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ORIGINAL ARTICLE

Blood and sputum microbiota composition in Afghan immigrants and Iranian subjects with pulmonary tuberculosis

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ABSTRACT

Background and Objectives: TB infection is one of the most challengeable epidemiological issues. Complex interactions between microbiota and TB infection have been demonstrated. Alteration in microbial population during TB infection may act as a useful biomarker. The present study examined the microbiota patterns of blood and sputum samples collected from Afghan immigrants and Iranian patients with active TB.

Materials and Methods: Sixty active pulmonary TB patients were enrolled in the study. Blood and sputum samples were collected. To detect phylum bacterial composition in the blood and sputum samples, bacterial 16S rRNA quantification by Real-Time qPCR was performed.

Results: A significant decrease in Bacteroidetes in Iranian sputum and blood samples of Afghan immigrants and Iranian TB active subjects were seen. While, sputum samples of Afghan immigrants showed no significant differences in Bacteroidetes abundance among TB active and control. Firmicutes were also presented no significant difference between sputum samples of the two races. Actinobacteria showed a significant increase in Iranian and Afghan sputum samples while this phylum showed no significant abundance in Iranian and Afghan TB positive blood samples. Proteobacteria also showed an increase in sputum and blood samples of the two races.

Conclusion: An imbalance in Bacteroidetes and Firmicutes abundance may cause an alteration in the microbiota composition, resulting in dysregulated immune responses and resulting in the augmentation of opportunistic pathogens during TB infection, notably Proteobacteria and Actinobacteria. Evaluation of human microbiota under different conditions of TB infection can be critical to a deeper understanding of the disease control.

Keywords: *Mycobacterium tuberculosis*; Bacteroidetes; Firmicutes; Actinobacteria; Proteobacteria

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INTRODUCTION

A common cause of infection in the human history, tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (MTB) (1). According to the latest World Health Organization (WHO) global TB report 2022, about 10.6 million (125-143 per 100 000 population) newly identified TB cases and a total of 1.6 million deaths were estimated to have been caused by TB (2). A potent reversal is noted in WHO report regarding progress towards the first End TB Strategy milestone. There was only a 5.9% reduction from 2015 to 2021, compared with a 35% reduction from 2015 to 2020. It is estimated that the COVID-19 pandemic disrupted essential TB services in 2021, resulting in an increase in TB deaths (3). Due to the high mortality rate associated with untreated TB (about 50%), reduced case detection has a noticeable impact on TB mortality within a short period of time (4). Among WHO regions (2), developing countries accounted for the majority of TB deaths in 2021 (2). It is possible that the failure to effectively control the source of infection, counts as one of the main reasons of the large percentage of the population being infected. To effectively control TB and achieve the "END TB" goals, it is crucial to acquire comprehensive information about this disease, especially for countries like Iran that have a large Afghan population (5). Studies on MTB have not been able to explain MTB control fully, and mechanisms for its progression have not been fully clarified. Hence, it is imperative to identify novel opportunities for improving control strategies.

For rapid and accurate diagnosis and treatment strategies, anew potent biomarkers with high sensitivity and specificity should be introduced. Recently, several studies have focused on the composition of microbial communities during different stages of the TB infection for applying as biomarkers (6, 7). As an emerging and interesting field, microbiota patterns and TB infection has been the subject of increasing studies in recent years. Human microbiota includes a multitude of bacterial, archaeal, viral, and fungal species. In the future, control strategies for MTB infection may be improved by considering dysbiosis of normal microbiota (8). Revealing the exact composition of human microbiome in lungs and blood of MTB infected patients will enable us for better understanding of the complex interactions between the host and the invader. While many studies have focused on the composition of gut microbiome, microbiome alteration pattern in other human body niches may be very meaningful. Others have emphasized that, MTB infection is not caused solely by a single infectious agent, and a complex interactions have been proposed with the contribution of other microbiota present in the niches (9). It has been recently discovered that the microbiota in sputum may be a potential diagnostic marker for drug-resistant TB. It is possible to detect resistant strains of TB in infected patients with both *Kingella* and *Chlamydophila*. Further, *Bordetella* can be used to diagnose TB patients who have developed multidrug resistance (10). More recently, however, the prospect of a blood microbiota has been of much interest to the scientific community. Nevertheless, clinical trials are currently employing a few microbiota-based biomarkers (8). The MTB infection is generally viewed as the outcome of a multipart interaction between human microbiota communities rather than a single infectious agent (9). As a result, microbiota analysis may contribute to the Stop TB Strategy goal of limiting the global TB burden. The current study analyzed the microbiota patterns of blood and sputum samples collected from Afghan immigrants and Iranian patients with active TB.

MATERIALS AND METHODS

Human subjects. Thirty Iranian and 30 Afghan patients were enrolled in the study. Demographic characteristics of all participants including age, gender, socioeconomic backgrounds, education, BMI, and underlying disease was recorded. The study was eligible for subjects who had typical pulmonary TB symptoms, such as fibrocavitary lung infiltrates and positive Ziehl-Neelsen acid-fast bacilli on sputum, as well as positive sputum cultures. After decontaminating and homogenizing with N-acetyl-cysteine (NAC), Lowenstein-Jensen (LJ) culture medium was inoculated with sputum samples. After incubation for the first week at 37°C, all culture tubes were observed once a week until eight weeks after incubation. For MTB diagnosis, isolated colonies from LJ medium were subjected to biochemical tests. As controls, sixty healthy racial, age, and sex-matched individuals were recruited. In the study, healthy controls were not infected with TB, including latent and active infection, and had no clinical symptoms of any infectious disease. Iranians and Afghans were both required to

submit sputum and blood samples simultaneously.

This study was approved by the ethics committee of the Pasteur Institute of Iran (IR.PII.REC.1398.046).

Sample preparation, DNA isolation, and DNA quality control. Every Iranian and Afghan participant was given anticoagulated whole blood and sputum. On ice, sterile laboratory tubes containing blood samples were shipped to the Pulmonary Department of the Pasteur Institute of Iran. More than 90% of the microbial DNA in the blood can be found in the buffy coat layer of blood samples, which is mainly composed of white blood cells and platelets. For DNA extraction, the sputum samples were liquified using NaOH and phosphate buffered saline was used for pH adjustment. Debris was removed using a centrifugation step and 200 µl from the supernatant underwent DNA extraction process using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). For buffy coat extraction, a 100 µl mixture of white blood cells (with few red blood cells) was aliquoted and extracted by the same protocol. Gel electrophoresis was used to check the DNA quality and the concentration of DNA extracted from sputum and buffy coat samples was determined using a Nanodrop spectrophotometer. Equal amount of the extracted DNA from each patients were aliquoted and analyzed.

Bacterial 16S rRNA quantification by Real-Time qPCR. Quantitative real-time PCR was used to quantify various bacterial genomic abundances in sputum and blood samples. With phylum-specific DNA primers, variable regions of bacterial 16S rRNA gene sequences were amplified. In order to assess the specificity of primers, nucleotide BLAST was used at NCBI. Primer sequences were presented (Table 1). The PCR reactions were performed in duplicate using the Roche LightCycler® 96 system (Roche, Switzerland). Each 20-µl PCR reaction contained 8 µl distilled water, 10 µl SYBR Green master mix (Takara, Japan), 1 µl DNA template, and 0.5 µl of forward and reverse primers (10 pmol/L). Following 1 min of heating at 95 °C, the mixture was subjected to 40 amplification cycles including denaturation (95°C for 15 s), annealing (55°C for 20 s), and extension (72°C for 20 s). In order to complete the melting curve analysis of the PCR product, the temperature of the product was cooled from 95°C to 60°C after amplification. In order to calculate the bacterial loads, serial dilutions of DNA extracts from standard strains *Escherichia coli* were prepared 10-fold. Using this standard curve, DNA concentrations for each bacterium were calculated from sputum and blood samples.

Statistical analysis. For checking the normality of data distribution, the Kolmogorov-Smirnov test was used. The LSD posthoc test and one-way analysis of variance were applied to normally distributed data. Non-parametric tests Kruskal-Wallis and Man-Whitney U Test were used for non-normally distributed data. For the comparison of bacterial abundance, the Mann-Whitney Test of SPSS version 25.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism® version 7 were employed. Mean rank differences (MRD) and adjusted P-values were presented in the text. A significance level below 0.05 was considered meaningful for the differences.

RESULTS

Characteristics of the study population. Many of the targeted participants were from low socioeconomic backgrounds and had only a high school education. Furthermore, more than half of them were household heads. It was not significantly different between Iranian (TB positive males: 21; healthy control males: 17) and Afghan (TB positive males: 14; healthy control males: 14) active TB groups and their respective healthy controls (P>0.05). Mean age of Iranian (median: 36.5; Q1: 20; Q3: 50) and Afghan (median: 22.5; Q1: 20; Q3: 50) TB positive groups related to their healthy controls (Iranian [median: 30; Q1: 22; Q3: 50]; Afghan [median: 31.5; Q1: 20; Q3: 50]) was not significantly different (P>0.05).

Weight and height of the participants were recorded on acceptance and body mass index (BMI) was calculated. Mean BMI was significantly lower in active TB patients compared to the controls (Iranian cases: 19 ± 0.29 against Iranian healthy controls: 21.2 ± 0.23; and Afghan cases: 18.2 ± 0.31 against Afghan controls: 21.2 ± 0.34, P<0.001). All participants were tested for HIV infection and all were negative. Clinical symptoms and signs of active TB patients were recorded. The commonly reported disease symptoms were coughing (98.3%), weight loss (83.3%), fever (78.3%), night sweats (69.8%) and hemoptysis (81.6%). There were no clinical signs of any infectious disease among the healthy controls included in the study.

Table 1. Universal and Phylum specific primer pairs

Phylum	Primer Sequences	Amplicon Size (bp)	References
16S rRNA	F: Eub338: ACTCCTACGGGAGGCAGCAG R: Eub518: ATTACCGCGGCTGCTGG	200	(11)
Firmicutes	F: Lgc353: GCAGTAGGGAATCTTCCG R: Eub518: ATTACCGCGGCTGCTGG	180	(12)
Proteobacteria	F: Eub338: ACTCCTACGGGAGGCAGCAG R: Bet680: TCACTGCTACACGYG	360	(13)
Actinobacteria	F: Actino235: CGCGGCCTATCAGCTTGTTG R: Eub518: ATTACCGCGGCTGCTGG	300	(12)
Bacteroidetes	F: Cfb319: GTACTGAGACACGGACCA R: Eub518: ATTACCGCGGCTGCTGG	220	(12)

Phylum bacterial composition in the blood samples. Phylum-level bacterial composition of blood samples was presented in Fig. 1. ΔCt value of each sample was plotted on vertical axis.

Phylum-level bacterial abundances in TB positive and healthy control subjects were calculated and presented as delta-Ct values ($\Delta Ct = Ct_{experimental} - Ct_{reference}$). Ct value of universal primers targeting Eubacteria was used as reference and subtracted from the Ct value of phylum-specific primers. Among the study groups, expression levels of universal 16S rDNA were almost equal between active TB cases and controls (Iranian-MRD: -37.30; P>0.05 and Afghan-MRD: -19.70; P>0.05). Nonetheless, when different bacterial phyla were analyzed in Afghan immigrants and Iranian subjects, a significant decrease in Bacteroidetes phylum in blood samples of Afghan immigrants (MRD: -57.50; P=0.02) and Iranian (MRD: -60.83; P=0.01) TB active subjects compared to their healthy controls were seen. Whereas, Firmicutes phylum were presented no significant difference between groups of two evaluated races (Iranian [MRD: -5.10; P>0.05]; Afghan [MRD: -26.0; P>0.05]). In addition, Proteobacteria phyla showed a significant increase in blood samples of Afghan TB active subjects compared to the related healthy controls (MRD: 0.941; P<0.001). While, in related to Iranian blood samples, an overexpression but not significant has been detected in Proteobacteria abundance (MRD: 0.398; P=0.27). On the other hand, Actinobacteria phylum showed no significant abundance in Iranian (MRD: 30.73; P>0.05) and Afghan (MRD: 33.00; P>0.05) TB positive blood samples compared to the healthy subjects.

Phylum bacterial composition in the sputum samples. Phylum-level bacterial composition of sputum samples has been presented in Fig. 2. Expression levels of the reference gene (Ct of 16S rDNA) were not statistically different among the groups (Iranian-MRD: -4.567; P>0.05; Afghan-MRD: -22.37; P>0.05). Nonetheless, analysis of different bacterial phyla highlighted some differences. A significant decrease in Bacteroidetes phylum in Iranian sputum samples compared to healthy control were seen (MRD: -54.43; P=0.04) while Afghan immigrants showed no significant differences in Bacteroidetes abundance among TB active and healthy subjects (MRD: -47.90; P>0.05). Firmicutes phylum were also presented no significant difference between groups of two evaluated races (Iranian [MRD: 26.63; P>0.05]; Afghan [MRD: 20.77; P>0.05]). In addition, Actinobacteria phylum showed a significant increase in Iranian (MRD: 99.47; P<0.001) and Afghan (MRD: 64.27; P=0.005) sputum samples compared to the related healthy subjects. Proteobacteria also showed an increase in sputum samples of two races compared to the related healthy subjects, but not significant (Iranian [MRD: -28.83; P>0.05]; Afghan [MRD: -24.03; P>0.05]).

DISCUSSION

It is becoming increasingly obvious that microbiota affect human health and is involved in human diseases, and that their clinical significance is becoming more apparent as well. The Human Microbiome Project was applied from 2008 to 2012 to progress understanding of healthy human microbiota (14). It is a mutualistic relationship between microbial com-

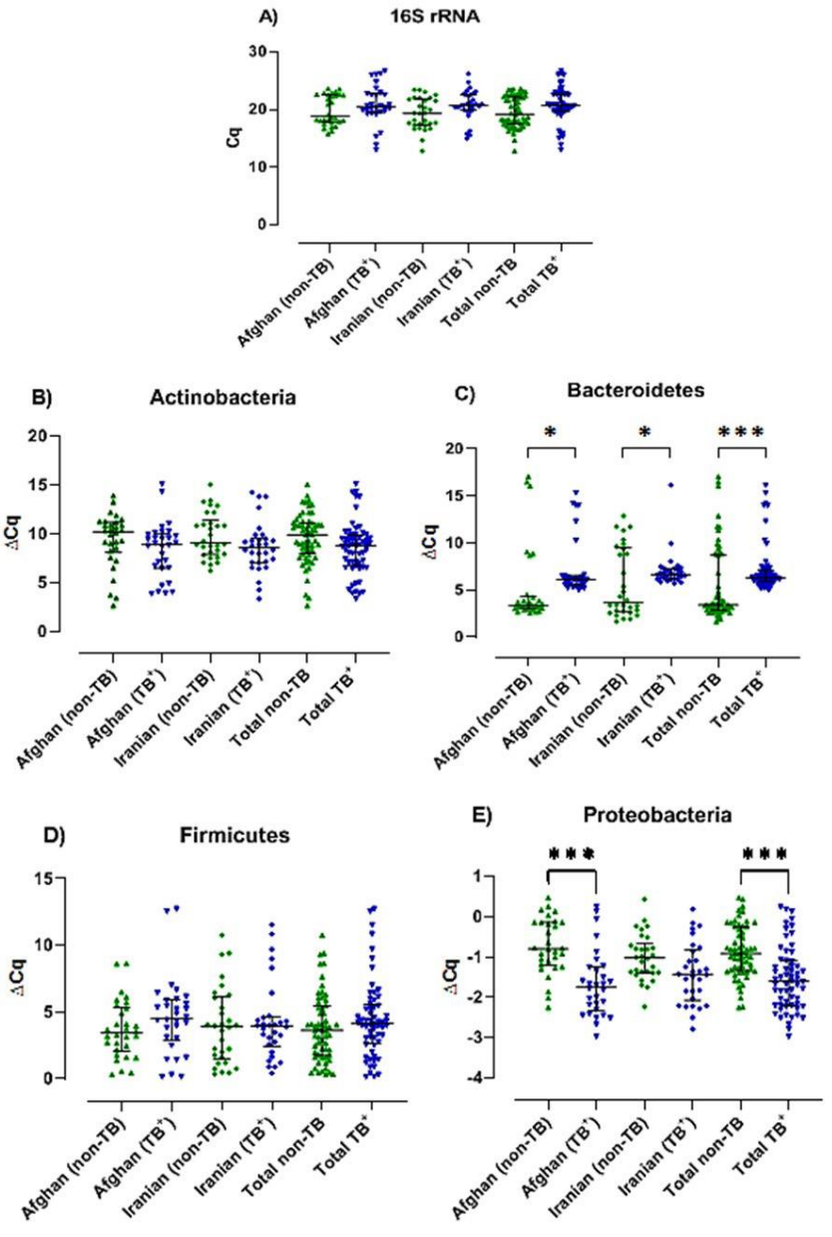

Fig. 1. Blood bacterial population by qPCR. phylum-level bacterial genome detection in Iranian and Afghan TB positive and related healthy control subjects. Delta-Ct values were calculated using experimental and reference genes. A) Reference gene (P=0.05). B) Actinobacteria (P=0.03), C) Bacteroidetes, D) Firmicutes (P=0.56), and E) Proteobacteria; Results were presented as median and interquartile range. *P<0.05, **P<0.01, ***P<0.001.

munities and their hosts. In the field of microbiota, most studies focus primarily on gut microbiota communities, while human epidemiological studies show gut microbial dysbiosis can impact lung microbiota in the long term. There is growing evidence that the gut microbiota influences the lung, a relationship that has been termed the gut-lung axis (15). However, the underlying mechanisms and pathways remain poorly understood. The gut-lung axis connects the gut niche with the lung niche, allowing bacteria and their metabolites to be transported into the bloodstream. It is important to note that the gut-lung axis operates in a bidirectional manner, a lung inflammation affecting the gut microbiota and blood can be induced (16). Many studies have examined the gut and lung microbiota in TB infection (10, 17, 18), but the blood

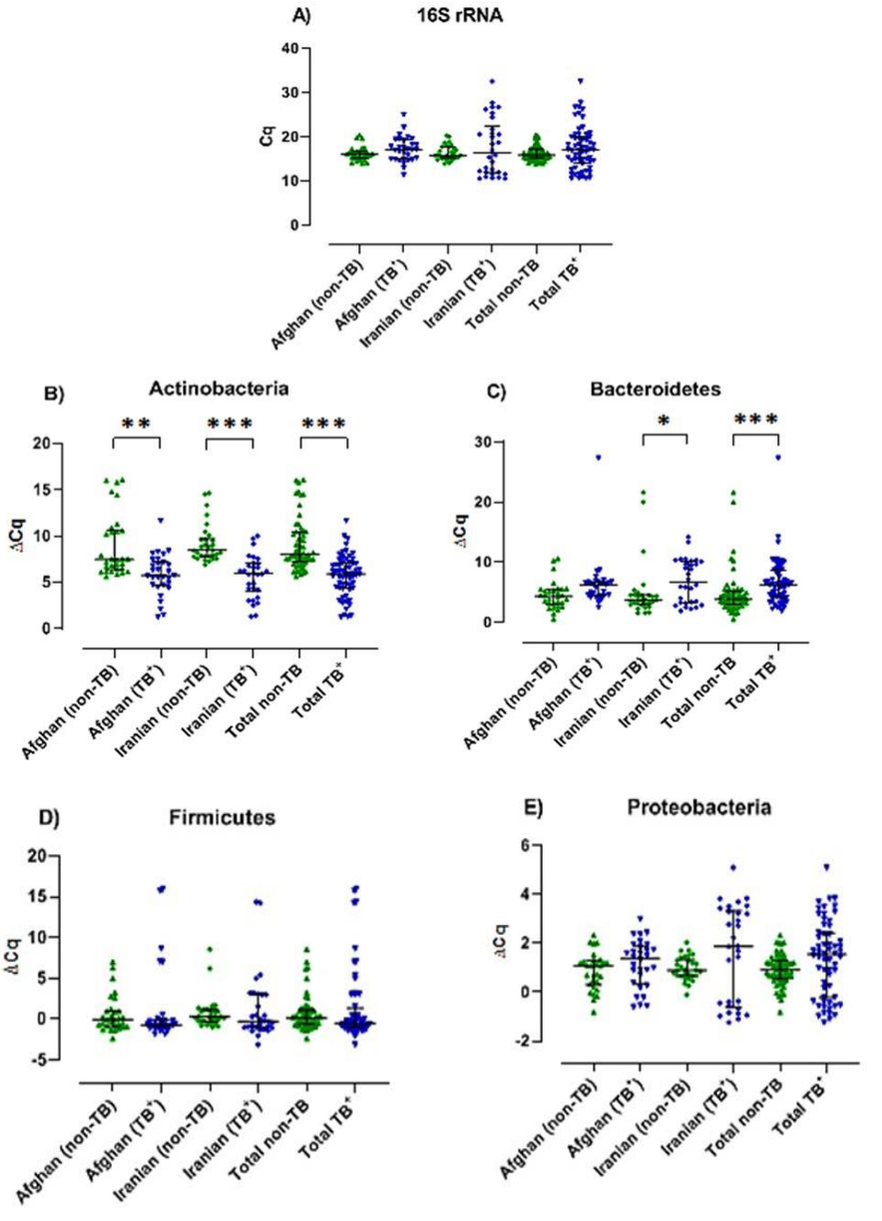

Fig. 2. Sputum bacterial population by qPCR. Phylum-level bacterial genome detection in Iranian and Afghan TB positive and related healthy control subjects. Delta-Ct values were calculated using experimental and reference genes. A) Reference gene (P=0.73), B) Actinobacteria, C) Bacteroidetes, D) Firmicutes (P=0.09), and E) Proteobacteria (P=0.11); Results were presented as median and interquartile range. *P<0.05, **P<0.01, ***P<0.001.

microbiota has received little attention. In general, the human microbiota plays a focal role in immunomodulation. It can prevent MTB transmission, reactivate from latency, affect disease severity, and lead to antibiotic resistance (19). A variety of changes in the gut, lung, and blood microbiota composition occur during TB infection, and their importance for effective MTB control attracts intense attention from researchers (19-21). Unraveling differences in microbial composition of TB patients among the two population, Iranian and Afghan, may bring new insights about TB pathogenesis, host-invader interactions and may lead to effective TB control. To assess the phylum-level bacterial composition in Iranian and Afghan active TB patients and healthy control subjects, blood and sputum samples were prepared and analyzed. Iranian sputum samples showed a significant down regulation of Bacteroidetes compared

to healthy controls (MRD: -54.43; P=0.04). Similar reductions in the abundances of Bacteroidetes in sputum samples were seen in Afghan active TB patients compared to the controls (MRD: -47.90; P>0.05). Additionally, this phylum was significantly downregulated in blood samples of Afghan immigrants (MRD: -57.50; P=0.02) and Iranians (MRD: -60.83; P=0.01) with TB. Among the bacteria in the human microbiota, Bacteroidetes are the second most abundant phylum, and they are also the major producers of the SCFAs acetate and propionate (22, 23). There was some conflicting evidence regarding the alteration of Bacteroidetes, but the most predominant finding was the down regulation of most abundant Bacteroidetes genera in sputum of TB patients (24-26). Members of this phylum of microbiota produce metabolites such as acetate and propionate. These metabolites are important because of their antimicrobial activity and enhancing antimicrobial peptides such as defensin (27). It has been found that defensin inhibits mycobacterial growth inside granulomas when expressed on airway epithelial cells (28). The presence of acetate also increases the phagocytosis of macrophages and neutrophils and their ability to kill bacteria (29). On the other hands, these metabolites may prevent T cell activation and proliferation, thereby, making the host more susceptible to the infection (30, 31). Overall, such alterations may occur through the gut-lung axis by suppressing systemic immune responses and providing a favorable environment for MTB pathogenesis in TB patients.

The Firmicutes phylum composition was not significantly different between Iranian [MRD: 26.63; P>0.05] and Afghan [MRD: 20.77; P>0.05] sputum samples and blood samples of two evaluated races (Iranian [MRD: -5.10; P>0.05]; Afghan [MRD: -26.0; P>0.05]). It is also well known that Firmicutes are implicated in the metabolism and energy balance of the microbiota, just as Bacteroidetes are (32). Having an imbalance in the Firmicutes/Bacteroides ratio may indicate disrupted homeostasis, pathogenic invasion, or unhealthy conditions (33). In several independent studies, the Firmicutes phylum was detected in significantly reduced numbers in sputum samples of TB patients (20, 26). While there is a knowledge gap in blood microbiota related researches. In the gut-lung axis, there could be a reciprocal causal relationship between imbalanced Firmicutes and TB infection. There is some evidence that the imbalanced Firmicutes composition may cause susceptibility to TB infection or the reactivation of latent TB infections. Meanwhile, dysregulated immunity caused by TB infection might also contribute to imbalanced Firmicutes (20). As with Bacteroidetes, Firmicutes is also a major producer of SCFAs (22). Following dysbiosis of the gut microbiota, Firmicutes imbalance in blood and sputum samples can increase the pathogenic potential of MTB (34). Briefly, alterations in the abundances of Bacteroidetes and Firmicutes, the two important bacterial phyla in the gut and lung with immunomodulatory potentials, during the TB infection may cause immune dysregulation and ultimately affects body defense against the infection. On the other hand, the Actinobacteria phylum exhibited a significant increase in Iranian (MRD: 99.47; P<0.001) and Afghan (MRD: 64.27; P=0.005) sputum samples in comparison to the related healthy subjects. This phylum also showed an increase in Iranian (MRD: 30.73; P>0.05) and Afghan (MRD: 33.00; P>0.05) TB positive blood samples compared to the healthy subjects. Proteobacteria also showed an increase in sputum samples of two races compared to the related healthy subjects, but not significant (Iranian [MRD: -28.83; P>0.05]; Afghan [MRD: -24.03; P>0.05]). In line with this evidence, Proteobacteria phyla showed a significant increase in blood samples of Afghan TB active subjects compared to the related healthy controls (MRD: 0.941; P<0.001). While, in related to Iranian blood samples, overexpression but not significant has been detected in Proteobacteria abundance (MRD: 0.398; P=0.27). Several studies have demonstrated an abundance of Proteobacteria and Actinobacteria in the lung microbiota of people with TB (20, 26, 35) and the results of our study are in line with these findings. There are several opportunistic pathogenic genera within the Actinobacteria and Proteobacteria, which disrupt mucosal barriers (36). As a result, the gut-lung axis may be responsible for the high abundance of these phyla in the blood and lung microbiota (37).

There are some limitations in this study that can influence on results interpretation. It cannot completely indicate the potential impact of microbiota prevalence in Iranian and Afghan TB patients because sample size is limited. In addition, because of the limited phyla evaluated in the current study, the possible effect of some bacterial genera and species could improve the results. Although the more developed, specific, and sensitive, but expensive molecular techniques, in particular next generation sequenc-

ing (NGS) could more accurately present and compare the microbiota profile in Iranian and Afghan participants. Finally, as with any research studies, restrictions associated with possible economical bias should be noted.

CONCLUSION

Our findings emphasized that an imbalance in Bacteroidetes and Firmicutes abundance may cause an imbalance in SCFA constitution and alter the microbiota environment, resulting in dysregulated immune responses and resulting in the augmentation of opportunistic pathogens during TB infection, notably Proteobacteria and Actinobacteria. In future MTB infection control strategies, dysbiosis of normal microbiota may be an essential topic to consider because it plays a major role in pathogenesis. Alteration in the microbial composition of gut and lung following anti-TB therapy have immunomodulatory effects and should be included in the treatment strategies. Evaluation of human microbiota during TB infection course may be critical to a deeper understanding of the interactions between the host and the invader.

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