

**The impact of long-term azithromycin treatment on sputum
bacteriome of African children with HIV-associated chronic lung
disease: an analysis from the double blind, placebo-controlled
BREATHE trial – Supplementary materials**

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SECTION 1. DETAILED DESCRIPTION OF METHODS

1.1.1. Differentially abundant taxa and SIMPER analysis.

Differentially abundant taxa were identified using ANCOM2[1] 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 below (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[2], Deseq2[3], Ancombc[4], Corncob[5], MaasLin2[6] with total sum scaling and log transformation, MaasLin2[6] 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. The input for all the methods is a phyloseq object merged at the genus level and 0.5% prevalence filtered. No rarefaction was applied to the feature tables used in the analysis below. In the first three methods, we applied Wilcoxon test on ASV counts normalised using three different methods to determine how different normalisations will affect the number of genera found to be differentially abundant.

a. Mann–Whitney–Wilcoxon test on Total sum scaled ASV counts

The ASV counts, previously merged at genus level and 0.5% prevalence filtered, were converted to relative abundances using total sum scaling equation – $x/\text{sum}(x)$, where x is ASV counts implemented using the *transform_sample_counts* function embedded in the phyloseq package. The resulting relative abundances of each genus of AZM and Placebo arms were then compared using the Wilcoxon test. Comparison of samples from different visit but within the same trial arm were done by Wilcoxon test for paired samples. The p values were then adjusted for multiple testing using Benjamin-Hochberg method to produce the q values. The false discovery rate was set to 0.05 hence only genera with q values < 0.05 were deemed differentially abundant.

b. Mann–Whitney–Wilcoxon test on variance stabilising transformed (VST) ASV counts

A pseudocount of one was applied to all ASVs before conversion from phyloseq to Deseq2 object. This was necessary so that log geometric means can be calculated when the *estimateSizeFactors* function embedded in Deseq2 is later applied. The phyloseq object was then converted to a Deseq2 object using the *phyloseq_to_deseq2* function so that VST normalisation can be conducted using a function from this package. Size factors (*estimateSizeFactors*) and dispersions (*estimateDispersions*) were estimated before variance stabilising transformation was applied to the data (*getVarianceStabilizedData*). The resulting variance stabilising transformed (VST) ASVs count of each genus of AZM and Placebo arms were then compared using the Wilcoxon test. Comparison of samples from different visit but within the same trial arm were done by Wilcoxon test for paired samples. The p values were then adjusted for multiple testing using Benjamin-Hochberg method to produce the q values. The false discovery rate was set to 0.05 hence only genera with q values < 0.05 were deemed differentially abundant.

c. Mann–Whitney–Wilcoxon test on centre log ratio transformed (CLR) ASV counts

Here a pseudocount of one was again applied to allow the calculation of log geometric means. The ASV counts were then CLR transformed using the *transform_sample_counts* function embedded in the phyloseq package with the function $x/\exp(\text{mean}(\log(x)))$, x is ASV counts. The resulting log ratios of each ASV within the genera of AZM and Placebo arms were then compared using the Wilcoxon test. Comparison of samples from different visit but within the same trial arm were

done by Wilcoxon test for paired samples. The p values were then adjusted for multiple testing using Benjamin-Hochberg method to produce the q values. The false discovery rate was set to 0.05 hence only genera with q values < 0.05 were deemed differentially abundant.

d. DESeq2

This analysis is based on negative binomial distribution and makes use of VST normalisation. It does not account for the compositional nature of the data. The phyloseq object was converted to `deseq2` using the function `phyloseq_to_deseq2`. The `DESeq` function was applied to this object with Wald test, local `fitType` and `poscounts` option for `sfType`. The `lfcShrink` function was applied with `coef = 2` and `type = "apeglm"`. The p values were then adjusted for multiple testing using Benjamin-Hochberg method to produce the q values. The false discovery rate was set to 0.05 hence only genera with q values < 0.05 were deemed differentially abundant.

e. Analysis of compositions of microbiomes with bias correction (ANCOM-BC)

The `ancombc` function in the ANCOMBC R package v 1.0.5 was applied to the genus-agglomerated and 0.5% prevalence-filtered ASV counts with the following options: p value adjustment was Benjamin-Hochberg, `library cut = 1000`, `structural zeros = TRUE`, `neg_lb = TRUE`, `conserve = TRUE`, `global = TRUE`. All other options were left as default. The false discovery rate was set to 0.05 hence only genera with q values < 0.05 were deemed differentially abundant.

f. ALDEx2

A data frame of the genera counts, and corresponding sample metadata were passed to the `aldex` function in ALDEx2 R package v 1.22.0 setting the `denom` parameter to `"iqlr"`. All other parameters were set to default. Both Wilcoxon (ALDEx2 Wilcoxon) and t-test (ALDEx2 t-test) were used for testing differences in genera relative abundances between AZM and Placebo or samples from different visits. The false discovery rate was set to 0.05. The function returned Benjamini-Hochberg (BH) FDR-corrected p values.

g. Corncob

The genus-merged and 0.5% prevalence filtered phyloseq object was passed to the `differentialTest` function of the `corncob` R package version 0.2.0. We selected Wald test for significance testing and false discovery rate to Benjamini-Hochberg. All other options were set to default. Since the false discovery rate was set to 0.05 hence only genera with q values < 0.05 were deemed differentially abundant.

h. MaAsLin 2 - Normalisation = TSS, transformation= LOG, fixed effects= trial_arm

A data frame of the genera counts, and corresponding sample metadata were passed to the `Maaslin2` function in MaAsLin2 R package v 1.4.0 setting the minimum prevalence to 0.0 because 0.5% prevalence filtered was already applied to the counts. Maximum significance was set to 0.05, `standardize` was set to `FALSE` and `fixed effect` was set as `trial arm` or `visit`. All other parameters were set to default. Since the false discovery rate was set to 0.05 hence only genera with q values < 0.05 were deemed differentially abundant.

i. MaAsLin 2 Normalisation = NONE, transformation= NONE, fixed effects= trial_arm

A data frame of the genera ASV counts that have previously been centred-log-ratio transformed and corresponding sample metadata were passed to the `Maaslin2` function in MaAsLin2 R package v 1.4.0 setting the minimum prevalence to 0.0 because 0.5% prevalence filtered was already applied to the counts. Maximum significance was set to 0.05, `standardize`

was set to FALSE and fixed effect was set trial arm or visit. Normalisation and transformation were set to “None”. All other parameters were set to default. Since the false discovery rate was set to 0.05 hence only genera with q values < 0.05 were deemed differentially abundant.

j. ANCOM-II

The genus-merged, 0.5% prevalence filtered ASV count table was inputted through the ANCOM-II[1] (<https://github.com/FrederickHuangLin/ANCOM>) *feature_table_pre_process* function which identified outlier and structural zeros. The trial arm or visit was specified as the group and main variable. The resulting feature table was then passed through the ANCOM function and p -values were FDR-corrected using the BH method (alpha set at 0.05). W statistics greater than or equal to 60% of the total number of genera tested were considered significant.

SECTION 2. SUPPLEMENTARY RESULTS

2.1. Results of Quality Control Steps

2.1.1. Introduction

A total of 1152 samples (78 biological samples belonging to a comparison group, 12 Zymobiomics extraction controls, 12 Zymobiomics sequencing control, 101 biological repeats, 43 non-template (negative or Primestore) controls and 906 biological samples included in the main trial) including controls were processed in three runs of 384 samples each. To ensure that the extraction step and sequencing steps are validated we included Zymobiomics mock community extraction controls (cells) (catalogue no. ZR D6300, Zymo Research Corp., Irvine, CA, United States) and sequencing controls (DNA) (catalogue no. ZR D6305, Zymo Research Corp., Irvine, CA, United States). We also repeated samples within plates and between plates in the same and between runs, to assess reproducibility. This action is to ensure there are no differences in bacteriome profiles introduced by batch effects. Furthermore, we compared profiles from biological samples with non-template control (in this case, Primestore, which was used as a storage medium of the samples) to assess background contaminating profiles. We then assess whether samples with low biomass (low 16S copies) clustered with the negative controls on a log-ratio biplot, suggesting background contamination rather than true biological signal. Also, we assess clustering of samples based on age, run number, study site (country), visit/timepoint, number of reads and 16S copy numbers which may introduce bias in our analysis. Finally, we used the *isContaminant* function within the DECONTAM R package to determine which ASVs are likely to be contaminants based on the Primestore profiles. This section of the supplementary material contains the results of each of these analyses.

a. Extraction and Sequencing Controls

The Zymobiomics mock community extraction controls (cells) (catalogue no. ZR D6300, Zymo Research Corp., Irvine, CA, United States) and sequencing controls (DNA) (catalogue no. ZR D6305, Zymo Research Corp., Irvine, CA, United States) were comparable to the theoretical compositions provided by the manufacturer (Figure S1, Table S1). However, the DNA profiles were more comparable than the cells highlighting a small bias in the extraction step probably from the lysis step. The mock communities were included on each plate in each run resulting in a total of 12 samples of the extraction controls and 12 of the sequencing controls.

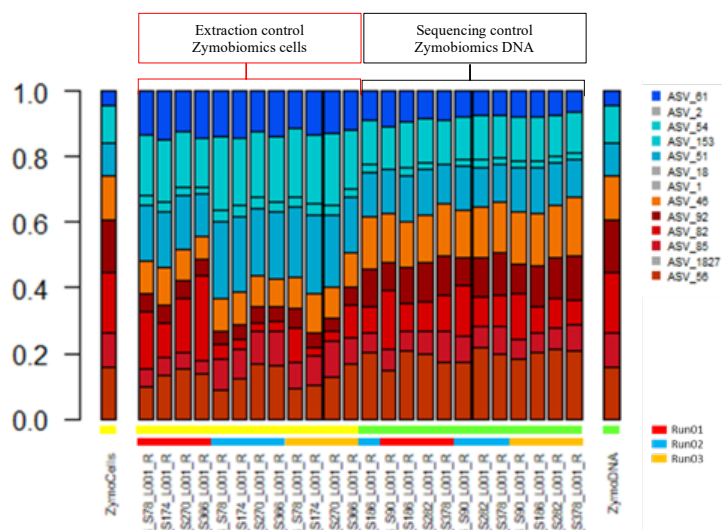


Figure S 1. A bar plot of the taxa and their relative abundance of the extraction and sequencing mock controls compared to manufacturer profiles.

Table S 1. The taxonomy of the ASVs in the extraction and sequencing control.

	Kingdom	Phylum	Class	Order	Family	Genus	Species
ASV_61	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	NA
ASV_2	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	NA
ASV_54	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Salmonella	NA
ASV_153	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Salmonella	enterica
ASV_51	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacterales	Enterobacteriaceae	Escherichia/Shigella	NA
ASV_18	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Neisseriaceae	Neisseria	NA
ASV_1	Bacteria	Proteobacteria	Gammaproteobacteria	Burkholderiales	Neisseriaceae	Neisseria	NA
ASV_46	Bacteria	Firmicutes	Bacilli	Staphylococcales	Staphylococcaceae	Staphylococcus	NA
ASV_92	Bacteria	Firmicutes	Bacilli	Lactobacillales	Listeriaceae	Listeria	NA
ASV_82	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA
ASV_85	Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	NA
ASV_1827	Bacteria	Firmicutes	Bacilli	Bacillales	NA	NA	NA
ASV_56	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA

b. Reproducibility within and between the three runs

A total of 101 biological specimens were repeated within the three runs with one sample repeated thrice. Of these 74 was repeated in the same run (WR) while 28 were repeated between runs (BR). Reproducibility as measured by R^2 was very high (> 0.9). Only one between run repeat had an R^2 value < 0.9 (0.69). With regards to reproducibility, no sample was excluded based on age, 16S copies or number of reads as none of these affected reproducibility Figure S2(A, B and C). Out of the 74 WR repeats, 54 were repeated on the same plate to assess intra-plate reproducibility, while 20 specimens were

repeated between plates in the same run to assess inter-plate reproducibility within the same run. Reproducibility for all 74 samples was very high (> 0.91) hence no sample was excluded (Figure S2, D, E and F).

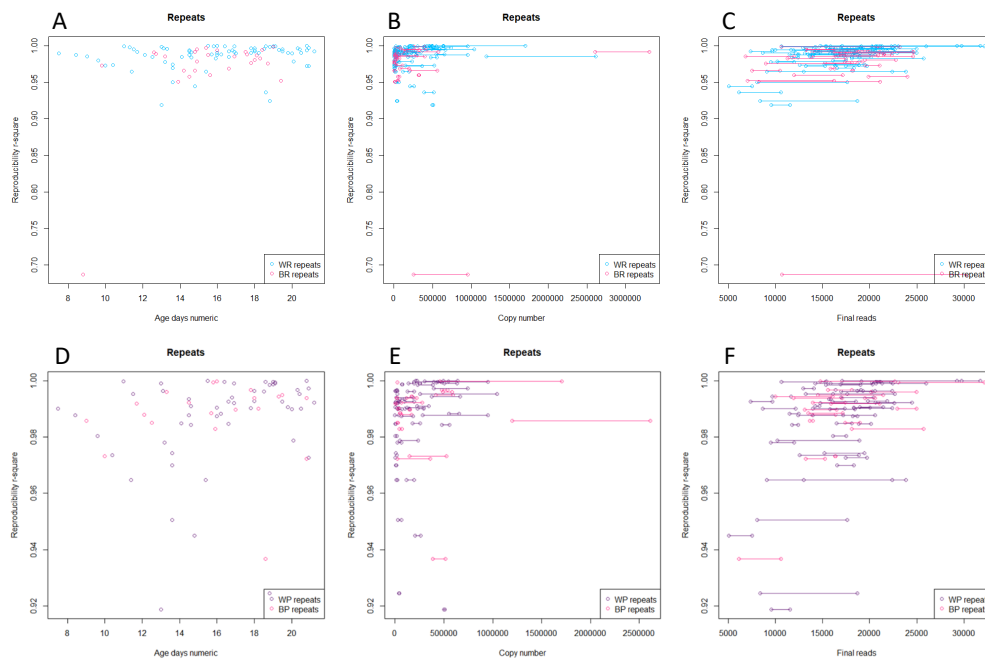


Figure S 2. A scatterplot showing the correlation between samples repeated within a run (WR, n = 74) and between runs (BR, n=28). A) shows reproducibility in relation to age of the participants, B) shows reproducibility in relation to 16S copy numbers and C) shows reproducibility in relation to number of final reads. The second row of the figure shows samples repeated within the same plate in the same run (WP, n=54) and between plates in the same run (BP, n=20). D) shows reproducibility in relation to age of the participants, E) shows reproducibility in relation to 16S copy numbers and F) shows reproducibility in relation to number of final reads.

c. Relationship between the biological samples and negative controls (Primestore) profiles

We included 43 Primestores (sample storage media) as negative controls across the three runs (Run 1= 13, Run2=16, Run3 = 14). The Primestores samples used were made up of two different batches used in sample storage to better account for batch-to-batch variations in background profiles. The number of biological samples analysed was 960. There was a total of 3219 ASVs that had greater than zero reads. As our previous experiences and that of others[7,8] has shown that age is positively correlated with biomass (16S copies) and this also correlates with the number of reads and sometimes with alpha diversity, we sort to assess this in our data. We found no correlation between the final reads and 16S copies ($r=0.05$) Figure S3 (A1 and A2) and none between age and 16S copies ($r=0.07$) Figure S3 (C1 and C2). However, we detected a slight negative correlation between Shannon diversity index and 16S copies ($r= -0.26$) Figure S3 (B1 and B2).

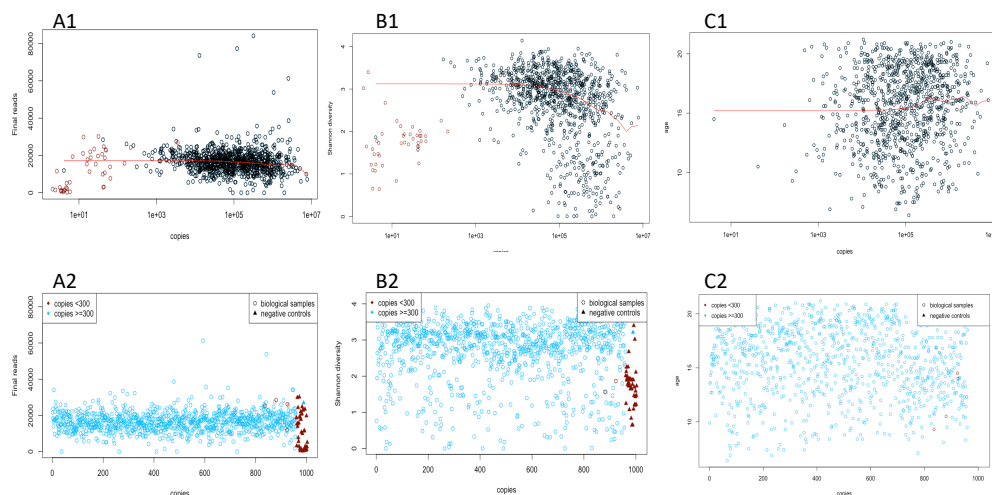


Figure S 3. A scatterplot showing the spread of biological samples (n=960) and the negative controls (primestore, n=43) 16S copies vs final number of reads (A1 and A2), Shannon alpha diversity index (B1 and B2) and age of participant in years (C1 and C2).

Next, we investigated whether our low biomass specimens (<500 / μ l copies, n = 4) shared bacteriome profiles with our negative controls (cluster together on ordination plots). We observed that this was not the case, Figure S4. However, we did excluded specimens with < 100 16S gene copies (n = 2). Our further analysis revealed that samples with low read counts (<1000 reads) may cluster with negative controls indicating similar bacteriome profiles, Figure S5. We therefore excluded these samples as well (read counts were 0 – 4, n = 5). As we and others have shown that these low biomass specimens produced poor reproducible sequencing profiles[7,8]. Four Primestores had <100 16S copies (n = 4) while a large number had >1000 reads (39 of the 43). Specimens collected at younger ages do not seem to cluster with negative controls, Figure S6. We assessed the relationship between the number of ASVs detected and the 16S copies and number of reads. We observed that the number of ASVs detected negatively correlated with low 16S copies (<500), Figure S7 and low number of reads, Figure S8. This observation further supports our exclusion of biological samples with <500 copies and/or <1000 reads.

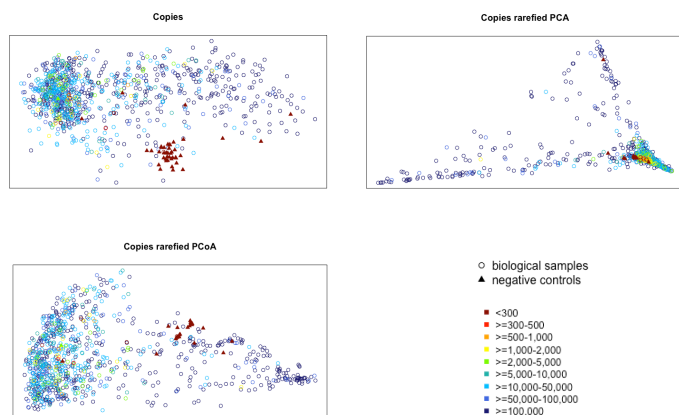


Figure S 4. Ordination plots of showing the spread of biological samples (n=960) and the negative controls (primestore, n=43) coloured by their 16S copies. Low biomass samples (low 16S copy numbers) did not seem to cluster with negative controls. Only four (4) biological samples had <300 16S copies, the remaining red data points represent Primestores.

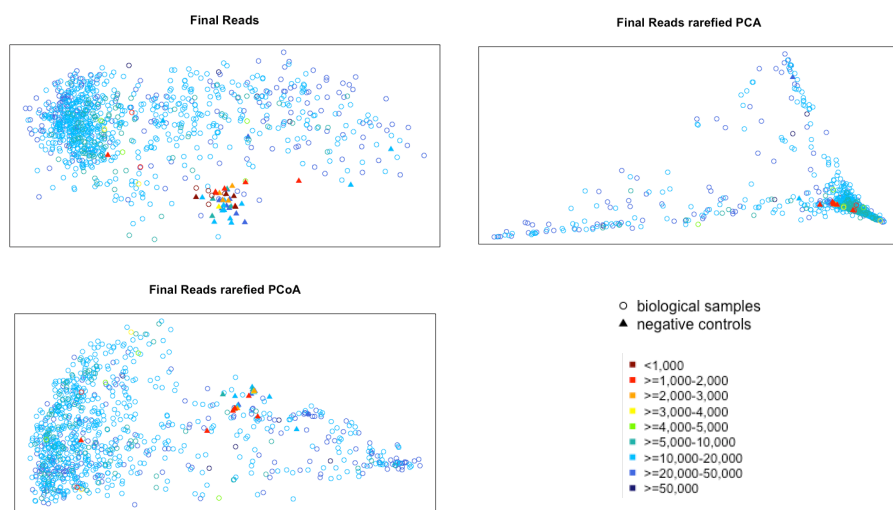


Figure S 5. Ordination plots showing the spread of biological samples (n=960) and the negative controls (primestore, n=43) coloured by their number of reads. Low read count samples (<1,000 reads) may cluster with negative controls

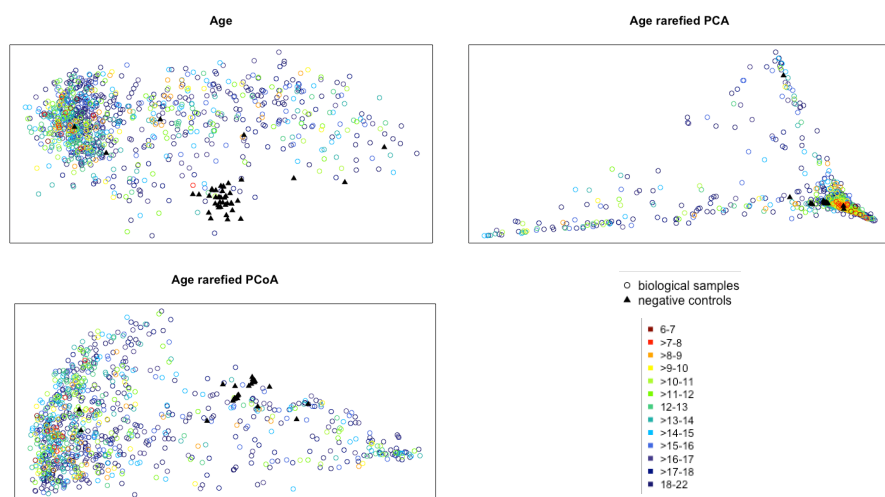


Figure S 6. Ordination plots showing the spread of biological samples (n=960) and the negative controls (primestore, n=43) coloured by the age of the participant. Specimens collected at younger ages do not seem to cluster with negative controls.

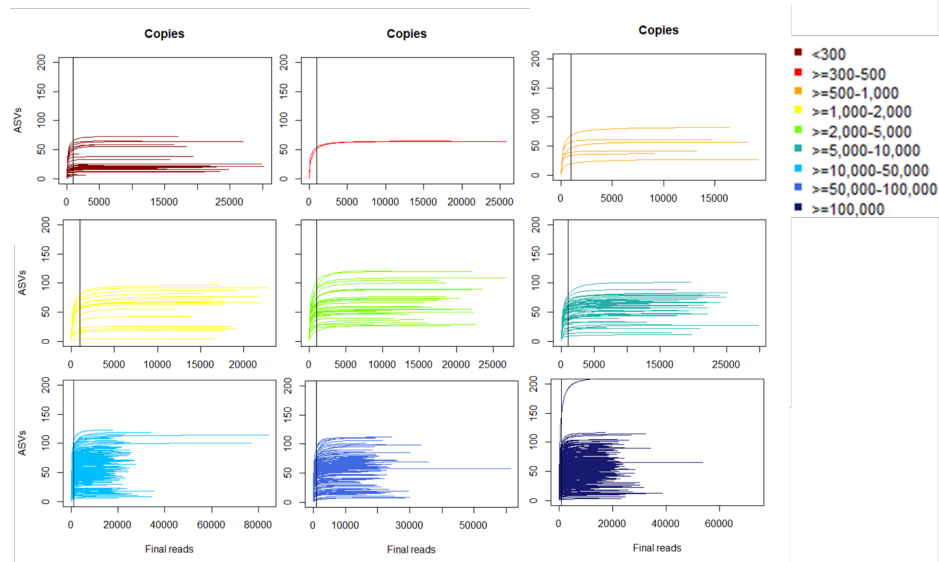


Figure S 7. Rarefaction curves showing number of ASVs detected and 16S copies of samples. The number of ASVs detected negatively correlated with low 16S copies (<500).

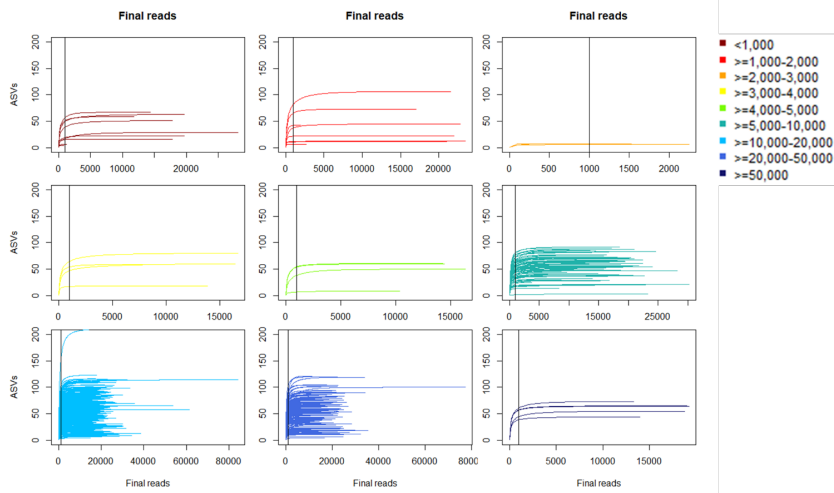


Figure S 8. Rarefaction curves showing number of ASVs detected and number of reads of samples. The number of ASVs detected negatively correlated with low read counts (<1000).

Next, we used a bar plot to visualise the ASV profiles of the two samples with less than 100 16S copies, Figure S9 and the ten samples with >100 to <1000 16S copies, Figure S10 in comparison to the primestore samples to further assess similarity in profiles. Finally, we constructed a ordination plots (log-ratio biplot, PCA and PCoA) on the subset of biological samples with low 16S copies, Figure S11 and low read counts, Figure S12 to assess whether they will cluster with the Primestore samples.

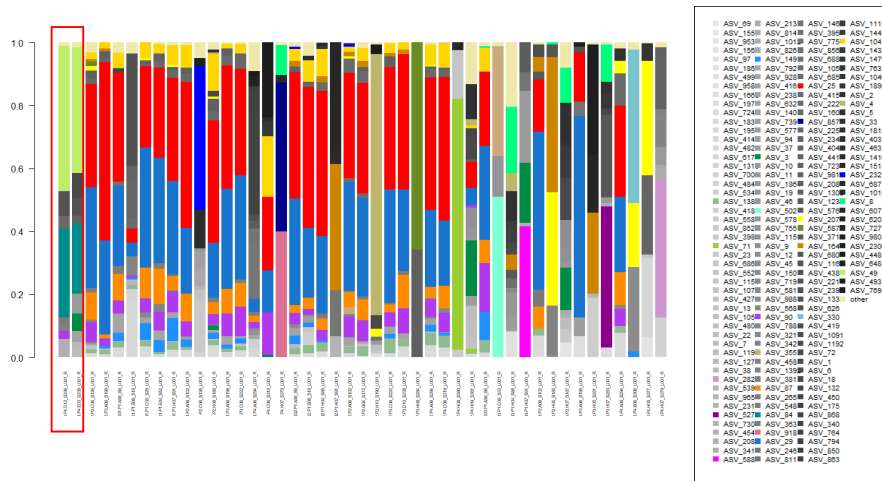


Figure S 9. Bar plot showing the profiles of biological samples with <100 16S copies (n=2) in comparison to Primestores profiles (n=43).

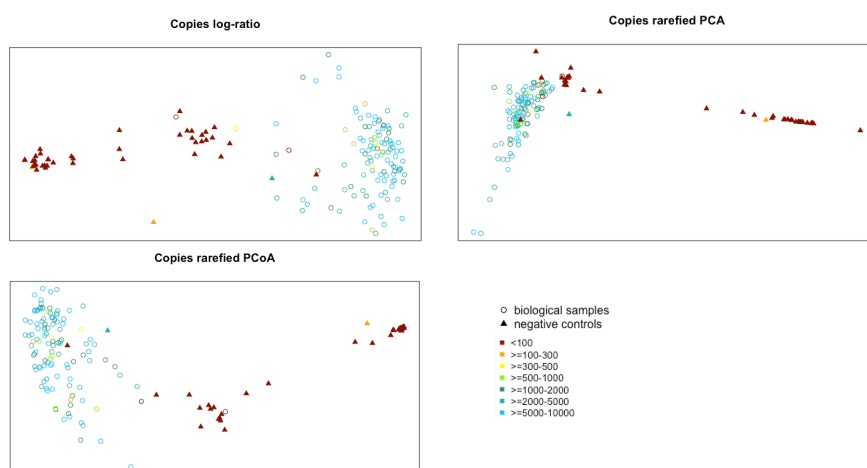


Figure S 12. Ordination plots showing the profiles of a subset of biological samples with low reads and the negative controls. There is a separation between the biological samples with low reads and the negative controls (primestore, n=43).

We continue to explore whether our specimens clusters together based on a) which of the three runs, the samples were processed in (run 1, 2 or 3), b) the country of origin of the sample (Zimbabwe or Malawi), c) the sampling time point or visit (baseline, 12 months (48 weeks) and 18 months (72 weeks)), d) sampling method (expectoration vs. induction) and their relationship with negative controls, e) age especially for younger ages (6-10 years and 11 to 19 years). We did not observe any clustering pattern based on run numbers (Figure S13), country (study site) from which the samples were collected (Figure S14) or the visit or timepoint at which the sample was collected, Figure S15. Specimens collected at younger ages do not seem to cluster with negative controls, Figure S16. We detected no clustering based on any of these variables nor with negative controls. Hence no sample was excluded on the basis on any of these criteria.

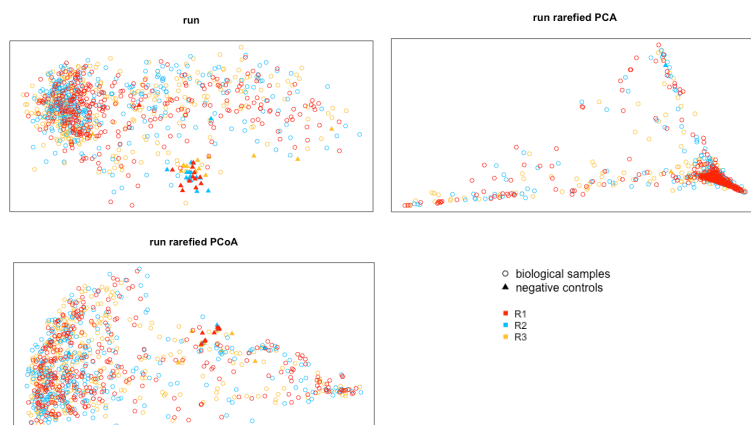


Figure S 13. Ordination plots showing the spread of biological samples (n=960) and the negative controls (primestore, n=43) coloured by the run in which the sample was processed. No clustering patterns based on run numbers.

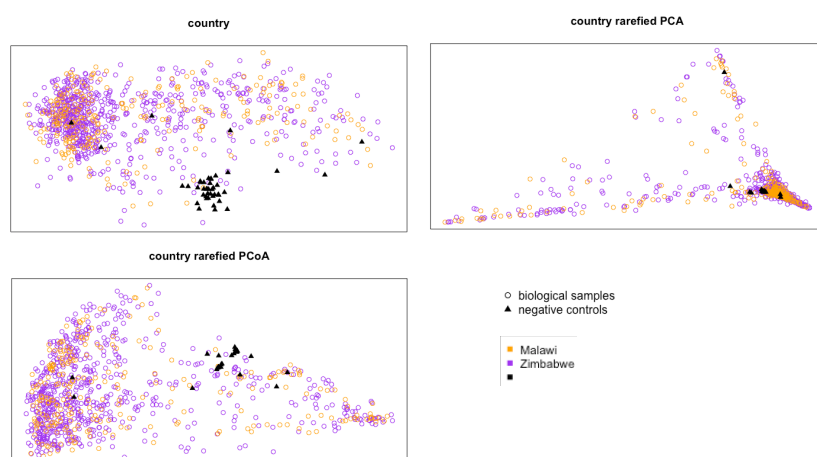


Figure S 14. Ordination plots showing the spread of biological samples (n=960) and the negative controls (primestore, n=43) coloured by the country of sampling. No clustering patterns based on country.

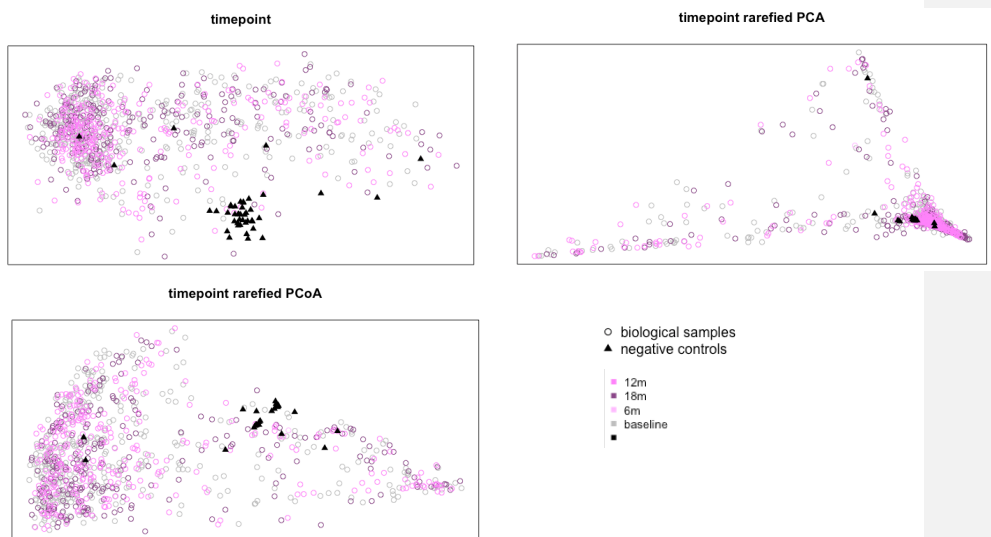


Figure S 15. Ordination plots showing the spread of biological samples (n=960) and the negative controls (primestore, n=43) coloured by visit. No clustering patterns based on visit.

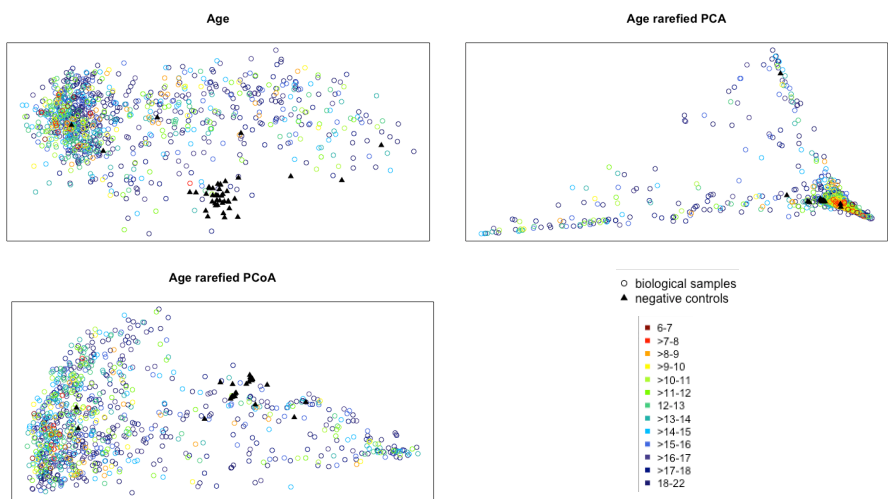


Figure S 16. Ordination plots showing the spread of biological samples (n=960) and the negative controls (primestore, n=43) coloured by the age at sampling. No clustering patterns based on age.

d. *In silico* correction of contamination and spurious ASVs

The number of biological samples remaining is 953 after excluding 7 samples (2<100 copies and 5<1000 reads). The analysis using the DECONTAM R package was conducted on 43 Primestores (negative controls) and 953 biological samples. (Figure S17). The exclusion of the seven biological samples reduced the initial number of ASVs with reads greater than zero from 3219 to 3216. A threshold of 0.4 was set for the *iscontaminant* function and 70 ASVs were returned as contaminants and were consequently removed. The number of ASVs used subsequently (>0 reads and none from negative controls if occurring only here) was 2829.

Spurious ASVs defined as having ≤ 10 reads were identified from these 2829 and removed. These were 1161 ASVs representing 41% of the 2829 ASVs analysed here. They also represent <0.001% of the profile of any given sample. Only 7 of these 1161 ASVs were detected in >1 sample (i.e 2 samples). 1668 ASVs remain from 953 biological samples for downstream analyses (15 ASVs are unclassified at phylum level). Of these 953 samples, 78 samples were from a comparison group not enrolled in the trial hence only 875 samples are reported in this paper.

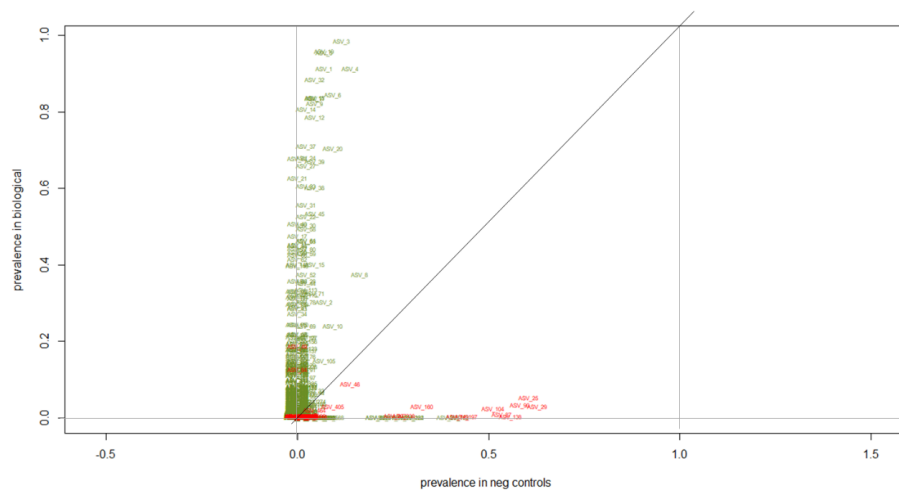


Figure S 17. Output from decontamination analysis using the DECONTAM R package. The contaminants ASVs are shown in red at the bottom right and non-contaminants in green at the top left of the plot. A total of 70 ASVs have been identified as potential contaminants and removed.

Table S 2. List of 70 ASVs detected by the DECONTAM R package as potential contaminants based on comparison between biological samples and negative controls.

	Genus
ASV_1344	Actinomyces
ASV_1299	Actinomyces
ASV_197	Mycobacterium
ASV_1520	Tropheryma
ASV_138	Yonghaparkia
ASV_558	Kocuria
ASV_398	Micrococcus
ASV_600	Rothia
ASV_758	Alloprevotella
ASV_789	Alloprevotella
ASV_483	Prevotella
ASV_510	Prevotella
ASV_658	Prevotella
ASV_800	Prevotella
ASV_1296	Prevotella
ASV_1376	Prevotella
ASV_1410	Prevotella
ASV_1701	Prevotella
ASV_946	Capnocytophaga
ASV_1184	Bergeyella
ASV_1353	Lentimicrobium
ASV_728	Campylobacter
ASV_735	NA
ASV_149	Bacillus
ASV_238	Psychrobacillus
ASV_739	Aerococcus
ASV_82	Lactobacillus
ASV_1084	Lactobacillus
ASV_46	Staphylococcus
ASV_1748	NA
ASV_1227	Selenomonas
ASV_841	Dialister
ASV_612	Veillonella
ASV_1072	Veillonella
ASV_584	Leptotrichia
ASV_825	Leptotrichia
ASV_1086	Leptotrichia
ASV_1142	Leptotrichia
ASV_1984	Oceanivirga
ASV_1322	TM7x
ASV_1654	NA
ASV_90	Bosea
ASV_87	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
ASV_464	Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
ASV_84	Bradyrhizobium
ASV_29	Paracoccus
ASV_25	Blastomonas
ASV_579	Novosphingobium
ASV_160	Sphingomonas
ASV_207	Ralstonia
ASV_1317	Brachymonas
ASV_522	Neisseria
ASV_1152	NA
ASV_104	Methyloversatilis
ASV_338	Klebsiella
ASV_357	Actinobacillus
ASV_424	Actinobacillus
ASV_1416	Acinetobacter
ASV_1085	Moraxella
ASV_317	Moraxella
ASV_446	Moraxella
ASV_1348	Moraxella
ASV_320	Pseudomonas
ASV_1208	Pseudomonas
ASV_405	Pseudoxanthomonas
ASV_49	Stenotrophomonas
ASV_1270	Treponema
ASV_1342	Treponema
ASV_1371	Treponema
ASV_1790	Treponema

e. Total bacterial load

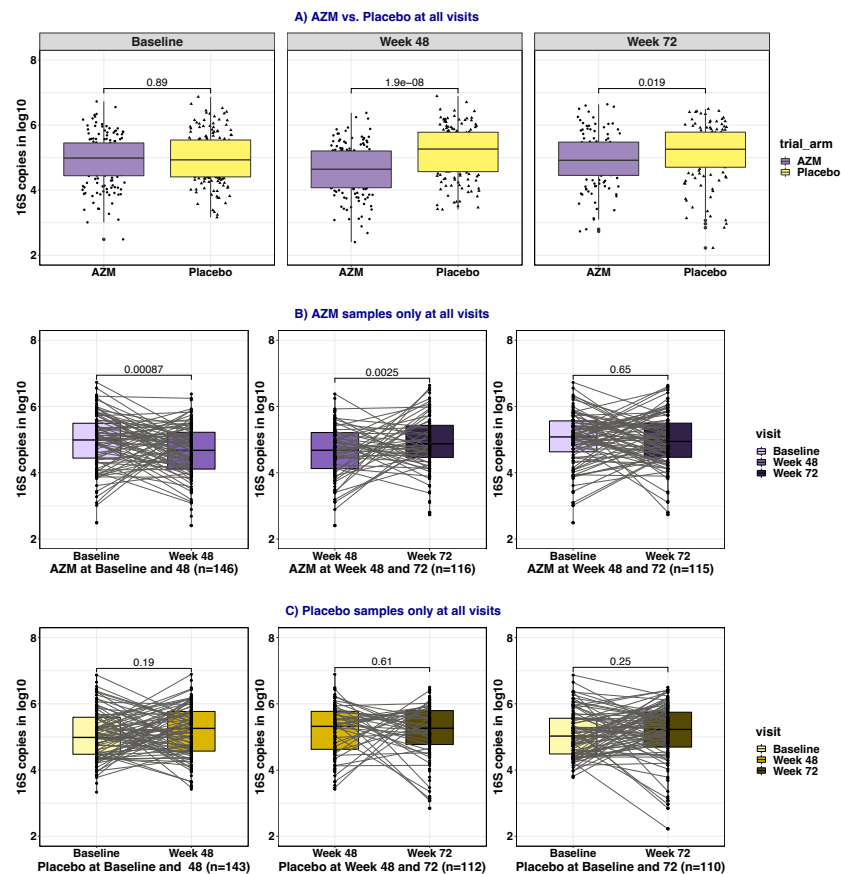


Figure S 18. Boxplot of bacterial load between trial arms at each visit (A) and between visits within AZM arm only (B) and Placebo arm only (C). 16S rRNA gene copy number, used as proxy for bacterial load, was measured with qPCR. The between arm comparisons were implemented using Wilcoxon signed rank test for unpaired samples while within-arm comparisons used Wilcoxon signed rank test for paired samples.

f. Alpha diversity

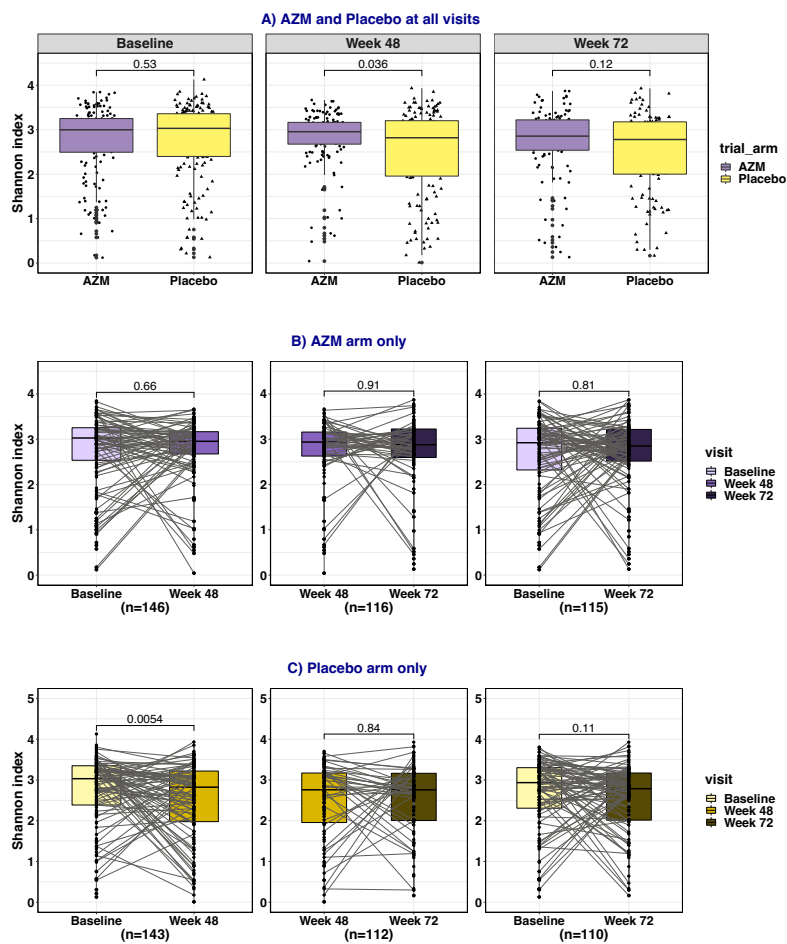


Figure S 19. Boxplot of Shannon alpha diversity index between trial arms at each visit (A) and between study visits in AZM (B) and Placebo (C) arms. The between trial comparisons were implemented using Wilcoxon signed rank test for unpaired samples while within-trial comparisons used Wilcoxon signed rank test for paired samples.

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g. Beta diversity- Azithromycin only

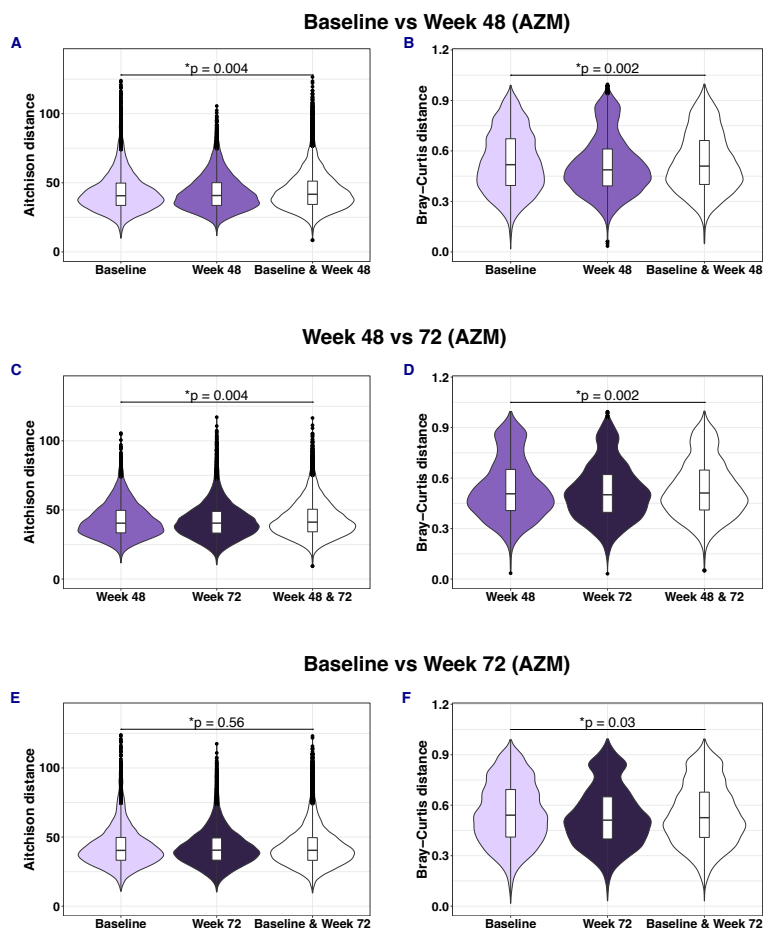


Figure S 20. Violin boxplot comparing two beta diversity metrics between samples collected from participants in the AZM arms at baseline and 48 weeks, 48 and 72 weeks and baseline and 72 weeks. PERMANOVA test used. A) Comparison of samples from baseline and 48 weeks using Aitchison distance. B) Comparison of samples from baseline and 48 weeks using Bray-Curtis distance on unrarefied ASV counts. C) Comparison of samples from 48- and 72-weeks using Aitchison distance. D) Comparison of samples from 48- and 72-weeks using Bray-Curtis distance on unrarefied ASV counts. E) Comparison of samples from baseline and 72 weeks using Aitchison distance. F) Comparison of samples from baseline and 72 weeks using Bray-Curtis distance on unrarefied ASV counts. *p values were adjusted using BH correction. The first two violin boxplots of each figure shows the distribution of the within group distances in the samples from the two visits. The third violin boxplots of each figure shows the distribution of the between group distance between the two visits. 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.

h. Beta diversity- Placebo only

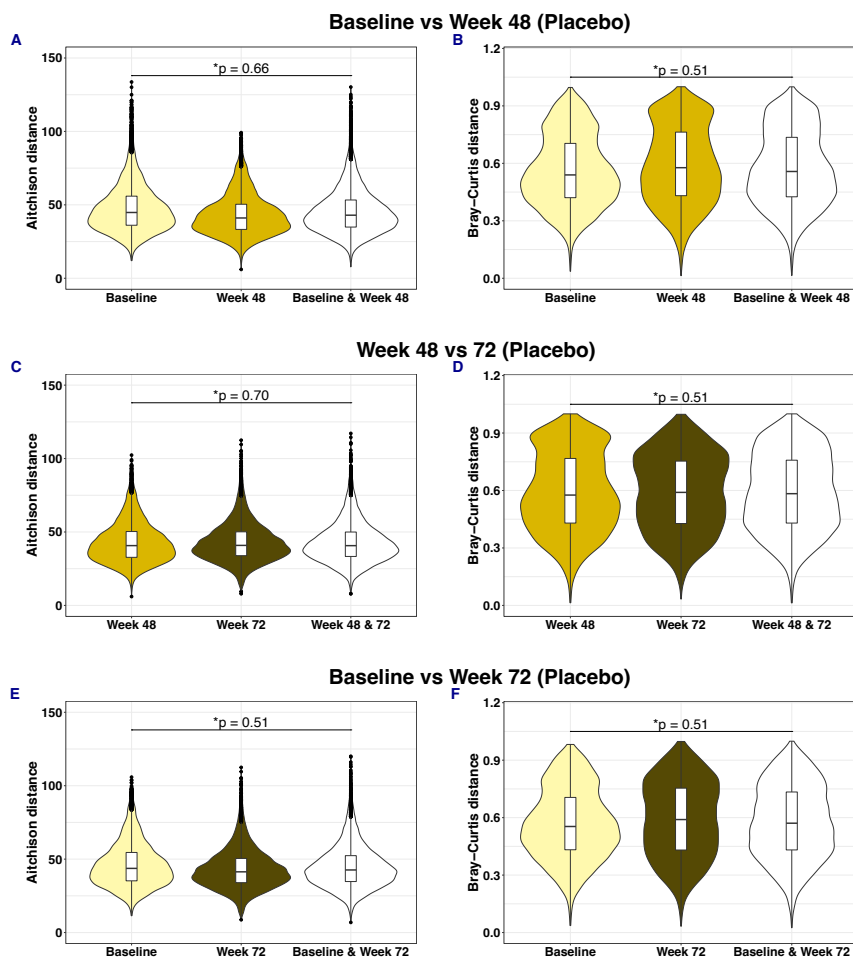


Figure S 21. Violin boxplot comparing two beta diversity metrics between samples collected from participants in the Placebo arms at baseline and 48 weeks, 48 and 72 weeks and baseline and 72 weeks. PERMANOVA test used. A) Comparison of samples from baseline and 48 weeks using Aitchison distance. B) Comparison of samples from baseline and 48 weeks using Bray-Curtis distance on unrarefied ASV counts. C) Comparison of samples from 48- and 72-weeks using Aitchison distance. D) Comparison of samples from 48- and 72-weeks using Bray-Curtis distance on unrarefied ASV counts. E) Comparison of samples from baseline and 72 weeks using Aitchison distance. F) Comparison of samples from baseline and 72 weeks using Bray-Curtis distance on unrarefied ASV counts. *p values were adjusted using BH correction. The first two violin boxplots of each figure shows the distribution of the within group distances in the samples from the two visits. The third violin boxplots of each figure shows the distribution of the between group distance between the two visits. 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.

i. Beta diversity- Azithromycin and Placebo

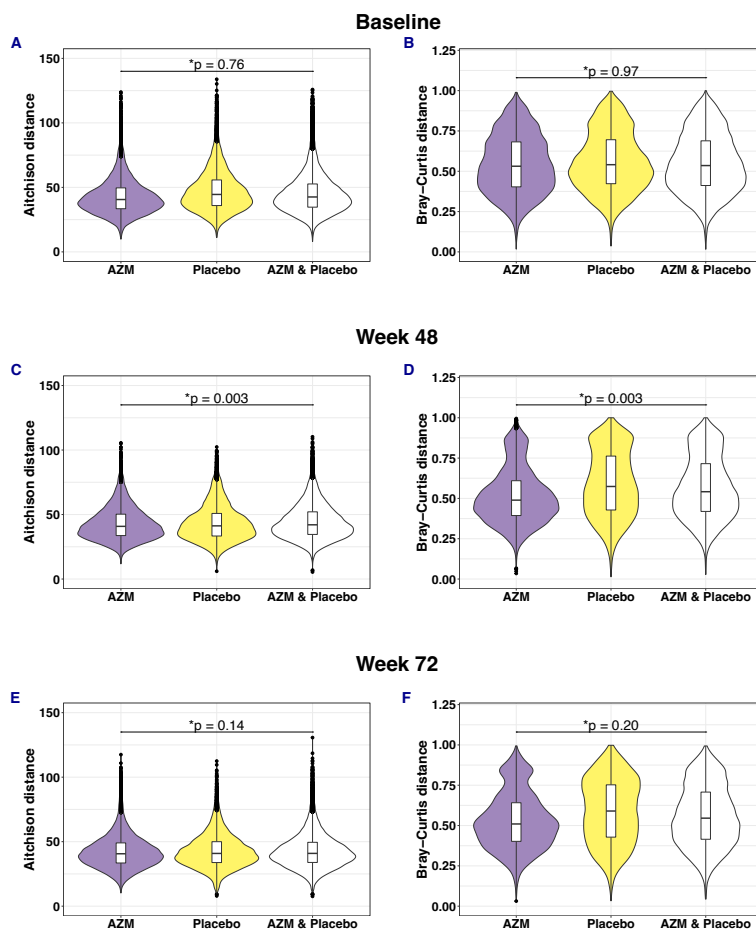


Figure S 22. Violin boxplot comparing two beta diversity metrics between trial arms at 0, 48 and 72 weeks. A) Comparison of AZM and Placebo at baseline using Aitchison distance. B) Comparison of AZM and Placebo at baseline using Bray-Curtis distance on unrarefied ASV counts. C) Comparison of AZM and Placebo at 48 weeks using Aitchison distance. D) Comparison of AZM and Placebo at 48 weeks using Bray-Curtis distance on unrarefied ASV counts. E) Comparison of AZM and Placebo at 72 weeks using Aitchison distance. F) Comparison of AZM and Placebo at 72 weeks using Bray-Curtis distance on unrarefied ASV counts. *p values were adjusted using BH correction. The first two violin boxplots of each figure shows the distribution of the within group distances in the AZM and Placebo arms respectively. The third violin boxplots of each figure shows the distribution of the between group distance between AZM and Placebo arms. 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.

j. Relative abundance of Phyla in all samples

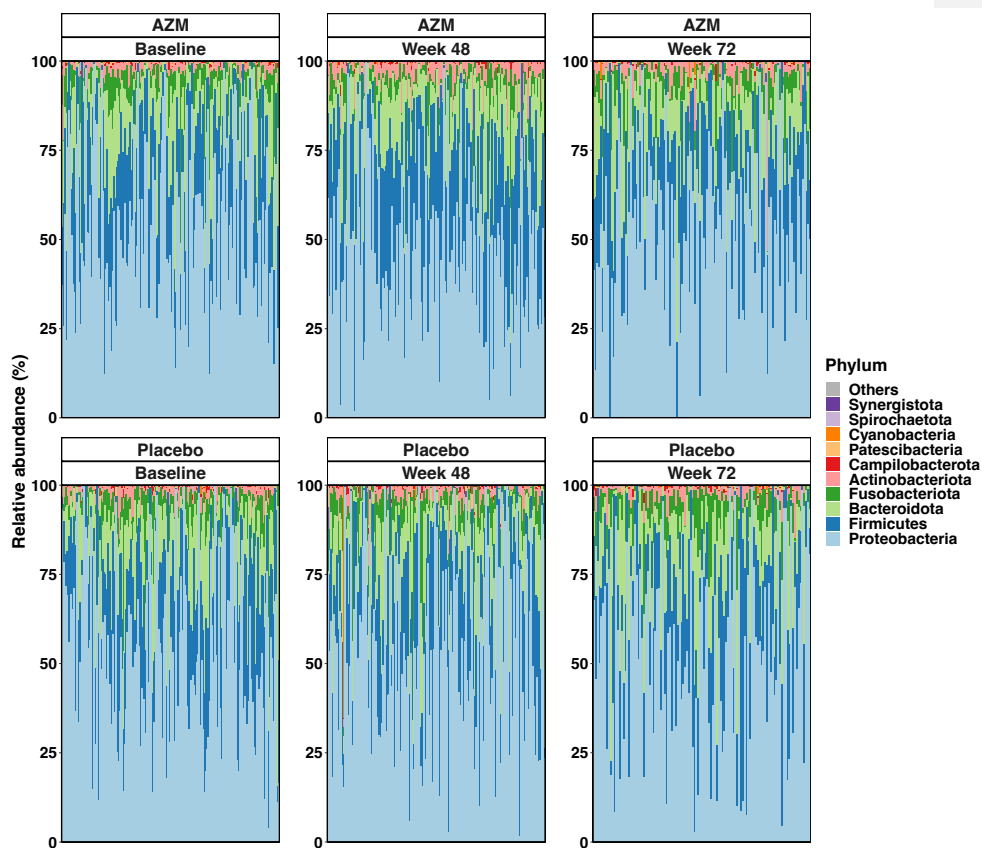


Figure S 23. Barplot of the relative abundances of the top 10 most prevalent phyla in all samples. Upper right, middle, and left panels show samples from participants in the AZM arm at baseline, 48 weeks and 72 weeks. The lower right, middle and lower left panels show samples from participants in the Placebo arm at baseline, 48 weeks and 72 weeks. “Others” refers all phyla that are not included in the top 10.

k. Relative abundance of Genera in all samples

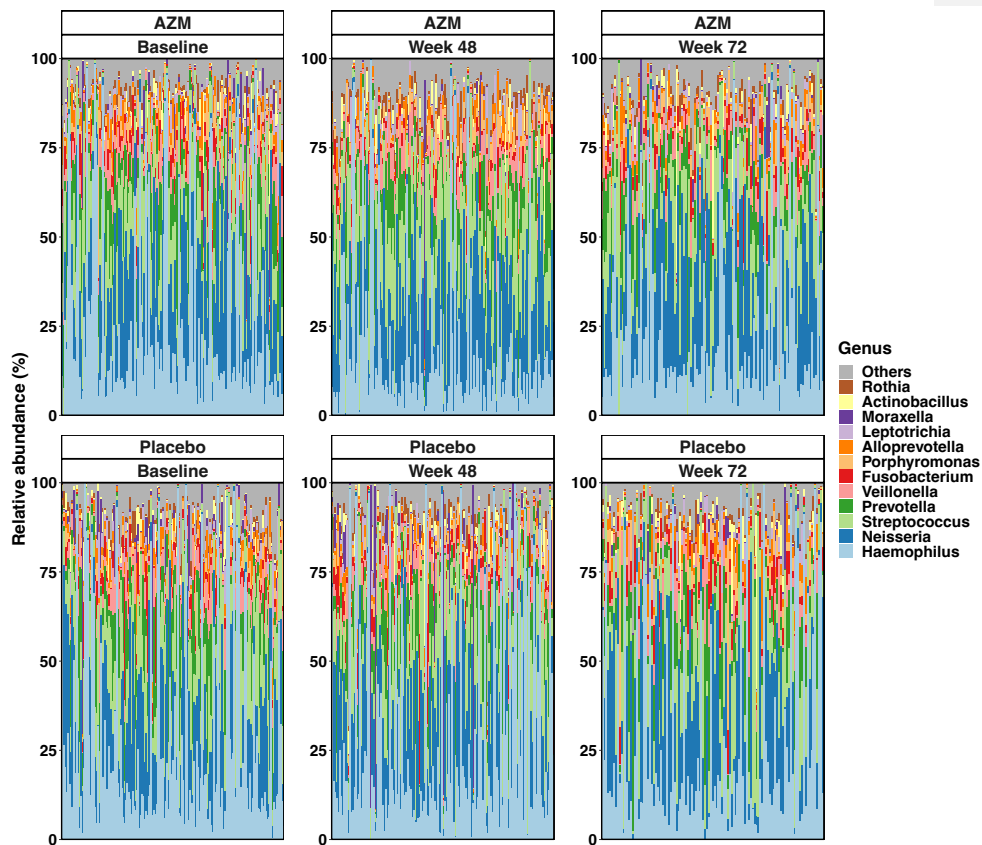


Figure S 24. Barplot of the relative abundances of the top 12 most prevalent genera in all samples. Upper right, middle, and left panels show samples from participants in the AZM arm at baseline, 48 weeks, and 72 weeks. The lower right, middle and lower left panels show samples from participants in the Placebo arm at baseline, 48 weeks, and 72 weeks. “Others” refers all other genera that are not included in the top 12.

2.2. Results of differential abundance of taxa testing

2.2.1. AZM and Placebo

a. AZM and Placebo at baseline

No taxon was found to be differentially abundant by any of the methods.

b. AZM and Placebo at 48 weeks

Results of all the methods are capture in Table S3 located at <https://figshare.com/s/6a4af26f8f696718de38> or <https://doi/10.25375/uct.19172150>

Table S 3. Results of differential abundance testing of bacterial taxa from AZM and Placebo samples from 48 weeks using 10 methods.

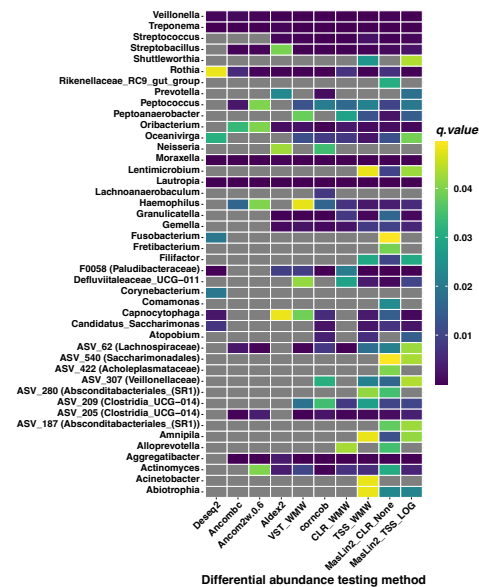


Figure S 25. Heatmap displaying the q values of the genera detected as differentially abundant between AZM and placebo arms at 48 weeks by 10 statistical methods. For ANCOM2, taxa with w 0.6, 0.7, 0.8 and 0.9 were assigned q value of 0.01, 0.001, 0.0001 and 0.00001 respectively. Five genera were detected as differentially abundant by all methods (*Lautropia*, *Moraxella*, *Rothia*, *Treponema* and *Veillonella*).

c. AZM and Placebo at 72 weeks.

Treponema was detected by Ancom2 and *Lautropia* by Deseq2 as differentially abundant taxa. None of the other methods detected a differentially abundant taxon.

Table S 4. Results of differential abundance testing of bacterial taxa from AZM and Placebo samples from 72 weeks using Deseq2

Genus	baseMean	log2FoldChange	lfcSE	p value	Adjusted p value
<i>Lautropia</i>	149.33	-1.42	0.35	1.44E-06	0.0002

Table S 5. Results of differential abundance testing of bacterial taxa from AZM and Placebo samples from 72 weeks using Ancom-II

Genus	W	detected_0.9	detected_0.8	detected_0.7	detected_0.6
<i>Treponema</i>	41	FALSE	TRUE	TRUE	TRUE

2.2.2. Azithromycin arm only

a. AZM at baseline and 48 weeks

Results of all the methods are capture in Table S6 located at <https://figshare.com/s/087eb661dba977926963> or <https://doi/10.25375/uct.19172162>

Table S 6. Results of differential abundance testing of bacterial taxa from samples from the AZM arm at baseline and 48 72 weeks using 10 methods.

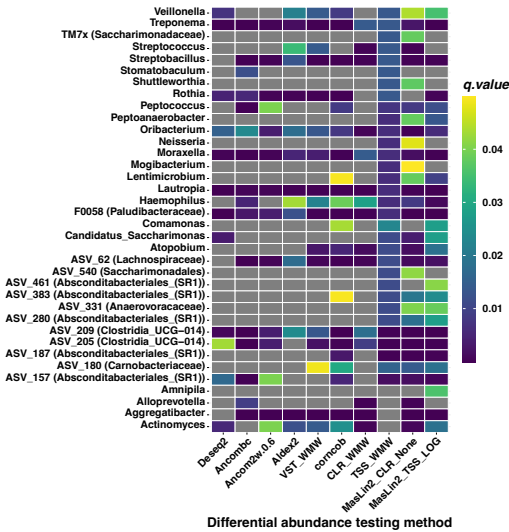


Figure S 26. Heatmap displaying the q values of the genera detected as differentially abundant within the AZM arm between baseline and 48-week samples by 10 methods. For ANCOM2, taxa with w 0.6, 0.7, 0.8 and 0.9 were assigned q value of 0.01, 0.001, 0.0001 and 0.00001 respectively. *Lautropia*, *Moraxella*, *Treponema*, *Oribacterium*, *F0058*, and ASV 209 were detected as differentially abundant by all methods.

b. AZM at 48 and 72 weeks

Results of all the methods are capture in Table S7 located at <https://figshare.com/s/378daaaed6e97aa00085> or <https://doi/10.25375/uct.19172165>

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Table S 7. Results of differential abundance testing of bacterial taxa from samples from the AZM arm at 48 and 72 weeks using 10 methods.

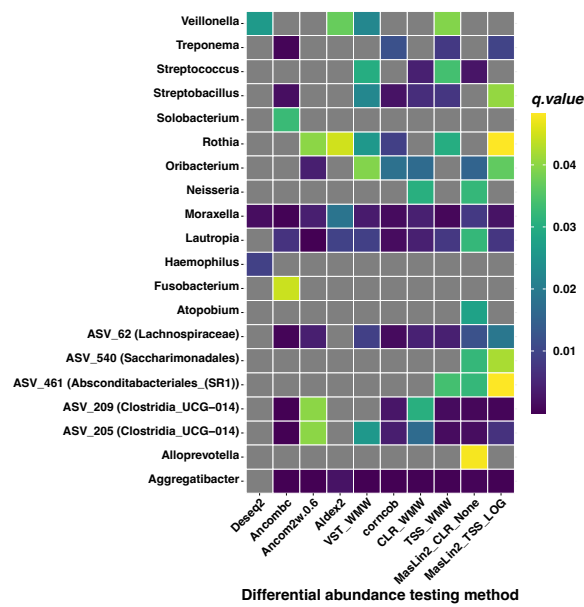


Figure S 27. Heatmap displaying the q values of the genera detected as differentially abundant within the AZM arm between 48- and 72-week samples by 10 methods. For ANCOM2, taxa with w 0.6, 0.7, 0.8 and 0.9 were assigned q value of 0.01, 0.001, 0.0001 and 0.00001 respectively. Only *Moraxella* was detected as differentially abundant by all methods.

c. AZM at baseline and 72 weeks

No taxon was found to be differentially abundant by any of the methods.

2.2.3. Placebo arm only

a. Placebo at baseline and 48 weeks

No taxon was found to be differentially abundant by any of the methods.

b. Placebo at 48 and 72 weeks

Moraxella was detected as differentially abundant by Deseq2. None of the other methods detected a differentially abundant taxon.

Table S 8. Results of differential abundance testing of bacterial taxa from Placebo samples from 48 and 72 weeks using Deseq2.

Genus	baseMean	log2FoldChange	lfcSE	p value	Adjusted p value
<i>Moraxella</i>	242.26	-9.05E-06	0.001	9.54E-07	1.31E-04

c. Placebo at baseline and 72 weeks

Moraxella detected by Deseq2. None of the other methods detected a differentially abundant taxon.

Table S 9. Results of differential abundance testing of bacterial taxa from Placebo samples from baseline and 72 weeks using Deseq2.

Genus	baseMean	log2FoldChange	lfcSE	p value	Adjusted p value
<i>Moraxella</i>	231.49	-2.42	0.59	3.82E-05	5.20E-03

2.3. Results of SIMPER analysis

Table S 10. Contributions of top genera to overall dissimilarity between AZM and Placebo arms at 48 weeks and, within the AZM arm, between Baseline and 48-week samples- SIMPER analysis.

Genus	AZM and Placebo at 48 weeks						Baseline and 48 weeks in the AZM arm					
	Average contribution to overall dissimilarity.	Standard deviation of contribution.	Mean abundance in AZM arm	Mean abundance in Placebo arm	Ordered cumulative contribution	*P value	Average contribution to overall dissimilarity.	Standard deviation of contribution.	Mean abundance at Baseline	Mean abundance at 48 weeks	Ordered cumulative contribution	
Haemophilus	12.8	14.2	28.4	17.5	23.6	0.003	11.4	13.2	25.8	17.9	22.6	
Neisseria	8.4	6.3	17.7	20.5	39.2	0.15	8.1	6.2	19.1	20.3	38.7	
Streptococcus	6.2	4.8	15.3	19.6	50.7	0.002	5.8	4.8	15.9	19.4	50.2	
Prevotella	5	4.4	8.1	10	59.9	0.07	5	4.3	9.4	10.1	60.1	
Moraxella	2.4	8	4.1	0.9	64.4	<0.0001	1.4	5	1.9	1	62.9	
Veillonella	2.3	1.9	3.4	5.4	68.7	<0.0001	2.1	1.8	3.8	5.3	67.2	
Porphyromonas	1.8	2.7	2.9	3	72	0.10	1.7	2.2	3.1	3.1	70.5	
Fusobacterium	1.7	2.4	3.5	2.2	75.2	0.10	1.8	2.5	3.6	2.2	74	
Leptotrichia	1.5	2.6	2	2.2	78	<0.0001	1.5	2.5	2	2.2	76.9	
Actinobacillus	1.4	2.5	1.8	1.7	80.6	0.22	1.3	2.2	1.6	1.7	79.4	
Lautropia	1.4	2	0.8	2.9	83.3	0.01	1.4	2	1.1	2.9	82.3	
Rothia	1.3	1.4	1.6	2.8	85.7	0.73	1.2	1.4	1.4	2.8	84.8	
Alloprevotella	1.3	1.3	2	2.3	88.1	0.83	1.6	1.5	3.1	2.2	87.9	
Actinomyces	0.6	0.8	0.7	1.1	89.1	<0.001						
Granulicatella	0.6	0.5	1.1	1.5	90.2	<0.001						
Gemella	0.6	0.6	1	1.3	91.2	<0.001						
Aggregatibacter	0.4	0.7	0.7	0.4	92.1	0.99						

Contributions by genus were assessed by similarity of percentages (SIMPER) analysis of Bray-Curtis distance. Average dissimilarity is a measure of dissimilarity accounted for by each genus between the sputum bacteriome composition between AZM and Placebo arms at 48 weeks or, within the AZM arm, between baseline and 48 week visits. Contribution (%) is the percentage of total dissimilarity that the contribution of each genus accounts for, calculated as the mean contribution divided by mean contribution of all genera across samples. Cumulative (%) is percentage of dissimilarity that is accounted for by all genera included in the model to this point. Mean abundance is the mean relative abundance of each genus. Only the taxa accounting for 92% of dissimilarity are shown. *p values for comparison of mean abundance between AZM and placebo at 48 weeks by Wilcoxon signed-rank test with BH correction.

2.4. Results of linear regression of within-participant change in beta diversity and lung function.

Table S 11. Univariate linear regression analysis of within-participant Aitchison distance (outcome) and within-participant change in lung function metrics (FVCz and FEV1z) between visits.

Trial arm	Within-participant change in FEV1z			Within-participant change in FVCz		
	coef	stderr	pval	coef	stderr	pval
AZM	1.05	0.45	0.02	0.95	0.42	0.02
Placebo	0.3	0.57	0.6	-0.71	0.46	0.13

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 beta diversity measured using Aitchison's distance 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) Number of samples in azithromycin (AZM) and placebo arms are 377 and 365 respectively.

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