Sputum bacterial load and bacterial composition correlate with lung function and are altered by long term azithromycin treatment in children with HIV-associated chronic lung disease

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Abstract

Background

Long-term azithromycin (AZM) treatment reduces the frequency of acute respiratory exacerbation in children and adolescents with HIV-associated chronic lung disease (HCLD). However, the impact of this treatment on the respiratory bacteriome is unknown.

Method

African children with HCLD (defined as forced expiratory volume in 1 second z-score (FEV1z) less than -1.0 with no reversibility) were enrolled in a placebo-controlled trial of once-weekly AZM given for 48-weeks (BREATHE trial). Sputum samples were collected at baseline, 48 weeks (end of treatment) and 72 weeks (six months post-intervention in participants who reached this timepoint before trial conclusion). Sputum bacterial load and bacteriome profiles were determined using 16S rDNA qPCR and V4 gene amplicon sequencing, respectively. The primary outcomes were within-participant and within-arm (AZM vs placebo) changes in the sputum bacteriome measured across baseline, 48 weeks and 72 weeks. Associations between clinical or socio-demographic factors and bacteriome profiles were also assessed using linear regression.

Results

In total, 347 participants (median age: 15.3 years, interquartile range [12.7–17.7]) were enrolled and randomised to AZM (173) or placebo (174). After 48 weeks, participants in the AZM arm had reduced sputum bacterial load vs placebo arm (16S rDNA copies/ml in log₁₀, mean difference and 95% confidence interval [CI] of AZM vs Placebo -0.54 [-0.71; -0.36]). Shannon alpha diversity remained stable in the AZM arm but declined in the placebo arm between baseline and 48 weeks (3.03 vs. 2.80, $p = 0.04$, Wilcoxon paired test). Bacterial community structure changed in the AZM arm at 48 weeks compared with baseline (PERMANOVA test $p = 0.003$) but resolved at 72 weeks. The relative abundances of genera previously associated with HCLD decreased in the AZM arm at 48 weeks compared with baseline, including *Haemophilus* (17.9% vs. 25.8%, $p < 0.05$, ANCOM $\omega = 32$) and *Moraxella* (1% vs. 1.9%, 47, $p < 0.05$, ANCOM $\omega = 47$). This reduction was sustained at 72 weeks relative to baseline. Lung function (FEV1z) was negatively associated with bacterial load (coefficient, [CI]: -0.09 [-0.16; -0.02]) and positively associated with Shannon diversity (0.19 [0.12; 0.27]). The relative abundance of *Neisseria* (coefficient, [standard error]: (2.85, [0.7], $q = 0.01$), and *Haemophilus* (-6.1, [1.2], $q < 0.001$) were positively and negatively associated with FEV1z, respectively. An increase in the relative abundance of *Streptococcus* from baseline to 48 weeks was associated with improvement in FEV1z (3.2 [1.1], $q = 0.01$) whilst an increase in *Moraxella* was associated with decline in FEV1z (-2.74 [0.74], $q = 0.002$).

Conclusions

AZM treatment preserved sputum bacterial diversity and reduced the relative abundances of the HCLD-associated genera *Haemophilus* and *Moraxella*. These bacteriological effects were associated with improvement in lung function and may account for reduced respiratory exacerbations associated with AZM treatment of children with HCLD.

Background

Macrolides, including azithromycin (AZM), are commonly used to manage chronic lung diseases (CLD), including cystic fibrosis [1], chronic obstructive pulmonary disease (COPD) [2], asthma [3], post-transplantation obliterative bronchiolitis [4] and bronchiectasis [5]. Long-term use of AZM is associated with improved lung function and survival; and reduced frequency of acute respiratory exacerbation, antibiotic administration and hospitalisation in CLDs [6]. We have previously shown that long-term AZM reduced the frequency of acute respiratory exacerbations and all-cause hospitalisation in children and adolescents with HIV-associated CLD (HCLD) [7].

Mechanisms for these beneficial effects may include a combination of the antimicrobial, anti-inflammatory and immunomodulatory effects of macrolides [6]. The direct antibacterial effect of AZM on pathogens may be enhanced by improved phagocytic competency of alveolar macrophages, which tend to be defective in CLD [8, 9]. Additional benefits of AZM in CLDs include antiviral activity, improved ciliary function, interference with *Pseudomonas aeruginosa* biofilm formation, mitigation of airway mucus hypersecretion and promotion of pulmonary epithelial cell healing [6].

We previously observed that a *Haemophilus*, *Moraxella* or *Neisseria* (HMN)-dominant sputum bacteriome was associated with a 1.5-fold increased risk of HCLD, compared to a *Streptococcus* or *Prevotella*-dominant bacteriome [10]. We also showed an association between HCLD and respiratory carriage of *Streptococcus pneumoniae* and *Moraxella catarrhalis* [11]. The therapeutic effect of AZM in COPD may be mediated by changes in the lung bacteriome along with associated changes in microbial metabolites that interact with the host immune system [2]. Huang *et al* [12] observed that, in COPD, acute exacerbations were associated with an increase in the relative abundance of the phylum Proteobacteria (HMN belong to this phylum). Antibiotic treatment reduced this relative abundance and resulted in the resolution of symptoms. Although similar mechanisms may apply in HCLD, the effect of AZM on the respiratory microbiome in HCLD has not been studied. An insight into how AZM affects the HCLD respiratory microbiome and how this may mediate its beneficial effects will contribute towards understanding the pathogenesis of HCLD and facilitate targeted therapeutics.

In this study, we determined the impact of long-term AZM treatment (weekly treatment for 48 weeks) on the diversity and composition of the sputum bacteriome of Zimbabwean and Malawian children and adolescents with HCLD. We also measured the persistence of bacteriome alterations six months post-treatment, which has not been previously investigated in other CLDs. Further, we assessed the association between bacteriome alterations and key clinical outcomes, including reduction in acute pulmonary exacerbations, all-cause hospitalisation, and lung function. Finally, we investigated whether AZM altered the airway bacteriome in favour of HCLD-protective taxa.

Methods

Study design, participants, setting, eligibility, and intervention

This was a sub-study of the Bronchopulmonary Function in Response to Azithromycin Treatment for Chronic Lung Disease in HIV-infected Children (BREATHE) trial [7, 13] (clinicaltrials.gov identifier NCT02426112). The trial protocol [13] and its main findings [7] have been previously described. Briefly, children and adolescents aged 6–19 years with perinatally-acquired HIV infection taking antiretroviral therapy (ART) for a minimum of six months were recruited from outpatient HIV clinics in Blantyre, Malawi and Harare, Zimbabwe. HCLD was defined as forced expiratory volume in 1 second (FEV1) z-score (FEV1z) less than –1.0 with no reversibility, i.e., <12% improvement in FEV1z after 200µg of salbutamol inhalation. FEV1z was calculated using the African American module of the Global Lung

Function Initiative 2012 reference equations [14] validated among children in Zimbabwe by our team [15]. Exclusion criteria included acute respiratory symptoms, tuberculosis, pregnancy, hypersensitivity to AZM, prolonged QTc interval, impaired hearing or renal clearance. Participants were randomised to receive 48 weeks of once-weekly, weight-based doses of oral AZM [13]. Placebo arm participants received identical tablets without the active drug. For both trial arms, this was followed by a six-month treatment-free period (to 72 weeks). Participant adherence was defined as “*not missing, on average, more than two of the 12 dispensed doses, as assessed by pill count, splitting time in the study into four 12-week periods, as per visit and study medication dispensing schedule*” [7]. All trial participants were analysed at 48 weeks. In this sub-study, only participants with sputum samples available from, at least, one of the three visits (baseline, 48 and 72 weeks) were included. Hospitalisation was defined as a period of stay in a hospital > 24 hours. Acute respiratory exacerbation was defined as new or worsening respiratory symptoms, as assessed by a physician.

Study outcomes

For this sub-study comparing AZM and placebo arms, the primary outcomes were within-participant change in bacteriome profiles (as defined by Aitchison distance, Bray-Curtis index and differentially abundant taxa) of the sputum samples from baseline to 48-week, 48- to 72-week, and from baseline to 72-week study visits. Secondary outcomes were 1) differences in sputum bacterial load and bacteriome at all visits between AZM and placebo arms; 2) associations between alpha and beta diversity metrics and selected covariates including drug adherence, age, sex, site, season of sampling, duration of ART, lung function, MRC dyspnoea score [16] and acute respiratory exacerbation; 3) differences in within-participant sputum beta diversity of participants who developed an adverse event during the study (acute respiratory exacerbation, hospitalisation or receipt of additional antibiotics other than study drug or cotrimoxazole) compared to those that did not; and 4) the differentially abundant taxa driving changes and differences in bacteriome profiles between trial arms and visits.

Sample and data collection

Sputa were collected from participants at baseline, 48 weeks (end of treatment) and 72 weeks (six months post-intervention in participants who reached this timepoint before trial conclusion). For participants who could not expectorate spontaneously, sputum was induced with hypertonic saline. Samples were stored in PrimeStore® Molecular Transport medium [Primestore] (Longhorn Vaccines & Diagnostics LLC, Bethesda, USA) immediately after collection, transported on ice and stored at -80°C at the study centres. This was followed by batch shipment on dry ice to Cape Town, South Africa, where samples were stored at -80°C until further processing. Clinical and socio-demographic data were collected at each visit using questionnaires administered by a study nurse and from participant hospital records.

Nucleic acid extraction and purification

Sputum samples stored at -80°C were thawed, vortexed for 10 seconds and 450µl transferred into ZR BashingBead™ Lysis Tubes containing 0.5mm beads (catalogue no. ZR S6002–50, Zymo Research Corp., Irvine, CA, United States). Mechanical lysis was done at 50 Hz for 5 minutes using the TissueLyser LTTM (Qiagen, FRITSCH GmbH, Idar-Oberstein, Germany). The lysate was centrifuged at 10000 rpm (10,640 g) (Eppendorf F-45-30-11, Merck KGaA, Darmstadt, Germany) for 2 minutes and 250 µl of the supernatant was loaded onto a QIA Symphony® SP instrument (Qiagen, Hombrechtikon, Switzerland). Nucleic acid was purified using the DSP Virus/Pathogen Mini Kit® (catalogue no. 937036, Qiagen GmbH, Hilden, Germany) and eluted into 70 µl. We included bacterial mock community cells (catalogue no. ZR D6300, Zymo Research Corp., Irvine, CA, United States) as template extraction controls on each of the four plates of the three sequencing runs. We also included neat Primestore and Milli-Q®

ultrapure water (exposed to UV for 30 minutes prior to use) (Millipore Sigma, Burlington, MA, USA) as no-template extraction controls.

16S rDNA library preparation and amplicon sequencing

The total bacterial load (16S rDNA gene copies) of each extracted sample was determined using a previously described qPCR [17]. Gene copy numbers were extrapolated from standard curves derived from *E. coli* strain JM109 DNA (catalogue no. ZR E2006, Zymo Research Corp., Irvine, CA, United States), using genome size (4.60×10^6) and number of 16S rDNA gene copies (seven). For bacteriome analysis, the V4 hypervariable region of the 16S rDNA gene underwent two-step amplification as previously described [18] with the following modifications: 5 ml of DNA was used as template for each step of the PCR with a final concentration of 8 pmol loaded on the Illumina MiSeq. We also included a bacterial mock community DNA standard (catalogue no. ZR D6305, Zymo Research Corp., Irvine, CA, United States) and no template extraction controls on all plates in each run. Randomly selected samples were repeated within or between sequencing runs to assess within or between-run reproducibility. Samples from the same participant were all processed on the same plate to avoid batch effects. Each run included a balanced number of samples from both trial arms and from both study sites.

Bioinformatics analysis of 16S rDNA metagenomic data

After the quality of demultiplexed paired-end reads was deemed acceptable via FastQC [19] and MultiQC [20], the DADA2 pipeline [21] (wrapped in the Nextflow algorithm [22]) was used to filter and trim reads, infer amplicon sequence variants (ASVs), and assign taxonomy to ASVs. Default parameters were applied for all DADA2 functions unless expressly mentioned. Briefly, forward reads were trimmed and truncated at 24 and 248 bases, and reverse reads were trimmed and truncated at 25 and 235 bases. The minimum read length after trimming and truncation was set to 250 bases. Sequencing reads were dereplicated and pooled, and ASVs were inferred for each sample via the DADA2 sample inference algorithm and the estimated error model. Denoised sequences were generated by merging forward and reverse reads with overlap length set to 20 and allowing for no mismatches in the overlap region. Chimeric sequences were identified through exactly reconstructing them by combining a left-segment and a right-segment of more abundant sequences, and then were removed from the ASV table of denoised merged sequences. Taxonomy was assigned to each ASV using the SILVA version 138 [23] species classifier implementation for DADA2 [21]. Phylogenetic alignment was done using *AlignSeqs* in the DECIPHER [24] package while a neighbour-joining phylogenetic tree was constructed using functions in the PHANGORN [25] package. The 16S rDNA gene sequencing datasets were submitted to the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository (Accession number PRJNA769290).

Quality control and *in silico* correction of contamination

Both experimental work and data analysis plan were completed before unblinding of study investigators. We used a previously published in-silico quality control approach to minimise experimental error as outlined below [26]. Spurious ASVs, defined as ASVs with < 5 reads across all sequenced biological specimens and no-template control, were removed. We assessed nucleic acid extraction and sequencing efficiency by comparing the mock bacterial community extraction and sequencing controls to the manufacturer profiles. Next, we assessed reproducibility within and between runs by comparing bacteriome profiles of within- and between-run repeats. To determine whether the bacteriome profile of the low biomass samples was background noise from the storage medium (Primestore) or amplicon contamination we assessed clustering of these samples with the no-template controls (Primestore) on a log-ratio biplot and Principal Coordinates Analysis (PCoA) plot. Next, biological specimens with

16S rDNA gene copy numbers $\leq 500/\mu\text{l}$ were removed, as we and others have shown that these low biomass specimens produce poorly reproducible sequencing profiles [26, 27]. We used the *isContaminant* function embedded in the DECONTAM [28] package in R to identify potential contaminants using sequence data from biological specimens and no-template controls (Primestore). The maximum proportions of each identified contaminant in the no-template controls were subtracted from the biological specimens. Detailed quality control and decontamination steps and results are described in the supplementary material (Supplementary Table S1 and S2, Supplementary Figure S1–S17).

Statistical analysis

Statistical analysis was performed using R statistical software (version 4.0.4). Categorical variables were reported as frequency distributions and continuous variables as medians with interquartile ranges (IQRs) if non-parametric or means with standard deviations (SD) if otherwise. Normality distribution of continuous variables was assessed using Shapiro Wilk test. Between trial arm comparisons of continuous outcomes were done using Mann-Whitney U or Student t-test where appropriate. Fisher's exact test or Chi-square test were used to compare categorical variables between arms where appropriate. We used Wilcoxon signed-rank matched-pairs test to compare continuous outcomes between visits within trial arms. Correction for multiple testing was with Benjamin/Hochberg (cut off = 0.05). Amplicon Sequence Variants (ASV) were merged to the genus level and 0.5% prevalence filtering was applied to samples before analysis. The number of ASVs in a single sample represented bacterial taxonomic richness. Shannon's index [29] was used to characterise richness and evenness (alpha diversity).

Beta diversity was determined for intra-individual differences in samples from different visits or inter-individual differences in samples collected at the same visit. Beta diversity was determined using Euclidean distances of centred log-ratio (CLR) transformed ASV counts (Aitchison distance) after applying a pseudo count of one or by Bray-Curtis dissimilarity index [30] on unrarefied ASV counts, all implemented using *distance* functions in the PHYLOSEQ R package. Principal Coordinates Analysis (PCoA) plots were used for the visualisation of beta diversity. We used Permutational multivariate analysis of variance (PERMANOVA) [31] implemented using the *adonis2* function in the VEGAN [32] R package to compare beta diversity between visits or trial arms. PERMANOVA tests were adjusted for age, sex, site, ART regimen, MRC dyspnoea score, FEV1z, cotrimoxazole prophylaxis, previous tuberculosis treatment and previous admission for chest problems within 12 months prior to enrolment. Permutational Analyses of Multivariate Dispersions (PERMDISP) (implemented using *betadisper* function in VEGAN) was used to assess the homogeneity of dispersion between visits and trial arms.

Differentially abundant taxa were identified using ANCOM2 [33] on unrarefied data with False Discovery Rate (FDR) Benjamini/Hochberg correction (cut off = 0.05). Nine other methods for detecting differentially abundant taxa were used for comparison and reported in the supplementary material (Wilcoxon signed-rank test on data after the following normalisation methods—total sum scaling, variance stabilising transformation, and centred log transformation after applying a pseudo count of one, Aldex2 [34], Deseq2 [35], ANCOM-BC [36], Corncob [37], MaasLin2 [38] with total sum scaling and log transformation, MaasLin2 [38] with centred log transformation after applying a pseudo count of one). The relative contribution of each taxon to overall dissimilarity was measured using SIMPER analysis on the Bray-Curtis distances between samples.

To determine the association between sputum bacterial load ($\log_{10}$ transformed 16S rDNA gene copies) or alpha diversity (Shannon index) and clinical and socio-demographic factors, we used linear mixed effect models (LME) via the LME4 and LMERTTEST R packages. For each LME, we included visit, and the interaction term visit-versus-trial-arm[39] as fixed effects and the individual participant identifier as a random effect. We included in a stepwise

manner, adherence, age, site, sex, season of sampling, Medical Research Council (MRC) dyspnoea score, body-mass-index (BMI), weight-for age-z-score, height-for age-z-score, BMI-for age-z-score, ART regimen, duration of ART, HIV viral load suppression, cotrimoxazole prophylaxis, previous tuberculosis treatment or previous hospitalisation for chest problems one year prior to study enrolment, forced vital capacity (FVC), forced vital capacity z-score (FVCz), FEV1z, FEV1 percentage predicted (FEV1pcpred), FVC percentage predicted (FVCpcpred) and FEV1/FVCz as covariates in the models. Continuous outcome variables included in the models were log-transformed where necessary to satisfy model assumptions. Adherence to AZM, age, site, sex, season of sampling, MRC dyspnoea score, HIV viral load suppression (baseline values were used as values at 72 weeks were unavailable), cotrimoxazole prophylaxis, previous tuberculosis treatment or previous hospitalisation for chest problems one year prior to study enrolment, ART regimen and duration were selected *a priori* to be adjusted for. Any other variable identified as an independent predictor in the univariate analysis ($p < 0.05$) was also included in the multivariate LME. The following were excluded from the multivariate model because of co-linearity: BMI-for-age z-score and weight-for-age (colinear with height-for-age), and CD4 count (colinear with viral load suppression). We also assessed the association between bacterial genera and FEV1z, FVCz and adverse events (hospitalisation, additional antibiotic use and acute respiratory exacerbation during intervention) using a linear mixed effect model implemented with MaasLin2 [38]. FEV1z, visit, interaction between trial arm and visit were included as fixed effects and participant as random effect with ASV counts total sum scaled and converted to 100% and without log transformation. We also used linear regression implemented with MaasLin2 [38] to investigate the association between the within-participant change in FEV1z and FVCz over time in each trial arm (fixed effects) and (i) within-participant change in the percentage relative abundance of the five bacterial genera of interest (*Streptococcus*, *Prevotella*, *Haemophilus*, *Neisseria* and *Moraxella*), (ii) within-participant (between visit) beta diversity (Aitchison distance). The results of the regressions were reported as beta coefficients and standard error of the models.

Results

Participant characteristics

The BREATHE trial took place between June 2016 and September 2019. The socio-demographic and clinical data have previously been published [7]. Briefly, 347 children and adolescents were randomised, 173 to the AZM group and 174 to the placebo arm. The median age of the participants at baseline was 15.3 years, interquartile range [12.7-17.7] and 49% (170/347) were female. The participants in the AZM arm were younger and had been more frequently treated for tuberculosis than the placebo arm. Other characteristics did not significantly differ between arms. Almost all participants (90%, 313/347) were receiving long-term cotrimoxazole prophylaxis. The baseline clinical, socio-demographic and microbiological features of the participants are presented in Table 1. During the intervention, fewer participants in the AZM arm experienced acute respiratory exacerbations (16, 9.2%) and hospitalisations (2, 1.2%) compared to placebo (exacerbations: 30, 17.2%, hospitalisation, 9, 5.2%) [7].

Number of samples

Ninety nine percent of all samples (869/875) were spontaneously expectorated while the remainder were induced (3 samples in each study arm). Nineteen percent of participants (66/347) were not followed up at 72 weeks due to logistical limitations. Details of the number of samples collected from each study site and at each visit and the numbers included and excluded are summarised in Figure 1.

Table 1. Characteristics of study participants at baseline

Characteristics	AZM arm N=173	Placebo arm N=174
Demographic Characteristics		
Age in years, Median (IQR)	14.7 (12.6 – 16.8)	15.8 (13.0 – 18.1)
Female Sex, No. (%)	80 (46.2)	90 (51.7)
Currently in school, No. (%) ^a	146 (84.5)	139 (79.9)
Site: Zimbabwe, No. (%)	120 (69)	121 (70)
HIV Characteristics		
Duration on ART in years, Median (IQR)	5.9 (3.8 – 9.0)	6.4 (3.9 – 8.2)
HIV viral load suppression, <1000 copies/ml, No. (%)	102 (59.0)	94 (54.0)
CD4 count category, >200 cells/ul, No. (%)	157 (91)	156 (89.7)
Antiretroviral regimen NNRTI, No. (%) ^a	127 (73)	131 (75)
Lung function		
FEV1 z-score, Median (IQR)	-1.94 (-2.5, -1.4)	-2.0 (-2.4, -1.5)
Clinical Characteristics		
Underweight, No. (%) ^b	98 (56.6)	83 (47.7)
Stunted, No. (%) ^b	95 (54.9)	80 (46.0)
History of TB, No. (%) ^a	58 (33.6)	39 (22.4)
Admitted for chest problems in last year, No. (%)	3 (1.7)	3 (1.7)
MRC dyspnoea Score		
1	89 (51)	96 (55)
2	64 (37)	62 (36)
3	12 (7)	11 (6.3)
4	7 (4)	4 (2.3)
5	1 (1)	1 (1)
Cotrimoxazole prophylaxis	157 (92) ^a	156 (90)
^dSputum culture		
<i>Streptococcus pneumoniae</i>	43 (26%)	38 (23%)
<i>Staphylococcus aureus</i>	51 (31%)	46 (28%)
<i>Haemophilus influenzae</i>	4 (2%)	7 (4%)
<i>Moraxella catarrhalis</i>	15 (9%)	15 (9%)

Any of the four bacteria above	82 (49%)	79 (48%)
Sputum total bacterial load		
Sputum bacterial count (16S copy numbers in log ₁₀), Median (IQR)	5.0 (4.45 – 5.45)	4.93 (4.41 – 5.54)
Sputum bacteriome alpha diversity		
Shannon-Wiener diversity, Median (IQR)	3.0 (2.5 – 3.3)	3.0 (2.4 – 3.4)
Observed Taxon richness, Median (IQR)	29 (22 – 35)	29 (23 – 36)
^eSputum bacteriome within-arm beta diversity		
Aitchison distance, Median (IQR)	34.2 (30.9 – 37.5)	35.0 (32.0 – 38.3)
Bray-Curtis index, Median (IQR)	0.69 (0.57 – 0.83)	0.71 (0.59 – 0.84)

^a Missing value: Currently attending school – n=1 AZM arm; Antiretroviral regimen, n=1, Placebo arm; History of TB– n=1, AZM arm;

Cotrimoxazole prophylaxis– n=2, AZM arm, ^b z-score <-2, ^c Only asked for those with current cough, FEV1: Forced Expiratory Volume in one Second; Participants with missing responses are excluded from that variable. ^dNumber of sputum samples is 166 (AZM) and 164 (Placebo). IQR: Interquartile range, NNRTI:Non-nucleoside Reverse Transcriptase Inhibitor. CLR = centred-log ratio. ^eCompared using PERMANOVA test.

AZM reduced sputum bacterial load among participants in the intervention arm

The median bacterial load (log₁₀ 16S rDNA gene copies/ul) of the 875 samples was similar between the two arms at baseline [median (IQR) AZM, 5.0 (4.45 – 5.45) vs Placebo 4.93 (4.41 – 5.54), p=0.89] but was lower in AZM than placebo at both the 48-week [median (IQR) AZM, 4.65 (4.08 – 5.21) vs Placebo 5.27 (4.57 – 5.78), p< 0.0001] and 72-week visits [median (IQR) AZM, 4.92 (4.46 – 5.48) vs Placebo 5.26 (4.71 – 5.79), p=0.019] (Figure S18A). Within the AZM arm, 16S rDNA gene copy numbers at 48 weeks 4.65 (4.08 – 5.21) were lower than at baseline (5.0 (4.45 – 5.45), p<0.001) and at 72 weeks (4.92 (4.46 – 5.48), p=0.003) (Figure S18B). Bacterial load remained similar across visits in the placebo arm (Figure S18C).

We used linear mixed effect modelling (LME), Table 2, to explore associations between bacterial load (16S rDNA copies) and clinical and socio-demographic characteristics. Although bacterial load remained constant in the placebo arm, it declined significantly at 48 weeks compared to baseline in the AZM arm (adjusted beta coefficient and 95% confidence interval: -0.5 log₁₀ copies per ml [-0.63; -0.29]). Participants from the Zimbabwean study site on average had a higher bacterial load than those from Malawi (0.3 log₁₀ copies per ml [0.11; 0.49]) at any visit. Better lung function was associated with lower bacterial load. Specifically, a one unit increase in FEV1z was associated with 0.1 log₁₀ copies per ml [0.02- 0.2] decrease in bacterial load at any visit. Participants who had received treatment for tuberculosis before enrolment had higher bacterial loads (0.2 log₁₀ copies per ml [0.03 – 0.32]) at any visit than those who were not previously treated.

Association between AZM treatment, clinical and socio-demographic characteristics and sputum alpha diversity

The median number of sequences per sample was 36,456 (IQR: 31,392–42,086) which reduced to 16,969 (IQR: 13,302–19,902) with a total of 1,665 ASVs, after quality control and *in-silico* decontamination steps. We used linear mixed effect modelling (LME), Table 3, to explore associations between within-sample diversity (Shannon diversity index) and clinical and socio-demographic characteristics. Although, the Shannon diversity in the AZM arm remained unchanged (Figure S19B), it was significantly higher than placebo at 48 weeks (0.25 [0.07; 0.42]) and 72 weeks (0.2 [0.01; 0.40]). Participants from the Zimbabwean study site, on average, also had a higher Shannon diversity index than those from Malawi (0.27 [0.06; 0.47]) at any visit. Also, participants with MRC dyspnoea score of two had on average 0.26 units [0.1; 0.42] higher Shannon diversity index (higher sputum bacteriome alpha diversity) than participants with a score of one. Participants with higher FEV1z at any visit had a more diverse sputum bacteriome (higher Shannon index) compared to those with lower scores (0.19 [0.12; 0.27]). Finally, participants who were previously treated for tuberculosis before enrolment had, on average, 0.2 units [-0.34; -0.04] lower Shannon diversity index at any visit than those who were never treated.

AZM treatment resulted in changes in the overall bacteriome profile

Beta diversity among samples from participants in the AZM arm between baseline and 48 weeks [PERMANOVA test, on Aitchison distance $p = 0.004$, Bray-Curtis, $p = 0.002$], or between 48 weeks and 72 weeks [Aitchison distance, $p = 0.004$, Bray-Curtis, $p = 0.002$], was greater than between samples within the same visit, showing within-participant changes in overall community structure across visits, Supplementary Figures S20. There was no difference in dispersion of samples between visits (PERMDISP, $p > 0.05$). In contrast, no difference in beta diversity between visits, compared to within visits, was observed within the placebo arm (Figures S21). At baseline, between-arm beta diversity did not differ significantly from within arm beta diversity in either study arm [PERMANOVA test on Aitchison distance, ($p = 0.76$), Bray-Curtis, ($p = 0.97$), Figure S22A, B, Figure 2A, B]. However, at 48 weeks, overall bacterial composition differed between the two trial arms, with greater diversity between arms than within arms [PERMANOVA test on Aitchison distance, ($p = 0.003$), Bray-Curtis, ($p = 0.003$) Figure S22C, D and Figure 2. A dispersion effect was absent when considering Aitchison distance (PERMDISP $p = 0.85$) but was present when considering Bray-Curtis distance (PERMDISP $p = 0.002$), with greater dispersion in the Placebo than in the AZM arm (Figure 2). The difference in overall bacterial composition was not evident at the 72-week visits [PERMANOVA test on Aitchison distance, ($p = 0.14$), Bray-Curtis, ($p = 0.2$), Figure S22E, F and Figure 2].

Table 2. The association between bacterial load (16S rDNA copies) and selected variables using linear mixed effects modelling

Variable	Levels	^a Number of observations (n=875)	Participants (n=346)	¹ Co-efficient (95% CI)	¹ p value	² Adjusted Co-efficient (95% CI)	² p value
Visit	Placebo at Week 48	150 (17.1%)	150 (43.4%)	Reference			
	AZM at Week 48	154 (17.6%)	154 (44.5%)	-0.48 [-0.65; -0.32]	<0.0001	-0.46 [-0.63; -0.29]	<0.0001
	Placebo at Week 72	117 (13.4%)	117 (33.8%)	Reference			
	AZM at Week 72	123 (14.1%)	123 (35.5%)	-0.17 [-0.35; 0.02]	0.08	-0.19 [-0.38; 0.0]	0.051
Adherence	Adherent	661 (75.5%)	246 (71.1%)	Reference			
	Non-adherent	214 (24.5%)	214 (61.8%)	-0.22 [-0.37; -0.07]	0.004	-0.1 [-0.26; 0.06]	0.24
Site	Malawi	233 (26.6%)	106 (30.6%)	Reference			
	Zimbabwe	642 (73.4%)	240 (69.4%)	0.38 [0.24; 0.52]	<0.0001	0.3 [0.11; 0.49]	0.003
Age in years		779 (89%)	346 (100%)	0.02 [0; 0.04]	0.05		
Sex	Female	420 (48%)	170 (49.1%)	Reference			
	Male	455 (52%)	176 (50.9%)	-0.06 [-0.19; 0.08]	0.41	-0.08 [-0.21; 0.05]	0.23
Season of sampling	May-Oct-Dry	465 (53.1%)	295 (85.3%)	Reference			
	Nov-Apr-Rainy	409 (46.7%)	277 (80.1%)	0.02 [-0.07; 0.12]	0.60	0.04 [-0.06; 0.13]	0.41
MRC dyspnoea score at Baseline	1	479 (54.7%)	184 (53.2%)	Reference			
	2	316 (36.1%)	126 (36.4%)	-0.2 [-0.34; -0.06]	0.02	-0.07 [-0.22; 0.09]	0.76
	3	53 (6.1%)	23 (6.6%)	-0.28 [-0.55; 0]		-0.04 [-0.36; 0.28]	
	4	23 (2.6%)	11 (3.2%)	-0.36 [-0.75; 0.04]		-0.18 [-0.62; 0.27]	
	5	4 (0.5%)	2 (0.6%)	0.12 [-0.81; 1.05]		-0.79 [-2.29; 0.71]	

FEV1z		862 (98.5%)	346 (100%)	-0.11 [-0.18; -0.04]	0.003	-0.09 [-0.16; -0.02]	0.02
Forced Vital Capacity (FVC)		852 (97.4%)	345 (99.7%)	0.02 [-0.08; 0.12]	0.76		
FVCz		852 (97.4%)	345 (99.7%)	-0.08 [-0.14; -0.02]	0.01		
FEV1/FVCz		852 (97.4%)	345 (99.7%)	-0.03 [-0.08; 0.02]	0.28		
% Predicted FVC		852 (97.4%)	345 (99.7%)	-0.01 [-0.01; 0]	0.01		
% Predicted FEV		862 (98.5%)	346 (100%)	-0.01 [-0.01; 0]	0.003		
BMI-for-age z-score		868 (99.2%)	346 (100%)	-0.03 [-0.09; 0.02]	0.21		
Weight-for-age z-score		868 (99.2%)	346 (100%)	-0.01 [-0.06; 0.03]	0.61		
Height-for-age z-score		868 (99.2%)	346 (100%)	0.02 [-0.04; 0.07]	0.55		
CD4 at enrolment		868 (99.2%)	346 (100%)	0 [0; 0]	0.14		
CD4 at all visits		631 (72.1%)	346 (100%)	0 [0; 0]	0.04		
Viral load at all visits		676 (77.3%)	343 (99.1%)	0 [0; 0]	0.89		
Viral load suppression at baseline	Suppressed	495 (56.5%)	193 (55.8%)	Reference			
	Unsuppressed (≥ 1000 copies/ ml)	377 (43.0%)	151 (43.6%)	0.09 [-0.04; 0.22]	0.18	0.1 [-0.04; 0.23]	0.16
Weight_for_age z score	Not underweight	407 (46.5%)	166 (48%)	Reference			
	Underweight	468 (53.5%)	180 (52%)	-0.04 [-0.17; 0.09]	0.54		
Height_for_age z score	Not stunted	434 (49.6%)	171 (49.4%)	Reference			
	stunted	441 (50.4%)	175 (50.6%)	-0.02 [-0.15; 0.12]	0.81	0.01 [-0.12; 0.15]	0.84
Acute	No	752 (85.9%)	300 (86.7%)	Reference			

exacerbation during intervention	Yes	123 (14.1%)	46 (13.3%)	0.15 [-0.04; 0.34]	0.13		
Hospitalised during intervention	No	852 (97.4%)	336 (97.1%)	Reference			
	Yes	23 (2.6%)	10 (2.9%)	0.14 [-0.27; 0.54]	0.5		
Additional antibiotics during intervention	No	843 (96.3%)	334 (96.5%)	Reference			
	Yes	32 (3.7%)	12 (3.5%)	-0.2 [-0.55; 0.15]	0.27		
³ Any events during intervention	No	724 (82.7%)	288 (83.2%)	Reference			
	Yes	151 (17.3%)	58 (16.8%)	0.1 [-0.08; 0.27]	0.28		
Cotrimoxazole prophylaxis at Baseline	No	88 (10.1%)	32 (9.2%)	Reference			
	Yes	783 (89.5%)	312 (90.2%)	-0.19 [-0.41; 0.04]	0.10	-0.14 [-0.35; 0.07]	0.20
Age group at Baseline	17-19y	258 (29.5%)	103 (29.8%)	Reference			
	13-16y	374 (42.7%)	149 (43.1%)	-0.05 [-0.21; 0.10]	0.02	-0.01 [-0.25; 0.24]	0.55
	10-12y	165 (18.9%)	63 (18.2%)	-0.3 [-0.49; -0.11]		-0.14 [-0.34; 0.06]	
	6-9y	78 (8.9%)	31 (9%)	-0.01 [-0.34; 0.15]		-0.01 [-0.16; 0.15]	
Ever admitted for chest problems in the past year before enrolment	No	863 (98.6%)	340 (98.3%)	Reference			
	Yes	12 (1.4%)	6 (1.7)	-0.08 [-0.62; 0.46]	0.76	-0.33 [-0.97; 0.3]	0.33
Ever treated for tuberculosis before enrolment	No	609 (69.8%)	248 (71.9%)	Reference			
	Yes	263 (30.2%)	97 (28.1%)	0.27 [0.13; 0.41]	<0.0001	0.17 [0.03; 0.32]	0.02
Duration of ART at Baseline	6m-2y	80 (9.1%)	33 (9.5%)	Reference			
	2-<4y	141 (16.1%)	58 (16.8%)	0.16 [-0.11; 0.43]	0.70	-0.04 [-0.3; 0.23]	0.71
	4y-<6y	187 (21.4%)	72 (20.8%)	0.12 [-0.14; 0.38]		-0.05 [-0.3; 0.21]	

6y+	442 (50.5%)	172 (49.7%)	0.11 [-0.13; 0.34]	-0.11 [-0.35; 0.12]
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Abbreviations: forced vital capacity (FVC), forced vital capacity z-score (FVCz), forced expiratory volume in 1 second (FEV1) z-score (FEV1z), FEV1 percentage predicted (FEVpcpred), FVC percentage predicted (FVCpcpred) and FEV1/FVCz ratio of FEV1 and FVC z-score, Body mass index (BMI). ^aThe difference between the number of observations and the total (875) represent number of missing observations for that variable. ¹The estimate of coefficient with 95% confidence intervals and p values were obtained from univariate linear mixed effect model with participant included as a random effect and each variable and trial arm: visit interaction term as explanatory variables and log₁₀ 16S rDNA copies of the sputum samples as dependent variable. ²The estimate of coefficient with 95% confidence intervals and p values were obtained from multivariate linear mixed effect model with participant included as a random effect, trial arm, visit and trial arm: visit interaction term and all variables that have values under the “Adjusted Coefficient” column as explanatory variables and log₁₀ 16S rDNA copies of the sputum samples as dependent variable. FEV1/FVCz, FVCz, FVCpcpred, FEVpcpred were excluded from the final model because of collinearity. Viral load and CD4 counts were excluded from the final model because data was not collected at 72 weeks, the values at baseline were used instead. ³Any event refers to either acute respiratory exacerbation; additional antibiotics other than interventional drug or cotrimoxazole, or hospitalisation during intervention.

Table 3. The association between Shannon diversity indices and selected variables using linear mixed effects modelling

Variable	Levels	^a Number of observations (n=875)	Participants (n=346)	¹ Co-efficient (95% CI)	¹ p value	² Adjusted Co-efficient (95% CI)	² p value
Visit	Placebo at Week 48	150 (17.1%)	150 (43.4%)	Reference			
	AZM at Week 48	154 (17.6%)	154 (44.5%)	0.28 [0.11; 0.45]	0.001	0.25 [0.07; 0.42]	0.01
	Placebo at Week 72	117 (13.4%)	117 (33.8%)	Reference			
	AZM at Week 72	123 (14.1%)	123 (35.5%)	0.20 [0.01; 0.39]	0.04	0.2 [0.01; 0.40]	0.04
Adherence	Adherent	661 (75.5%)	246 (71.1%)	Reference			
	Non-adherent	214 (24.5%)	214 (61.8%)	0.02 [-0.15; 0.18]	0.85	0.07 [-0.1; 0.24]	0.42
Site	Malawi	233 (26.6%)	106 (30.6%)	Reference			
	Zimbabwe	642 (73.4%)	240 (69.4%)	0.07 [-0.09; 0.23]	0.39	0.27 [0.06; 0.47]	0.01
Age in years		779 (89%)	346 (100%)	-0.02 [-0.04; 0]	0.09		
Sex	Female	420 (48%)	170 (49.1%)	Reference			
	Male	455 (52%)	176 (50.9%)	0.14 [-0.01; 0.28]	0.06	0.13 [-0.01; 0.27]	0.07
Season of sampling	May-Oct-Dry	465 (53.1%)	295 (85.3%)	Reference			
	Nov-Apr-Rainy	409 (46.7%)	277 (80.1%)	-0.06 [-0.16; 0.03]	0.20	-0.09 [-0.19; 0.01]	0.07
MRC dyspnoea score at Baseline	1	479 (54.7%)	184 (53.2%)	Reference			
	2	316 (36.1%)	126 (36.4%)	0.14 [-0.01; 0.29]	0.04	0.26 [0.1; 0.42]	0.04
	3	53 (6.1%)	23 (6.6%)	0.03 [-0.26; 0.33]		0.16 [-0.18; 0.49]	
	4	23 (2.6%)	11 (3.2%)	-0.11 [-0.53; 0.31]		0.25 [-0.21; 0.71]	
	5	4 (0.5%)	2 (0.6%)	-1.21 [-2.21; -0.21]		0.52 [-1.05; 2.09]	

FEV1z		862 (98.5%)	346 (100%)	0.21 [0.14; 0.29]	<0.001	0.19 [0.12; 0.27]	<0.001
Forced Vital Capacity (FVC)		852 (97.4%)	345 (99.7%)	0.05 [-0.06; 0.16]	0.37		
FVCz		852 (97.4%)	345 (99.7%)	0.1 [0.03; 0.16]	0.004		
FEV1/FVCz		852 (97.4%)	345 (99.7%)	0.12 [0.06; 0.18]	<0.001		
% Predicted FVC		852 (97.4%)	345 (99.7%)	0.01 [0; 0.01]	0.003		
% Predicted FEV		862 (98.5%)	346 (100%)	0.02 [0.01; 0.02]	<0.001		
BMI-for-age z-score		868 (99.2%)	346 (100%)	0.03 [-0.03; 0.08]	0.35		
Weight-for-age z-score		868 (99.2%)	346 (100%)	0.02 [-0.03; 0.07]	0.47		
Height-for-age z-score		868 (99.2%)	346 (100%)	0 [-0.06; 0.06]	0.98		
CD4 at enrolment		868 (99.2%)	346 (100%)	0 [0; 0]	0.06		
CD4 at all visits		631 (72.1%)	346 (100%)	0 [0; 0]	0.01		
Viral load at all visits		676 (77.3%)	343 (99.1%)	0 [0; 0]	0.04		
Viral load suppression at baseline	Suppressed	495 (56.5%)	193 (55.8%)	Reference			
	Unsuppressed (≥ 1000 copies/ ml)	377 (43.0%)	151 (43.6%)	-0.11 [-0.26; 0.03]	0.12	-0.06 [-0.2; 0.08]	0.40
Weight_for_age z score	Not underweight	407 (46.5%)	166 (48%)	Reference			
	Underweight	468 (53.5%)	180 (52%)	0.04 [-0.1; 0.18]	0.59		
Height_for_age z score	Not stunted	434 (49.6%)	171 (49.4%)	Reference			
	stunted	441 (50.4%)	175(50.6%)	0.04 [-0.1; 0.18]	0.57	0.09 [-0.05; 0.23]	0.23
Acute exacerbation during intervention	No	752 (85.9%)	300 (86.7%)	Reference			
	Yes	123 (14.1%)	46 (13.3%)	-0.22 [-0.42; -0.01]	0.04		

Hospitalised during intervention	No	852 (97.4%)	336 (97.1%)	Reference			
	Yes	23 (2.6%)	10 (2.9%)	-0.28 [-0.72; 0.15]	0.21		
Additional antibiotics during intervention	No	843 (96.3%)	334 (96.5%)	Reference			
	Yes	32 (3.7%)	12 (3.5%)	-0.1 [-0.49; 0.28]	0.60		
³ Any events during intervention	No	724 (82.7%)	288 (83.2%)	Reference			
	Yes	151 (17.3%)	58 (16.8%)	-0.23 [-0.41; -0.04]	0.02	-0.16 [-0.35; 0.03]	0.11
Cotrimoxazole prophylaxis at Baseline	No	88 (10.1%)	32 (9.2%)	Reference			
	Yes	783 (89.5%)	312 (90.2%)	-0.1 [-0.34; 0.15]	0.44	-0.13 [-0.36; 0.09]	0.26
Oxygen Saturation	Normal	840 (96.0%)	345 (99.7%)	Reference			
	Abnormal	26 (3.0%)	26 (7.5%)	-0.27 [-0.56; 0.01]	0.06		
Heart rate	Normal	810 (92.6%)	340 (98.3%)	Reference			
	Abnormal	56 (6.4%)	44 (12.7%)	-0.22 [-0.44; -0.01]	0.04	-0.1 [-0.32; 0.11]	0.36
Age group at Baseline	17-19y	258 (29.5%)	103 (29.8%)	Reference			
	13-16y	374 (42.7%)	149 (43.1%)	-0.02 [-0.02; 0.18]	0.24	0.03 [-0.13; 0.2]	0.47
	10-12y	165 (18.9%)	63 (18.2%)	0.19 [-0.2; 0.40]		0.17 [-0.04; 0.38]	
	6-9y	78 (8.9%)	31 (9%)	0.13 [-0.14; 0.40]		0.01 [-0.24; 0.27]	
Ever admitted for chest problems in the past year before enrolment	No	863 (98.6%)	340 (98.3%)	Reference			
	Yes	12 (1.4%)	6 (1.7)	-0.14 [-0.72; 0.44]	0.64	0.5 [-0.16; 1.17]	0.16
Ever treated for tuberculosis before enrolment	No	609 (69.8%)	248 (71.9%)	Reference			
	Yes	263 (30.2%)	97 (28.1%)	-0.24 [-0.39; -0.08]	0.003	-0.19 [-0.34; -0.04]	0.02
Duration of ART	6m-2y	80 (9.1%)	33 (9.5%)	Reference			

at Baseline	2-<4y	141 (16.1%)	58 (16.8%)	0.07 [-0.22; 0.35]	0.87	0.18 [-0.1; 0.46]	0.63
	4y-<6y	187 (21.4%)	72 (20.8%)	-0.01 [-0.28; 0.27]		0.11 [-0.16; 0.37]	
	6y+	442 (50.5%)	172 (49.7%)	-0.02 [-0.27; 0.23]		0.14 [-0.1; 0.39]	

Abbreviations: forced vital capacity (FVC), forced vital capacity z-score (FVCz), forced expiratory volume in 1 second (FEV1) z-score (FEV1z), FEV1 percentage predicted (FEV1pcpred), FVC percentage predicted (FVCpcpred) and FEV1/FVCz ratio of FEV1 and FVC z-score, Body mass index (BMI). ^aThe difference between the number of observations and the total (875) represent number of missing observations for that variable. ¹The estimate of coefficient with 95% confidence intervals and p values were obtained from linear mixed effect model with participant included as a random effect and each variable and trial arm: visit interaction term as explanatory variables and Shannon indices of the sputum samples as dependent variable. ²The estimate of coefficient with 95% confidence intervals and p values were obtained from multivariate linear mixed effect model with participant included as a random effect, trial arm, visit and trial arm: visit interaction term and all variables that have values under the “Adjusted Coefficient” column as explanatory variables and Shannon indices of the sputum samples as dependent variable. FEV1/FVCz, FVCz, FVCpcpred, FEV1pcpred were excluded from the final model because of collinearity. Viral load and CD4 counts were excluded from the final model because data was not collected at 72 weeks, the values at baseline were used instead. ³Any event refers to either acute respiratory exacerbation; additional antibiotics other than interventional drug or cotrimoxazole; or hospitalisation during intervention.

Within the placebo arm, the median within-participant Bray-Curtis distance between samples from participants who had an adverse event (acute exacerbation of respiratory illness, hospitalisation or required additional antibiotics, n= 115) was higher than between samples from participants who did not have an adverse effect (n=35), when comparing baseline to 48 weeks (Kruskal Wallis test, $p=0.005$), or baseline to 72 weeks (Kruskal Wallis test, $p=0.008$) (Figure 3B). Within the AZM arm, fewer participants developed an adverse event (n= 16) and there were no significant differences in within-participant Bray-Curtis distance across any of the timepoints when comparing with participants who did or did not have an adverse event, Figure 3A.

Composition of taxa within the sputum bacteriome of participants

Five phyla accounted for 99.2% of the mean relative abundances of all bacteria, Proteobacteria (53.4%), Firmicutes (23.4%), Bacteroidetes (14.2%), Fusobacteria (5.2%) and Actinobacteria (3.0%) (Figure 4A, S23). At genus level, the 10 most abundant genera at baseline included *Haemophilus* (25.5%), *Neisseria* (18.8%), *Streptococcus* (15.8%), *Prevotella* (9.5%), *Veillonella* (3.9%), *Fusobacterium* (3.2%), *Alloprevotella* (2.9%), *Porphyromonas* (2.7%), *Moraxella* (2.5%) and *Leptotrichia* (2.3%) (Figure 4B, S24).

To detect which genera were driving the changes in beta diversity observed between the trial arms at 48 weeks, we tested for differentially abundant taxa using ANCOM2. At baseline, no taxon was differentially abundant between the trial arms. At 48 weeks, 13 genera were differentially abundant between trial arms (Figure 5 and S21). *Moraxella*,

Haemophilus, *Treponema*, *Aggregatibacter*, *Streptobacillus*, *Peptococcus*, ASV 205 (*Clostridia*_UCG-014) and ASV-62 (*Lachnospiraceae*) had significantly lower relative abundance in the AZM arm compared to placebo (Figure 5). In contrast, *Veillonella*, *Rothia*, *Lautropia*, *Actinomyces* and *Oribacterium* had increased in relative abundance in the AZM arm (Figure 5A). Fifteen genera were detected to be differentially abundant in the samples from participants in the AZM arm between baseline and 48 weeks (Figure 5A). There was a high degree of concordance between those genera detected as differentially abundant between AZM and placebo arms at 48 weeks and those detected as differentially abundant between the baseline and 48 weeks samples in the AZM arm (12 out of 16 genera were concordant, all with the same direction of effect) Figure 5A.

When comparing baseline with 72 weeks, many of the same genera (*Lautropia*, *Treponema*, *Rothia*, *Peptococcus*, ASV_209 *Clostridia*, ASV_157 *Absconditabacteria*, *Aggregatibacter*, *Moraxella*, *Haemophilus*) that were differentially abundant when comparing baseline with 48 weeks remained differentially abundant, with the same direction of effect, however the magnitude of the difference (estimated by CLR mean difference) was generally reduced (Figure 5A). Interestingly, several genera (*Veillonella*, *Oribacterium*, *Streptobacillus*, ASV_205 *Clostridia*, ASV_62 *Lachnospiraceae*) showed opposite directions of effect when comparing baseline to 48 weeks with baseline to 72 weeks.

Concordance between differential abundance testing methods.

We used other statistical methods for comparison with the differential abundance results from ANCOM2, which generally showed strong concordance, supplementary material (Figure S25, Supplementary Table S3-S9). At 48 weeks, five genera were detected as differentially abundant between study arms by all methods (*Lautropia*, *Moraxella*, *Rothia*, *Treponema* and *Veillonella*) (Figure S25). *Lautropia*, *Moraxella*, *Treponema*, *Oribacterium*, *F0058*, and ASV 209 were detected as differentially abundant between the baseline and 48-week samples from participants in the AZM arm by all methods (Figure S26). *Moraxella* was the only genus detected as differentially abundant between the 48- and 72-week samples from participants in the AZM arm by all methods (Figure S27). There was also a high level of agreement between the differential abundance testing and the SIMPER analysis of the contribution of taxa to overall dissimilarity (Bray Curtis distance) Table S10.

Associations between relative abundance of bacteria genera and clinical measures

We used a linear mixed effect model to identify possible associations between lung function (FEV1z, FVCz) and bacterial genera. Relative abundance of *Neisseria* was positively associated with FEV1z (coefficient, 2.85, standard error, 0.7, $q=0.01$) while that of *Haemophilus* was negatively associated (coefficient, -6.1, standard error, 1.2, $q<0.001$). In a separate model each, the adverse events of hospitalisation, additional antibiotic use and acute respiratory exacerbation during intervention were not associated with any of the five bacterial genera previously identified as associated with HCLD (*Streptococcus*, *Prevotella*, *Haemophilus*, *Moraxella* and *Neisseria*) [10].

We next used a univariate linear regression effect model to study whether change in lung function between visits over the study period was associated with changes in the relative abundances of the five bacterial genera of interest or with change in within-participant beta diversity over time. In the AZM arm, within-participant increase in FEV1z was positively associated with within-participant increase in the relative abundance of *Streptococcus* (coefficient [standard error], 3.2 [1.11], $q=0.01$, Table 4). In contrast, an increase in FEV1z was negatively associated with within-

participant increase in the relative abundance of *Moraxella* (-2.74 [0.74], $q=0.002$, Table 4). Furthermore, in the AZM arm, an increase in within-participant Aitchison distance was positively associated with within-participant increase in both FEV1z (1.05 [0.45], $p=0.02$, Table S11) and FVCz (0.95 [0.42], $p=0.02$ Table S11).

Table 4. Univariate linear regression analysis of within-participant change in FEV1z and FVCz and within-participant change in relative abundance of selected genera (outcome) between visits.

Within-participant change in genus	Trial arm	Within-participant change in FEV1z				Within-participant change in FVCz			
		coef	stderr	pval	qval	coef	stderr	pval	qval
<i>Streptococcus</i>	AZM	3.22	1.11	0.004	0.01	2.66	1.04	0.01	0.08
	Placebo	-0.79	1.46	0.59	0.82	-0.42	1.2	0.72	0.93
<i>Prevotella</i>	AZM	0.1	0.86	0.91	0.97	0.59	0.78	0.45	0.63
	Placebo	-1.54	1.08	0.16	0.82	-1.56	0.87	0.07	0.51
<i>Haemophilus</i>	AZM	-0.92	1.95	0.64	0.89	-1.12	1.82	0.54	0.63
	Placebo	-0.76	2.86	0.79	0.9	0.15	2.3	0.95	0.95
<i>Moraxella</i>	AZM	-2.74	0.74	0.0003	0.002	-1.28	0.7	0.07	0.25
	Placebo	0.18	1.42	0.9	0.9	0.92	1.11	0.41	0.93
<i>Neisseria</i>	AZM	0.05	1.43	0.97	0.97	-0.34	1.33	0.8	0.8
	Placebo	1.5	1.83	0.41	0.82	2.66	1.04	0.01	0.08

Associations were tested with MaAsLin2 using a linear regression model with FEV1z or FVCz and trial arm as fixed effects and within-participant change in relative abundance of selected genera as outcome. Statistical significance was corrected for multiple testing using Benjamini/Hochberg correction. Columns correspond to the within-participant change in genus, trial arm, the coefficient estimate (coef) and standard error from the model (stderr), nominal p-value (pval), and FDR corrected p-value (qval). Number of samples in azithromycin (AZM) and placebo arms are 377 and 365 respectively.

Discussion

We investigated the impact of long-term AZM treatment on the diversity and composition of the sputum bacteriome of children and adolescents with HCLD, and the persistence of this effect six months post-intervention. AZM treatment was associated with reduced bacterial load and maintenance of within-sample (alpha) diversity compared with placebo. In turn, low bacterial load and high alpha diversity were associated with better lung function measures. Treatment with AZM was associated with a reduction in the relative abundance of several potentially pathogenic taxa at 48 weeks, including *Haemophilus* and *Moraxella*. These changes persisted, but were less marked, at 72 weeks. The relative abundance of *Haemophilus* was negatively associated with lung function (FEV1z), while an increase in the relative abundance of *Moraxella* over time was associated with a decline in lung function. In contrast, the bacterial genera *Neisseria* and *Streptococcus* were associated with improved lung function measures.

The reduction in the total sputum bacterial load after 48 weeks of AZM treatment is expected, given the antibacterial activity and immune modulatory effects of AZM [8,40] and may have contributed to the reduction in the frequency

of acute respiratory exacerbations in the AZM arm [7]. The difference in the bacterial load between the arms at 48 weeks represents an approximately 3-fold reduction in bacterial 16S rDNA gene copies and may be biologically relevant. Sputum bacterial load returned to baseline levels by 72 weeks. Interestingly, other studies of long-term administration of AZM did not observe any effect on total bacterial burden measured by 16 rDNA gene copy numbers [2,3,41] or quantitative culture [41]. The reasons for the inconsistent findings may include differences in dosage, duration and frequency of AZM treatment, varying samples types (bronchoalveolar lavage vs sputum), HIV-status, prior antibiotic exposure, smoking status, age and variations between different CLDs (COPD [2,41], asthma [3], cystic fibrosis [1]). For instance, AZM would likely have less effect on bacterial load if the dominant taxa, e.g., *Pseudomonas* or *Staphylococcus spp* (in cystic fibrosis), are resistant to AZM.

Low alpha diversity is a signal for dysbiotic bacterial communities in many diseases [42,43]. We observed a decline in alpha diversity in the placebo, but not the AZM arm, over time. High alpha diversity (Shannon index) was associated with enhanced lung function (FEV1z and FVCz). Our findings are consistent with those of Rogers et al [44] who showed that lower alpha diversity of the sputum bacteriome was associated with lower FEV1z in adult participants with non-cystic fibrosis bronchiectasis treated with twice-daily erythromycin for 12-months.

In our study, low alpha diversity was also associated with the development of adverse events in the placebo arm. The decrease in alpha diversity observed in the placebo arm at 48 weeks may, in part, have been a consequence of the additional antibiotic exposure due to the more frequent acute respiratory exacerbations and hospitalisations occurring in this arm. This additional antibiotic exposure may also explain our observation that the median within-participant change in the bacteriome profiles of placebo arm participants who experienced adverse events during the trial was greater than those that did not. Other studies have described changes in microbial profiles associated with treatment of acute exacerbations of chronic obstructive pulmonary disease [12].

Interestingly, AZM did not affect Shannon diversity (which is a measure of both species richness and evenness) of the sputum bacteriome at any visit. This observation is consistent with the findings of Acosta et al [1] in which two years of AZM treatment did not affect the alpha diversity of cystic fibrosis sputum bacteriome [1], but contrasts with the findings of Segal et al [2] who observed a reduction in Shannon alpha diversity in emphysema patients treated with AZM.

Bray-Curtis within-group dissimilarity was lower than between-group dissimilarity, when comparing the 48-week samples in the AZM arm with baseline samples in the AZM arm or the 48-week samples in the placebo arm. Our findings are consistent with previous studies in asthma [3], COPD [2] and bronchiolitis [45] participants. In contrast, Acosta [1] did not observe any effect of AZM on the beta diversity of the sputum bacteriome of cystic fibrosis participants. This difference in findings may be explained by the more prolonged duration of treatment – two years compared to 48 weeks or less in the other studies – over which period the effect of AZM may diminish due to macrolide resistance or poor drug adherence. In our study, beta diversity (within-participant Aitchison distance) in the AZM arm between study visits was also associated with improvement in lung function measures over the same period, suggesting that the AZM-mediated change in composition of the sputum bacteriome may contribute to improved lung function in this population.

We identified 13 differentially abundant taxa including *Moraxella*, *Haemophilus*, *Treponema* (reduced) and *Rothia*, *Veillonella* and *Lautropia* (enriched) in the AZM compared to placebo arms at 48 weeks. The high degree of concordance (12/13) between these taxa and those identified as differentially abundant between the baseline and 48-week samples in the AZM arm indicates that this finding is unlikely to represent false discovery. Many of the same taxa were identified by multiple different methods for differential abundance testing. These data are

supported by our previous analysis of sputum bacterial culture from this cohort, which showed a reduction in the prevalence of *Moraxella catarrhalis* and *Haemophilus influenzae* at 48 weeks [46] compared with baseline.

In a separate cross-sectional study [10], we identified increased risk for HCLD in children with sputum microbiota dominated by *Haemophilus*, *Moraxella* or *Neisseria* (HMN) compared with microbiota dominated by *Prevotella* or *Streptococcus*. We also showed that cell-free products of HMN sputum microbiota induced epithelial disruption and inflammatory responses in vitro [10]. Our findings from the present study support the central role of *Haemophilus* and *Moraxella* in HCLD. Improvement in FEV1z was negatively associated with *Moraxella* relative abundance. Interestingly, the reduced relative abundance of *Haemophilus* and *Moraxella* at 48 weeks in the AZM arm persisted, but with reduced magnitude, at 72 weeks. Further work is required to explore the persistent effect of AZM on these taxa beyond 72 weeks, and associated changes in lung function. The relative abundance of *Streptococcus* was positively associated with FEV1z, which is consistent with our earlier finding study [10] that this genus may be protective in HCLD. However, in the current study, the relative abundance of *Neisseria* was positively associated with FEV1z, in contrast to our previous findings.

Our study has several strengths. It is the first study to assess the effect of long-term AZM treatment on the sputum bacteriome of African children with HIV-associated chronic lung disease. The double-blinded randomised, placebo-controlled design allows more direct inference of causal relationships. It is also the first study to analyse the persistence of AZM effect on the sputum bacteriome 6 months after treatment cessation using this study design. Our study is limited by several factors. 16S rDNA amplicon sequencing is unable to resolve taxonomy to the species level, which may be important, for example given the widely varying pathogenicity of *Streptococcus* species. The use of sputum to assess the lower airway microbiota is limited by contamination from the upper airways. However, previous studies have demonstrated acceptable concordance between the sputum and bronchoalveolar lavage bacteriome, [47,48] in asthma [48] and cystic fibrosis participants [47]. The 16S RNA qPCR that we used is subject to bias due to varying copy number of 16S rDNA genes in bacteria.

Conclusion

In conclusion, 48 weeks of once-weekly AZM reduced the total bacterial load and preserved within-sample bacterial diversity of children and adolescents with HCLD, features that were associated with better lung function measures. Our results confirm and extend previous findings, from a separate cohort, that *Haemophilus* and *Moraxella* likely play a central role in the pathogenesis of HCLD whilst *Streptococcus* may be protective. Modulation of the airway microbiota, with a targeted reduction in disease-associated taxa, may be a strategy to ameliorate disease in people with HCLD.

Declarations

Ethical approval and consent to participate

The trial was approved by the London School of Hygiene and Tropical Medicine Ethics Committee, The Regional Committee for Medical and Health Research Ethics, University of Cape Town Human Research Ethics Committee and from each local regulatory board before project commencement. This sub-study was approved by the Human Research and Ethics Committee of the University of Cape Town - (HREC/REF: 092/2019). Written informed consent and assent were provided by guardians and participants under 18 years, respectively. Older participants (≥ 18 years)

consented independently at enrolment. All data obtained and generated during the study were kept confidential. This research was conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable

Availability of data and material

The 16S amplicon sequences, anonymised clinical , laboratory and socio-demographic data and analysis codes that support the findings of this study are available in Sequence Read Archive (accession number PRJNA769290) and GitHub repository BREATHE_Sputum_bacteriome_analysis.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

MPN and RAF conceived the study. REA conducted all laboratory experiments (DNA extraction, library preparation and sequencing), data analysis and wrote the first draft of the manuscript supervised by MPN and FSD. YX assisted REA with bioinformatics and statistical analysis of the data. SK conducted data preprocessing and bioinformatics analysis to generate Biom files from raw sequences. SCW assisted with laboratory workflow, supervised the

laboratory experiments and performed the in-silico quality control and decontamination steps. SGL provided the R scripts for in-silico quality control and decontamination steps. GM coordinated sample collection in Zimbabwe. LGN is the study coordinator in Malawi. AMR and VS are the trial statisticians, and they guided the reporting of results and interpretation of the data. BW and RH reviewed the manuscript. JOO and RAF are trial PIs. JOO secured funding from GLOBVAC on behalf of the consortium. All authors contributed to the manuscript, and all authors read and approved the final version.

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Figures

Figure 1

Flow chart of the number of samples collected from each study sites at each visit and the numbers included and excluded from final analysis. LTFU =Loss to follow up. 16S copies =16S rDNA copies

Figure 2

Principal Coordinates Analysis of Atchison (A) and Bray-Curtis (B) [on unrarefied ASV counts] distance matrixes between trial arms at each visit. The confidence ellipses define the region that contains 95% of all samples that can be drawn from the underlying “t” distribution for each arm.

Figure 3

Violin boxplot comparing the within-participant change in Bray-Curtis distance on CLR transformed ASV counts from baseline to 48 weeks, baseline to 72 weeks and 48 weeks to 72 weeks between participants who developed acute exacerbation, hospitalised or were administered additional antibiotics (yes) with those who did not (no), in the AZM arm (A) and Placebo arm (B). Wilcoxon test was used for all comparisons. The horizontal line in the middle of the box is the median. The box presents interquartile range. The whiskers show 95% confidence interval. The shape of the violin display frequencies of values.

Figure 4

Line plot of the mean relative abundances of the top five phyla (mean relative abundance $\geq 2\%$) (A) and top 10 genera (mean relative abundance $\geq 1\%$) (B) in all samples at all visits and between trial arms. The shape of the data points and the line color represents the phylum in A. The shape of the data points for genus corresponds to the phylum it belongs to in A while the line colours indicate the genus in B.

A)

Figure 5

Analysis of Composition of Microbiomes (ANCOM2) differential abundance testing for samples at each visit and between trial arms. Taxa identified as differentially abundant between trial arms at 48 weeks (A, left panel), between AZM arm at Baseline and 48 weeks (A, middle panel) and between AZM arm at 48 and 72 weeks (A, right panel). Associations were considered significant using an ANCOM detection level ≥ 0.6 . Stars on the right of each bar indicate ANCOM detection levels: levels ≥ 0.6 are presented by 1 star, ≥ 0.7 by 2 stars, ≥ 0.8 by 3 stars and ≥ 0.9 by 4 stars. The raw W statistic for each comparison is shown left of the stars. The horizontal length of each bar indicates the mean centered log ratio (CLR) difference in relative abundance. B) Boxplots of $\log_{10}$ relative abundances of bacterial genera detected as differentially abundant by ANCOM2 by trial arm at 48 weeks (B) and within AZM arm at Baseline, 48 and 72 weeks (C).

Supplementary Files

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