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Air Pollution Exposure is Associated with the Gut Microbiome as Revealed by Shotgun Metagenomic Sequencing

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Author Contributions

T.L.A. conceptualized this study, defined the research question, and wrote the manuscript. F.F. and C.M. analyzed data and wrote the manuscript. A.F., M.S., I.B., R.B.J., and Z.C. provided statistical input and contributed to the review and editing of the manuscript. M.B. and W.B.P. contributed to the review and editing of the manuscript. F.D.G. designed the original Meta-AIR study and contributed to the review and editing of the manuscript. J.S.K. assisted with primary data collection and the review and editing of the manuscript. F.L. provided exposure estimates and R.K. conducted sequencing studies and both individuals assisted with data interpretation. F.F. and T.L.A. had primary responsibility for final content. All authors read and approved the final manuscript.

Author Statement

Tanya L Alderete: Conceptualization, Funding Acquisition, Project Administration, Methodology, Writing - Original Draft Preparation. **Farnaz Fouladi:** Methodology, Formal Analysis, Data Curation, Writing - Original Draft Preparation. **Cameron Martino:** Formal Analysis, Writing - Review & Editing. **Anthony A. Fodor:** Resources, Software, Validation, Writing - Review & Editing. **Michael Sioda:** Software, Writing - Review & Editing. **Ivory C. Blakley:** Writing - Review & Editing. **Roshonda B. Jones:** Statistical Input, Writing - Review & Editing. **Zhanghua Chen:** Statistical Input, Writing - Review & Editing. **Maximilian J. Bailey:** Writing - Review & Editing. **William B. Patterson:** Writing - Review & Editing. **Frank D. Gilliland:** Funding Acquisition, Project Administration, Writing - Review & Editing. **Jeniffer S. Kim:** Project Administration, Investigation, Writing - Review & Editing. **Frederick Lurmann:** Data Curation, Methodology, Writing - Review & Editing. Rob Knight: Resources, Investigation, Writing - Review & Editing.

Competing Interests statement

The authors declare no competing interests.

Abstract

Animal work indicates exposure to air pollutants may alter the composition of the gut microbiota. This study examined relationships between air pollutants and the gut microbiome in young adults residing in Southern California. Our results demonstrate significant associations between exposure to air pollutants and the composition of the gut microbiome using whole-genome sequencing. Higher exposure to 24-hour O_3 was associated with lower Shannon diversity index, higher *Bacteroides caecimuris*, and multiple gene pathways, including L-ornithine de novo biosynthesis as well as pantothenate and coenzyme A biosynthesis I. Among other pollutants, higher NO_2 exposure was associated with fewer taxa, including higher *Firmicutes*. The percent variation in gut bacterial composition that was explained by air pollution exposure was up to 11.2% for O_3 concentrations, which is large compared to the effect size for many other covariates reported in healthy populations. This study provides the first evidence of significant associations between exposure to air pollutants and the compositional and functional profile of the human gut microbiome. These results identify O_3 as an important pollutant that may alter the human gut microbiome.

Keywords

air pollution; gut microbiome; whole genome sequencing

Introduction

Air pollution has a significant impact on population health and has been identified as the fifth leading risk factor for mortality worldwide (“State of Global Air 2019. Special Report. Boston, MA: Health Effects Institute.,” 2019). Most of the disease burden that is attributable to air pollution results from chronic noncommunicable diseases, including respiratory disease and type 2 diabetes. In addition to exposure-induced effects on mortality, work has shown that long-term exposure to particulate matter with an aerodynamic diameter ≤2.5 micrometers ($PM_{2.5}$), nitrogen dioxide (NO_2), ozone (O_3), and nitrogen oxides (NOx) are associated with greater risk for obesity, glucose dysregulation, and type 2 diabetes (Alderete et al., 2018a; Jerrett et al., 2017; 2014; McConnell et al., 2015; Miller et al., 2016; Vella et al., 2015). The mechanisms underlying these associations remain uncertain but are thought to include increased systemic levels of inflammation, alterations to adipose tissue metabolism, as well as impacts on the gut microbiota (Alderete et al., 2018b; Kelishadi et al., 2014; C. Liu et al., 2014; Matthews et al., 2016; Sun et al., 2009). For example, two studies have shown that increased near-roadway air pollution exposure (NOx) was correlated with the relative abundance of gut bacteria that have been associated with obesity and altered metabolism (Alderete et al., 2018b; Ley et al., 2006). Additionally, a recent population-based epidemiological study found that the gut microbiota partially mediated the effect of fine particulate matter (PM) on type 2 diabetes (T. Liu et al., 2019). To date, no human studies have examined the association between exposure to air pollutants with both the composition and functional potential of the gut microbiome using shotgun metagenomic sequencing.

Studies suggest that air pollutants may adversely affect the gastrointestinal tract (Beamish et al., 2011; Lomer et al., 2002) where ultrafine particles may reach the intestine through inhalation and diffusion from the terminal alveoli into systemic circulation or through ingestion of inhaled particles following mucociliary clearance from the airways (Beamish et al., 2011; Elder and Oberdörster, 2006; Salim et al., 2014). Once in the intestine, PM components may alter the composition and function of the gut microbiota by either supporting or inhibiting the growth of specific microbes (Adams et al., 2015; Gao et al., 2016; X. Li et al., 2019; Yasuyuki et al., 2010). Additionally, $PM_{2.5}$ and O_3 have been shown to have extrapulmonary effects that may alter the hypothalamic-pituitary-adrenal (HPA) axis through vagal nerve activation (Gackière et al., 2011) or effects on the hippocampus (Thomson, 2019), which can increase levels of catecholamines and steroid hormones. Animal and human studies have shown that increased exposure to O_3 resulted in increased plasma corticosterone levels (Thomas et al., 2018) as well as plasma cortisol and corticosterone concentrations (Miller et al., 2016), respectively. Thus, O_3 induced activation of the HPA axis may increase the production of cortisol and norepinephrine, which may alter the composition of the gut microbiota (Bassett et al., 2019; Lyte and Ernst, 1993; Petrosus et al., 2018) via receptors that have similar activity to adrenergic receptors (Hughes et al., 2009; Hughes and Sperandio, 2008). Norepinephrine may also induce changes in the enteric nervous system (Carabotti et al., 2015), which can alter gastrointestinal motility, mucus secretion, and ion transport (Corfield, 2018; Keely et al., 2012; Sandle and Binder, 1987; Schroeder, 2019; Sheppard, 2002; Wiles et al., 2016). Such changes in the luminal environment, could lead to alterations in the gut microbiota. Overall, this hypothesized mechanism is supported by the gut-brain axis, which allows for bidirectional communication (Lyte, 2014) that may result in changes in bacterial proliferation in the presence of norepinephrine (Lyte and Ernst, 1992) at the gut lamina propria.

Studies have shown that the gut microbiota may contribute to the development of obesity and type 2 diabetes (Ley et al., 2006; Qin et al., 2012; Ross et al., 2015; Turnbaugh et al., 2006; Vrieze et al., 2012). Emerging evidence also indicates that air pollutants may impact the relative abundance of gut bacteria (Alderete et al., 2018b; T. Liu et al., 2019). Despite this, it remains unknown whether air pollutants are associated with the composition and functional potential of the gut microbiome. The primary aim of this study was to determine whether residential based estimates of $PM_{2.5}$, particulate matter with an aerodynamic diameter ≤10 micrometers in diameter (PM_{10}), NO_2, O_3, or NOx exposures were associated with the gut microbiome in young adults before and after adjusting for potentially important confounding variables, which included body mass index (BMI), age, sex, race/ethnicity, season of testing, diet, and parental education. We hypothesized that higher prior year exposure to air pollutants would be associated with the relative abundance and the functional potential of gut bacteria using shotgun sequencing of stool samples. In an exploratory analysis, we sought to determine whether specific gut bacterial taxa found to be associated with exposures were also associated with BMI, body fat percent, and risk factors for type 2 diabetes (i.e., fasting glucose, fasting insulin, 2-hour glucose and insulin levels, glucose and insulin area under the curve, insulin resistance, and β-cell function) or markers of gut bacterial translocation (i.e., lipopolysaccharide-binding protein (LBP), sCD14).

Materials and Methods

Research Design

This microbiome study was composed of 101 participants who were recruited from the Meta-AIR (Metabolic and Asthma Incidence Research) study between 2014–2017 at the University of Southern California (USC). The Meta-AIR study was a sub study of the Southern California Children's Health Study (CHS), which was a large school-based cohort that began in 2002–2003. The primary aim of the Meta-AIR study was to investigate the impacts of ambient and near-roadway exposures on metabolic health and adiposity in young adults. Participants in the Meta-AIR study were recruited based on their overweight and obese status, which was determined at school visits in 2011–2012. This study also recruited participants to represent the extremes of residential NOx values in Southern California CHS communities using probability weighted sampling from addresses reported at their last school visit. The inclusion criteria for Meta-AIR included participation in Cohort E of the CHS, as well as age and sex adjusted BMI of ≥ 85th percentile in 2011–2012, and the absence type 1 or type 2 diabetes. The CHS study design has been described in detail previously (Chen et al., 2015; Gauderman et al., 2015; Urman et al., 2014). Briefly, children from Cohort E were followed from kindergarten or first grade to high school graduations with an original enrollment of 3,474 participants (Chen et al., 2015). Smoking was not an exclusion criterion, yet only 9.9% of participants in this study reported smoking in within the last month. Participants were excluded if they were using any medication or diagnosed with a condition known to influence insulin and/or glucose metabolism or body composition. Additionally, participants were excluded from the current study if they used antibiotics in the two weeks prior to their clinical visit. Prior to testing, informed written consent/assent was obtained from the parents/participant. The University of Southern California Institutional Review Board approved this study.

Clinical Assessments

As previously reported (Kim et al., 2019) Meta-AIR study participants underwent thorough phenotyping which included measures indicative of adiposity and metabolic outcomes, which were collected at the USC Diabetes and Obesity Research Institute and Clinical Trials Unit. These clinical measures included height, weight, waist circumference, blood pressure, heart rate and an oral glucose tolerance test (OGTT). BMI was calculated as weight in kilograms over the square of height in meters and body composition was assessed using a dual-energy X-ray absorptiometry (DEXA) scan. The Human Insulin ELISA Kit (EMD Millipore) was used to assay fasting insulin, and a hexokinase-mediated reaction assay with Roche Covas C501 was used to assay glucose tolerance. Insulin resistance was calculated by HOMA-IR [fasting glucose (mg/dL)*fasting insulin (μU/mL)/405]. β-cell function (HOMA-β) was calculated by (360*fasting insulin)/(fasting glucose-63). A subset of 81 participants' diets were assessed using 24-hour diet recalls, and this nutritional data was analyzed using the Nutrition Data System for Research (version 2014, University of Minnesota).

Regional Ambient and Near-Roadway Air Pollution Exposure Assessment

Residential history was collected during study visits which included move in and move out dates for each respective residence accounting for multiple places of residence. Latitude and

longitude coordinates were determined via parcel level geocoding of residential addresses using the Texas A&M geocoder (http://geoservices.tamu.edu/Services/Geocode/). As described in our previous study (Alderete et al., 2018b), the determined latitude and longitude coordinates were used to estimate average prior year residential ambient and near-roadway air pollutant exposure levels. For example, in the case that a resident inhabited more than one residence in the year prior to their study visit, time spent at each residence was determined and exposure estimates were weighted based on this time distribution.

Ambient monitoring stations were used to determine monthly air pollution exposure by downloading hourly air quality data from the U.S. Environmental Protection Agency's Air Quality System (https://www3.epa.gov/ttn/airs/airsaqs/). Using this method, exposure data for the 12 months prior to each visit were estimated for each participant and used to calculate prior year exposure. These air quality measurements provide a good characterization of relative regional air pollution gradients since monitoring stations are spaced 20–30 kilometers (km) apart. Federal Reference Method (FRM) monitors were used to measure gaseous pollutants, whereas FRM and Federal Equivalent Method (FEM) monitors were used to measure particulates. Monthly averages were calculated from daily data using 75% completeness criteria. To calculate monthly ambient exposures, parcel level data was used in the inverse distance-squared weighting (IDW2) algorithm which spatially interpolated air quality data from up to four monitoring stations within a 50 km radius of the participant's residence (Wong et al., 2004). Prior work by our group has shown that the IDW2 method in California was robust to a leave one out validation for monthly monitoring AQS site data and performs as well as more sophisticated models that are limited by shorter spatial-temporal coverage (Eckel et al., 2016).

In order to model residential near-roadway exposure for the year prior to each clinical visit, the California Line Source Dispersion Model (CALINE4) was used to estimate levels of nitrogen oxides (NOx) produced by motor vehicles on roads within 5 km of each residence. CALINE4 takes into account traffic counts, road geometry, emission factors, and local meteorology (e.g., wind speed/direction, mixing heights, atmospheric stability) (Benson, 1992). This model obtains traffic counts and road geometry from Caltrans and TeleAtlas/GDT. The CALINE4 model encompasses annual-average traffic impact estimates from all road classes (i.e. freeways or highways, major roadways, and minor roadways). These estimates were based on the Streetmap Premium database (ArcGIS 10.1, Environmental Systems Research Institute Inc., Redlands, CA). Total near-roadway exposures were defined as the sum of freeway and non-freeway sources of NOx. Non-freeway exposures were defined as the sum of NOx from major and minor roadways. Near-roadway air pollution is a complex mixture of particles and gases, including NOx, carbon monoxide, particulate matter, organic compounds, elemental carbon, and polycyclic aromatic hydrocarbons (Fujita et al., 2007) that differ by roadways type and traffic volumes (Clements et al., 2009; Zhu et al., 2009). Due to the complexity of this exposure, NOx was used as a marker for the mixture of near-roadway pollutants. The CALINE4 line source dispersion model has been evaluated against near-road hourly observations in numerous studies (Benson, 1992; Kenty et al., 2007; Levitin et al., n.d.; Yura et al., 2007). Thus, the CALINE4 model has demonstrated reasonable performance for a variety of inert pollutants in different roadway and meteorological setting.

Gut Microbiome

Fecal samples were collected using commercial collection kits (Second Genome, San Francisco, CA) that contain a preservative (Norgen Biotek, Canada) which stabilizes DNA and RNA at ambient temperatures. Within 2.4 days after collection, fecal samples were stored at −80°C until analysis. Genomic DNA was isolated from samples and amplified in accordance with Earth Microbiome Project standard protocols (van Esterik et al., 2015), and Genomic isolates were collected in a final 50 μl extraction volume. Shallow whole-genome sequencing (WGS) was conducted at the University of California, San Diego in Dr. Rob Knight's laboratory, and used to determine the relative abundance of bacterial taxa. WGS sequencing was performed on the Illumina HiSeq 4000 platform.

Metagenomic DNA sequences were decontaminated from human genome reads using KneadData (http://huttenhower.sph.harvard.edu/kneaddata). Taxonomic classification was performed using Kraken2 (Wood and Salzberg, 2014) through an automated pipeline BioLockJ (https://github.com/msioda/BioLockJ). Average number of reads per sample was 366,499 ± 161,679 (n=101). Abundance of microbial sequence read counts were normalized for each taxonomic group in order to account for varying sequencing depths across samples using the equation (McCafferty et al., 2013) below:

$$\log_{10}\left(\left[\frac{Raw\ count\ in\ sample\ (i)}{\#\ \ of\ sequences\ in\ sample\ (i)} \times Average\ \#\ \ of\ sequences\ per\ sample\right] + 1\right)$$

This normalization scheme attempts to mitigate the impact of the pseudo-count on samples of different sequencing depth. Microbial gene families and pathways were profiled using the HUMAnN2 pipeline (Franzosa et al., 2018). Briefly, HUMAnN2 characterizes the abundance of each pathway in a community as a function of the abundance of the pathway's component reactions and each reaction's abundance is computed as the sum over abundances of genes that catalyze a reaction. Gene family and pathway abundance reads per kilobase were normalized to copies per million (cpm). On average, 492768 ± 68943 cpm in each sample were uniquely annotated to a gene family using the UniRef90 database (Suzek et al., 2015), and 25,601 ± 3,681 cpm in each sample were functionally annotated to a gene pathway using the MetaCyc database (Caspi et al., 2018). In addition, HUMAnN2 uses the MetaPhlAn2 (Truong et al., 2015) classifier to assign taxonomy to organisms in a metagenomic sample. Taxonomic classification from MetaPhlAn2 classifier was further used to confirm the results from Kraken2.

Statistical Analysis

Descriptive statistics are presented as mean ± standard deviation (SD) for continuous variables and as frequency (percentage) for categorical variables. The Shannon diversity index (a measure of richness and evenness) was calculated using the vegan package in the R statistical program. Briefly, a community with a higher number of species and a homogeneous distribution of relative abundances has a higher Shannon diversity index. A higher diversity can be indicative of a healthier microbial ecosystem (Calle, 2019). Linear regression analysis was performed to examine the univariate associations between the gut microbiome and air pollutants (i.e., total NOx, $PM_{2.5}$, PM_{10}, NO_2, O_3). Multivariable linear

models were then used to study these associations after adjusting for potentially important covariates (i.e., age, BMI, energy intake, macronutrients, Hispanic ethnicity, sex, season of testing (warm/cold), and parental education as a proxy for socioeconomic status). Parental education was categorized as low (n=43), high (n=38), and missing/don't know (n=20). Low parental education was defined as <12th grade, completed grade 12, and some college or technical school while high parental education was defined as 4 years of college and some graduate training college. There was strong correlation among the air pollutants (Supplemental Table 1); however, sensitivity analyses were performed using multi-pollutant models for O_3 and NO_2 (Spearman's rank-order correlation $r = -0.42$; $p<0.001$). Following this, Kendall tests were used to determine if findings from the linear models were robust to non-parametric testing. For all univariate linear regression models and Kendall tests, rare taxa or gene pathways that were present in less than 25% in samples were removed. Additional analysis was performed to confirm the primary findings form the linear regression analysis in Songbird (v1.0.1) (Morton et al., 2019). This differential abundance analysis was performed on the species level Kraken2 results with respect to O_3. Briefly, Songbird accounts for the compositional nature of microbial data and uses a multinomial regression model to estimate differential ranks. Optimized model parameters were determined (differential prior = 1 and learning rate = 0.01) and compared to a baseline model of 1. The models are then compared by a Q^2-value defined as 1 – model CV/baseline CV, which is similar to a R^2-value used in ordinary linear regression. A Q^2-value close to one and greater than zero ensures that the covariates entered into the formula are improving the model fit. A tutorial on this procedure can be found at https://github.com/biocore/songbird. We obtained a Q^2-value of 0.37 when including the covariate O_3, indicating good model predictive accuracy. Next, we confirmed differently ranks by generating log-ratios through Qurro (Fedarko et al., n.d.) following the tutorial found at https://github.com/biocore/qurro. Based on this analysis, differential rankings confirmed that *Bacteroides* was highly ranked with increased O_3. From these highly ranked species, the log-ratio of *Bacteroides caecimuris* versus *Leuconostoc spp.* was compared to O_3. *Leuconostoc spp.* was selected as the denominator due to a mean rank close to zero, indicating little change compared to O_3.

Multidimensional scaling, a statistical technique to visualize dissimilarity between samples in a high dimensional dataset, was performed on WGS sequencing data using the "capscale" function in the vegan package with Bray-Curtis dissimilarity. In addition, the "envfit" function in the vegan package was used to visualize the correlation of air pollutant variables with the gut microbiota composition on the ordination plot. Lastly, the ADONIS test, a permutation-based multivariate analysis of variance using Bray-Curtis dissimilarity matrices with 10,000 permutations, was used to examine associations between the gut microbiota with air pollutants (i.e., total NOx $PM_{2.5}$, PM_{10}, NO_2, O_3) and participant characteristics (i.e., BMI, age, sex, Hispanic ethnicity, energy intake, season, and parental education). Effect modification by sex and obesity on the associations between the gut microbiome and air pollutants was further examined using interaction terms in these multivariate models.

For our explanatory aim, associations between gut bacteria and obesity (i.e., BMI, body fat percent), metabolic outcomes (i.e., fasting glucose, fasting insulin, 2-hour glucose and insulin levels, glucose and insulin area under the curves following OGTT, HOMA-IR, and

HOMA-β), and markers of gut microbial translocation (i.e., sCD14, LBP, ratio of sCD14/LBP) were examined using both simple univariate and multivariable linear regression models that adjusted for age, BMI, energy intake, Hispanic ethnicity, sex, season, and parental education. P-values from all statistical analyses were adjusted for multiple hypothesis testing using a false discovery rate of 5% with the Benjamini-Hochberg procedure. For each table the total number of hypotheses corrected was the product of all of the independent variables considered and the number of taxa examined. All analyses were conducted in R statistical package version 3.5.1 and figures were produced using RStudio.

Results

This study included 101 participants from the Meta-AIR Study. General characteristics and the average air pollutant exposures are reported in Table 1. The mean age of participants was 19.6 years (range 17.7 – 21.8), 57.4% were male, and approximately 54.5% self-identified as Hispanic ethnicity. On average, participants were overweight with an average BMI of 28.9 kg/m^2 (range 17.3 – 47.4). The gut bacterial community composition was dominated by the phylum *Bacteroidetes* (59.74% ± 13.97), followed by *Firmicutes* (31.51% ± 10.75), *Proteobacteria* (4.99% ± 6.34), and *Actinobacteria* (2.54% ± 2.51).

Air Pollutants were Associated with the Gut Microbiota using WGS

We first performed multi-dimensional scaling on the WGS data using Bray-Curtis dissimilarity at the species level (Figure 1). The ordination plot shows that O_3 exposure was significantly correlated with the first axis of the multi-dimensional scaling analysis, which explains 28% of variation in the data. Using a non-parametric multivariate ADONIS test with 10,000 permutations, we found that exposure to air pollutants explained the largest proportion of the variance in gut bacterial composition (Figure 2). The percent variation that was explained by exposure to air pollutants was 4.0% for total NOx (FDR corrected $p = 0.049$), 4.4% for NO_2 (FDR corrected $p = 0.049$), and 11.2% for O_3 concentrations (FDR corrected $p = 0.001$), which is large relative to the effect size for many other covariates reported in healthy populations (Falony et al., 2016). At the species level, O_3 exposure explained 5.4% of the variation in gut bacterial composition (FDR corrected $p = 0.001$). By contrast, the metadata variables sex, season, Hispanic ethnicity, BMI, age, parental education, and energy intake from 24-hour diet recalls all explained less than 4% of the variance and were not significantly associated with gut microbial community composition by the ADONIS test at a $p < 0.05$ threshold.

The above analysis suggests patterns of association between air pollutants and global measures of the entire microbial community but does not reveal which individual taxa drive these associations. We therefore built simple univariate linear regression models for ambient and near-roadway air pollutants including total NOx, NO_2, $PM_{2.5}$, PM_{10}, and O_3 exposure as well as potentially important confounding subject metadata including sex, BMI, age, energy intake, season, parental education, and Hispanic ethnicity (Figure 3). Of all of factors, O_3 exposure had the most significant associations at the phylum level (Supplemental Figure 1). Most of these phyla were low abundant taxa with the exception of a few common gut bacterial phyla including *Bacteroidetes* (FDR corrected $p = 0.022$, $R^2 = 0.11$),

Actinobacteria (FDR corrected p = 0.022, R^2 = 0.11) and *Proteobacteria* (FDR corrected p=0.038, R^2=0.10; Supplemental Figure 2A–C). In addition, the phylum *Firmicutes* was positively associated with NO_2 (FDR corrected p = 0.017, R^2 = 0.12; Supplemental Figure 2D). Similarly, O_3 exposure had the most significant associations at the family level. For example, O_3 was negatively associated with *Coriobacteriaceae* (FDR corrected p = 0.004, R^2 = 0.18), *Lactobacillaceae* (FDR corrected p = 0.004, R^2 = 0.17), *Acidobacteriaceae* (FDR corrected p = 0.005, R^2 = 0.16), and *Bacillaceae* (FDR corrected p = 0.024, R^2 = 0.11) and was positively associated with *Bacteroidaceae* (FDR corrected p = 0.006, R^2 = 0.15). A few taxa at the family level were also positively associated with NO_2, such as *Coriobacteriaceae* (FDR corrected p = 0.039, R^2 = 0.10) *Ruminococcaceae* (FDR corrected p = 0.041, R^2 = 10), *Acidobacteriaceae* (FDR corrected p = 0.048, R^2 = 0.09). These results were generally robust to the non-parametric Kendall test (Supplemental Figure 3).

At the genus level, WGS sequencing demonstrated significant positive associations between *Bacteroides* and O_3 (p = 0.009, R^2 = 0.16; Figure 4A). Utilizing the increased taxonomic resolution of WGS, we built linear models at the species level. Within *Bacteroides*, higher abundances of the bacterial species *Bacteroides caecimuris* were associated with higher exposure to O_3 (p = 0.037, R^2 = 0.15; Figure 4B). Overall, 128 bacterial species (R^2 = 0.14 to 0.25) were associated with O_3 (p_{all}<0.05). While only 4 (R^2= 0.14 to 0.17) and 5 (R^2 = 0.14 to 0.16) bacterial species were associated with NO_2 and total NOx, respectively (p_{all}<0.05) (Supplemental Figure 4). Differential abundance analysis using Songbird reveled that these findings were not driven by compositional artifacts (Morton et al., 2019). Specifically, differential rankings showed that at the genus level, *Bacteroides* was highly ranked with an increase in O_3 and that the log-ratio of the *Bacteroides caecimuris* versus *Leuconostoc spp*. had a significant relationship by Pearson correlation to O_3 (R^2 = 0.31; p = 0.007) but not to NO_2 (R^2 = −0.18; p = 0.123) (Supplemental Figure 5).

WGS also revealed that higher exposure to O_3 was associated with a lower bacterial evenness (p<0.001; R^2 = 0.15) and a lower Shannon diversity index (p<0.001, R^2 = 0.15) at the species level (Figure 4C and D). Results from our univariate linear regression modeling also indicated that age, sex, season, Hispanic ethnicity, energy intake, parental education, and BMI were not strongly associated with the relative abundance of gut bacterial taxa (Figure 3). Likewise, associations between the gut microbiota composition and bacterial diversity and O_3 exposure were unchanged after adjusting for each of these respective variables as well as dietary macronutrients (i.e., protein, fat, carbohydrate, fiber) using multivariable linear regression models (Supplemental Tables 2 and 3). Overall, there was no evidence to support differences in effects of exposure to air pollutants by sex (males versus females) or overweight/obese status (overweight/obese versus normal BMI) (Supplemental Table 4). Lastly, gut bacteria found to be associated with O_3 were not associated with BMI, glucose metabolism, insulin resistance, or markers of gut microbial translocation before or after adjusting for covariates, which included sex, BMI, age, energy intake, season, parental education, and Hispanic ethnicity (Supplemental Table 5 and 6).

Finally, in order to validate the results obtained from WGS data that were taxonomically classified by Kraken2, we compared the results to those obtained from MetaPhlAn2, which relies on a set of marker genes as opposed to the Kraken2 that uses all available sequences

(Lindgreen et al., 2016). As we might expect from previous literature, fewer taxa were identified by MetaPhlAn2 when compared to Kraken2 (68 versus 1184 genera). However, despite differences in taxonomy, similar results were observed with O_3. Prior year O_3 exposure was positively associated with *Bacteroides* (FDR corrected $p = 0.014$, $R^2 = 0.15$) and was negatively associated with Shannon diversity (FDR corrected $p<0.001$, $R^2 = 0.15$) and evenness (FDR corrected $p<0.001$, $R^2 = 0.16$) at the genus level (Supplemental Figure 6). Collectively, these results demonstrate that associations between O_3 and microbial composition and diversity are robust to different analysis pipelines (Kraken2 vs. MetaPhlAn2).

Air Pollutants were Associated with Gut Bacterial Function using WGS

We next expanded our analysis beyond taxa identification by using the HUMAnN2 pipeline on the WGS data to investigate which gene pathways were associated with increased exposure to O_3 (Figure 5A). Functional gene pathways related to cell growth and insulin release were found to be associated with O_3 exposure. These include pathways involved in coenzyme A biosynthesis, pantothenate and coenzyme A biosynthesis, L-ornithine de novo biosynthesis, glycolysis, mannitol cycle, and gondoate biosynthesis were positively associated with O_3 (FDR corrected $p<0.05$, $R^2 = 0.14–0.18$; Figure 5B). These associations were largely unchanged after adjusting for NO_2, BMI, age, sex, energy intake, season, Hispanic ethnicity, dietary macronutrients, and parental education (Supplemental Table 7). Further, simple linear regression models did not display functional associations with any of the other air pollutants, BMI, age, sex, energy intake, season, parental education, or Hispanic ethnicity (Figure 7A).

Discussion

In this cohort of young adults from Southern California, our results demonstrate significant associations between exposure to air pollutants and the composition of the gut microbiome using WGS. These findings were robust to adjusting for confounders such as age, sex, Hispanic ethnicity, BMI, season of study visit, parental education (proxy for socioeconomic status), energy intake, and macronutrients (i.e., protein, fat, carbohydrates, fiber). Notably, the percent variation that was explained by exposure to air pollutants was 4.0% for total NOx, 4.4% for NO_2, and 11.2% for O_3 concentrations. These air pollutants explained the highest amount of variation in the gut when compared to other factors known to impact the gut microbiome (i.e. BMI, and energy intake); however, this may be partially explained by the relatively small range of BMI, age, energy intake, and race/ethnicity in our study population. Additionally, we observed that the phylum *Firmicutes* was positively associated with NO_2, yet O_3 exposure had the most significant associations at the phylum and species level. This study also found that gene pathways involved in fatty acid synthesis/degradation, were enriched with higher O_3 exposure. These included L-ornithine de novo biosynthesis and pantothenate and coenzyme A biosynthesis pathways, which may play a role in gut barrier integrity (Leonardi and Jackowski, 2007) as well as obesity and type 2 diabetes (Bandyopadhyay et al., 2006; Davaapil et al., 2014; Webster et al., 2008). Collectively, these results provide novel evidence for significant associations between exposure to air pollutants with both the composition and functional potential of the human gut microbiome.

A growing body of work has found that increased exposure to air pollutants is associated with increased obesity, dysregulated glucose metabolism (Alderete et al., 2017; Lucht et al., 2018; Thiering et al., 2013; Toledo-Corral et al., 2018; Yang et al., 2018), and the composition of the gut microbiota (Alderete et al., 2018b; Kish et al., 2013; R. Li et al., 2017; T. Liu et al., 2019; Mutlu et al., 2018; Ribière et al., 2016; Wang et al., 2018). Additionally, the gut microbiota has also been linked with these adverse health outcomes (Ley et al., 2006; Qin et al., 2012; Ross et al., 2015; Suez et al., 2014; Turnbaugh et al., 2006; Vrieze et al., 2012; Walters et al., 2014). While studies in rodent models demonstrate significant associations between inhaled and ingested air pollutants with the composition of the gut microbiota (Kish et al., 2013; R. Li et al., 2017; Mutlu et al., 2018; Ribière et al., 2016; Wang et al., 2018), only two studies have examined these associations in humans (Alderete et al., 2018b; T. Liu et al., 2019). One of these was our previous pilot study that examined 43 participants from the Meta-AIR cohort using 16S rRNA sequencing. Results from this study found that increased near-roadway exposure (NOx) was correlated with the relative abundance of gut bacteria that have been associated with obesity and altered metabolism (e.g., *Coriobacteriaceae and Bacteroidaceae*) (Alderete et al., 2018b). Additionally, gut microbial taxa that were correlated with near-roadway air pollution exposure accounted for 24% and 29% of the correlation between exposure to air pollutants and fasting glucose levels (Alderete et al., 2018b). The second study examined 6,627 adults from south China and found that exposure to $PM_{2.5}$ and PM_{10} were associated with a decreased gut microbial alpha diversity. PM exposure was also negatively associated with *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia* and was associated with several taxa within the *Bacteroidetes* phyla. Additionally, impaired fasting glucose was positively associated with $PM_{2.5}$ and PM_{10} exposure and these associations were partially mediated by gut microbial diversity (T. Liu et al., 2019).

The current study found that exposure to air pollutants was associated with reduced gut bacterial diversity and numerous gut bacterial species. This could have important implications as several studies have shown that decreased gut bacterial diversity is associate with poor health outcomes (Turnbaugh and Gordon, 2009; Turnbaugh et al., 2009; Vangay et al., 2018). We also found that 128 bacterial species were associated with O_3 and 4 and 5 bacterial species were associated with NO_2 and total NOx, respectively. Most of these phyla were low abundant taxa with the exception of a few common gut bacterial phyla including *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. Further, within *Bacteroides*, higher abundances of the bacterial species *Bacteroides caecimuris* were associated with higher exposure to O_3. In addition, the phylum *Firmicutes* was positively associated with NO_2. Similar to our findings, the Liu et al. study found that a lower gut microbial diversity was associated with higher $PM_{2.5}$ and PM_{10} exposure (T. Liu et al., 2019). In our previous study, we found that a lower abundance of *Bacteroidaceae* and a higher abundance of *Coriobacteriaceae* was associated with higher NO_x exposure (Alderete et al., 2018b). In the current study, a lower abundance of *Bacteroidaceae* was also associated with higher NO_x exposure, but this association did not remain significant after adjusting for multiple comparisons (data not shown). Additionally, in the current study, higher NO_2 was associated with a higher abundance of *Coriobacteriaceae* and higher O_3 exposure was associated with a higher abundance bacterial species belonging to the *Bacteroidaceae* family. These mixed

findings may be due to differences in sample size, air pollutants examined, or different sequencing methods that allow for varying levels of taxonomic resolution to be examined.

Studies have shown that these bacterial taxa and reduced gut microbial diversity have been linked with obesity and type 2 diabetes (Qin et al., 2012; Turnbaugh et al., 2006; Vrieze et al., 2012). Despite these findings, we did not observe any significant associations between the gut microbiome and metabolic outcomes either before or after adjusting for important confounders (BMI, age, sex, energy intake, season, parental education, or Hispanic ethnicity). This may be partially due to the fact that our study included young adults that, despite being overweight, were largely metabolically healthy. This study builds on previous work by examining the functional potential of the gut microbiome using WGS. Our results show that increased exposure to average prior year O_3 was associated with enrichment of multiple gene pathways that have the potential to impact gut barrier integrity and/or host metabolism, including L-ornithine de novo biosynthesis and pantothenate and coenzyme A biosynthesis I. L-ornithine is a non-essential amino acid and precursor for polyamines and nitric acid (Selamnia et al., 1998) and polyamines are necessary for cell growth (Hölttä et al., 1993) and nitric acid has antiproliferative properties (Kumar and Kashyap, 2015). Additionally, pantothenate and CoA biosynthesis play a role in fatty acid synthesis/degradation, phospholipids synthesis, and serve as a cofactor for cell growth (Leonardi and Jackowski, 2007). CoA derivatives have also been shown to inhibit insulin release (Davaapil et al., 2014; Webster et al., 2008) and are elevated in obesity and type 2 diabetes (Bandyopadhyay et al., 2006). Thus, enrichment of these pathways may have important implications in gut bacterial communities and gut permeability through cell growth and cytotoxicity as well as changes in fatty acid and/or phospholipid synthesis and degradation.

$PM_{2.5}$ and O_3 have been shown to have extrapulmonary effects that may alter the HPA axis through vagal nerve activation (Gackière et al., 2011) or effects on the hippocampus (Thomson, 2019), which can increase levels of catecholamines and steroid hormones. While previous work has largely focused on the effects of PM or near-roadway exposure on the gut microbiota (Alderete et al., 2018b; Kish et al., 2013; R. Li et al., 2017; T. Liu et al., 2019; Ribière et al., 2016), O_3 has been largely overlooked. In the current study, we found that O_3 exposure was consistently and strongly associated with the composition and functional potential of the gut microbiome. While the mechanisms that link increased O_3 exposure and the gut microbiome have yet to be fully characterized, animal and human studies have shown that O_3 exposure increases plasma corticosterone levels (Thomas et al., 2018) as well as plasma cortisol and corticosterone concentrations (Miller et al., 2016), respectively. Thus, activation of the HPA axis may increase the production of cortisol and norepinephrine, which may induce changes in the gut microbiota (Petrosus et al., 2018). Additionally, the gut-brain axis allows for bidirectional communication (Lyte, 2014) that may result in changes in bacterial proliferation in the presence of norepinephrine (Lyte and Ernst, 1992).

Results from this study provide early evidence that elevated exposure to air pollutants may impact the composition and functional potential of the human gut microbiome. These findings were unchanged after adjusting for potentially important confounders, including age, sex, Hispanic ethnicity, BMI, season, parental education, and dietary factors known to influence the composition of the gut microbiota. Despite these study strengths, this work

was limited by the moderate sample size of 101, which may have reduced our statistical power. However, this study was able to detect 128 bacterial species that were associated with O_3 exposure as well as 6 functional pathways. The current study may also be limited by participant selection, which was based on being overweight or obese at their previous visit. This may limit the generalizability of the current study, be a potential source of selection bias, and may have reduced our ability to find associations between gut bacteria and metabolic markers. However, at the time of the current study, we observed a range of BMI values that included normal weight, overweight, and obese young adults. While exploratory analysis using multi-pollutant models showed that our significant findings from single pollutant models for O_3 were robust to NO_2 adjustment, a known limitation air exposure research is the moderate to high correlation among exposure variables. As such, our multi-pollutant models may be limited by bias amplification (Weisskopf et al., 2018). Residential based estimates of air pollution exposure may have resulted in exposure misclassification, yet exposure misclassification should be random across participants (non-differential) and likely would bias estimates towards the null (Nerriere et al., 2005). Although compositional data analysis used in human microbiome research may contribute to type I errors, we verified the main findings using Songbird that is a compositionally aware method (Morton et al., 2019). Lastly, differences in processing pipelines could impact the specific gut bacterial taxa found to be associated with air pollution exposure. However, we utilized two different classifiers for taxonomic assignment of WGS data and found that both of these pipelines identified significant associations between the gut microbiota and exposure to O_3.

Conclusions

Our results show that increased exposure to air pollution has the potential to impact the human gut microbiome, which remained robust after adjusting for potentially important confounders. These early findings, coupled with other epidemiological and animal studies, suggest that exposure to air pollutants may increase risk for obesity and type 2 diabetes through alterations to the gut microbiome. These findings have significant public health relevance since air pollution remains a challenge despite extensive efforts to improve air quality and O_3 pollution has worsened in much of the nation. Despite this, additional epidemiological and mechanistic studies are needed in order to examine the exact mechanisms by which pollutants impact the human gut microbiome and increase human disease risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Exposure to air pollutants was significantly associated with the composition of the gut microbiome using whole-genome sequencing.
- Higher exposure to 24-hour O_3 was associated with lower Shannon diversity index, higher *Bacteroides caecimuris* and multiple gene pathways, including L-ornithine de novo biosynthesis and pantothenate and coenzyme A biosynthesis I.
- This study provides the first evidence of significant associations between exposure to air pollutants and the compositional and functional profile of the human gut microbiome.

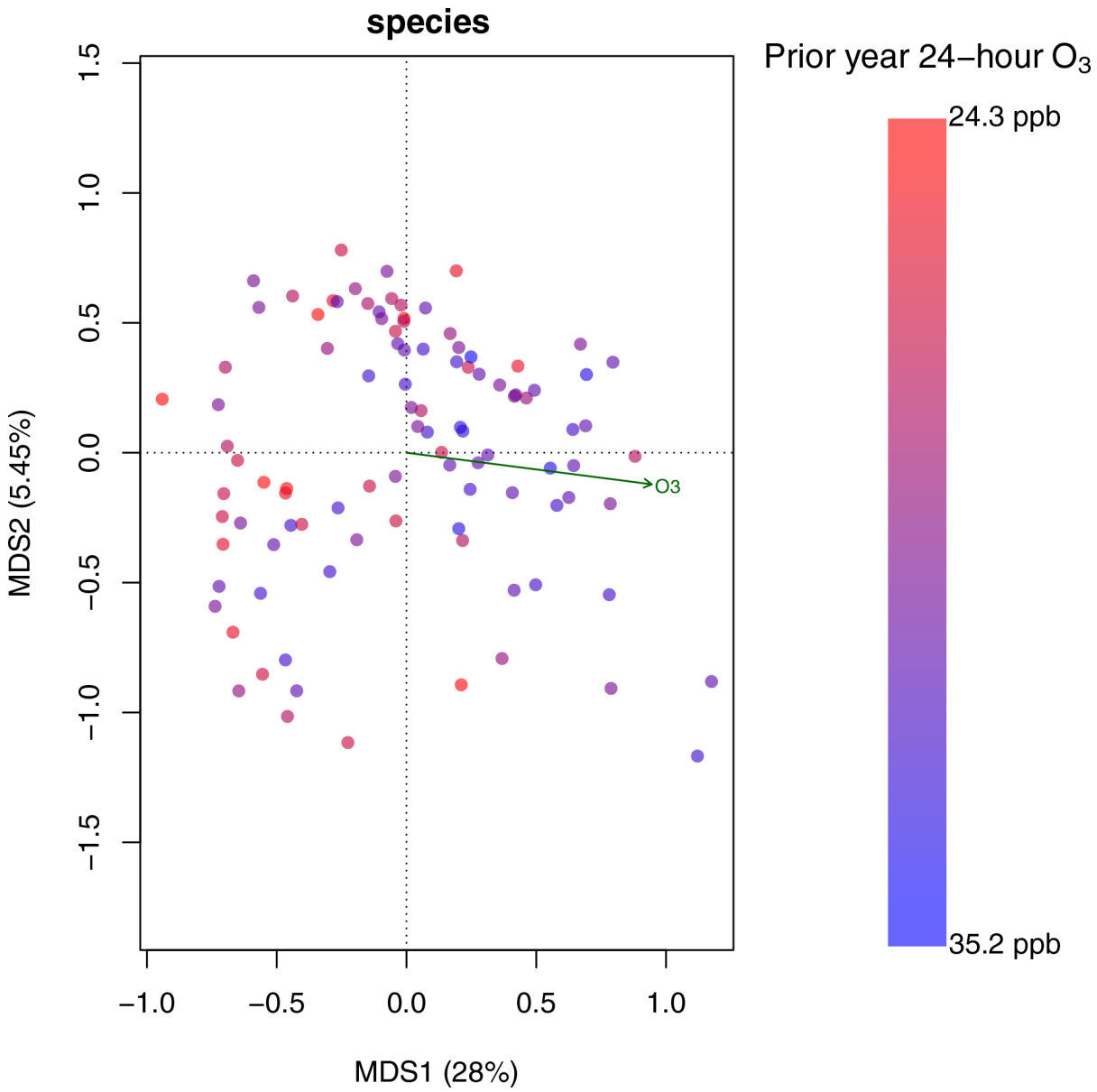

Figure 1. Exposure to O_3 was Significantly Correlated with Gut Bacterial Species on the First Axis of the Multi-dimensional Scaling Analysis using Whole Genome Sequencing (WGS)

Multi-dimensional scaling on the WGS data was performed using Bray-Curtis dissimilarity at the species level. Each point represents a sample and the gradient of O_3 exposure is color coded. This ordination plot shows that O_3 exposure was significantly correlated with the first axis of the multi-dimensional scaling analysis, which explains 28% of variation in the data ($p<0.001$, $R^2=0.17$).

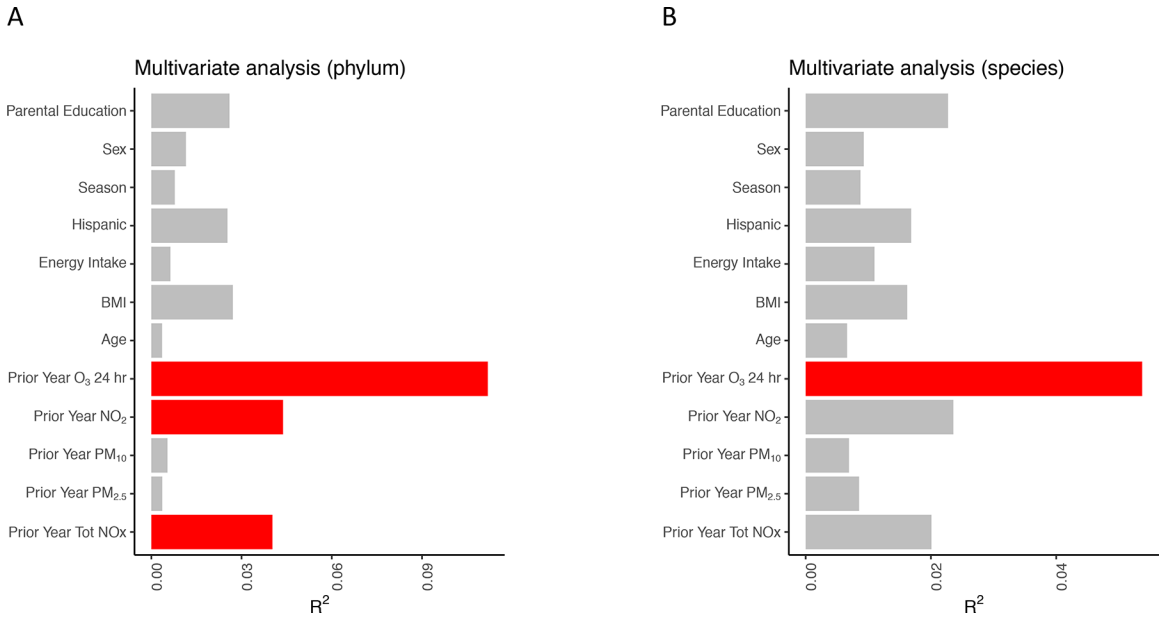

Figure 2. Exposure to Air pollutants was Associated with the Gut Microbiota at the Phylum and Species Level Using Whole Genome Sequencing (WGS)

Figure shows the results from a non-parametric multivariate ADONIS test with 10,000 permutations. For each explanatory variable, separate multivariate models were constructed to determine the association between the gut microbiota composition and each explanatory variable at the level of the phylum (A) and species (B). R^2 values correspond to the fraction of variation in the gut microbiota composition that is explained by each variable. Red bars indicate statistical significance (FDR<0.05).

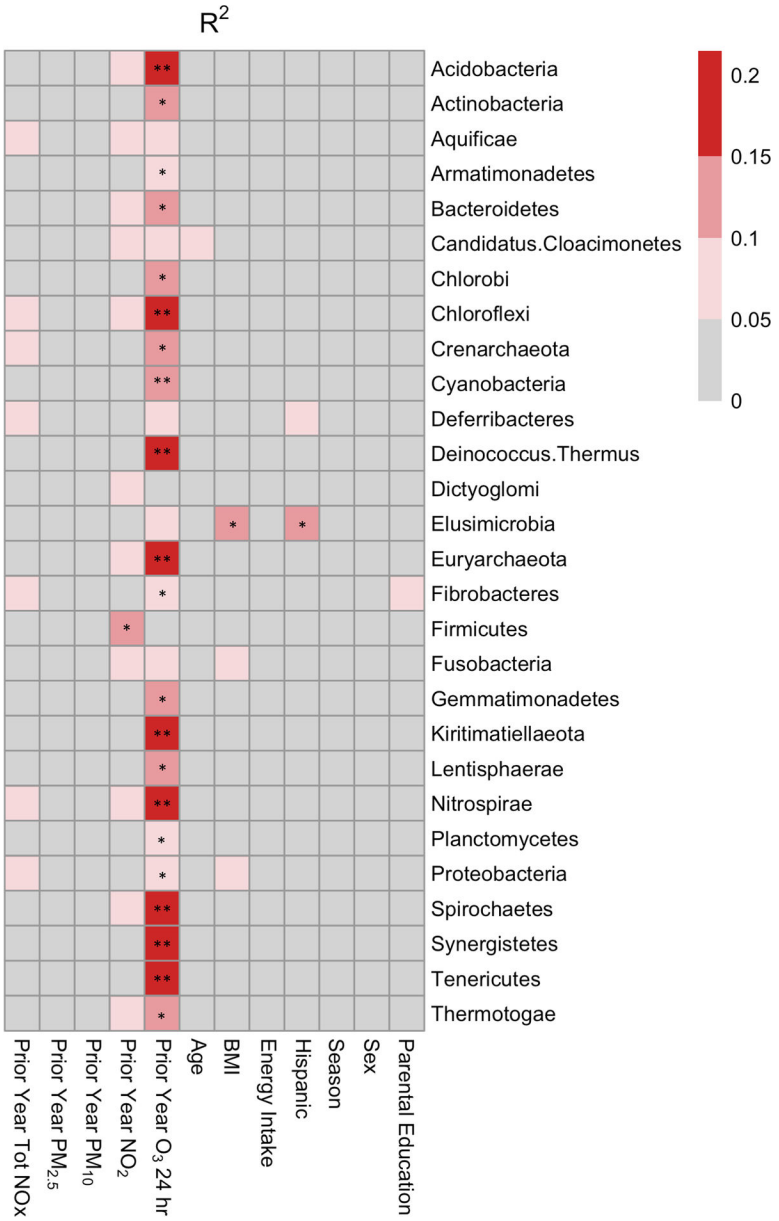

Figure 3. Exposure to O_3 was Associated with the Gut Microbiota at the Phylum Level Using Whole Genome Sequencing (WGS)

Univariate linear regression was used to determine the association between the relative abundance of gut bacterial phyla and each explanatory variable. The heatmap shows R^2 values, which are indicative of the amount of variation explained by each explanatory variable in univariate regression models. ** FDR-corrected p-value < 0.01, * FDR-corrected p-value < 0.05.

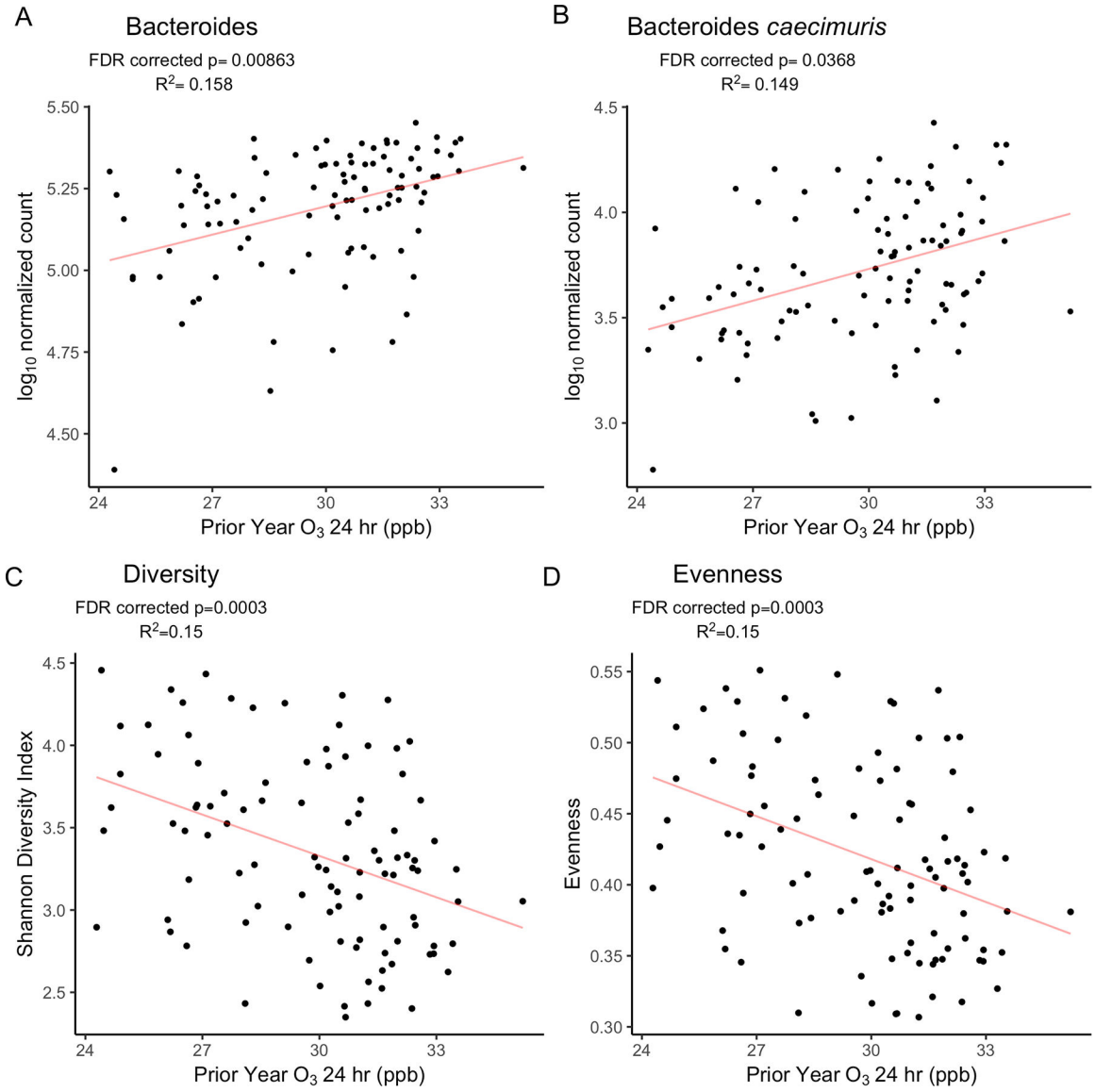

Figure 4. Exposure to O_3 was Positively Associated with the Relative Abundance of Taxa Belonging to the *Bacteroides* Genus and was Negatively Associated with Gut Bacterial Diversity using Whole Genome Sequencing (WGS) Taxonomically Classified by Kraken2

Higher exposure to O_3 was positively associated with the relative abundance of the genus *Bacteroides* (**A**) and the relative abundance of the bacterial species *Bacteroides caecimuris* (**B**). Higher exposure to O_3 was associated with a lower bacterial Shannon diversity index (**C**) and evenness (**D**) at the species level.

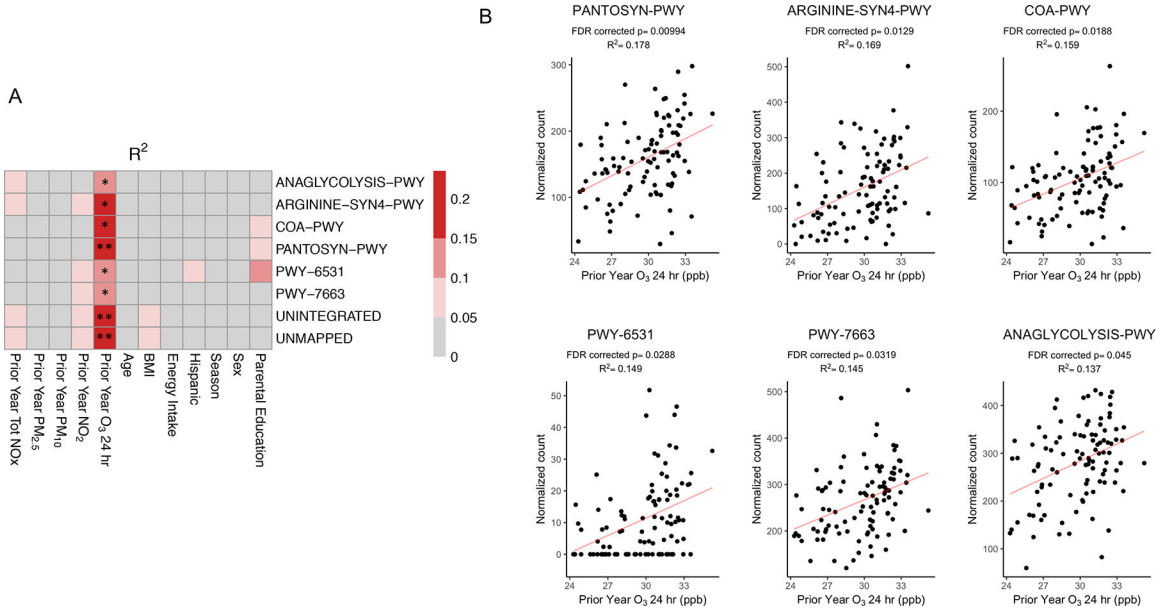

Figure 5. Exposure to O_3 was Significantly Associated with Multiple Gene Pathways Using Whole Genome Sequencing (WGS)

Univariate linear regression analysis was used to determine the associations between the relative abundance of microbial gene pathways and each explanatory variable. The heatmaps shows R^2 values (**A**), which are indicative of the amount of variation explained by each explanatory variable. ** FDR-corrected p-value < 0.01, * FDR-corrected p-value < 0.05. Scatter plots (**B**) show the associations between the relative abundance of six gene pathways and O_3 exposure using univariate linear models with the corresponding R^2 and FDR corrected p-values. PANTOSYN-PWY: pantothenate and coenzyme A biosynthesis I, ARGININE-SYN4-PWY: L-ornithine de novo biosynthesis, COA-PWY: coenzyme A biosynthesis I, PWY-6531: mannitol cycle, PWY-7663: gondoate biosynthesis (anaerobic), ANAGLYCOLYSIS-PWY: glycolysis III (from glucose). Pathway abundance is normalized to copies per million (cpm).

Table 1.

Baseline Characteristics and Prior Year Ambient and Near-Roadway Concentration in Young Adults from the Meta-AIR Study

	Mean ± SD	Missing Values
General Characteristics	**n=101**	
Age (years)	19.6 ± 0.9	0
Sex (Males/Females, %)	58/43, 57.4%	0
Ethnicity (Hispanic/Non-Hispanic, %)	55/46, 54.5%	0
BMI (kg/m^2)	28.9 ± 5.4	0
Parental Education		
Low (n, %)	43, 42.6%	0
High (n, %)	38, 37.6%	0
Unknown/Missing (n, %)	20, 19.8%	-
Metabolic Indices		
Fasting Glucose (mg/dL)	89.8 ± 8.7	4
Fasting Insulin (μU/mL)	10.6 ± 7.6	25
Body Fat Percent (%)	34.6 ± 8.8	17
2-Hour Glucose (mg/dL)	120.2 ± 28.6	19
2-Hour Insulin (μU/mL)	102.2 ± 100.6	19
Glucose Area Under the Curve	272.8 ± 46.5	19
Insulin Area Under the Curve	243.8 ± 167.2	38
HOMA-IR	2.4 ± 1.8	25
HOMA-B	163.4 ± 152.4	25
Energy Intake and Macronutrients		
Energy Intake (Kcal)	1994.0 ± 655.9	20
Carbohydrates (g)	241.4 ± 79.1	20
Fat (g)	80.4 ± 34.8	20
Protein (g)	81.6 ± 34.6	20
Fiber (g)	17.6 ± 6.7	20
Prior Year Average Exposure to Air Pollutants**		
NO_2 (ppb)	15.5 ± 3.9	1
PM_{10} (μg/m^3)	30.1± 7.1	0
$PM_{2.5}$ (μg/m^3)	12.0 ± 2.5	0
O_3 (ppb)	29.8 ± 2.5	0
Total NOx (ppb)	7.1± 7.1	1

** Average prior year exposure to ambient and near-roadway air pollutants based on residential addresses. Nitrogen oxides (NOx) in parts per billion (ppb) were used as a marker of traffic emissions. Data are reported as mean with standard deviation (SD). Parental education was defined as low (n=43), high (n=38), or unknown/missing (n=20). Low was defined as <12th grade, completed grade 12, and some college or technical school. High was defined as 4 years of college and some graduate training college.