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Alterations in the intestinal microbiota associated with active tuberculosis and latent tuberculosis infection☆

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ABSTRACT

Objectives: To study the characteristics of intestinal microbiota at different stages of *Mycobacterium tuberculosis* infection.
Methods: Fecal samples of 19 active tuberculosis (ATB) patients, 21 latent tuberculosis infection (LTBI) individuals, and 20 healthy controls (HC) were collected. Gut microbiota of all the participants were analyzed by 16S rDNA sequencing. Clinical information of ATB patients was also collected and analyzed.
Results: Both ATB and LTBI groups showed significant decreases in microbial diversity and decline of *Clostridia*. For ATB patients, bacteria within phylum Proteobacteria increased. While for LTBI individuals, genera *Prevotella* and *Rosburia* enriched. The abundance of *Faecalibacterium, Clostridia* and *Gammaproteobacteria* has the potential to diagnose ATB, with the area under the curve (AUC) of 0.808, 0.784 and 0.717. And *Prevotella* and *Rosburia* has the potential to diagnose LTBI, with the AUC of 0.689 and 0.689. Notably, in ATB patients, the relative abundance of *Blautia* was negatively correlated with the proportions of peripheral T cells and $CD8^+$T cells. And serum direct bilirubin was positively correlated with *Bacteroidales*, while negatively correlated with *Clostridiales* in ATB patients.
Conclusions: The specifically changed bacteria are promising markers for ATB and LTBI diagnosis. Some gut bacteria contribute to *anti*-MTB immunity through interactions with T cells and bilirubin.

1. Introduction

Tuberculosis (TB) is an old disease caused by *Mycobacterium tuberculosis* (MTB) [1]. About a quarter of the world's population is infected with MTB, despite the availability of chemotherapy and the bacilli Calmette-Guerin (BCG) vaccine. What's worse, the coronavirus disease (COVID-19) pandemic has set back the fight against TB [1]. The number of people newly diagnosed with TB has dropped from 7.1 million in 2019 to 5.8 million in 2020 [1]. Moreover, the number of people who died from TB in 2020 has returned to

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the level of 2017 [1]. To end this preventable and curable disease, intensified research is essential.

MTB spreads when active tuberculosis (ATB) patients expel bacteria into the air [1]. Inhalation of aerosol droplets containing MTB with subsequent deposition in the lungs leads to one of four possible outcomes: ① immediate clearance of the organism; ② primary disease: immediate onset of ATB; ③ latent tuberculosis infection, LTBI; ④ reactivation disease: onset of ATB following a period of LTBI [2]. LTBI is a state of persistent immune response to stimulation by MTB antigens, with no evidence of clinical manifestation of ATB [2]. LTBI can be detected by interferon-gamma release assay (IGRA) or tuberculin skin test (TST) [2]. It is estimated that about 2 billion people are LTBI, of which a relatively small proportion (5–10 %) will develop ATB in their lifetime [1]. In high-exposure settings, a sizable percentage of the population remains $TST^{-}/IGRA^{-}$ [3], suggesting that a large number of people are able to clear MTB despite the possible exposure. It is not clear why some people remain resistant to the initial infection. Nor is it clear why 85–95 % of those infected do not progress from LTBI to ATB.

In the history of human TB research, many potential risk factors have been identified [4]. Apart from factors such as nutrition [5], immune status [6], and host genetics [7], there's evidence that the composition and function of the microbiome are also responsible for MTB infection [8]. The results of animal model study showed that MTB aerosol infection could significantly change the intestinal microbiota of mice [9]. The microbiome has been reported to contribute to TB susceptibility primarily by influencing host immune status [10]. For example, intestinal microbiota dysregulation is related to increased early pulmonary colonization of MTB and decreased Th1 immunity in experimental mice [11,12]. The above evidence points to the regulatory role of intestinal microbiota in pulmonary immunity, known as the "gut-lung axis" [13–15].

However, studies [16–20] of changes in the intestinal microbiome of TB patients have yielded inconsistent results. One study [17] found that bacterial communities were more diverse in ATB patients than that in healthy controls, while other studies [16,18–20] came to the opposite conclusion. Similarly, different studies have found inconsistent changes in the diversity and composition of gut microbiota in MTB-infected mice [9,11,21]. Besides, the characteristics of the intestinal microbiome in LTBI patients have not been fully studied. Therefore, we aimed to compare the intestinal microbiota of LTBI and ATB patients.

Here, we report the intestinal microbiota characteristics of MTB infected people in China, an ethnic group with a high TB burden. By using 16S rDNA sequencing, we compared the taxonomic diversity of gut microbiota in 19 ATB patients, 21 LTBI individuals, and 20 healthy controls. The results showed that there were significant differences in composition and diversity of intestinal microbial among the three groups. These findings may provide insights into the role of gut microbes in different stages of the MTB infection cycle.

2. Materials and methods

2.1. Participants Recruitment and sample collection

Patients with newly developed pulmonary TB at Xi'an Chest Hospital were included in the ATB group (n = 19). Pulmonary TB was confirmed according to the Pulmonary TB Diagnostic Criteria (WS 288–2017, China). Healthcare workers without evidence of ATB or other diseases in the same hospital were recruited and divided into two groups according to the results of IGRA tests. IGRA negative healthcare workers were included in healthy control (HC) group (n = 20), while IGRA positive healthcare workers were included in LTBI group (n = 21). Exclusion criteria included receiving more than one week of antibiotic or probiotic treatment within the previous two months. Participants answered a detailed questionnaire regarding their age, gender, height, and weight. Clinical information of ATB patients was also collected, including clinical picture, laboratory results, and radiological examination results. Fecal samples from the three groups were collected aseptically and stored at −80 °C until DNA extraction.

Ethical statement

This study was approved by the Ethics Committee of the First Affiliated Hospital of the Fourth Military Medical University (KY20192083–F-1). Written informed consent of all participants was obtained.

2.2. Peripheral blood T cell detection

Peripheral blood samples were collected aseptically by venipuncture from the 19 ATB patients, using ethylenediamine-tetraacetic acid (EDTA) blood collection tubes. Anticoagulated blood stored at room temperature was stained within 48 h of draw and then analyzed within 24 h of staining. BD Multi-Check Control was used with each run to assess system performance. Lymphocyte subset percentage and absolute counts were enumerated with the BD Multitest CD3/CD8/CD45/CD4 reagent in BD Trucount Tubes and analyzed on the BD FACSCanto II Flow Cytometer (BD Biosciences) using BD FACSCanto clinical software version 2.0. The details are as follows: 20 μL of Multitest CD3/CD8/CD45/CD4 reagent and 50 μL of well-mixed, anticoagulated whole blood were mixed gently in a tube and incubated for 15 min in the dark at room temperature; then 450 μL of 1 × BD Multitest IMK Kit Lysing Solution was added to the tube and incubated for another 15 min in the dark at room temperature; finally, after the cells were vortexed thoroughly, the tube was installed on the cytometer to acquire the sample. Results were reported as the percentage of positive cells ($CD4^{+}$ T cells, $CD8^{+}$ T cells, and T lymphocytes) per lymphocyte population. BD Multitest™ CD3/CD8/CD45/CD4 contains FITC-labeled CD3, clone SK7; PE-labeled CD8, clone SK1; PerCP-labeled CD45, clone 2D1; and APC-labeled CD4, clone SK3.

2.3. Measurement of serum bilirubin

Fasting elbow venous blood was collected from the participants and centrifuged at 700×*g* for 10 min to separate the serum. Total bilirubin (TBIL), direct bilirubin (DBIL), and indirect bilirubin (IBIL) were assayed by chemical oxidation method using microtubes on an automated analyzer (Siemens Advia 2400).

2.4. DNA extraction and amplification

Genomic DNA was extracted from stool (n = 60) using a QIAamp Power Fecal DNA Kit (Qiagen). PCR was performed on DNA extracted from stool samples to amplify the 16S rDNA gene for sequencing. The 16S rDNA hypervariable regions V3+V4 were PCR-amplified using primers 338F ACTCCTACGGGAGGCAGCAG and 806R GGACTACHVGGGTWTCTAAT with the barcode. The PCR amplification program consisted of an initial heating step at 95 °C for 3 min; 27 cycles of 95 °C for 30s, 55 °C for 30s, and 72 °C for 45s; and a final extension step of 72 °C for 10min. All PCR reactions were carried out on ABI GeneAmp® 9700 (Thermofisher) with TransStart Fastpfu DNA Polymerase (TransGen). PCR products were performed electrophoresis on 2 % agarose gel to confirm the presence and size of amplicons.

2.5. Sequencing analysis

PCR products were purified by the AxyPrep DNA Gel Extraction Kit (Axygen). The TruSeq® DNA Sample Prep Kit (Illumina) was used to generate the sequencing libraries, following the manufacturer's recommendations. Then, the library was sequenced on an Illumina Miseq PE300 platform (Illumina) to generate 400–440 bp raw reads.

2.6. Sequence data analysis

Ribosomal Database Project (RDP) classifier (Version 2.11, http://sourceforge.net/projects/rdp-classifier/) Bayes algorithm was used to annotate the taxonomic information of operational taxonomic units (OTUs) with ≥97 % similarity. The relative abundance of each OTU was calculated at each classification level (kingdom, phylum, class, order, family, genus). The Alpha diversity of samples was calculated using Mothur (Version 1.3.1 http://www.mothur.org/wiki). Beta diversities were calculated by QIIME (Version 1.7.0, http://www.qiime.org). Non-parametric Wilcox test was carried out to determine the significant difference in species abundance between the sample groups by R (Version 3.4.3). OTUs with significant differences in each group were selected and clustered according to their abundance information in each group to draw a heat map by R (version 3.4.3). Linear Discriminant Analysis Effect Size (LEfSe) analysis was carried out on the samples according to different grouping conditions to find out bacterial taxa with different abundance in the pairwise analysis [22]. The identified features were then subjected to a linear discriminant analysis (LDA) model, with the LDA threshold logarithmic score set to 4.0 and ranked.

3. Statistics

The correlations between the relative abundance of specific microbes and T cell proportions and serum bilirubin concentrations, were analyzed by the Spearman correlation test. Receiver operating curve (ROC) analysis of intestinal bacteria for diagnosing ATB and LTBI were performed by GraphPad Prism software (Version 8.0.2).

4. Results

4.1. Participants characteristics

We enrolled 19 ATB patients, 21 LTBI individuals, and 20 healthy controls for the gut microbiome analysis. Detailed characteristics of participants are shown in Table 1. The median ages of participants in the ATB, LTBI, and HC groups were 30, 34, and 31 years, respectively. The proportion of male participants in the ATB, LTBI and HC groups was 57.9 %, 66.7 %, and 20.0 %, respectively. And there were no significant differences in age and sex composition among the three groups. Moreover, the average body mass index (BMI)

Table 1
Characteristics of subject for intestinal microbiota analysis.

	ATB (n = 19)	LTBI (n = 21)	HC (n = 20)	P values		
				ATB vs LTBI	ATB vs HC	LTBI vs HC
Age; (years)	30(23–34)	34(28–44)	31(28–33)	0.2103	>0.9999	0.7626
Gender; (male %)	57.9	66.7	20.0	0.7451	0.1760	0.4841
BMI (kg/m^2)	20.8 ± 2.2	23.9 ± 2.7	22.5 ± 2.9	0.0011	0.1168	0.1977
OTUs	43,440 ± 5137	46,657 ± 8609	46,818 ± 1909	0.3863	0.3595	0.9975

BMI: body mass index; The Kruskal Wallis test was used for pairwise analysis of age; Fisher's exact test was used for pairwise analysis of gender; And Ordinary one-way ANOVA was used for pairwise analysis of BMI.

of ATB group, LTBI group and HC group were 20.8, 23.9, and 22.5, respectively. The BMI of ATB group was significantly lower than that of LTBI. ($P = 0.001$). Furthermore, there was no significant difference in OTUs among the three groups **(Table 1)**.

5. MTB infection affects the bacterial diversity of intestinal microbiota

Species accumulation curves and the rarefaction curve are shown in Figs. S1 and S2. The alpha-diversity was assessed by parameters of the Abundance-based Coverage Estimator (ACE) index, Chao index, Shannon index, and Simpson index. ACE and Chao indices in ATB group were significantly reduced when compared with HC and LTBI groups (Fig. 1A and B). The Shannon index (positively correlated with the community diversity) of LTBI was significantly lower than that of HC, and that of ATB was significantly lower than that of LTBI. The trend of Simpson index (negatively correlated with the community diversity) was opposite to Shannon index among the three groups. (Fig. 1C and D). In a word, the community diversity (estimated by Shannon and Simpson) decreased significantly as MTB infection worsened, while the community richness (estimated by ACE and Chao) decreased significantly only in ATB patients.

The beta-diversity, assessed by weighted uniFrac-based principal co-ordinate analysis (PCoA) and analysis of similarities (ANOSIM), indicated that the intestinal communities of ATB group were different from that of LTBI group ($P = 0.048$) and HC group ($P = 0.001$) (Fig. 2A). Moreover, the heat map showed significant differences in the distribution of the top 50 intestinal genera among the three groups (Fig. S3).

6. MTB infection affects the bacterial composition of the gut microbiota

In healthy people, the four most abundant bacterial phyla were Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Fig. 2C). And the nine most abundant bacterial classes were *Clostridia*, *Bacteroidia*, *Actinobacteria*, *Negativicutes*, *Gammaproteobacteria*, *Bacilli*, *Erysipelotrichia*, *Fusobacteria*, and *Verrucomicrobiae* (Fig. S4A). And the top 10 genera were *Bacteroides*, *Prevotella*, *Bifidobacterium*, *Faecalibacterium*, *Agathobacter*, *Megamonas*, *Lactobacillus*, *Subdoligranulum*, *Blautia*, and *Escherichia-Shigella* (Fig. 3A).

To further understand the microbial composition alterations, the relative abundance of the three groups was compared pairwise at the bacterial phylum, class, and genus levels. At the phylum level, the relative abundance of Proteobacteria in ATB group was significantly increased compared with that in HC ($P = 0.033$) and LTBI ($P = 0.013$) groups (Fig. 2B). Similarly, compared with HC ($P = 0.032$) and LTBI($P = 0.012$) groups, the relative abundance of *Gammaproteobacteria* was significantly increased in ATB group

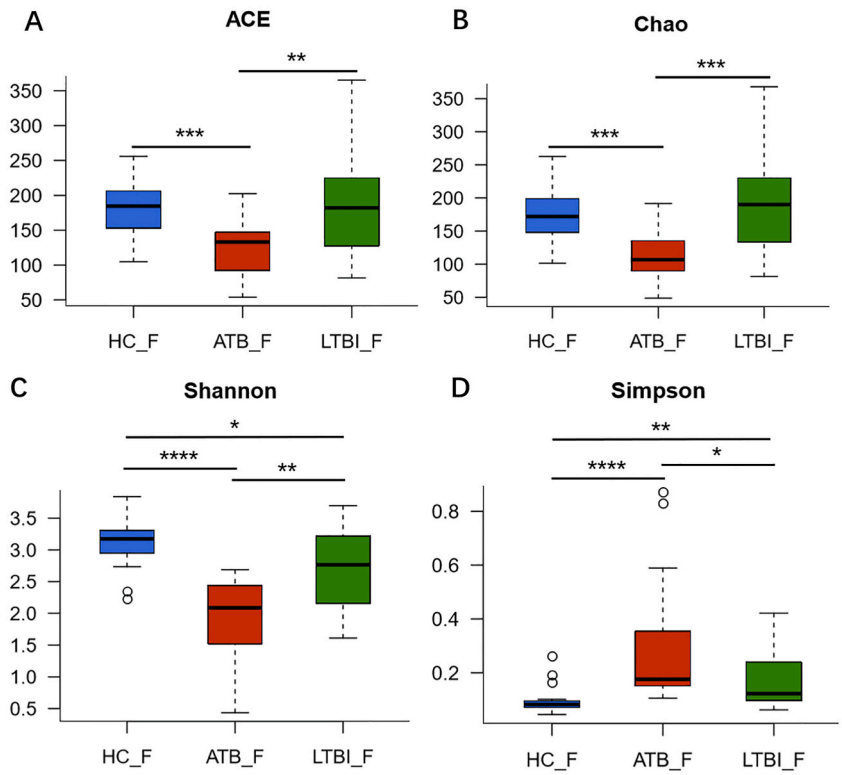


Fig. 1. | Alpha-diversity of fecal microbiota in the three groups. **(A, B)** Chao index and ACE index were used to determine fecal microbial richness. **(C, D)** Shannon index and Simpson index were used to determine fecal microbial diversity. The *P*-value was calculated using Wilcoxon two-tailed test. The statistical significance is displayed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

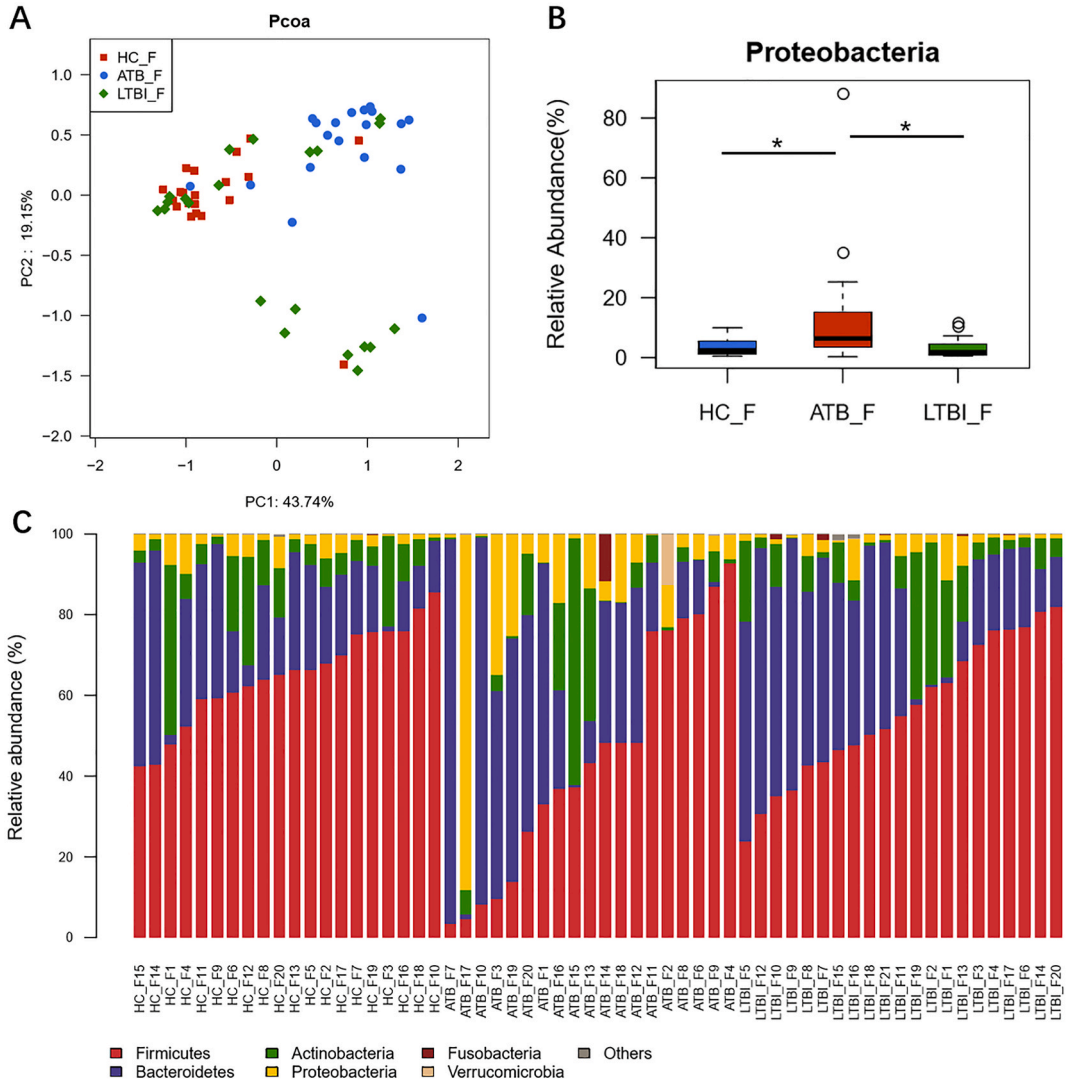


Fig. 2. | **(A)** Principal Co-ordinates Analysis (PCoA) for beta-diversity depicting the relationships between the fecal microbiomes of different groups based on weighted Unifrac. **(B)** Comparison of the relative abundance of the phylum Proteobacteria of the fecal microbiomes. **(C)** Stacked column bar graphs depicting the phylum level taxonomic abundance of the fecal microbiomes. Taxa were identified by name for the most abundant phyla or merge into the "Others" category for below 0.5 % abundance. The *P*-value was calculated using Wilcoxon two-tailed test. Statistical significance is displayed as * $P < 0.05$.

(Fig. S4C). Moreover, the relative abundance of *Clostridia* in LTBI group was significantly lower than that in HC group ($P = 0.0460$). And the relative abundance of *Clostridia* in ATB group was further significantly lower than that in LTBI group ($P = 0.027$) (Fig. S4B). Bacterial communities were further compared at the genus level (Fig. 3B–G). The relative abundance of *Prevotella* in LTBI group was higher than that in both ATB ($P = 0.044$) and HC ($P = 0.026$) groups (Fig. 3B). And compared with HC group, *Bifidobacterium* and *Blautia* were significantly decreased in ATB group ($P = 0.046$ and $P = 0.006$, respectively) (Fig. 3C and G). Furthermore, compared with LTBI ($P = 0.002$) and HC ($P = 0.001$) groups, the relative abundance of *Faecalibacterium* was significantly lower in ATB group (Fig. 3D and E). Also, compared with HC group, the relative abundance of *Subdoligranulum* was significantly reduced in ATB ($P = 0.007$) and LTBI ($P = 0.049$) groups (Fig. 3F). In addition, the relative abundance of *Agathobacter* in LTBI group was significantly lower than that in HC group ($P = 0.028$), and *Agathobacter* in ATB group was further significantly lower than that in LTBI group ($P = 0.020$). In brief, there were significant differences in the composition of intestinal flora among the three groups.

6.1. *Correlations of peripheral T cells and serum bilirubin with intestinal bacteria in ATB patients*

Since bilirubin has anti-inflammatory effects, we also explored potential associations between serum bilirubin levels and the relative abundance of fecal microbiota in ATB patients. The results showed that *Bacteroidales* was positively correlated with DBIL (r =

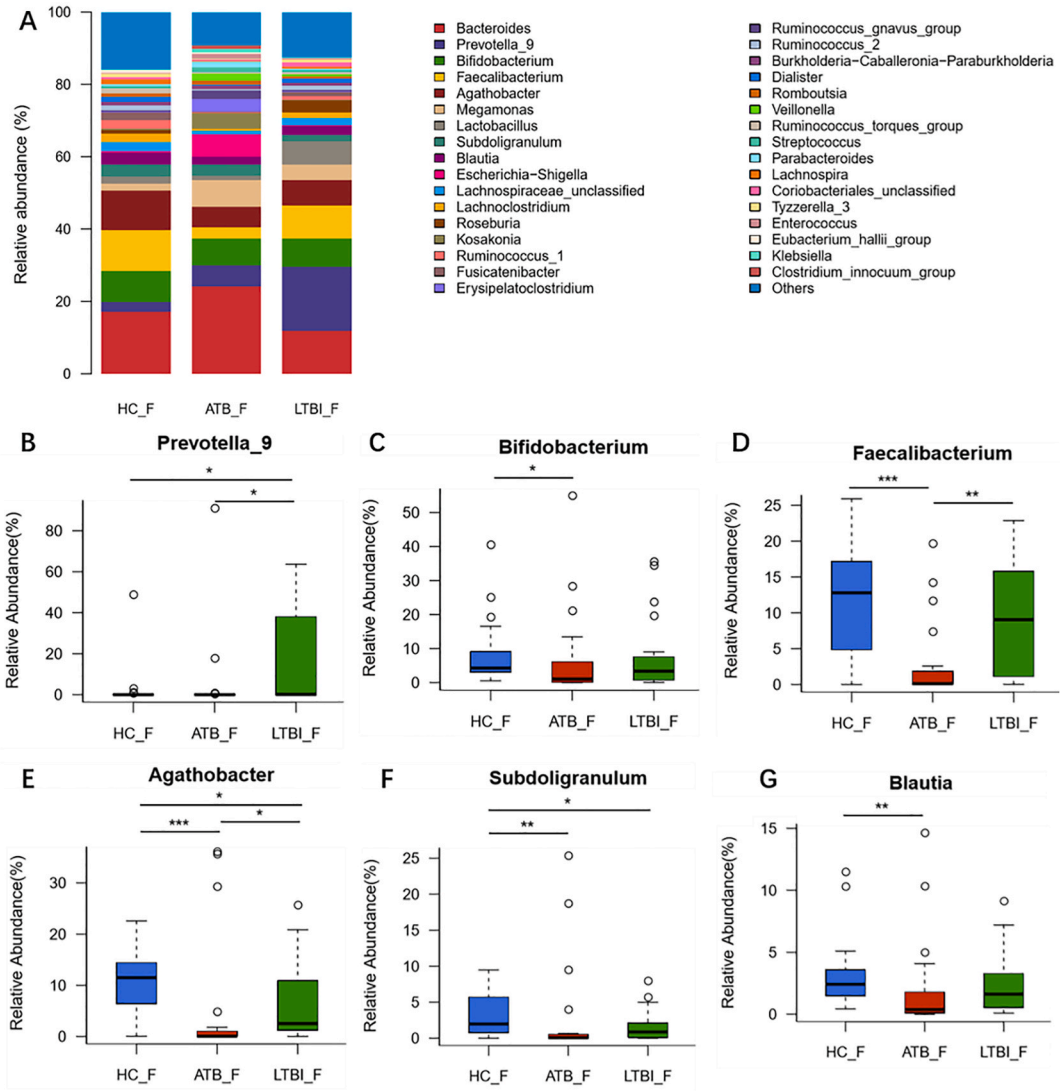


Fig. 3. | **(A)** Stacked column bar graphs depicting the genus level taxonomic abundance of the fecal microbiomes. Taxa were identified by name for the most abundant genera or merge into the "Others" category for below 0.5 % abundance. **(B–G)** Comparison of relative abundance of the genera *Prevotella, Bifidobacterium, Faecalibacterium, Agathobacter, Subdoligranulum* and *Blautia*. The *P*-value was calculated using Wilcoxon two-tailed test. Statistical significance is displayed as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

0.472, $P = 0.042$) (Fig. 4A). On the contrary, *Clostridiales* was negatively correlated with DBIL (r = −0.550, $P = 0.015$) (Fig. 4B). ATB patients were further divided into culture-negative group (CN, n = 7) and culture-positive group (CP, n = 12) according to whether sputum MTB culture was positive or not. And it turned out that DBIL in CN group (4.9 μmol/L) was significantly higher than that in CP group (2.7 μmol/L, $P = 0.023$), indicating that the decrease of serum DBIL may be a predictor of culture positivity in ATB patients.

T lymphocytes are the primary adaptive immune cells to resist MTB infection. So, Spearman correlation test was also used to measure the correlation between the relative abundance of bacterial taxa and the proportion of T cells in ATB patients. We found that the relative abundance of *Blautia*, which was lower in ATB group than that in HC group, was negatively correlated with T cells and $CD8^{+}$T cells (r = −0.502, $P = 0.029$; r = −0.495, $P = 0.031$) (Fig. 4C–D). Of interest, there was no significant difference in the proportion of peripheral T cells and $CD8^{+}$T cells between CN and CP groups ($P = 0.585$, $P = 0.338$). Considering that T cells are associated with *anti*-MTB immunity, *Blautia* may contribute to host immune status by influencing T cell proportions in ATB patients. Apart from *Bacteroidales* and *Clostridiales*, *Prevotella* and *Prevotellaceae* are also positively correlated with DBIL in ATB (Fig. S5). As the relative abundance of *Prevotella* and *Prevotellaceae* in most ATB patients were close to zero, the correlations were not believed to be clinically meaningful.

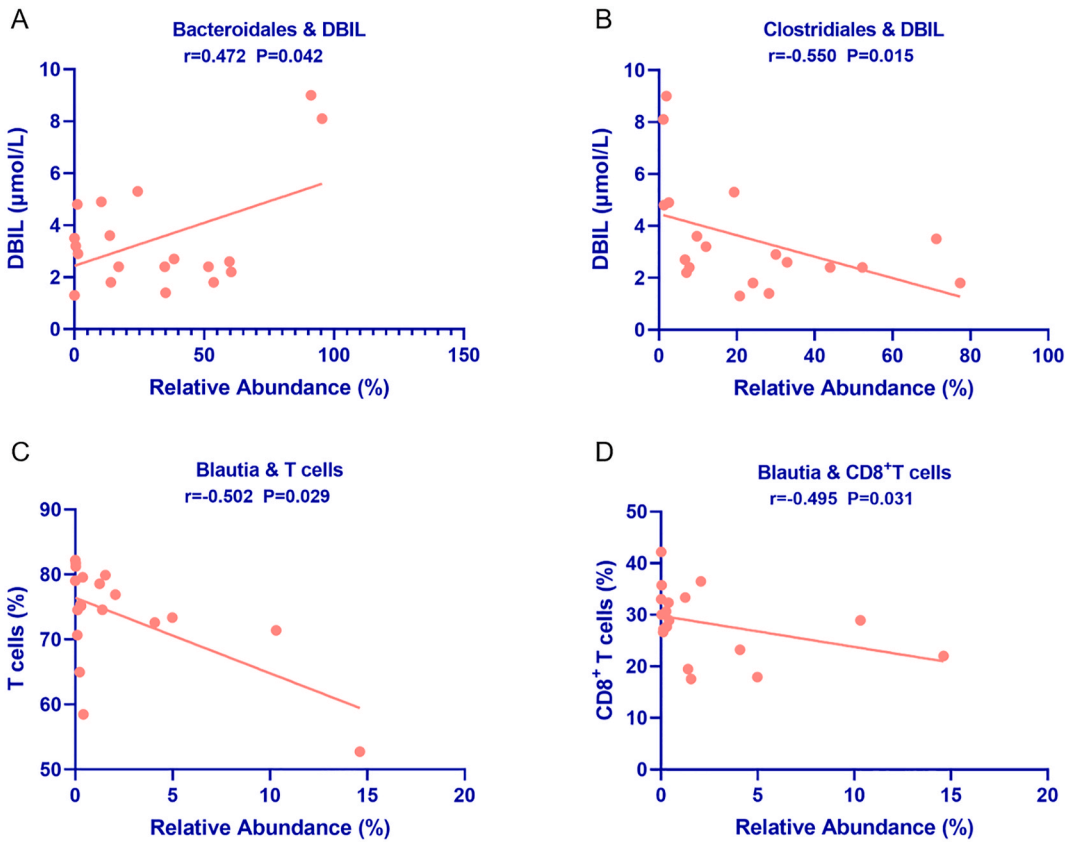


Fig. 4. | **(A–B)** Correlation between the relative abundance of *Bacteroidales* and *Clostridiales* and serum direct bilirubin concentration in ATB group. **(C–D)** Correlation between relative abundance of *Blautia* and peripheral T cells and $CD8^{+}$T cells in ATB group. The *P*-value was calculated using Spearman correlation test.

6.2. *The specific enrichment of gut microbiota varied in different groups*

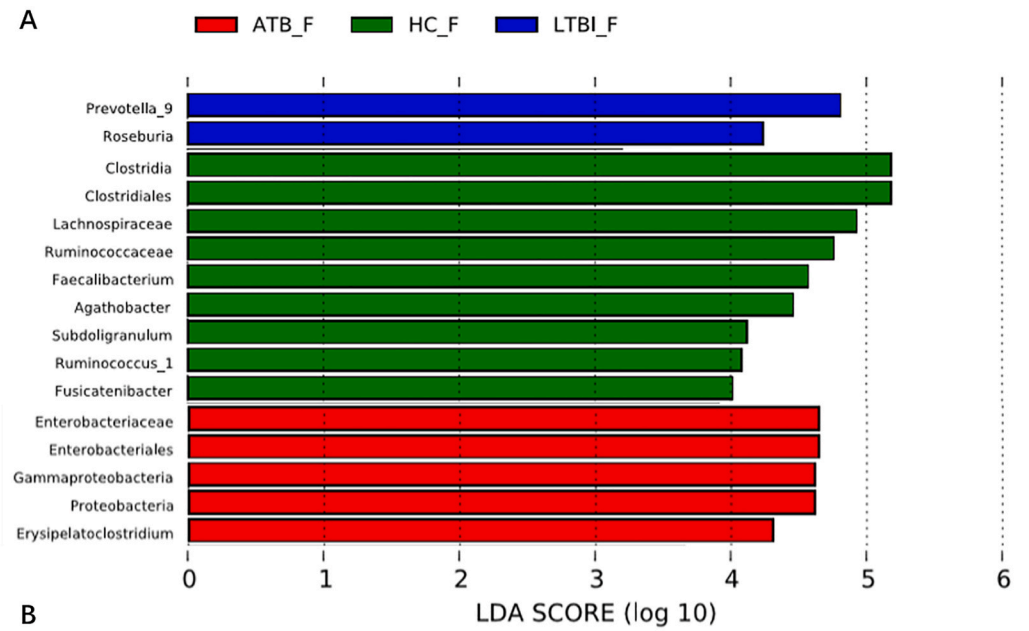
LEfSe analysis with LDA was performed to describe the bacterial taxa that enriched specifically in the three groups (LDA score >4.0). The unique enrichment taxa in ATB group were *Enterobacteriaceae*, *Enterobacteriales*, *Gammaproteobacteria*, *Proteobacteria*, and *Erysipelatoclostridium*. Whereas, in LTBI group, *Prevotella* and *Roseburia* were specifically enriched. Besides, nine unique taxa were identified in HC group, including *Clostridia*, *Clostridiales*, *Lachnospiraceae*, *Ruminococcaceae*, *Faecalibacterium*, *Agathobacter*, *Subdoligranulum*, *Ruminococcus* and *Fusicatenibacter* (Fig. 5A).

The differential enrichment of specific bacteria was pairwise compared and presented in histograms (LDA score >4.0) (Fig. 5B) as well as cladograms (LDA score >2.0) (Fig. S6). By comparing HC and ATB groups, the ATB group was enriched by *Enterobacteriaceae*, *Enterobacteriales*, *Gammaproteobacteria*, Proteobacteria, and *Erysipelatoclostridium*, while the HC group was enriched by *Bifidobacteriale*, *Bifidobacteriaceae*, *Bifidobacterium*, *Clostridia*, *Clostridiales*, *Oscillospiraceae*, *Ruminococcus*, *Faecalibacterium*, *Lachnospiraceae*, *Agathobacter*, and *Fusicatenibacter* (Fig. 5B). And when LTBI and ATB groups were compared, *Prevotella*, *Clostridia*, *Clostridiales*, *Oscillospiraceae*, *Faecalibacterium*, *Lachnospiraceae*, *Agathobacter*, and *Roseburia* were specifically enriched in LTBI, while *Enterobacteriaceae*, *Enterobacteriales*, *Gammaproteobacteria*, Proteobacteria, and *Erysipelatoclostridium* were still specifically enriched in ATB group (Fig. 5B). Moreover, the comparison between HC and LTBI groups showed that the HC group was enriched by *Clostridia*, *Clostridiales*, and *Agathobacter*, while the LTBI group was enriched by *Prevotella* (Fig. 5B).

6.3. *The specifically enriched bacteria are potential diagnostic markers*

ROC analysis was used to determine the diagnostic performance of the relative abundance of intestinal bacteria that were specifically enriched in ATB groups (LTBI + HC as controls) or LTBI (ATB + HC as controls). As shown in Fig. 6, when the relative abundance of *Faecalibacterium, Clostridia* and *Gammaproteobacteria* be used as markers of ATB, their area under the curve (AUC) are 0.808, 0.784 and 0.717; and when the relative abundance of *Prevotella* and *Rosburia* be used as markers of LTBI, their AUC are 0.689 and 0.689. As shown in Table 2, when the cut-off value of *Faecalibacterium* relative abundance is <7.558 %, the Yoden index of ATB diagnosis was the highest, with a sensitivity of 58.54 % and specificity of 84.21 %. When the cut-off value of *Prevotella* relative abundance was >0.035 %, the Yoden index of LTBI diagnosis was the highest, with a sensitivity of 69.23 % and specificity of 71.43 %.

A



B

Phylum	Class	Order	Family	Genus	ATB vs HC	LTBI vs HC	ATB vs LTBI
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium			
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella_9			
Firmicutes	Clostridia	Clostridiales	Oscillospiraceae	Ruminococcus_1			
				Faecalibacterium			
			Lachnospiraceae	Agathobacter			
				Roseburia			
				Fusicatenibacter			
			Clostridiaceae	Erysipelatoclostridium			
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae				

Fig. 5. | **(A)** Column diagram of LDA analysis of fecal microbiome in the three groups (HC, LTBI and ATB) ($P < 0.05$; LDA score >4.0). **(B)** Pairwise LDA effect size (LEfSe) analysis table of fecal microbiome. The phyla and subsequent taxonomic levels are sorted alphabetically, and the corresponding LDA score for each pairwise analysis is indicated in the column ($P < 0.05$; LDA score >4.0).

These results suggest that the relative abundance of intestinal bacteria may contribute to the diagnosis of ATB and LTBI.

7. Discussion

MTB infection is a result of sophisticated microbial community interactions [18]. Although pulmonary TB develops in the lung, the gut microbiota is a key factor affecting TB because of its vast size and well-known effect on the immune system. In the present study, we measured the gut microbiome of healthy controls, LTBI individuals, and pulmonary ATB patients by 16S rDNA gene sequencing. The results showed significant differences in gut microbiota between the three groups. Besides, associations between specific bacterial taxa and T cells, $CD8^+$T cells, and serum DBIL levels were found in pulmonary ATB patients. In addition, we preliminarily identified intestinal bacterial markers for pulmonary ATB and LTBI diagnosis.

Compared with the HC group, changes of gut microbiota in pulmonary ATB and LTBI groups had some similarities. In the current study, a significant decrease in the intestinal microbiota diversity was observed in LTBI and pulmonary ATB groups. This is consistent with previous studies that found a decrease of both species number and microbial diversity in MTB-infected patients compared with that in HCs [16,20]. Confounders leading to the decrease in intestinal bacterial diversity such as other diseases and oral drug interventions [23–25] were excluded in this study. In terms of the community composition, our results showed that class *Clostridia* was less abundant in LTBI and pulmonary ATB groups. The reduction of *Clostridia* has also been reported in newly diagnosed and recurrent

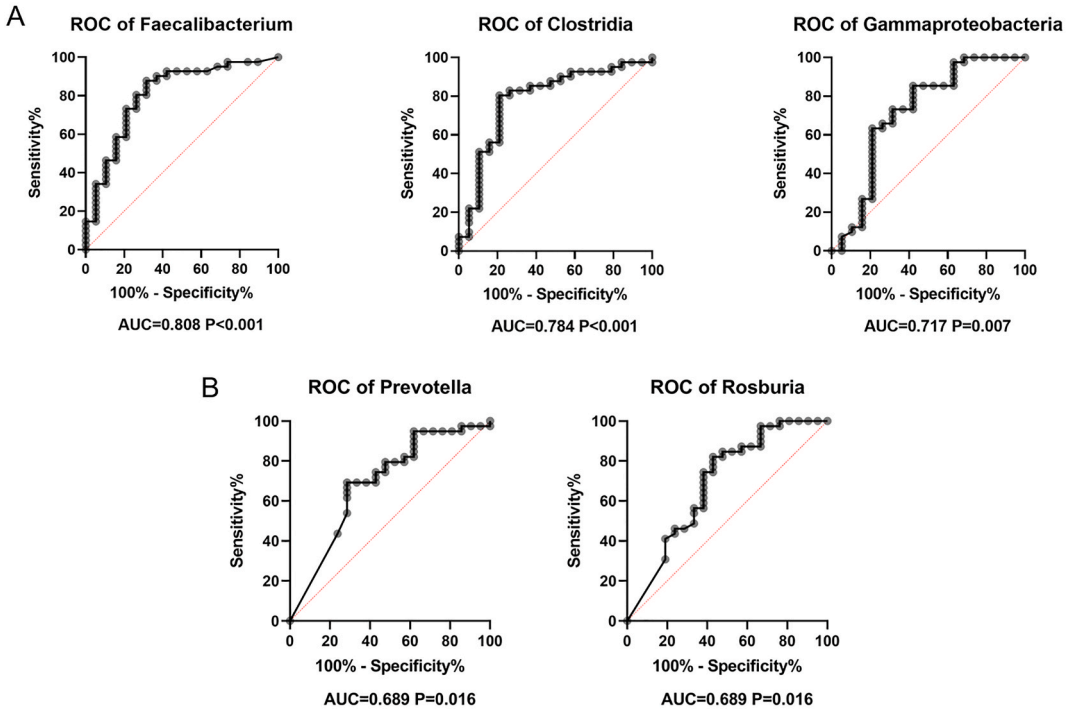


Fig. 6. | **(A)** ROC was performed on the relative abundance of intestinal bacteria *Faecalibacterium, Clostridia* and *Gammaproteobacteria,* which were specifically enriched in ATB group to diagnose ATB. **(B)** ROC was performed on the relative abundance of intestinal bacteria *Prevotella* and *Rosburia,* which were specifically enriched in the LTBI group to diagnose LTBI.

Table 2
Intestinal bacteria that can be used to diagnose ATB or LTBI.

		Cut-off value (%)	Sensitivity (%)	Specificity (%)	AUC	*P* value
To diagnose ATB	Faecalibacterium	<7.558	58.54	84.21	0.808	<0.001
	Clostridia	<52.34	51.22	89.47	0.784	<0.001
	Gammaproteobacteria	>2.94	63.41	78.95	0.717	0.007
To diagnose LTBI	Prevotella	>0.035	69.23	71.43	0.689	0.016
	Rosburia	>0.005	41.03	80.95	0.689	0.016

AUC: area under the curve; Cut-off value for diagnosis of ATB was determined with Receiver operating curve (ROC) analysis.

pulmonary TB patients [17] and MTB-infected mice [9]. *Clostridia* species are Gram-positive anaerobic bacteria that has been reported to contribute to gut homeostasis by maintaining intestinal barrier function and exerting anti-inflammatory and immunomodulatory properties [26]. A recent study found that physiological concentrations of butyrate [27] (one of the main metabolites of *Clostridia*) can suppress pro-inflammatory cytokine responses induced by MTB, while promote the production of an anti-inflammatory cytokine IL-10 [28]. Previous studies [29,30] have shown that *Clostridia* can reduce intestinal inflammation in mice by increasing regulatory T cells (Tregs) in the large intestine. And Treg can inhibit the clearance of MTB in the early stage of MTB infection [31–33]]. Thus, the reduction of *Clostridia* in MTB-infected populations may suppress intestinal anti-inflammatory immunity, leading to the predominance of pro-inflammatory immunity. The lung immunity to MTB may be promoted by the pro-inflammatory intestinal immunity through the gut-lung axis.

So far, there have been few studies exploring the intestinal microbiome in LTBI individuals. One study showed that LTBI individuals were 50 % less likely to develop ATB with the presence of *Helicobacter pylori* in their gut [34]. In our study, the alpha-diversity index showed that the richness and diversity of intestinal bacterial communities in the ATB group was lower than those in the LTBI group. These results indicate that intestinal bacterial diversity was further lost with the aggravation of MTB infection, which is consistent with the results observed in animal experiments [9]. Decreased intestinal microbial diversity has also been reported to be associated with some TB-related comorbidities, such as malnutrition [35]. Notably, no participants in our study were malnutrition, although BMI in the pulmonary ATB group was significantly lower than that in the LTBI group.

Furthermore, 14 taxa were found to be enriched differently in pulmonary ATB and LTBI groups. Among these taxa, 9 were more abundant in LTBI, while 5 were enriched in pulmonary ATB. Strikingly, 8 out of the 9 LTBI-enriched bacteria belong to the class *Clostridia.* And *Prevotella* was a non-*Clostridia* bacterium that was uniquely enriched in LTBI group. Interestingly, studies on sputum microbiota found that *Prevotella* was less abundant in pulmonary ATB patients than that in healthy controls [17,36]. And we have

demonstrated that *Prevotella* can induce the proliferation and accumulation of Th17 cells in the colon of mice [37]. On the contrary, four out of the five pulmonary ATB-enriched bacteria belong to the phylum Proteobacteria. The enrichment of Proteobacteria in pulmonary ATB patients has also been confirmed by other studies [17]. Proteobacteria are Gram-negative bacteria, including many opportunistic pathogens. This suggests that the intestinal microenvironment of pulmonary ATB patients may be more susceptible to be colonized by opportunistic pathogens. Not surprisingly, *Clostridia*, *Faecalibacterium* (genus within *Clostridia*) and *Gammaproteobacteria* (class within Proteobacteria) are promising potential markers for the diagnosis of pulmonary ATB, while *Prevotella* and *Roseburia* (genus within *Clostridia*) are promising potential markers for the diagnosis of LTBI.

Besides, *Clostridiales* was found to be negatively correlated with serum bilirubin in pulmonary ATB patients. Previous findings showed that species within *Clostridiales* have the ability to convert bilirubin [38,39] and bilirubin have anti-inflammatory effects on Th17 immune responses [40]. Given that both DBIL and *Clostridiales* have anti-inflammatory properties and were reduced in pulmonary ATB patients, anti-inflammatory factors may be suppressed in pulmonary ATB patients.

Another interesting finding was that in pulmonary ATB patients *Blautia* was negatively correlated with peripheral $CD8^+$T cells and T cells. *Blautia* has been found to be associated with reduced graft-versus-host diseases (GVHD)-related mortality [41]. In addition, it has been reported that antigen-experienced $CD8^+$T cells were positively correlate with mycobacterial smear grade [42]. So, the relatively low *Blautia* abundance in gut microbiota may contribute to enhancing host *anti*-MTB immunity by influencing T cell proportions in pulmonary ATB patients.

The mechanisms by which MTB interacts with the gut microbiota are still not well understood. Some have found that disturbance of the gut microbiota leads to increased susceptibility to MTB, and others have found that the gut microbiota is altered after MTB infection. In either case, we propose that MTB and the gut microbiota affect each other indirectly by influencing host immunity. For example, it was reported that gut dysbiosis in isoniazid-treated mice resulted in impairment of T-cell activation, proliferation, and memory T-cells generation, as well as decreased transcripts level of cytokines (such as IL-1β, IL-12, and IL-6) that are required for protective immune response against MTB [43]. We speculate that in ATB patients, a decreased "immunosuppressive" *Clostridia* and an increased "pro-inflammatory" Proteobacteria may favor a pro-inflammatory environment that restricts MTB growth. For LTBI populations, the increased "immunosuppressive" *Clostridia* and "immune-enhanced" *Prevotella* may help maintain the *anti*-MTB immune balance.

There are some limitations in this study. First, future multicenter studies with larger sample sizes are needed to explore the underlying reasons for the inconsistent results of intestinal microbiome changes in TB patients. Second, the auxiliary diagnostic value of the indicators found in this study for pulmonary ATB and LTBI needs to be further verified in IGRA positive respiratory tract infection patients without ATB in the future. Third, prospective cohort studies are needed to verify the causal relationship between MTB infection and alterations in gut microbiota.

Overall, MTB-infected subjects were characterized by a lower intestinal microbial diversity concurrent with lower relative abundance of "anti-inflammatory" microbes, such as *Clostridia*. LTBI was featured with an intermediate decrease in microbial diversity and *Clostridia* abundance, as well as a specific enrichment of *Prevotella*. While for pulmonary ATB patients, the microbial diversity and *Clostridia* abundance further reduced, whereas Proteobacteria abundance increased. Meanwhile, in pulmonary ATB patients, *Blautia* may be associated with *anti*-MTB immunity by interactions with peripheral T cell, while *Clostridiales* and *Bacteroidales* may be associated with *anti*-MTB immunity by interactions with serum DBIL.

Data availability statement

Data associated with our study hasn't been deposited into a publicly available repository yet. And they are available on request to the corresponding author.

Ethics statement

This study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of the Fourth Military Medical University, with the approval number: KY20192083–F-1. All participants provided informed consent to participate in the study.

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CRediT authorship contribution statement

Yuan Huang: Writing – original draft, Project administration, Formal analysis, Data curation, Conceptualization. **Jinhua Tang:** Formal analysis, Data curation. **Zheng Cai:** Formal analysis, Data curation. **Yun Qi:** Investigation. **Shen Jiang:** Investigation. **Tingting Ma:** Investigation. **Ying Yue:** Investigation. **Fang Huang:** Methodology. **Han Yang:** Methodology. **Yueyun Ma:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22124.

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