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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Gut microbiota in wheezing preschool children and the association with childhood asthma

To the Editor,

Reliable biomarkers to predict asthma in wheezing preschool children are lacking. Recently, the impact of gut microbial perturbations on the development of asthma gained widespread attention. Gut microbial dysbiosis in the first year of life was associated with asthma in multiple birth cohort studies.[1-5] Microbial metabolites might play a crucial role in maintaining an adequate immune balance and preventing asthma through its influence on regulatory T-cells (Tregs) and the *Foxp3* gene.[1,6] However, most data are derived from animal studies, whereas most human studies have focused on the association between infant gut microbiota and asthma-like symptoms at an age when a reliable diagnosis of asthma cannot yet be made. Furthermore, no studies have been performed that investigated gut microbial composition in wheezing children, and its association with subsequent development of asthma.

In the Asthma DEtection and Monitoring (ADEM) study (clinicaltrial.gov: NCT 00422747), 202 wheezing children and 50 healthy controls aged 2-4 years were prospectively followed until 6 years of age, when a definitive diagnosis of asthma was made. The study was approved by the Dutch national medical ethical committee, and written informed consent was given by all parents. A detailed study protocol was previously published.[7,8]

At inclusion, faecal and blood samples were collected. Faecal microbial composition was analysed by sequencing of the 16S rRNA V3-V4 gene region. In total, 230 samples (70 true asthmatics, 114 transient wheezers and 46 healthy controls) were successfully analysed (see flow chart, Figure S1). In blood, atopic sensitisation (Phadiatop Infant test), proportion of Tregs by flow cytometry ($CD4^+CD25^{high}CD127^-$), and *Foxp3* gene expression were assessed. See Appendix S1 for a detailed description of study methods. The baseline characteristics are displayed in Table S1.

First, we examined whether microbial richness and diversity at preschool age were predictive for future asthma development. Neither the microbial richness (OR 0.99 [95%CI 0.98-1.01]; P = .46), nor the microbial diversity as assessed by the Shannon index (OR 1.01 [0.98-1.04]; P = .53) were significantly different between transient wheezing

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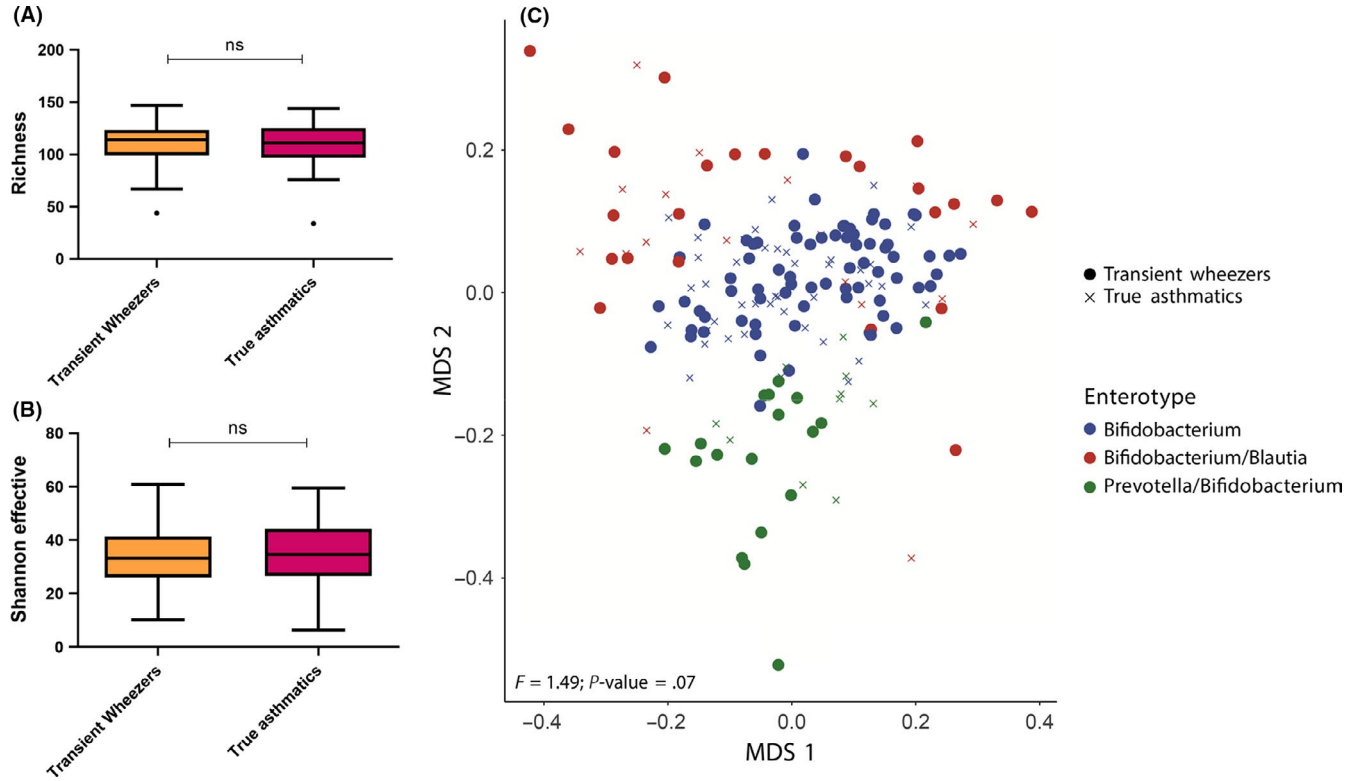

FIGURE 1 Microbial richness, diversity and community structure among preschool wheezing children who did (true asthmatics) or did not (transient wheezers) subsequently develop asthma. Microbial richness (observed species) (A) and diversity (Shannon effector index) (B) are not significantly different between transient wheezers and true asthmatics (ns: nonsignificant; Kruskal-Wallis). C, Multidimensional scaling (MDS) based on Bray-Curtis dissimilarity indicates three different enterotypes driven by Bifidobacterium, Bifidobacterium/Blautia and Prevotella/Bifidobacterium. Overall, microbial community structure is not statistically significantly different between transient wheezers and true asthmatics (permutational analysis of variance [PERMANOVA])

children and true asthmatics while adjusting for potential confounders (sex, breastfeeding, birth season, atopy parents, siblings, parental smoking status, day care attendance; Figure 1A-B). At preschool age, these indices were also not different between wheezers and healthy controls, while adjusting for potential confounders (Figure S2).

Next, we examined the overall microbial community structure (as assessed by the Bray-Curtis dissimilarity), which was neither significantly different between transient wheezers and true asthmatics (PERMANOVA P = .07, Figure 1C), nor between preschool wheezers and healthy controls (PERMANOVA P = .22, Figure S2). Microbial profiles of all children were clustered using Dirichlet Multinomial Mixture (DMM) modelling. Three distinct clusters (enterotypes) were identified, that is those that were driven by a relatively high abundance of *Bifidobacterium*, *Bifidobacterium* combined with *Blautia*, and *Prevotella* combined with *Bifidobacterium*, respectively. Neither the amount of wheezing children who developed asthma, nor the proportion of children with preschool wheeze were significantly different among the three enterotypes, while adjusting for multiple confounders in multivariable logistic regression analyses (Table S2 and S3). Altogether these results indicate that microbial diversity and overall microbial community structure are not predictive for subsequent asthma development among preschool wheezing children.

Furthermore, we examined whether the relative abundance of specific bacterial genera was predictive for future asthma development. Using multivariable logistic regression, we found that the relative abundance of the genera *Gemmiger* (P = .03) and *Escherichia* (P = .02) was significantly higher in wheezing children who developed asthma at age 6 years (Figure 2A-B). The risk of developing asthma was highest in those children who harboured the highest relative abundance of these two bacterial genera (Figure 2C-D). In particular, a high relative abundance of *Escherichia* was associated with 4.6-fold increased odds of asthma (P = .02, Figure 2D). When comparing preschool wheezers with healthy controls, the relative abundance of *Collinsella* (P = .01) and *Dorea* (P = .02) was significantly lower in wheezing children (Figure S3).

Finally, we examined whether gut microbial profiles were related to atopic sensitisation, Tregs and *Foxp3* gene expression. Besides a weak, yet statistically significant, positive correlation between *Foxp3* gene expression and bacterial diversity (Shannon index) within the entire study population (Spearman's rho = 0.16; P = .02), atopic sensitisation, Tregs and *Foxp3* gene expression were neither associated with the overall microbial community structure (Bray-Curtis dissimilarity) nor with the abundance of specific bacterial genera.

To our knowledge, this is the first study to examine the gut microbiota in wheezing preschool children and its association with asthma progression. Our findings suggest that at age 2-4 years, the microbiota perturbations associated with asthma might only be modest. This is in line with the proposed early window-of-opportunity, the first months of life, during which the microbiome is thought to have its strongest

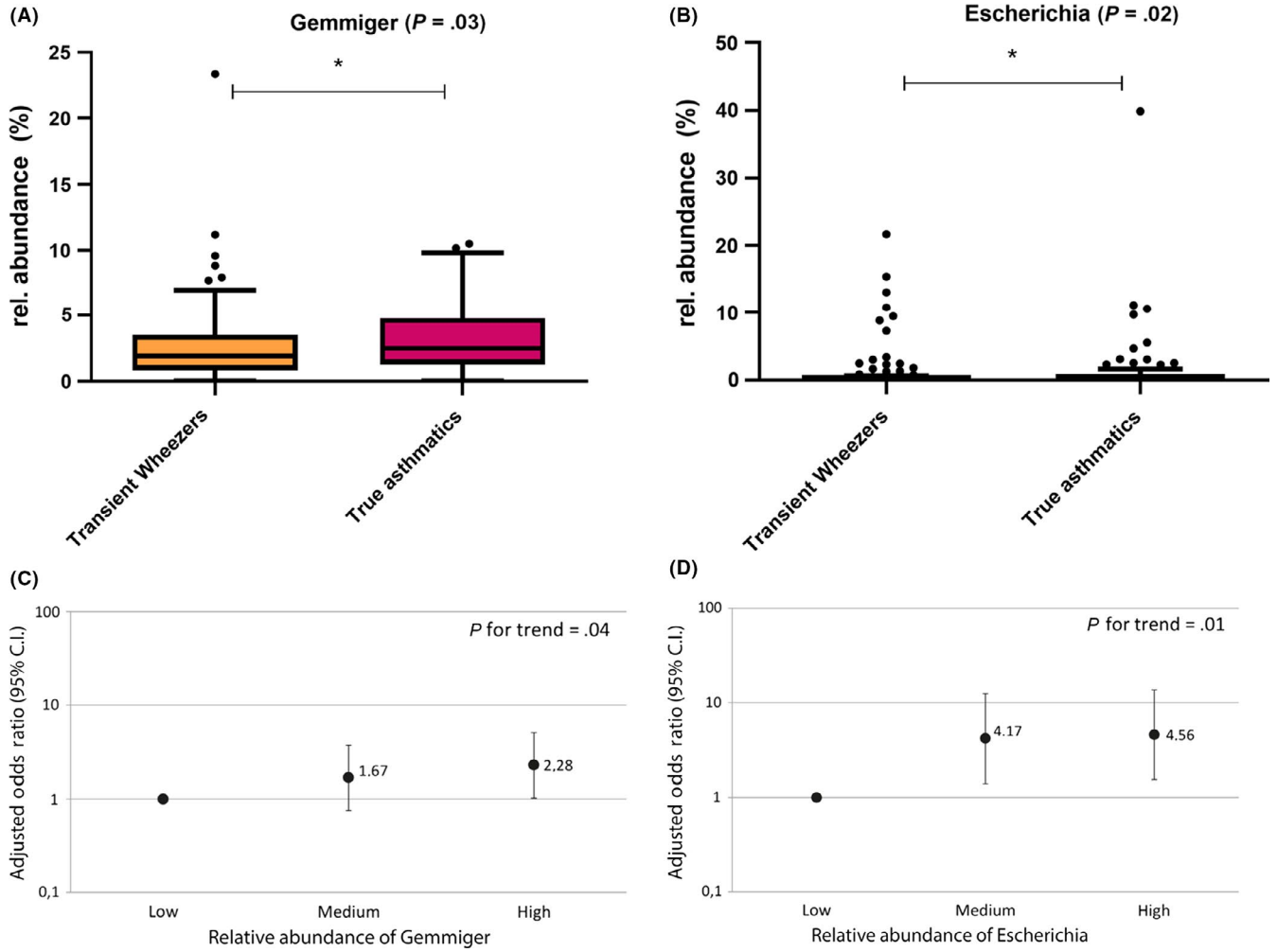

FIGURE 2 The relative abundance of specific microbial taxa increases the risk of subsequent asthma. The relative abundance of bacterial genera *Gemmiger* (*P* = .03) and *Escherichia* (*P* = .02) is higher in true asthmatic children compared with transient wheezers (A, B). Multiple logistic regression analyses show that the higher risk to develop asthma among children with a high abundance of *Gemmiger* and *Escherichia* remained statistically significant upon adjustment for sex, breastfeeding, birth season, atopy parents, siblings, parental smoking status and day care attendance (C, D)

impact on immune maturation and tolerance development and stabilises beyond infancy.[1,9] However, this early time-window might not be a suitable age to identify biomarkers for asthma prediction as early asthma symptoms may not have occurred yet. Moreover, the bacterial genera *Gemmiger* and in particular *Escherichia* were significantly associated with asthma, suggesting that some microbial dysbiosis might still exist at preschool age among wheezing children prone for developing asthma. In a recent paediatric study, *Escherichia* was one of three significantly predominant genera in children with asthma compared with healthy controls.[10] Furthermore, in a recent adult study, *Escherichia* was one of two genera that discriminated asthmatics with fixed airway obstruction from those with no airway obstruction.[11] These results are in line with our findings, potentially indicating that the abundance of *Escherichia* in particular may play a role in the early development of asthma. It has been suggested that an increased abundance of aerotolerant bacteria might be a nonspecific response to inflammatory conditions.[12] Recently, an increase in *Escherichia* appeared to reduce butyrate production, which was associated with asthma.[10] Potentially, *Escherichia* abundance has a wider influence on short-chain fatty acids production, thereby causing an immunological dysbalance leading to a Th2-response.

The strength of our study is that we assessed the gut microbial profiles of a large group of preschool wheezing children, using modern sequencing techniques. Another strength is a reliable asthma diagnosis at age 6 years based on symptoms, medication use and lung function measurements.[8]

The current study has several limitations. First of all, microbiome data are high dimensional, and it cannot be excluded that the observed associations are the result of multiple comparisons. Replication in future studies is therefore needed. Additionally, albeit being the first study to examine the gut microbiota in wheezing preschool children and its association with asthma progression, the sample size limits the power of stratified analyses. The number of sensitised preschool children might for example have been too low to detect significant associations when stratifying for atopic and nonatopic asthma. It is also plausible that microbial perturbations

especially impact the risk of asthma in children with certain genetic asthma risk loci, which requires stratified analyses for genotype. Finally, the number of children with eczema was substantial in our population, which might have been accompanied by dietary adaptations. Unfortunately, we had no additional information on the children's diets or restrictions.

In conclusion, gut microbial diversity and overall gut microbial community structure at age 2-4 years were not associated with preschool wheezing or future asthma development at age 6. When compared to microbial perturbations during infancy, microbial perturbations at preschool age appear to be only modestly associated with asthma. On a genus level, some bacterial genera were associated with wheezing (*Collinsella* and *Dorea)* or subsequent development of asthma *(Gemmiger* and *Escherichia)*, suggesting some microbial dysbiosis in children prone for developing asthma. The role of these genera in the development of asthma warrants further investigation.

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

MB and N.v.B. drafted the manuscript; N.v.B., LB and JP were responsible for gut microbial analysis; MB, N.v.B., LB, K.v.d.K, QJ, ED and JP contributed to the design of the study, data collection and interpretation of data. All authors read, revised and approved the final manuscript.

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