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Sex differences in the association between long-term ambient particulate air pollution and the intestinal microbiome composition of children

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ABSTRACT

The intestinal microbiome is essential for gastrointestinal and overall health, yet its response to air pollution in children remains underexplored. In a study involving 412 young children from the ENVIRONAGE cohort, stool samples were analysed via Illumina Miseq sequencing to assess microbiome alpha diversity (observed richness, species evenness, and Shannon diversity) and composition. Exposure to previous year particulate air pollution (black carbon, $PM_{2.5}$, coarse PM, and PM_{10}) was modeled using high-resolution spatial–temporal interpolation models. Multiple linear regression models were adjusted for a priori selected covariables and stratified by sex. Furthermore, we performed a differential relative abundance analysis at family and genus level, while accounting for the same covariables. Statistically significant effect modification by sex was apparent for several intestinal alpha diversity indices and air pollutants. In boys, we observed negative associations between particulate air pollution exposure and intestinal microbiome richness (estimates ranging from −5.55 to −9.06 per interquartile range (IQR) increase in particulate air pollution exposure) and Shannon diversity (estimates ranging from −0.058 to −0.095 per IQR increase). Differently, in girls non-significant positive associations were observed with species evenness (estimates ranging from 0.019 to 0.020 per IQR increase) and Shannon diversity (estimate 0.065 per IQR increase in black carbon). After multiple testing correction, we reported several bacterial families and genera (*Streptococcaceae*, *Clostridiales Incertae Sedis XIII*, *Coriobacteriaceae*, *Streptococcus*, and *Paraprevotella*) to be oppositely associated with particulate air pollution exposure in boys and girls. Our findings show a sex-dependent association between particulate air pollution exposure and intestinal microbiome composition, highlighting boys as potentially more vulnerable to diversity loss associated with childhood exposure to particulate pollution.

1. Introduction

From birth onwards, a symbiotic relationship develops between the intestinal microbiome and human cells, (Barker-Tejeda, 2024) maturing to an adult-like, more or less stable stage at the age of three. (Voreades et al., 2014) As the intestinal microbiome coevolves with its human host, it is of paramount importance for metabolism, gut integrity and permeability, immunity, and overall health. (Fusco, 2023; Finegold, 1969) Imbalance in its composition, known as intestinal dysbiosis, has been associated with short- and long-term health consequences, such as obesity, (DeGruttola et al., 2016) inflammatory bowel disease, (DeGruttola et al., 2016) hypertension, (Yang, 2015) and cognitive decline, potentially via the gut-brain axis. (Zare Sakhvidi, 2022) Early life changes in the intestinal microbiome might have long-term health

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consequences, (Sarkar et al., 2021) making this period crucial for intestinal microbiome development. Despite its importance, most microbiome research to date is focused on adult populations. (Derrien et al., 2019).

Factors, such as sex, (Kim et al., 2020) antibiotic use, (Lathakumari et al., 2024) and diet, (Rinninella, 2023) are known to influence the intestinal microbiome composition. However, they only account for a small portion of the inter-individual variation in intestinal microbiome composition, suggesting that other factors, such as environmental influences, may also contribute. (Falony, 2016) A systematic review of 12 studies in humans linked particulate air pollution exposure in general to reduced gut bacterial alpha diversity and altered specific bacterial taxa, (Van Pee et al., 2023) even though both positive (Gan, 2022) and negative (Liu, 2019) associations with alpha diversity were found. It is important to investigate the relationship between ambient air pollution exposure and the intestinal microbiome composition in children as they may be more vulnerable due to their developmental stage, (Capitani et al., 2023) higher breathing rate, (Fleming, 2011) and proximity to ground-level pollutants. (Wu et al., 2022) Nevertheless, the number of studies in children is low (Van Pee, 2023; Bailey, 2022; Kim, 2022; Zheng, 2020; Qiu, 2024) and mainly include children with a chronic disorder such as asthma (Zheng, 2020) or autism traits, (Kim, 2022) which limits an effective translation of the findings to the general population. Therefore, it is important that large-scale studies are performed in healthy children. Moreover, increasing evidence suggests that boys and men may be more sensitive to the effects of air pollution exposure (Shin et al., 2021; Li, 2022) and that their intestinal microbiome is more susceptible to external influences. (Cuevas-Sierra et al., 2021) Therefore, it is essential to investigate whether air pollution exposure has a sex-specific impact on the intestinal microbiome in children. Air pollution particulates might alter the intestinal microbiome via multiple mechanisms, including the direct translocation of inhaled and ingested particles to the intestines (Van Pee, 2024) or indirect via the induction of systemic inflammation. (Pope, 2016).

This study analyzed data from 412 four-to-twelve-year-old children enrolled in the ENVIRonmental influence ON early aging (ENVIR*ON*AGE) mother–child cohort (Janssen, 2017) to examine the association between previous year exposure to modeled black carbon, particulate matter with an aerodynamic diameter $\leq$ 2.5 μm ($PM_{2.5}$), particulate matter with an aerodynamic diameter $>$ 2.5 μm and $\leq$ 10 μm (coarse PM), and particulate matter with an aerodynamic diameter $\leq$ 10 μm (PM_{10}) and the gut microbiome composition, taking into account multiple covariables.

2. Material and Methods

2.1. Study population and sample collection

The ongoing ENVIR*ON*AGE cohort study, including over 2200 mother–child pairs, recruits the mother–child pairs at East-Limburg Hospital (ZOL; Genk, Belgium), and follows them longitudinally. (Janssen, 2017) The cohort is approved by the Ethical Committees of Hasselt University and East-Limburg Hospital (EudraCT B37120107805) and adheres to the Helsinki Declaration. During their stay at the maternity ward, mothers provide detailed socio-demographic information by completing questionnaires (*e.g.,* ethnicity, parity, and maternal education). Additional information is obtained through medical records (*e.g.,* newborn sex and delivery date). Covariates are classified in the Supplemental Methods. Follow-up examinations are performed when the child is between four and six or nine and eleven years old. Both age groups are included in the present study. These visits include clinical measurements (*e.g.,* height and weight measurements, out of which the body mass index (BMI) is calculated), biological sample collection, and a general (*e.g.,* breastfeeding and school address) and medical questionnaire (*e.g.,* antibiotic and probiotic use). Additionally, the mother completes a semi-quantitative food frequency questionnaire detailing the child's average monthly dietary intake. Based on this questionnaire, the NOVA food score (Monteiro et al., 2010) (energy intake (kcal) from ultra-processed food/day) was calculated as ultra-processed food intake has been associated with changes in the intestinal microbiome (Cuevas-Sierra et al., 2021) and metabolome. (Handakas, 2022) Additionally, whole grains intake was calculated (see Supplemental Methods and Table S1) and the frequency of consuming fruit and vegetables per week ($\leq$2 days/week, 3–4 days/week, 5–6 days/week, 1 time/day, $>$1 time/day) was extracted. Mothers also collected one stool sample of their child in a stool sample kit at home after the follow-up visit. The stool sample was stored in the home freezer (−18 °C on average) and collected by the study team on dry ice within the time frame of two weeks and stored at −80 °C until further analyses. Mothers also filled in the Bristol stool scale (Chumpitazi, 2016) (as a proxy of intestinal transit time) and reported the time between the current stool sample and the previous stool sample (referred to as 'time since last stool'). Written informed consent was obtained from the participating mothers at the maternity ward and again in the follow-up phases. The research reported on here is part of a bigger exposome framework, the Flexigut project. (Pero-Gascon, 2022).

2.2. Stool microbiome 16S rRNA gene sequencing and processing

As outlined before, fecal DNA was extracted from 427 frozen stool samples. (Samaey, 2024) In short, DNA was extracted from 150 to 200 mg frozen stool using the MagAttract PowerMicrobiome DNA/RNA KF kit (Qiagen) in accordance with the manufacturer's instructions. The V4 hypervariable region of the 16S rRNA gene was amplified using 515F/806R primers. The resulting amplicons were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced on the Illumina Miseq platform with the sequencing kit Miseq v2 to produce 250 bp paired-end reads. Sequencing data was analyzed using the DADA2 pipeline (Callahan, 2016), as explained elsewhere. (Samaey, 2024) Briefly, the first 30 bp were excluded from each read, and the sequence length was adjusted to 130 bp for the forward read, and 200 bp for the reverse read. The sequence error rate, dereplications, sample composition inference, and chimera removal were carried out with the default parameters of the DADA2 package. With the DADA2 RDP implementation (R packages "dada2" function "assignTaxonomy"), taxonomy was assigned for each read using the rdp_train_set_16 (https://zenodo.org/record/801828#.Xe-PctF7mQc) as reference input. Following this, the GTDB_bac120_arc122_ssu_r202_Species trainset (R packages "dada2" function "addSpecies") was used for the amplicon sequence variant (ASV) annotation. The resulting amplicon sequence variants (ASVs; in total 7971 ASVs) and taxonomy tables were combined with the metadata file into a phyloseq object (Phyloseq, version 1.26.1). (McMurdie and Holmes, 2013) Contaminant ASVs (n = 26 ASVs) were removed using the frequency (threshold is 0.1) method from the package Decontam (version 1.2.1). (Davis et al., 2018) Next, ASVs that were present in less than three samples and had less than five reads (n = 6484 ASVs) were omitted from the dataset. A total of 1461 ASVs remained after quality processing. Relative taxa abundances were computed at the family and genus level. Additionally, bacterial alpha diversity indices (observed richness, species evenness, and Shannon diversity) were calculated, for which samples were rarefied to the smallest number of reads present in a sample (*i.e.,* 10,533 reads). Fifteen children were omitted from the analyses due to missing covariable data, resulting in 412 children. The selection of ASVs and samples is depicted in Fig. S1.

2.3. Modeled air pollution exposure

Daily air pollutant concentrations at the child's residential and school address were obtained from the Belgian Interregional Environment Agency. In brief, a spatial–temporal interpolation method was used that considers land-cover data from satellite images (CORINE land-cover dataset) and pollution data from fixed monitoring stations

combined with a dispersion model that uses emissions from point and line sources. (Janssen, 2017) Hereby, daily exposure values can be calculated in high-resolution receptor grids (4x4 km^2). Overall model performance was evaluated by leave-one-out cross-validation, including 14 monitoring points for black carbon, 34 for $PM_{2.5}$, and 58 for PM_{10}, resulting in a spatiotemporally explained variance of 0.74 for black carbon and >0.80 for $PM_{2.5}$ and PM_{10}. (Janssen, 2017) Ambient air pollution exposure at the residence and school were combined based on the child's calculated proportion of time spent at school. The average black carbon, $PM_{2.5}$, and PM_{10} air pollution concentrations were calculated during the year preceding the stool sample collection while accounting for address changes. Coarse PM, defined as particles with diameters >2.5 μm and ≤10 μm, was calculated as the difference between the average PM_{10} and the average $PM_{2.5}$ concentration. The accuracy of the air pollution model was further supported by measured internal black carbon loads, as significant correlations were observed between previous year modeled black carbon exposure and the number of black carbon particles in children's urine ($r = 0.13$, $p = 0.03$). (Saenen, 2017) Furthermore, modeled black carbon exposure during the entire pregnancy correlated well with the number of black carbon particles in placental tissue ($r = 0.43$, $p = 0.06$) (Bove, 2019) and cord blood ($r = 0.50$, $p < 0.0001$). (Bongaerts, 2022).

2.4. Statistical analyses

All statistical analyses were performed using Rstudio (version 4.2.3; R Core Team). First, to visualize the impact of each covariable on the intestinal microbiome profile, a distance-based redundancy analysis was performed, using the RLdbRDA package developed in RaesLab (Leuven, Belgium). Principal Component Analyses were subsequently performed for the most important covariables. Linear regression models were used to assess individual associations between exposure to ambient particulate air pollution (black carbon, $PM_{2.5}$, coarse PM, and PM_{10}) during the previous year and intestinal microbiome alpha diversity (*i.e.,* observed richness, Shannon diversity, and species evenness), while adjusting for *a priori* selected covariables: the child's age (years), BMI (kg/m^2), parity (first, second, or third or more child), previous month antibiotic use (yes or no), season of stool sample collection (winter, spring, summer, or autumn), maternal education (low, middle, or high), Bristol stool scale (normal, diarrhea, or constipation), time since last stool (<12 h, 12–24 h, or >24 h), and the NOVA food score (kcal from ultra-processed food /day). As more and more research indicates sex differences in the intestinal microbiome composition, (Kim et al., 2020; Takagi, 2019) we tested whether there was effect modification by sex on the association between particulate air pollution exposure and the intestinal microbiome composition. As the interaction terms between sex and air pollution on the gut microbiome indices were statistically significant, we ran the analyses stratified by sex. Results were expressed as difference in intestinal microbiome alpha diversity index (with 95 % confidence interval (CI)) per interquartile range (IQR) increase in air pollutant. In sensitivity analyses, we assessed whether additionally adjusting for ethnicity (European or non-European), whole grains (% of the time), fruit and vegetable intake (frequency per week), and previous month probiotic use (yes or no), breast feeding (yes or no), or omitting children who took antibiotics in the previous month (n = 15) or children born via a cesarean section (n = 14) affected the observed associations. Additionally, we conducted sensitivity analyses assessing air pollution exposure over the previous month and exclusively at the home address. Lastly, raw bacterial family counts were used as input for the differential relative abundance analysis using the 'Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC2)' R package (version 2.0.2), which applies a log-transformation to the observed counts. We accounted for the covariables above. All options were kept as default, meaning that only bacterial families present in ≥10 % of all participants were included in the analyses. Adjustment for multiple testing was performed by restricting the false discovery rate (FDR) q-value to ≤0.05. Results were expressed as percentage difference in the relative abundance per IQR increase in air pollutant.

3. Results

3.1. Population characteristics

A total of 412 4-to-12-year-old children were enrolled in this study, out of which 190 (46 %) were boys with a mean ± standard deviation (sd) age of 7 ± 2 years and BMI of 17 ± 3 kg/m^3 (Table 1). Around half of the children were the firstborn (55 %), and the majority were of European ancestry (88 %), born via a natural delivery (97 %), and breastfed (77 %). Most children did not take antibiotics or probiotics in the month before the stool sample collection (96 % for both). As proxies for dietary intake, NOVA food score, whole grains intake, and frequency of eating fruit and vegetables were calculated. The average calculated NOVA food score was 560 ± 178 kcal from ultra-processed food/day. Furthermore, children ate whole grain variants when eating bread, crackers, cereals, cookies, pasta, or rice approximately 40 % of the time, and most consumed fruit (39 %) and vegetables (44 %) once a day. Approximately 50 % of the mothers had a high education degree. Stool samples were mainly collected in spring (33 %), followed by winter (26 %), summer (24 %), and autumn (17 %). Around 61 % of the children had a normal stool sample based on the Bristol stool scale, and 43 % collected the stool sample 12–24 h after the previous defecation. Table 1 depicts the population characteristics for the entire study population, and boys and girls separately. Table 2 shows the distribution of intestinal alpha diversity indices and particulate air pollution exposure combined at the residential and school addresses during the year prior to the collection of the stool sample. The distribution is shown for the entire study population, and boys and girls separately. The average exposure to black carbon, $PM_{2.5}$, coarse PM, and PM_{10} during the previous year were 0.62 $\mu g/m^3$, 10.79 $\mu g/m^3$, 8.33 $\mu g/m^3$, and 19.12 $\mu g/m^3$, respectively.

3.2. Gut microbiome composition of the study cohort

412 stool samples, with an average ± sd of 45,777 ± 15,071 reads and 158 ± 37 ASVs post-filtering, were included in the analyses. On average, each stool sample contained 158.31 species, with a species evenness of 1.71 and a Shannon diversity index of 3.74. Bacterial relative abundances were calculated at the family (56 families; Fig. 1A) and genus level (153 genera; Fig. 1B). The most abundant families were *Lachnospiraceae* (31 %), *Ruminococcaceae* (30 %), and *Bacteroidaceae* (15 %); the most abundant genera were *Faecalibacterium* (19 %), *Bacteroides* (15 %), and *Roseburia* (10 %). Fig. 2 shows the results of the distance-based redundancy analysis for boys (Fig. 2A) and girls (Fig. 2B). For boys, time since last stool ($p_{adj} = 0.02$) and previous year exposure to coarse PM ($p_{adj} = 0.04$) significantly influenced the intestinal microbiome profile. They had a cumulative effect size of around 1.3 %. Two factors that were borderline significant were the Bristol stool scale ($p_{adj} = 0.08$) and age ($p_{adj} = 0.08$). For girls, age at stool sample collection ($p_{adj} = 0.01$) and the Bristol stool scale ($p_{adj} = 0.02$) significantly influenced the intestinal microbiome profile. They had a cumulative effect size of around 1.6 %.

3.3. Particulate air pollution exposure and intestinal microbiome alpha diversity stratified by sex

As the multiplicative interaction terms between sex and air pollutants were statistically significant (Table S2), multivariable-adjusted linear regression models were stratified by sex. In boys, particulate air pollution exposure was negatively associated with observed richness and Shannon diversity (Fig. 3 and Table S2). For instance, each IQR increase in PM_{10} was associated with a 8.43 (95 % CI: −15.29 to −1.55; $p = 0.02$) lower observed richness and a 0.095 (95 % CI: −0.17 to −0.022; $p = 0.01$) lower Shannon diversity. Similar results were found

Table 1
Study population and stool sample characteristics.

Characteristics	All study participants (n = 412)	Boys (n = 190)	Girls (n = 222)	p-value boys-girls
CHILD				
Sex				
Boy	190 (46.12 %)			
Girl	222 (53.88 %)			
Age (years)	7.49 ± 2.34	7.48 ± 2.36	7.49 ± 2.32	0.91
BMI (kg/m^2)	16.67 ± 2.63	16.80 ± 2.58	16.49 ± 2.82	0.26
Ethnicity				
European	362 (87.86 %)	171 (90.00 %)	191 (86.04 %)	0.22
Non-European	50 (12.14 %)	19 (10.00 %)	31 (13.96 %)	
Previous month antibiotic use				
No	395 (95.87 %)	183 (96.32 %)	212 (95.50 %)	0.68
Yes	17 (4.13 %)	7 (3.68 %)	10 (4.50 %)	
Previous month probiotic use				
No	395 (95.87 %)	181 (95.26 %)	214 (96.40 %)	0.57
Yes	17 (4.13 %)	9 (4.74 %)	8 (3.60 %)	
NOVA food score (kcal from ultra-processed food/day)	560.01 ± 177.52	583.24 ± 184.91	540.12 ± 168.84	0.01
Whole grains intake (% of the time)	40.33 ± 27.85	41.97 ± 27.26	39.31 ± 28.95	0.35
Fruit intake				
≤2 days/week	24 (5.81 %)	12 (6.32 %)	12 (5.41 %)	0.84
3–4 days/week	52 (12.59 %)	24 (12.63 %)	28 (12.61 %)	
5–6 days/week	44 (10.65 %)	23 (12.11 %)	21 (9.46 %)	
1 time/day	161 (39.23 %)	73 (38.42 %)	88 (39.64 %)	
>1 time/day	131 (31.72 %)	58 (30.53 %)	73 (32.88 %)	
Vegetable intake				
≤2 days/week	25 (6.05 %)	16 (8.42 %)	9 (4.05 %)	0.25
3–4 days/week	54 (13.07 %)	24 (12.63 %)	30 (13.51 %)	
5–6 days/week	83 (20.10 %)	41 (21.58 %)	42 (18.92 %)	
1 time/day	181 (44.07 %)	88 (46.32 %)	93 (48.89 %)	
>1 time/day	69 (16.71 %)	21 (11.05 %)	48 (21.62 %)	
MATERNAL				
Parity				
First child	225 (54.61 %)	108 (56.84 %)	117 (52.70 %)	0.60
Second child	146 (35.44 %)	65 (34.21 %)	81 (36.49 %)	
Third or more child	41 (9.95 %)	17 (8.95 %)	24 (10.81 %)	
Partus				
Natural delivery	398 (96.61)	183 (96.32 %)	215 (96.85 %)	0.77
Cesarean section	14 (3.39)	7 (3.68 %)	7 (3.15 %)	
Breastfeeding				
Yes	317 (76.94 %)	144 (75.79 %)	173 (77.93 %)	0.61
No	95 (23.06 %)	46 (24.21 %)	49 (22.07 %)	
Maternal education				
Low	55 (13.35 %)	22 (11.58 %)	33 (14.86 %)	0.56
Middle	146 (35.44 %)	68 (35.79 %)	78 (35.14 %)	
High	211 (51.21 %)	100 (52.63 %)	111 (50.00 %)	
STOOL SAMPLES				
Season of stool sample				
Winter	106 (25.73 %)	45 (23.68 %)	61 (27.48 %)	0.20
Spring	137 (33.25 %)	65 (34.22 %)	72 (32.43 %)	
Summer	100 (24.27 %)	53 (27.89 %)	47 (21.17 %)	
Autumn	69 (16.75 %)	27 (14.21 %)	42 (18.92 %)	
Bristol stool scale				
Normal	251 (60.92 %)	125 (65.79 %)	126 (56.76 %)	0.08
Diarrhea	82 (19.90 %)	37 (19.47 %)	45 (20.27 %)	
Constipation	79 (19.17 %)	28 (14.74 %)	51 (22.97 %)	
Time since last stool				
<12 h	83 (20.15 %)	45 (23.68 %)	38 (17.12 %)	0.57
12–24 h	177 (42.96 %)	78 (41.05 %)	99 (44.59 %)	
>24 h	152 (36.89 %)	67 (35.26 %)	85 (38.29 %)	

Data is presented as mean ± standard deviation or total number (percentage). Ethnicity was based on the native country of the newborn's grandparents and classified as European when two or more grandparents were European or non-European when at least three grandparents were of non-European origin. Parity is categorized as mothers having their first, second, or third or more child. Maternal educational level was classified as "low" if the mother did not obtain a high school diploma, "middle" if the mother obtained a high school diploma, and "high" if the mother obtained a college or university degree. Time since last stool refers to the time between the current stool sample and the previous stool sample. BMI: body mass index.

Table 2
Descriptive statistics for particulate air pollution exposure during the year previous to stool sample collection and for intestinal microbiome alpha diversity indices.

	All children (n = 412)	Boys (n = 190)	Girls (n = 222)	p-value boys versus girls
	Mean ± sd	Mean ± sd	Mean ± sd	
Previous year particulate air pollution exposure				
Black carbon	0.62 ± 0.07	0.62 ± 0.06	0.62 ± 0.07	0.17
$PM_{2.5}$	10.79 ± 1.06	10.77 ± 1.04	10.84 ± 1.07	0.39
Coarse PM	8.33 ± 0.81	8.31 ± 0.79	8.34 ± 0.82	0.63
PM_{10}	19.12 ± 1.72	19.11 ± 1.66	19.18 ± 1.75	0.72
Intestinal microbiome alpha diversity				
Observed richness	158.31 ± 36.53	158.59 ± 36.28	157.23 ± 36.28	0.72
Species evenness	1.71 ± 0.11	1.71 ± 0.11	1.71 ± 0.11	0.65
Shannon diversity	3.74 ± 0.37	3.74 ± 0.37	3.74 ± 0.37	0.94

Results are shown for all study participants, and for boys and girls separately. Air pollution exposure is combined at the residential and school address. sd: standard deviation.

for black carbon, $PM_{2.5}$, and coarse PM. With regard to the analyses in girls, positive trends were found for species evenness (but not with coarse PM) (Fig. 3 and Table S2). For instance, each IQR increase in PM_{10} exposure was associated with a 0.019 (95 % CI: −0.003 to 0.040; p = 0.08) higher species evenness. Results for black carbon and $PM_{2.5}$ were comparable. Furthermore, each IQR increase in black carbon exposure was associated with a 0.065 (95 % CI: −0.002 to 0.13; p = 0.06) higher Shannon diversity. We showed that additionally adjusting for ethnicity, whole grains, fruit and vegetable intake, previous month probiotic use, breastfeeding, or assessing air pollution exposure over the previous month, or exclusively at the home address did not importantly change the effect estimates (Table S3). Similarly, results remained largely the same when children who took antibiotics in the previous month (n = 7 for boys and n = 8 for girls) or children born via a cesarean section (n = 7 for each sex) were excluded from the analysis (Table S3).

3.4. Particulate air pollution exposure and bacterial relative abundances

Raw bacterial family and genera counts were used as input in the ANCOM-BC2 R package to examine the association between previous year exposure to particulate air pollution and the relative abundance of bacterial taxa. Analyses were stratified by sex and corrected for multiple testing via FDR (Fig. 4 and Supplemental Figs. S2 and S3). For the analyses in boys, black carbon exposure was positively associated with the relative abundance of seven families (*Sutterellaceae*, *Streptococcaceae, Peptococcaceae* 1, *Bacteroidaceae, Bifidobacteriaceae*, *Enterobacteriaceae*, and *Eubacteriaceae*) and 12 genera (Streptococcus, *Anaerorhabdu*, *Eisenbergiella*, *Sutterella*, *Peptococcus*, *Bacteroides*, *Eggerthella*, *Bifidobacterium*, *Clostridium IV*, *Hungatella*, *Odoribacter*, *Butyricicoccus*, and *Flavonifractor*). Further, black carbon exposure was negatively associated with four families (*Coriobacteriaceae*, *Clostridiales Incertae Sedis XIII*, *Christensenellaceae*, and *Prevotellaceae*) and ten genera (*f_Clostridiales Incertae Sedis XIII g_NA*, *Sporobacter*, *Collinsella*, *Clostridium III*, *Barnesiella*, *Coprobacillus*, *Prevotella*, *Christensenella*, *Paraprevotella*, and *Anaerovorax*). In addition, $PM_{2.5}$ and PM_{10} exposure were both negatively associated with the relative abundance of *Victivallaceae*, *Rhodospirillaceae*, *Victivallis*, and *f_Rhodospirillaceae g_NA*. Additionally, PM_{10} exposure was negatively associated with *Methanobacteriaceae*, *Holdemanella*, *Parasutterella*, *Butyrivibrio*, and *Methanobrevibacter*, and

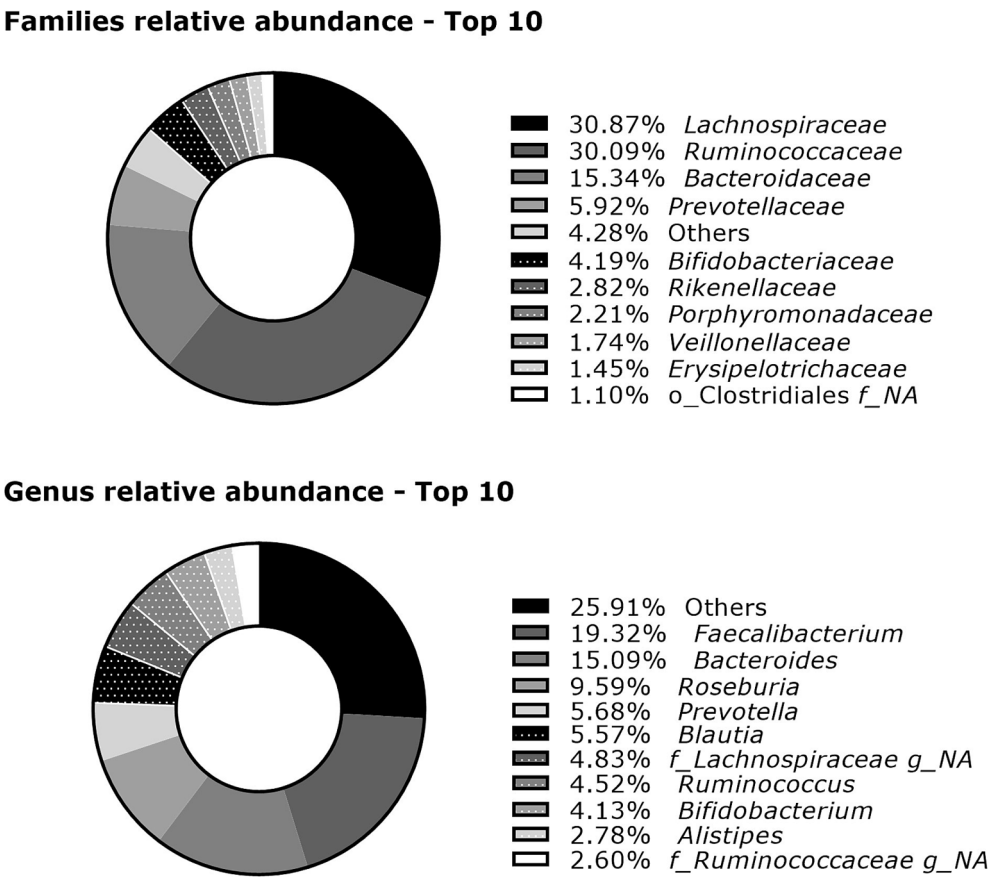

Fig. 1. Overview of the relative abundance (%) of the 10 most abundant bacterial (A) families and (B) genera in relation to all other taxa.

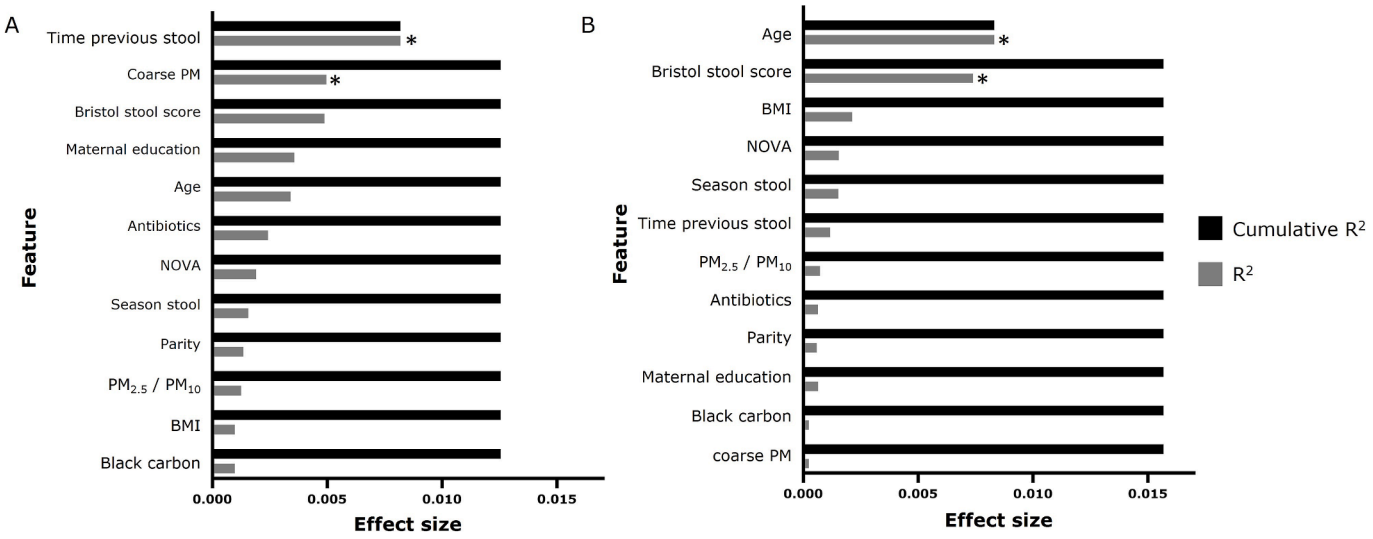

Fig. 2. Results of the distance-based redundancy analysis using the RLdbRDA package developed in RaesLab for (A) boys and (B) girls. BMI: body mass index. * indicate features that are statistically significantly associated with the intestinal microbiome profile.

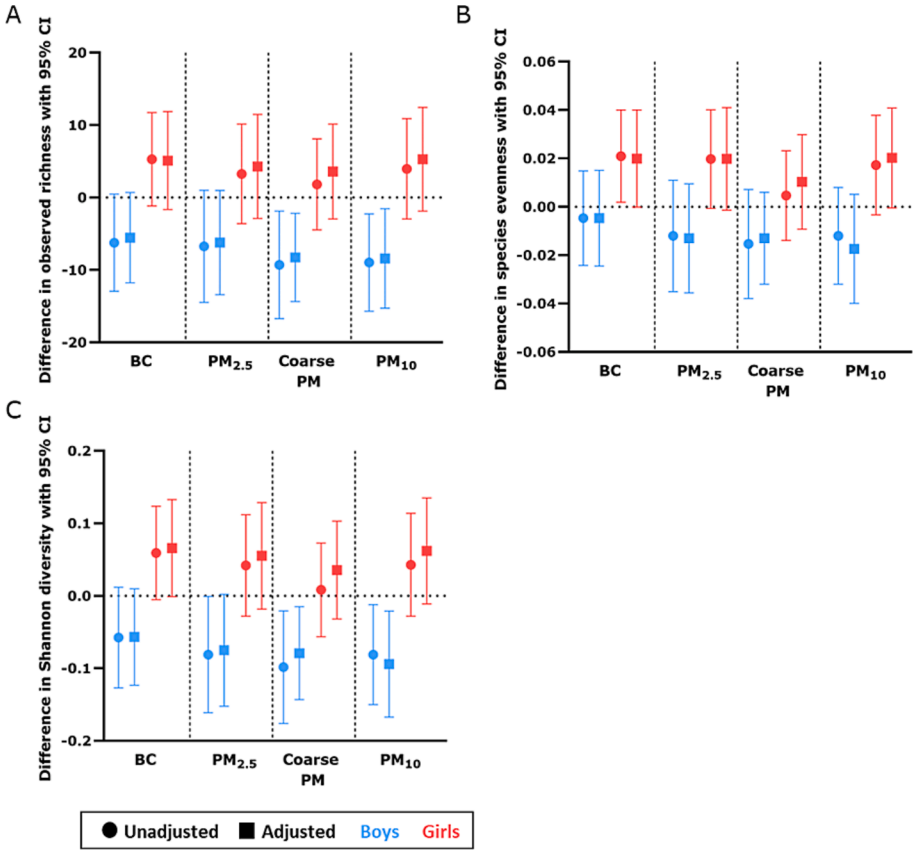

Fig. 3. Association between particulate air pollution exposure during the year previous to stool sample collection and (A) observed richness, (B) species evenness, and (C) Shannon diversity, stratified by sex. The studied air pollutants are black carbon (BC), particulate matter with an aerodynamic diameter ≤2.5 μm ($PM_{2.5}$), coarse PM, and particulate matter with an aerodynamic diameter ≤10 μm (PM_{10}). Results of unadjusted and adjusted models are shown. Multivariable-adjusted linear regression models were adjusted for the child's age, BMI, parity, previous month antibiotic use, season of stool sample, maternal education, Bristol stool scale, time since last stool, and NOVA food score. The estimates represent the difference in alpha diversity with a 95 % CI per IQR increase in air pollutant. BMI: body mass index; CI: confidence interval; IQR: interquartile range.

positively with *f_Erysipelotrichaceae g_NA, Flavonifractor*, and *Romboutsia*. Coarse PM was positively associated with *Flavonifractor* and *f_Erysipelotrichaceae g_NA*, but not with the relative abundance of any bacterial family.

In girls, black carbon exposure was positively associated with the relative abundance of six bacterial families (*Verrucomicrobiaceae*, *Rikenellaceae*, Desulfovibrionales *f_NA*, *Peptococcaceae 1*, *Clostridiales Incertae Sedis XIII*, and *Coriobacteriaceae*) and 20 genera (*Pseudoflavonifractor*,

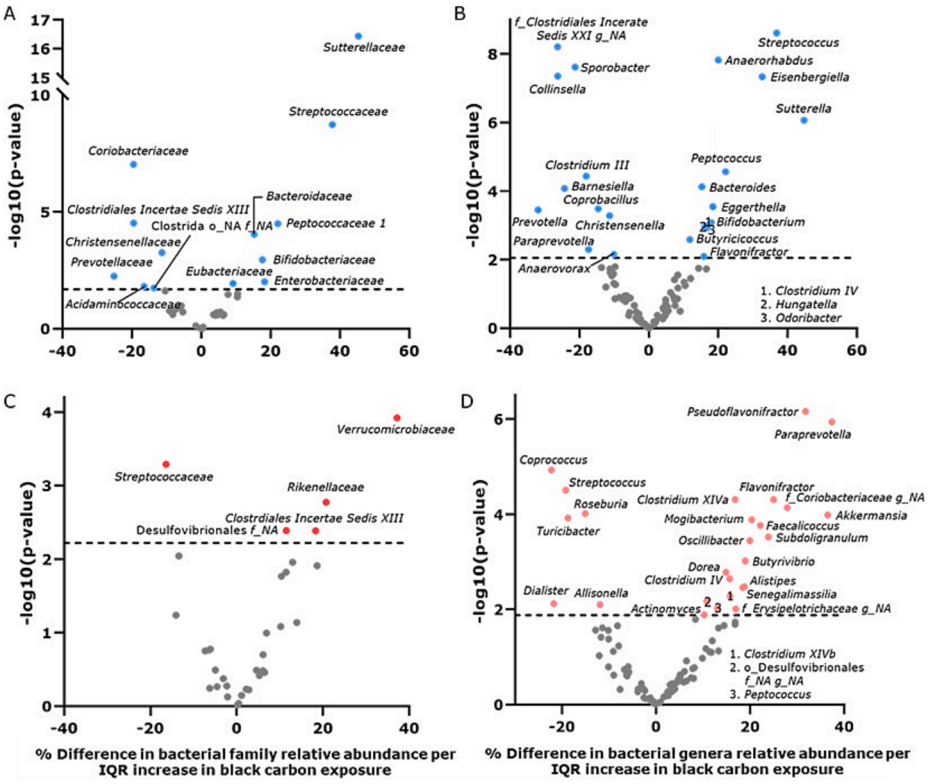

Fig. 4. Volcano plot of the associations between black carbon exposure and the relative abundance at bacterial family (A&C) and genera (B&D) level, stratified by sex. Multiple linear regression models were adjusted for the child's, age, BMI, parity, previous month antibiotic use, season of stool sample, maternal education, Bristol stool scale, time since last stool, and NOVA food score. Results are expressed as the difference in relative abundance (%) per IQR increase in black carbon. Results for boys and girls are indicated in blue and pink, respectively. $P_{adj} > 0.05$ are indicated in grey. $P_{adj} < 0.05$ are indicated in blue or pink. IQR: interquartile range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Paraprevotella, *Flavonifractor, Clostridium XIVa 16.80 % f_Coriobacteriaceae g_NA, Akkermansia, Mogibacterium, Faecalicoccus, Subdoligranulum, Oscillibacter, Butyrivibrio, Dorea, Clostridium IV, Alistipes, Senegalimassilia, Clostridium XIVb, o_Desulfovibrionales f_NA g_NA, Peptococcus, f_Erysipelotrichaceae g_NA*, and *Actinomyces*). Furthermore, black carbon exposure was negatively associated with two families (*Streptococcaceae* and *Pasteurellaceae*) and six genera (*Coprococcus*, *Streptococcus*, *Roseburia*, *Turicibacter*, *Dialister*, and *Allisonella*) in girls. Exposure to coarse PM was negatively associated with the relative abundance of *f_Bacteroidales g_NA*. No statistically significant associations were found with $PM_{2.5}$ and PM_{10} exposure. For details, see the Supplemental Results.

4. Discussion

This study reveals sex-specific associations between intestinal microbiome alpha diversity indices and long-term particulate air pollution exposure. Among boys, we found a negative association with intestinal microbiome alpha diversity, while in contrast, girls showed positive trends (although not statistically significant) with species evenness and Shannon diversity. These findings underscore the potential for air pollution to differentially impact the gut microbiome across sexes, highlighting boys as potentially more vulnerable to diversity loss due to particulate pollution exposure in childhood. Our findings were corroborated by multiple sensitivity analyses, showing the robustness of the findings. After correction for multiple testing, we reported several bacterial families and genera to be associated with particulate air pollution exposure, mainly with black carbon.

It is postulated that particulate ambient air pollution might impact the intestinal microbiome through different routes. First, particles (Schraufnagel, 2020) can be ingested via mucociliary clearance after deposition in the airways, subsequently reaching the intestines. (Uzeloto, 2021) Additionally, particles can accumulate in the intestinal lumen by ingesting particle-contaminated food and water. (Schraufnagel, 2020; Salim et al., 2014) Besides ingestion, small particles (<1 μm) can enter the lung alveoli, (Schraufnagel, 2020) cross the lung-blood barrier, (Schraufnagel, 2020; Chow, 2006) and translocate to distal body sites via the systemic circulation. Recently, air pollution-related particles were visualized in different intestinal tissue layers of human ileum and colon biopsies. (Van Pee, 2024) In the intestines, particles may cause inflammation, oxidative stress, or affect the intestinal barrier function, as demonstrated in several animal studies, possibly leading to alterations in the intestinal microbiome. For instance, Mutlu *et al.* (Mutlu, 2011) found that mice exposed to high levels of urban PM exhibited higher intestinal permeability, lower levels of the tight junction protein Zona Occuludens-1, higher levels of interleukin-6, and more intestinal cell apoptosis than control mice. Ambient air pollution exposure might also affect the intestinal microbiome through systemic pathways, such as systemic inflammation. (Araujo, 2010).

Till now, the majority of epidemiological studies, including our own study linking black carbon particles in biological fluids and tissues to the intestinal microbiome of four-to-six-year-old children, (Van Pee, 2023) reported negative associations between air pollution exposure and intestinal microbiome alpha diversity. (Liu, 2019; Van Pee, 2023; Sommer, 2022) Contradictory, one study in pregnant women found positive associations. (Gan, 2022) They reported that PM_{10} and $PM_{2.5}$ exposure during the entire pregnancy were associated with an higher observed richness, Chao1 richness, ACE richness, and Shannon diversity (the latter only for $PM_{2.5}$), based on the microbiome profile assessed during pregnancy. (Gan, 2022) However, the potential role of sex as an effect modifier on the microbiome has not yet been explored. Interestingly,

previous research on other health outcomes has suggested that susceptibility to air pollution may differ between boys/men and girls/women. Some studies suggest that men may experience more profound effects from air pollutants, such as decreased endothelial function. (Zhang, 2024) Furthermore, men were at higher risk of hospitalization than women following short-term $PM_{2.5}$ exposure (1.8 % versus 0.7 % per 10-unit increase in $PM_{2.5}$), (Shin et al., 2021) and pre-admission air pollution exposure correlated more strongly with hospitalization of male COVID patients than of female patients. (Vos, 2023) A study by Hoffmann *et al.* (Hoffmann, 2009) reported positive associations between long-term $PM_{2.5}$ exposure and two systemic inflammation markers (*i.e.,* high-sensitivity C-reactive protein and fibrinogen) in men, while no associations were found in women. In addition, a study on mice reported that *in utero* exposed male mice exhibited more severe intestinal alterations than female mice (*e.g.,* decreased villus and crypt length, and lower levels of tight junction protein ZO-1 in the ileum). (Guilloteau, 2022) Epidemiological research also showed sex-dependent associations between ambient air pollution exposure and gastrointestinal disorders. For instance, long-term exposure to PM_{10} and NO_2 was associated with an increased risk of Crohn's disease in men but not in women. (Li, 2022) One possible explanation for the sex-specific findings in our study is the differences in hormone levels between boys/men and girls/women. (Wang, 2022; Liao et al., 2023) Prepubertal girls already have higher levels of estradiol hormones than prepubertal boys. (Bay et al., 2004) For instance, estrogen levels were significantly lower in healthy American boys (9 years, 0.08 pg/mL) than in girls (8 years, 0.60 pg/mL). (Klein et al., 1994) An epidemiological study by Shin *et al.* (Shin, 2019) reported correlations between serum hormone levels and the relative abundance of specific intestinal bacterial genera. Especially estrogen hormones are known for their anti-inflammatory properties, as they, among others, can inhibit the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ). (Harding and Heaton, 2022; Kalaitzidis and Gilmore, 2005) Estrogen has been shown to inhibit NF-κB activity, which is an important immunomodulator involved in systemic inflammation regulation. (Xing, 2012; Sas, 2012) Systemic inflammation is known to affect the composition of the intestinal microbiome, although the relationship is potentially bidirectional. (Al Bander et al., 2020).

Our observed effect modification of sex between gut biodiversity and long-term air pollution exposure is unlikely to be explained by a difference in confounding structure, as only the NOVA food score and colonic transit time significantly differ between both sexes. The NOVA food score was notably lower in girls compared with boys, aligning with existing literature suggesting that girls and women typically consume more fruits, vegetables, and fibers, contributing to a healthier dietary profile. (Feraco, 2024) This strengthens the validity of our NOVA score as a relevant measure of dietary quality in this context. Furthermore, girls/women have a slower colonic transit time. (Degen and Phillips, 1996) This transit time is a plausible factor contributing to sex differences in inflammatory susceptibility mediated by the gut microbiome (*e.g.,* longer transit time may results in longer fermentation or favor growth of certain species).

We found some opposite associations between black carbon exposure and bacterial taxa in boys and girls. In boys, black carbon exposure was associated with higher levels of *Streptococcaceae* and *Streptococcus* but lower levels of *Clostridiales Incertae Sedis XIII*, *Coriobacteriaceae*, and *Paraprevotella*, while the opposite was observed in girls. *Streptococcaceae*, including *Streptococcus*, are involved in carbohydrate fermentation and lactic acid production, (Gillespie, 1994) contribute to energy absorption, (Hove et al., 1999) and supporting strictly anaerobic bacteria to produce short-chain fatty acids, (Berstad et al., 2016) important for intestinal epithelium integrity. (Liu, 2021) Some strains, however, can alleviate bacterial and viral-induced diarrhea, especially in infants, (Hove et al., 1999) or cause intestinal colitis. (Dinakaran, 2018) *Clostridiales Incertae Sedis XIII* is poorly studied, but overrepresentation of the Clostridiales order has been associated with irritable bowel syndrome. (Tap, 2017) Besides, its intestinal levels are reduced by probiotic use. (Liu, 2020) The family *Coriobacteriaceae* also showed sex-dependent associations and metabolizes glucose, bile salt, and steroids. (Zhao, 2018; Liu, 2018) Lower *Coriobacteriaceae* levels were observed in Crohn's disease and ulcerative colitis patients. (Pittayanon, 2020) Our previous publication also reported negative associations between intestinal *Coriobacteriaceae* levels and urinary black carbon. (Van Pee, 2023) Lastly, *Paraprevotella* strains in mice were found to promote trypsin autolysis, aiding protein digestion in the small intestine, but potentially disrupting the mucosal barrier and causing inflammation in the large intestine. (Li, 2022) Exposure to $PM_{2.5}$ and PM_{10} in boys was linked to lower levels of *Victivallaceae*; *Rhodospirillaceae*, *Victivallis*, and *f_Rhodospirillaceae g_NA*. *Victivallaceae*, to which *Victivallis* belongs, likely regulates immunity as decreased levels were associated with autoimmune responses. (Li, 2024; Zou, 2024) The genus *Victivallis* is naturally present in the gut, and is more abundant in healthy individuals compared with persons with hypercholesteremia. (Morales, 2022) Intestinal levels of *Rhodospirillaceae*, although less studied, have been linked to auto-immune disorders (Montgomery, 2023) and neurological diseases. (Zhou et al., 2021) Interestingly, our finding that black carbon exposure was negatively associated with the relative abundance of *Christensenellaceae* aligns with our previous study in children, where negative associations were also found with the urinary black carbon load. (Van Pee, 2023) Intestinal levels of *Christensenellaceae* negatively correlate with BMI, low-density lipoproteins levels in blood, and gastrointestinal disorder incidence. (Pittayanon, 2020; Goodrich, 2014; Fu, 2015) Both black carbon, coarse PM, and PM_{10} exposure were negatively associated with the relative abundance of *f_Erysipelotrichaceae g_NA* and *Flavonifractor* (black carbon only with the latter) in boys. In contrast to our findings, long-term metal exposure was found to be positively associated with *Erysipelotrichaceae* levels, (Shao and Zhu, 2020) a family associated with gastrointestinal inflammation, but with inconsistent levels in inflammatory bowel disease patients like Crohn's disease. (Kaakoush, 2015) Last, Flavonifractor plays a role in metabolizing catechins (Mikami, 2020) and a study in mice showed that oral administration of the strain can reduce intestinal and systemic inflammation. (Mikami, 2020; Ogita, 2020) These findings highlight the impact of air pollution on gut microbiota and potential pathways to disease outcomes.

We highlight several strengths of this study. To our knowledge, this is the first large-scale study assessing the association between long-term particulate air pollution exposure and the intestinal microbiome composition in healthy children. Exposure to particulate air pollution was combined at the residential and school addresses, and address changes were accounted for to ensure accurate exposure data. Second, we were able to adjust for numerous potential confounders, including often overlooked transit time, and conducted sensitivity analyses to show the robustness of our findings. Our relatively large sample size enabled us to stratify the analyses by sex, informing on multiple sex-specific findings. Our study has some limitations. We focused on the relative abundance at family and genus level and did not consider species level. Even though species levels might be more specific, information regarding the functional attributes of individual species is limited, and hence the true biological meaning of the observed associations in terms of human health and disease might remain difficult to grasp. Moreover, metagenomic shotgun analyses should be conducted to acquire more detailed information on the possible effect of particulate air pollution on the microbiome composition and functionality. Finally, we utilized time-weighted daily average air pollution data from both residential and school addresses. However, it is important to acknowledge that children's movement patterns are not uniform or fixed, and air pollution levels fluctuate throughout the day, introducing a potential bias that cannot be fully accounted for by modeled air pollution data. Nonetheless, a sensitivity analysis using only residential air pollution data yielded comparable results.

5. Conclusions

Our findings show a sex-dependent association between particulate air pollution exposure and intestinal microbiome composition, with negative associations in boys and positive (albeit not statistically significant) associations in girls. The results suggest an influential role of childhood exposure to particulate air pollution in establishing the intestinal microbiome composition, which is influenced by sex. More epidemiological research is needed to confirm the observed sex-specific associations between particulate air pollution exposure and intestinal microbiome composition and to explore potential mechanisms underlying these divergent associations.

CRediT authorship contribution statement

Thessa Van Pee: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Liesa Engelen:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Marthe De Boevre:** Writing – review & editing, Conceptualization. **Muriel Derrien:** Writing – review & editing, Formal analysis, Conceptualization. **Janneke Hogervorst:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Roger Pero-Gascon:** Writing – review & editing, Conceptualization. **Michelle Plusquin:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Giulia Poma:** Writing – review & editing, Conceptualization. **Arnau Vich I Vila:** Writing – review & editing, Formal analysis, Conceptualization. **Adrian Covaci:** Writing – review & editing. **Lynn Vanhaecke:** Writing – review & editing, Conceptualization. **Sarah De Saeger:** Writing – review & editing, Conceptualization. **Jeroen Raes:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Tim S. Nawrot:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109457.

Data availability

Data will be made available on request.

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