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Research paper

Short- and long-term impacts of azithromycin treatment on the gut microbiota in children: A double-blind, randomized, placebo-controlled trial

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ABSTRACT

Background: Macrolides are commonly prescribed for respiratory infections and asthma-like episodes in children. While their clinical benefits have been proved, concerns regarding the side-effects of their therapeutic use have been raised. Here we assess the short- and long-term impacts of azithromycin on the gut microbiota of young children.

Methods: We performed a randomized, double-blind, placebo-controlled trial in a group of children aged 12–-36 months, diagnosed with recurrent asthma-like symptoms from the $COPSAC_{2010}$ cohort. Each acute asthma-like episode was randomized to a 3-day course of azithromycin oral solution of 10 mg/kg per day or placebo. Azithromycin reduced episode duration by half, which was the primary end-point and reported previously. The assessment of gut microbiota after treatment was the secondary end-point and reported in this study. Fecal samples were collected 14 days after randomization ($N = 59$, short-term) and again at age 4 years ($N = 49$, long-term, of whom $N = 18$ were placebo treated) and investigated by 16S rRNA gene amplicon sequencing.

Findings: Short-term, azithromycin caused a 23% reduction in observed richness and 13% reduction in Shannon diversity. Microbiota composition was shifted primarily in the *Actinobacteria* phylum, especially a reduction of abundance in the genus *Bifidobacterium*. Long-term (13–39 months after treatment), we did not observe any differences between the azithromycin and placebo recipients in their gut microbiota composition.

Interpretation: Azithromycin treatment induced a perturbation in the gut microbiota 14 days after randomization but did not have long-lasting effects on the gut microbiota composition. However, it should be noted that our analyses included a limited number of fecal samples for the placebo treated group at age 4 years.

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1. Introduction

There has been a rapid rise in the use of antibiotics over the past decades [1]. Respiratory infections account for the majority of hospital visits during which antibiotics are prescribed [2]. Even though current guidelines do not recommend antibiotics for the treatment of asthma-like episodes in young children [3], they are among the most commonly prescribed drugs for this condition [4]. Macrolides are often prescribed to children in USA [5], especially to those with respiratory infections and penicillin allergies [6–9]. They are considered safe, well-tolerated and possess antimicrobial activity against gram-positive cocci, such as *Streptococcus pneumoniae*, and gram-negative cocci *Moraxella catarrhalis* and atypical pathogens such as *Mycoplasma pneumoniae* [7]. Besides these activities, azithromycin, a second-generation macrolide, shows antimicrobial activity against microorganisms that erythromycin has no or marginal effect on such as *Haemophilus influenzae* [10]. We recently reported a reduction in the duration of asthma-like symptoms by half after azithromycin treatment [11]. However, the use of antibiotics for reducing such episodes in children does raise concerns given the worldwide action plans to reduce per capita antibiotic consumption. On the one hand, it has been well documented that antibiotic consumption is the primary driver of antibiotic resistance

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Research in context

Evidence before this study

Findings from our previous studies showed that antibiotics such as azithromycin could shorten the duration of asthma-like symptoms in young children. While the clinical benefits of azithromycin intervention have been proved, the potential drawbacks of its use still remain. Considering the associations of gut microbiota with health problems, it is important to investigate the potential consequences introduced to the gut microbiota when azithromycin is prescribed in clinic. On Feb 4, 2018, we searched the scientific literature in PubMed (with no date or language restrictions) for the various combinations of the following search terms "antibiotics", "RCT", "intestinal", and "gut". We identified all previous studies regarding the influence of antibiotics on the gut microbiota in children. Only few publications were double-blind, randomized, placebo-controlled trial (DB-RCT) design, among which none had investigated the long-term effect of antibiotic administration on gut microbiota.

Added value of this study

This study, to our knowledge, is the first DB-RCT investigating both short- and long-term impacts of azithromycin treatment on the gut microbiota composition in children. These data showed a massive perturbation of gut microbiota composition shortly after azithromycin treatment, but the long-lasting adverse effects regarding such perturbations were not observed. However, our analyses did have a limited number of fecal samples for the placebo treated group at age 4 years.

Implications of all the available evidence

Even though our previous study proved the clinical benefits of azithromycin treatment, current guidelines do not recommend antibiotics for the treatment of asthma-like episodes in young children. Compared to the clearly observed disturbance of the gut microbiota composition shortly after azithromycin treatment, its long-term effects regarding such disturbance were not detected. These findings suggested that antibiotic intervention is a strong factor in influencing the gut microbiota for short-term, but the general concerns regarding the undesired, long-lasting impact are alleviated. However, for long-term effects, we were able to analyze only a limited number of fecal samples for the placebo treated group (N = 18) at age 4 years. Nevertheless, the impact of azithromycin treatment at the gene level, such as the gut resistome, and the correlations of such treatment with health problems later in life need to be investigated.

[12,13], and can lead to dysbiosis of the gut microbiota [14]. On the other hand, the treatment of recurrent asthma-like episodes in children represents a major unmet clinical need that has an impact on both the children's quality of life and healthcare resources. Naturally, the benefits and potential drawbacks of antibiotic use for acute management of asthma-like episodes represent a clinical dilemma. Whilst azithromycin is efficient at reducing episodes duration in young children with recurrent asthma-like symptoms, its potential long-term impact on the development of gut microbiota needs to be addressed.

The gut microbiota of adults is a complex and relatively stable community, involved in both host metabolic activity [15] and immune function [16]. However, the taxonomic composition and the structure of this community is highly variable during the first 2–3 years of life [17] and is continuously influenced by numerous factors [18–20], of which antibiotic use is suggested to have the most profound effects [21]. Trasande et al. [22] found that the earlier in life an antibiotic is prescribed, the greater its influence on body mass index (BMI). Studies have previously shown that the gut microbiota is important during the first year of life, as reduced diversity was associated with increased risk of allergic disease [23–25] and delayed maturation can trigger an inherited asthma risk [26]. Alterations of the gut microbiota during this critical window have been suspected to have long-lasting consequences [27], such as decreased richness of the gut microbiota [28]. Although the bacterial richness can recover rapidly in adults [29], high level of antibiotic resistance genes are still observed years later [30,31]. Furthermore, antibiotics can potentially induce the enrichment of antibiotic resistant strains [32], pathogen invasion facilitated by perturbation of non-target commensal gut microbes [33], and community-wide alterations in the gut microbiota composition [34].

Recently, two double-blind, randomized, placebo-controlled trials (DB-RCTs) have investigated the short-term impact of azithromycin treatment on the gut microbiota in children. Both studies found a decrease in richness and diversity of the gut microbiota and an altered taxonomic composition [35,36]. In contrast to the short-term impact of azithromycin on the gut microbiota in children, its long-term effects are not well known. One observational study suggested influences on children's gut microbiota for up to 2 years after macrolide treatment (s) [34]. We therefore explored these effects in a nested DB-RCT in the unselected Copenhagen Prospective Studies on Asthma in Childhood 2010 ($COPSAC_{2010}$) mother-child cohort [37]. Here, we investigate both short-term and long-term impact of azithromycin treatment on the gut microbiota in children. Our study aims to clarify concerns regarding the disturbance of gut microbiota composition when using azithromycin for acute management of recurrent asthma-like episodes in young children.

2. Materials and methods

2.1. Study design and participants

As part of the $COPSAC_{2010}$ cohort, the parents filled out a structured symptoms diary of their children's airway symptoms every day from birth. Parents of children, aged 12–36 months, were invited to participate in the DB-RCT if diagnosed with recurrent asthma-like symptoms, defined as: five episodes of troublesome lung symptoms within 6 months; 4 weeks of continuous symptoms; a severe acute episode needing oral prednisolone or hospital admission. Exclusion criteria were macrolide allergy, heart, liver, neurological, kidney disease and or one or more clinical signs of pneumonia. More details regarding cohort enrollment can be found in our previous publication [11].

Children participating in the DB-RCT were prescribed a 3-day course of oral azithromycin solution of 10 mg/kg per day or matching placebo at acute asthma-like episodes from 12 to 36 months and fecal samples were collected 14 days after randomization and no baseline samples were collected before treatment (Fig. 1a). Children could be included in the trial at a maximum of seven asthma-like episodes, with each treatment randomized independently of any prior treatments. Because participations of children in the DB-RCT were episode driven, children would be invited to participate again if they experienced later episodes. Therefore, additional participations in the trial occurred after a random time interval. Fecal sample was collected from each child when they were 4 years old in the same manner [26].

2.2. Study population

In the DB-RCTs, a total of 72 children (mean age 2·0 years [SD 0·6]) were recruited, each with one to seven episodes (Fig. 1). A total of 124 fecal samples from 62 children were received. After removing eight samples due to low sample quality, the remaining 116 samples from

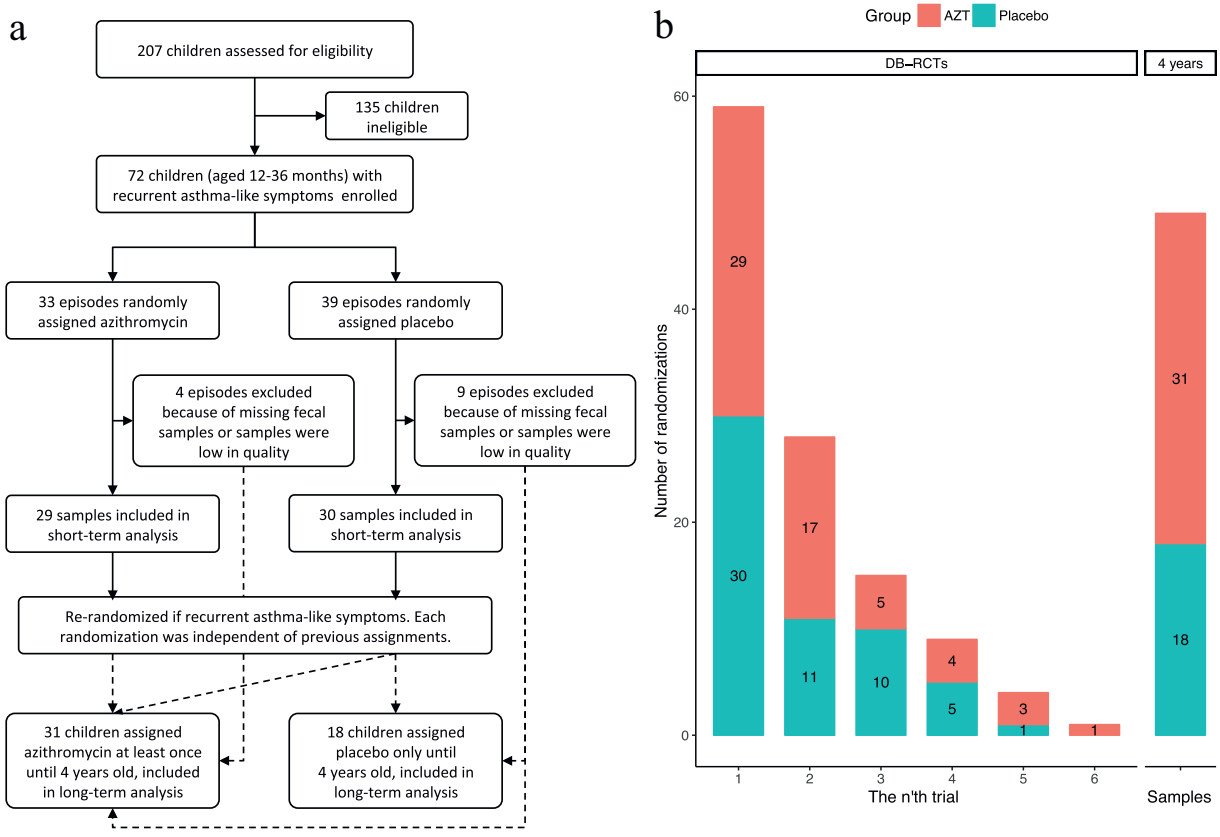

Fig. 1. (a) Trial profile showing the study design of the first participation specifically and all possible following participations. Dashed lines refer to possible sources a sample was from. (b) Barplot showing the distribution of treatments at the first to sixth participation in the DB-RCTs and at 4 years. AZT at 4 years corresponds to children with at least a single azithromycin treatment, whereas placebo are the children who did not receive any azithromycin treatments.

59 children were considered as the sample population in the DB-RCTs. Since some children participated in the DB-RCT more than once, to avoid within-child correlation, only samples collected at the first participation were used for short-term analysis and were grouped based on how children were treated (azithromycin or placebo) (Fig. 1b). Among these 59 children, at their first participation, 29 were from the azithromycin-treated (AZT) group and 30 were from the placebo group. The baseline characteristics of recruited children in the DB-RCT are shown in Supplementary Table 1 and none of the clinical covariates differed significantly between treatment groups.

At 4 years of age, fecal samples were collected from 49 children; of which 31 samples were from children who were treated with azithromycin at least once during the DB-RCTs and the remaining 18 samples were from children who only received placebo (Fig. 1b).

2.3. Randomization and masking

Each asthma-like episode was randomized to either azithromycin or placebo. Treatments were randomly allocated at the Pharmacy of Glostrup (Copenhagen, Denmark) with the computer generated random numbers in blocks of ten. The copies of randomized code were kept at the research site and the pharmacy in sealed envelopes. Investigators and participating families were masked to treatment assignment until children turned 3 years old. Those assessing primary outcome were masked; those doing secondary outcome, which is presented in this study, were not.

2.4. DNA extraction, sequencing, and bioinformatic analysis

DNA extraction and sequencing were performed as described by Mortensen et al. [38] Briefly, the microbial DNA was extracted using the PowerMag® Soil DNA Isolation Kit on the EpMotion® automated pipetting system, EpMotion 5075 (Eppendorf). The microbiota was investigated by 16S rRNA gene sequencing using a two-step PCR procedure targeting the V4 region (~290 bp; primers 515F [5′-GTGCCAGCMGCCGCGGTAA-3′] and 806R [5′-GGACTACHVGGGTWTCTAAT-3′]). Paired-end sequencing (2×250 bp) was performed on the Illumina MiSeq System (Illumina Inc., CA, USA) with the MiSeq Reagent Kits v2 (Illumina Inc., CA, USA); 5·0% PhiX was included as an internal control.

Bioinformatic analysis was performed as described by Stokholm et al. [26] Briefly, the raw Illumina MiSeq sequencing output was primer trimmed (biopieces), quality filtered and merged (UPARSE), and de-novo operational taxonomic unit (OTU) clustered at 97% (vsearch). A phylogenetic tree was built (QIIME) and the taxonomy was predicted against the Greengenes database (version of 2013).

We used rarefaction curves to determine the minimum sequencing depth necessary to describe the microbiota of each sample (Supplementary Fig. 1). The rarefaction curves showed that Shannon diversity reaches asymptotes for samples at 1000 sequences. Based on this, samples with less than 2000 sequences were excluded.

2.5. Statistical analysis

Continuous and categorical data of baseline characteristics were analyzed with *t*-test and chi-square test respectively. The sample size of this study was estimated based on the primary end-point (episode duration) and has been reported previously [11].

The effect of azithromycin on alpha diversity (Shannon index and observed richness) was assessed with two linear regression models (function "lm" in R-package "stats"): one for short-term effect of azithromycin treatment (14 days after randomization, at the first participation), age of a child was included as a covariate; one for long-term

effect (4 years of age), number of times a child participated in the DB-RCT was included as a covariate. To fulfill the assumptions of linear regression, Shannon index at 4 years of age was transformed with "boxcox" in R-package "MASS" because of the violation of normality. For beta diversity, comparisons of UniFrac distances (R-package "phyloseq") between groups were tested with Permutational Multivariate Analysis of Variance with adonis (R-package "vegan") (treatment and age were included as variables) [39,40]. Comparisons of relative abundance of taxa at all phylogenetic levels between treatment groups were assessed with permutation test [41].

To identify genera that were most correlated with treatment, a Random Forest model (named as "RF-1") was performed at genus level (R-package "randomForest") [42]. Its performance was validated via 20 cycles of 10-fold cross-validation (200 iterations in total), with 5000 trees per iteration. The parameter "mtry" was tuned by 10 cycles of 10-fold cross-validation (100 iterations in total) of all possible values.

To assess the recovery of gut microbiota, we built two Random Forest models at OTU level based on fecal samples collected at the first participation (RF-2 model) and 4 years of age (RF-3 model). These two models were performed with 5000 trees and the default value of parameter "mtry". The prediction accuracy of Random Forest models was obtained from the confusion matrix and Area Under the ROC Curve (AUC) was calculated.

2.6. Governance

The $COPSAC_{2010}$ study was approved by the Local Ethics Committee for Copenhagen (H-B-2008-093) and the Danish Data Protection Agency (2015–41–3696). This DB-RCT was approved separately by; the Local Ethics Committee (H-3-2010-065), the Danish Data Protection Agency (2010–41–5023), the Danish Health and Medicines Authority (2612–4329), and registered at ClinicalTrials.gov (NCT01233297). Parents of children gave written and oral informed consent before enrolment of participants. The complete COPSAC biobank is publicly available at the Danish National Biobank (www.biobankdenmark.dk). The entire COPSAC data, including the DB-RCT specific data, are currently being transferred to a publicly available database (the Danish Data Archive, www.sa.dk).

3. Results

3.1. Short-term: alteration of alpha and beta diversity at day 14

At day 14, after randomization, 30 AZT children had significantly lower richness in the fecal samples compared to the 29 placebo children (177·8 ± 56·0 [mean ± standard deviation] vs. 230·6 ± 61·2, respectively, $p = 0·0006$; Fig. 2). Similarly, Shannon diversity was significantly lower in the AZT group compared to the placebo group (2·96 ± 0·80 [mean ± standard deviation] vs. 3·41 ± 0·58, respectively, $p = 0·009$). Both alpha diversity indices increased over age, during which the discrepancies in diversity between groups reduced.

Based on UniFrac distance, the principal coordinates analysis (PCoA) plot illustrated that the AZT group partially overlapped with the placebo group; treatment accounted for a small but significant proportion of variance ($R^2 = 3·8\%$, $p = 0·027$ and $R^2 = 4·2\%$, $p = 0·0007$, weighted and un-weighted distance, respectively; Supplementary Fig. 2).

3.2. Short-term: alteration of taxonomic composition at day 14

Bacteroidetes and *Firmicutes* were the most abundant phyla (relative abundance 57·2% and 31·6%, respectively), followed by *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*; these five phyla had a combined

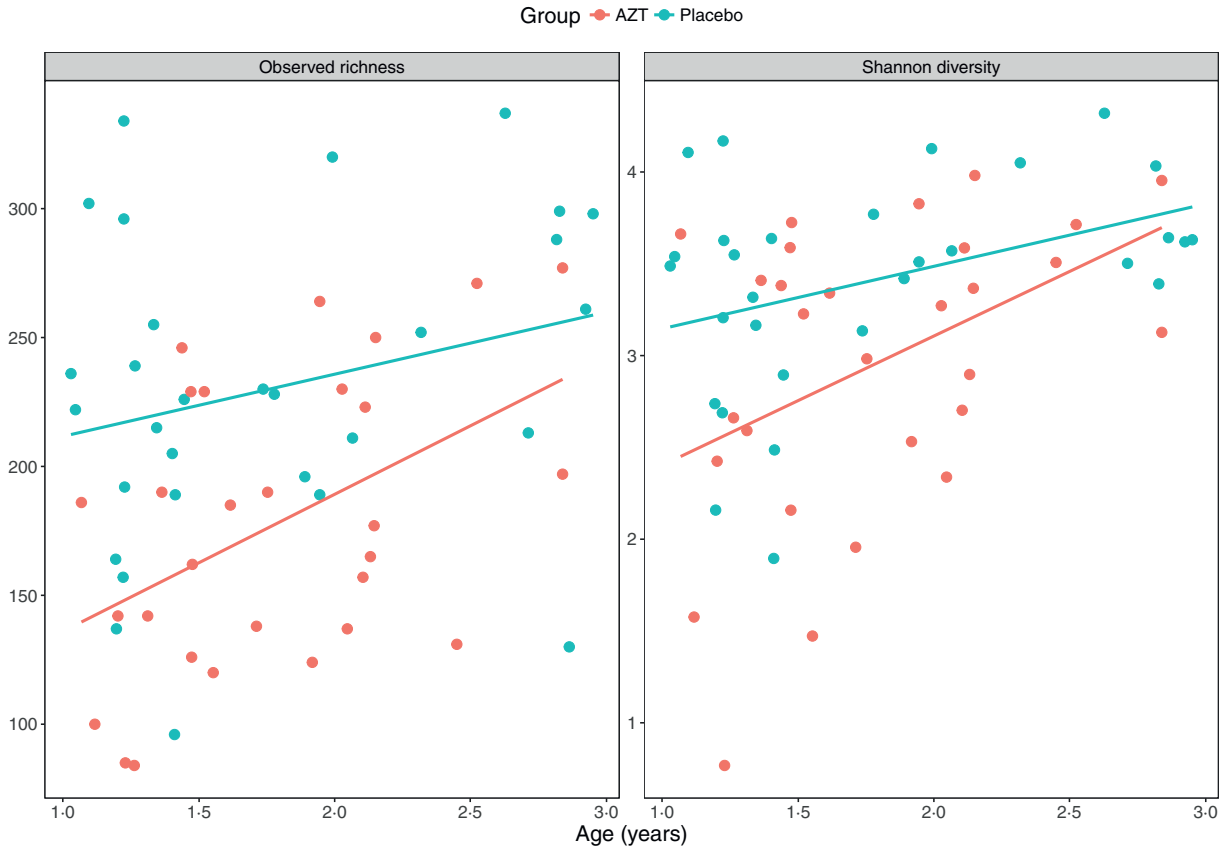

Fig. 2. Short-term effect: Alpha diversity over age between groups. Distribution of observed richness and Shannon diversity for the AZT (red lines) and the placebo (blue lines) groups. The line indicates the linear regression of the correlation between age and alpha diversity.

relative abundance of 99·7%. We observed a decrease in the relative abundance of *Actinobacteria* in the AZT group compared to the placebo group (Supplementary Table 2). Notably, *Bifidobacterium* accounted for the majority of composition changes in *Actinobacteria*, which was evident at all taxonomic ranks, particularly OTU level, where 17 of 21 significant OTUs belonged to *Bifidobacterium* and all were dramatically reduced in the AZT group.

3.3. Short-term: random forest models based on taxonomic composition at day 14

To further elucidate the impact of azithromycin treatment on the gut microbiota composition and to identify its recovery purely based on the gut microbiota, we built two Random Forest models, a supervised machine-learning algorithm, based on the 59 samples collected at the first participation. The first model (RF-1), built at genus level, produced an AUC of 0·89 ($p = 0$, by permutation test with 10,000 iterations), and was used to identify genera that were most affected by azithromycin treatment. The genera having best treatment-discriminatory performance were identified based on importance scores (Fig. 3), among which *Bifidobacterium* showed an exceedingly higher score than the remaining genera. The second model (RF-2), built at OTU level, produced an AUC of 0·92 ($p = 0$, by permutation test with 10,000 iterations), and was used to assess the recovery of gut microbiota at the second participation and 4 years of age.

3.4. Second participation: partial recovery of gut microbiota

After the first randomization, 28 children fulfilled the inclusion criteria again and participated in the DB-RCT for their second time and also had a fecal sample collected. Although the time intervals between two participations were variable (mean 223·3 days [SD 152·8]), the relatively longer time than 14 days enabled us to assess the recovery of gut microbiota after azithromycin treatment; 11 of these 28 children were treated with placebo at their second randomization, and the effect of their first treatment could be evaluated here. Among these 11 children, six were treated with azithromycin, and five were treated with placebo at their first randomization. No difference between groups was observed in either alpha diversity (median of observed richness, 183 ± 74·5 vs. 233 ± 64·0 for AZT and placebo, respectively, $p = 0·052$, Wilcoxon rank-sum test; median of Shannon, 3·43 ± 0·89 vs. 3·94 ± 0·47 for AZT and placebo, respectively, $p = 0·13$, Wilcoxon rank-sum test) or beta diversity (weighted Unifrac, $R^2 = 12·8\%$, $p = 0·22$). Next, we applied RF-2 model to assess the recovery of gut microbiota and we correctly identified the treatments for three of six samples in azithromycin-treated group and five of five samples in placebo group (AUC = 0·94, $p = 0$, by permutation test with 10,000 iterations). The prediction with Random Forest model indicated that half of the children who were treated with azithromycin at their first participation did not recover within this time interval.

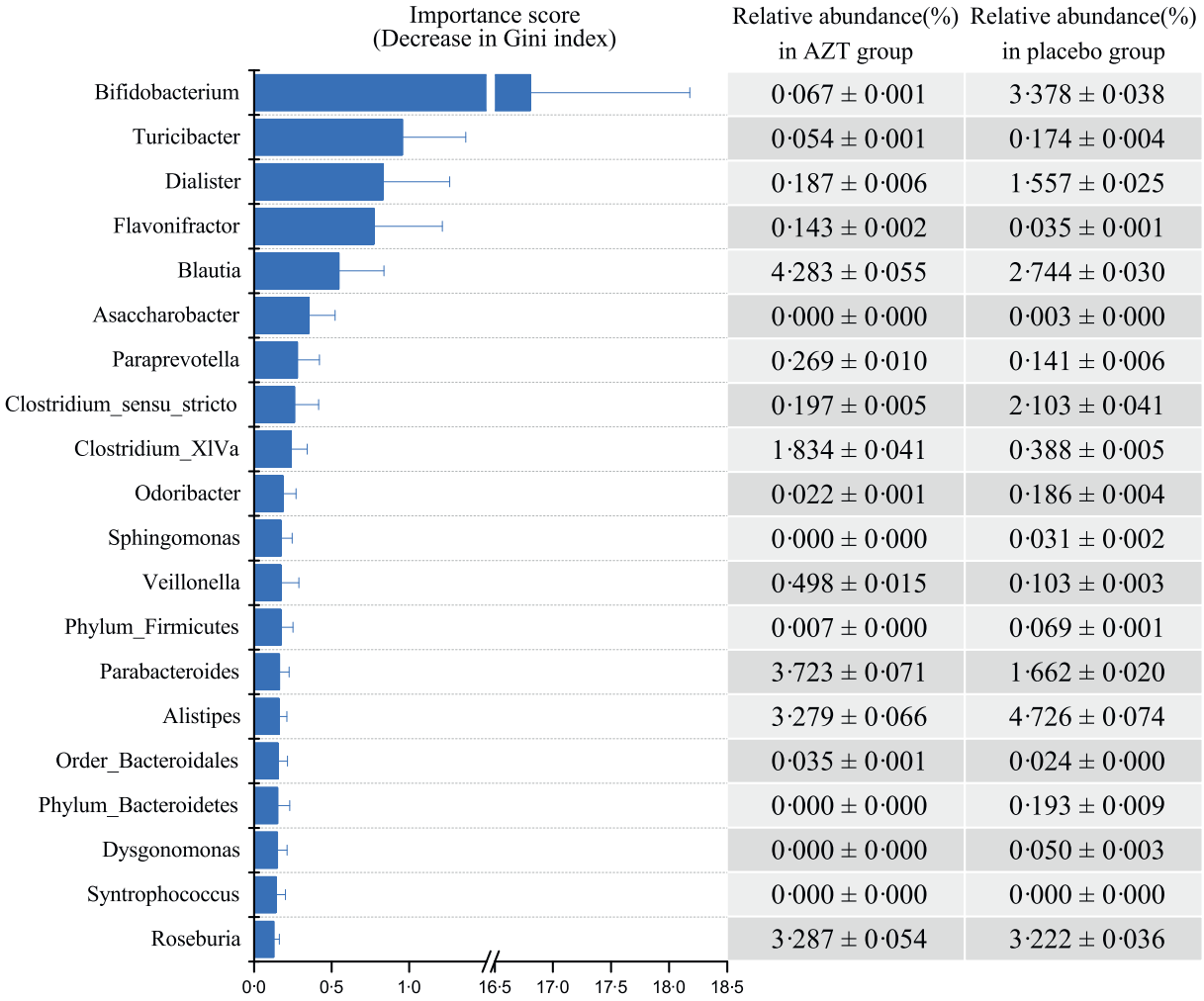

Fig. 3. Short-term effect: The top 20 taxa with the highest importance score (Gini index) by the Random Forest algorithm for distinguishing treatment groups and their corresponding relative abundances.

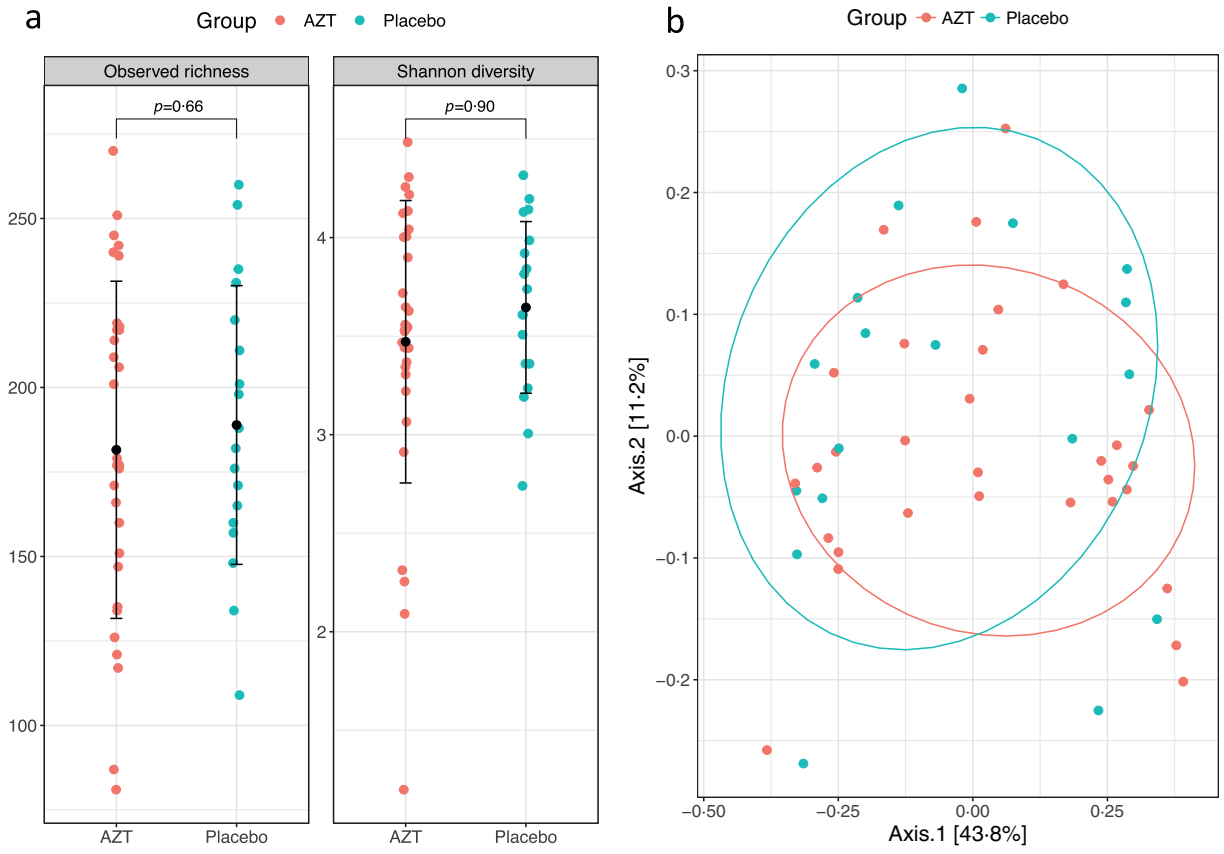

Fig. 4. Long-term effect: Alpha and beta diversity between groups. (a) Dotplot showing the distribution of observed richness and Shannon diversity for the AZT (red) and placebo (blue) groups. Black dots indicate the mean; error bars indicate the standard deviation. (b) Weighted UniFrac distances between AZT (red) and placebo (blue) groups, visualized by principal coordinates analysis (PCoA), with ellipses encircling 75% of samples from each group.

3.5. Long-term: recovery of gut microbiota at 4 years of age

To assess the long-term impact of azithromycin, we investigated the 49 fecal samples collected from AZT ($N = 31$) and placebo ($N = 18$) groups when children were 4 years old. We did not observe any significant differences in alpha diversity (mean of observed richness, 181·5 ± 49·9 vs. 188·9 ± 41·2 for AZT and placebo, respectively, $p = 0·66$; mean of Shannon, 3·47 ± 0·72 vs. 3·64 ± 0·44 for AZT and placebo, respectively, $p = 0·90$; Fig. 4a) or beta diversity between groups (weighted Unifrac, $R^2 = 2·0\%$, $p = 0·37$; Fig. 4b). Furthermore, we did not observe any OTUs differ significantly in relative abundance between groups. Next, the RF-2 model was applied to assess the recovery of these children based on the gut microbiota. Of the 31 children in AZT group, 26 were identified as placebo, resulting in an AUC of 0·69 ($p = 0·013$, by permutation test with 10,000 iterations). To further validate the result, we built a third Random Forest model for 4-year samples at the OTU level (RF-3 model) to differentiate the treatment groups. The RF-3 model produced an AUC of 0·56 ($p = 0·24$, by permutation test with 10,000 iterations).

4. Discussion

Azithromycin had a strong effect on the composition of gut microbiota 14 days post-treatment, but these effects did not persist to 4 years of age (13–39 months after the last treatment) in our DB-RCT of azithromycin in young children [11]. Current guidelines discourage the use of antibiotics during asthma-like episodes in early life due to lack of evidence of severe bacterial infections as main episode triggers [3], and adverse effects on the colonizing microbiota [43,44]. Recent evidence showed that bacteria are important triggers for asthmatic episodes [45], and that azithromycin reduced the duration of symptoms by half [11].

In the present study, the 3-day course of azithromycin resulted in a perturbation of the gut microbiota 14 days after randomization. Alpha diversity was significantly reduced and the microbiota composition was shifted. However, long-lasting impact of azithromycin on the gut microbiota composition was not observed.

In our study, 14 days after randomization, children in the AZT group had 23% lower richness and 13% lower Shannon diversity in their fecal samples compared to the placebo group. In particular, the relative abundance of *Actinobacteria* was reduced. Based on the taxonomic composition, the Random Forest model identified study arms with high accuracy, the genus *Bifidobacterium* was the most important contributor.

We observed increasing richness and Shannon diversity with age of the child, which represented an ongoing maturation of the gut microbiota. Of interest, the later the azithromycin prescribed to children, the smaller the difference in alpha diversity seemed between two treatment groups. This decreasing discrepancy may be attributed to early antibiotic administration having stronger microbiota perturbing effects in younger children where the microbiota is still developing [22] compared to the older children, who may recover faster because of a more mature baseline composition. However, this study did not provide sufficient statistical power to confirm a significant interaction between age and treatment, therefore further investigation is needed.

Long-term effects of azithromycin treatment were not observed. At 4 years of age (13–39 months after the last treatment), we could not distinguish children according to AZT or placebo group based on alpha

diversity, beta diversity, discriminant OTUs or by Random Forest models. The full recovery of children's gut microbiota in AZT group indicated that azithromycin treatment did not induce long-term compositional perturbations.

Our results are at odds with an observational study of children on the influence of macrolides on gut microbiota [34]. They observed lower richness for subjects who were exposed to macrolides within the preceding 2 years compared to the control group. The discrepancy may derive from some differences existing between our data and that of Korpela et al. The recovery time (13–39 months) of our subjects is longer compared to theirs (12–24 months); the age (median 2·0 years [IQR 1·0]) of our subjects is younger compared to theirs (median 5 years). Furthermore, observational studies may always have additional confounding factors, which drive both antibiotic use and microbial differences.

Bifidobacterium, the dominant genus in *Actinobacteria*, was one of the most affected genera by azithromycin treatment and had an exceedingly high importance score determined by Random Forest model. The relative abundance of *Bifidobacterium* in the AZT group was 50-fold lower compared to the placebo group and in many cases they were too low to be detected. *Bifidobacterium* has been shown to be one of the most affected genera by clarithromycin and metronidazole in the gut [31]. Most of the *Bifidobacterium* spp. strains are likely susceptible to macrolides and other antibiotics [46]. Similar results were observed in Korpela's study where the abundance of *Bifidobacterium* was reduced around 4-fold when a participant was treated with macrolides during the preceding 6 months. However, two recent DB-RCTs found no difference in *Bifidobacterium* abundance between groups [35,36]. These discrepancies may derive from the different characteristics of study population, since Parker's and Doan's populations were from south India and Niger, respectively, compared to our cohort from Denmark.

Our results revealed that azithromycin treatment for asthma-like symptoms in childhood led to a transient perturbation of the gut microbiota composition ($N = 59$, 12–36 months of age); however, long-term impact of azithromycin regarding such perturbations was not observed ($N = 49$, 4 years of age). Our study may alleviate concerns about adverse effects of azithromycin use in young children. Furthermore, considering the strength of DB-RCT and azithromycin likely being the main source of disturbance on gut microbiota, we speculate that our findings may also extend to non-asthmatic children (12 to 36 months of age) who have been prescribed azithromycin.

However, limitations should also be acknowledged. The children may have received antibiotics for other reasons during these first 4 years of life, but that would work against the null hypothesis. Even though we had 116 samples from the 12–36 months period, in order to avoid within-child correlations, only 59 samples from the first randomization were used for short-term analysis. Therefore, we may have low statistical power to distinguish the differences between treatment groups. A similar issue for the 4-year samples was that most children had been randomized to azithromycin at one point during the trial period, reducing the size of the placebo group compared to the AZT group. In addition, exclusions and loss to follow up also resulted in the reduction of sample size. Furthermore, since we did not collect baseline samples before randomization, we could only assess the alteration of gut microbiota at the group level instead of tracking individual child before and after treatment. For the recovery assessment of the gut microbiota at the second participation, we were limited by both a small sample size and variable time intervals between participations. Another limitation was the resolution of 16S rRNA gene sequencing techniques and perturbation caused by azithromycin at the gene level, such as antimicrobial resistance, could not be evaluated. Most OTUs were classified to genus level, but for some OTUs the resolution was insufficient for such classification, therefore the unclassified taxa might introduce bias for statistical analysis.

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The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

HB reports personal fees from Chiesi Pharmaceuticals, outside the submitted work. All other authors have nothing to disclose.

Author contributions

HB designed and carried out the study. SJS supervised the data acquirement. SW, MSM, ADB, JT, and MAR contributed to the statistical analysis. SW, MSM, JS, SJS contributed to the concept and interpretation of the data. SW drafted the manuscript, UT contributed to the writing and preparation. All authors made a substantial contribution in the revision of the manuscript.

Availability of data and material

The datasets analyzed and/or used in the present study are available from the corresponding author upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.11.035.

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