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The oral microbiome of newly diagnosed tuberculosis patients; a pilot study

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ABSTRACT

Background: Changes in oral microbiota composition (dysbiosis) have long been known to play a key role in the pathogenesis of oral and systemic diseases including respiratory diseases. However, till now, no study has assessed changes in oral microbiota following tuberculosis (TB) infection in humans.
Aims: This is the first study of its kind that aimed to investigate oral microbial dysbiosis in newly diagnosed, treatment naïve, TB patients.
Methods: Oral swab samples were collected from newly diagnosed TB patients ($n = 20$) and age, gender and ethnicity matched healthy controls ($n = 10$). DNA was extracted and microbiota analyzed by sequencing the hypervariable (V3–V4) region of the bacterial 16S rRNA gene using Illumina MiSeq platform. Bioinformatics and statistical analyses were performed using QIIME and R.
Results: Bacterial richness, diversity and community composition were significantly different between TB patients and healthy controls. The two groups also exhibit differential abundance at phylum, class, genus and species levels. LEfSe analysis revealed enrichment (LDA scores ($\log_{10}$) >2, $P < 0.05$) of *Firmicutes* (especially *Streptococcus*) and *Actinobacteriota* (especially *Rothia*) in TB patients relative to healthy controls. Gene function prediction analysis showed upregulation of metabolic pathways related to carbohydrates (butanoate, galactose) and fatty acids metabolism, antibiotics biosynthesis, proteosome and immune system signaling.
Conclusion: These observations suggest significant variations in diversity, relative abundance and functional potential of oral microbiota of TB patients compared to healthy controls thereby suggesting potential role of oral bacterial dysbiosis in TB pathogenesis. However, longitudinal studies using powerful metagenomic and transcriptomic approaches are crucial to more fully understand and confrim these findings.

1. Background

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis*, has remained a global public health concern over recent decades. In humans, TB infection occurs when an otherwise healthy person inhales infective aerosol droplets released by active TB patients during sneezing, coughing and talking [9]. Following inhalation, almost 90% of affected individuals do not develop any disease primarily due to an intact immune system. In the remaining cases, the host may either contain the infection and remain asymptomatic for years (latent TB) or develop an active TB disease [38]. Population level surveys indicate that latent TB affects nearly one fourth of the human population worldwide [8]. Following the recent Covid-19 pandemic, the number of active TB patients and TB-related deaths increased significantly. For example, the Global Tuberculosis report in 2022 reported 10.6 million new cases of TB across the globe in 2021 [45]. This figure is an addition to the 1.6 million TB-related deaths in the same year marking TB as the second most common cause of death from an infectious disease.

TB disease primarily affects the lungs (pulmonary TB) but around 15% of cases involved infection in other body sites (extrapulmonary TB) [2]. The most common sites for extrapulmonary TB include lymph nodes, central nervous system, bones and joints, abdomen and oral

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cavity. In the oral cavity, TB lesions commonly present as ulcers at any site, especially tongue and salivary glands. A recent metanalysis reported that in 249 (82%) out of 301 cases, oral lesion was the first manifestation of TB. Moreover, almost half of these cases have no lung involvement [10]. Pathogenesis of TB, whether pulmonary or extrapulmonary, involves three main stages: infection, latent or active TB disease, and treatment response or failure [32]. So far, several different risk factors responsible for progression of TB from one stage to the next have been documented. Recently, emerging research evidence suggests changes in microbiome composition at distant body sites, such as the gut, following infection with TB in the lungs [14,46].

The human microbiome is defined as a commensal consortium of microorganism including bacteria, fungi and viruses that colonizes different habitats on or within the human body (the gut, oral cavity, skin, nose, genitourinary tract, etc). Under normal circumstances, these commensal microbial ecosystems play an important role in nutrition, physiology and immune system development. The oral microbiome is the second most diverse ecosystem of the human body, behind the gut microbiome. By composition, the oral microbiome in humans consists of >700 different species of bacteria, fungi, viruses and protozoa [7]. The oral microbiome is crucial for maintaining oral and systemic health. However, there are times when the microbial diversity and or functional potential of the oral microbiome is disturbed resulting in microbial dysbiosis. Oral microbial dysbiosis has been implicated in several common oral pathologies such as dental caries, gingivitis, periodontitis and even oral cancer [31,44]. Similarly, current research suggests a link between systemic diseases such as cancer, Alzheimer's disease, rheumatoid arthritis, diabetes and preterm birth, and oral microbiome dysbiosis [35]. However, until now, there have been no studies that explore the oral microbiome in TB patients. Thus, the current study was performed to explore oral microbiome diversity in TB patients in comparison with age- and gender-matched healthy controls.

2. Methodology

2.1. Study design and participants recruitment

This study was conducted as part of a larger, longitudinal cohort study aiming to explore the role of the microbiome in TB pathogenesis, treatment response and outcome [40]. A case control study design was employed to recruit newly diagnosed, treatment naïve TB patients at the outpatient department of Medical Teaching Institute Khyber Teaching Hospital (MTI – KTH) Peshawar, Pakistan from November 2021 – May 2022. The study only included adults (age 18 years and above) who were diagnosed with TB after detailed history, clinical examination and laboratory assessments (sputum smear microscopy and Xpert MTB/RIF assay) following national guidelines for control of TB in Pakistan. The controls were healthy subjects with no history of pulmonary TB, matched for sex and age (± 3 years) and randomly selected from non-family neighbors of the TB patients. Participants who were previously diagnosed/treated for TB, who were severely anemic, currently or recently receiving broad-spectrum antibiotics or prescription mouthwashes or those who were pregnant and/or lactating at the time of enrollment were excluded from the study. All participants matching the eligibility criteria of the study were invited to participate in the study by providing a participant information sheet with detailed information about the study in an easy to understand local (Pashto) language. Those who agreed to participant in the study were asked to sign an informed consent form. Ethical approval for the study was obtained from the Ethical Review Board of Khyber Medical University, Pakistan (Ref Number: DIR/KMU-EB/PR/000858). Based on the inclusion and exclusion criteria, a total of 30 participants (20 TB patients and 10 healthy controls) were recruited.

2.2. Collection of demographic and oral health data

Socio-demographic characteristics and oral health and hygiene practice data were collected using an interviewer-administered, structured questionnaire. The demographic characteristics included age, gender, education and socioeconomic status information. Data regarding oral health and hygiene habits, and self-reported oral health status were also recorded using a previously validated questionnaire [37].

2.3. Samples collection

Oral rinse samples were collected from TB patients and control subjects following a standard protocol [47]. The samples were collected between 9 and 11 am. The participants were instructed to refrain from eating food, or from drinking flavoured juices or carbonated drinks for at least two hours prior to sample collection. The participants were asked to swish 10 mL of sterile phosphate buffer saline solution for one minute and expectorate the contents into a sterile, 50 mL Falcon tube. The collected samples were vortexed thrice for 30 s followed by centrifugation at 14000*g* for 10 min to obtain a pellet. The pellet was stored at −80 °C until analysis.

2.4. DNA extraction

The phenol chloroform (organic) method was used for DNA extraction. For this purpose, the saliva pellet was dissolved in 550 μL of lysis solution A (0.32 mM sucrose, 10 mM Tris at pH 7.5) 5 mM $MgCl_2$ and 1% Triton). Samples were centrifuged at 13000 rpm for 1 min followed by addition of 500 μL lysis solution B (10 mM Tris at pH 7.5, 400 mM NaCl, 2 mM EDTA) and gentle mixing to ensure homogenization. Samples were incubated for 30 min at 60 °C followed by addition of 15 μL protein kinase and 20% sodium dodecyl sulphate (SDS) solution and an overnight incubation at 56 °C. The next day, 500 μL of phenol, chloroform and isoamyl alcohol (25:24:1 ratio; PCI solution) were added to the samples and, following mixing, the samples were centrifuged at 13000 rpm for 10 min and the aqueous phase was transferred to a fresh tube. The aqueous layer was then treated with an equal volume of chloroform and isoamyl alcohol (24:1) and centrifuged again for 10 min at 13000 rpm. The aqueous layer was transferred to a fresh 1.5 mL centrifuge tube and 55 μL of 0.3 M sodium acetate and 500 μL of chilled isopropanol were added. After an overnight incubation at −20 °C, the samples were centrifuged at 13000 rpm for 10 min. The supernatant was discarded and the pellet was treated with 300 μL of 70% ethanol and centrifuged again at 10,000 rpm for 5 min in order to remove all impurities. The pellet was retained and air dried, while the supernatant was discarded. The DNA pellet was resuspended in TE Buffer (10 mM Tris, 1 mM EDTA, pH 8) and stored at 4 °C. DNA quality and quantity were assessed by 1% agarose gel electrophoresis and spectrophotometry (absorbance at 260/280 nm).

2.5. Next generation sequencing and bioinformatics

The V3–V4 region of the bacterial 16S rRNA gene was amplified with universal forward and reverse primers followed by library construction and quality control. The purified DNA was then subjected to high throughput, pair-end sequencing of the hypervariable (V3-V4) region using a Miseq™ PE300 sequencing platform (Illumina, San Diego, CA, USA). The raw sequencing files were demultiplexed and the resulting quality-filtered FASTQ files were analyzed using the Quantitative Insights into Microbial Ecology (QIIME2 v 2022.4) [4] package in Linux. The Manifest file method was used to import the reads and the quality checking of the reads was carried out using DADA2 which is a denoising tool in QIIME2 [6]. For improving of the data quality, chimeric sequences and sequences falling below the Phred Quality Score < 20 were filtered. QIIME2 also provided the representative sequences tables along

with the frequencies of the Amplicon Sequence Variants (ASVs). The q2-feature-classifier [3], which is based on a machine learning method called classify-sklearn naïve bayes, was used to assign taxonomy to the representative sequences. The classifier was trained on the basis of the V3-V4 region of 16S rDNA using SILVA v138 (http://www.arb-silva.de/), an OTU reference sequences database. Each sample's taxonomic composition was visualized using relative abundance at phylum, class, order, family, genus and specie level through taxa-bar-plots in R.

2.6. Statistical analysis

Descriptive statistics were performed by calculating frequencies and percentage for the categorical variables, and mean and standard deviation for the numerical variables. Depending on the type of the variable, differences in demographic characteristics and oral health and hygiene habits between the TB patients and healthy controls were assessed by chi square test and Student's *t*-test using SPSS version 25. A p-value of <0.05 was considered significant.

Diversity analysis of the data was carried out through the Phyloseq package in R (The R Foundation for Statistical Computing, Vienna, Austria). A total of 5 measures (ACE, Fischer, Observed, Shannon and Simpson) were used to calculate the alpha diversity (within sample diversity). The alpha diversity measures were compared for significance using the Wilcoxon rank-sum test. Beta diversity (diversity between samples) was calculated using the UniFrac method [27], which is a qualitative method to measure the dissimilarity between communities by incorporating the phylogenetic relationships between the features. Principle Co-ordinate Analysis (PCoA) was also incorporated along with the UniFrac method for computation of beta diversity. The permutational ANOVA (PERMANOVA) test was used for significance analysis of beta diversity measures. The DESeq2 [26] package in R language was used to explore the differentially expressed taxonomic hierarchy at phylum, genus and specie level. Linear discriminant analysis (LDA) effect size (LEfSe) was performed to determine the taxa which contribute to the effect size between the TB patients and healthy controls. For functional predictions, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States2 (PICRUSt2) [13] was used to determine the microbial associated functional capacity. The relative abundance of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) was predicted in each sample. The *ggpicrust2* program was used to annotate the KO IDs against the KEGG pathways.

3. Results

3.1. Demographic characteristics and oral health and hygiene habits of the participants

A total of 30 participants, including TB patients ($n = 20$) and healthy controls ($n = 10$), were recruited. No significant differences were observed among the participants in terms of gender, age, education level and household income (Table 1). All of the participants reported that they clean their teeth at least once per day (86.7%) in the morning (83.3%) using a tooth brush and tooth paste (73.3%). A small proportion of the participants (16.7%) also reported using Miswak as a teeth cleaning tool. Around two thirds of the participants reported that they have never visited a dentist. Around three in every four participants (73.3%) reported good oral health with no oral health issues (46.7%). However, 23.3% of the participants reported gum bleeding when brushing teeth or eating hard foods.

3.2. Bacterial community richness and diversity

To investigate whether the oral microbiome was altered following TB infection, the diversity indices (alpha and beta diversity) and relative abundance at different taxonomic levels were calculated using 16S rRNA gene sequence data. Alpha diversity, measuring the observed richness or evenness of bacterial species in a community, was assessed using different diversity indices. As shown in Fig. 1, all diversity indices (ACE, Fisher, Observed, Shannon and Simpson) were significantly different between the TB patients and healthy controls groups. The results further indicate that newly diagnosed TB patients had a significantly more diverse oral microbiome composition than healthy controls.

The same trend was also observed in the beta diversity indices, which is a measure of the variability in different samples community composition. For this purpose, PCoA analysis based on the Unweighted UniFrac method was used to assess differences between the two groups. As shown in the Fig. 2, core microbiomes in TB patients and healthy

Table 1
Socio-demographic characteristics and oral health and hygiene habits of the study participants.

Participant characteristics	Participant category		Total, $N = 30$ (%)	P-value
	TB patients, N 20 (%)	Healthy controls, N 10 (%)		
Participant gender				
Male	7 (35)	5 (50)	12 (40)	0.344
Female	13 (65)	5 (50)	18 (60)	
Age (± SD)	27.1 ± 8.8	25.4 ± 5.3		0.08
Education categories				
No formal education	6 (30)	2 (20)	8 (26.7)	
Primary school	8 (40)	3 (30)	11 (36.7)	
High school & College	2 (10)	3 (30)	5 (16.7)	0.566
University level	4 (20) 20.0%	2 (20) 20.0%	6 (20) 20.0%	
Monthly household income (± SD)	27,150 ± 12,201	43,500 ± 15,465		0.411
Total no of household members				
≤ 5 members	4 (20)	1 (10)	5 (16.7)	0.11
6–10 members	8 (40)	8 (80)	16 (53.3)	
> 10 members	8 (40)	1 (10)	9 (30)	
Do you clean your teeth regularly?				
Yes	20 (100)	10 (100)	30 (100)	a
No	0 (0)	0 (0)	0 (0)	
How often/times do u clean/brush your teeth per day? Think about one year ago				
1 time per day	17 (85)	9 (90)	26 (86.7)	0.53
Two times per day	1 (5)	1 (10)	2 (6.7)	
>2 times per day	2 (10)	0 (0)	2 (6.7)	
What time do you clean/brush your teeth? Multiple answers possible				
Morning only	17 (85)	8 (80)	25 (83.3)	0.874
Morning and before going to bed	1 (5)	1 (10)	2 (6.7)	
Other (e.g. prayer times)	2 (10)	1 (10)	3 (10)	
Which of the following you use to clean your teeth? These may not be mutually exclusive.				
Tooth brush and tooth paste	15 (75)	7 (70)	22 (73.3)	0.94
Miswak	3 (15)	2 (20)	5 (16.7)	
Dental floss	2 (10)	1 (10)	3 (10)	
Do you use fluoride tooth paste for teeth cleaning				
No	3 (15)	2 (20)	5 (16.7)	0.551
Yes	17 (85)	8 (80)	25 (83.3)	
How often do you visit a dentist?				
Regularly (every 6 months)	4 (20)	1 (10)	5 (16.7)	0.769
Only when there is need e.g. dental pain	3 (15)	2 (20)	5 (16.7)	
I have never visited dentist	13 (65)	7 (70)	20 (66.7)	
Self-reported oral health				
Fair/Poor	5 (25)	3 (30)	8 (26.7)	0.548
Good/Very good	15 (75)	7 (70)	22 (73.3)	
Currently or in the past 12 months, have you had experienced any issue related to your teeth				
No	14 (40)	6 (60)	14 (46.7)	0.426
Toothache	2 (10)	1 (10)	3 (10)	
Sensitivity	3 (15)	0 (0)	3 (10)	
Gum bleeding	4 (20)	3 (30)	7 (23.3)	
Other	3 (15)	0 (0)	3 (10)	

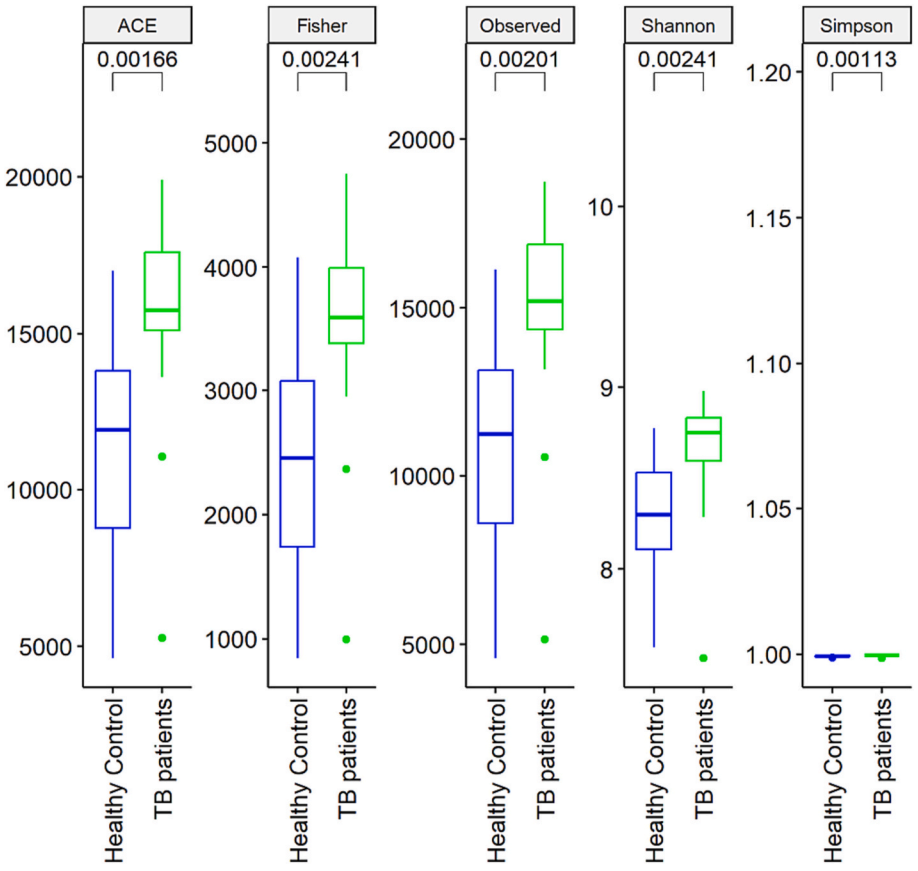

Fig. 1. A: Alpha diversity indices of the oral microbiota in TB patients and health controls. Each of the five alpha diversity indices (ACE, Fisher, Observed, Shannon and fSimpson) was found to be significantly different between the two groups as assessed by Wilcoxon test. From bottom to top, each box plot indicates minimum, first quartile, median, third quartile and maximum values.

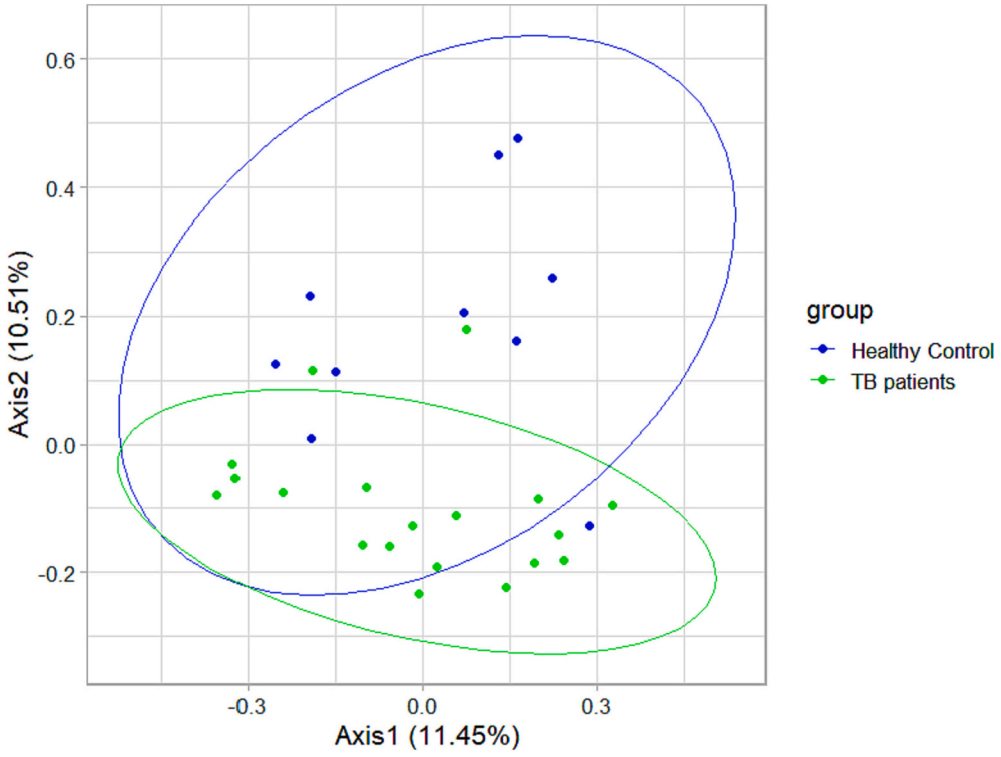

Fig. 2. Principal coordinate analysis (PCoA) of bacterial communities in TB patients and healthy controls. The first and second coordinates are represented by horizontal and vertical axes in the PCoA plot and represent the proportion of variance in bacterial communities between the two groups (shown by percentage).

controls cluster separately thus indicating that the oral bacterial community composition in TB patients is generally distinct from that of healthy controls.

3.3. Oral bacterial community composition in TB patients and healthy controls

Following quality control, a total of 10,073,899 sequences with an average of 347,375 reads and a total of 149,664 OTUs were obtained after clustering at 97% similarity. The OTUs were further grouped into phyla, classes, orders, families, genera and species. Overall, 27 phyla

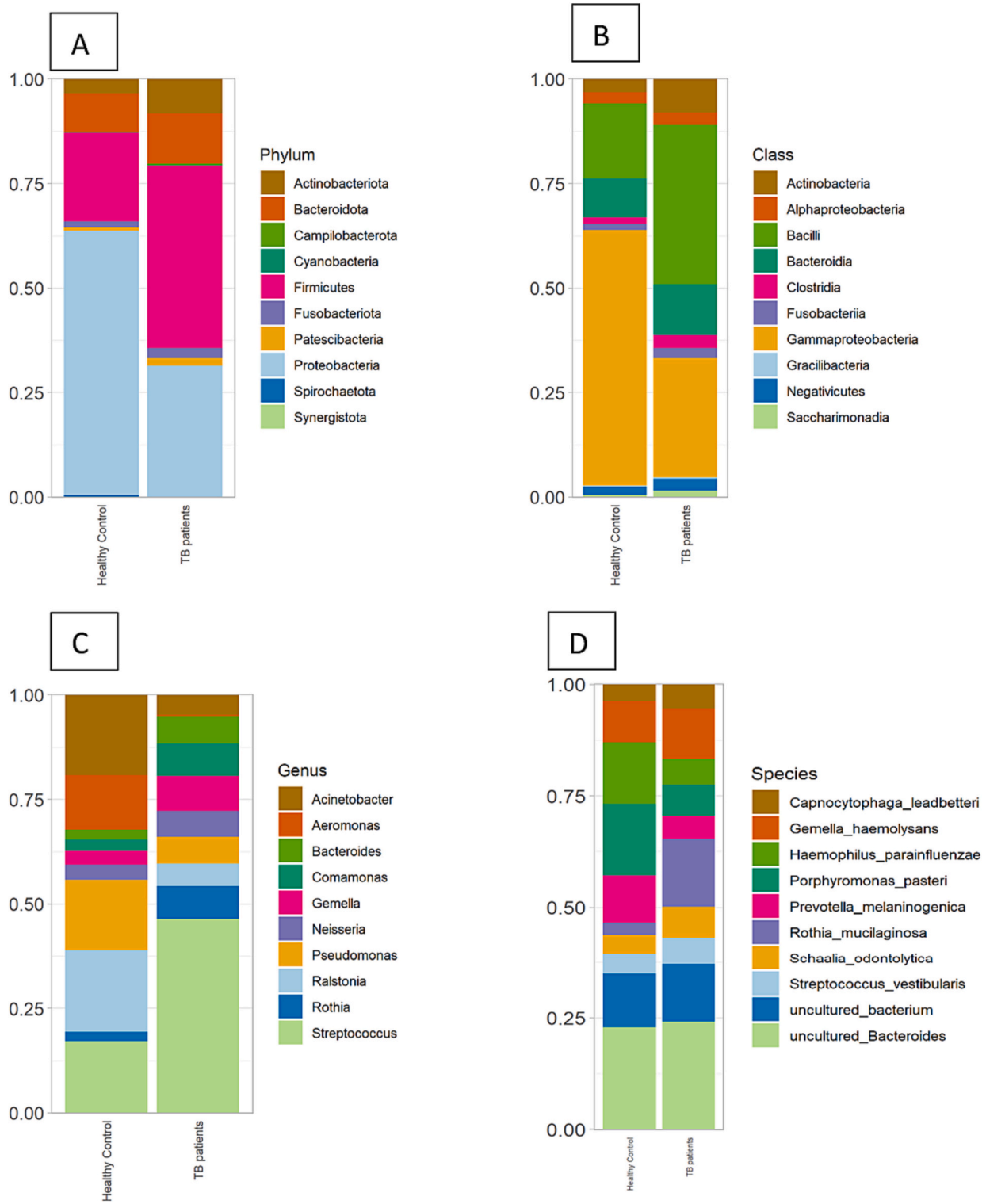

Fig. 3. The relative abundance of the ten most abundant bacterial taxa, at phylum, class, genus and species level, in the TB patients and healthy controls. A, B, C and D show the relative abundance of the top 10 phyla, classes, genera and species present in saliva samples of TB patients and healthy controls.

were identified in TB patients and 19 in healthy controls. Fig. 3 presents the relative abundance of the top 10 bacterial taxa (at phylum, class, genus and species level) in the TB patients and healthy controls.

Proteobacteria was the most abundant phylum accounting for 63% of bacteria in healthy controls followed by *Firmicutes* (21%) and *Bacteroidota* (9%). In contrast, *Firmicutes* were predominant (43%) in saliva samples of TB patients followed by *Proteobacteria* (31%) and *Bacteroidota* (12%). Marked differences were also observed in relative abundance of bacteria at class level. As shown in Fig. 3B, *Bacilli* was the predominant class, at 38%, in oral samples of TB patients followed by *Gammaproteobacteria* (28%) and *Bacterioidia* (12%). However, in the healthy control group, the most abundant bacterial class was the *Gammaproteobacteria* (60%) followed by the *Bacilli* (18%) and *Bacterioidia* (9%). Over 411 different bacterial genera were identified in TB patients and 275 in healthy controls. As shown in the Fig. 3C, *Streptococcus* is the predominant (28%) genus in TB patients followed by *Gemella* (5%) and *Rothia* (4%). However, in healthy controls, *Acinetobacter* (14%), *Ralstonia* (14%), *Pseudomonas* (12%) and *Aeromonas* (9%) were the most commonly identified genera. A total of 458 bacterial species were identified in TB patients and 342 in healthy controls, but only 22–24 had abundances of ≥1%. The oral microbiome of TB patients was dominated by *Rothia mucilaginosa* (7.78%), *Gemella haemolysans* (5.79%), *Schaalia odontolytica* (3.59) and *Porphyromonas pasteri* (3.57%). In contrast, *Porphyromonas pasteri, Acinetobacter junii, Haemophilus parainfluenzae, Prevotellan melaninogenica* and *Haemophilus parahaemolyticus* were the most abundant species in the oral microbiome of healthy controls. A heatmap of the relative abundance of the 30 most abundant species is presented in Fig. S1.

3.4. Alteration in bacterial taxa between TB patients and healthy controls

To further discern taxonomic differences between the two groups, LEfSe analysis was performed which showed significant differences in bacterial abundance between the TB patients and healthy controls at phylum and genus level with LDA scores ($\log_{10}$) >2, $P < 0.05$. The histograms in Fig. 4 represent potential biomarkers differentiating between the two groups. At phylum level, *Firmicutes* and *Actinobacteriota* were significantly more abundant in TB patients while *Proteobacteria, Spirochaetota* and *Synergistota* displayed a significantly higher relative abundance in healthy controls (Fig. 4A). The same trend was also followed at genus level wherein a total of 28 genera exhibited a significantly higher relative abundance in TB patients. Of these, *Streptococcus* was the most common bacterial genus with highest abundance in TB patients followed by *Comamonas, Rothia, Bacteriodes* and *Actinomyces*. However, *Ralstonia, Sphingomonas* and *Alloprevotella* were the most abundant bacterial genera in healthy control samples (Fig. 4B).

3.5. Differences in gene functions between TB patients and healthy controls

We further performed a PICRUST analysis to evaluate the effect of TB infection on oral microbiome functional potentials. The oral samples from the TB patients and controls gave distinct KEGG profile thus indicating dissimilar microbial functional potential. A total of 84 metabolic pathways that were differentially enriched between the two groups (TB patients and healthy controls) were identified and are presented in Table S1. Of these, 55 of the KO pathways identified in TB patients and healthy controls samples were metabolic pathways related to biosynthesis and degradation. Fig. 5 present the top 30 most altered metabolic pathways (Fig. 5) identified in TB patients and healthy controls. As shown in the figure, only 10 KO pathways were enriched in TB patients. Overall, metabolic (glutathione, tyrosine, riboflavin, butanoate, propnoate) and xenobiotic degradation pathways (benzoate, flourobenzoate, ethylbenzene, aminobenzoate, fatty acids, styrene, toluene) were enriched in healthy controls while antibiotic biosynthetic pathways (vancomycin, streptomycin, neomycin, kanamycin and gentamycin) were enriched in TB patients.

Rows indicate the corresponding KEGG pathways and KO terms, and columns indicate the TB and control participants. Dark blue and negative values indicate downregulation while red and positive values indicate upregulation.

Statistical differences in KEGG pathways between the TB patients

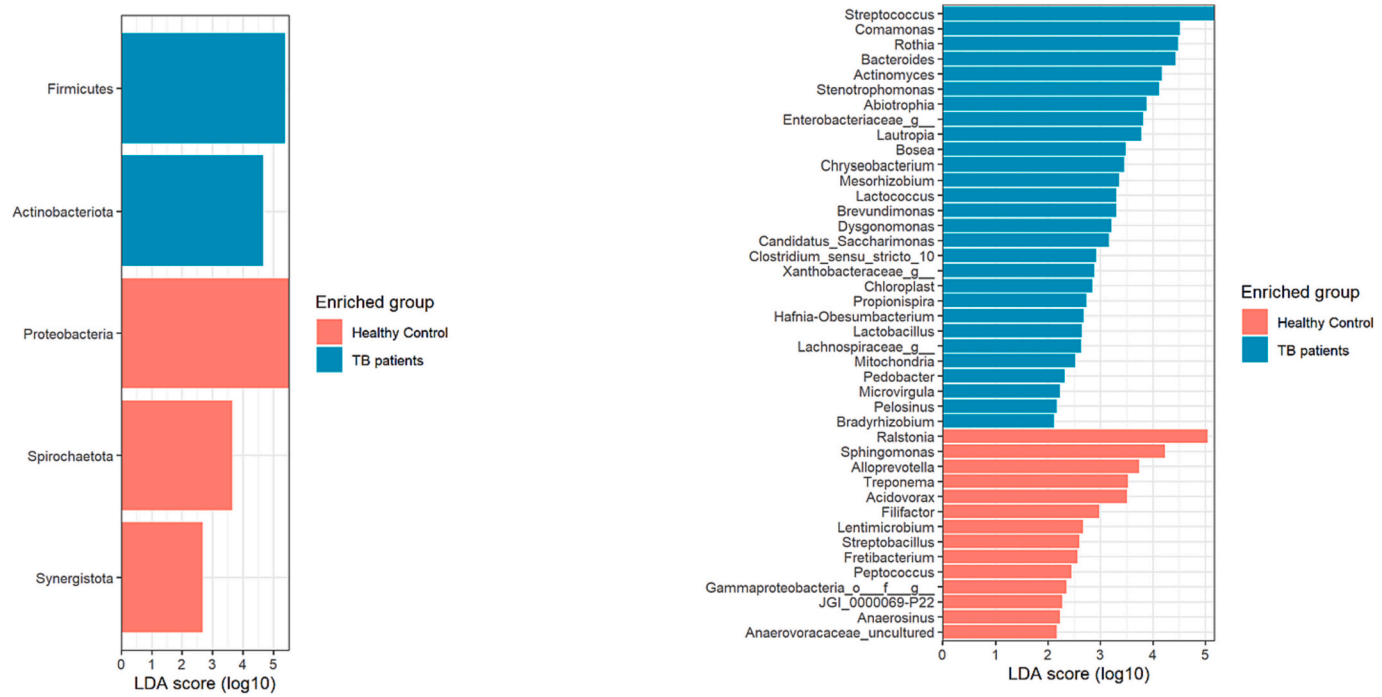

Fig. 4. Linear discriminant analysis Effect Size (LEfSe) at genus and phylum level between the two groups. An LDA score >2 was used to determine significant differences in abundance between the two groups. (A) Significant differences in abundance between TB patients and healthy controls at phylum level. (B) Significant differences in abundance between TB patients and healthy controls at genus level.

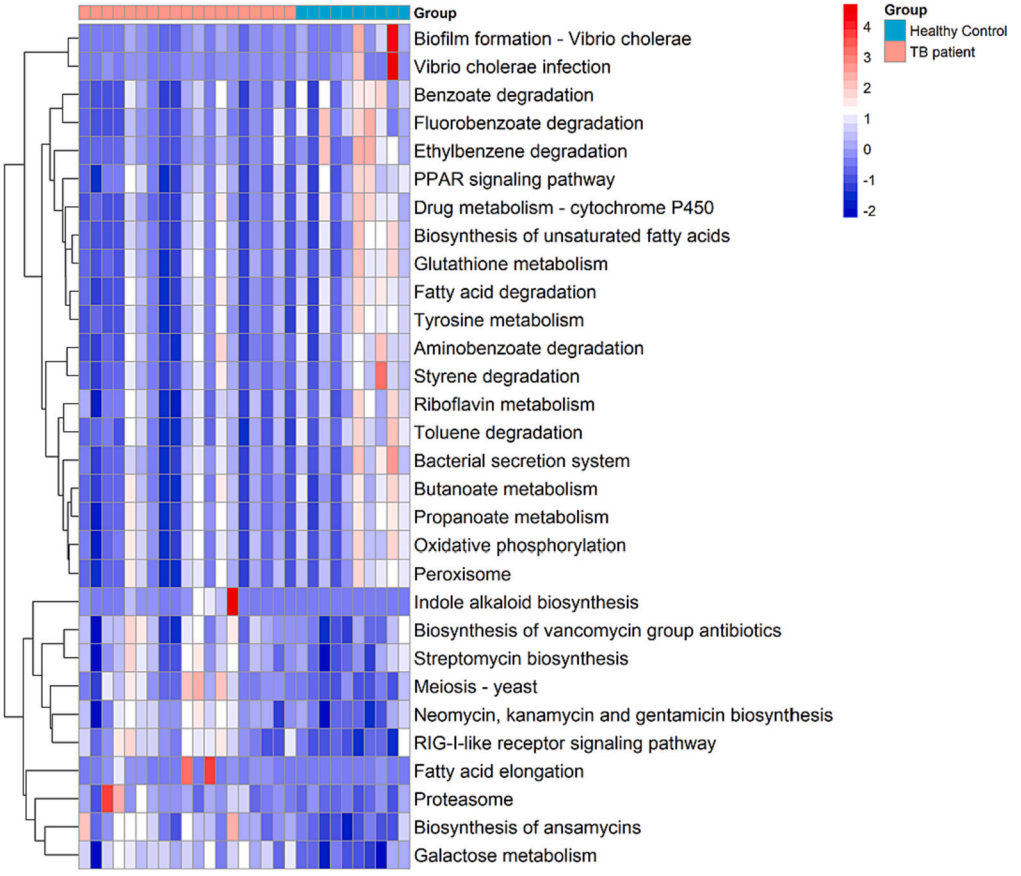

Fig. 5. Heatmap of the top 30 enriched KEGG pathways in TB patients and healthy controls.

and healthy control groups were also calculated. As shown in Fig. 6, significant differences in the relative abundance of the top 30 enriched pathways in TB patients and healthy controls were observed. Overall, the biosynthesis and degradation pathways were significantly more abundance in healthy controls. Only the relative abundance of functional genes related to metabolism of carbohydrates (butanoate, galactose) and fatty acids, antibiotics biosynthesis, proteosome and immune system signaling (Fig. 6) were found significantly more abundant in TB patients.

4. Discussion

Poor oral health has long been recognized as an important risk factor for systemic diseases including diseases of the lungs. The association between oral health and pulmonary diseases such as pneumonia, asthma and chronic obstructive pulmonary diseases is frequently reported in the literature [20]. However, the biological mechanism underlying these associations is largely unexplored and poorly understood. One of the possible mechanisms underpinning these association is the microbiome present in the oral cavity and lungs. The oral cavity harbors one of the

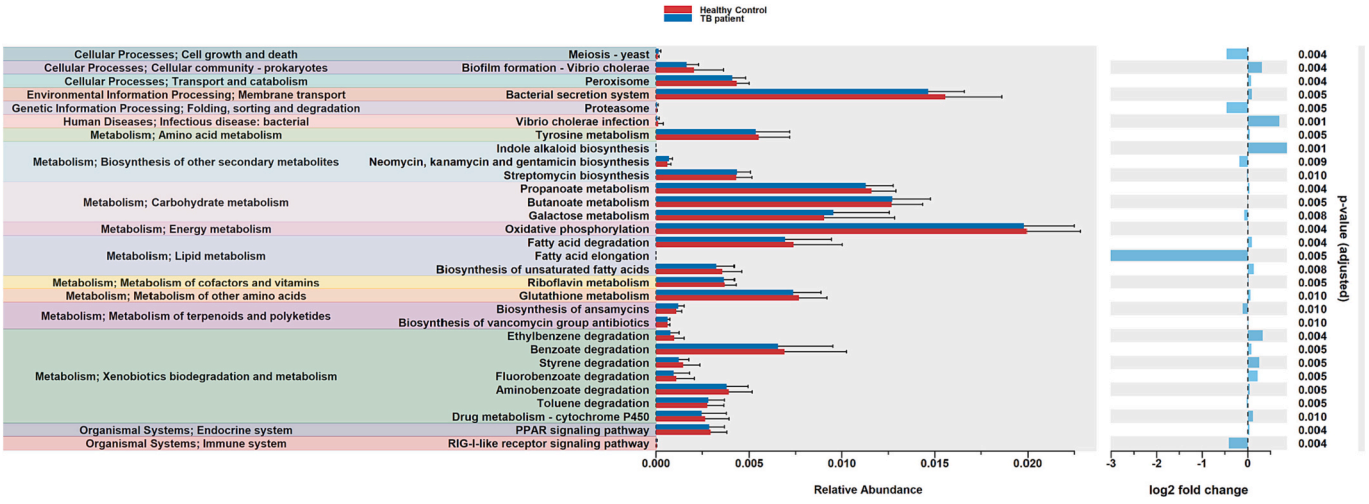

Fig. 6. Relative abundance of metabolic pathways encode in each input sample of TB patients and healthy control Student *t*-test was utilized to compare TB patients and healthy control using KEGG second level functional group.

most diverse microbial ecosystems (oral microbiome) in the human body owing to its large surface area, constant bathing in saliva, moderate pH and temperature, and the availability of oxygen and nutrients [22]. The oral cavity is also a gateway connecting the respiratory tract with the outside environment and the entry point of microbiota into the gut and lungs [29]. Despite the fact that the oral cavity and lungs are two different anatomical sites, striking similarities between the oral and lung microbiome are reported in the literature [1]. It is now believed that there exists an oral-lung axis wherein changes in the composition and functional potential of one microbiome results in the altered immune response and changes in the other [18]. So far, oral microbiome dysbiosis has been reported in pneumonia, cystic fibrosis, asthma, COPD and lung cancer [12].

In the current study, the oral microbiota samples of TB patients were found to exhibit higher alpha diversity (species richness and evenness) measures and to cluster significantly differently with respect to healthy controls. Although no study has previously explored the oral bacterial diversity in TB patients, there are a few reports comparing the oral microbiome diversity in other pulmonary diseases. To further explore the microbiome differences, we looked at unique and overlapping genera across TB (current study) and recently published reports on oral microbiome diversity in other pulmonary diseases: asthma, allergic rhinitis and chronic obstructive pulmonary disease [25,36]. A Venn diagram of the unique and share genera between these studies is presented in supplementary Fig. S2. As shown in the figure, there were 37 genera shared across these three different pulmonary diseases; the commonest being *Prevotella, Treponema, Lactobacillus, Veillonella, Gemella, Neisseria, Corynebacterium* and *Serratia*. The results of the current study confirm and reinforce the finding of recent studies reporting a higher diversity and distinct oral microbiota composition in COPD, allergic rhinitis and asthmatic patients than in healthy controls. However, there are some reports showing either no change or a lower oral bacterial diversity in patients with pulmonary disease with respect to healthy controls [15,16,42]. Given the discrepancy in alpha-diversity patterns among different pulmonary diseases and conditions, the possibility that oral alpha diversity measures are reliable indicator of TB status or pathogenesis would require a more extensive study.

At phylum level, the oral bacteriomes in the current study consisted mainly of *Proteobacteria, Firmicutes, Bacteroidota* and *Actinobacteria.* Although, these bacteria generally constitute the most abundant bacterial phyla in the oral microbiota [5,34,39], the relative abundance varied significantly between the TB and control groups as revealed by LefSe analysis with the threshold LDA scores of 2.0. *Firmicutes* were twofold more abundant (43.8%) in the oral microbiome of TB patients than in healthy controls (21.1%) samples. These results are in concordance with the findings with previous research showing a significantly higher abundance of *Firmicutes* (36.9%) in the salivary microbiome of COPD patients [24]. At genus level, LefSe analysis showed enrichment of the oral microbiome of TB patients with 28 distinct bacterial genera. The current study found a significantly higher abundance of streptococci in the oral microbiome of TB patients (28%) than healthy controls (12.5%). *Streptococcus* is a large genus of Gram-positive bacteria with excellent adaptation potential at various body sites including oral cavity, lung and upper respiratory tract. These finding are in contrast to some previous reports where a significantly higher relative abundance of streptococci was reported in healthy controls than in asthmatic patients [17]. However, such finding are not unexpected since several Streptococcal species, including *Streptococcus salivarius* and *Streptococcus oralis*, are commensal species abundant in the oral microbiome of healthy individuals and provide protection against pathogenic infections. Indeed, preliminary research suggests that nasal spray treatment containing streptococcal species are effective in reducing the occurrence of upper respiratory tract infections [33,43]. The oral microbiome of TB patients was also enriched with *Rothia,* a Gram-positive coccus belonging to the family Micrococcaceae. *Rothia mucilaginosa* is a member of the normal oral microflora of humans. Although this bacterium has a low virulence potential, the higher relative abundance of *R. mucilaginosa* in TB patients is alarming since it has been recognized as an opportunistic pathogen in immunocompromised patients [28]. For examples, *R. mucilaginosa* was reported as an opportunistic pathogen in almost 83% of cystic fibrosis patients [23]. Similarly, *Gemella haemolysans*, a Gram-positive facultatively anaerobic bacterium, was also significantly higher in abundance in TB patients (5.79%) than healthy controls (3.35%). *G. haemolysans* is among the most common bacterial species in the human oral microbiome with proven hemolytic activities [11]. A recent in vitro study has shown that *G. haemolysans* inhibits the growth of *Porphyromonas gingigivalis*, a common periodontal pathogen in the oral cavity [30]. The results presented herein are consistent with the view that a higher abundance of this bacterium may be responsible for the reduced abundance of *P. gingigivalis* in the oral microbiome of TB patients (0.03%) compared to healthy controls (0.5%).

Marked differences in the KO pathways, especially those related to metabolism, xenobiotic degradation and antibiotic synthesis, were observed in the oral microbiome of TB patients and healthy controls (Fig. 5, Table S1). An overall downregulation of the majority of metabolic pathways was observed in TB patients thereby suggesting a decrease in metabolic potential of the TB patients. These findings are in concordance with the study by *Yongfei Hu* et al. who reported decreased enrichment of metabolic pathways in the gut microbiome of TB patients compared to healthy controls [21]. It is worth noting that pathways related to carbohydrate metabolism and cellular processes such as cell growth and death were significantly enriched in TB patients thereby indicating the higher diversity in this group [41]. In contrast, glutathione metabolism was significantly decreased in TB patients. Glutathione is an antioxidant that can help reduce inflammatory response and cell damage in the host [19]. Reduced glutathione metabolism may indicate a key pathway for inflammatory damage in TB patients. Inflammation is also an important underlying mechanism connecting the oral microbiome with pulmonary diseases such as pneumonia [18]. Although not confirmed, the same may hold true for TB.

4.1. Strengths and limitations of the study

The major point of this study was to determine whether the oral microbiome of newly-diagnosed TB patients, with no history of broad-spectrum antibiotic use, may be linked with TB susceptibility or be impacted by TB. Since it is extremely difficult to find and recruit study participants with such strict inclusion and exclusion criteria, no previous study has been reported to discern any link between TB infection and the oral microbiome. The current study found a higher number of OTUs and oral microbiota diversity in TB patients, a finding that was enabled by the high quality of the collected samples and the NGS analysis. Furthermore, recruitment of an age and gender matched healthy control group from the same local area, and with similar socioeconomic backgrounds, oral health and hygiene habits would limit any study bias. However, the current study does incorporate some limitations. The first and most important is the small samples size due to the strict inclusion criteria and limited recruitment time making it difficult to generalize the study findings. Secondly, due to the cross-sectional nature of the study, it was not possible to explore the variation in oral microbiome diversity and functional potential at different stages of TB. Third, the use of 16S rRNA gene sequencing technology greatly limited the ability to identify bacteria at species and strain level. Finally, it was not possible to collect lower airway samples to compare with the oral microbiome in order to determine how the lung and oral microbiomes might interact with each other during TB. However, this is an ongoing study and such limitation will be addressed in future work.

5. Conclusion

To our knowledge, this is the first study assessing oral bacterial diversity in newly-diagnosed TB patients, and age and gender-matched

healthy controls. We show that the oral bacteriome of TB patients is significantly different in diversity, relative abundance and functional potential. The study findings suggests that oral bacterial dysbiosis and associated changes in functional potential may influence the occurrence of TB and/or be impacted by the disease. However, there is an urgent need for more extensive, longitudinal studies and the application of more powerful metagenomic and transcriptomic approaches to confirm and extend these findings, and to provide insight into the nature of the indicated link between the oral microbiome and TB.

Author statement

AI was not used in the composition of this manuscript.
The authors declare no competing interests.
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CRediT authorship contribution statement

Muhammad Shahzad: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition. **Muhammad Saeed:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Humaira Amin:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Nada Binmadi:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft. **Zafar Ullah:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft. **Sana Bibi:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft. **Simon C. Andrew:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2024.110816.

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