Environment International 169 (2022) 107497

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Full length article

Air pollution from livestock farms and the oropharyngeal microbiome of COPD patients and controls

Warner van Kersen [a], Alex Bossers [a], Wouter A.A. de Steenhuijsen Piters [b,c], Myrna M.T. de Rooij [a], Marc Bonten [b], Ad C. Fluit [b], Dick Heederik [a], Fernanda L. Paganelli [b], Malbert Rogers [b], Marco Viveen [b], Debby Bogaert [b,d], Helen L. Leavis [b], Lidwien A.M. Smit [a,*]

[a] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands
[b] University Medical Center Utrecht, Utrecht, the Netherlands
[c] Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
[d] University of Edinburgh Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, Edinburgh, United Kingdom

ARTICLE INFO

Handling Editor: Adrian Covaci

ABSTRACT

Air pollution from livestock farms is known to affect respiratory health of patients with chronic obstructive pulmonary disease (COPD). The mechanisms behind this relationship, however, remain poorly understood. We hypothesise that air pollutants could influence respiratory health through modulation of the airway microbiome. Therefore, we studied associations between air pollution exposure and the oropharyngeal microbiota (OPM) composition of COPD patients and controls in a livestock-dense area.

Oropharyngeal swabs were collected from 99 community-based (mostly mild) COPD cases and 184 controls (baseline), and after 6 and 12 weeks. Participants were non-smokers or former smokers. Annual average livestock-related outdoor air pollution at the home address was predicted using dispersion modelling. OPM composition was analysed using 16S rRNA-based sequencing in all baseline samples and 6-week and 12-week repeated samples of 20 randomly selected subjects (n = 323 samples). A random selection of negative control swabs, taken every sampling day, were also included in the downstream analysis.

Both farm-emitted endotoxin and PM_{10} levels were associated with increased OPM richness in COPD patients ($p < 0.05$) but not in controls. COPD case-control status was not associated with community structure, while correcting for known confounders (multivariate PERMANOVA $p > 0.05$). However, members of the genus *Streptococcus* were more abundant in COPD patients (Benjamini-Hochberg adjusted $p < 0.01$). Moderate correlation was found between ordinations of 20 subjects analysed at 0, 6, and 12 weeks (Procrustes $r = 0.52$ to 0.66; $p < 0.05$; Principal coordinate analysis of Bray-Curtis dissimilarity), indicating that the OPM is relatively stable over a 12 week period and that a single sample sufficiently represents the OPM.

Air pollution from livestock farms is associated with OPM richness of COPD patients, suggesting that the OPM of COPD patients is susceptible to alterations induced by exposure to air pollutants.

1. Introduction

Living in livestock dense areas has been associated with health effects in epidemiological studies worldwide. Particularly livestock-related air pollution at the residential level is suggested to be relevant for public health (Borlée et al., 2018, 2017a,2017b,2015; de Rooij et al., 2019; Douglas et al., 2018; Elliott, 2004; Mirabelli et al., 2006; Pavilonis et al., 2013; Radon et al., 2007; Rasmussen et al., 2017; Schinasi et al., 2011; Schulze et al., 2011; Sigurdarson and Kline, 2006; Simões et al., 2022; Smit et al., 2014; van Kersen et al., 2020). Adverse health effects reported in relation to livestock farm emissions such as ammonia (NH_3) and particulate matter (PM) include lung function deficits, as well as increased respiratory symptoms like coughing and wheezing (Borlée et al., 2017a; Radon et al., 2007; Schulze et al., 2011). Several studies have linked increased ambient NH_3 levels with lung function deficits in non-farming residents (Borlée et al., 2017a; Loftus et al., 2015; van Kersen et al., 2020). People with chronic obstructive pulmonary disease (COPD) were found to be especially vulnerable to livestock-related NH_3

* Corresponding author: Lidwien Smit, Institute for Risk Assessment Sciences, Yalelaan 2, 3584 CM, Utrecht, the Netherlands.
E-mail address: l.a.smit@uu.nl (L.A.M. Smit).

https://doi.org/10.1016/j.envint.2022.107497
Received 18 March 2022; Received in revised form 22 June 2022; Accepted 30 August 2022
Available online 5 September 2022

levels (van Kersen et al., 2020). Similarly, increased respiratory symptoms in COPD patients living near livestock farms have been reported (Borlée et al., 2015). Recently, exposure to livestock farm emitted PM was found to be associated with respiratory health effects, indicating endotoxin as a plausible etiologic agent (Beentjes et al., 2022; de Rooij et al., 2019; Farokhi et al., 2018) However, mechanisms behind adverse respiratory health effects in COPD patients associated with livestock farm emissions remain poorly understood. A biological mechanism that could play a role is alteration of the airway microbiota composition. Livestock operations are a potential source of microbes and air pollutants, both of which could act on the microbial composition of the airways.

The predominant determinants of the lung microbiota are thought to be explained by immigration and elimination processes of bacteria from the upper respiratory tract (URT) (Dickson et al., 2016, 2015). In contrast, the microbiota of diseased lungs is suggested to be determined by regional growth conditions like nutrient availability, competition and activation of host inflammatory cells. While disruptions of the airway microbiota have mainly been linked to severe disease, signs of functional distortion have been shown in the microbiota of mild COPD patients while the community composition remained indistinguishable from healthy volunteers. This indicates that the airway microbiota may be relevant in the earlier stages of COPD as well (Dickson et al., 2016). Therefore, studying changes in the airway microbiota in relation to the living environment, especially in patients with a respiratory condition, could help to explain the associations between environmental exposures and respiratory health. A similar mechanism of dysbiosis in airway microbiota was suggested to play a role by studies in the Netherlands and United States where exposure to air pollution from livestock farms was associated with an increased risk of pneumonia (Poulsen et al., 2018; Smit et al., 2017).

The goal of this explorative study is to investigate whether the oropharyngeal microbiota (OPM) of COPD patients differs from controls, and whether residential exposure to air pollution from livestock farms is associated with microbial community composition. We performed 16S rRNA gene amplicon sequencing of oropharyngeal samples from 99 COPD patients and 184 healthy controls living in an area with a high density of intensive livestock (mainly poultry, pig, cattle, goat and mink (Borlée et al., 2017a)) farms in the Netherlands. In analysing the resulting OPM compositions, we were particularly interested in differences associated with residential exposure to livestock farm-emitted endotoxin and particulate matter with a nominal aerodynamic diameter of 10 μm or less (PM_{10}). Lastly, we analyzed reproducibility of the individual OPM composition by sequencing samples collected

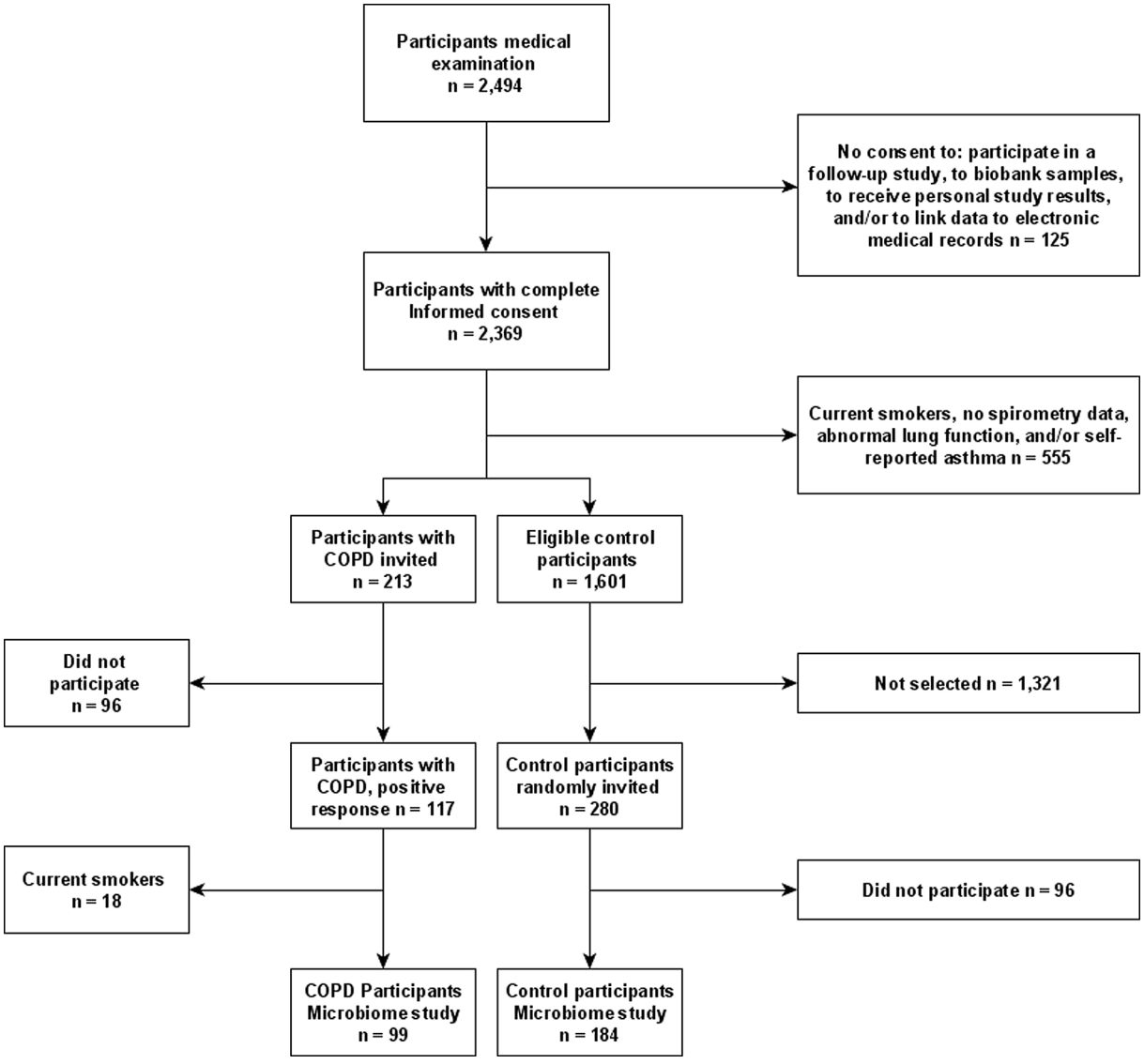

Fig. 1. Flowchart of the study population of 99 COPD cases and 184 control subjects.

repeatedly from the same individuals over a 12-week period.

2. Materials and methods

2.1. Study population and design

Study participants were selected from 2,369 participants of the cross-sectional Dutch Livestock Farming and Neighbouring Residents' Health study (VGO) population, of which the design, enrollment, and medical examination have been described (Borlée et al., 2017b, 2015). The selection procedure for the present case-control study is shown in a flowchart (Fig. 1). First, all COPD patients were selected by a lung function specialist, based on their spirometry values and curves. COPD was defined as a post-bronchodilator (BD) measurement of FEV_1/FVC below the lower limits of normal. We also invited subjects if they had a pre- or post- BD FEV_1/FVC below 0.7 in combination with at least one self-reported respiratory symptom (wheeze, shortness of breath) or if they reported doctor-diagnosed COPD. Current smokers were excluded. Control subjects were randomly selected from all non-smoking, non-asthmatic and non-COPD subjects with normal lung function. Both cases and controls were excluded post-enrollment when a smoking habit became apparent during home visits. The study protocol (no. 13/533) was approved by the Medical Ethical Committee of the University Medical Centre Utrecht. All participants signed informed consent.

2.2. Sampling procedure

Between February 2015 and July 2016, during home visits, oropharyngeal samples were collected from 99 COPD cases and 184 controls. In addition to the baseline (t0) sample, participants were sampled again after 6 (t1) and 12 weeks (t2). Samples were collected using Copan Eswabs and stored on ice in 1 ml liquid Amies Medium (483CE, Copan Diagnostics Inc., CA) during transport. DNA extraction and sequencing was done for all baseline (t0) samples, and for a random selection of t1 and t2 samples from 10 COPD cases and 10 controls. A random selection of field blanks (air swabs), taken every sampling day, together with unused swabs and laboratory controls was included in the downstream analysis.

2.3. DNA Extraction, 16S rRNA gene amplification and sequencing

The DNA isolation procedure was performed as previously described and can be found in the supplementary methods (Wyllie et al., 2014). A 469-bp (base pair) amplicon, encompassing the V3 and V4 hypervariable regions of the 16S rRNA gene, was amplified and sequenced using the Illumina MiSeq Reagent Kit v3 (600-cycle) on an Illumina MiSeq instrument according to Fadrosh *et al.* (Fadrosh et al., 2014). More details can be found in supplementary methods.

2.4. Bioinformatics

Sequencing primers and heterogeneity spacers were removed using cutadapt version 2.8 (Martin, 2011). Output sequences were processed in R version 4.0.2 using the dada2 package version 1.16, resolving the sequences into amplicon sequence variants (ASVs)(Callahan et al., 2016; R Core Team, 2020). For the sequence filtering step, forward and backward reads were truncated to high-quality regions at 200 and 250 base pairs respectively. After inspection of the read quality profiles, the maximum expected errors (maxEE) was set to 3. Taxonomy was assigned using the Silva reference database and the naïve Bayesian classifier, version 138 (Quast et al., 2013). Species level annotation was confirmed using BLASTnt against the REFSEQ targeted loci database at the National Center for Biotechnology Information ("Database resources of the National Center for Biotechnology Information," 2018). Further filtering steps are described in the supplemental methods.

2.5. Data analysis

Associations involving beta-diversity, measured by Bray-Curtis dissimilarity, were visualized using Principal Coordinates Analysis (PCoA) and tested by multivariable PERMANOVA. To explore which taxa drive the overall compositional differences between COPD cases and controls, or individuals with high or low livestock-emitted endotoxin concentrations (tertiles T3 vs T1) (de Rooij et al., 2019), differential abundance analysis was performed at ASV, genus, family and phylum level using DESeq2 version 1.28.1 and MaAsLin2 version 1.2.0 (Love et al., 2014; Mallick et al., 2021) (see supplemental methods).

Differences in alpha-diversity between COPD cases and controls were assessed using linear models. As response variables, species richness and the Shannon diversity index were used. Explanatory variables included case-control status, sex, age (continuous), education level (low, medium, high), lung medication (yes/no), antibiotic use (yes/no), COPD GOLD stage, former smoker (yes/no) and season (Winter Dec-Feb, Spring Mar-May, Summer Jun-Aug, fall Sep-Nov). To assess relations between OPM composition and livestock exposure, we included presence of a poultry farm within 1 km of the home address (Smit et al., 2017), and annual-average concentrations of livestock-emitted PM_{10} and endotoxin at home addresses (predicted by dispersion modelling as described in the supplementary methods; continuous variables) (de Rooij et al., 2019). Relationships were first explored in univariable analyses. Consecutively, predictors that yielded p-values < 0.2 were used in multivariable models to identify independent drivers while correcting for known confounders. To determine whether a single sample is representative for an individual's OPM and if its composition can be reproduced over a short period of time, multiple robustness analyses were performed as described in the supplemental methods.

3. Results

3.1. Study population

A detailed overview of participant characteristics, stratified by cases and controls, can be found in Table 1. Overall, 48.1 % of the participants were female, and mean age was 58.2 years. Cases were found to be slightly older with a mean age of 61.4 years compared to 56.5 years in controls ($p < 0.001$). Cases differed from controls in terms of smoking history with a mean difference of 6.1 pack-years ($p < 0.001$). The majority of cases were classified as mild COPD patients with 45 (45.5 %) and 30 (30.3 %) participants assigned to GOLD stage 1 and 2 respectively. The remaining 24 (24.2 %) cases were classified with GOLD stage 0. One third (33.3 %) of the cases used lung medication during the sampling period. Besides mono- or combination therapies with inhaled corticosteroids, bèta 2-sympathicomimetics and parasympatholytics, monotherapy with oral leukotriene antagonists also occurred.

3.2. Sequencing

Approximately 5,411,000 reads were generated with an average of 14,000 reads per sample, ranging from 430 to 54,000. Processing the raw data resulted in 2,705 ASVs. Compositional analyses were performed using 1,092 taxa that remained after filtering, as detailed in the supplemental methods.

3.3. Oropharyngeal microbiota composition

An overview of the taxonomic composition at family level can be found in Fig. 2. To summarise, relative abundance was dominated by Streptococcaceae (mean 47 %; range 5–91 %), Veillonellaceae (mean 16 %; range 0–44 %) and Prevotellaceae (mean 10 %; range 0–37 %). A comparison of the top 10 most abundant genera per sample type (including negative controls) can be seen in Supplementary Fig. 1. We explored whether the OPM composition differed between COPD cases

Table 1
Study population characteristics of COPD cases and healthy controls sampled for oropharyngeal microbiota analysis.

	Case (N = 99)	Control (N = 184)	Overall (N = 283)	p-value [a]
Sex, female	42 (42.4 %)	94 (51.1 %)	136 (48.1 %)	0.205
Age (y)	61.4 [28.9, 71.8]	56.5 [28.7, 71.7]	58.2 [28.7, 71.8]	<0.001
BMI*	26.8 [18.1, 48.9]	27.3 [17.2, 48.1]	27.1 [17.2, 48.9]	0.368
Education level				
low	24 (24.2 %)	40 (21.7 %)	64 (22.6 %)	0.738
medium	48 (48.5 %)	86 (46.7 %)	134 (47.3 %)	
high	27 (27.3 %)	58 (31.5 %)	85 (30 %)	
Pack-years of cigarettes smoked†	13.9 [0, 54.6]	7.74 [0, 127]	9.91 [0, 127]	0.001
COPD grade, GOLD				
0	24 (24.2 %)	184 100 %)	208 (73.5 %)	<0.001
1	45 (45.5 %)	0 (0.%)	45 (15.9 %)	
2	30 (30.3 %)	0 (0 %)	30 (10.6 %)	
Pre-BD measurements				
FEV_1 (l)	2.51 [0.88, 4.62]	3.23 [1.49, 5.12]	2.98 [0.88, 5.12]	<0.001
FVC (l)	4.00 [1.66, 8.02]	4.16 [2.09, 7.21]	4.10 [1.66, 8.02]	0.236
FEV_1/FVC (l)	0.63 [0.26, 0.90]	0.78 [0.66, 0.92]	0.73 [0.29, 0.92]	<0.001
Post BD measurements				
FEV_1 (l)	2.67 [1.03, 4.79]	3.34 [2.26, 5.39]	3.11 [1.03, 5.39]	<0.001
FVC (l)	4.10 [1.90, 7.87]	4.17 [2.61, 7.16]	4.15 [1.90, 7.87]	0.616
FEV_1/FVC	0.65 [0.31, 0.92]	0.80 [0.71, 0.90]	0.75 [0.31, 0.92]	<0.001
Uses lung medication	33 (33.3 %)	1 (0.5 %)	34 (12.0 %)	<0.001
Atopy	30 (30.3 %)	51 (27.7 %)	81 (28.6 %)	0.711
Childhood on farm	36 (36.4 %)	59 (32.1 %)	95 (33.6 %)	0.592
Respiratory symptoms during sampling	20 (20.2 %)	48 (26.1 %)	68 (24.0 %)	0.337
Antibiotic use within 4 weeks prior to sampling	13 (13.1 %)	5 (2.7 %)	18 (6.4 %)	0.002
Residential exposure to livestock farm emitted endotoxin (EU/m^3)[Ŧ]	0.23 [0.032, 1.26]	0.25 [0.032, 0.93]	0.24 [0.032, 1.26]	0.428
Residential exposure to livestock farm emitted PM_{10} ($\mu g/m^3$) [Ŧ]	0.29 [0.041, 1.22]	0.30 [0.036, 1.07]	0.30 [0.036, 1.22]	0.482
N farms within 500 m, tertiles				
no farms	33 (33.3 %)	62 (33.7 %)	95 (33.6 %)	0.984
1 or 2 farms	36 (36.4 %)	65 (35.3 %)	101 (35.7 %)	
>2 farms	30 (30.3 %)	57 (31 %)	87 (30.7 %)	
N farms within 1000 m, 50 % quantiles				
0–16 farms	87 (87.9 %)	164 (89.1 %)	251 (88.7 %)	0.904
>16 farms at 1 km	12 (12.1 %)	20 (10.9 %)	32 (11.3 %)	
Poultry exposure				
nearest poultry at > 1 km	41 (41.4 %)	76 (41.3 %)	117 (41.3 %)	1
poultry farm within 1 km	58 (58.6 %)	108 (58.7 %)	166 (58.7 %)	

Data are presented as mean [range] or n (%). Definition of abbreviations: BD = bronchodilator; COPD = chronic obstructive pulmonary disease; EU: endotoxin unit; FEV_1 = forced expiratory volume in 1 s; FVC = forced vital capacity; Education levels: low—lower secondary school or less; medium—intermediate vocational education or upper secondary school; high—higher vocational education or university. *BMI = mass(kg)/(height (m))^2. †Mean pack-years for former smokers. [Ŧ] Annual average concentration at the home address estimated by dispersion modelling. [a] Two sample *t*-test or Chi^2 test across cases and controls.

and controls in a multivariable analysis adjusting for age, gender, smoking history, season and antibiotic use (Supplementary Table 1). No compositional differences were shown in this analysis related to case-control status (PERMANOVA p = 0.26, R^2 = 0.004) or livestock-emitted endotoxin (PERMANOVA p = 0.31, R^2 = 0.012). Community composition differed slightly between males and females (PERMANOVA p < 0.01, R^2 = 0.007). Season (PERMANOVA p = 0.07;R^2 = 0.014) and farm childhood (PERMANOVA p = 0.09;R^2 = 0.005) were found to have a borderline significant effect.

3.4. Differential abundance analysis

As the compositional analysis revealed little biological variation, we chose to focus on the more abundant taxa in the differential abundance analysis. To this end, we removed taxa which accounted for <0.1 % relative abundance in <25 % of the samples. The remaining dataset consisted of 74 ASVs belonging to 26 genera, 19 families and 7 phyla. Using DESeq2, we found that the genus *Streptococcus* was significantly more abundant in COPD cases compared to controls (log2 fold change [LFC] = 0.68; Benjamini Hochberg [BH] adjusted p = 0.001). This association was confirmed by testing for differences in normalized counts using the Kruskal-Wallis test (Fig. 3). At species level, *Streptococcus salivarius*, *Streptococcus parasanguinis* and another undefined *Streptococcus* species were associated with COPD, although not statistically significant (Supplementary Fig. 2). In comparison to DESeq2, MaAsLin2 did not return any significant associations. It did, however, show a similar, but statistically non-significant association between *Streptococcus* abundance and COPD (Supplementary Table 2 β = 0.03, BH adjusted p = 0.38). Differential abundance analysis in relation to livestock-related endotoxin exposure revealed slight differences in Firmicutes and Actinobacteriota abundance between low and high exposed individuals (Supplementary Fig. 3). However, these associations were no longer statistically significant after adjusting for multiple testing.

3.5. Alpha-diversity

Differences in within-subject diversity were analysed using data rarefied at 5,000 sequences per sample, maintaining 177 controls and 94 cases in the dataset (96 % of all samples). Rarefaction curves of all samples can be found in Supplementary Fig. 4 We used multivariable linear models, adjusting for sex, smoking history, education level, season, and antibiotic use, after variable selection with univariable models. Univariable analysis of observed richness and Shannon indices did not show an association with COPD status (Supplementary Table 3).

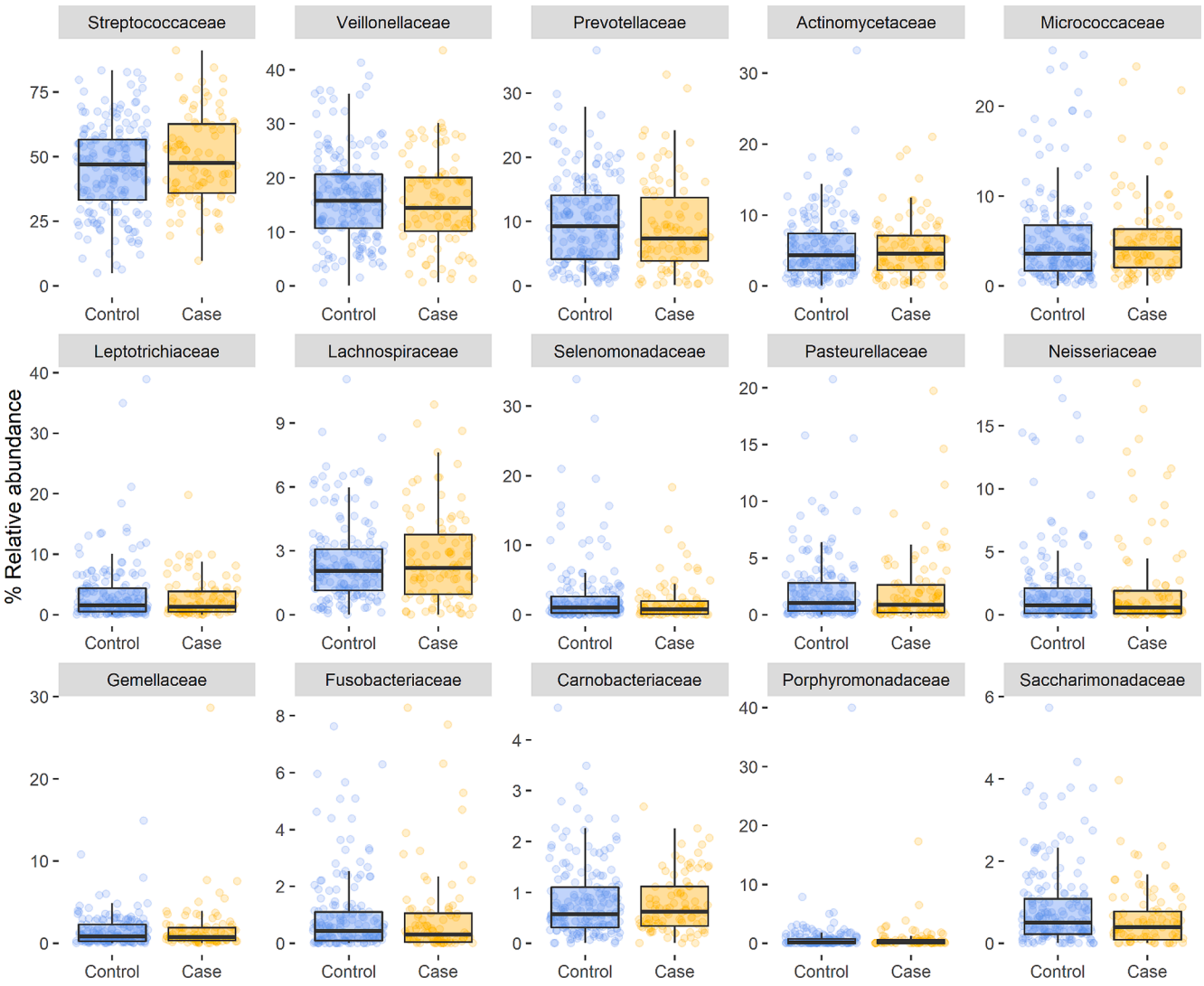

Fig. 2. Relative abundances at family level, showing taxa accounting for at least 0.5% (mean relative abundance) of the oropharyngeal microbiota.

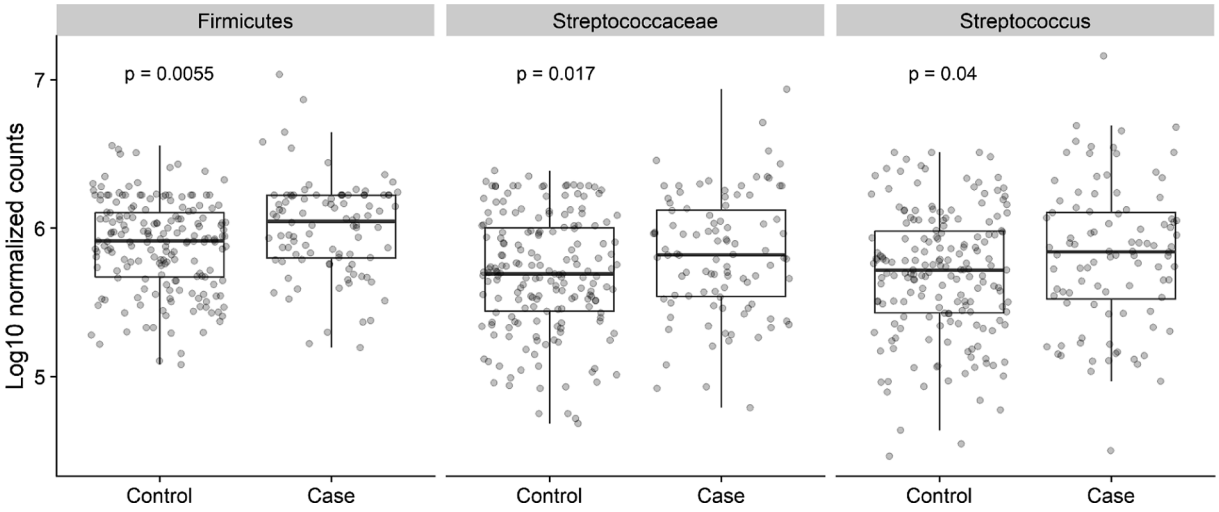

Fig. 3. DESeq2 normalized counts of differentially abundant taxa at phylum (LFC = 0.47), family (LFC = 0.53) and genus level (LFC = 0.68) between COPD cases and controls. P-values calculated using the Kruskal-Wallis test.

Table 2
Determinants of alpha-diversity indices of the oropharyngeal microbiota of COPD cases and controls in a multivariable linear model.

	Richness			Shannon diversity		
Variable	β	95 % CI	p value	β	95 % CI	p value
COPD case vs control	−2.20	−8.1:3.7	0.46	−0.041	−0.18:0.1	0.57
Gender female vs male	−0.76	−6.11:4.6	0.78	−0.002	−0.13:0.13	0.97
Education level						
Medium vs low	6.63	−0.09:13.35	0.05	0.21	0.04:0.37	0.01
High vs low	0.78	−6.56:8.11	0.84	0.10	−0.08:0.28	0.27
Smoking history former vs never	−0.35	−5.87:5.17	0.90	−0.05	−0.18:0.08	0.46
Residential exposure to livestock farm emitted endotoxin (EU/m^3)[Ŧ]	4.91	−0.1:9.92	0.06	0.081	−0.04: 0.2	0.19
Season						
Spring (Mar-May) vs winter (Dec-Feb)	0.15	−6.96:7.25	0.97	0.09	−0.08:0.26	0.29
Summer (Jun-Aug) vs winter	4.10	−3.02:11.21	0.26	0.17	−0.01:0.34	0.06
Fall (Sep-Nov) vs winter	−7.16	−17.23:2.9	0.16	−0.16	−0.4:0.08	0.19
Antibiotics within 4 weeks prior to sampling	−2.65	−13.47:8.18	0.63	0.16	−0.1:0.42	0.23

[Ŧ] Annual average concentration at the home address by dispersion modelling, scaled to the 10-90th percentile range. EU: endotoxin unit.

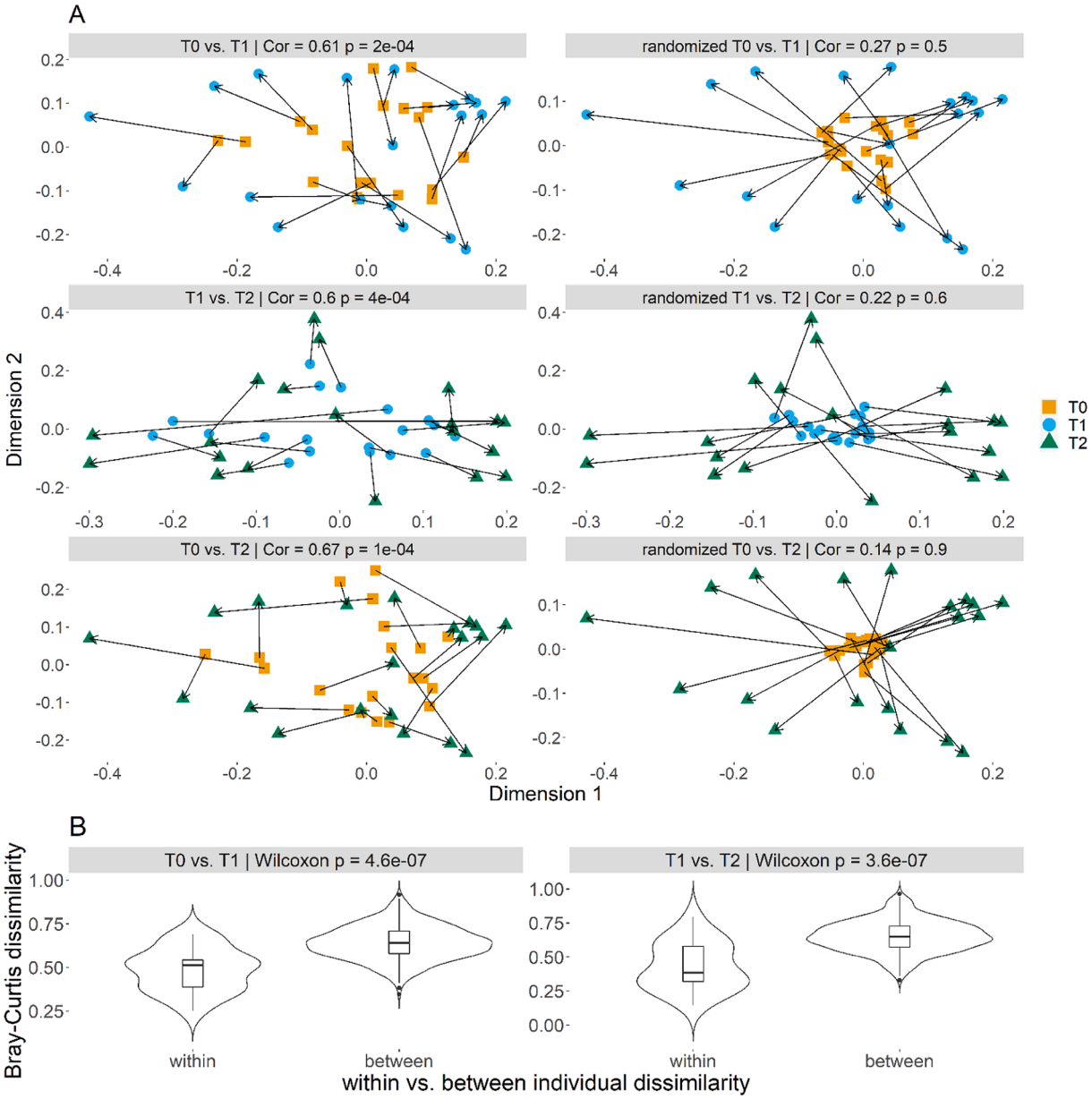

Fig. 4. A: Procrustes errors comparing ordinations (PCoA – Bray-Curtis) of the 20 individuals that had their oropharyngeal microbiota sampled at t0, t1 (after 6 wks) and t2 (after 12 wks). B: Within vs between individual Bray-Curtis distances over timepoints.

Likewise, in the multivariable linear model, COPD status, gender, smoking history, and antibiotic use were not associated with alpha diversity (Table 2). In the multivariable analysis, medium education level (vs low) was associated with both higher richness ($\beta = 6.63$;$p = 0.05$) and increased Shannon diversity ($\beta = 0.21$;$p = 0.01$). Shannon diversity appeared higher in samples taken during summer (Jun-Aug) compared to winter (Dec-Feb)($\beta = 0.17$;$p = 0.06$). Similarly, we found an association between residential exposure to livestock-emitted endotoxin and increased richness ($\beta = 4.91$; $p = 0.06$). When stratifying the model for COPD case-control status, the positive association of residential exposure to livestock-emitted endotoxin with richness is shown to be driven by the COPD cases (Supplementary Table 4, $\beta = 10.02$;$p = 0.02$). Residential exposure to livestock-emitted PM_{10} showed a similar relationship with increased richness (Supplementary Table 5, $\beta = 5.58$;$p = 0.08$) and COPD status (Supplementary Table 6, $\beta = 13.11$;$p = 0.01$).

3.6. Oropharyngeal microbial community stability over time

Procrustes analysis of 20 randomly selected subjects sampled at 0, 6, and 12 weeks showed moderate but significant within-individual correlation ($r = 0.50$–0.66, $p < 0.05$). This correlation was lost when the sample identifiers were randomized, resulting in a between-individual comparison ($r = 0.15$–0.23; $p > 0.6$; Fig. 4A). Repeating the analysis with randomized sample identifiers over 1,000 Monte Carlo iterations showed that this was not due to chance. Likewise, comparing Bray-Curtis dissimilarity of the repeated samples within and between individuals revealed significantly higher between (vs within) individual dissimilarity (Fig. 4B). Therefore, we could conclude that the individual OPM community is relatively stable, at least over a 12-week period.

4. Discussion

The aim of our study was to investigate whether the URT microbiota composition of community-based COPD patients differed from adults without COPD living in the same geographic region. Subsequently, we explored the potential role of residential exposure to livestock-emitted air pollution in shaping the OPM in subjects with and without COPD. We found evidence suggesting that residential exposure to livestock-emitted endotoxin and PM_{10} is associated with increased species richness in COPD patients. While the overall OPM composition did not differ between mild and non-exacerbating COPD cases and subjects with normal lung function and no diagnosis of asthma or COPD, *Streptococcus* spp. was more abundant in the OPM of COPD patients. However, the exploratory nature of our epidemiological study precludes further conclusions about direction of associations, causality, or underlying mechanisms.

We did not find differences in overall bacterial community composition related to COPD status or livestock exposure. This might be explained by the relative stability of the OPM compared to that of the nasopharynx which is known to be more susceptible to environmental exposures like pig farming (Flynn and Dooley, 2021). A study in Iowa (United States) reported significantly higher alpha-diversity in the nasal microbiome of 33 livestock workers compared to 26 non-livestock workers, though this was not observed for the oropharyngeal microbiome (Kates et al., 2019). Likewise, the OPM community structure in our study did not differ between former and never smokers. Although previous studies reported differences in URT microbiota due to smoking (Charlson et al., 2010; Morris et al., 2013), these studies included current smokers which were excluded in the present study.

Differential abundance analysis revealed that three ASV's, annotated to the genus *Streptococcus*, were more highly represented in subjects with COPD, compared to the controls. A BLAST search revealed that these belonged to *Streptococcus salivarius*, *Streptococcus parasanguinis* and an undefined *Streptococcus* spp. *Streptococcus* (especially *Streptococcus pneumoniae*) has been associated with COPD exacerbations and disease severity (Lee et al., 2016; Toraldo and Conte, 2019). In addition both *S. salivarius* and S. *parasanguinis* are opportunistic pathogens and have been associated with COPD and smoking (Turek et al., 2021). In an endotoxin-induced lung inflammation mouse model the lung microbiota shifted towards endogenous opportunistic pathogens, suggesting an immunological mechanism behind our observed results. (Poroyko et al., 2015). Thus, studying OPM may help to provide an explanation why COPD patients living in a livestock-dense area are at increased risk of wheezing, exacerbations and a lower lung function (Borlée et al., 2015; van Dijk et al., 2016). This, however, needs to be confirmed by longitudinal studies. Likewise, further experimental studies are needed to elucidate which taxa of the OPM could be influenced by air pollution exposure.

We report increased richness related to residential exposure to livestock farm-emitted endotoxin and PM_{10}, but only among COPD patients. We speculate that the airways of COPD patients are more susceptible to irritation by air pollutants, resulting in an environment that promotes bacterial growth. The similarity between the effect of endotoxin and PM_{10}, is explained by the fact that endotoxin was modeled as a fraction of the livestock farm-emitted PM_{10}. Our results suggest that the impact of mild COPD and residential livestock exposure on the microbial community of the oropharynx is subtle. However, it has been shown that in mild COPD patients the largely normal microbiome may still be distorted due to subtle changes in the presence or absence in rare key members and is therefore less able to react to and mediate changes in host inflammation (Dickson et al., 2016). These subtle changes in microbial community of COPD patients are different from changes in other respiratory diseases. Notable changes in the respiratory microbiota have been detected early on in asthma, whereas in COPD distortion of the microbiota is mainly associated with advanced disease (Dickson et al., 2016). While advanced COPD has been associated with a shift in microbiota composition from Bacteroidetes to Proteobacteria, a phylum known for its opportunistic pathogenic members like *Pseudomonas* and *Haemophilus*, shifts towards a Firmicutes dominant community have also been reported (Dickson et al., 2016). In combination with our reported association between mild COPD and increased Firmicutes abundance this suggest that changes in the respiratory microbiome in COPD patients occur more gradually than previously thought. This is also supported by a recent multi-omic meta-analysis which showed that Proteobacteria, Actinobacteria, and Firmicutes are main contributors to the biosynthesis of pro-inflammatory agents in COPD patients (Mallick et al., 2021).

Limitations of the present study include its exploratory nature. While this is one of the largest microbiome studies performed in COPD patients, further increasing the sample size might lead to identifying more associations, especially those with smaller effect sizes. The mainly mild, population-based COPD patients we included, probably do not show as much variation in their OPM compared to healthy controls as one might expect in more severe patients recruited from a clinical setting. Furthermore, given that the microbiota of diseased lungs is thought to be determined by regional growth conditions, traditional sampling techniques might be preferred. However, it has been shown that oropharyngeal swabs are an adequate proxy for traditional samples like sputum when studying COPD (Liu et al., 2017). In addition, oropharyngeal swabs are easier to standardize and less intrusive compared to sputum sampling. Lastly, the striking difference in output between DESeq2 and MaAsLin2 illustrates that these results should be carefully interpreted. We argue that the negative binomial model performed by DESeq2, compared to the linear model used by MaAsLin2, theoretically fits our case-control design best. More importantly, differences in abundance between groups were verified using a Kruskal-Wallis test on normalized counts.

Strengths of this study include the use of validated models that predict livestock-related endotoxin levels at the home address as a measure for livestock exposure of microbial origin (de Rooij et al., 2019). Further, our analysis of repeated samples confirmed stability of individual microbiota profiles within a 3-month period, although we

were unable to identify clear predictors that explain the between-subjects variability in overall community composition. Lastly, the use of multiple negative and community controls enabled us to minimise the influence of contamination which is a notorious source of bias when working with low-biomass samples.

In conclusion, we showed a relationship between residential exposure to livestock-related endotoxin and PM_{10} and OPM richness of COPD patients, suggesting that OPM of COPD patients is susceptible to alterations induced by exposure to air pollutants. While we did not find community structural differences in the OPM of mild COPD patients versus controls, results do suggest increased *Streptococcus* spp. abundance in COPD patients. The implications and the underlying mechanisms of these associations offer multiple angles for future research regarding health effects from air pollution.

5. Data availability

Availability of data and materials Raw sequence data were submitted into the Sequence Read Archive (SRA) at the NCBI under accession number PRJNA810336. The phyloseq object is available at https://doi.org/10.5281/zenodo.6303131.

6. Support statement

Data collection for this study was supported by a grant from the Lung Foundation Netherlands (Grant No: 3.2.11.022). Sequencing costs were covered by internal funding of the Institute of Risk Assessment Sciences, Utrecht University and the University Medical Center Utrecht.

CRediT authorship contribution statement

Warner van Kersen: Writing – original draft, Formal analysis, Visualization, Software. **Alex Bossers:** Methodology, Software, Writing – review & editing. **Wouter A.A. de Steenhuijsen Piters:** Methodology, Software, Writing – review & editing. **Myrna M.T. de Rooij:** Methodology, Software, Writing – review & editing. **Marc Bonten:** Conceptualization, Writing – review & editing. **Ad C. Fluit:** Conceptualization, Writing – review & editing. **Dick Heederik:** Conceptualization, Funding acquisition, Writing – review & editing. **Fernanda L. Paganelli:** Investigation, Writing – review & editing. **Malbert Rogers:** Methodology, Software. **Marco Viveen:** Investigation, Writing – review & editing. **Debby Bogaert:** Conceptualization, Writing – review & editing. **Helen L. Leavis:** Conceptualization, Writing – review & editing. **Lidwien A.M. Smit:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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 availability is described in the main text

Acknowledgements

We are grateful to Marieke Oldenwening, who performed the fieldwork, and Gerdit Greve and Betty Jongerius-Gortemaker for technical assistance (Institute for Risk Assessment Sciences, Utrecht University).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107497.

References

Beentjes, D., Shears, R.K., French, N., Neill, D.R., Kadioglu, A., 2022. Mechanistic insights into the impact of air pollution on pneumococcal pathogenesis and transmission. Am. J. Respir. Crit. Care Med. https://doi.org/10.1164/rccm.202112-2668TR.

Borlée, F., Yzermans, C.J., Krop, E.J.M., Maassen, C.B.M., Schellevis, F.G., Heederik, D.J.J., Smit, L.A.M., 2018. Residential proximity to livestock farms is associated with a lower prevalence of atopy. Occup Env. Med oemed-2017-104769. 10.1136/oemed-2017-104769.

Borlée, F., Yzermans, C.J., van Dijk, C.E., Heederik, D., Smit, L.A.M., 2015. Increased respiratory symptoms in COPD patients living in the vicinity of livestock farms. Eur. Respir. J. 46, 1605–1614. https://doi.org/10.1183/13993003.00265-2015.

Borlée, F., Yzermans, C.J., Aalders, B., Rooijackers, J., Krop, E., Maassen, C.B.M., Schellevis, F., Brunekreef, B., Heederik, D., Smit, L.A.M., 2017a. Air pollution from livestock farms is associated with airway obstruction in neighboring residents. Am. J. Respir. Crit. Care Med. 196, 1152–1161. https://doi.org/10.1164/rccm.201701-0021OC.

Borlée, F., Yzermans, C.J., Krop, E., Aalders, B., Rooijackers, J., Zock, J.-P., van Dijk, C. E., Maassen, C.B.M., Schellevis, F., Heederik, D., Smit, L.A.M., 2017b. Spirometry, questionnaire and electronic medical record based COPD in a population survey: Comparing prevalence, level of agreement and associations with potential risk factors. PLoS ONE 12, e0171494. https://doi.org/10.1371/journal.pone.0171494.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Charlson, E.S., Chen, J., Custers-Allen, R., Bittinger, K., Li, H., Sinha, R., Hwang, J., Bushman, F.D., Collman, R.G., 2010. Disordered microbial communities in the upper respiratory tract of cigarette smokers. PLoS ONE 5, e15216. https://doi.org/10.1371/journal.pone.0015216.

de Rooij, M.M.T., Smit, L.A.M., Erbrink, H.J., Hagenaars, T.J., Hoek, G., Ogink, N.W.M., Winkel, A., Heederik, D.J.J., Wouters, I.M., 2019. Endotoxin and particulate matter emitted by livestock farms and respiratory health effects in neighboring residents. Environ. Int. 132, 105009 https://doi.org/10.1016/j.envint.2019.105009.

Dickson, R.P., Erb-Downward, J.R., Freeman, C.M., McCloskey, L., Beck, J.M., Huffnagle, G.B., Curtis, J.L., 2015. Spatial variation in the healthy human lung microbiome and the adapted island model of lung biogeography. Ann. Am. Thorac. Soc. 12, 821–830. https://doi.org/10.1513/AnnalsATS.201501-029OC.

Dickson, R.P., Erb-Downward, J.R., Martinez, F.J., Huffnagle, G.B., 2016. The microbiome and the respiratory tract. Annu. Rev. Physiol. 78, 481–504. https://doi.org/10.1146/annurev-physiol-021115-105238.

Douglas, P., Robertson, S., Gay, R., Hansell, A.L., Gant, T.W., 2018. A systematic review of the public health risks of bioaerosols from intensive farming. Int. J. Hyg. Environ. Health 221, 134–173. https://doi.org/10.1016/j.ijheh.2017.10.019.

Elliott, L., 2004. Ecological associations between asthma prevalence and potential exposure to farming. Eur. Respir. J. 24, 938–941. https://doi.org/10.1183/09031936.04.00006404.

Fadrosh, D.W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R.M., Ravel, J., 2014. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome 2, 6. https://doi.org/10.1186/2049-2618-2-6.

Farokhi, A., Heederik, D., Smit, L.A.M., 2018. Respiratory health effects of exposure to low levels of airborne endotoxin – a systematic review. Environ. Health 17, 14. https://doi.org/10.1186/s12940-018-0360-7.

Flynn, M., Dooley, J., 2021. The microbiome of the nasopharynx. J. Med. Microbiol. 70, 001368 https://doi.org/10.1099/jmm.0.001368.

Kates, A.E., Dalman, M., Torner, J.C., Smith, T.C., 2019. The nasal and oropharyngeal microbiomes of healthy livestock workers. PLoS ONE 14, e0212949. https://doi.org/10.1371/journal.pone.0212949.

Lee, S.-W., Kuan, C.-S., Wu, L.-S.-H., Weng, J.-T.-Y., 2016. Metagenome and metatranscriptome profiling of moderate and severe COPD sputum in taiwanese han males. PLoS ONE 11, e0159066. https://doi.org/10.1371/journal.pone.0159066.

Liu, H.-Y., Zhang, S.-Y., Yang, W.-Y., Su, X.-F., He, Y., Zhou, H.-W., Su, J., 2017. Oropharyngeal and sputum microbiomes are similar following exacerbation of chronic obstructive pulmonary disease. Front. Microbiol. 8, 1163. https://doi.org/10.3389/fmicb.2017.01163.

Loftus, C., Yost, M., Sampson, P., Torres, E., Arias, G., Breckwich Vasquez, V., Hartin, K., Armstrong, J., Tchong-French, M., Vedal, S., Bhatti, P., Karr, C., 2015. Ambient ammonia exposures in an agricultural community and pediatric asthma morbidity. Epidemiol. Camb. Mass 26, 794–801. https://doi.org/10.1097/EDE.0000000000000368.

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8.

Mallick, H., Rahnavard, A., McIver, L.J., Ma, S., Zhang, Y., Nguyen, L.H., Tickle, T.L., Weingart, G., Ren, B., Schwager, E.H., Chatterjee, S., Thompson, K.N., Wilkinson, J. E., Subramanian, A., Lu, Y., Waldron, L., Paulson, J.N., Franzosa, E.A., Bravo, H.C., Huttenhower, C., 2021. Multivariable Association Discovery in Population-scale Meta-omics Studies. bioRxiv 2021.01.20.427420. 10.1101/2021.01.20.427420.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 10.14806/ej.17.1.200.

Mirabelli, M.C., Wing, S., Marshall, S.W., Wilcosky, T.C., 2006. Asthma symptoms among adolescents who attend public schools that are located near confined swine feeding operations. Pediatrics 118, e66–e75. https://doi.org/10.1542/peds.2005-2812.

Morris, A., Beck, J.M., Schloss, P.D., Campbell, T.B., Crothers, K., Curtis, J.L., Flores, S.C., Fontenot, A.P., Ghedin, E., Huang, L., Jablonski, K., Kleerup, E., Lynch, S.V.,

Sodergren, E., Twigg, H., Young, V.B., Bassis, C.M., Venkataraman, A., Schmidt, T. M., Weinstock, G.M., Lung HIV Microbiome Project, 2013. Comparison of the respiratory microbiome in healthy nonsmokers and smokers. Am. J. Respir. Crit. Care Med. 187, 1067–1075. 10.1164/rccm.201210-1913OC.

Pavilonis, B.T., Sanderson, W.T., Merchant, J.A., 2013. Relative exposure to swine animal feeding operations and childhood asthma prevalence in an agricultural cohort. Environ. Res. 122, 74–80. https://doi.org/10.1016/j.envres.2012.12.008.

Poroyko, V., Meng, F., Meliton, A., Afonyushkin, T., Ulanov, A., Semenyuk, E., Latif, O., Tesic, V., Birukova, A.A., Birukov, K.G., 2015. Alterations of lung microbiota in a mouse model of LPS-induced lung injury. Am. J. Physiol. - Lung Cell. Mol. Physiol. 309, L76–L83. https://doi.org/10.1152/ajplung.00061.2014.

Poulsen, M.N., Pollak, J., Sills, D.L., Casey, J.A., Nachman, K.E., Cosgrove, S.E., Stewart, D., Schwartz, B.S., 2018. High-density poultry operations and community-acquired pneumonia in Pennsylvania. Environ. Epidemiol. 2, e013 https://doi.org/10.1097/EE9.0000000000000013.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.

R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Radon, K., Schulze, A., Ehrenstein, V., van Strien, R.T., Praml, G., Nowak, D., 2007. Environmental exposure to confined animal feeding operations and respiratory health of neighboring residents. Epidemiology 18, 300–308. https://doi.org/10.1097/01.ede.0000259966.62137.84.

Rasmussen, S.G., Casey, J.A., Bandeen-Roche, K., Schwartz, B.S., 2017. Proximity to industrial food animal production and asthma exacerbations in Pennsylvania, 2005–2012. Int. J. Environ. Res. Public Health 14. https://doi.org/10.3390/ijerph14040362.

Database resources of the National Center for Biotechnology Information, 2018. Nucleic Acids Res. 46, D8–D13. 10.1093/nar/gkx1095.

Schinasi, L., Horton, R.A., Guidry, V.T., Wing, S., Marshall, S.W., Morland, K.B., 2011. Air pollution, lung function, and physical symptoms in communities near concentrated swine feeding operations. Epidemiol. Camb. Mass 22, 208–215. https://doi.org/10.1097/EDE.0b013e3182093c8b.

Schulze, A., Römmelt, H., Ehrenstein, V., van Strien, R., Praml, G., Küchenhoff, H., Nowak, D., Radon, K., 2011. Effects on pulmonary health of neighboring residents of concentrated animal feeding operations: exposure assessed using optimized estimation technique. Arch. Environ. Occup. Health 66, 146–154. https://doi.org/10.1080/19338244.2010.539635.

Sigurdarson, S.T., Kline, J.N., 2006. School proximity to concentrated animal feeding operations and prevalence of asthma in students. Chest 129, 1486–1491. https://doi.org/10.1378/chest.129.6.1486.

Simões, M., Janssen, N., Heederik, D.J.J., Smit, L.A.M., Vermeulen, R., Huss, A., 2022. Residential proximity to livestock animals and mortality from respiratory diseases in The Netherlands: a prospective census-based cohort study. Environ. Int. 161, 107140 https://doi.org/10.1016/j.envint.2022.107140.

Smit, L.A.M., Boender, G.J., de Steenhuijsen Piters, W.A.A., Hagenaars, T.J., Huijskens, E.G.W., Rossen, J.W.A., Koopmans, M., Nodelijk, G., Sanders, E.A.M., Yzermans, J., Bogaert, D., Heederik, D., 2017. Increased risk of pneumonia in residents living near poultry farms: does the upper respiratory tract microbiota play a role? Pneumonia 9. https://doi.org/10.1186/s41479-017-0027-0.

Smit, L.A.M., Hooiveld, M., Beer, F. van der S., Winden, A.W.J.O., Beekhuizen, J., Wouters, I.M., Yzermans, C.J., Heederik, D., 2014. Air pollution from livestock farms, and asthma, allergic rhinitis and COPD among neighbouring residents. Occup. Environ. Med. 71, 134–140. 10.1136/oemed-2013-101485.

Toraldo, D.M., Conte, L., 2019. Influence of the lung microbiota dysbiosis in chronic obstructive pulmonary disease exacerbations: the controversial use of corticosteroid and antibiotic treatments and the role of eosinophils as a disease marker. J. Clin. Med. Res. 11, 667–675. https://doi.org/10.14740/jocmr3875.

Turek, E.M., Cox, M.J., Hunter, M., Hui, J., James, P., Willis-Owen, S.A.G., Cuthbertson, L., James, A., Musk, A.W., Moffatt, M.F., Cookson, W.O.C.M., 2021. Airway microbial communities, smoking and asthma in a general population sample. EBioMedicine 71, 103538. https://doi.org/10.1016/j.ebiom.2021.103538.

van Dijk, C.E., Garcia-Aymerich, J., Carsin, A.-E., Smit, L.A.M., Borlée, F., Heederik, D.J., Donker, G.A., Yzermans, C.J., Zock, J.-P., 2016. Risk of exacerbations in COPD and asthma patients living in the neighbourhood of livestock farms: observational study using longitudinal data. Int. J. Hyg. Environ. Health 219, 278–287. https://doi.org/10.1016/j.ijheh.2016.01.002.

van Kersen, W., Oldenwening, M., Aalders, B., Bloemsma, L.D., Borlée, F., Heederik, D., Smit, L.A.M., 2020. Acute respiratory effects of livestock-related air pollution in a panel of COPD patients. Environ. Int. 136, 105426 https://doi.org/10.1016/j.envint.2019.105426.

Wyllie, A.L., Chu, M.L.J.N., Schellens, M.H.B., Gastelaars, J. van E., Jansen, M.D., Ende, A. van der, Bogaert, D., Sanders, E.A.M., Trzciński, K., 2014. Streptococcus pneumoniae in Saliva of Dutch Primary School Children. PLOS ONE 9, e102045. 10.1371/journal.pone.0102045.