INVESTIGATING THE EFFECTS OF TRAFFIC-GENERATED AIR-POLLUTION ON THE MICROBIOME AND IMMUNE RESPONSES IN LUNGS OF WILDTYPE MICE

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There is increasing evidence indicating that exposure to air pollutants may be associated with the onset of several respiratory diseases such as allergic airway disease and chronic obstructive pulmonary disorder (COPD). Many lung diseases demonstrate an outgrowth of pathogenic bacteria belonging to the Proteobacteria phylum, and the incidence of occurrence of these diseases is higher in heavily polluted regions. Within the human body, the lungs are among the first to be exposed to the harmful effects of inhaled pollutants and microbes. Research in the past few decades have expounded on the air-pollution-induced local and systemic inflammatory responses, but the involvement of the lung microbial communities has not yet been well-characterized. Lungs were historically considered to be sterile, but recent advances have demonstrated that the lower respiratory tract is replete with a wide variety of microorganisms - both in health and disease. Recent studies show that these lung microbes may play a significant role in modulating the immune environment by inducing IgA and mucus production.

Air pollutants have previously been shown to alter intestinal bacterial populations that increase susceptibility to inflammatory diseases; however, to date, the effects of traffic-generated air pollutants on the resident microbial communities on the lungs have not been explored. The microbiome is influenced by several factors, including diet and environmental exposures. A large percentage of the Western world population consumes a high-fat (HF) diet which has resulted in the epidemic of obesity. Consumption of an HF diet has been shown to alter the intestinal microflora and increase baseline inflammation. We aimed to understand whether diet might also contribute to the alteration of the commensal lung microbiome, either alone or related to exposure.

Thus, we investigated the hypothesis that exposure to air pollutants can alter the commensal lung microbiota, thereby promoting alterations in the lung's immune and inflammatory responses; in addition to determining whether these outcomes are exacerbated by a high fat-diet.

We performed two studies with exposures to different components of air pollutant mixtures on C57Bl/6 mice placed on either a control (LF) diet or a high-fat (HF) diet. Our first exposure study was performed on C57Bl/6 mice with a mixture of gasoline and diesel engine emissions (ME: 30 µg PM/m3 gasoline engine emissions + 70 µg PM/m3 diesel engine emissions) or filtered air (FA) for 6h/d, 7 d/wk for 30 days. The ME study investigated the alterations in immunoglobulin A (IgA), IgG and IgM, and lung microbiota abundance and diversity. Our results revealed ME exposures alongside the HF diet causes a decrease in IgA and IgG when compared to FA controls, thereby decreasing airway barrier protection. This was accompanied by the expansion of bacteria within the Proteobacteria phylum and a decrease in the overall bacterial diversity and richness in the exposed vs. control groups.

In our second study, we exposed C57Bl/6 mice to only the diesel exhaust particle component (35µg DEP, suspended in 35µl 0.9% sterile saline) or sterile saline only (control) twice a week for 30 days. We investigated immunoglobulin profiles by ELISA that revealed a significant increase in IgA and IgG in response to DEP. We also observed an increase in inflammatory tumor necrosis factor (TNF) - α, Interleukin (IL) -10, Toll-like receptors (TLR) - 2,4, nuclear factor kappa B (NF-κB) histologically and by RT-qPCR. Mucus production and collagen deposition within the lungs were also significantly elevated with DEP exposures. Microbial abundance determined quantitatively from the bronchoalveolar lavage fluid (BALF) by qPCR revealed an expansion of bacteria belonging to the Proteobacteria phylum in the DEP exposed groups on the HF diet. We also observed an increase in reactive oxygen and nitrogen species (ROS-RNS) products (nitrates),

within the groups that revealed an expansion of Proteobacteria. These observations are most likely due to the unique metabolic capabilities of Proteobacteria to proliferate in inflammatory environments with excess nitrates. We assessed if treatments with probiotics could attenuate the DEP-induced inflammation by supplementing a separate group of study animals on the HF diet with 0.3 g/day of Winclove Ecologic® Barrier probiotics in their drinking water throughout the study. With probiotic treatments, we observed a significant decrease in ROS-RNS that was accompanied by complete elimination of Proteobacteria suggesting that in the absence of nitrates, the expansion of Proteobacteria is curbed effectively. We also observed a decrease in proinflammatory TNF-α and collagen deposition with probiotic treatments, and an increase in IgA levels within the BALF, suggesting that probiotics aid in balancing proinflammatory responses and enhance beneficial immune responses to efficiently mediate the DEP-induced inflammation.

Both studies showed that air pollutants alter the immune defenses and contribute to lung microbial alterations with an expansion of Proteobacteria. The immunoglobulin profiles discordant between the two studies can be explained by the route and/or duration and composition of air pollutant exposure. Collectively these studies suggest that exposure to air pollutants alter immune responses and/or increase the availability of inflammatory by-products within the lungs that can enable the selective outgrowth of pathogenic bacteria. The observed detrimental outcomes are further exacerbated when coupled with the consumption of an HF diet. Importantly, these results may shed light on the missing link between air pollution-induced inflammation and bacterial expansion and also point to therapeutic alternatives to curb bacterial outgrowth in lung disease exacerbations observed in patient populations living and/or working in heavily polluted regions.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

1.1 Aims of Study

The primary focus of this dissertation is to investigate the effects of traffic generated air pollution mixtures – both gaseous and PM components on the lung's immune and inflammatory responses and to assess the impact on the resident microbial profile alongside high-fat diet consumption. A major consideration for the reader is that there are two different exposure studies and routes of exposure used for both studies detailed in the dissertation. Although the aims of both studies are similar in nature, the observed effects differ widely in the immune response generated. Despite the different routes of immune system activations, it is important to make note that with both exposure studies there are similar alterations in the commensal lung microbiota that is consistently observed. The aims of this dissertation were three-fold:

- Aim 1: Determine whether exposures to mixed vehicle emissions alter lung immunoglobulin levels and microbiota in C57Bl/6 wildtype mice, on either a low or high-fat diet. This Aim investigated the impact of mixed vehicle emissions and diet on immunoglobulins – IgA, IgG, and IgM in lung tissues by ELISA. Additionally, we performed microbiome analysis quantitatively by qPCR, and qualitatively by 16S Illumina sequencing, and assessed if there were alterations in the microbial diversity and richness between control vs. exposed animals.

- Aim 2: Examine whether exposures to diesel exhaust particles affect lung inflammatory responses and microbial profiles in C57Bl/6 wildtype mice, on either a low or high-fat diet. Diesel exhaust particles have been implicated to be among the most noxious elements in air pollution causing systemic inflammatory responses. Hence, we investigated whether diesel exhaust particles alongside diet insults can induce expression of inflammatory markers (TNF-α, IL-1β, TLR-2,

TLR-4, NF-κB) locally within the lungs by histology and RT-qPCR. We also performed morphological examination of lung tissues, assessed the presence of mucus by AB/PAS staining and collagen deposition by Masson's trichrome staining. We also measured the levels of IgA and IgG in the bronchoalveolar lavage fluid by ELISA and analyzed the expression of their respective receptors responsible for their transcytosis (polymeric IgA receptor - pIgR, neonatal Fc receptor - FcRn). Microbiome analysis was performed to identify alterations in major bacterial phyla in exposed vs. control animals by qPCR.

- Aim 3: Determine a potential mechanism causing alterations in the lung microbial profile in response to air-pollutant exposures in C57Bl/6 wildtype mice, on either a low or high-fat diet. This Aim investigated whether the synergistic effects of air pollution and diet cause the generation of ROS and RNS products, which serve as selective nutrients for the outgrowth of Proteobacteria. We also checked for the presence of macrophages that may contribute to the release of ROS and RNS by Immunofluorescence. We also analyzed if probiotic treatment could decrease DEP-induced generation of ROS-RNS and if it could attenuate the observed bacterial dysbiosis and inflammation. The effects of Probiotic treatment on collagen deposition and IgA levels were also investigated.

1.2 Lung Structure and Immune Response

The respiratory system is divided into the upper and lower respiratory tract**.** The upper respiratory tract includes nasal passages, pharynx, and larynx. The lower respiratory tract is composed of the trachea, bronchi, bronchioles, and alveoli. The conducting portion is made up of nasal passages, pharynx, larynx, trachea, bronchi, and bronchioles. The trachea branches into the two main bronchi, which further branch to give rise to the secondary and tertiary bronchi. These then branch even further to several orders of successive smaller airways termed bronchioles. The

terminal bronchioles are the final components of the conducting portion of the lower respiratory system that lead into the respiratory bronchioles that branch out into alveoli (Figure 1.1A) (Paxton et al., 2003). The respiratory portion contains bronchioles, alveolar ducts, alveolar sacs, and alveoli. The lungs are also part of the lower respiratory tract. They are spongy organs that begin at the lower part of the trachea and include bronchioles and alveoli. Lungs are located in the thoracic cavity, enclosed by the ribs and diaphragm.

The epithelial linings within the trachea, bronchi, and bronchioles are covered by cilia and a thin film of mucus that is produced by goblet cells. The mucus layer traps particulates and irritants, and the ciliary movement drives the mucus layer towards the pharynx where it is removed from the respiratory system either by swallowing or expectoration (Figure 1.1B) (Quinton, 2017). Below the epithelial layer is the lamina propria, a thin layer of loose connective tissue that is rich in immune cells.

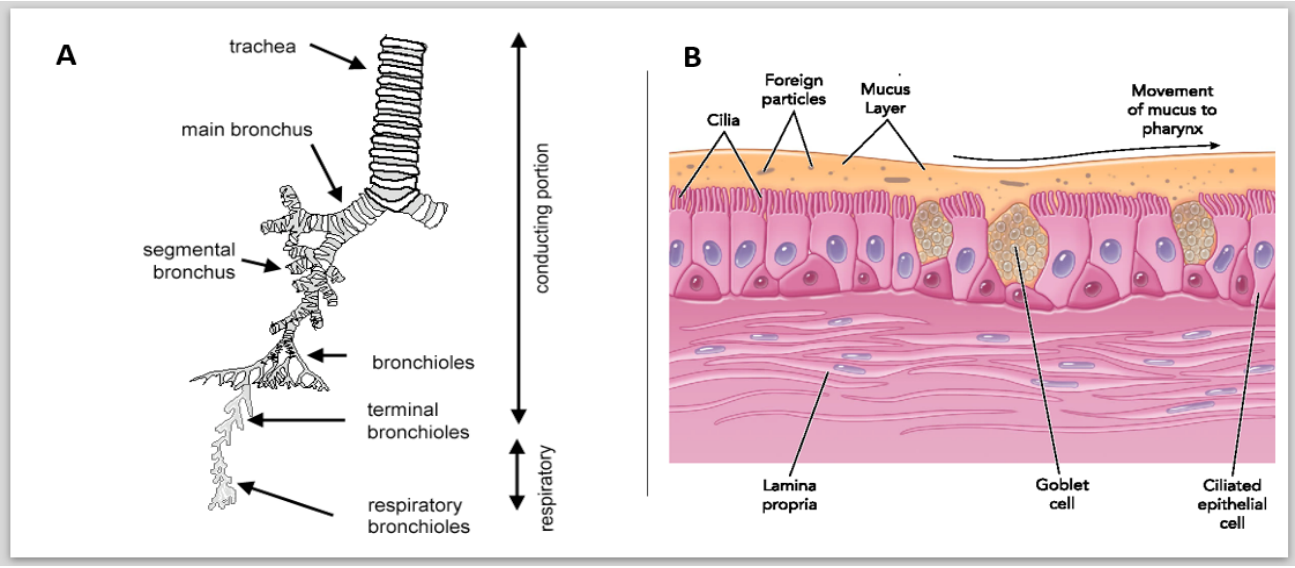

Figure 1.1: Diagram of the airway structure and mucociliary escalator: (A) components of the respiratory system (Paxton et al., 2003). (B) Airway surface ciliated mucus escalator (Quinton, 2017).

The lung's major function is gas exchange that occurs in the alveoli that are tiny air sacs at the tips of the terminal bronchioles. Oxygen in inhaled air entering the alveoli diffuses rapidly

across the epithelium into capillaries, organized in a single layer in the alveolar septum. Capillaries are separated from the air space by a thin layer of epithelial and interstitial components. The alveolar septum consists of Type I and Type II alveolar cells. Type I cells cover a large surface area and represent the gas exchange surface in the alveolus. Type II cells produce surfactant, a mixture of phospholipids and proteins that help reduce surface tension to prevent alveolar collapse during exhalation.

1.2.1 Airway Immune Responses

The airway epithelium represents a physical barrier that forms tight junctions, produces mucus, and maintains an antigen-specific secretory IgA (SIgA) barrier that protects the airway surface from continuous exposure to inhaled particulates and microorganisms. The respiratory epithelium can directly detect pathogens and activate immune signals ("The lungs at the frontlines of immunity," 2015). Lung-resident macrophages and dendritic cells also actively eliminate particulates, allergens, and microbes from inhaled air. Alveolar macrophages are the largest population of leukocytes in steady-state, and they effectively phagocytose and eliminate potential toxins that have evaded the mechanical defenses of the respiratory tract. Dendritic cells are antigen-presenting cells that act as mediators between the innate and adaptive arms of the immune system (Nicod, 2005). Dendritic cells situated in the basolateral surface of the epithelium sample antigens in the airway lumen, as well as travel to lymph nodes to present these antigens to T cells, which then sets the tone of immune responses. In the absence of an infectious organism, these responses alone are usually capable of maintaining a steady-state on the lungs in an anti-inflammatory mode ("The lungs at the frontlines of immunity," 2015). Lymphocytes are scarce in the airway and alveolar lumen, but in the event of infection and some pathologies, lymphocytes are detected in the bronchial submucosa (Pilette et al., 2001). Lymphocytes may also be organized

in bronchus-associated lymphoid tissue (BALT), when abundant. One of the main functions of lymphocytes in BALT is Immunoglobulin A (IgA) production (Chiavolini et al., 2010).

1.2.2 Major Immunoglobulins on the Lungs

IgA is the predominant immunoglobulin in mucosal secretions, and it functions to provide the first line of mucosal defense by neutralizing toxins and maintaining mucosal tolerance with commensals. IgA secreted is dependent on close cooperation between mucosal lymphoid tissue and the airway epithelium that allows the transport of polymeric immunoglobulins from the basolateral side to the apical side through polymeric IgA receptor (pIgR). A portion of pIgR called secretory component (SC) cleaves off as IgA translocates into the lumen. The SC reduces the susceptibility of the IgA molecule to proteolytic digestion (Pilette et al., 2001). Most of the IgA produced is transported via this mechanism into the airway lumen, where they perform their protective functions. SIgA recognizes antigens on the surfaces of bacteria and viruses and causes agglutination of pathogens inhibiting their ability to adhere to the epithelium and thereby colonize mucosal surfaces (Mantis and Forbes, 2010).

Immunoglobulin M (IgM) is the first immunoglobulin produced in the blood in response to an antigen. The pentameric structure and size of the IgM molecule prevent its diffusion into the lungs. IgM producing plasma cells are found to be present in the bronchial mucosa, but it is less frequent than IgG or IgA producing cells. IgM is also transported across the epithelium by pIgR mechanism (Borrok et al., 2018; Burnett, 1986). Immunoglobulin G (IgG) is mainly found in the blood, but plasma cells producing IgG have been located in the bronchial mucosa that is most likely the source of locally produced IgG that has been obtained from bronchoalveolar lavage fluid (BALF). IgG synthesized by plasma cells has been observed to be transported across the epithelium by the FcRn receptor on the bronchial epithelial cells (Spiekermann et al., 2002). Both

IgM and IgG function by neutralizing pathogens by fixing complement and opsonization (Horton and Vidarsson, 2013).

1.3 Effects of Air Pollutants on Lung Immune Responses

The pattern of toxicity of air pollutants depends on their composition and the site of deposition within the respiratory tract. Gases get rapidly diffused and are taken up by the circulation where they can cause systemic inflammatory effects. The size of the PM determines the region of deposition within the respiratory system. Coarse PM that has an aerodynamic diameter of 2.5 – 10 µm gets deposited in the large conducting airways. Fine $PM_{2.5}$ and ultrafine PM <0.1 µm gets deposited in the small airways and alveoli. The compartmental distribution of PM within the upper and lower respiratory tract is detailed in Figure 1.2 (Schwarze et al., 2013). The surface of PM has endotoxins and immunogenic substances such as pollen and fungal spores that can independently cause activation of Toll-like receptors (TLRs) and also cause exacerbation of asthma symptoms (Delfino et al., 1997; Shoenfelt et al., 2009a).

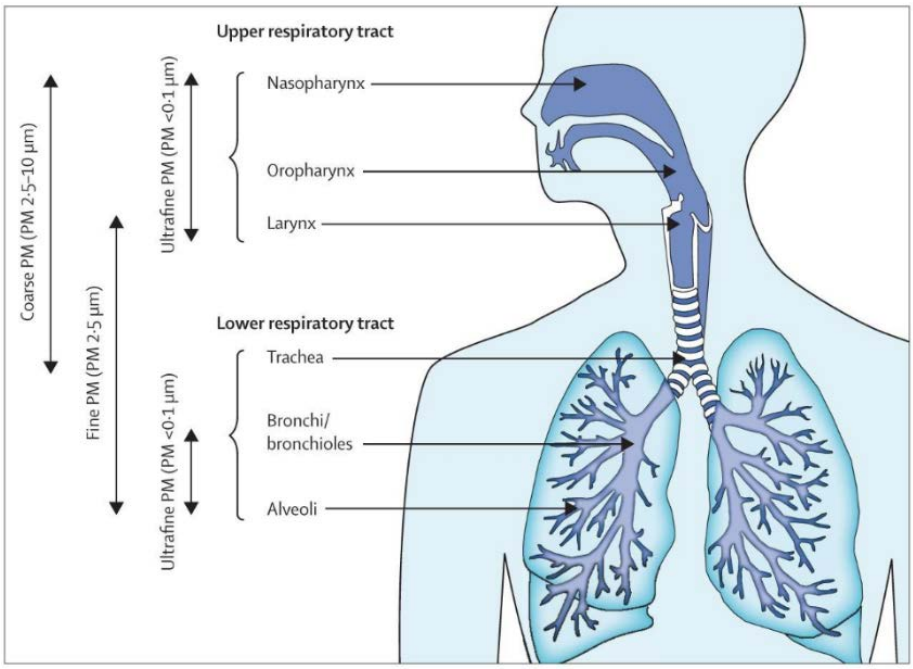

Figure 1.2: Compartmental deposition of particulate matter (PM) within the respiratory tract (Schwarze et al., 2013).

Exposure to air pollutants in humans have documented profound inflammatory changes in the airways (Grunig et al., 2014). Both particulate matter and gaseous pollutants can act on airways to initiate and worsen inflammation. The most common patterns after exposure to air pollutants are neutrophil infiltration and macrophage recruitment in BALF of both healthy and asthmatic individuals (Xu et al., 2013). These neutrophils and macrophages release proinflammatory cytokines and toxic proteases that contribute to epithelial damage (Schwarze et al., 2013). Among the traffic generated pollutants, diesel exhaust has been observed to be one of the main factors contributing to the allergy pandemic. Healthy volunteers exposed to diesel exhaust for only 18 hours had a significant increase in neutrophils and CD4+/CD8+ T cell ratio in the bronchoalveolar portion. They were also found to have reduced phagocytosis by macrophages (Yin et al., 2005). Suppression of alveolar macrophages leading to reduced IL-1β, TNF-α, and ROS production in response to bacterial lipopolysaccharide (LPS) challenge has also been demonstrated in animal models (Yin et al., 2005, Ambient particulate matter exposure was found to increase secretory IgA levels in rat lungs, indicating increased immune activity in response to certain pollutants (Li et al., 2017). A decrease in salivary IgA has also been observed in response to air pollution that predisposes individuals to oral disease (Mehrbani et al., 2016.). Both immune suppression and initiation of pronounced inflammatory pathways have been observed in response to different particulates in air pollution, all of which have severe consequences on the host.

1.4 Air Pollution Exposure-Mediated Alterations in Lung Structure and Function

Various chemicals, including trace metals and polycyclic aromatic hydrocarbons (PAHs), are absorbed onto the fine particulate matter, which translocates into blood circulation after particle deposition in the lungs initiating inflammation and adverse health effects (Gualtieri et al., 2009; Luo et al., 2014). Exposure to PM has been observed to cause morphological changes at terminal

bronchioles and adjacent alveolar sacs with significant infiltration of inflammatory cells in connective tissue (Sancini et al., 2014). After exposure, PM is observed to be engulfed in phagocytic cells along the lung parenchyma and within alveolar macrophages. $PM_{2.5}$ bypasses the mechanical defenses on the lung and reaches the alveoli causing significant damage to the epithelium, which results in swelling of the alveolar walls affecting the air-blood barrier integrity (Sancini et al., 2014).

Ambient PM has also been associated with small airway remodeling, low airflow rates, and impaired lung function. Several studies point to lower lung function in children exposed to air pollutants over time (Adam et al., 2015; Islam et al., 2007). Cumulative prolonged exposure to ambient PM_{10} and ozone was found to be associated with a decline in lung function in the elderly population measured by forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1), which are measurable quantitative parameters of respiratory health (Adam et al., 2015). In the adult population, it was observed that exposure to nitrogen dioxide, $PM_{10,}$ and traffic generated pollutants resulted in a reduced FEV1, which is a reliable marker indicating future morbidity and mortality (Young et al., 2007). Reduced FEV1 is a crucial marker in assessing the risk of COPD, lung cancer, coronary artery disease, thus indicating that exposure to pollutants can have detrimental effects on overall health (Young et al., 2007; Zaigham et al., 2016).

1.5 Air Pollution and Lung Diseases

Air pollution exposures alone result in around 7 million premature deaths in a year, of which 43% are deaths due to COPD, and 26% due to respiratory infection deaths (WHO Global Health Observatory data, 2016). There are close to 380 million people living with COPD, and it is expected to become the leading cause of death in 15 years (Quaderi and Hurst, 2018). Exposure to ambient particulate matter from biomass fuels and mixed vehicle emissions (ME) for 7 months

alone has been shown to induce airway cells to release multiple cytokines capable of inducing pronounced COPD in rat models (He et al., 2017). PM exposure from these sources also caused a reduction in lung function and local and systemic inflammation followed by mucus metaplasia and emphysema (He et al., 2017).

Air pollution is considered a primary risk factor for asthma and COPD, and the incidence of these diseases is higher in heavily polluted regions, suggesting that air pollutants play a crucial role in the development or exacerbation of lung diseases. Exposures to $PM_{2.5}$ are associated with asthma morbidity and mortality (Wagner et al., 2012). PM exposures in asthmatic rats have been shown to increase airway mucus, BALF neutrophils, and eosinophils and induce pronounced airway hyperresponsiveness.

Air pollution exposures have also been implicated in the increased incidence of idiopathic pulmonary fibrosis (IPF). IPF is a progressive and chronic fibrotic lung disease with a median survival of 3-5 years from diagnosis (Caminati and Harari, 2010). Although the mechanism of development of IPF is debatable, studies have shown that exposure to nitrogen dioxide, ozone, and PM is associated with increased mortality and decreased FVC, which suggests that these pollutants play a role in worsening IPF (Sesé et al., 2018). Some of the proposed mechanisms of IPF development include abnormal alveolar re-epithelialization caused by chronic inflammation and oxidant-antioxidant dysregulation (Sesé et al., 2018). Other proposed mechanisms include surfactant abnormalities and stress of the endoplasmic reticulum, all of which contribute to increased deposition of extracellular matrix components (ECM) (Johannson et al., 2015; Korfei et al., 2008).

1.6 Mechanism of Air-Pollution Mediated Toxicity

The generation of ROS and reactive nitrogen species (RNS) by macrophages is a well-

studied mechanism of air-pollution mediated toxicity (Lodovici and Bigagli, 2011). O_2^- produced by ROS and NO_3^- produced by RNS act as effectors against invading pathogens/pollutants, and they are beneficial if the physiological activity of antioxidant defenses balances these mechanisms. Oxidative stress occurs due to increased generation of reactive species or reduced activity of antioxidant defenses (Poljšak and Fink, 2014a). Other proposed mechanisms for development and exacerbation of asthma include inflammatory pathways that enhance the respiratory sensitization to allergens (Gowers et al., 2012). Traffic generated air pollutants, especially diesel-exhaust particles (DEP) have been associated with regulatory T cell (Treg) dysfunction through epigenetic mechanisms (Nadeau et al., 2010). Hypermethylation of CpG islands in *Foxp3*, a specific transcription factor for Treg cells, is found to occur with chronic exposures to DEP (Brunst et al., 2013). This results in the suppression of Treg cells that produce anti-inflammatory mediators. Air pollutants can also cause hypermethylation of interferon (IFN)-γ in T cells that results in shifting towards the Th2 allergic phenotype that possibly plays a role in the observed exacerbations of asthmatic patients (Kohli et al., 2012).

1.7 Lung Microbiota

Lungs were thought to be sterile until recently, mainly due to the inability to cultivate microbes using culture-based methods, low abundance, and the possibility of pharyngeal contamination when using a bronchoscope for sampling. Culture-independent techniques such as next-generation sequencing of the highly conserved 16S ribosomal RNA gene has facilitated the identification of previously uncultivable bacteria (Dickson et al., 2016). We now understand that the lower respiratory tract is replete with microorganisms both in health and diseases. The lung microbiome is highly variable due to the dynamic responses of inhalation, exhalation, mucociliary clearance, etc., regularly occurring within the lungs. Low density and continuous renewal are the

two important characteristics of the lung microbiota. Lung microbial abundance ranges from 10^3 – 10^5 CFU/g of lung tissue, much lower than the GI tract that harbors close to 10^{14} microbes (Mathieu et al., 2018; Remot et al., 2017). Microorganisms enter the lung via microaspiration – they travel from the microbe-filled oral cavity to the lungs in small saliva droplets and land on the carina where the airway branches into right and left lungs (Dickson et al., 2017). From here, some bacteria find their way into alveolar sacs. The lungs do not host a constant microbial community as there is continuous immigration of microbes due to microaspiration, mucosal dispersion, inhalation, and elimination via mucociliary clearance, coughing, and host immune responses. Mucus secretion by club and goblet cells in the bronchi trap inhaled toxins and expels pollutants and microbes through ciliary action and cough. Physiological features of the respiratory tract play a role in the adherence of specific bacterial populations. There are different gradients of pressure and temperature between the upper respiratory tract and the lower respiratory tract, which may affect bacterial colonization. In healthy individuals, a constant balance is maintained between microbial immigration and elimination. When this balance is disturbed, an alteration in lung microbial communities is observed due to the expansion of bacteria with a competitive advantage.

Most of the lung nutrients are obtained from host compounds such as immunoglobulins, cytokines, defensins, lactoferrins, and mucins. Changes in the availability of these metabolites may have a crucial impact on the colonization of selective bacteria (Mathieu et al., 2018). Excessive mucus production causes obstruction in airways in respiratory diseases such as pneumonia, asthma, and cystic fibrosis. The presence of excessive mucus can also cause selection of certain bacteria that can use mucins to produce propionate which can be used by *Pseudomonas aeruginosa*, an opportunistic pathogen (Flynn et al., 2016).

Despite the continuous renewal and replacement of microbial communities on the lung,

most of the microbes involved in these fluxes belong to 4 major phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Mathieu et al., 2018). These four major phyla are identical in both humans and mice. In mice, these four phyla dominate the lung microbiome in all stages of development, starting from 1 week to 8 weeks of life. The dominant genera at 1-2 weeks are *Defluvibacter*, *Lactobacillus*, and *Streptococcus*. Microbial diversity in mice lung increases as the mice grow from a neonate into an adult. The adult lung microbiota of mice has a significant abundance of *Lactobacillus, Streptococcus, Bacillus, Actinobacillus, Acinetobacter*, and *Propionibacterium* (N. Singh et al., 2017). Lung bacterial communities are highly variable among mice, and there is currently no consensus on the definition of a typical microbiota, which is present in a state of homeostasis with host cells. It is also unclear if certain bacteria serve as markers for good lung health (Dickson et al., 2018; Mendez et al., 2019).

Dysbiosis of the lung microbiome is observed in various pulmonary diseases. Microbiome analysis in COPD patients has revealed significant changes in the relative abundance of bacteria, namely *Haemophilus, Pseudomonas,* and *Moraxella* (Millares et al., 2014). A few studies also point to an increase in bacteria within the Proteobacteria phylum during COPD exacerbations (Huang et al., 2014). Studies in patients with asthma indicate significant alterations in the bacterial compositions with a predominance of the Proteobacteria phylum, specifically *Haemophilus* (Hilty et al., 2010*).* Asthmatic airways are also shown to have a decrease in Bacteroidetes, especially the *Prevotella* species, which is predominant in healthy airways (Marri et al., 2013). In asthma, alterations in the microbiome are observed even in mild cases and appear to be similar to the profiles in severe cases as well. Cystic Fibrosis (CF) is characterized by the development of bronchiectasis and airway obstruction. Exacerbations in CF are associated with infection caused by pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Ramsey, 1996). There

is no evidence linking increased bacterial density in CF, but few studies support a loss of diversity as the disease progresses. This loss in diversity was also found to increase with increasing age and cumulative antibiotic exposure (Cox et al., 2010; Zhao et al., 2012).

1.8 Receptors on the Lungs

The lungs are uniquely positioned to continuously interact with the external environment and the entire blood circulation. Therefore, it has several molecular mechanisms in place to initiate signaling pathways when exposed to foreign antigens. Both resident myeloid and structural cells of the lung express a complete repertoire of Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) (Kovach and Standiford, 2011). The TLR family disseminates the signal to intracellular transcription factors that regulate inflammatory gene expression (Figure 1.3) (Erin I Lafferty et al., 2010). The TLR family consists of 10 distinct members in humans: TLR1 – TLR10 and 12 in mice: TLR 1-9 and TLR 11-13 (Nabar et al., 2018). TLR1, TLR2, TLR4, and TLR5 recognize bacterial ligands such as lipoproteins and lipopolysaccharide, whereas TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids (Takeuchi and Akira, 2010). TLR signaling pathway originates from the cytoplasmic Toll/IL-1 receptor (TIR) domain and interacts with the adaptor protein MyD88. Upon stimulation, MyD88 activates IL-1 receptor-associated kinase by phosphorylation leading to the activation of NF-κB, which regulates gene expression of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6. TLR also causes activation in a MyD88 independent manner, which also results in the activation of NF-κB. Before activation, the NF-κB is bound to inactive κB (IκB) and is retained in an inactive form in the cytoplasm. There are five different NF-κB proteins named C-Rel, RelA (p65), RelB, NF-κB1(p105; precursor of p50) and NF-κB2 (p100; precursor of p52) (Kawai and Akira, 2007). NF-κB/Rel proteins exist as homo or heterodimers and stay bound to IκB in the cytoplasm. NF-κB

proteins regulate the expression of distinct but overlapping genes involved in inflammation. In TLR signaling, the most frequently activated form of NF-κB is the heterodimer with RelA and p50 (Hayden et al., 2006). When TLRs first sense a PAMP, they recruit a combination of Toll/Il-1 receptor (TIR-containing adaptor proteins such as (1) myeloid differentiation primary response gene 88 (MyD88), (2) TIR-containing adaptor protein (TIRAP), (3) TIR-domain-containing adaptor molecule 1 (TRIF) and (4) TIR domain-containing adaptor molecule (TRAM) (Akira and Takeda, 2004).

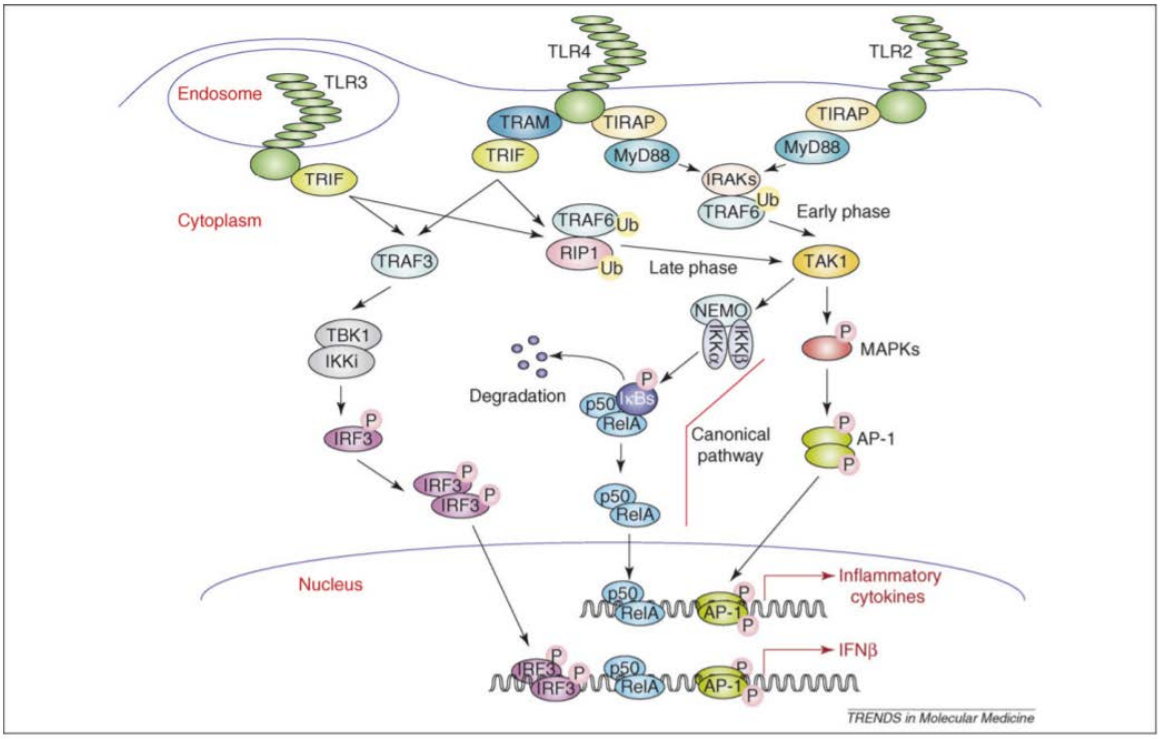

Figure 1.3: Signaling to NF-κB by toll-like receptors (Erin I Lafferty et al., 2010).

TLR signaling is divided into two pathways, such as MyD88- dependent and MyD88 independent or TRIF-dependent. Both these pathways activate NF-κB, but each of these pathways activates an additional pathway that determines its unique effector functions (Kawai and Akira, 2007). The adaptor protein MyD88 interacts with members of the IRAK (IL-1 receptor-associated kinase) family of protein kinases, which results in their phosphorylation and dissociation from

MyD88 and it interacts with TRAF6, which is a ubiquitin ligase that promotes polyubiquitination of target proteins including TRAF6 itself and NEMO ((NF-kB essential modifier) (Adhikari et al., 2007; West et al., 2006). NEMO and TRAF6 recruit protein kinase complex involving TAK1 (transforming growth factor-b-activated kinase-1) which activates two distinct pathways involving the IKK complex (inhibitory κB (IκB) proteins, which is triggered by two kinases, IkB kinase - IKKα and IKKβ and mitogen-activated protein kinase (MAPK) (Kawai and Akira, 2007). IκB is phosphorylated by kinases and ubiquitinated, releasing the P50/RelA heterodimer, which then translocates to the nucleus where it acts as a transcription factor by binding to regulatory DNA sequences.

NF-κB activation in a TRIF dependent manner is used by TLR4 and TLR3. TRIF directly binds to TRAF6, which then activates TAK1 in a similar pattern as the MyD88 dependent pathway. TRIF also recruits TRAF3 which interacts with two other IKK – related kinases TBK1(TRAF family member-associated NF-kB activator (TANK) binding kinase-1 and IKKi. These kinases cause the phosphorylation of interferon regulatory factor (IRF3), which dimerizes and translocates into the nucleus to regulate transcription (Kawai and Akira, 2007).

Increased activation of the NF-κB will result in the continuous production of proinflammatory cytokines that can lead to tissue damage if not controlled once the antigen is mediated. Hence, there is a need for transcriptional regulation. Termination of the NF-κB response involves re-synthesis of IκB that can enter the nucleus and remove NF-κB from the DNA and re-localize it to the cytoplasm. Some studies also point to the degradation of the promoter region bound to RelA, thereby silencing transcription.

1.9 The Gut-Lung Axis

The gut and lungs are both mucosa-lined luminal organs exposed to a wide variety of

foreign antigens and have a robust immune mechanism in place. Migration of microbes in the gut is unidirectional in the absence of gastrointestinal reflux, and it is serially interrupted by chemical and physical barriers. Conversely, the lungs have bi-directional movement of microbes and air without any physical barriers between the larynx and the most distant alveoli. Recent studies indicate a link between gut microbiota and lung immunity. Microbial metabolites produced in the gut are understood to influence lung immunity and lung diseases. Exposure to allergens or pollutants results in elevated immune responses in the lung, as well as immune signaling from the intestinal sites. In the gastrointestinal tract, a combination of signals from the microbes stimulates dendritic cells to activate various subsets of T cells such as Th1, Th17, Tregs within mesenteric lymph nodes (Figure 1.4) (Evsyutina et al., 2017).

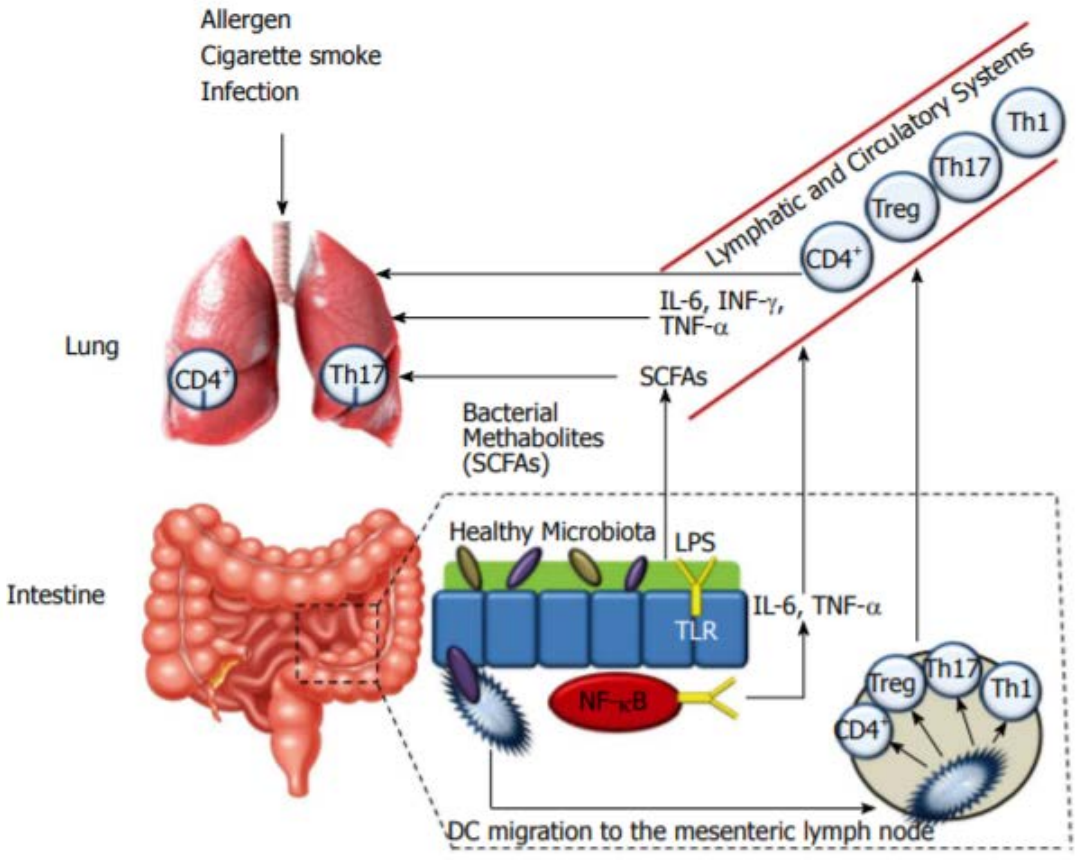

Figure 1.4: Intestinal microbiome effects on lung immunology (Evsyutina et al., 2017).

Following an immune challenge in the airways, T cells activated in the gastrointestinal-associated lymphoid tissue and mesenteric lymph nodes move into the respiratory mucosa, where they aid in airway immune defenses. Bacterial metabolites such as short-chain fatty acids (SCFAs) directly act on epithelial and immune cells and contribute to anti-inflammatory effects. SCFAs can also downregulate TLR stimulation by modulating the activity of NF-κB, thereby reducing the production of proinflammatory cytokines such as TNF-α and IL-6 (Evsyutina et al., 2017). Metabolites such as SCFA move through the bloodstream to affect immune responses on the lung, and different factors such as bacterial products digested by antigen-presenting cells on the lungs reach the mesenteric lymph nodes. Immune cells induced in both these organs move via the lymphatics, leading to the modulation of immune response across both organs (Figure 1.5) (Anand and Mande, 2018).

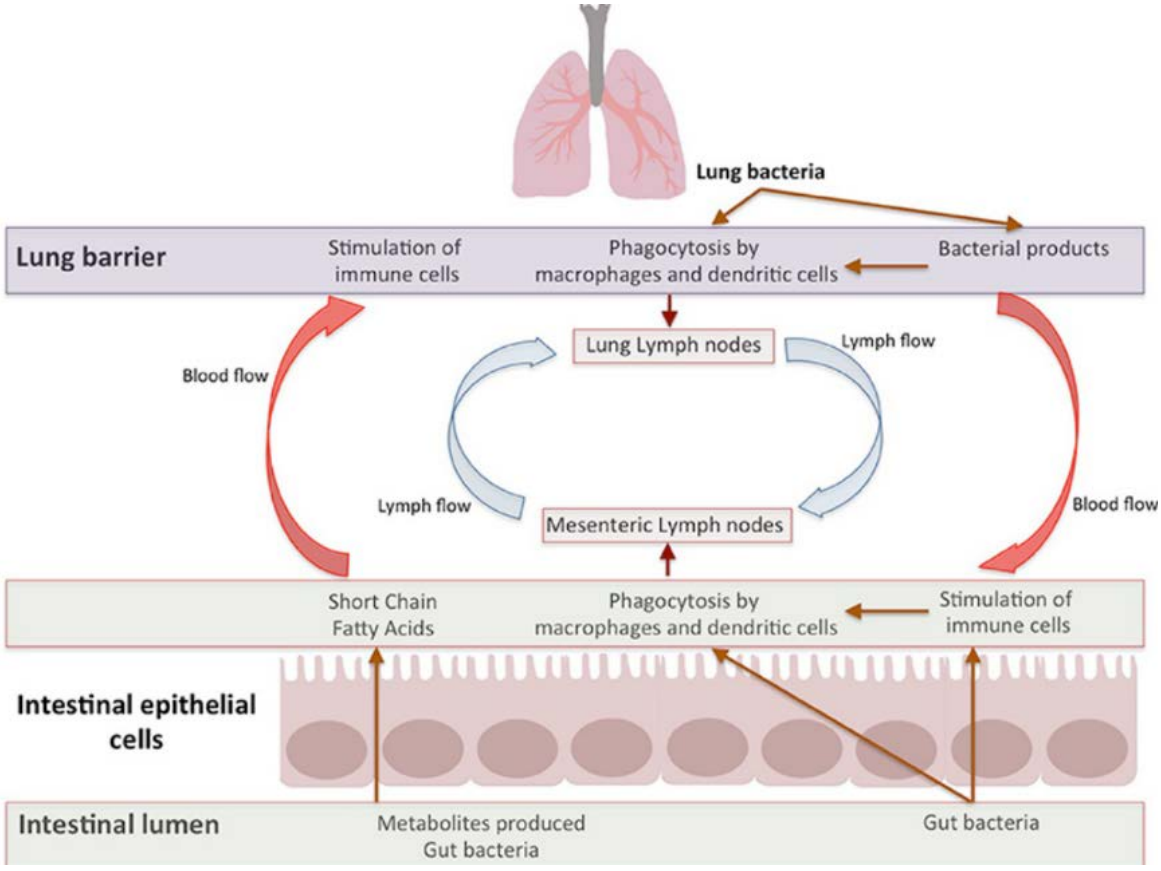

Figure 1.5: Bi-directional crosstalk between the lungs and the gut (Anand and Mande, 2018).

The bi-directional relationship between the gut and the lung is evident in intestinal disturbances that are observed to worsen lung diseases. Antibiotics mediated gut bacterial dysbiosis was associated with the worsening of allergic respiratory inflammation (Russell et al., 2013). Another study showed an increase in the susceptibility to influenza virus in the lungs after selective depletion of bacteria by the administration of neomycin (Looft and Allen, 2012). During respiratory diseases, dysbiosis of both intestinal and lung microbiota has been observed, which is manifested by an outgrowth of Proteobacteria and Firmicutes (Marsland et al., 2015). Progress of asthma in early life is associated with a decrease in *Bifidobacteria* and a subsequent increase in *Clostridia* in the intestine (Kalliomäki et al., 2001). Thus, it is evident that gut microbial disturbances contribute to alterations in lung immune defenses. This agrees with the concept of a "common mucosal response," which states that the gut microbial changes will influence immune responses at distal sites, including lung (McGhee and Fujihashi, 2012; Mestecky, 1987).

1.10 Probiotics in Lung Health

There is increasing evidence demonstrating the impact of diet and nutrition on the microbiome and how a "healthy microbiome" aids in improving overall health. Microbial dysbiosis is often associated with inflammatory diseases, and restoration to the "healthy microbiome" promotes the remission of diseases. Probiotic bacteria strains are used to treat many inflammatory diseases, including antibiotic-associated diarrhea (D'Souza et al., 2002). The use of probiotics in lung diseases is increasing in importance, with studies showing beneficial responses with probiotic intake. The immune response to probiotics is considered dependent on the strains used in the study, which will differ in the metabolites produced (Sharma et al., 2018). Probiotics have been found to differentially regulate the balance between proinflammatory and anti-inflammatory cytokines by maintaining the balance across different T cell responses such as -

Th2/Th1, Tregs, and Th17 (Mortaz et al., 2013). Probiotic treatments have been shown to alleviate allergic asthma, atopic dermatitis, and alleviate food allergies (Feleszko et al., 2007; Hougee et al., 2010). Studies have also shown the beneficial effects of probiotic intake in viral infections where they stimulate immune responses to reduce the duration of influenza and prevent the development of pneumonia (Harata et al., 2010; Morrow et al., 2010).

Few studies point towards improvement in chemotherapy symptoms following cancer treatment. A probiotic study using *Bacillus subtilis* in conjunction with chemotherapy in lung cancer patients showed a decrease in the rate of intestinal dyspepsia as a result of healthier gut microflora (Serkova et al., 2013). Another study showed that the administration of fermented milk to mice with cancer reduced tumor growth with less extravasation of tumor cells and lung metastasis. A decrease in the infiltration of macrophages and an increase in the antitumor response of CD8+ cells were also observed (Morimoto et al., 2005). Probiotic use in both in-vivo and in-vitro studies shows positive effects on cancer cell invasion and metastasis supporting their beneficial effects (Motevaseli et al., 2017). Fewer studies point to the direct effects of probiotics, but it is believed that the *Lactobacillus* and *Lactococcus* bacterial strains exert positive effects on the gut microbiome. Indirect effects of probiotics are thought to be in the modulations of NK cells, which are the major cells responsible for the prevention of uncontrolled cell division. Probiotics are understood to help remediate the effects of treatments by clearing harmful chemicals and heavy metals from the body (Sharma et al., 2018). Also, probiotics increase the activity of certain immune cells, enhancing immune defense against uncontrolled cell proliferation. They also cause an increase in antioxidant defenses and cytokine productions which leads to apoptosis of cancerous cells (Sharma et al., 2018). Probiotics were also found to decrease cancer-specific proteins and pro-carcinogenic enzymes, inhibiting tumor growth (Dasari et al., 2017).

1.11 Gaps in Knowledge

A few emerging studies that show inhaled air pollutants from both anthropogenic and natural sources can induce alterations in the major commensal bacteria present in the GI tract, increasing susceptibility to inflammation (Fitch et al., 2020; Mutlu et al., 2018). Air pollutants' impact on the GI tract is starting to gain attention in recent years due to the increase in the incidence of inflammatory gut diseases in heavily polluted regions. Few studies have also investigated the impact of air pollutants on the lung microbiome. Household air pollutants caused by the combustion of wood, charcoal, biomass fuels have been shown to affect the composition of the lung microbiome to contain higher abundances of pathogenic bacteria (Rylance et al., 2016). Exposure to TiO_2 nanoparticles has also been shown to induce airway hyperresponsiveness and increase inflammatory cells accompanied by alterations in the commensal lung microbial composition (Lee et al., 2019). There are currently no studies that have investigated the effects of traffic generated gaseous and particulate matter components on the lung microbiome. Additionally, diet is an important factor contributing to shaping the microbiome. Consumption of an HF diet has been shown to increase susceptibility to metabolic diseases and contribute to low-grade systemic inflammation (Duan et al., 2018). The average Western diet that consists of > 30% fat is present in much of the human population and contributes to the obesity epidemic. Much of the research focused on understanding the impact of air pollutants have not accounted for the underlying impact of the confounding dietary effects. Hence, with this study, we sought to investigate the hypothesis that exposure to air pollutants alongside a high-fat diet results in inflammatory responses that alters the commensal lung microbial profile that is significant in the development of lung diseases. The aims of this dissertation are to investigate the detrimental outcomes of traffic-generated air pollutants alongside the consumption of high-fat vs. low-fat diets in wildtype C57Bl/6 mice.

CHAPTER 2

TRAFFIC GENERATED EMISSIONS ALTER THE LUNG MICROBIOTA BY PROMOTING THE EXPANSION OF PROTEOBACTERIA IN C57BL/6 MICE PLACED ON A HIGH-FAT DIET

2.1 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 has 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 6h/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 16S sequencing. We observed a significant decrease in lung IgA in the ME-exposed animals on an HF diet, in comparison to the FA-exposed animals on an HF diet. Our results 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 results

in decreased immune surveillance and lung bacterial dysbiosis, which is of significance in lung diseases.

Keywords: Air pollution, lung microbiome, immunoglobulins, Proteobacteria

2.2 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). 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 16S 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 microbes 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, thereby 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), primary because these microbes have unique abilities to thrive in inflammatory environments (G. 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 alterations 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 wild-type 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 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.

2.3 Materials and Methods

2.3.1 Animals and Inhalational Exposure

Male C57Bl/6 mice (3-month-old) were placed either on a standard mouse chow (LF) or a high-fat (HF) diet (TD88137 Custom Research Diet, Harlan Teklad, Madison, WI; 45% kcal fat content by weight, 1.5g/kg cholesterol content) for 30 days prior to exposures. Mice were then exposed to whole-body inhalation to a mixture of gasoline and diesel engine exhaust (ME: 30 µg PM/m^3 gasoline engine emissions + 70 µg PM/m^3 diesel engine) for 6h/d, 7 d/wk, for a period of 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). The control groups were exposed to filtered air (FA) also for the same length of time. Particle size distribution, composition, and mass concentration were determined, as previously described in Suwannasual et al. 2018. The particle mass size distribution had a median of ~1 µm (range: <0.5 −20µm), 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 duration of the exposure protocol. Mice were kept in standard shoebox cages within AAALAC International-approved rodent housing facility (2m^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 hours 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.

2.3.2 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.

2.3.3 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 16S rRNA analysis, samples were normalized to Eubacteria utilizing known-concentration standards. Bacterial primers used are described in Table 2.1.

Table 2.1: Primer sequences used for qPCR analysis.

Bacteria	Sequence
Eubacteria FP	5′-ACTCCTACGGGAGGCAGCAGT-3′
Eubacteria RP	5′-ATTACCGCGGCTGCTGGC-3′
Enterobacteriaceae FP	5′-GTGCCAGCMGCCGCGGTAA-3′
Enterobacteriaceae RP	5′-GCCTCAAGGGCACAACCTCCAAG-3′
Bacteroidetes FP	5′-GGTTCTGAGAGGAGGTCCC-3′
Bacteroidetes RP	5′-GCTGCCTCCCGTAGGAGT-3′
Firmicutes FP	5′-GGAGYATGTGGTTTAATTCGAAGCA-3′
Firmicutes RP	5′-AGCTGACGACAACCATGCAC-3′
Actinobacteria FP	5′-CGCGGCCTATCAGCTTGTTG-3′
Actinobacteria RP	5′-CCGTACTCCCCAGGCGGGG-3′

2.3.4 Illumina MiSeq Sequencing

Genomic DNA was isolated using ZR Fecal DNA miniprep (Zymo Research) from lung homogenates (n=6 per group). 16S 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).

2.3.5 Bioinformatics Microbiota 16S and Statistical Analysis

Sequence reads obtained from the 16S 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, the paired-end reads, that is, the forward and the corresponding reverse reads obtained from paired-end sequencing were combined to form "contigs". Further, these sequence reads are from the V4 region (~ 250-300 bp) of the 16S 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 same sequence multiple times. These processed sequences were then aligned to the reference database, SILVA, containing the 16S rRNA gene sequences. The database was first customized to our regions of interest (V4 regions) using command pcr.seqs; this was performed for improvement of overall alignment. Following the alignment, any sequences that didn't 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

[*] https://rpubs.com/maddieSC/mothur_SOP_May_2018

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 more lower taxonomic level assignments and leaves less 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.

2.3.6 Statistics

Data were analyzed by two way ANOVA with Sidak-Holm multiple comparison test using GraphPad Prism 7. Data are expressed as mean ± SEM and a $p < 0.05$ was considered statistically significant.

2.4 Results

2.4.1 Exposure to ME Alters Immunoglobulin Levels within the Lungs of C57Bl/6 Wildtype Mice

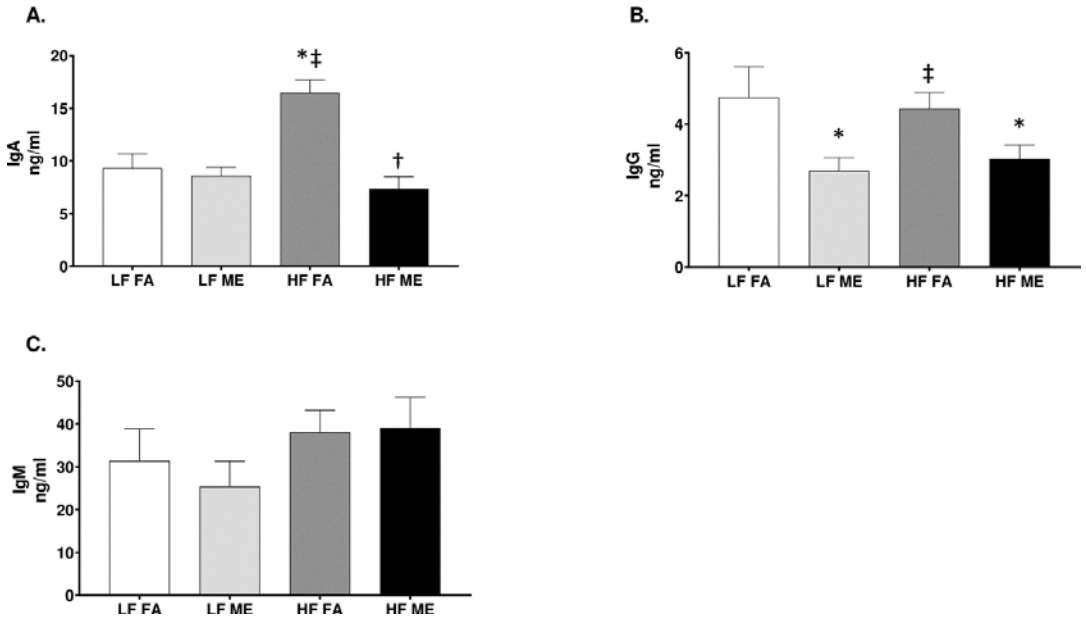

Figure 2.1: Decrease in lung IgG and IgA is observed in mice exposed to ME and HF diet. ELISA of (A) IgA, (B) IgG, and (C) IgM in lung tissue homogenates of C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body inhalation to a mixture of gasoline and diesel engine exhaust (ME: 30 µg PM/m3 gasoline engine emissions + 70 µg PM/m3 diesel engine) for 6h/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.

IgA is the predominant immunoglobulin in mucosal secretions that neutralizes antigens by immune exclusion (Stokes et al., 1975). IgG and IgM are also locally present within the lungs, and

they aid the exclusion of invading antigens by fixing complement (Pilette et al., 2001). 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. 2.1A, $p<0.001$). Interestingly, when compared to the HF FA, we observed a sharp decrease in IgA in the HF ME group (Fig. 2.1A, $p<0.001$). 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. 2.1B, $p = 0.023$, $F = 9.140$ for exposure), compared to the LF FA group. IgM levels were found to be unaltered across all groups (Fig. 2.1C).

2.4.2 Exposure to ME Results in an Increased Abundance of Proteobacteria in C57Bl/6 Mice on the HF Diet

The bacterial load on the lungs is considerably lower when compared to the gastrointestinal tract owing to the sparse availability of nutrients within the lungs. Bronchoalveolar lavage has been shown to contain bacterial loads that range from 4.5 to 8.25 log copies/ml, but these vary widely due to the dynamic responses within the lungs (L. Wang et al., 2017). 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. 2.2A).

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. 2.2B, C). Interestingly, we observed that the abundance of Proteobacteria was significantly elevated only in the HF ME groups (Fig. 2.2D, $p = 0.031$). 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. 2.2E).

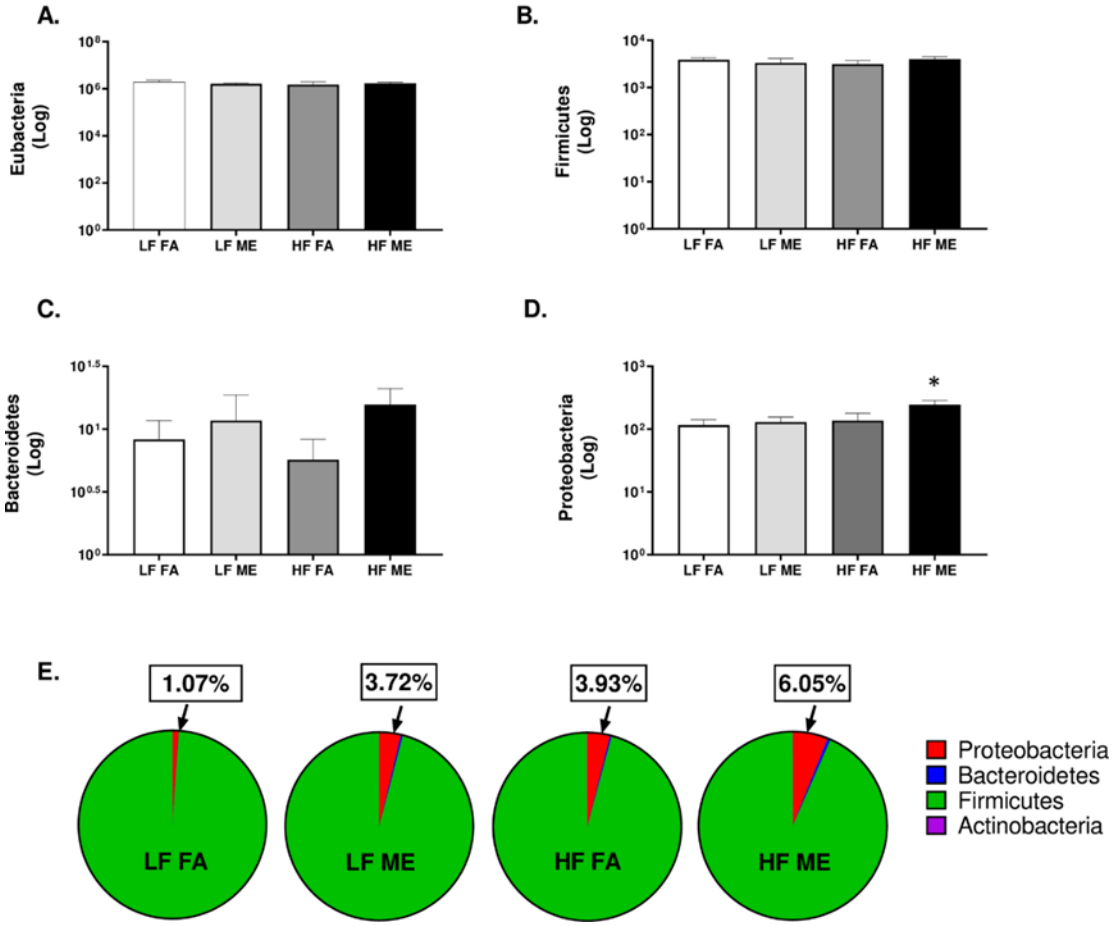

Figure 2.2: Exposure to ME results in increase in the abundance of Proteobacteria. qPCR of lung tissue homogenates for (A) total bacteria (Eubacteria) and phyla - (B) Firmicutes, (C) Bacteroidetes, (D) Proteobacteria, (E) pie charts representing all major phyla in C57Bl/6 mice placed either on regular 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 6h/d, 7 d/wk for a period of 30 days. *p<0.05 compared to LF FA by two way ANOVA.

2.4.3 Exposure to ME Results in 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 16S rRNA region. We observed a similar increase in the Proteobacteria phylum in the HF ME groups alone (Fig. 2.3A). Although we observed

Proteobacteria in the LF ME groups, they had very low reads and were removed during normalization of this dataset. Further classification revealed that most of the bacteria within the Proteobacteria phylum belonged to the Enterobacteriaceae family (Fig. 2.3B, 3C). Interestingly, we observed that *Lactobacillus* predominantly present in both the LF and HF control groups was absent in the ME exposed groups (Fig. 2.3B, 3D). We found 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. 2.3D). 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. 2.3C).

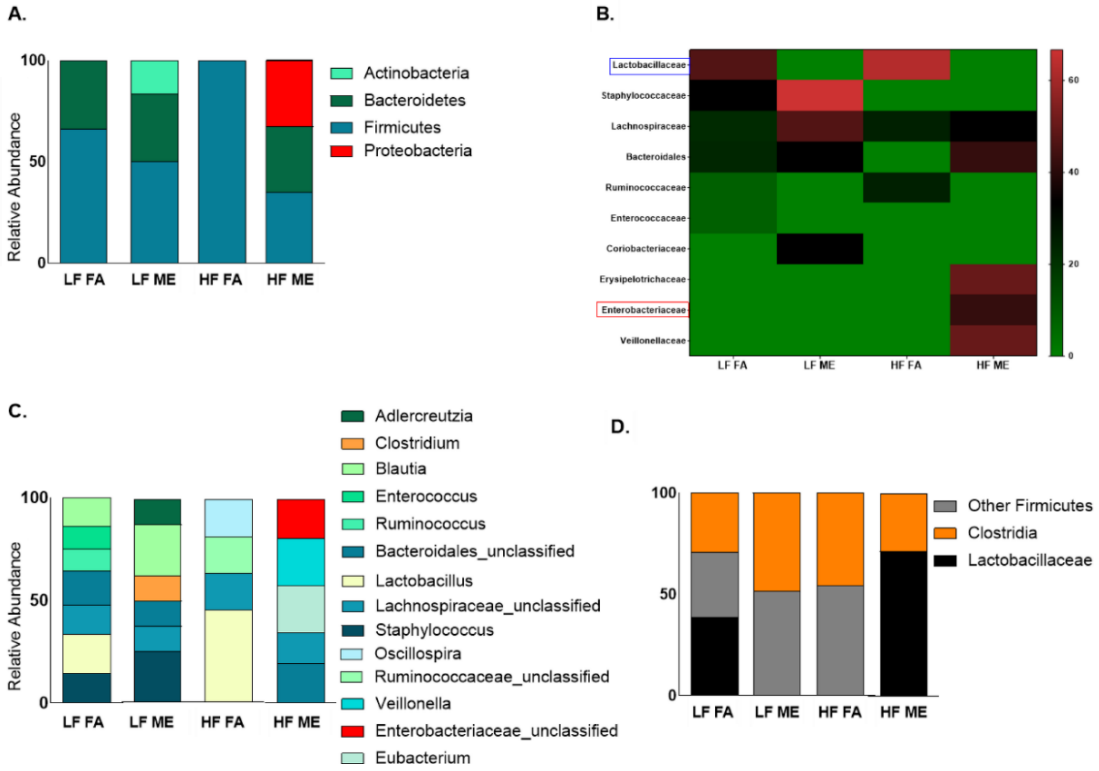

Figure 2.3: Exposure to ME and HF diet results in an increase in the relative abundance of Enterobacteriaceae belonging to the Proteobacteria phylum. 16S Illumina sequencing of the lung bacterial DNA at the (A) phylum and (C) genus level, (B) heatmap showing the relative abundance at the family level 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 6h/d, 7 d/wk for a period of 30 days.

2.4.4 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 calculated using the Shannon (diversity) index (Fig. 2.4A) and Chao1 (richness) index (Fig. 2.4B), 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 microbes.

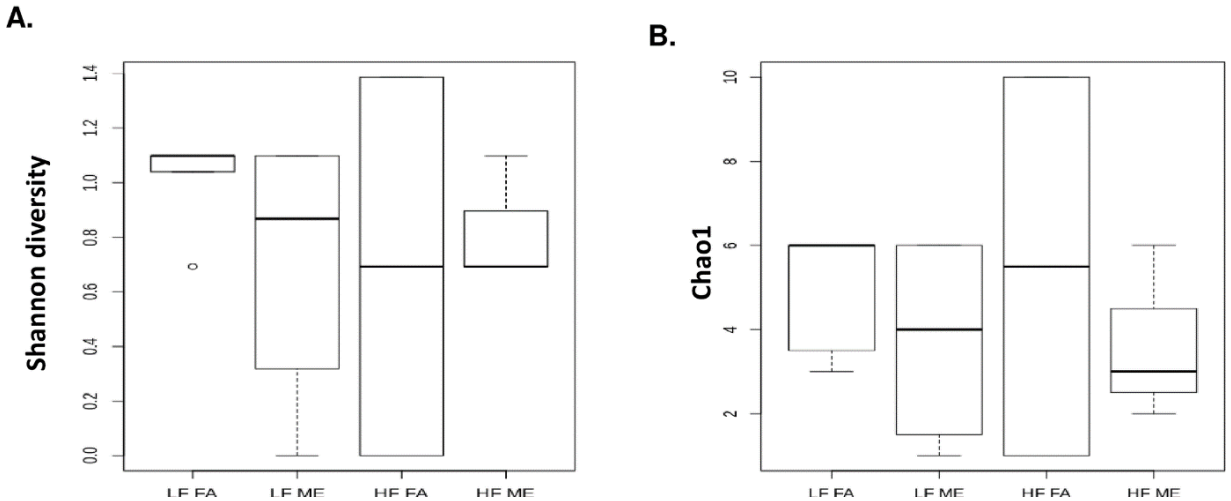

Figure 2.4: Bacterial alpha diversity analysis of exposure and diet groups. Alpha diversity analysis using (A) Shannon index (diversity) and (B) Chao1 index (richness) compared in C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body 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 6h/d, 7 d/wk for a period of 30 days.

We observed significant differences in β-diversity in lung microbiota profiles in the exposed and control groups. Fig. 2.5A is an unweighted PCoA plot constructed using unweighted UniFrac distances representing the total variance in bacteria. Each dot is representative 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 microbes within the lung. Despite these challenges, we still observe a clear separation between the ME exposed and FA control groups. Both the unweighted and weighted PCoA plot shows LF FA and HF FA samples are

dispersed away from the LF ME and HF ME groups (Fig. 2.5A, 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 2.2). A significant variation was also observed between the control groups on both diets (Table 2.2, p = 0.014).

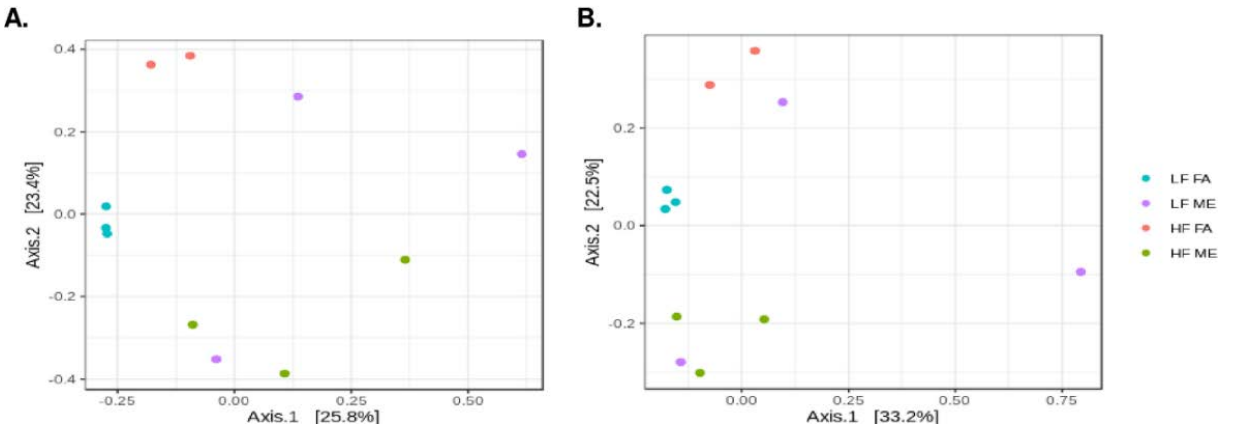

Figure 2.5: Bacterial diversity is reduced with ME exposures. β-diversity calculations using (A) unweighted and (B) weighted analyses. Each circle is representative of one animal in each of the groups of C57Bl/6 mice placed either on regular chow (LF) or a high-fat (HF) diet and exposed to either filtered 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 6h/d, 7 d/wk for a period of 30 days.

Table 2.2: 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		

Exposure Groups	Sum Sq	Df	Mean Sq	P- value
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

2.5 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; G. 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 a degradation in the major immunoglobulins IgA and IgG that play crucial roles in protecting the airways. 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). We suspect a decrease in IgA in the exposed groups on the HF diet would be associated with decreased immune surveillance leading to an unwarranted bacterial outgrowth of Proteobacteria. The decrease in IgA itself can 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). IgA, however, 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). We suspect a gut-lung axis mediated response that causes IgA to significantly increase 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. One such study with ambient particulate matter exposure was shown to increase secretory IgA levels but decrease IgG (Li et al., 2017). A decrease in salivary IgA has also been observed in response to smoke and lead

(Mehrbani et al., 2016). These discrepancies observed between different studies can be explained by 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 ; ,Lodovici and Bigagli, 2011). Although inflammation was traditionally understood to expel potential antigens, recent evidence suggests that certain 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. Baseline inflammation is possibly much higher in the HF ME groups as the HF diet alone can contribute to an increase in 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, and a possible gut-lung interplay may be contributing to an increased predominance of Firmicutes within the HF FA groups (Zhang and Yang, 2016). Beneficial microbes 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 to have pathogenic outcomes in several lung diseases (Carrabba et al., 2012; 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 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, which is a family that contains a large group of pathogenic bacteria implicated in several 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 activation of 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 could contribute to worsening lung diseases; however, there are a few limitations to note. 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 16S sequencing that affected our n-value, but 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.

2.6 Conclusion

In summary, our study demonstrates that exposure to traffic-generated air pollution can affect immune defenses and alter the lung microbial profile, which is exacerbated by concurrent consumption of an HF diet. 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 data, 2016). Understanding the lung microbiota shifts in response to the environment and/or 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.

2.7 Acknowledgments

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2.8 Conflict of Interest

Funding from a grant received from the National Institute of Environmental Health Sciences at National Institute of Health was used to conduct some of the studies described, herein; however, the authors declare no conflict of interest or financial gains to these entities associated with this publication.

2.9 Funding

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CHAPTER 3

EXPOSURE TO DIESEL EXHAUST PARTICLES RESULTS IN A ROS-RNS MEDIATED LUNG BACTERIAL DYSBIOSIS IN WILDTYPE MICE ON A HIGH-FAT DIET

3.1 Abstract

Traffic generated emissions have been associated with the development and exacerbation of inflammatory lung disorders such as chronic obstructive pulmonary disorder (COPD) and idiopathic pulmonary fibrosis (IPF). Although most of these lung diseases show an expansion of Proteobacteria, the role of traffic-generated particulate pollutants on microbial lung dysbiosis has not been well-characterized. Thus, we investigated the hypothesis that exposure to diesel exhaust particles (DEP) can alter commensal lung microbiota, thereby promoting alterations in the lung's immune and inflammatory responses. We aimed to understand whether diet might also contribute to the alteration of the commensal lung microbiome, either alone or related to exposure, and thus used male C57Bl/6 mice (4-6-week-old) on either regular chow (LF) or HF diet (45% kcal fat), randomly assigned to be exposed via oropharyngeal aspiration to 35µg DEP, suspended in 35µl 0.9% sterile saline or sterile saline only (control) twice a week for 30 days. A separate group of study animals on the HF diet were concurrently treated with 0.3 g/day of Winclove Ecologic® Barrier probiotics in their drinking water throughout the study. Our results show that DEP-exposure increases lung tumor necrosis factor (TNF)-α, interleukin (IL)-10, Toll-like receptor (TLR) 2, 4 and the nuclear factor kappa B (NF-κB) histologically and by RT-qPCR, as well as Immunoglobulin A (IgA) and Immunoglobulin G (IgG) in the bronchoalveolar lavage fluid (BALF), as quantified by ELISA. We also observed an increase in macrophages and ROS-RNS by immunofluorescence staining. Histological examinations revealed enhanced inflammation and collagen deposition. We observed an expansion of Proteobacteria in the DEP-exposed group on

the HF diet, which was diminished with probiotic intake by qPCR of bacterial 16S rRNA genes in BALF. Our findings suggest that exposure to DEP with the consumption of HF diet results in bacterial dysbiosis in the lung, associated with persistent and sustained inflammation, which is known to contribute to the development of lung diseases.

3.2 Introduction

The lungs are among the most extensive interface within the human body that are first exposed to particulates within inhaled air. Several studies have described the toxicological implications of particulates within the lungs leading to pronounced inflammation and reduced lung function (Bevan et al., 2018; Gerlofs-Nijland et al., 2007; Paulin and Hansel, 2016; Riediker et al., 2019). There is increasing evidence suggesting that exposure to air pollutants is a leading cause of many observed lung disorders such as asthma, chronic obstructive pulmonary disorder (COPD), bronchitis, and idiopathic pulmonary fibrosis (IPF) (Doiron et al., 2019; Guarnieri and Balmes, 2014; Johannson et al., 2014). The incidence of these diseases is also higher in polluted regions suggesting that air pollutants play a crucial role in either development or exacerbation of underlying lung conditions. Reactive oxygen species (ROS) produced by macrophages have been reported to be an essential driving factor in the air-pollutant mediated inflammatory response observed within the lungs (Lodovici and Bigagli, 2011). Exposure-induced inflammation is often characterized by the expression of several inflammatory markers, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 (Dobreva et al., 2015; Pope et al., 2016). Air pollutants have also been shown to induce Toll-like receptor 2 (TLR2) expression and subsequent activation of the nuclear factor kappa B (NF-κB) transcription factor that accelerates the inflammatory response (Bauer et al., 2012).

Although there has been extensive research in the observed inflammatory response

associated with air pollutant exposures, the involvement of the lung microbial communities has not yet been well-characterized. With emerging evidence on the presence of a commensal lung microbiome, it is becoming evident that these microbes are not mere bystanders, but they play a role in maintaining homeostasis, and their alterations may have detrimental outcomes in lung health.

The gut microbiome is associated with a wide variety of homeostatic functions, including nutrient absorption and immune system development (Jandhyala et al., 2015; Tibbs et al., n.d.). Alterations in the human gut microbiota have been associated with many gastrointestinal diseases including inflammatory bowel disease (Baker et al., 2009). On the other hand, the lung microbiome has not been explored extensively, nor are their functions in health understood. The lungs were always known to have bacterial colonization in diseases, but the discovery of the lung microbiome was delayed due to the long-held view that the lungs of healthy individuals were sterile (Dickson and Huffnagle, 2015). These misconceptions were mainly due to sampling difficulties of the low abundant lung microbiome, but advances in sequencing techniques have revealed that the lower respiratory tract is replete with a wide variety of microorganisms - both in health and diseases (Kumpitsch et al., 2019). Few studies show that these microbes help prime lung dendritic cells induce the production of Immunoglobulin A (IgA), the predominant antibody at mucosal surfaces (Ruane et al., 2016). Germ-free mice were found to have a reduced ability for IgA production, predisposing them to irritants and microbial challenges. These mice were also found to have reduced mucus production, thereby limiting their mucociliary defense functions (Yun et al., 2014).

The commensal flora arrives within the lungs from the oral cavity via microaspiration, (Huffnagle et al., 2017) but the lungs do not host a constant microbial community due to

continuous immigration and elimination as a result of host immune defenses (Dickson and Huffnagle, 2015). Despite these fluxes observed, most bacteria within the lungs belong to four major phyla – Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Mathieu et al., 2018). Unlike the gastrointestinal tract, nutrient availability within the lungs is sparse - mostly obtained from immunoglobulins, cytokines, and mucins. This decreased nutrient availability in the lung likely accounts for the low bacterial abundance ranging from 10^4 to 10^8 when compared to the GI tract that harbors close to 10^{12} microbes (L. Wang et al., 2017). Changes in the availability of these nutrients have a crucial impact on lung microbes. In many pulmonary disease states, there is an increase in mucus production, which favors the growth of certain opportunistic bacteria that can metabolize mucins and proliferate, especially bacteria belonging to the Proteobacteria phylum (Flynn et al., 2016). Proteobacteria are found to be present in higher amounts in many inflammatory diseases, mainly due to their unique abilities to utilize the byproducts of inflammation to proliferate (Gianenrico Rizzatti et al., 2017).

The microbiome is influenced by several factors, including diet and environmental exposures (Claus et al., 2016; R. K. Singh et al., 2017). A large percentage of the Western world population consumes a diet rich in fats (typically>30% fat) which has resulted in the epidemic of obesity (Hurt et al., 2010). Consumption of a high-fat (HF) diet has been shown to alter the intestinal microflora and increase baseline inflammation (Duan et al., 2018). Although the gut and the lungs are distinct anatomical sites, recent studies show evidence of a complex gut-lung cross-talk involving effector molecules that protect from environmental stimuli and infections at mucosal surfaces (Enaud et al., 2020). Dietary supplementation with probiotics has been shown to exert a wide range of beneficial effects with respect to reduced infections and attenuation of disease duration (Morimoto et al., 2005; Szajewska et al., 2013). Emerging studies show that probiotics

exert pleiotropic effects – protective roles in the GI tract by competing with local pathogens, and indirect effects by changing the host immune system to noninflammatory tolerogenic patterns by inducing IL-10 producing T regulatory (T_{reg}) cells (Oelschlaeger, 2010). The immunomodulatory effects of probiotics extend systemically and at distant mucosal sites including the lungs, as observed by improvements in allergic airway diseases (Zajac et al., 2015).

To date, the synergistic effects of both environmental pollutants and HF diet consumption on the lung microbiota profiles and inflammation have not been explored. Understanding the interactions between environmental exposures, diet, microbiome, and the immune system are significant in the development of therapeutic alternatives in diseases. Although there has been significant interest in probiotic influences in disease states, there are fewer studies that have investigated the effects of probiotics on air-pollution induced inflammation. Considering this gap in knowledge, we investigated whether exposure to diesel exhaust particles (DEP) results in inflammation and lung microbial dysbiosis in wild-type C57Bl/6 mice on either a standard mouse chow vs. HF diet.

3.3 Materials and Methods

3.3.1 Animals and Inhalational Exposure

Four-to-six week-old male C57Bl/6 mice from Taconic, C57BL/NTac were placed on either a control (LF) diet with matched sucrose levels containing 10 % fat, 70% carbohydrate and 20% protein (Research Diets # D124505H, n=24) or a high-fat (HF) diet containing 45% fat, 35% carbohydrates and 20% protein (Research Diets #D12451, n=24). A separate set of mice (n =24) were placed on the HF diet and treated with a dose of 0.3 g/day (~7.5 $x10^8$ cfu/day) of Ecologic® Barrier probiotics (Winclove Probiotics, Amsterdam, Netherlands) in the drinking water over the course of the exposures. This probiotic blend includes a mixture of *Bifidobacterium*,

Lactobacillus, and *Lactococcus strains*. Doses of probiotic were monitored through the use of low-drip metered water bottles, and concentrations adjusted as necessary, to maintain consistent dosing throughout the exposure period. Mice were randomly assigned to be exposed via oropharyngeal aspiration (OA) to 35µg diesel exhaust particles (DEP, n=36), obtained from NIST (Standard Reference Material #2975), suspended in 35µl 0.9% sterile saline or sterile saline only (control, n=36) twice a week for 30 days. For OA, mice were lightly anesthetized with 2% Isoflurane (Butler Schein Animal Health) and suspended at an approximately 60-degree angle on a surgery board (rodent intubation stand; Biolite RIS 100). 35 µl of either DEP suspended in sterile saline, or sterile saline only (vehicle), was pipetted into the oropharynx with a micropipette while the tongue was gently pulled forward with forceps to open the airway. The nostrils were then covered gently to induce aspiration into the lungs. Mice were monitored for any signs of distress during recovery from anesthesia. Mice were housed in standard shoebox caging and maintained at a constant temperature and humidity. Mice had access to chow and water *ad libitum* throughout the study period. All procedures were approved by the University of North Texas Institutional Animal Care and Use Committee (IACUC) and adhere 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).

The concentration for DEP exposure chosen was higher than what would be expected for daily environmental human exposure. This specific concentration for DEP exposure was chosen because it is similar to the concentration of total particulate matter (PM) used in preliminary analysis containing mixtures of whole vehicle exhaust (PM + gases) (Suwannasual et al., 2019). OA was chosen as the preferred route for exposure for these studies since it stimulates the route PM would take from the oropharynx region into the lungs and ensures homogenous delivery to the respiratory system.

At the end of the study, mice were anesthetized with Euthasol® and euthanized by exsanguination within 24 hours of the final exposure. The lungs were collected from all study animals, snap-frozen, and stored at -80°C for RNA analysis. Lung tissues were immediately harvested and prepared for histochemical, immunofluorescent, and protein examination of proposed endpoints. Bronchoalveolar lavage fluid (BALF) was collected by flushing the trachea with 2 ml of sterile saline. BALF was centrifuged at 200g for 5 min at 4 °C and the supernatant (1ml) was collected and stored at −80 °C for protein analysis. For microbiome analysis, BALF was again centrifuged at 15,000 *g* for 15min at 4 °C and the pellet was stored at −80 °C.

The nomenclature used are as follows: (a) LF Control: C57Bl/6 mice placed on LF diet and given sterile saline, (b) LF DEP: C57Bl/6 mice placed on LF diet and exposed to DEP, (c) HF Control: C57Bl/6 mice placed on HF diet and given sterile saline, (d) HF DEP: C57Bl/6 mice placed on HF diet and exposed to DEP, (e) HF Control – Probiotics: C57Bl/6 mice placed on HF diet, treated with probiotics and given sterile saline, (f) HF DEP – Probiotics: C57Bl/6 mice placed on HF diet, treated with probiotics and exposed to DEP.

3.3.2 Blood Differential Count

Tail vein blood was collected on day five after DEP exposures for a differential count. The blood sample was smeared on a microscopic slide and air-dried following fixation in methanol. Giemsa staining was done using automated slide Stainer (Hematek 3000 Siemens). Blood differential counts were performed as described in ref (Bjorner and Zhu, 2019).

3.3.3 Histology

For all histological endpoints in lung tissues, the whole lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm. Tissues were cleared with HistoChoice Clearing Agent three times for 10 min at RT, rehydrated, and stained. A minimum of

3-4 locations on each section (4 sections per slide), 3 slides, and n=3 per group were used for analysis; All histology sections were imaged with bright field illumination at 20x.

Alcian Blue/PAS staining: Overall mucus production was analyzed by staining with a combination of Alcian-blue/periodic acid-Schiff (AB/PAS) stain as per manufacturer protocols (Fisher Scientific #88043, #88016). The combination of Alcian blue and the PAS stain was used to quantify both neutral (magenta) and acidic (deep blue) mucins. Cells containing both neutral and acidic mucins stained purple. These slides were then scored by a blinded participant on a 15 point scale. The intensity of the colors magenta, deep blue, and purple were each quantified on a scale of 1-5 and the scores were added resulting in a total scoring range of 0 to 15.

H&E staining: Morphological analysis of lung tissue sections were done by H & E staining (American Mastertech stain kits, #KTHNEPT), following manufacturer recommendations. Histological changes were analyzed by a blinded participant using a 4-point scale as follows: No detectable inflammation - 0, bronchioles surrounded by a few inflammatory cells – 1, bronchioles surrounded by a layer one cell deep – 2, bronchioles surrounded by a layer 2-4 cells deep – 3, bronchioles surrounded by a layer more than four cells deep – 4.

Masson’s trichrome staining: Collagen deposition was analyzed by Masson’s trichrome staining, following manufacturer recommendations (Fisher Scientific, #87019). Scoring was performed by using a Cytation5 imaging reader (BioTek), Gen5 software. For Cytation scoring, each tissue section was divided into six quadrants and each quadrant was imaged as a 2x2 montage at 20x in color brightfield mode. Images were analyzed, in the red channel which allows for all fibrotic tissue (blue) to be subtracted from the image resulting in the percentage confluence for the red channel. The percentage of fibrosis was calculated by subtracting red percentage confluence from 100.

3.3.4 Immunofluorescence Analysis of the Lungs

Immunofluorescent staining was used to quantify TNF-α, IL-10, nitrotyrosine, MOMA-2, and NF-κB p65 proteins using techniques previously described by our laboratory (Lund et al., 2011). Tissues were stained using the following primary antibodies: TNF-α (1:250; Abcam, ab6671), IL-10 (1:200; Santa Cruz Biotechnology SC-365858), nitrotyrosine (1:200; Santa Cruz Biotechnology, SC-32757) MOMA-2 (1:500, Abcam ab33451), and NF-κB p65 (1:500, Abcam, ab86299). Secondary antibodies used were anti-rabbit Alexa Fluor 555, anti-mouse Alexa Fluor 546, anti-mouse Alexa Fluor 555, anti-rabbit Alexa Fluor Plus 488, and anti-rat Alexa Fluor 488. Slides were imaged under fluorescent microscopy at 40x with the appropriate excitation/emission filter, digitally recorded, RGB overlay signals were split and analyzed for specific fluorescence using image densitometry with Image J software (NIH). A minimum of 3-4 locations on each section (4 sections per slide), 3 slides, and n=3 per group were used for analysis; 40x images were used for image quantification.

3.3.5 Real Time RT-qPCR

Total RNA was isolated from the lungs (n=6 per group) using an RNAEasy Mini kit (Qiagen, Valencia, CA) per kit instructions, and cDNA was synthesized using an iScript cDNA Synthesis kit (Biorad, Hercules, CA; Cat. #170–8891). Real-time PCR analysis of markers of inflammation TNF-α, IL-10, pIgR, FcRn, TLR2, TLR4, NF-κB p65, IL-1β, IL-6 IL-17, as well as mucin genes Muc5b, Muc5ac, Muc4 was conducted using specific primers (Table 3.1) and SYBR green detection (Sso Advanced Universal SYBR Green Supermix, Biorad; Cat #172–5271), following manufacturer's protocol. Samples were processed on a Biorad CFX96, and $\Delta\Delta C_T$ values calculated and normalized (to GAPDH), as previously described by our laboratory (Lund et al., 2011, 2009).

Table 3.1: Primer sequences used for RT-qPCR analysis.

	Sequence
Muc5b FP	5′-CTGGCACCTGCTCTGTGCA-3′
Muc5b RP	5′-CACTGCTTTGAGGCAGTTCT-3′
Muc5ac FP	5′-ACGACACTTTTCAGTACCAATGAC-3′
Muc5ac RP	5′-GCTTCCTTACAGATGCAGTCCT-3′
pIgR FP	5′-AGTAACCGAGGCCTGTCCT-3′
pIgR RP	5′-GTCACTCGGCAACTCAGGA-3′
TNF-α FP	5′-CCACCACGCTCTTCTGTCTAC-3′
TNF-α RP	5′-TGGGCTACAGGCTTGTCACT-3′
NF-κB p65 FP	5′-CTTCCTCAGCCATGGTACCTCT-3′
NF-κB p65 RP	5′-CAAGTCTTCATCAGCATCAAACTG-3′
IL-1β FP	5′-CCTTCCAGGATGAGGACATGA-3′
IL-1β RP	5′-TGAGTCACAGAGGATGG-GCTC-3′
IL-6 FP	5′-CCGGAGAGGAGACTTCACAG-3′
IL-6 RP	5′-GGAAATTGGGGTAGGAAGGA-3′
IL-10 FP	5′-ATAACTGCACCCACTTCCCA-3′
IL-10 RP	5′-GGGCATCACTTCTACCAGGT-3′
TLR-2 FP	5′-GCCACCATTTCCACGGACT-3′
TLR-2 RP	5′-GGCTTCCTCTTGGCCTGG-3′
TLR-4 FP	5′-TTTATTCAGAGCCGTTGGTG-3′
TLR-4 RP	5′-CAGAGGATTGTCCTCCCATT-3′

3.3.6 DNA Isolation and qPCR

DNA from BALF and lung tissues were extracted using ZR Fecal DNA miniprep (Zymo Research). For bacterial 16S rRNA analysis, samples were normalized to Eubacteria utilizing known-concentration standards. Bacterial primers used are described in ref (Babu et al., 2018).

3.3.7 IgA and IgG ELISA

IgA and IgG concentrations were measured using IgA (Fisher Scientific #EMIGA) and IgG (Fisher Scientific #88-50400-22) in BALF following the manufacturer's recommended protocol. ELISA was read on a Cytation5 plate reader at 450 nm absorbance. 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.

3.3.8 Statistical Analysis

Data were analyzed by two way ANOVA with Sidak-Holm multiple comparison all-pairwise test using GraphPad Prism 8. Data are expressed as mean ± SEM and a $p < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Exposure to DEP Results in Systemic and Peri-Bronchial Inflammation, which is More Pronounced with the Consumption of the HF Diet in C57Bl/6 Wildtype Mice

Consumption of an HF diet has been shown to not only increase susceptibility to metabolic diseases but also contribute to low-grade systemic inflammation (Duan et al., 2018). To investigate whether subacute exposure to inhaled DEP resulted in acute inflammation systemically, we quantified total white blood cells and performed differential blood counts after 5 days of DEP exposures. We observed that the total white blood cell counts were significantly higher in the HF DEP group when compared to the LF Control ($p = 0.001$), LF DEP ($p = 0.030$), and HF Control ($p = 0.029$) groups (Fig 3.1A). The respective F values for the total white blood cell counts are: exposure = 7.539, diet = 7.402, exposure x diet interaction = 0.351. The differential counts for neutrophils were significantly higher in the HF DEP group when compared to LF Control, LF

DEP, and HF Control ($p < 0.001$) with F value of exposure = 17.490, diet = 8.503, exposure x diet interaction = 8.745 (Fig. 3.1B).

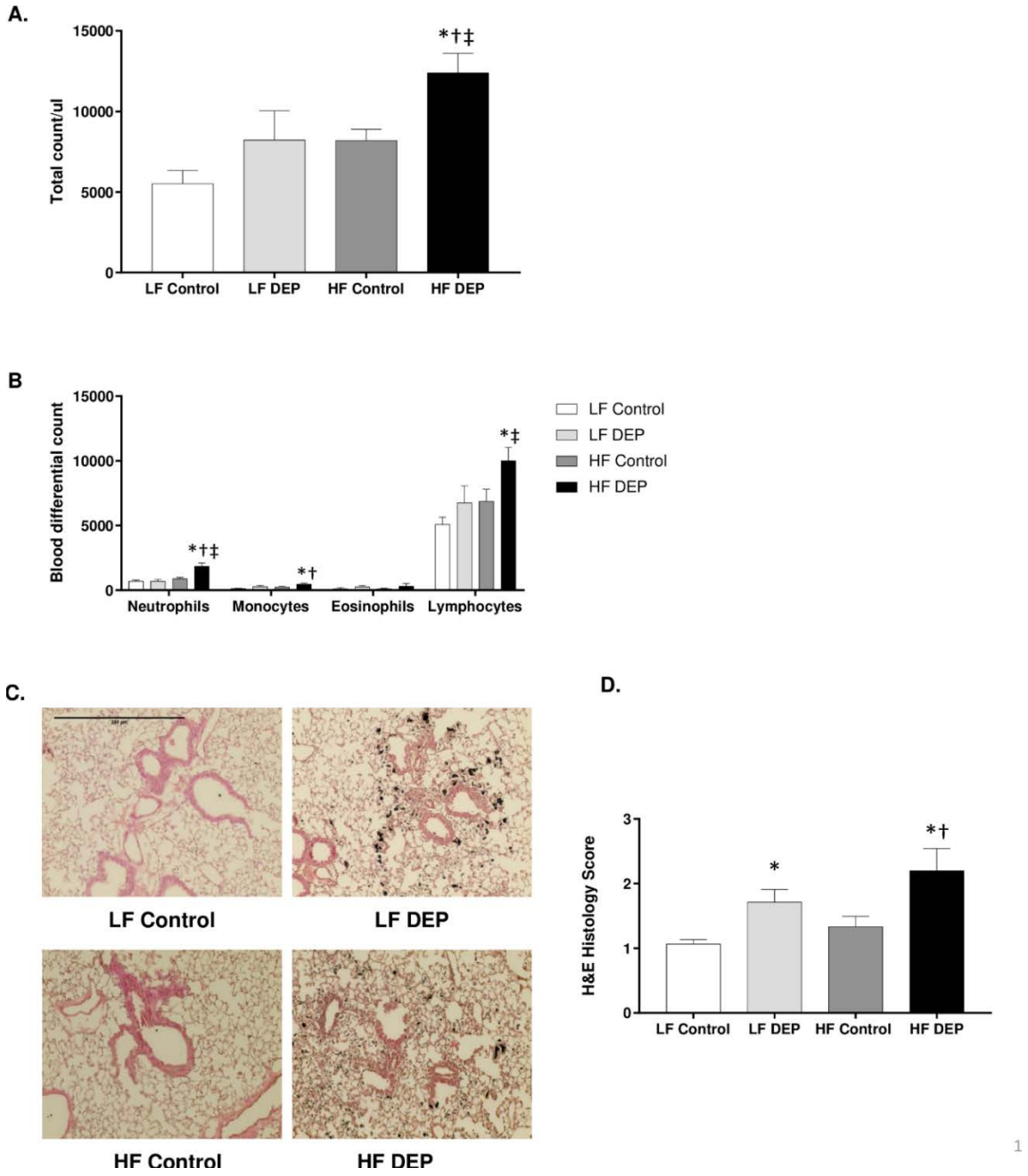

Figure 3.1: Exposure to DEP results in systemic and peri-bronchial inflammation. (A) Graphs representing total white blood cell count and (B) blood differential counts at one week following DEP exposure. (C) Representative images of H&E staining of lung sections in control and exposed groups. (D) Quantification of histological injury score in mice exposed to either DEP or saline. Images displayed are using 20X magnification. Scale bar = 240µm. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

Monocyte and lymphocyte counts were also higher in the HF DEP group compared to LF Control ($p = 0.004$), LF DEP ($p = 0.033$), and HF Control ($p = 0.033$), $F= 7.097$ for exposure (monocytes) and $F = 4.965$ for exposure (lymphocytes) (Fig. 3.1B). We did not detect any basophils in all the groups (data not shown) or any differences across the groups in eosinophils in the differential assessments. To assess whether inflammation persists within the lungs after 30 days of DEP exposure, we performed morphological examinations of the lung tissue sections. Compared to the controls, we observed significant peri-bronchial inflammation surrounding the bronchioles in mice that were exposed to DEP, regardless of the diet consumed (Fig. 3.1C). The histology scores for LF DEP were significantly higher when compared to LF Control ($p = 0.029$). The inflammation was found to be more pronounced in the HF DEP group when compared to the LF Control ($p <0.001$) and HF Control ($p = 0.005$) as indicated by the histology scores, $F = 12.99$ for exposure (Fig. 3.1D).

3.4.2 Exposure to DEP Results in Increased Levels of Immunoglobulins in the BALF of C57Bl/6 Mice

IgA is the predominant immunoglobulin in mucosal secretions and its main function is to provide the first line of defense by neutralization of toxins (Pilette et al., 2001). IgG, another important immune defense within airways functions in close cooperation with IgA by fixing complement and opsonization (Pilette et al., 2001). To observe if DEP exposure alters the levels of IgA and IgG, we performed ELISA on the BALF obtained from these mice. We observed a significant increase in IgA regardless of the diet consumed. IgA was higher in LF DEP and HF DEP compared to LF Control ($p <0.050$) and HF Control ($p = 0.010$), $F = 13.670$ for exposure (Fig. 3.2A). Compared to LF Control, IgG levels were also significantly elevated in the LF DEP ($p = 0.004$) and HF DEP ($p = 0.027$) (Fig 3.2B). We also observed a significant increase in IgG in the HF Control group compared to LF Control (Fig. 3.2B, $p = 0.045$). The respective F values for

BALF IgG levels are: exposure = 4.907, diet = 0.935, exposure x diet interaction = 4.286. To determine whether the upregulation of immunoglobulins in the lungs correlated to the expression of receptors responsible for transcytosis, we quantified the transcript expression of the polymeric IgA receptor (pIgR) responsible for the transcytosis of IgA as well as FcRn, the receptor for IgG transport into the airway lumen by RT-qPCR. We observed that the pIgR receptor expression was significantly elevated in the LF DEP and HF DEP groups compared to LF Control and HF Control ($p<0.001$), $F = 49.090$ for exposure (Fig. 3.2C). FcRn expression was upregulated only in LF DEP compared with LF Control ($p = 0.013$) (Fig. 3.2D).

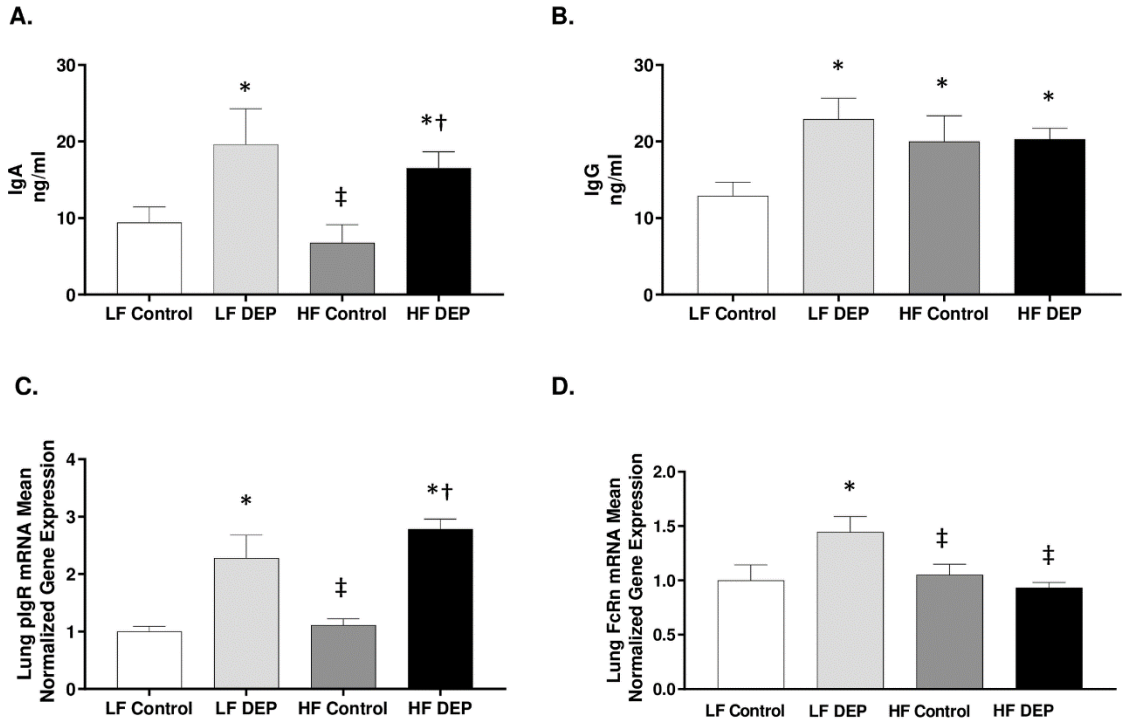

Figure 3.2: Exposure to DEP results in increased levels of immunoglobulins in the BALF. (A) Quantification of IgA (ng/ml) and (B) IgG (ng/ml), in the bronchoalveolar lavage fluid (BALF) of 4-6 week-old male C57Bl/6 wildtype mice, on either control (LF) or high-fat (HF) diet exposed to either saline (control) or diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days, by ELISA. (C) Mean normalized gene expression of IgA receptor - pIgR and (D) IgG receptor – FcRn in lung tissues, as determined by RT-qPCR. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

We did not observe an increase in FcRn expression in HF Control and HF DEP groups. The F values for FcRn expression levels are: exposure = 2.090, diet = 4.199, exposure x diet interaction = 6.252.

3.4.3 Exposure to DEP Increases TNF-α and IL-10 Expression in the Lungs of C57Bl/6 Mice

To investigate whether subchronic DEP-exposure results in persistent production of inflammatory cytokines, we analyzed the expression of proinflammatory cytokines - TNF-α, IL-1β, and IL-6. In comparison to the lungs from the LF (Fig. 3.3A-C) and HF (Fig. 3.3G-I) control animals, we observed a significant increase in TNF- α in the lungs from both the LF DEP (Fig. 3.3D-F, $p = 0.001$) and HF DEP (Fig. 3.3J-L, $p<0.001$) groups, $F = 42.48$ for exposure, as quantified in Fig. 3.3M. Real time RT-qPCR analysis showed similar trends in TNF-α mRNA transcript, with statistical increases being observed in the DEP-exposed lungs, independent of diet, compared to both the LF and HF controls (Fig. 3.3N, $p<0.01$; $F = 22.590$ for exposure). We did not detect any elevation in the expression of IL-1β and IL-6 (data not shown) across any of the groups. We suspected anti-inflammatory mediators to be present after a 30-day exposure. When we analyzed the expression of IL-10, an anti-inflammatory cytokine, we observed that in comparison to the lungs from the LF (Fig. 3.4A-C) and HF (Fig. 3.4G-I) control animals, there was a significant increase in IL-10 in the lungs from both the LF DEP (Fig.3.4D-F, $p = 0.002$), and HF DEP (Fig. 3.4J-L, $p<0.001$) groups, $F = 25.960$ for exposure, as quantified in Fig 3.4M. When analyzed by real time RT-qPCR, we observed significant increase in the IL-10 mRNA transcript in the DEP-exposed lungs, compared to the LF controls (Fig. 3.4N, $p<0.05$; $F = 10.530$ for exposure).

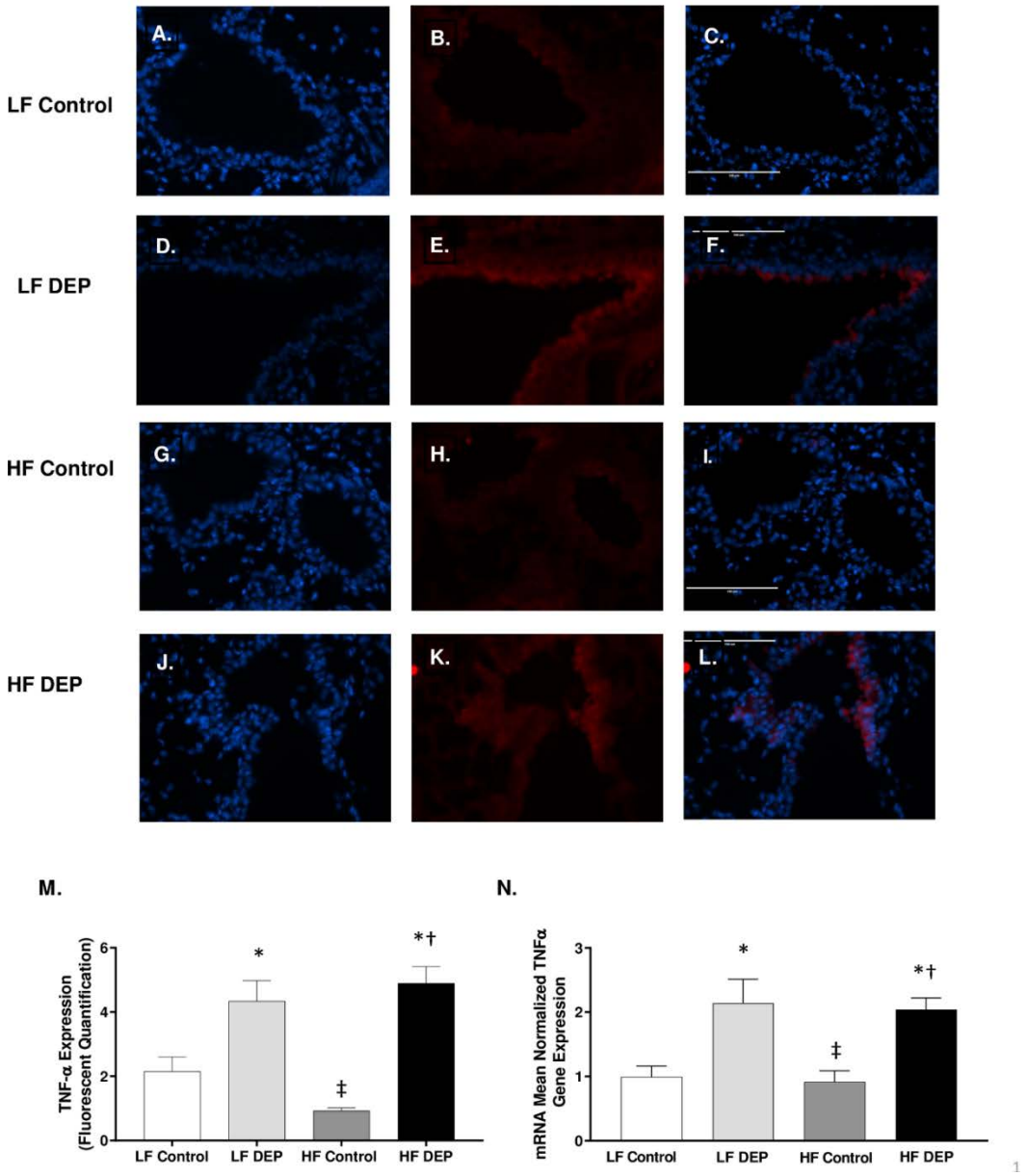

Figure 3.3: Exposure to DEP increases TNF-α expression in the lungs of C57Bl/6 mice. Representative images of tumor necrosis factor (TNF)-α expression in the lungs of C57Bl/6 mice on a control (LF; A-C) or high-fat (HF; G-I) diet exposed to saline (control) or on a LF (D-F) or HF diet (J-L) exposed to diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days. Red fluorescence indicates TNF-α expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (red; B, E, H, K) panels. (M) Graph of histology analysis of lung TNF-α fluorescence and (N) mean normalized gene expression of TNF-α mRNA transcript expression within the lungs, as determined by RT-qPCR. 40x magnification; scale bar = 100 µm. *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

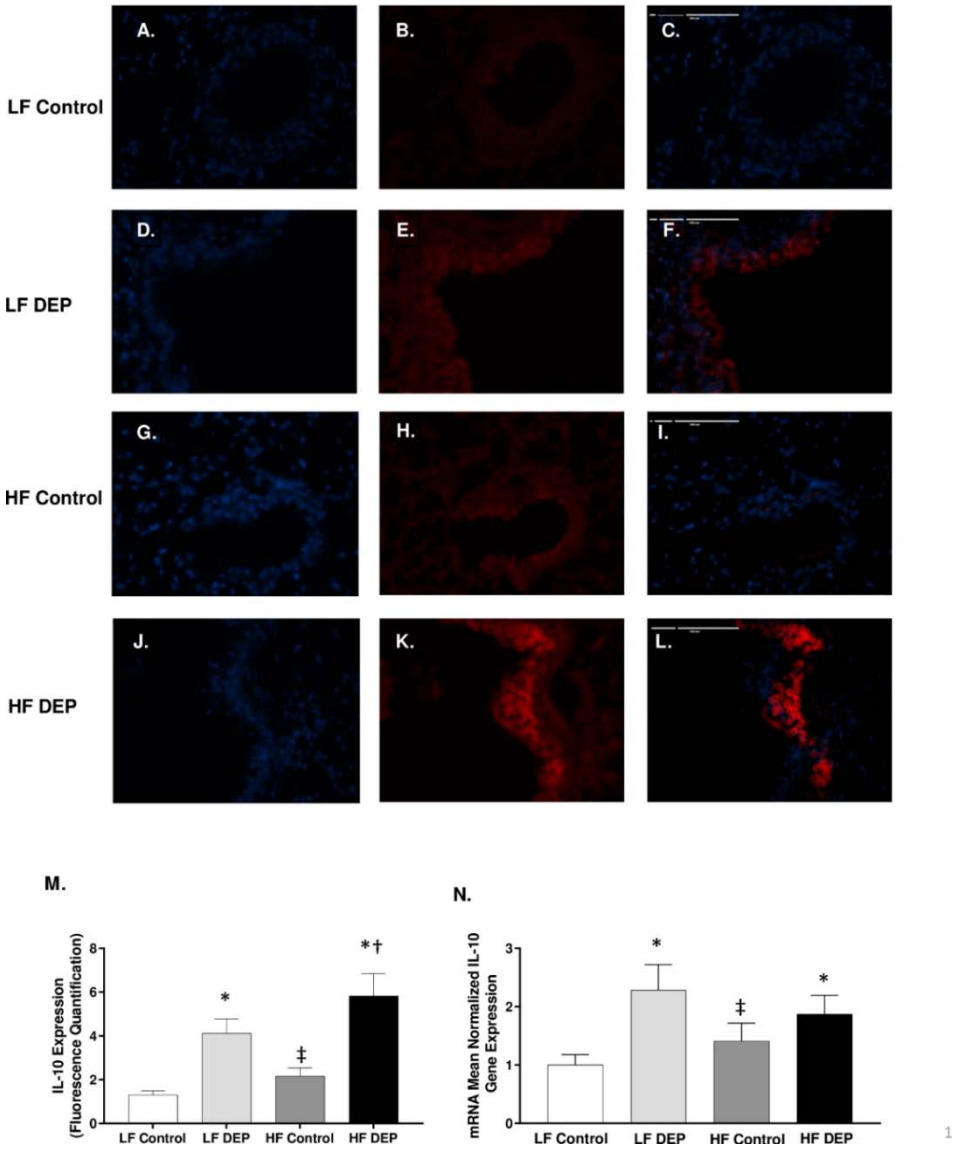

Figure 3.4: Exposure to DEP increases IL-10 expression in the lungs of C57Bl/6 mice. Representative images of Interleukin - 10 (IL-10) expression in the lungs of C57Bl/6 mice on a low-fat (LF; A-C) or high-fat (HF; G-I) diet exposed to saline (control) or on a LF (D-F) or HF diet (J-L) exposed to diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days. Red fluorescence indicates IL-10 expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (red; B, E, H, K) panels. (M) Graph of histology analysis of lung IL-10 fluorescence and (N) mean normalized gene expression of IL-10 mRNA transcript expression within the lung, as determined by RT-qPCR. 40x magnification, scale bar = 100 µm. *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

3.4.4 Exposure to DEP Results in Elevated Production of Mucus in C57Bl/6 Mice

A healthy mucus barrier maintained under physiological conditions is crucial in eliminating

pathogens and irritants within the lungs (Fahy and Dickey, 2010). When we investigated whether gel-bound mucins Muc5ac and Muc5b, which are considered to be integral components of the airway mucus are elevated with DEP exposures, we observed no alteration at the transcript level after a 30-day exposure duration (Fig. 3.5 A, B).

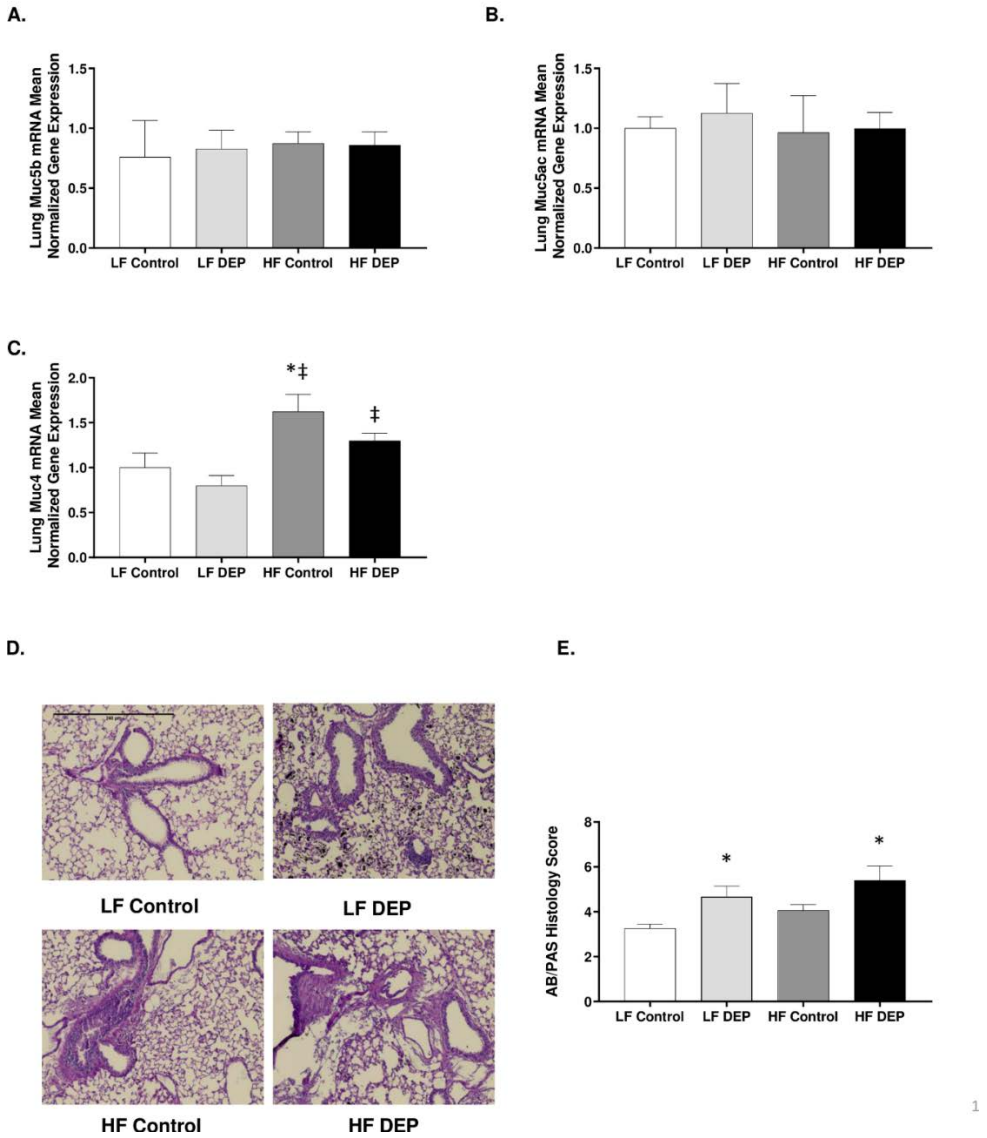

Figure 3.5: Exposure to DEP results in elevated production of mucus in C57Bl/6 mice. (A) Mean normalized gene expression of Muc5b, (B) Muc5ac and (C) Muc4 mRNA transcript expression within the lungs of C57Bl/6 wildtype mice, as determined by RT-qPCR, on either control (LF) or high-fat (HF) diet, exposed to either saline (control) or diesel exhaust particles (DEP –35 µg PM) twice a week for a total of 30 days. (D) Representative images of AB/PAS staining of lung sections and (E) quantification of histological mucus score. Images displayed are using 20X magnification, scale bar = 240 µm. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

Interestingly, we did observe a significant increase in the membrane-bound mucin Muc4 mRNA transcript in the lungs of the HF control compared to LF Control ($p = 0.006$) and LF DEP ($p < 0.001$). We also observed a significant increase in Muc4 in HF DEP compared to LF DEP ($p = 0.02$) (Fig 3.5C). The F values for Muc4 levels are: exposure = 3.316, diet = 15.200, exposure x diet interaction = 0.177; however, there was no alteration in expression of Muc4 mRNA transcript in the LF DEP group (Fig. 3.5C). When we stained for overall mucus produced within these lungs, compared to LF Control animals, we observed a significant increase in mucus production in the lungs of DEP exposed animals (Fig 3.5D), as quantified in Fig 3.5E ($p = 0.011$, $F = 8.353$ for exposure).

3.4.5 Exposure to DEP Results in an Increased Relative Abundance of Proteobacteria in C57Bl/6 Mice on the HF Diet

Inflammation and mucus are now understood to increase the availability of nutrients that can be used by selective bacteria to proliferate and outcompete the others (Zeng et al., 2017). To investigate whether there are bacterial alterations within the lungs in response to DEP exposures and/or fat content in the diet, we performed qPCR of the 16srRNA region and quantified the total bacterial abundance. We observed that the total bacteria (Eubacteria) were decreasing in the HF DEP study groups compared to LF Control ($p = 0.003$) and compared to LF DEP ($p = 0.017$) (Fig.3.6A) The respective F values for Eubacteria are: exposure = 2.490, diet = 9.228, exposure x diet interaction = 0.217. We also analyzed the quantitative abundance of individual phyla and observed that compared to LF Control, Firmicutes was significantly decreasing in the HF Control ($p = 0.02$) and HF DEP ($p = 0.002$). There was also a significant decrease in Firmicutes in the HF DEP group compared to the LF DEP ($p = 0.017$). (Fig. 3.6B). The F values for Firmicutes are: exposure = 1.645, diet = 12.040, exposure x diet interaction = 0.033. Compared to LF Control,

there was a significant decrease in Bacteroidetes as well, in both the HF Control and the HF DEP group ($p < 0.05$) (Fig. 3.6C).

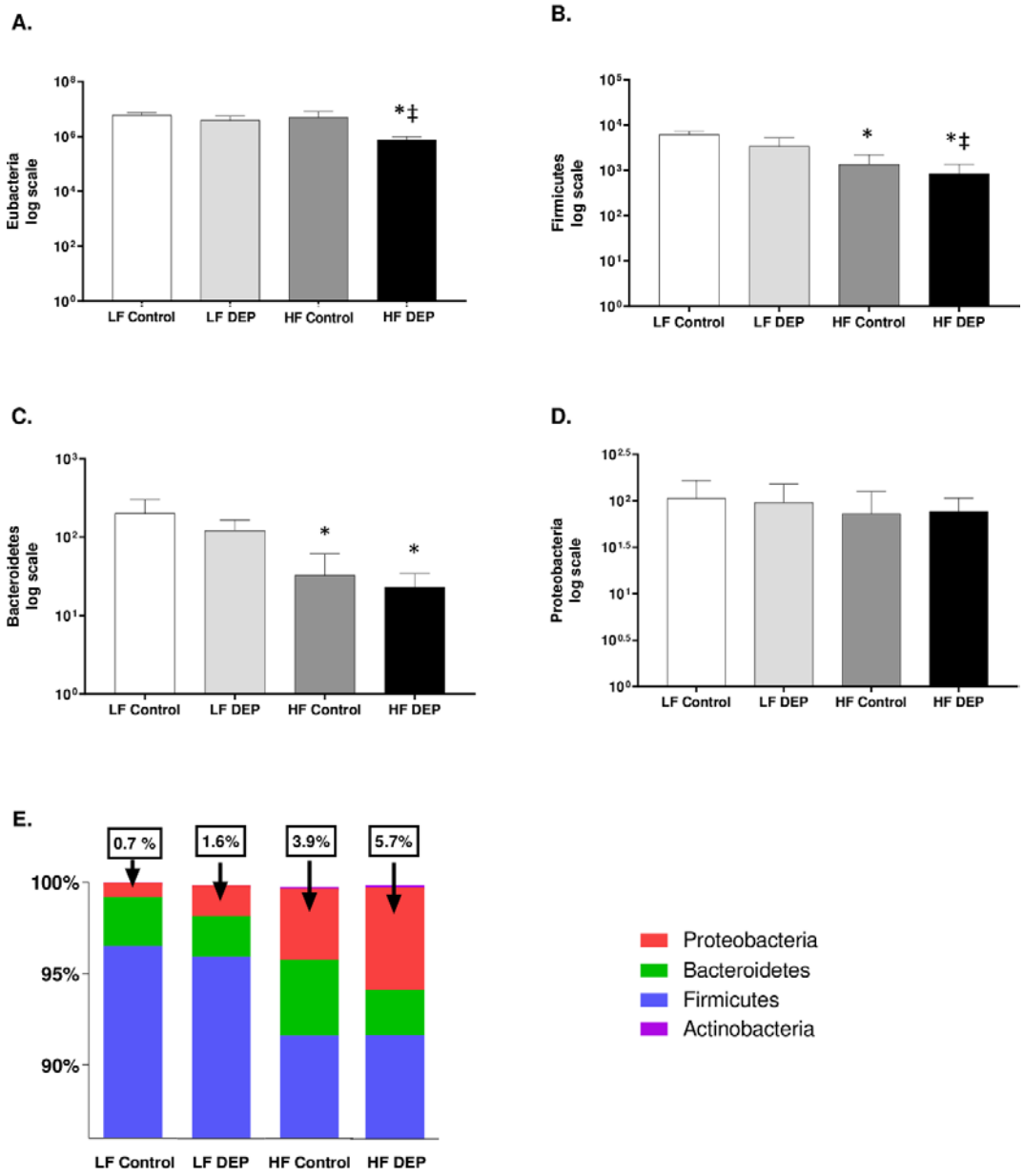

Figure 3.6: Exposure to DEP results in an increased relative abundance of Proteobacteria in C57Bl/6 mice on the HF diet. (A) Quantification in log scale of (A) Eubacteria (total bacteria), (B) Firmicutes, (C) Bacteroidetes and (D) Proteobacteria within the lungs of C57Bl/6 wildtype mice on either control (LF) or high-fat (HF) diet exposed to either saline (control) or diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days by qPCR. (E) 100% stacked columns representing the percentages of major lung phyla. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

The respective F values for Bacteroidetes are: exposure = 0.666, diet = 7.858, exposure x diet interaction = 0.356. There was no significant difference in Proteobacteria abundance noted in the lungs across any of the study groups (Fig.3.6D, p = 0.070). However, 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 group, likely due to the significant decrease in both Firmicutes and Bacteroidetes (Fig. 3.6E).

3.4.6 Exposure to DEP Induces ROS-RNS Production and Increases Macrophages within the Lungs of C57Bl/6 Mice

The generation of ROS and reactive nitrogen species (RNS) by macrophages is a well-understood mechanism of air-pollution mediated toxicity (Lodovici and Bigagli, 2011). O_2^- produced by ROS and NO_3^- produced by RNS act as effectors against invading pathogens/pollutants and they are beneficial if these mechanisms are balanced by the physiological activity of antioxidant defenses. Oxidative stress occurs as a consequence of an increased generation of reactive species or reduced activity of antioxidant defenses (Poljšak and Fink, 2014b). To investigate the presence of these radicals we analyzed the combined effects of DEP and HF diet to induce extracellular ROS and RNS. We stained lung sections with nitrotyrosine that stains for peroxynitrite, a stable ROS+RNS product. When compared to LF (Figs 3.7A-C) and HF (Figs. 3.7G-I) controls, we observed that nitrotyrosine was significantly expressed in the LF DEP (Figs. 3.7D-F, p =0.016), and HF DEP (Figs. 3.7J-L, p <0.001) exposed groups, as quantified in Fig. 3.7M. We also observed a significant increase in nitrotyrosine in the HF Control groups compared to LF Controls (p = 0.013). The F values for nitrotyrosine are: exposure = 30.76, diet = 22.00, exposure x diet interaction = 4.412.

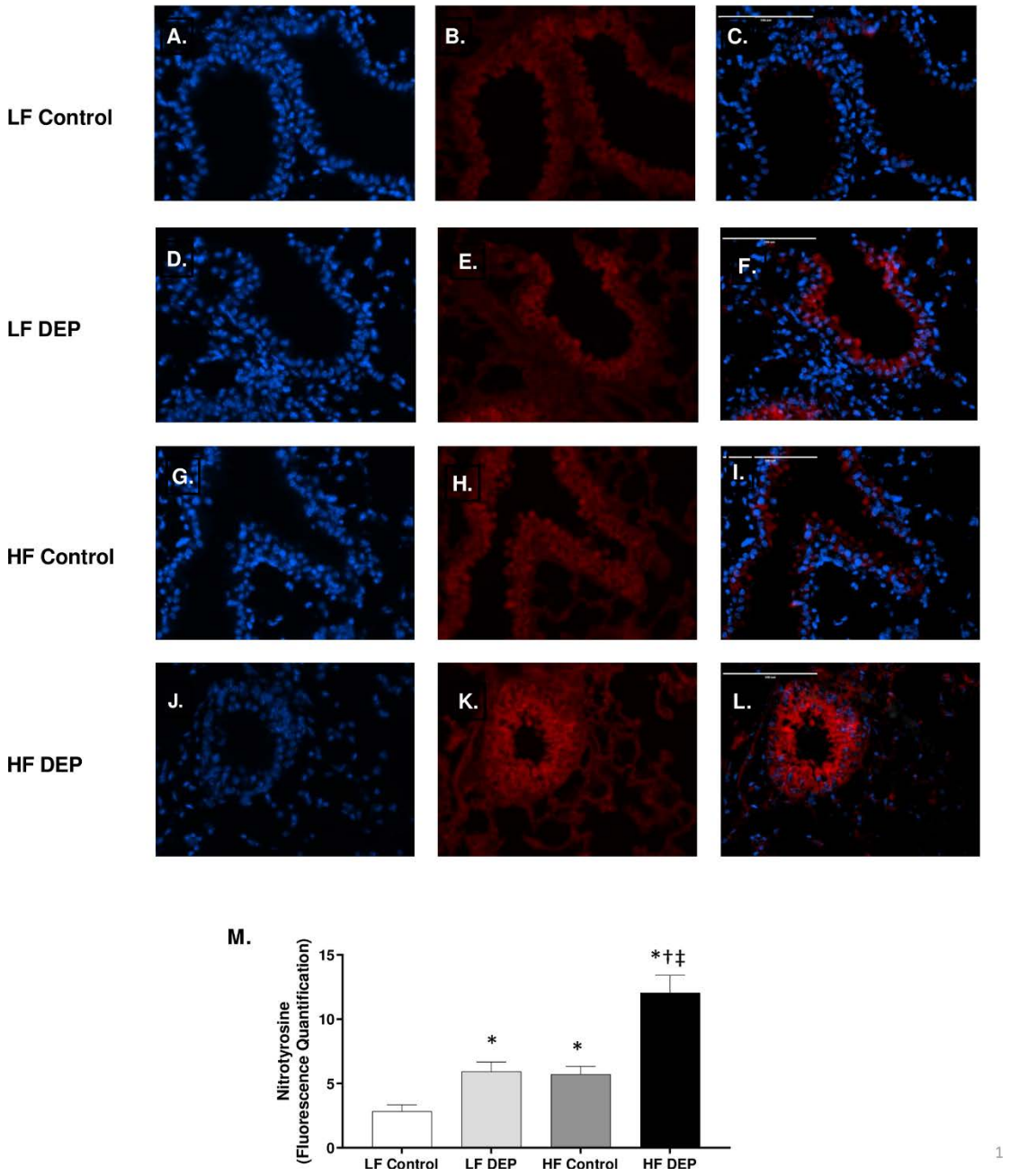

Figure 3.7: Exposure to DEP induces ROS-RNS production within the lungs of C57Bl/6 mice. Representative images of nitrotyrosine expression in the lungs of C57Bl/6 mice on a control (LF; A-C) or high-fat (HF; G-I) diet exposed to saline (control) or on a LF (D-F) or HF diet (J-L) exposed to diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days. Red fluorescence indicates nitrotyrosine expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (red; B, E, H, K) panels. (M) Graph of histology analysis of lung nitrotyrosine fluorescence. 40x magnification, scale bar = 100 µm. .Δp<0.05 compared with LF Control. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

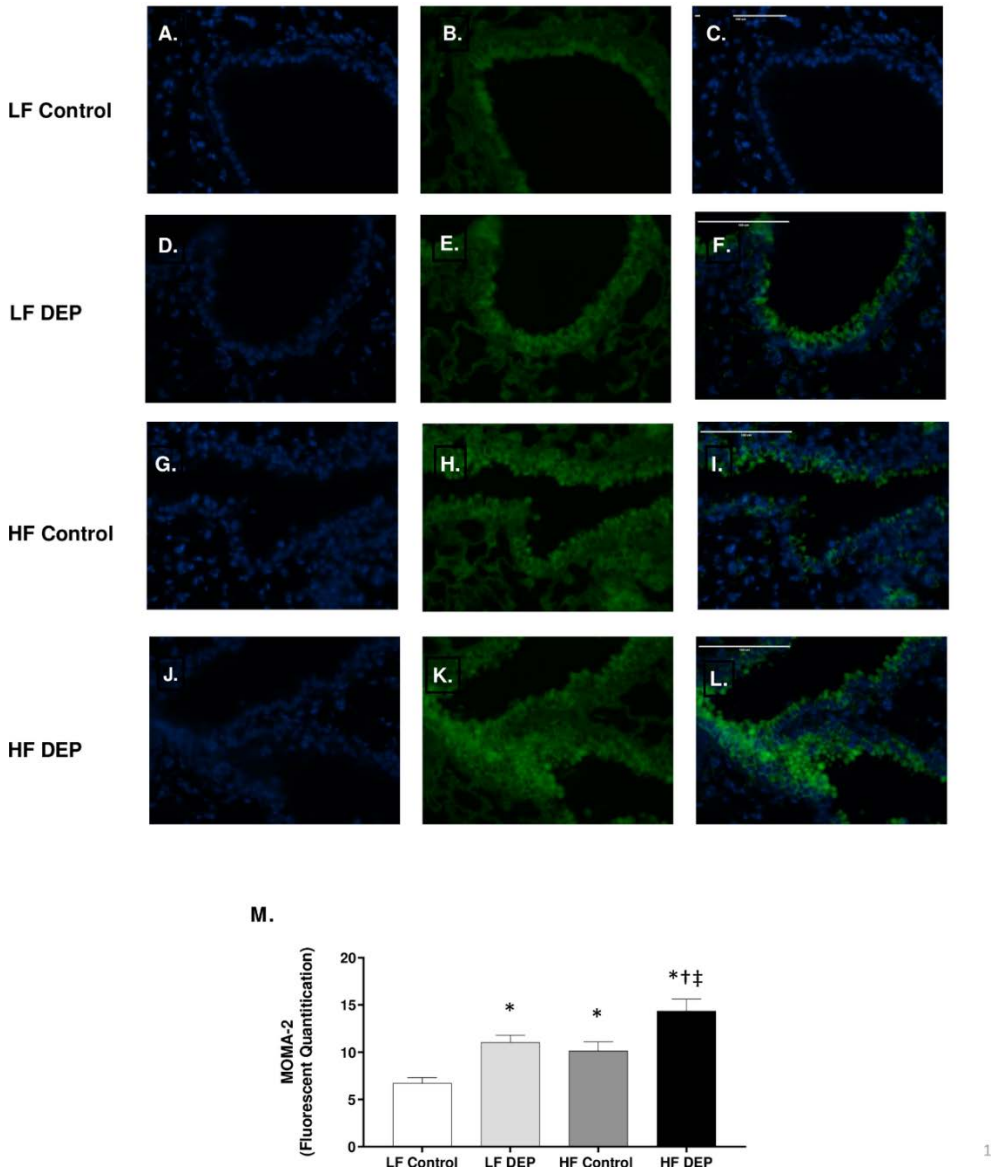

Figure 3.8: Exposure to DEP increases macrophages within the lungs of C57Bl/6 mice. Representative images of MOMA-2 expression in the lungs of C57Bl/6 mice on a control (LF; A-C) or high-fat (HF; G-I) diet exposed to saline (control) or on a LF (D-F) or HF diet (J-L) exposed to diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days. Green fluorescence indicates MOMA-2 expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (green; B, E, H, K) panels (M) Graph of histology analysis of lung MOMA-2 fluorescence. 40x magnification; scale bar = 100 µm. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

Since macrophages are the most abundant steady-state leukocyte, we suspected them to be involved in the observed oxidative stress response (Michael et al., 2013). To quantify if the

proportion of macrophages were higher in the exposed groups, we stained for monocyte and macrophage antibody-2 (MOMA-2). Compared to LF controls (Figs. 3.8A-C), we observed that MOMA-2 expression was increased in the LF DEP group (Figs. 3.8D-F, $p = 0.002$), the HF Control group (Figs. 3.8G-I, $p = 0.015$), and the HF DEP group (Figs. 3.8J-L, $p<0.001$), as quantified in Fig. 3.8M. The F values for MOMA – 2 are: exposure = 20.13, diet = 12.53, exposure x diet interaction = 0.002.

3.4.7 Exposure to DEP Results in Activation of TLR2 and TLR4 in C57Bl/6 Mice

Both resident myeloid and structural cells of the lung express a complete repertoire of TLRs that recognize pathogen-associated molecular patterns (PAMPs) (Kovach and Standiford, 2011). The TLR family disseminates the signal to intracellular transcription factor - NF-κB that regulates inflammatory gene expression (Erin I. Lafferty et al., 2010). TLR2 and TLR4 recognize bacterial ligands such as lipoproteins and lipopolysaccharide (LPS), respectively. Since we observed bacterial alterations within the airway lumen, we quantified the expression of TLRs as well as NF-κB p65 protein. We observed a significant increase in TLR2 regardless of the diet consumed. Compared to LF and HF Controls, we observed a significant increase in the expression of TLR2 mRNA transcript in the lungs of DEP-exposed animals (Figs 3.9A, $p<0.01$, $F = 30.68$ for exposure). Compared to the LF Control group, we also observed a statistical increase in TLR4 mRNA in the LF DEP ($p = 0.004$), HF Control ($p = 0.010$), and HF DEP ($p = 0.050$) group (Fig. 3.9B). The respective F values for TLR4 are: exposure = 3.607, diet = 1.321, exposure x diet interaction = 7.896.

Compared to LF controls (Figs. 3.10A-C), we observed that NF-κB p65 expression was increased in the LF DEP group (Figs. 3.10D-F), and the HF DEP group (Figs. 3.10J-L), as quantified in Fig. 3.10M ($p<0.001$, $F = 73.89$ for exposure). Real time RT-qPCR of the NF-κB

p65 mRNA transcript revealed statistical increases in the LF DEP compared to the LF controls (Fig. 3.10N, p = 0.01; F = 5.705 for exposure). However, we did not observe an increase in NF-κB p65 in the HF DEP group (Fig. 3.10N).

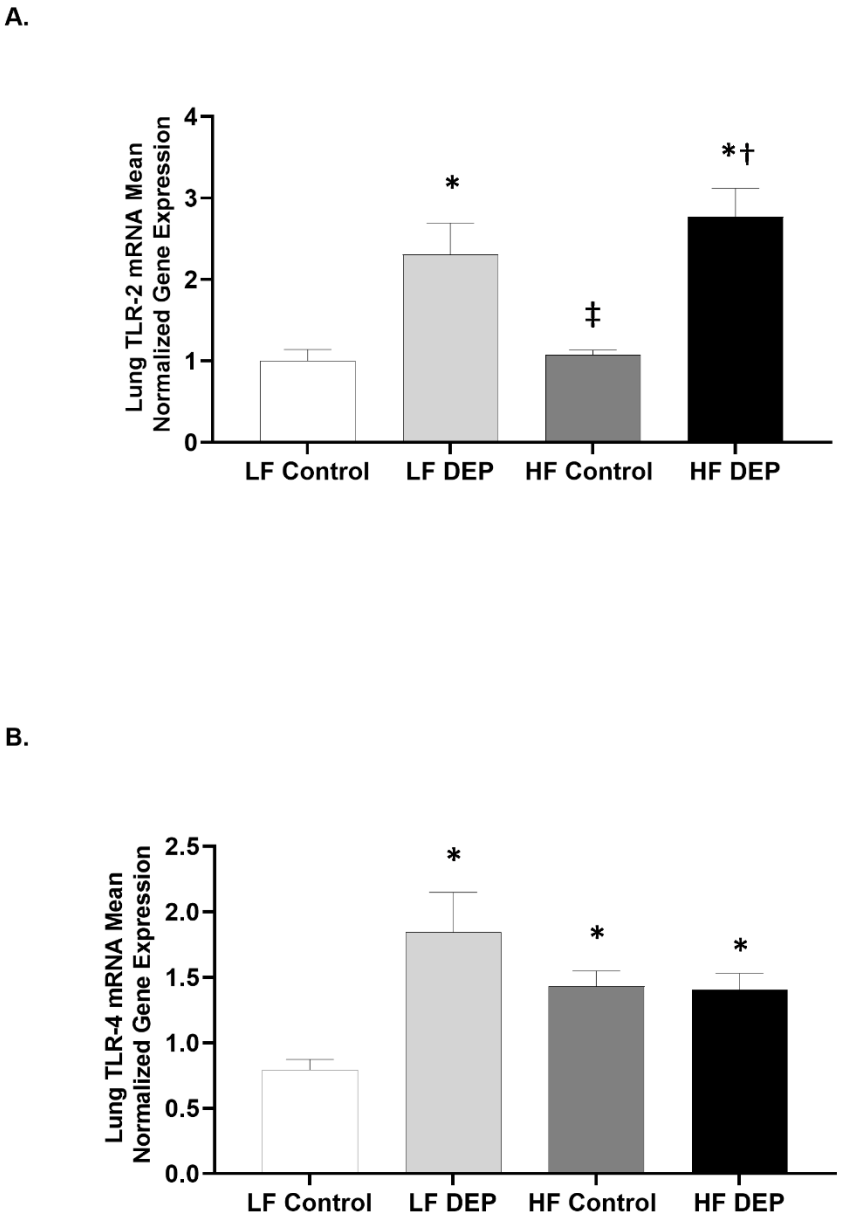

Figure 3.9: Exposure to DEP results in activation of TLR2 and TLR4 in C57Bl/6 mice. Mean normalized gene expression of (A)TLR2 and (B) TLR4 mRNA transcript expression within the lungs of C57Bl/6 wildtype mice, as quantified by RT-qPCR, on either control (LF) or high-fat (HF) diet, exposed to either saline (control) or diesel exhaust particles (DEP –35 µg PM) twice a week for a total of 30 days. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

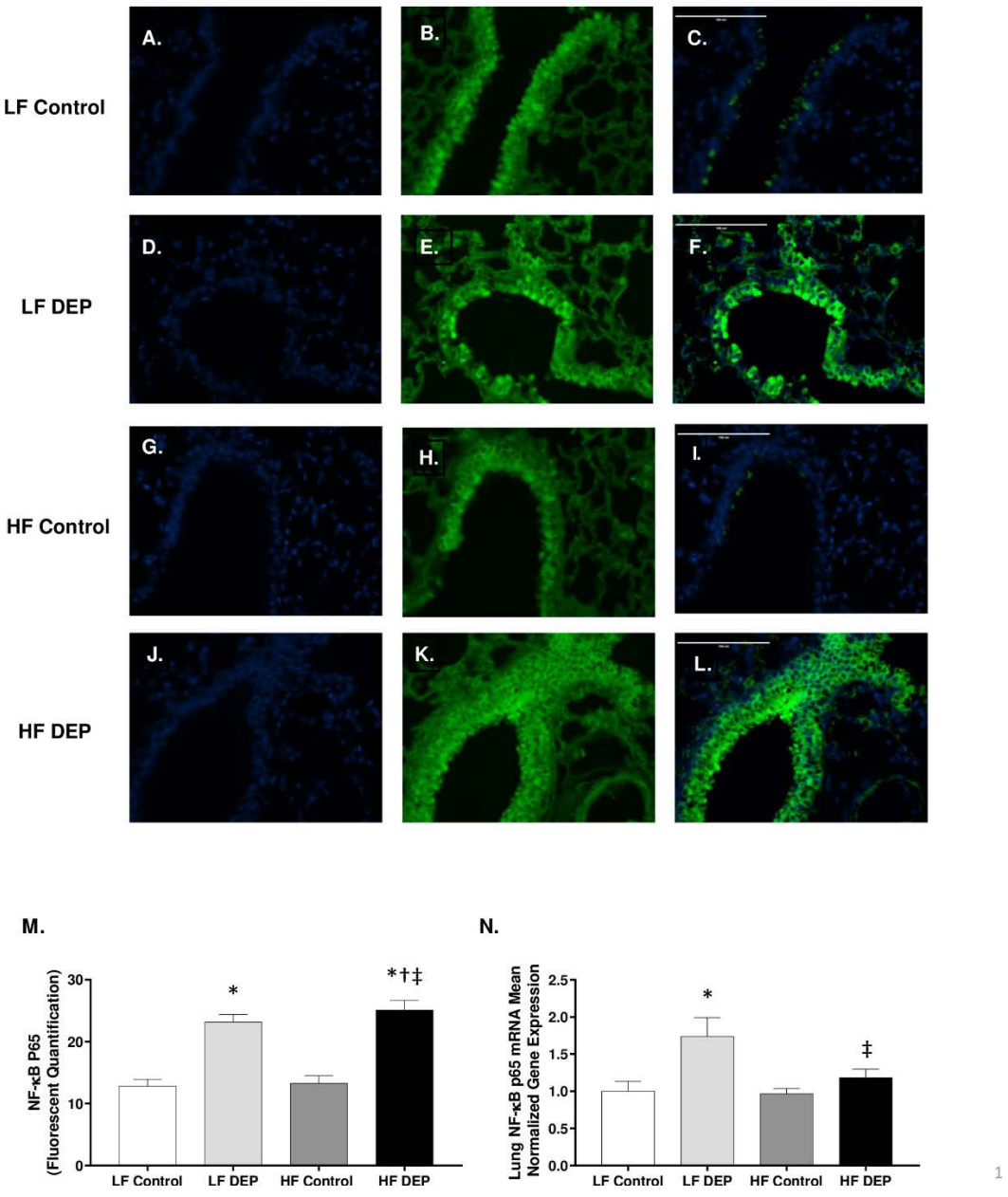

Figure 3.10: Exposure to DEP results in increased NF-κB p65 expression. Representative images of NF-κB p65 expression in the lungs of C57Bl/6 mice on a control (LF; A-C) or high-fat (HF; G-I) diet exposed to saline (control) or on a LF (D-F) or HF diet (J-L) exposed to diesel exhaust particles (DEP – 35 µg PM/m3) twice a week for a total of 30 days. Green fluorescence indicates NF-κB p65 expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (green; B, E, H, K) panels. (M) Graph of histology analysis of lung NF-κB p65 fluorescence. (N) mean normalized gene expression of NF-κB p65 mRNA transcript expression within the lung, as determined by RT-qPCR. 40x magnification, scale bar = 100 µm.Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

3.4.8 C57Bl/6 Mice Exposed to DEP Show Increased Collagen Deposition Surrounding the Bronchioles

Air pollutant exposures have been implicated in inducing and/or worsening fibrosis in chronic lung conditions (Conti et al., 2018; Goss et al., 2004); therefore, we stained for collagen deposition in the lungs following 30 days of subchronic DEP exposure. Compared to LF Controls, we observed increased collagen deposition surrounding the bronchioles in both the LF and HF DEP-exposed animals (Fig. 3.11A), as quantified in Fig. 3.11B ($p<0.01$, $F = 13.12$ for exposure), suggesting that subchronic exposures resulted in increased deposition of extracellular matrix components (ECM).

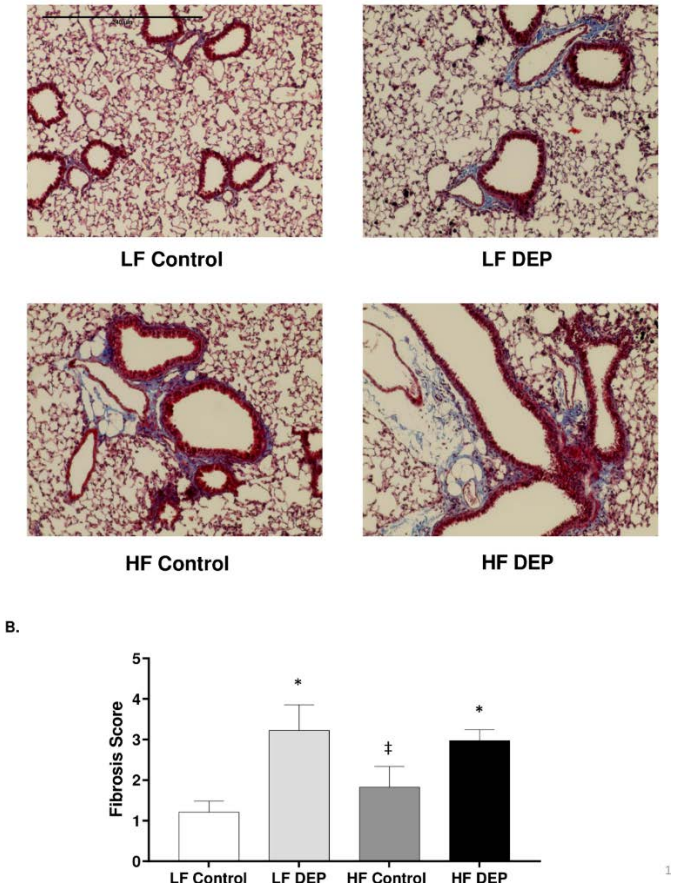

Figure 3.11: C57Bl/6 mice exposed to DEP show increased collagen deposition surrounding the bronchioles. (A) Representative images of lung tissue sections stained with Masson's trichrome in C57Bl/6 wildtype mice on either control (LF) or high-fat (HF) diet exposed to either saline (control) or diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days. Blue areas indicate collagen deposition. (B) Graph of histology scoring of Masson's trichrome staining. 20x

magnification, scale bar = 240µm. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

3.4.9 Probiotic Supplementation Decreases the Expansion of Proteobacteria in C57Bl/6 Mice Lungs

Probiotic supplementation has been shown to improve lung immune responses that have beneficial effects during respiratory infections (Forsythe, 2014). We investigated whether probiotic intake could aid in restoring the commensal phyla within the lungs. Interestingly, our results revealed an increase in the overall bacterial abundance (Fig 3.12A), compared to HF Control ($p<0.01$) and compared to HF DEP ($P<0.001$), with an increase in Firmicutes (Fig. 3.12B) in the probiotic groups. We also observed that the expansion of Proteobacteria was significantly attenuated in the lungs of HF Control and HF DEP-exposed animals, when compared to their counterparts with no probiotic supplementation (Fig. 3.12B).

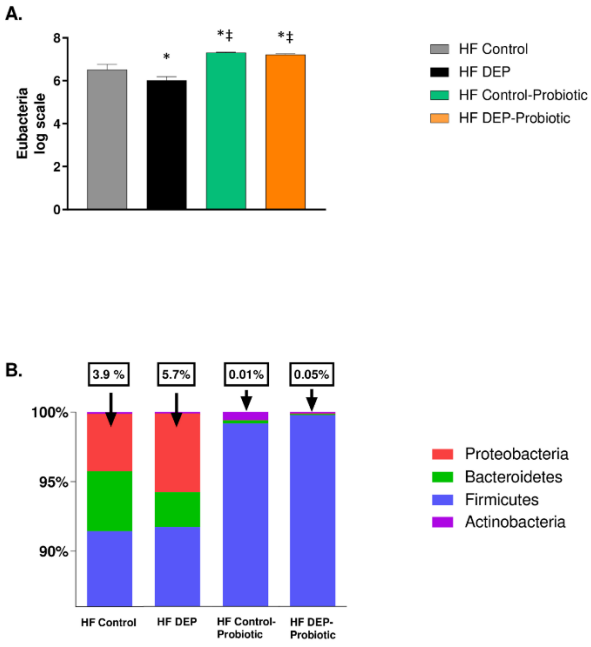

Figure 3.12: Probiotic supplementation decreases the expansion of Proteobacteria in C57Bl/6 mice lung. (A) Quantification by qPCR of (A) Eubacteria (log scale), (B) 100% stacked columns representing the percentages of major phyla within the lungs of C57Bl/6 wildtype mice on high-fat (HF) diet exposed to either saline (control) or diesel exhaust particles (DEP – 35 µg PM) twice a week

for a total of 30 days alongside a dose of 0.3 g/day (~7.5 x10^8 cfu/day) of Ecologic® Barrier probiotics in the drinking water over the course of the exposures. Data are depicted as mean ± SEM with *p<0.05 compared to HF Control, †p<0.05 compared to HF Control - Probiotics, ‡p<0.05 compared to HF DEP by two way ANOVA.

3.4.10 Probiotic Supplementation Decreases Inflammation and Collagen Deposition

Probiotics have been shown to improve inflammation observed in many lung diseases due to their immunomodulatory properties (Javanmard et al., 2018; Mortaz et al., 2013; Tian et al., 2019). Thus, we investigated whether the inflammation observed with DEP could be attenuated with probiotics. We observed that TNF-α expression, found to be significantly elevated with DEP exposures, were decreasing with probiotic supplementation. In comparison to the lungs from the HF DEP (Figs. 3.13D-F), we observed a significant decrease in TNF-α in the lungs from the HF Control – Probiotic (Figs. 3.13G-I) and HF DEP – Probiotics group (Figs. 4-13J-L, $p<0.001$, $F = 63.22$ for exposure). Although the decrease in TNF-α expression is significant, we notice that the expression in the HF DEP – Probiotic group is still significantly higher when compared to HF Control (Figs. 3.13A-C) and HF Control – Probiotic groups (Figs. 3.13 G-I, $p< 0.01$). The respective F values for TNF-α are: exposure = 63.22, probiotic treatment = 19.45, exposure x diet interaction = 15.11.

We observed a similar statistical decrease in nitrotyrosine in the HF Control – Probiotic (Fig. 3.14G-I) and HF DEP – Probiotic group (Fig. 3.14J-I) compared to the HF DEP (Fig. 3.14D-F, $p<0.001$) and HF Control (Fig. 3.14A-C, $p<0.01$) (Fig.3.14D-F). The F values for nitrotyrosine are: exposure = 22.81, probiotic treatment = 90.74, exposure x diet interaction = 15.86. When we checked to see if immunoglobulin levels were altered with probiotic treatments (Fig. 3.14 N), we observed that IgA was significantly elevated in the HF DEP – Probiotic group when compared to the HF Control ($p<0.001$), HF DEP ($p = 0.001$) and HF Control – Probiotic ($p<0.001$) groups. The

F values for IgA levels are: exposure = 28.74, probiotic treatment = 13.41, exposure x diet interaction = 1.016.

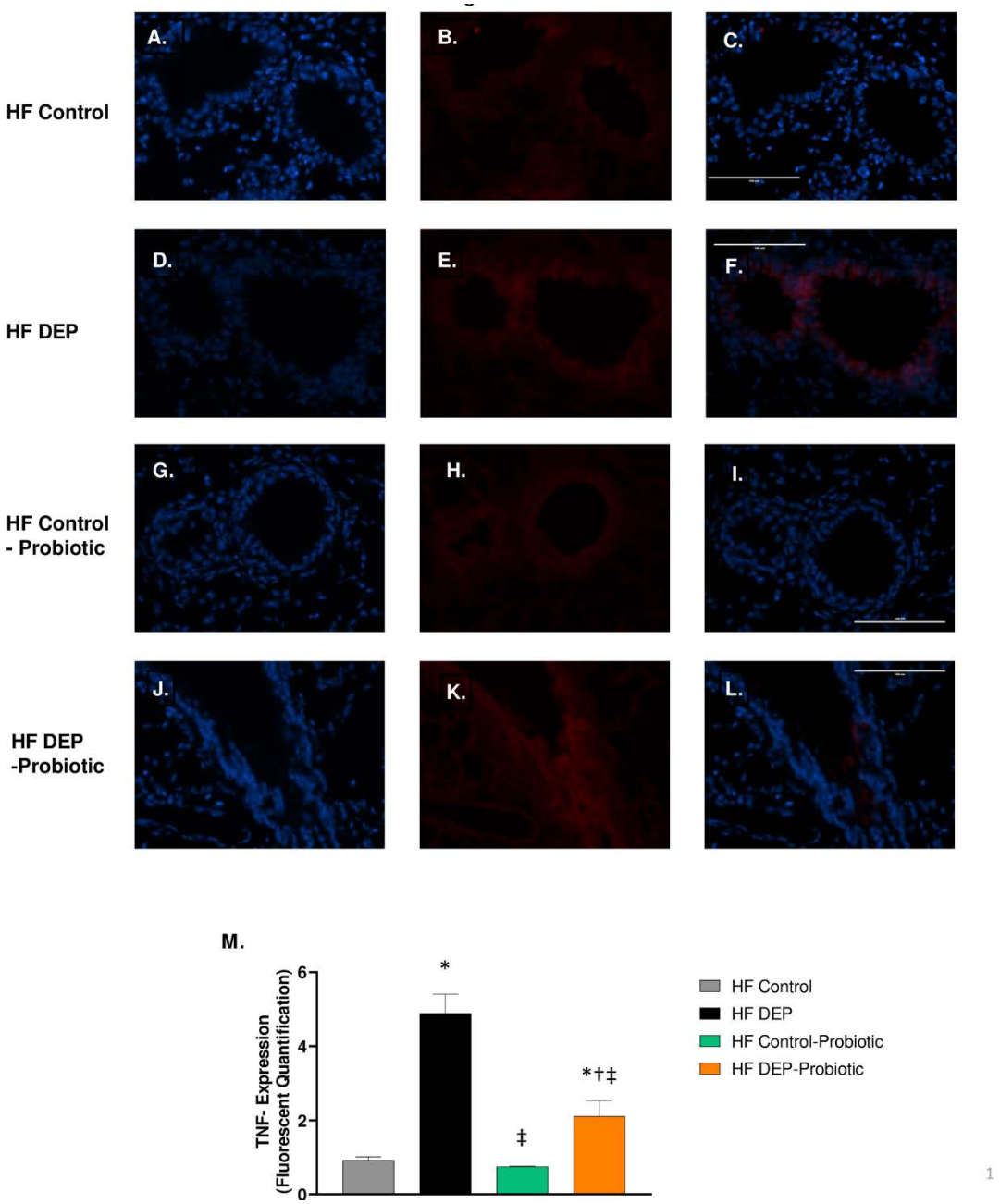

Figure 3.13: Probiotic supplementation decreases TNF-α expression. Representative images of TNF-α expression in the lungs of C57Bl/6 mice, on a high-fat (HF) diet exposed to either (A–C) saline, (D–F) DEP – 35 µg PM, or (G–I) saline and probiotics - 0.3 g/day (~7.5 x10^8 cfu/day) and (J-L) DEP and probiotics. Red fluorescence indicates TNF-α expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (red; B, E, H, K) panels. (M) Graph of histology analysis of lung TNF-α fluorescence. 40x magnification, scale bar = 100 µm. Data are depicted as mean ± SEM with *p<0.05 compared to HF Control, †p<0.05 compared to HF Control - Probiotics, ‡p<0.05 compared to HF DEP by two way ANOVA.

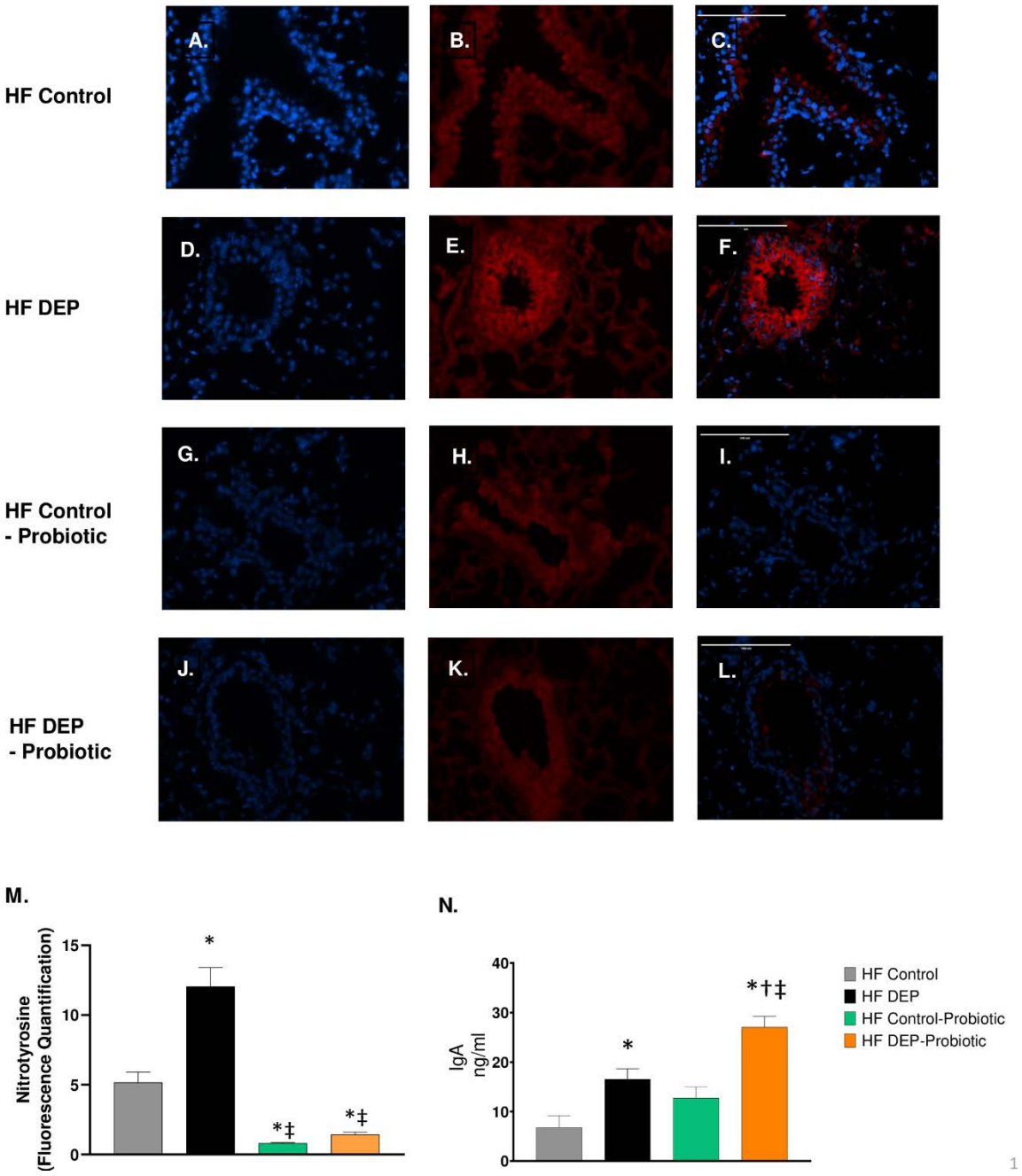

Figure 3.14: Probiotic supplementation decreases ROS-RNS product formation and increases production of IgA. Representative images of nitrotyrosine expression in the lungs of C57Bl/6 mice, on a high-fat (HF) diet exposed to either (A–C) saline, (D–F) DEP – 35 µg PM, or (G–I) saline and probiotics - 0.3 g/day (~7.5 x10^8 cfu/day) and (J-L) DEP and probiotics. Red fluorescence indicates nitrotyrosine expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (red; B, E, H, K) panels. (M) Graph of histology analysis of lung nitrotyrosine fluorescence. (N) Quantification of IgA (ng/ml) in the bronchoalveolar lavage fluid by ELISA. Data are depicted as mean ± SEM with *p<0.05 compared to HF Control, †p<0.05 compared to HF Control - Probiotics, ‡p<0.05 compared to HF DEP by two way ANOVA.

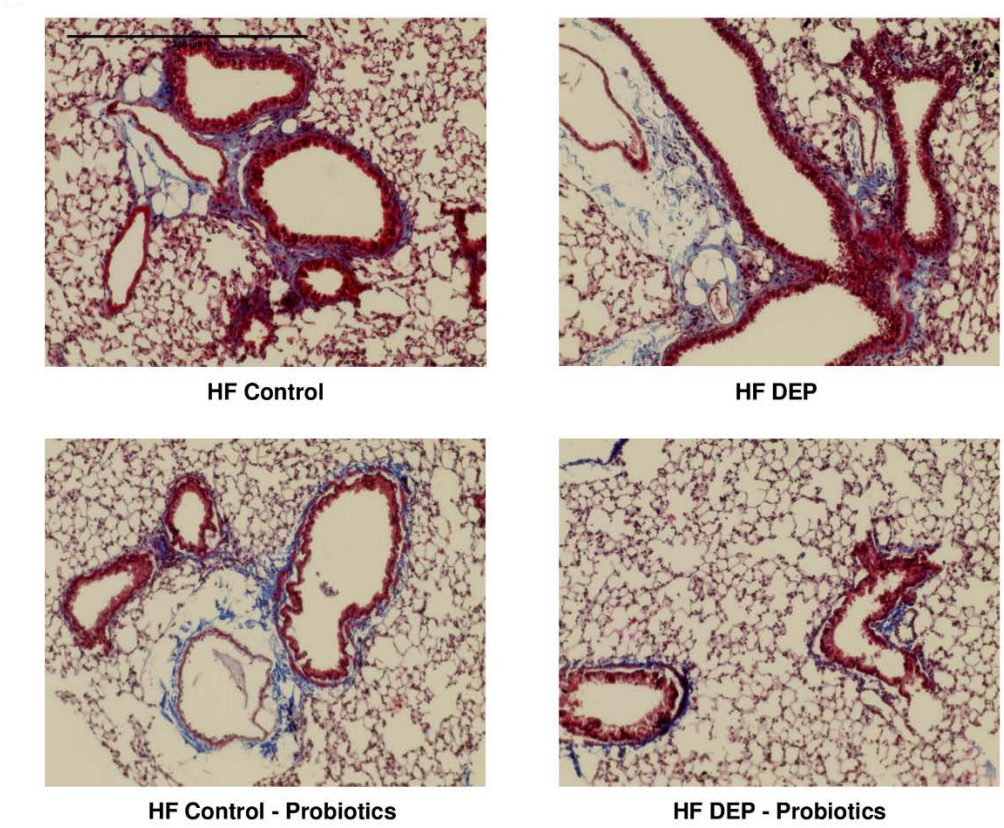

Figure 3.15: Probiotic supplementation decreases collagen deposition. (A) Representative images of lung tissue sections stained with Masson's trichrome in of 4-6 week-old C57Bl/6 wildtype mice on high-fat (HF) diet exposed to either saline (control) or diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days alongside a dose of 0.3 g/day (~7.5 x10^8 cfu/day) of Ecologic® Barrier probiotics in the drinking water over the course of the exposures. Blue areas indicate collagen deposition. (B) Graph of histology scoring of Masson's trichrome staining. 20x magnification, scale bar = 240µm. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

With the observed decrease in inflammation with probiotic supplementation, we investigated whether the pathological outcomes observed with DEP exposure could be attenuated. Interestingly, we observed that collagen deposition was significantly decreasing in the HF DEP – Probiotics group and HF Control – Probiotic group when compared to the HF DEP group (Fig. 3.15 B, $p = 0.002$). The F values for fibrosis score are: exposure = 2.191, probiotic treatment = 11.24, exposure x diet interaction = 1.821.

3.5 Discussion

Recent studies have demonstrated a strong correlation between exposure to air pollutants and an increase in the incidence and hospitalizations of respiratory conditions such as asthma and COPD. Lung diseases are often associated with persistent colonization of bacteria belonging to the Proteobacteria phylum (Hurst et al., 2010; Sze et al., 2014). Although alterations in the commensal lung microbiome are postulated to influence disease behaviors, a direct association between exposure to particulate air pollution components and bacterial lung dysbiosis has not been characterized. Additionally, while the consumption of the HF diet is understood to increase inflammation and cause gut microbial shifts, little information exists on the combined effects of HF diet and particulate pollution within the lungs. In this study, we report for the first time to our knowledge, that exposure to DEP alters the microbiome and inflammatory response within the lungs of C57Bl/6 mice on the HF diet.

The impact that the HF diet plays is observed in the neutrophil counts that reveal a significant interaction between diet and exposure suggesting that the synergistic effect of diet is responsible for the observed acute immune response. Although we observe an increase in monocytes and lymphocytes, the involvement of neutrophils is far more pronounced. Neutrophils are among the first cells to be recruited to the site of infection and we suspect they play a crucial

role in mediating acute immune responses with DEP exposures (Rosales et al., 2017). Few studies point to the elevation of several proinflammatory cytokines, including IL-1β, IL-6, and IL-8, after acute DEP exposures (Pacheco et al., 2001). However, in our study, most of these initial proinflammatory cytokines were not elevated, likely due to the longer study duration. We did, however, observe a significant increase in TNF-α and IL-10 in the lungs of DEP-exposed animals, regardless of the diet they were consuming. Thus, it is plausible that at the 30 day exposure time point, there may be activated anti-inflammatory mediators. We suspect these inflammatory cytokines are generated predominantly by macrophages that were found to be abundant in the DEP exposed groups. Based on our results, as well as previous studies, it is likely that there is macrophage polarization into proinflammatory M1 producing TNF-α and anti-inflammatory M2 producing IL-10 occurring within the lungs after 30 days of DEP exposure (Bazzan et al., 2017; Ji et al., 2018).

Acute exposure to DEP has been shown to upregulate Muc5ac and Muc5b (Na et al., 2019), but we were unable to detect these mucins at the 30 day exposure time point. However, we were able to detect increased overall mucus in the exposed groups by AB/PAS staining within the lungs which suggests that DEP exposure results in the sustained presence of mucus after 30 days of exposure. The presence of excessive mucus has been shown to cause the expansion of the opportunistic pathogen, *Pseudomonas aeruginosa* belonging to the Proteobacteria phylum, which is commonly observed in lung diseases (Flynn et al., 2016). In addition to mucus, the DEP-induced inflammation also likely changes the nutrient environment within the lungs. Although inflammation was traditionally understood to function as a means to expel potential antigens, it is now understood that inflammatory by-products such as ROS and RNS, result in the generation of nitrates that can be utilized only by bacteria within the Proteobacteria phylum (Scales et al., 2016).

Proteobacteria can efficiently metabolize nitrates since they have the highest nitrate reductase activity in their genomes, compared to the other commensals (Zeng et al., 2017). We observed a significant elevation in peroxynitrite levels in the lungs of our DEP-exposed animals, correlated with an increased abundance of Proteobacteria in the lung microbiome, suggesting that extracellular nitrate availability is aiding the proliferation of Proteobacteria. Firmicutes and Bacteroidetes do not possess nitrate-reducing metabolic capabilities, which we speculate may drive the decreased abundance in the HF groups. ROS-RNS are generated by many immune cells, including neutrophils and macrophages, as anti-microbial effectors (Fang, 2004). Although we see an increase in systemic neutrophils with acute exposures, it is likely most of the ROS-RNS produced is generated by macrophages since they are the most abundant within the lungs and have a longer life-span when compared to neutrophils (Simon and Kim, 2010). We noticed that the bacterial abundance was higher in the BALF when compared to the lung tissues, suggesting that most of the lung bacteria are present within the airway lumen (data not shown).

Endotoxins present on PM have been shown to upregulate TLR2, which signals the pIgR to facilitate increased IgA transport across the airway lumen (Shoenfelt et al., 2009b). Since we observe an increase in pIgR in the DEP exposed groups, it is plausible that the increase in IgA is in response to a TLR mediated signaling (Schneeman et al., 2005). The increase in TLR4 observed in the HF Control group is possibly due to the demonstrated ability of TLR4 signaling in the obesity-induced inflammation (Rogero and Calder, 2018). Although we do not observe an increase in IgA, IgG is elevated in the HF Control groups. This may be due to a gut-lung mediated response since increased IgG has been shown with HF diet consumption (Petta et al., 2018; Zhang et al., 2020). TLRs have also been shown to activate macrophages with the subsequent transcription of inflammatory mediators like TNF-α (Gallego et al., 2011). All TLRs converge into activating the

NF-κB transcription factor family, and NF-κB signaling is understood to be required for sustained cytokine expression in macrophages (Dorrington and Fraser, 2019). Thus, the increased expression of the NF-κB protein p65 observed within the lungs in response to DEP could be due to a TLR mediated signaling; however, additional studies are necessary to determine mechanistic signaling pathways involved.

TLR4 is activated in response to the gram-negative bacterial cell wall component - LPS (Takeuchi et al., 1999). The expansion of gram-negative Proteobacteria observed within the airway lumen could contribute to the activation of TLR4. There is likely a continuous cycle of inflammation throughout the duration of DEP exposures – first by DEP-induced inflammation, which increases nitrates within the lung environment selectively, enabling the outgrowth of Proteobacteria. Secondly, the expansion of Proteobacteria contributes to further TLR4 activation making this a vicious cycle (Fig. 3.16). This is analogous to the inflammation-dysbiosis model proposed in the exacerbation of chronic lung diseases (Dickson et al., 2014). We suspect that even in health, exposure to particulate pollutants can increase the susceptibility to develop lung conditions since we observe increased collagen deposition surrounding the bronchioles with 30 days of DEP exposures. Chronic inflammation resulting from immune responses that persist for longer durations in which inflammation, tissue remodeling, and repair processes coincide has been shown to induce fibrosis (Wynn, 2008). Oxidative stress has been demonstrated to contribute to the progression of IPF and other lung diseases (Bargagli et al., 2009). We noticed abnormal tissue elements in our morphological assessments, which were identified to be collagen deposition by Masson's trichrome staining. This suggests that there is sustained and persistent inflammation resulting in the deposition of ECM. Although the initial inflammatory response may be beneficial with DEP exposures, repeated exposure of these particles within the lungs leads to pathological

outcomes. The increase in ECM components is an observed phenomenon in lung diseases such as IPF as well as COPD (He et al., 2017; Zhao et al., 2019). Interestingly, both these diseases show persistent colonization of bacteria such as *Haemophilus influenzae* and *Pseudomonas aeruginosa* both of which belong to the Proteobacteria phylum (Beasley et al., 2012). We suspect that alterations in the commensal lung microbiome in health can increase inflammation and facilitate the proliferation of pathogenic bacteria if not mediated by host immune responses.

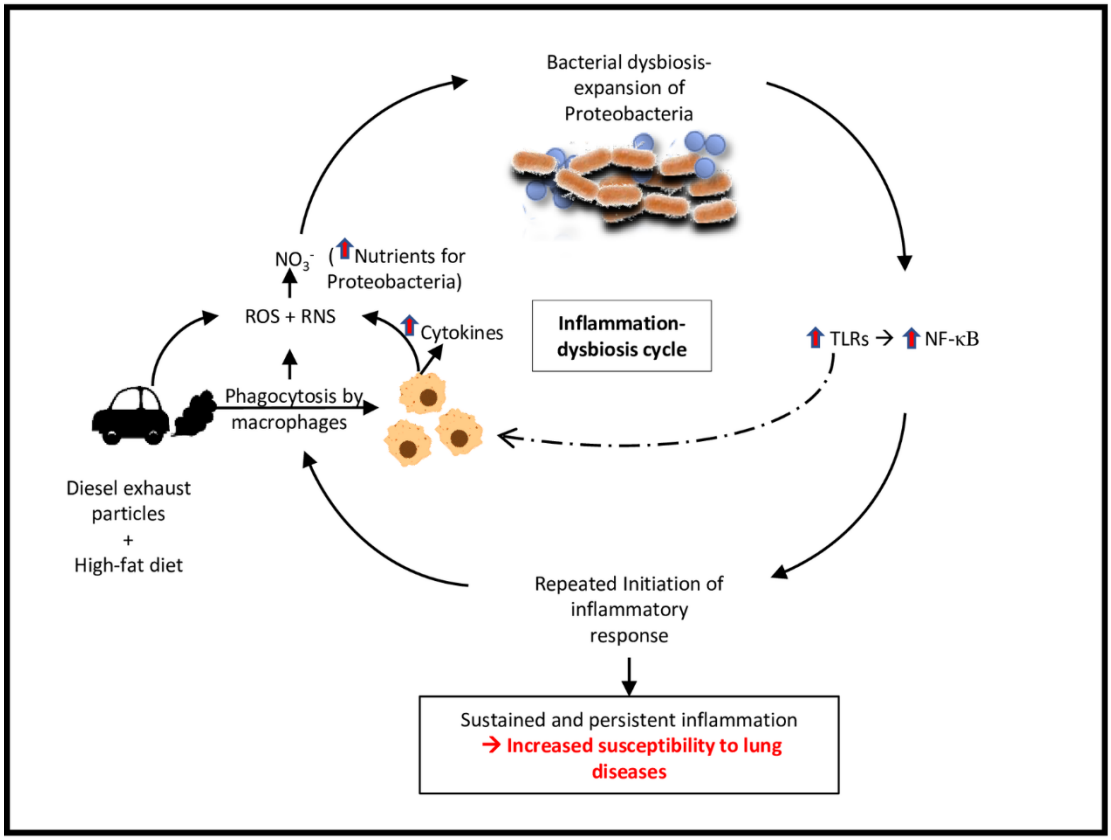

Figure 3.16: Graphical summary of DEP exposure study. Synergistic effects of diesel exhaust particles (DEP) and high-fat (HF) diet results in increased reactive oxygen and nitrogen species (ROS + RNS) produced possibly by macrophages, which increases nitrates within the lung environment. These nitrates provide nutrients for anaerobic respiration and selective growth of Proteobacteria within the lungs resulting in bacterial dysbiosis. Both DEP and the bacterial dysbiosis activate Toll-like receptors (TLRs) which results in the subsequent activation of NF-κB mediated inflammatory gene transcription. Since NF-κB signaling is understood to be required for sustained cytokine expression in macrophages, the production of inflammatory cytokines, ROS, and RNS is sustained, resulting in a continuous inflammation-dysbiosis cycle throughout the duration of DEP exposures. This sustained and persistent inflammation is a possible contributor and significant in the development of lung diseases.

Interestingly, and somewhat unexpectedly, with probiotic supplementation, we observe that the expansion of Proteobacteria is curbed with a decrease in nitrates in the lung environment, which indicates that the DEP-induced inflammation is attenuated. The mechanism by which probiotics help attenuate inflammation has been reported to involve T_{reg} cell-mediated balance of proinflammatory signaling (Mortaz et al., 2013). Thus, it is plausible that proinflammatory responses are balanced within the DEP or HF lungs with probiotics, as we observe a reduction in expression of the proinflammatory cytokine TNF-α. The reduced proinflammatory and oxidative stress responses may play a role in decreasing collagen deposition. Since the effects of probiotics are primarily thought to be via a gut-lung mediated response, further studies investigating how these probiotics modulate inflammatory responses within the gastrointestinal tract are currently being pursued by our lab. Although we suspect the pathogenic bacteria are eliminated (or "out-competed") in this process, it is important to consider whether a shift in the commensal microbiota is ideal. Proteobacteria have been shown to induce IgA responses in the gastrointestinal tract during the first week of life (Mirpuri et al., 2014a). Proteobacteria's healthy balance within the commensal microbial profile has also been shown to protect against sepsis (Wilmore et al., 2018). However, in our knowledge, there is no evidence of Proteobacteria inducing immune responses within the adult lungs. Despite this, we checked to see if there were alterations in IgA with probiotic exposures. We observed an increase in IgA with probiotic intake, which is in cooperation with other studies suggesting that immune defenses may not be affected with the elimination of Proteobacteria from the lungs (Maldonado Galdeano et al., 2019). However, further studies are warranted to determine if susceptibility to bacterial or viral challenges is influenced by probiotic supplementation. Since only the expansion of Proteobacteria is concerning, rather than causing a shift from the commensal profile, strategies to reduce Proteobacteria selectively could be explored.

Eliminating nitrates within the lungs by either using antioxidants to scavenge radicals or inhibiting molybdenum-dependent enzymes that are utilized by Proteobacteria for nitrate utilization are viable alternatives for further characterization (M. Rekdal and Balskus, 2018).

Although we observe an alteration within the lung microbes at a 30-day time point, these responses may occur in phases and our observations at a certain time point within a group may not necessarily be identical to another time point, especially considering the lung microbes are highly variable. 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. However, it is important to consider that these exposures were done twice a week, and not daily, which allowed for some clearance and immune mediation of these particles within the lungs. Also, exposures done twice a week as opposed to daily exposures is similar in nature to human exposures on days of high vs. acceptable levels of PM. We encountered challenges as we sequenced these bacteria due to the low abundance of the lung microbes, and hence, we were unable to determine the genus/species of the bacteria that are expanding with DEP, which is a further limitation to the current study. Identifying and specifically targeting bacterial species that are expanding may also be a suitable alternative to curb their outgrowth.

3.6 Conclusion

Our observations within the lungs lead us to hypothesize that DEP exposures cause inflammation and microbial dysbiosis in a ROS-RNS mediated fashion, which is exacerbated by concurrent consumption of an HF diet. DEP exposures contribute to a cycle of inflammation and dysbiosis, which could be associated with increased susceptibility to developing fibrosis and associated decrease in lung function with long-term exposures. Air pollution exposures alone result in around 7 million premature deaths in a year, of which 43% are deaths due to COPD, and 26%

due to respiratory infection deaths (WHO Global Health Observatory data, 2016). There are close to 380 million people living with COPD and it is expected to become the leading cause of death in 15 years (Quaderi and Hurst, 2018). Understanding the lung microbiota shifts in response to the environment and diet to be a possible contributor in the development and worsening of lung diseases is paramount to identifying the mechanistic pathways involved in air-pollutant mediated effects on lung diseases and overall health.

3.7 Conflict of Interest

Probiotics were provided by Winclove Probiotics and funding from a grant received from the National Institute of Environmental Health Sciences at National Institute of Health was used to conduct some of the studies described, herein; however, the authors declare no conflict of interest or financial gains to these entities associated with this publication.

3.8 Funding

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3.9 Acknowledgements

The authors would like to thank Dr. Saskia vanHemert and Jojanneke Schmidt, at Winclove Probiotics, for their assistance in providing the Ecologic Barrier Probiotics for this study. Additionally, we would like to thank Dr. Usa Suwannasual, JoAnn Lucero, Tyler Armstrong, and Nicholas Mitchell for their assistance with animal exposures, as well as Dr. Imelda Norton from UNT Vivarium for her assistance in the monitoring of the animals throughout the study duration. We thank Dr. Art Goven for his guidance and assistance in the blood differential assessments.

Special mention to Benjamin Dubansky and Brooke Dubansky for their assistance in the histological aspects of this study.

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

The mechanism by which air pollutants induce detrimental outcomes within the lungs has been described in several studies, with oxidative stress mediating many of the observed lower respiratory outcomes (Lodovici and Bigagli, 2011). The lungs are uniquely vulnerable to air pollution exposures since they are the most extensive interfaces within the human body to be first exposed to toxicants within inhaled air. The composition of air pollutants that consist of both gaseous and particulate matter (PM: 2,5-10 μm) and the site of deposition of PM within the respiratory tract determines their pattern of toxicity. Gases such as ozone (O_3), sulfur dioxide (SO2), nitrogen dioxide (NO2) and carbon monoxide (CO) are among the most commonly described gaseous pollutants in ambient air (Kurt et al., 2016). Exposure to ozone in animal studies has been shown to induce inflammation and emphysematous changes in lung tissue, which was accompanied by a decrease in antioxidant nuclear factor erythroid-related factor 2 (Nrf2) and superoxide dismutase (SOD) activity (Wiegman et al., 2014). SO2 has been shown to upregulate proinflammatory cytokine expression and cause a Th1/Th2 imbalance contributing to the increased risk of asthma development (Li et al., 2014). Exposure to PM2.5, NO2, and O_3 is associated with lower lung function, lower forced expiratory volume in one second (FEV1*)* and forced vital capacity (FVC) (Rice et al., 2013). Polluted environments with higher levels of fine particulate matter contain redox-active metals and organic aerosols that can increase reactive oxygen species (ROS: OH, O_2^-, O_3, H_2O_2) levels within the lungs. The linings within the lungs contain antioxidants and surfactant proteins. O_3 and OH radicals react with these antioxidants and surfactants to form secondary oxidants. The ROS mediated damage is initiated when redox-active

pollutants and ROS undergo several redox reaction cycles. This process begins with the transfer of electrons from antioxidants to redox-active components of PM, such as quinones forming semiquinones. The quinones are regenerated by reacting with O_2 resulting in the generation of O_2^- radicals that are further converted into hydrogen peroxide (H_2O_2) (Charrier et al., 2014). H_2O_2 plays a central role in the oxidative stress and radical cycles that occur within the respiratory tract

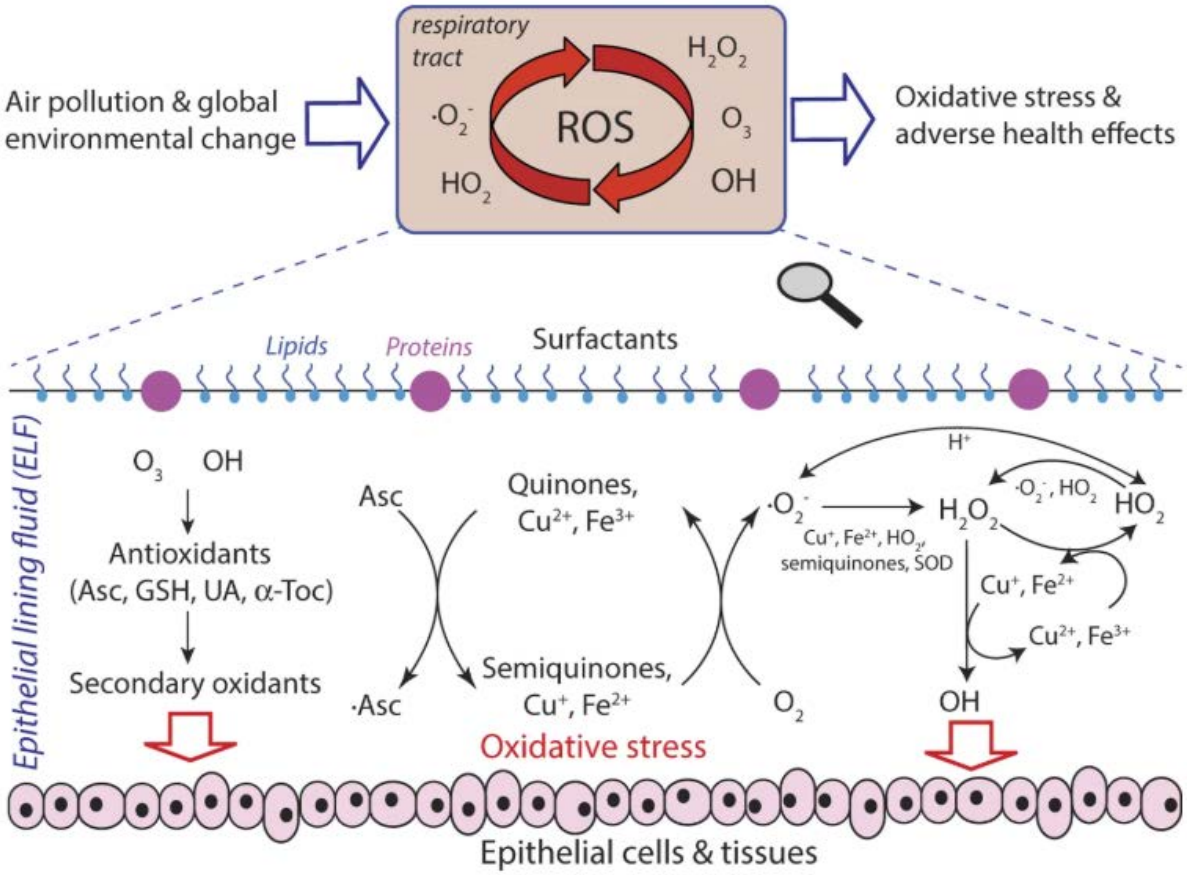


Figure 4.1: Reaction between environmental pollutants and reactive oxygen species (ROS) (Lakey et al., 2016).

Oxidative stress occurs as a consequence of an increased generation of reactive species or reduced activity of antioxidant defenses (Poljšak and Fink, 2014b). The toxicological consequences of oxidative stress are multifold if not compensated by antioxidant defenses. The pro-oxidants can damage cells or tissues, but the primary targets for oxidative damage are nucleic

acids, lipids, and proteins. Oxidation of DNA bases when not repaired has detrimental consequences on base pairing resulting in point mutations. Although such damage is usually repaired or cell death mechanisms are activated, in some instances, DNA oxidation is associated with tumor formation (Davalli et al., 2018). The significant consequences of oxidative protein damage are loss of catalytic function and impairment of structural protein activity (Reichmann et al., 2018). Oxidative stress also results in cellular membrane peroxidation and damage (Ramana et al., 2014). The lungs are highly predisposed to the generation of ROS due to the higher availability of oxygen within the airways. Like ROS, reactive nitrogen species (RNS) are also actively generated within the lungs during normal physiological events. In response to antigenic stimuli, activated immune cells such as macrophages and neutrophils produce extracellular ROS (O_2^-) and RNS (NO) as antimicrobial effectors. In our diesel exhaust particle (DEP) exposure study, we observed a significant increase in neutrophils acutely, whereas macrophages were present within tissues abundantly after 30 days of exposure to DEP. O_2^- and NO result in the formation of peroxynitrite ($ONOO^-$), a potent cytotoxic free radical (Figure 4.2) (Pacher et al., 2007).

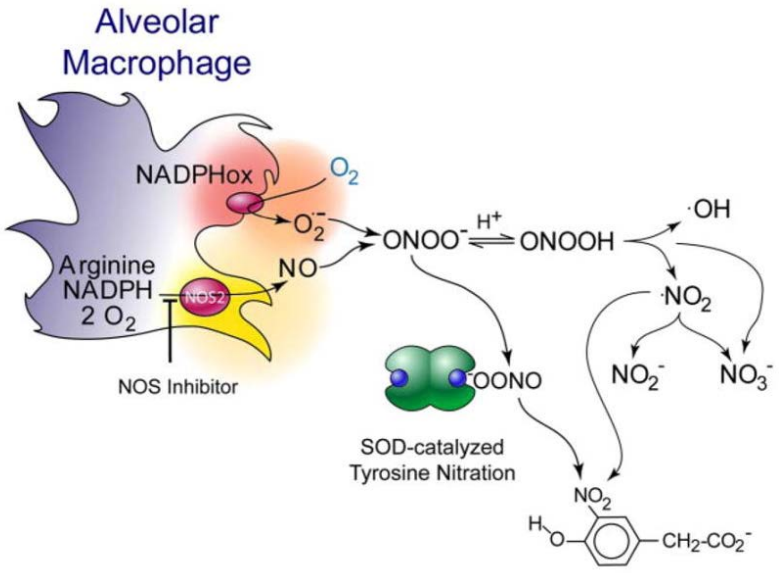


Figure 4.2: Peroxynitrite formation by alveolar macrophages (Pacher et al., 2007).

Under physiological conditions, peroxynitrite formation is low and antioxidant defenses minimize the oxidative damage. However, under conditions of toxicity, peroxynitrite being more reactive than its parent molecules causes pronounced cellular damage and cell death (Figure 4.3) (Pacher et al., 2007). Peroxynitrite formation has been implicated in many cell death signal transduction pathways including, mitogen-activated – protein kinases (MAPK) - ERK, JNK, and p38, that regulate many critical cellular functions (Figure 4.4) (Pesse et al., 2005). Our DEP exposure study reveals an increased presence of peroxynitrite within the lungs, most of which we suspect are present outside the cells due to the release from macrophages and neutrophils. At this time, we have not quantified intracellular ROS damage, but we suspect DEP exposures cause significant cellular damage as evidenced by other studies (Pan et al., 2004).

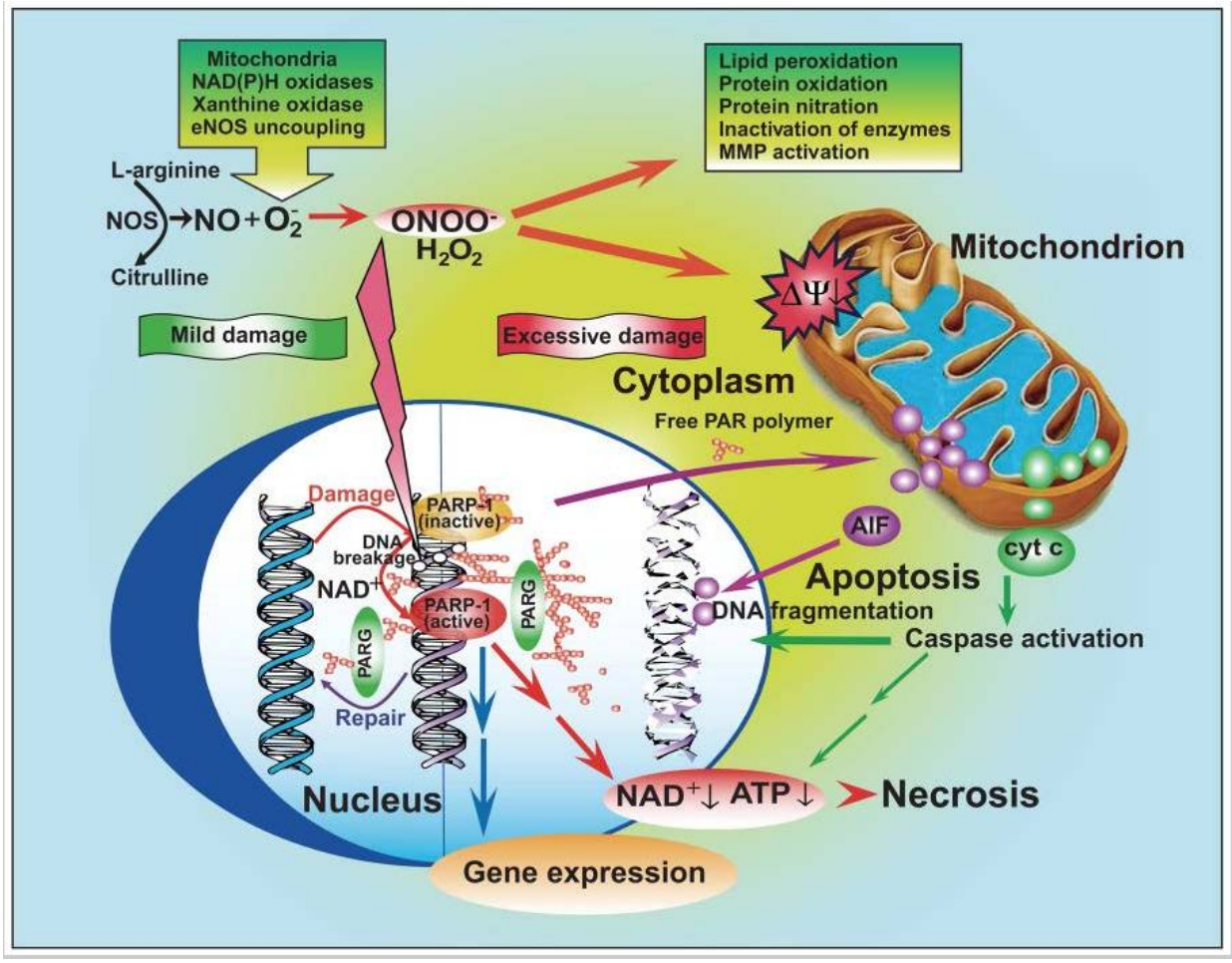


Figure 4.3: Molecular mechanisms involved in peroxynitrite-mediated cell death (Pacher et al., 2007).

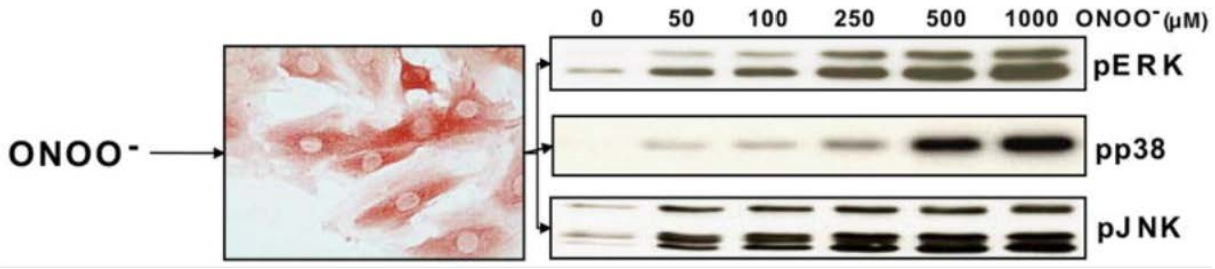


Figure 4.4: Western blots showing the activation pattern of the three MAPKs in cell line H9C2 by treatment with increasing concentrations of peroxynitrite.(Pesse et al., 2005)

Few studies have shown that reactive radicals and their products can regulate the lung's fibrotic process through directly activating fibroblasts (Hogaboam et al., 1998). It is plausible that the increase in peroxynitrite observed in our DEP exposure study likely contributed to pulmonary injury leading to the deposition of collagen surrounding the bronchial tubes.

With our mixed emissions (ME) study, we were unable to quantify the levels of peroxynitrite formation due to the unavailability of samples. Nevertheless, we suspect these radicals are present within these lungs causing many of the observed immune deficiencies. Neutrophilic influx is a characteristic feature observed in response to air pollutant exposures (Bauer et al., 2012). The gases in our ME exposure facilitate rapid diffusion of these particulates, possibly causing infiltration of neutrophils within the airway lumen. Neutrophils have been shown to produce vast quantities of ROS and RNS through their oxidant-generating systems – NADPH oxidase and nitric oxide synthase (NOS) (Grommes and Soehnlein, 2011). Neutrophils have a potent antimicrobial armor consisting of not only oxidant radicals but also proteinases such as neutrophil elastase. Under conditions of inflammation or repeated exposure to air pollutants as in our study, it is plausible that the release of this toxic protease aimed at destroying the irritant become unregulated to the extent that they cause tissue damage. Neutrophil elastase has been shown to induce pronounced lung tissue injury, and also degrade the secretory component of IgA. It is likely that increased neutrophilic recruitment within the lungs in response to ME is possibly

causing degradation of IgA in the groups exposed to high -fat (HF; 21% fat by content), resulting in reduced immune surveillance against the bacterial commensals present within the lungs. The diminished immune response may have resulted in the bacterial outgrowth of Proteobacteria within the lungs. Apart from the degradation of IgA observed with ME and HF exposures, we suspect the oxidative stress responses may increase nitrate availability providing selective nutrients for the outgrowth of Proteobacteria. Proteobacteria bloom has been documented to occur in inflammatory environments with excess ROS+RNS products (nitrates) (Scales et al., 2016). Our IgA results show that there is a significant interaction between HF diet and ME exposures suggesting that consumption of the HF diet is causing further harm to the host. High-fat diet consumption has been shown to induce higher levels of neutrophil recruitment, and the effects in combination with ME exposures may be causing an unregulated increase in neutrophil elastase leading to the degradation of IgA (Moorthy et al., 2016). Neutrophils are likely the primary cells responsible for both IgA degradation and oxidative stress responses, which could facilitate the outgrowth of pathogenic bacteria belonging to the Proteobacteria phylum. Although we were unable to test our hypothesis with the ME study, we were able to determine the presence of peroxynitrite and its involvement in the bacterial dysbiosis observed with the DEP exposure study.

In our DEP study, we observed an increase in peroxynitrite and detected increased expression of nuclear transcriptional factors and inflammatory gene expression. Oxidative stress has also been shown to induce the NF-κB mediated gene transcription (Kabe et al., 2005). The upregulation in NF-κB observed with DEP exposures may be either due to oxidative stress responses or due to the activation by Toll-like receptors (TLR) – 2 and 4. TLRs are activated in response to bacterial ligands such as gram-negative bacterial cell wall components - LPS and peptidoglycans from gram-positive bacteria. We suspect that the endotoxins present on the DEP

or the expansion of gram-negative Proteobacteria may stimulate TLR expression. Proteobacteria's expansion could be directly linked to the presence of oxidative products within the lungs in response to DEP exposure. Peroxynitrite formed as a result of ROS+RNS decomposes to release nitrates within inflammatory environments. Presence of extracellular nitrates aids in the anaerobic respiration of certain bacteria belonging to the Proteobacteria phylum. Nitrate reductase activity is crucial for the utilization of nitrates that are generated in excess in inflammatory environments. To determine the nitrate-reducing activity, a study was done to compare the genomes of bacterial phyla. The results showed that bacteria belonging to the Proteobacteria phylum had the highest nitrate reductase activity (Winter and Bäumler, 2014). These metabolic capabilities are restricted to only members of the Proteobacteria phyla since Firmicutes and Bacteroidetes, the major commensals in the GI tract in the absence of inflammation, have negligible nitrate reductase activities (Figure 4.5).

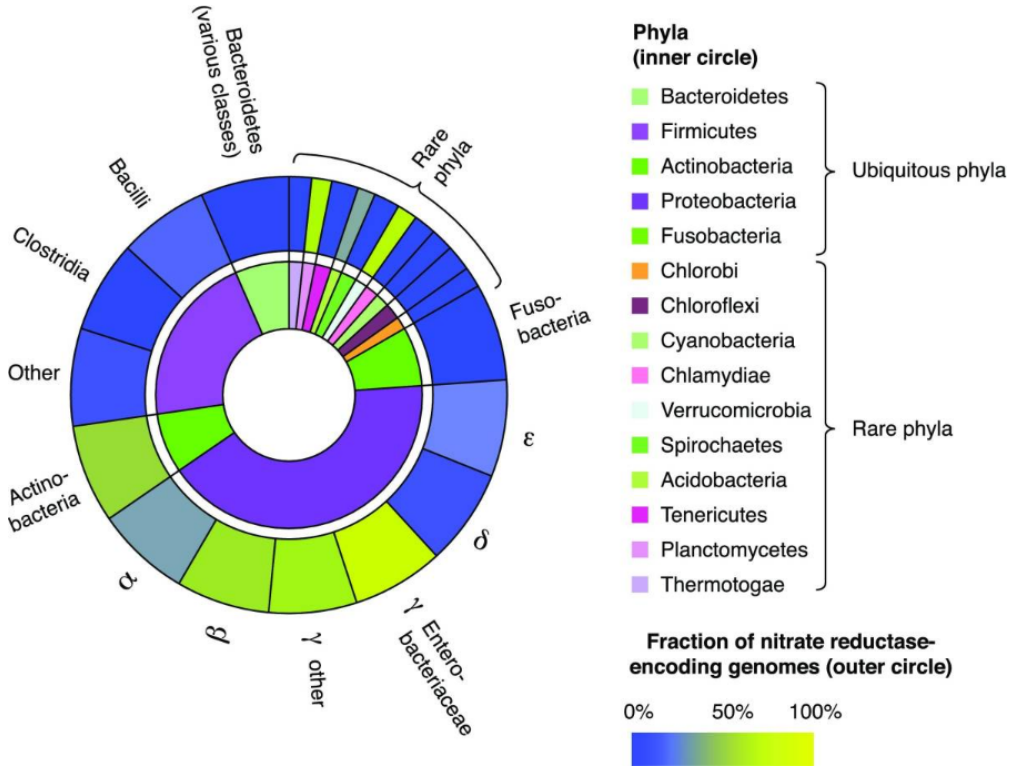


Figure 4.5: Nitrate reductase activity in gastrointestinal bacterial genomes (Winter and Bäumler, 2014)

Some of the opportunistic pathogens, such as *E.coli* and *P. aeruginosa,* have been shown to utilize nitrate in a 2-step nitrate reduction pathway where NO_3^- is first reduced to NO_2^- by NO_3^- reductase that is then followed by reduction of NO_2^- to ammonium by NO_2^- reductase (Figure 4.6) (Tiso and Schechter, 2015).

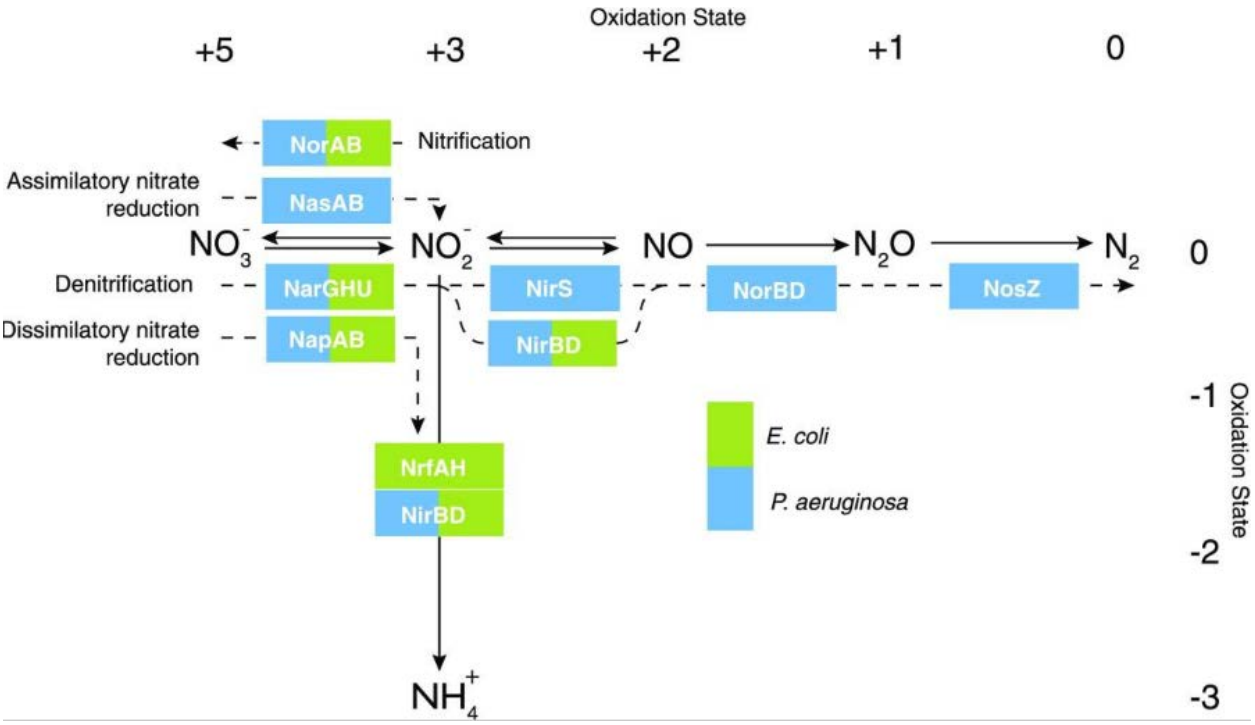


Figure 4.6: Nitrate utilization in *E.coli* and *P.aeruginosa* based on the information provided by KEGG (Tiso and Schechter, 2015). The rectangles represent an enzyme in either *E.coli* (light green) or *P.aeruginosa* (light blue). The letters inside the rectangle represent the proteins of the enzyme. Solid arrows represent the possible direction of the enzymatic reaction, whereas dotted arrows represent the actual direction of the pathway.

The expansion of Proteobacteria that was observed with both our exposure studies may be regulated by these mechanisms with increased nitrates providing excess nutrients for their selective outgrowth. Recent studies point to an inflammation – bacterial colonization feedback loop that is a continuous cycle present and possibly regulating inflammatory disease pathways. LPS present on gram-negative Proteobacteria have immunostimulatory properties that enable them to activate TLR-4 on the epithelial lining and myeloid cells, leading to increased phagocytosis. The same bacteria that can stimulate TLRs benefit the most from an inflammatory environment and

outcompete the other commensals taking advantage of the situation (Figure 4.7) (Scales et al., 2016).

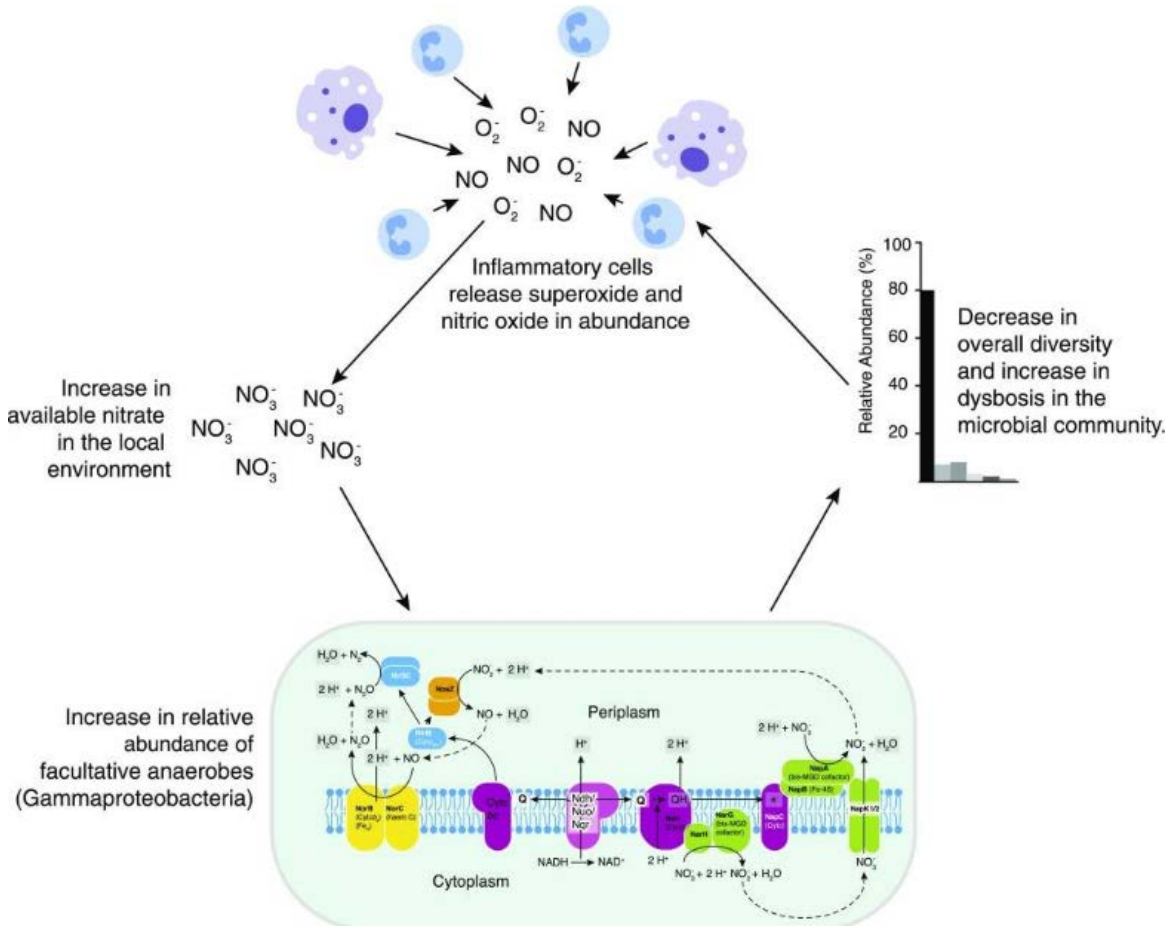


Figure 4.7: Proposed mechanism of Proteobacteria bloom in inflammation (Scales et al., 2016).

With both ME and DEP exposure studies, we show an expansion of the Proteobacteria phylum suggesting that air pollution exposures result in bacterial alterations within the lung. In the ME study, we also show that the bacterial diversity and richness is reduced due to the selective outgrowth of Proteobacteria. We observed Enterobacteriaceae belonging to the Gammaproteobacteria class to be expanding with our ME exposures. The expansion of Gammaproteobacteria is of concern due to the immunostimulatory properties of LPS on their cell walls. LPS contains a lipid – A moiety composed of a diglucosamine backbone attached with 5 or 6 acyl chains that are required for its endotoxic ability. Two genes *LpxL* (penta – acylated) and

LpxM (hexa-acylated) determine how LPS is acylated. The penta-acylated LPS is less immunostimulatory when bound to TLR4 compared to the hexa-acylated LPS (Brix et al., 2015). The *LpxL* gene is found in most gram-negative bacteria, but Gammaproteobacteria have both the *LpxL* and *LpxM* genes, and hence they are the more immunostimulatory version of LPS (Scales et al., 2016). The expansion of Proteobacteria is observed in many lung diseases, and studies have pointed to asthma patients having up to a 7-fold increase in the hexa-acylated LPS-producing bacteria (Brix et al., 2015), suggesting a link between the expansion of Proteobacteria and inflammatory lung diseases. Air pollution is a major risk factor for respiratory conditions and the fact that air pollutants in itself can cause an increase in Proteobacteria indicates their involvement in worsening lung conditions.

Our study has not been able to delineate the effects of air pollution-induced inflammation vs. Proteobacteria-induced inflammation since activation of NF-κB can occur directly due to endotoxins on PM and not just LPS on Proteobacteria. However, we were able to show that eliminating Proteobacteria was beneficial for inflammatory responses with the use of probiotics. At this time, we do not exactly know the mechanism by which probiotics mediate changes within the commensal lung phyla. We suspect that this could occur due to beneficial metabolites produced by probiotic strains that help to attenuate lung inflammation via the gut-lung axis. It is also plausible that there is active colonization of probiotic strains within the oral cavity, and by microaspiration, these microbes could travel within the lungs where they can exert their anti-inflammatory properties. Probiotics have been shown to induce antioxidant defenses, and also induce Tregs (Y. Wang et al., 2017; Zhao et al., 2013), both of which could be contributing to the decreased presence of peroxynitrite and proinflammatory TNF-α within the lungs. In the absence of peroxynitrite, the availability of nitrates reduces which impedes the outgrowth of

Proteobacteria. We also observed probiotics to increase immunoglobulin (IgA) within the lungs, which suggests that probiotics not only suppress inflammation, but also enhance airway defenses. The decrease in the expansion of Proteobacteria observed with probiotic treatment may also be due to the increase in immunosurveillance by IgA. Proteobacteria have been selectively shown to induce IgA producing B cells in the GI tract, especially during the first week of life (Mirpuri et al., 2014b). However, this has never been documented to occur within the lungs.

We also observed that the DEP-induced inflammation results in increased deposition of collagen surrounding the bronchioles. It is likely that the increase in TLR4 observed, and the subsequent activation of NF-κB proteins play a role in enhancing the inflammatory responses, and the continuous nature of inflammation may have resulted in the deposition of extracellular matrix components. TLR4 has been implicated in the pathophysiology of bleomycin-induced lung fibrosis (Li et al., 2013). We also observed an increase in macrophages within DEP and HF diet exposed lungs and we suspect macrophages to be involved in the increased expression of TNF-α. TLRs can activate macrophages with the subsequent transcription of inflammatory mediators like TNF-α (Gallego et al., 2011). NF-κB signaling is required for sustained cytokine expression in macrophages (Dorrington and Fraser, 2019). We observe TLR4 upregulated with high-fat diet consumption alone. Saturated fatty acids have been shown to stimulate an inflammatory response through the TLR-2 and TLR-4 signaling pathway (Hwang et al., 2016). Thus, it is plausible that most of the inflammatory responses observed with DEP-exposure are mediated via a TLR mediated NF-κB signaling; however, additional studies are necessary to determine mechanistic signaling pathways involved in this study.

4.2 Conclusion

Numerous studies in the past few decades have documented air pollution exposures to be

a significant risk factor associated with both development and exacerbation of many respiratory conditions. Although the inflammatory events occurring as a result of air pollutants have been studied extensively, the newly identified lung microbiome's involvement remains vastly unknown. The findings from this study suggest that exposure to both a mixture of gasoline and diesel emissions and diesel exhaust particles can result in lung microbial dysbiosis with an outgrowth of Proteobacteria. Additionally, with this study, we were also able to show the effects of high-fat diet consumption alongside air-pollution exposures that result in further exacerbation of underlying inflammatory responses. The mechanistic studies conducted revealed that the expansion of Proteobacteria was directly linked to the availability of air pollutant-induced ROS+RNS releasing nitrates within the lung environment. In the absence of these nitrates, the expansion of Proteobacteria was curbed efficiently. Pathogenic bacteria belonging to the Proteobacteria phylum are observed to be present in many lung diseases such as asthma, COPD, emphysema, etc. (Larsen et al., 2015). The expansion of Proteobacteria with air-pollution exposures is concerning due to their immunostimulatory properties that can increase inflammation within the lungs and promote inflammatory diseases. We observed activation of TLR-2 and 4, as well as the subsequent activation of NF-κB, which may be responsible for the chronic inflammation generated within these lungs that resulted in the progressive deposition of collagen. Our study also highlights a viable alternative to remediating the air-pollution induced inflammation within the lungs by using probiotics. The proposed mechanism of probiotic treatment is understood to be the initiation of antioxidant defenses and anti-inflammatory T regulatory cell responses since we observed a significant decrease in both ROS+RNS products and suppression of proinflammatory cytokines. Probiotic treatments helped to attenuate the inflammatory response, and also aided in enhancing immune surveillance by inducing IgA production that possibly played a role in curbing the

outgrowth of Proteobacteria as well.

Collectively, the results of this study suggest that environmental exposure to traffic-generated pollutants concomitant with HF diet consumption contributes to inflammation and oxidative stress, resulting in the expansion of Proteobacteria. Such findings may provide insights into therapeutic alternatives for the prevention and treatment of adverse outcomes in inflammatory lung diseases, especially in individuals living and working in heavily polluted regions.

APPENDIX:

ADDITONAL RESULTS

A.1 IgA ELISA of Lung Tissues of ApoE$^{-/-}$ Mice on a High-Fat Diet Exposed to Either Filtered Air or Mixed Vehicle Emissions

Lung tissues were homogenized in a beat beater with sterile saline and the supernatants were used for Immunoglobulin A (IgA) ELISAs. The concentration of IgA (Fisher Scientific, EMIGA) was measured in 10-fold diluted lung tissue homogenates 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.

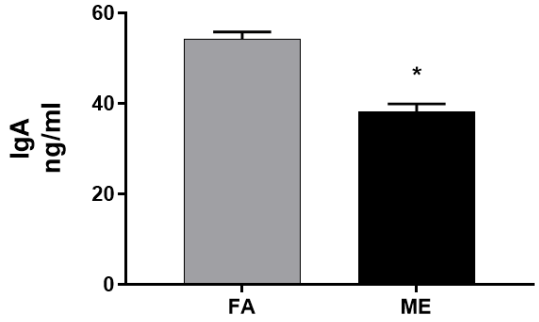


Figure A.1: IgA decreases in response to mixed vehicle emission exposures in ApoE$^{-/-}$ mice placed on a high fat diet. ELISA of IgA in lung tissue homogenates of ApoE$^{-/-}$ mice placed either on a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body inhalation to a mixture of gasoline and diesel engine exhaust (ME: 30 µg PM/m3 gasoline engine emissions + 70 µg PM/m3 diesel engine) for 6h/d, 7 d/wk for a period of 30 days. Data are depicted as the mean ± SEM with *p<0.05 compared to FA by 2-tailed student's *t* test.

We observed a similar decrease in IgA in the ME study done on C57Bl/6 mice in the groups that were exposed to high-fat diet. This decrease in IgA observed in both animal models may be due to a neutrophilic infiltration resulting in the degradation of the secretory IgA which is observed in many lung diseases especially COPD ((McCullagh et al., 2017; Pilette et al., 2003).

A.2 Quantification of Major Phyla in Lung Tissues of ApoE$^{-/-}$ Mice on a High-Fat Diet Exposed to Either Filtered Air or Mixed Vehicle Emissions

DNA from homogenized lung tissues 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 16S rRNA analysis, samples were normalized to Eubacteria utilizing known-concentration standards.

Table A.1: Primer sequences used for qPCR analysis.

Bacteria	Sequence
Eubacteria FP	5′-ACTCCTACGGGAGGCAGCAGT-3′
Eubacteria RP	5′-ATTACCGCGGCTGCTGGC-3′
Proteobacteria FP	5′-GTGCCAGCMGCCGCGGTAA-3′
Proteobacteria RP	5′-GCCTCAAGGGCACAACCTCCAAG-3′
Bacteroidetes FP	5′-GGTTCTGAGAGGAGGTCCC-3′
Bacteroidetes RP	5′-GCTGCCTCCCGTAGGAGT-3′
Firmicutes FP	5′-GGAGYATGTGGTTTAATTCGAAGCA-3′
Firmicutes RP	5′-AGCTGACGACAACCATGCAC-3′

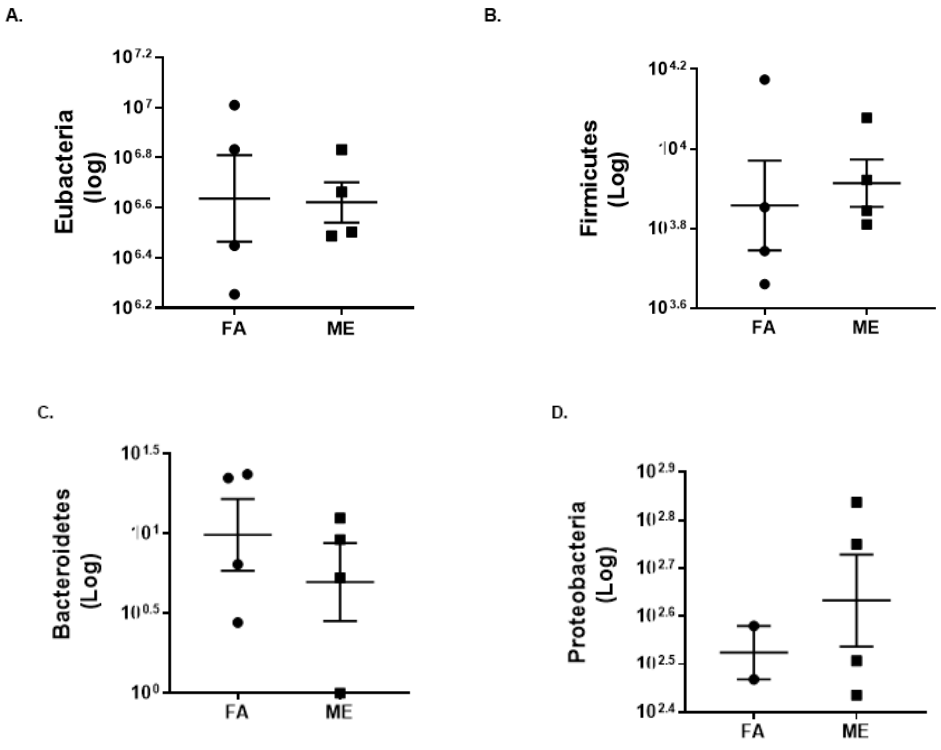


Figure A.2: Quantification of bacterial phyla after exposure to mixed vehicle emissions in ApoE$^{-/-}$ mice placed on a high fat diet. qPCR of lung tissue homogenates for (A) total bacteria (Eubacteria) and phyla - (B) Firmicutes, (C) Bacteroidetes, (D) Proteobacteria in ApoE$^{-/-}$ mice placed on a high-fat (HF) diet and exposed to either filtered air (FA) or whole-body inhalation to a mixture of gasoline

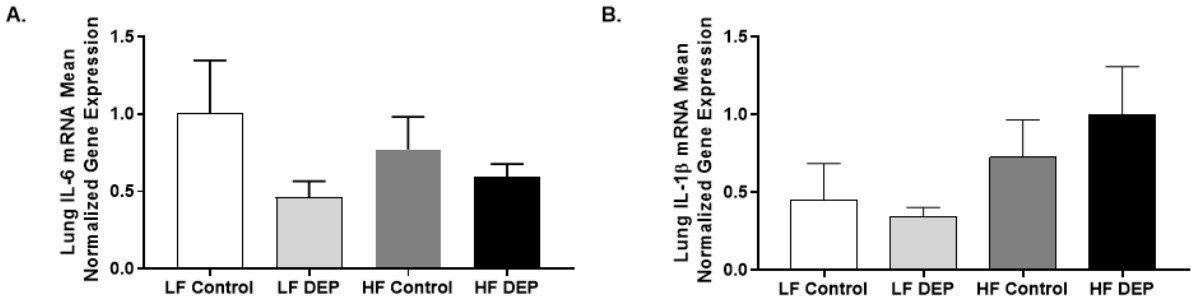
and diesel engine exhaust (ME: 30 µg PM/m3 gasoline engine emissions + 70 µg PM/m3 diesel engine) for 6h/d, 7 d/wk for a period of 30 days.

A.3 IL-1β, IL-6, IL-17 mRNA Expression in Lung Tissues of C57Bl/6 Mice Exposed to Diesel Exhaust Particles

Total RNA was isolated from the lungs (n=6 per group) using an RNAEasy Mini kit (Qiagen, Valencia, CA) per kit instructions, and cDNA was synthesized using an iScript cDNA Synthesis kit (Biorad, Hercules, CA; Cat. #170–8891). Real-time PCR analysis of markers of inflammation IL-1β, IL-6 IL-17 was conducted using specific primers (Table A.2) and SYBR green detection (Sso Advanced Universal SYBR Green Supermix, Biorad; Cat #172–5271), following manufacturer's protocol. Samples were processed on a Biorad CFX96, and $\Delta\Delta C_T$ values calculated and normalized (to GAPDH), as previously described by our laboratory (Lund et al., 2011, 2009).

Table A.2: Primer sequences used for qPCR analysis

	Sequence
IL-1β FP	5′-CCTTCCAGGATGAGGACATGA-3′
IL-1β RP	5′-TGAGTCACAGAGGATGG-GCTC-3′
IL-6 FP	5′-CCGGAGAGGAGACTTCACAG-3′
IL-6 RP	5′-GGAAATTGGGGTAGGAAGGA-3′
IL-17 FP	5′-TCCCTCTGTGATCTGGGAA-3′
IL-17 RP	5′-CTCGACCCTGAAAGTGAAGG-3′



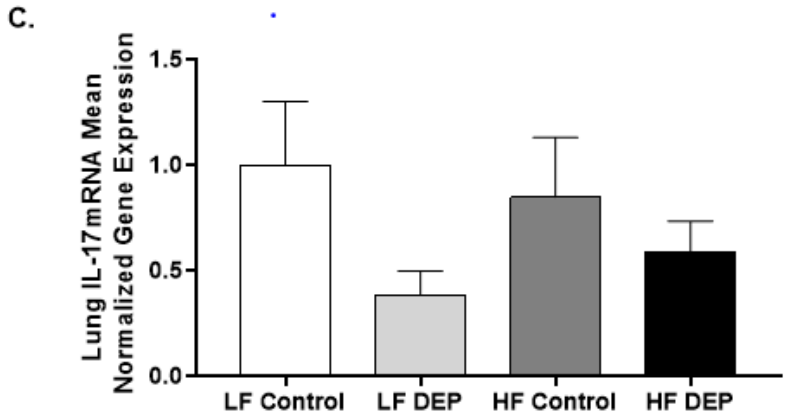


Figure A.3: Proinflammatory gene expression in lungs of C57Bl/6 mice exposed to diesel exhaust particles. Mean normalized gene expression of (A) IL-6, (B) IL-1β, and (C) IL-17 mRNA transcript within the lungs of male C57Bl/6 wildtype mice, on either control (LF) or high-fat (HF) diet exposed to either saline (control) or diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days as determined by RT-qPCR.

A.4 Antimicrobial Peptides mBD-2 and mBD-3 mRNA Expression in Lung Tissues of C57Bl/6 Mice Exposed to Diesel Exhaust Particles

Total RNA was isolated from the lungs (n=6 per group) using an RNAEasy Mini kit (Qiagen, Valencia, CA) per kit instructions, and cDNA was synthesized using an iScript cDNA Synthesis kit (Biorad, Hercules, CA; Cat. #170–8891). Real-time PCR analysis of antimicrobial peptides mBD-2 and mBD-3 was conducted using specific primers and SYBR green detection (Sso Advanced Universal SYBR Green Supermix, Biorad; Cat #172–5271), following manufacturer's protocol. Samples were processed on a Biorad CFX96, and $\Delta\Delta C_T$ values calculated and normalized (to GAPDH), as previously described by our laboratory (Lund et al., 2011, 2009).

Table A.3: Primer sequences used for qPCR analysis

	Sequence
mBD-2 FP	5′-AAGTATTGGATACGAAGCAG-3′
mBD-2 RP	5′-TGGCAGAAGGAGGACAAATG-3′
mBD-3 FP	5′-GCATTGGCAACACTCGTCAGA-3′
mBD-3 RP	5′-CGGGATCTTGGTCTTCTCTA-3′

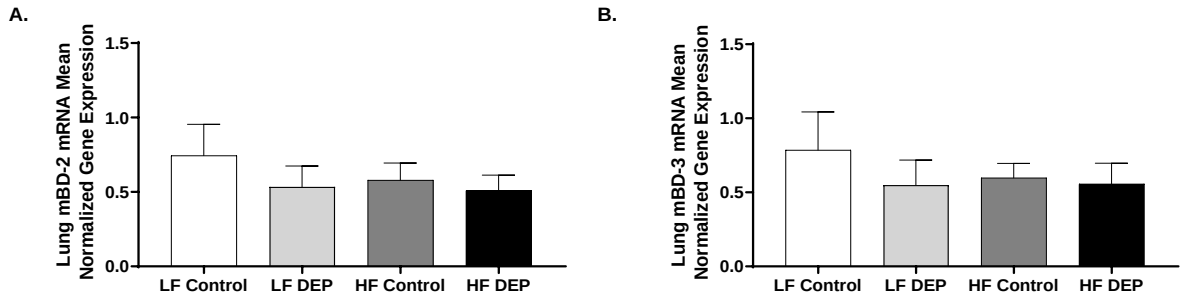


Figure A.4: Antimicrobial peptide expression in lungs of C57Bl/6 mice exposed to diesel exhaust particles. Mean normalized gene expression of (A) mBD-2 and (B) mBD-3 mRNA transcript expression within the lungs of male C57Bl/6 wildtype mice, on either control (LF) or high-fat (HF) diet exposed to either saline (control) or diesel exhaust particles (DEP – 35 µg PM) twice a week for a total of 30 days as determined by RT-qPCR.

A.5 Lipids in Lung Tissue Sections of C57Bl/6 Mice Exposed to Diesel Exhaust Particles

Lipids within the lungs were stained using nile red. Slides were imaged under fluorescent microscopy at 40x with the appropriate excitation/emission filter, digitally recorded, RGB overlay signals were split and analyzed for specific fluorescence using image densitometry with Image J software (NIH).

Studies have shown that exposure to certain air pollutants can result in lipid accumulation within the lungs that can affect the phagocytic ability of macrophages (Thimmulappa et al., 2012). We performed lipid staining to observe if there was excessive lipid accumulation within the lungs of DEP exposed mice. Interestingly we observed that there was a decrease in lipids present within these lungs. We suspect this may be due to damage caused due to the reactive oxygen species produced in the lungs in response to DEP. The surfactant fluid that helps to maintain surface tension within the alveolar regions are composed of a mixture of lipids and proteins. Studies have shown ROS can damage surfactant proteins and disrupt the biophysical activity that can alter the surfactant lipids (Andersson et al., 1999; Haddad et al., 1993). However, further studies are required to understand the underlying mechanisms.

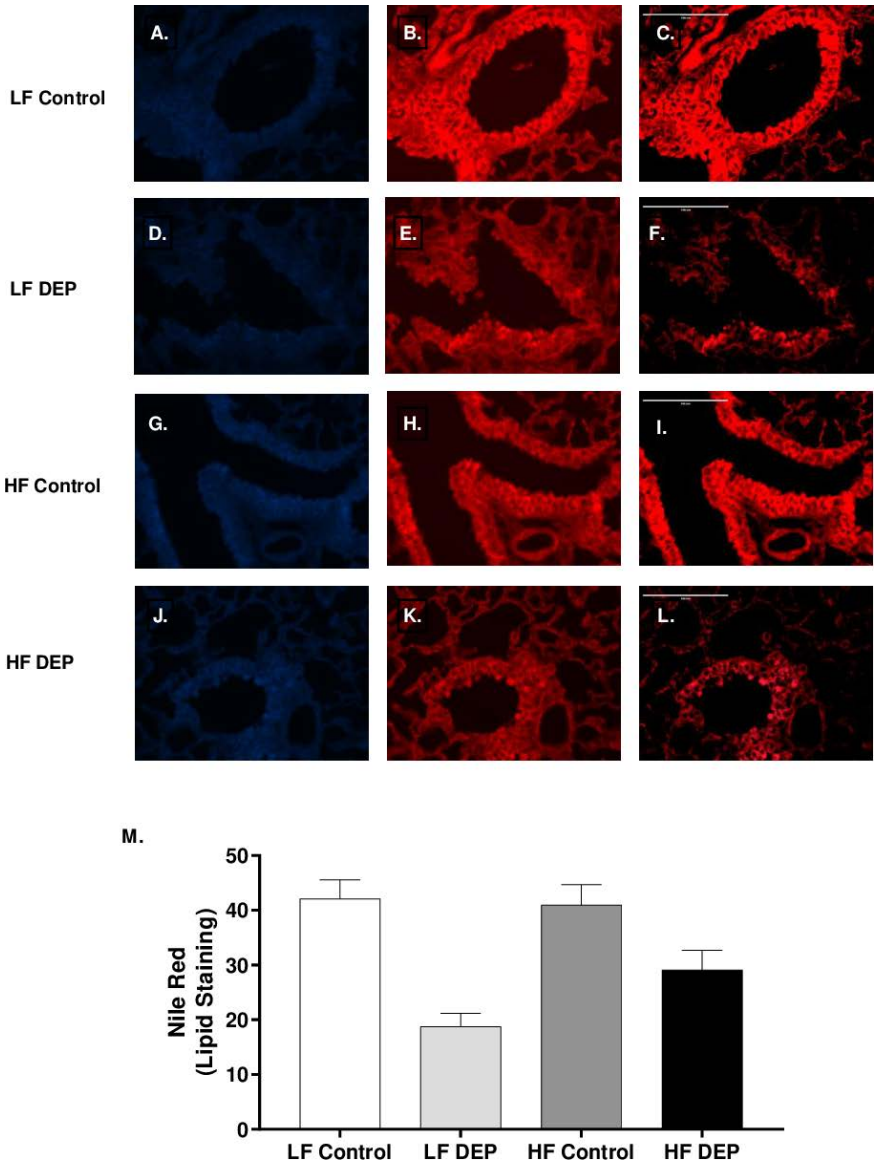


Figure A.5: Representative images of nile red (lipid) expression in the lungs of C57Bl/6 mice on a control (LF; A-C) or high-fat (HF; G-I) diet exposed to saline (control) or on a LF (D-F) or HF diet (J-L) exposed to diesel exhaust particles (DEP – 35 µg PM/m3) twice a week for a total of 30 days. Red fluorescence indicates nile red, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (green; B, E, H, K) panels. (M). Graph of histology analysis of lung nile red fluorescence. 40x magnification, scale bar = 100 µm. Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

A.6 ACE2 Receptor Expression in Lungs of C57Bl/6 Mice Exposed to Diesel Exhaust Particles

SARS-CoV-2, the virus causing the coronavirus disease (COVID), uses the angiotensin-converting enzyme 2 (ACE2) to facilitate entry into host cells. We sought to investigate if exposure

to diesel exhaust particles alters the expression of the ACE2 receptor. Lung tissues were stained with ACE2 antibody (1:250; Santa Cruz Biotechnology E-11) and anti-mouse Alexa Fluor 546. Slides were imaged under fluorescent microscopy at 40x with the appropriate excitation/emission filter, digitally recorded, RGB overlay signals were split and analyzed for specific fluorescence using image densitometry with Image J software (NIH).

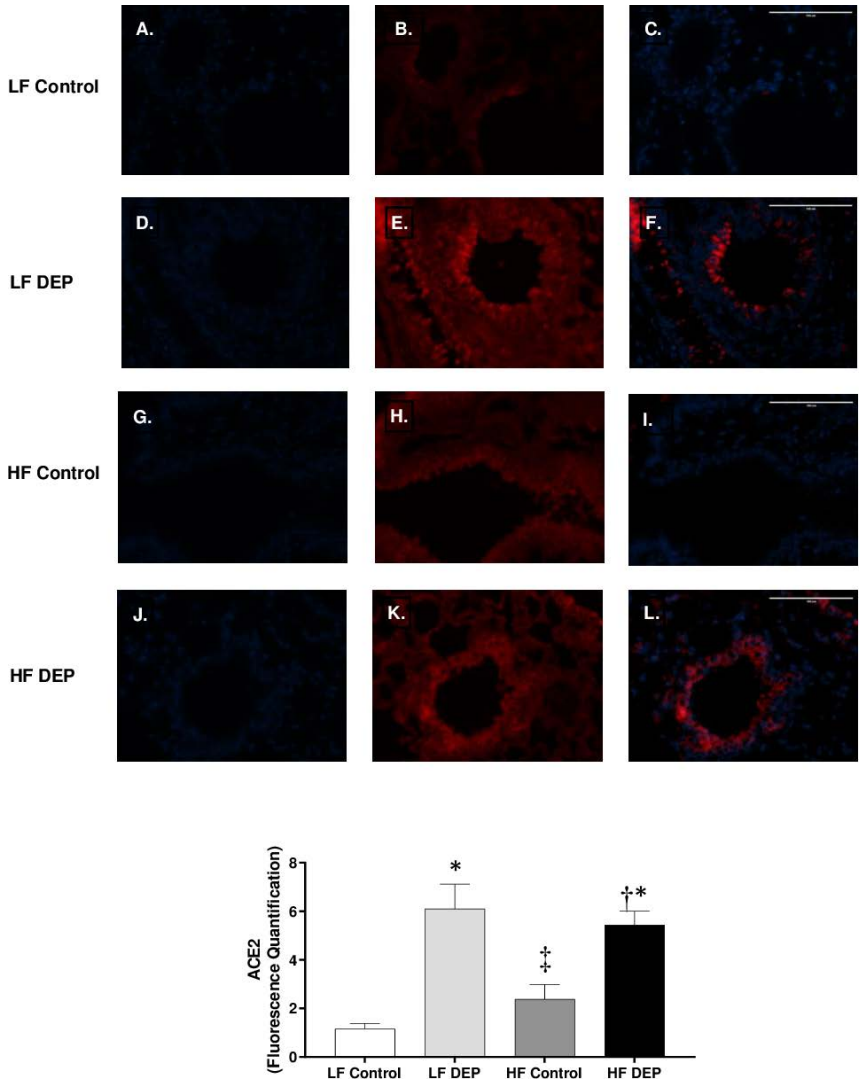


Figure A.6: Representative images of ACE2 receptor expression in the lungs of C57Bl/6 mice on a control (LF; A-C) or high-fat (HF; G-I) diet exposed to saline (control) or on a LF (D-F) or HF diet (J-L) exposed to diesel exhaust particles (DEP – 35 µg PM/m3) twice a week for a total of 30 days. Red fluorescence indicates ACE2 expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (red; B, E, H, K) panels. (M) Graph of histology analysis of lung ACE2 fluorescence. 40x magnification, scale bar = 100 µm.Data are depicted as mean ± SEM with *p<0.05 compared to LF Control, †p<0.05 compared to HF Control, ‡p<0.05 compared to LF DEP by two way ANOVA.

A.7 Probiotic Supplementation Decreases ACE2 Receptor Expression in Lungs of C57Bl/6 Mice Exposed to Diesel Exhaust Particles

Lung tissues were stained with ACE2 antibody (1:250; Santa Cruz Biotechnology E-11) and anti-mouse Alexa Fluor 546. Slides were imaged under fluorescent microscopy at 40x with the appropriate excitation/emission filter, digitally recorded, RGB overlay signals were split and analyzed for specific fluorescence using image densitometry with Image J software (NIH).

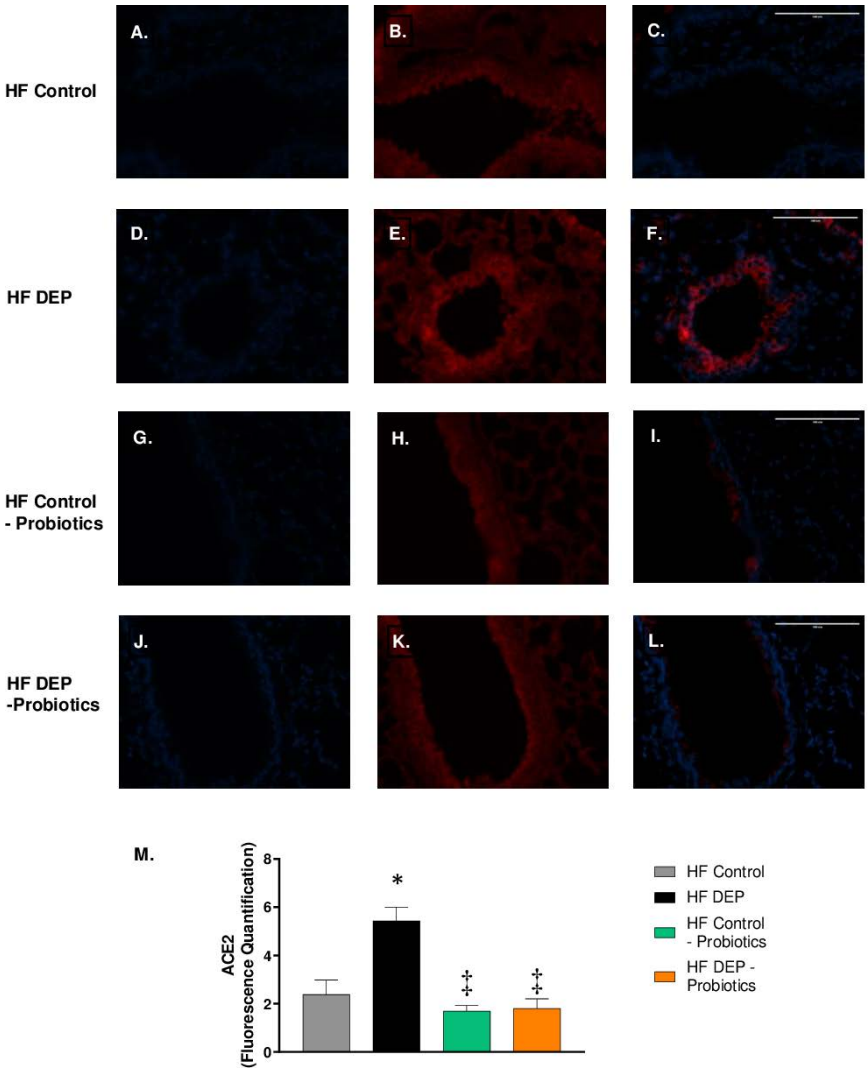


Figure A.7: Representative images of ACE2 receptor expression in the lungs of C57Bl/6 mice, on a high-fat (HF) diet exposed to either (A–C) saline, (D–F) DEP – 35 µg PM, or (G–I) saline and probiotics - 0.3 g/day (~7.5 x10^8 cfu/day) and (J-L) DEP and probiotics. Red fluorescence indicates ACE2 expression, blue fluorescence is nuclear staining (Hoechst). Right panels (C, F, I, L) are merged figures of left (blue; A, D, G, J) and center (red; B, E, H, K) panels. (M) Graph of histology analysis of lung ACE2 fluorescence. 40x magnification, scale bar = 100 µm. Data are depicted as mean ± SEM with *p<0.05 compared to HF Control, ‡p<0.05 compared to HF DEP by two way ANOVA.

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