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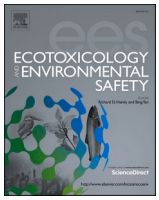

Exploring the impact of traffic-related air pollution on the gut microbiota of school-age children: A pilot study

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ABSTRACT

Over the last few years, investigating the changes of gut microbiota after the exposure to airborne pollution has gained increasing interest, due to the evidence that altered microbial communities may contribute to the development of chronic diseases.The aim of the present pilot study was to investigate the gut microbiota composition of school-age children in relation to their living environment. In particular, the impact of air pollution, diet, body mass index, and environmental tobacco smoke, on the richness and diversity of the gut microbiota of children, was evaluated. The gut microbiota composition of 44 children (10.5 ± 0.62 years) who lived in Lazio district (Italy) was investigated via 16 s rDNA sequencing. The main result was the observation that children living within 500 m from the closest highly trafficked road possessed a highly diverse and rich gut microbiota, as evidenced by the higher alpha-diversity indices (Faith's phylogenetic and Observed Features diversity) as compared to children exposed to low levels of vehicle traffic, via both uni- and multi- variate analyses ($p < 0.05$). By using the LEfSe analysis, several bacterial taxa were associated to the gut microbiota of children living near trafficked roads, such as, for example, the genera *Anaerotruncus* spp. and *Acutalibacter* spp., linked to chronic diseases for their pro-inflammatory activities. Our findings advance the knowledge on the complex interplay between air pollution and gut microbiota on children health, although long-read sequencing approaches will be helpful to better identify distinct microbial signatures potentially associated to the long-term exposure to air pollution.

1. Introduction

The gut microbiota is the largest microbial community in the human body with approximately 10^{14} bacteria represented mainly by *Firmicutes* (60–80 %) and *Bacteroidetes* (20 -40 %) while Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobiota are in the minority (Durack and Lynch, 2019). A balanced microbiota is essential for the integrity of the intestinal epithelial barrier and for maintaining normal physiological functions (Filardo et al., 2022a). Indeed, the gut microbiota contributes to important metabolic functions of the host organism, while also protecting against pathogenic challenges and modulating the physiological activities of the central nervous, endocrine, and immune systems (Durack and Lynch, 2019).

To date, it is well known that the intestinal microbiota composition vastly differs among individuals and, particularly, the abundance of specific bacteria varies in relation to the age; indeed, the gut microbiota begins to form at the time of birth, and its composition changes during the first years of life until the appearance of a stable microbial flora (Fan and Pedersen, 2021; Filardo et al., 2022a). From birth to the end of childhood, the gut microbiota is more susceptible to the effects of environmental and lifestyle-related factors such as dietary habits (excessive energy intake or poor eating habits), frequency and intensity of physical activity, the time spent indoor or in vehicles, etc; on this regard, a high fat diet shifts the gut microbiota composition to an over-representation of LPS-expressing bacteria, leading to a pro-inflammatory state, called "metabolic endotoxemia", involved in the development and progression of obesity-linked insulin resistance and type-2 diabetes mellitus (Malesza et al., 2021; Zmora et al., 2019).

Recently, several studies have supported the link between exposure to air pollution and alterations in the composition, diversity, and

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functionality of gut microbiota, suggesting a novel potential mechanism based on air pollution exposure in the pathogenesis of severe disorders such as asthma, obesity, type 2 diabetes, etc. (Brumberg et al., 2021; Liu et al., 2021a; Zheng et al., 2020). Particularly interesting is the evidence suggesting the changes in gut microbiota of asthmatic children following exposure to air pollution as potential factors triggering asthma (Zheng et al., 2020). Since then, other studies observed that the exposure to air pollution may affect the composition of gut microbiota during early and later life stages such as in infants, in children with autistic traits as well as in overweight and obese adolescents (Alderete et al., 2018; Kim et al., 2022). There is also evidence that indoor air pollution may influence gut microbiota and, hence, contribute to short and/or long-term effects on human health (Yuan et al., 2023); However, to date, few studies have investigated the effects of air pollution on gut microbiota of healthy children (Van Pee et al., 2025, 2023a).

In this scenario, the aim of the present pilot study was to investigate the gut microbiota composition of school-age children in relation to their living environment. In particular, the impact of outdoor and indoor air pollution, diet, body mass index, and environmental tobacco smoke, on the richness and diversity of the gut microbiota of children, was evaluated.

2. Materials and methods

2.1. Study design

In this cross-sectional study, children attending the 5th grade of Primary School in a small town of a province in central Italy (Lazio district), were selected for enrolment during a biomonitoring campaign in the early summer of 2023. The small town counted, in 2023, a total population of 13.297 inhabitants (population density 118.02 inhabitants/km^2); it sits at the crossroads of two main highly trafficked Roman roads, Aurelia and Cassia, and it possesses a surface of 113, 21 km^2.

This was a pilot study associated with the research project PON REACT EU 2014–2020 focused on green topics aimed to evaluate the impact of air pollution on the gut microbiota composition of school-age children. Inclusion criteria were: 1) attendance at the same school and class, and inhabitant of the same city; 2) resident at the same address for the last 5 years. All children who attended the same school and class but used to commute to the school from another city were excluded.

2.2. Environmental characteristics data collection

Personal and environmental characteristics were obtained by self-reported questionnaire which included questions about children' demographic data (age, sex), height (cm) and weight (kg) health status, the distance of children's house from the closest highly trafficked road, and behaviour features such as extracurricular sport activities, dietary habits, time spent indoor, time spent on vehicles. Dietary habits were assessed by KIDMED index (Mediterranean Diet Quality Index for children and adolescents) based on self-administered, 16-item questionnaire investigating the frequency of food consumption. Responses to questions that had a negative connotation to the Mediterranean diet were given a score equal to (−1) and the questions that constituted positive aspects were scored (+1); Mediterranean diet (MD) score: ≥ 8, high adherence; 4–7, moderate adherence; ≤ 3, low adherence. The KIDMED questionnaire used in this study was adapted based on the work by Altavilla and Caballero-Pérez (2019).

Child exposure to outdoor air pollution was evaluated on annual data obtained from near local air quality monitoring station of the Italian Regional Agency for Environmental Protection and Prevention (ARPA) specific for the Lazio region. In addition, the child exposure to traffic -related air pollution was estimated taking in account the distance of the children's house from the closest highly trafficked road; each child was classified in two distinct categories, "low-traffic" and "high-traffic", based on a distance cut-off of 500 m. A "highly-traffic road" was defined as a main road with continuous traffic at peak times and often travelled by heavy vehicles. Child exposure to indoor pollution was also evaluated taking in account some information on domestic heating devices and exposure to environmental tobacco smoke (ETS) including both second-hand and third-hand smoke. Lastly, some parent's characteristics (nation of birth, education level, occupational status) as well as features of living environment (house floor, house density) were included in the questionnaire.

A written informed consent as well as questionnaire and personal data processing authorization were signed by their parents. This study followed the project protocol approved by the Ethics Committee for Transdisciplinary Research of the University of Rome "Sapienza" (protocol n. 29/2023) and was conducted according to the guidelines of the Declaration of Helsinki.

2.3. Sampling design

In the first stage of the project, all students' parents and school-teachers received information about the aims and plans of the research proposal, the modalities to fill in the ad-hoc designed questionnaire, as well as to collect and store the faecal sample. On the day of the sampling, each participant collected a faecal sample in a sterile polypropylene container with spoon and stored them at 4–8°C; the parents fulfilled the questionnaire, the informed consent, and the personal data processing authorization. The next morning, each participant brought all the material to school, delivering it to the research team that was waiting for them at the school entrance. All samples, transported via refrigerated containers to the laboratory, were immediately stored at −80 °C until further analysis.

2.4. Metagenomic analysis

2.4.1. DNA isolation and quantification

After thawing, $\leq$ 150 mg of faecal samples were aliquoted and transferred into 2 mL screwcap tubes containing glass beads and BashingBeadTM lysis buffer (Zymo Research, USA). Tubes were, then, vortexed for 30 min on an iSWIX System (Neuation Technologies, India), and up to 400 μL of the supernatant was used for DNA extraction via the Quick-DNATM Fecal/Soil Microbe Miniprep Kit (Zymo Research, USA), according to the Manufacturer's Instructions. DNA was quantified by fluorescence spectroscopy (Quant-iT™ PicoGreen® dsDNA Assay Kit, Thermo Fisher, USA), and its integrity was checked by agarose gel electrophoresis.

2.4.2. 16 s rDNA amplification and Illumina MiSeq sequencing

Dual-indexed universal primers 341 F (CCTACGGGNGGCWGCAG) and 802 R GACTACHVGGGTATCTAATCC; Illumina, USA) were utilized for the two-step PCR amplification of the V3–V4 hypervariable regions of the 16 s rDNA (16S/ITS Nextera two-step PCR kit, Illumina Inc., USA), as previously described (Filardo et al., 2022b).

2.4.3. Sequencing data and bioinformatic analysis

The sequencing data were classified in operational taxonomic units (OTUs) via the software framework QIIME2 (version 2023.9-amplicon) (Bolyen et al., 2019) as previously described (Filardo et al., 2022b). The statistical analysis of the OTUs was performed in QIIME 2, normalizing all samples to the sample with the lowest read count for alpha and beta diversity comparisons. Shannon's Diversity, Faith's Phylogenetic Diversity, and Observed Features Diversity indexes were used as metrics for alpha rarefaction analysis. In particular, Shannon's diversity index accounts for both the abundance and evenness of the species present, Faith's phylogenetic diversity (PD) index is defined as the sum of the branch lengths of a phylogenetic tree connecting all species in the community, and Observed Features diversity index considers the number and abundance of the different species in the community. Relative

abundances of taxa were expressed as means ± standard error of means (SEM), whereas alpha diversity indexes as median (IQR).

Weighted and unweighted UniFrac analysis were used as a measure of beta-diversity; the Jackknifed principal coordinates analysis (PCoA) was used so to ensure that our rarefaction selection was not the cause of the observed clustering patterns (Lozupone and Knight, 2005; Lozupone et al., 2007). In particular, the weighted UniFrac analysis was based on sequence distances in the phylogenetic tree and their relative abundances, whereas the unweighted UniFrac analysis was based solely on the sequence distances.

2.5. Statistical analysis

Continuous data were expressed as mean ± standard deviation (SD), and Student's *t*-test was used in case of parametric data, or Mann-Whitney *U* test in case of non-parametric data. For alpha-diversity comparison, a linear regression model was used to account for the effect of potential confounders on the relationship between gut microbiota and traffic-related air pollution, whereas, for beta-diversity comparison, Adonis was used for category comparisons of beta diversity distance matrices, based on the permutational multivariate analysis of variance (PERMANOVA) (Caporaso et al., 2010). For the correlation analysis, the logarithmic transformation was applied to continuous data (BMI and distance from the closest highly trafficked road) in order to allow for a better fit to the Gaussian distribution; hence, the Pearson correlation coefficient (r) was computed. For taxa comparisons, relative abundances based on all obtained reads were used. Differential taxonomic units between groups were identified using the linear discriminant analysis (LDA) coupled with effect size measurement (LEfSe) and the Analysis of Composition of Microbiomes (ANCOM) as previously described (Mandal et al., 2015; Segata et al., 2011). Bonferroni correction was used to correct for multiple hypothesis testing when needed. The single or multiple inference significance level was set at 5 %.

3. Results

3.1. Study population

Overall, the parents of 137 fifth grade children, who lived in a small town in central Italy, were invited to participate in the biomonitoring campaign. Fifty-three children were enrolled in the study achieving a participation rate of about 39 %. Nine of them were excluded because of an amount of sample insufficient for microbiota analysis and, thus, 44 children (26 males and 18 female) were included in our study. None of the participants had any acute or chronic disease and none was on antibiotic and/or probiotic treatment in the last three months before the enrolment.

The personal and environmental characteristics of the studied population were reported in Table 1. The average age of children was 10.5 ± 0.62 years, 59.1 % were male and the mean weight and BMI were 38.4 ± 9.6 Kg and 18.0 ± 3.6 Kg/m^2, respectively. According to WHO cutoffs (https://www.who.int/tools/growth-reference-data-for-5–19-years/indicators/bmi-for-age, accessed on 18th December 2024), about 59 % had a healthy weight, almost 32 % were overweight or obese and 9 % were underweight. None of the children ate at school and according to the KIDMED score only 25 % of children showed dietary habits with a high adherence to the Mediterranean diet. Regarding outdoor environmental exposure factors, PM_{10} concentration, averaged over 5 years preceding gut microbiota evaluation of children was of 14.54 ± 2.92 μg/m^3. In particular '2023 was the best year, both in terms of exceeding the daily PM_{10} threshold (50 μg/m^3) and in terms of annual average values (40 μg/m^3), across the entire Italian national territory, since PM_{10} data became available (https://www.isprambiente.gov.it/, accessed on 18th December 2024). Most of the children (n = 28, 63.6 %) lived closer than 500 m from a highly trafficked road (HT), whereas 18 lived farther than 500 m from a highly trafficked road (LT). About 73 % of children spent less than an hour in the car daily; almost all the studied population spent more than 17 h indoor (40 children) and practiced at least one extra-curricular sport activity (43 children). Concerning household environment, 45.5 % of house are located on the ground, 52.3 % of them presents a density ≤ 30 m^2 per inhabitant and more than two-thirds of homes (68.2 %) are heated by methane or electric heaters. Considering ETS exposure, 36.4 % of the children lived with at least one cohabitant smoker (same percentage for mother and father). However, no information on the smoking habits of cohabiting parents emerged from the questionnaires. Lastly, as for characteristics of parents, the mother had generally a higher level of education (tertiary/higher, > 14 years) than the father (27.3 % vs. 6.8 %).

Table 1
Personal and environmental characteristics of the study population.

Characteristics		Total population (N = 44) %
Age (years)	10.5 ± 0.62	
Gender	Male	59.1 (n = 26)
	Female	40.9 (n = 18)
Ponderal status according to BMI[a]	Underweight	9.0 (n = 4)
	Healthy weight	59.1 (n = 26)
	Overweight	20.5 (n = 9)
	Obese	11.4 (n = 5)
Mediterranean Diet adherence[b]	Low	27.3 (n = 12)
	Moderate	42.7 (n = 21)
	High	25.0 (n = 11)
Distance from the closest highly trafficked road	Low (LT)	36.4 (n = 16)
	High (HT)	63.6 (n = 28)
Time spent indoor[c]	< 17 h	6.8 (n = 3)
	≥ 17 h	90.9 (n = 40)
	Unknown	2.3 (n = 1)
Time spent on vehicles[c]	≤ 60 min	72.7 (n = 32)
	> 60 min	11.4 (n = 5)
	Unknown	15.1 (n = 7)
Extra-curricular sport activities	No	2.3 (n = 1)
	Yes	97.7 (n = 43)
Exposure to ETS[d]	Not exposed	63.6 (n = 28)
	Exposed	36.4 (n = 16)
Domestic heating devices	Methane or electric heaters	68.2 (n = 30)
	Oil or wood heaters	31.8 (n = 14)
House density (m^2/inhabitant)	≤ 30	52.3 (n = 23)
	> 30	47.7 (n = 21)
House floor	0	45.5 (n = 20)
	≥ 1	54.5 (n = 24)
Maternal education	Basic (≤ 9 years)	25.0 (n = 11)
	Upper secondary (≤ 14 years)	47.7 (n = 21)
	Tertiary/higher (> 14 years)	27.3 (n = 12)
Paternal education	Basic (≤ 9 years)	25.0 (n = 11)
	Upper secondary (≤ 14 years)	68.2 (n = 30)
	Tertiary/higher (> 14 years)	6.8 (n = 3)

[a] BMI: body mass index (weight in kg/height squared in m)
[b] according to KIDMED index
[c] according to daily habits
[d] ETS: environmental tobacco smoke; HT, high-traffic, < 500 m from the closest highly trafficked road; LT, low-traffic, > 500 m from the closest highly trafficked road.

3.2. Composition of the gut microbiota in the study population

The metagenomic analysis of the hypervariable region V3–4 from the bacterial 16 s rDNA via Illumina sequencing provided an average of 11700.59 paired-end reads [median 7946 (interquartile range, IQR, 10116.5)] across all samples. After the removal of singletons and rare OTUs, an average number of 54.6 [56 (14.5)] OTUs was observed in the study population. Overall, the lowest read was 6657, and, hence, the

OTUs were randomly sub-sampled to this minimum read for diversity analysis to avoid bias.

The gut microbiota composition of the children enrolled in this study was analysed according to several parameters, including gender, BMI, distance to the closest highly trafficked road, smoking habits among close relatives living with them, and type of domestic heating devices.

According to the exposure to traffic-related air pollution, the HT children possessed a gut microbiota characterized by an increased relative abundance of Actinobacteriota (13.4 %) as compared to LT children (9.6 %, $p = 0.033$), as shown in Table 2. Verrucomicrobiota were also increased, whereas Firmicutes were slightly decreased in the gut microbiota of HT children as compared to the gut microbiota of LT children, although the differences did not reach statistical significance. At the genus level, significant differences could be observed for the genus *Vescimonas* spp., that resulted increased in HT children as compared to LT children ($p = 0.03$, Table 3). Furthermore, LT children showed increased relative abundance of *Faecalibacterium* spp., and decreased relative abundance of *Akkermansia* spp., *Gemmiger* spp., and *Bacteroides* spp., as compared to HT children, although they did not reach statistical significance. The groups of HT and LT children were comparable in respect to the adherence to the Mediterranean diet.

Different gut microbial community signatures could also be observed in relation to the BMI. At the phylum level, Verrucomicrobiota showed increasing relative abundance going from children with a BMI lower than the 5th percentile (underweight) to children with a BMI higher than the 95th percentile (obese) ($p < 0.05$) (Table 4). Interestingly, reduced Firmicutes, and increased Bacteroidota and Actinobacteriota could also be observed in underweight children as compared to all the other groups, although they did not reach statistical significance (Table 4). At the genus level, obese children were characterized by higher relative abundance of *Akkermansia* spp. ($p < 0.05$) and lower relative abundance of *Agathobacter* spp. ($p < 0.05$) than all other groups. Underweight children also showed significantly decreased *Bacteroides* spp. and *Waltera* spp., as compared to those in overweight children (BMI within the 85th and 95th percentiles, $p < 0.05$), and increased *Clostridium* spp. and *Turicibacter* spp. as compared to children with an ideal BMI (BMI within the 5th and 85th percentiles, $p < 0.05$, Table 5). Lastly, overweight children were characterized by increased relative abundance of *Waltera* spp. and *Marseilibacter* spp. ($p < 0.05$) and decreased *Escherichia* spp. ($p < 0.05$) as compared to children with an ideal BMI ($p < 0.05$) (Table 5).

No statistically significant differences in the composition of the gut microbiota were observed according to gender, smoking habits amongst the close relatives living with them, and the type of domestic heating devices.

3.3. Alpha- and beta- diversity indexes in school-age children

The diversity and richness of the gut microbiota was investigated in the enrolled children, via measures of alpha- and beta- diversity, according to the gender, BMI, distance from the closest highly trafficked road, and smoking habits among close relatives sharing the same household.

As for alpha-diversity measures, both the Faith's PD and the Observed Features diversity indices were significantly increased ($p < 0.05$) in the gut microbiota in HT children as compared to LT children, as shown in Fig. 1. No differences were observed when considering the gender, BMI and close relatives' smoking habits. Shannon's diversity index did not show any statistically significant differences for all the independent variables considered. Interestingly, both

Table 3
Composition of children gut microbiota in relation to the distance from the closest highly trafficked road at the genus level.

Genera	HT children (n = 28)			LT children (n = 16)			*p* values
Faecalibacterium (% ± rel. SE)	13.3	±	2.5	18.6	±	3.5	NS
Akkermansia (% ± rel. SE)	12.3	±	3.6	9.2	±	4.4	NS
Dorea (% ± rel. SE)	7.3	±	2.2	8.6	±	3.1	NS
Bifidobacterium (% ± rel. SE)	9.4	±	2.6	7.6	±	4.6	NS
Agathobacter (% ± rel. SE)	6.4	±	1.3	6.8	±	1.6	NS
Prevotella (% ± rel. SE)	0.7	±	0.4	6.0	±	3.8	NS
Gemmiger (% ± rel. SE)	10.1	±	2.3	5.9	±	1.5	NS
Bacteroides (% ± rel. SE)	5.5	±	1.5	3.7	±	0.6	NS
Catenibacterium (% ± rel. SE)	0.01	±	0.01	3.6	±	3.6	NS
Phocaeicola (% ± rel. SE)	2.3	±	0.6	3.2	±	0.6	NS
Alistipes (% ± rel. SE)	2.9	±	0.5	2.4	±	0.6	NS
Dialister (% ± rel. SE)	1.8	±	0.5	1.8	±	0.8	NS
Waltera (% ± rel. SE)	1.8	±	0.3	1.8	±	0.5	NS
Ruminococcus (% ± rel. SE)	1.9	±	0.8	1.7	±	0.8	NS
Clostridium (% ± rel. SE)	0.7	±	0.3	1.4	±	0.6	NS
Marseillibacter (% ± rel. SE)	1.1	±	0.3	1.4	±	0.4	NS
Collinsella (% ± rel. SE)	3.2	±	1.1	1.3	±	0.5	NS
Intestinimonas (% ± rel. SE)	1.4	±	0.4	1.1	±	0.3	NS
Peptacetobacter (% ± rel. SE)	1.0	±	0.2	1.1	±	0.2	NS
Gracilibacter (% ± rel. SE)	0.5	±	0.1	0.9	±	0.3	NS
Aristaeella (% ± rel. SE)	0.9	±	0.2	0.7	±	0.3	NS
Escherichia (% ± rel. SE)	0.5	±	0.2	0.6	±	0.3	NS
Agathobaculum (% ± rel. SE)	0.4	±	0.1	0.6	±	0.2	NS
Vescimonas (% ± rel. SE)	1.2	±	0.2	0.5	±	0.1	*0.03*
Ligilactobacillus (% ± rel. SE)	0.0	±	0.0	0.5	±	0.5	NS
Roseburia (% ± rel. SE)	0.3	±	0.1	0.5	±	0.3	NS
Turicibacter (% ± rel. SE)	0.2	±	0.04	0.5	±	0.3	NS
Parabacteroides (% ± rel. SE)	0.8	±	0.1	0.4	±	0.1	NS
Others (% ± rel. SE)	11.9	±	1.4	7.3	±	1.1	NS

HT, high-traffic < 500 m from the closest highly trafficked road; LT, low-traffic, > 500 m from the closest highly trafficked road; SE, Standard Error; NS, not significant.

Table 2
Composition of children gut microbiota in relation to the distance from the closest highly trafficked road at the phylum level.

Phyla	HT children (n = 28)			LT children (n = 16)			*p* values
Firmicutes (% ± rel. SE)	59.8	±	19.2	62.8	±	26.4	NS
Bacteroidota (% ± rel. SE)	13.4	±	11.5	16.9	±	14.4	NS
Actinobacteriota (% ± rel. SE)	13.4	±	14.1	9.6	±	18.4	*0.033*
Verrucomicrobiota (% ± rel. SE)	12.3	±	18.9	9.2	±	17.0	NS
Proteobacteria (% ± rel. SE)	0.6	±	1.0	0.9	±	1.1	NS
Pseudomonadota (% ± rel. SE)	0.2	±	0.5	0.4	±	1.3	NS
Patescibacteria (% ± rel. SE)	0.1	±	0.2	0.1	±	0.2	NS
Actinomycetota (% ± rel. SE)	0.03	±	0.1	0.02	±	0.1	NS
Thermodesulfobacteriota (% ± rel. SE)	0.1	±	0.1	0.02	±	0.04	NS
Desulfobacterota (% ± rel. SE)	0.02	±	0.1	0	±	0	NS
Nitrospinota (% ± rel. SE)	0.1	±	0.2	0	±	0	NS
Synergistota (% ± rel. SE)	0.03	±	0.1	0	±	0	NS

HT, high-traffic < 500 m from the closest highly trafficked road; LT, low-traffic, > 500 m from the closest highly trafficked road; SE, standard error; NS, not significant

Table 4
Composition of children's gut microbiota according to their BMI at the phylum level.

Phyla	Underweight (< 5 percentile) (*n*=4)	Ideal weight (from 5 to 85 percentile) (*n*=26)	Overweight (from 85 to 95 percentile) (*n*=9)	Obese (> 95 percentile) (*n*=5)
Firmicutes (% ± rel. SE)	34.4 ± 1.6	58.7 ± 0.2	57.8 ± 1.4	50.4 ± 2.6
Bacteroidota (% ± rel. SE)	20.1 ± 4.2	16.7 ± 0.1	14.9 ± 0.4	7.6 ± 10.0
Actinobacteriota (% ± rel. SE)	39.3 ± 7.0	12.2 ± 0.1	5.1 ± 0.3	11.3 ± 10.2
Verrucomicrobiota (% ± rel. SE)	4.9* ± 0.8	11.0** ± 0.2	21.8*** ± 2.1	29.9 ± 5.0
Proteobacteria (% ± rel. SE)	0.4 ± 0.03	1.0 ± 0.01	0.3 ± 0.01	0.3 ± 9.5
Pseudomonadota (% ± rel. SE)	0.8 ± 0.2	0.1 ± 0.002	0.0 ± 0	0.3 ± 20.0
Patescibacteria (% ± rel. SE)	0.2 ± 0.03	0.1 ± 0.001	0.0 ± 0.004	0.02 ± 16.9
Actinomycetota (% ± rel. SE)	0.0 ± 0	0.02 ± 0.001	0.1 ± 0.004	0.04 ± 12.2
Thermodesulfobacteriota (% ± rel. SE)	0.0 ± 0	0.1 ± 0.001	0.0 ± 0.002	0.01 ± 15.7
Desulfobacterota (% ± rel. SE)	0.0 ± 0	0.01 ± 0.0005	0.0 ± 0.005	0.0 ± 0.0
Nitrospinota (% ± rel. SE)	0.0 ± 0	0.05 ± 0.001	0.0 ± 0	0.1 ± 20.0
Synergistota (% ± rel. SE)	0.0 ± 0	0.02 ± 0.0003	0.0 ± 0.001	0.02 ± 20.0

SE, standard error; * $p = 0.016$, ** $p = 0.007$, and *** $p = 0.019$ vs obese children (BMI > 95 percentile).

Table 5
Composition of children gut microbiota according to their BMI at the genus level.

Genera	Underweight (< 5 percentile) (n = 4)	Ideal weight (from 5 to 85 percentile) (n = 26)	Overweight (from 85 to 95 percentile) (n = 9)	Obese (> 95 percentile) (n = 5)
Faecalibacterium (% ± rel. SE)	7.4 ± 2.9	17.5 ± 3.0	16.3 ± 3.4	7.8 ± 4.6
Akkermansia (% ± rel. SE)	4.7$^{\#}$ ± 2.7	8.5 ± 3.0	11.8$^{\#}$ ± 8.9	29.0* ± 6.7
Dorea (% ± rel. SE)	10.3 ± 8.1	7.6 ± 1.9	8.9 ± 5.8	4.5 ± 3.8
Bifidobacterium (% ± rel. SE)	24.8 ± 16.5	8.5 ± 2.5	0.8 ± 0.3	11.6 ± 6.7
Agathobacter (% ± rel. SE)	7.1 ± 3.5	7.8 ± 1.3	5.2 ± 2.2	2.4* ± 1.4
Prevotella (% ± rel. SE)	13.2 ± 12.1	0.6 ± 0.5	5.1 ± 4.4	0.3 ± 0.3
Gemmiger (% ± rel. SE)	8.1 ± 5.3	6.1 ± 1.3	9.4 ± 3.1	20.9 ± 9.2
Bacteroides (% ± rel. SE)	1.1*,$^{\$}$ ± 0.5	6.3 ± 1.5	3.9 ± 0.7	2.3 ± 1.2
Catenibacterium (% ± rel. SE)	0.0 ± 0.0	2.2 ± 2.2	0.04 ± 0.04	0 ± 0
Phocaeicola (% ± rel. SE)	1.5 ± 0.6	3.2 ± 0.6	2.2 ± 0.5	1.7 ± 1.3
Alistipes (% ± rel. SE)	1.3 ± 0.7	3.2 ± 0.6	2.8 ± 0.9	1.3 ± 0.7
Dialister (% ± rel. SE)	0.4 ± 0.3	2.0 ± 0.5	2.2 ± 1.3	0.6 ± 0.2
Waltera (% ± rel. SE)	0.7$^{\$}$ ± 0.3	1.3 ± 0.2	3.6* ± 0.7	2.1 ± 0.5
Ruminococcus (% ± rel. SE)	2.5 ± 2.4	2.1 ± 0.9	1.2 ± 0.6	1.1 ± 0.7
Clostridium (% ± rel. SE)	1.6*,$^{\#}$ ± 0.6	0.7 ± 0.3	1.9 ± 0.9	0.2 ± 0.1
Marseillibacter (% ± rel. SE)	0.2 ± 0.1	0.9 ± 0.2	2.8* ± 0.8	0.6 ± 0.2
Collinsella (% ± rel. SE)	1.8 ± 0.7	2.3 ± 0.6	4.6 ± 3.0	0.5 ± 0.2
Intestinimonas (% ± rel. SE)	0.6 ± 0.4	1.5 ± 0.4	1.4 ± 0.3	0.7 ± 0.3
Peptacetobacter (% ± rel. SE)	1.4 ± 0.6	1.0 ± 0.2	1.2 ± 0.3	0.7 ± 0.3
Gracilibacter (% ± rel. SE)	0.3 ± 0.1	0.7 ± 0.2	0.8 ± 0.4	0.3 ± 0.2
Aristaeella (% ± rel. SE)	0.2 ± 0.1	0.9 ± 0.2	1.1 ± 0.4	0.6 ± 0.3
Escherichia (% ± rel. SE)	0.4 ± 0.3	0.8 ± 0.3	0.1* ± 0.04	0.2 ± 0.1
Agathobaculum (% ± rel. SE)	0.2 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	0.1 ± 0.02
Vescimonas (% ± rel. SE)	0.6 ± 0.3	1.1 ± 0.2	0.7 ± 0.3	1.2 ± 0.5
Ligilactobacillus (% ± rel. SE)	0 ± 0	0.3 ± 0.3	0 ± 0	0.04 ± 0.04
Roseburia (% ± rel. SE)	0.1 ± 0.1	0.3 ± 0.1	0.9 ± 0.4	0.2 ± 0.2
Turicibacter (% ± rel. SE)	1.5* ± 1.2	0.2 ± 0.04	0.2 ± 0.1	0.04 ± 0.03
Parabacteroides (% ± rel. SE)	0.4 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
Others (% ± rel. SE)	8.1 ± 3.5	11.1 ± 1.5	9.5 ± 1.7	8.6 ± 1.5

SE, Standard Error; * $p < 0.05$ vs ideal BMI; $^{\#}$ $p < 0.05$ vs obese BMI; $^{\$}$ $p < 0.05$ vs overweight BMI.

Faith's PD and Observed Features diversity indexes showed a negative correlation with the distance from the closest highly trafficked road (expressed as logarithmic transformation of the distance in meters, r = -0.312, $p = 0.039$, and r = -0.317, $p = 0.036$, respectively, Fig. 2). No significant correlation was observed for all the alpha-diversity indices used (Faith's PD, Observed Features and Shannon's diversity indices) and the BMI as continuous variable.

To account for the effect of potential confounders, the statistical relationships between Faith's PD/Observed Features, and the distance from the closest highly trafficked road, were evaluated using a linear regression model, that included as covariates BMI (as continuous and binary variable), Diet (as binary variable), type of domestic heating device (as binary variable), and parents' smoking habits (as binary variable). As reported in Table 6, the adjustment for potential confounders does not substantially alter the effect of traffic exposure on Faith's PD index (p = 0.051 for the continuous variable, p = 0.057 for the binary variable) and on Observed Features diversity index (p = 0.056 for the continuous variable, p = 0.048 for the binary variable).

After excluding the effect of other variables, and particularly the traffic exposure as main effect, the interaction effect between BMI and traffic exposure was not statistically significant for either Faith's PD ($p = 0.195$) or Observed Features diversity ($p = 0.225$) indices.

Concerning the beta- diversity measures, PERMANOVA analysis was performed to investigate the effects of all the different variables, specifically the distance from the closest highly trafficked street, BMI, Diet, the type of domestic heating device, and parents' smoking habits, on unweighted and weighted UniFrac distance matrices. No significant differences were observed for each single variables in both weighted and unweighted UniFrac analyses, whereas the interactions between BMI and diet with the type of domestic heating device, and between BMI and the distance from the closest highly trafficked road, led to a statistically

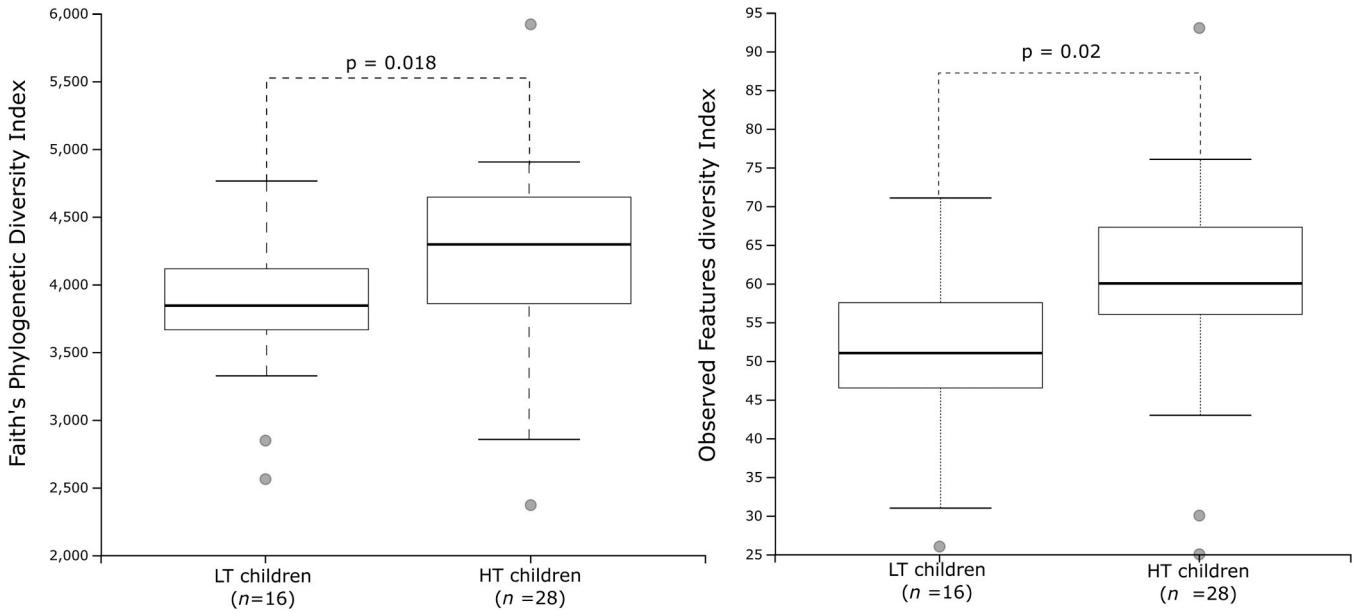

Fig. 1. Alpha- diversity of the microbial communities in the gut microbiota of school-age HT and LT children. Faith's phylogenetic diversity and Observed feature diversity indexes were used as measures of alpha-diversity within groups. The circles out of the range represent the outliers.

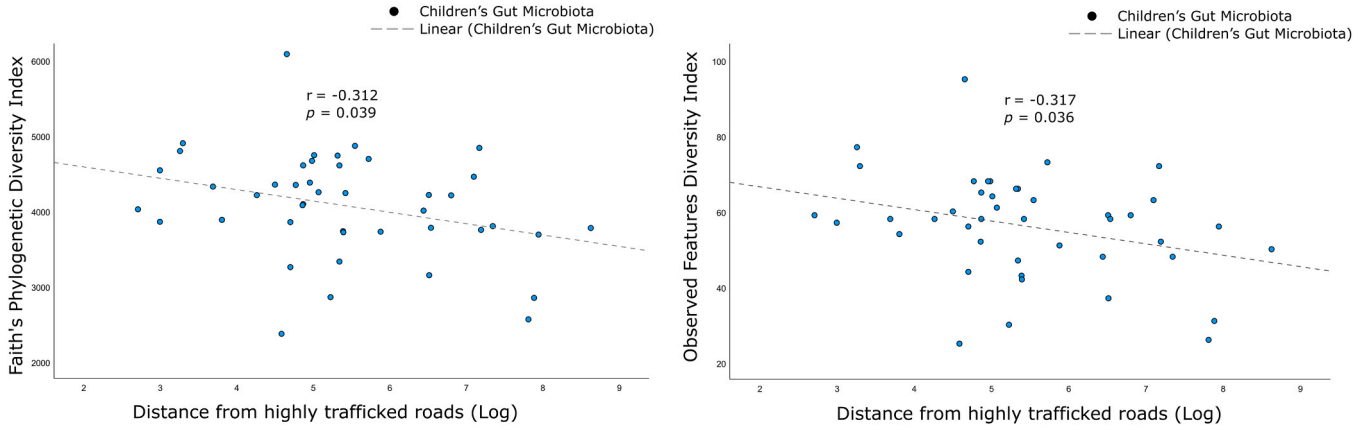

Fig. 2. Alpha- diversity correlations in school-age children according to the distance from the closest highly trafficked road. Pearson correlation coefficient (r) was used as a measure of the strength of the relationship between the two datasets.

Table 6
Linear regression model for the relationship between Faith's PD/Observed Features and the distance from the closest highly trafficked road, accounting for potential confounders.

	Dependent variable: Faith's PD				**Dependent variable: Observed Features**			
	B	*SE*	*t*	*p*	*B*	*SE*	*t*	*p*
BMI (log)	572.1	569.2	1.005	0.321	12.4	11.3	1.099	0.279
Diet (binary)	111.9	231.7	0.483	0.632	3.3	4.6	0.713	0.48
Heating (binary)	−156.2	223.9	−0.698	0.49	−1.7	4.4	−0.372	0.712
Smoking (binary)	212.5	217.3	0.978	0.334	3.0	4.3	0.687	0.497
Distance (log)	−149.1	73.8	−2.019	*0.051*	−2.9	1.5	−1.971	*0.056*
BMI (binary)	90.6	227.5	0.398	0.693	2.8	4.5	0.623	0.537
Diet (binary)	127.7	238.4	0.535	0.595	3.7	4.7	0.79	0.434
Heating (binary)	−214.4	223.9	−0.958	0.344	−2.9	4.4	−0.645	0.523
Smoking (binary)	160.9	217.1	0.741	0.463	2.0	4.3	0.469	0.642
Distance (binary)	423.3	215.9	1.961	*0.057*	8.7	4.3	2.045	*0.048*

BMI, body mass index; Diet, adherence to mediterranean Diet via the KIDMED index; Heating, domestic heating devices; Smoking, parents' smoking habits; Distance, distance expressed in metres from the closest highly trafficked road; SE, standard error; B, regression coefficient (slope); SE, standard error of B; t, *t*-test; p, p-value.

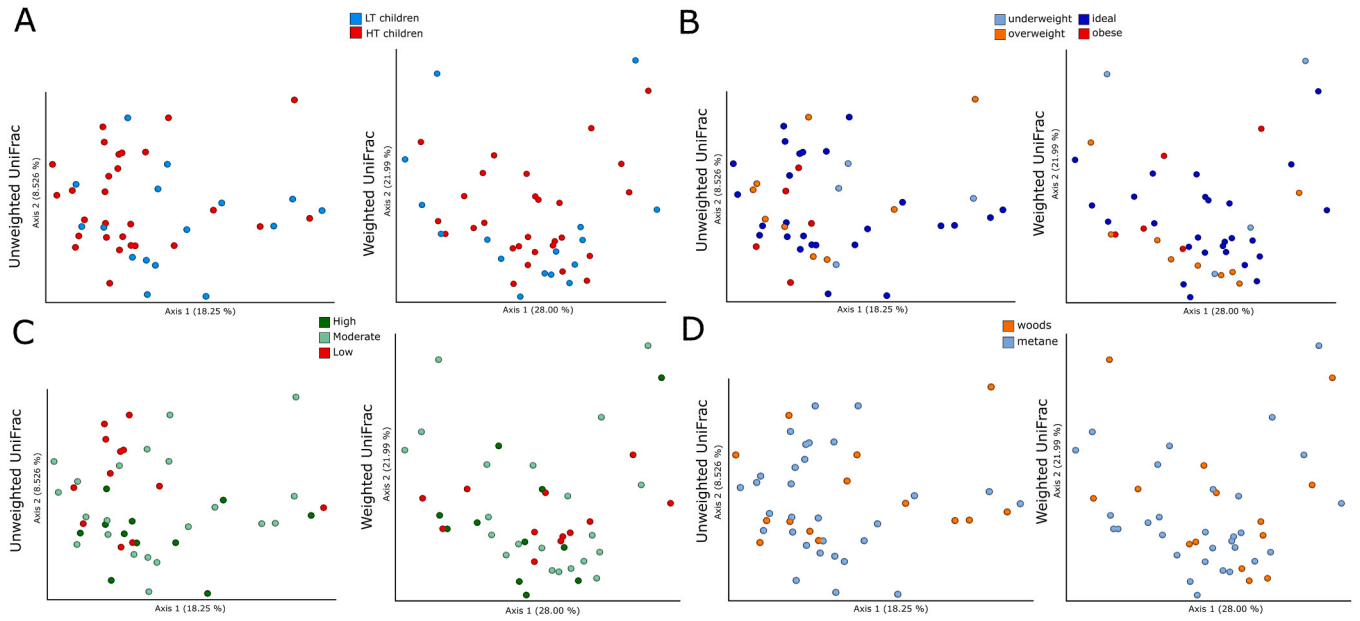

Fig. 3. Comparison of the beta- diversity of the gut microbiota according to the different independent variables. Principal coordinate analysis (PCoA) plots of beta-diversity clustering according to the distance from the closest highly trafficked road (A), BMI (B), adherence to the mediterranean diet (C) and the type of domestic heating device (D). Unweighted and weighted UniFrac analysis are used as measure of beta- diversity. Each dot represents the gut bacterial community composition of one individual, and the groups were compared using Adonis for beta-diversity measures.

significant clustering in the weighted UniFrac analysis only (Fig. 3 and tables S1 and S2).

3.4. Identification of specific taxa as potential microbial signatures

The linear discriminant analysis (LDA) coupled with effect size measurement (LEfSe) and the Analysis of Composition of Microbiome (ANCOM) approaches were used to identify specific network of bacterial taxa that characterize the different microbial communities of the gut microbiota in the school-age children enrolled in this study, in relation to gender, BMI, distance from the closest highly trafficked road, and close relatives' smoking habits.

As shown in Fig. 4A and B, concerning the exposure to vehicle traffic, HT children showed a gut microbiota associated to a wider variety of bacterial genera than the gut microbiota in LT children. Specifically, HT children presented a strong positive association with *Vescimonas* spp. (LDA>3.0), *Alistipes* spp. (LDA>2.5), *Anaerotruncus* spp. (LDA>2.0), *Acutalibacter* spp. (LDA>2.0), and *Dethiosulfovibrio* spp. (LDA>2.0), whereas LT children only showed a negative association with *Rothia* spp. (LDA> −2.0) and *Clostridium* spp. (LDA> −2.0).

Concerning BMI, different bacterial profiles presented a strong association with each BMI-based body types (Fig. 4C, D); specifically, the gut microbiota in underweight children was positively associated with the presence of *Senegalimassilia* spp. (LDA>3.0) and *Rothia* spp. (LDA>2.0); overweight children, instead, presented a strong positive association with *Walteria* spp. (LDA>4.0), *Marseilibacter* spp. (LDA>4.0), and *Clostridium* spp. (LDA>3.5); obese children showed a gut microbiota positively associated to the presence of *Akkermansia* spp. (LDA>5.0) and *Catonella* spp. (LDA>4.0). Lastly, children with an ideal BMI had a gut microbiota characterized by the positive association with *Bacteroides* spp. (LDA>4.0).

The ANCOM analysis did not reveal any significant differentially abundant taxa in the school-age children enrolled in this study according to either the BMI, or the exposure to vehicle traffic.

4. Discussion

The main result of our pilot study is the observation of significant differences in the diversity and composition of the gut microbiota of healthy school-age children in relation to their living environment. Specifically, children living within 500 m from the closest highly trafficked road possessed a highly diverse and rich gut microbiota, as evidenced by the increased alpha-diversity indices (Faith's phylogenetic and Observed Features diversity index) as compared to the children exposed to low levels of vehicle traffic ($p < 0.05$). Furthermore, after adjusting for potential confounders (BMI, diet, domestic heating system and parents' smoking habits) via multivariate analysis, the statistical significance of the effect of traffic-related air pollution on the biodiversity of the gut microbiota in children was preserved.

Concerning the bacterial composition, in our study, a high number of taxa were identified as potential microbial signatures by the LEfSe analysis, including *Vescimonas* spp., *Alistipes* spp., *Anaerotruncus* spp., *Acutalibacter* spp., and *Dethiosulfovibrio* spp., in the gut microbiota of children living close to trafficked roads. Similarly, Cruells et al. observed consistent increases of *Alistipes* spp. with increasing exposure to traffic-related air-pollutants, considering this association as a protective response to the epithelial damage in the gastro-intestinal tract (Cruells et al., 2024). Indeed, many studies indicate that *Alistipes* spp. are mucin-degrading bacteria promoting epithelial development and contributing to the maintenance of intestinal homeostasis (Gavin et al., 2018; Glover et al., 2022; Kim et al., 2021). Furthermore, the association of specific bacterial taxa, as those found in our study, with the exposure to traffic-related air pollution may lead to a predisposing condition for the development of chronic disorders later in life; in fact, *Anaerotruncus* spp. and *Acutalibacter* spp. have been linked to lung and colorectal cancers, as well as ulcerative colitis, for their pro-inflammatory activities (Feng et al., 2024; Wan et al., 2023; Zhu et al., 2023).

Nowadays, vehicle emissions are regarded as a primary contributor to air pollution and related adverse health effects. Indeed, cars emit several pollutants including $PM_{10,}$ increasing the risk for chronic diseases such as cardiovascular diseases, lung cancer, and diabetes, leading causes for premature death worldwide (https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death, accessed on 18th December 2024). In our study, the children lived in an area where the PM_{10} concentration (14.54 ± 2.92 μg/m^3 averaged value over 5 years) was always under the daily limit indicated in the European Directive

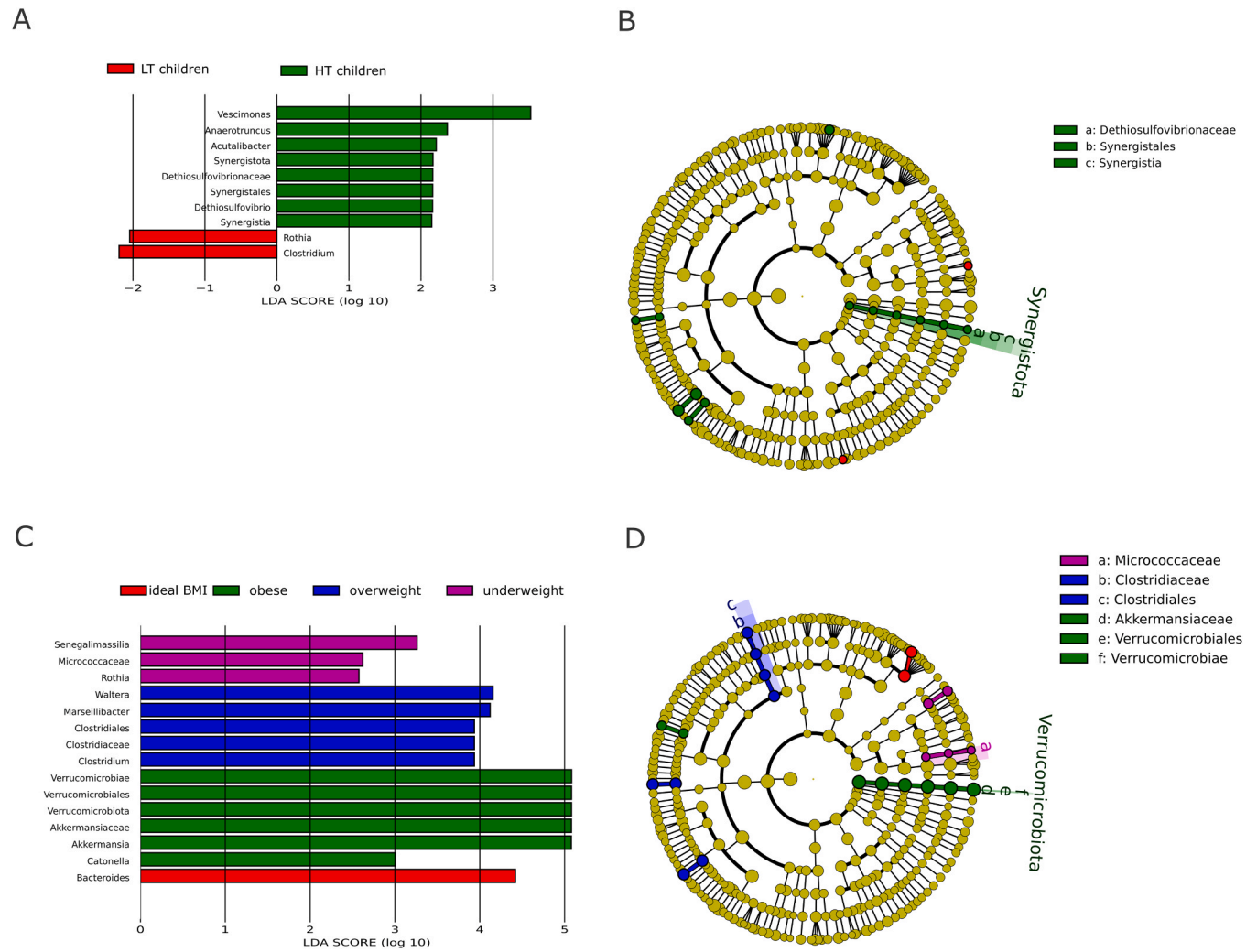

Fig. 4. Linear discriminant analysis with effect size measurement (LEfSe) of the gut microbiota in school-age children in relation to the exposure to traffic-related air pollution and the BMI. Histograms of the LDA scores in relation the proximity to the closest highly trafficked road (HT, high-traffic <500 m, LT, low-traffic >500 m) (A) and to the BMI (C) were computed for statistically significant differentially abundant taxonomic units among the groups. The cladograms related to the exposure to vehicle traffic (high or low) (B) and to the BMI (D) highlights the relationship of the significantly different taxonomic units between the groups. Differences are represented in the colour of the most abundant class, and each circle's diameter is proportional to the taxon's abundance.

2008/50/EC (50 μg/m^3) on air quality in order to avoid, prevent or reduce harmful effects on adult health (https://environment.ec.europa.eu/topics/air/air-quality/eu-air-quality-standards_en, accessed on 18th December 2024). As a result, the highly diverse and rich gut microbiota observed in children living close to trafficked roads may be due to their high susceptibility to air pollutants, even below limit values. Indeed, compared to adults, children breathe more rapidly and inhale more air relative to their body weight and, hence, adsorb more pollutants (Lau et al., 2024). In addition, the body of children, as well as their organs and immune system, is still in active development during childhood and, hence, they are at a higher risk of developing, in the future, chronic conditions (https://www.eea.europa.eu/publications/air-pollution-and-childrens-health, accessed on 18th December 2024). Also, the long-term exposure to low concentrations of pollutants may be responsible for the alteration of their gut microbiota observed in our study, since the included children have lived at the same address for the last 5 years. In this regard, several studies have demonstrated the association between long-term exposure to air pollution and the increased risks of cardiovascular events, asthma, chronic obstructive pulmonary disease, even below the current limit values for air pollutants (Brunekreef et al., 2021; Liu et al., 2021b).

To the best of our knowledge there are few studies investigating the impact of air pollution on healthy children microbiota (Van Pee et al., 2025). Differently from our evidence, indicating increased diversity of the gut microbiota in children who lived close to highly trafficked roads, Van Pee et al. found, in male children, a negative association between alpha diversity indices and air particulate exposure, although, in female children, non-significant positive associations were observed (Van Pee et al., 2025). These differences may be due to several factors, including the different age range of the study population, the different eating habits as well as difference in the methods used to evaluate exposure in children. As for the age range, it is known that gut microbiota composition undergoes substantial changes during childhood, particularly when exposed to different environmental factors (e.g. diet and outdoor and indoor air pollution). Van Pee et al. included, indeed, children from 4 to 12 years of age, with an average age of 7.49 ± 2.3 years, whereas we included only children from a narrower age range (9–12 years), with an average age of 10.5 ± 0.62. Concerning dietary habits, less of 45 % children enrolled in the study of Van Pee et al. eat fruit and vegetables once a day and whole grains approximately 40 % of the time, whereas more than 70 % children included in our study have dietary habits with moderate-high adherence to the Mediterranean diet, characterized by a high intake of plant-based foods such as fruits, vegetables, legumes, nuts, and whole grains, along with the predominant use of extra virgin

olive oil as the main source of fat (Rovira et al., 2024). As for the assessment of air-pollution exposure, Van Pee et al. used a model interpolating land cover data from satellite images with pollution data from fixed monitoring stations, whereas we considered five-year average PM_{10} concentration from local air quality monitoring station and the proximity to the closest highly trafficked road as a proxy measure for child exposure to traffic-related air pollutants. Lastly, other important information such as indoor air pollution exposure (ETS, domestic heating devices), known to influence the composition of gut microbiota (Yuan et al., 2023), was not considered in the study of Van Pee et al. (Van Pee et al., 2025).

Discordant results related to microbiota composition and air pollution have also been described in other populations including general and vulnerable individuals ranging from infants to the elderly; specifically, negative, positive or no associations between particulate air pollution exposure and intestinal microbiome biodiversity were reported in a recent systematic review (Van Pee et al., 2023b), and this suggests the complexity of the interaction between the exposure to different air pollutants and gut microbiota diversity and composition.

In our study, potential bacterial signatures in relation to the BMI were found; the most unexpected finding is the prevalence of *Akkermansia* spp. in obese children, since its increased abundance in adults, mainly *Akkermansia muciniphila*, is correlated with positive health outcomes, including the protection from obesity (Liu et al., 2024; Mo et al., 2024). A recent study has evidenced a great genotypic and phenotypic diversity among *A. muciniphila* strains isolated by obese children suggesting that *Akkermansia* isolates may differentially impact metabolic health resulting in beneficial or harmful effect (Becken et al., 2021). In addition, we may also hypothesize that bacterial species belonging to *Catonella* spp., evidenced in the obese children enrolled in our study, may negatively interfere with the protective effects of *Akkermansia* spp. To date, literature data regarding the pathogenic characteristics of *Catonella spp*. and other genera observed in overweight and obese children including *Walteria spp.*, *Marseilibacter* spp. is limited, as these are relatively recently characterized microorganisms (Antezack et al., 2021; Ndongo et al., 2017).

Our study has several strengths: it is the first study investigating the gut microbiota in Italian healthy school-age children in relation to traffic-related air pollution, taking into account relevant confounders, including dietary habits, indoor air pollution, and BMI. To date, in fact, there are studies on infant gut microbiota in relation to pre- post-maternal exposure to air pollution (Bailey et al., 2022; Cruells et al., 2024; Qiu et al., 2024), and most of the studies investigating the impact of air pollution on the gut microbiota focused on populations living in regions of the world with the highest exposure to air pollutants including China (Qiu et al., 2024; Sun et al., 2022; Wu et al., 2020; Zhao et al., 2022; Zheng et al., 2020). A further strength of our study is that the exposure to school-related air pollution and seasonal changes in dietary habits were also controlled by enrolling children attending the same school, and collecting the stool specimens at the same day. On the other hand, the main limitation of our study is the low sample size together with the lack of monitoring of individual exposure to air pollutants, and the study's cross-sectional design; indeed, ours is a pilot study providing preliminary data from an ongoing wider research project.

In conclusion, our findings advance the knowledge on the complex interplay between air pollution and gut microbiota on children health and, at the same time, opens interesting challenges, such as, for example, the study of other sources of indoor and outdoor pollution, like non-exhaust traffic emissions. Similarly, long-read sequencing approaches, including full-length 16 s rDNA sequencing and whole-genome sequencing via shotgun sequencing of all DNA fragments in a microbial community, will allow a deeper characterization of the bacterial signatures potentially associated to the long-term exposure to air pollution.

CRediT authorship contribution statement

Rosa Sessa: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Patrizio Pasqualetti:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Simone Filardo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marisa Di Pietro:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Matteo Albano:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Matteo Vitali:** Writing – review & editing, Validation, Supervision, Resources, Investigation, Formal analysis, Conceptualization. **Carmela Protano:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Arianna Antonucci:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation.

Consent to participate

Written informed consent was obtained from all individual participants included in the study.

Ethics approval

This study followed the project protocol approved by the Ethics Committee for Transdisciplinary Research of the University of Rome "Sapienza" (protocol n. 29/2023) and was conducted according to the guidelines of the Declaration of Helsinki.

Clinical trial number

Not applicable.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Simone Filardo reports financial support was provided by University of Rome "La Sapienza". If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118801.

Data availability

Raw data from the 16S rDNA amplicon sequencing of gut microbiota are deposited at NCBI sequence read archive (SRA, https://www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA1201587

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