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Gut microbiota associated with pulmonary tuberculosis and dysbiosis caused by anti-tuberculosis drugs

Yongfeng Hu[a,1], Qianting Yang[d,1], Bo Liu[a,1], Jie Dong[a], Lilian Sun[a], Yafang Zhu[a], Haoxiang Su[a], Jian Yang[a], Fan Yang[a,***], Xinchun Chen[c,**], Qi Jin[a,b,d,*]

[a] MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China
[b] Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, the First Affiliated Hospital, Zhejiang University, Hangzhou 310003, China
[c] Department of Pathogen Biology, Shenzhen University School of Medicine, Shenzhen 518060, China
[d] Shenzhen Engineering Center for Tuberculosis, Shenzhen Key Lab. of Infection & Immunity, Shenzhen Third People's Hospital, Shenzhen University, Shenzhen 518112, China

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SUMMARY

Background: An improved understanding of the gut microbiota could lead to better strategies for the diagnosis, therapy and prophylaxis of tuberculosis (TB). The impact of both *Mycobacterium tuberculosis* (Mtb) infection and anti-TB treatment on the gut microbiota has rarely been studied.

Methods: We characterized the diversity and composition of the gut microbiota in pulmonary TB patients as well as the effects of anti-TB drugs on the gut microbiota.

Results: Pulmonary Mtb infection led to a minor decrease in the α diversity of the gut microbiota when compared to healthy controls, which mainly resulted from changes in the relative abundance of the members of genus *Bacteroides*. Anti-TB therapy caused a rapid, significant alteration in the community structure. The relative abundance of members of genus *Clostridiales* of the phylum *Firmicutes* significantly decreased during anti-TB treatment, while many members of genus *Bacteroides*, including *Bacteroides* OTU230 and *Bacteroides fragilis*, were among the taxa that increased. OTU8 and OTU2972 assigned to family Erysipelotrichaceae of the phylum *Firmicutes* showed a dramatic increase 1 week after the start of therapy, while the other members of this family decreased.

Conclusions: Pulmonary TB and anti-TB treatment caused a distinct dysbiosis of the gut microbiota. Our study contributes valuable information implying potential links between the gut microbiota and TB.

Introduction

Tuberculosis (TB), caused by the *Mycobacterium tuberculosis* (Mtb) complex, is one of the oldest illnesses in history and remains a major health problem worldwide.[1] In 2016, the WHO estimated a third of the global population to be latently infected with *Mycobacterium tuberculosis* (Mtb), with 10.4 million active TB cases and 1.7 million deaths annually. An estimated 53 million lives were saved through TB diagnosis and treatment between 2000 and 2016.[2]

The microbiota plays an important role in human health and disease, which is involved in energy harvest and storage as well as a variety of metabolic functions, and even more importantly, the gut microbiota interacts with the immune system, providing signals to promote the maturation of immune cells and the normal development of immune functions.[3,4] Immunomodulatory roles of the gut microbiota may prove to be critical in the host response against TB, including preventing TB infection, reducing progression from latency, mitigating disease severity, and lowering the incidence of drug resistance and co-infections.[5]

* Corresponding author at: MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China.
** Corresponding author at: Department of Pathogen Biology, Shenzhen University School of Medicine, Shenzhen 518060, China.
*** Corresponding author at: MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China.
E-mail addresses: yangfan@ipbcams.ac.cn (F. Yang), chenxinchun@szu.edu.cn (X. Chen), zdsys@vip.sina.com (Q. Jin).
[1] These authors contributed equally to this work.

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Table 1
Characteristics of the enrolled study patients. Data are divided into the study groups described in the text. HCs are IGRA−; people with LTBI are IGRA+. [1] means active TB patients receiving 1-week anti-TB therapy, while [2] means active TB patients receiving 2-week anti-TB therapy. Active pulmonary TB was finally confirmed by positive examinations for Mtb in sputum or BAL samples.

Group	Healthy control (HC, $n=13$)	Active TB (TB, $n=28$)	Latent TB (LTBI, $n=10$)	TB[1] (T1, $n=13$)	TB[2] (T2, $n=10$)	TB cured (Tc, $n=10$)
Female, n (%)	6 (46)	15 (54)	6 (60)	5 (38)	6 (60)	5 (50)
Age, mean ± SD, yr	37.6 ± 2.6	41.3 ± 3.7	42.6 ± 4.8	42.6 ± 1.3	39.2 ± 6.7	40.1 ± 5.3
Anti-TB duration	/	Naïve	/	1 week	2 weeks	6 months
No. of reads	61621 ± 12598	67762 ± 10536	70577 ± 7548	72380 ± 5210	70571 ± 7484	72334 ± 6615
No. of OTUs	414 ± 69	391 ± 52	377 ± 72	352 ± 38	315 ± 34	305 ± 28
Diagnosis	Mtb-/IGRA−	Mtb +	Mtb-/IGRA+	–	–	–

In the lung, resistance to multiple bacterial and viral pathogens is enhanced by the gut microbiota, which has been reported to protect against respiratory infection by *Streptococcus pneumoniae* or *Klebsiella pneumoniae* via GM-CSF signalling.[6] In addition, pulmonary infection with influenza virus can significantly alter the intestinal microbiota profile through a mechanism dependent on type I interferons,[7,8] However, knowledge about whether and how TB induces changes in the gut microbiota is limited, given that the intestinal tract is not the primary site of infection for Mtb.[9] In addition, isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) are the four drugs in the first-line antimicrobial regimen used clinically to treat drug-susceptible TB. While INH, PZA, and EMB are thought to specifically target mycobacteria, RIF is a broad-spectrum antibiotic with potency against many gram-positive and gram-negative bacteria.[2] Effective treatment of drug-susceptible TB requires at least 6 months of daily therapy with multiple orally administered antibiotics. Thus, the standard multidrug TB treatment with so long a duration may have a wide range of effects on the gut microbiota, as has been documented with other antibiotic treatments.[10] However, little is known about the effects of first-line TB antibiotics on the intestinal microbiome.

In this study, we performed a cross-sectional study, which included five groups of active TB patients (TB group), latent TB patients (LTBI group), active TB patients with a 1-week (T1 group) or 2-week (T2 group) treatment and cured TB patients (Tc group), to characterize the intestinal microbiota changes in response to Mtb infection (including active and latent infection) and anti-TB therapy in people from China.

Materials and methods

Study design and ethics statement

We recruited five groups of individuals for a cross-sectional research study designed to characterize the gut microbiomes associated with TB patients from China. Active pulmonary TB was suspected in patients with one or more of the following clinical symptoms as described previously: persistent fever and cough, haemoptysis, weight loss, night sweats, poor appetite, fatigue and a chest radiograph showing unilateral upper zone infiltrates.[11] A diagnosis of active TB was finally confirmed by positive examinations for Mtb on all initial samples, including sputum and bronchoalveolar lavage (BAL) samples.[11] A diagnosis of LTBI was made if an individual had no symptoms but had a positive interferon gamma release assay (IGRA) test.

The five groups of individuals consisted of 13 healthy controls (HCs, IGRA-), 10 patients with LTBI (LTBI, IGRA+), 28 active TB patients (TB group), 13 TB patients with 1-week anti-TB therapy, including INH, RIF, EMB, and PZA (T1 group), 10 TB patients with 2-week anti-TB therapy (T2 group), and 10 patients who were cured of active TB (Tc group). All subjects were HIV negative. Clinical characteristics of the study population are depicted in Table 1. Generally, the age and sex were not significantly different among the groups.

Stool processing and DNA isolation

Stool samples were collected from the recruited subjects, immediately refrigerated, and transported to the laboratory, where they remained in the refrigerator until processing for storage at −80 °C. Metagenomic DNA was extracted from an aliquot of ~100 mg from each stool sample using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., USA) following the manufacturer's protocol, with modifications as outlined in the Earth Microbiome Project (version 4_13). DNA samples were stored at −20°C until further processing.

16S rRNA amplification and deep sequencing

The V4 hypervariable regions of the bacterial 16S rRNA gene, a small and highly conserved locus of the bacterial genome that permits genus- and species-level identification, were amplified from each sample using the 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers as previously described prior to sequencing.[12] AccuPrime High-Fidelity *Taq* DNA polymerase (Invitrogen, USA) was used for PCR. The following primary PCR cycling conditions were used: (1) initial denaturation at 95 °C for 2 min; (2) 20 cycles of touchdown PCR at 95 °C for 20 s, 60 °C for 20 s (decreasing by 0.3 °C each cycle), and 72 °C for 5 min; (3) 20 cycles of standard PCR at 95 °C for 20 s, 55 °C for 15 s, and 72 °C for 5 min; and (4) a final extension at 72 °C for 10 min. Barcoded libraries for multiplex high-throughput sequencing were constructed to identify the reads from different samples after sequencing. Deep sequencing was performed by using the HiSeq 2500 platform and the HiSeq Reagent Kit V2 (500 cycles; Illumina, San Diego, CA) according to the manufacturer's instructions.

Data analysis

The sequencing data were processed as described previously using mothur v.1.36.1 software (Schloss Laboratory, University of Michigan, Ann Arbor, MI) according to the standard operating procedures for HiSeq sequencing data, using a minimum sequence length of 250 base pairs. A shared community file and a phylotyped (genus-level grouping) file were generated by using operational taxonomic units (OTUs) ("species") binned at 97% identity. The OTU numbers were assigned during the binning process, and the classification was performed by using the mothur implementation in the Ribosomal Database Project Classifier and the Ribosomal Database Project taxonomy training set.

All statistical analyses were performed using packages 'vegan' v2.4-1 and 'ggplot2' v2.2.1 in R 3.4.1 (https://www.r-project.org/) and GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). The unsupervised ordination data-visualization technique (nonmetric

multidimensional scaling, NMDS) was used to compare the overall structure of the gut microbiome of subjects among different groups. This method can identify the "average" of a given specimen type as its centroid and provide a 2-dimensional visualization of the differences among the members of a population, with similar members grouping together. Significant differences in community composition were determined by using permutational ANOVA (PERMANOVA), specifically running the adonis function in vegan with 1000 permutations. We compared the means by using an unpaired t-test, using Tukey's multiple-comparisons test when appropriate.

Results

A total of 84 stool samples were collected and sequenced from HCs ($n = 13$), LTBI patients ($n = 10$), active TB patients (TB, $n = 28$), TB patients with 1-week front-line anti-TB therapy (T1, $n = 13$), TB patients with 2-week anti-TB therapy (T2, $n = 10$), and cured TB patients (Tc, $n = 10$). Across all of the gut samples, a total of 5774, 193 high-quality 16S rRNA V4 gene sequences were obtained and classified into 1,581 OTUs (excluding singletons), of which 120 OTUs were supported by >1000 reads each. The number of reads per sample was $68{,}740 \pm 9848$ (mean $\pm$ SD). The number of reads and OTUs in each group are listed in Table 1.

Pulmonary Mtb infection induces minor changes in the gut microbiota

To determine whether and how the intestinal microbiota change in response to pulmonary Mtb infection, we performed a cross-sectional analysis of 16S rRNA (V4 region) in three groups of subjects, those with active pulmonary TB (TB, $n = 28$), those with latent TB (LTBI, IGRA+, $n = 10$) and HCs ($n = 13$), to analyse the composition of the gut microbiota.

A minor decrease in α diversity was observed in patients with Mtb infection compared with that in HCs, as assessed by the observed number of OTUs, Shannon index and Pielou's evenness index (Fig. 1A), which measure the total number of OTUs and, in the case of the Shannon index and Pielou's evenness index (J), the richness, abundance, and evenness of the OTU distribution. The value of J′ ranges from 0 to 1, with larger values representing more even distributions in abundance among OTUs. We then used NMDS to compare groups of samples based on phylogenetic or count-based distance metrics. The clustering driven by active and latent Mtb infections was not statistically significant based on NMDS (Fig. 1B).

We next compared the composition of the microbiome to identify bacterial taxa that differed among the three groups. We observed trends in differential relative abundance mainly in members of the genus *Bacteroides* of the phylum *Bacteroidetes* and certain members of the phylum *Firmicutes* among the three groups, namely, TB, LTBI and HC (Fig. 1C). However, none of these differences were significant. Together, these findings above revealed that Mtb infection, regardless of active or latent status, caused only minor changes in the composition of the gut microbiota, in line with previous studies on murines.[10,13]

Anti-TB treatment causes a significant alteration in the gut microbiota

Next, we examined the effects of anti-TB therapy on gut microbiota in active Mtb-infected patients in a four-way comparison of the TB, TB with 1-week front-line TB antibiotics (INH, RIF, PZA, and EMB, T1), TB with 2-week front-line TB antibiotics (T2) and TB cured (Tc) groups using the same methodology described above.

The observed number of OTUs, the Shannon index and the Pielou's evenness index were significantly reduced in TB patients with anti-TB treatment, especially with a more than 2-week duration of therapy, versus the active TB controls, which demonstrated that the loss of microbial diversity was significant after one week of treatment. When the groups of T1, T2 and Tc were compared with each other, we found that the observed number of OTUs in the T1 group was statistically greater than that in the T2 and Tc groups, but the Shannon index and Pielou's evenness index were not. There were no significant differences between the T2 and Tc groups in terms of the observed number of OTUs, the Shannon index and Pielou's evenness index. The results indicated that the anti-TB therapy could result in a diversity imbalance within two weeks (Fig. 2A). We next compared the overall community structure and composition of the microbiota in the naïve and treatment TB groups. NMDS analyses revealed a highly separated clustering of samples collected from TB patients from those of both TB patients receiving therapy and cured TB patients (Fig. 2B).

Further, we wanted to determine which taxa were significantly affected by anti-TB therapy. A significant decrease was observed in the treatment group in the relative abundances of *Ruminococcus sp. 39BFAA, Ruminococcus gnavus*, and *Faecalibacterium* (OTU 15), all belonging to genus *Clostridiales* of the phylum *Firmicutes* (Fig. 2C). In contrast, the relative abundances of *Bacteroides* (OTU230), *Bacteroides* (OTU 1513), *Bacteroides fragilis, Bacteroides plebeius, Bacteroides caccae, Bacteroides coprophilus,* and *Parabacteroides distasonis*, which all belong to genus *Bacteroides* of the phylum *Bacteroidetes,* were significantly enriched in the treatment group. In addition, OTU8 and OTU2972 assigned to the family *Erysipelotrichaceae* and *Enterococcus* in family *Enterococcaceae* of the phylum *Firmicutes* were significantly enriched in TB patients with 1 week front-line TB antibiotics, while other members of this family, such as *Erysipelatoclostridium ramosum*, were reduced compared to those in naïve TB patients. The genus *Bifidobacterium* was significantly reduced in the T1 group but significantly enriched in the Tc group when compared with the TB group. The alterations in the bacterial community structure and composition induced by antibiotic treatment were comparable in the T1, T2 and Tc groups. Together, these observations revealed that treatment with conventional anti-TB drugs may cause a significant alteration in the diversity and the community structure and composition of the gut microbiota.

Discussion

Studies of the microbiota are altering the way that we think about human diseases. There is a vital cross-talk between the mucosal tissues of our body, as exemplified by intestinal complications during respiratory disease and vice versa.[6,14] The dysbiosis of gut microbiota during Mtb infection or anti-TB treatment may play an important role in TB pathophysiological processes. This study examined the gut microbiota of active pulmonary TB, latent pulmonary TB and healthy subjects using Illumina HiSeq analysis of 16S rRNA-encoding genes. Further, we determined the effect of anti-TB treatment on the gut microbiota. The results showed that both active and latent Mtb infections induced only minor changes in the intestinal microbiota, while anti-TB therapy led to a rapid alteration in the microbiota as quickly as one week after treatment. The gut microbial α diversity was significantly reduced, and the overall structure of the gut microbiota composition was significantly distinct before and after anti-TB therapy. Our study contributes valuable information about the gut microbiota in pulmonary TB patients. These observations may imply potential links between gut microbiota changes and TB, and such changes may

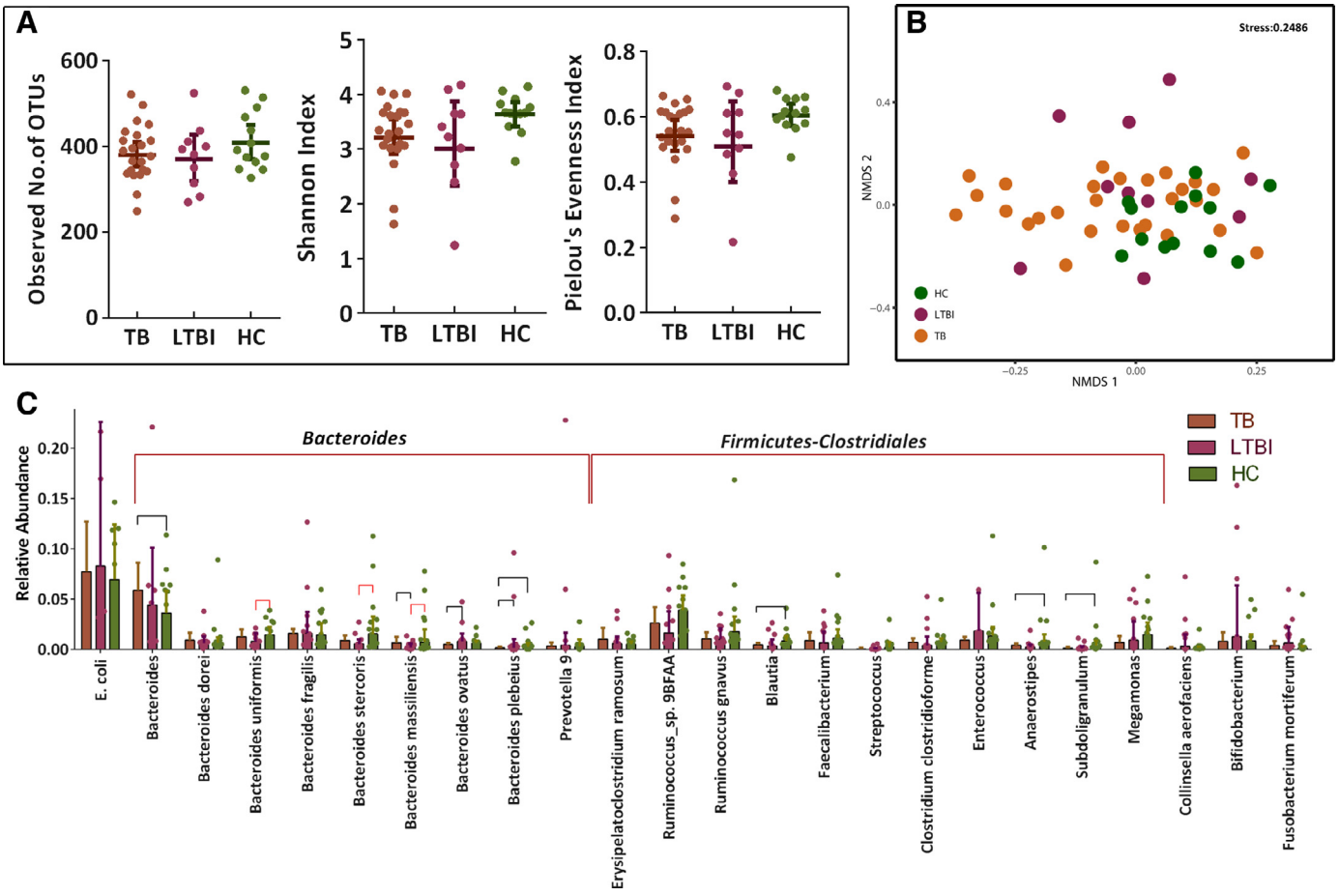

Fig. 1. Gut microbiota richness and diversity associated with pulmonary TB infection. (A) Alpha diversity was calculated by the number of observed genera, the Shannon index and Pielou's evenness index, which all revealed significant differences among the active TB (TB), latent TB (LTBI) and healthy controls (HC) groups. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Beta diversity using the ordination of gut bacterial communities. (1) Unsupervised ordination (nonmetric multidimensional scaling, NMDS) from TB specimens versus HC specimens. The analysis is based on OTUs from the sequences of the V4 regions of the 16S rRNA. NMDS analysis demonstrated differences in OTUs among active TB, LTBI and healthy subjects ($P < 0.072$). (C) Relative abundances of the most common organizational taxonomic units identified in stool samples from TB, LTBI and HC subjects. OTUs were compared by the Holm–Sidak method *t*-test for all OTUs, with average relative abundances of greater than 1% across all samples, and significant differences are demonstrated by *asterisks* ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Some genus designations appear more than once because multiple OTUs have the same consensus taxonomy.

constitute a risk factor for the progression and prognosis of pulmonary TB.

In agreement with previous studies using mouse models,[10] we found that pulmonary infection with Mtb causes alterations largely focused in the members of genus *Bacteroides* of phylum *Bacteroidetes* and the members of genus *Clostridiales* of phylum *Firmicutes*. However, these changes were minor in α or β diversity when active TB patients, latent TB patients and HCs were compared with one another. The minor changes induced by Mtb infection in the gut microbiota are not surprising given that the intestinal tract is not the primary site of infection for this pathogen. Pulmonary influenza infections can significantly alter the intestinal microbiota profile through a mechanism dependent on type I interferons (IFN-Is) produced in the lungs.[7,8] Moreover, many studies have suggested important roles for chemokines, including IFNα/IFNβ/IFN-γ, in host defence,[15,16] and it is possible that these cytokines in TB act as IFN-Is in pulmonary influenza infection.

Furthermore, in a mouse model, aerosol Mtb infection caused a rapid loss of diversity in the gut microbiota as rapidly as 6 days following lung infection.[9] Another longitudinal comparison of the microbiota of mice infected with Mtb by aerosol inhalation showed a slight but significant decrease in diversity that was evident until week 12 after infection.[10] In a cross-sectional study, researchers demonstrated that active and latent TB had no detectable effect on the intestinal microbiome composition. The number of observed OTUs was significantly lower in the treatment group when compared to the Mtb uninfected controls. This result may be due to anti-TB therapy but not TB infection.[17] The TB-associated gut microbiota show a greater diversity and richness of species compared with those of healthy household controls.[18] In another investigation, the differences in the gut microbiota among new TB, recurrent TB and healthy controls were characterized. There were no significant differences among the groups in terms of the Simpson and Shannon indexes.[13] In our study, no significant changes in alpha diversity were found. As described previously,[5,19] several variables, such as sampling time, number of samples, DNA extraction techniques, regions of 16S rRNA, sequencing methods, and analysis, can affect the evaluation of the gut microbiota, and no one standard method exists, which may underlie some of the differences between these studies.

Another important part of the present study was to observe the effects of the anti-TB drugs administered during pulmonary TB on the gut microbiota. Despite a number of studies describing the impact of antibiotics on the gut microbiota,[20] to the best of our knowledge, the effects of the front-line drugs used in anti-TB treatment have been rarely described.[10,17] Effective treatment of drug-susceptible TB requires at least 6 months of daily therapy, making it one of the longest courses of antibiotic therapy required to

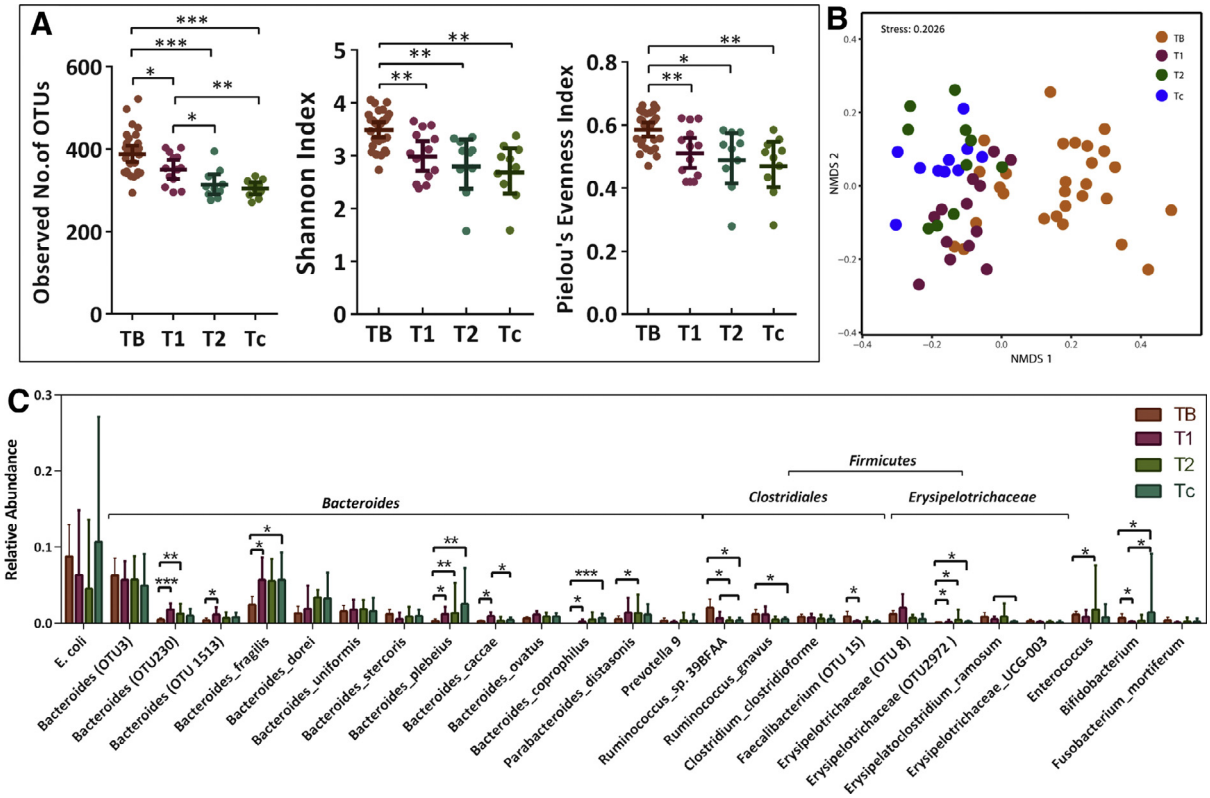

Fig. 2. Gut microbiota richness and diversity associated with anti-TB therapy (A) Alpha diversity was calculated by the number of observed genera, the Shannon index and Pielou's evenness index, which all revealed significant differences among the naïve active TB (TB), TB treated for 1 week (T1), TB treated for 2 weeks (T2) and cured TB (Tc) groups. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Beta diversity using the ordination of gut bacterial communities. Unsupervised ordination (nonmetric multidimensional scaling, NMDS) from the naïve active TB (TB), TB treated for 1 week (T1), TB treated for 2 weeks (T2) and cured TB (Tc) groups. The analysis is based on OTUs from the sequences of the V4 regions of the 16S rRNA. NMDS analysis demonstrated significant differences in OTUs among different groups ($P < 0.05$). (C) Relative abundances of the most common organizational taxonomic units identified in stool samples from TB, T1, T2 and Tc subjects. OTUs were compared by the Holm-Sidak method *t*-test for all OTUs with average relative abundances of greater than 1% across all samples, and significant differences are demonstrated by *asterisks* ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Some genus designations appear more than once because multiple OTUs have the same consensus taxonomy.

treat an infectious disease. This long treatment duration sets the stage for dysbiosis of the gut microbiota. Indeed, our study demonstrated that anti-TB antibiotics caused a significant alteration in the diversity and the community structure and composition of the gut microbiota. Although dysbiosis after the cessation of TB therapy was not investigated in this study, the alterations induced by anti-TB drugs likely lasted for a long time, as described by other studies.[10,15]

In conclusion, the gut microbiota sampled by stool in naïve and treated TB patients were analysed, and the gut bacterial communities significantly differed depending on the TB infection and anti-TB therapy. These data serve as a basis for further investigations into the role of the gut microbiome in TB treatments and outcomes.

Conflict of interest

None of the authors have a conflict of interest with the information presented in this manuscript.

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