsiella granulomatis* and *Klebsiella pneumonia. Klebsiella variicola* was the most predominant species in all samples with 12-30% of reads. K*lebsiella granulomatis* was more dominant on AF samples with significant difference between sites (MW p=<0.001). *Klebsiella pneumonia* which is a component of skin normal flora and is a potential pathogen consisted of 2.26% of total reads and 12% of sequences associated to *Klebsiella* genus.

The *Bacillus* genus showed high species level diversity with 72 species in all dataset. *Bacillus safensis, B. firmus, B. siralis and B. smithii* were predominant species and represented 98.5% of Bacillus reads.

Other known skin residents with clinical significance were *Streptococcus* and *Staphylococcus* genera. These genera were detected with low proportions in comparison to the previous genera. Sequences assigned to *Staphylococcus* genus represented with average 1.8% of reads in which *S. haemolyticus* and *S. caprae* were dominant over other *Staphylococcus* species and consisted of 50% of *Staphylococcus* populations.

Streptococcus genus also detected at low abundance and detected with more abundance in AF with two fold proportion in comparison with PF (MW p=<0.001). *S. tigurinus*, belongs to the *S. mitis* group and originally isolated from a patient with infective endocarditis (44), it was found with proportions ranging from 13 to 67% of Streptococcal populations.

The numbers of unclassified sequences were increased with the taxonomic classification at steady rate 1.1%, 1.7% and 4.7% for order, family and genus level respectively. At species level, unclassified sequences were found to be 25.4% of all sequences.

Bacterial species richness and diversity

Alpha diversity

Rarefaction curves were generated for samples and groups reflected remarkable differences in bacterial diversity between AF and PF (Fig. 3). Using ecological diversity indices with each sample, a total of 15,367 OTUs were detected at 97% similarity according to the observed clusters (S_{obs}); the maximum number of detected OTUs was in sample (P5). For sites, the largest number of OTUs was detected in PF and it was 1847 OTUs. Richness and diversity indices were in the same trend with Sobs except Shannon diversity index where sample (P4) was the highest with 3.59 (Table 1). According to Shannon evenness estimator all samples had uneven relative abundance of predominant species reflecting no homogeneity of communities. Sample (A4) was found to be the most even sample, while PF was the most even site with $E_{H'}$ 0.463.

Beta diversity

The average values of pairwise comparisons between different samples were 0.77 for Jaccard and 0.26 for Theta. These findings revealed less similarity between samples.

Intrasubject variation was inferred from pairwise comparisons between two sites in the same subject, where the average of pairwise comparisons was 0.757 and 0.267 for Jaccard and Theta respectively.

The mean of pairwise comparisons between the two

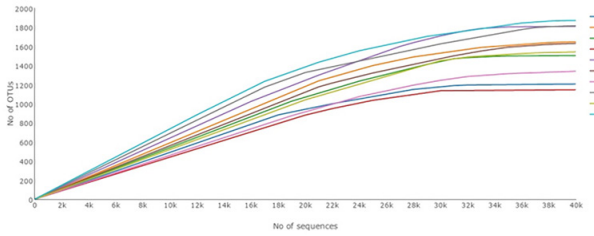

Figure 3. Individual rarefaction curves for each sample. X-axis represents the number of detected operational taxonomic units (OTUs) at different sampling process. Y-axis represents the number of sampled sequences.

Table 1. Alpha-diversity indices at 97% of similarity: coverage, Sobs, invSimpson, Chao parameters H' and $E_{H'}$

Alpha diversity indices for each sample							
Sample code	N_{seqs}	Goods coverage (%)	S_{obs}	Chao1	Invsimpson	H'	$E_{H'}$
A1	40000	0.978409	1196	2631.2	4.71	2.65	0.373938
P1	40000	0.968649	1648	3592.64	5.21	3.44	0.464406
A2	40000	0.958238	1499	3147.9	4.53	3.02	0.412988
P2	40000	0.969665	1114	1947.946	4.69	3.12	0.444716
A3	40000	0.974598	1797	3433.775	6.52	3.52	0.469717
P3	40000	0.964282	1619	3441.242	7.09	3.01	0.407331
A4	40000	0.968808	1302	2838.36	5.50	3.48	0.485244
P4	40000	0.967529	1798	4027.52	4.04	3.59	0.479022
A5	40000	0.960183	1525	3278.75	7.44	3.27	0.446127
P5	40000	0.974302	1869	3962.28	9.69	3.56	0.472577
Alpha diversity indices for the combined group of each site							
AF	40000	0.989479	1571	3001.9254	5.36	3.02	0.41202
PF	40000	0.987965	1847	3925.8636	5.43	3.49	0.463285

N_{seqs} = Number of sequences in the sample; S_{obs} = Number of observed OTUs; Chao1 = Richness index ; InvSimpson = Inversed Simpson's; H' = Shannon diversity index and $E_{H'}$ = Shannon equitability index; A1 for example = antecubital fossa of subject 1; P1 for example = popliteal fossa of subject 1; AF= all samples from antecubital fossa; PF= all samples from popliteal fossa.

site groups using Jaccard and Theta were 0.73 and 0.19 respectively. These results revealed intersubject variation according to body site (Fig. 4). Relations between different sites and samples were assessed using UniFrac based principle component analysis in which there was clustering with high divergence of PF samples, while principle coordinate analysis based on UniFrac showed that clustering of samples was made according to sampling site rather than subject (Fig. 5).

Correlation between members of bacterial communities

Spearman correlation coefficient (SpCC) was used to assess the correlation between bacterial taxa at different taxonomic levels. A total of 95,652 (r=>0.6 and < -0.6) significant positive and negative correlations were detected which revealed competition between community members .The strongest correlations (r= -0.86) were found between the most predominant phyla in all samples *Proteobacteria*, *Actinobacteria* and *Firmicutes*.

Taxa within the same family in many cases showed positive SpCC but the majority detected with negative SpCC mainly between OTUs belonged to *Enterobacteriaceae*, while between genera, the most significant positive SpCC were found between OTUs assigned to *Klebsiella* and *Bacillus* genera. At species level, OTUs that assigned to *Acinetobacter* genus had the strongest negative correlation (r= -0.97, P = 0.004) with the majority of dominant species while existed with positive correlation (r = 0.91, P= <0.001) with the rare species.

Discussion

Evidence to support the role of microbial normal flora in the wealth/ well-being of the human skin has been demonstrated previously (45), Members of human skin associated populations showed exponential increase in number at different taxonomic levels according to the fast development of different molecular approaches. Exploration of human microbiome started in the last century from ordinary culture based approach (6, 8), reaching the first culture independent sequencing using chain termination (46), till currently high throughput sequencing (47).

Many studies were conducted in many countries such as the United States, Japan, the United Kingdom and recently in China (3, 27, 48, 49). To our knowledge, this is the first study to be conducted in one of the Middle East

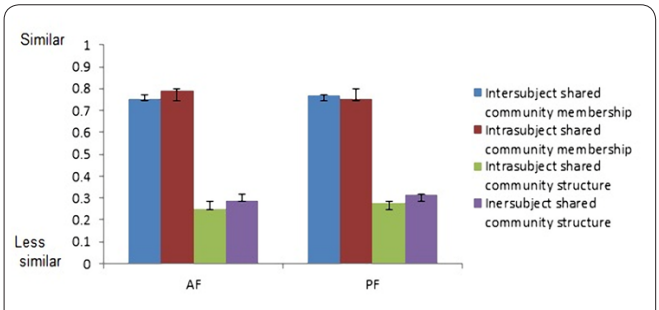

Figure 4. Intrapersonal and interpersonal similarity of antecubital fossa and popliteal fossa. Y-axis represents Jaccard and Theta values; a value of 1 implies identical community membership and structure, while a value of 0 implies dissimilar community membership and structure. Error bars represent SE.

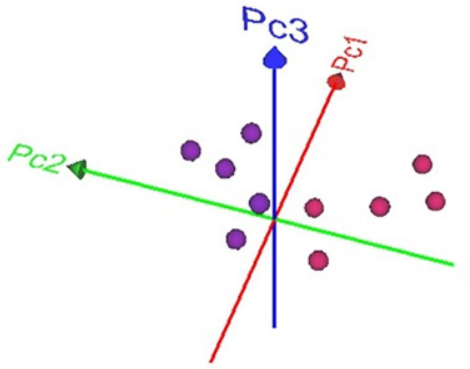

Figure 5. A three-dimensional plot of principle coordinate analysis (PCoA) of weighted UniFrac scores. Clustering of skin samples was performed using PCoA of weighted UniFrac distances. Colors represent different skin sites. Samples with similar principal coordinates appear closer together. Plot was generated using the pairwise weighted UniFrac distances (where PC1= variability at 52.4%, PC2= variability at 10.7% and PC3 at 8.7%). Balls color represents the sampling sites, where pink balls for samples from AF, while violet balls for samples from PF.

countries, Egypt. Although the number of samples was limited, many previous pilot studies were performed on similar number of samples which were suitable with respect to evaluation of human microbiome (50, 51).

At phylum level, *Actinobacteria*, *Firmicutes*, and *Proteobacteria* were found to be the most predominant phyla of the human skin microbiome with different proportions; they were accounted for 94.6% of the clones (20). While in another study these three phyla accounted over 82% of the sequences (52). Studying cutaneous bacterial communities of Amerindians in the Venezuelan Amazon detected 20 phyla with three predominating phyla*; Proteobacteria, Firmicutes* and *Actinobacteria* (53).In our study, 29 phyla were detected and the top three phyla were in the same trend but accounted for over 95.6%. Gao, Fierer and Grice reported that *Propionibacterium*, *Staphylococcus, Acinetobacter, Streptococcus,* and *Corynebacterium* were detected as the most predominant genera while Leung found the same trend in addition to *Enhydrobacter*(20, 22, 51) .

In the current study, at genus level, there was a noticed predominance of *Klebsiella* genus in all subjects. Over the last decades there was a noticed alteration in temperature in Egypt (54). Elevated temperature as well as humidity provide favorable condition for certain microorganisms especially gram negative bacilli (8). Previous studies were conducted for the analysis of bacterial load in drinking water and River Nile branches in which pathogenic species were detected such as gram negative bacilli (55-57). *Klebsiella* genus was present with the highest proportions in all samples including *Klebsiella variicola*, a plant associated microorganism which was reported as a frequent cause of blood stream infections (62, 63). Our finding of the predominance of *Klebsiella* genus in healthy skin may suggest the role of life style (daily habits such as ablution which is washing of face, arms and legs prior to prayers several times per day) as well as environmental factors including mainly weather on the composition of skin associated bacteria (58). *Bacillus, Pseudomonas, Escherichia, Meiothermus* and *Corynebacterium* were also abundant in both sites in all subjects.

Opportunistic or pathogenic skin colonizer were characterized in many studies, the most detected species belonged to *Staphylococcus, Streptococcus* or *Propionibacterium* genera which are present in abundant proportions. In the present study, these genera were detected in low proportions. Non-aureus *Staphylococci* such as *S. haemoluticus, S. capare , S. cohnii* and *S. hominis* were isolated from many sites of the human body as axillae, perineum, and inguinal areas(59). These non-aureus species were the most predominant over other *Staphylococcus* species such as *S .aureus* or *S. epidermidis* that were characterized as a main residents of human skin with proportion ranging of 0.1-40% of bacterial populations (3, 49, 52, 60) while in this study, they were detected with low relative abundance 0.0 17% of reads.

Many parameters and factors can shape composition and diversity of bacterial community which vary between sites in the same person (3, 49, 52, 60). Our results was found to be in the same trend as previous studies. Subjects showed intersubject variation between the same sites. PF was more rich and diverse than AF. Members of bacterial populations drive the composition of these communities as noticed from SpCC results through competition and mutualism between dominant phyla or between species in the same genera. Relations between them were not only restricted to more abundant members but also included rare or minor members as *Acinetobacter* genus.

The structure of human microbiome generally is considered as a personalized signature (17, 61). Across all human microbiome studies there were no genera or species that can be called core genera. In our study the proportions of genera that were shared between all samples were few and each subject had a unique species. Moreover, Human microbiome is very dynamic and its variability resulted from geographical, personal, topographical, temporal, environmental and racial variation (53). The current study provides new insight into the skin microbiome not only in its composition but also in the effect of some factors such as environmental and personal factors in the structure of the skin microbiome in Egypt. The field of metagenomic needs more focus to discover the dim area of unclassified bacteria or sequences that are not assigned at different taxonomic levels. These results can be considered as a baseline for further investigations of human microbiome in healthy as well as in diseased individuals.

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