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1. Introduction

We are just beginning to understand how early life exposure to diverse bacterial communities train the immune system. This early life training plays a critical role in screening and facilitating appropriate responses to external stimuli. For instance, urban living, a western diet, and antibiotic use are all associated with a decrease in microbial diversity (Sonnenburg and Sonnenburg, 2019). All three of these factors have steadily increased in the United States and Europe while coinciding with an increase in atopic disease. In contrast, previous research has shown that lifestyles associated with rural environments with higher microbial exposure via soil results in a lower risk for developing allergic diseases (Ruokolainen et al., 2017) and murine asthma models show that soil exposure-induced increases in microbial diversity decrease experimental asthma symptoms (Ottman et al., 2018).

Exposure to diverse microbial communities has also been demonstrated to modulate the immune response to acute infectious diseases. Previous studies have measured the immune response following an influenza challenge that either utilized antibiotics to decrease gut microbial diversity (Gonzalez-Perez and Lamouse-Smith, 2017; Gonzalez-Perez et al., 2016; Abt et al., 2012) or a fecal transplant to increase gut microbial diversity (Rosshart et al., 2017). These studies have consistently shown that mice with greater gut diversity had a less severe response to infection. Thus, these studies indicate that it is beneficial to the host to harbor a diverse gut microbiome prior to exposure to influenza.

Influenza severity and mortality are driven by excessive pulmonary inflammation and pro-inflammatory cytokine production (Bradley-Stewart et al., 2013). It is believed that environmentally acquired microbiota can help to modulate the immune response to influenza, and thereby prevent an excessive immune response. Several studies have suggested mechanisms for this gut microbiome driven immune modulation. Lin et al. (Lin et al., 2020) showed that environment can outweigh genetics as an immunological driver by comparing the effect of rewilding of mice versus *Atg16l1*^{T316A/T316A}, *Atg16l1*^{T316A/+} and *Nod2*^{-/-} genetic variants on variation in T cell populations. Zhou et al. (Zhou et al., 2016) found significantly lower total serum immunoglobulin E level in mice upon exposure to soils. Despite data supporting associations between gut microbiota and immune responses, more recent research suggests that in the lung, lung microbial communities may have a more dominant effect on lung immune activation than gut communities. This is evidenced by data showing that baseline inflammatory cytokine levels, as markers of immune activation, were more strongly correlated with variation in lung microbiome than the gut microbiome (Dickson et al., 2018). Therefore, when considering microbiome effects on lung immune responses, a greater focus is needed on changes in the lung microbiome that direct lung immunity effects.

While soil exposure has been shown to influence allergic inflammation and cytokine release, to date no study has addressed the impact of soil exposure on acute pulmonary infection responses or the effect of exposure to different soils with varying levels of microbial diversity (e.g. urban vs rural soil) in juvenile mice. In this study, we test the hypothesis that host gene expression in response to an influenza challenge will be modulated by soil exposure. To explore this, we exposed mice to one of several different soil types for five weeks (before, during and after an influenza challenge), and monitored changes in the fecal and lung microbiomes by longitudinal 16S amplicon sequencing and RNA-Seq to determine immune system homeostasis alterations based on urban vs. rural soil exposure and their effects following influenza infection. This study provides evidence for how individual microbial exposure may be influencing immune system homeostasis and its implications for acute pulmonary infection.

2. Methods

2.1. Study design and sample collection

This study was completed with the approval of Duke University Institutional Animal Care and Use Committee (IACUC) under protocol A102–18-04. Male 4-week-old BALB/c mice were split evenly into one of four cohorts: Control, River, Pine, and Road. The Control mice were raised with standard corn cob bedding whereas the remaining mice were raised with clean bedding amended with 300 mL of one of three different types of soil. The soil exposure continued throughout the experiment and 300 mL of new soil was added with bi-weekly cage changes. The soils used to amend the cage bedding were characterized as having high (Pine), medium (River), and low (Road) microbial diversity. The River and Pine soil were collected from Duke Forest and the Road soil was collected adjacent to Highway 15–501 in Chapel Hill, North Carolina. The GPS locations for the soil sample sites are available in the supplemental material (Supplemental Table 1). Prior to introduction into the cages, the soils were screened via PCR for mouse pathogens (Supplemental Table 2). The soils were collected all at once and divided into 300 mL aliquots, and stored sealed at 4 °C until added to the cages. To reduce cross contamination between cages, the mice were placed in isolator cages and gloves were changed between handling mice from different environmental exposure. All mice were given a standard diet and the cages were distributed reverse osmosis treated water through a centralized Lixit® system that was fed to each cage in parallel.

After 32 days of standard rearing with amended soils, the mice were exposed via intra-nasal instillation to either live influenza A (PR8) virus or heat inactivated (HI) virus at a concentration of 250 PFUs in 40 µL. The original virus stock was purchased from Charles River (Norwich, CT, Cat# 10100374). Heat inactivation was achieved by heating aliquots at 60 °C for 1 h and immediately cooled on ice prior to –80 °C storage. The heat inactivation was confirmed by infecting MDCK cells with the HI or PR8 virus at 10 multiplication of infection. There were plaques observed with the PR8 virus while the HI virus had no effect. To achieve the 250 PFU concentration, a plaque assay was performed with 10-fold serial dilutions plated on confluent MDCK cells in duplicate. Then after virus PFU was calculated from the assay the stock solution was appropriately diluted. Additionally, the PR8 stock has been utilized in prior studies (Drury et al., 2023; Vose et al., 2021) and HI virus was utilized to ensure the observed inflammatory/immune response was due to an active infection. Mice were initially weighed at time of HI or PR8 exposure and subsequently on days 3, 5, and 7 post exposure, then euthanized. Day 7 post exposure was chosen as the final timepoint as it is when the inflammatory response is most robust. The mice were anesthetized with isoflurane and euthanized with 250 µL of urethane at 250 mg/mL. Then, a bronchoalveolar lavage (BAL) with a 1 × PBS solution was performed as previously described (Tighe et al., 2018). The BAL fluid was centrifuged to collect the cells and the supernatant was removed. The cells were then treated with 0.5 mL 1 × RBC lysis buffer and resuspended in 0.5 mL PBS. Total cell counts were collected with a Cellaometer K2 (Nexcelom Biosciences, Lawrence, MA). A 100 µL sample of the cells were then placed on a cytospin 4 (Thermo Scientific, Waltham, MA), dried and stained with Hema 3 and examined under a light microscope to define the total number of macrophages, leukocytes, eosinophils, and neutrophils. The right superior and inferior lung lobes were used for RNA-Seq and viral qPCR while the right middle lobe was used for DNA sequencing. All tissue samples were flash frozen in liquid nitrogen and stored at –80 °C until further processing. The study utilized 24 mice divided equally into the soil by HI or PR8 exposure groups (*n* = 3) and then repeated with two more 24 mouse cohorts twice, giving a final total sample size of 72. Fig. 1 depicts a summary of the study.

Fecal samples were collected at days 0, 14 and 35 (3 days post HI or PR8 exposure). Each mouse was placed into a large cup, weighed, and then kept until at least two fecal pellets were collected. The cups were cleaned with a 0.5 % hydrogen peroxide solution between the weighing

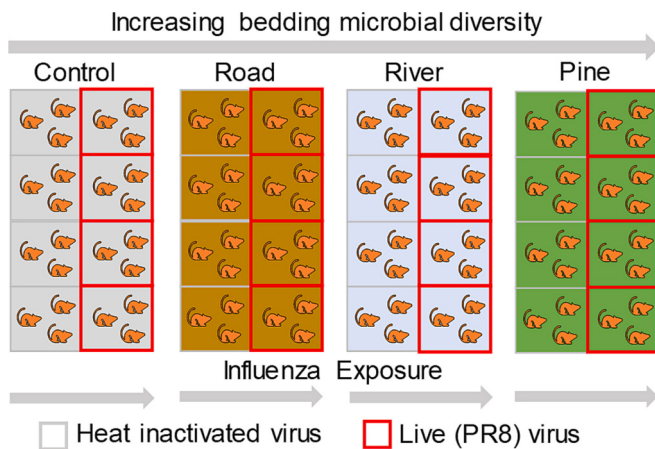


Fig. 1. Pictorial representation of the study cohorts/design. Each box represents one cage with three mice housed per cage. The bedding conditions are indicated by the difference in background shading (control, low (road), medium (river), and high (pine) microbial diversity soil bedding). The red outline indicates those mice that were exposed to a live influenza virus (PR8), the non-outlined mice were exposed to heat inactivated (HI) virus.

of each mouse. Approximately 1 g of bedding from each cage were also collected on the same days that fecal samples were collected (days 0, 14, and 35). After collection, these samples were stored in microcentrifuge tubes at -80°C until sample DNA was extracted.

2.2. Extraction of genomic DNA, RNA and sequencing

Total genomic DNA was extracted from each sample using a phenol/chloroform extraction protocol outlined in the Supplemental Material. The isolated DNA was used as a template for PCR amplification of the V4 region of the 16S rRNA gene (515F FWD: GTGYCAGCMGCCGCGGTAA; 806R REV: GGACTACNVGGGTWTCTAAT, Earth Microbiome Project). Samples were prepared using the standard Illumina workflow protocol for 16S amplicon library preparation (Illumina, San Diego, California). Then 250 bp paired-end sequencing was performed on an S-Prime flowcell with an Illumina NovaSeq 6000 at the Duke Sequencing and Genomic Technologies Core Facility (Duke University, Durham, NC).

Raw FASTQ files were quality filtered, trimmed, denoised, merged, checked for chimeras, and assigned taxonomy to generate amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomic assignments for the 16S amplicons were made using the Silva v138 database (Quast et al., 2013). Contaminating sequences were detected from negative control samples and removed using the package Decontam (Davis et al., 2018). Further sequences were removed if they were present in more than two of the eight blank samples with more than three reads per blank sample.

Total RNA was extracted from whole lung tissue using a RNeasy kit (Qiagen, Hilden, Germany) with the following modifications. Prior to beginning the standard RNeasy protocol, TRIzolTM (Invitrogen, Waltham, MA) was added to the sample at 1 mL per 0.1 g. The sample was then homogenized with a tissue-tearor on ice for 1 min. Then 0.2 mL of chloroform was added per mL of TRIzolTM, the sample was shaken vigorously, and allowed to sit at room temperature for 3 min. After sitting, the sample was centrifuged at 10,000 $\times$ g for 18 min at 4°C . The top aqueous phase was then removed and utilized for the RNeasy extraction protocol.

At the end of the extraction, the eluate was split into two: one half was used for RNA-Seq and the other was used for cDNA synthesis for the qPCR assay. A library of 24 lung RNA extractions was sequenced in duplicates. The Duke Sequencing and Genomic Technologies Core Facility (Duke University, Durham, NC) performed RNA-Seq library preparation and sequencing. Libraries were prepared using the KAPA

Stranded mRNA kit (Roche, Basel, Switzerland) from 24 of the lung RNA samples with 3 mice representing each environment-flu exposure condition. RNA-Seq libraries were sequenced on an S-Prime flowcell with an Illumina NovaSeq 6000 to generate 50 bp paired-end reads. The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Then 20 μL qPCR reactions were run on a Bio-Rad CT1000 with the following reaction mixture: 4 μL of cDNA, 0.5 μL 10 μM Forward and Reverse Primers, 5.5 μL molecular grade water, and 2 $\times$ SYBR Green PCR Master Mix. The primers were designed to target the matrix protein 1 of influenza A (van Elden et al., 2001) with the following sequences: forward 5'-GGACTGCAGCGTA-GACGCTT-3' and reverse 5'-CATCTGTTGTATATGAGGCCCAT-3'.

2.3. Data analysis

All data analysis was performed using the R v4.0.3 programming language. Several R packages were used for data analysis including: dada2 v1.18 (Callahan et al., 2016) for creating amplicon sequence variants, phyloseq v1.34 (McMurdie and Holmes, 2013) for generating ordination plots and computing alpha and beta diversity, stats for running statistical analyses, microbiome v1.12 (Lahti, 2017) for center log ratio transformations, and ggplot2 v3.3.5 (Wickham, 2016) for the generation of visual plots. Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) v1.0.5 was used to identify differentially abundant ASVs and functional pathways (Lin and Peddada, 2020). PERMANOVA and Bray-Curtis distances were calculated using vegan v2.5-7 (Jari Oksanen et al., 2018).

Normality of alpha diversity values for richness and Shannon was confirmed using the Shapiro-Wilk test before subsequent statistical testing. For those normally distributed data, significance testing with ANOVA was performed followed by pairwise testing with Tukey's HSD. Since species richness for the cage bedding samples was not normally distributed, these were log transformed prior to ANOVA and pairwise testing. Also, fecal microbiome samples' species richness was not normally distributed and normality could not be achieved with a transformation so the non-parametric Wilcoxon Ranked Sign test with False Discovery rate (Benjamini-Hochberg) corrected values was used. Statistical significance in microbial communities between groups was tested for significance via homogeneity of dispersion and PERMANOVA (Anderson, 2017) calculated using Bray-Curtis distances. Functional pathways were predicted using PICRUST2 v2.4.0 (GM, D., et al., 2020).

The RNA-Seq FASTQ files were quality filtered with fastq-mcf (Aronesty, 2013), then mapped to the Genome Reference Consortium Mouse Build 38 (Consortium, 2020) using STAR v2.7.6a (Dobin et al., 2013). DESeq2 v1.30.1 (Love et al., 2014) was then used to identify differentially expressed genes. Genes were considered differentially expressed if they met the criteria of an adjusted p value <0.1 and a log 2-fold change >1 . Pathway analysis was performed using the gage v 2.38.3 (Luo et al., 2009) R package to identify Gene Ontology (GO) (Gene Ontology, 2021; Ashburner et al., 2000) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2021) with significantly different expression between groups.

2.4. Code and data availability

The code used to generate results can be accessed via GitHub through the following url: <https://github.com/alexmccumber/mouseenv>. The RNA-Seq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Ron Edgar and Lash, 2002) and are accessible through GEO Series accession number GSE215292 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE215292>). The 16S rRNA data have been deposited with links to BioProject accession number PRJNA865133 in the NCBI BioProject database (<https://www.ncbi.nlm.nih.gov/bioproject/>).

3. Results

3.1. Cage environment diversity is influenced by the introduction of soil

Prior to their introduction into the mouse cages, an initial characterization of the soils with PERMANOVA indicated that there was a significant difference in community composition between the soil microbiomes ($R^2 = 0.27$, $p < 0.001$). The diversity and richness profiles were as expected, with Pine soils having the highest Shannon diversity and richness and the Road soils having the lowest (Supplemental Fig. 1A and B). ANOVA results indicate that there was not a significant difference in Shannon diversity by soil type ($p = 0.16$) but there was a significant difference in richness by soil type ($p = 0.02$). Pairwise testing for richness showed that the Pine soils had significantly higher richness than the Road soils ($p = 0.016$).

Of all the soil, fecal and lung microbiome samples, the lungs had the least overall richness with 2101 unique ASVs identified. The fecal samples had 3866 unique ASVs and the soil samples had the highest number with 49,143 ASVs. A summary of the total ASVs and sequencing depth for the sample types is provided in Supplementary Table 3. For both the lung and fecal samples, the top 5 phyla at day 14 were Proteobacteria, Verrucomicrobiota, Actinobacteriota, Bacteroidota, and Firmicutes. These five phyla made up the majority (>95 %) of the lung and fecal microbiomes. The top 5 phyla in soil samples at day 14 were Proteobacteria, Planctomycetes, Acidobacteria, Acintobacteria, and Firmicutes, with a significant proportion of the soil ASVs (>25 %) belonging to phyla other than these top five (Fig. 2). Differences in soil exposures were not found to affect growth rates of the mice during the five-week growth period (data not shown).

For the day 14 and 35 cage bedding samples, the overall variance in community composition between soil types remained similar to the initial characterization as indicated by PERMANOVA ($R^2 = 0.26$, $p < 0.001$). Unlike the fresh soils, ANOVA indicated that Shannon diversity was significantly different by bedding type ($p = 0.05$, Supplemental Fig. 2A). Pairwise testing of Shannon diversity showed that Road bedding had significantly higher diversity than Control bedding ($p = 0.05$); however, the River vs. Control comparison was not significant (p -value = 0.06). For richness, the values were log transformed to meet the assumption of normality prior to running ANOVA. Like Shannon diversity comparisons, pairwise testing of log transformed richness showed that Road bedding had significantly higher richness than Control bedding ($p = 0.02$, Supplemental Fig. 2B) but Pine vs. Control was not significantly different (p -value = 0.08).

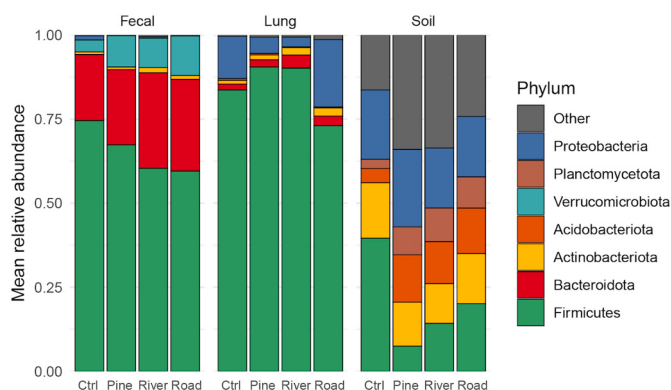


Fig. 2. Mean relative abundance of the top 5 most abundant phyla from each sample type for the lung, day 14 fecal, and day 14 soil exposure type. Those phyla not in the top 5 most abundant were group together as Other.

3.2. Soil and influenza exposure influence beta but not alpha diversity in the lung

To examine whether influenza exposure, comparison of the live vs. heat inactivated virus groups, or cage bedding affected alpha diversity of the lung microbiome, we compared the richness and Shannon diversity index for the lung microbiomes of mice in each exposure group; we did not observe a significant group difference for either metric. We explored differences in bacterial communities among the treatment groups using ordination plots and PERMANOVA. While there was no obvious clustering by soil type on the NMDS plot (Supplemental Fig. 3), statistical testing using PERMANOVA revealed significant differences in community compositions by soil exposure ($p < 0.001$, $R^2 = 0.11$). The PERMANOVA test also showed that PR8 exposure and the interaction between PR8 exposure and soil exposure was significant ($p < 0.001$). PR8 exposure only accounted for a small amount of the overall variance in the lung microbiome ($R^2 = 0.03$). A greater amount of the variance in the microbiome due to PR8 exposure depends on the soil exposure ($R^2 = 0.08$) and cage effects ($R^2 = 0.36$). To further explore if cage diversity influenced lung microbiome diversity, the Pearson correlation coefficient was calculated between the Shannon diversity index of day 35 cage samples and the corresponding lung tissue microbiome samples. There was no correlation found with either the HI or PR8 exposed group which suggests that lung diversity and richness are independent of environmental diversity.

3.3. The gut microbiome is more resistant to changes from soil exposure than the lung

We found that soil and PR8 exposure both resulted in statistically significant differences in gut microbiome between treatment groups. As with the lungs, we assessed the effects of soil exposure and time on the alpha diversity of the gut microbiome by evaluating the richness and Shannon diversity index for the gut microbiomes of mice in each exposure group. ANOVA showed that soil ($p < 0.001$), time ($p = 0.007$), PR8 ($p = 0.003$), and the interaction between soil, PR8 and time ($p = 0.019$) had a significant effect on the Shannon diversity index. There were no other significant interaction effects. Pairwise testing with Tukey's HSD showed that the Shannon diversity index was significantly higher in all soil exposed groups compared to Control ($p < 0.001$); comparisons among the soil types found that the River exposed mice had significantly higher values for Shannon diversity index than the Road exposed mice ($p = 0.008$). Pairwise testing for the effect of time found that the Shannon diversity index was significantly higher for day 14 compared to day 0 ($p = 0.007$), but not 35; and the PR8 exposed cohort had significantly lower Shannon diversity index compared to the HI cohort. Soil exposure had a statistically significant effect on richness of the mouse fecal microbiome. Pairwise testing with a Wilcoxon test showed that the median for each soil was significantly different from Control ($p < 0.05$, Bonferroni corrected), but there were no significant differences between soil groups. The PR8 exposed cohort had gut microbiomes with significantly lower richness than the HI exposed cohort ($p = 0.01$).

For the effects of soil and time on the between-sample differences for the gut, an NMDS plot showed no observable, distinct clustering. Statistical testing using PERMANOVA revealed that there are significant differences in community compositions by the main effects of soil exposure ($p < 0.001$, $R^2 = 0.07$), time ($p < 0.001$, $R^2 = 0.05$), and PR8 exposure ($p < 0.001$, $R^2 = 0.01$) (Supplemental Fig. 4). The interaction effects of soil by time ($p < 0.001$, $R^2 = 0.06$) and soil by flu ($p < 0.001$, $R^2 = 0.05$) were also significant. There was also a significant and large effect by cage ($p < 0.001$, $R^2 = 0.37$).

Overall, the variance attributed to soil exposure and PR8 in the gut was much lower than that of the lungs, suggesting that the lung microbiome is more susceptible to alterations due to soil and PR8 exposure.

3.4. *Lachnospiraceae* ASVs drive differences in both lung and gut microbiome composition as detected by ANCOM-BC

Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) was used to identify differentially abundant ASVs in each soil exposed lung microbiome compared to the Control group. The number of differentially abundant species varied by soil exposure. The Pine soil exposed group had 31 differentially abundant ASVs (Table 1). Several of these were Firmicutes and 22 belong to the Family *Lachnospiraceae*. The same pattern was observed in the Road soil exposed group, which had 70 differentially abundant ASVs, 38 of which were *Lachnospiraceae*. The River soil exposed group had 57 differentially abundant ASVs; 35 belonging to *Lachnospiraceae*. Thus, differences in lung microbiome composition appear to be largely driven by ASVs from the *Lachnospiraceae* family. However, ANOVA indicated there was no overall difference in *Lachnospiraceae* abundance in the lung microbiome after the data were aggregated at the family level and centered log-ratio (CLR) transformed.

To determine whether soil microbiome composition differences may lead to microbiome differences in the lung, ANCOM-BC was again used to identify differentially abundant ASVs between the Control bedding and the soil amended bedding for the day 14 and 35 samples. Then, the list of differentially abundant bedding ASVs was cross referenced with the list of differentially abundant lung ASVs. Only 4 ASVs were significantly different in both the lung and the cage bedding for the Pine soil exposed samples. A similar pattern was observed with the Road and River soil exposed samples, with 1 and 5 significant ASVs in both lung and cage bedding samples, respectively. This result suggests that the significant differences in ASVs in the cage bedding microbiomes are not present in the lung microbiome of mice.

Similar to the lung microbiome, ANCOM-BC determined that most of the differentially abundant ASVs in the fecal microbiome belong to the family *Lachnospiraceae* (Table 1). ANCOM-BC identified 148 significantly different ASVs in the Pine soil exposed fecal microbiome. A total of 37 ASVs were significant in both the fecal and soil samples, with 21 belonging to *Lachnospiraceae*. ANCOM-BC also identified 133 differentially abundant ASVs in the River exposed fecal samples (65 *Lachnospiraceae*), and 166 in the Road soil exposed samples (60 *Lachnospiraceae*). Unlike the lung *Lachnospiraceae*, there was a significant difference in the CLR transformed values of the Control group compared to the River and Road exposed soil group ($p < 0.05$, Tukey's HSD), but not the Pine exposed group ($p = 0.08$, Supplemental Fig. 5). The Pine soil exposed group was also significantly different from the River exposed group ($p = 0.01$).

3.5. No differences in weight loss, viral load, or immune cell counts by soil exposure were observed

The weight of the mice was recorded on days 3, 5, and 7 following intranasal influenza exposure. The HI virus exposed group did not lose significant weight over time, while the PR8 group lost significant weight from initial influenza exposure to day 7 (Supplemental Fig. 6). There

was no significant difference in weight lost by soil exposure. Infection was confirmed with qPCR as the PR8 group had a significantly higher viral load compared to the HI challenged group (Supplemental Fig. 7). There was no significant difference in viral load by soil type.

At 7 days post influenza exposure, we determined cell counts of macrophages and neutrophils in the collected BAL fluid (Fig. 3). For macrophages, there were a significant effects for both independent variables soil and PR8 exposure as determined by ANOVA ($p < 0.05$) with the PR8 exposed group having a higher macrophage count compared to the HI exposed group. However, neither the interaction between PR8 exposure and soil exposure nor any pairwise comparisons were significant. For neutrophils, there was a significant effect of PR8 exposure, with the PR8 exposed group having a higher neutrophil count compared to the HI exposed group, but neither soil ($p = 0.09$) nor the interaction between soil and live influenza ($p = 0.08$) reached significance as measured by ANOVA.

3.6. Soil exposure alters the immune response to influenza exposure

To determine if there were significant changes in gene expression from soil exposure, RNA was extracted and sequenced from the whole lung tissue of 24 mice at 7 days post PR8 or HI exposure, 3 from each soil by exposure group. With this data we identified gene expression specific to PR8 influenza exposure by comparing the HI and PR8 groups to the Control cohort. We also identified gene expression patterns that were specific to certain soil exposure groups. Principal component analysis of the differential expression data shows significant separation along the x-axis between the HI and PR8 exposed groups (Fig. 4). Within each of the PR8 and HI exposed groups, there was further clustering by soil exposure. Comparisons of the number of differentially expressed genes reflect the patterns observed in the PCA plot. Within the HI exposed group, there were no differentially expressed genes for the Road soil exposed group compared to the Control. The Pine and River soil exposed groups displayed differentially expressed genes; however, there were no GO terms or KEGG pathways with significantly different expression. We identified 61 biological process GO terms with differential expression between the PR8-Pine group and the PR8-Standard bedding group, several of which are immune system processes or related functions (e.g. B cell activation) that were upregulated. The full list of GO terms and KEGG pathways can be found in the GitHub repository.

Comparing the gene expression between the PR8-River and HI-River treated mice identified 186 biological process GO terms that were upregulated in the PR8-River treated mice. Several of these GO terms represented immune system processes or related functions. This comparison also identified four KEGG pathways upregulated in PR8-River treated mice including cytokine-cytokine interactions and cell cycling.

Compared to the Control group, the Pine soil exposed group had significantly downregulated inflammation associated genes (*HIF3A* and *FGFBP1*) but significantly upregulated *CX3CL1*, a gene suspected to regulate leukocyte adhesion and migration. The River soil exposed group had several upregulated interleukin genes: *IL2RG*, *IL11*, *IL18BP*, *IL4RA*, *IL3RA*, *IL10RA*, and *IL12B*. However, *IL17RD* was downregulated. Several chemokines were also upregulated: *CXCR3*, *CXCL12*, *CSCL16*, and *CCL5*, while *CCL17* and *TAF1A* were downregulated. The River soil exposed group was also significantly upregulated in the gene known to inhibit hemagglutinin mediated viral entry of influenza A, *IFTM3* (Bailey et al., 2012) (Supplemental Fig. 8). Table 2 summarizes the total number of differentially expressed genes by soil exposure type for either the HI, PR8, or change in expression from the HI to PR8 (Delta) groups.

3.7. Soil exposure alters the functional profile of the gut microbiome

To determine whether the functional profile of the gut microbiome changes upon Soil exposure, PICRUST2 was used to predict metagenome function. PICRUST2 uses the amplicon sequence to infer a community

Table 1

The number of significant lung microbiome ASVs identified by ANCOM-BC in lung only or both lung and cage bedding samples (for the combined HI and PR8 exposed groups within each soil condition). The number in parentheses is the number of those ASVs that are *Lachnospiraceae*.

		Sig. host ASVs (<i>Lachnospiraceae</i>)	Sig. host and bedding ASVs (<i>Lachnospiraceae</i>)
Lung	Pine	31 (22)	4 (2)
	Road	70 (38)	1 (0)
	River	57 (35)	5 (4)
Gut	Pine	148 (72)	37 (21)
	Road	166 (60)	16 (0)
	River	133 (65)	4 (2)

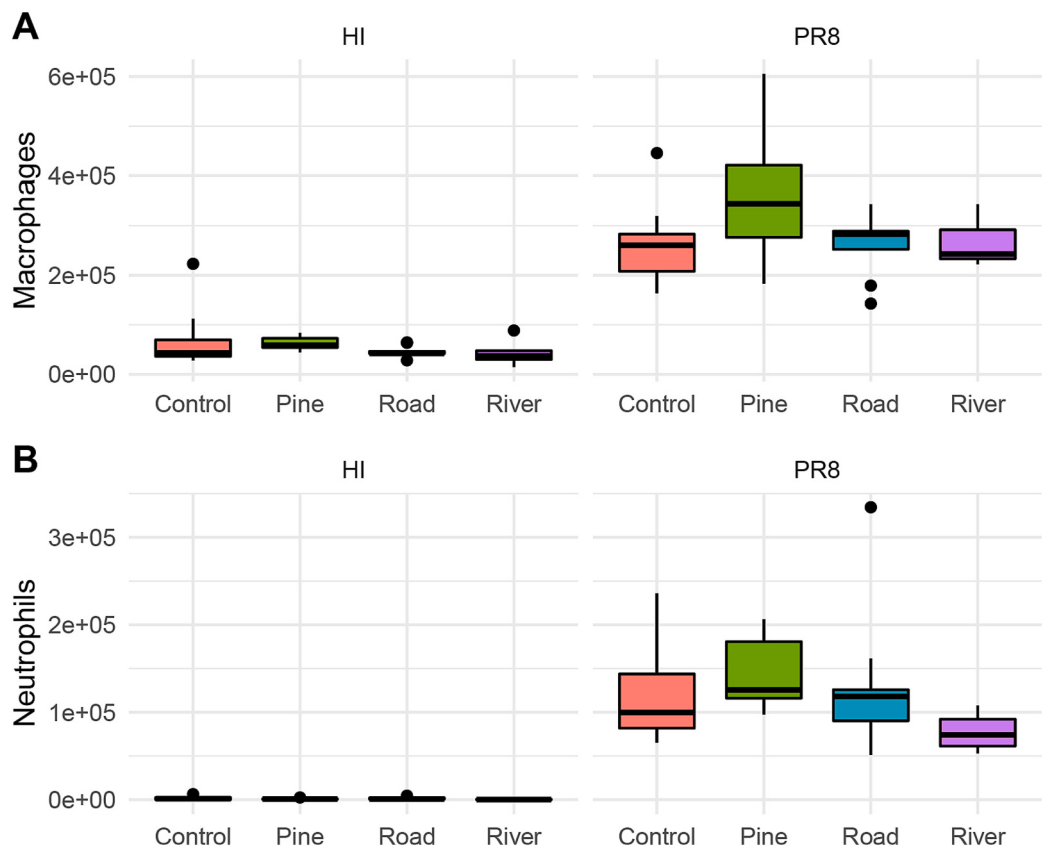


Fig. 3. Box and whisker plots of the total number of A) macrophages and B) neutrophils counted per mL of bronchioalveolar lavage fluid by soil and heat inactivated (HI) or live influenza (PR8) virus exposure. For the boxplots, the middle line represents the median, the upper and lower boxes represent the first and third quartiles, the lines represent the minimum and maximum, and individual points are outliers.

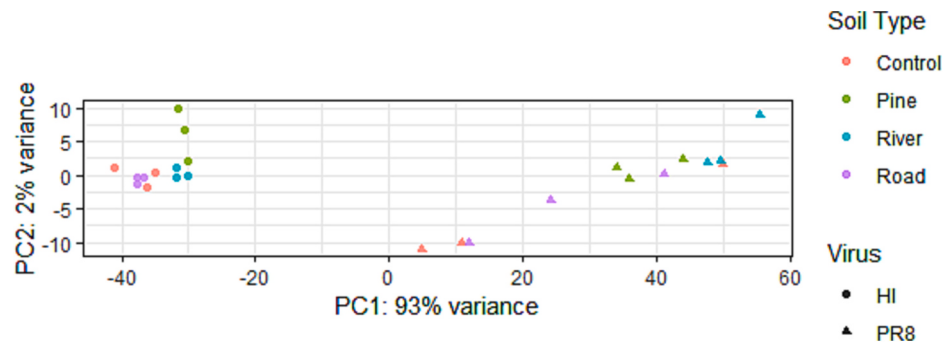


Fig. 4. Principle component graph of the DESeq2 normalized lung tissue RNA-seq data count matrix for each sample. Color indicates soil exposure group and shape represents either heat inactivated (HI) or live influenza (PR8) virus exposure. Principal component (PC) 1 captures 93 % of the variance while PC2 captures 2 % of the variance.

Table 2
Total number of differentially expressed genes from RNA-seq data from the lung tissue samples identified by DESeq2 by soil exposure type relative to control for either the heat inactivated (HI), live influenza (PR8) virus exposure, or change in expression from the HI to PR8 (Delta) groups.

	HI	PR8	Delta
Pine	225	407	144
River	41	1884	1226
Road	0	28	0

metagenome, which is then used to infer the MetaCyc (Caspi et al., 2014) pathway abundances. A principal component graph of the CLR transformed inferred MetaCyc pathway abundances was created to visualize differences in the predicted metagenomes (Supplemental Fig. 7). Overall, PERMANOVA showed soil exposure resulted in significant differences in pathways ($p < 0.05$) and accounted for approximately 7.5 % of MetaCyc pathway variance. As the pathway data are compositional, ANCOM-BC was used to identify pathways that are differentially abundant. There were 44 MetaCyc pathways that were significantly different for the Pine soil exposed group compared to Control, most of which were for amino acid or sugar metabolism. For the Road soil exposed group there were 71 significantly different pathways and all belonged to the quinol and quinone biosynthesis superclass. The

River soil exposed group had 41 significantly differentially abundant pathways. Like the Pine and Road exposure groups, several of the River soil exposure groups identified pathways were amino acid and sugar metabolism as well as quinol and quinone biosynthesis pathways. In addition, there were three pathways that were of potential interest due to the observed differential immune response: L-glutamate degradation VIII (to propanoate), (S)-propane-1,2-diol degradation, and 2-methylcitrate cycle II (propanoate degradation). The first two pathways belong to the superclass “fermentation to short-chain fatty acids” and the third to “propanoate degradation”, compounds of interest due to their potential to regulate immune system function. The model coefficients for ANCOM-BC indicate that while the (S)-propane-1,2-diol degradation pathway is present in greater abundance in the Pine soil exposed samples, the 2-methylcitrate cycle II (propanoate degradation) is lower in River soil exposed mice (Table 3). L-glutamate degradation VIII (to propanoate) was lower in all three soil exposed mice compared to the Control mice.

4. Discussion

Soil exposure can alter the functional profile of animal microbiomes by changing community compositions which, in turn, alters the immune response to a viral challenge. Soil exposure significantly impacted the composition of both the gut and lung microbiomes and these differences seem to have been driven by the family *Lachnospiraceae*. We found that 11 % of the variance of lung microbiome composition can be accounted for by different soil exposures, and that this soil exposure effect was greater on the lungs than the gut. This was expected given previous observations that the lung microbiome is mostly comprised of transient members, while in the gut newly introduced bacteria not only have to survive passage through the stomach but also must outcompete native taxa to establish themselves as part of the host microbiome (Corcoran et al., 2005). Additionally, the gut is known to have higher alpha diversity, so even if soil microbes became established in both sites, the effect of this alteration is likely to be less impactful on the gut (Dickson et al., 2017). We were able to observe changes in the gut microbiome over time through longitudinal fecal sampling, which was not possible for the lungs; however, time accounted for less gut microbiome variance than soil exposure. This is not a surprising result as known regulators of gut microbiome composition, such as diet, remained constant. In humans, longitudinal metagenomic and culturing studies have shown that >50 % of the members of the gut microbiome are retained for at least 1 year (Faith et al., 2013; Schloissnig et al., 2013; Hildebrand et al., 2021).

While we observed an impact of soil exposure on overall lung microbiome composition, soil exposures did not seem to have a significant effect on the alpha diversity of lung microbiomes: neither Shannon diversity nor richness of the lung microbiome correlated with the cage bedding microbiome diversity or richness. A similar trend has been observed in a horse model, where grazing at pasture or being housed indoors did not impact Shannon diversity or richness of the lung microbiome (Fillion-Bertrand et al., 2019). Similarly, Megahed et al. (Megahed et al., 2019) found no difference in Hill diversity or Chao1 richness in the lung microbiome of pigs grown with slatted flooring compared to standard straw bedding. Though neither of these studies provided samples of environmental diversity, these results show that the lung microbiome diversity and richness may not be significantly altered

by environmental differences and remains at a steady state, though individual membership within the lung microbiome changes with environmental exposures. Thus, these data suggest that the lung is able to regulate microbiome diversity independent of the environment but not community composition.

Firmicutes was the dominant phylum in all lung and fecal samples regardless of soil exposure. ANCOM-BC suggests that ASVs from the Firmicutes based family *Lachnospiraceae* drive most of the compositional differences we observed between microbiomes and that these changes were a result of differences in soil exposure. While there were several individual *Lachnospiraceae* ASVs different between lung microbiomes by soil exposure, this study found no observable pattern between the soil exposure and Control groups' CLR of *Lachnospiraceae* in the lung microbiome. However, *Lachnospiraceae* were in greater abundance in the fecal microbiome of the Control group compared to all the soil exposed groups. This is in line with a previous study that found *Lachnospiraceae* was lower in relative abundance in the gut microbiome of mice that were exposed to soil compared to controls (Ottman et al., 2018). *Lachnospiraceae* are spore-forming, common gut and soil microbiome members (Huang et al., 2019; Li et al., 2019). In a study classifying gut bacteria by dispersal strategies, Hildebrand et al. (Hildebrand et al., 2021) identify *Lachnospiraceae* as hereditarily persistent, or a group that is inheritable from parent to child but also has a high turnover rate in the gut and is dependent upon a continuous cycle of reinfection. Considering most of the differentially abundant ASVs were originally identified in the day 0 fecal microbiomes, the results suggest that the soils may play a role in selection of specific *Lachnospiraceae* ASVs as they cycle through the host and environment where some soils may provide a distinct advantage to a particular *Lachnospiraceae* ASV. Overall, this family is highly diverse and thought to be mainly gut associated and there is currently no known literature to suggest which mechanisms may be causing this selection.

Lachnospiraceae are currently of interest as they are known to produce short chain fatty acids (SCFAs) that are thought to be beneficial to host health (Vacca et al., 2020). One of these SCFAs, propionate, has been shown to reduce lung inflammation, potentially acting through the gut-lung axis (Liu et al., 2021). In a sterile lung injury model in mice, the gut microbiomes associate with lower inflammation had higher abundances of *Lachnospiraceae* (Tian et al., 2019). *Lachnospiraceae* has been a topic of particular interest in immunocompromised populations. In HIV patients, airways with higher *Lachnospiraceae* have been associated with lower bacterial burden and reduced expression of TNF- α and matrix metalloproteinase-9 (Iwai et al., 2014). The presence of *Lachnospiraceae* in the lungs has also been associated with longer duration of ventilator days and acute respiratory distress syndrome (Dickson et al., 2020). The presence of *Lachnospiraceae* in the mouse lung microbiome may be a reasonable expectation since coprophagy may provide a direct route of introduction for feces associated bacteria into the lung.

While soil exposure did not confer any specific protective or susceptible role in terms of weight loss or viral titers following PR8 vs. HI exposure, soil exposure significantly affected gene expression. The role the microbiome plays in the host immune response is complex and its mechanisms are an active area of research. Bradley et al. (Bradley et al., 2019) found that interferon levels decreased after giving mice antibiotics, facilitating earlier entry of influenza viral particles into epithelial cells. This effect was reversed after a fecal transplant, implicating the gut microbiome in the regulation of an important antiviral host system. In the present study, a similar effect was observed for Interferon Induced Transmembrane Protein 3 (*IFITM3*) expression. While baseline levels of *IFITM3* were not significantly different between any of the different soil exposure groups, overall *IFITM3* had higher expression levels in soil exposed groups compared to the Control group. Further, *IFITM3* expression has been shown to be positively correlated with antibody development following an influenza infection (Lei et al., 2020). Thus, our data suggest that while soil exposure may not play a role in protection against acute influenza infection in this model system, soil

Table 3

ANCOM-BC beta coefficients for the mouse fecal samples PICRUST2 inferred pathways by soil exposure type (stars indicate significance).

Pathway	Pine	River	Road
(S)-propane-1,2-diol degradation	1.76*	1.31	1.10
L-Glutamate degradation VIII (to propanoate)	-1.38	-1.11	-1.64
2-Methylcitrate cycle II (propanoate degradation)	-0.67	-0.35	-0.95*

exposure may potentially impact long term immune development and response to re-infection. This observation is consistent with the broader understanding that taking antibiotics lowers antibody titers (Zimmermann and Curtis, 2018) and that microbiome diversity may play a role in vaccine efficacy rates (McCoy et al., 2019). Importantly, Hagan et al. (Liu et al., 2021) showed antibiotic administration prior to influenza vaccine administration impaired antibody binding to H1N1 in human subjects with low pre-existing antibody titers while Ichinohe et al. (Hagan et al., 2019; Ichinohe et al., 2011) found that mice treated with antibiotics had lower influenza specific antibody titers 2-weeks post infection. Both these studies utilized broad spectrum antibiotics that would likely kill *Lachnospiraceae*. In mice, the relative abundance of *Lachnospiraceae* has previously been positively associated with antibody titers following a rabies vaccine (Zhang et al., 2020). This relationship merits further investigation as there continue to be trends between *Lachnospiraceae* and antibody development. There are limitations to our data that complicate interpretation: gene expression data was collected at a single time point, so while higher expression of *IFIT3* expression at 7 days post exposure is believed to be beneficial, it is possible that higher *IFIT3* expression is due to a delayed clearance of the viral infection compared to the other groups. However, the lack of difference in viral titers between the soil exposure groups suggests that this is not the case.

Along with an upregulated interferon gene, indicating increased antiviral activity, cytokine and chemokine genes were generally also upregulated in the soil exposed mice. This finding is consistent with a previous study where some OTUs, including a *Lachnospiraceae* OTU, were positively associated with serum cytokine and chemokine levels (Bartley et al., 2017). While we are not aware of any study concerning the role of the lower respiratory tract microbiome, bacteria in the upper respiratory tract (Kaul et al., 2020) and gut (Rosshart et al., 2017) have been shown to regulate influenza inflammation. Several of the genes previously inferred to be modulated by upper respiratory tract and gut microbiome are anti-inflammatory cytokines or cytokine associated genes including: interleukin 4 (*IL4RA*), interleukin 10 (*IL10RA*), and interleukin 11 (*IL11*). This finding is consistent with the Rosshart et al. (Rosshart et al., 2017) study which found higher IL10 protein levels in the lung tissue of mice that were given a fecal transplant from a wild mouse (that would have had direct contact with soils) compared to lab-reared mice at four days post influenza exposure. The inferred upregulation of the propionate production pathway in the gut microbiome of the River soil exposed mice supports a role for the gut microbiome in regulating the immune response. This inferred upregulation appears to be mainly driven by *Lachnospiraceae* ASVs that had significantly higher abundance in the River soil exposed mice. The inferred upregulation of propionate production could be driving the upregulation of anti-inflammatory genes and increase in neutrophils that we observed in this group. Previously, Tian et al. (Tian et al., 2019) found that *Lachnospiraceae* relative abundance was positively correlated with gut propionate concentrations, which reduced lung inflammation following a lung ischemia reperfusion injury in mice. In another mouse model, the authors showed that intratracheal exposure to higher propionate concentrations resulted in greater lung injury due to reduced beneficial inflammation following a *Staphylococcus aureus* pneumonia challenge. Thus, while preventing excessive inflammation may be beneficial to prevent lung injury following influenza exposure, it could leave the host more susceptible to bacterial co-infection. This is potentially important clinically as secondary bacterial infections are a significant cause of increased mortality and morbidity in influenza patients (Morris et al., 2017).

There are several limitations to this study. The introduction of different soils into cage bedding was designed to model living in different environments with different microbial communities of different complexity; as with all models, this one was imperfect. While the microbial communities of the soils differed prior to introduction into cage bedding (Supplemental Fig. 9), they became more similar over time and the alpha diversity of each decreased. There are several possible

explanations, including colonization of cage bedding by the fecal pellet microbial community and the loss of specific taxa after being removed from the supportive environment provided by their natural ecosystem. Further, we have not investigated the possibility that abiotic factors in soil samples may have affected the mice. As for the differential gene expression data, it is possible that the microbiome is impacting the kinetics of immune responses following the virus. This could lead to early or delayed gene responses. Therefore, by sampling only one time point, which was the point of peak inflammation, it is possible that changes in kinetics would not be observed.

We cannot rule out the possibility that contamination during processing contributed to some of the observed community variation. We took particular caution to include several negative control samples, but lungs have very low microbial biomass and are particularly susceptible to contamination (Drengenes et al., 2019). Future studies should attempt to measure total microbial burden, which is likely an important consideration in studying the lung microbiome.

Because not all *Lachnospiraceae* produce SCFAs, a follow-up experiment with shotgun metagenomic sequencing may be able to elucidate this family's role in SCFA production by not only improving taxonomic resolution to distinguish SCFA-producing taxa from non-producing taxa, but also by directly quantifying the relative abundance of SCFA pathway genes. Lastly, conducting the lung lavage to retrieve immune cells for phenotyping may have altered the lung microbiome community, and therefore may have altered communities observed at the final timepoint.

5. Conclusions

Alterations in host lung gene expression and the gut and lung microbiomes following soil and influenza exposure were assessed. Key findings from this study are that the lung regulates microbiome diversity independent of the environment but not community composition and soil exposure influenced gene expression in the lungs but had no effect on viral load or weight loss. *Lachnospiraceae* ASVs accounted for a significant proportion of the differences between gut and lung microbiomes as detected by ANCOM-BC and their relationship with the host and its immune system development/maturation warrants further investigation.

CRedit authorship contribution statement

Alexander W. McCumber: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yeon Ji Kim:** Writing – original draft, Visualization, Validation, Methodology, Formal analysis. **Joshua Granek:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis. **Robert M. Tighe:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Claudia K. Gunsch:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

data links are included in manuscript

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Appendix A. Supplementary data

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