

Azithromycin exposure induces transient microbial composition shifts and decreases the airway microbiota resilience from PM_{2.5} stress in healthy adults: a randomized, double-blind, placebo-controlled trial.

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Research

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Abstract

Background

Azithromycin, widely used in recent years, can change the airway microbiota in patients with chronic lung diseases. Little data exists regarding the effects of azithromycin administration on airway microbiota among healthy adults. Therefore, we conducted a randomized, double-blind, placebo-controlled trial to assess the process of variation and re-establishment of the airway microbiota after azithromycin exposure in healthy adults.

Methods

Forty-eight healthy volunteers were enrolled and randomly assigned into two groups. 500mg azithromycin or placebo was administered once daily for 3 days. We collected the induced sputum at the day before the drugs administration (D0), the day after the treatment course was completed (D4), 14 days, 30 days and 60 days post-dosing. 16S rRNA gene sequencing and quantification were applied to the induced sputum samples. We collected the environmental information including air quality data [particulate matter (PM 2.5) and PM 10 , air quality index (AQI) values] that might have an influence on the airway microbiota during the study. The subjects' respiratory tract infection (RTI) events during sampling were recorded.

Results

Azithromycin didn't alter bacterial load but significantly reduced species richness and Shannon index. Azithromycin exposure resulted in decrease in the detection rate and relative abundance of different families belonging to *Veillonellaceae* , *Pasteurellaceae* , *Leptotrichiaceae* , *Neisseriaceae* and *Fusobacteriaceae* . By contrast, the relative abundance of taxa belonging to *Streptococcus* increased immediately after azithromycin intervention. The shifts in the microbial community composition require about 14 days to recover while alpha-diversity recovered later. The high concentration of PM 2.5 contributed to a novel variability in microbial community composition of azithromycin group at D30 (30 days after baseline). The network analysis found that azithromycin altered the microbial interactions within airway microbiota. The influence was still obvious at D14 when the relative abundance of most taxa had returned to the baseline level.

Conclusions

Azithromycin has transient effect in airway microbiota of healthy adults and decreases the ability of the airway microbiota resilient from PM 2.5 stress. The influence of azithromycin on microbial interactions is noteworthy though the airway microbiota has returned to the near-baseline level.

Background

Human airway microbiota forms a complex, balanced ecosystem and serves as the gatekeeper to respiratory health[1]. In both animal models and human studies, it has been shown that the respiratory microbiota is related to the maturation of alveolar architecture and shapes the local immune system[2–5]. A longitudinal study proves that, among the healthy children, the early-life healthy airway microbiota development enforces the defense against respiratory tract infections[6]. Besides, the respiratory microbiota may have a role in dealing with the inhaled pollutants[7]. The dysbiosis of airway microbiota, driven by decreased microbial diversity and overgrowth of certain bacterium, may play a role in the occurrence and development of respiratory diseases[8, 9].

Antibiotic use is suggested to be one of the most important perturbations for the airway microbiota. In recent years, macrolides are widely used in infection conditions and a range of chronic inflammatory airway disorders, including cystic fibrosis, bronchiectasis, chronic obstructive pulmonary disease and persistent uncontrolled asthma[10–14]. Previous randomized placebo-controlled trials have shown that after exposure to a long-term macrolide therapy, a significant reduction in the diversity of respiratory microbiota and *Haemophilus influenzae* load can be observed in lung disease patients[15, 16]. Although previous analyses well establish that macrolide exposure can change the respiratory microbiota in patients with lung diseases, little data exists regarding the variation in airway microbiota after azithromycin administration among healthy adults, as well as the longitudinal description about the recovery trajectory of microbiota shifts after the antibiotic perturbation.

Given that the respiratory tract is open to airflow, the airway microbiota may be influenced by the environmental factors. Recent healthy murine studies show that lung communities clustered significantly by various environmental sources (vendors, cages and shipments)[3]. The exposure to elevated levels of $PM_{2.5}$ contributes to the change in sputum and pharyngeal microbiota composition, even in the healthy cohort[17, 18].

In this work, we focus on the airway microbiota dynamic variation in the healthy adults in the longitudinal study. The 16S rRNA gene sequencing was applied to the induced sputum samples collected before administration of the drugs (azithromycin and placebo) and at four timepoints over 60 days post-dosing. We aim to track the process of variation and re-establishment of biodiversity, microbial interactions and community composition of the airway microbiota after a 3-days administration of azithromycin. We also estimate the potential influence of environmental factors, such as the concentration of $PM_{2.5}$, PM_{10} and temperature data during the follow-up on the airway microbiota.

Results

Baseline characteristics

The recruitment of the study began on October 8, 2018 and lasted for 2 weeks. We enrolled 48 healthy volunteers with an average age of 26.6 years old (Table 1). The follow-up visits took place from October 2018 to December 2018 (Fig. 1). In total, 221 induced sputum samples were collected (Table 2). All the

healthy volunteers resided in Beijing, China and never left Beijing during the study period. Thirty-eight subjects (79.2%) completed the five timepoints. One subject was diagnosed with acute laryngitis after timepoint D0. Six and three subjects had common cold at timepoints D30 and D60 respectively. All dropouts were lost to follow-up right after the last visit. The induced sputum samples were analyzed by sequencing of the 16S rRNA genes, yielding 24,027,321 reads after quality filtering with on average $108,720 \pm 107,741$ (12,525 ~ 719,237) reads per sample. Thirty-six negative control samples were sequenced alongside the sputum samples obtaining $4,149 \pm 6,288$ reads per sample.

Table 1
Demographics of healthy volunteers.

Parameter	Azithromycin Group (n = 24)	Placebo Group (n = 24)	P Value
Male sex, n (%)	12(50)	12(50)	
Age, yr, median \pm IQR	25 \pm 3.00	25 \pm 3.25	0.60 ^d
Height, cm, mean \pm SD	168.75 \pm 7.57	167.21 \pm 8.52	0.51 ^e
Weight, kg, mean \pm SD	62.71 \pm 12.27	61.27 \pm 9.55	0.65 ^e
Race, n (%)			
Han	23(95.83)	22(91.67)	1 ^f
Others	1(4.17)	2(8.33)	
Residence ^a , n (%)			
Chaoyang District	20(83.33)	19(79.17)	1 ^f
Others	4(16.67)	5(20.83)	
Occupation, n (%)			
Students	15(62.5)	19(79.17)	0.20 ^h
Others ^b	9(37.5)	5(20.83)	
Microbial diversity ^c			
a-Diversity, mean \pm SD			
Richness	405.71 \pm 89.66	403.5 \pm 87.81	0.93 ^e
Shannon	5.98 \pm 0.43	6.00 \pm 0.32	0.81 ^e
β -Diversity			
Unweighted Unifrac distance	NA	NA	0.74 ⁱ
Weighted Unifrac distance	NA	NA	0.78 ⁱ
a: All the participants were resided in Beijing, China, most of them lived in Chaoyang District, others in Changping District, Shijingshan District, Haidian District, Dongcheng District and Fengtai District.			
b: Other occupations included physicians, company employees, and civil servants.			

Parameter	Azithromycin Group (n = 24)	Placebo Group (n = 24)	P Value
c:	Diversity of the baseline sputum microbiota of the healthy volunteers using samples collected before drug administration.		
d:	Compared by two-sided Wilcoxon rank sum test.		
e:	Compared by two-sided Student's t-test.		
f:	Compared by two-sided Chi-squared test with continuity correction.		
h:	Compared by two-sided Pearson's Chi-squared test.		
i:	Compared by PERMANOVA.		
	IQR is short for interquartile range.		
	SD is short for standard deviation.		

Table 2

Data of sputum samples collected at different timepoints^a.

Timepoints	Azithromycin Group (n = 114)	Placebo Group (n = 107)
D0	24	24
D4	24	23
D14	24	23
D30	21	20
D60	21	17
a:	The sputum samples were collected before the day of administration of the drugs (D0), the day after the treatment course was completed (D4), 14 days (D14), 30 days (D30) and 60 days (D60) post-dosing. D: day	
b:	Thirty-eight of the all subjects completed the five timepoints. Six subjects had a cold at timepoint D30. Three subjects had common cold at timepoint D60. One subject was diagnosed with acute laryngitis after timepoint D0. All dropouts were lost to follow-up right after the last visit.	

Minimal influence of procedural contaminations on sputum samples sequencing

It was important to identify the impact of contamination on sequencing results, because the consumables and reagents used in the experiments, such as DTT, DNA isolation and library preparation

might contain bacterial DNA. The microbial community composition between sputum samples and control samples was quite distinct based on both unweighted UniFrac and weighted UniFrac distance (PERMANOVA, $R^2=0.40, 0.28$; $P=0.001, 0.001$ Additional file 1: Figure S1a-b). The five Zero-radius Operational Taxonomic Units (ZOTUs, 100% sequence similarity) detected in sputum samples with the highest abundance comprised 23.9% of all sequences, while comprising 9.8% of all sequences detected in control samples (Additional file 1: Figure S1c). The most abundant ZOTU detected in control specimens (ZOTU103) accounted for only 0.029% of all sequences detected in sputum specimens (Additional file 1: Figure S1d). We performed microbial ecology analysis using the decontam package whose function was to identify contaminants in sequencing data based on statistical approach[19]. We used both the frequency and prevalence methods with threshold 0.1 and 0.5 respectively and confirmed only 3 contaminants ZOTUs (ZOTU1864, ZOTU2014, ZOTU2236) in our data (Additional file 1: Figure S1e-f, Additional file 2: Table S1). However, the abundance of the 3 ZOTUs were very low (comprising $1.96 \times 10^{-4}\%$, $6.24 \times 10^{-5}\%$, $4.58 \times 10^{-5}\%$ of all sputum samples sequences). In conclusion, we found few evidences of procedural contaminations influencing the species detected in sputum and put all ZOTUs into the microbial ecology analysis.

Stable bacterial DNA burden in healthy volunteers' airways after azithromycin administration

We quantified the bacterial DNA of every sputum sample using droplet digital

PCR system. There was considerable variation across individual sputum samples (the first quartile and the third quartile, $1.8 \times 10^7 \sim 9.7 \times 10^7$ copies/g). However, we found no significant difference in sputum bacterial DNA burden across different timepoints in either azithromycin or placebo group (Wilcoxon signed-rank test, Bonferroni adjusted P values (q) >0.05 for all Fig. 2).

The shifts in sputum phylogenetic diversity after antibiotic exposure

The baseline sputum microbiota of healthy volunteers (D0) showed no significant difference between the two groups (two-sided Student's t-test and PERMANOVA, $P>0.05$ for all Table 1). Immediately after the azithromycin course was completed (D4), both the richness and Shannon index were dramatically reduced compared to D0 (two-sided paired t-test, $q=8.718 \times 10^{-6}$ and $q=5.111 \times 10^{-6}$, respectively Fig. 3a-b). By D14, the species richness still remained decreased, but Shannon index significantly increased compared to D4 (two-sided paired t-test, $q=1, q=1.024 \times 10^{-3}$ Fig. 3a-b), suggesting that the relative abundance of surviving microorganisms affected by antibiotic started to recover. During the two-month follow-up, the species richness significantly increased at D30 compared to D14 and completely returned to the baseline microbial profiles at D60 (two-sided paired t-test, $q=1.88 \times 10^{-4}$, $q=0.426$ Fig. 3a).

Meanwhile, we found no significant differences in richness and Shannon index among the healthy volunteers in the placebo group across the five timepoints. (Additional file 3: Figure S2a-b).

Although pairwise PERMANOVA testing using unweighted UniFrac distance showed significant compositional differences between timepoint D0 and timepoints D4, D14, D30 and D60 ($R^2=0.078, 0.107, 0.082, 0.056$; $q=0.01, 0.01, 0.01, 0.05$), principal coordinate analysis (PCoA) demonstrated that the sputum microbial compositions gradually returned towards their initial composition after profound differences at D4 (Fig. 3c). The microbial profiles recovery was also reflected by a highest unweighted UniFrac distance observed at D4, along with significantly descending magnitude over time compared to the placebo group (Fig. 3d). When we used weighted UniFrac distance accounting for relative abundance to perform pairwise PERMANOVA testing in azithromycin group, the significant change in microbial community composition compared to D0 was identified at D4, but not D14 ($R^2=0.103, 0.035$; $q=0.01, 0.92$ Fig. 3e).

Microbial taxonomic variation during 60 days' follow-up

We investigated the microbial taxonomic variation across the five timepoints by comparing the detection rate and relative abundance of the ZOTUs. We involved 292 ZOTUs whose relative abundance was greater than 0.01% and detection rate was greater than 50% at baseline to analyze. After exposing to azithromycin, the detection rate of 109 ZOTUs decreased at D4. There was a depletion of some families, such as *Veillonellaceae* (17.4%), *Leptotrichiaceae* (10.1%), *Fusobacteriaceae* (8.3%), *Neisseriaceae* (7.3%), *Pasteurellaceae* (6.4%), *Actinomycetaceae* (6.4%), *Prevotellaceae* (6.4%) and *Lachnospiraceae* (5.5%). By D14, 72 (66.1%) of the ZOTUs whose detection rate decreased significantly between D0 and D4 exhibited significant differences. By contrast, only 36 (33%) of the above mentioned ZOTUs showed differences in their relative abundance, suggesting that the loss of species usually recovered later than the abundance of them. Most of the ZOTUs returned to D0 level during the follow-up, but several ZOTUs, such as *Fusobacteriia* ZOTUs, *Pasteurellaceae* ZOTUs, *Neisseria* ZOTU and *Leptotrichiaceae* ZOTUs remained low detection rate at D30, and had not been back to the initial state by the end of observational period (Fig. 4, Additional file 4: Table S2). We observed an enrichment of the family *Streptococcaceae* ZOTUs in the relative abundance at D4, immediately post-administration. Most of them rapidly decreased and returned to the baseline level at D14 (Fig. 4, Additional file 4: Table S2).

Among 292 ZOTUs, the detection rate of 205 ZOTUs returned to the D0 level at D14 and only 2.9% of them exhibited significant differences compared to D0 again at D30 (Additional file 5: Figure S3a-b, Additional file 6: Table S3). The relative abundance of 222 ZOTUs were no significant differences between D0 and D14 while 9% of those ZOTUs had novel variation in the relative abundance at D30 (Additional file 5: Figure S3c). At family and genus levels, the relative abundance of 96.08% ($n=49$) of families and 81.82% ($n=81$) of genera had been not significantly different compared to baseline at D14 while 14 families and 8 genera had a shift at D30 again (Additional file 5: Figure S3d).

Attenuated resilience in response to environmental factors disturbance

Higher biodiversity richness was observed in samples collected at D30 but there was no significant difference in Shannon index between D14 and D30 (two-sided paired t-test, $q=1$ Fig. 3b). Besides, a significantly compositional shift was also observed (pairwise PERMANOVA, $R^2=0.081$, $q=0.01$ Fig. 3e), in line with an increased weighted UniFrac distance (0.285 ± 0.068 Fig. 3f) at this timepoint, suggesting that some variation occurred to the abundance of sputum taxa and prevented the recovery. We found that the daily mean concentrations of $PM_{2.5}$, PM_{10} , AQI values, daily mean humidity and temperature were significantly different (Kruskal-Wallis rank sum test, $P<0.05$ Additional file 7: Figure S4) among the five periods. The distance-based redundancy analysis (db-RDA) was performed to explain the environmental stresses on the sputum microbiota variation. By D4, not only the antibiotic, but also humidity had a significant influence on the structure of the microbiota ($R^2=0.026$, $q=0.004$; $R^2=0.053$, $q=0.004$ Fig. 5a). After the variance decomposition, we found that the antibiotic could individually explain 2.6% of microbiota variance ($P=0.001$) and humidity could individually explain 2.3% of microbiota variance ($P=0.001$). Hence, we concluded that administration of azithromycin was related to sputum microbiota, but the environmental factor, humidity, might also shape the microbial communities. By D30, db-RDA showed that the high concentration of $PM_{2.5}$ significantly contributed to the variability in microbiota composition, but not antibiotics ($R^2=0.026$, $q=0.004$; $q=0.052$ Fig. 5b), suggesting environmental factors served as a novel shock to disturb the sputum microbiota which had recovered to the near-baseline microbial community composition.

The effects of azithromycin on airway microbial interactions within the microbial community network

To explore the changes of microbial interaction after exposure to azithromycin, a bacterial community network analysis was performed. The azithromycin group networks became less complicated and stabilized after the antibiotic exposure regarding decreases in the total number of vertices, edges, connectance, average degree, average clustering coefficient and centralization closeness (Additional file 8: Figure S5). The co-occurrence patterns of microbial communities before azithromycin administration and after were quite distinct. We identified the importance of ZOTUs using closeness centralization scores. ZOTUs belonging to families *Fusobacteriaceae* and *Porphyromonadaceae* set a central position at D0 and those assigned to families *Prevotellaceae*, *Lachnospiraceae* and *Veillonellaceae* played the important roles as well. After exposure to antibiotics, families *Veillonellaceae* and *Streptococcaceae* came to the first while families *Fusobacteriaceae* and *Lachnospiraceae* superiority in the network declined. By D60, families *Fusobacteriaceae* and *Lachnospiraceae* returned to their original position again (Fig. 6a). Along with the decrease in detection rate or relative abundance of the major species after administration of antibiotics, the synergistic effect in the co-occurrence networks occupied the main status (Fig. 6a). Despite having a few overlapped edges between timepoint D0 and other timepoints

networks, the closeness of shared nodes was quite different. Though, by D14, the relative abundance of most ZOTUs showed no significant difference compared to D0, the closeness of them still remained decreased (Fig. 6b-c). In control group, families *Fusobacteriaceae*, *Lachnospiraceae*, *Prevotellaceae* and *Veillonellaceae* kept occupying the important positions within the framework along with time (Additional file 9: Figure S6a). The co-occurrence patterns of the microbial communities were quite similar, showing that they contained more overlapped edges and the closeness of shared nodes exhibited very small differences among the five timepoints networks (Additional file 9: Figure S6b-c). It was interesting to see that both positive and negative correlation existed in the networks, suggesting that the interactions among the species made the bacterial community stable.

Discussion

This is the first randomized, double-blind, placebo-controlled trial that investigates the microbial shifts in airways after exposure to azithromycin among healthy adults. The core findings of our study are that airway microbiota of healthy volunteers show resilience and abilities for recovery from azithromycin perturbation. There is a depletion of families Veillonellaceae, Pasteurellaceae, Leptotrichiaceae, Neisseriaceae and Fusobacteriaceae while an enrichment of family Streptococcaceae immediately after azithromycin intervention. The shifts in the microbial community composition require about 14 days to recover while alpha-diversity recovers later. Azithromycin exposure alters the microbial interactions within airway microbial community networks and decreases the ability of the airway microbiota resilient from PM_{2.5} stress. The influence of azithromycin on microbial interactions is noteworthy though the relative abundance of most taxa has returned to the baseline level.

In our study, we find that exposure to azithromycin resulted in decrease in the detection rate and relative abundance of different families belonging to Veillonellaceae, Pasteurellaceae, Leptotrichiaceae, Neisseriaceae, which are sensitive to azithromycin[20]. Those taxa may act as a major ecological change causing the decrease in alpha-diversity after antibiotic administration immediately. The finding is similar to the previous researches that macrolides have a selective impact on *H. influenzae* or species assigned to Pasteurellaceae within airway microbiota both in asthma and non-cystic fibrosis bronchiectasis patients and cause significantly reduced diversity after antibiotic exposure[15, 16]. By contrast, the relative abundance of taxa belonging to Streptococcus increases immediately after azithromycin intervention. Among the asthma patients, the azithromycin administration is correlated with an increase in cultured azithromycin-resistant Viridans streptococci, in line with the increased abundance of Streptococcus reported here[15]. The macrolide resistance in Streptococcus spp is increasing around the world in recent years[21–23]. In China, more than 90% of the Streptococcus pneumoniae isolates are resistant to macrolide both in children and adults[24, 25]. The high antibiotic-resistance may provide those species with an advantage to effectively colonize the airway after intervention. Fortunately, the upgradations of Streptococcus species are transient and return to the D0 level after antibiotic ceases in our study. Our results suggest that the effects of azithromycin on airway microbiota composition are modest. The microbial biodiversity in our study requires about 14–60 days to recover, while it takes

6 months for gut microbiota after antibiotic disturbance[26]. Besides, azithromycin plays few roles in altering the total bacterial load in the airways.

PM_{2.5} exposure is associated with increased all-cause and respiratory mortality, impaired lung function, exacerbations of chronic lung diseases, lung cancer, even Acute Respiratory Distress Syndrome (ARDS) [27–33]. Some studies have found that PM_{2.5} exposure could alter human pharyngeal[18], airways[17] and gut microbiota composition[34] because atmospheric particulate matter commonly carries microbes rich in Firmicutes, Proteobacteria and Bacteroidetes phyla[35]. During our study period, the concentration of PM_{2.5} is very high in the week around timepoint D30. However, we do not observe the variation in airway microbiota community composition of placebo group subjects. One of the reasons may be that all the subjects work indoor for most of time and the duration of daily pollutant exposure is short. What's more, the microbiota communities can show remarkable stability against perturbations[36] and the respiratory microbiota normally has the ability to deal with the inhaled pollutants[7]. The microbiota stability may comprise two concepts. One is resistance, meaning that the composition and function of a given microbiota stand unchanged in response to a disturbance and the other is resilience, which describes that the microbiota is able to return to the predisturbed state[37]. On the contrary, the airway microbiota of azithromycin group subjects, an almost re-established microbial ecology after exposure to antibiotics, cannot bear the same burden of PM_{2.5} pollution and has a novel shift in microbial community composition at D30. From the previous studies, we find that the individuals with dysregulated respiratory tract condition, such as respiratory illness and smoker, or disequibrated airway ecology, are particularly susceptible to the environmental stress or disturbance[38, 39]. Therefore, we assume that, in our study, the stability of airway microbiota decreases because of azithromycin exposure, which results in attenuated resilience in response to environmental factors.

We perform network analysis to support above-mentioned assumption. The functional stability of microbial communities depends on the complicated microbe-microbe interactions[40]. Studies about soil microbiota confirm that the network complexity is consistent with the ability to cope with the diverse environmental changes[41]. After exposure to azithromycin, the airway microbiota networks in our study become simple, indicating the attenuated resilience. The combination of both mutualistic and antagonistic correlations embraced in the networks is important to keep ecological equilibrium[42]. The microorganisms within the communities can cooperate and compete for nutrition to prevent the growth or decline of certain one[36]. In our results, the weakening antagonistic effects due to losing some species contribute to the novel variation in airway microbiota when the environment factors change. It is reported that the mode of action of keystone taxa which mediates multiple interactions within networks is associated with the stability of the microbial communities[43]. Our study finds that antibiotic perturbation targets the crucial taxa by altering the position of them in the framework, which may, in turn, precipitate the microbiota structure fragility. Moreover, the influence of azithromycin on microbial interactions within airway microbial communities is noteworthy though the relative abundance of most taxa has returned to the baseline level.

Our study has following limitations. Firstly, we use induced sputum to investigate the variation in airway microbiota after antibiotic intervention. The induced sputum is a variable mixture that may contain materials from human airways and oral cavity. We ask the subjects to fast for 4–6 hours and clean oral cavity. To determine the contribution of each source, oral washes were also performed by having the subjects gargle with 10 ml sterile 0.9% saline for 60 seconds immediately before sputum induction. However, the concentration of DNA in oral washes is very low (data is not shown) and we aren't able to prepare the 16S rRNA gene library of oral washes along with the sputum samples successfully. Although previous studies show that the salivary microbiota composition remains stable after exposure to antibiotics[44] and the effect of erythromycin on the oropharyngeal microbiota limits to *Actinomyces* genus[45]. The lack of oral cavity microbiota data is still a limitation in current investigation. Secondly, human airway microbiome may be influenced by numerous factors. To avoid deviations as much as possible, we strictly execute a randomized, double-blind, placebo-controlled pattern across the entire study and experimentation process. The stability in airway microbiota of placebo group subjects gives us evidence to believe our conclusions drawn from that of azithromycin group. At last, we only follow up the volunteers for 60 days, the longer-term effects after azithromycin administration demand more attention in future.

Conclusions

In summary, we conclude that azithromycin exposure has transient effect in airway microbial community composition of healthy adults. Azithromycin alters the microbial interactions within airway microbial community networks and decreases the airway microbiota resilience from PM_{2.5} stress. The influence of azithromycin on microbial interactions is noteworthy though the relative abundance of most taxa has returned to the baseline level.

Methods

Study design and population

We designed a randomized, double-blind, placebo-controlled trial to explore the influence of azithromycin on the airway microbiota of healthy adults. Inclusion criteria for the healthy volunteers were men and women aged over 18 years old and had no history of smoking. Detailed inclusion and exclusion criteria are provided in (Additional file 10: Table S4). Finally, forty-eight healthy volunteers were enrolled into this study and stratified by gender and randomly assigned to either the azithromycin or the placebo group (1:1) using a computer-generated random number table. 500mg azithromycin or 500mg starch tablets was administered once daily for 3 days. The drugs were contained in an identical opaque white capsule respectively and packed in sealed kraft paper bags. All healthy volunteers, investigators and study research staff were masked to intervention allocation.

Sputum collection

The healthy volunteers were asked to fast for 4-6 hours and clean oral cavity, including brushing teeth and gargling with 10ml 0.9% saline at our laboratory immediately before the sputum induction. And then, they were subjected to inhale 4.5% sterile hypertonic saline nebulized from an inhalation delivery (PARI, Germany) for 10 minutes and endeavored to cough throughout the process. Most subjects could expectorate an adequate sample (sputum ≥ 1 ml with as little saliva as possible) after the first inhalation. For those who failed to meet the demands, we extended the duration of aerosol inhalation cumulatively up to 60 minutes with pause every 10 minutes or allowed them to cough for the second time later. Sputum was collected in sterile containers, weighed and 2 ml of 0.1% dithiothreitol (DTT) (Sigma-Aldrich, Poole, UK) in phosphate-buffered saline (PBS) was added for liquefaction. The 0.1% DTT-PBS in sputum collection cups and sterile saline in the atomization device served as negative control samples. After incubated at room temperature for 15 minutes on a rolling mixer, all the samples were stored in sterile tubes at -80°C immediately until processed. The sputum samples were collected at the day before the drugs administration (D0), the day after the treatment course was completed (D4), 14 days, 30 days and 60 days post-dosing. We asked the participants to maintain a consistent time point in induced sputum collecting within individual longitudinal samples timepoint.

Environmental and volunteers' follow-up information collection

We collected the environmental information that might have an influence on the airway microbiota, including air pollution data, humidity, temperature and history of leaving Beijing city during the study period. We obtained the daily mean concentration of $\text{PM}_{2.5}$ and PM_{10} , air quality index (AQI) values, daily mean humidity and temperature in Beijing city from China air quality online monitoring platform (The data is available from <https://www.aqistudy.cn/>). Relevant information recorded before every sampling included whether the subjects developed respiratory tract infection symptoms and history of antibiotic administration.

DNA extraction and 16S rRNA gene sequencing

DNA extraction from sputum, paralleled with control samples, was performed using the Maxwell® RSC Whole Blood DNA Kit (Promega, USA). 750 μl sample was transferred to the eppendorf tube and centrifuged for 5 minutes at 14,000g. The supernatant was discarded and precipitation was re-suspended in PBS to 720 μl with 80 μl proteinase K (Tiangen, China) added into. Then, we transferred re-suspended material to a lysing matrix A tube (Qbiogene, Carlsbad, CA). Cells were lysed by bead beating using a FastPrep system (Qbiogene, Carlsbad, CA) for 3 cycles of 30 seconds at 6.0 m/sec and the mixture was subsequently incubated at 56°C for 1 hour. After centrifugation for 5 minutes at 14,000 rpm, 500 μl supernatant was then transferred to the 1# well of the cartridge. 70 μl of Elution Buffer was added to the bottom of each elution tube. Extracted DNA was quantified using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) and stored at -20°C

The V3-V4 hypervariable region of the 16S rRNA gene of all samples was amplified by PCR according to the 16S Metagenomic Sequencing Library Preparation guide provided by Illumina technology[46]. The amplicon primers were Forward Primer =5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and Reverse Primer=5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. The PCR conditions were 95°C for 3 min; followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and then 72°C for 5 min. The amplicons were performed in 25 µL reactions with 12.5ng template DNA. Index PCR was the 2nd stage PCR step that attached dual indices and Illumina sequencing adapters using the Nextera XT Index Kit, Index Primer 1(N7xx) and Index Primer 2(S5xx) (Illumina, San Diego, CA) in 50 ul reaction. The PCR conditions were 95°C for 3 min, 8 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, then 72°C for 5 min. The resulting amplicons were purified using Agencourt AMPure XP (Beckman Coulter) and quantified using a Qubit 3.0 Fluorometer. The pooled library, including negative controls, was sequenced on an Illumina Miseq platform (Illumina, San Diego, CA, United States) using pair-end sequencing (2×300bp).

Bacterial DNA quantification

Bacterial DNA of sputum samples was quantified using a QX200 Droplet Digital PCR System (BioRad, Hercules, CA). Two replicates were used per sample. Primers, cycling conditions and workflow were performed according to a previously published protocol[3].

Statistical analysis

The sequencing data were processed and analyzed using the software VSEARCH version 2.7.1[47], USEARCH version 10.0[48]. The sequencing data were merged (minimum 50 bp overlap), trimmed of primers and indexes, and quality filtered (fastq maxee rate 0.01) with VSEARCH version 2.7.1. Reads were denoised into Zero-radius Operational Taxonomic Units (ZOTUs) with UNOISE3[49] and chimeras were filtered using SILVA version 123 reference database. Taxonomic assignment of ZOTUs was performed by SINTAX[50].

Statistical analysis was performed in R version 3.6.1 via the Rstudio interface. To identify the contaminating DNA coming from experiment operation, the decontam package was used. The feature table covering all samples and the concentration of DNA in each eligible library as measured by fluorescent intensity were input to the function. Species richness, Shannon index, unweighted UniFrac distance, weighted UniFrac distance were computed and ordination analyses were conducted using vegan, ape, ggplot2 and GUniFrac packages with all ZOTUs. Principal coordinates analysis and permutational analysis of variance (PERMANOVA) were performed based on both unweighted UniFrac distance and weighted UniFrac distance. Distance-based redundancy analysis (db-RDA) was performed using weighted UniFrac distance. The diversity comparisons between the same group were calculated by paired t-test or Wilcoxon signed-rank test. The diversity comparisons between the different groups were

calculated by Student's t-test or Wilcoxon rank sum test. We performed linear mixed model and McNemar-Bowker test to compare the variation in relative abundance and detection rate of the ZOTUs whose relative abundance was greater than 0.01% and detection rate was greater than 50% at baseline, respectively. Network analysis was used to explore co-occurrence patterns of ZOTUs within the groups. We involved the ZOTUs whose relative abundance was greater than 0.01% and detection rate was greater than 50% at baseline to obtain Spearman rank correlation matrix ($r \geq 0.6$ and $q \leq 0.05$). The networks were constructed and visualized using igraph package. P values were adjusted using the Bonferroni-control procedure. The heatmaps were visualized by ComplexHeatmap package[51].

Abbreviations

RTIs: respiratory tract infections; D0: baseline; D4: four days after baseline; D14: fourteen days after baseline; D30: thirty days after baseline; D60: sixty days after baseline; PM: particulate matter; AQI: air quality index; DTT: dithiothreitol; PBS: phosphate-buffered saline; PERMANOVA: Permutational multivariate analysis of variance; ZOTUs: Zero-radius Operational Taxonomic Units; PCR: polymerase chain reaction; PCoA: principal coordinate analysis; db-RDA: distance-based redundancy analysis.

Declarations

Ethics approval and consent to participate

The study was carried out in China-Japan Friendship Hospital, Beijing, China. Ethical approval was obtained from the China-Japan Friendship Hospital Ethics Committee (Approval No. 2018-120-K86). All subjects provided written consents. The study was registered at Chinese Clinical Trial Registry (ChiCTR1800018494).

Consent for publication

Not applicable

Availability of data and materials

The sequence data have been deposited in the NCBI Sequence Read Archive under accession PRJNA565553. (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA565553>). Feature, taxonomy, metadata tables and a reproducible workflow of the analysis are available for download at https://github.com/Zoey-Du/Sputum_microbiota.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design: BC, SSD. Acquisition of data: SSD, XYD, LHS, AHS, SRM, YMW, XXF. Analysis and interpretation of data: SSD, XHZ, JKZ, XYD, SL. Drafting or revising of manuscript: SSD, XHZ, JKZ, XYD, LHS, BBL, CLW. Final approval of manuscript: SSD, LHS, BHL, YML, BC.

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Supplemental Information

Additional file 1: Figure S1. Procedural contaminations on sputum samples sequencing analysis. (A) PCoA plot based on unweighted UniFrac distance (A) and weighted UniFrac distance (B) between sputum samples and negative control samples. The ellipses represent the 68% confidence interval for each timepoint. ZOTUs are ranked in descending order of mean relative abundance of sputum samples (C) and control samples (D). Decontam package confirms 3 contaminants ZOTUs (ZOTU1864, ZOTU2014, ZOTU2236) in our data using both the frequency (E) and prevalence (F) methods with threshold 0.1 and 0.5 respectively. ** a noncontaminant ZOTU.

Additional file 2: Table S1. Procedural contaminations on sputum samples sequencing analysis.

Additional file 3: Figure S2. Airway microbial diversity and community composition in placebo group. (A) Species richness. (B) Shannon index. The boxplots represent the diversity measures for the subjects (centre line, mean; box limits, \pm standard deviation; whisker limits, maximum/minimum). The points are connected across timepoints by grey lines. (C) PCoA plot based on unweighted UniFrac distance. (D) PCoA plot based on weighted UniFrac distance. The ellipses represent the 68% confidence interval for each timepoint.

Additional file 4: Table S2. The mean relative abundance and detection rate of 292 ZOTUs.

Additional file 5: Figure S3. Sankey plots to describe the number of ZOTUs whose detection rate (A) and relative abundance (B) shifts or returns to the baseline level across the five timepoints. For the taxa whose relative abundance has been of no significant difference from the D0 level at D14, novel shifts in their relative abundance at timepoint D30 are shown at ZOTU (C), family and genus levels (D). Red represents that there is a significant difference in the relative abundance of the species at D30 compared to D0 level and blue represents that there is a significant difference in the relative abundance of the species at D30 compared to D14.

Additional file 6: Table S3. The number of ZOTUs whose relative abundance or detection rate shifts or returns to the baseline level across the five timepoints.

Additional file 7: Figure S4. The daily mean air pollution data and temperature and humidity during the sputum samples collecting. * AQI ($161 \pm 73 \text{ ug/m}^3$), the concentration of $\text{PM}_{2.5}$ ($90 \pm 36 \text{ ug/m}^3$) and PM_{10} ($213 \pm 159 \text{ ug/m}^3$) are highest at D30 among the five timepoints (Nemenyi test, $q < 0.05$).

Additional file 8: Figure S5. The topological properties of microbial networks in azithromycin and placebo groups.

Additional file 9: Figure S6. The network analysis in placebo group. (A) Networks of co-occurring ZOTUs in airway microbiota for timepoints D0, D4, D14, D30 and D60. Nodes are colored by ZOTU families, with size proportional to mean relative abundance, and edge width proportional to confidence score. (B) The number of shared edges between network D0 and timepoints D4, D14, D30, D60. (C) The closeness centralization of shared nodes between network D0 and timepoints D4, D14, D30, D60.

Additional file 10: Table S4. The inclusion and exclusion criteria for the subjects

Figures

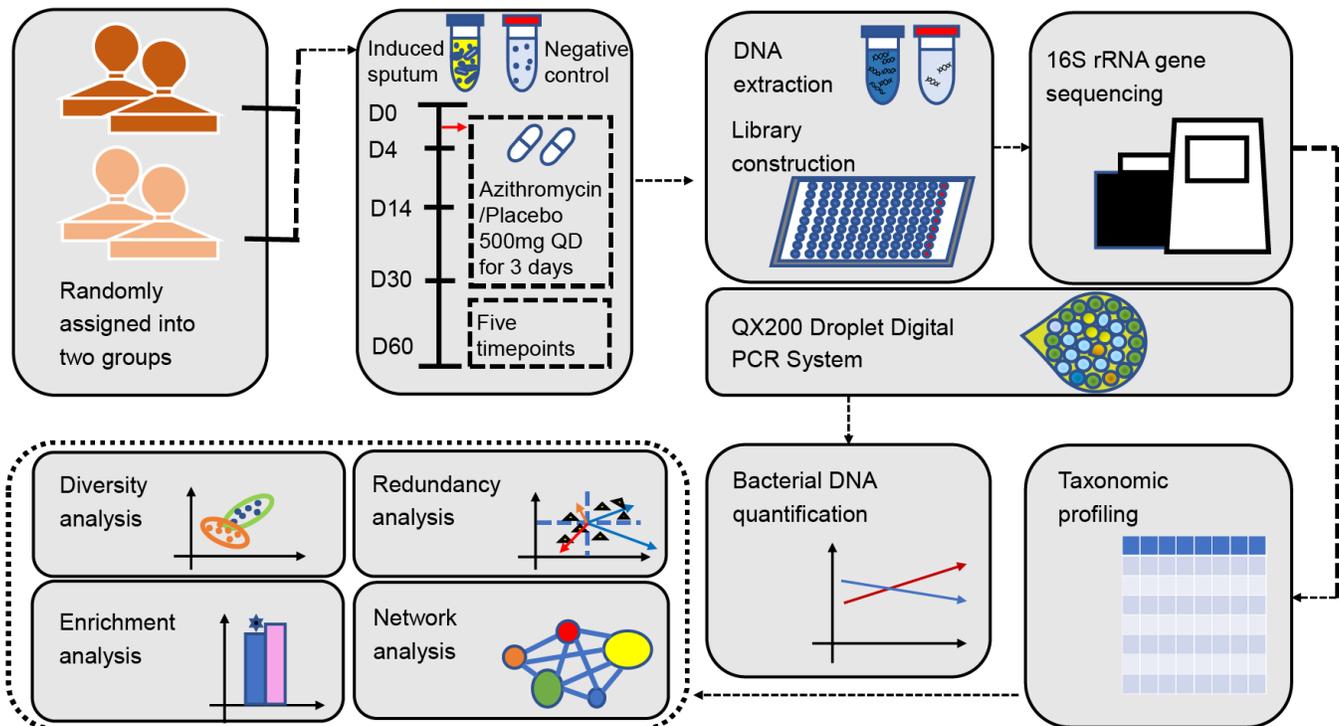


Figure 1

Overview of the study design and sample collection. The healthy volunteers (n=48) enrolled in this study are stratified by gender and randomly assigned to either the azithromycin or the placebo group (1:1). 500mg azithromycin or 500mg starch tablets which are contained in an identical opaque white capsule is administered once daily for 3 days. The sputum samples are collected at the day before the drugs administration (D0), the day after the treatment course is completed (D4), 14 days, 30 days and 60 days post-dosing. 16S rRNA gene sequencing is applied to the induced sputum samples and negative control samples. Bacterial DNA of sputum samples is quantified using a QX200 Droplet Digital PCR System. The microbial ecology analysis is performed on the data produced from the study later.

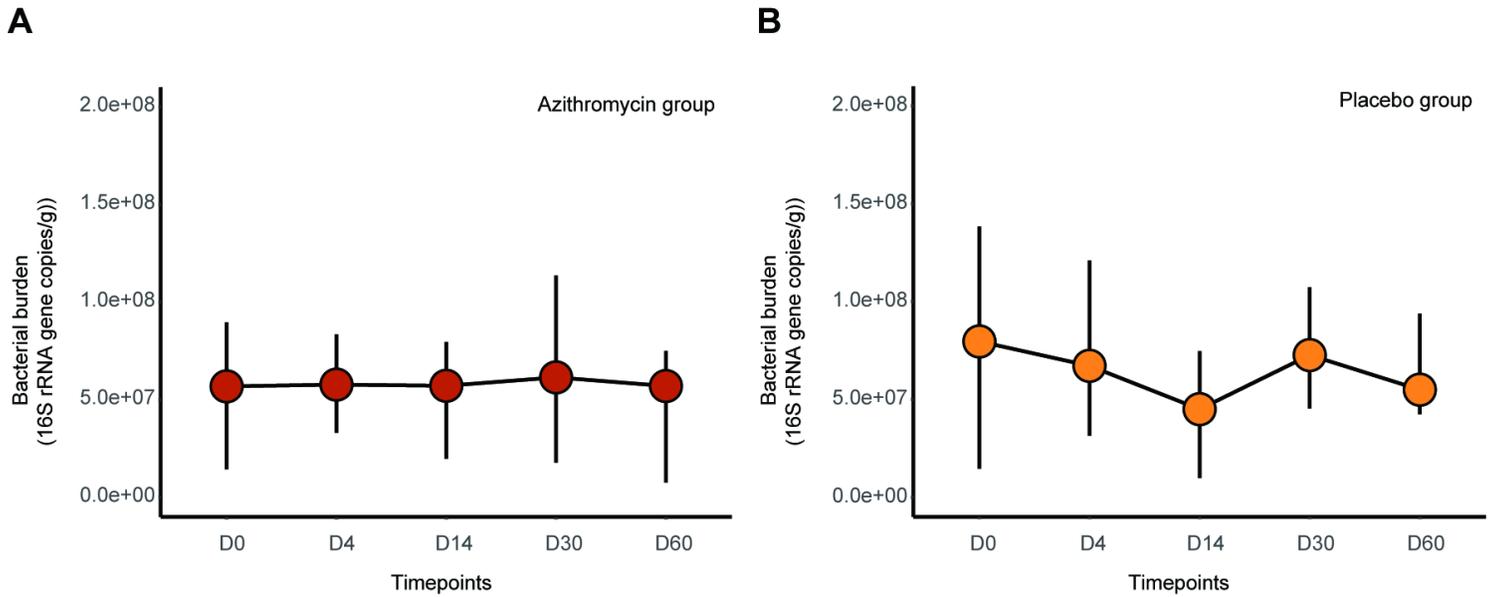


Figure 2

Sputum bacterial DNA quantification using droplet digital PCR of the 16S rRNA gene. There is no significant difference in sputum bacterial DNA burden across different timepoints in either azithromycin group (A) or placebo group (B) (Wilcoxon signed-rank test, Bonferroni adjusted P values (q) > 0.05 for all).

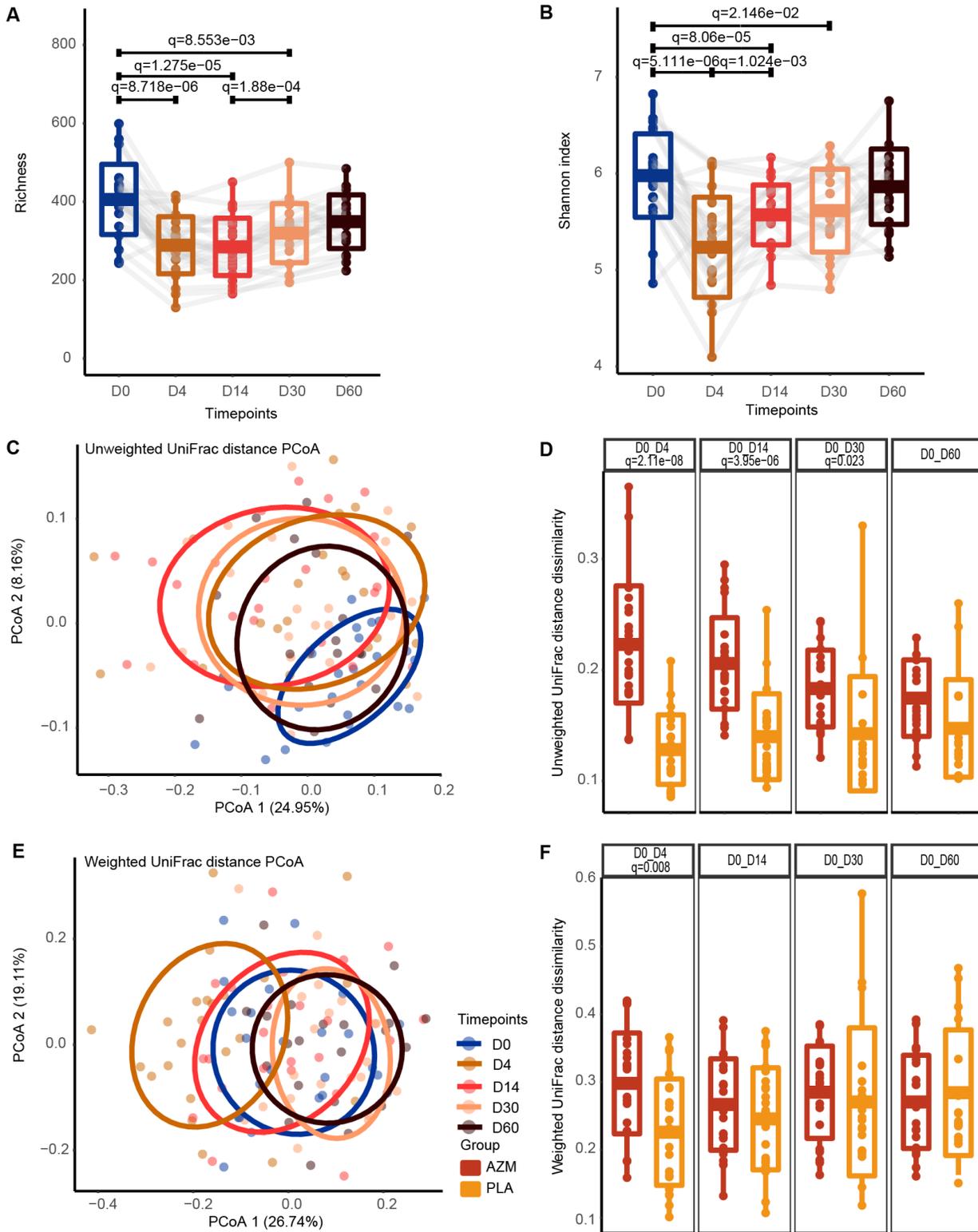


Figure 3

Airway microbial diversity and community composition changes in azithromycin group. (A) Species richness. (B) Shannon index. The boxplots represent the diversity measures for the subjects (centre line, mean; box limits, \pm standard deviation; whisker limits, maximum/minimum). The points are connected across timepoints by grey lines. PCoA plot based on unweighted UniFrac distance (C) and weighted UniFrac distance (E). The ellipses represent the 68% confidence interval for each timepoint. The boxplots

show the unweighted UniFrac distance (D) or weighted UniFrac distance (F) dissimilarities between same-subject samples collecting from two different timepoints (centre line, mean; box limits, \pm standard deviation; whisker limits, maximum/minimum).

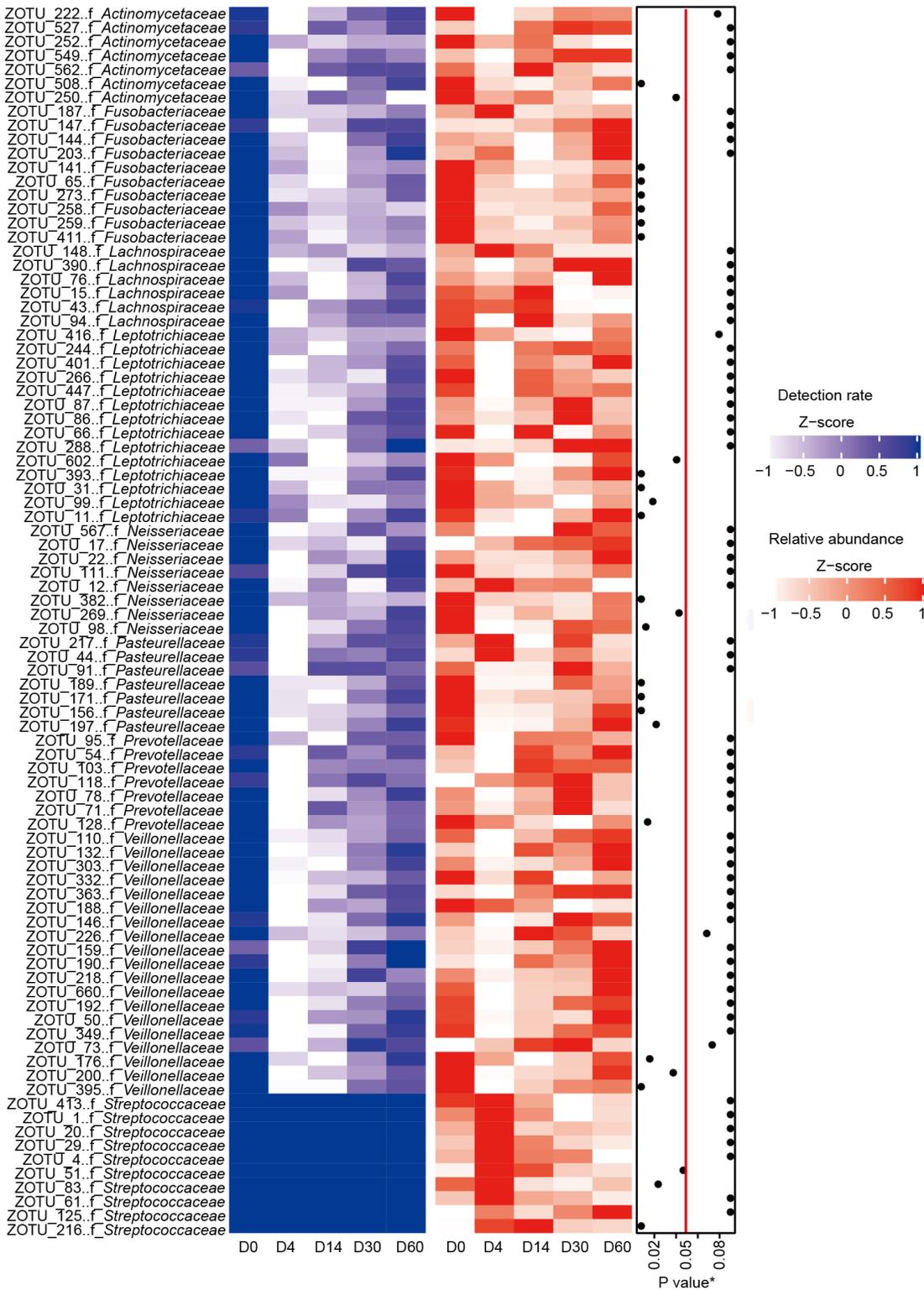


Figure 4

Microbial taxonomic variation after azithromycin administration during 60 days' follow-up. * P value ≥ 0.05 : there isn't a significant difference in the relative abundance of the species at D14 compared

to D0; P value < 0.05: there is a significant difference in the relative abundance of the species at D14 compared to D0.

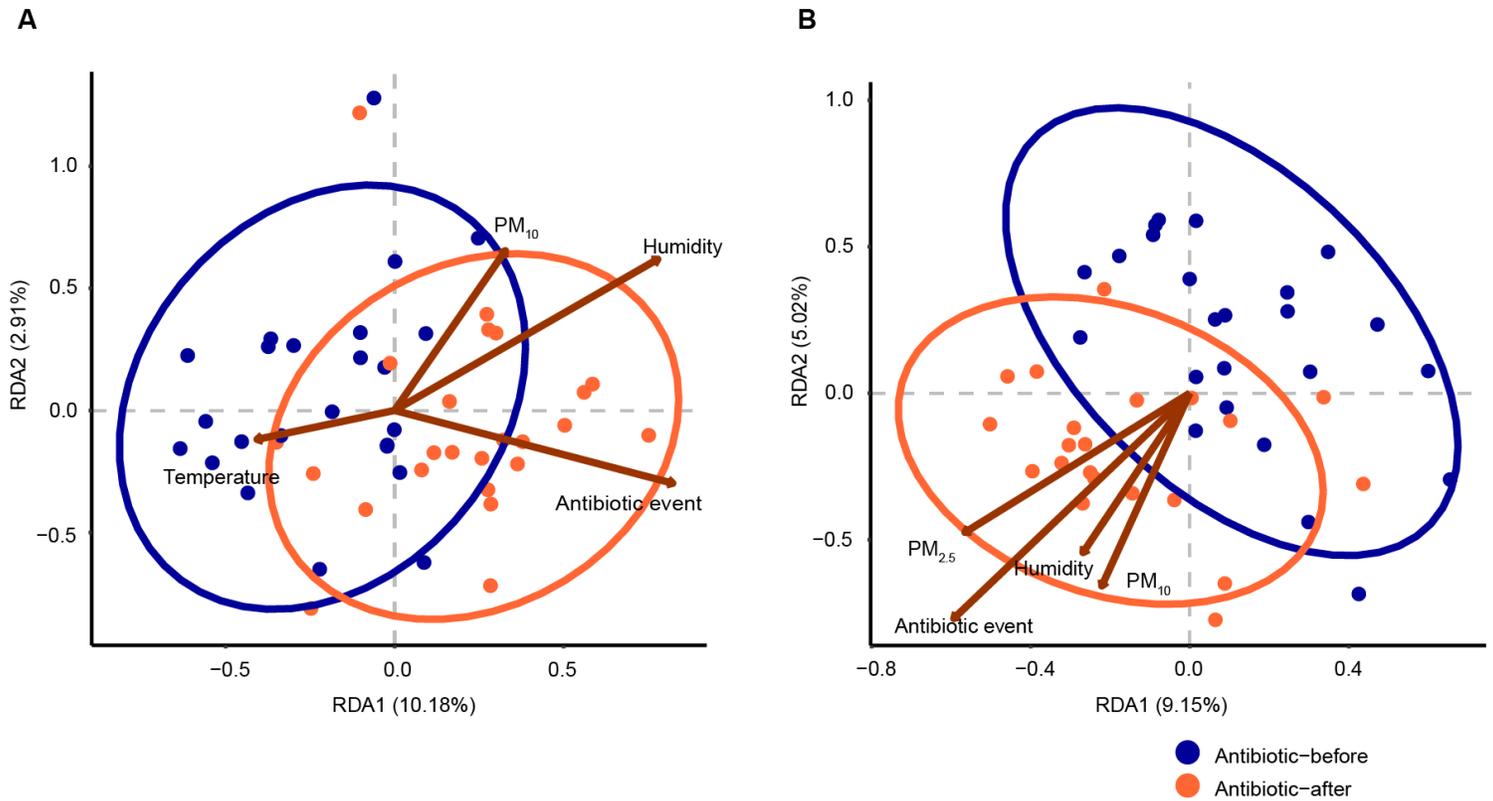


Figure 5

Distance-based redundancy analysis (db-RDA) based on weighted UniFrac distance. (A) The relation of the environmental factors, antibiotic event and the sputum microbiota variation at D4. (B) The relation of the environmental factors, antibiotic event and the sputum microbiota variation at D30. The ellipses represent the 95% confidence interval for each timepoint.

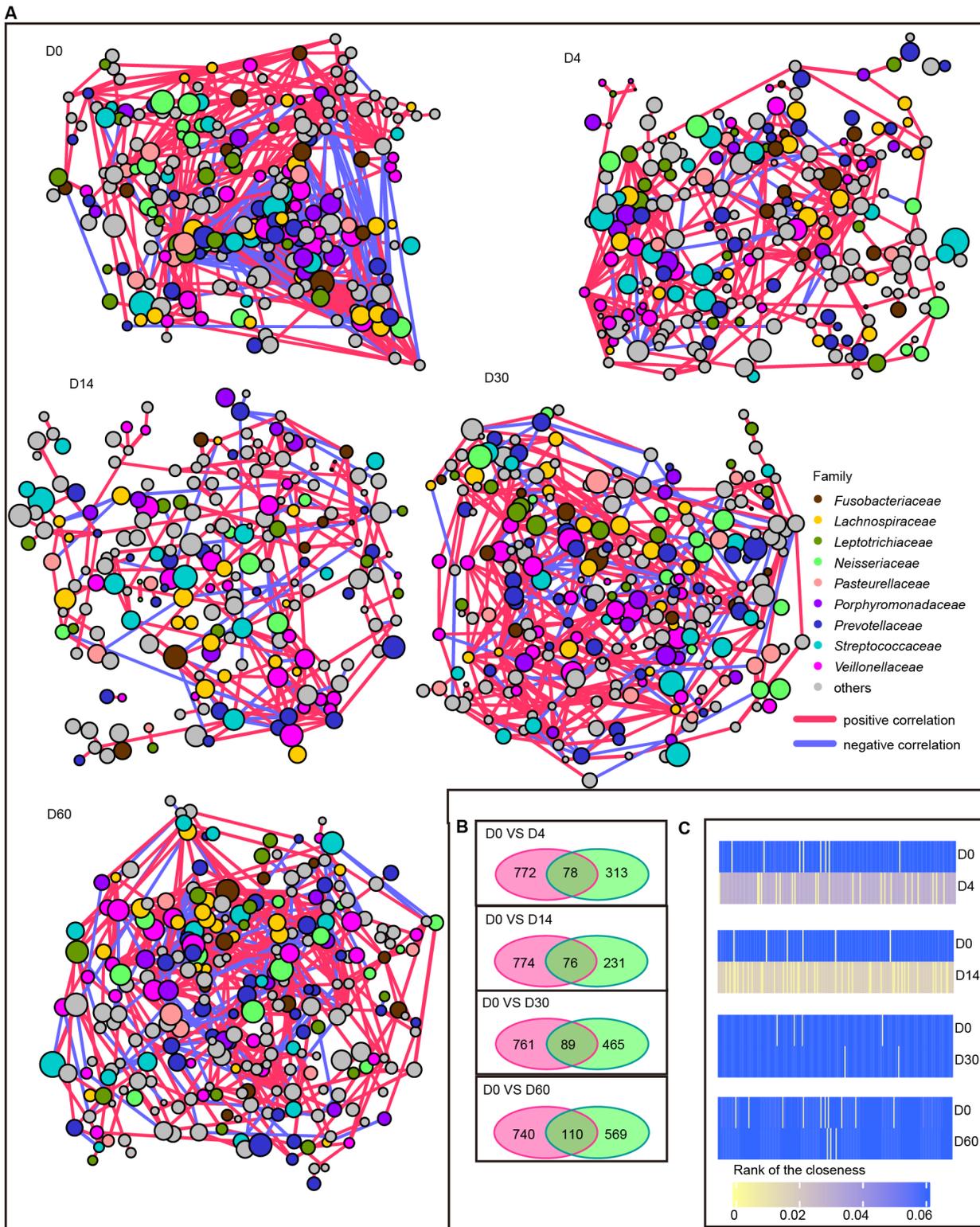


Figure 6

The network analysis in azithromycin group. (A) Networks of co-occurring ZOTUs in airway microbiota for timepoints D0, D4, D14, D30 and D60. Nodes are colored by ZOTU families, with size proportional to mean relative abundance, and edge width proportional to confidence score. (B) The number of shared edges between network D0 and timepoints D4, D14, D30, D60. (C) The closeness centralization of shared nodes between network D0 and timepoints D4, D14, D30, D60.

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