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Inhalational Exposure to Particulate Matter Air Pollution Alters the Composition of Microbiome in Small Bowel and Colon

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Abstract

Recent studies suggest an association between particulate matter (PM) air pollution and gastrointestinal (GI) disease. In addition to direct deposition, PM can be indirectly deposited in oropharynx via mucociliary clearance and upon swallowing of saliva and mucus. Within the GI tract, PM may alter the GI epithelium and gut microbiome. Our goal was to determine the effect of PM on gut microbiota in a murine model of PM exposure via inhalation. C57BL/6 mice were exposed via inhalation to either concentrated ambient particles or filtered air for 8-hours per day, 5-days a week, for a total of 3-weeks. At exposure's end, GI tract tissues and feces were harvested, and gut microbiota was analyzed. Alpha-diversity was altered with increased richness diversity in PM-exposed mice compared to air-exposed mice in the GI tract. Most importantly, PM-induced alterations in the microbiota were very apparent in beta-diversity comparisons throughout the GI tract and appeared to increase from the proximal to distal parts. Changes in genera were seen and suggest that distinct bacteria may have the capacity to bloom with PM exposure. Exposure to PM

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Availability of data and material

All data generated or analyzed during this study are included in this published article [and its supplementary information files]. Additional datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Raw sequence data are deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under BioProject ID: PRJNA397037.

alters the microbiota throughout the GI tract which maybe a potential mechanism that explains PM induced inflammation in the GI tract.

Graphical abstract

Keywords

air pollution; microbiota; gastrointestinal; intestine; feces

Introduction

Particulate Matter (PM) is a component of air pollution and has been linked with cardiovascular diseases (Brook et al., 2010; Dai et al., 2014), lung cancer (Hamra et al., 2014), impairment of lung development and a decrease in lung function (Paulin and Hansel, 2016), community acquired pneumonia (Neupane et al., 2010b), deep vein thrombosis (Baccarelli et al., 2008), and lower verbal learning performance (Gatto et al., 2014). Recent studies also revealed a link between PM and gastrointestinal (GI) disease including appendicitis (Kaplan et al., 2009), colorectal cancer (Lopez-Abente et al., 2012) and increased hospitalization of patients with inflammatory bowel disease (IBD) (Ananthakrishnan et al., 2011). These findings strongly suggest an association between PM exposure and inflammatory diseases of GI tract (Kaplan et al., 2010). World Health Organization has ranked PM air pollution as the 13th most common cause of overall mortality in the world and attributed over 3 million premature deaths per year to outdoor pollution in 2012 (World Health Organization 2014; World Health Organization, 2016). The impact of air pollution on human mortality has recently been confirmed by another study showing that PM air pollution, leads to 3.3 million premature deaths per year worldwide (Lelieveld et al., 2015). These findings underscore the magnitude of the health effects that PM exposure may potentially cause (World Health Organization 2014; World Health Organization, 2016).

After initial inhalation, where inhaled particles are deposited depends on their size. Most of the larger particles are sequestered in the upper airway or in conducting lower airways such as trachea and larger bronchi (Kreyling et al., 1999; Moller et al., 2004; Oberdorster, 1993). Smaller size particles particularly those that are less in 2.5 microns in mean diameter (PM_{2.5}) can reach the bronchioles and alveolar spaces where they are phagocytosed by alveolar macrophages (Kreyling et al., 1999; Moller et al., 2004; Oberdorster, 1993). Particles sequestered in macrophages and directly in the mucus layer in lower airways are subsequently transported up to the oropharynx and then swallowed into the GI tract

(Beamish et al., 2011; Oberdorster, 1993; Semmler-Behnke et al., 2007). Furthermore, PM can also be ingested directly by consumption of food and water contaminated by PM (Beamish et al., 2011; Commission, 2002; De Brouwere et al., 2012; Kampa and Castanas, 2008; Oberdorster, 1993; Salim et al., 2014b). It has been estimated that 10^{12} - 10^{14} particles are ingested per day by an individual on Western diet (Lomer et al., 2004; Lomer et al., 2002). Collectively, the GI tract can be exposed to significant amounts of PM through these direct and indirect routes of exposure (Beamish et al., 2011; De Brouwere et al., 2012; Oberdorster, 1993; Salim et al., 2014b).

We have previously examined the effect of PM on GI permeability and pro-inflammatory cytokine production (Mutlu et al., 2011). In that study, we demonstrated that exposure to PM increased gut permeability both in cell-based and animal models. Treatment of gut epithelial cells with PM caused increased production of mitochondrial reactive oxygen species (ROS), release of inflammatory cytokines and induced apoptosis of colonocytes (Mutlu et al., 2011). While our murine model confirmed the effect of PM that we observed on enterocytes *in vitro*, the translation of these findings to human exposure was limited as we had used a single dose instillation of PM via gastric lavage to evaluate the effects of PM on GI tract.

The unwanted health effects of PM on the GI tract may not be limited to its effects on the GI epithelium. When PM enters the GI tract, it not only gets in contact with the GI epithelial and immune cells, but also with more than 10^{14} microbes residing there. Growing evidence suggest that alterations in the composition and diversity of gut microbiota may play a role in the development of GI diseases such as IBD as well as other inflammatory disorders of the GI tract.

In this study, we aimed to determine whether exposure to inhaled PM at clinically relevant doses alters the bacterial composition throughout the gastrointestinal tract in mice. This is the first study that investigated the effects of PM on microbiome composition of GI tract using a clinically relevant model of PM exposure via inhalation.

Materials and Methods

Animals

The research protocol was evaluated and approved by the Animal Care and Use Committee of Northwestern University, and the University of Chicago in Chicago, Illinois. The mice were 20-25 g, male, 8-12 weeks old and these C57BL/6 mice were obtained from Jackson Laboratories. Ten mice were allocated into each study group. Mice received the 2918 Teklad global 18% protein rodent diet (Envigo, Indianapolis, IN) prior to exposure and during the times when they are not being exposed to PM or filtered air in the exposure chambers. During the 8-hour exposure to PM or FA, they received Diet Gel 76A (ClearH2O, Westbrook, ME).

Inhalational exposure to PM_{2.5}

We exposed mice to PM_{2.5} concentrated from ambient air in Chicago 8 hours per day for 5 days a week for three consecutive weeks in a chamber connected to Versatile Aerosol Concentration Enrichment System (VACES) (Budinger et al., 2011; Chiarella et al., 2014).

The VACES system draws approximately 100 liters per minute of ambient air from which PM is condensed and then resuspended for delivery to a chamber designed specifically to ensure uniform distribution of the particles. We exposed control mice to filtered air in an identical chamber connected to the VACES in which a Teflon filter was placed on the inlet valve to remove all particles. We estimated ambient PM_{2.5} concentrations as the mean of reported values from the 4 EPA monitoring locations closest to our location (State of Illinois Environmental Protection Agency, 2014). Particle counts in the chamber were measured with a TSI 3775 particle counter (Shoreview) and used to determine the enrichment in the chamber compared with the ambient air as previously described (Budinger et al., 2011; Chiarella et al., 2014). The mean daily ambient PM_{2.5} concentration in Chicago was $16.3 \pm 0.85 \mu\text{g}/\text{m}^3$ during the study period, and the mean concentration in the PM exposure chamber was $135.4 \pm 6.4 \mu\text{g}/\text{m}^3$. Chicago is the third largest city in the US, with approximately 2.7 million and 9.5 million residents in the city and metropolitan area, respectively. Interstate highways, railroads, and 2 major airports connect the city to other urban areas in the region. Major point sources of particulate air pollution include 2 coal-fired power plants and metal processing, paint, and solvent factories (the last being in the southern and southeast parts of the city) (Binaku et al., 2013). Mobile source emissions account for the majority of atmospheric nitrogen compounds, while refineries, coal burning, and steel manufacturing are responsible for sulfur compounds (Binaku et al., 2013). The composition of airborne PM is primarily sulfate and organic carbons and secondary nitrates. Particulate NO₃⁻, SO₄²⁻, and elemental carbon concentrations (2.5, 2.9, and 1.5 $\mu\text{g}/\text{m}^3$, respectively) approximate those in other major American cities (Babich et al., 2000).

Characterization of PM

To determine the chemical composition of PM_{2.5} that our mice were exposed, four blank and four PM-exposed Teflon filters (PTFE, 37 mm, 2 μm pore; PALL Life Sciences, Ann Arbor, MI) with a mean ($\pm$ SD) particle mass of $1.1 \pm 0.5 \text{ mg}$ were agitated for 1 hour in 3.0 mL 1.0 N HCl. Supernatants were analyzed for metals (in duplicates) using inductively-coupled plasma optical emission spectrometry (ICP-OES; Model Optima 4300D, Perkin Elmer, Norwalk, CT) operated at two separate wavelengths for each metal. Chemical composition of PM_{2.5} is shown in Table 1.

Harvesting of tissues

Following the completion of exposure to PM_{2.5} or filtered air for three weeks as described above, tissues (stomach (S), small intestine (SI), cecum (CC), colon (CL)) were harvested using sterile instruments for each individual animal and site. In addition, fecal (F) samples were also collected for evaluation of gut microbiome composition in feces. Comparison groups included PM_{2.5} exposure (n=10) and filtered air inhalation (n=10) groups.

Sequencing and Sequence Quality Assessment

DNA was extracted from gastrointestinal tract organs and feces by FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH 44139 USA) according to the manufacturer's protocol. Extracted DNA samples were tested with fluorometric quantitation (Qubit, Life Technologies, Grand Island, NY 14072) to verify their adequacy in amount and samples with inadequate amounts of template DNA were not used for further sequencing process. We

used the 28F forward primer, 5'-GAGTTTGATCCTGGCTCAG-3' and 519R reverse primer, 5'-GTNTTACNGCGGCKGCTG-3' to pyrosequence 16S rDNA on a 454 GS FLX platform, with barcoding and using titanium kits to perform high throughput sequencing at Research and Testing Labs, Inc. (Lubbock, Texas, USA) for the analysis of the bacterial 16S rRNA phylotypes (Smith et al., 2010). Sequence processing and quality assessment were achieved by using custom C# and Python scripts at Research and Testing Labs, Inc. in addition to python scripts within the QIIME in addition to python scripts within the QIIME (Quantitative Insights Into Microbial Ecology) software pipelines (VirtualBox versions 1.5, 1.6, and 1.7) (<http://qiime.org>) (Caporaso et al., 2010b; Ishak et al., 2011; Sen et al., 2009; Wolcott et al., 2009). The sequence outputs were filtered to eliminate low-quality sequences (defined as any sequences that are <200 bps or >1,000bps, sequences with any nucleotide mismatches to either the barcode or primer, sequences with homo polymer runs >6, sequences with an average quality score of <25, and sequences with ambiguous bases >6) and were truncated at the reverse primer. Sequences were denoised using USEARCH (Edgar, 2010), chimeric sequences were filtered with UCHIME (Edgar et al., 2011) and Chimera Slayer (Haas et al., 2011). Operational taxonomic units (OTUs) were selected using UCLUST (Edgar, 2010) at a 97% similarity level, and representative sequences were selected. Sequences were aligned with PyNAST (Caporaso et al., 2010a) and filtered alignments were classified according to their taxonomic annotation in QIIME using the RDP (Ribosomal Database Project) classifier (Wang et al., 2007) at an 80% confidence threshold against the Greengenes database as implemented in QIIME (version gg_otus-12_10-release). A hundred samples (80 tissue and 20 fecal samples) were analyzed.

We gathered a total of 696,279 raw sequences, and 232,165,658 raw bases with an average of 6962 sequences per sample at an average length of 333.43 bps per sequence. In the PM-exposed group, one of the small bowel tissue samples had a very low coverage with 396 sequences obtained from the entire sample and therefore this sample was omitted from any further analyses. After completion of quality filtering as described above, we were able to obtain 309,143 total sequences with an average of 3127 sequences per sample which were denoised, greater than 250-bp-long, demultiplexed, reverse-primer truncated and chimera-filtered and these were used for the rest of the analysis. The higher quality sequences were rarified to the minimum of 980 sequences in all samples to conduct alpha- and beta-diversity analyses.

Statistics

Alpha diversity and beta diversity were measured at the OTU level. Biodiversity was calculated at the OTU level because taxon-based methods based on evolutionary theory may cause misinterpretation of the findings as not all bacteria even at species level are not evenly related genetically and phenotypically (Lozupone and Knight, 2008).

Alpha Diversity—Alpha diversity describes the distribution of taxa within the same community i.e. in a single sample. Count-based alpha diversity indices give us information about alteration of bacterial composition in terms of the presence or abundance of different sequences binned to different OTUs. However, count based alpha diversity indices don't demonstrate whether the changes observed within a sample are in phylogenetically related

OTUs or unrelated ones. Divergence-based methods (such as PD-Whole Tree as implemented in QIIME) come handy in this regard and provide us the summation of total branch length of each species in the phylogenetic tree and put an emphasis on their relatedness. Besides, the latter index also has the property to demonstrate sufficiently taxon richness within the same community like count-based methods since index value of this phylogenetic method increases by adding a new species (Lozupone and Knight, 2008; Morgan and Huttenhower, 2012). Therefore, in our study alpha diversity was calculated using both count-based and divergence-based methods using the following four indices: Chao1 index, number of OTUs within the sample also known as Richness, and the Shannon index and a Phylogenetic-Diversity-Whole-Tree (Lozupone and Knight, 2008; Morgan and Huttenhower, 2012). Further information regarding alpha diversity indices are also described in our previous study (Shobar et al., 2016).

QIIME VB1.7 was used to generate the initial α -rarefaction curves. Publication quality alpha diversity rarefaction graphs were recreated using Microsoft Excel (Lozupone et al., 2006; Lozupone and Knight, 2005; Lozupone et al., 2011; Martin, 2002). Statistical comparisons for alpha-diversity measures were conducted using SPSS (Version 23.0.0, Chicago, IL). Variables were checked for normality assumptions using both descriptive statistics such as skewness, kurtosis and normality tests. Independent t-tests or non-parametric Mann-Whitney U tests were used as appropriate for the comparisons.

Beta Diversity—While alpha diversity (i.e. within sample diversity) pertains to a single sample and its microbial composition, beta diversity (i.e. between sample diversity) provides information about the differences in microbial communities in different samples and compares them (Mandal et al., 2015). Beta diversity can be measured by either methods that take into consideration the relative abundance of each taxon or by methods which measure only presence/absence of each taxon. (Lozupone et al., 2007). Unifrac is a distance metric that is used to measure beta diversity by taking phylogenetic information of OTUs into account in contrast to other beta diversity measures that give equal weight to each OTU in the calculation. Unifrac measures branch length of bacterial populations with a phylogenetic tree to determine similarity in bacterial composition in different samples (i.e. communities). Unweighted Unifrac takes each different OTUs into account in terms of its presence or absence. Bray-Curtis similarity index is another measure of beta diversity which is based on the count of bacterial taxa (OTUs in our case) that are common between two different samples (Bray and Curtis, 1957). While Unifrac provides information of phylogenetic relatedness, the Bray-Curtis similarity index cannot. Therefore, we used both Unifrac and the Bray-Curtis similarity indices to analyze the data.

Ordination and clustering techniques are helpful to visually illustrate the differences in beta diversity measures in multiple samples at the same time. We used principal coordinates analysis (PCoA) as the ordination method in our analyses. By placing PCoA values into three visual axes, we were able to create a three-dimensional data plot demonstrating the Unifrac distances and Bray-Curtis similarities of different communities. Each dot on the PCoA plot represents an individual sample and samples that are farther apart on the graph are more dissimilar. QIIME VB1.7 was used to obtain Bray-Curtis similarity, Unifrac distances and PCoA coordinate values at the level of OTUs. KingViewer KiNG (Kinemage

Next Generation) Display Software (Richardson Lab, Duke University, Durham, NC) was used to generate 3D data plotters.

Stacked histograms of the bacterial taxa from the phylum to the genus level were generated using Microsoft Excel. The linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011) was used to discover differentially abundant taxa between the air-exposed and PM-exposed mice and the analysis was conducted using Galaxy modules provided by the Huttenhower lab (<https://huttenhower.sph.harvard.edu>). The alpha values for the factorial Kruskal-Wallis test among classes (in our case air exposure vs PM exposure) and the alpha values for the pairwise Wilcoxon test between subclasses (in our case the sites of sample collection i.e. stomach, small intestine, cecum, colon, and fecal samples) were set at 0.05. The threshold for log LDA used was 2 logs and the analysis strategy was chosen as strict “all-against-all”. Taxa present in less than 4 samples and taxa with very low abundance (less than 1% in majority of the samples) were not reported as significant.

Results

Exposure to particulate matter induces significant increases in diversity in the small intestine, colon and feces

Alpha diversity was analyzed using Richness (i.e. number of observed OTUs), Chao1, phylogenetic diversity-whole tree (PD-WT) and Shannon indices (Lozupone and Knight, 2008) at the OTU level. As shown in (Fig. 1), Richness, and Chao1 and Shannon indices were increased in the feces obtained from PM-exposed mice compared to air-exposed mice ($p=0.016$, 0.011 , 0.002 , respectively). Similarly, Richness and Shannon indices were increased in the small intestinal samples from the PM-exposed mice compared to air-exposed mice ($p=0.019$, 0.004 , respectively), with an increased trend in the Chao1 index ($p=0.065$). There was no difference in the PD-WT index in fecal and small intestinal samples suggesting that the observed changes in alpha diversity were not due to phylogenetically distinct or environmentally unique bacteria acquired with PM exposure. We did not find any statistically significant changes in alpha diversity in the stomach. In the distal GI tract, we found no changes in alpha diversity in the cecum; however, there was an increase in the Shannon index in colon tissues obtained from PM-exposed mice compared to the air-exposed mice ($p=0.049$). Collectively, these results suggest that changes in alpha diversity are modest and seem most apparent in the fecal environment, implying a predominant or cumulative effect of PM on the microbiota in the GI tract lumen.

Particulate matter-induced differences in the composition of bacterial community increase distally along the GI tract

In order to examine the differences in global bacterial composition throughout the GI tract, we used the Bray-Curtis similarity and the unweighted Unifrac metrics. Undirected ordination of the samples using the above metrics in a principal coordinates analysis demonstrated significant differences in community composition, causing a clear separation of the PM-exposed samples from air-exposed samples in all sites of the GI tract and in feces (Figs. 2 and 3). The visual differences were also statistically significant at all sites for both the unweighted Unifrac and the Bray-Curtis similarity metrics (Table 2). Interestingly, the

degree of deviation from control microbiota (obtained from air-exposed mice) was the least in the stomach, but gradually increased from proximal to distal GI tract reaching the highest level in the feces. These results suggest PM-induced changes in microbiota increase along the GI tract with a more profound effect in the distal compared to the proximal GI tract.

Particulate matter-induced changes in bacterial composition occur globally throughout the GI tract, are evident at both the highest and lowest taxonomic levels with a consistent pattern of reduction in Firmicutes

We searched for consistent and global changes induced by PM exposure in the composition of microbiota throughout the GI tract by conducting a LEfSe analysis in all samples (In other words, air- exposed samples vs. PM-exposed samples was the primary grouping, with each sample site being the secondary subgrouping). Compared to air-exposed samples, we found a significant reduction in Firmicutes, in the PM-exposed group, at all sites at the phylum level (Fig. 4a). These differences persisted at the family level (Supplemental Information Fig. S1a). *Staphylococcaceae* and other families within the Bacilli class within the Firmicutes phylum were higher in the air-exposed mice (Supplemental Information Fig. S1b and 1c); whereas these families were replaced by *Rikenellaceae* and other Bacteroidales order within the Bacteroidetes phylum, which were higher in the PM-exposed mice (Supplemental Information Fig. S1d–e). At the genus level, the reductions within the Firmicutes were again observed (Fig. 4b) and the differences noted in various genera (corresponding to the above listed family level changes) were also preserved across all sites (Fig. 5a–g). Another significant change was within the genera in the *Lactobacillaceae* family, especially in samples from the proximal GI tract. In the air-exposed mice, the genus *Lactobacillus* was observed, but in the PM-exposed mice, an unnamed genus within the *Lactobacillaceae* was detected in the samples (Fig. 5c and 5g). In summary, these data suggest that PM exposure has a global effect bacterial microbiota composition at every site of the GI tract and some bacterial taxa can be seen consistently throughout, regardless of the location where the sample was obtained.

Multiple taxa differ between the air-exposed and PM-exposed animals at each site

While it is important to look for consistent changes throughout the GI tract, it is also well-known that different parts of the GI tract constitute highly different environments in terms of pH, oxygenation, and motility. Considering the differences in the environments, we therefore postulated that some of the changes in microbiota may occur only at one particular site and not others. To explore this possibility, we conducted additional comparisons between the air-exposed and PM-exposed groups separately for each sampling area within the GI tract. We observed the following differences: In the stomach, at the phylum level, Firmicutes were modestly increased in the air-exposed group (LDA score $\log(10) = 4.81$). Similar to the global changes observed for all samples, at the genus level, in the PM-exposed group, other genera within *Lactobacillaceae* and other genera within the Bacteroidales order were increased, whereas in the air-exposed group, the genus *Lactobacillus* was increased (Supplemental Information Fig. S2a–c). In the small intestine, at the phylum level, Firmicutes were higher in the air-exposed group (LDA score $\log(10) = 5.52$), whereas Bacteroidetes were higher in the PM-exposed group (LDA score $\log(10) = 5.36$). At the genus level, in the PM-exposed group, an unnamed genus within *S24_7* family within the

Bacteroidetes phylum, other genera within the Bacteroidales order, and an unnamed genus within the *Lactobacillaceae* were increased (Supplemental Information Fig. S3a–c); whereas in the air-exposed group *Staphylococcus*, *Lactobacillus* and an unnamed genus within the *Aerococcaceae* were increased (Supplemental Information Fig. S3d–f). In the cecum, at the phylum level, Firmicutes were higher in the air-exposed group (LDA score $\log(10) = 5.13$), whereas Bacteroidetes were higher in the PM-exposed group (LDA score $\log(10) = 5.16$). At the genus level, in the PM-exposed group, an unnamed genus within the *Rikenellaceae*, an unnamed genus within the *S24_7* family within the Bacteroidetes phylum, other genera within the Bacteroidales order were increased (Supplemental Information Fig. S4a–c); whereas in the air-exposed group, *Bacteroides*, *Staphylococcus*, *Turicibacter*, *Ruminococcus* and other genera within the *Ruminococcaceae* family, *Oscillospira*, and other bacteria within the Clostridiales class were increased (Supplemental Information Fig. S4d–j). In the colon, at the phylum level, Firmicutes were higher in the air-exposed group (LDA score $\log(10) = 5.29$), whereas Bacteroidetes were higher in the PM-exposed group (LDA score $\log(10) = 5.31$). At the genus level, in the PM-exposed group, an unnamed genus within *S24_7* family within the Bacteroidetes phylum and *Parabacteroides* were increased (Supplemental Information Fig. S5a–b); whereas *Turicibacter*, *Anaeroplasma*, *Clostridium*, other genera within *Firmicutes*, and other genera within *Mollicutes* were increased in the air-exposed group (Supplemental Information Fig. S5c–g). In the feces, at the phylum level, Firmicutes were higher in the air-exposed group (LDA score $\log(10) = 4.99$), whereas Bacteroidetes were higher in the PM-exposed group (LDA score $\log(10) = 4.99$). At the genus level, in the PM-exposed group, an unnamed genus within the *Rikenellaceae*, an unnamed genus within the *S24_7* family within the Bacteroidetes phylum, other genera within the Bacteroidales order, an unnamed genus within the *Lactobacillaceae*, an unnamed genus and other genera within the *Lachnospiraceae* were increased (Supplemental Information Fig. S6a–f); whereas in the air-exposed animals, *Bacteroides*, *Staphylococcus*, *Lactobacillus*, and *Turicibacter* were increased (Supplemental Information Fig. S6g–j). In summary, these observed differences point toward enhancement of some bacterial taxa such as unnamed genera within *Lactobacillaceae*, *Rikenellaceae*, *S24_7* families after exposure to PM. Furthermore, in parallel to the ordination results, more bacterial taxa are observed to be differentially abundant moving from the proximal to the distal GI tract. Together, these results suggest that PM induces dysbiosis throughout the GI tract which is prominent distally but observable also in the proximal GI tract.

Exposure to PM induces elevated TNF- α expression only in colon and does not affect the expression of other cytokines

To determine whether PM-induced changes in bacterial composition is associated with inflammation in GI tract, we measured mRNA expression of *Tnfa*, *Il6* and *KC* (*Il8* equivalent in mice) in stomach, small bowel, cecum and colon harvested from FA- and PM-exposed mice. We found that compared to FA-exposed mice, PM-exposed mice exhibited increased expression of *Tnfa* mRNA in colon, whereas *Tnfa* expression was not different at other sites (Fig. 6a). There was no difference in the expression of *Il6* and *KC* between FA-exposed and PM-exposed mice (Fig 6b,c).

Discussion

Particulate matter air pollution is a global environmental health problem causing 3.7 million premature deaths annually, representing 6.7% of all deaths worldwide (2014; Lelieveld et al., 2015; Organization, 2016). The mortality is largely due to increased cardiopulmonary events, including pneumonia (Brook et al., 2010; Budinger et al., 2011; Chiarella et al., 2014; Downs et al., 2007; Gauderman et al., 2004; Krewski et al., 2004; McCreanor et al., 2007; Miller et al., 2007; Mills et al., 2007; Mutlu et al., 2007; Mutlu et al., 2006; Neupane et al., 2010a; Pope et al., 2009; Soberanes et al., 2009). Recent evidence suggests that exposure to PM may also play a role in gastrointestinal diseases particularly those conditions characterized by inflammation such as appendicitis and IBD (Beamish et al., 2011; Laden et al., 2006). In this study, we demonstrate that PM significantly increases gut microbial diversity in the small bowel, colon and the feces and alters gut microbiota composition along the GI tract. Importantly, PM-induced alterations in the composition of bacteria increased from the proximal to distal parts of the GI tract. Furthermore, observed changes are present starting at the top of the taxonomic classification with a significant reduction in *Firmicutes*, which is in the same direction as the changes reported in patients with inflammatory diseases of the GI tract such as IBD (Matsuoka and Kanai, 2015; Sartor and Mazmanian, 2012). Lastly, it is also important to note that while site differences exist, there are also specific changes in genera seen across all samples, suggesting that some distinct bacteria may have the capacity to bloom with PM exposure throughout the GI tract.

Particulate matter can get access to GI tract via several ways (Beamish et al., 2011). Although a significant portion of PM can be inhaled into the lungs, there is substantial deposition in the nasopharynx and oropharynx. Particles deposited in the conducted airways may be cleared out of the lungs via mucociliary clearance within 24 hours (Oberdorster, 1993). A considerable portion of smaller size PM (PM_{2.5}) may reach alveoli upon inhalation. The particles are phagocytosed by alveolar macrophages and subsequently moved back to oropharynx by mucociliary clearance (Gerrity et al., 1983; Lippmann et al., 1980; Moller et al., 2004; Oberdorster, 1993; Semmler-Behnke et al., 2007). Particulate matter that is directly deposited in the nasopharynx and oropharynx as well as indirectly deposited via mucociliary clearance can subsequently be swallowed allowing the PM to enter the stomach and reach the rest of the GI tract (Beamish et al., 2011; Kreyling, 1993; Moller et al., 2004; Oberdorster, 1993; Semmler-Behnke et al., 2007). Direct dietary ingestion is another means of exposure to PM via ingestion of food (fruits and vegetables) or water that are contaminated by PM (Commission, 2002); however, the relative contribution of dietary contamination vs inhalation has not been quantified. Prior studies trying to demonstrate the fate of inhaled PM in the body using radioactive labelling have provided conflicting results likely due to methodological issues since radioactive label itself may change the behavior of the particles and their size and importantly may disassociate from the particle leading to inaccurate conclusions (Nemmar et al., 2002). Therefore, it is not possible to quantify how much PM exactly reaches the various portions of the GI tract. However, the health effects of PM in the GI tract have been investigated and demonstrated by multiple groups of scientists (Ananthakrishnan et al., 2011; Beamish et al., 2011; Kaplan et al., 2010; Kaplan et al., 2012; Kaplan et al., 2013; Salim et al., 2014a; Salim et al., 2014b).

Once in the GI tract, PM has been reported to exert its health effects via multiple mechanisms (Beamish et al., 2011). First, PM may have a direct effect on GI epithelial cells. As the primary physical barrier of the gut, intestinal epithelial cells regulate permeability (DeMeo et al., 2002). Tight junctions between intestinal epithelial cells, strengthened by intracellular cytoskeletal proteins constitute a valuable part of this defense system. This barrier function might be affected by intestinal epithelial injury induced by PM. In our previous studies, we reported that PM causes a dose-dependent increase in the generation of mitochondrial ROS, which were required for alveolar epithelial cell dysfunction following exposure to PM *in vitro* (Mutlu et al., 2006). PM has been shown to enhance paracellular permeability in alveolar epithelial cell monolayers after exposure to PM via reduction in tight junction proteins (Caraballo et al., 2011). The effect of PM on alveolar epithelial cell permeability could be reversed by the overexpression of mitochondrial antioxidant enzymes (Caraballo et al., 2011). Similar to the effects of PM on alveolar epithelial cells, we reported that PM induces mitochondrial ROS production in colonic epithelial cells and consequently causes apoptosis of colonic epithelial cells and increases permeability (Mutlu et al., 2011). Our *in vivo* experiments in mice also confirmed that PM exposure caused an increase in gut permeability associated with disruption of tight junctions characterized by decreased expression of tight junction proteins in colonic epithelium (Mutlu et al., 2011). Secondly, PM may also exert a direct or indirect effect on gut microbiome, which may also contribute to gut permeability and inflammation. When administered orally, PM may lead to a significant inflammatory response via ROS production and NF- κ B activation in GI tract (Chiarella et al., 2014; Mutlu et al., 2011). Furthermore, long-term exposure of mice to PM orally not only increases pro-inflammatory cytokines but also leads to infiltration of the colonic lamina propria with neutrophils and this effect appears to be pronounced in *IL10*^{-/-} mice, an important murine model of IBD in which there are also alterations in the colonic microbiota (Kish et al., 2013; Salim et al., 2014a). While these observed effects of PM in the gut provide some insights into how PM contributes to gut permeability and inflammation, it should also be noted clearly that exploration of the gastrointestinal effects of PM is still at its infancy considering that the exposures to PM in the above mice studies were orally via gastric gavage as opposed to inhalational exposure, limiting the applicability of findings to real life.

Influence of bacterial composition on the host has become an important field of investigation with the rapid development of high throughput sequencing techniques (Larsen and Dai, 2015; Morgan and Huttenhower, 2012). Our study is the first to evaluate the effect of a clinically relevant PM exposure via inhalation on the gut microbiome throughout the GI tract from stomach to the colon as well as in feces. Our results revealed a trend towards a statistically significant increase in alpha diversity throughout the GI tract except the cecum where we have seen a decrease in all four alpha diversity indices, although this was not statistically significant. In addition, PM exposure also significantly altered the composition along the entire GI tract, favoring some bacterial taxa over others.

To date, two studies evaluated the effect of PM on the gut microbiome in the literature (Kish et al., 2013; Salim et al., 2014a). One of these studies looked at the PM-induced changes in fecal microbiome in *IL10*^{-/-} murine model of colitis after daily gavage of PM for 7-14 days (Kish et al., 2013). Using the terminal restriction fragment length polymorphism (T-

RFLP), the study demonstrated that the fecal microbiome clustered differently for both the *II10*^{-/-} and background control (129SvEv) mice at the beginning and end of the experiment (as colitis was developing), although the bacterial taxa responsible for this difference in clustering was not well-clarified due to the inherent limitations of T-RFLP. In fact, the only notable change in the background strain was a rise in *Verrucomicrobia*. Only in the *II10*^{-/-} mice, there was a decrease in *Bacteroidetes*, and rise in *Firmicutes* as the mice developed colitis (Kish et al., 2013). This study modeled PM exposure via oral ingestion limiting the applicability of these findings to human disease. These findings are different from a later study published by the same group of investigators: In this second study, only *II10*^{-/-} mice were examined. *II10*^{-/-} mice were fed PM₁₀ as part of their chow diet (PM-fed group) and were compared to mice fed with chow diet only (control group) (Mouse Diet 9F, Lab Diet, St. Louis) at the weeks they are expected to develop colitis. The fecal microbiome composition was studied with MiSeq Illumina sequencing, which did not reveal any differences in the Shannon diversity index at the family level in the fecal samples studied (Salim et al., 2014a). Unlike the author's previous study, no differences were noted in the *Bacteroidetes* and *Firmicutes* and *Verrucomicrobia*, but a relative decrease in *Bifidobacterium* was seen in all PM-fed data points in PM-fed *II10*^{-/-} mice. Comparatively, our results did not show differences in *Verrucomicrobia*, or *Actinobacteria*.

The differences between our study and the other two studies perhaps could be explained by several factors including the differences in (1) the mouse strain, (2) food composition, (3) mode and duration of exposure to PM and (4) techniques to assess microbiome. First, there is a notable difference in the genetic background of mice that were used in the prior study compared to ours. We used only wild-type mice with intact immune system not lacking *II10* expression and therefore should mount a full inflammatory response when exposed to PM compared to *II10*^{-/-} mice. Because *II10*^{-/-} mice are on 129/SvEv background, Kish et al used 129/SvEv mice as their control strain, which has a different genetic background compared to C57BL/6 mice (Kish et al., 2013). Second, there were differences in the type of food that were given to mice between our study and the other two studies, which used two different diets (Laboratory Rodent 5001 (Kish et al., 2013) and Mouse Diet 9F (Salim et al., 2014a), LabDiet, St. Louis, MO). Third, we used a clinically relevant model of PM exposure using inhalation compared to other studies in which mice were exposed to PM orally via administration of PM as a mixture in diet (Kish et al., 2013; Salim et al., 2014a) or by oral gavage (Kish et al., 2013). In addition to the mode of exposure (inhalational vs. oral), there were differences in the duration of exposure between our study and the other two studies. We exposed our mice to PM via inhalation 8 hours per day, 5 days per week for 3 weeks. In contrast, Kish et al. orally administered PM to 6-week old female mice for 7-14 days (short-term) and 35 days (long-term) (Kish et al., 2013). In addition, mice exposed to PM for short-term received the PM in a single bolus via gavage, while mice exposed to PM for long-term received it constantly mixed with food. In the study by Salim et al, mice were exposed to PM orally mixed with food prenatally and postnatally for 20 weeks. (Salim et al., 2014a). Fourth, the only prior study that included wild type (129/SvEv) mice used a fingerprinting technique to determine bacterial composition (Kish et al., 2013), and fingerprinting techniques in general can be considerably inferior to sequencing in resolution and depth, and many of the results obtained by fingerprinting cannot be accurately attributed to a single

bacterial taxon in complex samples such as feces. Lastly, study by Kish et al. examined the effect of PM only in feces, which is a major limitation, while we examined the effect of PM not only in feces but also in the entire GI tract including mucosa-associated bacteria (Kish et al., 2013). Therefore, findings observed in our study reflects not only what happens in the lumen but also provides for exploring possible pathophysiological links between PM and gastrointestinal diseases at the tissue level. Alterations observed in our study may also be a reflection of disturbed gut permeability as a consequence of direct injury in the PM-exposed group.

One of the bacterial taxon that is observed to be differentially abundant in our dataset is the members of Bacteroidales order. They are among the most abundant of the cultured gram-negative organisms in human gut, and some have been associated with beneficial properties to their host whereas others have been associated with periodontal disease (Coyne and Comstock, 2008). Within this order, we observed increases in both unnamed genera as well as in the *S24_7* family. This latter family constitutes an uncultured group of bacteria that have recently been shown to have extensive glycan metabolizing capability (varying in their preference for dietary glycans to host glycans) by genomic analysis, probably creating distinct spatial niches within the gut of homeothermic animals (Ormerod et al., 2016). The metabolic capacity to degrade host glycans (which includes gastrointestinal mucous which is important for the gut barrier function) could have an effect on gastrointestinal permeability or could be a consequence of it. Some members of the *S24_7* family also have been shown to have urease (Ormerod et al., 2016), which is unusual for the Bacteroidales order and which in turn has been associated with virulence in other bacterial organisms (Mora and Arioli, 2014). In our experiments, another altered member of Bacteroidales order is *Rikenellaceae*, which has been shown to be associated with benzo(a)pyrene exposure in another murine study (Ribiere et al., 2016). Benzo(a)pyrene is an environmental polycyclic aromatic hydrocarbon, which is an important component of air pollution with significant adverse health effects (Lewtas, 2007; Perera, 1981). We also observed changes in the beneficial bacteria with PM exposure within the *Lactobacillaceae*, such as the disappearance of the *Lactobacillus* genus which has been traditionally considered to have a positive impact on intestinal health and has been a component of many probiotics. Instead, we noted appearance of unnamed genera which are likely to be among the environmental members of the *Lactobacillaceae* family that degrade environmental carbohydrate-containing substrates in water, soil and sewage (Felis and Pot, 2014).

Although we used a clinically more relevant model of PM exposure compared to the two previous studies that evaluated the effect of PM on microbiome by administering it directly into the GI tract, there may be some limitations that need to be taken into consideration. The concentration of PM that we exposed our mice is similar to what we and other investigators have previously reported (Chiarella et al., 2014; Liu et al., 2014; Liu et al., 2017). While the PM_{2.5} levels in the US and western Europe have declined, many people in the rest of the world are exposed to PM_{2.5} levels that are similar to what we used in our studies (World Health Organization 2018; World Health Organization, 2016). Additionally, the concentration of PM_{2.5} in the chamber is 10-fold higher than what a human will be exposed to in Chicago, but the total number of particles inhaled by a human is much greater than a mouse because the minute ventilation of a 70-kg man is ~200-300 fold higher than an 8-12-

week old mouse (Depledge, 1985). Lastly, we exposed animals to PM for 8 hours per day; however, this may not be representative of human exposure unless the individual has a profession that requires him or her to spend significant portion of their time outside. Despite these limitations, our studies show for the first time that inhalational exposure to PM to levels that are found in many cities in the developing world affect bacterial composition in GI tract.

Conclusions

In summary, dysbiosis, an imbalance of gut microbiome composition, is associated with obesity (Ley et al., 2005), type I and II diabetes mellitus (Gulden et al., 2015; Larsen et al., 2010), irritable bowel syndrome (Staudacher and Whelan, 2016), inflammatory bowel disease (Tamboli et al., 2004) and hypertension (Yang et al., 2015). As an environmental toxicant that can get access to the GI tract directly and indirectly, PM has been increasingly recognized as a contributor to the development of GI diseases. In this study for the first time, we reveal that exposure to PM via inhalation alters gut microbiome composition throughout the GI tract. Our results also provide a possible mechanistic link to the previously observed, increased inflammatory response with PM exposure. These findings upon further exploration can enhance our understanding of how air pollution contributes to GI disease or other systemic diseases in which gastrointestinal microbiome or excess gut permeability is postulated to play a role. In fact, if confirmed in human studies, additional knowledge that is obtained may change our approach to treatment of certain gastrointestinal and various other diseases linked to PM and may also result in policy changes in public health strategies in regard to control of environmental PM exposure.

As an environmental factor that has been linked with significant health effects and mortality, our results suggest that PM may affect the entire GI tract through changes in microbiota composition and can thereby be postulated to play a major role in either initiation or worsening of existing GI or metabolic conditions in which alterations in the GI microbiota have been shown to play a major role.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Capsule

Exposure to particulate matter air pollution alters the gut's microbial composition, which could be a potential mechanism that supports particulate matter induced inflammation in the gastrointestinal tract.

Highlights

- Examine the alterations in microbial composition throughout the gastrointestinal tract and feces following inhalational exposure to particulate matter (PM) mimicking real life exposures in humans.
- Gut microbiome at five sampling sites (stomach, small intestine, cecum, colon, feces) between PM-exposed vs. air-inhaled mice were examined.
- PM-induced gut microbial changes which may lead to either initiation or exacerbation of existing inflammatory diseases of the gastrointestinal tract.

Fig 1. Rarefaction plots of PM-exposed vs. air-exposed samples by five sampling sites
 PM-exposed samples are colored in red; air inhaled samples are colored in blue. Diversity analyzed using four indices as follows: The Y- axes show the Chao1 index (panels (a), (e), (i), (m), (q)), Richness i.e. Number of OTUs (panels (b), (f), (j), (n), (r)), PD-WT index (panels (c), (g), (k), (o), (s)), and the Shannon diversity index (panels (d), (h), (l), (p), (t)). The X-axes show the number of sequences obtained from the samples. Results from samples extracted from stomach are shown in panels (a-d); those from small intestine are shown in panels (e-h); those from cecum are shown in panels (i-l); those from the colon are shown in panels (m-p); those from stool are shown in panels (q-t).

Fig 2. Significant difference in clustering of bacterial communities in the PM-exposed and air-exposed samples

Beta diversity was analyzed by Bray-Curtis similarity between samples at OTU level. PCoA was used to create three-dimensional data-plots. Each blue dot represents one sample obtained from air-inhaled mice; each red dot represents one sample obtained from PM-inhaled mice. Plots of samples collected from (a) stomach, (b) small intestine, (c) cecum, (d) colon and of (e) feces. A significant separation of air-exposed samples vs. PM-exposed samples was noted in PCoA plots and appeared be more pronounced going distally from stomach to feces. In panel (f) all available samples are plotted, and each sample is represented as one dot.

Fig 3. Significant difference in clustering of bacterial communities in the PM-exposed and air-exposed samples

Beta diversity was analyzed by unweighted Unifrac between samples at OTU level using QIIME. PCoA was used to create three-dimensional data-plots. Each blue dot represents one sample obtained from air-inhaled mice; each red dot represents one sample obtained from PM-inhaled mice. Plots of samples collected from (a) stomach, (b) small intestine, (c) cecum, (d) colon and of (e) feces. A significant separation of air-exposed samples vs. PM-exposed samples was noted in PCoA plots and appeared to be more pronounced going distally from stomach to feces. In panel (f) all available samples are plotted, and each sample is represented as one dot.

Fig 4. Stacked histograms demonstrating the relative microbial abundances by site and by inhalational exposure

Each column in the stacked histogram represents one sample and has different color bars within it, and these bars are proportional to the percent relative bacterial abundance of each taxa within the sample summing up to %100. Stacked histograms show the visible alterations in microbiome composition in the GI tract between PM-exposed mice and air-exposed mice. In panel (a), taxonomic representation is made at phylum level; in panel (b), taxonomic resolution is shown at the genus level. For panel b, with each tone denotes a different genus; each color corresponds to the color of the phylum shown in panel a; least abundant taxa (<1%) have been filtered for better visualization. The samples are collected from the stomach (STC), small intestine (SI), cecum (CC), colon (CL) and feces (F). In both

stacked histograms, of the major phyla, Firmicutes are given the tones of blue, Bacteroidetes are given the tones of red, and Proteobacteria are given the tones of yellow.

Fig 5. LefSe analysis of all samples revealed genera altered with PM exposure throughout the GI tract

In panel (a), LefSe scores are shown for the statistically significant and differentially abundant genera between the PM-exposed and air-exposed samples. Blue bars indicate the genera increasing in abundance in all air-inhaled sampling sites; red bars indicate the genera increasing in abundance in all PM-exposed samples across the all of five sampling sites (stomach, small intestine, cecum, colon, feces). The X-axis shows the linear discriminant analysis score in log 10. In panels (b), (c), (d), (e), (f), (g) histograms of the relative abundance of these six genera are shown individually for each site; each bar represents the abundance in a single sample; and if the abundance is zero, no bar is shown on the panel corresponding to that particular sample. Panel (b) shows the results for unidentified genera within the *Bacteroidales* order; Panel (c) shows the results for an unidentified genus within the *Lactobacillaceae* family; Panel (d) shows the results for an unidentified genus within the *Rikenellaceae* family; Panel (e) shows the results for unidentified genera within the *Bacilli* class; Panel (f) shows the results for *Staphylococcus*, and Panel (g) shows the results for *Lactobacillus*. In panel (b-g), continuous and dotted black horizontal lines represent mean

and median values, respectively; and each pink bar indicates the relative abundance of the indicated genus in one sample collected from the cecum; each green bar indicates the relative abundance of the indicated genus in one sample collected from the colon; each yellow bar indicates the relative abundance of the indicated genus in one fecal sample; each purple bar indicates the relative abundance of the indicated genus in one sample collected from the small intestine; and each turquoise bar indicates the relative abundance of the indicated genus in one sample collected from the stomach.

Figure 6. Exposure to PM induces increased expression of *TNF- α* in colon and does not affect the expression of other cytokines

To determine whether PM-induced changes in bacterial composition is associated with inflammation in GI tract, we measured mRNA expression of (a) *Tnfa*, (b) *Il6* and (c) *KC* in stomach, small bowel, cecum and colon harvested from FA- and PM-exposed mice. N=4-5, *p<0.05

Table 1

Chemical composition of PM

	Blank filter (mean $\pm$ SD)	PM_{2.5} (mean $\pm$ SD)
[Ca] (ppm)	0.121 $\pm$ 0.040	9.628 $\pm$ 4.825
[K] (ppm)	0.021 $\pm$ 0.009	1.373 $\pm$ 0.151
[Mg] (ppm)	0.005 $\pm$ 0.004	2.637 $\pm$ 1.460
[Na] (ppm)	0.033 $\pm$ 0.018	9.287 $\pm$ 0.815
[Cd] (ppm)	BDL	BDL
[Cu] (ppm)	0.0003 $\pm$ 0.001	0.202 $\pm$ 0.058
[Fe] (ppm)	0.072 $\pm$ 0.044	3.878 $\pm$ 1.086
[Mn] (ppm)	0	0.134 $\pm$ 0.014
[Pb] (ppm)	0.0035 $\pm$ 0.001	0.666 $\pm$ 0.423
[Zn] (ppm)	0.0110 $\pm$ 0.003	1.211 $\pm$ 0.217

BDL, below detection limit

Table 2

ANOSIM statistics for beta-diversity

Samples	Unweighted Unifrac		Bray-Curtis	
	R	p-value *	R	p-value *
Stomach	0.4029	0.001	0.2794	0.003
Small intestine	0.5267	0.001	0.5353	0.001
Cecum	0.7020	0.001	0.7651	0.001
Colon	0.8158	0.001	0.7963	0.001
Feces	0.9789	0.001	0.9933	0.001
All samples	0.5096	0.001	0.6128	0.001

* Reflects the p-values after 999 permutations