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Traffic generated emissions alter the lung microbiota by promoting the expansion of Proteobacteria in C57Bl/6 mice placed on a high-fat diet

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Abstract

Air pollution has been documented to contribute to severe respiratory diseases like asthma and chronic obstructive pulmonary disorder (COPD). Although these diseases demonstrate a shift in the lung microbiota towards Proteobacteria, the effects of traffic generated emissions on lung microbiota profiles have not been well-characterized. Thus, we investigated the hypothesis that exposure to traffic-generated emissions can alter lung microbiota and immune defenses. Since a large population of the Western world consumes a diet rich in fats, we sought to investigate the synergistic effects of mixed vehicle emissions and high-fat diet consumption. We exposed 3-month-old male C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF: 45% kcal fat) diet to mixed emissions (ME: 30 μg PM/m^3 gasoline engine emissions + 70 μg PM/m^3 diesel engine emissions) or filtered air (FA) for 6 h/d, 7 d/wk for 30 days. Levels of pulmonary immunoglobulins IgA, IgG, and IgM were analyzed by ELISA, and lung microbial profiling was done using qPCR and Illumina 16 S sequencing. We observed a significant decrease in lung IgA in the ME-exposed animals, compared to the FA-exposed animals, both fed a HF diet. Our results

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CRediT authorship contribution statement

Sarah Daniel: Conceptualization, Investigation, Formal analysis, Writing. **Vaidehi Pusadkar**: Formal analysis, Data curation, Writing - review & editing. **Jacob D. McDonald**: Methodology, Validation, Supervision, Resources, Writing - review & editing. **Julie Mirpuri**: Resources, Methodology, Writing - review & editing. **Rajeev K. Azad**: Software, Formal analysis, Data curation, Resources, Writing - review & editing. **Art Goven**: Resources. **Amie K. Lund**: Conceptualization, Validation, Investigation, Project administration, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

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

also revealed a significant decrease in lung IgG in the ME-exposed animals both on the LF diet and HF diet, in comparison to the FA-exposed animals. We also observed an expansion of Enterobacteriaceae belonging to the Proteobacteria phylum in the ME-exposed groups on the HF diet. Collectively, we show that the combined effects of ME and HF diet result in decreased immune surveillance and lung bacterial dysbiosis, which is of significance in lung diseases.

Keywords

Air pollution; Lung microbiome; Immunoglobulins; Proteobacteria

1. Introduction

The lungs are among the first organs to be exposed to the harmful effects of inhaled air pollution. Air pollutants including particulate matter (PM), polycyclic aromatic hydrocarbons, gaseous mixtures of nitrogen dioxide, carbon monoxide, and volatile organic compounds have all been implicated in causing severe lung damage (Marino et al., 2015; Moorthy et al., 2015; Xing et al., 2016). Exposure to air pollutants is associated with the exacerbation of several respiratory diseases such as asthma, bronchitis, and chronic obstructive pulmonary disorder (COPD) (Andersen et al., 2011; Faustini et al., 2013). Air pollution exposures result in around 7 million premature deaths in a year, of which 43% are deaths due to COPD, and 26% due to respiratory infections (WHO Global Health Observatory, 2016). The incidence of the occurrence of these diseases is also higher in heavily polluted regions suggesting that air pollutants play a role in either development or exacerbation of underlying lung conditions (Kim et al., 2018). Air pollutants have been documented to affect immune response at the mucosal surfaces by altering immunoglobulin production and releasing inflammatory mediators (Hiraiwa and van Eeden, 2013; Li et al., 2017). Although there has been a lot of interest in the immunological consequences of air pollution, we are only beginning to explore the impact of these pollutants on the newly identified lung microbiome.

Lungs were historically considered to be sterile, but recent advances in sampling techniques and 16 S rRNA sequencing have demonstrated that the lower respiratory tract is replete with a wide variety of microorganisms– both in health and disease (Dickson et al., 2016). The healthy lung microbiome is variable due to the dynamic responses of inhalation, exhalation, mucociliary clearance, host-immune responses, etc., that occur continuously within the lungs. Despite these fluxes, most of the bacteria in the healthy lungs belong to 4 major phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Mathieu et al., 2018). The commensal microbial diversity is crucial in maintaining several homeostatic functions such as immune system development and regulation. With advances in lung microbiome studies, we now understand that these microorganisms are not mere bystanders, but they play a significant role in modulating the immune environment on the lungs. Studies in germ-free mice have demonstrated that IgA production is significantly reduced within their airway lumen, making them vulnerable to antigen challenges (Ruane et al., 2016). In the absence of microbial stimulation in germ-free mice, they were also found to have decreased mucus production, which severely impedes their mucociliary defense mechanism (Yun et al., 2014).

In disease states, it is observed that the diversity of commensal bacteria is often affected when certain bacteria with selective advantages proliferate and outcompete the others. In many inflammatory diseases of the lung, a shift in the microbiota profile towards Proteobacteria is observed (Hilty et al., 2010; Molyneaux et al., 2013), primarily because these microorganisms have unique abilities to thrive in inflammatory environments (Rizzatti et al., 2017). The human microbiome is understood to be influenced by several factors, including diet and environmental exposures (Tasnim et al., 2017). There are a few emerging studies that show inhaled air pollutants from both anthropogenic and natural sources can also induce alteration in the Firmicutes: Bacteroidetes ratio in the GI tract, which increases susceptibility to inflammation (Fitch et al., 2020; Mutlu et al., 2018).

A large percentage of the Western world consumes a diet rich in fats, which has contributed to the epidemic of obesity, characterized by low-grade inflammation (Duan et al., 2018). High-fat (HF) diet consumption alone has been documented to cause microbial shifts with an increase in Firmicutes in the gastrointestinal tract (Murphy et al., 2015). However, to date, the synergistic effects of traffic-generated air pollutant mixtures and HF diet on the lung microbiota have not been characterized. To address this gap in knowledge, we investigated the hypothesis that exposure to a mixture of gasoline and diesel emissions can alter the lung microbiota and immune defenses in wildtype mice placed on an HF diet. The interactions between environmental exposures, diet, microbiome, and the immune system are vital in understanding the development of diseases. In the following experiments, we exposed C57Bl/6 wildtype mice to a mixture of gasoline and diesel emissions and placed them on either a standard mouse chow or HF diet, and analyzed immunoglobulin levels and lung microbiota profiles.

2. Materials and methods

2.1. Animals and inhalational exposure

Three-month-old C57Bl/6 male mice (C57BL/6NTac, Taconic, Germantown, NY) were placed either on a standard mouse chow (LF, containing 12% fat, n = 12) or a high-fat (HF, n = 12) diet (TD88137 Custom Research Diet, Harlan Teklad, Madison, WI; 21.2% fat content by weight, 45% kcal from fat, 1.5 g/kg cholesterol content) for 30 days prior to exposures. Mice were then exposed to whole-body inhalation to either filtered air (FA, n = 6 per diet) or a mixture of gasoline and diesel engine exhaust (ME: 30 μg PM/m^3 gasoline engine emissions + 70 μg PM/m^3 diesel engine; n = 6 per diet) for 6 h/d, 7 d/wk, for 30 days. ME was created by combining exhaust from a 1996 GM gasoline engine and a Yanmar diesel generator system, and exposures chemistries and PM characterized, as previously reported (Lucero et al., 2017; Lund et al., 2011; McDonald et al., 2004, 2008; Mumaw et al., 2016; Oppenheim et al., 2013). Particle size distribution, composition, and mass concentration were determined, as previously described (Suwannasual et al., 2018). The particle mass size distribution had a median of 1 μm (range: < 0.5–20 μm), particle number size distribution for this exposure had a median size of approximately 60 nm; with total particle mass for the mixture measured at 102.5 ± 20.9 μg/m^3 over the 30 d study. Particle mass concentration by gravimetric analysis of Teflon membrane filters at the inlet of the chamber and inside the exposure chamber was conducted once/wk throughout the exposure protocol. The FA

exposure consisted of HEPA-filtered ambient air. Mice were kept, 4 to a cage, in standard shoebox cages within AAALAC International-approved rodent housing facility (2 m^3 exposure chambers) for the entirety of the study, which maintained a constant temperature (20–24 °C) and humidity (30–60% relative humidity). Chow and water were provided ad libitum, except during daily exposures when chow was removed. All procedures were approved by the Animal Care and Use Committee at the Lovelace Respiratory Research Institute and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Tissue Collection: Animals were sacrificed 14–16 h after their last exposure. Mice were anesthetized with Euthasol (0.1 ml per 30 g mouse) and euthanized by exsanguination. The lungs were dissected and immediately snap-frozen in liquid nitrogen.

The nomenclature used are as follows: (a) LF FA: C57Bl/6 mice placed on LF diet and exposed to FA, (b) LF ME: C57Bl/6 mice placed on LF diet and exposed to ME, (c) HF FA: C57Bl/6 mice placed on HF diet and exposed to FA, (d) HF ME: C57Bl/6 mice placed on HF diet and exposed to ME.

2.2. ELISA

Lung tissues (n = 6 per group) were homogenized in a beat beater with sterile saline and the supernatants were used for Immunoglobulin ELISAs. The concentration of IgA (Fisher Scientific, EMIGA), IgG (Fisher Scientific, 88-50400-22), and IgM (Fisher Scientific, 88-50470-22) were measured in 10-fold diluted lung tissue homogenates using ELISA according to the manufacturer's recommendations. The samples were processed in triplicates, and values were determined from a known value standard curve, using a sigmoidal four-parameter logistic (4-PL) curve-fit.

2.3. qPCR

DNA from homogenized lung tissues (n = 6 per group) was extracted using ZR Fecal DNA miniprep (Zymo Research). qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and the CFX96 Real-Time system (Bio-Rad). For bacterial 16 S rRNA analysis, samples were normalized to Eubacteria utilizing known-concentration standards. Bacterial primers used are described in Table 1.

2.4. Illumina MiSeq sequencing

Genomic DNA was isolated using ZR Fecal DNA miniprep (Zymo Research) from lung homogenates (n = 6 per group). 16 S rRNA genes (variable region 4, V4) were amplified using a composite forward primer and a reverse primer with a unique 10-base barcode used to tag PCR products from respective samples, as described in Fan et al. (2015).

2.5. 16 S Bioinformatics and statistical analyses

Sequence reads obtained from the 16 S rRNA sequencing were analyzed by Mothur Software (version: 1.39.5) from the pipeline of MiSeq SOP (Schloss et al., 2009); standard procedure of this pipeline was followed. In this pipeline (https://rpubs.com/maddieSC/mothur_SOP_May_2018), the paired-end reads, that is, the forward and the corresponding

reverse reads obtained from paired-end sequencing, were combined to form “contigs.” Furthermore, these sequence reads are from the V4 region (~ 250–300 bp) of the 16 S rRNA sequences; however, due to the PCR errors, sequence reads longer than 300 bp, and sequences with ambiguous base calls could be generated (Kozich et al., 2013). These low-quality sequences were removed from further analysis. Next, following the standard protocol of Mothur, the duplicate sequence reads were merged as it is not computationally useful to align the same sequence multiple times. These processed sequences were then aligned to the reference database, SILVA, containing the 16 S rRNA gene sequences. The database was first customized to our regions of interest (V4 regions) using command pcr.seqs; this was performed for the improvement of overall alignment. Following the alignment, any sequences that did not align to V4 region sequences in the customized database were removed. After the alignment, parts of sequences overhanging at both ends were removed. Gap characters (“−”) inserted during the alignment were removed. Further, these sequences were de-noised via a pre-clustering step (sequences were sorted based on the abundance and clustered based on their nucleotide difference less than 2) and removal of chimeras. These sequences were clustered based on species-level (97% or more) similarity to form Operational Taxonomic Units (OTUs), hence giving the absolute abundance matrix. Once the OTUs were established, taxonomic identity was assigned to each OTU. We used the Greengenes database for the taxonomic classification as the usage of this database is known to provide lower taxonomic level assignments and leaves fewer sequences unclassified. Additionally, a consensus confidence threshold was set at the 80% classification cut-off (default in Mothur) to specify the taxonomic identities. Finally, normalized taxonomic abundance for OTUs in each sample was obtained by dividing the abundance values by the total number of sequences in the sample, using the “normalized.shared” function. Normalized values were then approximated to the nearest integer. Note that the samples with very low sequence count (< 3) were eliminated from the further statistical analysis.

These data were further used as the input for the downstream statistical analyses performed. Alpha diversity quantifying the diversity of microbial species within a sample was estimated using the Chao1 index (for species richness) and Shannon index (for species diversity; the more the richness of the species and the more the species evenly distributed in a sample, the greater the Shannon diversity) (Chao, 1984; Chazdon et al., 1998; Shannon and Weaver, n.d.). Beta diversity representing the diversity in microbial species between different samples was calculated in the form of UniFrac weighted and unweighted principal coordinate analysis (PCoA) plot (Lozupone and Knight, 2005). Unweighted UniFrac is a qualitative measure that estimates the distance between two microbial communities based on the fraction of the branch length in a phylogenetic tree leading to descendant taxa in exclusively one or the other community (Lozupone et al., 2007). Weighted UniFrac, an extension of the unweighted Unifrac, also takes into account the relative abundance of taxa represented in the communities. Alpha diversity analysis was performed and the UniFrac (weighted and unweighted) PCoA plots were generated in the R environment using the Phyloseq package. The diversity estimators including AMOVA and ANOVA were implemented with mothur.

2.6. Statistics

Data were analyzed by two-way ANOVA with a Sidak-Holm multiple pairwise comparison post-hoc test using GraphPad Prism 7 for Fig. 1 and Fig. 2A-D. Data are expressed as mean ± SEM, and a $p < 0.05$ was considered statistically significant.

3. Results

3.1. Exposure to ME alters immunoglobulin levels within the lungs of C57Bl/6 wildtype mice

Although IgA is the predominant immunoglobulin in mucosal secretions, IgG and IgM are also locally present within the lungs and aid in the exclusion of invading antigens. To investigate whether exposure to inhaled vehicle emissions can alter airway defenses, we quantified the levels of IgA, IgG, and IgM within lung homogenates by ELISA. When compared to LF FA and LF ME groups, we observed IgA to significantly increase in the HF FA group (Fig. 1A, $p < 0.001$). Interestingly, when compared to the HF FA, we observed a significant decrease in IgA in the HF ME group (Fig. 1A, $p < 0.001$). The respective F values for IgA levels are: exposure = 17.020, diet = 6.021, exposure x diet interaction = 12.27. We also observed a significant decrease in IgG in the LF ME and HF ME groups (Fig. 1B, $p = 0.023$, $F = 9.140$ for exposure), compared to the LF FA group. IgM levels were found to be unaltered across all groups (Fig. 1C).

3.2. Exposure to ME results in an increased abundance of Proteobacteria in C57Bl/6 mice on the HF diet

To determine whether the bacterial abundance within the lungs was altered with our exposures, we quantified the total bacterial load using qPCR. We obtained a total of 6 log copies/ml of bacterial DNA (Fig. 2A). However, there were no statistical differences observed in the total bacterial abundance across all groups. There were also no significant alterations observed within Firmicutes and Bacteroidetes between the groups (Fig. 2B, C). Interestingly, we observed that the abundance of Proteobacteria was significantly elevated only in the HF ME groups (Fig. 2D, $p = 0.031$). The respective F values for Proteobacteria are: exposure = 2.499, diet = 3.492, exposure x diet interaction = 1.165. Actinobacteria was barely detected by qPCR (data not shown). When we measured the percentages of the individual phyla, contributing to the total bacterial abundance, for each of the study groups, we observed that the overall percentage of Proteobacteria was much higher in the lungs of the HF ME group (Fig. 2E).

3.3. Exposure to ME results in the increased relative abundance of Enterobacteriaceae in C57Bl/6 mice on the HF diet

To confirm the expansion of Proteobacteria observed by qPCR, we performed Illumina MiSeq sequencing analysis of the 16 S rRNA region. We observed a similar increase in abundance in the Proteobacteria phylum in the HF ME groups alone (Fig. 3A). Although we observed Proteobacteria in the LF ME groups in the absolute abundance file, they had very low reads and were removed during the normalization of this dataset. Further classification revealed that most of the bacteria within the Proteobacteria phylum belonged to the

Enterobacteriaceae family (Fig. 3B, C). Interestingly, we observed that Lactobacillus predominantly present in both the LF and HF control groups was absent in the ME exposed groups (Fig. 3B, D). We found that bacterial alterations are occurring within the Firmicutes phyla as well. Clostridia were found to be expanding in the ME exposed groups on both LF and HF diets (Fig. 3D). Although most of these bacteria within the Clostridia class belong to the Lachnospiraceae family, only the LF ME group showed the presence of *Clostridium* species (Fig. 3C).

3.4. Bacterial diversity is altered with exposure to ME in C57Bl/6 mice

To investigate exposure mediated shifts in lung microbial diversity, we performed α-diversity and β-diversity calculations. The α-diversity was estimated using the Shannon (diversity) index (Fig. 4A) and Chao1 (richness) index (Fig. 4B), both of which showed a decrease in the bacterial diversity in the HF ME group. However, these results were not statistically significant by ANOVA due to the low abundance of these microorganisms. We observed significant differences in β-diversity in lung microbiota profiles in the exposed and control groups. Fig. 5A is an unweighted PCoA plot constructed using unweighted UniFrac distances representing the total variance in bacterial communities. Each dot is representative of the lung microbiome of one animal in each of the four groups. Although we had six animals in each group, we were unable to obtain reads for all the samples due to the low abundance of microorganisms within the lungs. Despite these challenges, we still observe a clear separation between the ME exposed and FA control groups. Both the unweighted and weighted PCoA plots show LF FA and HF FA samples are dispersed away from the LF ME and HF ME groups (Fig. 5A, B). Analysis of Molecular Variance (AMOVA) was performed to assess the variations among different groups. AMOVA showed UniFrac distances with a significant p-value of 0.006 between LF FA and LF ME and a p-value of 0.007 between the LF FA and HF ME groups (Table 2). A significant variation was also observed between the control groups on LF or HF diets (Table 2, $p = 0.014$).

4. Discussion

Recent studies point to an increase in the incidence of lung diseases in heavily polluted regions, with mounting evidence implicating air pollution to be a primary risk factor for increased hospitalizations of individuals with respiratory diseases, such as asthma and COPD (Kurt et al., 2016; Moore et al., 2016; Raji et al., 2020). Many of these lung diseases have been associated with an increase in bacteria belonging to the Proteobacteria phylum (Dickson et al., 2013; Rizzatti et al., 2017). However, to date, an association between air pollution-mediated lung microbial alterations or their effects on the onset or progression of lung diseases has not been made. In this study, we report for the first time to our knowledge that exposure to a mixture of gasoline and diesel emissions in combination with the HF diet affects immune defenses and causes lung bacterial dysbiosis with an expansion of Proteobacteria. Both the gaseous and the PM component of traffic generated air pollutants were incorporated in our study to determine the overall impacts of vehicle derived pollutants. We utilized the HF diet component alongside our exposures since the standard Western diet that constitutes > 30% fat is present in much of the human population. Using

this model, we sought to analyze the synergistic effects of inhaled air pollutants and the HF diet.

The results obtained indicate that exposures to mixed emissions cause a decrease or degradation of immunoglobulins that play crucial roles in protecting the airways. ME-exposure resulted in decreased pulmonary IgG in both the LF and HF-diet animals, while pulmonary IgA was only decreased with ME exposure within the HF-diet group and was not statistically different from the LF FA or LF ME groups. In addition to viral neutralization, IgA functions to prevent bacterial adherence on the surface of airway epithelial cells, thereby preventing unwarranted activation of immune responses (Mantis and Forbes, 2010). It is possible that with concurrent consumption of a HF diet, ME exposure mediates a reduction in pulmonary IgA that promotes decreased immune surveillance leading to an unwarranted bacterial outgrowth of Proteobacteria within the lungs of those animals. The decrease in IgA may be associated with degradation caused by toxic proteases released by neutrophils that rapidly infiltrate the airway lumen when exposed to pollutants (Pilette et al., 2003). Importantly, IgA was found to be significantly elevated in the HF FA groups. An increase in intestinal IgA in response to HF diets has been documented (Kunisawa et al., 2014); however, diet outcomes on IgA expression in the lung are not as well characterized. It is plausible that a gut-lung axis mediated response may contribute to alterations in IgA expression in the lungs of mice on the HF diet (Enaud et al., 2020). A decrease in IgG in ME exposed groups on both LF and HF diet suggests that the decrease is exposure mediated. A previous study of ambient particulate matter exposure on Sprague–Dawley rats increased secretory IgA levels but decreased IgG (Li et al., 2017). A decrease in salivary IgA has been observed in children living in polluted regions (Mehrbani et al., 2016). These discrepancies observed between different studies can be explained by several reasons, including different study models, variations in the components of exposed particulates and gaseous emissions, as well as the duration and intensity of exposures. Both immune suppression and initiation of pronounced inflammation have been observed in response to different particulates in air pollution, all of which have detrimental consequences on the host.

In response to air pollutant exposures, immune cells have been documented to generate reactive oxygen and nitrogen species (ROS, RNS) to mediate the challenge, and it is a well-understood mechanism of air-pollution mediated toxicity (Laskin et al., 2010; Lodovici and Bigagli, 2011). Although inflammation was traditionally understood to expel potential antigens, recent evidence suggests that specific microbial communities can exploit byproducts of inflammation to proliferate and worsen inflammatory responses (Scales et al., 2016). For example, macrophages and neutrophils release ROS in the form of O_2^- and RNS in the form of NO_3^- as antimicrobial effectors that decompose to generate nitrates in the process of eliminating antigens. These nitrates can be utilized selectively by bacteria belonging to the Proteobacteria phylum for anaerobic respiration and growth (Winter and Bäumler, 2014). We suspect that air pollution-induced oxidative stress, along with the degradation of IgA, may be responsible for the observed increase in Proteobacteria in the HF ME group of study animals. Baseline inflammation may be higher in the HF ME groups as the HF diet alone are reported to increase inflammation; however, this was not assessed in the current study (Duan et al., 2018).

The bacterial composition was found to be altered in the HF FA groups with an abundance of Firmicutes. HF diet has been shown to increase Firmicutes in the gastrointestinal tract. Thus, a possible gut-lung interplay may contribute to an increased predominance of Firmicutes within the HF FA groups (Zhang and Yang, 2016). Beneficial bacteria such as Lactobacillus that has anti-inflammatory properties (Mortaz et al., 2013) were found to decrease in the ME exposed groups. This was accompanied by an increase in Clostridia in the ME exposed groups, with LF ME groups exhibiting an increase in the *Clostridium* species. Many bacteria in the Clostridia class are beneficial commensals, but *Clostridium* species and their outgrowth have been associated with having pathogenic outcomes in several lung diseases (Palmacci et al., 2009; Shu et al., 2008). Actinobacteria was also found to be elevated only in the LF ME group by sequencing. However, we are unaware if an increase in Actinobacteria has pathogenic outcomes within the lungs. The roles of these commensal lung bacteria are not well-known, considering our rudimentary knowledge of the lung microbiome.

The shifts observed in the Firmicutes and Proteobacteria phylum may be causing the reduced bacterial diversity observed in our results in the HF ME groups as there is an outgrowth of bacteria with selective advantages. Although not statistically significant, the Shannon diversity index shows reduced bacterial diversity in the HF ME group. The Chao1 index that measures richness of the microbial profile also revealed a decrease in the abundance of bacteria in the HF ME groups, suggesting opportunistic pathogens may be replacing commensals. The PCoA plots show a distinct separation of each group, reinstating our observations that exposures cause shifts in the microbial profile.

We also notice the expansion of Enterobacteriaceae within the Proteobacteria phylum. Bacteria belonging to the Proteobacteria phylum have been associated with many mucosal diseases (Yang and Jobin, 2014). It is plausible that the expansion of these bacteria could potentially enhance the inflammatory response, possibly due to a lack of IgA responses to safeguard against unwarranted bacterial adherence. In the absence of IgA, Proteobacteria can proliferate and adhere to epithelial linings and induce inflammatory signaling by activating Toll-like receptors (Tana et al., 2003). Thus, with decreasing IgA observed in the lungs of the HF ME-exposed animals in our study, there could be unwarranted expansion and adherence of Proteobacteria on epithelial linings that could enhance inflammation (Huffnagle et al., 2017).

With this study, we hope to shed light on the effects that air pollutants can have on the lungs and how they may exacerbate lung diseases; however, there are a few limitations to note. First, the concentration of ME used in the current study (100 $\mu g/m^3$ PM) would be considered a high environmental exposure scenario. However, this PM concentration is within (or below) the range of PM observed in near roadway, occupational, and heavily populated urban area exposure scenarios (Costa et al., 2017; IQAir, 2021; Pronk et al., 2009). Moreover, a high degree of variability is observed across all the groups, which we believe is characteristic of the lung microbiome profile owing to dynamic responses within the lungs. The microbial composition is constantly renewing, and our observations at a certain time point within a group may not necessarily be identical at another time point. These exposures were done for a total of 30 days, and the reported findings are taken from

only the one-time point, which is a limitation to this study. We were unable to detect bacteria in many of our samples by 16 S sequencing that affected our n-value. However, the qPCR results from the same samples helped to confirm the expansion of Proteobacteria. We were also unable to confirm inflammation and generation of ROS-RNS with this study due to the lack of available tissues; however, subsequent studies are currently underway to investigate these mechanisms.

5. Conclusion

Considering the increasing number of lung disorders that occur in urban populations exposed to high air pollution levels, we sought to determine whether traffic-generated air pollutants can affect lung immunoglobulin levels and microbiota with or without concurrent consumption of an HF diet. Our study demonstrates that high-fat diet and/or exposure to traffic-generated air pollution can affect pulmonary immunoglobulin levels and alter the lung microbial profile. Understanding the lung microbiota shifts in response to the environment and diet as a possible contributor(s) to the pathophysiology of lung diseases is paramount to identifying mechanistic pathways involved in air-pollutant mediated effects on pulmonary disorders and overall human health.

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Abbreviations:

4-PL	Four-parameter logistic
AMOVA	Analysis of molecular variance
COPD	Chronic obstructive pulmonary disorder
ELISA	Enzyme linked immunosorbent assay
FA	Filtered air
HF diet	High-fat diet
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M

LF	Regular chow
ME	Mixture of gasoline and diesel engine exhaust
OTUs	Operational Taxonomic Units
PCoA	Principal coordinate analysis
PM	Particulate matter
qPCR	Quantitative PCR
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

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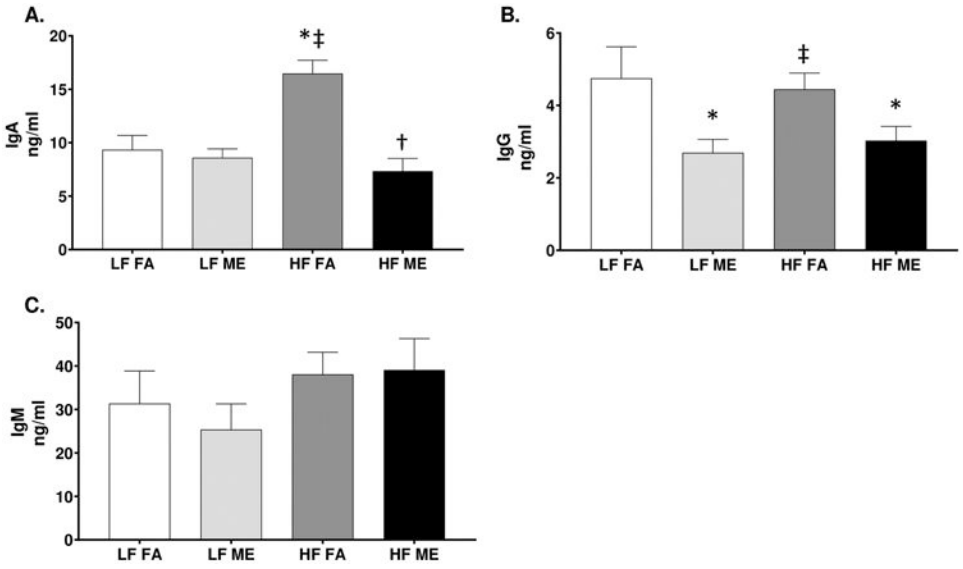

Fig. 1.

A decrease in lung IgG and IgA is observed in mice exposed to ME and HF diet. ELISA of (A) IgA, (B) IgG, and (C) IgM in lung tissue homogenates of C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body inhalation to a mixture of gasoline and diesel engine exhaust (ME: 30 μg PM/m3 gasoline engine emissions + 70 μg PM/m3 diesel engine) for 6 h/d, 7 d/wk for a period of 30 days. Data are depicted as the mean ± SEM with *$p < 0.05$ compared to LF FA, †$p < 0.05$ compared to HF FA, ‡$p < 0.05$ compared to LF ME by two way ANOVA.

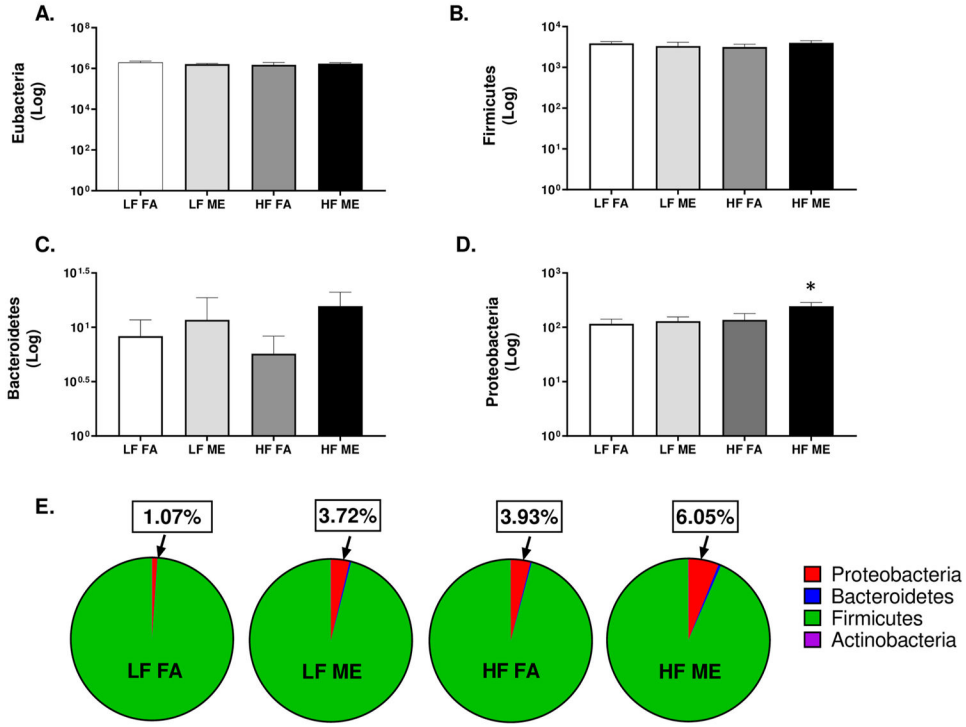

Fig. 2.

Exposure to ME results in an increase in the abundance of Proteobacteria. qPCR of lung tissue homogenates for (A) total bacteria (Eubacteria) and phyla - (B) Firmicutes, (C) Bacteroidetes, (D) Proteobacteria, (E) pie charts representing all major phyla in C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body inhalation to a mixture of gasoline and diesel engine exhaust (ME: 30 μg PM/m3 gasoline engine emissions + 70 μg PM/m3 diesel engine) for 6 h/d, 7 d/wk for a period of 30 days. *$p < 0.05$ compared to LF FA by two way ANOVA.

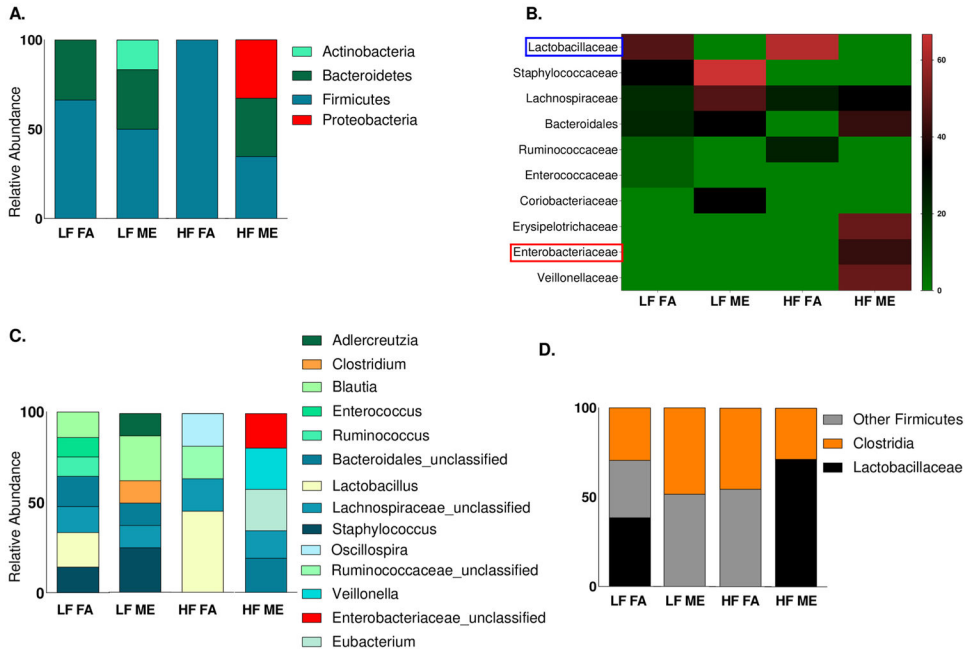

Fig. 3.

Exposure to ME and HF diet increases the relative abundance of Enterobacteriaceae belonging to the Proteobacteria phylum. 16 S Illumina sequencing of the lung bacterial DNA at the (A) phylum and (C) genus level, (B) heatmap showing the relative abundance at the family level and (D) relative abundance of major bacteria in the Firmicutes phlya in lung tissues of C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body inhalation to a mixture of gasoline and diesel engine exhaust (ME: 30 μg PM/m3 gasoline engine emissions + 70 μg PM/m3 diesel engine) for 6 h/d, 7 d/wk for a period of 30 days.

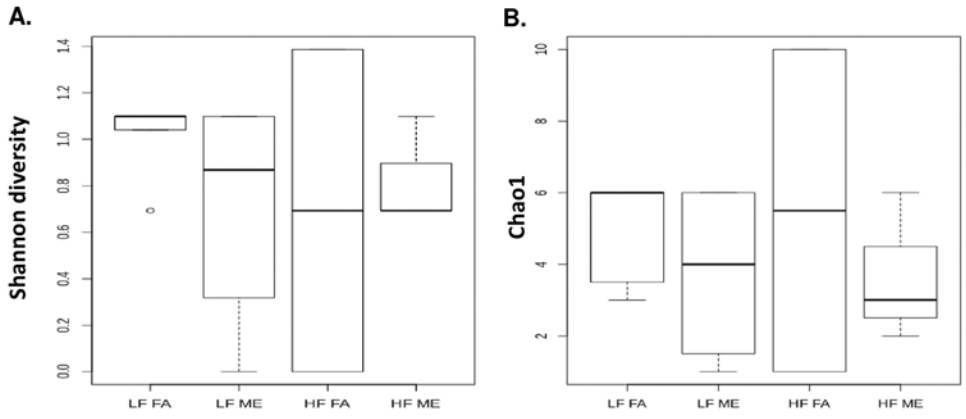

Fig. 4.

Bacterial alpha diversity analysis of exposure and diet groups. Alpha diversity analysis using (A) Shannon index (diversity) and (B) Chao1 index (richness) compared in C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body inhalation to a mixture of gasoline and diesel engine exhaust (ME: 30 μg PM/m3 gasoline engine emissions + 70 μg PM/m3 diesel engine) for 6 h/d, 7 d/wk for a period of 30 days.

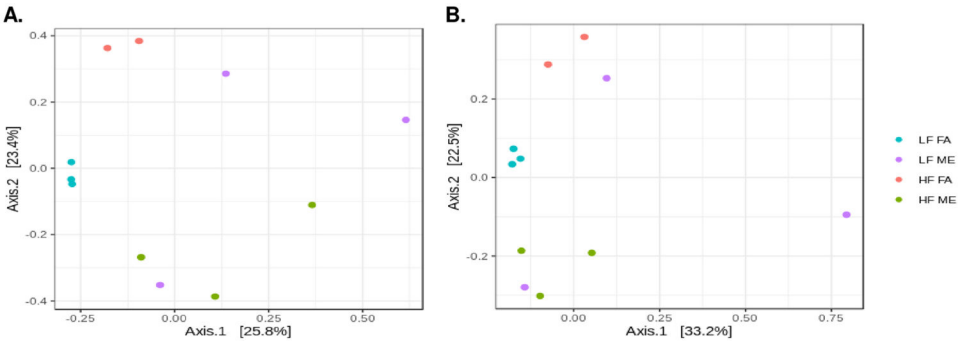

Fig. 5.

Differential clustering of bacterial groups is observed with ME exposures. β-diversity calculations using (A) unweighted and (B) weighted analyses. Each circle is representative of one animal in each of the groups of C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body inhalation to a mixture of gasoline and diesel engine exhaust (ME: 30 μg PM/m3 gasoline engine emissions + 70 μg PM/m3 diesel engine) for 6 h/d, 7 d/wk for a period of 30 days.

Table 1

Primer sequences used for qPCR analysis.

Primer	Sequence	Tm
Eubacteria FP	5′-ACTCCTACGGGAGGCAGCAGT-3′	68.4 °C
Eubacteria RP	5′-ATTACCGCGGCTGCTGGC-3′	70.7 °C
Enterobacteriaceae FP	5′-GTGCCAGCMGCCGCGGTAA-3′	76.3 °C
Enterobacteriaceae RP	5′-GCCTCAAGGGCACAACCTCCAAG-3′	73.6 °C
Bacteroidetes FP	5′-GGTTCTGAGAGGAGGTCCC-3′	62.7 °C
Bacteroidetes RP	5′-GCTGCCTCCCGTAGGAGT-3′	64.4 °C
Firmicutes FP	5′-GGAGYATGTGGTTTAATTCGAAGCA-3′	63.9 °C
Firmicutes RP	5′-AGCTGACGACAACCATGCAC-3′	66.5 °C
Actinobacteria FP	5′-CGCGGCCTATCAGCTTGTTG-3′	69.9 °C
Actinobacteria RP	5′-CCGTACTCCCCAGGCGGGG-3′	74.9 °C

FP, forward primer; RP, reverse primer; Tm, melting temperature.

Table 2

AMOVA analysis of microbiota profiles from lungs of C57Bl/6 mice exposed via inhalation to mixed emissions.

Exposure groups	Sum Sq	Df	Mean Sq	P-value
LF FA vs. LF ME				0.006
Among groups	0.970628	1	0.970628	
Within groups	3.84797	10	0.384797	
Total	4.81859	11		
HF FA vs. HF ME				0.106
Among groups	0.58214	1	0.58214	
Within groups	2.72784	6	0.45464	
Total	3.30998	7		
LF FA vs. HF ME				0.007
Among groups	0.778862	1	0.778862	
Within groups	3.14873	9	0.349858	
Total	3.92759	10		
HF FA vs. LF FA				0.014
Among groups	0.766791	1	0.766791	
Within groups	2.42088	7	0.345841	
Total	3.18767	8		
HF FA vs. LF ME				0.504
Among groups	0.510557	1	0.510557	
Within groups	3.42708	7	0.489583	
Total	3.93764	8		
HF ME vs. LF ME				0.088
Among groups	0.603311	1	0.603311	
Within groups	4.15492	9	0.461658	
Total	4.75824	10		

FA, filtered air; ME, mixed vehicle emissions; LF, standard mouse chow; HF, high-fat diet; Df, degrees of freedom; Sum Sq, sum of squares; Mean Sq, mean of squares.