The First Report of Diversity Analyses of Skin Microbiome In Indonesian Leprosy Patient

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Abstract

Background

Skin microbiome is quiet diverse. There are several factors influencing the skin microbiome, such as skin diseases. However, the effects of leprosy on the skin microbiome remain unclear and there are only a few studies about skin microbiome on leprosy. The aim of this study was to investigate the alpha diversity of skin microbiome on lesional site of multibacillary (MB) leprosy patients who visited the top referral hospital in West Java Indonesia. Here in this study we characterize the skin microbiome in leprosy patient in compared to healthy individual by using next generation 16S rRNA sequencing. A total 18 skin swab samples were collected from 18 samples (14 leprosy patients, 4 healthy individuals).

Results

Taxonomic analysis of leprous skin lesions revealed main five phyla: *Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes,* and *Cyanobacteria*. *Proteobacteria* and *Firmicutes* were overrepresented in leprosy patients, while *Actinobacteria, Bacteroidetes*, and *Cyanobacteria* were diminished in leprosy patients compared to healthy individuals. The main five genera in leprous skin lesions were *Staphylococcus, Acinetobacter, Corynebacterium, Micrococcus,* and *Propionibacterium. Staphylococcus, Acinetobacter*, and *Micrococcus* were enriched in leprosy patients, while *Corynebacterium* and *Propionibacterium* which have a protective role in normal skin, were diminished in leprosy patients when compared with healthy individuals. Twenty-five species were found in leprous skin lesions that were not typical in human skin and considered as potentially pathogenic. The alpha diversity analysis showed that leprous skin lesions is less diverse than that of the healthy skin microbiome.

Conclusion

As a conclusion, the skin microbiome on lesional site of leprosy patient show alteration and less diverse compare to healthy individuals. This suggest that leprosy can affects skin microbiome profile or otherwise.

Introduction

Leprosy is a chronic granulomatous infectious disease caused by *Mycobacterium leprae*,[1,2] it primarily affects peripheral nerve and skin.[2] Leprosy still became a significant health problem in several countries,[3] including Indonesia, which is the third country with the highest cases of leprosy in the world after India and Brazil.[4,5] In 1982, World Health Organization (WHO) divided leprosy into two classifications, namely paucibacillary (PB) and multibacillary (MB) leprosy.[4] Disability due to leprosy is more common in MB leprosy compared to PB.[6] It is known that leprosy is thought to affect the skin microbiome. To date, there are only two publications from Brazil[7,8] and one publication from India[9] regarding skin microbiome in leprosy, but there is no publication from Indonesia.

Microbiome is all the microorganisms, their genomes, and the surrounding environmental conditions present in a particular ecosystem.[10] It is present in various locations in the body, for example the eyes, nose, mouth, ears, lungs, and skin. The microbiome found in the skin is referred to as the skin microbiome,[11] which play a role in human health.[8] Various factors can affect the skin microbiome, for example diseases[8,12–14] Several cutaneous diseases such as atopic dermatitis, acne, psoriasis, and leprosy can affect microbiome although the effects on pathophysiology of these diseases is unclear.[7,8,15] The interaction of microbiota inter and intraspecies can modulate the innate immune response of the host.[16] The microbiome plays a role in the function and polarization of macrophages towards proinflammatory (M1) as well as anti-inflammatory (M2).[17] Macrophage is one of the keys in the pathogenesis of leprosy. M1 type of macrophages were dominated in PB leprosy, while an M2 type of macrophages were dominated in MB type leprosy.[18]

This is the first report of the diversity and composition of the skin microbiome in Indonesian leprosy patients as compared to healthy individuals by using next generation 16S rRNA sequencing. We report data from 18 study participants, including 3 leprosy patients before treatment, 4 leprosy patients during treatment, 3 leprosy patients during treatment with reversal reaction, and 4 leprosy patients who have been released from treatment, and 4 healthy individuals.

Materials And Methods

Study design

This research was a descriptive study using a cross-sectional method conducted in Dr. Hasan Sadikin Hospital Bandung, West Java, Indonesia. The aim of this study was to investigate the alpha diversity of skin microbiome on lesional site of MB leprosy patients who visited the top referral hospital in West Java Indonesia. The Research Ethical Committees of the Dr. Hasan Sadikin Hospital Bandung approved the study with approval number LB.02.01/X.6.5/325/2019. After being detailed informed about the study, all the participants gave their written consent between January 2020 and March 2020.

Inclusion criteria

1. Leprosy patients, including before treatment, under treatment, in leprosy reaction, and release from treatment (RFT).
2. Age ≥15 years.
3. Active skin lesions on the patients back (scapular and lumbar regions)

Exclusion criteria

1. Individuals with other skin diseases, such as atopic dermatitis, acne vulgaris, and psoriasis vulgaris based on clinical manifestation

2. Individuals who have used moisturizer 24 hours before skin swab and any topical antibiotic or corticosteroid in previous 7 days
3. Individuals who have taken systemic antibiotic other than multidrug therapy (MDT) MB in previous 10 days

Specimen and DNA extraction

Person collecting samples wore a fresh pair of gloves and facemask for every participant to avoid contamination. Swabs of lesional skin from leprosy patients and healthy skin from healthy individuals on back area were collected using Puritan® DNA/RNA Shield™ collection tube with swab device. The sampling procedure was undertaken as follows: skin areas were selected and stretched with one hand and the other hand was holding a swab which was already soaked in wetting buffer solution on Puritan® DNA/RNA Shield™ collection tube. Swabbing was performed in one direction for fifty times with a firm pressure and make sure that each side of the swab were in contact with the skin. The collected swab samples were stored in -4 °C until DNA extraction. Total genome DNA from the samples was extracted using ZymoBIOMICS™ DNA Miniprep Kit No D4300 protocol. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water.

16S rRNA gene V3–V4 hypervariable regions amplification, library preparation, and sequencing.

The primers 515F (5’-CCTAYGGGRBGCASCAG-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT) was used to amplify V3-V4 hypervariable regions. All polymerase chain reaction (PCR) reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Mix same volume of 1x loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. Samples with bright main strip between 400 bp – 450 bp were chosen for further experiments. PCR products was mixed at equal density ratios. The mixed PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The libraries generated with NEBNext® Ultra™ DNA Library Prep Kit for Illumina and quantified via Qubit and Q-PCR, would be analysed by Illumina platform

Bioinformatics analysis

Paired-end reads was assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/). Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the Qiime (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) quality controlled process. The tags were compared with the reference database (Gold database,http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (UCHIME Algorithm,http://www.drive5.com/usearch/manual/uchime_algo.html) to detect chimera sequences (https://drive5.com/usearch/manual/chimeras.html). And then the chimera sequences were removed. Then the Effective Tags finally obtained.

Sequences analysis were performed by Uparse software (Uparse v7.0.1001 http://drive5.com/uparse/) using all the effective tags. Sequences with ≥ 97% similarity were assigned to the same Operational Taxonomic Units (OTUs). Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database (http://www.arb-silva.de/) for species annotation at each taxonomic rank (Threshold:0.8 ~ 1) (kingdom, phylum, class, order, family, genus, species). To obtain the phylogenetic relationship of all OTUs representative sequences, the MUSCLE (Version 3.8.31,http://www.drive5.com/muscle/) can compare multiple sequences rapidly. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity was performed basing on this output normalized data. The alpha diversity was assessed by Shannon diversity index.

Results

The bacterial composition of leprous skin lesions and healthy individuals normal skin was investigated using NGS. This is the first study on the diversity and composition of leprous skin lesions and normal skin healthy individuals through the deep sequencing of 16S rRNA genes in Indonesia. A total of 2,033,254 effective tags of 18 samples (3 leprosy patients before treatment (KB), 4 leprosy patients during treatment (KP), 3 leprosy patients during treatment with reversal reaction (KR), and 4 leprosy patients who have been release from treatment (RFT), and 4 healthy individuals (Ko)) was observed.

Skin microbiome composition in leprous skin lesions compared to healthy individuals

In this study, it was known that 51 phyla were identified, although there were some microbiota whose phylum had not been identified. The main five phyla in all of the samples were *Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes*, and *Cyanobacteria.* In leprous skin lesions, the most abundant phyla were *Proteobacteria* (41.8%) while in healthy individuals were *Actinobacteria* (41.5%). *Proteobacteria* and *Firmicutes* were enriched in leprosy patients, while *Actinobacteria, Bacteroidetes*, and *Cyanobacteria* were diminished in leprosy patients compared to healthy individuals. Interestingly, *Cyanobacteria* phylum were slightly overrepresented in KB group compared to other group and it was slightly more abundance than *Bacteroidetes* phylum in KB group. A phylum level taxonomic profile of the healthy skin and leprous skin lesion microbiome is shown in Figs. 1 and 2.

In this study, it was known that 872 genera were identified, although there were some microbiota whose genera had not been identified. The top ten genera in all of the samples were *Staphylococcus* (*Firmicutes*), *Acinetobacter, Pseudomonas, Ensifer* (*Proteobacteria*), *Corynebacterium, Micrococcus, Propionibacterium, Dietzia* (*Actinobacteria*), *Sphingobacterium, Chryseobacterium* (*Bacteroidetes*). In leprous skin lesions, the most abundance genera were *Staphylococcus* (20.8%) while in healthy individuals were *Corynebacterium* (15.6%). *Staphylococcus, Acinetobacter*, and *Micrococcus* were enriched in leprosy patients, while *Corynebacterium* and *Propionibacterium* were diminished in leprosy

patients when compared with healthy individuals. Leprous skin lesions and normal skin in healthy individuals were present many similar genera with varied frequency. However, in this study, we also found 22 genera which were only found in leprous skin lesion and was not found in the skin of the healthy individuals, namely *Methyloparacoccus, Micropruina, Prevotellaceae UCG-003, Cnuella, Vagococcus, Gluconobacter, hgcl clade, Salinicola, Johnsonella, Solibacillus, Butyrivibrio, Oceanobacillus, Odoribacter, unidentified Methylobacteriaceae, Pontibacter, Pleurocapsa, Candidatus Soleaferrea, Selenomonas 3, Saccharomonospora, Nevskia, Myroides*, and *Fluviicoccus.* A genera level taxonomic profile of the healthy skin and leprous skin lesion microbiome is shown in Figs. 3 and 4.

In this study participants, it was known that 479 species from leprous skin lesions and 454 species from healthy skin individuals were identified, although there were some microbiota whose species had not been identified. The top five species in all of the samples were *Acinetobacter johnsonii, Micrococcus luteus, Moraxella atlantae, Pseudomonas stutzeri*, and *Ensifer adhaerens. Acinetobacter johnsonii, Micrococcus luteus, Moraxella atlantae*, and *Pseudomonas stutzeri* were enriched in leprosy patients, while *Ensifer adhaerens* was diminished in leprosy patients when compared with healthy individuals. The relative frequency at the species level of the leprous skin lesion and healthy individuals is shown in **Table 1**.

Table 1. Top five taxa relative abundance in species of each leprosy patients and healthy

individuals

Phylum	Genera	Species	Leprosy patients	Healthy individuals
Proteobacteria	*Acinetobacter*	Acinetobacter johnsonii	0.0854	0.0218
Actinobacteria	*Micrococcus*	Micrococcus luteus	0.0684	0.0367
Proteobacteria	*Moraxella*	Moraxella atlantae	0.0410	0.0148
Proteobacteria	*Pseudomonas*	Pseudomonas stutzeri	0.0372	0.0201
Proteobacteria	*Ensifer*	Ensifer adhaerens	0.0343	0.0613

In this study, we also found 25 species which were only found in leprous skin lesion and was not found in the skin of the healthy individuals, namely *Deinococcus radiophilus, Actinomyces odontolyticus, Vibrio cholerae, Myxococcus virescens, Leptotrichia shahii, Legionella norrlandica, Vagococcus fluvialis, Leptotrichia wadei, Gluconobacter frateurii, Solibacillus silvestris, Rheinheimera aquimaris, Actinomyces massiliensis, Lactobacillus mucosae, Actinomyces orihominis, Rahnella aquatilis, Methylobacteriaceae bacterium YIM 100770, Bacterium Ellin6519, Flavobacterium sp.HME7856, Arcobacter cryaerophilus, Cupriavidus gilardii, Actinobacteria bacterium IMCC26256, Myroides marinus, Geitlerinema sp. PCC 7407, Saccharomonospora viridis*, dan *Blautia coccoides.*

Alpha diversity analysis

The alpha diversity was assessed by Shannon diversity index. In this study, it was known that the alpha diversity of leprous skin lesion (5.09) was higher than healthy individual group (5.25). From each group, it was known that the highest alpha diversity was a KB group (5.47), followed by RFT (5.18), KP 5.10, Ko (5.09), and KR (4.63). The p values were calculated by Wilcoxon rank sum. However, there were no significant differences between both group ($p > 0.05$) (Fig. 5.) In summary, the alpha diversity analysis showed that the healthy skin microbiome is more diverse than that of leprous skin patient.

Discussion

Recent investigations have highlighted the dysbiosis of the skin microbiome in leprosy patients. All of those studies are conducted in Brazil and India. From a 16S rRNA gene dataset, we provide the first description of Indonesian leprous skin lesions both before treatment, during treatment, during treatment with reversal reaction, and release from treatment compared to healthy individuals.

Leprous skin lesions revealed five dominant phyla represented by *Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes*, and *Cyanobacteria*. The same first four phyla were consistent with the previous studies on the skin microbiome of leprosy patients in Brazil and India.[7,8,9] However, the fifth phyla (*Cyanobacteria*) has not been mentioned in the previous studies of skin microbiome in leprous skin lesions. *Cyanobacteria* (blue-green algae) are common inhabitants of water (fresh, brackish, and marine) and terrestrial environments throughout the world. This phylum produces a broad spectrum of secondary metabolites– biologically active products, which could be toxic (cyanotoxins). The cutaneous adverse effects of *cyanobacteria* and their cyanotoxins are often underdiagnosed. *Cyanobacteria* has been reported can cause an irritant and allergic contact dermatitis and also generalized urticarial rash.[19] However, until recently the relationship between *Cyanobacteria* and leprosy is still unknown.

In this study, the result of *Proteobacteria* which was slightly overrepresented in leprosy patients and *Actinobacteria* which was underrepresented in leprosy patients compared to healthy individuals were consistent with the previous studies by Silva et al. in 2015. In this study, *Firmicutes* was overrepresented in leprosy patients and *Bacteroidetes* was underrepresented in leprosy patients compared to healthy individuals. These findings were different with previous studies by Silva et al. in 2015 and 2018, and also Bayal in 2019. The differences in the results of these studies are thought to be due to differences in the anatomical locations of the study samples. In this study, all samples were taken from the back, because all leprosy patients had skin lesions on the back, whereas in the study conducted by Silva et al.[7,8] samples were taken from the volar forearm. In addition, other factors that can affect the microbiome such as gender, age, diet, ethnicity, climate, and geographic location can also cause differences in the results of this study.[8,12–14]

Staphylococcus and *Acinetobacter* were the highest abundance genera in leprous skin lesions. These results were different from a previous study conducted by Silva et al.[7] in 2015 in Brazil, which was

Bacillus genera was the highest genera found in the leprous skin lesions. From the results of a study conducted by Bayal et al.[9] it was found that the group of leprosy patients during treatment in Hyderabad and Miraj areas of India had a significantly different skin microbiome. Based on a study conducted by Blaser et al.[20] in the United States and Venezuela in 2012 which compared the skin microbiome on the forearm between a population of healthy individuals in the United States and Venezuela. It was found that there were differences in the skin microbiome of the two populations. The difference in results between this study and that of Silva et al. and Bayal et al. presumably because the skin microbiome can be influenced by ethnicity, lifestyle, and/or geographic location.[8,12–14]

Propionibacterium and *Corynebacterium* genera were diminished in leprous skin lesions compared to healthy individuals. Similar results were shown in previous study conducted by Silva et al.[7] *Propionibacterium* and *Corynebacterium* were the dominant genera in healthy skin.[21,22] Both of these genera have a protective function in the skin of healthy individuals.[7] One of the species of the genus *Propionibacterium*, namely *Propionibacterium acnes* can inhibit the growth of pathogenic bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes* through the formation of free fatty acids and propionic acid, as well as the secretion of bacteriocins, such as thiopeptide.[13] Therefore, the decreased number of *Propionibacterium* and *Corynebacterium genera* in the leprous skin lesions are thought to be due to interference with the skin's protective function.[7]

Leprosy reactions can occur due to changes in the immune system in leprosy patients.[23] In reversal reaction, the role of T helper (Th)1 is more dominant than Th2. The existence of dysregulation of the immune system such as increased Th1 activity can cause changes in the composition of the microbiota and otherwise.[24] Based on the results of this study, there were differences in the order of the microbiota phylum (Firmicutes and Actinobacteria) and genera (*Micrococcus* and *Propionibacterium)* composition in leprosy patients during treatment with and without reversal reaction. This result indicate the possibility of reversal reaction influence on microbiome composition. To the best of our knowledge, this was the first report of the skin microbiome in leprosy with reversal reaction..

The top five species in all of groups in this study were *Acinetobacter johnsonii, Micrococcus luteus, Moraxella atlantae, Pseudomonas stutzeri*, and *Ensifer adhaerens*, with the first four species were enriched in leprosy patients, while *Ensifer adhaerens* was diminished in leprosy patients when compared with healthy individuals. *Acinetobacter johnsonii, Micrococcus luteus, Moraxella atlantae*, and *Pseudomonas stutzeri* have been reported previously as a pathogen bacteria that can cause some diseases, such as meningitis (*Acinetobacter johnsonii, Micrococcus luteus, Pseudomonas stutzeri)*, bacteriemia in adenocarcinoma patient (*Moraxella atlantae)*, and ecthyma gangrenosum (*Pseudomonas stutzeri).*[25–28] Factors that cause the four species mentioned above are found to be more in the leprous skin lesions than healthy individuals is not known, but it is possibly due to autonomic nerve damage in leprous skin lesions which causes dry skin and impaired protective role of the skin.[1] In contrast to the four previous species, *Ensifer adhaerens* was higher in healthy individuals compared to leprous skin lesions. *Ensifer adhaerens* is a Gram-negative bacteria from soil and has lysis properties for other Gram-

positive and negative bacteria.[29] The presence of this species on human skin has not been previously reported.

Mycobacterium leprae was not found in this study, which was same as the previous study.[7,8] It is possibly because of the skin swab as a sampling procedure, since this bacteria is an obligate intracellular pathogen of macrophages.[1,2,8]

Based on this study, there were 22 genera and 25 species of microbiota, which were only found in the leprous skin lesions. These genera and species are not commonly found on human skin. According to those circumstances, these thought to be a potential pathogen, and its existence is probably caused by an impaired skin barrier of protective function in leprosy patients.

The alpha diversity in the leprous skin lesions in this study was lower than that of healthy individuals. These results were similar to previous study conducted by Silva et al.[8] in 2018 in Brazil and Bayal et al.[9] in 2019 in India. Studies on alpha diversity in skin microbiome have been conducted in several other skin diseases, such as atopic dermatitis and psoriasis. Therefore, it was known that the diversity of the microbiome was found to be lower in these two diseases than in healthy individuals.[30,31] In atopic dermatitis, the decrease in the diversity of the microbiome is related to the degree of disease severity and increased colonization of pathogenic bacteria, such as *Staphylococcus aureus*.[32] This proves that some diseases can reduce the diversity of the skin microbiome including in leprosy.

Conclusion

The differences of the skin microbiome in leprous skin lesion between Indonesia and previous studies were observed. These findings indicate the importance of studying skin microbiome in leprosy patients from distinct geographical and cultural settings. There are a taxonomic changes and different abundance levels of the skin microbiome in leprous skin lesions compared to healthy individuals. The alpha diversity in the leprous skin lesions in this study was lower than that of healthy individuals and leprosy with reversal reactions was the lowest diversity of the microbiome compared to the other groups. Altogether, these results contribute to our knowledge of the global human skin microbiome and provide new insights into *M. leprae* and reversal reaction effects on the microbiome. However, it should be kept in mind that this study represents a minor portion of the Indonesian population. Future studies on a larger number of patients will be needed to strengthen and expand our conclusions.

Abbreviations

Healthy individuals
Ko
Leprosy patients before treatment
KB
Leprosy patients during treatment

KP
Leprosy patients during treatment with reversal reaction
KR
Leprosy patients who have been release from treatment
RFT
Multibacillary
MB
Multidrug Therapy
MDT
Operational Taxonomic Units
OTU
Paucibacillary
PB
Polymerase Chain Reaction
PCR
Release From Treatment
RFT
World Health Organization
WHO

Declarations

- **Ethics approval and consent to participate**

The Research Ethical Committees of the Dr. Hasan Sadikin Hospital Bandung approved the study with approval number LB.02.01/X.6.5/325/2019. Written informed consent was obtained from the patients for this research.

- **Consent for publication**

Not applicable

- **Availability of data and material**

All data generated or analysed during this study are included in this published article

- **Competing interests**

The authors declare that they have no competing interests

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- **Authors' contributions**

HG, PJ, DA contributed in data collection of skin swab procedures. HG, PJ, OS, DA performed a DNA extraction, sequencing, and analysis. ES, RF, RHi, were a contributing in determine manuscript composition. HG, PJ, OS, DA, ES, RF, RHi were contributing in writing the manuscript. All authors approved that final manuscript as submitted. Each author believes that the manuscript represents honest work.

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Figures

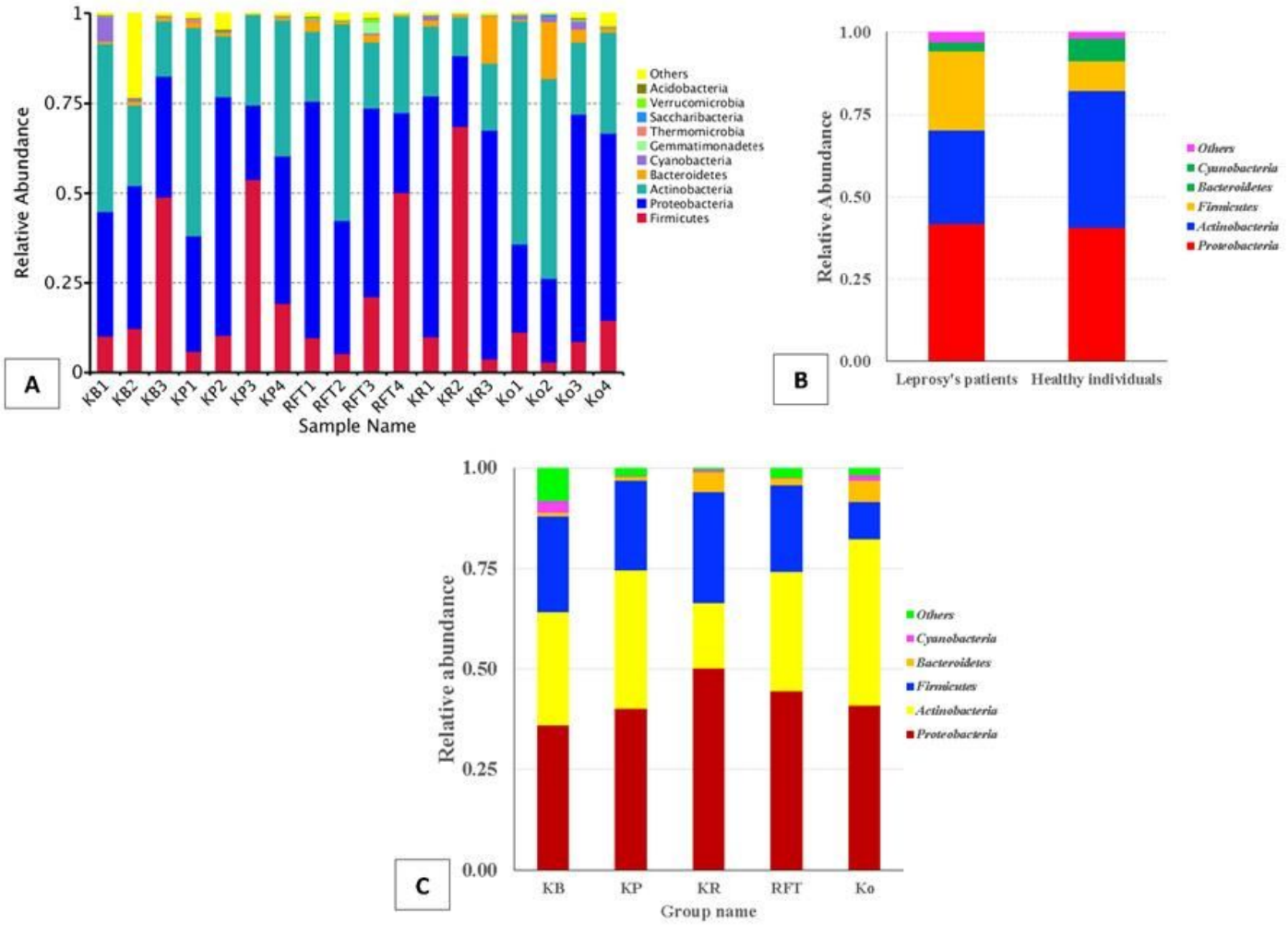

Figure 1

Relative abundance phyla in samples from study participant. (a) Top 10 phyla in each samples (b) Top 5 phyla in leprosy's patient and healthy indivudials (c) Top 5 phyla in each group. Patient groups are named for the before treatment (KB), during treatment (KP), during treatment with reversal reaction (KR), release from treatment (RFT), and healthy individual (Ko)

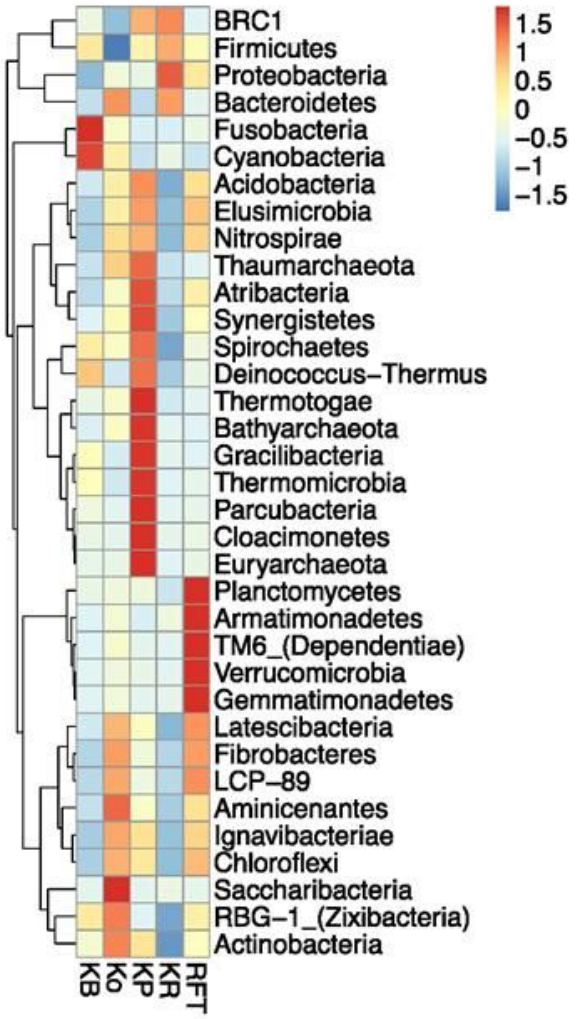

Figure 2

Phyla heatmap in each group. Patient groups are named for the before treatment (KB), during treatment (KP), during treatment with reversal reaction (KR), release from treatment (RFT), and healthy individual (Ko)

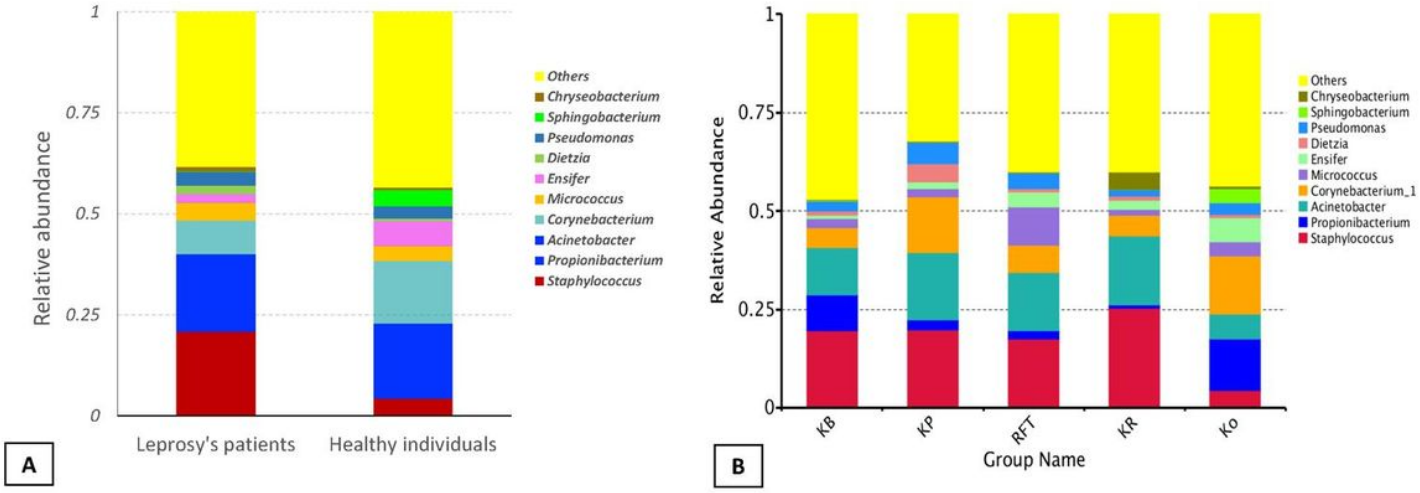

Figure 3

Relative abundance genera in samples from study participant. (a) Top 10 genera in leprosy's patients and healthy individuals. (b) Top 10 genera in each group. Patient groups are named for the before treatment (KB), during treatment (KP), during treatment with reversal reaction (KR), release from treatment (RFT), and healthy individual (Ko)

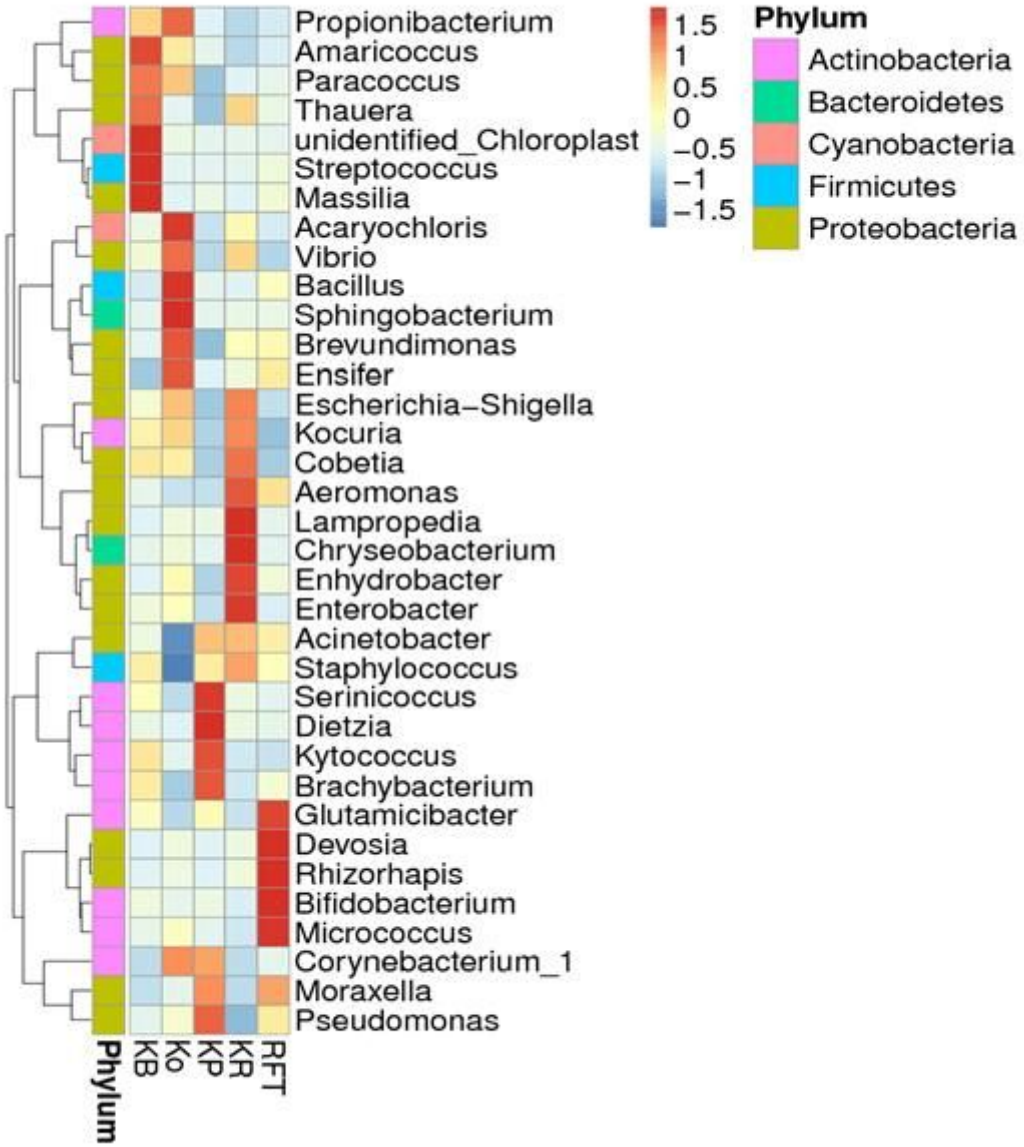

Figure 4

Genera heatmap in each group. Patient groups are named for the before treatment (KB), during treatment (KP), during treatment with reversal reaction (KR), release from treatment (RFT), and healthy individual (Ko)

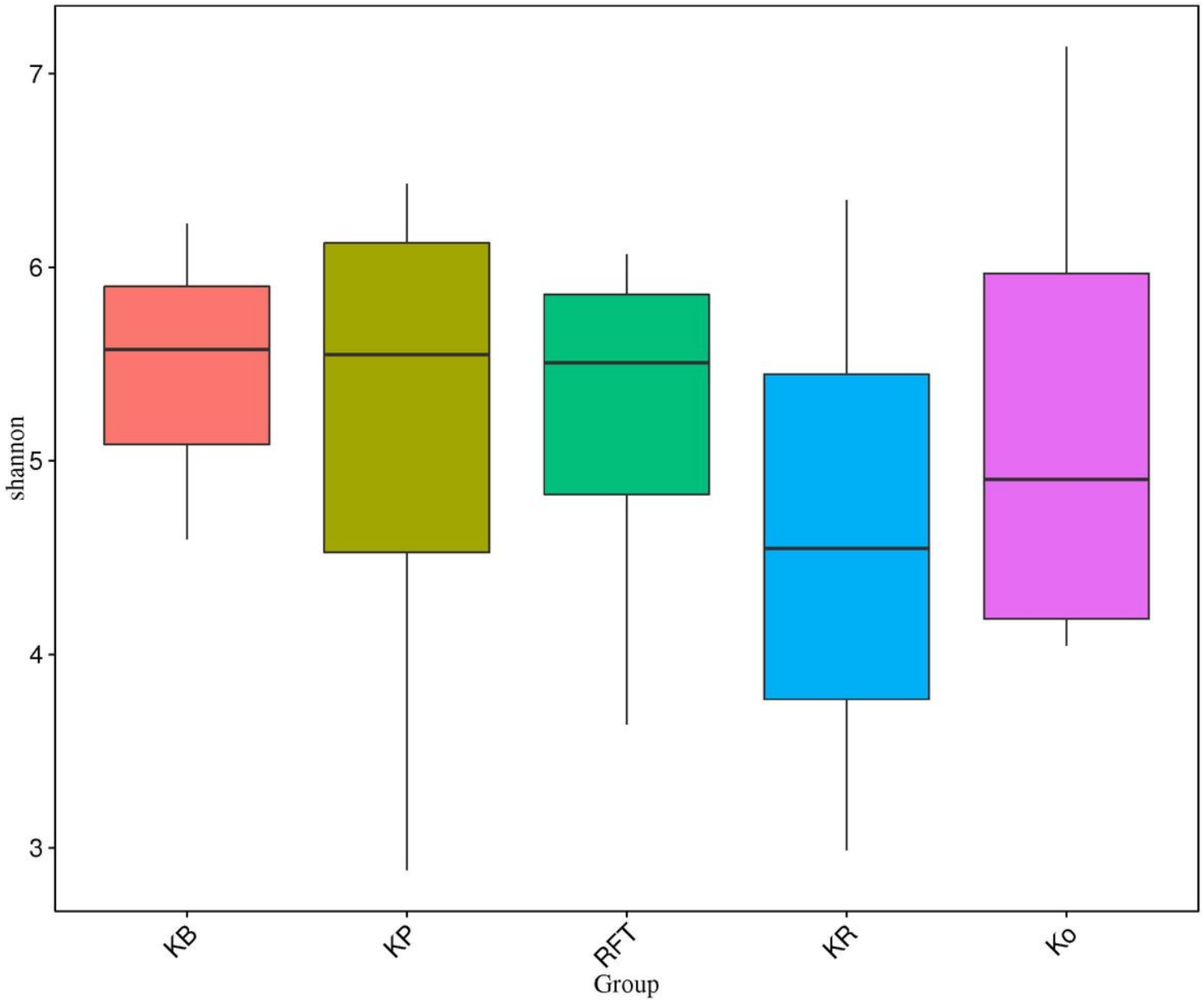

Figure 5

Alpha diversity indices of the skin microbiome from each group. Boxplot based on Shannon diversity index from each group. For the boxplots, the centerlines indicate the median, with the lower and upper edges of each box representing the first and third quartiles, respectively. Patient groups are named for the before treatment (KB), during treatment (KP), during treatment with reversal reaction (KR), release from treatment (RFT), and healthy individual (Ko)