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RESEARCH REPORT

Early-Life Air Pollution Exposure Is Associated with the Infant Gut Microbiome and Fecal Metabolome in the First Two Years of Life

Tanya L. Alderete, Elizabeth A. Holzhausen, Donghai Liang, Roshonda B. Jones, Fredrick Lurmann, Michael I. Goran, Howard H. Chang, and Jeremy A. Sarnat

INCLUDES A COMMENTARY BY THE INSTITUTE'S REVIEW COMMITTEE

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with a Commentary by the HEI Review Committee

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ABOUT HEI

The Health Effects Institute is a nonprofit corporation chartered in 1980 as an independent research organization to provide high-quality, impartial, and relevant science on the effects of air pollution on health. To accomplish its mission, the Institute

- identifies the highest-priority areas for health effects research
- competitively funds and oversees research projects
- provides an intensive independent review of HEI-supported studies and related research
- integrates HEI's research results with those of other institutions into broader evaluations
- communicates the results of HEI's research and analyses to public and private decision-makers.

HEI typically receives balanced funding from the US Environmental Protection Agency and the worldwide motor vehicle industry. Frequently, other public and private organizations in the United States and around the world also support major projects or research programs. HEI has funded more than 390 research projects in North America, Europe, Asia, and Latin America, the results of which have informed decisions regarding carbon monoxide, air toxics, nitrogen oxides, diesel exhaust, ozone, particulate matter, and other pollutants. These results have appeared in more than 275 comprehensive reports published by HEI, as well as in more than 2,500 articles in peer-reviewed literature.

HEI's independent Board of Directors consists of leaders in science and policy who are committed to fostering the public–private partnership that is central to the organization. The Research Committee solicits input from HEI sponsors and other stakeholders and works with scientific staff to develop a Five-Year Strategic Plan, select research projects for funding, and oversee their conduct. The Review Committee or Panel, which has no role in selecting or overseeing studies, works with staff to evaluate and interpret the results of funded studies and related research.

All project results and accompanying comments by the Review Committee or Panel are widely disseminated through HEI's website (*www.healtheffects.org*), reports, newsletters, annual conferences, and presentations to legislative bodies and public agencies.

ABOUT THIS REPORT

Research Report 237, *Early-Life Air Pollution Exposure Is Associated with the Infant Gut Microbiome and Fecal Metabolome in the First Two Years of Life*, presents a research project funded by the Health Effects Institute and conducted by Dr. Tanya L. Alderete at Johns Hopkins Bloomberg School of Public Health in Baltimore, Maryland. This research was funded under HEI's Walter A. Rosenblith New Investigator Award Program, which provides support to promising scientists in the early stages of their careers. The report contains three main sections:

- The **HEI Statement**, prepared by staff at HEI, is a brief, nontechnical summary of the study and its findings; it also briefly describes the Review Committee's comments on the study.
- The **Investigators' Report**, prepared by Alderete and colleagues, describes the scientific background, aims, methods, results, and conclusions of the study.
- The **Commentary**, prepared by members of the Review Committee with the assistance of HEI staff, places the study in a broader scientific context, points out its strengths and limitations, and discusses remaining uncertainties and implications of the study's findings for public health and future research.

This report has gone through HEI's rigorous review process. When an HEI-funded study is completed, the investigators submit a draft final report presenting the background and results of the study. Outside technical reviewers first examine this draft report. The report and the reviewers' comments are then evaluated by members of the Review Committee, an independent panel of distinguished scientists who are not involved in selecting or overseeing HEI studies. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, as necessary, to revise their report. The Commentary reflects the information provided in the final version of the report.

Although this report was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR–83998101 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and may not necessarily reflect the views of the Agency; thus, no official endorsement by it should be inferred. The contents of this report also have not been reviewed by private party institutions, including those that support the Health Effects Institute, and may not reflect the views or policies of these parties; thus, no endorsement by them should be inferred.

HEI STATEMENT

Synopsis of Research Report 237

Exploring the Link Between Early Life Air Pollution Exposures and the Infant Microbiome and Metabolome

BACKGROUND

Several studies have linked early-life environmental exposures, such as outdoor air pollution, to body mass index and overweight status in children, which are well-known risk factors for long-term adverse health outcomes, including heart disease and diabetes. The biological mechanisms underlying this relationship are not well understood, but recent research has suggested that air pollution exposures might contribute to obesity and other adverse outcomes through changes in the gut microbiome (the microbiota, including bacteria, fungi, viruses, and their genes, found in the human gastrointestinal tract) and their byproducts in the fecal metabolome (the collection of small molecules found in feces) (**Statement Figure**).

In response to HEI's *Request for Applications 18-2*: Walter A. Rosenblith New Investigator Award, Dr. Tanya Alderete of Johns Hopkins University submitted an application to HEI titled "Air Pollutants and the Gut Microbiota and Metabolome During Early Life: Implications for Childhood Obesity." Dr. Alderete proposed to examine whether prenatal and postnatal outdoor air pollution exposures, including traffic-related air pollution, can change infant gut bacteria and fecal metabolites. Such changes might alter infant growth trajectories in the first 2 years of life — a finding that could potentially provide new insights into the biological mechanisms through which air pollution might contribute to obesity.

APPROACH

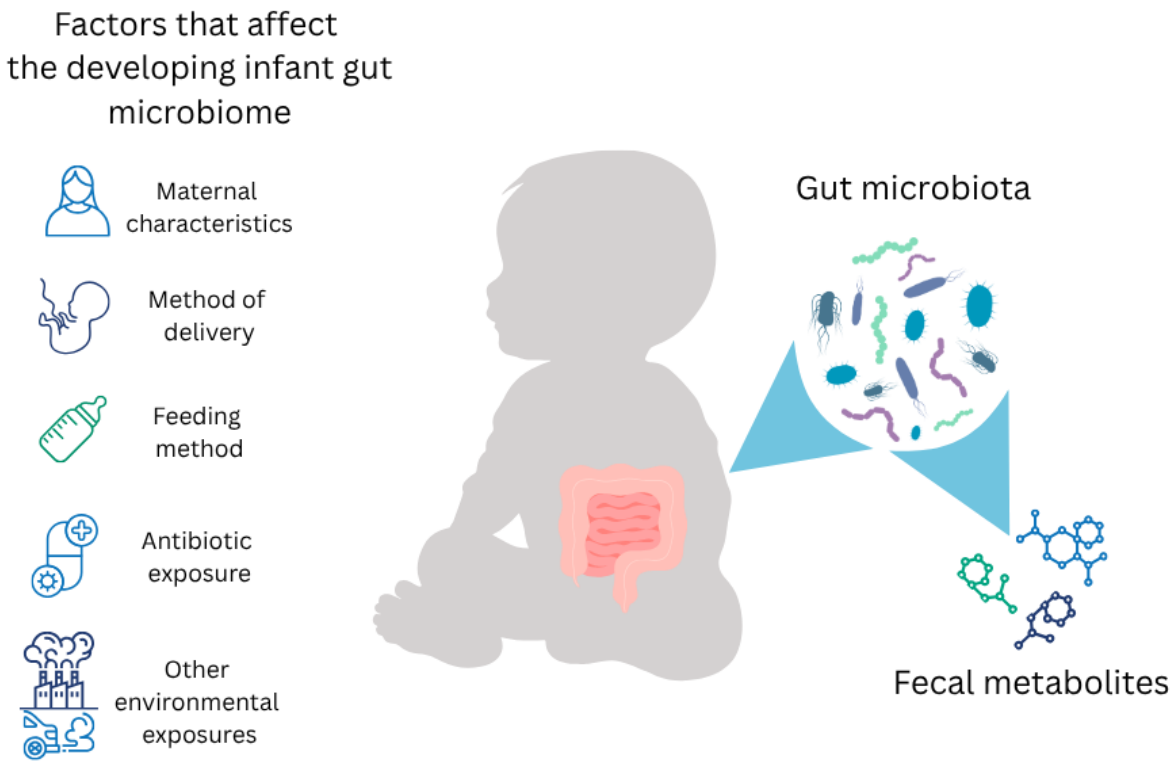
Dr. Alderete aimed to determine whether prenatal or postnatal exposure to air pollution is associated with a) lower diversity and altered relative abundances of gut bacteria and b) levels of specific fecal metabolites. She measured these endpoints at 1, 6, 12, 18, and 24 months after birth (Aim 1) and averaged these endpoints up to 24 months (Aim 2).

What This Study Adds

- This study examined whether prenatal or postnatal exposures to air pollution were associated with changes in the infant gut microbiome and fecal metabolome during the first 2 years of life.
- The team used a unique dataset of infant gut microbiota and fecal metabolites from a cohort of Hispanic mother–infant pairs in Southern California.
- Prenatal and postnatal air pollution exposures were associated with changes in the abundances of gut bacteria and levels of several fecal metabolites in infants during the first 2 years of life.
- Among participants with higher air pollution exposures, the team found some evidence of fewer beneficial gut bacteria, more potentially detrimental gut bacteria, and higher levels of metabolites indicative of oxidative stress and inflammation.
- This study provides a comprehensive set of exploratory analyses that contribute to our understanding of the relationships between air pollution and the infant gut microbiome and fecal metabolome.

Alderete and colleagues used a study cohort of 219 Hispanic mother–infant pairs participating in the Southern California Mother's Milk Study. Participants were enrolled at 1 month after birth and made several clinical visits up to 24 months after birth. The cohort included detailed information on the mother (such as age and socioeconomic status) and the infant (what their sex was, whether they were fed with human milk or formula, and when they started eating solid food). At each clinical visit, an infant stool sample was collected. Gut microbiome data were obtained from 207 infants, and fecal metabolome data were obtained from a subset of 127 infants. Stool samples were processed and analyzed using standard DNA sequencing and chemical analysis techniques.

This Statement, prepared by the Health Effects Institute, summarizes a research project funded by HEI and conducted by Dr. Tanya L. Alderete at Johns Hopkins Bloomberg School of Public Health in Baltimore, Maryland, and colleagues. Research Report 237 contains the detailed Investigators' Report and a Commentary on the study prepared by the HEI Review Committee.



Statement Figure. Overview of infant gut microbiome and fecal metabolome.

Based on the mothers' residential address histories, the team estimated prenatal and postnatal exposures to coarse particulate matter, fine particulate matter, nitrogen dioxide, ozone, and nitrogen oxides (a proxy for traffic-related air pollution) for each mother–infant pair. Prenatal exposure was based on the average of monthly air pollution exposure for the 9 months before birth. Postnatal exposure was based on air pollution exposures over short-term and long-term periods. Both prenatal and postnatal exposures were estimated using monthly concentrations of outdoor air pollutants derived from government monitoring data. Monthly traffic-related air pollution levels (nitrogen oxides) were estimated using an atmospheric dispersion model.

Alderete and colleagues used a combination of statistical models to evaluate associations between air pollution exposures and several outcomes of interest, including abundance (relative proportion of different types) and diversity (number of different types and distribution) of gut bacteria, as well as the identity and level of fecal metabolites.

KEY RESULTS

Gut Bacterial Abundance Alderete and colleagues reported that prenatal and postnatal exposures to several air pollutants were associated with short-term and long-term changes in the abundances of different gut bacteria. For instance, at various time points in early life, higher levels of prenatal exposure to coarse particulate matter, nitrogen dioxide, and nitrogen oxides were associated with a lower abundance of the beneficial gut bacterium *Bifidobacterium*. They also found that higher levels of prenatal exposure to nitrogen oxides were associated with a higher abundance of the potentially detrimental gut bacterium *Lelliottia amnigena*. In general, however, there were no clear trends or patterns across timepoints, specific gut bacterial abundances, or pollutant exposures.

Gut Bacterial Diversity Some postnatal air pollution exposures were associated with either increased or decreased diversity of gut bacteria, depending on infant age and the pollutant examined. For example, higher postnatal coarse particulate matter exposure was associated with greater gut bacterial diversity at 1 month after birth, whereas higher nitrogen oxide exposure was associated with reduced diversity at 6 months of age. More broadly, fewer associations were observed for diversity compared with gut bacterial abundance.

Fecal Metabolites The investigators found that exposures to coarse and fine particulate matter and nitrogen dioxide were associated with levels of several fecal metabolites at specific timepoints and altered levels over time up to 2 years. For instance, higher prenatal and postnatal exposures to fine particulate

matter were associated with lower average levels of metabolites involved in histidine metabolism during the first 2 years of life — a finding that potentially indicates gut inflammation. However, there was no overlap in metabolite levels across pollutants.

INTERPRETATION AND CONCLUSIONS

In its independent review of the study, the HEI Review Committee concluded that this research provides a set of exploratory analyses investigating potential mechanistic links between air pollution and the gut microbiome and fecal metabolome in infants, with possible implications for childhood obesity.

Alderete and colleagues found that estimated prenatal and postnatal exposures to outdoor air pollution were associated with lower abundances of some beneficial species of gut bacteria, higher abundances of certain detrimental species of gut bacteria, and metabolites that indicate oxidative stress or gut inflammation. However, no clear patterns were evident across pollutants, timepoints, or outcomes examined.

The Committee identified the collection of a unique dataset on the infant gut microbiome and fecal metabolome, along with analyses at multiple timepoints after birth, as key strengths of the study. However, the Committee noted that the small size of the study cohort limits statistical power and, thus, the reliability of the results. Future studies could expand the scope by analyzing the current dataset for other microbiota, such as fungi and viruses, and by considering additional functional characteristics of the gut microbiota.

Early-Life Air Pollution Exposure Is Associated with the Infant Gut Microbiome and Fecal Metabolome in the First Two Years of Life

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ABSTRACT

Introduction Obesity is a major public health concern because it increases the risk of numerous diseases, including cardiovascular disease and type 2 diabetes. Ambient and near-roadway air pollution has been associated with childhood obesity risk, independent of diet and physical activity. However, the biological mechanisms underlying these relationships remain unclear. Based on our previous work and existing literature, we hypothesized that exposure to air pollutants alters the developing infant gut microbiome and fecal metabolome, with implications for childhood obesity risk. In this study, we aimed to determine whether prenatal or early-life exposure to ambient air pollution and near-roadway air pollution is associated with the gut microbiome and fecal metabolome during the first 2 years of life.

Methods Our analysis had two components, both of which examined participants from the Southern California Mother's Milk Study, a Latino cohort in which we collected detailed information regarding maternal and child health during the first 24 months of life. Residential-based estimates of exposure to ambient particulate matter (particulate matter ≤2.5 µm and ≤10 µm in aerodynamic diameter: $PM_{2.5}$ and PM_{10}, respectively*), nitrogen dioxide (NO_2), and ozone (O_3), as well as near-roadway air pollution (NO_x), were modeled using residential address histories. High-throughput metagenomics and metabolomics were performed on stool samples collected at 1, 6, 12, 18, and 24 months of age. Overall, our sample included 207 unique individuals with gut microbiome data and 127 unique individuals with fecal metabolomics data. In the first analysis component, we examined the *cross-sectional* associations of pre- and postnatal exposure to ambient and near-roadway pollutants with the infant gut microbiome and fecal metabolome at 1, 6, 12, 18, and 24 months of age. In the second analysis component, we examined the *longitudinal* associations of pre- and postnatal exposure to air pollutants with the trajectory of the developing infant gut microbiome and fecal metabolome.

Results Our findings indicate that exposure to air pollutants during prenatal and postnatal periods is associated with significant changes in the developing gut microbiome and its metabolic output, as evidenced by perturbations in the fecal metabolome. These molecular alterations were evident in both cross-sectional and longitudinal analyses. The results suggest that early-life exposure to air pollution can disrupt the developmental trajectory of the gut microbiome, potentially leading to changes with substantial health implications. These findings underscore the importance of mitigating air pollution exposure during critical developmental periods to protect and promote gut health and overall well-being in infants.

Conclusions We identified gut microbes and fecal metabolites associated with early-life exposure to air pollution. Many of these markers of gut bacterial composition and function have been linked to childhood obesity. These findings contribute to our understanding of mechanisms underlying the obesogenic effects of air pollutants in early life. Future work in this cohort will include integrated mixture and multi-omics analyses to explore the joint impact of air pollution exposure on the gut microbiome and fecal metabolome.

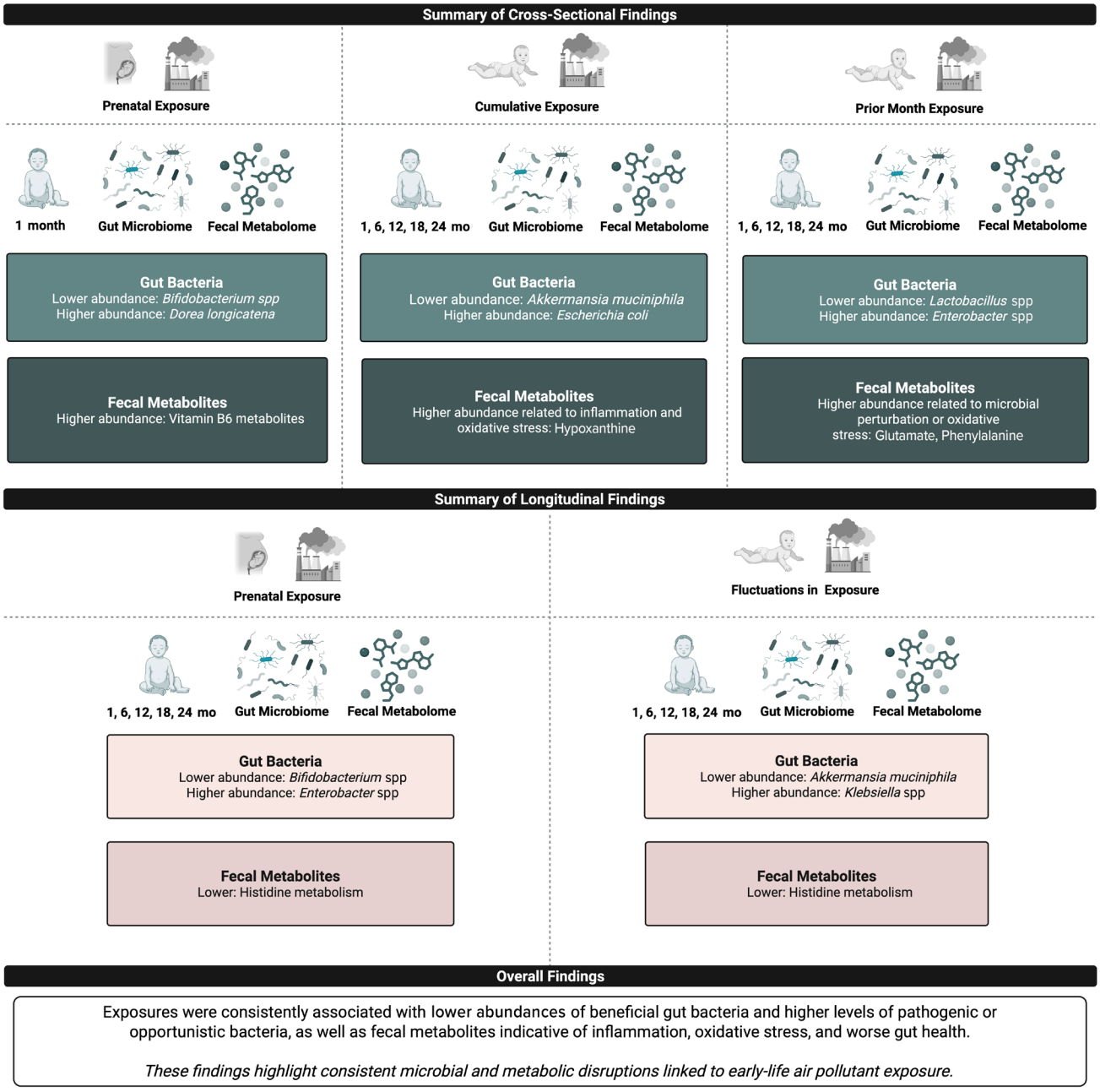
INTRODUCTION

Over the past 30 years, the prevalence of overweight and obesity has significantly increased in the United States.[1] Obesity has critical health implications due to its associations with adverse cardiometabolic outcomes in children and adults.[2,3] Without effective prevention strategies, nearly 38%

This Investigators' Report is one part of Health Effects Institute Research Report 237, which also includes a Commentary by the Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Tanya L. Alderete, Johns Hopkins Bloomberg School of Public Health, 615 N. Wolfe Street, Baltimore, MD 21205; email: *taldere1@jhu.edu*. No potential conflict of interest was reported by the authors.

Although this report was produced with partial funding by the United States Environmental Protection Agency under Assistance Award CR–83998101 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and may not necessarily reflect the views of the Agency; thus, no official endorsement by it should be inferred. This report has also not been reviewed by private party institutions, including those that support the Health Effects Institute, and may not reflect the views or policies of these parties; thus, no endorsement by them should be inferred.

*A list of abbreviations and other terms appears at the end of this report.



Source: Created in BioRender. Holzhausen EA and Alderete TL (2025). *https://BioRender.com/m57g481*.

of the US adult population is expected to remain overweight or obese.[4] An understanding of factors that contribute to obesity is crucial.

In addition to poor diet, reduced physical activity, and lower socioeconomic status, increased exposure to ambient and near-roadway air pollution may independently contribute to obesity.[5,6] Prenatal ambient and near-roadway exposures have been linked to low birth weight, which is separately associated with increased infant weight gain and a higher risk of childhood obesity.[7] For instance, children aged 5 to 11 years exposed to the highest levels of near-roadway pollution experienced a 13.6% greater annual body mass index (BMI) growth than those with the lowest exposure.[5] Additionally, our previous longitudinal work demonstrated that an increase in nitrogen dioxide (NO_2) and fine particulate matter (particulate matter ≤2.5 µm in aerodynamic diameter [$PM_{2.5}$]) exposure was associated with higher BMI at age 18 years.[6] However, not all studies show that pre- and postnatal exposures increase growth rates or childhood obesity.[8-10]

Although the exact mechanisms are unclear, air pollutants may adversely affect the gastrointestinal tract.[11-14] There is increasing evidence that air pollution exposure can lead to obesity, type 2 diabetes, and cardiovascular disease through alterations in gut microbial profiles[13-16] and gut bacteria–derived circulating metabolites.[15,17-19]

Our previous research in adolescents revealed a correlation between increased near-roadway air pollution exposure and the relative abundances of gut bacteria such as *Bacteroidaceae* and *Coriobacteriaceae*,[13] which are linked to obesity and altered metabolism.[20] Animal model studies also support a connection between air pollution exposure and the gut microbiota, suggesting that ultrafine particles reach the intestines through various pathways.[11,21] Increased exposure to air pollutants not only alters gut microbiome composition but also affects gut bacterial function, including the production of metabolites related to obesity. For example, gut bacteria, fecal lipid, and amino acid metabolites have been associated with increased BMI and abdominal adiposity in adults.[22] In mice, ingestion of ultrafine particles has been shown to alter gut microbiota and fecal cholesterol metabolites.[15] Furthermore, specific gut bacteria are involved in the metabolism of short-chain fatty acids,[23] lipids,[24] amino acids,[22,25-28] bile acids,[29,30] and tryptophan[31,32]; all of these processes have been linked to gut barrier integrity, satiety, body weight, and adipose tissue inflammation.

The first 1,000 days of life, including the pre- and postnatal periods, are critical developmental windows that considerably influence long-term health outcomes. During this time, exposure to air pollutants can affect infant and childhood growth trajectories.[33] Studies have also shown that the composition of the developing infant gut microbiome predicts rapid infant growth and childhood BMI.[34,35] However, human studies are limited in their ability to determine whether changes in the gut microbiome precede and contribute to increased BMI, or whether obesity itself leads to alterations in microbiome composition.[36,37] Consistent with this limited ability, although bacterial communities differ between lean and obese individuals, more than 95% of bacterial genetic material can be assigned to functional groups with shared metabolic activities observed in all individuals.[38] Nonetheless, emerging evidence indicates that early-life perturbations in the gut microbiota[34,35] have extensive implications for postnatal growth and childhood obesity risk. This relationship is biologically plausible given that the gut microbiome is involved in numerous metabolic processes, producing various diffusible metabolites that regulate gut barrier integrity, satiety, cell signaling, adiposity, body weight, and adipose tissue inflammation. In mice, exposure to ultrafine particulate matter increased circulating lipid metabolites,[15] and our recent study in infants identified a diverse array of metabolites linked with air pollution in fecal samples.[39] Our recent studies, and others, have shown that exposure to traffic pollutants is associated with alterations in circulating metabolites (e.g., histidine, tryptophan) produced by gut bacteria,[19,40,41] which are linked to gut microbiome dysbiosis and obesity.[34,35] Consequently, air pollutant exposure may disrupt the gut microbiome and fecal metabolome through toxic effects on bacteria, potentially impacting infant growth trajectories and increasing the risk of childhood obesity.

The goal of this Walter A. Rosenblith New Investigator Award is to determine whether prenatal or early-life exposure to air pollutants affects the infant gut microbiome and fecal metabolome. We performed parallel analyses of the gut microbiota and fecal metabolites to gain deeper insights into how prenatal and early-life exposure to air pollutants affects the composition and function of the gut microbiome. This study was conducted in a cohort of Latino mother–infant pairs from Southern California who were assessed at 1, 6, 12, 18, and 24 months of age. We hypothesized that increased exposure to air pollutants during pregnancy and early life would result in altered gut microbial profiles and fecal metabolites in infants. We also hypothesized that bacteria and metabolites associated with exposures would have known biological implications related to infant growth and development.

SPECIFIC AIMS

In this study, we explored the potential impact of early-life exposure to ambient and near-roadway air pollution on the developing gut microbiome and fecal metabolome in the first 2 years of life. Although exposure to air pollutants has been linked to lower birth weight and increased risk of childhood obesity, the biological mechanisms underlying these relationships remain uncertain. We hypothesized that elevated exposure to ambient and near-roadway air pollution during the prenatal period and early life alters infant growth trajectories via changes to the gut microbiome and fecal metabolome. Therefore, we sought to address the following two interrelated aims:

Aim 1. Determine whether prenatal and early-life exposure to ambient and near-roadway air pollution is associated with (1a) lower gut bacterial diversity and altered relative abundances of gut microbial taxa and (1b) fecal metabolites, separately at 1, 6, 12, 18, and 24 months of infant age. The infant gut microbiome undergoes rapid development in the first 2 to 3 years of life. Therefore, we first aimed to examine the cross-sectional relationships between prenatal and postnatal exposure to air pollutants and the infant gut microbiome and fecal metabolome.

Aim 2. Determine whether prenatal and early-life exposure to ambient and near-roadway air pollution is associated with the trajectory of (1a) the developing infant gut microbiome (lower diversity, altered relative abundance) and (1b) fecal metabolites. Building on Aim 1, we conducted a longitudinal analysis to track changes over time, providing a more comprehensive understanding of how continuous and cumulative exposure to air pollutants influences the development and dynamics of the gut microbiome and metabolome throughout early childhood.

and

Aim 3 (not included in this final report). Determine whether infant gut bacteria and fecal metabolites associated with increased ambient and near-roadway exposure are also associated with infant growth trajectories (e.g., weight-for-length z-score, waist circumference) under a mediation framework. This aim was not included in the final report due to substantial delays and disruptions caused by the COVID-19 pandemic. The pandemic greatly affected our ability to collect and analyze data within the planned time frame. Consequently, we were unable to fully explore the mediation framework linking environmental exposures to infant growth outcomes via gut microbiome and metabolome alterations. However, we intend to pursue this analysis in future studies outside of the final HEI report.

METHODS AND STUDY DESIGN

STUDY LOCATION AND OVERALL DESIGN

The longitudinal Mother's Milk Study is an ongoing prospective cohort study focusing on Latino mother–infant pairs from Southern California, aimed at examining early-life growth and development.[33,34,39,40,42,43] Participant recruitment began in 2016 at Los Angeles County maternity clinics associated with the University of Southern California. The inclusion criteria for mothers were being ≥18 years old at delivery; having a healthy, term, singleton birth; enrolling by approximately 1 month postpartum; self-identifying as Hispanic/Latino; intending to breastfeed for at least 6 months postpartum; and having a literacy level of at least 5th grade in either English or Spanish to comprehend study procedures. Exclusion criteria for mothers were medical conditions or medications that could potentially impact physical or mental health, nutritional status, or metabolism; tobacco use (defined as smoking more than one cigarette in the past week); recreational drug use; preterm or low birth weight; and clinically diagnosed fetal abnormalities. The recruitment strategy aimed to achieve a balanced representation across prepregnancy BMI categories (normal weight [BMI: 18–24.9 kg/m^2], overweight [BMI: 25–29.9 kg/m^2], and obese [BMI: >30 kg/m^2]). The study protocols received approval from the Institutional Review Boards at the Children's Hospital of Los Angeles and Johns Hopkins University. Participants provided written informed consent before enrollment in and any study-related procedures.

CLINICAL ASSESSMENTS

Participants were enrolled around 1 month postpartum and attended follow-up visits at 6, 12, 18, and 24 months postpartum. Initially, 219 mother–infant dyads were enrolled in the Mother's Milk Study. Socioeconomic status was estimated using a modified Hollingshead Index, as previously described.[33] Questionnaires assessed infant feeding practices, including the frequency of human milk and formula feeding and the age at which solid foods were introduced. Infants were classified as exclusively breastfed if parents reported no formula use; otherwise, they were classified as not exclusively breastfed. The Healthy Eating Index was calculated for infants after the introduction of solid foods, serving as a composite measure to assess dietary intake alignment with the Dietary Guidelines for Americans. This HEI Walter A. Rosenblith New Investigator Award supported the high-resolution metabolomics analysis of approximately 600 fecal samples in the present cohort. Specifically, a subset of 127 participants was selected for fecal metabolomics analysis to maximize the number of repeated samples within the first 2 years of life. Overall, 101 infants completed fecal metabolomics sampling at all five timepoints; the remaining 26 infants completed sampling at four of the five timepoints (Supplemental Figure 1; see Additional Materials on the *HEI Website*).[42]

RESIDENTIAL ADDRESS HISTORIES

Residential address histories were obtained via questionnaire during the baseline study visit and at each subsequent clinical research visit (1, 6, 12, 18, and 24 months). These address histories included the prenatal period and incorporated move-in and move-out dates for each respective residence, as well as multiple addresses, to ensure accurate exposure assessment. Each address was geocoded at the street level using the Texas A&M Geocoder,[44] which assigned latitude and longitude coordinates for each participant's residence. The Google Earth geocoder was used to confirm or correct locations with less accurate geocoding. Participants provided a total of 1,037 residential addresses spanning the pre- and postnatal periods. Of these, 91 addresses were matched at the address-point level, 942 were matched to the parcel level (using the parcel centroid), 26 were matched to address-range interpolations, and two were geocoded as ZIP Code Tabulation Areas; one address was classified as unknown. The two addresses classified as ZIP Code Tabulation Areas were excluded from further analysis. All other addresses were successfully geocoded with high quality (i.e., with address range interpolation or better).

AMBIENT AIR POLLUTION EXPOSURES

Residential exposure to ambient air pollutants, including particulate matter ($PM_{2.5}$ and particulate matter ≤10 µm in aerodynamic diameter [PM_{10}]), NO_2, and ozone (O_3) during the pre- and postnatal periods, was modeled for all mother–infant pairs. $PM_{2.5}$ and PM_{10} were measured in micrograms per cubic meter (µg/m^3); NO_2 and O_3 were assessed in parts per billion (ppb). Monthly averages of ambient pollutant exposures were estimated using data from the US Environmental Protection Agency's Air Quality System (*https://www.epa.gov/aqs*), which provides hourly and daily air quality data from ambient monitoring stations. To estimate air quality at unmeasured locations and create a continuous layer, spatial interpolation of up to four monitoring stations within 50 km of participants' homes was performed using inverse distance-squared weighting algorithm.[45,47] Mother–infant pairs were evenly

distributed across Southern California, with participants largely clustered in urban centers of Los Angeles, including South Central Los Angeles, where spatial coverage of the air monitoring network is robust. Participants for whom air pollution estimates could not be calculated for a given exposure window or study visit were excluded from analyses requiring those estimates; they were retained in analyses for which air pollution estimates could be calculated.

NEAR-ROADWAY AIR POLLUTION EXPOSURES

Near-roadway exposures were estimated using the California Line Source Dispersion Model (CALINE4) as point estimates at each participant's residential location.[46] The CALINE4 line-source dispersion model estimated concentrations of nitrogen oxides (NO_x) at the residence using traffic emissions (calculated within a 5-km buffer of the residence), traffic volume, roadway geometry, and meteorological conditions. These meteorological conditions included wind speed and direction, pollution mixing heights, and atmospheric stability.[47] Traffic volumes and speeds were obtained from machine-learning models developed by Bentley Systems, Inc. (2019), which provide more accurate and complete data for moderate and smaller roads than conventional traffic data sources. Vehicle emission factors were determined annually for each roadway link using the California Air Resources Board's EMFAC2021 model, based on traffic volumes, speeds, and the proportion of heavy-duty trucks. Meteorological conditions were obtained from the National Oceanic and Atmospheric Administration/National Centers for Environmental Prediction Real-Time Mesoscale Analysis model, a high-spatial (5 × 5 km) and high-temporal (1-hour) resolution analysis/assimilation system for near-surface weather conditions.[48,49] NO_x was used as a surrogate for the complex mixture of gases and particles emitted by vehicles, commonly referred to as traffic-related air pollution (TRAP).

EXPOSURE WINDOWS FOR AIM 1 (CROSS-SECTIONAL ANALYSES)

In Aim 1, we sought to understand the relationships of air pollution exposure with the gut microbiome and fecal metabolome at a single timepoint. Prenatal exposure was modeled based on the cumulative 9-month average before the infant's birth. Monthly average exposure estimates for each pollutant were available dating back 12 months from the 1-month postpartum study visit. Cumulative postnatal air pollution exposure was defined as the cumulative estimate of air pollution exposure from the infant's birth until the study visit (i.e., at 1, 6, 12, 18, and 24 months). Short-term exposures were defined as the estimated air pollution exposure during the month before each clinical visit.

EXPOSURE WINDOWS FOR AIM 2 (LONGITUDINAL ANALYSES)

In Aim 2, we sought to understand longitudinal associations of air pollution exposure with the gut microbiome or fecal metabolome, allowing both exposure (i.e., air pollution) and outcome (i.e., microbiome or metabolome) to vary over time. Longitudinal analyses examined both long-term exposures and fluctuations in exposure across the first 2 years of life. Long-term exposure was defined as the grand mean of prior-month air pollution exposures across all timepoints. Fluctuations in exposure were calculated as the grand mean minus the prior month's air pollution exposure, enabling examination of short-term deviations and their potential impacts during the follow-up period. This approach was selected to evaluate both the effects of chronic exposure and fluctuations in exposure on the gut microbiome and fecal metabolome.[6,50]

The correlation structure between each of the exposure windows used in this study is summarized in Supplemental Figure 2.

INFANT GUT MICROBIOME

DNA isolation was performed on infant stool samples collected at 1, 6, 12, 18, and 24 months of age, along with control samples, using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Catalog #D4300) in accordance with the manufacturer's protocol. Control samples included water, the ZymoBIOMICS Microbial Community Standard (Zymo Research, Catalog #D6300), and the ZymoBIOMICS Microbial Community Standard II Log Distribution (Zymo Research, Catalog #D6310). Lysis steps in the manufacturer's protocol were carried out using 2-mL Bashing Bead Tubes (Zymo Research) on a Vortex Genie 2 (Scientific Industries, Catalog #SI-0236) with a Microtube Adaptor (Scientific Industries, Catalog #S5001-7). DNA concentrations were measured using a Qubit Fluorometer (Thermo Fisher Scientific).

Indexed libraries were prepared from stool DNA using the Illumina Nextera XT DNA Library Prep Kit (Illumina, Catalog #FC-131-1096) and Illumina IDT for DNA/RNA UD Indexes Sets A, B, C, and D (Illumina, Catalog #20027213, #20027214, #20042666, and #20042667), following the manufacturer's protocols. Library quality was assessed using an Agilent Bioanalyzer 2100 (Agilent) with High Sensitivity DNA kits (Agilent, Catalog #5067-4626). Libraries were then pooled, and paired-end sequencing (2 × 150 bp) was performed using the Illumina NovaSeq platform. To reduce the likelihood of batch effects, samples were randomized before sequencing, DNA isolation, and library preparation. All batches were balanced with respect to participant characteristics, including infant sex, weight-for-age z-score, and weight-for-length z-score. Principal coordinates analysis was used to visualize potential batch effects (Supplemental Figure 3). Permutational multivariate analysis of variance (PERMANOVA) showed that sequencing batch was significantly associated with Bray–Curtis dissimilarity ($P = 0.001$). However, because the batch was not associated with air pollution exposure, it is unlikely to confound the exposure–outcome relationship. Sensitivity analyses adjusting for batch (data not shown) produced results similar to those of the primary models.

Therefore, to avoid unnecessary loss of statistical power, the batch was excluded from the final models.

Microbiome Data Processing

Trimmomatic[51] (v0.39) was used to validate pairs of paired-end sequence reads, remove adaptors (maximum mismatches = 2; palindrome clip threshold = 30; simple clip threshold = 10), trim reads (leading and trailing quality score ≥20; sliding window trimming with window size = 4 and minimum quality score ≥20), and remove reads with a length less than 50 bp. Hostile[52] (v1.0.0) was used to align trimmed reads against the human genome (index = "human-t2t-hla-argos985"); reads aligning to the human genome were removed. To assign taxonomy, cleaned, trimmed reads were aligned to the RefSeq database of bacterial, viral, plasmid, human, and vector sequences via Kraken 2[53] (v2.1.2; Standard Database, March 14, 2023). Species-level relative abundances of microbes in the samples were predicted using Bracken[54] (v2.9) with default settings. Taxa mapping to nonbacterial kingdoms were removed from further analysis. The average number of reads per sample was 17,625,883 (range: 6–131,105,466). After the removal of samples with read depth below 1,000,000 (n = 2), the average number of reads per sample was 17,667,879 (range: 1,975,654–131,105,466) [Supplemental Figure 4]; in the context of fecal metagenomics, this number of reads can be considered a form of shallow sequencing. To reduce the influence of outlying points, microbiome observations with values greater than or equal to 3 standard deviations above the population mean were considered missing.

Alpha- and Beta-Diversity Measures

Sample sequence read counts, which had previously been assigned taxonomy, were rarefied to 1,000,000 reads per sample. Alpha-diversity metrics (i.e., species richness, Pielou's evenness, and Shannon diversity index) were calculated using the rarefied data. Cameron et al. showed that repeated rarefaction is robust against variation in diversity dependent on library size while minimizing the data loss that occurs with a single rarefaction.[55] Accordingly, rarefaction was repeated 100 times, and the means of the alpha-diversity indices across these iterations were used for subsequent analyses. The same 100 rarefaction iterations were used to calculate beta-diversity among samples. Specifically, Bray–Curtis dissimilarity matrices were calculated for each iteration and averaged across the 100 iterations. The mean Bray–Curtis dissimilarity matrix was then used to derive principal coordinate analysis axes using the pcoa function within the vegan package in R software.

INFANT FECAL METABOLOME

As previously described,[39,42] OmniGene GUT kits were used to collect infant stool samples at 1, 6, 12, 18, and 24 months of age. The Emory Clinical Biomarkers Laboratory conducted untargeted high-resolution analysis using established protocols, as detailed in our earlier studies.[39,42] Briefly, fecal samples were mixed with ice-cold acetonitrile to precipitate proteins, stored on ice for 30 minutes, and centrifuged at 14,000 × g for 10 minutes. The supernatants were stored at 4°C until analysis. Extracts were analyzed in triplicate via liquid chromatography coupled with high-resolution mass spectrometry using a Dionex Ultimate 3000 and Thermo Scientific Orbitrap Fusion system.

Instrumentation and Analytical Conditions

Hydrophilic interaction liquid chromatography (HILIC) was performed using a Waters XBridge BEH Amide XP HILIC column (2.1 × 50 mm, 2.6 µm particle size) with positive electrospray ionization (ESI); reverse-phase (C18) chromatography was conducted using a Higgins Targa C18 column (2.1 × 50 mm, 3 µm particle size) with negative ESI to enhance the detection of metabolic features. For HILIC, analyte separation involved mobile phases of water, acetonitrile, and 2% formic acid, following a gradient elution: 22.5% water, 75% acetonitrile, and 2.5% formic acid for the initial 1.5 minutes; increasing linearly to 75% water, 22.5% acetonitrile, and 2.5% formic acid by 4 minutes; and finally holding for 1 minute. For C18 chromatography, analyte separation used water, acetonitrile, and 10 mM ammonium acetate, with a gradient elution starting at 60% water, 35% acetonitrile, and 5% ammonium acetate for the first minute; increasing linearly to 0% water, 95% acetonitrile, and 5% ammonium acetate by 3 minutes; and finally holding for 2 minutes. The mobile phase flow rate was 0.35 mL/min for the first minute, then increased to 0.4 mL/min for the remaining 4 minutes for both HILIC and C18 columns. Liquid chromatography coupled with high-resolution mass spectrometry was operated in full scan mode at 120k resolution, with a mass-to-charge ratio range of 85 to 1,275. Tuning parameters for sheath gas were set at 45 arbitrary units for positive ESI and 30 for negative ESI. For positive ESI, auxiliary gas was set at 25 arbitrary units and spray voltage at 3.5 kV; for negative ESI, auxiliary gas was set at 5 and spray voltage at –3.0 kV. Internal standards included pooled stool samples and standard reference materials for human metabolites, added at the beginning and end of each 20-sample batch for quality control and standardization.

Metabolite Confidence and Identification

Data from HILIC positive ESI and C18 negative ESI were analyzed separately. Raw files were converted to .mzXML format; metabolomic signals (i.e., metabolic features) were extracted and aligned using the R package apLCMS with modifications from xMSanalyzer to ensure quality control and mitigate batch effects after instrument analysis.[56,57] Briefly, a two-stage approach was used: each batch was initially processed individually, generating a batch-level feature matrix. Across these batch-level matrices, a second round of retention time and feature alignment was conducted. Metabolic features with a coefficient of variation greater than 30 were excluded, and the intensities of metabolic features were averaged across triplicates. Features detected in fewer than

10% of samples were removed from the analysis. Metabolic features were annotated and confirmed based on the Metabolomics Standards Initiative criteria. Level-1 confidence was assigned to features whose mass-to-charge ratio and retention time matched authentic standards analyzed with tandem mass spectrometry under identical conditions (within 10 ppm and 50 seconds). Principal component analysis was used to visually inspect the composition of the fecal metabolome (Supplemental Figure 5).

DATA ANALYSIS

ANALYSIS AIM 1

Cross-Sectional Associations of Air Pollution (Pre- and Postnatal) with the Infant Gut Microbiome and Fecal Metabolome We conducted an extensive analysis of individual cross-sectional associations of pre- and postnatal air pollution exposure with the gut microbiome and fecal metabolome, each described in more detail below. All analyses were conducted using R version 4.2.0. **Figure 1** summarizes the various data analyses and exposure windows utilized in this project.

Gut Microbiome (Aim 1, Cross-Sectional Analyses) We used negative binomial models to examine associations between air pollutant exposure and the infant gut microbiome. To reduce the potential influence of outliers, air pollution exposure values greater than three standard deviations above the population mean were truncated to the mean plus three standard deviations. We examined the cross-sectional associations of (1) prenatal air pollution exposure, (2) long-term postnatal exposure (cumulative exposure from the infant's birth to the study visit date), and (3) short-term postnatal exposure (prior month) with gut bacterial diversity and abundances at each clinical visit. At the 1-month visit, models were adjusted for infant age, infant sex, socioeconomic status, season of visit, mother's age, breastfeedings per day, formula feedings per day, mode of delivery, and maternal prepregnancy BMI. All adjustment sets were determined using a directed acyclic graph (**Figure 2**), where socioeconomic status, season, and mother's age were identified as conventional confounders; infant diet, mode of delivery, and maternal prepregnancy BMI were

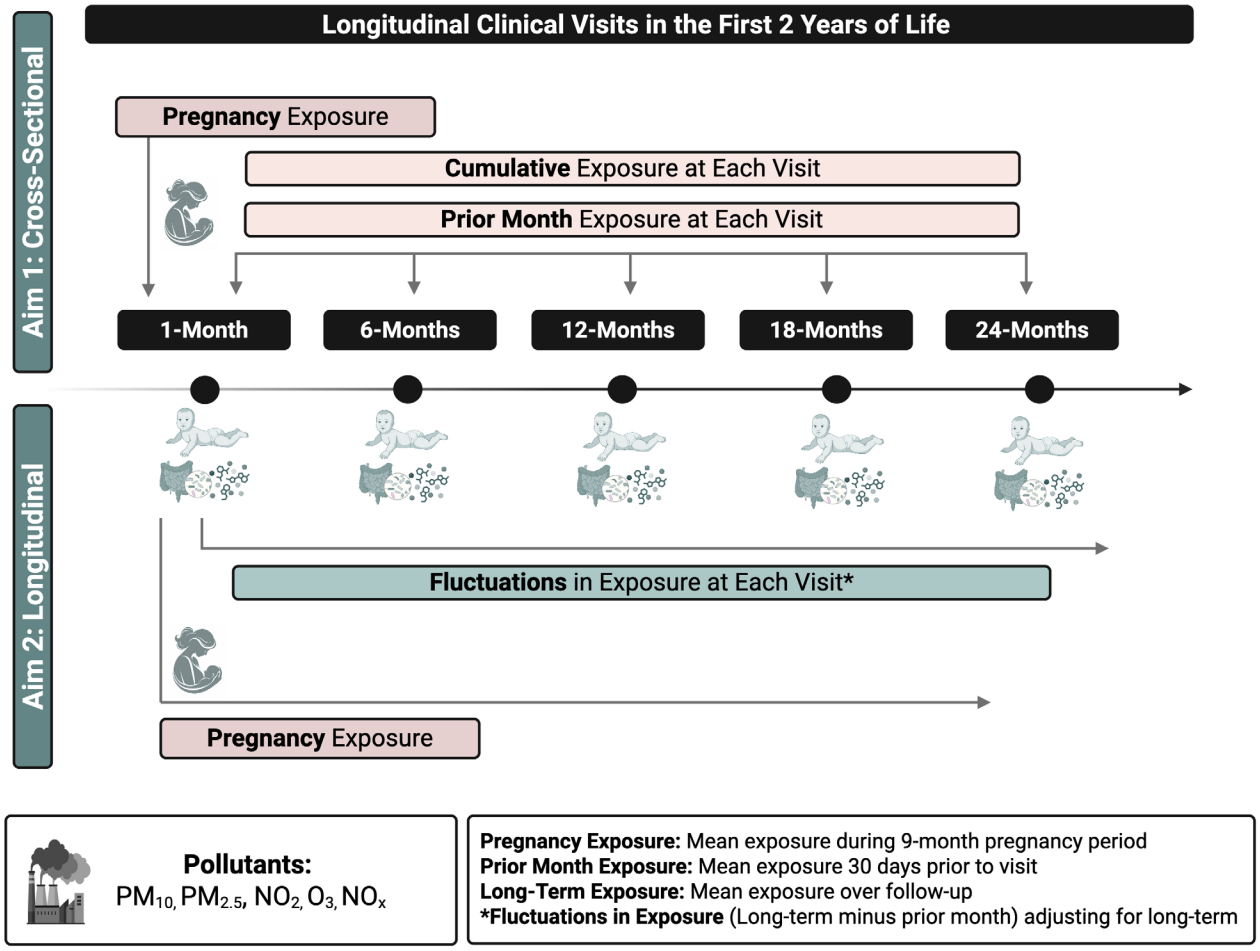


Figure 1. Summary of statistical analyses. Source: Created in BioRender. Holzhausen EA and Alderete TL (2025). *https://BioRender.com/m57g481*.

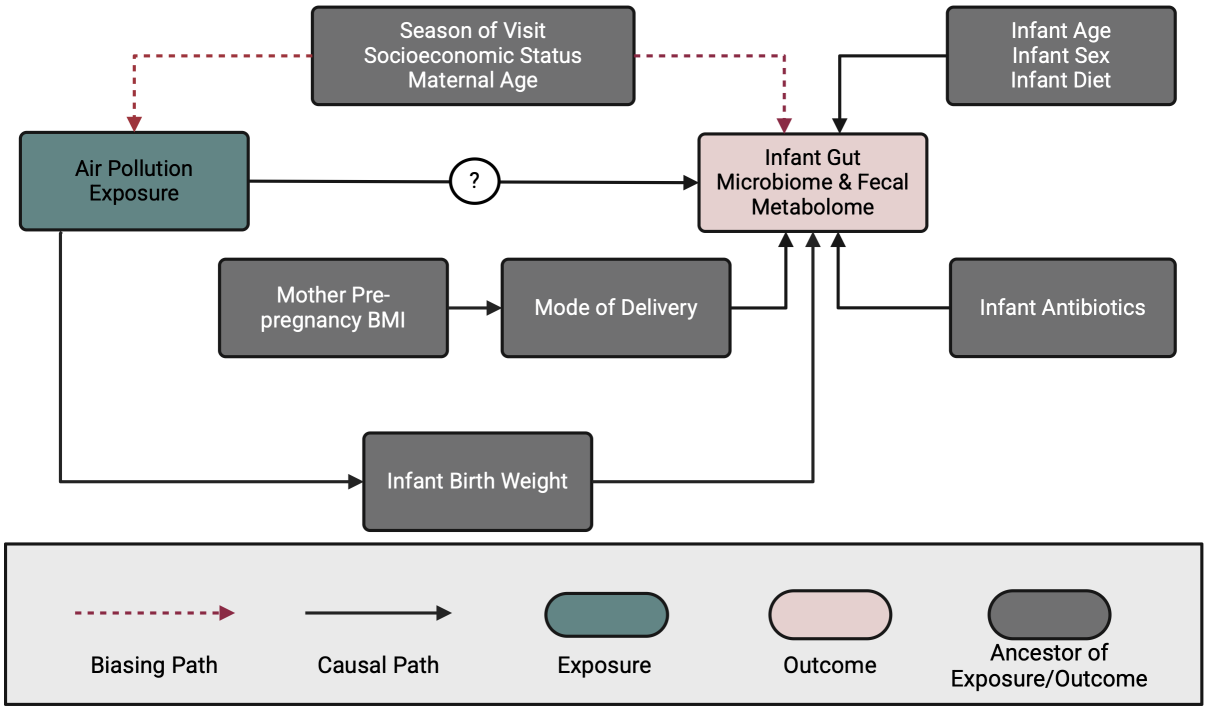


Figure 2. Directed acyclic graph summarizing the data generation process. Based on this directed acyclic graph, we identified season of visit, socioeconomic status, and maternal age as confounders. In addition to these confounders, all analyses were adjusted for infant age, infant sex, infant diet, maternal prepregnancy BMI, and mode of delivery, given their known importance in the development of the infant gut microbiome and fecal metabolome. Source: Created in BioRender. Holzhausen EA and Alderete TL (2025). *https://BioRender.com/r18j290*.

considered precision variables because they are important predictors of the infant microbiome. We did not adjust for infant antibiotic use because — as shown in Figure 2 — antibiotics were regarded as a precision variable, rather than a confounder. Additionally, fewer than 10% of infants were exposed to antibiotics, and our previous studies have shown that adjustments for antibiotic use do not meaningfully affect our findings.[34] We also assessed the overall associations of gut microbiome composition with maternal and infant characteristics via PERMANOVA (Supplemental Table 1). An offset term was included to adjust for the log of the total number of microbial counts for each sample. At the 6-month visit, models were additionally adjusted for whether infants had begun eating solid foods. At all subsequent timepoints, models were also adjusted for diet quality using the infant Healthy Eating Index. The Benjamini–Hochberg (BH) procedure was used to adjust for multiple testing across all microbiome analyses.

Fecal Metabolomics (Aim 1, Cross-Sectional Analyses) We conducted three unique but complementary analyses using multivariable linear models to examine associations of (1) prenatal air pollution exposure, (2) long-term postnatal exposure (cumulative exposure from birth to each respective visit date), and (3) short-term postnatal exposure (defined as air pollution exposure during the month before the study visit) with fecal metabolite intensity at 1, 6, 12, 18, and 24 months. All models were adjusted for infant age, infant sex, socioeconomic status, season of visit, mother's age, and frequency of breastfeeding and formula feedings per day. At the 1-month timepoint, none of the infants had begun eating solid foods; at subsequent timepoints, models were additionally adjusted for whether infants had begun solid food intake. This adjustment set was informed by our previous work in the cohort.[39] The BH procedure was used to adjust for multiple testing in all metabolomics analyses.

ANALYSIS AIM 2

Longitudinal Associations of Air Pollution (Pre- and Postnatal) with the Infant Gut Microbiome and Fecal Metabolome We conducted a longitudinal analysis of the associations of pre- and postnatal air pollution exposure with the gut microbiome and fecal metabolome, each described in more detail below. All analyses were performed using R version 4.2.0.

Gut Microbiome (Aim 2, Longitudinal Analyses) To visualize the overall composition of the microbiome samples, we used principal coordinates analysis (Supplemental Figure 6). Next, we utilized longitudinal negative binomial models to assess the association between prenatal air pollution exposure and the postnatal gut microbiome (i.e., taxonomic abundance). These models included an offset for the log-transformed total number of sequence counts and a random intercept to adjust for repeated measures within infants. Models were also

adjusted for infant age and sex, socioeconomic status, season, mother's age, human milk and formula feeding frequencies, mode of delivery, maternal prepregnancy BMI, infant mean Healthy Eating Index, and whether infants had begun eating solid foods. Covariates were selected based on our previous work and a review of the literature.[34,43]

We used linear mixed-effects models to assess the longitudinal association between postnatal air pollution exposure and gut microbiome alpha-diversity, incorporating a random intercept to adjust for repeated measures within individuals. We used negative binomial models to evaluate the association between fluctuations in postnatal air pollution exposure (i.e., the difference between the grand mean of prior-month air pollution exposure from 1 month to 2 years of infant age and the prior month's exposure at each timepoint) and the gut microbiome (i.e., taxonomic abundance), with an offset for the log of total sequence counts and a random intercept for repeated measures. To reduce the influence of outliers, air pollution exposure values greater than three standard deviations above the population mean were truncated to the mean plus three standard deviations. Models were adjusted for infant age and sex, socioeconomic status, season of the study visit, mother's age, frequency of human milk and formula feedings per day, mode of delivery, maternal prepregnancy BMI, whether infants had begun eating solid foods (yes/no), and the mean Healthy Eating Index after introduction of solid foods. Models were also adjusted for long-term air pollution exposure (i.e., the grand mean of each participant's pollutant exposure across all study visits). Analyses included all taxonomic levels (i.e., phylum, class, order, family, genus, and species).

Fecal Metabolome (Aim 2, Longitudinal Analyses) We used linear mixed-effects models to assess whether prenatal air pollution exposure was longitudinally associated with the log-transformed intensity of fecal metabolic features, incorporating a random intercept to adjust for repeated measures. Models were adjusted for infant age and sex, socioeconomic status, season of study visit, mother's age, frequency of human milk and formula feedings per day, and whether infants had begun eating solid foods.

Next, we used linear mixed-effects models to assess whether fluctuations in postnatal air pollution exposure (i.e., the difference between the grand mean of prior-month air pollution exposure from 1 month to 2 years of infant age and the prior month's exposure at each timepoint) were associated with postnatal fecal metabolite intensity. Because we aimed to independently assess the associations of fluctuations in air pollution exposure and long-term air pollution exposure with fecal metabolites, we also adjusted models for long-term mean air pollution exposure. Our adjustment set, informed by previous analyses,[39] included infant age and sex, socioeconomic status, season of the study visit, mother's age, frequency of human milk and formula feedings per day, and whether infants had begun eating solid foods.

RESULTS

AIM 1 GUT MICROBIOME

Population characteristics of participants included in the microbiome analyses are described in **Table 1**. Briefly, participants attended study visits at approximately 1, 6, 12, 18, and 24 months of infant age. At the 1-month study visit, the average infant age was 33 ± 5 days, 46% of infants were male, and 25% had been born by cesarean section. Changes in non-time-varying characteristics over time reflect missing follow-up data for some participants. Overall, gut microbiome data were available for 196 infants at 1 month of age, 157 at 6 months, 155 at 12 months, 143 at 18 months, and 171 at 24 months. At 1 month, parents reported an average of 6.7 ± 2.2 breastfeedings per day. At 6 months, parents reported that infants had begun eating solid foods at an average age of 6.0 ± 1.8 months.

Prenatal Air Pollution Exposure and the Gut Microbiome

We assessed whether prenatal air pollution exposure was associated with abundances of taxa (i.e., phylum, class, order, family, genus, and species) at 1 month of infant age (**Figure 3**). We found that prenatal exposure to air pollution was associated with differences in taxonomic abundance. Figure 3 summarizes the observed associations: each concentric circle in the figure represents a different taxonomic level, with the kingdom at the center and the species at the outer edge. Statistically significant associations (P_{BH} <0.2) are shown in red (negative associations) and blue (positive associations), with darker shading indicating larger beta estimates. For example, we found that higher prenatal PM_{10} exposure was associated with lower abundances of beneficial microbes, including 10 species from the genus *Bifidobacterium*. Similarly, higher prenatal NO_2 and NO_x exposures were both associated with lower abundances of several *Bifidobacterium* species. Higher prenatal NO_x exposure was additionally associated with higher abundances of *Lelliottia amnigena* and *Dorea longicatena*. Plots illustrating the observed statistically significant associations between prenatal air pollution exposure and microbial species abundances can be found in Supplemental File 1 [PM_{10}], Supplemental File 2 [$PM_{2.5}$], Supplemental File 3 [NO_2], Supplemental File 4 [O_3], and Supplemental File 5 [NO_x].

Cumulative Air Pollution Exposure and the Gut Microbiome

We next explored whether there were associations between gut microbiome alpha-diversity (i.e., Shannon, richness, evenness, and Simpson indices) and cumulative air pollution exposure (PM_{10}, $PM_{2.5}$, NO_2, O_3, $O_3 + NO_2$, and total NO_x), where cumulative air pollution exposure was defined as the total exposure from birth to the visit date (**Table 2**). We found that PM_{10} exposure was associated with higher Shannon diversity, evenness, and Simpson diversity at 1 month (b = 0.02, 0.002, 0.005; P = 0.04, 0.02, 0.02, respectively). At 1 month, O_3

Table 1. Characteristics of Mother–Infant Dyads with Infant Microbiome Data from the Southern California Mother's Milk Study, Enrollment from 2016 to 2019[a]

Variable	1 Month (*N* = 196)	6 Months (*N* = 157)	12 Months (*N* = 155)	18 Months (*N* = 143)	24 Months (*N* = 171)
Maternal characteristics	**Mean ± SD; *n* (%)**	**Mean ± SD; *n* (%)**	**Mean ± SD; *n* (%)**	**Mean ± SD; *n* (%)**	**Mean ± SD; *n* (%)**
Age (years)	29.0 ± 3.1	29.4 ± 6.2	29.8 ± 6.2	30.3 ± 6.2	30.8 ± 6.3
Prepregnancy BMI (kg/m^2)[b]	28.3 ± 5.7	28.8 ± 6.2	28.5 ± 5.7	28.3 ± 5.7	28.5 ± 5.8
SES[b,c]	26.8 ± 12.4	26.0 ± 11.9	26.2 ± 11.9	26.7 ± 12.6	27.2 ± 12.1
Antibiotics since delivery (yes, no, %yes)[b]	20, 171, 10.5%	14, 136, 9.3%	15, 133, 10.1%	14, 124, 10.1%	15, 148, 9.2%
Infant characteristics					
Age (days)	32.6 ± 4.7	185.5 ± 8.8	368.1 ± 10.4	551.5 ± 18.3	753.5 ± 46.8
Sex[b] (male, female, %male)	90, 106, 45.9%	73, 84, 46.5%	72, 83, 46.5%	65, 78, 45.5%	78, 93, 45.6%
Delivery mode[b] (CS, vaginal, %CS)	48, 148, 24.5%	40, 115, 25.8%	38, 116, 24.7%	32, 110, 22.5%	39, 129, 23.2%
Breastfeedings per day	6.7 ± 2.2	3.1 ± 3.3	2.1 ± 2.8	1.9 ± 2.7	1.7 ± 2.4
Formula feedings per day	2.2 ± 2.6	3.0 ± 2.9	2.3 ± 2.4	1.0 ± 1.7	1.1 ± 1.9
Age at solid food introduction (months)[b]	--	6.0 ± 1.8	5.8 ± 1.5	5.9 ± 1.6	5.9 ± 1.7
Healthy Eating Index	--	46.0 ± 6.7	60.8 ± 9.2	67.2 ± 9.9	68.1 ± 9.5
Antibiotics since birth[b] (yes, no, %yes)	19, 176, 9.7%	14, 140, 9.1%	15, 138, 9.8%	14, 128, 9.9%	18, 149, 10.8%
Season of visit[d] (warm, cool, %warm)	98, 98, 50%	74, 83, 47.1%	89, 66, 57.4%	70, 73, 49.0%	82, 89, 48.0%
Air pollution measures					
Prenatal PM_{10} ($\mu g/m^3$)b	29.8 ± 4.1	30.4 ± 3.7	29.8 ± 3.9	30.2 ± 40.1	29.5 ± 3.9
Prenatal $PM_{2.5}$ ($\mu g/m^3$)[b]	11.9 ± 1.3	12.1 ± 1.3	11.8 ± 1.1	12.0 ± 1.3	11.8 ± 1.2
Prenatal NO_2 (ppb)[b]	17.9 ± 2.8	18.0 ± 2.5	18.0 ± 2.8	18.2 ± 2.6	17.8 ± 2.7
Prenatal O_3 (ppb)[b]	42.6 ± 3.9	42.7 ± 3.9	42.8 ± 3.4	43.0 ± 3.8	42.5 ± 3.8
Prenatal NO_x (ppb)[b]	3.9 ± 2.1	3.9 ± 2.1	4.0 ± 2.2	4.0 ± 2.2	3.9 ± 2.1
Individual mean postnatal PM_{10} ($\mu g/m^3$)[b]	28.9 ± 3.9	28.5 ± 3.8	29.4 ± 3.3	29.2 ± 3.0	28.7 ± 3.8
Individual mean postnatal $PM_{2.5}$ ($\mu g/m^3$)[b]	11.8 ± 1.9	11.8 ± 1.7	11.9 ± 1.8	11.8 ± 1.7	11.9 ± 1.7
Individual mean postnatal NO_2 (ppb)[b]	16.3 ± 3.2	16.6 ± 2.4	16.7 ± 2.7	16.7 ± 2.5	16.5 ± 2.7
Individual mean postnatal O_3 (ppb)[b]	42.7 ± 5.0	42.4 ± 4.1	42.4 ± 4.5	41.8 ± 4.3	42.6 ± 4.8
Individual mean postnatal NO_x (ppb)[b]	3.9 ± 2.5	4.0 ± 2.6	3.5 ± 1.8	3.5 ± 1.9	4.2 ± 3.2
Fluctuation in PM_{10} ($\mu g/m^3$)	2.0 ± 6.3	1.9 ± 5.9	1.1 ± 5.2	-3.4 ± 5.1	-3.4 ± 5.6
Fluctuation in $PM_{2.5}$ ($\mu g/m^3$)	0.2 ± 3.1	0.7 ± 3.8	0.1 ± 2.9	-0.8 ± 2.8	-0.3 ± 2.7
Fluctuation in NO_2 (ppb)	1.3 ± 5.4	1.5 ± 7.3	-0.9 ± 4.9	-0.9 ± 6.3	-1.3 ± 4.3
Fluctuation in O_3 (ppb)	0.7 ± 6.5	-0.5 ± 7.8	0.2 ± 5.2	-0.7 ± 8.1	0.1 ± 6.1
Fluctuation in NO_x (ppb)	-0.3 ± 1.9	-0.5 ± 1.6	-0.1 ± 0.5	0.1 ± 0.8	0.9 ± 3.2
Cumulative PM_{10} ($\mu g/m^3$)	30.7 ± 6.9	30.9 ± 5.2	31.8 ± 3.6	30.7 ± 3.0	29.2 ± 3.4
Cumulative $PM_{2.5}$ ($\mu g/m^3$)	11.7 ± 2.6	12.3 ± 2.0	12.5 ± 1.3	12.2 ± 1.1	12.2 ± 0.8
Cumulative NO_2 (ppb)	17.5 ± 6.8	18.3 ± 5.0	18.2 ± 2.7	17.7 ± 2.6	17.2 ± 2.1
Cumulative O_3 (ppb)	43.3 ± 8.1	41.8 ± 4.6	42.1 ± 3.2	41.4 ± 2.9	41.9 ± 2.8
Cumulative NO_x (ppb)	3.4 ± 1.5	3.3 ± 1.4	3.3 ± 1.3	3.2 ± 1.3	3.3 ± 1.3
Prior-month PM_{10} ($\mu g/m^3$)	30.9 ± 7.4	30.5 ± 6.8	30.5 ± 6.9	25.9 ± 4.9	25.7 ± 7.0
Prior-month $PM_{2.5}$ ($\mu g/m^3$)	12.0 ± 3.7	12.5 ± 4.3	12.0 ± 3.4	11.0 ± 2.6	11.6 ± 3.4
Prior-month NO_2 (ppb)	17.6 ± 7.1	18.1 ± 6.9	15.9 ± 6.3	15.7 ± 5.3	15.2 ± 5.5
Prior-month O_3 (ppb)	43.4 ± 8.4	42.0 ± 8.1	42.6 ± 7.7	41.1 ± 8.1	42.7 ± 8.4
Prior-month NO_x (ppb)	3.5 ± 1.7	3.5 ± 1.7	3.3 ± 1.5	3.5 ± 2.0	3.6 ± 2.1

CS = cesarean section; ppb = parts per billion; SD = standard deviation; SES = socioeconomic status.

[a]Data are reported as mean and SD unless otherwise noted.

Table 1. (*continued*)

[b]Non-time-varying (i.e., assessed at a single timepoint and not expected to change over time); differences across time are due to participant missingness at specific timepoints ($P_{all} \geq 0.2$).
[c]SES was estimated using a modified version of the Hollingshead Index. Range for study population: 3–63.
[d]Study visits occurring between April 1 and September 30 were considered warm season; all other visits were considered cool season.

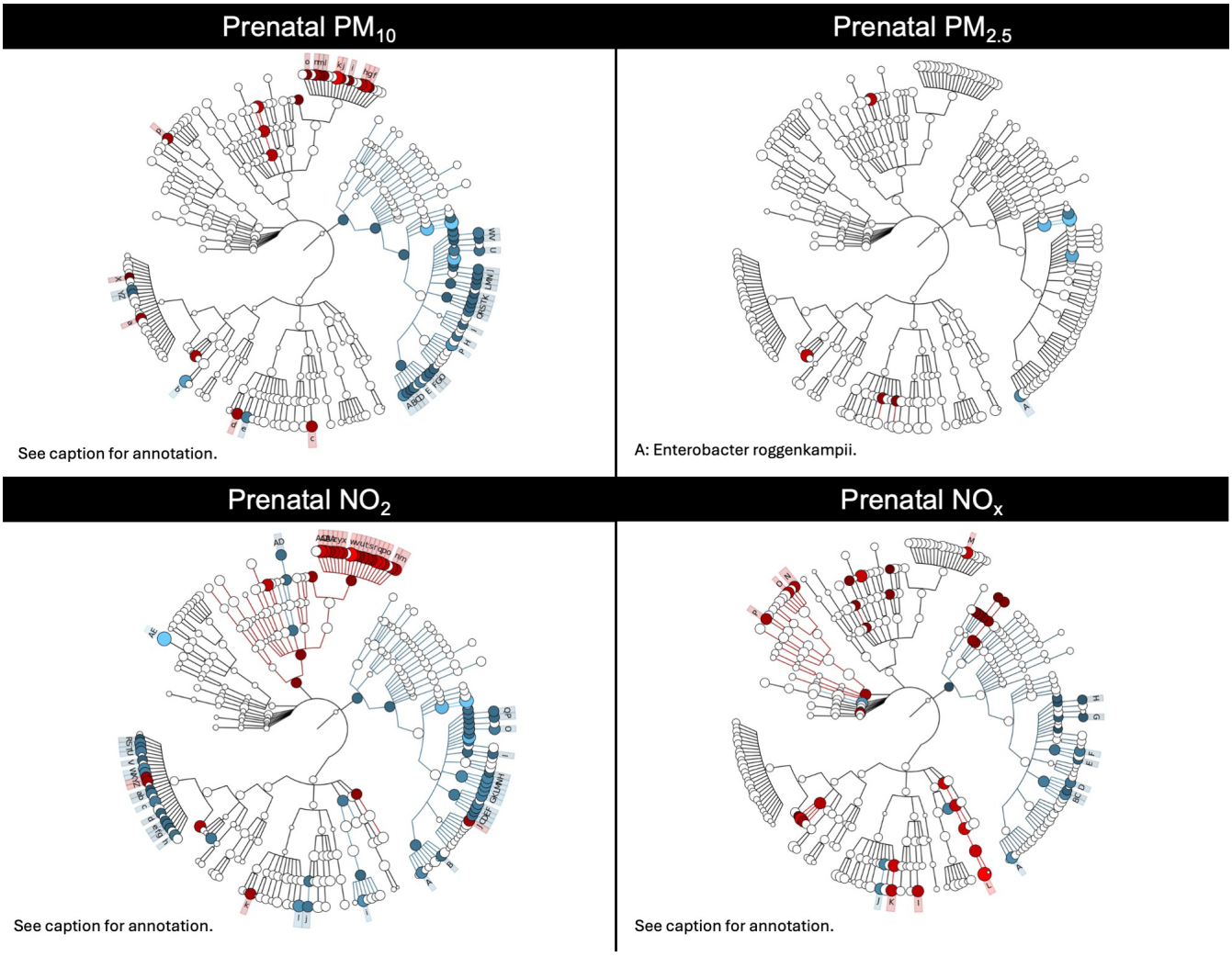


Figure 3. Associations of prenatal PM_{10}, $PM_{2.5}$, NO_2, and NO_x exposures with gut microbial taxa at 1 month. Estimates were obtained using negative binomial models where the exposure of interest was prenatal air pollution exposure, and the outcome was the abundance of each gut microbial taxon at 1 month of infant age. Models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeeding frequency, formula feeding frequency, mode of delivery, and maternal prepregnancy BMI. An offset was included to control for the log of the total number of microbial counts in each sample.

Annotations for PM_{10} species: A: Enterobacter roggenkampii, B: E. asburiae, C: E. mori, D: E. cloacae, E: E. cancerogenus, F: E. ludwigii, G: E. hormaechei, H: Citrobacter freundii, I: C. sp. R56, J: Cronobacter sakazakii, K: Shigella flexneri, L: S. dysenteriae, M: Leclercia adecarboxylata, N: L. sp. Colony189, O: Klebsiella aerogenes, P: K. grimontii, Q: Escherichia fergusonii, R: E. marmotae, S: E. coli, T: E. albertii, U: Cedecea neteri, V: Salmonella enterica, W: Lelliottia amnigena, X: Streptococcus agalactiae, Y: S. vestibularis, Z: S. sp. HSISM1, a: S. anginosus, b: Enterococcus faecalis, c: Clostridium perfringens, d: Blautia wexlerae, e: Mediterraneibacter gnavus, f: Bifidobacterium eulemuris, g: B. sp. TKU, h: B. longum, i: B. thermophilum, j: B. lemurum, k: B. bifidum, l: B. saguini, m: B. choerinum, n: B. adolescentis, o: B. asteroides, p: Phocaeicola dorei.

Annotations for NO_2 species: A: Enterobacter asburiae, AA: Bifidobacterium adolescentis, AB: B. subtile, AC: B. asteroides, AD: Rothia mucilaginosa, AE: Segatella copri, B: Enterobacter ludwigii, C: Citrobacter freundii, D: C. amalonaticus, E: C. portucalensis, F: C. sp. R56, G: C. braakii, H: Shigella flexneri, I: Leclercia sp. Colony189, J: Klebsiella michiganensis, K: Escherichia fergusonii, L: E. marmotae, M: E. coli, N: E. albertii, O: Cedecea neteri, P: Salmonella enterica, Q: Lelliottia amnigena, R: Streptococcus pneumoniae, S: S. sp. LPB0220, T: S. lactarius, U: S. constellatus, V: S. sp. A12, W: S. vestibularis, X: S. sp. HSISM1, Y: S. sp. HSISS2, Z: S. sp. HSISS3, a: S. mitis, b: S. gordonii, c: S. parasanguinis, d: S. oralis, e: S. sp. oral taxon 431, f: S. ilei, g: S. suis, h: S. australis, i: Veillonella parvula, j: Faecalibacterium prausnitzii, k: Blautia wexlerae, l: Enterocloster bolteae, m: Bifidobacterium pullorum, n: B. pseudolongum, o: B. eulemuris, p: B. sp. TKU, q: B. longum, r: B. catenulatum, s: B. angulatum, t: B. thermophilum, u: B. animalis, v: B. lemurum, w: B. bifidum, x: B. scardovii.

Annotations for NO_x species: A: Enterobacter asburiae, B: Citrobacter portucalensis, C: C. sp. R56, D: C. braaki, E: Shigella flexneri, F: S. dysenteriae, G: Cedecea neteri, H: Lelliottia amnigena, I: Clostridium perfringens, J: Dorea longicatena, K: Enterocloster bolteae, L: Thomasclavelia ramosa, M: Bifidobacterium longum, N: Bacteroides thetaiotaomicron, O: B. uniformis, P: Parabacteroides distasonis.

Table 2. Cross-Sectional Associations of Cumulative Air Pollution Exposure with Gut Microbiome Alpha-Diversity at 1, 6, 12, 18, and 24 Months of Infant Age[a]

	1 Month		6 Months		12 Months		18 Months		24 Months	
	b **(SE)**	**P**	***b*** **(SE)**	**P**	***b*** **(SE)**	**P**	***b*** **(SE)**	**P**	***b*** **(SE)**	**P**
Shannon										
PM_{10}	0.02 (0.008)	0.04	–0.003 (0.01)	0.8	0.02 (0.01)	0.2	–0.006 (0.02)	0.7	–0.02 (0.01)	0.1
$PM_{2.5}$	0.02 (0.02)	0.3	0.01 (0.03)	0.6	0.002 (0.04)	1.0	–0.02 (0.04)	0.7	0.06 (0.04)	0.1
NO_2	0.01 (0.01)	0.4	0.01 (0.01)	0.3	0.005 (0.02)	0.8	0.004 (0.02)	0.8	–0.001 (0.02)	1.0
O_3	0.01 (0.008)	0.1	–0.01 (0.01)	0.3	0.01 (0.02)	0.5	–0.02 (0.02)	0.7	–0.009 (0.01)	0.5
$O_3 + NO_2$	0.02 (0.009)	0.1	–0.008 (0.02)	0.6	0.01 (0.02)	0.5	–0.02 (0.02)	0.4	–0.01 (0.01)	0.5
Total NO_x	0.02 (0.04)	0.7	–0.08 (0.04)	0.03	0.04 (0.04)	0.3	0.02 (0.04)	0.7	–0.03 (0.03)	0.4
Richness										
PM_{10}	7.6 (12.0)	0.5	–5.6 (19.3)	0.8	41 (31.9)	0.2	–21.2 (39.23)	0.6	–36.63 (26.02)	0.2
$PM_{2.5}$	9.2 (31.3)	0.8	2.4 (48.8)	1.0	12.4 (83.5)	0.9	–46.9 (98.3)	0.6	86.66 (97.69)	0.4
NO_2	10.0 (20.1)	0.6	11.9 (20.1)	0.6	33.8 (45.7)	0.5	19.3 (43.8)	0.7	12.85 (43.49)	0.8
O_3	8.1 (11.6)	0.5	–3.4 (23.8)	0.9	40.2 (38.9)	0.3	–35.3 (42.4)	0.4	–12.07 (31.83)	0.7
$O_3 + NO_2$	11.6 (12.4)	0.4	7.5 (29.4)	0.8	40.7 (38.9)	0.3	–34.0 (48.3)	0.5	–11.56 (31.99)	0.7
Total NO_x	–13.8 (54.8)	0.8	–108.5 (71.8)	0.1	75.1 (87.0)	0.4	112.8 (93.1)	0.2	36.31 (66.23)	0.6
Evenness										
PM_{10}	0.002 (0.0009)	0.02	–0.0002 (0.001)	0.9	0.002 (0.001)	0.2	–0.0004 (0.002)	0.8	–0.002 (0.001)	0.1
$PM_{2.5}$	0.003 (0.002)	0.2	0.002 (0.003)	0.5	0.0004 (0.004)	0.9	–0.002 (0.004)	0.7	0.007 (0.004)	0.1
NO_2	0.001 (0.002)	0.4	0.002 (0.001)	0.2	0.001 (0.002)	0.9	0.0003 (0.002)	0.9	–0.0003 (0.002)	0.9
O_3	0.002 (0.0009)	0.1	–0.002 (0.001)	0.2	0.001 (0.002)	0.5	–0.002 (0.002)	0.4	–0.001 (0.001)	0.5
$O_3 + NO_2$	0.002 (0.001)	0.03	–0.001 (0.002)	0.5	0.001 (0.002)	0.5	–0.002 (0.002)	0.4	–0.001 (0.001)	0.5
Total NO_x	0.002 (0.004)	0.6	–0.009 (0.004)	0.03	0.004 (0.004)	0.4	0.0008 (0.004)	0.9	–0.004 (0.003)	0.2
Simpson										
PM_{10}	0.005 (0.002)	0.02	0.001 (0.002)	0.6	0.003 (0.002)	0.1	0.0009 (0.002)	0.7	–0.001 (0.001)	0.3
$PM_{2.5}$	0.007 (0.005)	0.2	0.008 (0.006)	0.2	–0.001 (0.006)	0.8	–0.003 (0.005)	0.6	0.01 (0.004)	0.02
NO_2	0.001 (0.003)	0.7	0.005 (0.002)	0.06	0.001 (0.003)	0.7	0.0006 (0.002)	0.8	0.001 (0.002)	0.6
O_3	0.004 (0.002)	0.04	–0.005 (0.003)	0.1	0.005 (0.003)	0.1	–0.002 (0.002)	0.4	–0.002 (0.001)	0.2
$O_3 + NO_2$	0.004 (0.002)	0.02	–0.003 (0.003)	0.5	0.005 (0.003)	0.1	–0.002 (0.003)	0.4	–0.002 (0.001)	0.2
Total NO_x	0.003 (0.009)	0.8	–0.02 (0.009)	0.02	0.006 (0.006)	0.3	0.003 (0.005)	0.6	–0.003 (0.003)	0.2

b = beta estimate; SE = standard error.

[a]Estimates were generated using linear models in which the outcome of interest was alpha-diversity (i.e., Shannon, richness, evenness, and Simpson indices) at the 1-, 6-, 12-, 18-, and 24-month study visits; the predictor of interest was cumulative air pollution exposure from birth to each study visit. The 1-month models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, and maternal prepregnancy BMI. At the 6-month timepoint, models were additionally adjusted for the introduction of solid foods; at subsequent timepoints, models were also adjusted for the infant Healthy Eating Index. Bolded cells indicate statistical significance ($P < 0.05$).

Table 3. Cross-Sectional Associations of Quartile of Cumulative Air Pollution Exposure with Overall Microbiome Composition (Estimated Via Bray–Curtis Dissimilarity)

	1 Month		6 Months		12 Months		18 Months		24 Months	
	R^{2a}	P	R^{2a}	P	R^{2a}	P	R^{2a}	P	R^{2a}	P
PM_{10}	0.02	0.2	0.02	0.8	0.02	0.2	0.02	1.0	0.02	0.2
$PM_{2.5}$	0.02	0.4	0.02	0.3	0.02	0.3	0.02	0.9	0.02	0.5
NO_2	0.02	0.5	0.01	1.0	0.02	0.2	0.02	0.9	0.03	0.005
O_3	0.02	0.1	0.01	1.0	0.03	0.1	0.02	0.6	0.02	0.7
$O_3 + NO_2$	0.03	0.3	0.03	1.0	0.05	0.2	0.04	0.9	0.04	0.03
Total NO_x	0.01	0.7	0.03	0.06	0.02	0.5	0.02	0.7	0.02	0.03

[a] R^2 represents the proportion of variance in Bray–Curtis dissimilarity explained by quartile of cumulative air pollution exposure (i.e., from birth to each study visit) at 1, 6, 12, 18, and 24 months of infant age. Results were unadjusted, except for the $O_3 + NO_2$ model, in which cumulative exposure to NO_2 was included as a covariate. Bolded cells indicate statistical significance ($P < 0.05$).

exposure was associated with higher Simpson diversity both before and after adjustment for NO_2 ($b_{both} = 0.004$, $P_{both} < 0.04$). After adjustment for NO_2, O_3 was also associated with higher evenness ($b = 0.002$, $P = 0.03$). At 6 months, total NO_x exposure was associated with lower Shannon diversity, evenness, and Simpson diversity ($b = –0.08, –0.009, –0.02$; $P = 0.03, 0.03, 0.02$, respectively). We did not observe statistically significant associations between alpha-diversity measures and cumulative air pollution exposure at 12, 18, or 24 months of infant age.

Next, we used PERMANOVA to estimate the proportion of variability in overall gut microbiome composition (determined via Bray–Curtis dissimilarity) attributable to quartiles of cumulative air pollution exposure at each timepoint (**Table 3**). We found that NO_2 exposure at 24 months of age explained 3% of the variability in Bray–Curtis dissimilarity ($P = 0.005$). We also found that the model including both NO_2 and O_3 was significant, explaining 4% of the variability in Bray–Curtis dissimilarity ($P = 0.03$). We did not observe statistically significant associations between overall microbiome composition and cumulative air pollution exposure for other pollutants or at other study visits.

We subsequently used negative binomial models to assess whether the abundances of gut microbial species were cross-sectionally associated with cumulative air pollution exposure (**Table 4**). The greatest number of statistically significant associations (i.e., $P_{BH} < 0.2$) was observed between cumulative PM_{10} exposure and gut microbial species abundances at 1 month of infant age — there were 37 significant associations.

In **Figure 4**, we summarize the associations of cumulative $PM_{2.5}$ exposure with gut microbial taxa abundances at 1, 6, 18, and 24 months of infant age using dendrograms, where each branch represents a different taxonomic level; annotations are added at the species level. Although we did not

Table 4. Numbers of Statistically Significant Cross-Sectional Associations Between Cumulative Air Pollution Exposure and Gut Microbial Species at 1, 6, 12, 18, and 24 Months of Infant Age[a]

	1 Month		6 Months		12 Months		18 Months		24 Months	
	$P_{BH} < 0.2$	$P_{BH} < 0.05$	$P_{BH} < 0.2$	$P_{BH} < 0.05$	$P_{BH} < 0.2$	$P_{BH} < 0.05$	$P_{BH} < 0.2$	$P_{BH} < 0.05$	$P_{BH} < 0.2$	$P_{BH} < 0.05$
PM_{10}	37	3	6	1	21	3	5	2	0	0
$PM_{2.5}$	0	0	8	0	4	1	14	3	33	18
NO_2	20	3	10	4	10	3	16	8	3	3
O_3	17	3	3	1	16	8	13	4	7	2
$O_3 + NO_2$	32	10	6	3	22	10	5	1	22	15
Total NO_x	8	1	6	2	12	2	19	4	17	5

[a] Cumulative air pollution exposure was defined as the total exposure from birth to each timepoint (i.e., 1, 6, 12, 18, and 24 months). Cells indicate numbers of statistically significant associations after correction for multiple testing using the BH method at thresholds of $P_{BH} < 0.2$ and $P_{BH} < 0.05$. Results were generated using negative binomial models, in which the outcome was the abundance of each gut microbial species. The 1-month models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, and maternal prepregnancy BMI; an offset was included to control for the log of total microbial counts in each sample. At the 6-month timepoint, models were additionally adjusted for the introduction of solid foods; at subsequent timepoints, models were also adjusted for the infant Healthy Eating Index. The numbers of species analyzed were 132 at 1 month, 188 at 6 months, 370 at 12 months, 541 at 18 months, and 802 at 24 months.

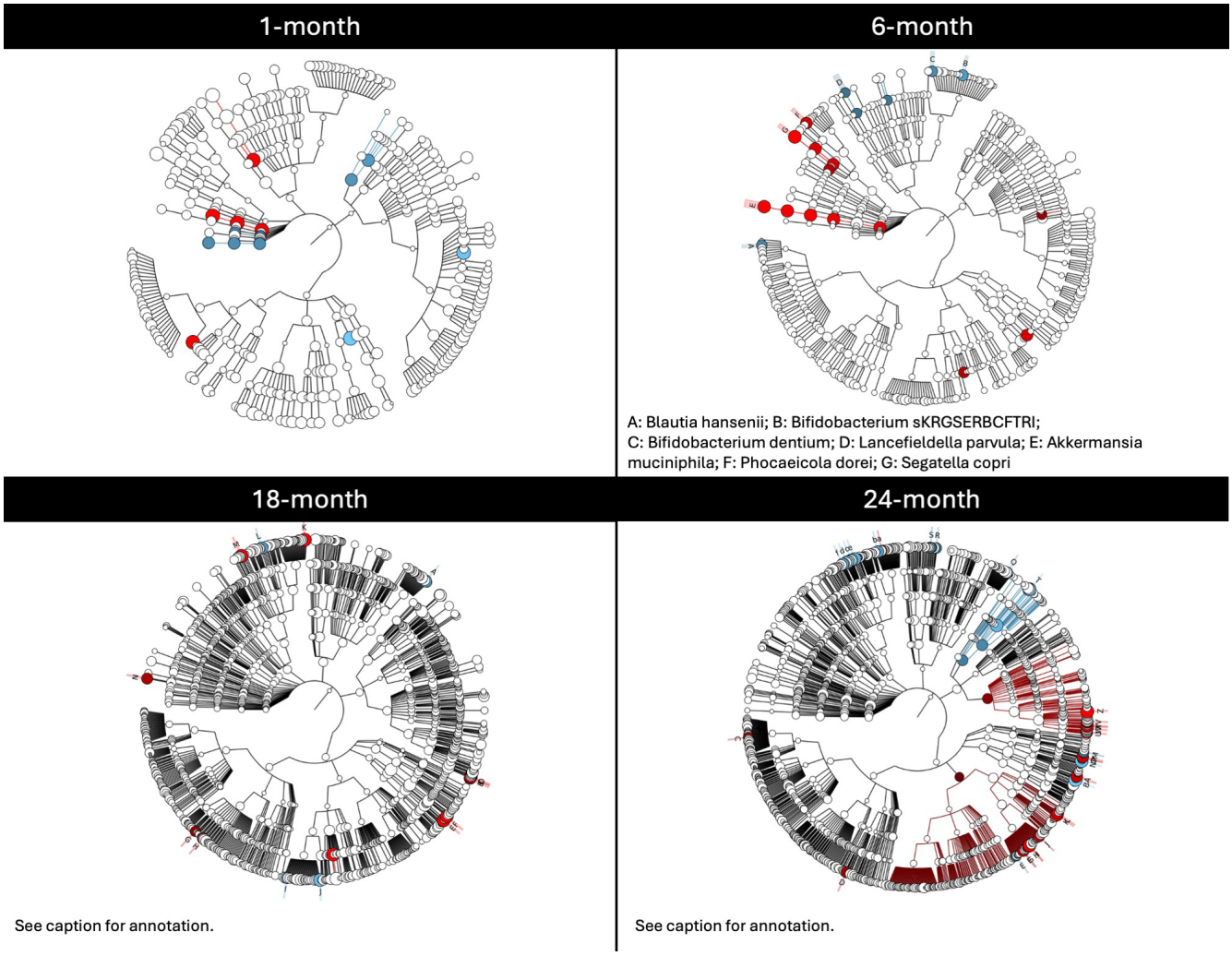


Figure 4. Associations of cumulative $PM_{2.5}$ exposure from birth to study visit with gut microbial taxa at 1, 6, 18, and 24 months of infant age. Estimates were obtained using negative binomial models in which the exposure of interest was cumulative PM2.5 exposure and the outcome was the abundance of each gut microbial taxon. The 1-month models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, and maternal prepregnancy BMI; an offset was included to control for the log of total microbial counts in each sample. At the 6-month timepoint, models were additionally adjusted for the introduction of solid foods; at subsequent timepoints, models were also adjusted for the infant Healthy Eating Index. Negative associations are shown in red and positive associations in blue; darker colors indicate stronger associations. Annotations are provided at the species level.

Annotations for 18-month species: A: Bifidobacterium angulatum; B: Klebsiella quasipneumoniae; C: Klebsiella variicola; D: Klebsiella pneumoniae; E: Megamonas funiformis; F: Phascolarctobacterium succinatutens; G: Lachnoclostridium sp. YL32; H: Enterocloster clostridioformis; I: Streptococcus anginosus; J: Streptococcus lutetiensis; K: Bacteroides intestinalis; L: Paraprevotella xylaniphila; M: Butyricimonas virosa; N: Fusobacterium ulcerans.

Annotations for 24-month species: A: Acidaminococcus intestini; B: Phascolarctobacterium succinatutens; C: Clostridium baratii; D: Romboutsia sp. CE17; E: Streptococcus anginosus; F: Streptococcus equinus; G: Streptococcus lactarius; H: Streptococcus sp. HSISS2; I: Streptococcus sp. HSISS3; J: Streptococcus thermophilus; K: Enterococcus avium; L: Enterococcus faecalis; M: Finegoldia magna; N: Catenibacterium mitsuokai; O: Turicibacter sanguinis; P: Turicibacter bilis; Q: Bifidobacterium adolescentis; R: Eggerthella sp. YY7918; S: Berryella intestinalis; T: Sutterella wadsworthensis; U: Klebsiella variicola; V: Citrobacter portucalensis; W: Enterobacter cloacae; X: Enterobacter hormaechei; Y: Enterobacter asburiae; Z: Raoultella ornithinolytica; a: Bacteroides nordii; b: Bacteroides caccae; c: Alistipes dispar; d: Alistipes ihumii; e: Paraprevotella xylaniphila; f: Parabacteroides goldsteinii.

observe statistically significant associations between $PM_{2.5}$ and species abundances at 1 month, we observed several associations between $PM_{2.5}$ exposure and gut microbial species abundances at 6, 18, and 24 months of age. For example, postnatal exposure to $PM_{2.5}$ was inversely associated with *Akkermansia muciniphila* abundance at 6 months. At 24 months, cumulative $PM_{2.5}$ exposure was inversely associated with the abundance of *Romboutsia* sp. CE17 and positively associated with the abundances of *Alistipes dispar* and *Alistipes ihumii*. Plots illustrating the observed statistically significant associations between $PM_{2.5}$ exposure and microbial species abundances can be found in Supplemental File 6 [6 months], Supplemental File 7 [18 months], and Supplemental File 8 [24 months].

Associations of cumulative NO_x exposure with gut microbial taxa at 1, 6, 18, and 24 months of infant age are summarized in **Figure 5**. Each branch in the figure represents a different taxonomic level, and species-level annotations are displayed. For example, at 1 month of infant age, cumulative NO_x exposure was positively associated with the abundances of *Dorea longicatena* and *Enterobacter asburiae*. At 6 months, higher cumulative NO_x exposure was associated with lower abundance of *Coprococcus comes*. At 18 months, higher cumulative NO_x exposure was associated with higher abun-

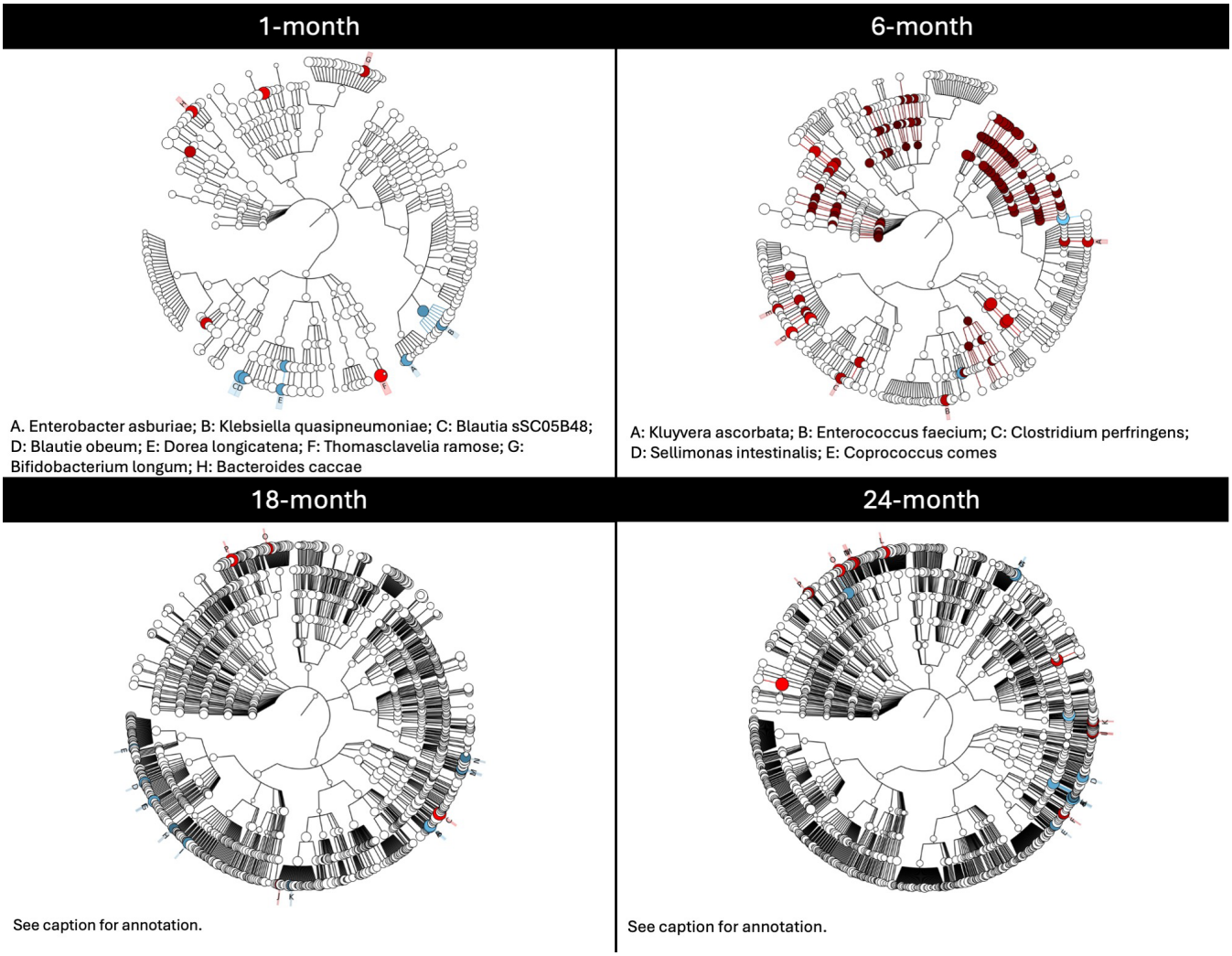


Figure 5. Associations of cumulative NO_x exposure from birth to study visit with gut microbial taxa at 1, 6, 18, and 24 months of infant age. Estimates were obtained using negative binomial models in which the exposure of interest was cumulative NOx exposure, and the outcome was the abundance of each gut microbial taxon. The 1-month models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, and maternal prepregnancy BMI; an offset was included to adjust for the log of total microbial counts in each sample. At the 6-month timepoint, models were additionally adjusted for whether solid foods had been introduced; at subsequent timepoints, models were also adjusted for the infant Healthy Eating Index. Negative associations are shown in red and positive associations are shown in blue; darker colors indicate stronger associations. Annotations are provided at the species level.

Annotations for 18-month species: A: Megasphaera massiliensis; B: Megasphaera elsdenii; C: Phascolarctobacterium succinatutens; D: Vescimonas coprocola; E: Clostridium neonatale; F: Eubacterium limosum; G: Eubacterium callanderi; H: Blautia argi; I: Coprococcus sp. ART55/1; J: Streptococcus anginosus; K: Streptococcus alactolyticus; L: Escherichia fergusonii; M: Escherichia coli; N: Salmonella enterica; O: Bacteroides ovatus; P: Parabacteroides sp. CT06.

Annotations for 24-month species: A: Megasphaera massiliensis; B: Megasphaera elsdenii; C: Megasphaera hexanoica; D: Acidaminococcus intestini; E: Ligilactobacillus ruminis; F: Enterococcus faecalis; G: Bifidobacterium angulatum; H: Bifidobacterium animalis; I: Eggerthella lenta; J: Klebsiella pneumoniae; L: Citrobacter freundii; M: Bacteroides caccae; N: Alistipes ihumii; O: Alistipes finegoldii; P: Odoribacter splanchnicus; Q: Desulfovibrio piger.

dances of several pathogenic gut bacterial species, including *Clostridium neonatale*, *Escherichia fergusonii, Escherichia coli,* and *Salmonella enterica.* As observed with cumulative $PM_{2.5}$ exposure, cumulative NO_x exposure at 24 months was associated with higher abundances of *Alistipes ihumii* and *Alistipes finegoldii.* Plots illustrating the observed statistically significant associations between NO_x exposure and microbial species abundances can be found in Supplemental File 9 [1 month], Supplemental File 10 [6 months], Supplemental File 11 [18 months], and Supplemental File 12 [24 months].

In the next analysis, we explored possible overlap between observed associations of cumulative air pollutant exposure and gut microbial species abundances at 1, 6, 12, 18, and 24 months of infant age (**Figure 6**). Overall, we detected minimal overlap in associations according to infant age. For example, we identified 33 microbial species uniquely associated with cumulative PM_{10} exposure at 1 month, five at 6 months, 17 at 12 months, and four at 18 months. There were two overlapping associations between the 1- and 6-month timepoints, two between the 1- and 12-month timepoints, and one between the 12- and 18-month timepoints.

Prior-Month Air Pollution Exposure and the Gut Microbiome

We investigated whether short-term air pollution exposure (i.e., prior-month exposure at each study visit) was associated

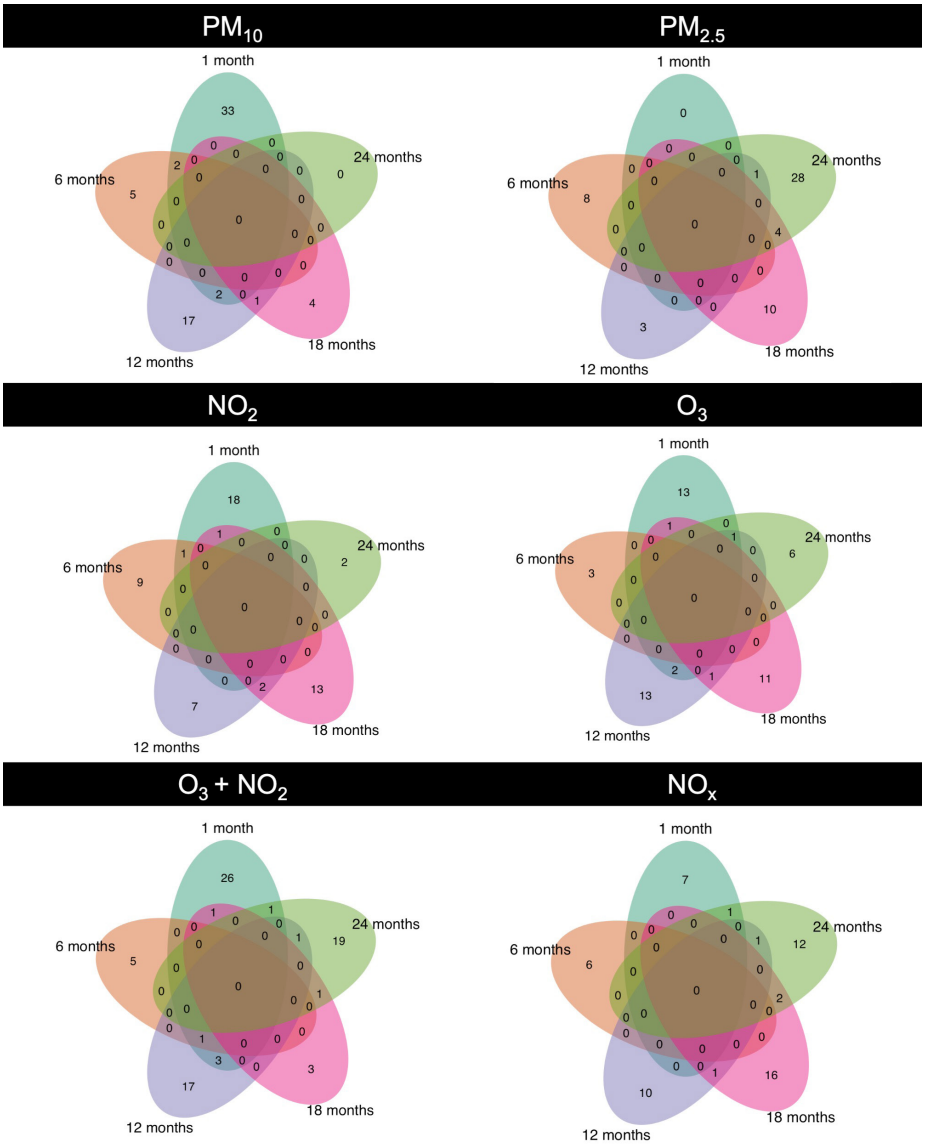


Figure 6. Venn diagrams summarizing the numbers of statistically significant associations between cumulative air pollution exposure and gut microbial species abundance across timepoints. These Venn diagrams summarize the number of statistically significant associations across different timepoints. For example, in the top right diagram, there were 33 significant associations between gut microbial species abundance and PM_{10}, indicated by the green circle. The 1-month models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, and maternal prepregnancy BMI. An offset was included to adjust for the log of the total number of microbial counts in each sample. At the 6-month timepoint, we additionally adjusted for whether solid foods had been introduced; at subsequent timepoints, we also adjusted for the infant Healthy Eating Index. Findings were considered statistically significant if PBH < 0.2.

with the gut microbiome. **Table 5** presents the number of gut microbial species significantly associated ($P_{BH} < 0.2$ or $P_{BH} < 0.05$) with prior-month exposure to each air pollutant. Among the gut microbial species associated with prior-month PM_{10}, NO_2, and O_3 exposures at 1 month, all were also associated with cumulative PM_{10} exposure at 1 month. At 6 months, *Bifidobacterium dentium* abundance was inversely associated with prior-month PM_{10} and $PM_{2.5}$ exposures; this species was also positively associated with cumulative PM_{10} exposure at 24 months. Similarly, *Klebsiella michiganensis* was associated with both cumulative and prior-month NO_2 exposures. At 18 months, there were 18 significant associations between PM_{10} exposure and species abundance, only one of which (*Fusobacterium ulcerans*) was also identified in the cumulative analyses. Among the four significant associations between $PM_{2.5}$ exposure and species abundance at 18 months, two — *Klebsiella variicola* and *Bifidobacterium angulatum* — were also identified in the cumulative analyses. Of the 19 statistically significant associations between prior-month NO_2 exposure and species abundance, only one (*Bacteroides intestinalis*) overlapped with cumulative exposure findings. In contrast, all species significantly associated with NO_x exposure were also identified in the cumulative analysis. Among nine statistically significant associations between O_3

Table 5. Numbers of Statistically Significant Cross-Sectional Associations Between Prior-Month Air Pollution Exposure and Gut Microbial Species at 1, 6, 12, 18, and 24 Months of Infant Age[a]

	1 Month		6 Months		12 Months		18 Months		24 Months	
	$P_{BH} < 0.2$	$P_{BH} < 0.05$	$P_{BH} < 0.2$	$P_{BH} < 0.05$	$P_{BH} < 0.2$	$P_{BH} < 0.05$	$P_{BH} < 0.2$	$P_{BH} < 0.05$	$P_{BH} < 0.2$	$P_{BH} < 0.05$
PM_{10}	9	5	6	0	1	1	18	6	27	12
$PM_{2.5}$	4	4	3	3	8	0	4	2	20	13
NO_2	6	4	8	5	8	4	19	2	18	8
O_3	11	4	3	0	14	6	9	7	22	7
$O_3 + NO_2$	27	4	1	0	15	2	6	6	13	6
NO_x	7	1	10	3	29	2	9	4	20	12

[a] Prior-month air pollution exposure was defined as the exposure during the month preceding each study visit (i.e., at 1, 6, 12, 18, and 24 months). Cells indicate the number of statistically significant results after correction for multiple testing using the BH method at $P_{BH} < 0.2$ and $P_{BH} < 0.05$. Results were generated using negative binomial models in which the outcome was the abundance of each gut microbial species. The 1-month models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, and maternal prepregnancy BMI; an offset was included to control for the log of total microbial counts in each sample. At the 6-month timepoint, models were additionally adjusted for the introduction of solid foods; at subsequent timepoints, models were also adjusted for the infant Healthy Eating Index. The numbers of species analyzed were 132 at 1 month, 188 at 6 months, 370 at 12 months, 541 at 18 months, and 802 at 24 months.

exposure and species abundance, three were also observed in the cumulative analyses. Overall, the 24-month findings were similar to those at 18 months, showing limited overlap between species associated with cumulative air pollution exposure and those associated with prior-month exposure.

We also assessed the associations of prior-month air pollutant exposure with alpha-diversity measures, including Shannon, richness, evenness, and Simpson indices (**Table 6**). At 1 month of age, O_3 exposure was associated with higher Simpson diversity (adjusted for NO_2 exposure) ($P = 0.04$). At 6 months of age, total NO_x exposure was associated with lower Shannon diversity, evenness, and Simpson diversity ($P_{all} \leq 0.03$). Next, we assessed the associations of prior-month air pollution exposure with overall microbiome composition, using the beta-diversity measure Bray–Curtis dissimilarity. We found that quartiles of prior-month NO_2 exposure explained 5% of the variation in Bray–Curtis dissimilarity at 24 months of infant age ($P = 0.007$) (**Table 7**).

AIM 1 FECAL METABOLOME

Population characteristics of participants included in the fecal metabolomics analyses are shown in **Table 8**. Study visits occurred at approximately 1, 6, 12, 18, and 24 months of infant age. At the initial study visit, participants with fecal metabolomics data were 46% male, and 73% had been born vaginally. Participants were selected to maximize the number of complete fecal samples across time. Changes in non-time-varying characteristics over time reflect missing follow-up data for some participants. At the 1-month timepoint, three participants were missing fecal metabolomics data; at 6 months, 11 participants were missing these data; at 12 months, seven participants were missing these data; at 18 months, four participants were missing these data; and at 24 months, one participant was missing these data (Supplemental Figure 1). At 1 month of age, infants received an average of 6.6 human milk feedings per day (range: 0–8); they were introduced to solid foods at a mean age of 5.9 months (range: 2–12 months).

Prenatal Air Pollution Exposure and the Fecal Metabolome at 1 Month

As previously described,[39] prenatal exposures to PM_{10}, $PM_{2.5}$, NO_2, and NO_x were associated with the intensities of 51 Level-1 metabolites (**Figure 7**). For instance, prenatal PM_{10} exposure was positively associated with pyridoxamine intensity; prenatal PM_{10}, $PM_{2.5}$, and NO_2 exposures were positively associated with 4-hydroxy-phenylglycine/pyridoxal intensity. Pyridoxamine and 4-hydroxy-phenylglycine/pyridoxal are both involved in vitamin B6 metabolism.[58] Prenatal PM_{10} and $PM_{2.5}$ exposures were both positively associated with the intensities of thymidine and beta-alanine/sarcosine, metabolites involved in pyrimidine metabolism.[58] Finally, prenatal $PM_{2.5}$ and NO_2 exposures were inversely associated with the intensities of 3-methoxy-4-hydromandelate/vanillylmandelate and tyrosine, which are involved in tyrosine metabolism.[58] We also explored the associations of prenatal NO_x exposure with 1-month metabolite intensities, revealing that arabinose/xylose/ribose intensities were inversely associated with prenatal NO_x exposure ($P_{BH} = 0.001$). Associations of prenatal O_3 exposure with fecal metabolite intensity at 1 month were also assessed; no statistically significant associations were detected, either before or after adjustment for prenatal NO_2 exposure.

Cumulative Air Pollution Exposure and the Fecal Metabolome

Linear multivariate models were used to assess whether cumulative postnatal exposures to PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x were associated with the intensities of Level-1 metabolites at 1, 6, 12, 18, and 24 months of age (**Table 9, Table 10**). At the 6-month study visit, PM_{10} exposure was positively

Table 6. Cross-Sectional Associations of Prior-Month Air Pollution Exposure with Gut Microbiome Alpha-Diversity at 1, 6, 12, 18, and 24 Months of Infant Age[a]

	1 Month		6 Months		12 Months		18 Months		24 Months	
	b (SE)	P	*b* (SE)	P	*b* (SE)	P	*b* (SE)	P	*b* (SE)	P
Shannon										
PM_{10}	0.01 (0.008)	0.1	–0.008 (0.008)	0.3	0.002 (0.007)	0.8	–0.02 (0.01)	0.2	0.007 (0.007)	0.3
$PM_{2.5}$	0.008 (0.02)	0.6	–0.02 (0.01)	0.2	–0.01 (0.02)	0.5	–0.01 (0.02)	0.6	0.03 (0.01)	0.048
NO_2	0.008 (0.01)	0.6	0.001 (0.01)	0.9	–0.02 (0.01)	0.1	0.02 (0.01)	0.2	–0.01 (0.009)	0.2
O_3	0.01 (0.007)	0.2	0.0006 (0.008)	0.9	0.01 (0.007)	0.1	–0.0009 (0.007)	0.9	0.008 (0.005)	0.1
$O_3 + NO_2$	0.01 (0.008)	0.1	0.002 (0.009)	0.9	0.008 (0.008)	0.3	0.003 (0.008)	0.7	0.006 (0.005)	0.3
Total NO_x	–0.004 (0.03)	0.9	–0.07 (0.03)	0.03	0.03 (0.04)	0.4	0.007 (0.03)	0.8	–0.02 (0.02)	0.4
Richness										
PM_{10}	3.0 (11.2)	0.8	–15.5 (14. 6)	0.3	11.9 (16.3)	0.5	–24.3 (25.1)	0.3	22.9 (15.5)	0.1
$PM_{2.5}$	–1.4 (22.0)	1.0	–24.2 (23.0)	0.3	–6.1 (33.9)	0.9	–6.4 (47.2)	0.9	63.8 (28.5)	0.03
NO_2	5.6 (18.2)	0.8	–15.1 (20.5)	0.5	–29.0 (25.2)	0.2	36.7 (30.8)	0.2	–16.8 (19.9)	0.4
O_3	4.6 (10.8)	0.7	–2.3 (14.5)	0.9	27.6 (16.2)	0.1	–8.2 (16.1)	0.6	12.1 (10.7)	0.3
$O_3 + NO_2$	5.7 (11.5)	0.6	–8.9 (16.8)	0.6	24.4 (17.2)	0.2	0.8 (17.6)	1.0	9.2 (11.8)	0.4
Total NO_x	–28.2 (48.1)	0.6	–86.3 (58.5)	0.1	61.0 (78.8)	0.4	4.7 (61.4)	0.9	–0.08 (43.9)	1.0
Evenness										
PM_{10}	0.002 (0.0009)	0.08	–0.0008 (0.0008)	0.4	0.0001 (0.0007)	0.9	–0.002 (0.001)	0.1	0.0006 (0.0007)	0.4
$PM_{2.5}$	0.001 (0.002)	0.5	–0.002 (0.001)	0.2	–0.001 (0.002)	0.4	–0.001 (0.002)	0.5	0.002 (0.001)	0.1
NO_2	0.0009 (0.001)	0.6	0.0004 (0.001)	0.7	–0.002 (0.001)	0.1	0.002 (0.001)	0.3	–0.001 (0.0009)	0.2
O_3	0.001 (0.0009)	0.1	0.00002 (0.0008)	1.0	0.001 (0.0007)	0.2	–0.000003 (0.0007)	1.0	0.0008 (0.0005)	0.1
$O_3 + NO_2$	0.002 (0.0009)	0.09	0.0003 (0.001)	0.8	0.0007 (0.0008)	0.4	0.0004 (0.0008)	0.6	0.0006 (0.0005)	0.3
Total NO_x	–0.0001 (0.004)	1.0	–0.008 (0.003)	0.02	0.003 (0.004)	0.4	0.0008 (0.003)	0.8	–0.002 (0.002)	0.3
Simpson										
PM_{10}	0.003 (0.002)	0.1	–0.002 (0.002)	0.3	0.0005 (0.001)	0.7	–0.002 (0.001)	0.1	0.0006 (0.0007)	0.4
$PM_{2.5}$	0.002 (0.004)	0.5	–0.004 (0.003)	0.2	–0.002 (0.002)	0.4	–0.003 (0.002)	0.2	0.002 (0.001)	0.1
NO_2	0.0004 (0.003)	0.9	0.0009 (0.002)	0.7	–0.002 (0.002)	0.2	0.001 (0.002)	0.4	–0.0006 (0.0009)	0.5
O_3	0.003 (0.002)	0.1	–0.0006 (0.002)	0.7	0.002 (0.001)	0.1	0.00007 (0.0008)	0.9	0.0005 (0.0005)	0.3
$O_3 + NO_2$	0.004 (0.002)	0.04	–0.0003 (0.002)	0.9	0.002 (0.001)	0.2	0.0005 (0.0009)	0.6	0.0003 (0.0005)	0.6
Total NO_x	–0.004 (0.008)	0.7	–0.02 (0.007)	0.02	0.0003 (0.005)	1.0	0.0008 (0.003)	0.8	–0.003 (0.002)	0.2

b = beta estimate; SE = standard error.

[a] Estimates were generated using linear models in which the outcome of interest was alpha-diversity (i.e., Shannon, richness, evenness, and Simpson indices) at the 1-, 6-, 12-, 18-, and 24-month study visits; the predictor of interest was air pollution exposure during the month preceding the visit. The 1-month models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, and maternal prepregnancy BMI. At the 6-month timepoint, models were additionally adjusted for the introduction of solid foods; at subsequent timepoints, models were also adjusted for the infant Healthy Eating Index. Bolded cells indicate statistical significance ($P < 0.05$).

associated with the intensities of glycerate, alpha-aminoadipate/methyl-glutamate, acetyl-glutamic acid, omega-hydroxydodecanoic acid, and hexyl-glutathione. At the 12-month study visit, PM_{10} exposure was positively associated with the intensity of trans-cinnamaldehyde. At 6 months, cumulative NO_2 exposure was positively associated with the intensities of butyrate/isobutyrate, glycerate, glutamic acid/methyl-aspartic acid, acetyl-glutamic acid, and anserine; it was inversely associated with monoglyceride(14:0/0:0/0:0). At 18 months, cumulative O_3 exposure was inversely associated with the intensity of omega-hydroxydodecanoic acid. At 24 months, cumulative O_3 exposure was positively associated with the intensities of butyrate/isobutyrate, succinate/methylmalonic acid, and dihydroxymandelic acid, both before and after adjustment for cumulative NO_2 exposure. At the 1-month study visit, NO_x exposure was inversely associated with the intensity of arabinose/xylose/ribose. At 6 months, NO_x exposure was inversely associated with the intensities of 17 metabolites, including hypoxanthine, indole-3-acetic acid, and hexanoylcarnitine. At 24 months, NO_x exposure was inversely associated with the intensity of acetylputrescine.

Prior-Month Air Pollution Exposure and the Fecal Metabolome

We also explored whether prior-month exposures to PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x were associated with metabolite

Table 7. Associations of Quartile of Prior-Month Air Pollution Exposure with Overall Microbiome Composition (Estimated Via Bray–Curtis Dissimilarity) at 1, 6, 12, 18, and 24 Months

	1 Month		6 Months		12 Months		18 Months		24 Months	
	R^{2a}	P	R^{2a}	P	R^{2a}	P	R^{2a}	P	R^{2a}	P
PM_{10}	0.04	0.051	0.02	0.5	0.02	0.3	0.02	0.3	0.03	0.7
$PM_{2.5}$	0.009	0.7	0.02	0.6	0.02	0.7	0.02	1.0	0.03	0.7
NO_2	0.01	0.7	0.03	0.1	0.02	0.7	0.03	0.1	0.05	0.007
O_3	0.009	0.7	0.02	0.5	0.02	0.2	0.03	0.2	0.03	0.8
$O_3 + NO_2$	0.01	0.9	0.05	0.2	0.04	0.5	0.05	0.2	0.07	0.06
Total NO_x	0.001	0.8	0.02	0.3	0.02	0.7	0.02	0.8	0.02	0.9

[a] R^2 represents the proportion of variance in Bray–Curtis dissimilarity explained by quartile of exposure to air pollution during the month prior to each study visit at 1, 6, 12, 18, and 24 months of infant age. Results were unadjusted, except for the $O_3 + NO_2$ model, in which cumulative exposure to NO_2 was included as a covariate. Bolded cells indicate statistical significance ($P < 0.05$).

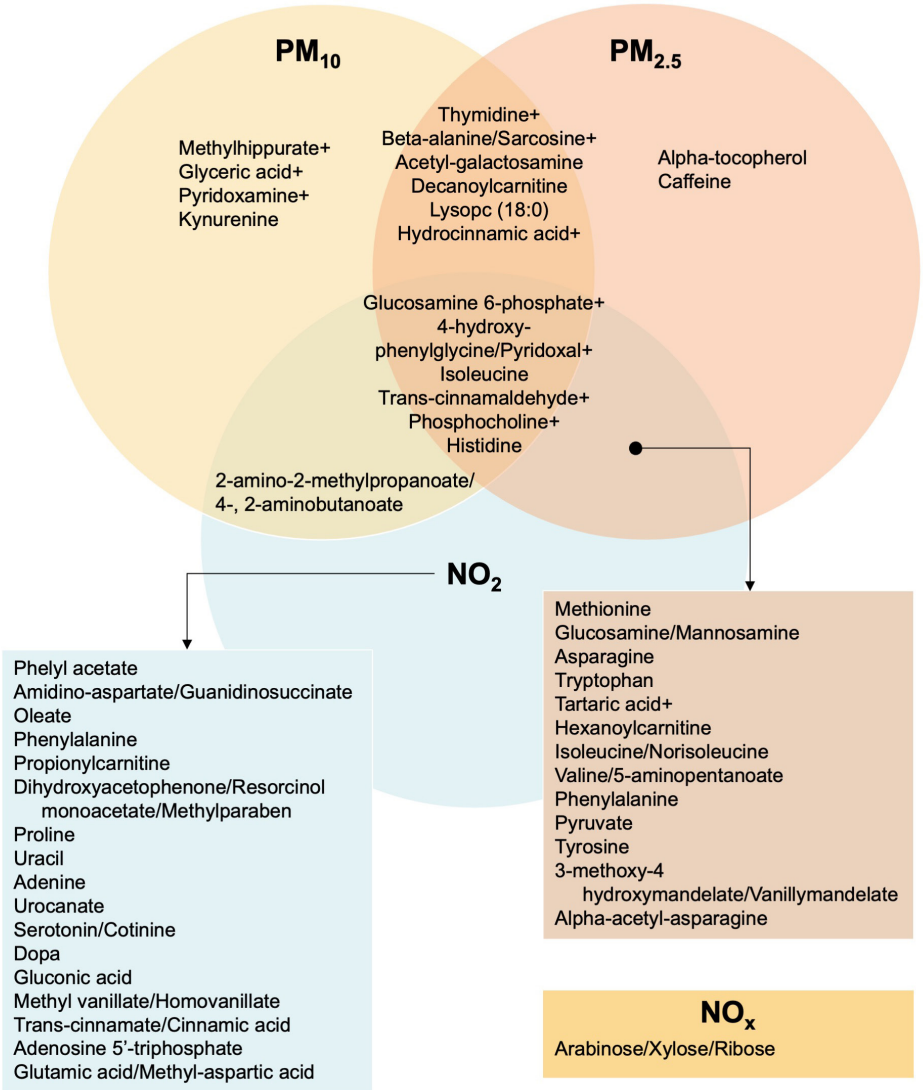


Figure 7. Prenatal PM_{10}, $PM_{2.5}$, NO_2, and NO_x exposures were associated with level-1 metabolites at 1 month of infant age. Plus (+) denotes metabolites that were positively associated with air pollution exposure. All other metabolites were inversely associated. Results were generated using multivariable linear models adjusted for infant age, infant sex, socioeconomic status, season of visit (warm vs. cold), maternal age, and breastmilk and formula feedings per day. Only results significant at PBH < 0.2 are shown. The association between prenatal O_3 exposure and fecal metabolite intensity was also assessed, but no statistically significant associations were identified regardless of adjustment for prenatal NO_2 exposure. (Source: Adapted with permission from *Holzhausen et al. 2024*; Creative Commons license *CC BY-NC-ND 4.0*.)

Table 8. Characteristics of Mother–Infant Dyads with Fecal Metabolomics Data from the Southern California Mother's Milk Study, Enrollment from 2016 to 2019[a]

Variable	1 Month (*N* = 124)	6 Months (*N* = 116)	12 Months (*N* = 120)	18 Months (*N* = 123)	24 Months (*N* = 126)
Maternal characteristics	**Mean ± SD; *n* (%)**	**Mean ± SD; *n* (%)**	**Mean ± SD; *n* (%)**	**Mean ± SD; *n* (%)**	**Mean ± SD; *n* (%)**
Age (years)	29.0 ± 6.3	29.4 ± 6.3	29.8 ± 6.4	30.4 ± 6.4	30.7 ± 6.2
Prepregnancy BMI (kg/m^2)[b]	28.6 ± 5.8	28.4 ± 5.7	28.6 ± 5.9	28.7 ± 5.8	28.6 ± 5.7
SES[b,c]	26.6 ± 12.1	26.4 ± 11.9	27.1 ± 12.1	26.8 ± 12.0	26.6 ± 12.0
Infant characteristics					
Age (days)	32.6 ± 3.3	186.0 ± 8.9	367.8 ± 10.6	551.5 ± 19.2	751.9 ± 46.6
Sex[b] (male, female, %male)	57, 67, 46%	55, 61, 47%	60, 60, 50%	59, 64, 48%	59, 67, 47%
Delivery mode[b] (CS, vaginal, %CS)	33, 91, 27%	31, 85, 27%	30, 90, 25%	32, 91, 26%	32, 94, 25%
Breastfeedings per day	6.6 ± 2.4	3.1 ± 3.4	2.2 ± 2.8	2.0 ± 2.7	1.6 ± 2.4
Formula feedings per day	2.4 ± 2.7	3.1 ± 2.9	2.2 ± 2.4	0.9 ± 1.7	0.9 ± 1.8
Age at solid food introduction (months)[b]	--	5.9 ± 1.6	5.8 ± 1.6	5.9 ± 1.6	5.9 ± 1.6
Season of visit[d] (warm, cool, %warm)	55, 69, 44%	54, 62, 47%	65, 55, 54%	61, 62, 50%	58, 68, 46%
Air pollution measures					
Prenatal PM_{10} ($\mu g/m^3$)[e]	30.3 ± 4.0	31.7 ± 6.9	30.9 ± 7.1	30.5 ± 4.0	30.3 ± 4.0
Prenatal $PM_{2.5}$ ($\mu g/m^3$)[e]	12.0 ± 1.2	11.9 ± 1.2	11.8 ±1.2	12.0 ± 1.2	11.9 ± 1.2
Prenatal NO_2 (ppb)[e]	18.2 ± 2.5	18.1 ± 2.5	18.0 ± 2.5	18.1 ± 2.5	18.1 ± 2.5
Prenatal O_3 (ppb)[e]	43.0 ± 3.7	43.0 ± 3.7	43.0 ± 3.7	43.1 ± 3.7	43.0 ± 3.6
Prenatal NO_x (ppb)	4.2 ± 2.4	4.2 ± 2.4	4.2 ± 2.4	4.2 ± 2.4	4.2 ± 2.4
Individual mean PM_{10} ($\mu g/m^3$)[b]	29.6 ± 3.2	29.6 ± 3.2	29.7 ± 3.2	29.5 ± 3.2	29.6 ± 3.2
Individual mean $PM_{2.5}$ ($\mu g/m^3$)[b]	11.9 ± 1.6	11.8 ± 1.6	11.8 ± 1.7	11.8 ± 1.6	11.8 ± 1.6
Individual mean NO_2 (ppb)[b]	17.1 ± 2.4	16.9 ± 2.2	17.1 ± 2.3	17.0 ± 2.4	17.0 ± 2.4
Individual mean O_3 (ppb)[b]	41.8 ± 4.1	42.0 ± 4.0	41.9 ± 4.1	41.9 ± 4.1	41.9 ± 4.1
Individual mean NO_x (ppb)[b]	3.6 ± 1.8	3.6 ± 1.8	3.6 ± 1.9	3.6 ± 1.8	3.6 ± 1.8
Fluctuation in PM_{10} ($\mu g/m^3$)	2.8 ± 6.8	2.2 ± 6.4	1.2 ± 5.4	-3.9 ± 5.2	-3.7 ± 5.7
Fluctuation in $PM_{2.5}$ ($\mu g/m^3$)	0.6 ± 3.5	0.7 ± 3.6	0.2 ± 2.8	-1.0 ± 2.8	-0.5 ± 2.4
Fluctuation in NO_2 (ppb)	1.9 ± 6.1	1.5 ± 7.6	-0.9 ± 4.9	-1.1 ± 6.2	-1.2 ± 4.5
Fluctuation in O_3 (ppb)	0.9 ± 7.3	01 ± 7.9	0.0 ± 5.6	-0.7 ± 8.2	-0.3 ± 6.3
Fluctuation in NO_x (ppb)	0.1 ± 0.9	-0.06 ± 0.9	-0.1 ± 0.6	0.1 ± 0.9	0.0 ± 0.8
Cumulative PM_{10} ($\mu g/m^3$)	32.3 ± 7.1	32.3 ± 4.9	32.2 ± 3.6	31.0 ± 3.1	30.0 ± 2.7
Cumulative $PM_{2.5}$ ($\mu g/m^3$)	12.4 ± 3.9	12.5 ± 2.0	12.6 ± 1.4	12.3 ± 1.1	12.1 ± 0.9
Cumulative NO_2 (ppb)	18.9 ± 7.2	19.0 ± 5.1	18.4 ± 2.6	17.9 ± 2.7	17.4 ± 2.1
Cumulative O_3 (ppb)	42.7 ± 8.5	41.8 ± 4.9	41.9 ± 3.0	41.2 ± 2.8	41.4 ± 2.2
Cumulative NO_x (ppb)	3.6 ± 1.9	3.6 ± 1.8	3.5 ± 1.8	3.5 ± 1.8	3.5 ± 1.8
Prior-month PM_{10} ($\mu g/m^3$)	32.4 ± 7.6	31.7 ± 6.9	30.9 ± 7.0	25.8 ± 5.0	26.4 ± 6.9
Prior-month $PM_{2.5}$ ($\mu g/m^3$)	12.5 ± 4.2	12.4 ± 4.1	12.0 ± 3.2	10.8 ± 2.6	11.4 ± 3.0
Prior-month NO_2 (ppb)	18.9 ± 7.5	18.4 ± 7.1	16.2 ± 6.1	15.9 ± 5.3	15.8 ± 5.6
Prior-month O_3 (ppb)	42.7 ± 8.7	42.1 ± 8.4	41.9 ± 7.9	41.2 ± 8.0	41.5 ± 8.1
Prior-month NO_x (ppb)	3.7 ± 2.0	3.5 ± 2.0	3.5 ± 1.9	3.6 ± 1.9	3.5 ± 1.7

[a]Data are reported as mean and SD unless otherwise noted. (Source: Adapted with permission from *Holzhausen et al. 2024*; Creative Commons license *CC BY-NC-ND 4.0*.)

[b]Non-time-varying (i.e., assessed at a single timepoint and not expected to change over time); differences across time are due to participant missingness at specific timepoints ($P_{all} \geq 0.2$).

[c]SES was estimated using a modified version of the Hollingshead Index. Range for study population: 3–63.

[d]Study visits occurring between April 1 and September 30 were considered warm season; all other visits were considered cool season.

[e]Grand mean of prior month air pollution for each participant visit.

Table 9. Cross-Sectional Associations of Cumulative Postnatal PM_{10}, NO_2, and O_3 Exposures with Metabolite Intensities at 6, 12, 18, and 24 Months of Infant Age [a]

	6 Months		12 Months		18 Months		24 Months	
	Metab.	P_{BH}	Metab.	P_{BH}	Metab.	P_{BH}	Metab.	P_{BH}
PM_{10}	Glycerate	↑ 0.07	Trans-cinnamaldehyde	↑ 0.07				
	Alpha-aminoadipate/methyl-glutamate	↑ 0.15						
	Acetyl-glutamic acid	↑ 0.15						
	Omega-hydroxydodecanoic acid	↑ 0.15						
	Hexyl-glutathione	↑ 0.15						
NO_2	Butyrate/isobutyrate	↑ 0.08						
	Glycerate	↑ 0.08						
	Glutamic acid/methyl-aspartic acid	↑ 0.08						
	Acetyl-glutamic acid	↑ 0.08						
	Anserine	↑ 0.08						
	Monoglyceride(14:0/0:0/0:0)	↓ 0.18						
O_3					Omega-hydroxydodecanoic acid	↓ 0.11	Butyrate/isobutyrate	↑ 0.09
							Succinate/methylmalonic acid	↑ 0.09
							Dihydroxymandelic acid	↑ 0.09
O_3 + NO_2							Butyrate/isobutyrate	↑ 0.09
							Succinate/methylmalonic acid	↑ 0.09
							Dihydroxymandelic acid	↑ 0.09

Metab. = metabolite.

[a]Results were generated from multivariable linear models in which the outcome of interest was log-transformed metabolite intensity and the primary predictor was cumulative air pollution exposure (from birth to the timepoint of interest). Models were adjusted for infant age (in days), infant sex, socioeconomic status, season of visit, maternal age, formula and breastfeeding frequency, and whether the infant had begun to eat solid foods. Arrows represent the direction of association. Shaded cells indicate no statistically significant associations for that exposure and timepoint. No statistically significant results were observed for $PM_{2.5}$ or any exposure at 1 month of infant age. Results shown were significant after multiple testing correction using the BH procedure ($P_{BH} < 0.2$). Results from both HILIC and C18 columns are included.

intensities at 1, 6, 12, 18, and 24 months of age (**Table 11**). At 6 months, PM_{10} exposure was inversely associated with the intensities of two metabolites, including 4-hydroxy-phenylglycine/pyridoxal. $PM_{2.5}$ exposure at 6 months was also inversely associated with the intensities of 4-hydroxy-phenylglycine/pyridoxal and five other metabolites. At 18 months, prior-month $PM_{2.5}$ exposure was inversely associated with the intensities of beta-alanine/sarcosine/alanine and melatonin. Prior-month NO_2 exposure was inversely associated with the intensities of two metabolites at 6 months and six metabolites at the 12-month study visit. Before adjustment for NO_2 exposure, O_3 exposure was positively associated with histidine intensity at 12 months. After adjustment for NO_2 exposure, O_3 exposure was inversely associated with the intensities of arabinose/xylose/ribose (6 months) and 2,6-dihydroxypyridine (24 months); it was positively associated with cadaverine and carnitine intensities at 24 months. Similar to cumulative NO_x exposure, we found that prior-month NO_x exposure was inversely associated with the intensities of 19 metabolites (**Table 12**).

Table 10. Cross-Sectional Associations of Cumulative Postnatal NO_x Exposure with Metabolite Intensities at 1, 6, and 24 Months of Infant Age[a]

	1 Month		6 Months		24 Months	
	Metab.	P_{BH}	**Metab.**	P_{BH}	**Metab.**	P_{BH}
NO_x	Arabinose/xylose/ribose	↓ 0.003	Aminophenol (2, 3, or 4)	↓ 0.18	Acetylputrescine	↓ 0.18
			Indole	↓ 0.18		
			Imidazoleacetate	↓ 0.18		
			Asparagine[b]	↓ 0.18		
			Hypoxanthine	↓ 0.18		
			Urocanate	↓ 0.18		
			Glutamic acid/aspartate	↓ 0.18		
			Methionine	↓ 0.18		
			Alpha-aminoadipate/ methyl-glutamate	↓ 0.18		
			Pyridoxine/noradrenaline	↓ 0.18		
			Indole-3-acetic acid	↓ 0.18		
			Methylhippurate	↓ 0.18		
			Succinyl-homoserine	↓ 0.18		
			Hexanoylcarnitine	↓ 0.18		
			Linoleate	↓ 0.18		
			Glutamic acid/methyl-aspartic acid	↓ 0.12		
			Dethiobiotin	↓ 0.12		

Metab. = metabolite.
[a]Results were generated from multivariable linear models in which the outcome of interest was log-transformed metabolite intensity and the primary predictor was cumulative air pollution exposure (from birth to the timepoint of interest). Models were adjusted for infant age (in days), infant sex, socioeconomic status, season of visit, maternal age, formula and breastfeeding frequency, and whether the infant had begun to eat solid foods. Arrows represent the direction of association. No statistically significant associations were identified at 12 or 18 months of infant age. Results shown were significant after correction for multiple testing using the BH procedure ($P_{BH} < 0.2$). Results from both HILIC and C18 columns are included.
[b]Consistent with previous publications.

AIM 2 GUT MICROBIOME

Prenatal Air Pollution Exposure and the Longitudinal Gut Microbiome

We first assessed whether associations existed between prenatal air pollution exposure and the longitudinal abundances of gut microbial taxa via longitudinal negative binomial models; we incorporated an offset for the log of total bacterial counts and a random intercept to adjust for repeated measures among infants. We found that prenatal exposures to PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x were associated with longitudinal changes in the abundances of several microbial species (**Figure 8**). For instance, higher prenatal PM_{10} exposure was associated with lower abundance of *Finegoldia magna* and higher abundances of three *Enterobacter* species. Higher prenatal $PM_{2.5}$ exposure was associated with lower abundances of three *Megasphaera* species and two *Roseburia* species. Higher prenatal NO_2 exposure was associated with lower abundances of *Akkermansia muciniphila* and three *Megasphaera* species, as well as increased abundances of three *Klebsiella* species. Finally, higher prenatal O_3 exposure was associated with lower abundances of several *Bifidobacterium* species.

Postnatal Air Pollution Exposure and the Longitudinal Gut Microbiome

We next assessed whether postnatal fluctuations in air pollution exposure were associated with alpha-diversity (i.e., Shannon diversity, richness, evenness, and Simpson diversity) using linear mixed-effects models. In these models, the outcome of interest was alpha-diversity, and the predictor of interest was the deviation in prior-month exposure from the individual's long-term mean (i.e., the grand mean of prior-month air pollution exposure). Overall, we did not find statistically significant associations ($P_{all} > 0.05$) between prior-month deviations in air pollution exposure and alpha-diversity (**Table 13**).

Table 11. Cross-Sectional Associations of Prior-Month PM_{10}, $PM_{2.5}$, NO_2, and O_3 Exposures with Metabolite Intensities at 6, 12, 18, and 24 Months of Infant Age [a]

	6 Months		12 Months		18 Months		24 Months	
	Metabolite	P_{BH}	Metab.	P_{BH}	Metab.	P_{BH}	Metab.	P_{BH}
PM_{10}	4-Hydroxy-phenyl-glycine/pyridoxal	↓ 0.08						
	2-,4-Quinolinecar-boxylic acid	↓ 0.08						
$PM_{2.5}$	4-Hydroxy-phenyl-glycine/pyridoxal	↓ 0.11			Beta-alanine/ sarcosine/alanine	↓ 0.14		
	Indole-3-acetic acid	↓ 0.11						
	Gamma-linolenic acid	↓ 0.15						
	Aminophenol (2, 3, or 4)	↓ 0.18			Melatonin	↓ 0,11		
	Methylvanillate	↓ 0.18						
	Phosphocholine	↓ 0.18						
NO_2	Pantothenic acid (B5)	↓ 0.06	Methoxytyramine Salsolinol	↓ 0.14 ↓ 0.14				
	Linoleate	↓ 0.06	Maleamate Valine/norvaline Pyridoxate Methoxytyrosine	↓ 0.198 ↓ 0.198 ↓ 0.198 ↓ 0.198				
O_3			Histidine	↑ 0.19				
O_3 + NO_2	Arabinose/xylose/ ribose	↓ 0.17					Cadaverine 2,6-Dihydroxy-pyridine Carnitine	↑ 0.12 ↑ 0.12 ↑ 0.19

Metab. = metabolite.

[a]Results were generated from multivariable linear models in which the outcome of interest was log-transformed metabolite intensity and the primary predictor was cumulative air pollution exposure (from birth to the timepoint of interest). Models were adjusted for infant age (in days), infant sex, socioeconomic status, season of visit, maternal age, formula and breastfeeding frequency, and whether the infant had begun to eat solid foods. Arrows represent the direction of association. Shaded cells indicate no statistically significant associations for that exposure and timepoint. No statistically significant associations were identified at 1 month of infant age. Results shown were significant after correction for multiple testing using the BH procedure ($P_{BH} < 0.2$). Results from both HILIC and C18 columns are included.

Next, we assessed the relationships of postnatal fluctuations in air pollution exposure and gut microbial taxa abundances via longitudinal negative binomial models (**Table 14**). After correction for multiple testing using the BH procedure, we found the greatest number of statistically significant associations at the species level. Specifically, each pollutant examined was associated with gut bacterial species abundances ($P_{BH} < 0.05$), including PM_{10} (n = 18 species), $PM_{2.5}$ (n = 6 species), NO_2 (n = 9 species), O_3 (n = 2 species, without adjustment for NO_2), O_3 (n = 8 species, with adjustment for NO_2), and NO_x (n = 6 species).

In **Figure 9**, we summarize the longitudinal associations of fluctuations in postnatal air pollution exposure with the abundances of infant gut microbial taxa during the first 2 years of life for selected air pollutants. Overall, we observed statistically significant associations between fluctuations in PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x exposures and the abundances of several gut microbial taxa. For example, PM_{10} exposure was inversely associated with the abundances of *Akkermansia muciniphila* and *Dysosmobacter welbionis*; it was positively associated with the abundance of *Clostridium neonatale*. Fluctuations in NO_2 exposure were positively associated with the abundances of *Klebsiella michiganensis* and *Raoultella ornithinolytica*. O_3 exposure fluctuations were positively associated with *Klebsiella pneumoniae* and *Klebsiella variicola* abundances; they were inversely associated with *Lactococcus lactis* abundance, both before and after adjustment for NO_2. Finally, NO_x exposure fluctuations were positively associated with the abundance of *Raoultella ornithinolytica*.

Table 12. Cross-Sectional Associations of Prior-Month NO_x Exposure with Metabolite Intensities at 1, 6, 12, and 18 Months of Infant Age[a]

	1 Month		6 Months		12 Months		18 Months	
	Metab.	P_{BH}	Metab.	P_{BH}	Metab.	P_{BH}	Metab.	P_{BH}
NO_x	Arabinose/ xylose/ribose	↓ 0.001	Aminophenol (2, 3, or 4)	↓ 0.09	Tyrosine	↓ 0.14	Indole-3-acetic acid	↓ 0.10
			Hypoxanthine	↓ 0.09				
			Indole-3-acetic acid	↓ 0.09				
			Pyridoxine/ noradrenaline	↓ 0.10				
			Succinyl-homoserine	↓ 0.15				
			4-Hydroxy-phenylglycine/ pyridoxal	↓ 0.17				
			Methyl-ecgonine	↓ 0.17				
			Indole	↓ 0.19				
			Phenethylamine	↓ 0.19				
			4-Pyridoxate	↓ 0.19				
			Hexanoyl carnitine	↓ 0.19				
			Guanosine 5'-diphosphomannose	↑ 0.19				
			Dihydrouracil (5, 6)	↓ 0.19				
			Glutamic acid/ methyl-aspartic acid	↓ 0.09				
			Dethiobiotin	↓ 0.09				
			Glycerate	↓ 0.09				

Metab. = metabolite.

[a]Results were generated from multivariable linear models in which the outcome of interest was log-transformed metabolite intensity and the primary predictor was cumulative air pollution exposure (from birth to the timepoint of interest). Models were adjusted for infant age (in days), infant sex, socioeconomic status, season of visit, maternal age, formula and breastfeeding frequency, and whether the infant had begun to eat solid foods. Arrows represent the direction of association. No statistically significant associations were identified at 24 months of infant age. Results shown were significant after correction for multiple testing using the BH procedure ($P_{BH} < 0.2$). Results from both HILIC and C18 columns are included.

AIM 2 FECAL METABOLOME

Prenatal Air Pollution Exposure and the Longitudinal Fecal Metabolome

We subsequently assessed the associations of prenatal exposures to PM_{10}, $PM_{2.5}$, NO_2, O_3, and total NO_x with longitudinal postnatal fecal metabolite intensity (**Figure 10**). Overall, we observed that prenatal exposures to $PM_{2.5}$ and NO_2 were longitudinally associated with fecal metabolite intensities. However, there were no statistically significant associations ($P_{BH} < 0.2$) between prenatal PM_{10}, NO_x, or O_3 exposures and fecal metabolite intensity. Higher prenatal exposures to NO_2 and $PM_{2.5}$ were each associated with lower intensities of phenylalanine, histidine, and tyrosine. Higher NO_2 exposure was additionally associated with lower intensity of methionine.

Postnatal Air Pollution Exposure and the Longitudinal Fecal Metabolome

Finally, we explored whether fluctuations in air pollution exposure (i.e., PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x) were associated with fecal metabolite intensities from 1 to 24 months of

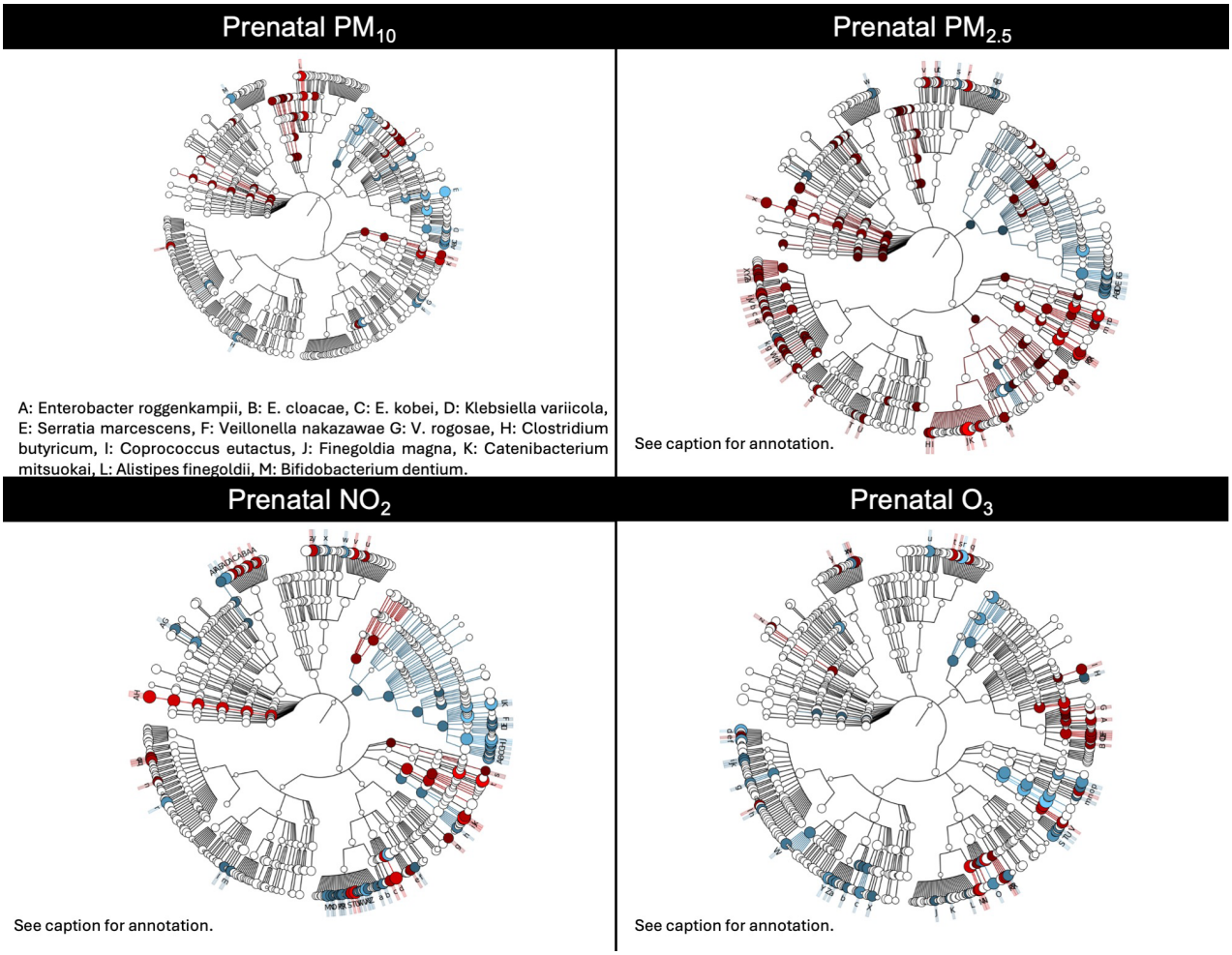


Figure 8. Prenatal air pollution was longitudinally associated with the abundances of infant gut microbial taxa. Estimates were obtained using longitudinal negative binomial models where the exposure of interest was prenatal air pollution exposure, and the outcome was the abundance of each gut microbial taxon. Models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeeding frequency, formula feeding frequency, mode of delivery, maternal prepregnancy BMI, infant mean Healthy Eating Index, and whether the infant had begun solid foods. An offset was included to control for the log-transformed total number of sequence counts.

Annotations for $PM_{2.5}$: A: Enterobacter roggenkampii, B: E. cloacae, C: E. kobei, D: E. ludwigii, E: Klebsiella aerogenes, F: K. quasipneumoniae, G: K. variicola, H: Streptococcus pyogenes, I: S. equinus, J: S. pasteurianus, K: S. gallolyticus, L: S. lutetiensis, M: Lactobacillus johnsonii, N: Listeria monocytogenes, O: Paenibacillus polymyxa, P: Megasphaera hexanoica, Q: M. elsdenii, R: M. stantonii, S: Vescimonas coprocola, T: Clostridium estertheticum, U: C. sporogenes, V: Butyrivibrio fibrosolvens, W: Lachnoanaerobaculum umeaense, X: Blautia obeum, Y: B. wexlerae, Z: B. pseudococcoides, a: B. argi, b: Anaerocolumna sedimenticola, c: Anaerostipes rhamnosivorans, d: Novisyntrophococcus fermenticellae, e: Roseburia intestinalis, f: R. hominis, g: Dorea formicigenerans, h: Anaeromicrophila herbilytica, i: Coprococcus catus, j: C. eutactus, k: Enterocloster asparagiformis, l: Pseudobutyrivibrio xylanivorans, m: Faecalitalea cylindroides, n: Thomasclavelia spiroformis, o: Faecalibacillus intestinalis, p: Bacteroides intestinalis, q: B. sp. DH3716P, r: B. stercoris, s: Phocaeicola dorei, t: Parabacteroides merdae, u: P. goldsteinii, v: Alistipes finegoldii, w: Bifidobacterium sp. FKU, x: Campylobacter jejuni.

Annotations for NO_2: A. Enterobacter roggenkampii, AA: Bifidobacterium catenulatum, AB: B. bifidum, AC: B. breve, AD: B. adolescentis, AE: B. dentium, AF: Rothia mucilaginosa, AG: Gordonibacter pamelaeae, AH: Akkermansia muciniphila, B: Enterobacter cloacae, C: E. ludwigii, D: Citrobacter freundii, E: C. portucalensis, F: Cronobacter sakazakii, G: Klebsiella aerogenes, H: K. pneumoniae, I: K. quasipneumoniae, J: K. variicola, K: Lelliottia amnigena, L: Raoultella ornithinolytica, M: Streptococcus constellatus, N: S. sp. A12, O: S. cristatus, P: S. acidominimus, Q: S. sp. oral taxon 061, R: S. sp. HSISM1, S: S. anginosus, T: S. pasteurianus, U: S. gallolyticus, V: S. oralis, W: S. sp. oral taxon 431, X: S. parasanguinis, Y: S. lutetiensis, Z: S. sp. LPB0220, a: S. sanguinis, b: Lactococcus lactis, c: Lactobacillus johnsonii, d: Ligilactobacillus ruminis, e: Enterococcus raffinosus, f: Granulicatella adiacens, g: Listeria monocytogenes, h: Veillonella parvula, i: Megasphaera hexanoica, j: M. elsdenii, k: M. stantonii, l: Flavonifractor plautii, m: Pusillibacter faecalis, n: Roseburia intestinalis, o: Coprococcus sp. ART55/1, p: C. catus, q: C. eutactus, r: Enterocloster asparagiformis, s: Finegoldia magna, t: Faecalibacillus intestinalis, u: Bacteroides caccae, v: B. stercoris, w: Phocaeicola dorei, x: Parabacteroides merdae, y: Alistipes shahii, z: A. finegoldii.

Annotations for O_3: A: Shigella flexneri, B: Klebsiella quasipneumoniae, C: Escherichia fergusonii, D: E. coli, E: E. marmotae, F: E. albertii, G: Lelliottia amnigena, H: Pseudomonas aeruginosa, I: Stenotrophomonas maltophilia, J: Streptococcus maltophilia, J: S. acidominimus, K: S. thermophilus, L: S. infantis, M: Lactococcus cremoris, N: L. lactis, O: Ligilactobacillus ruminis, P: Enterococcus casseliflavus, Q: E. faecalis, R: E. raffinosus, S: Veillonella nakazawae, T: V. dispar, U: V. rogosae, V: Megasphaera elsdenii, W: Faecalibacterium prausnitzii, X: Flintibacter sp. KGMB00164, Y: Clostridium butyricum, Z: C. cadaveris, a: C. sp. C1, b: C. sporogenes, c: Eubacterium ventriosum, d: Blautia sp. SC05B48, e: B. parvula, f: B. sp. KLE 1732 HM 1032, g: Anaerostipes caccae, h: Dorea longicatena, i: Coprococcus comes, j: C. sp. ARG55/1, k: C. catus, l: Enterocloster clostridioformis, m: Longicatena caecimuris, n: Thomasclavelia ramosa, o: T. spiroformis, p: Catenibacterium mitsuokai, q: Bacteroides sp. D2, r: B. caccae, s: B. sp. M10, t: B. fragilis, u: Prevotella melaninogenica, v: Bifidobacterium sp. FKU, w: B. longum, x: B. catenulatum, y: B. imperatoris, z: Eggerthella guodeyinii.

infant age (**Figure 11**). Higher PM_{10} exposure was associated with lower intensities of urocanate, beta-alanine/sarcosine/alanine, histamine, and histidinol. Higher $PM_{2.5}$ exposure was also associated with lower urocanate intensity. These metabolites are all involved in histidine metabolism.[58] Higher exposures to PM_{10} and NO_2 were additionally associated with lower intensities of 3-methoxytyramine and 4-pyridoxate, involved in tyrosine metabolism and vitamin B6 metabolism, respectively.[58] Higher O_3 exposure was associated with lower hypoxanthine intensity; fluctuations in NO_x exposure were not associated with statistically significant changes in the intensities of any fecal metabolites.

Table 13. Longitudinal Associations of Prior-Month Fluctuations in Air Pollution Exposure with Gut Microbiome Alpha-Diversity from 1 to 24 Months of Infant Age[a]

	Beta (SE)	P
Shannon		
PM_{10}	–0.002 (0.004)	0.6
$PM_{2.5}$	–0.0005 (0.007)	1.0
NO_2	–0.003 (0.005)	0.6
O_3	0.005 (0.004)	0.2
$O_3 + NO_2$	0.004 (0.004)	0.3
Total NO_x	0.01 (0.01)	0.3
Richness		
PM_{10}	–7.5 (7.4)	0.3
$PM_{2.5}$	–2.8 (13.4)	0.8
NO_2	–7.2 (10.1)	0.5
O_3	4.2 (6.8)	0.5
$O_3 + NO_2$	2.3 (7.6)	0.8
Total NO_x	4.2 (20.9)	0.8
Evenness		
PM_{10}	–0.0001 (0.0004)	0.8
$PM_{2.5}$	0.00002 (0.0008)	1.0
NO_2	–0.0002 (0.0006)	0.7
O_3	0.0005 (0.0004)	0.2
$O_3 + NO_2$	0.0005 (0.0004)	0.3
Total NO_x	0.002 (0.001)	0.2
Simpson		
PM_{10}	–0.0002 (0.0008)	0.8
$PM_{2.5}$	–0.0004 (0.001)	0.8
NO_2	–0.0005 (0.001)	0.7
O_3	0.0006 (0.0007)	0.4
$O_3 + NO_2$	0.0005 (0.0008)	0.6
Total NO_x	0.002 (0.002)	0.4

[a]Estimates were generated using linear mixed-effects models in which the outcome of interest was alpha-diversity (i.e., Shannon, richness, evenness, and Simpson indices) at each study visit; the predictor of interest was the prior-month deviation in air pollution exposure from the long-term mean (i.e., grand mean of individual prior-month air pollution exposure). Models were adjusted for long-term air pollution exposure, infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, whether solid foods had been introduced, infant mean Healthy Eating Index, and maternal prepregnancy BMI. Random intercepts were included to control for repeated measures among participants.

DISCUSSION AND CONCLUSIONS

SUMMARY

This study provides a comprehensive examination of the associations of pre- and postnatal exposure to air pollutants with the composition and function of the infant gut microbiome. Utilizing a multi-omics approach, our analysis incorporates microbiome profiling and fecal metabolomics data to offer novel insights into how air pollution exposure might influence gut health from early life stages. These dual omics layers enable a detailed understanding of the bacterial composition of the gut microbiome and its functional implications, as reflected in fecal metabolite profiles. Overall, our findings indicate that exposure to common air pollutants during both the prenatal and postnatal periods is associated with substantial alterations in the gut microbiome and its metabolic output. These alterations were evident in both cross-sectional and longitudinal analyses, underscoring the persistent and potentially cumulative impact of air pollution over time. We found that air pollution exposure was consistently associated with lower abundances of beneficial species such as *Akkermansia muciniphila* and higher abundances of pathogenic or opportunistic bacteria, as well as fecal metabolites indicative of inflammation, oxidative stress, and disrupted gut health. Our results highlight how early-life exposure to air pollutants can disrupt the delicate balance of the gut microbiome, leading to changes that may have long-term health implications. These findings emphasize the importance of mitigating air pollution exposure during critical developmental periods to protect and promote gut health and overall well-being in infants.

GUT BACTERIA ASSOCIATED WITH AIR POLLUTANT EXPOSURE

Air pollutant exposures were associated with several gut bacteria that may impact infant health. Notably, the genus *Bifidobacterium*, a core constituent of the infant gut, plays a critical role in newborn and infant development.[59,60] In our study, *Bifidobacterium* abundance was inversely associated with prior-month PM_{10} and $PM_{2.5}$ exposures, suggesting that higher levels of these pollutants negatively affect the presence of this beneficial bacterium in the infant gut. Conversely, $PM_{2.5}$ exposure was inversely associated with the abundances of several *Klebsiella* species, indicating a possible decrease in colonization or proliferation of these pathogenic species in the infant gut microbiome.[61] Particulate matter exposures were also inversely associated with the genus *Alistipes*, consistent with our previous work in this cohort involving 16S rRNA amplicon sequencing.[43] Moreover, higher prenatal $PM_{2.5}$ exposure was associated with lower levels of the genus *Romboutsia*, which is involved in fermentation processes and the production of short-chain fatty acids.[62-64] Additionally, higher pre- and postnatal exposures to particulate matter were associated with a lower abundance of *Akkermansia muciniphila*, a species with known anti-inflammatory properties.[65-67]

Table 14. Numbers of Statistically Significant Longitudinal Associations Between Fluctuations in Prior-Month Air Pollution Exposure and Gut Microbial Species from 1 to 24 Months of Infant Age

	$P_{BH} < 0.05$	$P_{BH} < 0.2$
Phylum		
PM_{10}	1	1
$PM_{2.5}$	1	1
NO_2	1	1
O_3	1	1
$O_3 + NO_2$	1	1
Total NO_x	0	0
Class		
PM_{10}	1	1
$PM_{2.5}$	2	2
NO_2	1	1
O_3	1	1
$O_3 + NO_2$	0	1
Total NO_x	0	0
Order		
PM_{10}	0	3
$PM_{2.5}$	3	3
NO_2	2	2
O_3	0	0
$O_3 + NO_2$	0	0
Total NO_x	0	1
Family		
PM_{10}	1	3
$PM_{2.5}$	2	3
NO_2	4	4
O_3	0	0
$O_3 + NO_2$	0	0
Total NO_x	0	1
Genus		
PM_{10}	2	14
$PM_{2.5}$	2	6
NO_2	5	6
O_3	2	2
$O_3 + NO_2$	2	5
Total NO_x	2	3
Species		
PM_{10}	18	37
$PM_{2.5}$	6	23
NO_2	9	22
O_3	2	12
$O_3 + NO_2$	8	15
Total NO_x	6	13

[a] Cells indicate the number of statistically significant results after correction for multiple testing using the BH method at $P_{BH} < 0.2$ and $P_{BH} < 0.05$. Results were generated using negative binomial models in which the outcome was the abundance of each gut microbial species. Models were adjusted for infant age, infant sex, socioeconomic status, season of visit, maternal age, breastfeedings per day, formula feedings per day, mode of delivery, whether solid foods had been introduced, infant mean Healthy Eating Index, and maternal prepregnancy BMI. An offset was included to adjust for the log of the total number of microbial counts in each sample, and random intercepts were included to control for repeated measures among participants. In total, 290 species, 220 genera, 135 families, 74 orders, and 21 phyla were included in these analyses.

In the context of NO_x exposure, several identified bacteria have been linked to human health. For example, at 1 month of infant age, we found that postnatal NO_x exposure was associated with higher abundances of *Dorea longicatena* and *Enterobacter asburiae.* Higher *Dorea longicatena* abundance has been observed in individuals with overweight or obesity[68] and has been positively correlated with fasting blood glucose levels in children with diabetes.[69] Additionally, *Enterobacter asburiae* is an opportunistic pathogen previously isolated from infant formula.[70] We found that higher cumulative postnatal NO_x exposure was associated with lower abundance of the beneficial species *Coprococcus comes*.[71-73] Longitudinal models also revealed that beneficial bacteria such as *Akkermansia muciniphila* and *Ligilactobacillus ruminis* were more abundant among infants with lower PM_{10} exposure.[74-76]

FECAL METABOLITES ASSOCIATED WITH AIR POLLUTANT EXPOSURE

Prenatal PM_{10} exposure was positively associated with the fecal metabolite pyridoxamine, suggesting an impact on vitamin B6 metabolism.[39,77,78] Another metabolite involved in vitamin B6 metabolism, 4-hydroxy-phenylglycine/pyridoxal, was positively associated with prenatal PM_{10}, $PM_{2.5}$, and NO_2 exposures. These findings suggest disruption of essential nutrient pathways due to early-life air pollution exposure. Prenatal PM_{10} and $PM_{2.5}$ exposures were positively associated with the intensities of thymidine and beta-alanine/sarcosine, which are involved in pyrimidine metabolism,[39,79] highlighting a potential impact on nucleic acid damage and repair mechanisms. Conversely, prenatal $PM_{2.5}$ and NO_2 exposures were inversely associated with tyrosine and 3-methoxy-4-hydromandelate/vanillylmandelate, supporting prior findings that dysregulated tyrosine metabolism is linked to fetal growth restriction and preeclampsia.[39,80] Several metabolites showed significant associations with postnatal air pollution exposures. Postnatal PM_{10} and NO_2 exposures were positively associated with glycerate, an endogenous metabolite that we previously linked to formula feeding in this cohort.[81] Postnatal PM_{10} and NO_2 exposures were also positively associated with butyrate and isobutyrate, both of which are vital immune regulators.[82-84] Postnatal NO_x exposure was inversely

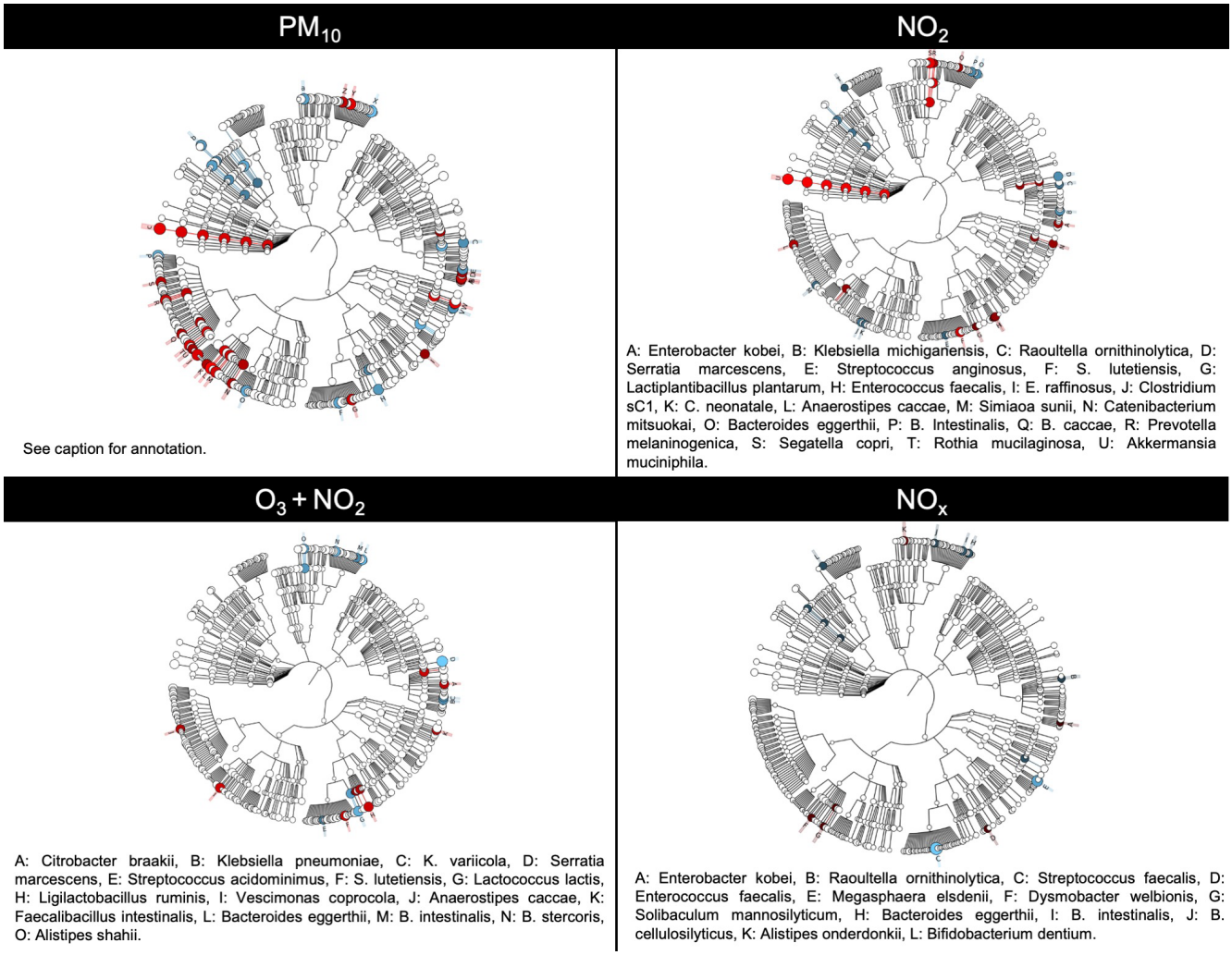


Figure 9. Fluctuations in postnatal air pollution exposure were associated with the longitudinal abundances of infant gut microbial taxa in the first 2 years of life. Estimates were obtained using longitudinal negative binomial models, in which the exposure of interest was postnatal fluctuations in air pollution exposure and the outcome was the abundance of each gut microbial taxon. Models were adjusted for infant age, infant sex, socioeconomic status, season, maternal age, human milk and formula feeding frequencies, mode of delivery, maternal prepregnancy BMI, infant mean Healthy Eating Index, and whether the infant had begun solid foods. Models also included an offset to control for the log-transformed total number of sequence counts.

Annotations for PM_{10}: A. Enterobacter cloacae, B: Enterobacter kobei, C: Leclercia adecarboxylata, D: Klebsiella aerogenes, E: K. pneumoniae, F: Streptococcus pasteurianus, G: S. lutetiensis, H: Ligilactobacillus ruminis, I: Listeria monocytogenes, J: Faecalibacterium prausnitzii, K: F. duncaniae, L: Vescimonas coprocola, M: Dysosmobacter welbionis, N: Pusillibacter faecalis, O: Clostridium neonatale, P: Blautia sp. SC05B48, Q: Anaerobutyricum hallii, R: Anaerostipes hadrus, S: Coprococcus sp. ART55/1, T: Lachnospira eligens, U: Pseudobutyrivibrio xylanivorans, V: Thomasclavelia ramosa, W: Faecalibacillus intestinalis, X: Bacteroides eggerthii, Y: B. caccae, Z: B. fragilis, a: Alistipes finegoldii, b: Schaalia turicensis, c: Akkermansia muciniphila.

associated with indole-3-acetic acid, which is thought to be microbially derived[85]; it has been shown to attenuate oxidative stress and inflammation and improve nonalcoholic fatty liver disease.[86,87] Furthermore, postnatal NO_2 exposure was inversely associated with pantothenic acid (vitamin B5) and linoleate — both essential nutrients that may contribute to brain development and infant growth.[88,89] Finally, prenatal exposures to PM_{10} and $PM_{2.5}$ were inversely associated with fecal histidine and positively associated with phosphocholine, which are important for metabolism and brain function, respectively.[90,91]

STRENGTHS AND LIMITATIONS

Air pollution exposures were assessed based on residential address histories, which present both strengths and limitations. A key strength of this method is that it includes the prenatal period and early life — critical windows for developmental exposure assessment. The database of air quality observations for PM, NO_2, and O_3 in Southern California is among the best in the United States. This approach leverages multiple data sources to enhance the accuracy of exposure estimation. Additionally, exposure to the mixture of near-roadway air pollution was characterized using the

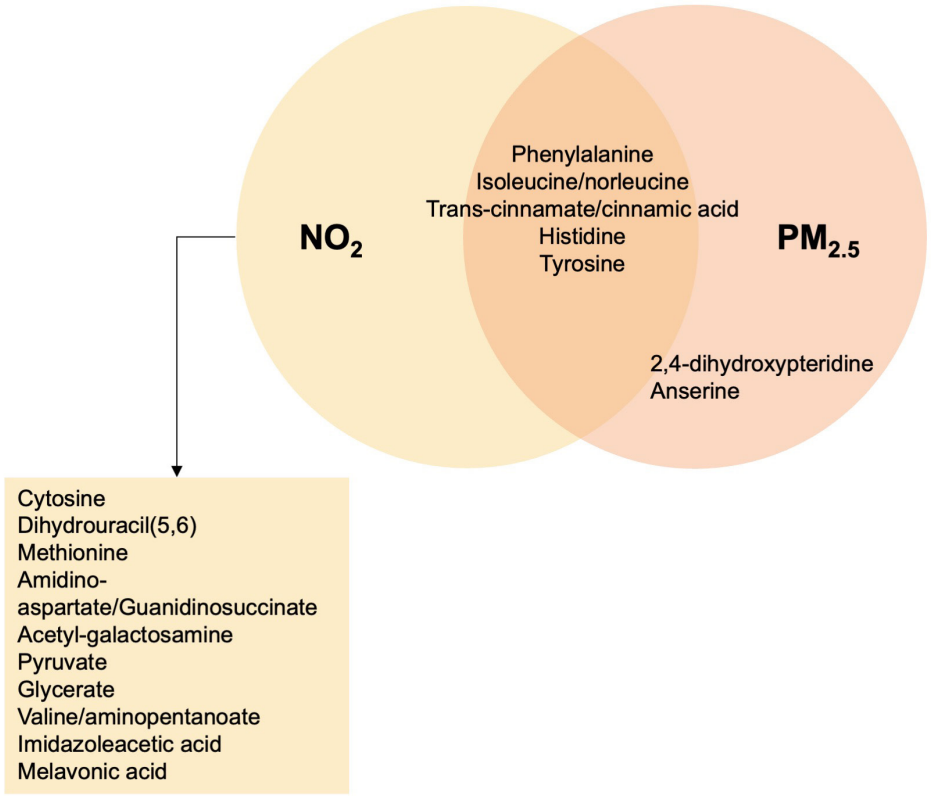


Figure 10. Prenatal air pollution exposure was longitudinally associated with the postnatal intensities of fecal metabolites. All associations were inverse, indicating that higher prenatal air pollution exposure was associated with lower metabolite intensity. Results were generated using linear mixed-effects models with a random participant-level intercept to control for repeated measures and adjusted for study visit (i.e., 1, 6, 12, 18, and 24 months), infant sex, socioeconomic status, season of visit (warm vs. cold), maternal age, introduction of solid foods, and breastmilk and formula feedings per day. Results shown were significant at PBH < 0.2. We also assessed whether prenatal PM_{10}, NO_x, O_3, and O_3 exposures — adjusted for prenatal NO_2 exposure — were associated with fecal metabolite intensities, but no associations met the threshold of PBH < 0.2.

CALINE4 air quality dispersion model, which incorporates detailed parameters such as roadway geometry, vehicle counts, emission rates, and atmospheric conditions.[47] This comprehensive approach provides a more robust exposure assessment. Despite these strengths, there are potential limitations. One notable concern is the possibility of exposure misclassification based on the amount of time participants spent away from their residences. Previous studies suggest that such misclassification bias tends to skew results toward the null hypothesis,[92] potentially underestimating the true exposure effect. Furthermore, the reliance on ambient data does not consider exposure to indoor-origin pollutants or other microenvironments, such as environmental tobacco smoke. Although mothers who smoked were excluded from the study, smoking by other household members could not be assessed, potentially leading to residual confounding.

The study cohort exclusively consisted of Latino participants, limiting the generalizability of the findings to other ethnic and racial groups. This lack of diversity may affect the applicability of the results to broader populations. However, there is no reason to believe that the biological mechanisms at play would differ according to race or ethnicity. A strength of this population is their relatively high exposure to air pollution and elevated rates of obesity, already observed by 2 years of age. Furthermore, many of our findings were consistent with studies involving more representative populations.[93] Therefore, although specific exposure levels and outcomes might vary, the underlying biological responses to air pollution are likely to be similar across different groups. The relatively small sample size across all aims of the study may have limited the statistical power, potentially affecting the ability to detect significant associations, especially those of small magnitude. Additionally, this study involved high-dimensional data that are strongly correlated, resulting in numerous statistical tests. Management and interpretation of such data is challenging because it increases the risk of false discoveries due to multiple comparisons. To address this risk, we adjusted for multiple hypothesis testing via the BH false discovery rate. However, this adjustment method may be overly conservative given the correlations among microbiome and metabolomic data. Thus, the small sample sizes and the correction method may each have increased the risk of Type II errors, where true associations could have been missed.

The study design incorporated both cross-sectional and longitudinal analyses, each with inherent limitations. Cross-sectional analyses can only determine associations, rather than causality. Longitudinal analyses, although stronger in suggesting temporal relationships, cannot definitively establish causation. Additionally, untargeted metabolomics was used in the study, leading to some uncertainty regarding the exact identity of many metabolic features beyond Level 1. This ambiguity is compounded by the fact that fecal

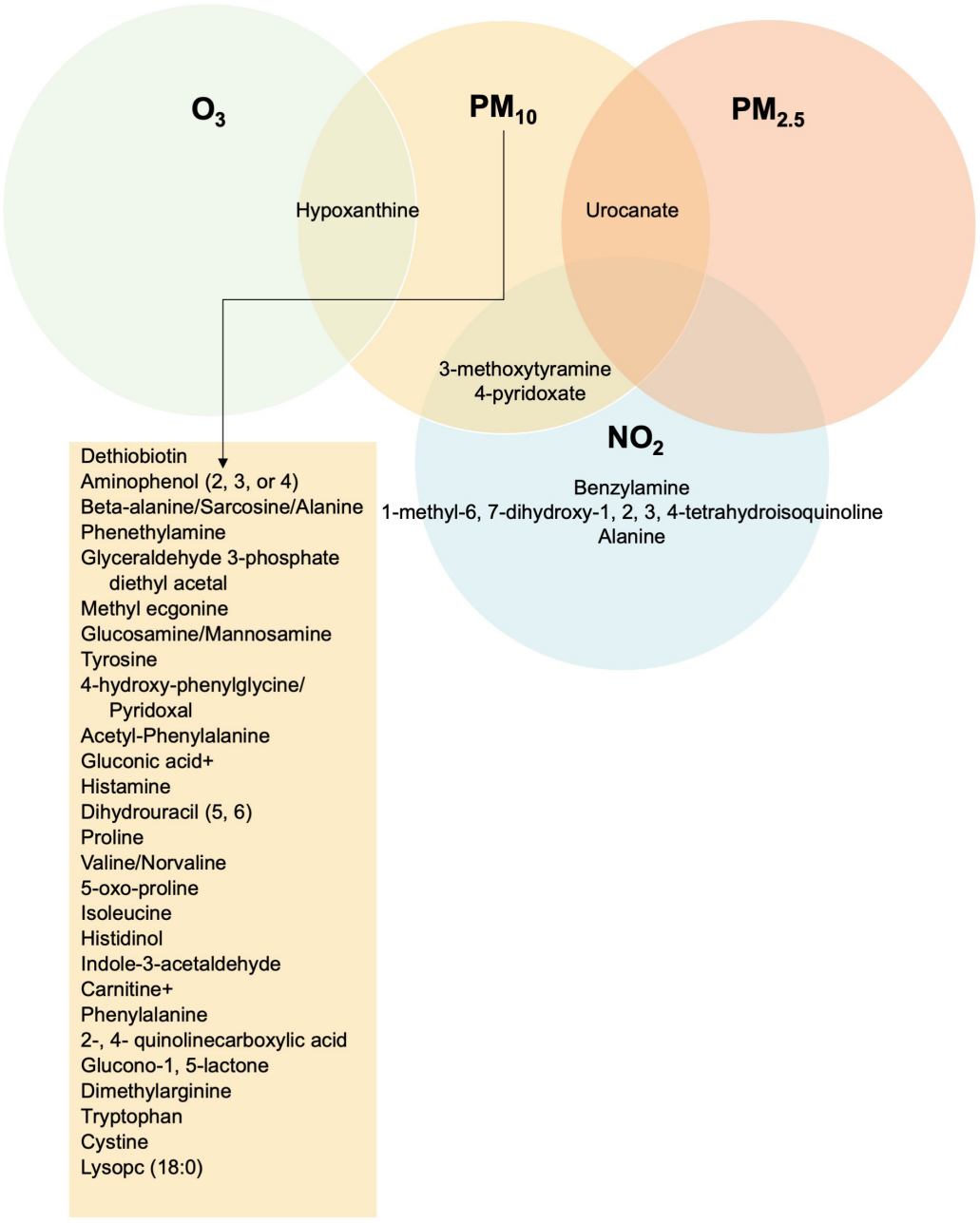


Figure 11. Fluctuations in prior-month PM_{10}, $PM_{2.5}$, and NO_2 exposures were associated with level-1 metabolites in the first 2 years of life. Plus (+) denotes metabolites that were positively associated with air pollution exposure. All other metabolites were inversely associated. Results were generated using linear mixed-effects models with a random participant-level intercept to control for repeated measures and adjusted for long-term air pollutant exposure (i.e., individual mean exposure to PM_{10}, $PM_{2.5}$, or NO_2, respectively), infant age, infant sex, socioeconomic status, season of visit (warm vs. cold), maternal age, introduction of solid foods, and breastmilk and formula feedings per day. Results shown were significant at PBH < 0.2. We also assessed whether fluctuations in NO_x exposure were associated with fecal metabolite intensity, but no associations met the threshold of PBH < 0.2. (Source: Adapted with permission from *Holzhausen et al. 2024*; Creative Commons license *CC BY-NC-ND 4.0*.)

metabolites could originate from gut bacteria or dietary sources, complicating the interpretation of the findings. Whereas untargeted approaches are comprehensive, they pose challenges in pinpointing precise biological pathways and metabolite sources, which may be addressed with future advances in metabolite annotation.

IMPLICATIONS OF THE FINDINGS

Disparities in exposures to ambient and near-roadway air pollution are prominent concerns, particularly for communities of color.[94,95] These communities often reside in areas with higher levels of pollution due to historical and socioeconomic factors.[94,96] Our findings highlight the critical need to address these environmental inequities — they are not only matters of environmental justice but also public health. Children in these communities are disproportionately exposed to harmful pollutants,[94,97] potentially influencing the composition and functional potential of their gut microbiome in the first 2 years of life. This early-life exposure can set the stage for various adverse health outcomes, underscoring the

importance of targeted interventions to mitigate exposure in these vulnerable populations.

Environmental exposures, particularly air pollution, have substantial effects on human health.[98] Our research provides evidence that exposures to ambient and near-roadway air pollution during critical developmental windows can alter the gut microbiome, which is crucial for various bodily functions, including digestion, immune response, and neurocognitive development.[99] The composition and function of the gut microbiome are essential for maintaining health, and early-life disruptions can have long-term consequences.[100,101]

Potential mechanisms linking air pollution to changes in the gut microbiome are multifaceted. Pollutant inhalation can lead to systemic inflammation, oxidative stress, and immune system modulation, which subsequently influence gut microbial composition.[102,103] Pollutants may also be directly ingested, further impacting the gut environment.[104] The biological plausibility of these associations is supported by existing literature (both animal and human studies), demonstrating that inhaled pollutants can affect the gut microbiome and fecal metabolome.[103-105] Our findings add to this body of knowledge by providing specific evidence that early-life exposure to air pollution can alter gut microbiome development, potentially leading to adverse health outcomes.

In the context of multiple hypothesis testing, it is essential to balance the risk of false positives with the potential for generating new hypotheses. Although our study presents numerous associations, we acknowledge the possibility of false positives, particularly because we selected a relatively lenient false discovery rate of 20% after correction for multiple testing. Therefore, future studies with targeted hypotheses are needed to validate our findings. Nevertheless, the present hypothesis-generating study provides a foundation for further investigation. Such studies are critical for the discovery and identification of new pathways and mechanisms that can be explored in subsequent research, ultimately contributing to a more comprehensive understanding of the interplay between environmental exposures and health. The presentation of raw and adjusted *P*-values supports a nuanced interpretation of our results.

SUMMARY AND FUTURE DIRECTIONS

The present results have important public health implications. Air pollution exposure is a modifiable risk factor, and interventions during critical developmental windows — such as the prenatal and postpartum periods — may reduce the burden of diseases, including obesity. By characterizing the impacts of air pollution on the gut microbiome and infant fecal metabolome, our research underscores the need for policies and practices aimed at reducing pollution exposure, particularly among vulnerable populations. This approach addresses environmental justice while promoting long-term health and well-being in children, paving the way for healthier future generations.

Several future studies will build on this rich dataset and extend the current analyses. Our next steps include investigating whether the impacts of air pollution exposure on the gut microbiome and fecal metabolome mediate the associations of higher air pollution exposure with infant growth trajectories and risk of childhood obesity. Additionally, we plan to utilize more sophisticated multi-omics approaches to better integrate our assessments of gut bacterial composition with fecal metabolites. This comprehensive strategy will enhance our understanding of the complex interactions between environmental exposures and health outcomes, ultimately informing more effective intervention strategies. Although this study focused on gut microbiome composition, metagenomic sequencing also allows predictions of microbial function based on genes and gene pathways, along with information regarding viruses and fungi that comprise the gut microbiome. Our future work will aim to fully utilize existing comprehensive data by incorporating these additional measures. For example, we plan to integrate the multi-omics layers available in this cohort by combining predicted microbial function based on genes and gene pathways with fecal metabolomics data.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author, T.L.A., upon reasonable request. The data are not publicly available because they contain information that could compromise the privacy of research participants.

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HEI QUALITY ASSURANCE STATEMENT

The conduct of this study was subjected to an independent audit by Westat staff members, including experienced quality assurance (QA) auditors with expertise in statistical modeling, OMICs, epidemiology, exposure assessment, maternal and child health, and geographic information systems analysis. The Westat QA audit team consisted of Dr. Daniel Chacreton, Dr. Sarah Ball, Dr. Sarah Reese, Mr. Michael Giangrande, and Ms. Rebecca Birch. These staff members are highly experienced in quality assurance oversight across various relevant domains. The QA oversight program consisted of a remote audit of the final report and the data processing steps. Key details of the dates of the audit and the reviews performed are listed below.

Date: May 2025 – September 2025

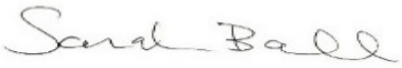
Remarks: The Alderete et al. study underwent an independent quality assurance (QA) audit by five Westat auditors with quality assurance oversight experience and expertise relevant to exposure assessment, OMICs, maternal and child health, epidemiological methods, geospatial analysis, and statistical analysis. The Westat QA audit of the final Alderete et al. report focused on adherence to the study protocol, appropriateness of the documentation of the study methods (e.g., data processing, exposure modeling, and statistical modeling), whether study assumptions and limitations were adequately addressed, and whether the investigators' conclusions were reasonable given the study findings and in consideration of the limitations. The QA team also reviewed the code and evaluated whether the report was easy to understand.

The Westat QA audit team provided a written report to HEI and the study investigators. The Westat QA auditors concluded that the study was well conducted in accordance with the study protocol and that the report was well written. The auditors also provided HEI and the investigator team with specific recommendations for improvement. Recommendations included clarifications between text descriptions and table annotations, adding text to clarify figures, and enhancing descriptions of statistical methodologies.

Alderete et al. responded to the QA recommendations and incorporated the feedback from the QA auditors in a final report that HEI provided to Westat. The Westat QA audit team attests that the final report appears to be representative of the study conducted.

Daniel Chacreton, PhD,
Statistician, Quality Assurance Auditor

Michael Giangrande, MGIS, Geographic Information System Analyst, Quality Assurance Auditor



Sarah Ball, ScD, Maternal and Child Health, Epidemiologist, Quality Assurance Auditor

Sarah Reese, PhD, Biostatistician, OMICs Expert, Quality Assurance Auditor

Rebecca Jeffries Birch, MPH, Epidemiologist, Quality Assurance Auditor

Date: October 1, 2025

ADDITIONAL MATERIALS AVAILABLE ON THE HEI WEBSITE

The Additional Materials contain supplemental figures, tables, and plots not included in the main report. They are available on the *HEI website*.

ABOUT THE AUTHORS

Tanya L. Alderete is an associate professor in the Department of Environmental Health and Engineering at Johns Hopkins University in the Bloomberg School of Public Health. Previously, she was an assistant professor at the University of Colorado Boulder (2018–2024) and a postdoctoral research scholar at the University of Southern California (2014–2018). Alderete received her PhD in Integrative Biology of Disease from the University of Southern California in 2014. Her research focuses on the effects of human exposure to environmental toxicants across the lifespan, with an emphasis on multi-omics techniques to gain deeper insights into the biological mechanisms linking exposure with disease risk.

Elizabeth A. Holzhausen is a research assistant professor in the Department of Environmental Health and Engineering at Johns Hopkins University in the Bloomberg School of Public Health. Previously, she was a postdoctoral research scholar at the University of Colorado Boulder (2021–2024). Holzhausen received her PhD in Epidemiology from the University of Wisconsin-Madison in 2021. She has experience in multidisciplinary biomedical and translational research, including performing epidemiological studies and using microbiome and metabolomics research methods. Holzhausen's research focuses on the early origins and long-term consequences of gut microbiome development during early life.

Donghai Liang is an associate professor in the Gangarosa Department of Environmental Health at Emory University in the Rollins School of Public Health. Liang received his PhD in Environmental Health Sciences from Emory University in 2018. His current research focuses on studying various ubiquitous pollutants, including air pollution and persistent organic pollutants, and their associated health effects in underserved and vulnerable populations. Using cutting-edge exposure assessment and high-throughput omics technologies, Liang has incorporated high-resolution metabolomics and multi-omics integration into pioneering investigations on the molecular mechanisms and disease etiology associated with environmental exposures.

Roshonda B. Jones is an assistant professor in the Department of Biology at North Carolina Agricultural and Technical State University. Before this, she was a research faculty member in the Department of Environmental Health and Engineering at Johns Hopkins University in the Bloomberg School of Public Health (2024). She received her PhD in Bioinformatics and Computational Biology in 2016 from the University of North Carolina at Charlotte. She has extensive postdoctoral experience and a strong track record in microbiome analysis and omics techniques, including next-generation sequencing. Jones's research focuses on understanding the intricacies of microbial communities and gene pathways in host systems, particularly in relation to nutrition and health outcomes such as obesity, diabetes, and neurodevelopmental disorders.

Fredrick Lurmann is the manager of Exposure Assessment Studies at Sonoma Technology. With more than 35 years of professional experience in air quality, he is a nationally recognized expert in air quality and exposure assessment. Lurmann provides technical direction and program management for projects sponsored by government agencies, universities, and nonprofit organizations. He frequently utilizes his expertise to determine prospective and retrospective air pollution exposure assignments for individuals participating in epidemiological studies of air pollution health effects. His research involves measurements, data analysis, and modeling of air pollution exposures and human time-activity. Much of Lurmann's recent work focuses on neighborhood-scale assessments, where proximity to mobile source emissions can substantially affect exposure.

Michael I. Goran is vice chair for Research and professor of Pediatrics at the Children's Hospital Los Angeles. Goran has conducted research in pediatric nutrition, obesity, energy metabolism, body composition, diet, physical activity, and insulin resistance in various studies for over 30 years. These studies have examined the etiology of obesity in numerous longitudinal and intervention studies in children and young adults, as well as the subsequent impact of obesity on chronic disease risk across various ethnic groups. Goran is the principal investigator of the Mother's Milk Study and an expert in infant growth trajectories and childhood obesity.

Howard H. Chang is an associate professor in the Department of Biostatistics and Bioinformatics at Emory University in Atlanta, Georgia. He earned his PhD in Biostatistics from Johns Hopkins University in Baltimore, Maryland. His current research interests include spatial epidemiology, environmental statistics, and Bayesian methods. For the current study, Chang served as the statistician, contributing to study design and data analysis.

Jeremey A. Sarnat is an associate professor of Environmental Health at the Rollins School of Public Health at Emory University in Atlanta, Georgia. He served as the principal investigator for the HEI DRIVE study. His research primarily focuses on measuring exposures and acute health responses to urban air pollution in various populations. He earned his BA in Anthropology from the University of Michigan in Ann Arbor, Michigan, and his MPH and ScD from the Harvard School of Public Health in Boston, Massachusetts. He serves on the Science Advisory Boards for Nitrogen Oxides and Particulate Matter in the US Environmental Protection Agency's Clean Air Scientific Advisory Committee. Sarnat served as the primary mentor on this Walter A. Rosenblith New Investigator Award.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

Note: *Publications denoted with an asterisk (*) are directly related to the HEI Rosenblith specific aims, and the HEI Rosenblith Investigator is denoted in bold text.*

Alderete TL, Jones RB, Shaffer JP, Holzhausen EA, Patterson WB, Kazemian E, et al. 2021. Early-life gut microbiota is associated with rapid infant growth in Hispanics from Southern California. Gut Microbes 13:1961203, *https://doi.org/10.1080/19490976.2021.1961203*.

*Bailey MJ, Holzhausen EA, Morgan ZEM, Naik N, Shaffer JP, Liang D, et al. 2022. Postnatal exposure to ambient air pollutants is associated with the composition of the infant gut microbiota at 6 months of age. Gut Microbes 14:2105096, *https://doi.org/10.1016/j.envres.2020.109130*.

Chalifour B, Holzhausen EA, Lim JJ, Yeo EN, Shen N, Jones DP, et al. 2023. The potential role of early life feeding patterns in shaping the infant fecal metabolome: implications for neurodevelopmental outcomes. NPJ Metab Health Dis 1:2, *https://doi.org/10.1038/s44324-023-00001-2*.

*Holzhausen EA, Chalifour BN, Tan Y, Young N, Lurmann F, Jones DP, et al. 2024. Prenatal and early life exposure to ambient air pollutants is associated with the fecal metabolome in the first two years of life. Environ Sci Technol 58:14121–14134, *https://doi.org/10.1021/acs.est.4c02929*.

Holzhausen EA, Kupsco A, Chalifour BN, Patterson WB, Schmidt KA, Mokhtari P, et al. 2023. Human milk EV-miRNAs: a novel biomarker for air pollution exposure during pregnancy. Environ Res Health. 2023 1:035002, *https://doi.org/10.1088/2752-5309/ace075*.

Holzhausen EA, Shen N, Chalifour B, Tran V, Li Z, Sarnat JA, et al. 2023. Longitudinal profiles of the fecal metabolome during the first 2 years of life. Sci Rep 13:1886, *https://doi.org/10.1038/s41598-023-28862-z*.

Morgan ZEM, Bailey MJ, Trifonova DI, Naik NC, Patterson WB, Lurmann FW, et al. 2023. Prenatal exposure to ambient air pollution is associated with neurodevelopmental outcomes at 2 years of age. Environ Health 22:11, *https://doi.org/10.1186/s12940-022-00951-y*.

Naik NC, Holzhausen EA, Chalifour BN, Coffman MM, Lurmann F, Goran MI, et al. 2024. Air pollution exposure may impact the composition of human milk oligosaccharides. Sci Rep 14:6730, *https://doi.org/10.1038/s41598-024-57158-z*.

Patterson WB, Glasson J, Naik N, Jones RB, Berger PK, Plows JF, et al. 2021. Prenatal exposure to ambient air pollutants and early infant growth and adiposity in the Southern California Mother's Milk Study. Environ Health 20:67, *https://doi.org/10.1186/s12940-021-00753-8*.

Research Report 237, *An Investigation of Early-Life Air Pollution Exposure and Its Effect on the Infant Gut Microbiome and Fecal Metabolome*, by T.L. Alderete et al.

INTRODUCTION

In 2021, over 40% of the United States population was estimated to be overweight or obese.[1,2] This statistic highlights a critical public health issue because being overweight or obese is a well-known risk factor for multiple diseases, such as cardiovascular disease and type 2 diabetes.[3] Understanding the factors that might contribute to the risks of having overweight and obesity is therefore important, particularly in children, where combined rates of these health statuses have increased linearly since 1990.[2]

Some studies have demonstrated associations between ambient and traffic-related air pollution and body mass index (BMI) in children and adolescents.[4,5] However, the biological mechanisms underlying these associations are not well understood. Recent research has suggested that ambient air pollution exposures might contribute to obesity and other adverse health outcomes through alterations in the gut microbiome (microorganisms, including bacteria, fungi, viruses, and their genes, within the gastrointestinal tract) and associated bacteria-derived metabolites in the fecal metabolome (the collection of small molecules produced by gut bacteria and found in feces).[6–9]

To evaluate the potential effects of early-life exposures to ambient and traffic-related air pollution on the developing gut microbiome and fecal metabolome, Dr. Tanya L. Alderete of Johns Hopkins University submitted an application to HEI titled "Air Pollutants and the Gut Microbiota and Metabolome During Early Life: Implications for Childhood Obesity" in response to HEI's *Request for Applications 18-2:* Walter A. Rosenblith New Investigator Award. This award was established to support an outstanding new investigator at the assistant professor level in conducting research on air pollution and health; it is unrestricted with respect to the specific research topic. Dr. Alderete proposed to examine whether prenatal and postnatal exposures to ambient air pollution, including traffic-related air pollution, affect the infant gut microbiota and fecal metabolome, potentially altering infant growth trajectories in the first 2 years of life. HEI's Research Committee recommended funding Dr. Alderete's application because the study had the potential to provide new insights into the mechanisms through which air pollution might contribute to obesity, with potential implications for precision prevention and treatment. The study began in 2020.

This Commentary provides the HEI Review Committee's independent evaluation of the study. It is intended to aid the sponsors of HEI and the public by highlighting both the strengths and limitations of the study and by placing the results presented in the Investigators' Report into a broader scientific and regulatory context.

SCIENTIFIC AND REGULATORY BACKGROUND

Several studies have shown that exposure to ambient air pollutants emitted by traffic and other sources — such as particulate matter ≤2.5 µm in aerodynamic diameter ($PM_{2.5}$), particulate matter ≤10 µm in aerodynamic diameter (PM_{10}), nitrogen dioxide (NO_2), and nitrogen oxides (NO_x) — is associated with higher BMI and increased risk of obesity in children.[4,5,10] However, other studies have demonstrated no association between ambient or traffic-related air pollution exposures and childhood obesity,[11,12] and the overall evidence for this relationship remains mixed.[13,14] Studies have also shown that ambient air pollution is associated with low birth weight,[15,16] and infant birth weight is closely linked to the composition of the infant gut microbiome.[17] This connection between weight and the gut microbiome might help explain the mixed evidence regarding the relationship between early-life air pollution exposures and obesity.

The mechanisms and risk factors linking air pollution exposures and obesity — both generally and specifically in children — are not well understood. Potential mechanisms include the effects of air pollution on changes in gene expression that occur without altering DNA sequences (i.e., epigenetic modulation), oxidative stress and inflammation, and disruption of neuroendocrine pathways, which can alter metabolic processes and appetite regulation.[13,18] Additionally, recent work has suggested that ambient air pollution exposures might contribute to obesity by affecting metabolic health through changes in the gut microbiome and fecal metabolome (**Box 1**), due to alterations in gut bacteria composition and function.[7,9] For example, a study in adolescents demonstrated correlations between higher exposures to traffic-related air pollution and the abundances of gut bacteria previously

Dr. Tanya L. Aldrete's 3-year study, "Air Pollutants and the Gut Microbiota and Metabolome During Early Life: Implications for Childhood Obesity," began in May 2020. Total expenditures were $500,000. The draft Investigators' Report was received for review in October 2024. A revised report, received in March 2025, was accepted for publication in April 2025. During the review process, the HEI Review Committee and the investigators had the opportunity to exchange comments and clarify issues in the Investigators' Report and its Commentary. Review Committee member Kiros Berhane did not partake in the review of the report due to a conflict of interest.

*A list of abbreviations and other terms appears at the end of this report.

linked to changes in metabolism and obesity.[6] Other research has shown associations between ambient air pollution and changes in gut bacteria during early life with childhood BMI and obesity risk.[19,20] Nonetheless, at the time Dr. Alderete's study began, few studies had examined mechanisms linking prenatal and postnatal ambient air pollution exposures with the gut microbiome and microbiome-derived metabolites among infants in the context of providing potential insights for childhood obesity.

In the United States, regulatory efforts have sought to moderate the health effects of $PM_{2.5}$ and NO_2. The National Ambient Air Quality Standards, established by the United States Environmental Protection Agency (US EPA), limit the 3-year annual average $PM_{2.5}$ concentration to 9 $\mu g/m^3$ and the annual average NO_2 concentration to 53 parts per billion.[21,22] In the most recent integrated science assessments for both particulate matter and oxides of nitrogen, obesity was considered a risk factor for air pollution-related health outcomes.[23,24] In those assessments, the US EPA concluded that existing evidence suggests an increased risk for $PM_{2.5}$-related health effects among individuals with obesity compared with non-obese individuals, and that evidence remains inadequate to determine NO_2-related health effects.

STUDY OBJECTIVES

The overall objective of Dr. Alderete's study was to evaluate whether prenatal or postnatal exposures to ambient air pollution, including traffic-related air pollution, affect the infant gut microbiome and fecal metabolome during the first 2 years of life. Using stool samples collected longitudinally from infants aged 1, 6, 12, 18, and 24 months, the team sought to explore two specific aims:

Aim 1. Determine whether prenatal or postnatal exposure to air pollution is associated with a) lower gut bacterial diversity and altered abundances of gut bacteria and b) levels of fecal metabolites, at each timepoint (cross-sectional analyses).

Aim 2. Determine whether prenatal or postnatal exposure to air pollution is associated with a) the developmental trajectory of the infant gut microbiota (i.e., lower average bacterial diversity or altered average relative abundances of gut bacteria) and b) changes in average fecal metabolite levels over time during the first 2 years of life (longitudinal analyses).

For ease of comprehension, various terms used throughout this Commentary that refer to the outcomes and exposures assessed in the study are defined in **Box 2**.

Box 1: An Introduction to the Infant Gut Microbiome and Fecal Metabolome

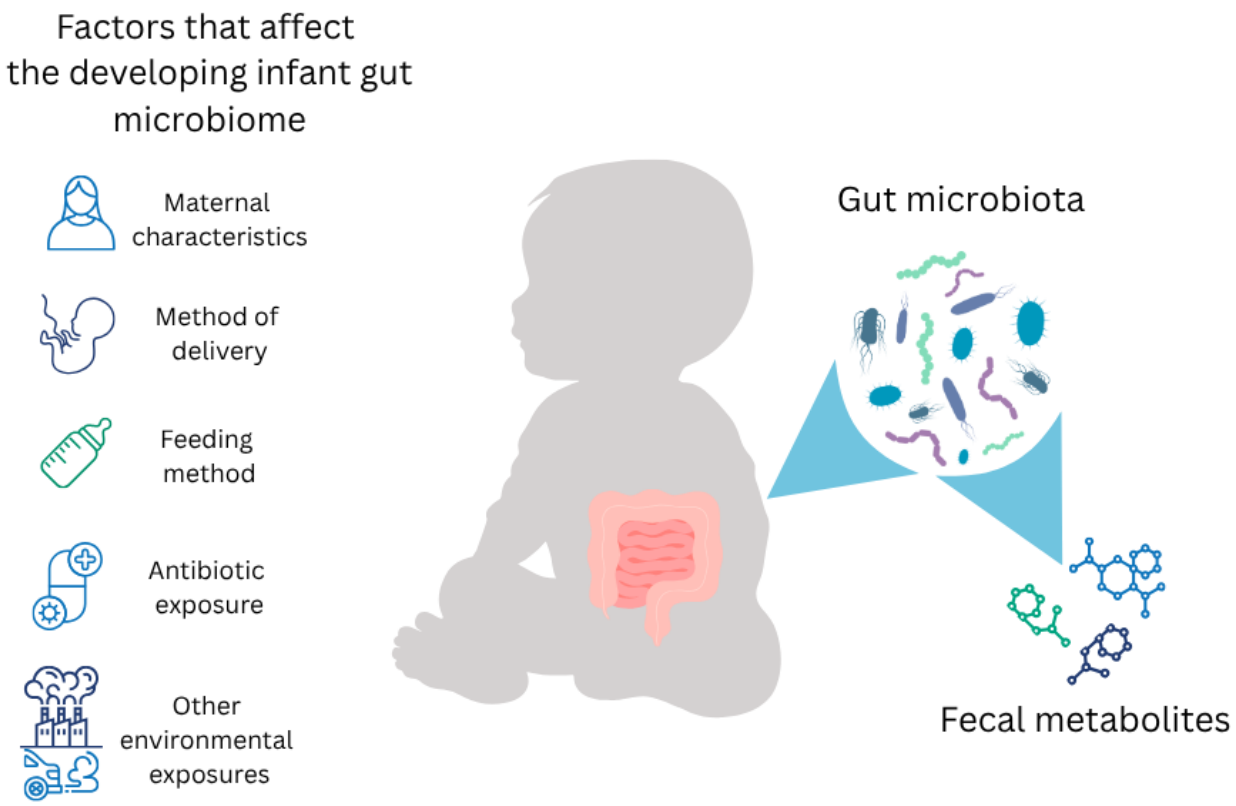
The infant gut microbiome consists of the microbiota, including bacteria, fungi, and viruses, found in the infant gastrointestinal tract. The infant fecal metabolome refers to the collection of metabolites, or small molecules, that reflect diet and metabolism, as well as metabolites produced by gut bacteria and fungi, or influenced by viral activity, as part of metabolic processes. It thus can partly provide a functional readout of the infant gut microbiome[25] (**Commentary Figure 1**).

Studies of the infant gut microbiome and fecal metabolome are important because the first 1,000 days after birth represent a critical period for the growth and development of the gut microbiome, with broad effects on the infant's immune system, metabolism, and neurodevelopment.[26,27] An array of factors influences this development, including maternal characteristics (such as diet, weight, and age) and, more importantly, the type of birth (vaginal delivery or cesarean section), the feeding method (human milk or formula), antibiotic exposure, and infant diet after the introduction of solid foods.[26–28] Additionally, other early-life exposures, such as exposures to air pollution, pets, metals, and chemicals, have been linked to alterations in the infant gut microbiota.[29–31]

Metagenomics and metabolomics are common methods used to study the gut microbiome and fecal metabolome, respectively. Generally, both approaches rely on stool samples, which are processed and analyzed using sequencing and mass spectrometry techniques, respectively.[32]

Metagenomics provides an overview of the composition, diversity, and function of the entire genomes of bacterial, fungal, and viral members of the microbiome by randomly sequencing DNA fragments within a sample. Metagenomic sequencing can provide phylogenetic information about the gut microbiota in a sample, including the abundance and diversity of various microbes,[25] and information related to the functional potential of the microbiome based on the presence of genes with specific known functions.[33] Abundance refers to the amount of a given type of microbe within a sample, whereas diversity refers to both the number of species (richness) and their distribution or relative abundance (evenness). Microbial diversity can be measured using alpha-diversity and beta-diversity metrics. Alpha-diversity focuses on microbial richness and evenness within a given sample.[34] Examples of alpha-diversity measures used in this study include the Shannon and Simpson indices. Beta-diversity focuses on differences in microbial composition between samples and was measured in this study using the Bray–Curtis dissimilarity metric.[35]

Fecal metabolomics provides insight into the metabolic processes occurring in the distal gut, which are partly driven by microbial metabolism. This approach involves identifying and quantifying metabolites within a sample using mass spectrometry. Fecal metabolic intensity reflects the relative levels of metabolites in a stool sample. The identification and quantification of metabolites can be either targeted (focusing on specific classes of compounds such as amino acids or fatty acids) or untargeted (aiming to identify as many metabolites as possible).[25]



Commentary Figure 1. Schematic of the infant gut microbiome and fecal metabolome.

Box 2: Defining Terms Used Throughout This Commentary

- Gut bacterial abundance: The amount of a specific gut bacterium within a sample.
- Gut bacterial diversity: A measure of the richness (number of unique bacterial types) or evenness of their distribution within a sample.
- Identity and levels of fecal metabolites: The type and quantity of small molecules detected in fecal samples, potentially produced by gut bacteria as byproducts of metabolism (referred to as "fecal metabolite intensity" in the Investigators' Report).
- Prenatal air pollution exposure: The average of monthly air pollutant concentrations for the 9 months before an infant's birth.
- Postnatal air pollution exposures: This study assessed three measures:
  - Short-term air pollution exposure: The average air pollutant concentration in the month before an infant's clinical visit.
  - Cumulative air pollution exposure: The average of monthly air pollutant concentrations from birth to a clinical visit.
  - Fluctuation from long-term air pollution exposure: The difference between the average of short-term air pollutant concentrations across all clinical visits and the monthly average concentration in the month before a clinical visit.

To address the study aims, Alderete and colleagues used a cohort of more than 200 Hispanic mother–infant pairs participating in the Southern California Mother's Milk Study. Pairs were enrolled at 1 month postpartum and attended subsequent clinical visits at various timepoints up to 24 months postpartum. Participants were all located in Southern California, and most resided in the Los Angeles area. Detailed information was available regarding both the mother and infant (such as age, sex, and socioeconomic status), as well as infant feeding practices. At each clinical visit, an infant stool sample was collected. All data were processed using metagenomic and metabolomic analysis techniques.

Based on the mothers' residential address histories, the team assigned estimates of prenatal and postnatal exposures to PM_{10}, $PM_{2.5}$, NO_2, ozone (O_3), and NO_x (used as a proxy for traffic-related air pollution) for each mother–infant pair. A cumulative 9-month average of air pollutant concentrations before birth was used to estimate prenatal exposure. Postnatal exposures in cross-sectional analyses included both

short-term (i.e., prior-month) and cumulative estimates of air pollution concentrations, whereas postnatal exposures in longitudinal analyses were based on fluctuations from long-term air pollution concentrations during the first 2 years of life. All exposure estimates were calculated using monthly concentrations of ambient air pollutants (PM_{10}, $PM_{2.5}$, NO_2, O_3) derived from US Environmental Protection Agency (US EPA) monitoring data and were spatially interpolated between central monitors. Monthly concentrations of traffic-related air pollutants (NO_x) were estimated using an air quality dispersion model.

Alderete and colleagues used a combination of negative binomial models and linear mixed-effects models to evaluate associations between air pollution exposure estimates and several outcomes of interest, including the abundances and diversity of gut bacteria in the infant gut microbiome and the identities and levels of fecal metabolites in the infant fecal metabolome.

The study also originally included a third aim to use mediation analysis to determine whether infant gut microbiota or fecal metabolites associated with higher estimated ambient or traffic-related air pollution exposures mediated changes in infant growth trajectories over time. However, this analysis could not be conducted due to substantial delays in the study related to the COVID-19 pandemic.

SUMMARY OF METHODS AND STUDY DESIGN

MOTHER'S MILK STUDY POPULATION

The study cohort was drawn from the Southern California Mother's Milk Study, a prospective cohort study of 219 Hispanic mother–infant pairs designed to examine the effects of human milk feeding on early-life growth and development. Eligible individuals were self-identifying Hispanic mothers who were at least 18 years old at the time of delivery; had a healthy, term (≥37 weeks) singleton birth; and intended to breastfeed for at least 6 months. Mother–infant pairs were enrolled at 1 month postpartum and attended follow-up visits at 6, 12, 18, and 24 months postpartum. Each visit included infant stool sample collection and completion of questionnaires on infant feeding practices (e.g., frequency of human milk feeding and age at which solid foods were introduced). Overall, the study sample included 207 infants with gut microbiome data; 127 of these infants were included in the high-resolution fecal metabolomics analysis.

The outcomes of interest for Dr. Alderete's study were 1) abundances of gut bacteria, 2) diversity of gut bacteria, and 3) identities and levels of fecal metabolites.

EXPOSURE ASSESSMENT

The investigators estimated monthly concentrations of PM_{10} (μg/m^3), $PM_{2.5}$ (μg/m^3), NO_2 (ppb), and O_3 (ppb) through spatial interpolation of monitoring data from the US EPA Air Quality System,[36] based on participants' residential address histories. NO_x was used as a proxy for traffic-related air pollution, and monthly concentrations of NO_x within 5 km of participants' homes were estimated using the California Line Source Dispersion Model (CALINE4),[37] an air quality model that integrates information regarding traffic emissions, traffic volume, roadway geometry, and meteorology to estimate pollutant concentrations near roadways.

Alderete and colleagues computed several measures of air pollutant exposure for each mother–infant pair, broadly categorized into prenatal and postnatal exposures, as previously defined in Textbox 1.

STOOL SAMPLE ANALYSIS AND DATA PROCESSING

For the gut microbiome analysis, the investigators performed DNA extraction and sequencing on infant stool samples. DNA sequence reads were taxonomically classified using the RefSeq database of bacterial, viral, plasmid, human, and vector sequences[38]; these reads were used to identify the relative abundances of gut bacterial taxa (categories used to classify bacteria based on shared biological characteristics) at the species level. The average number of reads per sample was about 17 million, which is considered relatively low in the context of fecal metagenomics. Shallow sequencing, in which a smaller amount of DNA is sequenced, provides a less detailed analysis of the microbiome compared with deep sequencing methods but is often more cost-effective. Gut bacterial diversity was assessed by calculating "alpha-diversity measures" to quantify species diversity within each sample (Shannon index, species richness, species evenness, and Simpson index) and "beta-diversity measures" to quantify differences in microbiome composition between samples (Bray–Curtis dissimilarity).

For the fecal metabolome analysis, stool samples were analyzed using liquid chromatography–high-resolution mass spectrometry (LC-HRMS). Fecal metabolites were profiled and analyzed using an untargeted approach (i.e., as many metabolites as possible were identified and quantified without prior knowledge of their identity or profile).

MAIN STATISTICAL ANALYSES

Alderete and colleagues conducted multiple statistical analyses to address their study aims. For brevity, see **Commentary Table 1** for a summary of the study population, exposure assessment, and statistical methods used.

Aim 1. Associations of Air Pollution Exposures with the Infant Gut Microbiome and Fecal Metabolome at Each Timepoint (Cross-Sectional Analyses)

The investigators used negative binomial and linear models and a variance test to evaluate associations between estimated prenatal and postnatal air pollution exposures and the infant gut microbiome. In these analyses, associations of estimated prenatal, cumulative, and short-term exposures to all air pollutants with gut bacterial abundances and diversity were examined for each infant follow-up visit. For the fecal metabolome analysis, associations of estimated prenatal,

Commentary Table 1. Summary of Main Statistical Analyses Conducted in This Study

Aim 1. Cross-Sectional Analyses				
Analysis	**Study Population**	**Estimated Exposures**	**Method**	**Outcome**
Infant gut microbiome	N = 207 infants with gut microbiome data 1-month visit (N = 196) 6-month visit (N = 157) 12-month visit (N = 155) 18-month visit (N = 143) 24-month visit (N = 171)	Prenatal, cumulative, and short-term estimates of PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x	1. Negative binomial models[a] 2. Linear models[a] 3. Permutational multivariate analysis of variance (PERMANOVA)	1. Abundances of gut bacterial taxa at each timepoint 2. Alpha-diversity of gut bacterial taxa at each timepoint 3. Beta-diversity of gut bacterial taxa at each timepoint
Infant fecal metabolome	N = 127 infants with fecal metabolite data 1-month visit (N = 124) 6-month visit (N = 116) 12-month visit (N = 120) 18-month visit (N = 123) 24-month visit (N = 126)	Prenatal, cumulative, and short-term estimates of PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x	Linear models[a]	Identity and levels of fecal metabolites at each timepoint
Aim 2. Longitudinal Analyses				
Analysis	**Study Population**	**Estimated Exposures**	**Method**	**Outcome**
Infant gut microbiome	N = 207 infants with gut microbiome data	Prenatal estimates of PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x	Negative binomial models[a]	Average abundances of gut bacterial taxa across timepoints
		Fluctuations from long-term early-life estimates of PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x	1. Negative binomial models[b] 2. Linear mixed-effects models[b]	1. Average abundances of gut bacterial taxa across timepoints 2. Alpha-diversity of gut bacterial taxa across timepoints
Infant fecal metabolome	N = 127 infants with fecal metabolite data	Prenatal and fluctuations from long-term early-life estimates of PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x	Linear mixed-effects models[b]	Average levels of fecal metabolites across timepoints

[a]All models were adjusted for infant age and sex, maternal age, maternal prepregnancy BMI, socioeconomic status, human milk feedings per day, formula feedings per day, type of delivery, whether solid foods had been introduced (6-month timepoint and after), and diet quality (12-month timepoint and after).

[b]All models were adjusted for infant age and sex, maternal age, maternal prepregnancy BMI, socioeconomic status, human milk or formula feedings per day, type of delivery, whether solid foods had been introduced, diet quality, and long-term early-life air pollution exposure.

cumulative, and short-term air pollution exposures with the identities and levels of fecal metabolites were assessed using multivariable linear models.

All models were adjusted for factors such as maternal and infant demographic characteristics, infant diet, and maternal BMI. All models also included adjustment for multiple testing using the Benjamini–Hochberg procedure to control the false discovery rate.

Aim 2. Associations of Air Pollution Exposures with the Infant Gut Microbiome and Fecal Metabolome Across All Timepoints (Longitudinal Analyses)

In longitudinal analyses of the infant gut microbiome, Alderete and colleagues used negative binomial models to evaluate associations between estimated prenatal exposure and fluctuations from long-term early-life air pollution exposures with the average abundances of gut bacterial taxa across

all timepoints. Linear mixed-effects models were used to assess associations between fluctuations from long-term early-life air pollution exposures and the average diversity of gut bacterial taxa, as well as the average level of fecal metabolites across all timepoints.

All models were adjusted for a suite of characteristics, such as maternal and infant demographic characteristics, infant diet, maternal BMI, and average long-term early-life air pollution concentrations. A random intercept was included in all models to adjust for repeated measures within infants.

SUMMARY OF KEY RESULTS

POPULATION CHARACTERISTICS AND EXPOSURE ASSESSMENT

The study included 207 infants with gut microbiome data and a subset of 127 infants with fecal metabolomics data from the Southern California Mother's Milk cohort. Sample sizes varied across timepoints because some participants missed follow-up visits (Commentary Table 1). The average prepregnancy BMI of the mothers was 28.3 kg/m^2, and the average maternal age at the 1-month follow-up visit was 29 years. About 55% of infants in the cohort were female, and about 25% had been delivered by cesarean section — both percentages are similar to rates in the overall US population.

Average estimated prenatal and postnatal exposures to air pollutant concentrations among participants across all timepoints are summarized in **Commentary Table 2**. Average estimated prenatal exposures were broadly consistent with average estimated cumulative and short-term exposures.

PRENATAL AND POSTNATAL AIR POLLUTION EXPOSURES AND THE INFANT GUT MICROBIOME AND FECAL METABOLOME: MAIN STATISTICAL ANALYSES

Overall, Alderete and colleagues found that both estimated prenatal and postnatal exposures to PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x demonstrated some associations with short-term and longer-term changes in the abundances of gut bacterial taxa in the infant gut microbiome. Similarly, they found that PM_{10}, $PM_{2.5}$, and NO_2 demonstrated some associations with changes in the level of specific fecal metabolites in the infant fecal metabolome. **Commentary Figure 2** provides an overview of the study's main findings.

Aim 1. Associations Between Air Pollution Exposures and the Infant Gut Microbiome and Fecal Metabolome at Each Timepoint (Cross-Sectional Analyses)

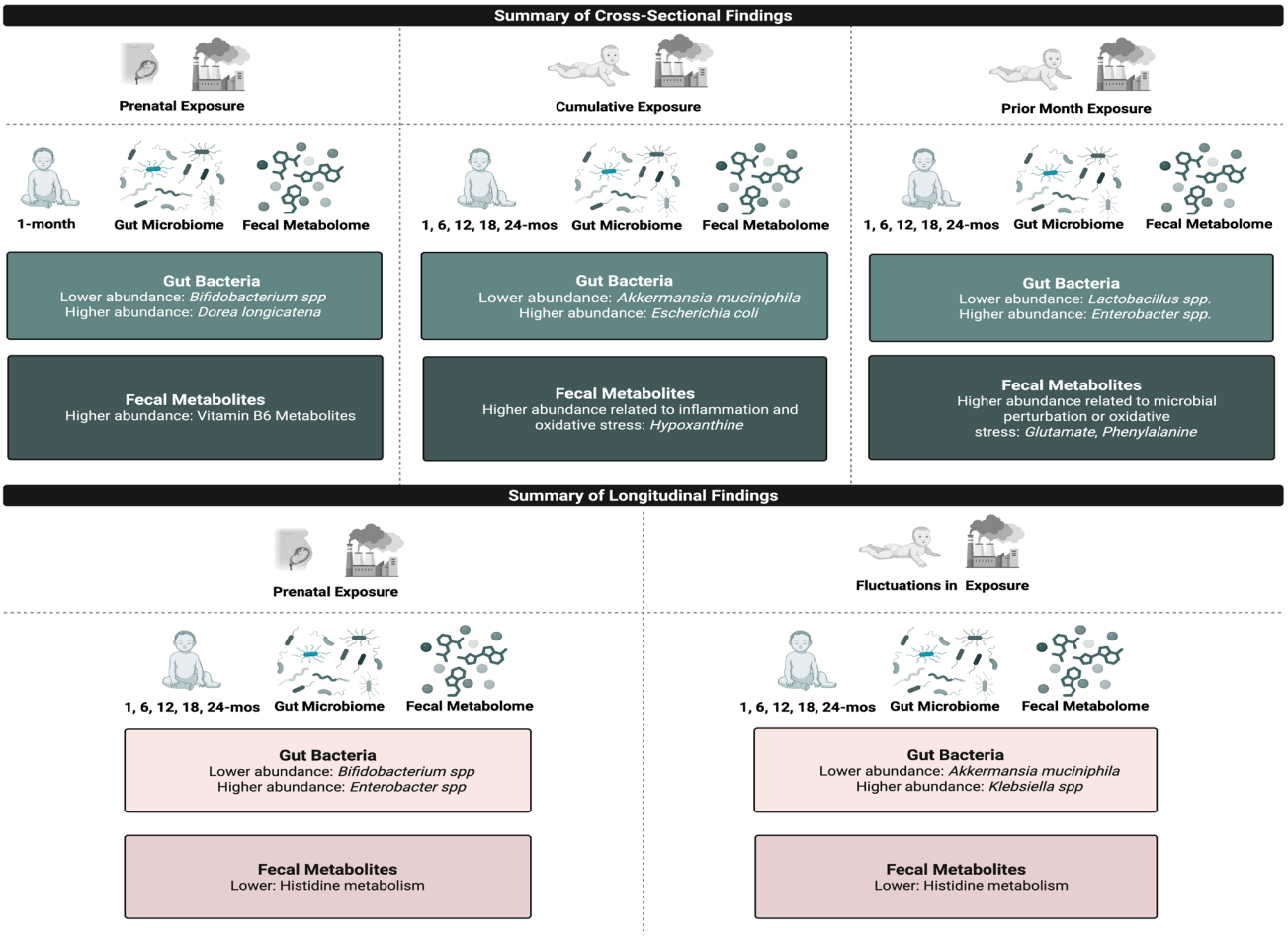
Gut Bacterial Abundance The team conducted cross-sectional analyses to evaluate associations between estimated prenatal exposures to all ambient and traffic-related air pollutants and gut bacterial abundance. They observed a mix of positive and inverse associations with the abundances of various gut bacterial taxa, potentially indicating a shift toward fewer beneficial bacteria. For example, increased PM_{10}, NO_2, and NO_x exposures were all associated with a lower abundance of *Bifidobacterium*, which is typically considered to promote gut health. Increased NO_x exposure was associated with a higher abundance of the potentially detrimental gut bacterium *Lelliottia amnigena*. Associations between estimated short-term (i.e., prior-month) and cumulative exposures to all air pollutants examined with gut bacterial abundances varied in both direction and magnitude; there were no clear patterns across timepoints, specific taxa, or pollutants.

Gut Bacterial Diversity The investigators reported statistically significant associations between estimated short-term and cumulative exposures to PM_{10}, O_3 (with and without adjustment for NO_2), and NO_x with various measures of alpha-diversity, such as the Shannon and Simpson indices. These findings indicated both greater and lesser fecal alpha-diversity, depending on the exposure metric and pollutant examined. Such associations were observed only at the 1-month and/or 6-month timepoints (see Investigators' Report Tables 2 and 6 for additional details). Only estimated short-term and cumulative NO_2 exposures were significantly associated with beta-diversity (i.e., Bray–Curtis dissimilarity), indicating differences in overall microbiome composition linked to NO_2 exposure.

Commentary Table 2. Average Estimated Exposures to Ambient Air Pollutants Across Cohort Participants for Each Exposure Measure Across the Study Period[a]

Ambient Air Pollutant	Prenatal Exposure Concentrations	Cumulative Exposure Concentrations	Short-Term (Prior Month) Exposure Concentrations	Fluctuations from Long-Term Exposure Concentrations
$PM_{2.5}$ (μg/m^3)	11.9	12.2	11.8	0
PM_{10} (μg/m^3)	29.9	30.7	28.7	−0.4
NO_2 (ppb)	18.0	17.8	16.5	−0.1
O_3 (ppb)	42.7	42.1	42.4	0
NO_x (ppb)	3.9	3.3	3.5	0

[a]Adapted from Investigators' Report Table 1.



Commentary Figure 2. Overview of main study findings. Source: Adapted from Holzhausen EA and Alderete TL (2025): *https://BioRender.com/m57g481*.

Fecal Metabolites In analyses of prenatal exposures and fecal metabolites, Alderete and colleagues found that increased exposures to PM_{10}, $PM_{2.5}$, and NO_2 were all associated with higher relative levels of fecal metabolites involved in vitamin B6 metabolism and brain function. Higher estimated prenatal NO_x exposures were associated with lower levels of metabolites linked to the breakdown of dietary sugars. No associations were observed for estimated prenatal O_3 exposures. The majority of statistically significant associations between short-term exposures (PM_{10}, $PM_{2.5}$, O_3 [adjusted for NO_2], and NO_x) or cumulative exposures (PM_{10}, NO_2, and NO_x) and levels of fecal metabolites were observed at the 6-month timepoint, although there was no apparent pattern in metabolite identity across pollutants.

Aim 2. Associations Between Air Pollution Exposures and the Infant Gut Microbiome and Fecal Metabolome Across All Timepoints (Longitudinal Analyses)

Gut Bacterial Abundance In longitudinal analyses, Alderete and colleagues evaluated estimated prenatal exposures to all ambient and traffic-related air pollutants and average gut bacterial abundances during the first 2 years of life. They observed that PM_{10}, $PM_{2.5}$, NO_2, O_3, and NO_x were associated with changes in the average abundances of gut bacterial taxa over time. Generally, associations among air pollutants and specific gut bacterial taxa varied, except for estimated prenatal $PM_{2.5}$ and NO_2 exposures, which were both associated with lower abundances of gut bacteria from the genus *Megasphaera*.

Similar to the cross-sectional analyses, some ambient air pollutants were associated with higher abundances of potentially detrimental gut bacteria. For instance, higher estimated NO_2 concentrations were associated with a higher abundance of *Klebsiella* in fecal samples. Estimated postnatal fluctuations from long-term early-life exposures to NO_2 and O_3 (with and without adjustment for NO_2) were also associated with higher abundances of certain *Klebsiella* species. In general, estimated postnatal exposures for all air pollutants showed statistically significant associations with changes in the average abundances of several gut bacterial taxa, again without an apparent trend according to pollutant or taxon. The investigators did not observe associations between estimated postnatal fluctuations from long-term early-life exposures and gut bacterial diversity (assessed using alpha-diversity measures) in longitudinal analyses.

Fecal Metabolites In longitudinal analyses of estimated prenatal exposures and average fecal metabolite levels over time, only $PM_{2.5}$ and NO_2 were associated with levels of fecal metabolites, specifically those involved in histidine and tyrosine metabolism. Regarding estimated postnatal fluctuations from long-term early-life exposures, statistically significant associations were observed for all pollutants examined except NO_x. For example, higher postnatal $PM_{2.5}$ and PM_{10} exposures were both associated with lower levels of metabolites involved in histidine metabolism. Overall, there were no obvious patterns in the associations between average levels of specific fecal metabolites and pollutant exposures.

HEI REVIEW COMMITTEE'S EVALUATION

This study leveraged a unique dataset from a southern California Hispanic mother–infant cohort to evaluate potential associations between prenatal and early-life air pollution exposures and infant gut bacteria and fecal metabolites. Alderete and colleagues observed that, in both cross-sectional and longitudinal analyses, estimated prenatal and postnatal exposures to ambient and traffic-related air pollution demonstrated some associations with changes in the abundances and diversity of infant gut bacteria and the identities and levels of infant fecal metabolites, with some indication of a shift toward fewer beneficial gut bacteria.

In its independent evaluation, the HEI Review Committee concluded that this study provides a detailed set of exploratory analyses that contribute to understanding potential mechanistic links between air pollution and the gut microbiome in infants, with a possible connection to childhood obesity. The Committee also emphasized that the dataset collected for this study is highly valuable and has strong potential for use in future research. Details on the strengths and limitations of the study are discussed below.

STUDY DESIGN, DATASETS, AND ANALYTICAL APPROACHES

The Committee identified the collection of a novel dataset on the infant gut microbiome and fecal metabolome as a key strength of the study. They also appreciated the thorough initial analyses, which considered both cross-sectional and longitudinal associations.

Several limitations were highlighted by the Committee. First, the sample size for the study cohort was relatively small (207 mother–infant pairs), and only 127 infants were included in the fecal metabolomics analysis, thus limiting the statistical power of the cross-sectional analyses. Second, given the number of variables in the dataset, multiple statistical tests were conducted, which greatly increased the potential for false positives. Alderete and colleagues appropriately acknowledged these limitations and applied the Benjamini–Hochberg procedure to adjust for multiple testing.

Third, the Committee noted that the investigators conducted shallow sequencing (millions of reads per sample) rather than deep sequencing (tens to hundreds of millions of reads per sample) and therefore did not leverage the full potential of the dataset. Fourth, the outcomes selected for this study primarily focused on phylogeny, emphasizing the composition of different species or taxa in the microbiome. The Committee suggested that the inclusion of outcomes related to gut microbial function would have provided additional insights into the relationship between early-life air pollution exposures and gut health.[39] Finally, the Committee stated that future research would benefit from consideration of the viral and fungal microbiomes, rather than focusing solely on the bacterial microbiome.

FINDINGS AND INTERPRETATION

In the cross-sectional and longitudinal analyses, Alderete and colleagues observed that estimated prenatal and early-life exposures to ambient and traffic-related air pollution demonstrated some associations with lower abundances of potentially beneficial gut bacterial species and higher abundances of detrimental gut bacterial species. However, no single ecological or molecular mechanism or pattern was evident across pollutants, outcomes, or timepoints during the first 2 years of life. The investigators also found that increased prenatal or early-life exposures to PM_{10}, $PM_{2.5}$, and NO_2 were generally associated with higher levels of several fecal metabolites, some of which might indicate oxidative stress or gut inflammation (e.g., histidine). However, similar to the metagenomic findings, the fecal metabolome analyses did not reveal clear patterns across pollutants, outcomes, or timepoints.

Given the varied findings and study design limitations, the Committee determined that this work represents a comprehensive set of exploratory analyses and a valuable contribution, but it emphasized that further research is needed. The Committee appreciated that the investigators appropriately characterized this study as hypothesis-generating, with potential for further exploration in future research.

The Committee also recognized that the investigators thoughtfully outlined several future directions, including evaluation of the potential mediating effects of the gut microbiome and fecal metabolome on associations between

ambient air pollution exposure and infant growth trajectories, as well as the use of more advanced multi-omics analytical approaches to explore gut bacterial function based on genes and gene pathways. Finally, the Committee highlighted that the dataset collected for this study represents an excellent resource for other researchers to conduct additional studies.

CONCLUSIONS

In summary, Alderete and colleagues examined whether prenatal or early-life exposures to ambient and traffic-related air pollution were associated with changes in the infant gut microbiome and fecal metabolome during the first 2 years of life. They found that both prenatal and early-life air pollution exposures demonstrated some associations with alterations in the abundances of gut bacteria in the infant microbiome and in the identities and levels of fecal metabolites in the infant metabolome. Although no substantial or conclusive patterns emerged, some associations indicated lower abundances of beneficial gut bacteria, higher abundances of potentially detrimental gut bacteria, and higher levels of metabolites that might indicate oxidative stress and inflammation. Ultimately, this study represents an extensive set of exploratory analyses that can be used in future research aimed at understanding the links between air pollution and the infant gut microbiome and fecal metabolome. Moreover, future research can benefit from this study's unique dataset, which can serve as a valuable resource for additional studies in this field.

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ABBREVIATIONS AND OTHER TERMS

b	beta estimate
BH	Benjamini–Hochberg
BMI	body mass index
C18	reverse-phase chromatography
CS	cesarean section
ESI	electrospray ionization
HILIC	hydrophilic interaction liquid chromatography
Metab.	metabolite
NO_2	nitrogen dioxide
NO_x	nitrogen oxides
O_3	ozone
PERMANOVA	permutational multivariate analysis of variance
PM	particulate matter
$PM_{2.5}$	particulate matter ≤2.5 μm in aerodynamic diameter
PM_{10}	particulate matter ≤10 μm in aerodynamic diameter
SD	standard deviation
SE	standard error
SES	socioeconomic status
US EPA	United States Environmental Protection Agency

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