Effect of seven anti-tuberculosis treatment regimens on sputum microbiome: a retrospective analysis of the HIGHRIF study 2 and PanACEA MAMS-TB clinical trials

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Summary

Background Respiratory tract microbiota has been described as the gatekeeper for respiratory health. We aimed to assess the impact of standard-of-care and experimental anti-tuberculosis treatment regimens on the respiratory microbiome and implications for treatment outcomes.

Methods In this retrospective study, we analysed the sputum microbiome of participants with tuberculosis treated with six experimental regimens versus standard-of-care who were part of the HIGHRIF study 2 (NCT00760149) and PanACEA MAMS-TB (NCT01785186) clinical trials across a 3-month treatment follow-up period. Samples were from participants in Mbeya, Kilimanjaro, Bagamoyo, and Dar es Salaam, Tanzania. Experimental regimens were composed of different combinations of rifampicin (R), isoniazid (H), pyrazinamide (Z), ethambutol (E), moxifloxacin (M), and a new drug, SQ109 (Q). Reverse transcription was used to create complementary DNA for each participant's total sputum RNA and the V3-V4 region of the 16S rRNA gene was sequenced using the Illumina metagenomic technique. Qiime was used to analyse the amplicon sequence variants and estimate alpha diversity. Descriptive statistics were applied to assess differences in alpha diversity pre-treatment and post-treatment initiation and the effect of each treatment regimen.

Findings Sequence data were obtained from 397 pre-treatment and post-treatment samples taken between Sept 26, 2008, and June 30, 2015, across seven treatment regimens. Pre-treatment microbiome (206 genera) was dominated by Firmicutes (2860 [44%] of 6500 amplicon sequence variants [ASVs]) at the phylum level and *Streptococcus* (2340 [36%] ASVs) at the genus level. Two regimens had a significant depressing effect on the microbiome after 2 weeks of treatment, $HR_{20mg/kg}ZM$ (Shannon diversity index p=0·0041) and $HR_{35mg/kg}ZE$ (p=0·027). Gram-negative bacteria were the most sensitive to bactericidal activity of treatment with the highest number of species suppressed being under the moxifloxacin regimen. By week 12 after treatment initiation, microbiomes had recovered to pre-treatment level except for the $HR_{35mg/kg}ZE$ regimen and for genus *Mycobacterium*, which did not show recovery across all regimens. Tuberculosis culture conversion to negative by week 8 of treatment was associated with clearance of genus *Neisseria*, with a 98% reduction of the pre-treatment level.

Interpretation $HR_{20mg/kg}ZM$ was effective against tuberculosis without limiting microbiome recovery, which implies a shorter efficacious anti-tuberculosis regimen with improved treatment outcomes might be achieved without harming the commensal microbiota.

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Introduction

Respiratory microbiota has been described as a gatekeeper of respiratory health that modulates host immunity and the resistance to colonisation by pathogens.[1] Different disease states and exposure to antibiotics have been shown to cause dysbiosis of the microbiome.[2] It has been shown that treatment with a standard first-line anti-tuberculosis regimen of 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol (HRZE) followed by 4 months of isoniazid and rifampicin, does not perturb overall microbiome diversity but depletes some immunologically important commensal bacteria, an outcome that might have long-term consequences on an individual's health.[3] Short-term tuberculosis regimen courses could shorten the length of microbiome exposure to antibiotics and reduce the risk of long-term damage to the microbiome. Accordingly, novel short-term tuberculosis regimen courses are being investigated, including the 4 month rifapentine–moxifloxacin-containing regimen that was recommended by WHO in 2022.[4]

The human lung is not sterile, even in healthy individuals. The generally diverse core composition of

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Research in context

Evidence before this study

Tuberculosis is treated with combinations of antibiotics in first-line and second-line regimens, depending on the drug sensitivity profile of the tuberculosis bacteria. We asked what impact these antibiotics, particularly the new clinical trial regimens, have on the respiratory microbiome and treatment outcome. We assessed high-dose rifampicin plus novel combinations, including moxifloxacin and SQ109, for efficacy in treating tuberculosis in two clinical trials, HIGHRIF study 2 and PanACEA MAMS-TB clinical trials. These studies showed that high-dose rifampicin was safe and efficacious and that including moxifloxacin increased early mycobactericidal activity without affecting safety. We searched PubMed, MEDLINE, and EMBASE using search terms "respiratory microbiome", "microbiota", "anti-tuberculosis antibiotics", "tuberculosis treatment response", and "antibiotic impact on microbiome" for studies published between July 1, 2003, and March 30, 2022, in the English language. The available literature described the microbiome of people who were healthy compared with people with tuberculosis and the effects of the first-line anti-tuberculosis regimen on the respiratory microbiota. No information was available on the new combinations of antibiotics to treat tuberculosis. Therefore, we assessed the impact of the new regimens on the sputum microbiome in comparison with the standard first-line regimen.

Added value of this study

This study has revealed the value and knowledge of using RNA, rather than DNA, in studying microbiome and how it responds to antibiotic therapy. RNA is a molecule that reflects cell viability, and, thus, is crucial for investigating the effect of antibiotics on microbiota. These findings have improved understanding of how new anti-tuberculosis regimens affect the microbiome, particularly the high sensitivity exhibited by the microbiome in the first two weeks of treatment.

Implications of all the available evidence

Although the microbiome recovers following exposure to antibiotics, it happens at different rates under different regimens. Broad-spectrum antibiotics in regimens have a large depressing effect on the microbial community, but this can be reduced by optimising the dosage. Therefore, it is crucial that microbiome analysis is considered as one of the parameters to evaluate and approve new antibiotics or treatment combinations. Future studies considering large sample size and long-term clinical outcomes will be crucial in establishing microbiome analysis as a drug safety parameter to include in clinical trials. Further investigation is needed to ascertain whether recovery of the microbiome while still under antibiotic pressure is influenced by replenishment from dietary sources or acquisition of genes for antibiotic resistance.

the microbiota in the human lung varies between people in relative abundance and prevalence.[5] The microbiome can vary based on age (neonates, infants, young people, and adults), diet, and disease.[6] Diseases such as lung cancer and tuberculosis have been associated with changes in the microbiome characterised by an increase or decrease of some taxa. The lung is the predilection site for *Mycobacterium tuberculosis* infection, and its microbiota are associated with various states of tuberculosis. This means that an intervention, such as antibiotic treatment, adds an extra level of pressure on the microbiome, which can further alter the composition and function of the lung microbiota.[7] The effect of tuberculosis regimens on lung microbiota is likely to be more associated with antibiotics that have activity against a wide range of Gram-positive and Gram-negative bacteria.[8] It is thus necessary to investigate the effect of the novel tuberculosis regimen courses, especially those incorporating broad-spectrum high-dose antibiotics.[9–11]

We used sputum to investigate the effect of standard-of-care and investigational tuberculosis regimen courses on the lung microbiota and their implications on treatment outcomes.

Methods

Study design

We did a retrospective analysis of the effect of anti-tuberculosis drugs on sputum microbiome using samples that were collected during two clinical trials: PanACEA MAMS-TB (NCT01785186; conducted between May 7, 2013, and March 25, 2014)[12] and the HIGHRIF study 2 (NCT00760149; conducted between Sept 26, 2008 and Sept 8, 2013).[13]

Sputum samples for the microbiome analysis were obtained from participants in the PanACEA MAMS-TB and HIGHRIF study 2 clinical trials in Tanzania. Approval of the MAMS study was obtained from the Tanzania National Institute for Medical Research (NIMR)-Mbeya Medical Research Centre ethics review committee, the NIMR-National Health Research ethics committee (NatHREC), and University of Munich (study sponsor) ethics committee. HIGHRIF study 2 was approved by the Kilimanjaro Christian Medical College Research Ethics and Review Committee, the Ifakara Health Institute Institutional Review Board, and NatHREC. Both studies were conducted according to Good Clinical Practice guidelines. Participants gave oral informed consent for inclusion in this follow-up analysis.

Study setting and participants

PanACEA MAMS-TB trial

The PanACEA MAMS-TB trial was a five-arm trial assessing four experimental treatment regimens, including isoniazid and 10 mg/kg of rifampicin plus pyrazinamide and ethambutol ($HR_{10mg/kg}ZE$; standard-of-

	Dosage and duration
PanACEA MAMS-TB clinical trial[12]	
Group 1 (HR$_{35mg/kg}$ZE)	35 mg/kg per day rifampicin, 15–20 mg/kg per day ethambutol, 5 mg/kg per day isoniazid, and 25 mg/kg per day pyrazinamide for 12 weeks followed by 5 mg/kg per day isoniazid and 10 mg/kg per day rifampicin for 14 weeks
Group 2 (HR$_{20mg/kg}$ZM)	20 mg/kg per day rifampicin, 400 mg/kg per day moxifloxacin, 5 mg/kg per day isoniazid, and 25 mg/kg per day pyrazinamide for 12 weeks followed by 5 mg/kg per day isoniazid and 10 mg/kg per day rifampicin for 14 weeks
Group 3 (HR$_{20mg/kg}$ZQ)	20 mg/kg per day rifampicin, 300 mg/kg per day SQ109, 5 mg/kg per day isoniazid, and 25 mg/kg per day pyrazinamide for 12 weeks followed by 5 mg/kg per day isoniazid and 10 mg/kg per day rifampicin for 14 weeks
Group 4 (HR$_{10mg/kg}$ZQ)	10 mg/kg per day rifampicin with 300 mg SQ109, 5mg/kg per day isoniazid, and 25 mg/kg per day pyrazinamide for 12 weeks followed by 5 mg/kg per day isoniazid and 10 mg/kg per day rifampicin for 14 weeks
Control (HR$_{10mg/kg}$ZE)	10 mg/kg rifampicin, 5 mg/kg isoniazid, 25 mg/kg pyrazinamide, and 15–20 mg/kg ethambutol daily
HIGHRIF study 2 clinical trial[13]	
600 mg (HR$_{600mg}$ZE)	Four capsules of fixed-dose combination dose (each capsule containing rifampicin 150 mg, isoniazid 75 mg, pyrazinamide 400 mg, and ethambutol 275 mg) plus two capsules of placebo
900 mg (HR$_{900mg}$ZE)	Four capsules of fixed-dose combination dose (each capsule containing rifampicin 150 mg, isoniazid 75 mg, pyrazinamide 400 mg, and ethambutol 275 mg) plus one capsule with 300 mg rifampicin plus one capsule of placebo
1200 mg (HR$_{1200mg}$ZE)	Four capsules of fixed-dose combination (each capsule containing rifampicin 150 mg, isoniazid 75 mg, pyrazinamide 400 mg, and ethambutol 275 mg) plus two capsules with 300 mg rifampicin
All participants	Each participant received 300 mg isoniazid and 600 mg rifampicin daily for 4 months of post-intensive phase treatment

Table 1: Drug dosing and duration under the PanACEA MAMS-TB and HIGHRIF study 2 clinical trials

care; isoniazid and 10 mg/kg of rifampicin plus pyrazinamide and SQ109 (HR$_{10mg/kg}$ZQ); isoniazid and 20 mg/kg of rifampicin plus SQ109 (HR$_{20mg/kg}$ZQ); isoniazid and 20 mg/kg of rifampicin plus pyrazinamide and moxifloxacin (HR$_{20mg/kg}$ZM); and isoniazid and 35 mg/kg of rifampicin plus pyrazinamide and ethambutol (HR$_{35mg/kg}$ZE; table 1).

The trial was conducted in three sites in Tanzania and four research institutes affiliated with hospitals in South Africa and is described elsewhere in more detail.[12] For PanACEA MAMS-TB, only samples from participants in Mbeya, Tanzania, were included in the analysis. All patients who responded well clinically to the treatment or had at least one negative culture towards the end of treatment follow-up were included in this study. Patients were followed up until 6 months after the end of treatment by telephone calls or on-site visits if participants were unwell. The study collected sputum samples taken weekly up to week 12. An outcome of cure, no information, or relapse was assigned at the end of the follow-up. Bacterial load was measured by Mycobacteria growth indicator tube time to positivity and by tuberculosis molecular bacterial load assay (TB-MBLA).[12]

HIGHRIF study 2
The HIGHRIF2 study investigated the pharmacokinetics, tolerability, and bacteriological response of rifampicin administered at varying doses. Details of the study design, sites, and selection of the study participants were published by Aarnoutse and colleagues.[13] Briefly, the study was conducted between Sept 26, 2008, and Sept 8, 2013, in Kilimanjaro and Dar es Salaam, Tanzania. Participants were given a fixed dose of standard tuberculosis drugs containing rifampicin at 600 mg

(HR$_{600mg}$ZE; standard-of-care), 900 mg (HR$_{900mg}$ZE), or 1200 mg (HR$_{1200mg}$ZE) daily (table 1). Study medications were taken together once daily in the morning for 7 days per week. After the intensive treatment phase, all patients were treated according to Tanzanian guidelines for 4 months.

Sputum was collected once per week up to week 12, and at weeks 14, 17, 22, and 26 during the treatment course. Sputum total RNA was extracted as previously described.[14] To prevent RNA loss after expectoration, sputa from the PanACEA MAMS-TB study were preserved in guanidine thiocyanate containing β-mercaptoethanol, whereas sputa from HIGHRIF study 2 were preserved in Oragene cups (DNA Genotek, Stittsville, ON, Canada). Sputa from both studies were frozen at –80°C until RNA extraction, applying the chloroform–phenol method. RNA extract (4 µL) was used to measure *M tuberculosis* bacillary load by TB-MBLA.

Procedures
Total RNA was reverse transcribed to complementary DNA (cDNA) using QuantiTect Reverse Transcription Kit (Qiagen, Manchester, UK). The reverse transcription reaction mixture was prepared by adding reverse transcription enzyme (1 µL), transcription buffer (4 µL), and transcription primer mix (1 µL) to the thawed RNA sample before incubation at 95°C for 3 mins. Resulting cDNA was quantified using a high-sensitivity single-stranded DNA Qubit assay (Fisher Scientific UK, Loughborough, UK). The cDNA was diluted with nuclease-free water to ensure the starting template concentration was not too high for PCR.

Primers targeting the V3–V4 region of the 16S rRNA gene were used to amplify the cDNA, as described by

Klindworth and colleagues.[15] Briefly, 2·5 μL of cDNA was added to 17·7 μL of the amplicon PCR master mix containing Taqmix (Qiagen, Manchester, UK; 9·5 μL), forward primer (4 μL), and reverse primer (4 μL). The amplicon PCR conditions were set up as follows: 95°C for 3 min, 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and then 72°C for 5 min. The amplicons were cleaned up using AMPure XP reagent (Beckman Coulter, Wycombe, UK) and 10mM tris(hydroxy) methylaminomethane buffer at pH 8·5 and ethanol (appendix p 2).[16]

For library preparation, index PCR was used to label the amplicons using variable DNA adapters following the Illumina metagenomic sequencing protocol. Briefly, 10 μL of cDNA amplicons were added to the index PCR master mix containing KAPA Hifi hot start ready mix (25 μL; Sigma Aldrich, Glasgow, UK) and PCR grade water (10 μL). Index PCR products were cleaned (appendix p 2), quantified using Qubit assay, and then labelled by adding unique indices.

Amplicon concentration was normalised to ensure equal concentration before pooling into one library. Amplicon quality and specificity were assessed using gel electrophoresis with 2·5% agarose gel, SYBRsafe dye (Qiagen, Manchester, UK), and 50 bp ladder. A single band of 600 bp was obtained indicating the specificity of the amplicons in the pooled library (appendix p 2). The concentration of the pooled library was measured and found to be 2·47 ng/μl. Background or cross-contamination was checked by running negative control samples, nuclease-free water, and master mixes for cDNA synthesis and amplicon PCR. These controls were run from in vitro cDNA synthesis to sequencing. The absence of detectable sequences was confirmation of no background contamination.

High-throughput amplicon sequencing was performed at the Integrated Microbiome Resource (Edinburgh Genomics Centre, Edinburgh, UK) on HiSeq 2000 platform. Before sequencing, a bioanalyser quality check confirmed sufficient material for sequencing with no appreciable adapter dimers. Raw reads were filtered, trimmed, and dereplicated, paired reads were merged, denoising was done, and chimaeras were removed using the DADA2 pipeline (1.8) within QIIME2 (version 2020.2). Taxa were allocated to amplicon sequence variants (ASVs) using the SILVA 132 database. Sequences assigned to eukaryotes and archaea were removed. Analyses were performed on the SILVA 132 database for total bacteria and sequences assigned to photosynthetic cyanobacteria were extracted and analysed separately. Alpha diversity metrics (amplicon sequence variant richness [taxa relative abundance], Faith's phylogenetic diversity [summation of length and number of phylogenetic tree units], Shannon diversity index [number of species scaled by their distribution in the community], and Pielou's evenness index [distributions of different species in the community]) were calculated for treatment medians within QIIME2 at a rarefaction of 1500 reads after ensuring all samples had reached the rarefaction curve plateau.

Statistical analysis

We grouped participants based on their region of origin and the difference in their microbiome diversity tested using a Mann-Whitney test. Similarly, we applied a Mann-Whitney test to calculate the difference between pre-treatment and post-treatment alpha diversity under different regimens. We used Spearman's rank correlation to calculate the correlation between *Mycobacterium* relative abundance and tuberculosis bacterial load measured by TB-MBLA. We applied a one-way ANOVA to test the variation in taxa richness over 8 weeks of treatment among those whose tuberculosis culture was negative, positive, or indeterminate. Indeterminate was defined as a culture in which the tuberculosis status could neither be called positive or negative. All calculations were done in GraphPad Prism (version 9) and statistical significance was accepted at $p < 0.05$.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Of the 394 samples, 122 (31%) were from $HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$, 40 (10%) were from $HR_{10mg/kg}ZQ$, 48 (12%) were from $HR_{900mg/kg}ZE$, 52 (13%) were from $HR_{1200mg/kg}ZE$, 45 (11%) were from $HR_{35mg/kg}ZE$, 42 (11%) were from $HR_{20mg/kg}ZQ$, and 45 (11%) were from $HR_{20mg/kg}ZM$ treatment regimens. Demographic data for the participants who provided sputa that were included in the current study are summarised in the appendix (p 2).

A total of 6500 ASVs from individuals treated for tuberculosis were analysed. Pre-treatment taxa were dominated by Firmicutes (2860 [44%]), followed by Bacteroidetes (1170 [18%]), Proteobacteria (845 [13%]), and Actinobacteria (52 [0·8%]). When the microbiome was divided by region of participant origin, Firmicutes remained dominant at 672 (42%; 1600 ASVs) in north–southeast and 2597 (53%; 4900 ASVs) in southwest Tanzania. The proportion of pre-treatment Actinobacteria was higher in north–southeast (304 [19%]) ASVs compared with in southwest (245 [5%]). Bacteroidetes (882 [18%]) and Proteobacteria (735 [15%]) ASVs were proportionally more abundant in southwest than in north–southeast (appendix p 3).

At the genus level, the four most abundant phyla were represented by *Streptococcus* (2340 [36%]), *Neisseria* (650 [10%]), *Veillonella* (390 [6%]), and *Mycobacterium* (325 [5%]) ASVs. When divided by sample origin, *Mycobacterium*, with 208 (13%) ASVs, was the second most dominant genus in north–southeast Tanzania surpassing *Neisseria* and *Veillonella* (appendix p 4).
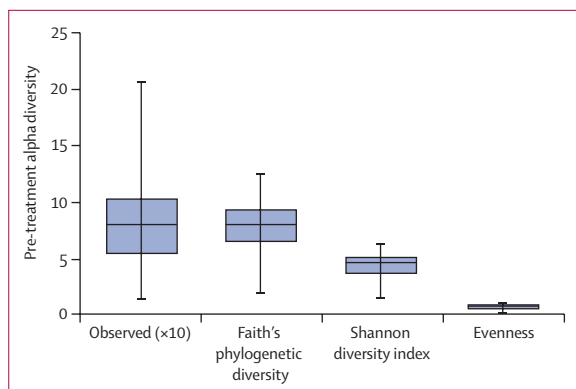
Figure 1: The pre-treatment microbiome richness, distribution, and diversity
Some taxa were more represented than others as indicated by low evenness. The scale of observed sequence variants (sample richness) was × 10 of the y-axis value.

Figure 2: Change in alpha diversity under different regimens following initiation of treatment
The highest fall in alpha diversity occurred in the first 2 weeks of treatment, after which alpha diversity began to recover. See table 1 for regimen details. ASV=amplicon sequence variant.

Before treatment, the alpha diversity of the whole cohort was median 81 (IQR 17–205) sample richness, 7·9 (2–12) Faith's phylogenetic diversity, 4·7 (1·6–6·2) Shannon diversity index, and 0·7 (0·3–0·9) Pielou's evenness. Evenness was comparatively lower than Shannon diversity index and Faith's phylogenetic diversity, suggesting an unevenly distributed microbiome in which some taxa dominated others (figure 1).

After establishing the pre-treatment alpha and beta diversity, we then explored how this diversity changed under different regimens after the initiation of treatment. In all regimens, the highest reduction in abundance and diversity occurred in the first 2 weeks of treatment. After the first 2 weeks of treatment, abundance and diversity began to recover, achieving pre-treatment level by week 8 after treatment began in most of the regimens. There was a fall-and-rise pattern of alpha diversity induced differently by different regimens. $HR_{10mg/kg}ZQ$ was responsible for the smallest reduction of the alpha diversity across all regimens (appendix pp 5–7). $HR_{20mg/kg}ZM$ caused the largest depression of evenness, dropping from 0·75 to 0·55. Evenness rose during the $HR_{10mg/kg}ZE$ regimen. Overall, the moxifloxacin–rifampicin containing regimen ($HR_{20mg/kg}ZM$), followed by high-dose rifampicin regimen $HR_{35mg/kg}ZE$, caused the largest depression of alpha diversity (figure 2A–D).

Using the Mann–Whitney test, we assessed whether the change in alpha diversity was significantly different between pre-treatment and post-treatment microbiome across the treatment course. Evenness significantly increased in the standard regimen $HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$ from 0·72 at baseline to 0·80 at week 12 of treatment (p=0·035 at week 2, p=0·024 at week 8, and p=0·034 at week 12 of treatment). Furthermore, the standard regimen did not cause a significant reduction of richness, Faith's phylogenetic diversity, or Shannon diversity index across the treatment period. The high-dose rifampicin regimen, $HR_{35mg/kg}ZE$ reduced richness only at week 2 (p=0·026), while Faith's phylogenetic
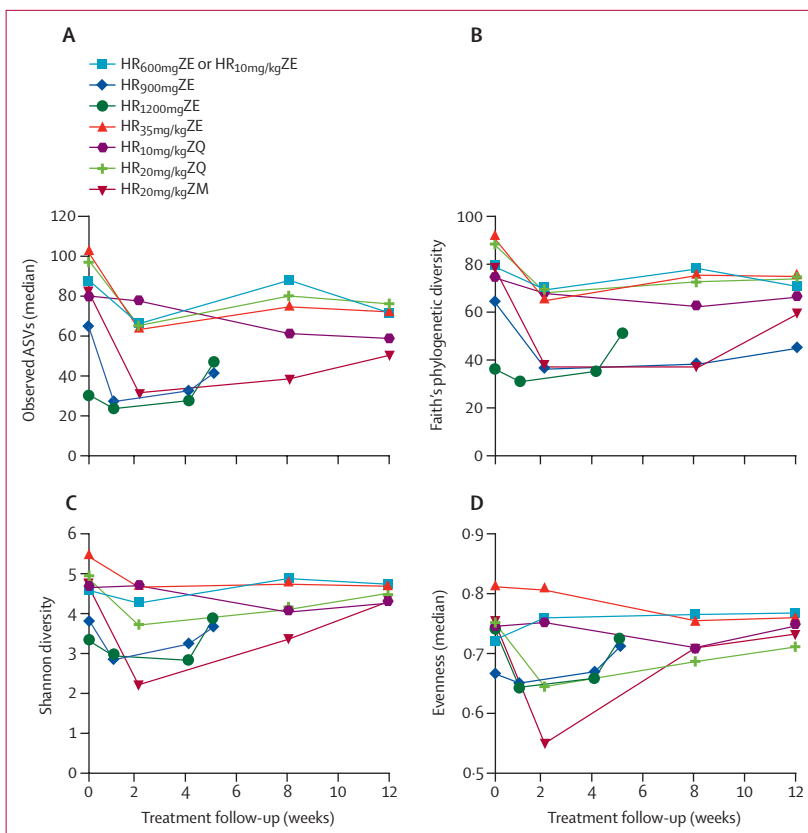
diversity (p=0·0055 at week 2, p=0·015 at week 8, and p=0·014 at week 12) and Shannon diversity index (p=0·027 at week 2, p=0·021 at week 8, and p=0·011 at week 12) were reduced across the follow-up period.

There was no significant reduction of evenness with $HR_{35mg/kg}ZE$ across the treatment course. The rifampicin–moxifloxacin regimen, $HR_{20mg/kg}ZM$, had an early effect on all alpha diversity measures, reducing richness (p=0·0007 at week 2 and p=0·0023 at week 8), Faith's phylogenetic diversity (p=0·0078 at week 2 and p=0·0025 at week 8), Shannon diversity index (p=0·0040 at week 2 and p=0·015 at week 8), and evenness (p=0·015 at week 2). Although there was an early reduction in richness in those given the $HR_{20mg/kg}ZM$ regimen, microbiome recovery was observed in all diversity measures at week 12 and from week 8 of treatment for evenness. $HR_{900mg/kg}ZE$ only had reduced richness at week 1 (p=0·031) and $HR_{20mg/kg}ZQ$ had reduced evenness at week 12 (p=0·0093) of treatment. No significant reduction or increase was observed with $HR_{10mg/kg}ZQ$ and $HR_{1200mg}ZE$ in all alpha diversity indices across the treatment period (table 2, table 3).

We sought to identify which taxa changed and how they changed under the treatment regimens that induced a significant change in alpha diversity measure. Taxa were

	Observed ASVs			Faith's phylogenetic diversity			Shannon diversity index			Evenness		
	Week 2	Week 8	Week 12	Week 2	Week 8	Week 12	Week 2	Week 8	Week 12	Week 2	Week 8	Week 12
HR$_{600mg}$ZE or HR$_{10mg/kg}$ZE												
Baseline (n=19)	88	88	88	8·02	8·02	8·02	4·68	4·68	4·68	0·72	0·72	0·72
Follow-up (n=16–26)	66	88	71·5	7·04	7·94	7·16	4·38	4·97	4·83	0·76	0·76	0·77
p value	0·090	0·92	0·61	0·09	>0·99	0·50	0·97	0·27	0·50	0·035*	0·024*	0·034*
HR$_{35mg/kg}$ZE												
Baseline (n=9)	102	102	102	9·23	9·23	9·23	5·49	5·49	5·49	0·81	0·81	0·81
Follow-up (n=8–9)	63	74·5	72	6·58	7·66	7·6	4·76	4·83	4·78	0·8	0·75	0·76
p value	0·026*	0·070	0·089	0·0055*	0·015*	0·014*	0·027*	0·021*	0·011*	0·54	0·17	0·063
HR$_{10mg/kg}$ZQ												
Baseline (n=7)	80	80	80	7·55	7·55	7·55	4·75	4·75	4·75	0·74	0·74	0·74
Follow-up (n=8)	77·5	61	58·5	6·88	6·33	6·75	4·79	4·16	4·37	0·75	0·71	0·74
p value	0·63	0·29	0·52	0·34	0·39	0·61	0·96	0·78	0·87	0·39	0·78	0·55
HR$_{20mg/kg}$ZQ												
Baseline (n=8)	98	98	98	8·96	8·96	8·96	4·96	4·96	4·96	0·75	0·75	0·75
Follow-up (n=7–9)	65	80	76	6·92	7·39	7·5	3·85	4·22	4·61	0·64	0·69	0·71
p value	0·16	0·15	0·34	0·094	0·075	0·19	0·014*	0·059	0·24	0·0093*	0·14	0·33
HR$_{20mg/kg}$ZM												
Baseline (n=9)	82	82	82	7·96	7·96	7·96	4·77	4·77	4·77	0·75	0·75	0·75
Follow-up (n=7–9)	31	38	50	3·76	5·08	6·032	2·42	3·51	4·41	0·55	0·71	0·73
p value	0·0007*	0·0023*	0·29	0·0078*	0·0025*	0·41	0·0040*	0·015*	0·41	0·015*	0·24	0·76

Baseline and follow-up are in median, unless specified. Except for upward trend of evenness under the HR$_{600mg}$ZE or HR$_{10mg/kg}$ZE regimen, in most cases microbiome took a downward trend that was significant in the HR$_{35mg}$ZE or HR$_{20mg/kg}$ZM regimens. See table 1 for regimen details. ASV=amplicon sequence variants. *p<0·05.

Table 2: Mann-Whitney test of the difference between pre-treatment and post-treatment microbiome under different regimens at weeks 2, 8, and 12

	Observed ASVs			Faith's phylogenetic diversity			Shannon diversity index			Evenness		
	Week 1	Week 4	Week 5	Week 1	Week 4	Week 5	Week 1	Week 4	Week 5	Week 1	Week 4	Week 5
HR$_{900mg}$ZE												
Baseline (n=6)	64·5	64·5	64·5	6·52	6·52	6·52	3·84	3·84	3·84	0·67	0·67	0·67
Follow-up (n=7–14)	26·5	32	41	3·66	3·88	4·58	3·02	3·39	3·79	0·65	0·67	0·71
p value	0·031*	0·25	0·33	0·091	0·18	0·35	0·091	0·23	0·85	0·90	>0·99	0·28
HR$_{1200mg}$ZE												
Baseline (n=7)	30	30	30	3·67	3·67	3·67	3·48	3·48	3·48	0·74	0·74	0·74
Follow-up (n=6–11)	23	27	46·5	3·13	3·58	5·19	3·12	3·01	4·01	0·64	0·66	0·72
p value	0·20	0·29	0·63	0·29	0·37	0·95	0·15	0·21	0·95	0·33	0·25	0·73

Baseline and follow-up are in median, unless specified. Except for upward trend of evenness under the HR$_{600mg}$ZE or HR$_{10mg/kg}$ZE regimen, in most cases microbiome took a downward trend that was significant in the HR$_{35mg}$ZE or HR$_{20mg/kg}$ZM regimens. See table 1 for regimen details. ASV=amplicon sequence variants. *p<0·05.

Table 3: Mann-Whitney test of the difference between pre-treatment and post-treatment microbiome under different regimens at weeks 1, 4, and 5

sorted based on their relative abundance (abundance), with more than 1% abundance named, while the ones with abundance between 0·0–0·9% were grouped under others. Along the course of treatment, some of the species with more than 1% abundance were reduced to less than 1% and were replaced by those from the others group, whose relative abundance rose to more than 1%. In general, the most abundant species never fell to less than 1% abundance across the treatment period in all regiments (see appendix p 8 for alpha diversity).

Distinctive regimen-induced taxa changes were observed in the microbiota with less than 1% abundance.

Considering that the changes in the taxa with more than 1% abundance were less distinctive between regimens, we analysed the taxa with less than 1% abundance (0·01–0·99 relative abundance). In the first-line regimen, HR$_{600mg}$ZE or HR$_{10mg/kg}$ZE, 50 taxa had less than 1% relative abundance before treatment, of which seven (14%) of 50 were reduced to undetectable levels by week 2 of treatment. In the experimental regimens, HR$_{35mg/kg}$ZE and HR$_{20mg/kg}$ZM, there were 56 and 62 pre-treatment taxa at less than 1% relative abundance, of which 24 (45%) of 53 and 32 (52%) of 62 were undetectable by week 2 of treatment, respectively. Gram-negative genera were the most

represented in the taxa cleared by regimens, five (71%) of seven for $HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$, 12 (50%) of 24 for $HR_{35mg/kg}ZE$, and 19 (59%) of 32 for $HR_{20mg/kg}ZM$, compared with Gram-positive genera, two (27%) of seven in $HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$, five (21%) of 24 in $HR_{35mg/kg}ZE$, and six (19%) of 32 in $HR_{20mg/kg}ZM$, cleared by the same regimens (appendix p 9).

The relative abundance of *Mycobacterium* spp continuously reduced and did not recover to pre-treatment levels in all treatment regimens. The reduction in relative abundance of *Mycobacterium* spp was correlated with the *M tuberculosis* bacillary load measured by the ribosomal RNA-based reverse transcriptase quantitative PCR test, TB-MBLA, across the period of treatment ($r=0.74$, 95% CI 0.49–0.87, $p<0.0001$; figure 3).

Participants were grouped according to their tuberculosis liquid culture results at week 8 of treatment: culture negative (early conversion), culture positive (non-converters), and indeterminate (culture grows a contaminant but tuberculosis cannot be definitively ruled in or out). We observed that the effect of the regimen–microbiome interaction was associated with the culture test outcome (appendix p 10). Contingency plots of their microbiome at phylum–class and family–genus levels revealed Proteobacteria of class Gammaproteobacteria most distinctively changed between the treatment outcome groups. Gammaproteobacteria include the most implicated pathogenic bacteria: *Neisseria* spp, *Pseudomonas* spp, *Moraxella* spp, and *Haemophilus* spp. By week 8 of treatment, abundance of Gammaproteobacteria was substantially reduced, of which genus *Neisseria* was decreased by 98% in sputum samples in the culture negative group (early conversion group). In the non-converted (culture positive) group, Gammaproteobacteria abundance stayed relatively stable, including a 2% increase in genus *Neisseria*. The indeterminate group had a small pre-treatment abundance of Gammaproteobacteria that remained small but was dominated by genus *Neisseria*, which increased by 25% from baseline to week 8 of treatment (figure 4A, B).

Discussion

In this study, we systematically assessed the effect of different combinations of anti-tuberculosis antibiotics on respiratory microbiomes and the implication of this effect on treatment outcomes. Unlike most studies that use DNA, microbiome was drawn from RNA, enabling us to accurately assess the effect of antibiotics on microbiome viability over the course of treatment. We show that, once exposed to anti-tuberculosis antibiotics, the microbiome diversity and abundance takes a fall and rise pattern akin to that of predator–prey relationships, reflecting the sensitivity and resilience of the microbiome under antibiotic pressure. In a longitudinal follow-up of 12 weeks, a significant reduction in microbiome diversity was observed in the first 2 weeks of treatment,
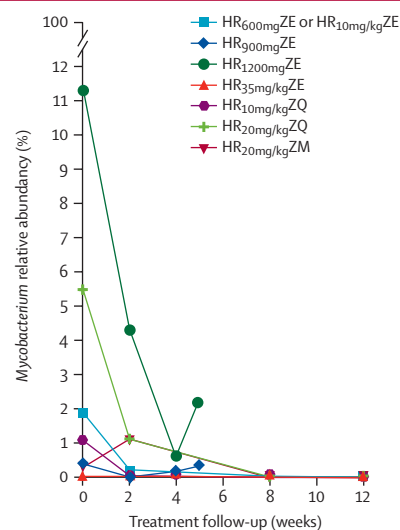

	0 weeks	2 weeks	4 weeks	5 weeks	8 weeks	12 weeks
$HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$	1.89	0.23			0.048	0.014
$HR_{900mg}ZE$	0.4	0.019	0.18	0.34		
$HR_{1200mg}ZE$	11.32	4.33	0.62	2.27		
$HR_{35mg/kg}ZE$	0.07	0.03			0.003	0.004
$HR_{10mg/kg}ZQ$	1.08	0.057			0.011	0.004
$HR_{20mg/kg}ZQ$	5.46	1.12			0.031	0.012
$HR_{20mg/kg}ZM$	0.31	1.13			0.007	0.018

Figure 3: The relative abundance of *Mycobacterium* over the treatment course
There was no recovery to pre-treatment levels in all regimens.

recapitulating the early bactericidal activity of anti-tuberculosis antibiotics. The regimens that contained moxifloxacin and rifampicin 35 mg/kg were responsible for the largest reduction of microbiome diversity that was significantly different from pre-treatment levels. Although most of the taxa recovered, genus *Mycobacterium* did not show recovery, suggesting a unique sensitivity to anti-tuberculosis antibiotics. The trend of *Mycobacterium* spp elimination was measured by TB-MBLA, a reverse transcriptase quantitative qPCR-based assay that specifically quantifies viable bacilli from the *M tuberculosis* complex.[16] It is important to note that, despite causing tuberculosis disease, *Mycobacterium* was never the most abundant taxa in any of the patients. Future studies could explore the existence of *M tuberculosis* as commensal bacteria, particularly in asymptomatic or healthy individuals, and the threshold or trigger by which *M tuberculosis* becomes pathogenic.

Pre-treatment microbiome was more diverse in the southwest of Tanzania than in north–southeast, suggesting a geographical influence on host microbiome. Geographical location, which encapsulates environment and the kind of diet, has been shown to shape the microbiome acquired.[1] However, participants in both regions had uneven microbiome, dominated by
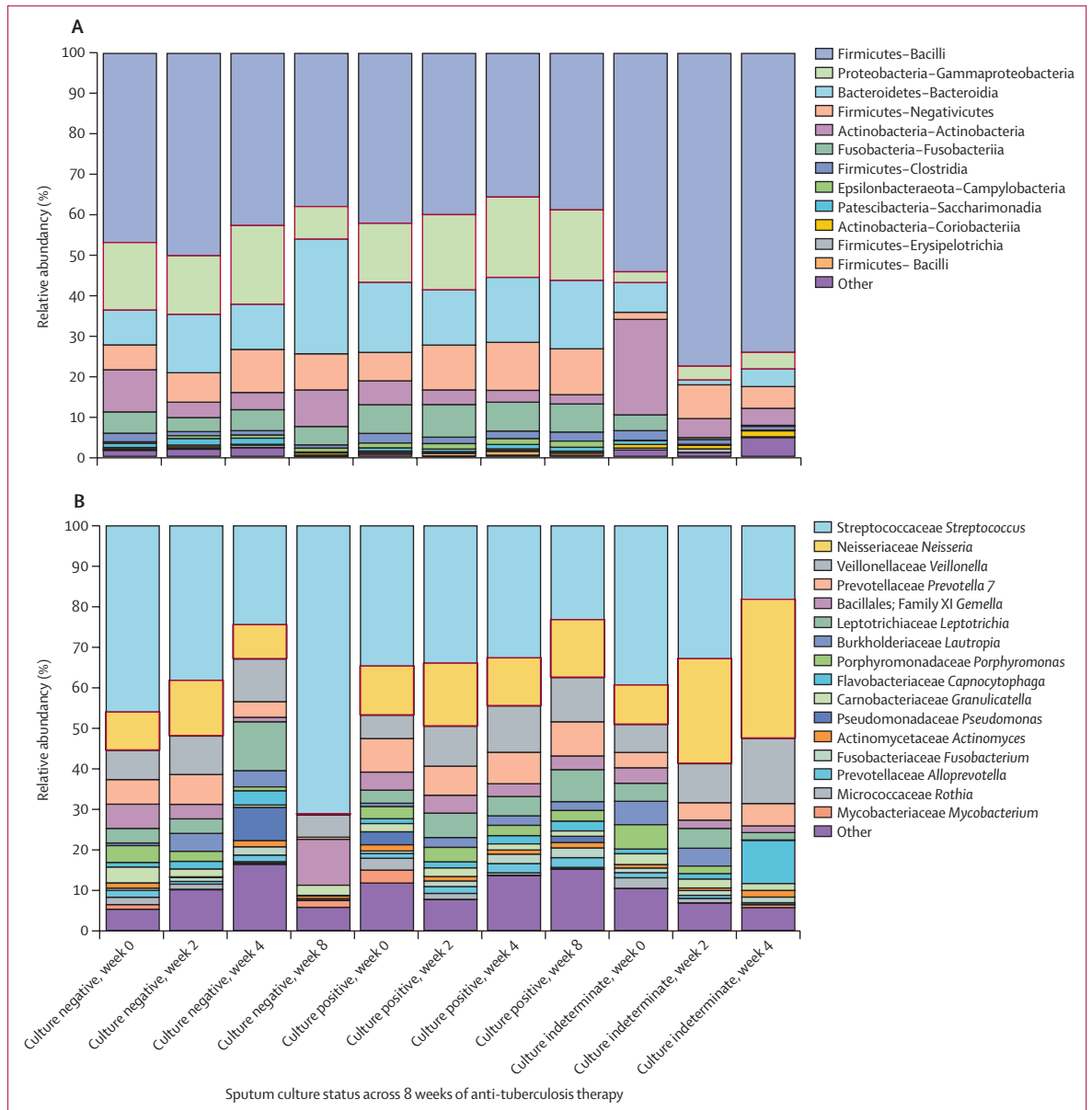
Figure 4: Association of taxa change and treatment outcome at week 8 of treatment
(A) Phylum–class level plot showing change in Proteobacteria–Gammaproteobacteria in culture negative, culture positive, and culture indeterminate participants.
(B) Genus level association showing changes in genus *Neisseria* among participants in culture negative, culture positive, and culture indeterminate groups. *Neisseria* was reduced by 98% in participants whose culture converted by week 8 of treatment.

Firmicutes (*Streptococcus* spp), Bacteroidetes (*Veillonella* spp), Proteobacteria (*Neisseria* spp), and Actinobacteria (*Mycobacterium* spp). Dominance of *Streptococcus* spp, *Viellonela* spp, and *Neisseria* spp in respiratory microbiota have been reported by other studies.[1]

Although all regimens exhibited a reduction in richness, diversity, and evenness, it was significant under $HR_{20mg/kg}ZM$ and $HR_{35mg/kg}ZE$ regimens, which had an effect across alpha diversity metrics. While the effect of the moxifloxacin regimen was strong until week 8 of treatment,

the suppressive effect of $HR_{35mg/kg}ZE$ on taxa diversity was sustained up to 12 weeks of treatment, suggesting a long-term impact of rifampicin. These observations are in line with the reported rapid action and sterilising effect of moxifloxacin and rifampicin.[12,17] Of the two regimens, only the moxifloxacin regimen reduced evenness in the first 2 weeks of treatment. The $HR_{10mg/kg}ZQ$ regimen also had an effect on phylogenetic diversity and evenness in the same 2-week period. By contrast, there was an increase in evenness by taxa under the standard $HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$ from week 2 through to week 12 of treatment.

The insignificant reduction of evenness by $HR_{35mg/kg}ZE$ and gain exhibited by $HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$ are an indication that the rifampicin bactericidal effect might be evenly spread across taxa, which consequently evens out taxa distribution rather than elimination. The effect might be different at a rifampicin dose higher than 35 mg/kg. The PanACEA MAMS-TB clinical trials found the $HR_{35mg/kg}ZE$ regimen more effective at causing stable culture conversion and recommended it for phase 3 studies. Since HIGHRIF study 2 found that a fixed dose of rifampicin 1200 mg was not superior to standard rifampicin 600 mg at causing culture conversion, it is plausible to speculate that the effect we have observed in the $HR_{20mg/kg}ZE$ regimen is most likely contributed by moxifloxacin.

A few taxa (n=12) had relative abundance of more than 1%, leaving the majority with an abundance of less than 1% (0·10–0·99%). The over 1% abundant taxa remained relatively stable, with minimal reduction by antibiotic action. There was a notable reduction in relative abundance under the moxifloxacin regimen, where *Streptococcus* spp was reduced by 15% and displaced by *Prevotella* as the second most abundant and *Neisseria* was reduced by 14% reduction from second to seventh position, taking up to week 12 to recover its abundance. By contrast, there was a 7% increase in *Streptococcus* spp and a 4% increase in *Neisseria* spp during the rifampicin 35 mg/kg regimen in the first 2 weeks of treatment and only 3% and 1% reduction of the same genera during the $HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$ regimens. This effect demonstrates the strong early bactericidal activity of the moxifloxacin containing regimen. The strong early bactericidal activity implies that supplementing rifampicin with moxifloxacin has stronger and faster antibacterial action than just increasing rifampicin dose alone.

The relatively stable taxa that had more than 1% abundance could not explain the reduction in richness and diversity observed in the first 2 weeks of treatment. A deeper analysis of the taxa that had a relative abundance of less than 1% abundance revealed the members of the community had reduced in abundance to below detection by week 2 of treatment. More than half of the taxa that were reduced to sub-detectable levels were Gram-negative bacteria (36 [57%] of 63, of which 19 [53%] were reduced under the regimen containing moxifloxacin). It has long been demonstrated that fluoroquinolones, such as moxifloxacin, have strong action against Gram-negative bacteria (including *Neisseria meningitidis*), Gram-positive *Streptococcus pnuemoniae*, and generally anaerobic bacteria.[18–20]

Although the rest of the taxa recovered during treatment, members of the genus *Mycobacterium* did not recover across all regimens, suggesting some form of selective elimination. A similar reduction of *Mycobacterium* spp with regard to recovery of other taxa was reported by Katete and colleagues[21] in patients with tuberculosis given the standard $HR_{10mg/kg}ZE$ regimen. The sustained elimination of *Mycobacterium* spp might be explained by

constituents of the anti-tuberculosis regimen (isoniazid, pyrazinamide, and ethambutol), which act specifically on *Mycobacterium* spp possibly enhanced by broad-spectrum rifampicin or moxifloxacin.[22,23]

We explored whether antibiotic–microbiome interactions impacted tuberculosis treatment outcomes. Proteobacteria genus *Neisseria* was reduced to a sub-detectable level in patients who converted to negative tuberculosis culture by week 8 of treatment. By contrast, there was a 2% rise in *Neisseria* spp in patients who remained culture positive and a 25% rise in *Neisseria* spp in patients who remained indeterminate by week 8 of treatment.

The sample size is small when divided by the number of analysed regimens. However, the pre-treatment core microbiome covered in this study is consistent with and representative of the respiratory microbiota composition published by other studies conducted in east Africa and outside Africa.[24,25] This implies that the core microbiome is similar between participants and might not vary much irrespective of the number of participants. Secondly, our study did not examine from which source the recovering taxa came from. Additional studies should investigate whether recovery was due to regrowth of suppressed taxa or due to replenishment from dietary sources and investigate the impact on long-term clinical and health outcomes.

In summary, we have shown that different anti-tuberculosis regimens and dosages have different effects on the sputum microbiome. The standard first-line regimen, $HR_{600mg}ZE$ or $HR_{10mg/kg}ZE$, appeared to affect the microbiome only marginally, causing an increase in taxa evenness and no significant reduction of diversity. Within the backbone of the standard regimen, increasing the dose of rifampicin alone to 35 mg/kg achieved a significant reduction of the microbiome, which did not recover to pre-treatment level by month 3 of treatment follow-up. A lower rifampicin dose of 20 mg/kg supplemented with 400 mg moxifloxacin resulted in a significant reduction of microbiome diversity, which recovered to pre-treatment level by month 3 of treatment. The drug, SQ109, appeared to have added no significant value to the performance of anti-tuberculosis regimen or its effect on the microbiome. Most importantly, *M tuberculosis* did not show recovery across regimens, an effect that implies that novel optimal anti-tuberculosis regimens could shorten treatment course without irreversible damage to the beneficial respiratory microbiome. Furthermore, large-scale longitudinal studies will be needed to ascertain whether it is only *M tuberculosis* that is eliminated and what implications this has on the recovering microbiome and treatment outcome.

References
1. Man WH, de Steenhuijsen Piters WA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol* 2017; **15:** 259–70.
2. Reyman M, van Houten MA, Watson RL, et al. Effects of early-life antibiotics on the developing infant gut microbiome and resistome: a randomized trial. *Nat Commun* 2022; **13:** 893.
3. Wipperman MF, Fitzgerald DW, Juste MAJ, et al. Antibiotic treatment for tuberculosis induces a profound dysbiosis of the microbiome that persists long after therapy is completed. *Sci Rep* 2017; **7:** 10767.
4. Uplekar M, Weil D, Lonnroth K, et al. WHO's new end TB strategy. *Lancet* 2015; **385:** 1799–801.
5. Quinn RA, Adem S, Mills RH, et al. Neutrophilic proteolysis in the cystic fibrosis lung correlates with a pathogenic microbiome. *Microbiome* 2019; **7:** 23.
6. Edwards DK, Jasny E, Yoon H, et al. Adjuvant effects of a sequence-engineered mRNA vaccine: translational profiling demonstrates similar human and murine innate response. *J Transl Med* 2017; **15:** 1–18.
7. Pechal JL, Schmidt CJ, Jordan HR, Benbow ME. A large-scale survey of the postmortem human microbiome, and its potential to provide insight into the living health condition. *Sci Rep* 2018; **8:** 5724.
8. Brennan P, Young D. Isoniazid. *Tuberculosis (Edinb)* 2008; **88:** 112–16.
9. Hong BY, Maulén NP, Adami AJ, Granados H, Balcells ME, Cervantes J. Microbiome changes during tuberculosis and antituberculous therapy. *Clin Microbiol Rev* 2016; **29:** 915–26.
10. Cheung MK, Lam WY, Fung WYW, et al. Sputum microbiota in tuberculosis as revealed by 16S rRNA pyrosequencing. *PLoS One* 2013; **8:** e54574.
11. Harris JK, De Groote MA, Sagel SD, et al. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. *Proc Natl Acad Sci USA* 2007; **104:** 20529–33.
12. Boeree MJ, Heinrich N, Aarnoutse R, et al. High-dose rifampicin, moxifloxacin, and SQ109 for treating tuberculosis: a multi-arm, multi-stage randomised controlled trial. *Lancet Infect Dis* 2017; **17:** 39–49.
13. Aarnoutse RE, Kibiki GS, Reither K, et al. Pharmacokinetics, tolerability, and bacteriological response of rifampin administered at 600, 900, and 1,200 milligrams daily in patients with pulmonary tuberculosis. *Antimicrob Agents Chemother* 2017; **61:** e01054–17.
14. Sabiiti W, Azam K, Farmer E, et al. Tuberculosis bacillary load, an early marker of disease severity and treatment response: the utility of tuberculosis Molecular Bacterial Load Assay. *Thorax* 2020; **75:** 606–08.
15. Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* 2013; **41:** e1–1.
16. Illumina. Metagenomic sequencing library preparation. Preparing 16S ribosomal RNA gene amplicons for the Illumina MiSeq system. https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf (accessed Sept 11, 2023).
17. Gillespie SH, Crook AM, McHugh TD, et al. Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *N Engl J Med* 2014; **371:** 1577–87.
18. Cottagnoud P, Täuber MG. Fluoroquinolones in the treatment of meningitis. *Curr Infect Dis Rep* 2003; **5:** 329–36.
19. Edmiston CE, Krepel CJ, Seabrook GR, et al. In vitro activities of moxifloxacin against 900 aerobic and anaerobic surgical isolates from patients with intra-abdominal and diabetic foot infections. *Antimicrob Agents Chemother* 2004; **48:** 1012–16.
20. Baxter M, Jacobson K, Albur M. Moxifloxacin should not be discounted in the treatment of bacterial meningitis. *J Infect* 2016; **73:** 173–74.
21. Kateete DP, Mbabazi MM, Nakazzi F, et al. Sputum microbiota profiles of treatment-naïve TB patients in Uganda before and during first-line therapy. *Sci Rep* 2021; **11:** 24486.
22. Unissa AN, Subbian S, Hanna LE, Selvakumar N. Overview on mechanisms of isoniazid action and resistance in *Mycobacterium tuberculosis. Infect Genet Evol* 2016; **45:** 474–92.
23. Goude R, Amin AG, Chatterjee D, Parish T. The arabinosyltransferase EmbC is inhibited by ethambutol in *Mycobacterium tuberculosis. Antimicrob Agents Chemother* 2009; **53:** 4138–46.
24. Dicker AJ, Lonergan M, Keir HR, et al. The sputum microbiome and clinical outcomes in patients with bronchiectasis: a prospective observational study. *Lancet Respir Med* 2021; **9:** 885–96.
25. Spottiswoode N, Bloomstein JD, Caldera S, et al. Pneumonia surveillance with culture-independent metatranscriptomics in HIV-positive adults in Uganda: a cross-sectional study. *Lancet Microbe* 2022; **3:** e357–65.