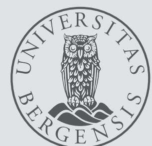
Indoor airborne microbiome, environmental and household determinants and respiratory health in Northern Europe

Hesham Amin

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2023

UNIVERSITY OF BERGEN



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Thesis for the degree of Philosophiae Doctor (PhD)
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Date of defense: 22.09.2023

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Year: 2023

Title: Indoor airborne microbiome, environmental and household determinants and respiratory health in Northern Europe

Name: Hesham Amin

Print: Skipnes Kommunikasjon / University of Bergen

Scientific environment

During the PhD period, I was affiliated with the BRuSH (Oral bacteria as determinants for respiratory health) at the Department of Clinical Science, Faculty of Medicine, University of Bergen, Norway. The laboratory work was conducted during my research stay at the Section for Microbiology, Department of Biology, Aarhus University, Aarhus, Denmark. The thesis was conducted as part of the ECRHS III (European Community Respiratory Health Survey) study.

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This PhD project was funded by the European Research Council (ECR) under the European Union's Horizon 2020 research and innovation programme (grant agreement n° 804199).

// Acknowledgements

I am thankful to the European Research Council (ERC) for financing this thesis through the European Union's Horizon 2020 research and innovation program (grant agreement n° 804199). The work for this thesis was carried out at the Department of Clinical Science at UIB, the Section for Microbiology, Department of Biology at Aarhus University, and the Department of Public Health at Aarhus University. I am grateful for the working facilities and support provided.

I would like to thank all my wonderful supervisors, without whom I would not have been able to conduct this research. To main supervisor Randi Jacobsen Bertelsen, thank you for your patience, deep expertise, motivation, and encouragement throughout the project. I have learned so much under your guidance. Vivi schlünssen, Thanks for your excellent supervision, warm welcome, hospitality, and interesting discussion. Tina Santl-Temkiv Thanks for your lab expertise, for your guidance, and for sharing your knowledge in air microbiology. Rajesh Shigdel Thanks for sharing your expertise.

Cecilie Svanes, Thank you for all your valuable practical and scientific input and for all the dinners, the piano concert, and your hospitality. And for finding funding for me. I would like to thank Ian Marshall for providing bioinformatic and statistical guidance and for taking the time to answer my technical questions. Kai Finster, thank you for your scientific thought, quick response, and "to the point" comments. Torben Sigsgaard provided insightful discussion and guidance during the project. Christine Cramer thanks you for your close cooperation, your useful discussions, and our joint time together during your research stay.

I would like to thank the data managers during my PhD project, Vilde Sørbye, Bente Skottvoll, and Anders Viken, for their patience and for providing me with clean and updated data sets. I would like to thank the members of the BRuSH research group. I am grateful for having the opportunity to work with all of you. You offer a good environment for learning and growing. A big applause to the RHINESSA research groups for all the inspiring meetings, team-building events, and social gatherings.

Finally, I would like to thank all my friends and family for their support and for believing in me, as well as my dear parents for always encouraging me and giving me so many opportunities in life.

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Abstract in English

Background: Human transition from outdoor environment to the built environments have reduced our exposure to microbial diversity. Nowadays we spend on average up to 90 % of our time indoors. Previous studies have indicated that exposure to airborne bacteria might prevent or facilitate the development of respiratory disease such as asthma. The studies also showed that children growing up on farm environments have lower risk of immune-mediated diseases than children growing up in urban areas probably due to exposure to farm-related microbiota through contact with livestock animals.

Objectives: We attempted to increase the efficiency of the extraction of airborne dust, DNA, and endotoxin from the EDC (Electrostatic Dust Collector), a sampling device used for addressing airborne bacterial exposure. We aimed to investigate the difference in airborne bacterial composition between the farmers' homes and suburban homes and to study bacterial transfer between livestock stables and farmers' homes. In Paper III, we attempted to identify factors associated with the composition of the indoor bacterial communities. In Paper IV, we aimed to investigate the association between indoor bacterial profiles (diversity and load), lung function, and airway inflammation. Furthermore, we aimed to determine whether individual bacterial taxa in the indoor microbiome affect lung function and airway inflammation.

Methods: Airborne dust particles were sampled with EDCs over a period of 14 days. The dust washed from the EDCs' cloths was used to extract DNA and endotoxin. We compared the DNA extraction efficiency of bacterial components from EDCs using five extraction techniques. A quantitative PCR (qPCR) was used to assess bacterial abundance. The V3–V4 region of the bacterial 16S rRNA gene was amplified and sequenced using Ilumina MiSeq to determine the composition of the bacterial communities. The Divisive Amplicon Denoising Algorithm (DADA2) algorithm was used for the inference of amplicon sequence variants from amplicon data. The kinetic chromogenic LAL assay was used for endotoxin measurement. The association between dependent and independent variables was studied using adjusted multivariate regression models.

Results: In paper I, we found that airborne bacteria were significantly more abundant and more diverse in farmers' homes than suburban homes ($p < 0.001$). In particular, the putatively beneficial bacterial taxa of intestinal origin were more abundant in the farmers' homes and originated mainly from surrounding environment rather than livestock stables.

In paper II, we found that two techniques outperformed the rest. Furthermore, we developed protocols to simultaneously extract both DNA and endotoxin from a single EDC cloth.

In paper III, the indoor airborne bacteria differed across five Northern European countries. Higher indoor bacterial diversity and load were associated with higher abundance of outdoor bacterial taxa which might be because of low precipitation and higher wind speed outdoor. Multivariate regression models showed that alpha diversity indices and bacterial and endotoxin loads were positively associated with the age of the occupants, the number of occupants, the cleaning frequency, the presence of dogs, and the age of the house.

In paper IV, we found that indoor bacterial diversity was associated with higher lung function in males and higher eosinophilic airway inflammation in females. We further found that cells affiliated with *Actinobacteriota* were associated with better lung function, while cells affiliated with *Clostridia* were associated with lower lung function. We further found that higher FeNO levels were negatively associated with *Cellulomonas* and positively associated with *Campylobacter*.

Conclusion: We showed that airborne bacteria in farmers' homes, especially cow farmers' homes, was characterized by high bacterial diversity compared to suburban homes. Additionally, it appears that the indoor airborne bacterial community in farmers' homes is influenced by intestinal animal microbiota from manure.

In Paper II, we were able to develop a joint protocol for DNA and endotoxin extraction. Our technique promotes a high quality-to-price ratio and may be employed in large epidemiological studies addressing airborne bacterial exposure where a large number of samples are needed.

In paper III, we concluded that indoor airborne bacteria are geographically patterned, and the contribution of outdoor bacterial taxa to indoor air might be affected by weather events. Furthermore, our results suggest that general lifestyle choices such as the number of occupants, types of pets, frequency of cleaning, and use of disinfectants impact the indoor microbiome.

Different associations were found between indoor bacteria and lung function and airway inflammation in males and females, suggesting sexual dimorphism in response to airborne bacterial exposure. Furthermore, the association patterns between the indoor bacterial communities and lung function and airway inflammation were different. The results from Paper IV provide new insights into understanding the complicated relationship between indoor bacterial exposures and respiratory health.

Abstract in Norwegian

Bakgrunn: Overgangen fra utemiljø til bygde miljøer har redusert vår eksponering for mikrobielt mangfold. I dag tilbringer det moderne mennesket opptil 80% av tiden innendørs. Tidligere studier har vist at eksponering for luftbårne bakterier kan minske risikoen for å utvikle luftveissykdommer som astma. Studiene viser også at barn som vokser opp på gård har lavere risiko for å utvikle immunmedierte sykdommer enn barn som vokser opp i urbane områder. Grunnen til dette kan være eksponering for gårdsrelatert mikrobiota gjennom kontakt med husdyr.

Mål: Vi ønsket å forbedre protokollen for analyse av bakterier og endotoksin fra EDC (Electrostatic Dust Collector), en prøvetakingsenhet som brukes til å måle luftbåren bakteriell eksponering. Videre ville vi undersøke forskjellen i luftbåren bakteriesammensetning mellom boliger til bønder og boliger i bystrøk, og å studere bakterieoverføring mellom husdyrstaller og boliger. I Paper III søkte vi å identifisere faktorer assosiert med sammensetningen av bakterier fra boliger fra fem forskjellige nordiske studiesentre. I Paper IV hadde vi som mål å undersøke sammenhengen mellom innendørs bakterieprofiler (mangfold og mengde) og typer av bakterier, lungefunksjon og lungeinflammasjon.

Metoder: Luftbårne støvpartikler ble samlet inn med EDC-er over en periode på 14 dager. Bakterielt DNA og endotoksin ble ekstrahert fra prøvene. Vi sammenlignet DNA-ekstraksjonseffektiviteten til bakterielle komponenter fra EDC-er med fem forskjellige teknikker. En kvantitativ PCR (qPCR) ble brukt for å vurdere mengde bakterier. V3–V4-regionen til det bakterielle 16S rRNA-genet ble amplifisert og sekvensert ved bruk av Ilumina MiSeq for å bestemme sammensetningen av bakteriesamfunnene. Den kinetiske kromogene LAL-analysen ble brukt for endotoksinmåling. Sammenhengen mellom avhengige og uavhengige variabler ble studert ved bruk av multivariate regresjonsmodeller.

Resultater: I artikkel I fant vi at luftbårne bakterier var betydelig mer tallrike og mer mangfoldige i bønders boliger enn i boliger i urbane strøk ($p < 0,001$). De antatt gunstige bakteriene var mer tallrike i bøndenes hjem og har sin opprinnelse hovedsakelig fra husdyrgjødsel og det ytre miljø, men ikke nødvendigvis direkte fra dyrene selv.

I artikkel II fant vi at to teknikker ga bedre resultat enn de tre øvrige teknikkene som ble testet. Videre utviklet vi protokoller for samtidig å trekke ut både DNA og endotoksin fra et EDC filter.

I artikkel III har vi beskrevet store geografiske forskjeller for sammensetningen av de innendørs luftbårne bakteriene. Høyere innendørs bakteriediversitet og mengde var assosiert med høyere forekomst av bakterier som har opphav fra utemiljø, som kan skyldes mindre nedbør og høyere vindhastighet utendørs. Multivariate regresjonsmodeller viste at alfa-diversitetsindekser og bakterie- og endotoksinkonsentrasjoner var positivt assosiert med beboernes alder, hvor mange personer som bor i boligen, rengjøringsfrekvens, hundehold og boligens alder.

I artikkel IV fant vi at innendørs bakteriediversitet var assosiert med god lungefunksjon hos menn, men med mer lungeinflammasjon hos kvinner. Vi fant videre at *Actinobacteriota* bakterier var assosiert med bedre lungefunksjon, mens *Clostridia* bakterier var assosiert med lavere lungefunksjon. Høyt FeNO-nivå var negativt assosiert med *Cellulomonas* og positivt assosiert med *Campylobakter* bakterier.

Konklusjon: Vi viste at luftbårne bakterier i bøndenes hjem, spesielt storfebøndenes boliger, var preget av høy bakteriediversitet sammenlignet med boliger i urbane strøk. Vår studie (artikkel I) viser at bakteriesammensetningen i bøndenes hjem har sin opprinnelse fra husdyrgjødsel og bidrarytterligere til verifiseringen av hypotesen om mikrobielt mangfold.

I artikkel II beskriver vi en felles protokoll for DNA- og endotoksinekstraksjon. Teknikken vår bidrar til et høyt kvalitet-til-pris-forhold og kan brukes i store epidemiologiske studier som tar for seg luftbåren bakteriell eksponering der et stort antall prøver skal analyseres.

I artikkel III konkluderte vi med at innendørs luftbårne bakterier viser store geografiske forskjeller, og bidraget fra utendørs bakterietaxa til inneluft påvirkes av værforhold. Videre antyder resultatene våre at generelle livsstils valg som yrke, typer kjæledyr, hyppighet av rengjøring og bruk av desinfeksjonsmidler påvirker innendørs mikrobiomet.

Det ble funnet forskjellige assosiasjoner mellom innendørs bakterier og lungefunksjon og lungeinflammasjon hos menn og kvinner, noe som tyder på seksuell dimorfisme som respons på luftbåren bakteriell eksponering. Videre var assosiasjonsmønstrene mellom innendørs bakteriesamfunn og lungefunksjon og lungeinflammasjon forskjellige. Resultatene fra artikkel IV gir ny innsikt i å forstå det kompliserte forholdet mellom innendørs bakterieeksponering og luftveishelse.

List of publications

Paper I

Amin H, Šantl-Temkiv T, Cramer C, Vestergaard DV, Holst GJ, Elholm G, Finster K, Bertelsen RJ, Schlunssen V, Sigsgaard T, Marshall IP. *Cow Farmers' Homes Host More Diverse Airborne Bacterial Communities Than Pig Farmers' Homes and Suburban Homes*. **Frontiers in Microbiology, 17 June 2022.**

Paper II

Amin H, Marshall I. P, Bertelsen R. J, Wouters I. M, Schlünssen V, Sigsgaard T, & Šantl-Temkiv T (2023). *Optimization of bacterial DNA and endotoxin extraction from settled airborne dust*. **Science of the Total Environment, 14 October 2022.**

Paper III

Amin H, Šantl-Temkiv T, Cramer C, Finster K, F Gómez, Gislason T, Holm M, Janson C, Jogi NO, Jogi R, Malinovschi A, Marshall I. P, Modig L, Norbäck D, Shigdel R, Sigsgaard T, Svanes C, Thorarinsdottir H, Wouters I. M, Schlunssen V, Bertelsen RJ. *Indoor Airborne Microbiome and Endotoxin: Meteorological Events and Occupant Characteristics are Important Determinants.* **Environmental Science & Technology 31 July 2023.**

Paper IV

Amin H, Cramer C, Drengenes C, Finster K, F Gómez, Gislason T, Holm M, Janson C, Jogi NO, Jogi R, Malinovschi A, Marshall I. P, Modig L, Norbäck D, Shigdel R, Sigsgaard T, Svanes C, Thorarinsdottir H, Wouters I. M, Šantl-Temkiv T, Schlunssen V, Bertelsen RJ. *Association between indoor bacterial communities, lung function and airway inflammation.* **Manuscript**.

Terms and Abbreviation

16S rRNA	16 ribosomal RNA
Alpha diversity	Diversity within-sample
Amplicon	DNA product of amplification via polymerase chain reaction
ANCOM BC	Analysis of Compositions of Microbiomes with Bias Correction
ANOSIM	Analysis of similarity
ASV	Amplicon Sequence Variant
Beta diversity	Diversity presents between the communities
COPD	Chronic Obstructive Pulmonary Disease
DADA2	Divisive Amplicon Denoising Algorithm 2
ECRHS	European Community Respiratory Health Survey
EDC	Electrostatic Dust Collector
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
FeNO	Fractional Exhaled Nitric Oxide
IgE	Immunoglobulin E
LPS	Lipopolysaccharide
Microbiome	A characteristic microbial community occupying a reasonable well-defined habitat which has distinct physio-chemical properties
PCoA	Principal Coordinates Analysis
PFW	Pyrogen Free Water
PM	Particulate matter
Taxonomy	The classification of groups of biological organisms based on shared characteristics

1. Introduction

The global trend toward industrialization and urbanization has resulted in an increasing number of people living and working indoors. According to some studies, people in industrialized countries spend up to 90% of their time indoors [1, 2], so in terms of duration, the inhalation exposure takes place largely indoors. In recent decades, several studies have highlighted the role of ambient airborne particulate matter (PM) as an important environmental pollutant [3]. Current research on airborne particles focuses on the health effects of PM and the microorganisms associated with PM [4].

Bacteria are occurring everywhere in the environment surrounding humans including indoor environment, some bacterial groups are more common indoors compared to outdoors [5]. The indoor environment, bacteria, and humans all contribute to an interconnected system that affect each other [6]. Our health may be affected by exposure to airborne bacteria. Effects of indoor airborne bacteria, whether harmful or beneficial, depend on to the composition, amount, and diversity of the microbial exposure as well as to the duration, timing, and source of the exposures [7, 8]. For example, it has been demonstrated that the airborne microbial community can differ between environments that promote and inhibit the development of asthma and allergies [9]. Environments rich in microbes, such as farms can have beneficial health effects and protect from allergy and asthma among children [10, 11]. Differences in airborne bacterial composition have been linked to lower allergy risk such as higher abundance of gram-positive bacteria [12]. Lynch et al [13] discovered that compared to children with atopy or wheeze, the home environment of healthy children were more likely to be characterized by specific bacteria from the families *Prevotellaceae*, *Lachnospiraceae*, and *Ruminococcaceae*. According to these findings, exposure to a specific group of airborne bacteria may help to reduce the risk of developing respiratory disease and allergies.

Association between bacterial exposure and development of respiratory diseases such as asthma is well known. The prevalence of asthma and allergy have increased during the past decades especially in Northern Europe [14]. In this thesis, we studied bacterial exposures across different indoor environments, such as livestock stables, farmers' homes, and suburban homes. We studied the factors affecting the indoor airborne bacterial composition as well as their association with lung function and airway inflammation. We also optimized bacterial DNA and endotoxin extraction from settled airborne dust, which is considered the most popular approach for measuring microbiological airborne exposure in the indoor environment.

1.1. The Hygiene, biodiversity, and microbiome rewilding hypotheses.

Since the 1980s, developed countries have seen an increase in atopic disease and immune dysregulation disorders while experiencing a decrease in infectious diseases. The developing countries in contrast appear to exhibit the opposite effect, with less immune dysregulation and a higher prevalence of infectious disease. After observing that hay fever was less common in children with older siblings, Strachan proposed the hygiene hypothesis in connection with allergic disease in 1989 [15]. Strachan hypothesized that increases prevalence of infections in childhood could suppress the development of allergic disease later in the life [16]. Since then, it has been established that early childhood exposure to microbes and parasites can lower the risk of developing allergic diseases. Several studies have supported the hygiene hypothesis. It has been discovered that living in a microbe-rich environment, such as a farm, can protect against allergic diseases and asthma [11, 17].

The modification of the initial hygiene hypothesis, i.e., the biodiversity hypothesis, suggests that a high microbial diversity around us can also increase richness of the human microbiome which have beneficial effect on the immune system [18]. The exposure to a high microbial diversity appears to be critical in protective scenarios for allergy and inflammatory disorders [19, 20]. According to recent studies, not only diversity, but also more specific indoor airborne microbiome composition, may play a role for better respiratory heath [17, 21]. Recently, a microbiome rewilding hypothesis has been proposed, aimed at urban habitat restoration to increase the microbial biodiversity and micro-ecological processes in urban areas by promoting nature around us to be wild and diverse rather than cultured and 'tamed' in order to achieve the best benefits for human health [22].

1.1.1. Farm environment

Several studies have found that the exposure to environments rich in microorganisms such as farms protect against the development of childhood asthma and allergies [23-25]. These studies link contact with livestock animals to a lower risk of immunoregulatory disorders. Frequent contact with farm animals during early childhood seems to be an important determining factor for protection against immunoregulatory disorders [26]. This observation is in line with to the biodiversity hypothesis that state that limited exposure to microbial diversity plays a key role aetiology of immune-mediated disease.

The air in livestock stables is highly contaminated with numerous species of microorganisms. Normand et al [26] found that children living on farms are exposed to numerous fungal and bacterial taxa that originate from livestock stables. Pasanen et al., found that airborne microorganisms may be indirectly transmitted from cow stables to farmhouses on workers' clothing [27]. Microbial dispersal from the farmers' working place to the home environment occurs through airflow or direct transport by family members that interact with livestock. Beside animal exposure the outdoor environmental bacteria from sources such as soil, water, and plants in farm areas might be responsible for the increase of bacterial diversity in the farmers' homes [28].

Different livestock, livestock stable design, and animal practice may result in different microbial exposure and, as a result, possible different health consequences for farmers working in these environments, as well as transmission of these microorganisms to farmers homes. For example, Danish regulations require farmers working in mechanical ventilated pig stables to wash their hands and change clothes to prevent the spread of zoonotic pathogens compared to farmers working in open-air and less precautioned cow stables [29]. Other factors that could lead to different bacterial exposure in cow stables compared to pig stables include reduced use of antibiotics in cow farming compared to pig farming.

A protective effect associated with exposure to the farm environment may not be limited to bacteria or bacterial compounds such as endotoxin (cell wall competent of gram-negative bacteria) which has been associated with a reduced prevalence of allergic and respiratory disease such as atopic asthma [30]. Furthermore, non-microbial molecules such as N-glycolylneuraminic acid can protect against airway inflammation through influencing the human immune system. N-glycolylneuraminic acid is a sialic acid molecule found in non-human mammalian cells such as present in farm animals [31].

1.1.2. Urban environment

According to the United Nations, more than half of the world's population lives in urban area, and by 2050, that number is expected to rise to more than two-thirds [32]. Compared to the farm environment, the indoor environment in urban and suburban areas is characterized by a less diverse microbial exposure [17]. The difference in bacterial diversity between the two indoor environments could be attributed to the greater variety of outdoor microbial sources such as plants, soil, and livestock animals in a farm environment, whereas humans themselves is the main source of the indoor air microbiome in urban environments [7, 26]. Karelia, a

region on the border between Finland and Russia, has a similar climate and vegetation but is socioeconomically and culturally distinct, with a more modern and urbanized lifestyle in Finland and a rural lifestyle in Russia. According to studies conducted on the population of Karelia, respiratory diseases and atopy are more common in Finnish Karelia than in Russian Karelia [26] Higher indoor bacterial diversity as well as the animal-associated bacterial species are much more prevalent in Russian house dust than in Finnish houses [12].

1.2. Indoor bacterial exposure

Microbes, such as bacteria, are ubiquitous, and they are found everywhere in our built environment (BE) [33]. Humans have extensive interactions with bacteria that are circulating in the air or on the surfaces of accessible objects in BE [34]. As a result, BE, humans, and bacteria form a system of interacting ecosystems that influence one another. Understanding the interactions between these systems is critical for developing effective management strategies for the BE and its inhabitants, which will provide a knowledge base for the development of intervention strategies for better control of respiratory diseases. Indoor bacterial communities are complex entities, and variety of factors influence their composition and cell concentrations in BE [35]. The indoor bacteria community composition have been found to be considerably impacted by the occupants and building elements such as ventilation and building materials, as well as outdoor environmental characteristics including geographic characteristics, vegetation, and soil type [36].

1.2.1. Occupant and occupant behaviour

Human and non-human occupants (pets) are considered the major sources of indoor bacteria [7]. Humans introduce a significant amount of bacteria into the indoor environment by shedding them from their skin and other body parts or transporting them through their clothes and shoes from outdoor [1]. As a result, an increase in human occupancy is associated with an increase in human-associated microorganisms in indoor air. A great number of indoor airborne bacteria can be traced back to human skin. It has been estimated that humans shed roughly a billion skin cells daily, of which many are associated with bacteria [37], other significant sources of indoor airborne bacteria are human oral and respiratory fluid bacteria emitted via coughing, sneezing, talking, and breathing. The microbial clouds emitted by humans could differ between individuals [7]. The occupant's gender and health status, for example, have been shown to influence the indoor bacterial composition [38, 39].

Occupant behaviour, such as time spent indoors or activities performed in BE, appears to influence indoor microbes [33]. For example, the less frequently organic waste was emptied, the higher the microbial levels in the indoor environments [40]. Cleaning is another important factor with opposing effects: a low frequency of cleaning increases microbial levels on home surfaces [41], whereas regular cleaning significantly reduces microbial levels on surfaces [42]. Furthermore, the immediate effect of the act of cleaning temporarily increase the levels of bacteria in the indoor air by disturbances of settled particles [43].

Pets are another important microbial reservoir that may influence the composition of the indoor airborne bacteria [44]. Studies have shown that bacterial diversity increases significantly with the presence of a dog in a household [44, 45]. Several studies reported that contact with pet animals might be linked to a lower risk of asthma and allergies. However the studies are conflicting [46]. One large study found that pet ownership had a significant protective effect against asthma, but the effect was moderate when compared to exposure to farm animals [47].

1.2.2. Ventilation

Ventilation (e.g., the exchange with outdoor air) influences the diversity and abundance of microbial communities found indoors [5]. The most direct impact of ventilation on indoor microbiomes is to facilitate outdoor microorganisms entering the BE. In contrast to natural ventilation such as windows and doors, mechanical ventilation usually uses filters which prevent some of the outdoor microbes and particulates from entering the BE [48]. Therefore, naturally ventilated rooms have indoor microbiomes more similar to those in the outdoor environment. As a result, natural ventilation is proven to mitigate sick building syndrome [49], which has shown positive health effects. However, natural ventilation also introduces undesirable contaminants such as allergens which make challenge to the broader application of natural ventilation. [33].

1.2.3. Moisture

The indoor air community's survival is strongly influenced by moisture [50]. The moisture in BE may come from a variety of sources including indoor water vapor, plumbing problems, leaks from the building roof systems [51]. Moisture in the air may also impact the indoor microorganisms in different ways. Relative humidity is often used to measure moisture saturation in the air. Low relative humidity will increase the aerosolization and resuspension

of microorganisms from surfaces into air due to occupant movements [52]. On the other hand, high relative humidity can create niches for microbial growth and facilitates the direct contact transfer from fomite to occupants [50].

Living in buildings with moisture damage and dampness has been linked to negative health outcomes, specifically respiratory symptoms, and infections, as well as asthma exacerbation and new onset [53]. These associations are supported by strong epidemiological evidence that has been thoroughly examined and analysed [54]. The actual agents responsible for these associations, as well as the mechanisms underlying them, are unknown. However, changes in fungal and bacterial exposures in indoor air and dust in response to moisture problems have been reported [53].

1.2.4. Building materials

All building materials may be subject to microbial growth. The properties of the building materials, coupled with temperature, humidity, and ventilation as well as the occupants, can affect microorganisms indoors [33]. In two ways, material properties can aid microbial colonization. First, the building materials' ingredients can serve as potential substrates and nutrients for microorganisms [55]. Second, building materials, such as wallpaper glue, paint, and textiles which are characterized by rough and porous surface, facilitate the adherence of dust and organic compounds. In addition, porous surfaces may also retain moisture. As a result, rough and porous building materials could facilitate microbial growth due to increased levels of organics and moisture [56].

1.2.5. Outdoor characteristics

The indoor microbiome is influenced by different outdoor characteristics, including geographic characteristics, latitude, relative humidity, and precipitation. According to an inter-continental microbial survey in hotel rooms, the structure of bacterial and fungal communities differed depending on latitude, relative humidity, and proximity to the sea [57]. Danko et al [58] carried out a worldwide-scale metagenomic survey in 60 cities throughout the world. The authors found global microbiome differed spatially, with each city having its own microbial profile. A study of traditional Japanese homes revealed a correlation between the microbial content on indoor surfaces and relative humidity [59].

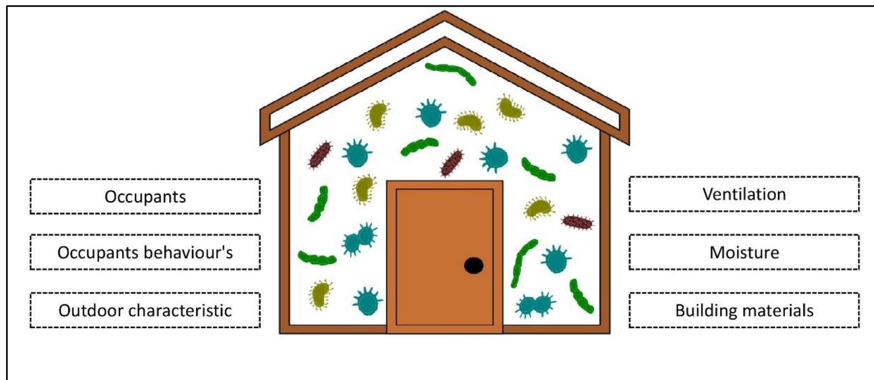

Figure 1: Main factors effecting indoor airborne bacteria.

1.3. Sampling methods for assessing indoor airborne microbial exposure

A variety of samples and collection devices have been used to investigate microbial exposure indoors and the health effects associated with it. In large epidemiological studies microbial sampling approaches in indoor environments typically need to be simple and feasible. The selection of the method depends on the purpose of the microbial measurement or the study question.

1.3.1. Active air sampling

Air sampling methods are intended to collect both culturable and nonculturable microbes. The active air sampling methods are based on several physical principles and methods. All of these methods necessitate stationary or personal pumps and trained personnel. Air samples are typically collected for short periods of time (from minutes to hours). As a result, it is unsuitable for assessing long-term exposure [60]. Furthermore, because airborne microbial concentrations vary both temporally and spatially, a single measurement does not provide a reliable picture of microbial exposure in indoor environments [61] making active air sampling unsuitable for assessing long-term microbial exposure in epidemiological studies, especially when a large number of samples are required.

1.3.2. Passive air sampling (dust sampling)

Dust samples are a better tool for measuring long-term microbial exposure indoors than short-term active air sampling. Dust samples describe the airborne microbial exposure through mechanisms of deposition and resuspension of dust. Dust samples have mostly been used in

large epidemiological studies where it is critical that the sampling is simple and inexpensive [62].

1.3.2.1. Reservoir dust samples

Long-term microbial exposure is often studied using reservoir dust samples such as floor and mattress dusts. Mattress dust samples have been used as a surrogate for microbial exposure in indoor environments, specifically when sleeping. In theory, mattress dust can be used to estimate an individual's long-term microbial exposure. However, mattress dust is dominated by a person’s own skin microbiota [37]. Another type of reservoir dust used to describe long-term indoor exposure is floor dust samples. Participants collecting floor dust samples from the floor or carpet using a vacuum cleaner is an easy and inexpensive method. One disadvantage of floor dust is that it can contain unknown amounts of particles that have never been airborne, such as soil or food.

There are two major limitations with the use of reservoir dust samples. First, dust accumulated over an unknown time period, particularly in the case of floor dust samples, which limits its usability when answering research questions that require fixed time periods of exposure [63]. Second reservoir dust may contain material that never or only partly contributes to resuspension. As a result, reservoir dusts may not accurately represent indoor airborne exposure.

1.3.2.2. Settled dust samples

The settled dust samples may represent better microbial inhalation exposure than reservoir dust [64]. Standardized surfaces are typically used to collect particles that fall from the air and settle on the surface over a set and standard amount of time, usually days to weeks. Settled dust samples aim to represent indoor air exposure, but microbial amounts in settled dust samples are generally lower than in floor dust or mattress dust samples due to lower concentration of airborne particles [62].

Among the different techniques, EDCs (Electrostatic Dust fall collectors) have been employed as a standard for passive sampling of settled airborne dust. The EDC is a sampling device made up of a polypropylene folder that holds two or four electrostatic cloths [64]. They are ideal for large epidemiological studies because they can be mailed to, deployed by, and returned by study participants without the need for specialized equipment or field personnel

[65]. Traditional biological exposure markers such as allergens, endotoxins, and glucan exposure have been studied using dust collected by EDCs [66-68]. With advances in next-generation sequencing techniques, EDCs were used to collect microbial cells for DNA sequencing analysis. However, low DNA yields have been a problem when using EDC in epidemiological studies, particularly for indoor urban samples which only contains a small amount of settled dust.

1.4. Bacterial agents targeted in epidemiological studies

Various bacterial products are targeted with chemical analytical methodology for determination of microbial exposure in the indoor environments. In this thesis we studied indoor bacterial exposure using endotoxin and bacterial DNA.

1.4.1. Endotoxin

Lipopolysaccharides (LPS), referred to as endotoxins, are large, biologically active molecules that form the outer membrane of Gram-negative bacteria. Endotoxin is by far the most commonly used cell wall marker in studies of health effects related to microbial exposure. The association between endotoxin exposure and respiratory health effect is complex. The protective effect of endotoxin is found in younger school-aged, non-allergic children [69]. However, negative health effects have been reported in occupational settings, in particular for occupations where the endotoxin exposure is high, such as for waste collecting, livestock farming, agriculture, and in the textile industry [70]. There are several types of analyses to measure endotoxin, but endotoxin is typically quantified with the Limulus Amoebocyte Lysate (LAL) assay [71]. This is an enzymatic assay that quantifies the activity of lipopolysaccharide in a sample by triggering an enzymatic cascade.

1.4.2. Muramic acid

Muramic acid (N-acetyl muramic acid) is an amino sugar and a constituent of the peptidoglycan layer of the cell walls of bacteria. The amount of peptidoglycan is much higher in Gram-positive bacteria (30 – 70% of the cell wall) than in Gram-negative bacteria (< 10% of the cell wall). Hence, muramic acid has been used as a chemical marker of Gram-positive bacteria in several epidemiological studies [72-74].

1.4.3. Viable cells

Cultivation is used to determine the number of viable microbes (colony forming units (CFU)) and to identify bacterial colonies. Microbes are either collected directly on the growth media or into a filter and then suspended in liquid medium [75]. Cultivation is selective because of the growth media used, and only those bacteria are detected that are capable to utilize the nutrients supplied by the medium. Identification of microbial isolates via microscopic or macroscopic evaluation requires training and a high level of expertise.

Exposure to viable bacteria indoors has been mainly determined from air samples. The culturable bacteria in house dust have only been analysed in a few studies. The viable bacterial biota in dust seems to be dominated by Gram-positive bacteria such as *Staplylococcus, Corynebacterium, and Lactococcus* [43].

1.4.4. Bacterial DNA

The first step in all DNA-based methods is the extraction of DNA from a sample. In the case of EDCs, this happens in two steps: the extraction of bacterial cells from the sample matrix, and the extraction of DNA from the cell envelopes either mechanically, enzymatically, and/or chemically to free the intracellular DNA [76]. Selecting single protocol to efficiently extract both Gram-positive and Gram-negative bacteria could be tricky due to the different cell wall characteristic. Unlike Gram-negative species that are readily lysed by standard protocols, the Gram-positive bacteria are more resistant to cell lysis because of greater concentration of peptidoglycan in their cell wall [77].

Targeting bacterial DNA allows the description of bacterial exposure quantitatively or qualitatively, depending on the methods applied. Culture independent detection methods have the advantage of detecting non-culturable and non-viable bacteria alongside with viable and culturable ones and thus provide a more complete picture of the bacteria, potentially health relevant exposure.

Quantitative Polymerase Chain Reaction (qPCR) provides quantitative information on the amount of a specific taxonomic group targeted as compared to chemical makers such as endotoxin measurements. Using qPCR with universal bacterial primers gives a general estimate of bacterial cells equivalents in a sample. Primers and probes are designed for the detection of a taxonomic group, genus, or a single species of interest. For quantifying total bacteria in

samples, the 16S ribosomal RNA (rRNA) genes are targeted, since they contain sequence regions that are highly conserved between members of the same genus or species [78]. In studies of the indoor environments, qPCR method has been frequently used to target bacterial groups from indoor dust samples [79-81].

In last decade, the microbiome of indoor environments has been studied with next generation sequencing (NGS) approaches, which allow a virtually complete characterization of complex bacterial communities in indoor environment. Microbial communities are primarily assessed with bacterial 16S rRNA gene amplicon sequencing techniques. Sequencing costs have been drastically reduced over the last decade, so that microbial community analyses from indoor samples is feasible even in large-scale studies. Progress with applying these approaches to indoor studies improved our understanding of the microbial ecology in BE. NGS-results have confirmed that human occupancy is one of the main sources for microbes in indoor environments [7, 82]. However. relatively few studies have yet applied these methods to study health effects of indoor bacterial exposure [83-85].

1.5. Indoor microbial exposures and respiratory health

Indoor microbial exposures have been associated with the development or exacerbation of respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [86]. According to recent microbiome studies, the pandemic of such respiratory diseases may partly be caused by reduced human exposure to environmental and commensal bacteria [87, 88]. Consistent with the "biodiversity hypothesis," which states that exposure to diverse microbial environments enriches the human microflora, enhances its immunomodulatory capacity, and protects against allergic and inflammatory diseases [18] In line with the biodiversity hypothesis, several studies have shown that higher indoor bacterial diversity is associated with better respiratory health [11, 84, 89, 90]. For example, Campbell et al (2017) [90] found increasing proxies of high microbial diversity during childhood to be associated with higher lung function in adulthood. Other studies, however, showed that bacterial diversity has not been associated with lung health [91, 92]. As a result, the link between indoor bacterial diversity and respiratory health is still unclear. On the other hand, indoor exposure to endotoxin has sparked heated discussion about whether it is harmful, neutral, or beneficial to lung health. For example, in the case of asthma, while a significant number of studies have found that endotoxin exposure protects against the development of asthma or its symptoms [93-96], an

equal number have found indications of harmful effects associated with endotoxin exposure [97-100], and some have found no association [101, 102].

Recent epidemiological studies relying on culture independent techniques report that potential beneficial indoor microorganisms are mainly found within the *Alphaproteobacteria* and *Actinobacteria*, and the less beneficial microorganisms are mainly from *Bacteroidia and Clostridia* [17, 36, 84, 91]. A major issue was that the health-associated microorganisms were different among studies and showed strong geographic variation. Most of the epidemiological studies looking into the association between indoor bacterial exposure and respiratory disease relies on questionnaire data and self-reported symptoms. In the current thesis, we studied indoor bacterial exposure in relation to markers of respiratory health such as lung function and airway inflammation.

1.5.1. Lung function and spirometry

Lung function tests, also known as pulmonary function tests, are non-invasive tests that examine how effectively the lungs operate and can thus aid in the investigation of breathing problems. Lung function tests can assist in the screening and diagnosis of lung disorders such as asthma and COPD. In addition, they can monitor lung diseases, and assess the efficacy of therapies [103]. There are different types of lung function tests, among them is spirometry, which is the most common and basic type of lung function test. Spirometry measures how much air the lungs can retain. The Spirometry also measures how forcefully one can empty air from the lungs [104].

Spirometry data from a patient must be compared to data from peers who are not smokers and have healthy lungs. Normal lung size and function are determined by four key factors: age, gender, height, and race [105]. Lung growth is complete by early adulthood and then decline by ageing. As a consequence, expected spirometry values should be calculated using an accurate age. Sex is a major correlate to lung function. Males with the same age, height, and race as females have larger lungs. Height is also used to calculate expected spirometry values as an estimate of chest size [106].

The three most commonly used indices in spirometry interpretation are forced vital capacity (FVC), forced expiratory volume in the first second (FEV1), and FEV1/FVC ratio [106]. Respiratory clinicians and epidemiological researchers often express and interpret lung function test results as a percentage (%). The % predicted values are calculated by dividing the

observed measurement (absolute values in L) by a predicted value multiplied by 100 (% predicted (observed/predicted) × 100) [107]. The main disadvantage of using % predicted is that it ignores the fact that the natural variability of spirometry outcomes in health is highly age dependent [107, 108]. The expression of results as Z-scores is a valid alternative method of reporting lung function. The Z-score (also known as standardised residual scores or SRS) is a mathematical combination of the percent predicted and the between-subject variability that results in a single number that accounts for the expected age- and height-related lung function variability among comparable healthy individuals [109].

1.5.2. Airway inflammation and Fractional Exhaled Nitric Oxide (FeNO)

Airway inflammation is thought to be an important component of asthma, chronic obstructive pulmonary disease (COPD), and other respiratory disease [110]. There are different techniques to assess airway inflammation. Among the techniques that are well developed and widely used in clinical trials to measures airway inflammation is exhaled nitric oxide (FeNO) where the concentration of nitric oxide (NO) in exhaled air is used to provide information about the presence of eosinophilic airway inflammation [111]. During inhalation, inducible nitric oxide synthase (NOS) enzyme expression in eosinophil and epithelial cells of the airways produces pathological NO [112]. FeNO has the advantage of being safe, straightforward, simple to replicate and provides an immediate result [111].

2. *Aim of thesis*

2.1. Main objective

The main goal of the thesis was to study the indoor airborne bacterial communities inside homes in Northern Europe, how they differ between homes in rural and suburban areas, what factors affect the indoor airborne bacterial communities, as well as their association with respiratory health markers.

2.2. Specific objectives

- In Paper I, we aimed to investigate the difference in airborne bacterial composition between the farmers' homes and suburban homes and to study bacterial transfer between livestock stables and farmers' homes.
- In Paper II, we attempted to increase the efficiency of the extraction of airborne dust, DNA, and endotoxin from the EDC.
- In Paper III, we attempted to identify factors associated with the composition of the indoor bacterial communities.
- In Paper IV, we aimed to investigate the association between indoor bacterial profiles (diversity and load), lung function, and airway inflammation, and furthermore, we aimed to determine whether individual bacterial taxa in the indoor microbiome affect lung function and airway inflammation.

3. *Material and Methods*

3.1. Study populations

This thesis is based on samples from four studies: the Sund Stald (SUS) study, the Health2006 cohort study, a pilot study population, and ECRHS III (European Community Respiratory Health Survey III). Paper I is based on the Sund Stald "(SUS) study and the Health2006 cohort. Paper II is based on a pilot study population. Papers III and IV are based on the ECRHS III study.

3.1.1. Sund Stald" (SUS) study (Paper I)

The SUS study began in 1992 among farming apprentices with the goal of describing the occurrence of respiratory symptoms in a farming environment and investigating the impact of farming on the development of allergies, asthma, and respiratory diseases [113]. In Jutland, Denmark, settled air dust was collected in 2007-08 during the 15-year follow-up from the homes of cow and pig farmers, as well as associated livestock stables. The decision to focus on farms in Jutland was motivated by the fact that Jutland accounts for the vast majority of Danish livestock production (roughly 80%).

3.1.2. Health2006 cohort study (Paper I)

For comparison with farmers' homes and associated livestock stables, settled air dust samples from suburban homes were collected as part of a cross-sectional study nested within the Health2006 cohort. Sampling was carried out in Greater Copenhagen. The people who lived in 11 municipalities south-west of Copenhagen and were from an urban background made up the Health2006 baseline cohort [114].

3.1.3. Pilot study population (Paper II)

Ten participants (faculty members from Aarhus University) from Aarhus, Denmark, took part in the study. Each participant collected settled airborne dust using EDCs from their living room and bedrooms. Eight EDC replicates were collected per sampling location (16 EDC clothes per participant); four EDCs were collected in the bedroom and four EDCs in the living room.

3.1.4. ECRHS III (Paper III and IV)

The ECRHS was initiated in 1991–1993 (ECRHS I), when over 18 000 young adults were randomly recruited from available population-based registers. Two examinations (at 27–57 years (ECRHS II, 1999–2003) and 39–67 years (ECRHS III, 2010–2014) have since taken place. The present study is comprised of 1038 subjects participating in the ECRHS III from Aarhus (Denmark), Bergen (Norway), Reykjavik (Iceland), Tartu (Estonia), and Uppsala (Sweden) (see Figure 2). From 2011 to 2014, the participants received an EDC after the main ECRHSIII questionnaires and clinical examinations to collect settled airborne dust from their bedrooms. Information about environmental determinants and respiratory symptoms were extracted from the ECRHS III interview questionnaire. The study protocols were approved by the local ethics committees at each of the centres. For detailed information about all questions, see appendix.

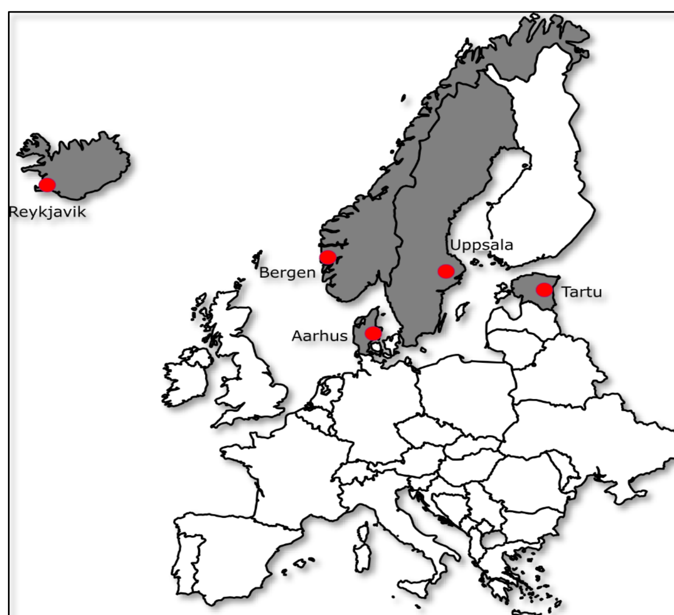

Figure 2: Study centres from ECRHS III included in paper III and IV.

3.2. Dust sampling (Paper I-IV).

An EDC (Electrostatic Dust Fall Collector) contain two EDC clothes each with a 209 cm^2 exposure area was used to collect settled dust from the air (Figure 3). The EDCs were placed approximately 1.5 m above the floor and exposed for 14 days [114]. The participants received a pre-paid envelope and were asked to return the EDC by mail to the research unit. All EDCs' cloths and samples were stored at -20°C until dust extraction.

Figure 3: The EDC sampler is composed of two electrostatic clothes placed in a plastic folder that is left open in a horizontal position for 14 days to allow dust to settle. The folder was kept closed before and after sampling and during transport and storage.

3.3. *Dust extraction (Paper I-IV).*

For paper I, the dust was extracted from one EDC cloth as follows: The EDC cloth was carefully placed in a sterile stomacher bag and mixed with 20 ml of PFW and 0.05% Tween 20 extraction buffer. The sample was processed in a stomacher at full speed for 10 minutes. The washed-off dust-containing fluid was then transferred to a sterile 50ml Falcon tube. This procedure was repeated until 40 ml of suspended dust was extracted from the EDC. Centrifugation was used to collect the dust pellet. After discarding the supernatant, the pellets were resuspended in 1.5 ml of Tween 20 extraction buffer. Unexposed EDC clothes were used for negative control extractions. The dust samples were kept at -20°C until DNA extraction.

In paper II, the extraction method was optimized and then later applied in paper III. The dust from the EDC cloth was extracted as follows: the EDC cloth was placed in a sterile stomacher bag and mixed with 20 ml of extraction buffer (PFW and 0.05% Tween 20). The samples were then processed in the stomacher for 3 minutes. The extracted fluid was stored on ice in a 50 ml falcon tube. This procedure was carried out again with 30 ml of PFW. On average, the total volume extracted from EDC was 45 ml. Approximately 4.5 ml (10%) were transformed into a 15 ml falcon tube for endotoxin extraction. The remaining 90% of the dust extract was used to collect dust into a 0.22 m pore size polyethersulfone membrane filter using glass filtration equipment. The membrane filter was transferred aseptically into pre-filled bead tubes and stored at -20°C until DNA extraction. For more information see dust extraction section in Paper I, II and III.

3.4. *DNA extraction (Paper I-IV).*

In the first publication, PowerLyzer PowerSoil DNA Isolation kit (MO BIO Laboratories, a Qiagen Company,Germany) was used to extract DNA from the dust pellets following the manufacturer's instructions.

In the second publication, five DNA extraction methods were used to extract DNA from settled airborne dust collected on EDC cloth to compare their performance. We compared three commercially available DNA extraction kits, DNeasy PowerSoil Pro Kit, DNeasy PowerWater Kit, and MagAttract PowerWater DNA/RNA Kit (MO BIO Laboratories, a Qiagen Company, Hilden, Germany), with two previously described non-commercial DNA extraction methods. The first was used to extract DNA from airborne cells collected on Sterivex filter columns [115], while the second was used to extract DNA from sediment samples [116]. The three

commercial kits were used according to the manufacturers' instructions. The detailed DNA extraction of dust using two non-commercial DNA extraction methods can be found in the second publication.

Based on method optimization from the second publication, the DNeasy PowerSoil Pro Kit (MO BIO Laboratories, a Qiagen Company,Germany) was used for DNA extraction from the dust in the third publication following the manufacturer's instructions with minor modifications. For more information see DNA extraction section Paper II and Paper III.

3.5. Endotoxin extraction and LAL assay (Paper II-IV)

Ten percent of the 45 ml liquid containing dust from the EDC cloth was transferred to a 15 ml Falcon tube for endotoxin extraction. Centrifugation at 1000 × g for 15 minutes removed the cell debris, and the supernatants were stored in glass vials as aliquots at -20 C until analysis. The supernatants were diluted in PFW 50 times before being tested using the quantitative kinetic chromogenic LAL assay (Kinetic-QCL 50-650 U kit, Lonza, Walkersville, Maryland, USA). Endotoxin from Escherichia coli O55:B5 was used as a reference standard. For the standard curve, thirteen concentrations ranging from 25 EU/ml to 0.006 EU/ml were used. The kinetic LAL assay detection limit for Vmax was defined as the average of the assay blanks plus two times the standard deviation of these blanks. The results were presented in EU m^2.

3.6. Mock community (Paper II)

In the second paper, ZymoBIOMICSTM Standardized Microbial Community (D6300) (mock community) (Zymo Research,Irvine, California,USA) was used to assess the DNA extraction efficiency. First, the mock community was used to assess the efficiency of DNA extraction as a function of different cell loads by double washing EDC clothes with 0.05% Tween 20 buffer. Secondly, the mock community was used to test the effect of additional rinsing with PFW on DNA yield. Finally, we used a mock community to test the ability of the combined extraction method (single washing EDC cloth with 0.05% Tween 20 buffer followed by rinsing once with PFW) to extract DNA across bacterial taxa using 16S RNA sequencing. For more details on the use of mock community and how the efficiency of the DNA extraction methods was calculated, see Paper II.

3.7. *Quantification of bacterial abundance (Paper I-IV)*

The bacterial load was measured using qPCR targeting 16S rRNA genes. Briefly, the reactions were carried out in a 20 ul reaction volume, which included 10 ul SYBR Green 1Master-2x, 2 ul bovine serum albumin (BSA; 10 mg/ml), 1 ul forward primer Bac908F (50-AAC TCA AAK GAA TTG ACG GG-30), 1 ul reverse primer Bac1075R (50-CAC GAG CTG ACG ACA RCC-30) (10 pmol/ml) [117], and 4 ul ddH2O (double-distilled water). Controls were created by replacing the DNA template with ddH2O. Serial dilutions of a plasmid containing a full-length 16S rRNA gene related to Sphingomonadales were used to generate standard curves. A detailed qPCR protocol is included in Paper I.

3.8. *16S rRNA amplicon sequencing (Paper I-IV)*

The 16S rRNA gene V3 and V4 regions were amplified using Bac341F (5'-CCT ACG GGN GGC WGC AG-3') and Bac805R primers (5'-GAC TAC GGT ATC TAA TCC-3'). The Illumina protocol (16S Metagenomic Sequencing Library Preparation) was used, with the modifications described in Vestergaard et al [118]. Three PCR reactions were used in the library preparation. The first PCR used bacteria-specific primers to amplify the V3 and V4 regions of the 16S rRNA gene. The second PCR included Illumina overhang adaptors, and the third PCR used Nextera XT Index primers. After each PCR step, AMPure XP magnetic beads were used to clean the PCR products. The Quant-iTTM dsDNA assay kit and the FLUOstar Omega fluorometric microplate reader (BMG LABTECH, Ortenberg, Germany) were used to determine the concentration of the PCR products. Following that, the samples were pooled and diluted to around 3 ng/ml DNA. Finally, the samples were sequenced using MiSeq sequencing (Illumina, San Diego, California, USA).

3.9. *Bioinformatic and statistical analysis (Paper I-IV)*

Bioinformatic and statistical analysis were performed in the R environment. The paired end read raw sequences were trimmed using the cutadapt package [119]. The open-source software package DADA2 [120] was used for error correction and modelling of the sequenced data, mostly by following the DADA2 pipeline. The shortread package [121] randomly subsamples all sequences to 20,000 reads (Papers I, III, and IV) and 50.000 reads (Paper II) to make richness comparisons accurate, as DADA2 tends to inflate richness estimates linearly with an increasing number of reads. The ASVs were taxonomically classified to the species

level using the SILVA database [122] reference database version 138. The decontam package [123] was used to eliminate ASVs contaminating reads in exposed EDC samples.

The ampvis2 package [124] was used to produce heatmaps showing the relative abundance of different taxa. The phyloseq package [125] was used to assess the alpha diversity using two diversity measures: observed (the number of individual bacterial taxa) and the Shannon index (the index reflects both richness and the relative abundance of each taxon in the data). The Wilcoxon Rank Sum test was used to compare the alpha diversity indices of different indoor environments and to investigate the differences between qPCR and the LAL assay measurements of bacterial and endotoxin load.

The ordination plots were carried out to compare the microbial communities in different indoor environments based on the Aitchison dissimilarity matrix and ape package [126]. Pairwise statistical comparisons were made between different indoor environments using the analysis of similarity (ANOSIM) test from the vegan package [127] based on the Aitchison dissimilarity matrix.

We used Analysis of compositions of microbiomes with bias correction (ANCOM-BC) [128] to identify the bacterial taxa whose abundances differ between different environmental types. For each bacterial taxon, ANCOM BC provides a statistically valid test with a q value (adjusted p-value) and confidence intervals (log fold change: natural logarithm). ANCOM BC was used for genera with relative abundances equal to or greater than 0.01%.

To study bacterial transfer between farmers' homes and associated livestock stables in Paper I. The "dist" function in the coda.base package [129] was used to construct Aitchison dissimilarity matrix dissimilarities between a farmer's home and the relevant stable (i.e., the sample pair represents where a farmer lived and worked). For more information, see paper I.

In papers III and IV, to study associations between dependent (outcome) and independent variables (exposure) we used adjusted multivariate linear regression. For model details about different models used see Paper III and IV. Table 1 lists the aims, characteristics, and methods of the four studies included in the thesis.

Table 1: Aims, characteristics of study populations, and methods used in papers I-IV.

	SUS & Health 2006 cohort study (Paper I)	Pilot study population (Paper II)	ECRHS III study (Paper III)	ECRHS III study (Paper IV)
Main objectives	Comparing types of bacteria and bacterial transfer between cow and pig stables and their farmers' homes. Comparing type of bacteria present between farmers' homes and suburban homes.	Improve DNA extraction from settled dust collected by EDC. Combine DNA and endotoxin extraction from EDC	To describe indoor airborne bacteria composition in Northern Europe. To determine factors affecting indoor bacteria in Northern Europe	To investigate the associations between indoor airborne bacteria, lung function and airway inflammation in adults in Northern Europe
Number of samples	Total number: 357 Pig farmer homes n= 84 Pig stables n=83 Cow farmer homes n=49 Cow stables n=41 Suburban homes n=100	Total number: 155 * EDCs collected from living room and the bedrooms of ten participants in Aarhus.	Total number: 1038 Aarhus n=160 Bergen n=300 Reykjavik n=346 Tartu n=84 Uppsala n=148	Total number: 1038 Aarhus n=160 Bergen n=300 Reykjavik n=346 Tartu n=84 Uppsala n=148
Laboratory & clinical examination	16s rRNA sequencing qPCR	16s rRNA sequencing qPCR LAL assay Mock community	16s rRNA sequencing qPCR LAL assay	16s rRNA sequencing qPCR LAL assay Spirometry test FeNO measurements
Statistical analysis	Wilcoxon rank sum test PCoA ANOSIM ANCOM BC	Wilcoxon rank sum test PCoA	ANOSIM ANCOM BC Adjusted multivariate linear regression. Spearman's correlation	ANOSIM ANCOM BC Adjusted multivariate linear regression.

* We successfully collected 155 out of 160 EDC clothes. Five EDC clothes were removed from the study because participants reported that the EDC fell on the floor or that a pet had been sitting on the EDC.

4. Summary of papers

4.1. Paper I: Cow Farmers' Homes Host More Diverse Airborne Bacterial Communities Than Pig Farmers' Homes and Suburban Homes.

A lower risk of immunoregulatory disorders including asthma and allergies has been associated with living on a farm. The increase in immunoregulatory diseases is believed to be caused by a decline in the diversity and composition of indoor microbial communities, with airborne bacteria contributing to achieve this effect. However, the composition of this microbial community in various farm and suburban indoor environments is still to be characterized.

In the first paper, our first aim was to compare the types of bacteria found in stables and farmers' homes, second to determine the difference between microbial communities in farmers' homes and suburban homes. Finally, to search for differences in taxonomic groups of putatively beneficial bacteria between livestock stables, associated farmers' homes, and suburban homes.

Over the period of 14 days, we used electrostatic dust collectors (EDCs) to collect settled airborne dust from stables and the homes of the associated farmers as well as from suburban homes. Then, the bacterial abundance was assessed using qPCR. In order to assess microbial diversity, the V3-V4 region of the bacterial 16S rRNA gene was amplified and sequenced using an Ilumina MiSeq. The inference of amplicon sequence variants from amplicon data was performed using the DADA2 algorithm.

We found that airborne bacteria were significantly more abundant in farmers' indoor environments than in suburban homes ($P < 0.001$). Cow farmers' homes had significantly higher bacterial diversity than pig farmers' and suburban homes ($P < 0.001$). Cow stables have higher bacterial diversity ($P < 0.001$) and abundance ($P < 0.001$) than pig stables. *Prevotellaceae*, *Lachnospiraceae*, and *Lactobacillus* bacterial taxa, as well as putative beneficial intestinal bacterial species such as *Lactobacillus amylovorus*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii*, were significantly more prevalent in farmers' homes than in suburban homes. We further found that higher similarity between bacterial communities in individual farmers' homes and their associated cow stables than for pig stables.

In conclusion, the results demonstrate that there are significant differences between suburban and farm homes in terms of airborne bacterial abundance, alpha and beta diversity, and community composition. Farmers' homes, particularly cow farmers' homes, were characterized by high bacterial diversity compared to suburban homes, which were dominated by bacteria from human sources and had low bacterial diversity. Furthermore, the gut microbiome of the farm animals contributed to the indoor airborne bacterial communities in farmers' homes, especially in the case of pig farmers' homes.

4.2. *Paper II: Optimization of bacterial DNA and endotoxin extraction from settled airborne dust.*

In the first study, one third of samples from suburban homes were excluded from analysis due to low DNA yield. The EDC is a passive dust sampling device that has been established for assessing airborne exposures, mainly for endotoxins. With advances in next-generation sequencing techniques, EDCs were used to collect microbial cells for DNA sequencing. However, low DNA yields have been problematic when employing passive sampling with EDC, especially in indoor environments that are characterized by a low airborne bacterial load, such as suburban and urban homes, a problem we faced in our first study (Paper I).

In the second paper, we aimed to optimize dust and DNA extraction from airborne dust collected using EDCs and establish a method to jointly extract DNA and endotoxin from a single EDC cloth. Using replicate EDC cloths that were simultaneously collected as well as a standardized mock community, we attempted to increase the efficiency of bacterial cell extraction from EDCs by employing filtration instead of centrifugation to concentrate the cells from the wash solution. We compared DNA extraction efficiency from cells collected on filters using five extraction techniques (Figure 4) by measuring the abundance, diversity, and structure of bacterial communities using qPCR and amplicon sequencing targeting 16S rRNA genes. Furthermore, we tested the co-extraction of DNA and endotoxin from a single EDC cloth and compared the endotoxin yield to the established method described by Noss et al [64]. Thirdly, we tested three different buffer systems (Figure 4) to achieve the optimal amount of DNA and endotoxin from the same extraction procedure.

We found that two DNA extraction methods outperformed the others. Furthermore, we showed that using a single EDC cloth is sufficient to accurately determine DNA and endotoxin exposure. Our protocol promotes a high quality-to-price ratio and may be employed in large epidemiological studies addressing airborne exposure when a large number of samples are needed.

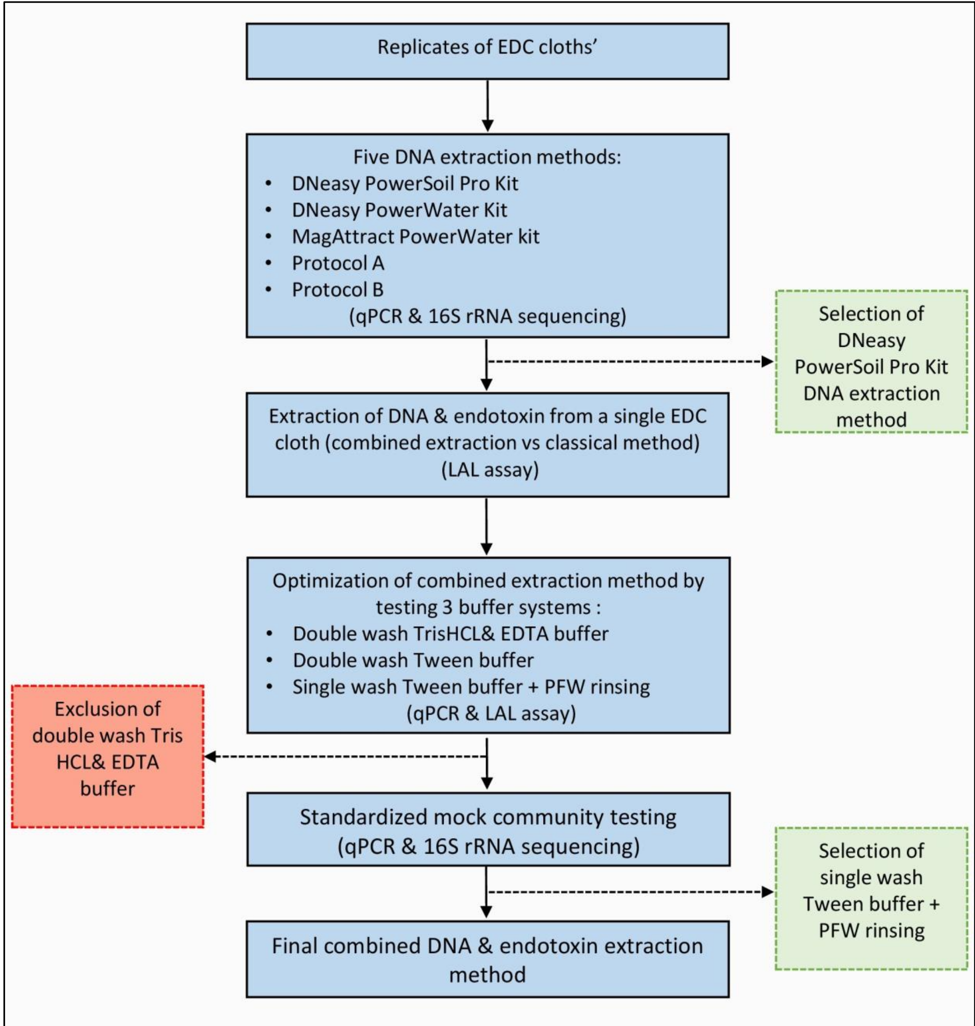

Figure 4: Overview figure summarizing the studies, methodology, and results in the second paper.

4.3. *Paper III: Indoor Airborne Microbiome and Endotoxin: Meteorological Events and Occupant Characteristics are Important Determinants.*

The indoor air environment contains diverse bacterial cells that originated from different sources. The exposure to these cells can impact human health, affecting the quality of life of many people. Despite their major role, little is known about the environmental determinants that contribute to the identity, diversity, and abundance of airborne bacterial cells.

In the third paper, we investigated the airborne microbiomes in the homes of 1038 European Community Respiratory Health Survey (ECRHS) III participants from five cities in Northern Europe: Aarhus (Denmark), Bergen (Norway), Reykjavik (Iceland), Tartu (Estonia), and Uppsala (Sweden). The settled airborne dust was collected in participants' bedrooms over a 14-day period using EDCs. The EDC clothes were handled based on protocol developed in the second paper, where dust, endotoxin, and DNA extraction from EDC clothes were optimized to obtain a comprehensive representation of the airborne bacterial communities. The DNA extracts were used for qPCR measurements to estimate bacterial load and 16S rRNA gene sequencing to determine microbial community composition, while the kinetic chromogenic LAL test was employed to quantify endotoxin. The association between bacterial profiles (such as diversity and load) and environmental determinants was studied using multivariate regression models.

Our results showed that households in Aarhus and Tartu were characterized by a higher bacterial load, diversity, and abundance of outdoor bacterial taxa compared to households in Bergen, Reykjavik, and Uppsala. Pairwise comparison between the Nordic cities showed the largest difference in beta diversity between Bergen and Tartu households (ANOSIM $R = 0.304$, $P = 0.001$). Meteorological factors associated with the different locations had an impact on the indoor airborne bacterial community, most likely through their impact on outdoor airborne bacteria. While precipitation had a negative correlation with the diversity and load of indoor airborne bacteria, windspeed had a positive correlation.

We found that the diversity of the indoor airborne microbiome increased while the bacterial load decreased with increasing age of the occupants. According to the multivariate regression models, alpha diversity indices and bacterial and endotoxin loads were positively associated with the number of occupants, the cleaning frequency, the presence of dogs, and the age of the house.

In conclusion, higher abundance of outdoor bacteria in Tartu and Aarhus households could be due to different weather events during the sampling period in these study centres. We also conclude that age of the occupant was strongly linked to the composition of the indoor bacteria community. Furthermore, we conclude that general lifestyle choices such as the number of occupants, types of pets, cleaning frequency of the household, and use of chemical disinfectants significantly impact the diversity of the indoor microbiome.

4.4. *Paper IV: Association between indoor bacterial communities, lung function and airway inflammation.*

Indoor bacterial communities have been linked to asthma in children, but little is known about the role of specific bacterial taxa in relation to lung function and lung inflammation in adults. We aimed to study the association between indoor bacterial exposure and lung function and airway inflammation in adults.

Settled airborne dust samples from the bedrooms of 1038 participants in the ECRHS III study were used to study the association between indoor bacterial communities (exposure) and spirometry and FeNO measurements (outcomes) using adjusted linear regression models stratified by the sex of the participants.

Indoor bacterial diversity and richness were associated with high lung function in males, but with elevated airway inflammation in females ($P < 0.05$). Most of the bacterial genera associated with better lung function were from the *Actinobacteriota* phylum. Where *Bacteroidia*, and *Clostridia* were found to be inversely associated with lung function. Bacterial genera considered to be part of the core oral microbiome, such as *Streptococcus* and *Veillonella*, were negatively associated with lung function. Furthermore, several bacterial genera were negatively associated with airway inflammation, only *Cellulomonas* (phylum *Actinobacteriota) was associated with lower* airway inflammation.

In conclusion, the indoor microbiome was associated with lung function and airway inflammation, modifying the outcomes differently in men and women. Lung function and airway inflammation showed very different patterns of association with the indoor microbiome.

5. *Discussion*

In this section, the main finding and implications of these studies are discussed and compared to existing knowledge in the field.

5.1. Indoor airborne microbiome between farmer's home and suburban homes

Several studies have found that children raised on farms have a lower incidence of allergy sensitization than those raised in cities. These studies correlate exposure to farm-related microbiota via interaction with livestock animals to a lower risk of allergies [17, 130, 131]. According to the hygiene hypothesis, minimal microbial exposure plays an important role in the development of immune-mediated diseases [132]. Ege et al. found that children raised on farms were exposed to a broader spectrum of environmental microbes than children raised in suburban environments [11]. One of our objectives in the first study was to evaluate the airborne bacterial communities of cow and pig farmers' homes and determine whether and to what extent the microbial communities in farmer's homes and suburban homes differ. Using qPCR, we found no significant variation in bacterial abundance between cow and pig farmers' homes. In contrast, there was a considerably higher bacterial abundance in the two types of farmer's homes compared to suburban homes, as previously shown by Pakarinen et al. [12].

The bacterial diversity differed significantly between the three home environments. In comparison to pig farmers' and suburban homes, cow farmers' homes had the highest bacterial diversity. Transport of microorganisms from surrounding environments such as livestock stables, plants, and soils may explain increases in bacterial load and diversity in farmer's homes when compared to suburban homes, where people are the primary source of the indoor environment. According to Lis et al., airborne microorganisms in farmers' residences were a mix of microorganisms from farm buildings including livestock stables [133]. They hypothesized that microorganisms are spread from agricultural buildings to residences via employees' clothing and bodies. We hypothesize that the higher bacterial diversity and richness in cow farmers' homes compared to pig farmers' homes is due to Danish regulations requiring farmers working in pig stables to wash their hands and change clothes to prevent the spread of zoonotic pathogens from pigs, whereas farmers working in open-air and less precautioned cow stables would transmit more bacteria to their homes [29].

Our findings support the various anticipated health effects associated with different indoor environments based on bacterial diversity and abundance. A reduced prevalence of immunoregulatory diseases has been associated to increased bacterial diversity in the indoor environment. Exposure to a variety of microorganisms has been inversely associated with the risk of asthma and atopy [134].

The bacterial community composition in suburban homes was distinct, whereas the pig and cow farmers' homes had minor overlap. Bacterial families that might have protective effects against allergy and asthma were found to be much more common in farmer's homes than in suburban homes. These include members of the Firmicutes families: *Lachnospiraceae*, *Lactobacillaceae*, *Ruminococcaceae* [135, 136]. The difference in indoor bacterial community composition between suburban and farmers' homes is in line with previous research that found a difference in microbiota between farm and non-farm homes [12, 17]. Despite the fact that the community composition of cow and pig farmers' homes overlaps, there was a considerable variation in the community composition of cow and pig farmers' homes indicating that the putative protective effects of airborne microbiomes in the homes of cow and pig farmers might differ.

5.2. Animal intestinal microbiota contribute to the indoor bacteria in farmers' homes.

In the first study, the beneficial taxa of the gut microbiome were shown to be more common in farmers' homes, particularly pig farmers' homes, than in suburban homes. Animal manure used as fertilizer in fields near farmers' homes might be a source of the presence of animal gut microbiota in the farmers' homes indoor air (Figure 5). Several bacterial species that are typically associated with the animal gut microbiome and were found to be abundant in farmers' homes have protective effects against inflammation, IBD, insulin resistance, and atopy according to recent animal, experimental, and epidemiological studies. *Faecalibacterium prausnitzii* which represents approximately 5% of the total faecal microbiota in healthy adults [137], was more abundant in farmers' homes than suburban homes. *F. prausnitzii* transplantation has been widely employed in dysbiosis of the gut flora associated with inflammation, autoimmune disease, and infectious disorders [138]. Bacterial species belonging to the *Bifidobacterium* genus were more abundant in cow farmers' homes than pig farmers' and suburban homes. Different strains of *Bifidobacterium* are frequently used as probiotics for

treatment of various medical conditions, including gastroenteritis and inflammatory bowel syndrome [139]. *Lactobacillus* species were more abundant in farmers' homes than in suburban homes. *Lactobacillus amylovorus* is known to be a probiotic associated with reduced obesity and have shown to alter body adiposity through modification of the gut microflora [140]. *Lactobacillus amylovorus* was also able to inhibits the TLR4 (Toll-like receptors) inflammatory signalling triggered by enterotoxigenic *Escherichia coli* in intestinal cells via modulation of TLR2 and cytokine regulation [141]. TLRs recognize a wide range of microbial molecules as well as airborne environmental allergens and act as adjuvants that influence positively or negatively allergic sensitization. Stimulation of TLR4 signalling can significantly exacerbate asthma through cytokine production [141]. Ability of *Lactobacillus amylovorus* to inhibit TLR4 signaling might explain the lower prevalence of allergic asthma and atopy and in rural areas.

Figure 5: Liquid manure spreader spreading cow manure on a hayfield [142].

5.3. Bacterial transfer from the livestock stables and the farmers' homes

In general, bacteria in the farmers' homes did not come from the cow or pig stables where they were working. This means that putatively beneficial bacteria in the air of farmers' homes are transported from outdoor sources in the environment surrounding the farmers' homes rather than from the farmers' own pig or cow stables (Figure 5). However, we found a stronger correlation between the bacterial communities found in farmers' homes and their related cow stables than between the bacterial communities found in farmers' homes and their

associated pig stables. This may be due to Danish legislation that mandate pig stable workers to wash their hands, change clothes, and disinfect equipment to minimize zoonotic disease transmission [29].

5.4. Five DNA extraction methods

In the second paper, out of the five DNA extraction methods tested, the best DNA yields were obtained using the PowerSoil kit and the DNA extraction method (Protocol A) reported by Lever et al. (2015) [115]. To investigate the consistency of DNA extraction in terms of DNA yield, bacterial richness, and bacterial composition, we repeated the DNA extraction process using these two methods on replicate EDCs. In comparison to the PowerSoil kit, DNA yields were more consistent while using Protocol A. When using the PowerSoil kit, however, the yield of DNA recovered was sufficient and above the detection limit. Protocol A demonstrated higher bacterial diversity and richness for all EDC cloths, indicating the method's ability to extract more bacterial taxa from EDC cloths than the PowerSoil kit. The PowerSoil kit, on the other hand, outperformed protocol A in detecting gram-positive bacteria like *Firmicutes* and *Actinobacteria*, possibly because the bead-beating method breaks down the cell wall of the gram-positive bacteria. We suggest prioritizing one of the two methods depending on the study's scale. Protocol A is more appropriate for deep metagenomic sequencing, where absolute quantification and consistency are critical. The PowerSoil kit, on the other hand, is suitable for large-scale epidemiological studies that may require a huge number of samples due to its simple and quick approach. In the third paper, which involved over 1000 EDC clothes, we chose the PowerSoil kit over Protocol A to reduce the time and cost required for the analysis of the samples.

5.5. Combined DNA & endotoxin extraction method

In the second paper, we tested the co-extraction of DNA and endotoxin to reduce the processing time and cost when analysis of large number of samples, as in the third paper. Therefore, we tested the co-extraction of DNA and endotoxin from a single EDC cloth and compared the endotoxin yield to the established method described by Noss et al [64]. Replicate EDCs showed largely similar results. This is probably because both methods employ the same extraction buffer (0.05% Tween 20 buffer). Therefore, we conclude that a single EDC cloth

can be used for DNA extraction and endotoxin extraction, providing more options for study design in epidemiological investigations and shortening the time needed to analyse samples.

5.6. Optimization of protocol using three buffer system

In the second paper, after we selected the PowerSoil kit and combined DNA and endotoxin extraction, we tried to further optimize the protocol to increase the amount of dust extracted from an EDC cloth and thereby increase the amount of DNA and endotoxin yield. Replicate EDCs were extracted using three buffer systems i) Double washing of the EDC with Tris-HCL EDTA buffer, ii) double washing with Tween 20 buffer, and iii) single wash with Tween 20 buffer followed by rinsing the EDC with PFW. The use of Tween 20 buffer resulted in a higher DNA and endotoxin yield compared to Tris HCL EDTA buffer. This could be due to the ability of the surfactant (Tween 20) to separate dust particles from fibrous material such as the EDC cloths. We also showed that rinsing EDC cloth with PFW instead of second wash with Tween 20 buffer increases both DNA and endotoxin yields. This suggests that the washing step with PFW increases motion of soil particles of dust particles out of the EDC cloth as explained by Shin et al [143]. The authors emphasized the importance of surfactant gradients that establish via rinsing with fresh water for soil particle removal from the fibrous materials. In the third paper we choose to extract dust from EDC cloths by first washing the EDC cloth with Tween buffer followed by rinsing the EDC cloth with PFW.

5.7. Indoor airborne microbiome sources

In the third paper we investigated the indoor airborne bacterial communities in homes from five cities in Northern Europe. Tartu and Aarhus households were characterized by a higher bacterial load and diversity compared to Bergen and Reykjavik households. In the five cities the main source of the indoor bacteria was the humans themselves. The Gram-positive bacteria, such as *Staphylococcus*, *Micrococcus*, *Corynebacterium* dominated the indoor air. In addition to the human associated taxa, the bacteria that stem from outdoor sources such as *Rhodococcus*, *Sphingomonas*, *Arthrobacter* contribute to the composition of the home microbiome in all cities[144-146].

The taxa from outdoor sources were more abundant in Tartu and Aarhus household compared to other cities, which might explain the increase of bacterial diversity and bacterial load in these two cities' households as outdoor bacteria representing additional sources of

microorganisms to indoor air. An increase in the relative abundance of Gram-negative bacterial taxa that mainly originate from outdoor sources such as *Protobacteria*, *Acinetobacter* [7, 147] explain the higher endotoxin (a cell component of Gram-negative bacteria) load in Tartu and Aarhus compared to other cities' household.

5.8. The effect of weather on the contributions of outdoor bacterial taxa to the indoor environment

During sampling the indoor dust, the average monthly precipitation rate was highest in Bergen. The lowest precipitation rate was found in Aarhus and Tartu. Wind speed was significantly greater in Aarhus and Tartu than in other cities. Recently, Fu et al. (2022) [36] reported that the microbial community within a building is influenced by various outdoor environmental factors, such as geographical characteristics and precipitation. Therefore, the meteorological measurements in Aarhus and Tartu may explain why outdoor bacterial taxa are more prevalent in households in Aarhus or Tartu than in other cities. Tartu and Aarhus were distinguished by low precipitation rates and stronger winds. In this study, wind speed was positively correlated with bacterial load and diversity, whereas precipitation was negatively correlated with bacterial diversity, bacterial load, and endotoxin load. In Aarhus and Tartu, high wind speeds may have increased the infiltration of outdoor bacterial taxa into indoor air. Thus, bacterial diversity and load are greater in these two cities' households. Yafeng et al. (2015) measured PM2.5 concentrations, an important bacterial carrier medium. The authors discovered a strong relationship between indoor and outdoor PM2.5 concentrations. The authors also discovered a positive correlation between PM2.5 indoor infiltration rate and outdoor wind velocity [148]. Households in Bergen were characterized by higher precipitation than other cities, which may explain the lower abundance of outdoor bacterial taxa in Bergen compared to the rest of the cities through decreased infiltration of outdoor air particles.

The rain scavenges out bacteria-associated particles and transport them to the ground in a process known as wet deposition [149]. But the impact of raindrops on various surfaces on earth triggers the emission of surface-associated bacteria into the air column [150]. Huffman et al (2013) [151] found that the concentration of airborne biological particles in a forest ecosystem increased significantly during rain. Xueying et al (2021) [152] investigated the effect of rain on PM2.5 concentrations in urban areas before and after 15 precipitation events

and discovered reductions in PM2.5 concentrations up to 90%. In the current study, indoor dust samples were collected from urban areas, which is similar to the latter case.

Asthma and atopy risk has been shown to decrease with increased exposure to diverse microorganisms [11, 89]. Kirjavainen et al. (2019) [17] found that the "farm-like" microbiome protective against asthma and atopy had a greater abundance of outdoor-associated bacterial taxa, such as *Sphingobacetria*, and *Alphaproteobacteria*. These taxa were less abundant in Bergen than in Aarhus and Tartu, which may be due to higher precipitation and slower wind speeds, which make it difficult for outdoor taxa to enter Bergen households. With global warming, precipitation intensity is anticipated to increase [153]. Wet deposition of outdoor particulates and particles associated with bacteria will increase with an increase in precipitation rate, leading to fewer bacteria in the indoor air and fewer exposures to environmental bacteria and endotoxins, both of which are critical for the establishment of a tolerogenic immune status.

5.9. Occupants' impact on indoor airborne microbiome

In the third paper, we observed that occupants impact the indoor air microbiome. The occupant's age was associated with an increase in bacterial diversity, a reduction in bacterial load, and a change in the composition of the bacterial community. Human skin microbiota is considered a principal source of indoor airborne bacteria [7]. The human skin microbiome changes with age, reflecting underlying age-related changes in the cutaneous structure and physiological function of skin [154]. As a result, changes in the indoor microbiome with increasing occupant age is a reflection of changes in the skin microbiome due to changes in skin biology. Several studies have shown that bacterial diversity increase with age [155, 156]. Howard et al. (2022) [156], investigated the skin microbiome of 158 participants and showed that bacterial diversity increased with age. A comparable study of 37 individuals found higher alpha diversity in older adults compared to younger adults [155]. The bacterial load on the skin tends to decrease with age, which also supports the results of our study. Lyden et al. (1975) [157] found that sebum secretion levels decrease with age. Sebum is rich in free fatty acids, and this leads to a decline in nutrients and consequently to a decrease in bacterial numbers.

Increases in human occupancy were associated with increases in bacterial diversity. This is consistent with previous research showing that occupancy increases the accumulation of human-associated microorganisms. The increase in bacterial diversity with increases in human occupancy could be attributed to several reasons. First, the microbial clouds emitted by

occupants may differ between individuals. Males and females, for example, have distinct microbial clouds [158]. Second, more humans indoors mean more activity and thus more resuspension of settled dust, as well as more transport of outdoor bacteria via their clothes and shoes [159].

Dogs allowed inside the bedroom significantly altered the composition of the indoor air microbiome, while this was not true in the case of cats. The dog significantly increased bacterial diversity which is consistent with previous findings of increased bacterial diversity in dog-owning households [35, 44, 160]. The difference in diversity between the two pets could be due to dogs being allowed outside (while many cats are not), thus bringing more environmental bacteria from outside [35]. The results of ANCOM BC support this observation. The increase in diversity associated with dog ownership was associated with an increase in different bacterial genera. These bacterial genera are either carried in by the dogs from the outdoor environment (such as *Rhodococcus*, *Sphingomonas*, and *Arthrobacter*) [7, 144-146] or originate from the dogs' own microbiota (such as *Moraxella* and *Fusobacterium*), which have been identified as common members of the canine oral and gastrointestinal tract microbiota [45, 161]. Dog ownership was found to be significantly associated with higher endotoxin load. This is in line with Elaine et al.'s study that found that higher endotoxin concentrations were associated with dog keeping. *Moraxella* and *Fusobacterium* , two gram-negative bacteria found to be the most abundant in the indoor air of the dog owner, may account for the increased endotoxin load associated with dog ownership, in addition to the gram-negative environmental bacteria brought in by the dog from the outdoors.

5.10. Impact of the occupants' behaviour on indoor airborne microbiome

Cleaning and use of disinfectants were identified as two of the most important occupant behaviours influencing the indoor airborne microbiome in the third paper. Increased cleaning frequency was associated with an increase in bacterial diversity and load. Cleaning may cause resuspension of settled dust and air mixing, increasing the number and types of bacterial taxa collected by EDCs. The frequency of cleaning does not appear to affect the composition of airborne particles. However, the use of cleaning and disinfecting agents significantly reduced the abundance of several bacterial taxa, particularly bleach (sodium hypochlorite). Our samples were collected between 2011 and 2014. However, since the COVID-19 pandemic, the use of chemical disinfectants such as sodium hypochlorite has increased exponentially in the indoor

environments [162]. As a result, it might be intriguing to study the effects of increased disinfectant use on the bacterial communities in indoor air.

5.11. Indoor characteristics and indoor airborne microbiome

In the third paper, in addition to the geographical location and the occupants, the indoor characteristics were associated with indoor bacterial profiles. The age of the house was found to be associated with an increase in bacterial diversity. The reason behind that might be because older houses have leaky plumbing systems compared to newer houses. In support of this, older built houses in the current study showed an increased abundance of bacterial taxa mostly belonging to water environments, such as *Friedmanniella, Ilumatobacter*, *Microlunatus, and Shimwellia* [163-166]. The presence of a rug in the bedroom where the EDC was placed was associated with an increased number of bacterial taxa in the indoor air; correspondingly, a rug was associated with increases in the abundance of three bacterial genera that are usually found in outdoor environments: *Sphingomonas*, *Pseudonocardia*, and *Friedmanniella* [7, 163, 167]. The majority of rugs are made of high-porosity textile materials, which aid in the adhesion of dust and organic compounds. Furthermore, the pores may be able to retain sufficient moisture [56]. As a result, increased levels of organics and moisture may facilitate microbial growth, explaining the higher abundance of environmental taxa in bedrooms with rugs.

Condensation on windows, which is a sign of increased air relative humidity, was associated with a decrease in bacterial diversity. High relative humidity in the air reduces the possibility of dust particles being resuspended in the air and influencing microorganism aerosolization from indoor surfaces [89, 168]. The presence of vents in the bedroom wall was associated with lower bacterial diversity. A wall vent is a mechanical ventilation unit that is used to supply fresh air to a home. Mechanically ventilated rooms have less diverse microbial communities than naturally ventilated rooms, according to Kembel et al [169]. The use of filters in mechanical ventilation prevents some of the outdoor bacteria and particulates from entering the home, resulting in lower microbial diversity than in natural ventilation systems [170].

5.12. The association between Indoor bacterial exposure and lung function and inflammation differ by sex

In the fourth paper, we explored the association between indoor airborne bacterial communities, lung function, and airway inflammation, using adjusted linear regression models stratified by sex. Our findings suggest that the effects of airborne bacterial exposure on lung function and inflammation differ by sex. The association between lung function and indoor airborne bacterial exposure was mostly found in males, while the association between airway inflammation and indoor airborne bacterial exposure was found in females only. Sex differences have been well established in respiratory diseases [171]. For example, asthma is more prevalent in boys than girls during childhood [172]. Recently, Renjin et al. reported that sex modifies the link between the airway microbiome and asthma [173]. Earlier studies reported differential responses to a range of environmental exposures in males and females in association with respiratory health outcomes such as tobacco smoke, farm upbringing, and air pollution [90, 174, 175]. Genetic factors, immune responses, and sex hormones are apparently the main mediators of these differences. Another explanation might be social and behavioural differences between men and women, such as in cleanliness, hygiene, and employment types.

5.13. Indoor bacterial exposure and lung function

Higher bacterial diversity was linked to a better lung function, which is consistent with the hygiene hypothesis and biodiversity hypothesis [15, 176]. These hypotheses claim that the increasing incidence of metabolic and immune diseases, including respiratory diseases such as asthma, is associated with a loss of biodiversity, particularly microbial diversity, in the outdoor and indoor environment [176]. In line with our finding, as study based on the ECRHS II population, found increasing proxies of microbial diversity during childhood to be associated with higher lung function in adulthood [90]. The increase in indoor airborne bacterial load was associated with lower lung function. In previous research higher airway bacterial loads have been associated with decreased lung function in COPD patients [177, 178]. Although we do not investigate the relationship between asthma and indoor bacterial exposure, a previous study discovered a negative association between the amount of bacteria in the air and the prevalence of asthma [36]. To study the association between indoor bacterial exposure and lung diseases, including asthma and COPD, previous studies mainly depended on the load of cell wall

compounds produced by Gram-negative bacteria (endotoxin / lipopolysaccharides [LPS]). In our study, there was a trend toward a negative association between endotoxin and lung function Z score. However, we didn't find a significant association. Our finding is in line with other cross-sectional studies that have found that mattress dust endotoxin levels are not associated with lung function [179, 180].

On the phylum level, positive associations were found between *Actinobacteriota* and lung function, while negative associations were found for *Bacteroidota*, which have previously been reported to increase the risk of asthma [36]. Higher abundance of *Actinobacteriota* in the indoor environment and the human respiratory tract was associated with a lower prevalence of asthma. We found that most bacterial genera that were positively associated with lung function belong to the *Actinobacteriota* phylum. Similarly, Karvonen et al [84] reported the abundance of several bacterial genera, mostly from the *Actinobacteriota*, to be associated with lower asthma risk. The taxa richness and abundance in *Clostridia* was associated with lower lung function. Fu et al. (2021) [91], recently reported that the abundance of several *Clostridia* genera and richness within *Clostridia* class was positively associated with asthma. *Streptococcus*, *Veillonella*, *Fusobacterium*, and *TM7* which are all considered to be components of the oral microbiome [181, 182] were negatively associated with lung function. Several studies have reported an increase in the abundance of *Streptococcus* and *Fusobacterium* indoors to be associated with lung diseases [17, 85, 178]. The increased prevalence of members of the oral microbiome in the indoor air of people with lower lung function might be explained by an increase in coughing in association with reduced lung function, suggesting that these bacterial taxa are a reflection of poor respiratory health rather than potential risk taxa, as was previously suggested [17].

5.14. Indoor bacterial exposure and airway inflammation

Our results indicated that the association between indoor air bacterial exposure and airway inflammation was different compared to the association between indoor air bacterial exposure and lung function. Contrary to the lung function, most associations between indoor bacterial exposure and FeNO were found in females, not males. In addition, we found different bacterial taxa to be associated with FeNO. To our knowledge, Fu et al. (2021) [91] is the only study which used culture-independent techniques to study the association between indoor bacterial exposure and airway inflammation using FeNO as marker. The authors did not find

an association between indoor exposure and FeNO [91]. We found that increased FeNO levels was associated with both increased bacterial diversity and endotoxin load. Lidwien et al (2015) found that increased occupational endotoxin exposure in a farming population was associated with increased FeNO levels [183]. Similarly, Yoda el al found that higher indoor and outdoor levels of endotoxin was associated with higher FeNO levels in young adults [184].

We found a significant association between increased relative abundance of the *Campylobacter* genus and higher FeNO levels. *Campylobacter* is a gram-negative bacterium and is the leading cause of human food-borne bacterial gastroenteritis [185]. The colonization of the gastrointestinal system by *Campylobacter* relies on numerous cellular defences. For example, an important antimicrobial tool of the mammalian innate immune system is the generation of harmful oxidative molecules such as the inducible nitric oxide synthase (iNOS) enzyme that produces NO [186], which might explain the association between increased abundance of *Campylobacter* in indoor air and FeNO levels. *Pseudomonas* and *Fusobacterium* were the only two bacterial genera that showed a positive association with airway inflammation as well as negative association with lung function, indicating that higher abundances of these bacterial genera in indoor air may pose a risk to respiratory health. Only *Cellulomonas*, from the *Actinobacteriota* phylum, was found to be related to lower FeNO levels. The probiotic potential of different *Cellulomonas* species has already been evaluated [187]. *Cellulomonas* have peroxidase activity, with ability to overcome oxidative stress [188] which might explain the association between increased abundance of *Cellulomonas* in indoor air and lower FeNO levels indicating that species affiliated to *Cellulomonas* could be potential protective agents against airway inflammation.

6. Conclusions

1. The indoor air in the farmers' homes, particularly cow farmers' homes, was characterized by higher bacterial diversity compared to suburban homes which were dominated by bacteria from human sources.

2. The intestinal animal microbiota from manure used in the fields appears to contribute to the indoor airborne microbiome in farmers' homes.

3. After a series of pilot studies using different buffer systems and different DNA extraction methods, we finalized a protocol for DNA and endotoxin extraction using a single EDC cloth. The combined extraction of DNA and endotoxin would significantly reduce the time and expense necessary for sample analysis in large epidemiological studies addressing airborne exposure.

4. Geographical differences in the indoor bacterial microbiome were significant, and we came to the conclusion that the increased abundance of outdoor bacterial taxa in Tartu and Aarhus households may be related to different weather conditions, particularly wind speed and precipitation.

5. The occupant age was associated with higher bacterial diversity and lower bacterial load in the indoor air. We propose that this is due to changes in the skin microbiome caused by skin aging.

6. The indoor microbiome is influenced by general lifestyle choices such the number of occupants, types of pets, frequency of household cleaning, and use of disinfectants.

7. Indoor bacterial exposure was associated with lung function and airway inflammation. The fact that the associations were different for males and females suggest sex difference in how lung function and airway inflammation respond to indoor bacterial exposure.

8. Lung function and airway inflammation showed very different patterns of association with the indoor airborne bacterial communities. Several bacterial genera that belong to

Actinobacteria were associated with high lung function, while *Bacteroidota*, *Clostridia,* and bacterial genera considered to be part of the core oral microbiome, including *Streptococcus* and *Fusobacterium,* were associated with low lung function. Higher FeNO levels were positively associated with *Campylobacter* and negatively associated with *Cellulomonas*.

7. *Future Perspectives*

1. In our first article we observed differences in bacterial community composition and abundance of potentially beneficial bacterial taxa between farmers' homes and suburban homes, supporting the concept that the bacterial composition in rural and farming environment contribute to better respiratory heath. Further studies, including experimental animal models and immunological studies, are needed to demonstrate the possible beneficial effects of specific bacterial taxa, which are abundant in rural environments.

2. In our third paper, we hypothesized that meteorological events, especially precipitation, would have an impact on the indoor airborne microbiome, most likely by decreasing the contribution of outdoor bacterial taxa to indoor air through wet deposition. With global warming, the intensity of precipitation is expected to rise, and if our assumption is right, fewer outdoor bacteria will contribute to the indoor microbiome, potentially having a negative impact on the development and maintenance of a tolerogenic immunological state. We propose that future studies should sample indoor and outdoor particles and collect meteorological data from different geographical locations to better understand how meteorological events affect the composition of the indoor airborne bacterial community, especially in light of future challenges such as global warming and an increase in atopic diseases.

3. In our third paper, we found that the use of disinfectants, especially bleach (sodium hypochlorite), was associated with a lower abundance of several bacterial taxa. In the third paper, indoor samples were collected between 2011 and 2014. With the advent of the COVID-19 pandemic, the use of chemical disinfectants such as sodium hypochlorite has increased in indoor environments. As a result, it would be interesting to investigate the effects of increased disinfectant use on the bacterial communities in indoor air.

4. In the fourth paper, we identified several bacterial genera positively and negatively associated with lung function and airway inflammation, which are valuable for understanding indoor bacterial exposure in relation to respiratory health. However, with the 16S rRNA sequencing, we were not able to characterize the strains and functional

gene of bacterial communities. Therefore, further studies aiming at functional profiling, such as through metagenomics, metabolomics, or animal studies, are needed to characterize the potential protective properties of these bacterial genera.

8. Reference

1. Kelley, S.T. and J.A. Gilbert, *Studying the microbiology of the indoor environment.* Genome biology, 2013. **14**(2): p. 1-9.
2. Fujiyoshi, S., D. Tanaka, and F. Maruyama, *Transmission of airborne bacteria across built environments and its measurement standards: a review.* Frontiers in microbiology, 2017. **8**: p. 2336.
3. Zhu, C., et al., *Role of atmospheric particulate matter exposure in COVID-19 and other health risks in human: a review.* Environmental Research, 2021. **198**: p. 111281.
4. Valavanidis, A., K. Fiotakis, and T. Vlachogianni, *Airborne particulate matter and human health: toxicological assessment and importance of size and composition of particles for oxidative damage and carcinogenic mechanisms.* Journal of Environmental Science and Health, Part C, 2008. **26**(4): p. 339-362.
5. Meadow, J.F., et al., *Indoor airborne bacterial communities are influenced by ventilation, occupancy, and outdoor air source.* Indoor air, 2014. **24**(1): p. 41-48.
6. Rai, S., D.K. Singh, and A. Kumar, *Microbial, environmental and anthropogenic factors influencing the indoor microbiome of the built environment.* Journal of Basic Microbiology, 2021. **61**(4): p. 267-292.
7. Hospodsky, D., et al., *Human occupancy as a source of indoor airborne bacteria.* PloS one, 2012. **7**(4): p. e34867.
8. Vuitton, D.A. and J.-C. Dalphin, *From farming to engineering: the microbiota and allergic diseases.* Engineering, 2017. **3**(1): p. 98-109.
9. Haahtela, T., et al., *Hunt for the origin of allergy–comparing the Finnish and Russian Karelia.* Clinical & Experimental Allergy, 2015. **45**(5): p. 891-901.
10. Jatzlauk, G., et al., *Influences of environmental bacteria and their metabolites on allergies, asthma, and host microbiota.* Allergy, 2017. **72**(12): p. 1859-1867.
11. Ege, M.J., et al., *Exposure to environmental microorganisms and childhood asthma.* New England Journal of Medicine, 2011. **364**(8): p. 701-709.
12. Pakarinen, J., et al., *Predominance of Gram-positive bacteria in house dust in the low-allergy risk Russian Karelia.* Environmental microbiology, 2008. **10**(12): p. 3317-3325.
13. Lynch, S.V., et al., *Effects of early-life exposure to allergens and bacteria on recurrent wheeze and atopy in urban children.* Journal of Allergy and Clinical Immunology, 2014. **134**(3): p. 593-601. e12.
14. Janson, C., et al., *Change in the prevalence asthma, rhinitis and respiratory symptom over a 20 year period: associations to year of birth, life style and sleep related symptoms.* BMC pulmonary medicine, 2018. **18**(1): p. 1-8.
15. Strachan, D.P., *Hay fever, hygiene, and household size.* BMJ: British Medical Journal, 1989. **299**(6710): p. 1259.
16. Stiemsma, L.T. and S.E. Turvey, *Asthma and the microbiome: defining the critical window in early life.* Allergy, Asthma & Clinical Immunology, 2017. **13**(1): p. 1-9.
17. Kirjavainen, P.V., et al., *Farm-like indoor microbiota in non-farm homes protects children from asthma development.* Nature medicine, 2019. **25**(7): p. 1089-1095.
18. Von Hertzen, L., I. Hanski, and T. Haahtela, *Natural immunity: biodiversity loss and inflammatory diseases are two global megatrends that might be related.* EMBO reports, 2011. **12**(11): p. 1089-1093.
19. Heederik, D. and E. von Mutius, *Does diversity of environmental microbial exposure matter for the occurrence of allergy and asthma?* Journal of Allergy and Clinical Immunology, 2012. **130**(1): p. 44-50.
20. Hanski, I., et al., *Environmental biodiversity, human microbiota, and allergy are interrelated.* Proceedings of the National Academy of Sciences, 2012. **109**(21): p. 8334-8339.

21. Lee, M.K., et al., *Exposures related to house dust microbiota in a US farming population.* Environmental health perspectives, 2018. **126**(6): p. 067001.
22. Mills, J.G., et al., *Urban habitat restoration provides a human health benefit through microbiome rewilding: the Microbiome Rewilding Hypothesis.* Restoration ecology, 2017. **25**(6): p. 866-872.
23. TEAM, S., *Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community.* Clinical & Experimental Allergy, 1999. **29**(1): p. 28-34.
24. Ernst, P. and Y. Cormier, *Relative scarcity of asthma and atopy among rural adolescents raised on a farm.* American journal of respiratory and critical care medicine, 2000. **161**(5): p. 1563-1566.
25. Klintberg, B., et al., *Fewer allergic respiratory disorders among farmers' children in a closed birth cohort from Sweden.* European Respiratory Journal, 2001. **17**(6): p. 1151-1157.
26. Normand, A.-C., et al., *Airborne cultivable microflora and microbial transfer in farm buildings and rural dwellings.* Occupational and environmental medicine, 2011. **68**(11): p. 849-855.
27. PASANEN, A.-L., et al., *Fungi carried from farmers' work into farm homes.* American Industrial Hygiene Association Journal, 1989. **50**(12): p. 631-633.
28. Panthee, B., et al., *Environmental and Human Microbiome for Health.* Life, 2022. **12**(3): p. 456.
29. Denver, S., et al., *The costs of preventive activities for exotic contagious diseases—a Danish case study of foot and mouth disease and swine fever.* Preventive veterinary medicine, 2016. **131**: p. 111-120.
30. Eduard, W., et al., *Do farming exposures cause or prevent asthma? Results from a study of adult Norwegian farmers.* Thorax, 2004. **59**(5): p. 381-386.
31. Frei, R., et al., *Exposure to nonmicrobial N-glycolylneuraminic acid protects farmers' children against airway inflammation and colitis.* Journal of Allergy and Clinical Immunology, 2018. **141**(1): p. 382-390. e7.
32. Cohen, B., *Urbanization, City growth, and the New United Nations development agenda.* Cornerstone, 2015. **3**(2): p. 4-7.
33. Li, S., et al., *Understanding building-occupant-microbiome interactions toward healthy built environments: A review.* Frontiers of Environmental Science & Engineering, 2021. **15**(4): p. 1-18.
34. Lax, S., et al., *Longitudinal analysis of microbial interaction between humans and the indoor environment.* Science, 2014. **345**(6200): p. 1048-1052.
35. Kettleson, E.M., et al., *Key determinants of the fungal and bacterial microbiomes in homes.* Environmental research, 2015. **138**: p. 130-135.
36. Fu, X., Z. Ou, and Y. Sun, *Indoor microbiome and allergic diseases: from theoretical advances to prevention strategies.* Eco-Environment & Health, 2022.
37. Täubel, M., et al., *The occupant as a source of house dust bacteria.* Journal of Allergy and Clinical Immunology, 2009. **124**(4): p. 834-840. e47.
38. Hoisington, A.J., et al., *The microbiome of the built environment and mental health.* Microbiome, 2015. **3**(1): p. 1-12.
39. Luongo, J.C., et al., *Microbial analyses of airborne dust collected from dormitory rooms predict the sex of occupants.* Indoor air, 2017. **27**(2): p. 338-344.
40. Wouters, I.M., et al., *Increased levels of markers of microbial exposure in homes with indoor storage of organic household waste.* Applied and Environmental Microbiology, 2000. **66**(2): p. 627-631.
41. Hyvärinen, A., et al., *Characterizing microbial exposure with ergosterol, 3-hydroxy fatty acids, and viable microbes in house dust: determinants and association with childhood asthma.* Archives of environmental & occupational health, 2006. **61**(4): p. 149-157.

42. Sordillo, J.E., et al., *Home characteristics as predictors of bacterial and fungal microbial biomarkers in house dust.* Environmental Health Perspectives, 2011. **119**(2): p. 189-195.
43. Rintala, H., M. Pitkäranta, and M. Täubel, *Microbial communities associated with house dust*, in *Advances in applied microbiology*. 2012, Elsevier. p. 75-120.
44. Fujimura, K.E., et al., *Man's best friend? The effect of pet ownership on house dust microbial communities.* Journal of Allergy and Clinical Immunology, 2010. **126**(2): p. 410-412. e3.
45. Mäki, J.M., et al., *Associations between dog keeping and indoor dust microbiota.* Scientific reports, 2021. **11**(1): p. 1-11.
46. Lødrup Carlsen, K.C., et al., *Does pet ownership in infancy lead to asthma or allergy at school age? Pooled analysis of individual participant data from 11 European birth cohorts.* 2012.
47. Fall, T., et al., *Early exposure to dogs and farm animals and the risk of childhood asthma.* JAMA pediatrics, 2015. **169**(11): p. e153219-e153219.
48. Hospodsky, D., et al., *Characterizing airborne fungal and bacterial concentrations and emission rates in six occupied children's classrooms.* Indoor air, 2015. **25**(6): p. 641-652.
49. Seppanen, O. and W. Fisk, *Relationship of SBS-symptoms and ventilation system type in office buildings.* 2002.
50. Hoeksma, P., A.J.A. Aarnink, and N. Ogink, *Effect of temperature and relative humidity on the survival of airborne bacteria= Effect van temperatuur en relatieve luchtvochtig-heid op de overleving van bacteriën in de lucht*. 2015, Wageningen UR Livestock Research.
51. Adan, O.C. and R.A. Samson, *Fundamentals of mold growth in indoor environments and strategies for healthy living*. 2011: Springer.
52. Wu, T., et al., *Infant and adult inhalation exposure to resuspended biological particulate matter.* Environmental science & technology, 2018. **52**(1): p. 237-247.
53. Jayaprakash, B., et al., *Indoor microbiota in severely moisture damaged homes and the impact of interventions.* Microbiome, 2017. **5**(1): p. 1-17.
54. Fisk, W.J., Q. Lei-Gomez, and M.J. Mendell, *Meta-Analyses of the Associations of Respiratory Health Effectswith Dampness and Mold in Homes.* Indoor air, 2006. **17**(LBNL-59363).
55. Lugauskas, A., L. Levinskait, and D. Pečiulyt, *Micromycetes as deterioration agents of polymeric materials.* International biodeterioration & biodegradation, 2003. **52**(4): p. 233-242.
56. Hoang, C.P., et al., *Resistance of green building materials to fungal growth.* International Biodeterioration & Biodegradation, 2010. **64**(2): p. 104-113.
57. Fu, X., et al., *Continental-scale microbiome study reveals different environmental characteristics determining microbial richness, composition, and quantity in hotel rooms.* Msystems, 2020. **5**(3): p. e00119-20.
58. Danko, D., et al., *A global metagenomic map of urban microbiomes and antimicrobial resistance.* Cell, 2021. **184**(13): p. 3376-3393. e17.
59. Kokubo, M., et al., *Relationship between the microbiome and indoor temperature/humidity in a traditional japanese house with a thatched roof in Kyoto, Japan.* Diversity, 2021. **13**(10): p. 475.
60. Chew, G., et al., *Dustborne and airborne fungal propagules represent a different spectrum of fungi with differing relations to home characteristics.* Allergy, 2003. **58**(1): p. 13-20.
61. Hyvärinen, A., et al., *Temporal and spatial variation of fungal concentrations in indoor air.* Aerosol Science & Technology, 2001. **35**(2): p. 688-695.
62. Leppänen, H.K., et al., *Quantitative assessment of microbes from samples of indoor air and dust.* Journal of exposure science & environmental epidemiology, 2018. **28**(3): p. 231-241.
63. Adams, R.I., et al., *Passive dust collectors for assessing airborne microbial material.* Microbiome, 2015. **3**(1): p. 1-11.
64. Noss, I., et al., *Evaluation of a low-cost electrostatic dust fall collector for indoor air endotoxin exposure assessment.* Applied and environmental microbiology, 2008. **74**(18): p. 5621-5627.

65. Kilburg-Basnyat, B., N. Metwali, and P.S. Thorne, *Effect of deployment time on endotoxin and allergen exposure assessment using electrostatic dust collectors.* Annals of Occupational Hygiene, 2015. **59**(1): p. 104-115.
66. Jacobs, J., et al., *Endotoxin levels in settled airborne dust in European schools: the HITEA school study.* Indoor Air, 2014. **24**(2): p. 148-157.
67. Samadi, S., et al., *Allergen and endotoxin exposure in a companion animal hospital.* Occupational and environmental medicine, 2010. **67**(7): p. 486-492.
68. Krop, E.J., et al., *Allergens and β-glucans in dutch homes and schools: characterizing airborne levels.* PloS one, 2014. **9**(2): p. e88871.
69. Lawson, J.A., et al., *Endotoxin as a determinant of asthma and wheeze among rural dwelling children and adolescents: a case–control study.* BMC pulmonary medicine, 2012. **12**(1): p. 1-10.
70. Liebers, V., T. Brüning, and M. Raulf, *Occupational endotoxin exposure and health effects.* Archives of toxicology, 2020. **94**(11): p. 3629-3644.
71. MILTON, D.K., et al., *Endotoxin measurement: aerosol sampling and application of a new Limulus method.* American Industrial Hygiene Association Journal, 1990. **51**(6): p. 331-337.
72. van Strien, R.T., et al., *Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health.* Journal of Allergy and Clinical Immunology, 2004. **113**(5): p. 860-867.
73. Laitinen, S., et al., *Evaluation of exposure to airborne bacterial endotoxins and peptidoglycans in selected work environments.* Annals of Agricultural and Environmental Medicine, 2001. **8**(2).
74. Sordillo, J.E., et al., *Multiple microbial exposures in the home may protect against asthma or allergy in childhood.* Clinical & Experimental Allergy, 2010. **40**(6): p. 902-910.
75. Nevalainen, A., M. Täubel, and A. Hyvärinen, *Indoor fungi: companions and contaminants.* Indoor air, 2015. **25**(2): p. 125-156.
76. de Bruin, O.M., et al., *A novel chemical lysis method for maximum release of DNA from difficult-to-lyse bacteria.* Microbial pathogenesis, 2019. **126**: p. 292-297.
77. Liu, Y., et al., *The development of an effective bacterial single-cell lysis method suitable for whole genome amplification in microfluidic platforms.* Micromachines, 2018. **9**(8): p. 367.
78. Stetzenbach, L.D., M.P. Buttner, and P. Cruz, *Detection and enumeration of airborne biocontaminants.* Current opinion in biotechnology, 2004. **15**(3): p. 170-174.
79. Adhikari, A., et al., *Dustborne and airborne Gram-positive and Gram-negative bacteria in high versus low ERMI homes.* Science of the Total Environment, 2014. **482**: p. 92-99.
80. Kärkkäinen, P.M., et al., *Determination of bacterial load in house dust using qPCR, chemical markers and culture.* Journal of Environmental Monitoring, 2010. **12**(3): p. 759-768.
81. Torvinen, E., et al., *Real-time PCR detection of environmental mycobacteria in house dust.* Journal of microbiological methods, 2010. **82**(1): p. 78-84.
82. Ross, A.A. and J.D. Neufeld, *Microbial biogeography of a university campus.* Microbiome, 2015. **3**(1): p. 1-12.
83. Dannemiller, K.C., et al., *Influence of housing characteristics on bacterial and fungal communities in homes of asthmatic children.* Indoor air, 2016. **26**(2): p. 179-192.
84. Karvonen, A.M., et al., *Indoor bacterial microbiota and development of asthma by 10.5 years of age.* Journal of Allergy and Clinical Immunology, 2019. **144**(5): p. 1402-1410.
85. Lee, M.K., et al., *House dust microbiota in relation to adult asthma and atopy in a US farming population.* Journal of allergy and clinical immunology, 2021. **147**(3): p. 910-920.
86. Wolf, M. and P.S. Lai, *Indoor microbial exposures and chronic lung disease: from microbial toxins to the microbiome.* Clinics in chest medicine, 2020. **41**(4): p. 777-796.
87. Borbet, T.C., et al., *The role of the changing human microbiome in the asthma pandemic.* Journal of allergy and clinical immunology, 2019. **144**(6): p. 1457-1466.

88. Loverdos, K., et al., *Lung microbiome in asthma: current perspectives.* Journal of Clinical Medicine, 2019. **8**(11): p. 1967.
89. Hyytiäinen, H., et al., *Microbial diversity in homes and the risk of allergic rhinitis and inhalant atopy in two European birth cohorts.* Environmental research, 2021. **196**: p. 110835.
90. Campbell, B., et al., *The effects of growing up on a farm on adult lung function and allergic phenotypes: an international population-based study.* Thorax, 2017. **72**(3): p. 236-244.
91. Fu, X., et al., *Derived habitats of indoor microbes are associated with asthma symptoms in Chinese university dormitories.* Environmental research, 2021. **194**: p. 110501.
92. Dannemiller, K.C., et al., *Indoor microbial communities: influence on asthma severity in atopic and nonatopic children.* Journal of Allergy and Clinical Immunology, 2016. **138**(1): p. 76-83. e1.
93. Braun-Fahrländer, C., et al., *Environmental exposure to endotoxin and its relation to asthma in school-age children.* New England Journal of Medicine, 2002. **347**(12): p. 869-877.
94. Campo, P., et al., *Influence of dog ownership and high endotoxin on wheezing and atopy during infancy.* Journal of allergy and clinical immunology, 2006. **118**(6): p. 1271-1278.
95. Carlsten, C., et al., *Association between endotoxin and mite allergen exposure with asthma and specific sensitization at age 7 in high-risk children.* Pediatric allergy and immunology, 2011. **22**(3): p. 320-326.
96. El-Sharif, N., et al., *Childhood asthma and indoor aeroallergens and endotoxin in Palestine: a case-control study.* Journal of Asthma, 2006. **43**(3): p. 241-247.
97. Bolte, G., et al., *Early endotoxin exposure and atopy development in infants: results of a birth cohort study.* Clinical & Experimental Allergy, 2003. **33**(6): p. 770-776.
98. Perzanowski, M.S., et al., *Endotoxin in inner-city homes: associations with wheeze and eczema in early childhood.* Journal of allergy and clinical immunology, 2006. **117**(5): p. 1082-1089.
99. Lai, P.S., et al., *School endotoxin exposure and asthma morbidity in inner-city children.* Chest, 2015. **148**(5): p. 1251-1258.
100. Smit, L.A., et al., *Occupational endotoxin exposure reduces the risk of atopic sensitization but increases the risk of bronchial hyperresponsiveness.* International archives of allergy and immunology, 2010. **152**(2): p. 151-158.
101. Ege, M.J., et al., *Not all farming environments protect against the development of asthma and wheeze in children.* Journal of Allergy and Clinical Immunology, 2007. **119**(5): p. 1140-1147.
102. Gehring, U., et al., *Respiratory symptoms in relation to indoor exposure to mite and cat allergens and endotoxins.* European respiratory journal, 2001. **18**(3): p. 555-563.
103. Ranu, H., M. Wilde, and B. Madden, *Pulmonary function tests.* Ulster Med J, 2011. **80**(2): p. 84-90.
104. Enright, P.L., M.D. Lebowitz, and D.W. Cockroft, *Physiologic measures: pulmonary function tests.* American journal of respiratory and critical care medicine, 2012.
105. Barroso, A.T., et al., *Factors affecting lung function: a review of the literature.* Archivos de Bronconeumología (English Edition), 2018. **54**(6): p. 327-332.
106. Haynes, J.M., *Basic spirometry testing and interpretation for the primary care provider.* Canadian journal of respiratory therapy: CJRT= Revue canadienne de la therapie respiratoire: RCTR, 2018. **54**(4).
107. Stanojevic, S., et al., *The Global Lung Function Initiative: dispelling some myths of lung function test interpretation.* Breathe, 2013. **9**(6): p. 462-474.
108. Quanjer, P.H., et al., *Measurement of FEF25–75% and FEF75% does not contribute to clinical decision making.* European Respiratory Journal, 2014. **43**(4): p. 1051-1058.
109. Stanojevic, S., A. Wade, and J. Stocks, *Reference values for lung function: past, present and future.* European Respiratory Journal, 2010. **36**(1): p. 12-19.
110. Barnes, P.J., et al., *Asthma and COPD: basic mechanisms and clinical management.* 2009.

111. Spahn, J. and J. Malka, *Exhaled nitric oxide as a biomarker for asthma management.* Personalizing Asthma Management for the Clinician, 2018: p. 49-58.
112. Ghosh, S. and S.C. Erzurum, *Nitric oxide metabolism in asthma pathophysiology.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2011. **1810**(11): p. 1008-1016.
113. Schlünssen, V., et al., *Exposure levels, determinants and IgE mediated sensitization to bovine allergens among Danish farmers and non-farmers.* International journal of hygiene and environmental health, 2015. **218**(2): p. 265-272.
114. Juel Holst, G., et al., *Household dampness and microbial exposure related to allergy and respiratory health in Danish adults.* European clinical respiratory journal, 2020. **7**(1): p. 1706235.
115. Lever, M.A., et al., *A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types.* Frontiers in microbiology, 2015. **6**: p. 476.
116. Xiao, K.-Q., et al., *Concurrent methane production and oxidation in surface sediment from Aarhus Bay, Denmark.* Frontiers in microbiology, 2017. **8**: p. 1198.
117. Ohkuma, M. and T. Kudo, *Phylogenetic analysis of the symbiotic intestinal microflora of the termite Cryptotermes domesticus.* FEMS microbiology letters, 1998. **164**(2): p. 389-395.
118. Vestergaard, D.V., et al., *Pig farmers' homes harbor more diverse airborne bacterial communities than pig stables or suburban homes.* Frontiers in microbiology, 2018. **9**: p. 870.
119. Martin, M., *Cutadapt removes adapter sequences from high-throughput sequencing reads.* EMBnet. journal, 2011. **17**(1): p. 10-12.
120. Callahan, B.J., et al., *DADA2: High-resolution sample inference from Illumina amplicon data.* Nature methods, 2016. **13**(7): p. 581-583.
121. Morgan, M., et al., *ShortRead: a bioconductor package for input, quality assessment and exploration of high-throughput sequence data.* Bioinformatics, 2009. **25**(19): p. 2607-2608.
122. Quast, C., et al., *The SILVA ribosomal RNA gene database project: improved data processing and web-based tools.* Nucleic acids research, 2012. **41**(D1): p. D590-D596.
123. Davis, N.M., et al., *Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data.* Microbiome, 2018. **6**(1): p. 1-14.
124. Andersen, K.S., et al., *ampvis2: an R package to analyse and visualise 16S rRNA amplicon data.* BioRxiv, 2018: p. 299537.
125. McMurdie, P.J. and S. Holmes, *phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data.* PloS one, 2013. **8**(4): p. e61217.
126. Paradis, E., J. Claude, and K. Strimmer, *APE: analyses of phylogenetics and evolution in R language.* Bioinformatics, 2004. **20**(2): p. 289-290.
127. Dixon, P., *VEGAN, a package of R functions for community ecology.* Journal of Vegetation Science, 2003. **14**(6): p. 927-930.
128. Lin, H. and S.D. Peddada, *Analysis of compositions of microbiomes with bias correction.* Nature communications, 2020. **11**(1): p. 1-11.
129. Comas-Cufí, M., *A Basic Set of Functions for Compositional Data Analysis. R package version 0.3.1.* 2020.
130. Basinas, I., et al., *Exposure to inhalable dust and endotoxin among Danish livestock farmers: results from the SUS cohort study.* Journal of environmental monitoring, 2012. **14**(2): p. 604-614.
131. Vedanthan, P.K., et al., *Effect of animal contact and microbial exposures on the prevalence of atopy and asthma in urban vs rural children in India.* Annals of Allergy, Asthma & Immunology, 2006. **96**(4): p. 571-578.
132. Brooks, C., N. Pearce, and J. Douwes, *The hygiene hypothesis in allergy and asthma: an update.* Current opinion in allergy and clinical immunology, 2013. **13**(1): p. 70-77.
133. Lis, D.O., G. Mainelis, and R.L. Górny, *Microbial air contamination in farmhouses–quantitative aspects.* Clean–Soil, Air, Water, 2008. **36**(7): p. 551-555.

134. Chiu, C.-Y., et al., *Airway microbial diversity is inversely associated with mite-sensitized rhinitis and asthma in early childhood.* Scientific reports, 2017. **7**(1): p. 1820.
135. Karimi, K., et al., *Lactobacillus reuteri–induced regulatory T cells protect against an allergic airway response in mice.* American journal of respiratory and critical care medicine, 2009. **179**(3): p. 186-193.
136. Huang, F., et al., *Early-life exposure to Clostridium leptum causes pulmonary immunosuppression.* PloS one, 2015. **10**(11): p. e0141717.
137. Martín, R., L.G. Bermúdez-Humarán, and P. Langella, *Searching for the bacterial effector: The example of the multi-skilled commensal bacterium Faecalibacterium prausnitzii.* Frontiers in Microbiology, 2018. **9**: p. 346.
138. He, X., S. Zhao, and Y. Li, *Faecalibacterium prausnitzii: A next-generation probiotic in gut disease improvement.* Canadian Journal of Infectious Diseases and Medical Microbiology, 2021. **2021**: p. 1-10.
139. Mangin, I., et al., *Oral administration of viable Bifidobacterium pseudolongum strain Patronus modified colonic microbiota and increased mucus layer thickness in rat.* FEMS microbiology ecology, 2018. **94**(11): p. fiy177.
140. Omar, J.M., et al., *Lactobacillus fermentum and Lactobacillus amylovorus as probiotics alter body adiposity and gut microflora in healthy persons.* Journal of functional foods, 2013. **5**(1): p. 116-123.
141. Finamore, A., et al., *Lactobacillus amylovorus inhibits the TLR4 inflammatory signaling triggered by enterotoxigenic Escherichia coli via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants.* PLoS One, 2014. **9**(4): p. e94891.
142. acdamey, L.c., *How cow manure replenish nutrients*. 2000. p. https://www.lawn-care-academy.com/cow-manure.html#gallery[pageGallery]/1/.
143. Shin, S., P.B. Warren, and H.A. Stone, *Cleaning by surfactant gradients: Particulate removal from porous materials and the significance of rinsing in laundry detergency.* Physical Review Applied, 2018. **9**(3): p. 034012.
144. Korthals, M., et al., *Application of PCR-SSCP for molecular epidemiological studies on the exposure of farm children to bacteria in environmental dust.* Journal of microbiological methods, 2008. **73**(1): p. 49-56.
145. Bowers, R.M., et al., *Spatial variability in airborne bacterial communities across land-use types and their relationship to the bacterial communities of potential source environments.* The ISME journal, 2011. **5**(4): p. 601-612.
146. Brodie, E.L., et al., *Urban aerosols harbor diverse and dynamic bacterial populations.* Proceedings of the National Academy of Sciences, 2007. **104**(1): p. 299-304.
147. Rintala, H., et al., *Diversity and seasonal dynamics of bacterial community in indoor environment.* BMC microbiology, 2008. **8**(1): p. 1-13.
148. Wang, Y., et al., *Experimental investigation on indoor/outdoor PM2. 5 concentrations of an office building located in Guangzhou.* Procedia Engineering, 2015. **121**: p. 333-340.
149. Wang, Y., W. Xia, and G.J. Zhang, *What rainfall rates are most important to wet removal of different aerosol types?* Atmospheric Chemistry and Physics, 2021. **21**(22): p. 16797-16816.
150. Jang, G.I., C.Y. Hwang, and B.C. Cho, *Effects of heavy rainfall on the composition of airborne bacterial communities.* Frontiers of Environmental Science & Engineering, 2018. **12**: p. 1-10.
151. Huffman, J.A., et al., *High concentrations of biological aerosol particles and ice nuclei during and after rain.* Atmospheric Chemistry and Physics, 2013. **13**(13): p. 6151-6164.
152. Tian, X., et al., *Effects of rain and snow on the air quality index, PM2. 5 levels, and dry deposition flux of PCDD/Fs.* Aerosol and Air Quality Research, 2021. **21**(8): p. 210158.
153. Tabari, H., *Climate change impact on flood and extreme precipitation increases with water availability.* Scientific reports, 2020. **10**(1): p. 1-10.

154. Li, Z., et al., *New insights into the skin microbial communities and skin aging.* Frontiers in microbiology, 2020. **11**: p. 565549.
155. Kim, H.-J., et al., *Aged related human skin microbiome and mycobiome in Korean women.* Scientific reports, 2022. **12**(1): p. 2351.
156. Howard, B., et al., *Aging-associated changes in the adult human skin microbiome and the host factors that affect skin microbiome composition.* Journal of Investigative Dermatology, 2022. **142**(7): p. 1934-1946. e21.
157. Leyden, J.J., et al., *Age-related changes in the resident bacterial flora of the human face.* Journal of Investigative Dermatology, 1975. **65**(4): p. 379-381.
158. Meadow, J.F., et al., *Humans differ in their personal microbial cloud.* PeerJ, 2015. **3**: p. e1258.
159. Hyytiäinen, H.K., et al., *Crawling-induced floor dust resuspension affects the microbiota of the infant breathing zone.* Microbiome, 2018. **6**(1): p. 1-12.
160. Lehtimäki, J., et al., *Skin microbiota and allergic symptoms associate with exposure to environmental microbes.* Proceedings of the National Academy of Sciences, 2018. **115**(19): p. 4897-4902.
161. Sitarik, A.R., et al., *Dog introduction alters the home dust microbiota.* Indoor air, 2018. **28**(4): p. 539-547.
162. Dhama, K., et al., *The role of disinfectants and sanitizers during COVID-19 pandemic: advantages and deleterious effects on humans and the environment.* Environmental Science and Pollution Research, 2021. **28**(26): p. 34211-34228.
163. Maszenan, A., et al., *Friedmanniella spumicola sp. nov. and Friedmanniella capsulata sp. nov. from activated sludge foam: Gram-positive cocci that grow in aggregates of repeating groups of cocci.* International Journal of Systematic and Evolutionary Microbiology, 1999. **49**(4): p. 1667-1680.
164. Guo, J., et al., *The seasonal variation of microbial communities in drinking water sources in Shanghai.* Journal of Cleaner Production, 2020. **265**: p. 121604.
165. Yuan, M., et al., *Phylogenetic diversity and biological activity of actinobacteria isolated from the Chukchi Shelf marine sediments in the Arctic Ocean.* Marine drugs, 2014. **12**(3): p. 1281-1297.
166. Jang, J., et al., *Environmental Escherichia coli: ecology and public health implications—a review.* Journal of applied microbiology, 2017. **123**(3): p. 570-581.
167. Riahi, H.S., P. Heidarieh, and M. Fatahi-Bafghi, *Genus Pseudonocardia: What we know about its biological properties, abilities and current application in biotechnology.* Journal of Applied Microbiology, 2022. **132**(2): p. 890-906.
168. Frankel, M., E.W. Hansen, and A.M. Madsen, *Effect of relative humidity on the aerosolization and total inflammatory potential of fungal particles from dust-inoculated gypsum boards.* Indoor Air, 2014. **24**(1): p. 16-28.
169. Kembel, S.W., et al., *Architectural design influences the diversity and structure of the built environment microbiome.* The ISME journal, 2012. **6**(8): p. 1469-1479.
170. Li, S., et al., *Understanding building-occupant-microbiome interactions toward healthy built environments: A review.* Frontiers of environmental science & engineering, 2021. **15**: p. 1-18.
171. Silveyra, P., N. Fuentes, and D.E. Rodriguez Bauza, *Sex and gender differences in lung disease*, in *Lung Inflammation in Health and Disease, Volume II*. 2021, Springer. p. 227-258.
172. Fuseini, H. and D.C. Newcomb, *Mechanisms driving gender differences in asthma.* Current allergy and asthma reports, 2017. **17**: p. 1-9.
173. Chen, R., et al., *Sex effects in the association between airway microbiome and asthma.* Annals of Allergy, Asthma & Immunology, 2020. **125**(6): p. 652-657. e3.
174. Mészáros, D., et al., *Domestic airborne pollutants and asthma and respiratory symptoms in middle age.* Respirology, 2014. **19**(3): p. 411-418.
175. Mann, J.K., et al., *Short-term effects of air pollution on wheeze in asthmatic children in Fresno, California.* Environmental health perspectives, 2010. **118**(10): p. 1497-1502.

176. Bello, M.G.D., et al., *Preserving microbial diversity.* Science, 2018. **362**(6410): p. 33-34.
177. Garcha, D.S., et al., *Changes in prevalence and load of airway bacteria using quantitative PCR in stable and exacerbated COPD.* Thorax, 2012. **67**(12): p. 1075-1080.
178. Ghosh, B., et al., *Bacterial load and defective monocyte-derived macrophage bacterial phagocytosis in biomass smoke-related COPD.* European Respiratory Journal, 2019. **53**(2).
179. Mendy, A., et al., *Exposure and sensitization to pets modify endotoxin association with asthma and wheeze.* The Journal of Allergy and Clinical Immunology: In Practice, 2018. **6**(6): p. 2006-2013. e4.
180. Leung, T.F., et al., *Indoor determinants of endotoxin and dust mite exposures in Hong Kong homes with asthmatic children.* International archives of allergy and immunology, 2010. **152**(3): p. 279-287.
181. Dewhirst, F.E., et al., *The human oral microbiome.* Journal of bacteriology, 2010. **192**(19): p. 5002-5017.
182. Ruan, X., et al., *The salivary microbiome shows a high prevalence of core bacterial members yet variability across human populations.* npj Biofilms and Microbiomes, 2022. **8**(1): p. 85.
183. Smit, L.A., et al., *Exhaled nitric oxide in endotoxin-exposed adults: effect modification by smoking and atopy.* Occupational and environmental medicine, 2009. **66**(4): p. 251-255.
184. Yoda, Y., et al., *Association of airborne endotoxin concentrations with pulmonary function and airway inflammation among students.* Environmental Epidemiology, 2019. **3**: p. 456.
185. Kelly, D., *The physiology and metabolism of Campylobacter jejuni and Helicobacter pylori.* Journal of Applied Microbiology, 2001. **90**(S6): p. 16S-24S.
186. Flint, A., A. Stintzi, and L.M. Saraiva, *Oxidative and nitrosative stress defences of Helicobacter and Campylobacter species that counteract mammalian immunity.* FEMS Microbiology Reviews, 2016. **40**(6): p. 938-960.
187. Yadav, M., et al., *Characterization of Cellulomonas sp. HM71 as potential probiotic strain for human health.* Frontiers in Cellular and Infection Microbiology, 2023. **12**: p. 1981.
188. Habib, M.H., H.J. Rozeboom, and M.W. Fraaije, *Characterization of a new DyP-peroxidase from the alkaliphilic cellulomonad, Cellulomonas bogoriensis.* Molecules, 2019. **24**(7): p. 1208.

9. Papers I- IV & Appendix

I

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ORIGINAL RESEARCH
published: 17 June 2022
doi: 10.3389/fmicb.2022.883991

Cow Farmers' Homes Host More Diverse Airborne Bacterial Communities Than Pig Farmers' Homes and Suburban Homes

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Microbial Symbioses, a section of the journal Frontiers in Microbiology

Received: *28 February 2022*
Accepted: *05 May 2022*
Published: *17 June 2022*

Citation:
Amin H, Šantl-Temkiv T, Cramer C, Vestergaard DV, Holst GJ, Elholm G, Finster K, Bertelsen RJ, Schlünssen V, Sigsgaard T and Marshall IPG (2022) Cow Farmers' Homes Host More Diverse Airborne Bacterial Communities Than Pig Farmers' Homes and Suburban Homes.
Front. Microbiol. 13:883991.
doi: 10.3389/fmicb.2022.883991

Living on a farm has been linked to a lower risk of immunoregulatory disorders, such as asthma, allergy, and inflammatory bowel disease. It is hypothesized that a decrease in the diversity and composition of indoor microbial communities is a sensible explanation for the upsurge in immunoregulatory diseases, with airborne bacteria contributing to this protective effect. However, the composition of this potentially beneficial microbial community in various farm and suburban indoor environments is still to be characterized. We collected settled airborne dust from stables and the associated farmers' homes and from suburban homes using electrostatic dust collectors (EDCs) over a period of 14 days. Then, quantitative PCR (qPCR) was used to assess bacterial abundance. The V3–V4 region of the bacterial 16S rRNA gene was amplified and sequenced using Ilumina MiSeq in order to assess microbial diversity. The Divisive Amplicon Denoising Algorithm (DADA2) algorithm was used for the inference of amplicon sequence variants from amplicon data. Airborne bacteria were significantly more abundant in farmers' indoor environments than in suburban homes ($p < 0.001$). Cow farmers' homes had significantly higher bacterial diversity than pig farmers' and suburban homes ($p < 0.001$). Bacterial taxa, such as Firmicutes, Prevotellaceae, Lachnospiraceae, and *Lactobacillus* were significantly more abundant in farmers' homes than suburban homes, and the same was true for beneficial intestinal bacterial species, such as *Lactobacillus amylovorus*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii*. Furthermore, we found a higher similarity between bacterial communities in individual farmers' homes and their associated cow stables than for pig stables. Our findings contribute with important knowledge on bacterial composition, abundance, and diversity in different environments, which is highly valuable in the discussion on how microbial exposure may contribute to the development of immune-mediated diseases in both children and adults.

Keywords: cow, pig, dust, airborne bacteria, 16S rRNA gene, indoor environment

HIGHLIGHTS

- Cow farmers' homes have higher bacterial diversity than pig farmers' homes and suburban homes.
- Cow stables have higher bacterial diversity and abundance than pig stables.
- Animal intestinal microbiota appear to contribute to the indoor bacteria in farmers' homes.
- Putative beneficial bacterial taxa are more abundant indoors in farmers' homes than in suburban homes.
- Bacterial communities in individual farmers' homes and cow stables are more similar than pig stables.

INTRODUCTION

Several studies have shown that children growing up on farms have a lower risk of immune-mediated diseases than children growing up in urban areas. These studies link exposure to farm-related microbiota through contact with livestock animals to a lower risk of immunoregulatory disorders, such as allergy, asthma, Irritable Bowel Diseases (IBD), and type 1 diabetes mellitus (Vedanthan et al., 2006; Basinas et al., 2012; Heikkinen et al., 2013; Timm et al., 2014, 2016; Elholm et al., 2016; Stein et al., 2016; Kirjavainen et al., 2019). These observed correlations have contributed to the hygiene hypothesis, which states that low exposure to microorganisms plays a key role in the aetiology of immune-mediated diseases (Okada et al., 2010). Supporting this hypothesis, Ege et al. (2011) found that children who lived on farms were exposed to more diverse environmental microorganisms than the children in the suburban areas.

Microbial dispersal from the farmers working places to the home environment occurs through airflow or direct transport by family members that interact with livestock, soil, water surface, and plants. Thus, the microbial diversity in the home environment may be increased and the composition of the airborne bacterial community in farmers' homes can be altered compared to suburban homes where humans and, to a lesser extent, pets are the main sources of the indoor air microbiome (Lis et al., 2008; Normand et al., 2011; Hospodsky et al., 2012). Low microbial diversity in urban areas might be the reason for cases of immune dysfunction, poor immune tolerance, and finally may lead to autoimmune disease. However, few studies have characterized the microbial community composition in various farms and farmhouses.

In the current study, we report on results obtained from settled dust from cow stables and cow farmers' homes collected on an electrostatic dust fall collector (EDC). We compared these results with data obtained using the same approach in pig stables, pig farmers' homes, and suburban homes (Vestergaard et al., 2018). We focused on the composition, abundance, and diversity of the airborne bacterial communities in cow stables and cow farmers' homes in comparison to the other indoor environments. The study aimed at (1) comparing type of bacteria present in stables and the farmers' homes, (2) determining if microbial communities in farmers' homes differ from suburban homes, and (3) searching for differences in taxonomic groups of putative beneficial bacteria between livestock stables, associated farmers' homes, and suburban homes.

Abbreviations: ASVs, Amplicon sequence variants; DADA, Divisive Amplicon Denoising Algorithm; EDC, Electrostatic dust collector; IBD, Irritable Bowel Diseases; qPCR, Quantitative PCR.

MATERIALS AND METHODS

Dust Sampling

Electrostatic dust collector was used to collect settled dust from the air with an exposure area of 209 cm^2 (Juel Holst et al., 2020). Sampling was conducted as part of a previous study in Jutland, Denmark, where settled air dust was collected from the farmers' homes and associated livestock stables (Vestergaard et al., 2018). Similarly, dust was collected in the suburban homes with the EDCs in the greater Copenhagen area (Juel Holst et al., 2020). During winter (November–April), 25 samples were collected from farmers' homes and 23 samples from the associated cow stables where the cow farmers were working. During the summer, (May–October), the numbers were 24 and 18, respectively. The EDCs were placed at 1.5 m above the floor, and the sampling period was 14 days. EDCs were kept at −20°C until DNA extraction.

Dust and DNA Extraction

The EDCs were processed as previously described by Vestergaard et al. (2018). They were carefully placed in a sterile stomacher bag and mixed with 20 ml of extraction buffer, consisting of pyrogen-free water and 0.05% Tween-20. The sample was processed in a stomacher (Star Blender LB 400, Seward, Worthing, United Kingdom) for 10 min at maximum speed. Thereafter, the fluid containing the washed-off dust was transferred to a sterile 50 ml Falcon tube. This procedure was repeated once more, until a total of 40 ml of suspended dust was extracted from the EDC. The dust was collected by centrifugation at $4{,}700 \times g$ for 15 min at 5°C. The supernatant was discarded, and the pellets were resuspended in 1.5 ml of 0.05% Tween-20 extraction buffer. Unexposed EDCs were used for negative control extractions. The dust samples were kept at −20°C until DNA extraction.

The PowerLyzer PowerSoil DNA Isolation kit (MO BIO Laboratories, an Qiagen Company, Germany) was used to extract DNA from the dust pellets following the manufacturer's instructions with minor modifications including prolonged bead-beating using a TissueLyser bead-beating machine for 2×5 min at 50 s^{-1} and prolonged centrifugation steps $13{,}000 \times g$ for 5 min at room temperature following the bead beating step. Negative control extractions were carried out using the same procedures.

PCR Amplification

Twenty samples from each indoor environment were randomly selected for quantitative PCR (qPCR) to quantify bacterial abundance. Briefly, the qPCR reactions were carried out in a 20 µl reaction volume containing 10 ml SYBR Green 1Master-2x, 2 ml bovine serum albumin (BSA; 10 mg/ml), 1 ml forward primer Bac908F (5′-AAC TCA AAK GAA TTG ACG GG-3′), and 1 ml reverse primer Bac1075R (5′-CAC GAG CTG ACG ACA RCC-3′; 10 pmol/ml; Ohkuma and Kudo, 1998). Controls were obtained by substituting DNA template with ddH2O. Serial dilutions of a plasmid encoding a full-length 16S rRNA gene linked to Sphingomonadales were used to generate standard curves. Thermal cycling and fluorescence measurements were carried out using an MX3005p qPCR machine (Agilent, Santa Clara, CA, United States; RRID:SCR_019526). One cycle of initial denaturation at 95°C for 5 min was followed by 45 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 20 s, and 80°C for 7 s.

Illumina MiSeq Sequencing

The 16S rRNA gene was amplified from 114 samples (90 samples, 12 negative control samples, and 12 technical replicates using the same DNA extract). Bac341F (5′-CCT ACG GGN GGC WGC AG-3′) and Bac805R (5′-GAC TAC GGT ATC TAA TCC-3′) bacteria-specific primers were used to amplify V3 and V4 regions (Klindworth et al., 2013). The steps for amplification of the 16S rRNA gene were carried out according to the Illumina protocol (16S Metagenomic Sequencing Library Preparation), with few modifications. The protocol included three PCR steps. In the first PCR, bacteria-specific primers were used to amplify the V3 and V4 regions of the 16S rRNA gene. The PCR mixture containing 2 µl template DNA was used for cow stable samples and 3 µl template DNA was used for farmers' home samples, 2 × KAPA HiFi Hotstart polymerase (KAPA Biosystems, Wilmington, MA, United States), 0.2 µM forward primer, 0.2 µM reverse primer, and BSA (4 g/L). The variation in the DNA template volume was due to different concentrations of bacteria and PCR inhibitors in the two indoor environments. The thermal cycling was performed in the following steps: an initial denaturation at 95°C for 3 min, 25 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s, and a final elongation at 72°C for 5 min. In the second PCR, the Illumina overhang adaptors were added using the same PCR conditions for the first PCR, albeit without added BSA and with only 10 amplification cycles instead of 25. The third PCR Nextera XT Index primers from the Nextera XT Index kit were used. Each reaction contained 2.5 µl Index primer 1 (N7XX) and 2.5 µl Index primer 2 (S5XX), 12.5 µl KAPA HiFi HotStart ReadyMix, and 5 µl dH2O with the same PCR thermal cycling program described above. Following each PCR step, AMPure XP magnetic beads were used for cleaning of the PCR products.

To determine the concentration of the PCR products, the Quant-iTTM dsDNA assay kit and a FLUOstar Omega fluorometric microplate reader (BMG LABTECH, Ortenberg, Germany) were used. Thereafter, the samples were diluted to approximately 3 ng/ml DNA and pooled. The DNA concentrations of pooled samples were measured with a Quant-iTTM dsDNA BR assay kit and on a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) before paired end 2 × 300 bp sequenced with a MiSeq sequencer (Illumina, San Diego, CA, United States; RRID:SCR_016379).

Bioinformatic and Statistical Analysis

The MiSeq-derived sequences from 84 pig farmer homes, 83 pig stables, and 100 suburban homes, along with associated metadata deposited by Vestergaard et al. (2018), were downloaded from the NCBI sequence read archive (SRA) under study number SRP124427. These data were combined with the data obtained in the present study. All sequence data processing, statistical analyses, and visualizations were carried out in RStudio version 1.4.1103 with R version 4.0.4 (R Core Team, 2013).

The sequences were trimmed using the cutadapt package version 1.16 (Martin, 2011) Open-source software package DADA2 version 1.18.0 (Callahan et al., 2016) was used for error correcting and modelling of the sequenced data, mostly by following the tutorial.[1] One major change was using the shortread package version 1.48.0 (Morgan et al., 2009) to randomly subsample all sequences to 20,000 reads following quality filtering in order to make richness comparisons accurate, as DADA2 tends to inflate richness estimates linearly with an increasing number of reads. Sequences belonging to the forward and reverse read libraries were merged together after primer trimming and quality filtering, and only sequences with a length greater than 430 base pairs were used, which was the expected amplicon length based on the primers. The ASVs were taxonomically classified into species using the DADA2 package's "assignTaxonomy" and "addSpecies" functions. The reference database used in the current study was the SILVA (RRID:SCR_006423) database version 138 (Quast et al., 2012). To eliminate ASVs contaminating reads in exposed EDC samples, the decontam package version 1.10.0 (Davis et al., 2018) was used. The "prevalence" method was used in the decontam package for contaminant detection. In the prevalence method, the prevalence (presence/absence across samples) of each sequence feature in true exposed EDC sample is compared to the prevalence in negative controls to identify contaminants.

The ampvis2 package version 2.6.8 (Andersen et al., 2018) was used to generate heatmaps and phyloseq version 1.27.6 (McMurdie and Holmes, 2013) and was used to assess the alpha diversity by calculating two diversity measures: observed (the number of individual bacterial taxa) and the Shannon index, which reflects both richness and the relative abundance of each taxon. The Wilcoxon Rank Sum test, implemented in the "wilcox.test" function in R version 4.0.4, was used for the comparison of the alpha diversity indices between different indoor environments as well as to investigate the differences in bacterial abundance measured by qPCR.

[1]benjjneb.github.io/dada2/tutorial_1_8

Ordination was carried out to compare the microbial communities in different indoor environments, based on the Aitchison dissimilarity matrix calculated using the "dist" function in coda.base package version 0.3.1 (Comas-Cufí, 2020). Principal coordinate analysis (PCoA) was carried out using the ape package version 5.5 (Paradis et al., 2004). Pairwise statistical comparisons were run between different indoor environments using analysis of similarity (ANOSIM) from the vegan package version 2.5-7 (Oksanen et al., 2015) based on the Aitchison dissimilarity matrix.

To identify specific bacterial taxa whose abundances significantly differ between different environmental types, we applied analysis of compositions of microbiomes with bias correction (ANCOM BC) version 1.0.5 (Oksanen et al., 2015). ANCOM BC provides a statistically valid test with a q value (adjusted value of p) and confidence intervals (log fold change: natural logarithm) for each bacterial taxon. ANCOM BC was performed for bacterial phyla, families, genera, and species levels with a relative abundance equal to or higher than 0.01%. The function "aggregate_taxa" from microbiome package version 1.15.0 (Yang et al., 2022) was used to aggregate taxa to a certain taxonomic level prior to ANCOM BC analysis. For each taxon in the data, ANCOM BC analysis results reported a coefficient value (log fold change) and a q value. A negative log fold change indicates that the taxa are less abundant compared to the reference group, and a positive log fold change indicates that the group has a higher abundance compared to the reference group. A q value equal to or less than 0.05 indicates a significant difference in abundances of the taxa between the two groups.

The "dist" function in the coda.base package version 0.3.1 was used to construct Aitchison dissimilarity matrix between a farmer's home and the relevant stable (i.e., the sample pair represents where a farmer lived and worked). The similarity of each pair was ranked among all non-matching home–stable pairs, with the final rank showing how similar associated home–stable pairs were compared to random association between any farmer's home and any stable. A rank of 1 indicates that there has been substantial bacterial transfer between the farmer's home and the stable, whereas a random ranking indicates that there has been no link.

Data Availability

The MiSeq-derived sequences used in this study were deposited in the NCBI under BioProject ID: PRJNA801418.[2]

RESULTS

Quality Filtering

Quality filtering and down sampling to 20,000 reads per sample, retain 65 suburban home samples out of 100, 40 cow stable samples out of 41, 38 cow farmers' homes samples out of 49, 81 pig stable samples out of 83, 82 pig stable samples out of 84, and 43 negative control samples (extraction blank and unexposed EDC samples) out of 52.

Bacterial Abundance

We found no significant difference in bacterial abundance between cow and pig farmers' homes as determined by qPCR ($p = 0.82$; **Figure 1**). In contrast, there was a significantly higher bacterial abundance in the two types of farmers' homes compared to the suburban homes ($p < 0.001$), i.e., the total number of airborne bacterial cells was higher in farmers' homes than in suburban homes, as the observed differences in bacterial abundance were beyond what could be explained solely by copy number variation. Livestock stables showed higher bacterial abundance than all indoor home environments ($p < 0.001$). Cow stables had a higher airborne bacterial load than pig stables ($p < 0.001$) and home environments ($p < 0.001$; **Figure 1**).

Alpha Bacterial Diversity

In terms of observed richness (numbers of bacterial taxa), the dust from cow farmers' homes had a significantly higher bacterial richness than pig farmers' homes and suburban homes (**Figure 2A**). The livestock stables had lower bacterial richness than farmers' homes ($p < 0.001$) with the lowest number of bacterial taxa found in dust collected from pig stables (**Figure 2A**). The Shannon index (a metric for bacterial diversity), which is an estimate of both the richness and uniformity of bacterial communities (**Figure 2B**), showed the same trend, with cow farmers' homes having richer and more uniform airborne bacterial community than pig farmers' and suburban homes ($p < 0.001$) and cow stables harboring a significantly higher bacterial diversity than pig stables ($p < 0.001$).

Beta Diversity of Indoor Environments

The PCoA using the Aitchison dissimilarity matrix, as well as the ANOSIM test, were used to investigate differences in airborne bacterial composition between different indoor environments. The airborne bacterial communities of farmers' homes and suburban homes were significantly different based on the ANOSIM test. The PCoA analysis revealed that the microbial community composition of suburban homes clustered separately, while there was a slight overlap between pig and cow farmers' homes (**Figure 3**). Despite the overlap between the farmers' homes, the difference in community composition between cow and pig farmers' homes was statistically significant (ANOSIM $R = 0.49$, $p = 0.001$). The ANOSIM test also revealed that the difference was greater between cow farmers' homes and suburban homes (ANOSIM $R = 0.57$, $p = 0.001$) compared to pig farmers' homes and suburban homes (ANOSIM $R = 0.45$, $p = 0.001$). The microbial community composition was more similar between pig stables and pig farmers' homes (ANOSIM $R = 0.14$, $p = 0.001$) than between cow stables and cow farmers' homes (ANOSIM $R = 0.29$, $p = 0.001$). The largest pairwise difference across all indoor environments was observed for pig and cow stables (ANOSIM $R = 0.75$, $p = 0.001$). All

[2]https://www.ncbi.nlm.nih.gov/sra/PRJNA801418

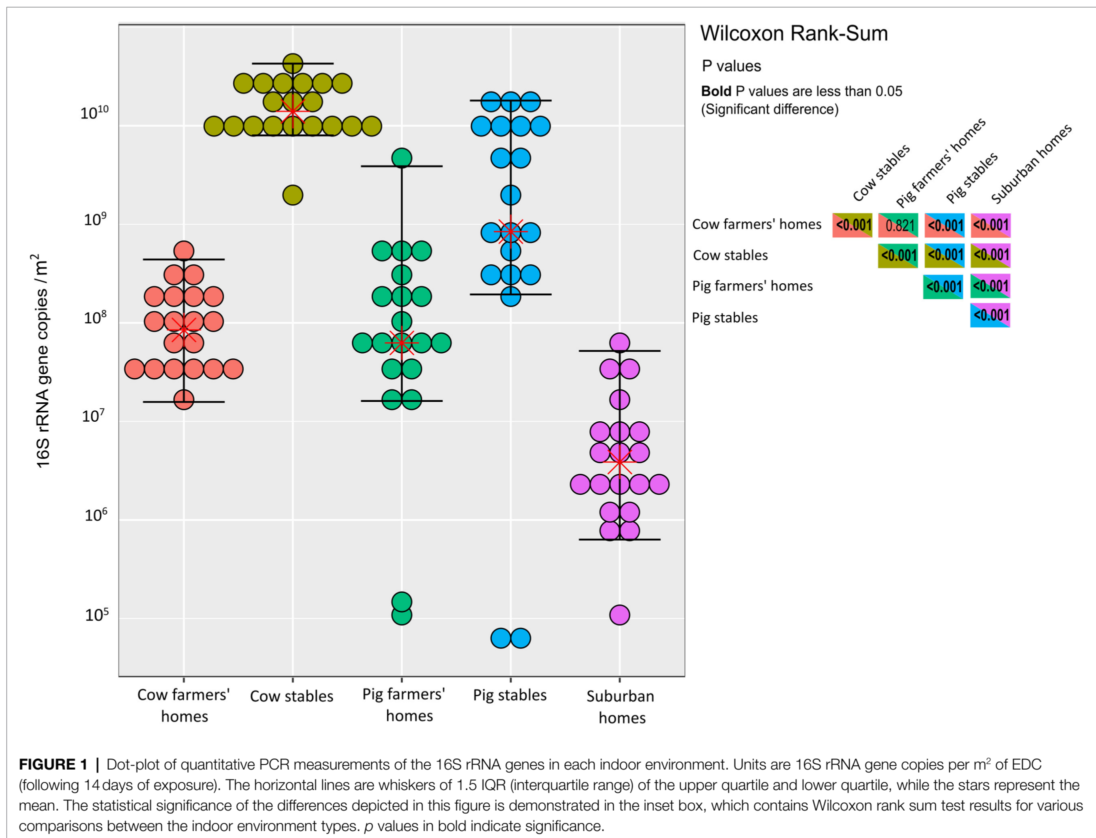

FIGURE 1 | Dot-plot of quantitative PCR measurements of the 16S rRNA genes in each indoor environment. Units are 16S rRNA gene copies per m^2 of EDC (following 14 days of exposure). The horizontal lines are whiskers of 1.5 IQR (interquartile range) of the upper quartile and lower quartile, while the stars represent the mean. The statistical significance of the differences depicted in this figure is demonstrated in the inset box, which contains Wilcoxon rank sum test results for various comparisons between the indoor environment types. *p* values in bold indicate significance.

distinctions between the different indoor environments are visible in the spatial organization of samples plotted using PCoA (**Figure 3**).

Seasonal Effects on Bacterial Community Composition and Abundance

The effect of season on the indoor airborne bacterial community in the cow farmers' home and cow stables was limited. Season had no significant effect on bacterial load (**Supplementary Figure 1**) or bacterial community composition (**Supplementary Figure 2**). Bacterial diversity and richness of cow stables were not affected by the season, whereas bacterial richness (number of bacterial taxa) was significantly higher in cow farmers' homes in the summer compared to the winter ($p = 0.03$; **Supplementary Figure 3**).

Bacterial Community Composition

Almost all samples were dominated by four bacterial phyla independent of the sampling location: Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes (**Figure 4A**). Firmicutes were more abundant in pig farmers' homes than in any other indoor home environment. Proteobacteria and Actinobacteria were the most prevalent in suburban homes, with 24.4 and 26.7%, respectively. Bacteroidetes were found in greater abundance in cow farmers' homes than in pig farmers' or suburban homes. These tendencies in phylum abundance were found to be significant by ANCOM BC analysis, except in the case of Bacteroidetes abundance (**Supplementary Tables 1–3**). Members of the Firmicutes families, Lachnospiraceae, Lactobacillaceae, Ruminococcaceae, and Peptostreptococcaceae were significantly more abundant in the farmer's homes than in the suburban homes. Apart from Peptostreptococcaceae, the other three families were relatively more abundant in farmers' homes than in livestock stables (**Figure 4B**). However, not all of them were significant between farmers' homes and stables (**Supplementary Tables 4, 5**). Rikenellaceae and Prevotellaceae families that belong to the Bacteroidetes phylum were found to be more abundant in farmers' homes than in suburban homes. Rikenellaceae, Prevotellaceae, Peptostreptococcaceae, and Lachnospiraceae were significantly more abundant in pig farmers' homes than in cow farmers' homes. However, Ruminococcaceae and Lactobacillaceae

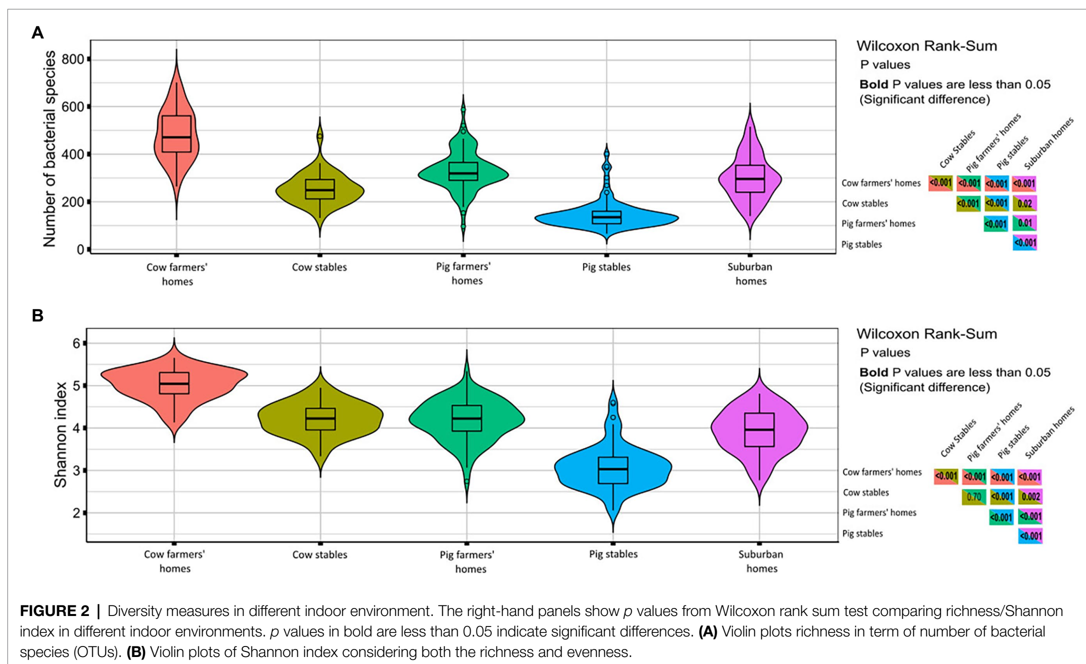

FIGURE 2 | Diversity measures in different indoor environment. The right-hand panels show *p* values from Wilcoxon rank sum test comparing richness/Shannon index in different indoor environments. *p* values in bold are less than 0.05 indicate significant differences. **(A)** Violin plots richness in term of number of bacterial species (OTUs). **(B)** Violin plots of Shannon index considering both the richness and evenness.

did not show a significant difference in abundance between the two types of farmers' homes (**Supplementary Tables 4**, **5**). The Gram-positive bacterial families, such as Staphylococcaceae, Corynebacteriaceae, Micrococcaceae, and Streptococcaceae dominated the airborne microbial communities in suburban homes (**Figure 4B**; **Supplementary Table 6**).

On the genus level, bacterial genera associated with the animal gut, including *Lactobacillus*, *Turicbacter*, *Intestinibacter*, *Terrisporobacter*, *Lachnospiraceae* UCG-007, and *Romboustia* based on AMDB: database of animal gut microbial communities (Yang et al., 2022), were found to be significantly more abundant in pig stables, followed by the pig farmers' home, than in any other indoor environment, including cow stables (**Figure 4C**). Some of these bacterial genera, such as *Lactobacillus and Turicbacter*, did not show a significant difference between the cow farmers' homes and suburban homes (**Supplementary Tables 7–9**). Species level identification also showed several bacterial species of intestinal origin (Yang et al., 2022), such as *Eubacterium hallii*, *Faecalibacterium prausnitzii*, *Lactobacillus amylovorus*, and *Clostridium butyricum* to be significantly more abundant in farmers' homes than in suburban homes. Apart from *Clostridium butyricum*, all the above-mentioned bacterial species were found to be significantly more abundant in farmers' homes than in livestock stables (**Supplementary Tables 10–12**). Bacterial species that were associated with animal and livestock environments, such as *Saccharopolyspora rectivirgula*, *Staphylococcus sciuri*, and *Streptococcus suis* were found in significantly greater abundance in associated farmers' homes than in suburban homes (**Supplementary Tables 10–12**).

Similarity of Bacterial Community Between the Farmer's Home and the Associated Stable

We performed a pairwise analysis of similarity between bacterial communities in livestock stables and associated farmer's homes (i.e., the pair of locations where the farmer worked and lived) to investigate whether the two associated indoor environments were more similar to each other than was the general similarity between unassociated stables and farmers' homes. We found that nine out of 29 (31%) associated cow stable–cow farmer's home pairs were more similar than non-associated stable–home pairs (**Supplementary Figure 4**). While we found only 14 out of 77 (18%) associated pig stable–pig farmers' home pairs had substantial bacterial transfer (**Supplementary Figure 5**). The quantitative pairwise distance values between the home and stables pairs are shown in **Supplementary Tables 13–16**. These values show that the number of shared bacteria between farmers' homes and cow stables is higher than between farmers' homes and pig stables. However, in general, the indoor air bacterial community in a specific farmer's home was more likely to be similar to the indoor air bacterial community in another farmer's stable than to the indoor air bacterial community in his or her own stable.

DISCUSSION

In this study, we investigated the airborne bacterial communities of five indoor environments: pig and cow farmers' homes, suburban homes, and pig and cow stables.

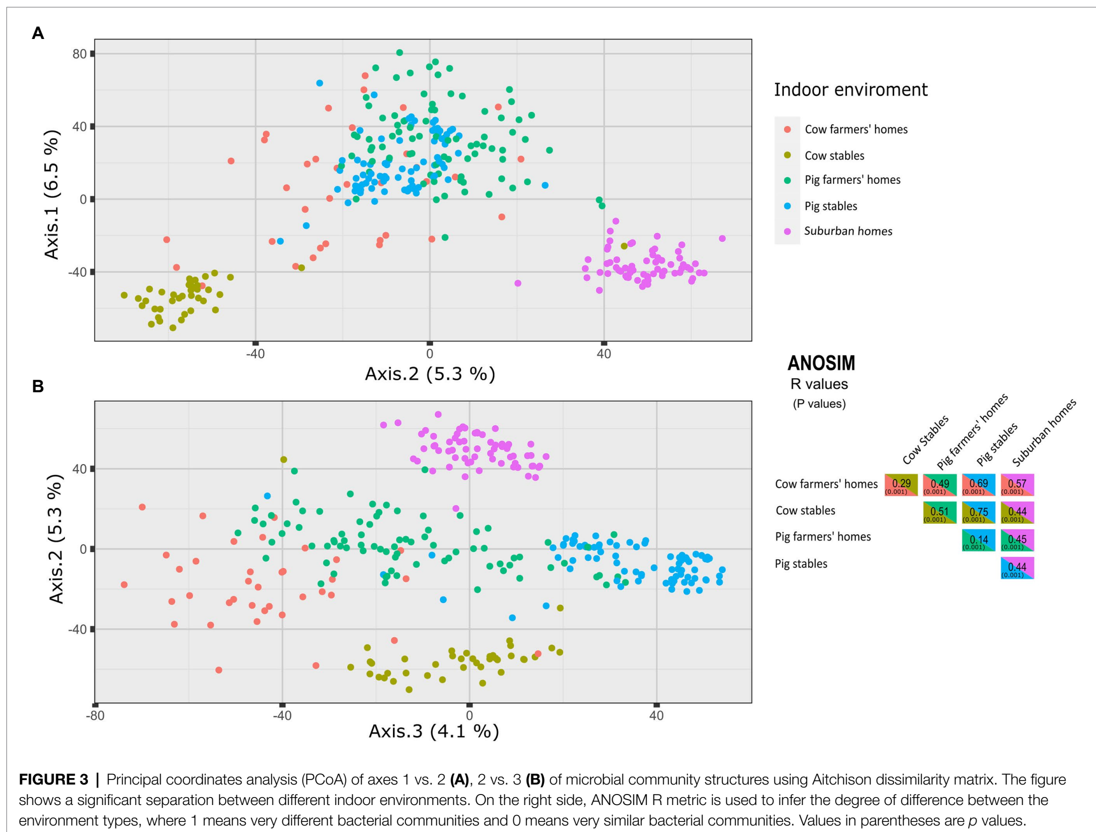

FIGURE 3 | Principal coordinates analysis (PCoA) of axes 1 vs. 2 **(A)**, 2 vs. 3 **(B)** of microbial community structures using Aitchison dissimilarity matrix. The figure shows a significant separation between different indoor environments. On the right side, ANOSIM R metric is used to infer the degree of difference between the environment types, where 1 means very different bacterial communities and 0 means very similar bacterial communities. Values in parentheses are *p* values.

The results demonstrate that the abundance, alpha and beta diversity, and community composition of airborne bacteria differ significantly between farmers' and suburban homes. We also showed that the gut microbiome of the farm animals contributed to the indoor airborne bacterial communities in farmers' homes, especially in the case of pig farmers' homes.

Higher Indoor Airborne Bacterial Abundance in Farmers' Home Than Suburban Homes

We found significantly higher bacterial abundance in cow and pig farmers' homes than in suburban homes (**Figure 1**), as previously reported by Pakarinen et al. (2008). In rural areas, a greater variety of outdoor microbial sources such as plants, soil, and livestock animals might explain the higher prevalence of microbes in farmers' homes compared to suburban homes. Bacterial abundance was 10–100 times higher in livestock stables than in the home environments (**Figure 1**). Increased bacterial abundance in livestock stables is consistent with prior research that found higher bacterial abundance in livestock-stable air compared to other indoor environments (Dungan et al., 2011; Hong et al., 2012). Aerosolization of dust particles and bacteria associated with animal skin and faces might explain the increase in bacterial abundance in livestock stables compared to home environments. According to Wei et al. attachment of airborne bacteria to the dust particles increases their viability, abundance, and metabolic capability thus alter the fate of bacterial cells in the air due to protection by the dust particles from harsh environmental conditions such as stables (Hu et al., 2020).

Cow stables exhibited a larger bacterial abundance in the air than pig stables. We cannot exclude the possibility that the difference in bacterial load could be due to differences in the number of animals as well as the design of the two types of livestock stables. Kembel et al. (2012) showed that natural ventilation significantly increased bacterial abundance compared to mechanically ventilated indoor environments. In the present study, the natural ventilation in the cow stable compared to mechanical ventilation in the pig stable may also have contributed to the difference in bacterial abundance.

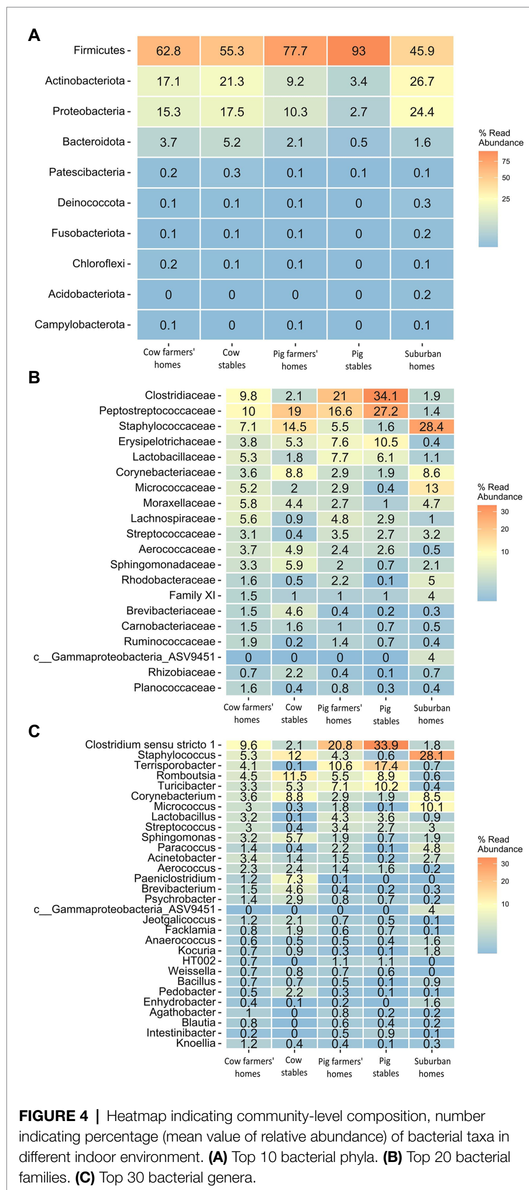

FIGURE 4 | Heatmap indicating community-level composition, number indicating percentage (mean value of relative abundance) of bacterial taxa in different indoor environment. **(A)** Top 10 bacterial phyla. **(B)** Top 20 bacterial families. **(C)** Top 30 bacterial genera.

Cow Farmers' Homes Have a High Level of Alpha Bacterial Diversity

The difference in bacterial richness and bacterial diversity (Shannon index) between the three home environments was significant. We demonstrate for the first time that cow farmers' homes have the highest bacterial diversity compared to the pig farmers' homes and suburban homes (**Figure 2**). The difference in airborne bacterial diversity between the two types of farmers' homes and suburban homes could be attributed to bacterial exposure from their livestock stables and bacterial dispersal from the outdoors to the indoor environment through ventilation. Lis et al. (2008) found that airborne microorganisms in farmers' homes consisted of a mixture of microorganisms from farm buildings, including livestock stables. They suggested that bacteria and fungi are transported from farm buildings to homes *via* workers' clothes and bodies. Pasanen et al. (1989) also concluded that airborne microorganisms may be indirectly transmitted from cow stables to farmhouses *via* workers' clothing. We speculate that the higher bacterial diversity and richness in cow farmers' homes compared to pig farmers' homes might be due to the microbes from pig and humans being more similar than those of cows and humans due to a more similar diet for pigs and humans compared to cows and humans. Another explanation could be because Danish regulations require farmers' working in pig stables to wash their hands and change clothes to prevent the spread of zoonotic pathogens from pigs are more strict compared to farmers working in open-air and less precautioned cow stables, so cow farmers would transmit more bacteria to their homes than pig farmers (Denver et al., 2016).

Seasonal differences in airborne bacterial richness but not bacterial diversity in cow farmers' homes were significant (**Supplementary Figure 3**). We found higher bacterial richness in the summer compared to the winter. This could be due to the fact that Denmark, where samples were collected, is located in Northern Europe, and experiences a temperate climate. Because air-conditioning systems are not common, people normally ventilate their homes by opening windows, as they would do more in summer when central heating is not running. Thus, it would bring in more bacteria taxa from the surrounding environment, which would lead to a rise in bacterial richness. In addition, the diversity of outdoor bacterial communities in Scandinavia was found to be higher in the summer compared to winter (Karlsson et al., 2020).

Increased bacterial diversity in the indoor environment has been linked to a lower prevalence of immunoregulatory disorders, including IBD, atopy, asthma, and type 1 diabetes mellitus. Timm et al. (2014) discovered that being born and living on a livestock farm for the first 5 years of life was associated with a lower risk of IBD when compared to being born and living in the city. It was hypothesized by the authors that the association could be due to decreased microbial diversity (Timm et al., 2014). Exposure to a variety of microorganisms has been inversely associated with the risk of asthma and atopy (Ege et al., 2011). Similarly, Valkonen et al. (2015) found that bacterial diversity was inversely related to atopy but not asthma. Others have suggested that exposure to a broad variety of non-pathogenic environmental microorganisms during childhood might have a protective effect against type 1 diabetes mellitus (Heikkinen et al., 2013). Our finding supports the different putative health outcomes between different indoor environments based on different levels of bacterial diversity.

Our results show that the bacterial diversity is higher in cow stables as compared to pig stables. The natural ventilation

in cow stables, as opposed to the mechanical ventilation and highly controlled, closed nature of the pig stables, might explain the increased indoor airborne bacterial diversity in the cow stables compared to pig stables, where the bacteria in the air will mainly come from a limited source, the pigs, and their feed. Other factors that could explain higher bacterial diversity in cow stables compared to pig stables include reduced use of antibiotics in cow farming compared to pig farming. Illi et al. (2012) reported that cow exposure is the farm exposure that protects against asthma and atopy. In the same study, the exposure to pigs did not show a protective effect against asthma and atopy (Illi et al., 2012).

Beta Diversity of the Indoor Airborne Bacterial Communities

According to the PCoA analysis, suburban homes had a distinct bacterial community, while the pig and cow farmers' homes showed a minor overlap (**Figure 3**). Different indoor bacterial community composition between suburban and farmers' homes is in line with previous studies, which showed a difference in microbiota between farm and non-farm homes (Kirjavainen et al., 2019; Fu et al., 2021). Even though there is an overlap in the community composition of cow and pig farmers' homes, there was a significant difference in the community composition between cow and pig farmers' homes. As a result, it is feasible that the putative protective effects of airborne microbiomes in the cow and pig farmers' homes might be different. The microbial communities were more similar between pig stables and pig farmers' homes than between cow stables and cow farmers' homes (**Figure 3**). As Stein et al. (2016) showed, distance to home and farmers' work might be an important factor explaining this similarity, but this information was not available in our study.

The PCoA analysis and ANOSIM showed that pig and cow stables had different indoor bacterial community compositions. This indicates that farmers working in these environments (stables) have different microbial exposure and, therefore, may experience different health consequences. Several studies report an inverse relationship between animal contact and the prevalence of atopy and respiratory allergy in childhood (Naleway, 2004; Vedanthan et al., 2006). In Denmark, Elholm et al. (2013) showed that exposure to farm animals protects against the development of atopy not only in childhood but also in young adulthood. They found that being exposed to cows, pigs, or combinations of these animals was associated with a decreased risk of new-onset sensitization when compared to participants without livestock exposure. The exposure to endotoxin has been associated with a reduced prevalence of sensitization to common allergens in a highly exposed adult farming (Portengen et al., 2005). In Alpine farm environments, the GABRIEL Study found that children who were exposed to cows, but not pigs, were protected against asthma, atopic sensitization, and hay fever (Illi et al., 2012). The lower number of pigs per farm in the alpine region compared to Jutland, Denmark, might explain why the association differs between the two studies with regard to exposure to pigs.

Indoor Airborne Bacterial Composition Between Rural and Suburban Areas

All samples, regardless of the environmental type, were dominated by four bacterial phyla: Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes. This is largely consistent with prior studies that showed the predominance of the four bacterial phyla in cow and pig stables and indoor home environments (Hong et al., 2012; Boissy et al., 2014; Zhang et al., 2019). The higher abundance of Firmicutes and lower abundance of Proteobacteria in farmers' homes relative to suburban homes is basically in line with prior epidemiological findings suggesting that Firmicutes decrease the risk of atopic sensitization (Lee et al., 2021). In contrast, Proteobacteria have been associated with allergy and found to be more common in the airways of neutrophilic asthma patients (Yang et al., 2018). Bacterial families that might have protective effects against allergy, IBD, and asthma were found to be significantly more abundant in farmers' homes than in suburban homes. These include members of the Firmicutes families, Lachnospiraceae, Lactobacillaceae, Ruminococcaceae (Lynch et al., 2014; Huang and Boushey, 2015), and Peptostreptococcaceae (Pekkanen et al., 2018). With the exception of Peptostreptococcaceae, the other three families that were suggested to have a protective effect against autoimmune disease were relatively more abundant in farmers' homes than in livestock stables, implying that the surrounding outdoor environment in rural areas could be the source of these bacterial families. This is consistent with the suggestion by Dimich-Ward et al. that some aspects of the protective effect of the farm environment are not attributable to contact with livestock (Dimich-Ward et al., 2006).

Rikenellaceae and Prevotellaceae families that belong to the Bacteroidetes phylum were found to be more abundant in farmers' homes, especially cow farmers' homes, than in suburban homes. Members of these two families are frequently found in cattle's gastrointestinal microbiota (Mao et al., 2015). Rikenellaceae and Prevotellaceae have been associated with protection against allergic asthma and allergy, with a possible explanation that the inhaled or ingested bacteria serve as a kind of an anti-allergy adjuvant for the allergens inhaled or ingested, a concept supported by recent research showing commensal bacteria protect against food allergen sensitization (Huang and Boushey, 2015).

We found beneficial taxa of gut microbiome to be more abundant in farmers' homes, especially pig farmers' homes, than in suburban homes. Recent animal and epidemiological studies have found that certain bacterial taxa have protective effects against inflammation, IBD, insulin resistance, and atopy (Radman et al., 2015; Udayappan et al., 2016; He et al., 2021). Oral treatment of diabetic mice with *Eubacterium hallii* leads to an improvement in insulin sensitivity (Udayappan et al., 2016). *Faecalibacterium prausnitzii* was discovered to boost the secretion of IL-10 thereby inhibit the creation of proinflammatory cytokines, such as TNF-α, IL-6, and IL-12 (He et al., 2021). Another bacterial species that showed higher relative abundance in farmers' homes than in suburban homes was *Lactobacillus amylovorus*. Using intestinal human and intestinal pig cells as substrate, *L. amylovorus* was able to be inhibit the TLR4 (Toll-like receptors) inflammatory signaling

via modulation of TLR2 and cytokine regulation (Finamore et al., 2014).

The majority of the bacterial species mentioned above were significantly more abundant in farmers' homes than in livestock stables. This could indicate that the environment surrounding farmers' homes might be the source of these bacterial taxa. The animal manure used in fields as fertilizer where farmers' homes are located might be a source for the presence of animal gut microbiota in the indoor air of the farmers' homes.

Bacterial Transfer From Livestock Stables to the Farmers' Homes

Overall, the microbial communities were more similar between pig stables and pig farmers' homes (ANOSIM $R = 0.14$) than between cow stables and cow farmers' homes (ANOSIM $R = 0.29$; **Figure 3**). However, we found a higher similarity in bacterial communities established in individual farmers' homes and their associated cow stables than pig stables, indicating more bacterial transfer from the cow stable than pig stables to the associated farmers' homes. Lower rate of bacterial transfer rate between pig stables and their associated farmer homes were previously reported and discussed by Vestergaard et al. (2018). It seems at first paradoxical that the airborne bacterial communities in pig farmers' homes are generally more similar to pig stables, while individual cow farmers' homes are more similar to their corresponding stables, but if we separate the concepts of similarity and transfer between stable and home then it makes more sense. It means that there is some general property of pig stables or pig farmers' homes (perhaps the porcine microbiota is more similar to the human than the bovine?) that makes them more similar but given microbial community data from a specific cow stable one would be more likely to accurately pair it to a specific cow farmer's home than for pigs, which is a separate concept.

Higher similarity in bacterial communities established in individual farmers' homes and their associated cow stables than for homes and pig stables. This could be a result of the strict Danish regulations that require employees present in pig stables to disinfect their hands, change clothes, and disinfect equipment to prevent the transmission of zoonotic diseases from pigs compared to less pre-cautioned and open-air cow stables (Denver et al., 2016).

In most cases, the indoor airborne bacteria in the farmers' homes did not originate from the cow or pig stables where they were working. This might imply that putatively beneficial bacteria in the farmers' homes air are transported from outdoor sources in the environment surrounding the farmers' homes rather than from the farmers' own pig or cow stables. This is consistent with the findings of Dimich-Ward et al., who suggested that some aspects of the farm environment, other than contact with livestock, were protective of respiratory and allergic conditions (Dimich-Ward et al., 2006). Outdoor environmental sources are responsible for increasing bacterial diversity in farmers' homes compared to suburban homes. Among these potential sources are plants, soil, water, and pig manure. In Denmark, pig manure is commonly used as a low-cost natural fertilizer for agricultural soil to increase crop yield and maintain soil fertility (Sommer and Knudsen, 2021). We found several bacteria taxa related to gut microbiota to be significantly more abundant in farmers' homes than in suburban homes (**Figure 4C**). This suggests an indirect transfer of microbes from the gut of the pigs to the indoor air of the farm home, which might explain the putatively beneficial bacteria common in the air of both farmers' homes and pig stables.

CONCLUSION

The settled airborne dust in farmers' homes, especially cow farmers' homes, was characterized by high bacterial diversity compared to suburban homes that were dominated by bacteria from human sources and had low bacterial diversity. Furthermore, intestinal animal microbiota from manure appears to contribute to the indoor airborne bacterial community in farmers' homes. All the observed differences in bacterial community composition, diversity, and abundance of specific types of bacteria found in this study support the concept that the bacterial composition in farmers' homes, and to a lesser extent, livestock stables, further contribute to a verification of the microbial diversity hypothesis. Further studies, including experimental animal models and immunological studies, are needed to demonstrate the possible beneficial effects of specific bacterial taxa, which are abundant in rural environments.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

GE, VS, and TS designed and carried out sample collection from cow stables and farmers' homes under the SUS12 study. DV, GH, KF, TS, TŠ-T, and IM planned qPCR and 16S rRNA gene sequencing. DV and GH extracted cells from EDC filters. DV extracted DNA from cells and carried out PCR and sequencing. HA and IM analyzed the data from sequencing and qPCR. HA, RB, IM, and TS wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by Realdania and by the Danish National Research Foundation (grant no. DNRF104). Additionally, this project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement no. 804199).

ACKNOWLEDGMENTS

We would like to thank Biochemical engineers at Section for Microbiology, Department of Biology, Aarhus University department Susanne Nielsen, Britta Poulsen, and Anne Stentebjerg for the technical assistance in generating 16S rRNA gene amplicon libraries and Martin Täubel and Anna Hyvarinnen National Institute for Health and Welfare (THL), Kuopio, Finland, for helping us to set up the extraction procedure for the dust from the EDC cloths.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.883991/full#supplementary-material

Supplementary Figure 1 | Dot-plot of quantitative PCR measurements of cow farmers' homes and cow stables based on season. The 10 dots in the dot-plot represents the results of 10 measurements. The horizontal lines are whiskers of 1.5 IQR of the upper quartile and lower quartile The significance of the differences depicted in this figure is demonstrated in the inset box, which contains Wilcoxon rank sum test results. P values less than 0.05 is significant.

Supplementary Figure 2 | Principal coordinates analysis (PCoA) of microbial community structures of cow farmers' homes and cow stables based on season using Aitchison dissimilarity matrix. On the right side ANOSIM R metric is used to infer the degree which the environment, where 1 means very different communities and zero means very similar communities. A significant difference ($P < 0.05$).

Supplementary Figure 3 | Diversity measures of cow farmers' homes and cow stables based on season. P values from Wilcoxon rank sum test comparing richness/Shannon index in different indoor environments. P values in bold are less than 0.05 indicate significant differences. **(A)** Violin plots richness in term of number of bacterial species (OTUs). **(B)** Violin plots of Shannon index considering both the richness and evenness.

Supplementary Figure 4 | Plots showing the similarity rank of associated cow stable — cow farmers' home pairs in a ranked list of all possible cow stable-home pairs. A similarity rank of 1 (Green bar) indicates that the airborne bacterial community in a given cow farmer's home is more similar to the cow stables where that farmer works than any other cow stable. Summer samples pairs showed 3 out of 12, while winter showed 6 out of 17 pairs were more similar than non-associated stable–home pairs.

Supplementary Figure 5 | Plots showing the similarity rank of associated pig stable — pig farmers' home pairs in a ranked list of all possible pig stable-home pairs. A similarity rank of 1 (Green bar) indicates that the airborne bacterial community in a given pig farmer's home is more similar to the pig stables where that farmer works than any other pig stable. Summer samples pairs showed 10 out of 40, while winter showed 4 out of 37 pairs were more similar than non-associated stable–home pairs.

REFERENCES

Andersen, K. S., Kirkegaard, R. H., Karst, S.M., and Albertsen, M. (2018). ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. BioRxiv [Preprint]. doi: 10.1101/299537

Basinas, I., Sigsgaard, T., Heederik, D., Takai, H., Omland, Ø., Andersen, N. T., et al. (2012). Exposure to inhalable dust and endotoxin among Danish livestock farmers: results from the SUS cohort study. *J. Environ. Monit.* 14, 604–614. doi: 10.1039/C1EM10576K

Boissy, R. J., Romberger, D. J., Roughead, W. A., Weissenburger-Moser, L., Poole, J. A., and LeVan, T. D. (2014). Shotgun pyrosequencing metagenomic analyses of dusts from swine confinement and grain facilities. *PLoS One* 9:e95578. doi: 10.1371/journal.pone.0095578

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

Comas-Cufí, M. (2020). Coda.base: A Basic Set of Functions for Compositional Data Analysis. R package version 0.3.1.

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., and Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* 6:226. doi: 10.1186/s40168-018-0605-2

Denver, S., Alban, L., Boklund, A., Houe, H., Mortensen, S., Rattenborg, E., et al. (2016). The costs of preventive activities for exotic contagious diseases—a Danish case study of foot and mouth disease and swine fever. *Prev. Vet. Med.* 131, 111–120. doi: 10.1016/j.prevetmed.2016.07.010

Dimich-Ward, H., Chow, Y., Chung, J., and Trask, C. (2006). Contact with livestock–a protective effect against allergies and asthma? *Clin. Exp. Allergy* 36, 1122–1129. doi: 10.1111/j.1365-2222.2006.02556.x

Dungan, R., Leytem, A., and Bjorneberg, D. (2011). Concentrations of airborne endotoxin and microorganisms at a 10,000-cow open-freestall dairy. *J. Anim. Sci.* 89, 3300–3309. doi: 10.2527/jas.2011-4002

Ege, M. J., Mayer, M., Normand, A. C., Genuneit, J., Cookson, W. O., Braun-Fahrländer, C., et al. (2011). Exposure to environmental microorganisms and childhood asthma. *N. Engl. J. Med.* 364, 701–709. doi: 10.1056/NEJMoa1007302

Elholm, G., Linneberg, A., Husemoen, L. L. N., Omland, Ø., Grønager, P. M., Sigsgaard, T., et al. (2016). The Danish urban–rural gradient of allergic sensitization and disease in adults. *Clin. Exp. Allergy* 46, 103–111. doi: 10.1111/cea.12583

Elholm, G., Schlünssen, V., Doekes, G., Basinas, I., Bibby, B. M., Hjort, C., et al. (2013). Become a farmer and avoid new allergic sensitization: adult farming exposures protect against new-onset atopic sensitization. *J. Allergy Clin. Immunol.* 132, 1239–1241. doi: 10.1016/j.jaci.2013.07.003

Finamore, A., Roselli, M., Imbinto, A., Seeboth, J., Oswald, I. P., and Mengheri, E. (2014). Lactobacillus amylovorus inhibits the TLR4 inflammatory signaling triggered by enterotoxigenic Escherichia coli via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants. *PLoS One* 9:e94891. doi: 10.1371/journal.pone.0094891

Fu, X., Ou, Z., Zhang, M., Meng, Y., Li, Y., Wen, J., et al. (2021). Indoor bacterial, fungal and viral species and functional genes in urban and rural schools in Shanxi Province, China–association with asthma, rhinitis and rhinoconjunctivitis in high school students. *Microbiome* 9:138. doi: 10.1186/s40168-021-01091-0

He, X., Zhao, S., and Li, Y. (2021). *Faecalibacterium prausnitzii*: a next-generation probiotic in gut disease improvement. *Can. J. Infect. Dis. Med. Microbiol.* 2021, 1–10. doi: 10.1155/2021/6666114

Heikkinen, S. M., Pitkäniemi, J. M., Kilpeläinen, M. L., and Koskenvuo, M. J. (2013). Does farm environment protect against type 1 diabetes mellitus? *Diab. Vasc. Dis. Res.* 10, 375–377. doi: 10.1177/1479164112474907

Hong, P. Y., Li, X., Yang, X., Shinkai, T., Zhang, Y., Wang, X., et al. (2012). Monitoring airborne biotic contaminants in the indoor environment of pig and poultry confinement buildings. *Environ. Microbiol.* 14, 1420–1431. doi: 10.1111/j.1462-2920.2012.02726.x

Hospodsky, D., Qian, J., Nazaroff, W. W., Yamamoto, N., Bibby, K., Rismani-Yazdi, H., et al. (2012). Human occupancy as a source of indoor airborne bacteria. *PLoS One* 7:e34867. doi: 10.1371/journal.pone.0034867

Hu, W., Murata, K., Fan, C., Huang, S., Matsusaki, H., Fu, P., et al. (2020). Abundance and viability of particle-attached and free-floating bacteria in dusty and nondusty air. *Biogeosciences* 17, 4477–4487. doi: 10.5194/bg-17-4477-2020

Huang, Y. J., and Boushey, H. A. (2015). The microbiome in asthma. *J. Allergy Clin. Immunol.* 135, 25–30. doi: 10.1016/j.jaci.2014.11.011

Illi, S., Depner, M., Genuneit, J., Horak, E., Loss, G., Strunz-Lehner, C., et al. (2012). Protection from childhood asthma and allergy in alpine farm environments—the GABRIEL advanced studies. *J. Allergy Clin. Immunol.* 129, 1470–1477.e6. doi: 10.1016/j.jaci.2012.03.013

Juel Holst, G., Pørneki, A., Lindgreen, J., Thuesen, B., Bønløkke, J., Hyvärinen, A., et al. (2020). Household dampness and microbial exposure related to allergy and respiratory health in Danish adults. *Eur. Clin. Respir. J.* 7:1706235. doi: 10.1080/20018525.2019.1706235

Karlsson, E., Johansson, A. M., Ahlinder, J., Lundkvist, M. J., Singh, N. J., Brodin, T., et al. (2020). Airborne microbial biodiversity and seasonality in northern and southern Sweden. *PeerJ* 8:e8424. doi: 10.7717/peerj.8424

Kembel, S. W., Jones, E., Kline, J., Northcutt, D., Stenson, J., Womack, A. M., et al. (2012). Architectural design influences the diversity and structure of the built environment microbiome. *ISME J.* 6, 1469–1479. doi: 10.1038/ismej.2011.211

Kirjavainen, P. V., Karvonen, A. M., Adams, R. I., Täubel, M., Roponen, M., Tuoresmäki, P., et al. (2019). Farm-like indoor microbiota in non-farm homes protects children from asthma development. *Nat. Med.* 25, 1089–1095. doi: 10.1038/s41591-019-0469-4

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Lee, M. K., Wyss, A. B., Carnes, M. U., Richards, M., Parks, C. G., Beane Freeman, L. E., et al. (2021). House dust microbiota in relation to adult asthma and atopy in a US farming population. *J. Allergy Clin. Immunol.* 147, 910–920. doi: 10.1016/j.jaci.2020.06.013

Lis, D. O., Mainelis, G., and Górny, R. L. (2008). Microbial air contamination in farmhouses–quantitative aspects. *Clean Soil Air Water* 36, 551–555. doi: 10.1002/clen.200800003

Lynch, S. V., Wood, R. A., Boushey, H., Bacharier, L. B., Bloomberg, G. R., Kattan, M., et al. (2014). Effects of early-life exposure to allergens and bacteria on recurrent wheeze and atopy in urban children. *J. Allergy Clin. Immunol.* 134, 593–601.e12. doi: 10.1016/j.jaci.2014.04.018

Mao, S., Zhang, M., Liu, J., and Zhu, W. (2015). Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function. *Sci. Rep.* 5:16116. doi: 10.1038/srep16116

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* 17, 10–12. doi: 10.14806/ej.17.1.200

McMurdie, P. J., and Holmes, S. (2013). Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Morgan, M., Anders, S., Lawrence, M., Aboyoun, P., Pages, H., and Gentleman, R. (2009). ShortRead: a bioconductor package for input, quality assessment and exploration of high-throughput sequence data. *Bioinformatics* 25, 2607–2608. doi: 10.1093/bioinformatics/btp450

Naleway, A. L. (2004). Asthma and atopy in rural children: is farming protective? *Clin. Med. Res.* 2, 5–12. doi: 10.3121/cmr.2.1.5

Normand, A.-C., Sudre, B., Vacheyrou, M., Depner, M., Wouters, I. M., Noss, I., et al. (2011). Airborne cultivable microflora and microbial transfer in farm buildings and rural dwellings. *Occup. Environ. Med.* 68, 849–855. doi: 10.1136/oem.2010.061879

Ohkuma, M., and Kudo, T. (1998). Phylogenetic analysis of the symbiotic intestinal microflora of the termite *Cryptotermes domesticus*. *FEMS Microbiol. Lett.* 164, 389–395. doi: 10.1111/j.1574-6968.1998.tb13114.x

Okada, H., Kuhn, C., Feillet, H., and Bach, J. F. (2010). The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clin. Exp. Immunol.* 160, 1–9. doi: 10.1111/j.1365-2249.2010.04139.x

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, MHH, Oksanen, MJ, et al. (2015). Vegan: community ecology package. Ordination methods, diversity analysis and other functions for community and vegetation ecologists. R package ver: p. 2.3-1.

Pakarinen, J., Hyvärinen, A., Salkinoja-Salonen, M., Laitinen, S., Nevalainen, A., Mäkelä, M. J., et al. (2008). Predominance of gram-positive bacteria in house dust in the low-allergy risk Russian Karelia. *Environ. Microbiol.* 10, 3317–3325. doi: 10.1111/j.1462-2920.2008.01723.x

Paradis, E., Claude, J., and Strimmer, K. (2004). APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* 20, 289–290. doi: 10.1093/bioinformatics/btg412

Pasanen, A. L., Kalliokoski, P., Pasanen, P., Salmi, T., and Tossavainen, A. (1989). Fungi carried from farmers' work into farm homes. *Am. Ind. Hyg. Assoc. J.* 50, 631–633. doi: 10.1080/15298668991375272

Pekkanen, J., Valkonen, M., Täubel, M., Tischer, C., Leppänen, H., Kärkkäinen, P. M., et al. (2018). Indoor bacteria and asthma in adults: a multicentre case–control study within ECRHS II. *Eur. Respir. J.* 51:1701241. doi: 10.1183/13993003.01241-2017

Portengen, L., Preller, L., Tielen, M., Doekes, G., and Heederik, D. (2005). Endotoxin exposure and atopic sensitization in adult pig farmers. *J. Allergy Clin. Immunol.* 115, 797–802. doi: 10.1016/j.jaci.2004.11.046

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

R Core Team (2013). R: A Language and Environment for Statistical Computing.

Radman, M., Golshiri, A., Shamsizadeh, A., Zainodini, N., Bagheri, V., Arababadi, M. K., et al. (2015). Toll-like receptor 4 plays significant roles during allergic rhinitis. *Allergol. Immunopathol.* 43, 416–420. doi: 10.1016/j.aller.2014.04.006

Sommer, S. G., and Knudsen, L. (2021). Impact of Danish livestock and manure management regulations on nitrogen pollution, crop production, and economy. *Front. Sustain.* 2:20. doi: 10.3389/frsus.2021.658231

Stein, M. M., Hrusch, C. L., Gozdz, J., Igartua, C., Pivniouk, V., Murray, S. E., et al. (2016). Innate immunity and asthma risk in Amish and Hutterite farm children. *N. Engl. J. Med.* 375, 411–421. doi: 10.1056/NEJMoa1508749

Timm, S., Frydenberg, M., Janson, C., Campbell, B., Forsberg, B., Gislason, T., et al. (2016). The urban-rural gradient in asthma: a population-based study in northern Europe. *Int. J. Environ. Res. Public Health* 13:93. doi: 10.3390/ijerph13010093

Timm, S., Svanes, C., Janson, C., Sigsgaard, T., Johannessen, A., Gislason, T., et al. (2014). Place of upbringing in early childhood as related to inflammatory bowel diseases in adulthood: a population-based cohort study in Northern Europe. *Eur. J. Epidemiol.* 29, 429–437. doi: 10.1007/s10654-014-9922-3

Udayappan, S., Manneras-Holm, L., Chaplin-Scott, A., Belzer, C., Herrema, H., Dallinga-Thie, G. M., et al. (2016). Oral treatment with *Eubacterium hallii* improves insulin sensitivity in db/db mice. *NPJ Biofilms Microbiomes* 2:16009. doi: 10.1038/npjbiofilms.2016.9

Valkonen, M., Wouters, I. M., Täubel, M., Rintala, H., Lenters, V., Vasara, R., et al. (2015). Bacterial exposures and associations with atopy and asthma in children. *PLoS One* 10:e0131594. doi: 10.1371/journal.pone.0131594

Vedanthan, P. K., Mahesh, P. A., Vedanthan, R., Holla, A. D., and Liu, A. H. (2006). Effect of animal contact and microbial exposures on the prevalence of atopy and asthma in urban vs rural children in India. *Ann. Allergy Asthma Immunol.* 96, 571–578. doi: 10.1016/S1081-1206(10)63552-1

Vestergaard, D. V., Holst, G. J., Basinas, I., Elholm, G., Schlünssen, V., Linneberg, A., et al. (2018). Pig farmers' homes harbor more diverse airborne bacterial communities than pig stables or suburban homes. *Front. Microbiol.* 9:870. doi: 10.3389/fmicb.2018.00870

Yang, X., Li, H., Ma, Q., Zhang, Q., and Wang, C. (2018). Neutrophilic asthma is associated with increased airway bacterial burden and disordered community composition. *Biomed. Res. Int.* 2018:9230234. doi: 10.1155/2018/9230234

Yang, J., Park, J., Jung, Y., and Chun, J. (2022). AMDB: a database of animal gut microbial communities with manually curated metadata. *Nucleic Acids Res.* 50, D729–D735. doi: 10.1093/nar/gkab1009

Zhang, W., Li, W., Ma, C., Wu, X., Li, X., Zeng, J., et al. (2019). Characterization of microbial communities in a dairy farm matrix in Ningxia, China, by 16S rDNA analysis. *Int. J. Genom.* 2019:3827360. doi: 10.1155/2019/3827360

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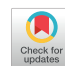
Science of the Total Environment 857 (2023) 159455

Contents lists available at ScienceDirect

Science of the Total Environment

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

Optimization of bacterial DNA and endotoxin extraction from settled airborne dust



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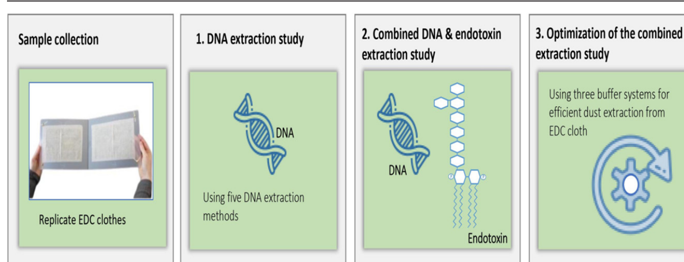
HIGHLIGHTS

- The DNeasy Power soil Kit had the best DNA extraction yield and time invested.
- Rinsing EDC cloth with pyrogen-free water promoted removal of dust particles.
- A protocol for DNA and endotoxin extraction using a single EDC was developed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Dimitra A Lambropoulou

Keywords:
DNA extraction method
Lipopolysaccharide
Airborne microbiome
Passive sampling
Buffer
EDC

ABSTRACT

Collecting and obtaining sufficient amount of airborne particles for multiple microbial component assessments can be challenging. A passive dust sampling device, the electrostatic dust fall collector (EDC) has been established for assessing airborne exposures including endotoxin and glucans. Recently, with advances in next-generation sequencing techniques, EDCs were used to collect microbial cells for DNA sequencing analysis to promote the study of airborne bacterial and fungal communities. However, low DNA yields have been problematic when employing passive sampling with EDC. To address this challenge, we attempted to increase the efficiency of extraction. We compared DNA extraction efficiency of bacterial components from EDCs captured on filters through filtration using five extraction techniques. By measuring the abundance, diversity and structure of bacterial communities using qPCR and amplicon sequencing targeting 16S rRNA genes, we found that two techniques outperformed the rest. Furthermore, we developed protocols to simultaneously extract both DNA and endotoxin from a single EDC cloth. Our technique promotes a high quality to price ratio and may be employed in large epidemiological studies addressing airborne bacterial exposure where a large number of samples is needed.

1. Introduction

Active air sampling is thought to provide an accurate representation of airborne bioaerosol populations due to the high volume of air that is sampled. Active sampling, on the other hand, underestimates microbial diversity by saturating the filter and desiccating the microorganisms, and requires skilled handling of materials and equipment, making it difficult

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http://dx.doi.org/10.1016/j.scitotenv.2022.159455
Received 29 July 2022; Received in revised form 3 October 2022; Accepted 11 October 2022
Available online 14 October 2022
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to collect a large number of samples at different locations (Normand et al., 2009; Ghosh et al., 2015; Eduarda and Heederik, 1998). Unlike active air sampling, sampling of settled dust allows for simple, non-invasive, cumulative sampling of bioaerosol over a period of several days. While short-term temporal dynamics of bioaerosols cannot be assessed due to low temporal resolution, the extended period is beneficial for assessing a representative sampling of microbial exposure (Normand et al., 2009). Due to its simplicity, settled dust sampling is among the most popular approaches for measuring microbiological airborne exposure in the indoor environment (Viegas et al., 2018; Viegas et al., 2019a; Viegas et al., 2019b; Viegas et al., 2020a; Viegas et al., 2020b).

Among the different techniques, EDCs (Electrostatic Dust fall collectors) have been employed as a standard for passive sampling of settled airborne dust. The EDC is a sampling device consisting of a polypropylene folder holding 2 or 4 electrostatic cloths (Kilburg-Basnyat et al., 2016; Noss et al., 2008). They are easy to use and are ideal for large population studies because they can be mailed to, deployed by, and returned by study participants without the need for elaborate equipment and field staff (Kilburg-Basnyat et al., 2016; Kilburg-Basnyat et al., 2015). Since the introduction of EDCs, the dust collected on EDC cloths has been used to study traditional markers of biological exposure, like allergens, endotoxins and glucan exposure (Jacobs et al., 2014a; Samadi et al., 2010; Jacobs et al., 2014b; Sander et al., 2018; Krop et al., 2014; Schlünssen et al., 2015), as well as culturing of bacteria and fungi (Hoppe et al., 2012; Spilak et al., 2015). More recent molecular markers have been used to identify microbial communities (Vestergaard et al., 2018; Adams et al., 2021) and to identify antimicrobial resistance genes in occupational environments (Luiken et al., 2020; Van Cleef et al., 2014; van Cleef et al., 2015; Van Cleef et al., 2016). Recently EDCs have also been used to collect settling dust to study environmental contamination of SARS-CoV-2 infection in mink farms, meat processing plants, nursing homes and secondary schools (de Rooij, 2021a; de Rooij, 2021b; Jonker, 2022; Linde, 2022).

After EDCs were established to assess indoor air quality, the feasibility of EDCs for endotoxin measurements were validated by several studies of urban and farm homes. Factors that can affect EDC sampling efficiency have been assessed and include heating, mailing, electrostatic charge, storage, and deployment time (Kilburg-Basnyat et al., 2016; Kilburg-Basnyat et al., 2015). In addition, the effect of the extraction procedure and the effect of extraction media on the concentration of endotoxin from EDC has been studied. Noss et al. (2010) and Spaan et al. (2008) recommend using 0.05 % Tween 20 (surfactant) in pyrogen-free water (PFW) as the extraction medium rather than PFW alone for the extraction of endotoxin from settled dust. However, Hoppe Parr et al. (2017) showed that higher endotoxin concentrations were obtained by extraction of organic dust with Tris-HCL EDTA buffer compared to Tween 20 buffer. Shin et al. (2018) revealed the mechanism of soil particle removal from fibrous materials cloth by diffusiophoresis, the directed motion of soil particles by chemical gradients of surfactant. The authors emphasized the importance of surfactant gradients that establish via rinsing with fresh water for soil particle removal. They concluded that rinsing with fresh water is the key to the effective cleaning.

The use of culture-independent methods and in particular the use of 16S rRNA gene and ITS sequencing techniques for the characterization of bacterial and fungal communities has received increasing attention in airborne exposure studies due to the ability to identify a large range of bacterial and fungal taxa that elude culture-based studies (Vestergaard et al., 2018; Adams et al., 2021; Dannemiller et al., 2014; Lai et al., 2018). Thus, culture-independent methods together with EDCs became an important tool in epidemiological studies that aim to establish a link between airborne microbial exposure and human health. However, many of the DNA extraction techniques employed so far have resulted in low DNA yields, Therefore, several amplification steps are required for sufficient starting material for DNA sequencing with the risk of introducing sequence artifacts and contamination from the environment or the reagents used (Salter et al., 2014; Castelino et al., 2017). Therefore, increasing the amount of dust extracted from EDC cloth and finding an optimal method for DNA extraction from settled dust are needed.

In the current study, we first aimed to compare the efficiency and comparability of five DNA extraction methods using replicate EDC samples collected in parallel and standardized bacterial community. Secondly, we aimed to combine bacterial DNA and endotoxin extraction. Thirdly, we tested three buffer systems to increase the amount of dust extracted from EDC cloth to achieve an optimal amount of DNA and endotoxin extraction.

2. Material and methods

2.1. Study design

The settled airborne dust was collected using EDCs from the living room and the bedrooms of ten participants in Aarhus, Denmark. For each participant eight EDC replicates per sampling location (16 EDC cloths per participant) were collected; four EDCs in the bedroom and four EDCs in the living room. Each EDC cloth had an exposure area of 0.0209 m^2 and were placed about 150 cm above the ground for a period of 14 days. We successfully collected 155 out of 160 EDC cloths that were used for the analysis.

2.2. Dust extraction

Dust extraction was performed in a clean lab for nucleic acid work to avoid contamination. All the tools used were either sterilized before use or were single use sterile tools. Dust extraction from EDC cloths was performed as described previously by Adams et al. (2015). Briefly EDC cloth were placed in sterile stomacher bag and mixed with 20 ml extraction buffer consisting of 0.05 % Tween 20 (Sigma Aldrich, Missouri, United States) in pyrogen free water (PFW, Milli-Q® A10 Ultrapure Water). The samples were then processed in stomacher (VWR type Star Blender LB400) for 10 min at maximum speed. The extracted fluid was collected in a 50 ml Falcon tube and kept on ice. This procedure was repeated once more until a total volume of 40 ml was extracted from the filter cloth. The dust suspended in Tween 20 buffer was collected into a 25 mm 0.22 μm pore size polyethersulfone membrane filter (Merck, New Jersey, United States). The glass-vacuum filtration device was rinsed with hydrochloric acid and ethanol and autoclaved between runs. Clean autoclaved glass funnel was used for each filter cloth extract. The membrane filter containing the concentrated dust samples was aseptically transferred into pre-filled bead tubes/Falcon tubes and stored at −20 °C until DNA extraction. An overview figure represents steps of extraction of the settled airborne dust from the EDC cloth presented in Fig. 1. For the DNA extraction study (Section 2.3), stomacher Star Blender LB400 (VWR, Radnor, Pennsylvania, USA) was used and for the remaining part of the study, low noise Smasher™ (bioMérieux, Marcy-lÉtoile, France) was used with adjustments: the samples were processed for 3 min at the fast mode (620 stroke/min). Further modifications were added to the dust extraction process to improve its efficiency (Sections 2.4 and 2.5).

2.3. DNA extraction

Earlier methods used centrifugation to concentrate dust extracted from the EDC cloths prior to DNA extraction (Vestergaard et al., 2018; Adams et al., 2015). In this study we instead collected the dust extracted from EDC cloths onto 0.22 μm polyethersulfone filters to improve the efficiency of particle retention, as we in a pilot study revealed a higher number of 16S rRNA gene copies using a filtration-based approach compared to a centrifugation-based approach (Supplementary Fig. 1). Five DNA extraction methods were then used on these filters to compare their performance. We compared three commercially available DNA extraction kits, DNeasy PowerSoil Pro Kit (named PowerSoil kit), DNeasy PowerWater Kit and MagAttract PowerWater DNA/RNA Kit (MO BIO Laboratories, a Qiagen Company, Hilden, Germany) with two previously described non-commercial DNA extraction methods, the first was used before to extract DNA from Sterivex filter columns (named protocol A) (Lever et al., 2015) while the second was used to extract DNA from sediment samples (named protocol B) (Xiao, 2017).

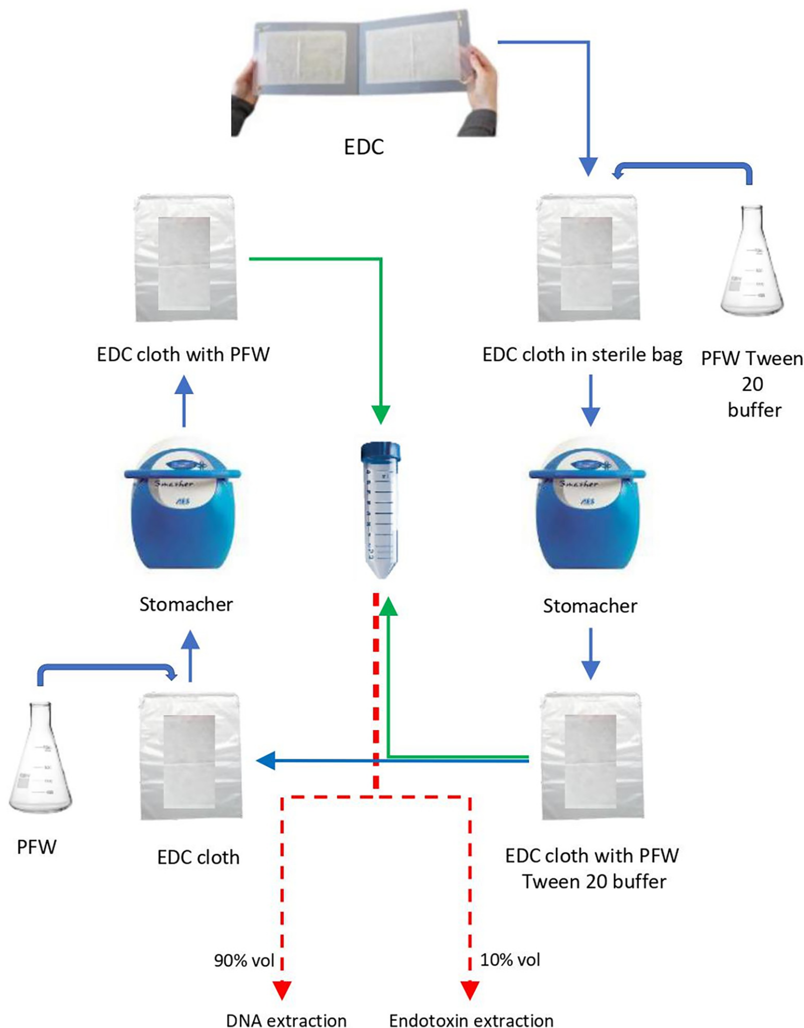

Fig. 1. Overview figure represent steps of extraction of the settled airborne dust from single EDC cloth. Bule arrows represent the direction of dust extraction from EDC cloth, green arrows represent collection of extracted fluid, and red arrows represent dividing of extracted fluid for downstream analysis.

In protocol A, membrane filters were placed into 15 ml Falcon tubes and entirely soaked with 0.1 ml of 10 mM dNTP and 1 ml of cell lysis solution consisting of: 30 mM Tris-HCl, 30 mM EDTA, 1 % Triton X-100, and 800 mM guanidium hydrochloride. The tubes were subjected to vortexing for 10 min at maximum speed. Following the lysis step, the tubes were frozen completely at −80C (at least 40 min). Then they were incubated on a thermoshaker (600 rpm) for 1 h at 50 °C. DNA was purified twice with 1 × sample volume of chloroform:isoamyl alcohol (24:1, vol:vol; Sigma Aldrich, Missouri, United States) with centrifugation steps at 10,000 ×*g* for 10 min in between the washes. Following DNA purification, 20 μg/ml of Linear polyacrylamide, 1.5 × volume isopropanol, and 0.1 volume 5 M NaCl were used to precipitate DNA overnight at −20 °C, then centrifuged at 4 °C for 30 min at 14,000 ×*g*. The pellets were washed with 70 % ethanol and dried for 5–7 min with a SpeedVac pre-heated to 50 °C. After that, the pellets were resuspended in 80 ul TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and purified with the Clean All RNA/DNA Clean-Up and Concentration Kit (Norgen Biotek, Ontario, Canada) to remove PCR inhibitors.

In the protocol B, membrane filters were placed at 2 ml screw cap micro tubes containing 0.25 ml zirconia beads (0.1 mm diameter, BioSpec, Oklahoma, United States) and lysis buffer mixture containing 200 ul TNS buffer (50 mM Tris, 150 mM NaCl, 10 % Sucrose, pH 8.0) and 650 ul sodium phosphate buffer solution (112.9 mM Na_2HPO_4, 7.1 mM NaH_2PO_4), the tubes were subjected to bead beating at 50 oscillations s^{-1} for 1 min using a TissueLyser LT 2500 (Qiagen Company, Hilden, Germany), followed by incubation in a thermomixer with 600 rpm at 50 °C. After lysis step, the mixture was centrifuged for 10 min at 19,000 ×*g* at 4 °C. The nucleic acids from the supernatant were purified with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, vol:vol:vol; Sigma Aldrich, Missouri, United States),

followed by purification with an equal volume of chloroform: isoamyl alcohol (24:1, vol:vol; Sigma Aldrich, Missouri, United States). One ml polyethylene glycol 8000 (Sigma Aldrich, Missouri, United States) was used to precipitate DNA at 4 °C overnight, then centrifuged at 19,000 ×*g* for 30 min. The precipitates were rinsed with an ice cold 70 % ethanol solution, dried in the air, and dissolved in 80 ul TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before being kept at −20 °C.

The three commercial kits were used according to manufacturers' instructions with the following refinement: the bead-beating step was carried out in a TissueLyser bead-beating machine for 10 min at 50 s^{-1}.

Two DNA extraction studies were performed. In the first DNA extraction study, the dust collected from EDC cloth simultaneously was used for the five DNA extraction methods and DNA yields were determined by quantitative PCR (qPCR). In the second DNA extraction study, the two most efficient methods were compared using qPCR and MiSeq sequencing of the V3-V4 region of bacterial 16S rRNA genes.

2.4. Combined DNA and endotoxin extraction

After achieving the first aim of the study, improving the amount of DNA extraction from settled dust. In this study we tested co-extraction of DNA and endotoxin from a single EDC cloth and compared the endotoxin yield to the established method described by Noss et al. (2008).

In the Combined DNA and endotoxin extraction, 10 % of 40 ml liquid containing the dust extracted from the EDC cloth were transferred to a 15 ml Falcon tube for endotoxin analysis. The cell debris was removed by centrifugation at 1000 ×*g* for 15 min and the supernatants were stored in glass vial as aliquots at −20 °C until analysis.

Following the classical method described by Noss et al. (2008), the EDC cloth was placed in glass Erlenmeyer flasks containing 20 ml 0.05 % Tween 20 buffer and shaken on a horizontal shaker (160 reciprocations/min) for 60 min at room temperature. 10 % of the extraction volume (2 ml) was harvested, centrifuged at 1000 ×*g* for 15 min, and supernatants were stored as aliquots in glass vials at −20 °C until analysis. The supernatants from the two extraction methods were analysed with the quantitative kinetic chromogenic LAL assay (Lonza, Basel, Switzerland).

2.5. Optimization of the combined extraction method

After establishing the combined extraction method, we aimed at optimizing the method by changing extraction buffers. Dust was extracted from replicate EDC clothes collected in the living room. Replicate EDCs were extracted by (i) double washing of the EDC with 100 mM Tris-10 mM EDTA buffer, (ii) double washing the EDC with 0.05 % Tween 20 buffer, (iii) first washing EDC with 0.05 % Tween 20 buffer followed by rinsing the EDC with 30 ml PFW. To compare between different buffers used, the DNA was extracted by PowerSoil kit and DNA yield was determined by qPCR. The endotoxin concentrations were measured by the kinetic chromogenic LAL assay.

2.6. Mock community

The ZymoBIOMICS™ Standardized microbial community (D6300) (mock community) (Zymo Research,Irvine, California,USA) with total cell concentration of ~1.4×10^{10} cells/ml was used to assess the DNA extraction efficiency a of different buffer system used. The bacterial cell numbers were estimated using qPCR targeting16S rRNA genes. The 16S rRNA operon copy number of 4.2 (Větrovský and Baldrian, 2013) was used to convert 16S rRNA gene copies obtained from by the qPCR into bacterial cell numbers. Across protocols that were tested, the DNA was eluted in 80 ul of TE buffer. The efficiency of the DNA extraction methods as a function of bacterial cell number was calculated according to the formula below. The expected bacterial cell number loaded on the unexposed EDC cloth were compared to actually retrieved bacterial cell number calculated by the formula.

$$\textit{Bacterial cell number} = \frac{\text{16S rRNA gene copies per ul} \times 80}{4.2}$$

First, the mock community was used to assess efficiency of DNA extraction as a function of different cell loads by double washing EDC cloths with 0.05 % Tween 20 buffer. The efficiency was determined using qPCR targeting 16S rRNA genes. The unloaded EDC cloths were spiked with known bacterial cell loads spanning between 7.71×10^4 and 7.71×10^8 cells per EDC cloth. Three EDC clothes for each cell load were extracted. Secondly, the mock community was used to test the effect of additional rinsing with PFW on DNA yield, therefore 12 blank EDC cloths were spiked with 7.71×10^6 bacterial cells and extracted using the following two buffer systems: i) double washing only with 0.05 % Tween 20 buffer or ii) single washing with 0.05 % Tween 20 buffer followed by 1–3 rinses with 30 ml PFW. Thirdly the mock community was used to test the efficiency of PowerSoil kit to extract DNA by adding 7.71×10^6 bacteria directly into pre-filled bead tubes.

Additionally, the mock community was used to test the ability of the combined extraction method (single washing EDC cloth with 0.05 % Tween 20 buffer followed by rinsing once with PFW) to extract DNA across bacterial taxa using 16S RNA sequencing.

2.7. Quantification of bacterial abundance

The bacterial abundance was measured by 16S qPCR as previously described by Vestergaard et al. (2018). The reactions were performed using a MX3005p qPCR machine (Agilent, Santa Clara, California, United States) and carried out in a 20 ul reaction volume containing 10 ul SYBR Green 1Master-2×, 2 ul bovine serum albumin (BSA; 10 mg/ml), 1 ul forward primer Bac908F (50-AAC TCA AAK GAA TTG ACG GG-30), 1 ul reverse primer Bac1075R (50-CAC GAG CTG ACG ACA RCC-30) (10 pmol/ml) (Ohkuma and Kudo, 1998), 4 ul dH2O, and 2 ul template DNA. Controls were obtained by substituting DNA template with ddH2O (double-distilled water). Standard curves were obtained using serial dilutions of a plasmid containing a full-length 16S rRNA gene related to Sphingomonadales. The thermal cycling conditions were one cycle of initial denaturation for 5 min at 95 °C, followed by 45 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 20 s, and 80 °C for 7 s.

2.8. 16S rRNA amplicon sequencing

Using Bac341F (5′-CCT ACG GGN GGC WGC AG-3′) and Bac805R primers (5′-GAC TAC GGT ATC TAA TCC-3′), the 16S rRNA gene V3 and V4 region was amplified. (Krop et al., 2014). The Illumina protocol (16S Metagenomic Sequencing Library Preparation) was followed with changes described in Vestergaard et al. (Kilburg-Basnyat et al., 2015). The library preparation included three PCR reactions. The first PCR amplified the 16S rRNA gene's V3 and V4 regions using bacteria-specific primers. The Illumina overhang adaptors were added in the second PCR, and the Nextera XT Index primers were used for the third PCR. AMPure XP magnetic beads were employed to clean the PCR products after each PCR step. The Quant-iTTM dsDNA assay kit and a FLUOstar Omega fluorometric microplate reader (BMG LABTECH, Ortenberg, Germany) were used to measure the concentration of the PCR products. Following that, the samples were diluted to around 3 ng/ml DNA and pooled together prior to being sequenced with MiSeq sequencing (Illumina, San Diego, California, United States).

2.9. Bioinformatic and statistical analysis

The sequencing data was processed in R (version 4.1.2) first by primer trimming using the cutadapt wrapper (Martin, 2011). The trimmed sequences were further processed using DADA2 (Divisive Amplicon

Denoising Algorithm 2) pipeline version 1.18.0 (Callahan et al., 2016) which used to infer true bacterial sequences from reads following the online tutorial. We added one modification into the DADA2 analysis pipeline using the shortread package version 1.48.0 (Morgan et al., 2009) to randomly subsample all sequences to 50,000 reads following quality filtering in order to make richness comparisons accurate. The ASVs were taxonomically classified up to the species level. The reference database used in the current study was the SILVA database version 138 (Quast et al., 2012). The decontam package version 1.10.0 (Davis et al., 2018) was used to eliminate ASVs from contaminating reads. For contaminate detection, the decontam package employed the "prevalence" method, where the identification of contaminates was based on the presence or absence taxa in the true positive samples (exposed EDC cloths) compared to the prevalence of these taxa in negative controls (unexposed EDC clothes and template-free PCR controls).

Heatmaps depicting the relative abundance of distinct bacterial taxa were created using the Ampvis2 package version 2.6.8. (Andersen, 2018) Phyloseq version 1.27.6 (McMurdie and Holmes, 2013) was used to generate two diversity measures for alpha diversity observed and the Shannon index. The Aitchison dissimilarity matrix was constructed using the "dist" function in the coda.base package version 0.3.1 and used to compare the microbial communities between different samples. PCoA ordination was carried out using the ape package version 5.5 (Paradis et al., 2004). According to Shapiro-Wilks test, the data were not normally distributed, so the Wilcoxon Rank Sum test which is included in the "wilcox.test" function in R version 4.1.2, was used to investigate the differences in bacterial abundance measured by qPCR and endotoxin yield measured by the LAL assay.

2.10. LAL assay

The supernatants from the two extraction methods (combined and classical method) were analysed without prior dilution. To study the effect of Tween 20 on LAL assay 10 supernatants were measured in three dilutions as 25×, 50× and undiluted. The 25× and 50× dilution showed higher endotoxin yield than undiluted supernatants. However, no significant differences were seen (Supplementary Fig. 2). The supernatants from the other experiments were diluted 50-times in PFW and analysed with the quantitative kinetic chromogenic LAL assay (Kinetic-QCL 50-650 U kit, Lonza, Walkersville, Maryland, USA). *Escherichia coli* O55:B5 reference standard endotoxin was used. Twelve concentrations spanning between 25 EU/ml and 0.012EU/ml were used for the standard curve. The detection limit for Vmax obtained by the kinetic LAL Assay was defined as the average of the assay blanks plus two times the standard deviation of these blanks. Results were given in EU m-2.

3. Results

3.1. DNA extraction studies

In the first DNA extraction study we compared amount of DNA yield by qPCR between the five DNA extraction methods. We found that protocol A and PowerSoil kit resulted in the highest DNA yields among the five DNA extraction methods (Fig. 2). The second DNA extraction study was conducted to compare the two best performing extraction techniques using qPCR and 16S rRNA bacterial gene sequencing. Protocol A resulted in more consistent DNA yields than PowerSoil kit when the first and the second DNA extraction study were compared (Fig. 3). After the decontamination procedures, samples retained on average (mean) 97 % of their reads, with a minimum of 85 % and a maximum of 100 %. The PowerSoil kit produced less contamination by fraction reads compared to protocol A, on average samples extracted by the PowerSoil kit retained 98 % of their reads compared to 95 % for protocol A. One sample extracted by the PowerSoil kit was removed from subsequent analysis due to a high (>50 %) contamination rate. (Supplementary Fig. 3).

In terms of bacterial composition, all samples from a specific location clustered together, while there was no clustering based on the method used. This indicates that variation between samples is greater than the variation caused by the two methods (Supplementary Fig. 4). The community composition at the phylum level was reproducible across the two methods for each home and contained a mixture of *Actinobacteria*, *Bacteroidetes*,

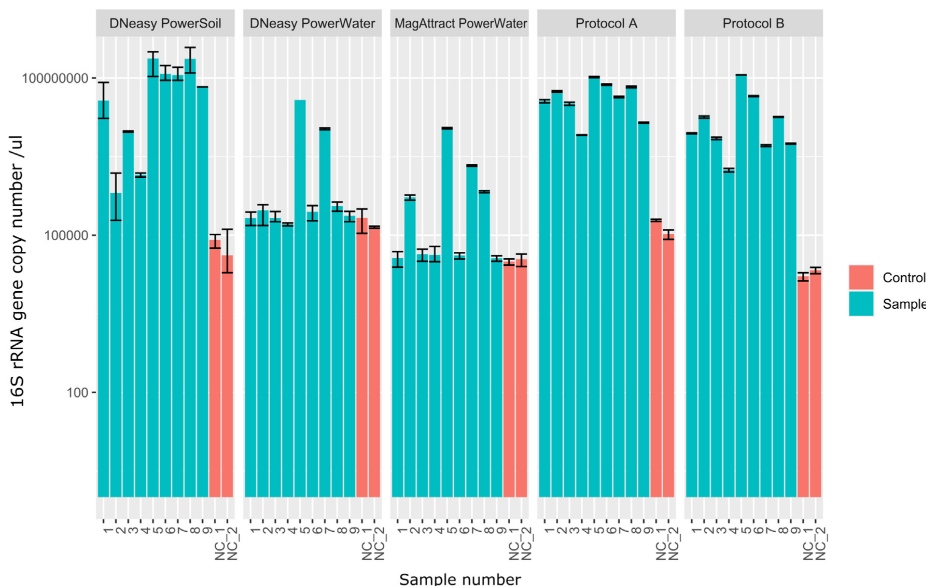

Fig. 2. Quantitative PCR measurements of the 16S rRNA of five EDC batches (each contain 9 EDC clothes) extracted by five DNA extraction methods. NC_1 and NC_2: Negative controls (unexposed EDC cloth).

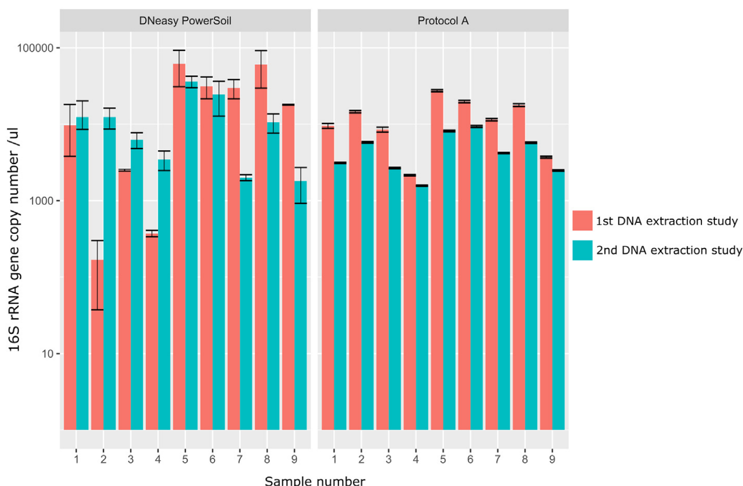

Fig. 3. Quantitative PCR measurements of the 16S rRNA genes using two replicate EDC batches extracted by PowerSoil kit and protocol A.

Cyanobacteria, *Firmicutes*, and *Proteobacteria*. The PowerSoil kit yielded a higher fraction of *Actinobacteria* and *Firmicutes* extraction, while protocol A generally yielded a higher fraction of *Proteobacteria* (Supplementary Fig. 5).

Protocol A consistently resulted in a higher Shannon diversity index and raw richness and in terms of reproducibility protocol A also performed better (Fig. 4). Contamination did not explain the higher bacterial diversity, or the higher bacterial richness obtained by protocol A as compared to the PowerSoil kit (Supplementary Figs. 6 and 7).

Comparing DNA yield obtained from the centrifugation-based protocol where the dust pellet resuspends in 1.5 ml 0.05 % Tween 20 before DNA extraction using the PowerSoil kit in Vestergaard et al. (2018) with the filtration-based protocol using the same DNA extraction kit showed that collecting the dust pellet on a membrane filter before DNA extraction significantly increased DNA yield (P value = 0.001) (Supplementary Fig. 1). Both samples from the two studies were from suburban homes.

3.2. Combined DNA and endotoxin extraction study

The combined method generally yields higher endotoxin concentrations, although the difference was not statistically significant (P = 0.51)

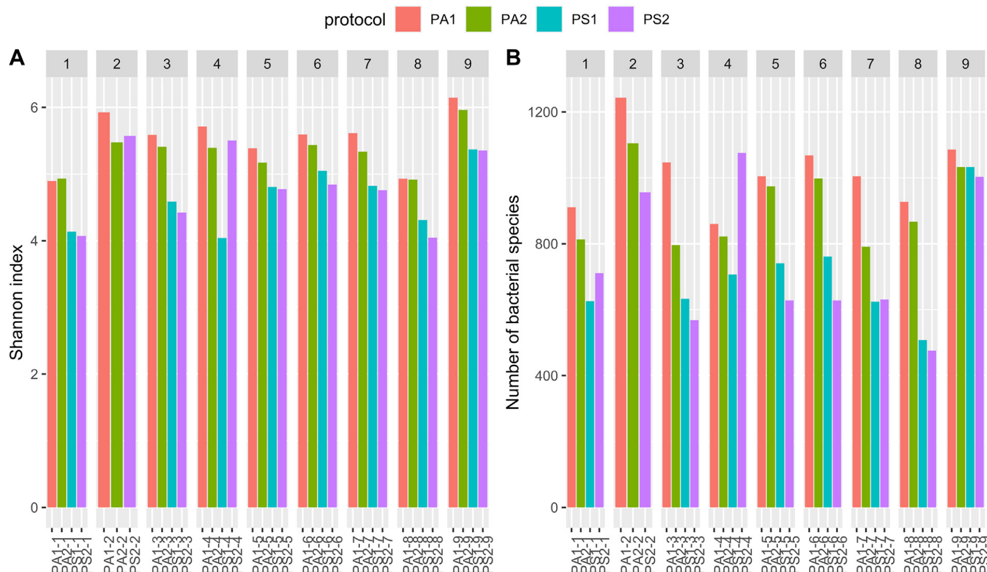

Fig. 4. Diversity measures calculated for two replicate EDC batches extracted by protocol A (PA) and PowerSoil kit (PS). PA1 & PA2: batch one and two extracted by protocol A, PS1 & PS2: batch one and two extracted by PowerSoil kit. (A) Bar plots of Shannon index considering both the richness and evenness (B) Bar plots of richness in term of number of bacterial species (OTUs).

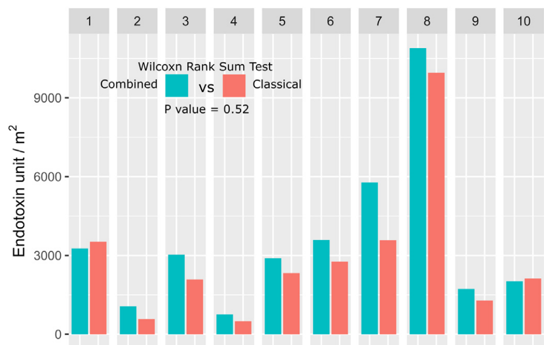

Fig. 5. Bar-plot of endotoxin concentration extracted by classic endotoxin extraction and combined extraction method.

compared to method commonly used for endotoxin extraction by Noss et al. (2008). However, the patterns were preserved (Fig. 5).

3.3. Optimization of the combined extraction study

We obtained a higher DNA and endotoxin yield when the EDC cloths were extracted using 0.05 % Tween 20 buffer in comparison to 100 mM Tris-HCl 10 mM EDTA buffer (Fig. 6). The 50-x dilution for Tris-HCl EDTA supernatants were just below detection limit of the LAL assay (0.012 EU/ml) compared to the 50-x dilution for Tween 20 supernatants that was above the detection limit of the LAL assay. We also showed that exchanging 0.05 % Tween 20 buffer with PFW during the second wash increased both DNA and endotoxin yield.

3.4. Mock community

Using a standardized mock community, we found that the efficiency of DNA extraction by double washing the EDC with 0.05 % Tween 20 buffer ranged from 2 to 4.4 %, with higher bacterial cell numbers giving higher DNA yields (data not shown). In a second mock community study, rinsing with PFW after single wash with 0.05 % Tween 20 buffer resulted in higher DNA yields (7.3 %) than washing twice with 0.05 % Tween 20 (5.8 %). A second rinsing step with PFW resulted in same range DNA yield (7.4 %), and a third rising step led to a decreased DNA yield (5.4 %) (Fig. 7). We also tested the efficiency of the PowerSoil kit to extract DNA directly from a standardized bacterial community and found that the PowerSoil kit was able to extract 57 % of the mock community's bacterial DNA.

Using the final proposed combined extraction method which included single washing of the EDC cloth with Tween 20 buffer, rising with PFW and DNA extraction using the PowerSoil kit, we could extract DNA from all bacterial taxa from the mock bacterial community (Fig. 8).

4. Discussion

The aim of this study was to optimize dust and DNA extraction from airborne dust collected using EDCs and to establish a method to jointly extract DNA and endotoxin from a single EDC cloth. We optimized DNA yields by rinsing an EDC cloth with PFW after the wash with 0.05 % Tween 20 buffer. We also show that deploying a single EDC cloth is sufficient to accurately determine DNA and endotoxin exposure. This possibility promotes cost and time-efficient analysis in large epidemiological studies.

Previously, DNA has been extracted from settled dust collected by EDC cloths as described by Adams et al. (2015): these were centrifugation-based protocols where the dust from the EDC was collected as pellets and subsequently resuspended in 0.05 % Tween extraction buffer for DNA extraction. This method, however, results in low DNA yields, especially in studies of suburban homes that characterized by low dust exposure compared to farmers homes and stables as showed by Amin et al. (n.d.). In this study,

Fig. 6. A) Quantitative endotoxin measurement. B) quantitative PCR measurements of the 16S rRNA genes. Extracted by 1) Single washing with Tween 20 buffer followed by rinsing with PFW (Tween 20 & PFW), 2) Double washing 0.05 % Tween 20 buffer (Tween 20), 3) Double washing with 100 mM Tris 10 mM EDTA buffer (TrisHCL/EDTA). The significance of the differences depicted in this figure is demonstrated by the Wilcoxon Rank Sum test results.

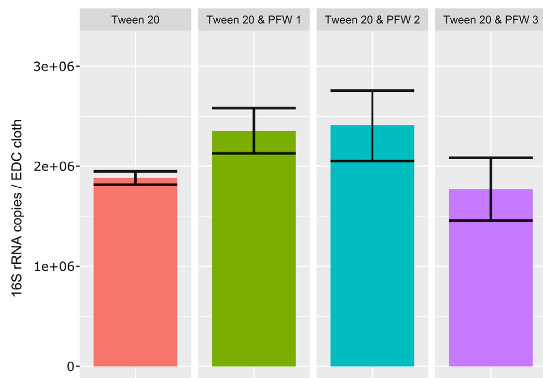

Fig. 7. 16S rRNA genes copy numbers obtained from EDC cloths spiked with 7.71 × 10^6 bacterial cells (32.3 × 10^6 16S rRNA gene copies) and extracted with four ways: Double washing with 0.05 % Tween 20 buffer, single washing with Tween 20 buffer followed by 1–3 rinsing with PFW. Each bar represent average of three replicates.

35 out 100 samples were removed after the filtration quality step duo to low number of reads. Switching to a filtration-based DNA extraction protocol, i.e., by collecting extracted dust pellets on membrane filters, showed a significant increase in DNA yield measured by quantitative PCR using similar DNA extraction methods.

In the DNA extraction study, the PowerSoil kit and DNA extraction method (protocol A) described by Lever et al. (2015) showed the highest DNA yields. Therefore, we performed another round of the DNA extraction using these two methods on replicate EDCs to study reproducibility of the DNA extraction in terms of DNA yield, bacterial richness, and bacterial composition. Protocol A resulted in more consistent DNA yields compared to the PowerSoil kit. However, the DNA yield obtained with the PowerSoil kit was sufficient and above the detection limit. Protocol A showed higher

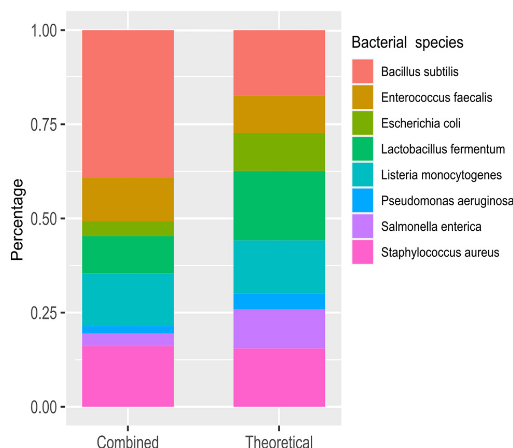

Fig. 8. Comparing the community composition of combined extraction method with the theoretical composition of the ZymoBIOMICS™ Microbial Community Standard. The combined stacked bar plot shows average community-level composition of species retrieved from 6 EDC clothes spiked with 10^6 bacterial cells from the standardized mock community and extracted by PowerSoil kit. For comparison, we show the theoretical composition of the 16S rRNA gene abundance for the standardized mock community.

diversity and richness for all EDC cloths, which indicates the ability of the method to extract more bacterial taxa from EDC cloths compared to the PowerSoil kit. Community composition obtained through both methods was comparable. However, the PowerSoil kit seemed to be better in detecting gram-positive bacteria such as *Firmicutes* and *Actinobacteria* than protocol A, suggesting that PowerSoil is able to break down the harder cell wall of gram-positive bacteria which is likely due to the bead-beating procedure. Depending on the downstream analysis and the scale of the study, we would recommend prioritizing one of the two protocols. Protocol A is better suited for deep metagenomic sequencing and for smaller studies where absolute quantification and reproducibility is of major importance. On the other hand, the PowerSoil kit's straightforward and fast procedure makes it amenable to large-scale epidemiological studies that may involve a large number of samples.

Comparing the combined extraction of DNA and endotoxin with the classical method for extraction of endotoxin (Noss et al., 2008) using replicate EDCs showed largely similar results. This is likely due to same extraction buffer used in the two methods (0.05 % Tween 20). Therefore, we conclude that a single EDC cloth can be used for extraction of both DNA and endotoxin allowing more flexibility in study design for epidemiological studies and reduced time required for sample analysis.

Optimization of the combined extraction method testing different buffers showed that Tween 20 resulted in a higher DNA and endotoxin yield compared to Tris HCL EDTA buffer. This could be due to the ability of surfactant (Tween 20) to separate dust particles from fibrous material such as the EDC cloths. Hoppe Parr et al. (2017) reported approximately two-fold increase in endotoxin units measured by LAL assay when using Tris HCL EDTA buffer compared to Tween 20 buffer (Hoppe Parr et al., 2017). However, they used house and barn dust collected by a high-volume small surface sampler and brushed off horizontal surfaces, respectively, while our study used airborne dust passively settled on the EDC cloths and therefore a low volume of material (Hoppe Parr et al., 2017). This indicates that Tween 20 is better for extracting dust from electrostatic EDC cloths compared to Tris HCl EDTA buffer.

Using a standardized mock community, we discovered that exposing EDC clothes to more bacterial cells result in higher relative DNA yields. However, in general, washing the EDC cloth with 0.05 % Tween 20 buffer twice produced a low DNA yield. Therefore, we further optimized the extraction for higher DNA yields. The dust extraction consists of two rounds washing the EDC cloth with 0.05 % Tween 20 buffer to extract the dust particles out of the EDC cloth. Recently, Shin et al. (2018) revealed the mechanism behind the removal of soil particles from fibrous materials using surfactant gradients produced via rinsing with fresh water that drives diffusiophoresis of soil particles out of fibrous materials (Shin et al., 2018). Using replicate EDC cloths, we showed that rinsing EDC cloth with PFW instead of second wash with Tween 20 buffer increases both DNA and endotoxin yields. This suggests that the washing step with PFW increases diffusiophoresis of dust particles out of the EDC cloth as explained by Shin et al. (2018). Subsequent washing steps did not further increase the yield and excessive washing with PFW actually decreased the DNA yield. Therefore, we chose to rinse the EDC cloth once with PFW after one single Tween 20 buffer wash to save time and decrease risk of contamination.

EDC cloths analysed using our developed protocol were successfully able to detect all bacterial taxa in the standardized mock community indicating the ability of the developed protocol to identify different bacterial taxa on the EDC cloths. However, the PowerSoil method revealed more gram-positive bacteria such as *Bacillus subtilis*. Previous studies have demonstrated that the microbial composition is primarily influenced by the efficacy of cell lysis rather than DNA recovery (Salonen et al., 2010; Scupham et al., 2007). A higher representation of *Bacillus subtilis* could be due to a prolonged bead beating (cell lysis) step, which could result in gram-negative genomic DNA shredding and thereby lower their representativeness. This is also in line with our observation that PowerSoil extraction of indoor dust resulted in a higher proportion of gram-positive Phyla *Firmicutes* and *Actinobacteria*. Thus, further optimization of the PowerSoil

DNA extraction method by decreasing the duration of the bead beating step might lead to a better species abundance representation.

Despite the ability of the developed protocol to extract different bacterial cell types without affecting the endotoxin quantification, one current limitation of the current study is that it does not test for the viability of bacteria collected on the membrane filter. Therefore, if the method is to be extended to studies of pathogen exposure, which rely on understanding the viability of pathogenic microorganisms, techniques such as cultivation or differential staining coupled with flow cytometry or fluorescent microscopy should be included. The main focus of the study was to address our ability to analyse airborne bacterial loads, diversity and community composition simultaneously with endotoxin analysis. Hence, we have not focused on other parts of the microbiota, such as fungi and glucans.

The developed protocol for simultaneous extraction of DNA and endotoxin from a single EDC cloth was planned as part of a large epidemiological study where hundreds of EDCs were collected. The detailed final protocol and materials used for combined DNA and endotoxin extraction from a single EDC cloth can be found in the supplementary material (see also Fig. 1).

5. Conclusion

After a series of pilot studies using different DNA extraction methods and different buffer systems, we finalized a joint protocol for DNA and endotoxin extraction using a single EDC cloth. Combined extraction of DNA and endotoxin would notably reduce the time and cost required for analysis of samples as well as allow other EDC cloths to be used in studying other analytes. Overall, in large epidemiological investigations addressing airborne exposure, that require a large number of samples, our technique supports a high quality to price ratio.

Funding

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement n° 804199).

Credit authorship contribution statement

VS, TS, IM, RB, and TŠ-T designed and carried out sample collection. IM, and TŠ-T planned the DNA extraction study. All authors planned optimization of DNA and endotoxin from a single EDC. HA extracted dust from EDC filters and carried out qPCR, LAL assays and the sequencing. HA, IW, and IM analysed the data from sequencing, qPCR and LAL assay. HA, RB, IM, and TŠ-T wrote the manuscript. All authors revised the manuscript.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Randi J. Bertelsen reports financial support was provided by European Research Council.

Acknowledgments

We would like to thank Susanne Nielsen and Britta Poulsen from the Section for Microbiology, Department of Biology, Aarhus University, for their excellent technical assistance in generating 16S rRNA gene amplicon libraries and quantitative PCR measurements. We are grateful to Kirsten Østergaard at the Department of Public Health, Environment, Work and Health, Danish Ramazzini Center, Aarhus University for performing the endotoxin measurements.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159455.

References

Adams, R.I., et al., 2015. Passive dust collectors for assessing airborne microbial material. Microbiome 3 (1), 1–11.
Adams, R.I., et al., 2021. Microbial exposures in moisture-damaged schools and associations with respiratory symptoms in students: a multi-country environmental exposure study. Indoor Air 31 (6), 1952–1966.
Amin et al., n.d., Amin, H., et al., Cow Farmers' Homes Host More Diverse Airborne Bacterial Communities than Pig Farmers' Homes and Suburban homes. Frontiers in Microbiology: p. 1892.
Andersen, K.S., 2018. ampvis2: An R Package to Analyse and Visualise 16S rRNA Amplicon Data. BioRxiv, p. 299537.
Callahan, B.J., et al., 2016. DADA2: high-resolution sample inference from illumina amplicon data. Nat. Methods 13 (7), 581–583.
Castelino, M., et al., 2017. Optimisation of methods for bacterial skin microbiome investigation: primer selection and comparison of the 454 versus MiSeq platform. BMC Microbiol. 17 (1), 1–12.
Dannemiller, K.C., et al., 2014. Next-generation DNA sequencing reveals that low fungal diversity in house dust is associated with childhood asthma development. Indoor Air 24 (3), 236–247.
Davis, N.M., et al., 2018. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6 (1), 1–14.
de Rooij, M.M., 2021. Occupational and Environmental Exposure to SARS-CoV-2 in and Around Infected Mink Farms. medRxiv.
de Rooij, M.M., 2021. Potential Environmental Transmission Routes of SARS-CoV-2 Inside a Large Meat Processing Plant Experiencing COVID-19 Clusters. medRxiv.
Eduarda, W., Heederik, D., 1998. Methods for quantitative assessment of airborne levels of noninfectious microorganisms in highly contaminated work environments. Am. Ind. Hyg. Assoc. J. 59 (2), 113–127.
Ghosh, B., Lal, H., Srivastava, A., 2015. Review of bioaerosols in indoor environment with special reference to sampling, analysis and control mechanisms. Environ. Int. 85, 254–272.
Hoppe Parr, K.A., et al., 2017. Modification of sample processing for the Limulus amebocyte lysate assay enhances detection of inflammogenic endotoxin in intact bacteria and organic dust. Innate Immun. 23 (3), 307–318.
Hoppe, K.A., et al., 2012. Assessment of airborne exposures and health in flooded homes undergoing renovation. Indoor Air 22 (6), 446–456.
Jacobs, J., et al., 2014. Endotoxin levels in settled airborne dust in european schools: the HITEA school study. Indoor Air 24 (2), 148–157.
Jacobs, J., et al., 2014. Dampness, bacterial and fungal components in dust in primary schools and respiratory health in schoolchildren across Europe. Occup. Environ. Med. 71 (10), 704–712.
Jonker, L., 2022. SARS-CoV-2 Outbreaks in Secondary School Settings in the Netherlands During Fall 2020; Silent Circulation. medRxiv.
Kilburg-Basnyat, B., Metwali, N., Thorne, P.S., 2015. Effect of deployment time on endotoxin and allergen exposure assessment using electrostatic dust collectors. Ann. Occup. Hyg. 59 (1), 104–115.
Kilburg-Basnyat, B., Metwali, N., Thorne, P.S., 2016. Performance of electrostatic dust collectors (EDCs) for endotoxin assessment in homes: effect of mailing, placement, heating, and electrostatic charge. J. Occup. Environ. Hyg. 13 (2), 85–93.
Krop, E.J., et al., 2014. Allergens and β-glucans in dutch homes and schools: characterizing airborne levels. PloS one 9 (2), e88871.
Lai, P.S., et al., 2018. The classroom microbiome and asthma morbidity in children attending 3 inner-city schools. J. Allergy Clin. Immunol. 141 (6), 2311–2313.
Lever, M.A., et al., 2015. A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. Front. Microbiol. 6, 476.
Linde, K.J., 2022. Detection of SARS-CoV-2 in Air and on Surfaces in Rooms of Infected Nursing Home Residents. medRxiv.
Luiken, R.E., et al., 2020. Farm dust resistomes and bacterial microbiomes in european poultry and pig farms. Environ. Int. 143, 105971.
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. 17 (1), 10–12.
McMurdie, P.J., Holmes, S., 2013. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one 8 (4), e61217.
Morgan, M., et al., 2009. ShortRead: a bioconductor package for input, quality assessment and exploration of high-throughput sequence data. Bioinformatics 25 (19), 2607–2608.
Normand, A.-C., et al., 2009. Assessment of dust sampling methods for the study of cultivable-microorganism exposure in stables. Appl. Environ. Microbiol. 75 (24), 7617–7623.
Noss, I., et al., 2008. Evaluation of a low-cost electrostatic dust fall collector for indoor air endotoxin exposure assessment. Appl. Environ. Microbiol. 74 (18), 5621–5627.
Noss, I., et al., 2010. Passive airborne dust sampling with the electrostatic dustfall collector: optimization of storage and extraction procedures for endotoxin and glucan measurement. Ann. Occup. Hyg. 54 (6), 651–658.
Ohkuma, M., Kudo, T., 1998. Phylogenetic analysis of the symbiotic intestinal microflora of the termite Cryptotermes domesticus. FEMS Microbiol. Lett. 164 (2), 389–395.
Paradis, E., Claude, J., Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20 (2), 289–290.
Quast, C., et al., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41 (D1), D590–D596.

Salonen, A., et al., 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: effective recovery of bacterial and archaeal DNA using mechanical cell lysis. J. Microbiol. Methods 81 (2), 127–134.
Salter, S.J., et al., 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol. 12 (1), 1–12.
Samadi, S., et al., 2010. Allergen and endotoxin exposure in a companion animal hospital. Occup. Environ. Med. 67 (7), 486–492.
Sander, I., et al., 2018. Indoor allergen levels in settled airborne dust are higher in day-care centers than at home. Allergy 73 (6), 1263–1275.
Schlünssen, V., et al., 2015. Exposure levels, determinants and IgE mediated sensitization to bovine allergens among danish farmers and non-farmers. Int. J. Hyg. Environ. Health 218 (2), 265–272.
Scupham, A., Jones, J., Wesley, I., 2007. Comparison of DNA extraction methods for analysis of Turkey cecal microbiota. J. Appl. Microbiol. 102 (2), 401–409.
Shin, S., Warren, P.B., Stone, H.A., 2018. Cleaning by surfactant gradients: particulate removal from porous materials and the significance of rinsing in laundry detergency. Phys. Rev. Appl. 9 (3), 034012.
Spaan, S., et al., 2008. Effect of extraction and assay media on analysis of airborne endotoxin. Appl. Environ. Microbiol. 74 (12), 3804–3811.
Spilak, M.P., et al., 2015. Impact of dwelling characteristics on concentrations of bacteria, fungi, endotoxin and total inflammatory potential in settled dust. Build. Environ. 93, 64–71.
Van Cleef, B., et al., 2014. Dynamics of methicillin-resistant Staphylococcus aureus and methicillin-susceptible Staphylococcus aureus carriage in pig farmers: a prospective cohort study. Clin. Microbiol. Infect. 20 (10), O764–O771.
van Cleef, B.A., et al., 2015. Livestock-associated MRSA in household members of pig farmers: transmission and dynamics of carriage, a prospective cohort study. PloS one 10 (5), e0127190.
Van Cleef, B.L., et al., 2016. Health and health-related quality of life in pig farmers carrying livestock-associated methicillin-resistant Staphylococcus aureus. Epidemiol. Infect. 144 (8), 1774–1783.
Vestergaard, D.V., et al., 2018. Pig farmers' homes harbor more diverse airborne bacterial communities than pig stables or suburban homes. Front. Microbiol. 9, 870.
Větrovský, T., Baldrian, P., 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. PloS one 8 (2), e57923.
Viegas, C., et al., 2018. Organic dust exposure in veterinary clinics: a case study of a small-animal practice in Portugal. Arh. Hig. Rada Toksikol. 69 (4), 309–315.
Viegas, C., et al., 2019. Characterization of occupational exposure to fungal burden in portuguese bakeries. Microorganisms 7 (8), 234.
Viegas, C., et al., 2019. Electrostatic dust collector: a passive screening method to assess occupational exposure to organic dust in primary health care centers. Air Qual. Atmos. Health 12 (5), 573–583.
Viegas, C., et al., 2020. Assessment of children's potential exposure to bioburden in indoor environments. Atmosphere 11 (9), 993.
Viegas, C., et al., 2020. Settleable dust and bioburden in portuguese dwellings. Microorganisms 8 (11), 1799.
Xiao, K.-Q., 2017. Concurrent methane production and oxidation in surface sediment from Aarhus Bay, Denmark. Front. Microbiol. 8, 1198.

III

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Article

Indoor Airborne Microbiome and Endotoxin: Meteorological Events and Occupant Characteristics Are Important Determinants

Published as part of the Environmental Science & Technology virtual special issue "The Exposome and Human Health".

Hesham Amin,* Tina Šantl-Temkiv, Christine Cramer, Kai Finster, Francisco Gomez Real, Thorarinn Gislason, Mathias Holm, Christer Janson, Nils Oskar Jõgi, Rain Jogi, Andrei Malinovschi, Ian P. G. Marshall, Lars Modig, Dan Norbäck, Rajesh Shigdel, Torben Sigsgaard, Cecilie Svanes, Hulda Thorarinsdottir, Inge M. Wouters, Vivi Schlünssen, and Randi J. Bertelsen[##]

Cite This: https://doi.org/10.1021/acs.est.3c01616

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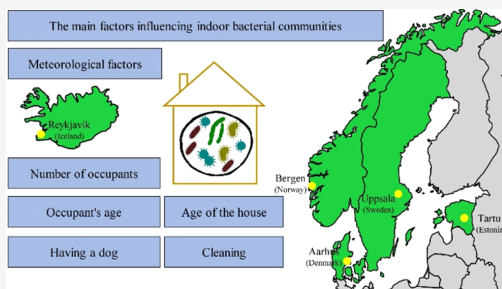
ABSTRACT: Airborne bacteria and endotoxin may affect asthma and allergies. However, there is limited understanding of the environmental determinants that influence them. This study investigated the airborne microbiomes in the homes of 1038 participants from five cities in Northern Europe: Aarhus, Bergen, Reykjavik, Tartu, and Uppsala. Airborne dust particles were sampled with electrostatic dust fall collectors (EDCs) from the participants' bedrooms. The dust washed from the EDCs' clothes was used to extract DNA and endotoxin. The DNA extracts were used for quantitative polymerase chain (qPCR) measurement and 16S rRNA gene sequencing, while endotoxin was measured using the kinetic chromogenic limulus amoebocyte lysate (LAL) assay. The results showed that households in Tartu and Aarhus had a higher bacterial load and diversity than those in Bergen and Reykjavik, possibly due to elevated concentrations of outdoor bacterial taxa associated with low precipitation and high wind speeds. Bergen-Tartu had the highest difference (ANOSIM $R = 0.203$) in β diversity. Multivariate regression models showed that α diversity indices and bacterial and endotoxin loads were positively associated with the occupants' age, number of occupants, cleaning frequency, presence of dogs, and age of the house. Further studies are needed to understand how meteorological factors influence the indoor bacterial community in light of climate change.



KEYWORDS: *Northern Europe, airborne microbiome, meteorological data, 16S rRNA and occupants' age*

1. INTRODUCTION

Today most humans have largely removed themselves from the outdoor environments in which they evolved, and spend >90% of their time indoors, i.e., in houses, offices, and schools.[1] Exposure to bacterial communities inside the indoor environment can impact human health.[2] Early life exposure to increased microbial load and diversity has been shown to be protective against allergic outcomes such as allergic asthma.[3] Researchers have used endotoxin concentrations as a proxy measure of bacteria exposure to understand the link between bacterial exposure and health outcomes in farming and nonfarming populations.[4,5] Using next-generation sequencing techniques, it has been shown that specific bacterial taxa are associated with asthma and atopy in both children and adults.[6−8] Studies of differences in house dust microbiome composition between farm and nonfarm homes of Finnish and German birth cohorts showed that the protective microbiome against asthma and atopy had a low abundance of *Streptococcaceae* relative to outdoor-associated bacterial taxa such as *Sphingobacteria* and endotoxin-producing bacteria belonging to the *Alphaproteobacteria* class.[9]

The indoor air environment is populated by different bacterial communities that originated from different sources, including human and animal occupants as well as outdoor air.[10−12] Despite the proposed importance of the indoor microbiome on health, the relative contributions of these sources, as well as factors influencing the composition of the

Received: March 2, 2023
Revised: July 13, 2023
Accepted: July 13, 2023

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Table 1. Characteristics of the Study Population

	Aarhus (N = 160)	Bergen (N = 300)	Reykjavik (N = 346)	Tartu (N = 84)	Uppsala (N = 148)	Total (N = 1038)[a]
season of sampling						
summer	21	123	150	70	63	427 (41.5%)
winter	139	175	194	14	78	600 (58.5%)
no. of occupants in the home						
one	23	34	33	15	17	122 (12.6%)
two or more	137	216	312	69	106	840 (87.4%)
occupant's age mean (SD)	53 (±6.5)	53 (±6.8)	55 (±7.1)	52 (±7.1)	56 (±7.2)	54 (±7.0)
dog in bedroom	31	25	47	6	10	119 (12.4%)
cat in bedroom	15	23	36	19	18	111 (11.4%)
kitchen fan use						
never	8	7	89	36	4	144 (15.2%)
sometimes	37	66	164	20	36	323 (34.1%)
all of the time	109	174	89	28	80	480 (50.7%)
window open at night						
never	94	97	56	63	83	393 (42.3%)
sometimes	12	42	102	12	21	189 (20.4%)
all of the time	53	107	187	9	18	374 (40.2%)
cleaning frequency						
less than 1 time per week	60	62	103	13	27	265 (27.7%)
1−3 times per week	83	163	188	63	86	583 (60.8%)
4−7 times per week	17	21	54	8	10	110 (11.5%)
use of bleach	26	106	86	1	22	241 (33.4%)
use of ammonia	23	85	6	1	9	124 (14.7%)
house age (years, mean (SD))	50 (±36)	41 (±34)	34 (±22)	41 (±26)	49 (±28)	41 (±30)
mattress age (years, mean (SD))	7.1 (±5.5)	7.7 (±5.8)	8.0 (±5.5)	7.7 (±8.8)	6.4 (±5.1)	7.5 (±5.9)
central heating	143	11	338	51	102	645 (67.1%)
ducted heating	5	23	1	1	24	55 (5.7%)
electric heating	11	238	0	40	31	320 (33.3%)
open coal heating	11	78	0	7	16	112 (11.6%)
radiator in bedroom	138	5	326	53	117	639 (66.5%)
air condition	0	41	5	14	10	70 (7.3%)
airbrick bedroom	34	5	0	1	78	118 (11.3%)
damp spots in bedroom	8	7	11	0	1	27 (2.9%)
condensation on window	76	48	49	30	22	225 (23.5%)
mold odor	18	10	23	11	4	66 (6.9%)
mold	43	35	34	23	16	151 (15.8%)
water damage	45	65	98	43	39	290 (31%)
no. of rooms						
one	1	2	0	8	1	12 (1.3%)
two	8	12	10	18	6	54 (5.6%)
three or more	151	235	334	58	115	894 (93.1%)
floor level						
ground floor	1	20	9	0	2	32 (3.3%)
first floor	86	94	147	26	47	400 (41.6%)
higher than first floor	73	136	189	58	73	529 (55.1%)
rug in bedroom	33	52	49	53	69	256 (26.7%)
fitted carpet in bedroom	40	13	3	9	3	68 (7.1%)
bedroom size (m^2, mean (SD))[b]	15 (±7.1)	13 (±3.9)	15 (±5.8)		15 (±4.9)	14 (±5.4)
floor heating[b]	22	34	18		1	75 (9.4%)
bedroom wallpaper[b]	4	88	10		102	204 (27.4%)
painted fiberglass[b]	15	72	4		9	120 (16.4%)
wall vent[b]	34	156	233		31	454 (52%)
ceiling exhaust[b]	2	6	14		17	39 (4.8%)
house type[b]						
apartment building	27	82	123		44	276 (30.8%)
detached house	76	138	116		65	395 (44.1%)
farmhouse	8	5	5		4	22 (2.4%)
terraced house	38	63	83		19	203 (22.7%)
precipitation rate (mm/day, mean (SD))	1.8 (±0.94)	8.3 (±3.7)	4.4 (±2.3)	1.9 (±0.70)	2.2 (±1.1)	4.7 (±3.6)
temperature (C°, mean (SD))	6.6 (±4.8)	2.5 (±5.2)	4.1 (±3.6)	6.0 (±7.1)	3.7 (±7.9)	4.1 (±5.5)

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Table 1. continued

	Aarhus (N = 160)	Bergen (N = 300)	Reykjavik (N = 346)	Tartu (N = 84)	Uppsala (N = 148)	Total (N = 1038)[a]
relative humidity (%, mean(SD))	91 (±3.6)	90 (±4.9)	86 (±5.8)	89 (±6.8)	90 (±8.1)	89 (±6.1)
wind speed (m/s, mean (SD))	6.2 (±0.75)	3.1 (±0.53)	5.3 (±1.5)	5.2 (±0.84)	2.2 (±0.21)	4.3 (±1.7)

[a]Information was missing for season (n = 11, 1%), All of the other characteristics were missing for around 80 participants (7−9%). [b]Variables extracted from the EDC questionnaire.

indoor airborne bacterial communities, are largely unknown. Understanding the determinants of the indoor bacterial community is pivotal to be able to influence the indoor microbiome and ultimately prevent negative health effects. A better understanding of the relationship between exposure to specific microorganisms and asthma and allergies is urgently needed, particularly in regions such as northern Europe, where the prevalence of allergic diseases and asthma has increased dramatically in recent decades.[13]

Hitherto, studies of the indoor microbial community and environmental determinants associated with the indoor environment have been limited to single geographical sites and small sample sizes (∼100).[14−18] Many environmental determinants such as building materials, occupant behaviors, and climate factors such as the precipitation rate and relative humidity affecting outdoor bacterial taxa are rather uniform within single geographical sites.[19] To identify the factors that affect indoor bacterial community variation, studies on a regional scale with complementary and comprehensive environmental data are required. Therefore, we studied the indoor bacterial air community in more than 1038 homes in ECRHS III. The goals of our study, in which we focused on the bacterial community and endotoxin, were (1) to make an inventory of the indoor airborne bacterial community composition, including α and β diversity, in five medium-sized northern European cities, and (2) to identify environmental factors associated with the composition of the bacterial communities and endotoxin concentration indoor.

2. MATERIALS AND METHODS

2.1. Study Populations. The present study initially comprised 1080 homes of the participants of the ECRHS III from Aarhus (Denmark), Bergen (Norway), Reykjavik (Iceland), Tartu (Estonia), and Uppsala (Sweden). The ECRHS (European Community Respiratory Health Survey) is an international multicentre population-based study aiming to determine the prevalence of and risk factors for the development of asthma and allergic diseases in adults living in Europe and Australia.[20] The participants were between 22 and 44 years at baseline around 1990. From 2011 to 2014, all of the participants invited for ECRHS III clinical examinations and interview questionnaires were asked to collect settled dust using an electrostatic dust fall collector (EDC) (Supporting Figure 1). Except for the Tartu study center, all participants filled in a short questionnaire related to the EDC (EDC questionnaires). The samples where participants reported that the EDC fell on the floor (23 samples) as well as samples that did not reach defined quality standards (better number of reads) in 16S rRNA amplicon sequencing (19 samples) were removed from the analysis. As a result, the total number of persons and samples included in the analysis was 1038. Information about environmental determinants was extracted from ECRHS III interviews and the EDC questionnaires. Local ethics committees at each center approved the study protocols. For detailed information about questionnaires, we refer to the official ECRHS website: http://www.ecrhs.org/. The study centers, number of participants from each center, and other environmental determinants of the study object are listed in Table 1.

2.2. Dust Sampling. Between March 2011 and January 2014, settled airborne dust was collected in participants' bedrooms over a 14-day period using EDCs (Supporting Figure 1) with an exposure area of 209 cm^2. The EDCs were placed 1.5 m above the floor.[21] The participants were instructed to return the EDCs by mail, along with the EDC questionnaires. All EDC samples were stored at −20 °C until dust extraction.

2.3. Dust, Endotoxin, and DNA Extraction. In 2022, the EDC clothes were handled as described previously, where dust, endotoxin, and DNA extraction from EDC clothes were optimized to obtain a comprehensive representation of the airborne bacterial communities.[22] For a detailed description of the dust, endotoxin, and DNA extraction, see the methods section in the Supporting Information.

2.4. 16S rRNA Amplicon Sequencing. 16S rRNA genes from the samples (including 35 control samples and 20 PCR controls) were amplified using the bacteria-specific primers targeting the V3 and V4 regions of the 16S rRNA gene. The Illumina protocol (16S Metagenomic Sequencing Library Preparation) was used for amplification of the 16S rRNA gene. The detailed description of the primers and the protocol for 16S rRNA gene sequencing is described in the methods section in the Supporting Information.

2.5. Quantitative PCR. The qPCR reactions targeting 16S rRNA genes were carried out using an MX3005p qPCR machine (Agilent, Santa Clara, CA). For a detailed description of the primers, the qPCR reaction components, and thermal cycling conditions, see the methods section in the Supporting Information.

2.6. LAL Assay. Each extract was diluted 50 times in PFW before analysis with the quantitative kinetic chromogenic LAL assay to overcome the masking effect of Tween 20 on the assay[22] (Kinetic-QCL 50−650 U kit, Lonza, Walkersville, Maryland). Endotoxin from *Escherichia coli* O55:B5 was used as a standard. To create a standard curve, 13 serial dilutions were employed, covering a range of values between 25 and 0.006 EU/mL. The cut-off signals (V_{max}) of the kinetic LAL Assay were defined as the average of the assay blanks plus two times the standard deviation of these blanks. The results were presented in EU m-2 units.

2.7. Bioinformatic and Statistical Analysis. All sequence data processing and statistical analyses were carried out in R version 4.2.1.[23] The raw data processing is described in detail in the Supporting Information.

Microbiome version 1.15.0[24] was used to assess α bacterial diversity (Shannon index, which reflects both richness and the relative abundance of each taxon), and bacterial richness (observed number of ASVs). The relative abundances of Gram-positive and Gram-negative bacteria were assigned,

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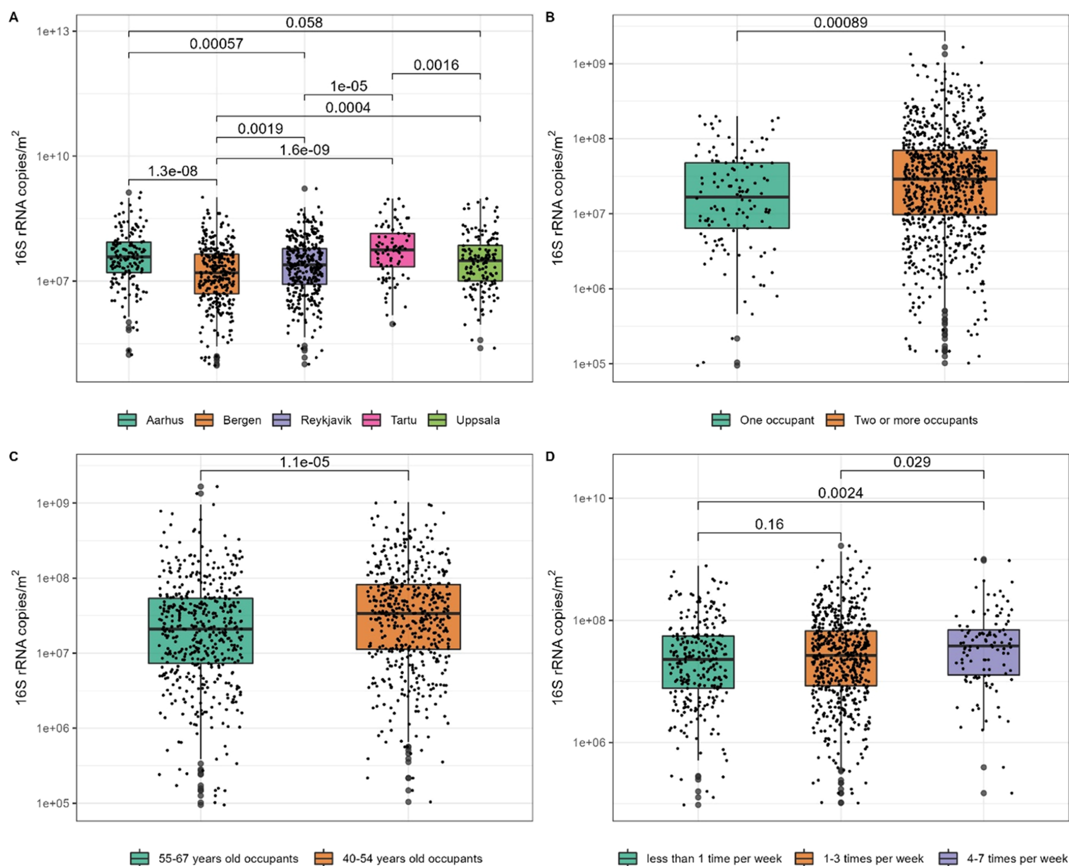

Figure 1. Boxplot of qPCR results of (A) cities' households, (B) number of occupants per household (one vs two or more), (C) older occupant age group (55−67 years old) compared vs younger age group (40−54 years old), (D) cleaning frequency. *P* values based on pairwise sample comparison in Wilcoxon signed-rank test. Only the significant pairwise comparison is shown.

based on relative abundances of phyla across samples from the five cities (Supporting Table 1).To identify specific bacterial taxa (genus level) whose abundances significantly differ between different environmental determinants (e.g., city and season), we applied analysis of compositions of microbiomes with bias correction (ANCOM BC) version 1.6.2.[25] We removed genera that accounted for less than 0.01% relative abundance and adjusted for the variables that showed association with Analysis of similarity (ANOSIM) test. The remaining taxa are affiliated to 201 bacterial genera. The ANOSIM test from the vegan package version 2.5-7[26] that is based on the Aitchison dissimilarity matrix was used to compare bacterial community structures between different environmental determinants. Continuous variables such as age of the house and the occupants' age were dichotomized based on the median.

The sampling period was split into two seasons, based on the monthly average temperature in the five cities obtained from the weather-base website (https://www.weatherbase.com/). The coldest months were assigned to winter (November, December, January, February, March, April), and the warmest months were assigned to summer (May, June, July, August, September, October).

To study the association between normally distributed dependent variables, i.e., bacterial diversity (Shannon index, Supporting Figure 2A) and bacterial richness (Number of bacterial taxa, Supporting Figure 2B), and independent variables, i.e., environmental determinants, we used multiple linear regression (stats package version 4.0.4[23]) based on two approaches to ensure robust regression analysis. In the first approach, we performed univariate analysis for all independent variables, and in the next step, we ran a multivariate model including the variables that showed associations ($P \leq 0.25$ as arbitrary value) with the dependent variables.

In the second approach, we included the environmental determinants in three consecutive models. For each model, variables that showed association ($P \leq 0.25$) with the dependent variables were kept in the model. In the first model, we included key determinants (city and season), while in the second model, we further included occupant and occupant-related behavior determinants (the presence of dog and cat, the number of occupants, the occupant's age, and

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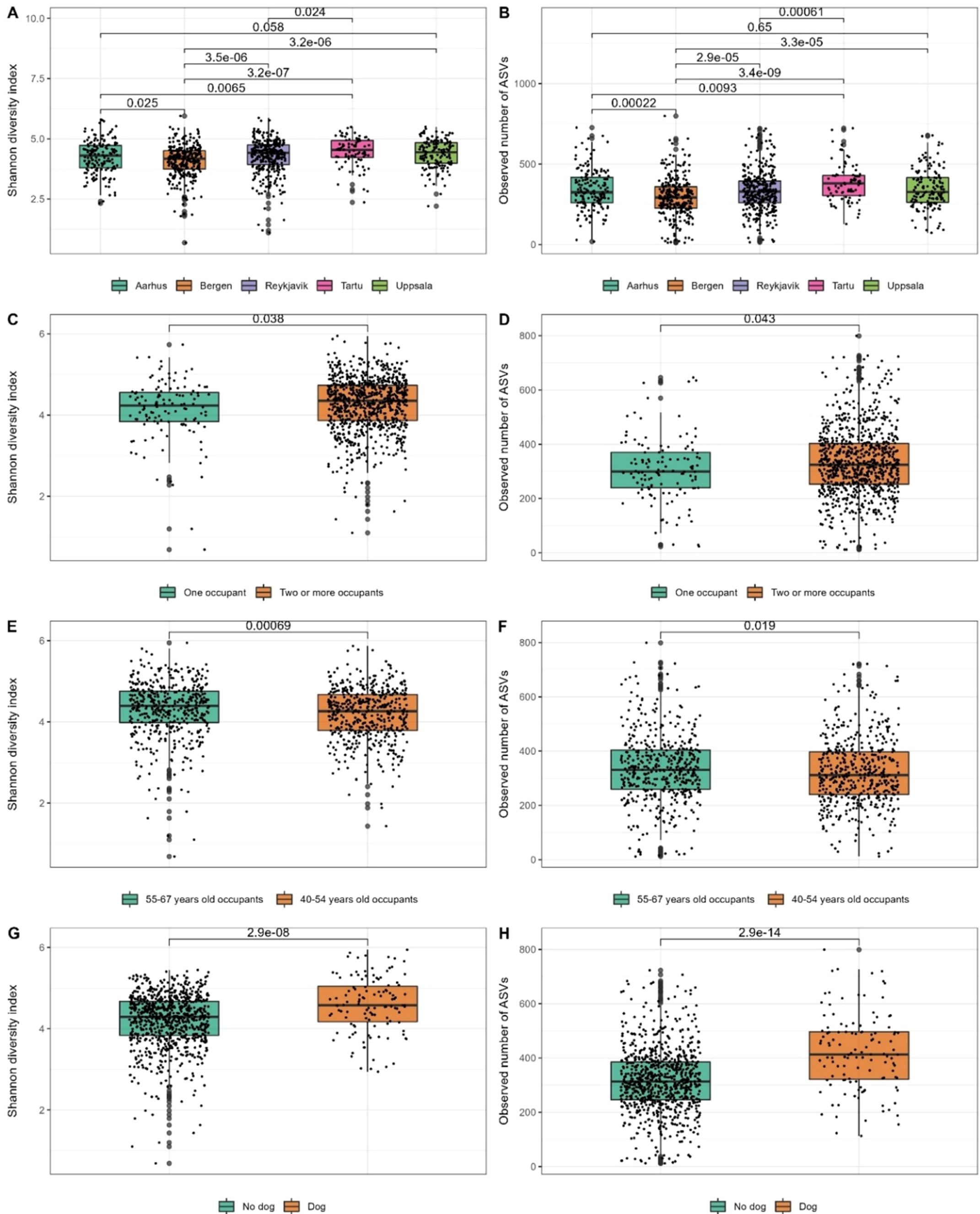

Figure 2. Box plots of α bacterial diversities indices. (A) Shannon index and (B) observed number of ASVs for the five cities' households. (C) Shannon index and (D) observed number of ASVs for the number of occupants (one vs two or more). (E) Shannon index and (F) observed number of ASVs for older occupant age group (55−67 years old) vs younger age group (40−54 years old). (G) Shannon index and (H) observed number of ASVs for dog in bedroom (no vs yes). *P* values are derived from pairwise sample comparison in Wilcoxon signed-rank test (only reported for statistically significant pairwise comparison).

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cleaning frequency). The third model considers indoor factors such as house age, type of heating system, presence of mold, condensation on the window, and ventilation. The reason behind the sequence of the models mentioned is that we expected that key determinants would be the ones with the strongest effect on indoor bacterial profiles, followed by occupant and indoor determinants based on the literature.[1,27]

To study the association between non-normal distributed dependent variables (bacterial load (16S rRNA gene copies/m^2, Supporting Figure 2C) and endotoxin load (EU/m^2, Supporting Figure 2D)) and environmental determinants, we used quantile regression from package "quantreg" version 5.86[28] and followed the same two approaches as for the multivariate linear regression models mentioned earlier.

2.8. Meteorological Data. The monthly average meteorological data for the precipitation rate (mm/day), temperature (C°), relative humidity (%), and wind speed (m/s) for each sample were extracted from the NASA Langley Research Center POWER Project (https://power.larc.nasa.gov/) based on the city of sample collection and the EDC opening date reported by the study participants between 2011 and 2014. The precipitation rate (mm/day) represents the total depth of rainwater (mm) for 24 h.

3. RESULTS

3.1. Quality Filtering and Study Characteristics. After quality filtering and downsampling to 20,000 reads per sample, a total of 1038 EDCs from Aarhus (n = 160), Bergen (n = 300), Reykjavik (n = 346), Tartu (n = 84), and Uppsala (n = 148) were included in the analysis. When performing analyses utilizing the EDC questionnaire, the Tartu samples were excluded since this questionnaire was not filled out by the Tartu participants. Thus, yielding a subgroup of 954. The characteristics of the study population based on the ECRHS III interview, the EDC questionnaire, and meteorological data during sampling the indoor dust are presented in Table 1.

3.2. Bacterial Load. In the first approach based on the univariate regression (Supporting Table 2), the multivariate quantile regression model showed the following determinants to be significant: cities, number of occupants, and occupant age (Supporting Table 3). Similar results were shown when the determinants were introduced in three consecutive models. Additionally, we found that cleaning more than 4 times per week was associated with a higher bacterial load compared to cleaning less than 1 time/week (Supporting Table 4).

Bergen households showed significantly lower bacterial load compared to those from other Nordic cities. There was no significant difference in bacterial load between Tartu and Aarhus and between Reykjavik and Uppsala households (Figure 1A). Reporting more than 1 person in the house was significantly associated with higher bacterial load (P = 0.01) (Figure 1B). The occupants within the youngest age group (40−54 years old) showed a significantly higher bacterial load compared to the older age group (55−67 years old) (Figure 1C). A high cleaning frequency was associated with a significantly higher bacterial load (Figure 1D).

3.3. Bacterial Diversity and Richness. With the first approach to study the association between indoor determinants and bacterial diversity (Shannon index) and richness (observed number of ASVs), based on the univariate regression (Supporting Table 5), the multivariate regression revealed that study site (the cities), keeping a dog in the bedroom, number of occupants, occupants' age, and age of the house to be significantly associated with both indices (Supporting Table 6). Season, condensation of water on window, and cleaning frequency (less than one time per week vs 4−7 times per week) showed significant association with the Shannon index only (Supporting Table 6).

Similar results were shown when the determinants were introduced in three consecutive models. We further found that the presence of mold was associated with increased bacterial diversity and that a rug in the bedroom increased the number of bacterial taxa (Supporting Table 7).

In a complete case analysis with data from both ECRHS III main interviews and EDC questionnaires, we found that bedroom size was significantly associated with increasing bacterial richness and diversity, while wall vent was associated with a decrease in bacterial diversity (Supporting Table 8).

In terms of Shannon index and number of bacterial ASVs, Bergen households had the lowest bacterial diversity while Tartu households had the highest bacterial diversity and bacterial richness (Figure 2A,B). The number of occupants in the house (Figure 2C,D) was significantly associated with both bacterial diversity and bacterial richness. Older age of the occupants (Figure 2E,F) and the presence of a dog in the bedroom (Figure 2G,H) were both associated with increased bacterial richness and diversity.

3.4. Dissimilarity of Bacterial Communities (β Diversity). Aitchison's dissimilarity matrix, as well as the ANOSIM test for categorical variables and Mantel tests for continuous variables were used to investigate differences in the composition of the airborne bacterial community composition as a function of environmental determinants. We found a statistically significant difference in β diversity between all five cities' households using pairwise comparisons (Supporting Table 9). The pairwise comparison between cities showed the highest difference in β diversity between Bergen and Tartu households (ANOSIM R = 0.304, P = 0.001) followed by Reykjavik vs Tartu households (ANOSIM R = 0.203, P = 0.001) while the lowest difference in β diversity was found between Bergen and Reykjavik households (ANOSIM R = 0.042, P = 0.001). The difference in the β diversity between all cities' households was significant (R = 0.1803, P value = 0.001).

The presence of a dog in the bedroom was associated with a significant difference in β diversity (ANOSIM R = 0.296, P = 0.001), whereas the presence of a cat in the bedroom was not (ANOSIM R = 0.0507, P = 0.09). Determinants which also showed significant association with β diversity of the indoor microbiomes were cleaning frequency, having the window open during night, wall vent, having a rug in the bedroom, and the number of rooms in the house (Supporting Table 9). Mantel test for continuous variables revealed that the occupants' age (Mantel R = 0.04, P = 0.002) and the age of the house (Mantel R = 0.04, P = 0.01) showed significant association with β diversity of indoor microbiome (Supporting Table 10).

3.5. Bacterial Community Composition and Differential Abundance Analysis. The indoor airborne bacterial communities in the five cities' households were dominated by five phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Myxococcota*, and *Bacteroidetes*, which made up about 97% of the total communities (Supporting Figure 3A). We found higher relative abundance for *Actinobacteria* in Bergen and Reykjavik households, whereas the relative abundance of *Proteobacteria* was higher in Aarhus and Tartu households. Family-level

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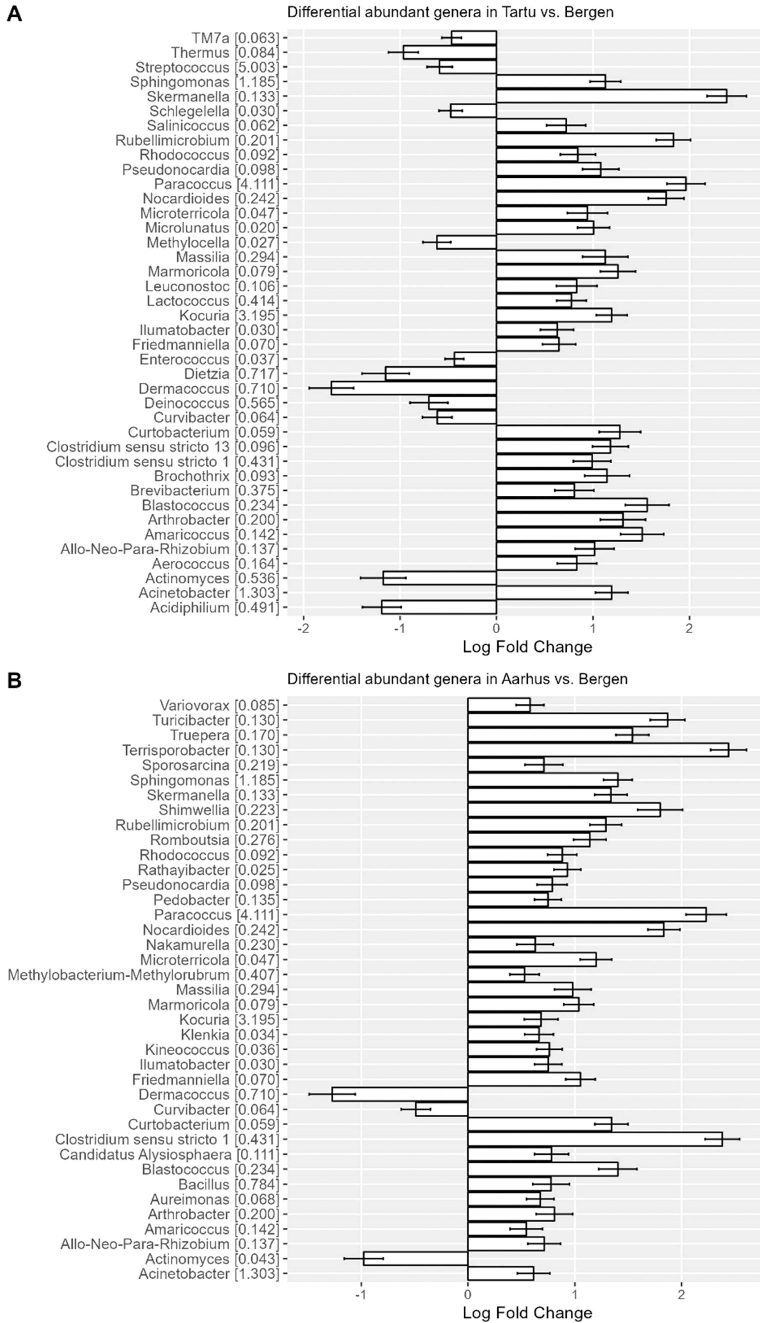

Figure 3. Differential abundant bacterial genera (A) in Tartu compared to Bergen households and (B) in Aarhus compared to Bergen households. Number in parentheses shows the relative abundance of the bacterial genera in the total number of samples. Positive log fold changes indicate an increase, and negative log fold changes indicate a decrease in the abundance of bacterial taxa compared to the reference group.

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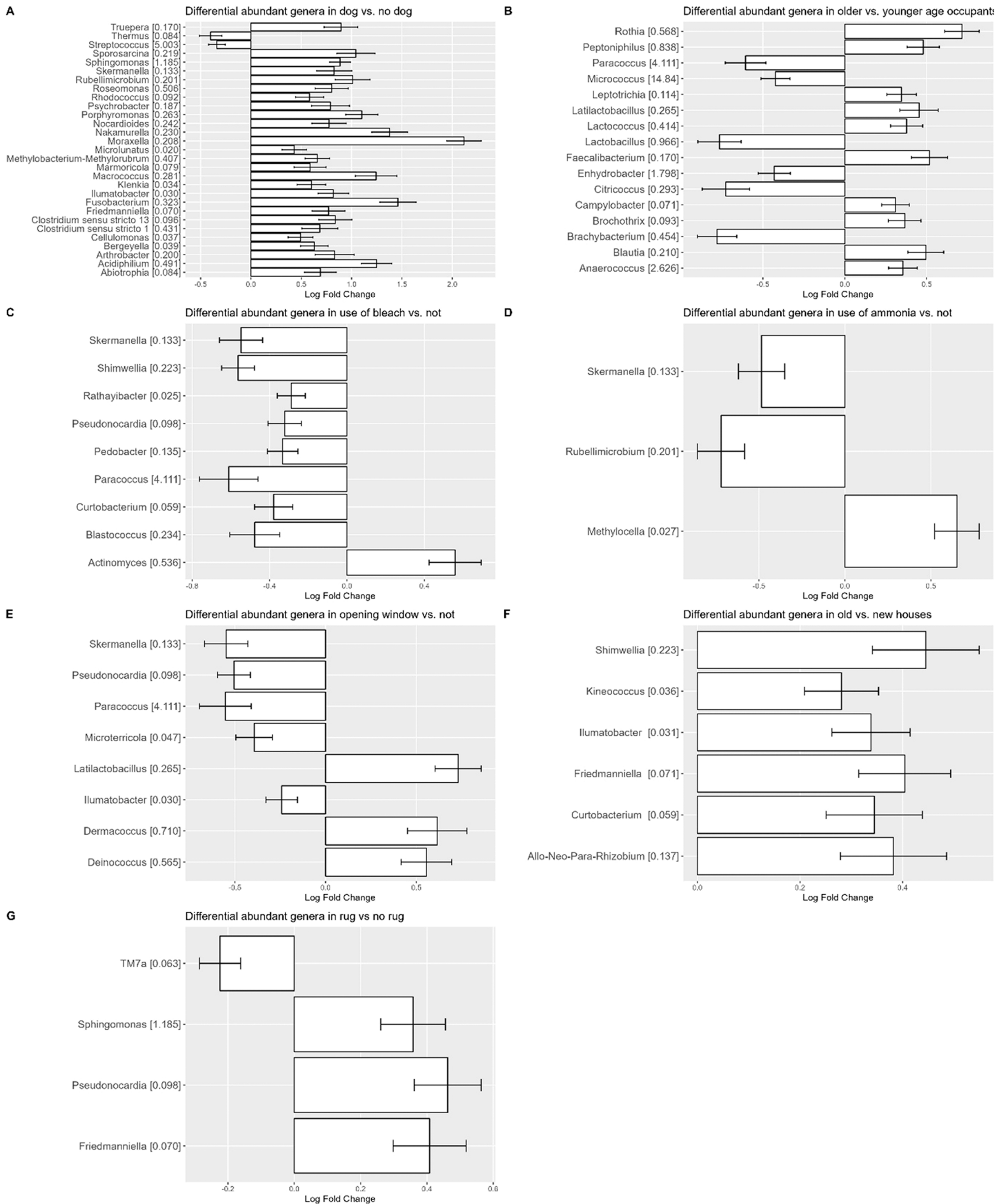

Figure 4. Differential abundant bacterial genera: (A) presence of dog in bedroom compared to absence, (B) older occupant age group compared to younger age group, (C) using bleach compared to not using bleach, (D) using ammonia compared to not using ammonia, (E) opening window compared to not opening the window, (F) old compared to new houses (G) presence of rug in bedroom compared to absence. Positive log fold changes indicate an increase, and negative log fold changes indicate a decrease in the abundance of bacterial taxa compared to the reference group.

composition showed that Gram-negative bacterial families such as *Rhodobacteraceae* and *Sphingomonadaceae* are more abundant in Aarhus and Tartu households (Supporting Figure 3B) than in Bergen, Reykjavik, and Uppsala households. The three

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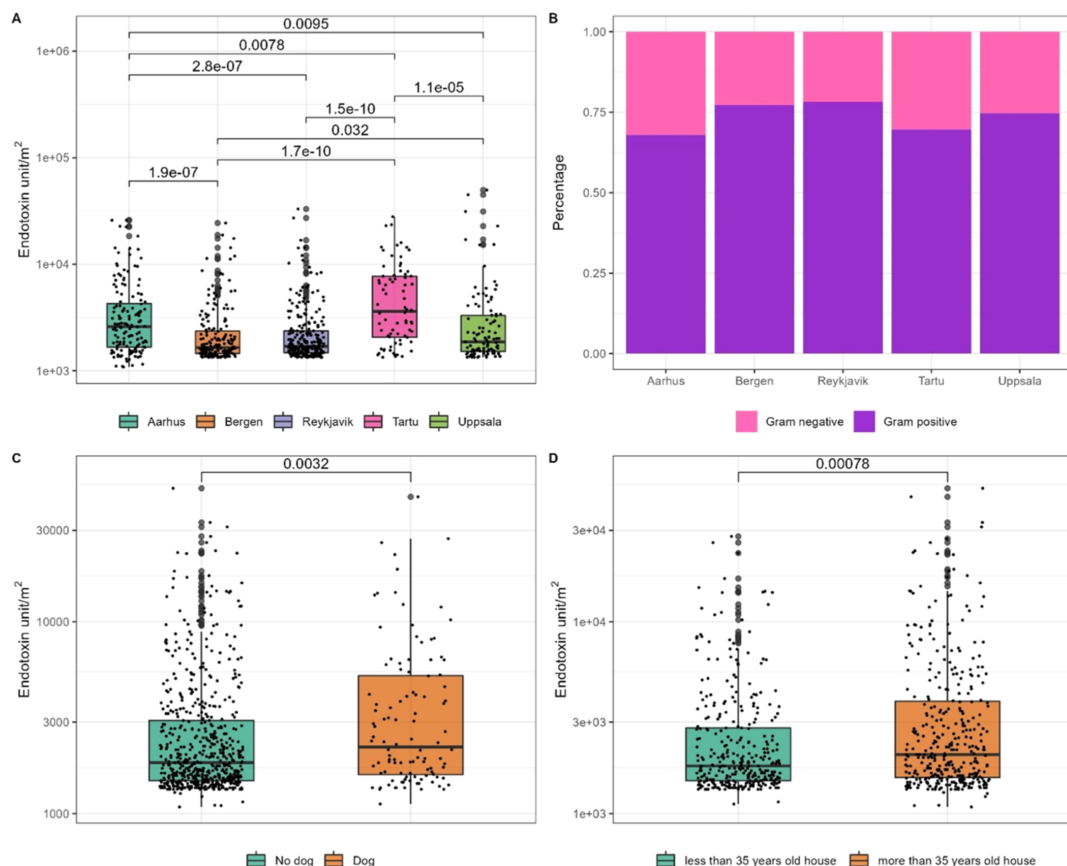

Figure 5. (A) Boxplot of endotoxin result of five cities' households; (B) relative abundance of Gram-negative and Gram-positive bacteria of the five cities; (C) boxplot of endotoxin measurements of dog in bedroom (no vs yes); (D) boxplot of endotoxin result for older houses compared to more recently built houses. *P* values based on pairwise sample comparison in Wilcoxon signed-rank test. Only the significant pairwise comparison is shown.

most abundant bacterial families in the five cities were Gram-positive: *Micrococcaceae*, *Staphylococcaceae*, and *Corynebacteriaceae* (Supporting Figure 3B). On the genus level, the three most abundant genera were *Micrococcus*, *Staphylococcus*, and *Corynebacterium*, which belong to the three most abundant bacterial families (Supporting Figure 3C).

For the determination of the differential abundance of bacterial genera, we focused on the environmental determinants that showed significant association with β diversity (ANOSIM and Mantel tests) as well as other determinants such as water damage, season of sampling, and number of occupants, based on the literature.[1,27,29]

We found 40 out of 201 bacterial genera to be differentially abundant between Bergen and Tartu households (Figure 3A). Many of the bacterial genera which were differentially abundant between Bergen and Tartu households were also differentially abundant between Bergen and Aarhus households (Figure 3B). In general, members of the phylum *Proteobacteria*, such as *Acinetobacter, Skermanella, Paracoccus*, and *Sphingomonas* genera, were significantly higher in abundance in Aarhus and Tartu households compared to other cities' households. Other pairwise differential abundance analysis between the five cities' households can be found in Supporting Figures 4−7.

The determinants related to the occupants which showed association with genera that expressed differential abundances were dog in bedroom and occupants' age. The presence of a dog in the bedroom was associated with a higher abundance of 25 bacterial genera (Figure 4A). There was no difference in the abundance of genera when a cat was present in the bedroom. Ten bacterial genera were more abundant within the older age group (55−67 years old) and 6 genera were less abundant compared to the younger age group (40−54 years old) (Figure 4B). Occupant behavior, such as cleaning frequency, did not affect the composition of the bacterial communities. However, the use of cleaning agents such as bleach and ammonia was associated with the abundance of nine and three bacterial genera, respectively (Figure 4C,D). Opening the window at night was associated with the abundance of several bacterial genera. Having the window open all the time compared to never was associated with differences in the abundance of 8

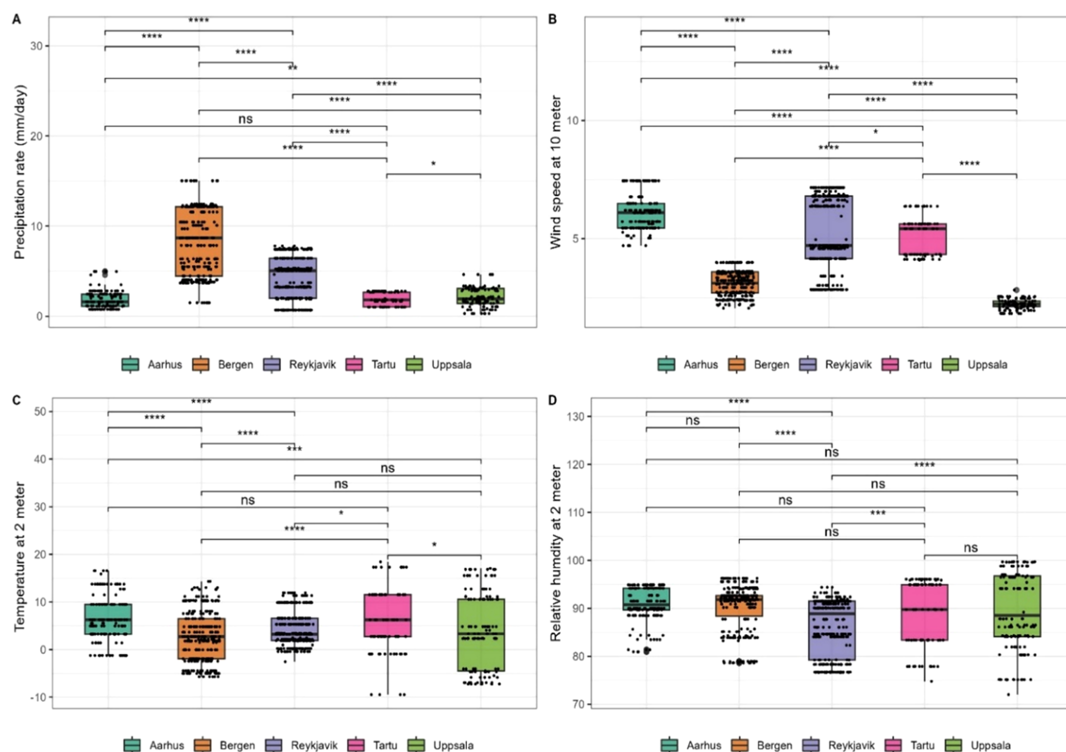

Figure 6. Box plots of monthly average meteorological data for the five cities during sampling of settled indoor airborne dust: (A) precipitation rate (mm/day), (B) wind speed (mm/s), (C) temperature (°C), and (D) relative humidity (%). An asterisk (*) indicates a significant pairwise comparison (P value ≤ 0.05). The greater the number of asterisks, the lower the P value. Nonsignificant pairwise comparison between cities, indicated by (ns).

genera (Figure 4E). The indoor determinants associated with differentially abundant genera were house age and having a rug in the bedroom. Houses that were >35 years old showed 6 more abundant genera than houses that were <35 years old (Figure 4F). The presence of a rug in the bedroom was associated with an increase in the abundance of three bacterial genera (Figure 4G).

3.6. Endotoxin Load. Out of 1038 samples, extracts from 758 samples (73%) had endotoxin concentrations above the background level (unexposed EDC cloths). 16 covariates were identified from the univariate analyses (Supporting Table 11). Using the first approach, only cities and age of the house showed significant association with endotoxin load (Supporting Table 12). These results were confirmed by the second approach, in which the environmental determinants were introduced in three consecutive models. (Supporting Table 13). A sensitivity analysis with complete data from both the ECRHS III main interview and the EDC questionnaires (without Tartu) showed that a dog in the bedroom was significantly associated with a higher endotoxin load (Supporting Table 14).

Bergen households had a significantly lower endotoxin load than the other cities except Reykjavik households. Tartu households, on the other hand, had significantly higher endotoxin load compared to the other four cities' households (Figure 5A). A higher relative abundance of Gram-negative bacteria was found in the Tartu and Aarhus households than in the other cities (Figure 5B). We found endotoxin concentration to be significantly correlated with the relative abundance of the three most abundant Gram-negative phyla, *Proteobacteria* (r = 0.32) followed by *Bacteroidota* (r = 0.17) and *Myxococcota* (r = 0.071) (Supporting Figure 8). Based on the Wilcoxon signed-rank test, there was a significant increase in endotoxin concentration in the indoor dust when dogs were allowed inside the bedroom and in older house groups compared to newer buildings (Figure 5C,D).

3.7. Meteorological Data. The average monthly precipitation rate during sampling of settled indoor dust was significantly higher in Bergen compared to the other cities. There was no statistically significant difference between precipitation rates in Aarhus and Tartu (Figure 6A). Wind speed and temperature were significantly higher in Aarhus and in Tartu compared to other cities (Figure 6B,C). The relative humidity was not significantly different between the cities except for Reykjavik, which had significantly lower relative humidity than other cities (Figure 6D).

The precipitation rate was negatively correlated with indoor air bacterial diversity, bacterial load, and endotoxin load. On the other hand, wind speed was positively correlated with both bacterial and endotoxin load. Bacterial diversity was found to

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Table 2. Spearman Rank Correlation Coefficients between the Meteorological Data and Indoor Airborne Bacterial Measurement

	Shannon index		Bacterial load		Endotoxin load	
	r value	*P* value	*r* value	*r* value	*P* value	*r* value
precipitation rate	−0.16	<0.001	−0.13	<0.001	−0.19	<0.001
wind speed	−0.003	0.91	0.11	<0.001	0.12	0.001
temperature	0.14	<0.001	0.08	0.008	−0.02	0.9
relative humidity	−0.10	0.001	−0.02	0.49	0.06	0.1

be positively correlated with temperature and negatively correlated with relative humidity (Table 2). Scatter plots of the correlation coefficient between the meteorological data and indoor air bacterial diversity, bacterial load, and endotoxin load can be found in Supporting Figures 9−11.

4. DISCUSSION

We investigated the role of occupants and indoor determinants on the bacterial microbiome of airborne indoor dust from 1038 households in five Nordic cities and showed that the variation in the airborne bacterial community is associated with six environmental determinants: geographical location, occupant's age, number of occupants, presence of a dog, cleaning, and house age. Furthermore, we found a meteorological characteristic to be correlated with the indoor airborne bacterial community. Here we emphasize precipitation, which was negatively correlated with the diversity and the load of the indoor airborne bacterial community.

4.1. Sources of Indoor Airborne Microbiome. In all five cities, the human body microbiome was the major contributor to the indoor bacterial microbiome. Indoor dust samples were dominated by Gram-positive bacteria, including a subset of bacterial genera known to be associated with humans (*Staphylococcus*, *Streptococcus*, *Micrococcus*, *Corynebacterium*, and *Lactobacillus*). Not surprisingly, many bacterial genera could be traced back to the human skin, although gut and oral environments also contribute.[10,15,30,31] Outdoor bacteria for example *Sphingomonas*, *Rhodococcus*, and *Arthrobacter* contributed to the composition of the indoor microbiome in all cities.[32−35] These bacteria may enter houses via windows and doors or could be transferred from shoes onto floors and carpets and then become resuspended in indoor air. This is in line with previous studies that showed that both the occupants and outdoor environments are the major sources of microorganisms found indoors.[10,11]

The taxa from outdoor sources such as *Sphingomonas*, *Rhodococcus*, and *Arthrobacter* were more abundant in Tartu and Aarhus household compared to other cities, which might explain the higher bacterial load and bacterial diversity in these two cities' households as outdoor bacteria are among key sources of bacteria in indoor. An increase in the relative abundance of Gram-negative bacterial taxa that mainly originate from outdoor sources such as *Protobacteria*, *Acinetobacter*, and *Skermanella*[16,36] and the increase in the bacterial load might together explain the higher endotoxin (i.e., a cell component of Gram-negative bacteria) load in Tartu and Aarhus compared to other cities' households that were characterized by fewer outdoor bacterial taxa.

The weaker link between endotoxin and indoor characteristics, compared to bacterial diversity and load observed throughout the current study, may be due to the dominance of Gram-positive bacteria from human skin indoors, which lack endotoxin. Factors related to humans and their behavior such as cleaning frequency, number of occupants, and occupants' age explain variations in bacterial diversity and load indoors but not endotoxin levels. In contrast, outdoor bacteria, rich in Gram-negative bacteria (containing endotoxin), contribute to higher endotoxin levels, influenced by outdoor activities like owning a dog.

4.2. Geographical Location and Meteorological Data. The meteorological factors, which are known to impact outdoor microbial communities,[37] might explain why there are different amounts of outdoor bacterial taxa in households located in different cities. In a previous study using wipes from the external surfaces of approximately 1200 households located across the United States, the authors found continental-scale distributions of the outdoor bacteria and suggested that change could be related to climate factors.[38] In the current study, Tartu and Aarhus were characterized by lower precipitation rates and higher wind speeds compared to other cities, while the temperature and relative humidity were within similar ranges. Fu et al. recently reported that the microbial community inside a building is affected by different outdoor environmental factors, such as geographical characteristics, precipitation, and relative humidity.[39] In the current study, wind speed and temperature were positively correlated with bacterial load and diversity, while precipitation was negatively correlated with bacterial diversity, bacterial load, and endotoxin load. High wind speeds might have increased the outdoor bacterial concentrations and thus the amount of outdoor bacterial taxa that infiltrated from outdoor air into indoor air in Aarhus and Tartu. Thus, indoor bacterial diversity and load were both higher in these two cities. Yafeng et al. measured the outdoor and indoor PM2.5 (Particulate Matter 2.5) concentrations, which is an important carrier medium for bacteria. The author found the indoor infiltration rate of PM2.5 to be positively correlated with outdoor wind speed and temperature.[40,41] Bergen was characterized with higher precipitation than other cities which might explain the lower abundance of outdoor bacterial taxa in Bergen households compared to the households in other cities through decreased infiltration of outdoor air particles. Rainfall is known to scavenge atmospheric particles, including bacteria, and transport them to the ground in a process known as "wet deposition," which increases with rainfall intensity.[42] However, the impact of raindrops on various surfaces on the ground might triggers the emission of surface-associated bacteria into the atmosphere,[19,43] which likely depends on the type of source environments.[44] So, it is likely the combinations of these two processes that will determine the concentration and type of airborne bacteria in outdoor air. Huffman et al. found that in a forest ecosystem the concentration of airborne biological particles increased significantly due to rainfall.[45] Tian et al.[46] established that the concentration of coarse aerosol particles (>2.5 μm in diameter) in urban environments was reduced by rain,[40,46] which fits well with our observation

https://doi.org/10.1021/acs.est.3c01616
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that heavier rainfall is associated with reduced outdoor bacteria indoors. In addition, rainfall was found to alter the composition of airborne bacterial community at a suburban site with an increase in the relative abundance of *Actinobacteria* and a decrease in the relative abundance of *Proteobacteria*, which matches the bacterial profile of Bergen, a city known for heavy rainfall.[19]

Exposure to a variety of microorganisms has been inversely associated with the risk of developing asthma and atopy.[8,47−49] With this in mind, Kirjavainen et al.[9] found that the protective "farm-like" microbiota against asthma and atopy had a higher abundance of outdoor-associated bacterial taxa, including *Sphingobacetria* and *Alphaproteobacteria* bacteria. These taxa were less abundant in Bergen compared to Aarhus and Tartu which might be related to higher precipitation and lower wind speeds that hinder the outdoor taxa to enter the homes in Bergen. The intensity of precipitation is expected to intensify with global warming,[50,51] and if our assumption is correct, this will increase wet deposition of outdoor particulates and particles associated with bacteria. As a result, fewer outdoor bacteria will contribute to the indoor microbiome and the intensity of exposures to environmental bacteria and endotoxins will decrease, with possible negative consequences for the development and maintenance of a tolerogenic immune status.[52]

4.3. Occupants' Age. Human skin microbiota is considered a principal source of indoor airborne bacteria.[16] The occupant's age was for the first time associated with an increase in bacterial diversity, a reduction in bacterial load, and a change in the composition of the bacterial community. However, we are aware that in the current study, only the age of the participant in the ECRHS study was known, while the age of other occupants who used the same bedroom where settled dust samples were collected was unknown.

The human skin microbiome undergoes age-associated changes that reflect underlying age-related alteration in the cutaneous structure and the physiological function of the skin.[53] Several studies have shown that bacterial species richness and diversity increase gradually with advancing age.[53−55] Howard et al. investigated the skin microbiome of 158 females aged 20−74 years old and showed that bacterial diversity increased with age. The authors also found a change in the relative abundance of several bacterial taxa between different age groups.[55] This supports our ANCOM BC results showing that 16 bacterial genera were differentially abundant between the two age groups in the current study. The number of bacteria on the skin tends to decrease with age, which also supports the results of our study. According to Lyden et al. sebum secretion levels decrease with age. As sebum is rich in triglycerides and free fatty acids, this leads to a decline in nutrients and consequently to a decrease in bacterial numbers.[56]

4.4. Level of Occupancy. The number of occupants was associated with an increase in both bacterial diversity and richness. This is in line with previous results, demonstrating that high occupancy leads to an accumulation of human-associated microorganisms.[18,31,57] The increase in bacterial diversity with increased human occupancy could be attributed to several causes: (1) bacteria emitted from occupants could differ between individuals[58,59] and (2) a higher density will lead to enhanced activity and thus, more resuspension of floor dust particles, in addition to more transport of outdoor bacteria attached to clothes and shoes.[60,61] In the present study, increasing occupancy was associated with an increase in bacterial load, which has also been shown in other studies.[10,16,62] Qian et al., studying the microbiome of classrooms, found that the bacterial load was much higher during the active school days than during vacation.

4.5. Pets. While dogs significantly contributed to the indoor airborne bacterial community both in terms of composition and diversity, cats had little influence on the indoor microbiome. These results are consistent with previous reports on the impact of cats and dogs.[27,63,64] An ANCOM BC analysis showed an increased abundance of several bacterial taxa we assume are either introduced by the dogs from the outdoor environment such as *Rhodococcus*, *Sphingomonas*, and *Arthrobacter*[16,32−34] or stem from the dogs' own microbiome itself such as *Moraxella* and *Fusobacterium*, common members of a dog's oral and gastrointestinal tract microbiome.[65,66] This is in line with the finding of Dunn et al. who found that households with dogs had a higher relative abundance of bacterial taxa associated with dog microbiota.[12] The presence of a dog in a household was also associated with a higher endotoxin load. This is in line with Fuertes et al. reporting that endotoxin concentration in air was associated with dogs but not with cats.[67] In the current study, higher endotoxin loads might be explained by the dog's own microbiota, such as *Moraxella* and *Fusobacterium*[65,66] These Gram-negative bacteria were found to be the most abundant taxa in the indoor air of the dog owners' households, in addition to the Gram-negative environmental bacteria brought in by the dog from the outdoors.

4.6. Cleaning and Use of Disinfectant. Higher cleaning frequencies were associated with an increase in bacterial diversity and load of the indoor air. Cleaning might lead to resuspension of settled dust and air mixing, thus increasing the number of bacterial taxa collected by the EDCs. This could explain the increase in bacterial diversity and load associated with higher cleaning frequency. Thus, cleaning frequency is one of the behavioral choices that can influence our daily exposure to different bacterial species. Sordillo et al.[68] observed that frequent cleaning increases muramic acid levels in indoor air, a component of Gram-positive bacteria's cell wall, which is consistent with our current finding.

Use of cleaning and disinfecting agents was related to a lower abundance of several Gram-negative and Gram-positive taxa, especially when bleach (sodium hypochlorite) was used. Due to the lack of selectivity, common disinfection practices such as the use of sodium hypochlorite, would indiscriminately kill indoor air microorganisms.[1] In the current study, samples were collected between 2011 and 2013. However, with the advent of the COVID-19 pandemic, the deployment of chemical disinfectants such as sodium hypochlorite has increased dramatically in various building environments.[69] In a recent study conducted during the COVID-19 pandemic, regularly disinfecting school classrooms by spraying disinfectant and wiping indoor surfaces was found to reduce airborne bacteria. which is in line with our findings.[70] Yet, it is necessary to conduct further research to understand the implications of altering the microbiome through intensified disinfection use on the health of individuals occupying the space.

4.7. House Age and Indoor Characteristics. In the present study, the age of the house was associated with an increase in bacterial diversity and richness. Previously, Kettleson et al.[27] showed that an increase of fungal diversity was associated with the age of the building. They did not find

https://doi.org/10.1021/acs.est.3c01616
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the same association with bacterial diversity. However, the small sample size (n = 35) compared to our study (n = 1038) might have masked some of the patterns. An increase in bacterial diversity in older houses may be caused by leaky plumbing systems, providing access for bacteria that will be further transferred to the indoor air through the ventilation system.[10,17] We have shown previously in ECRHSII that old buildings have more dampness and water leakages.[71] In the present study, the differential abundance analysis showed that in older buildings, there was an increased abundance of bacterial taxa belonging mostly to aquatic environments, including *Friedmanniella*, *Ilumatobacter*, and *Microlunatus*.[72−75] This implies that differences in the plumbing systems between old and new houses may affect the composition of the indoor airborne microbiome. Additionally, the age of the house was associated with an increased endotoxin load. Similarly, in a nationwide-scale study in the United States involving more than 800 homes, the authors found that the age of buildings was an important predictor of endotoxin concentration.[76]

Two indoor characteristics were associated with an increase in bacterial richness: type of bedroom floor and bedroom size. Maybe a bigger room size is accompanied by a bigger or larger window, which would increase the infiltration of outdoor bacterial taxa. Small-scale structured floors (i.e., rugs) contained more bacterial taxa than uniform surfaces such as fitted carpets. This was also reported by Weikl et al. who found that floor dust from rugs had a more diverse bacterial community composition than samples from carpets.[29] The composition of airborne indoor bacterial communities showed a significant association with the presence of a rug in the bedroom. Studies report a significant increase in the abundance of three bacterial genera: *Sphingomonas*, *Pseudonocardia*, and *Friedmanniella*, which are also found outdoors.[16,77,78] Most rugs are made of textile materials with high porosity, which facilitates the adherence of dust and organic compounds. In addition, the pores may also retain sufficient moisture.[79] In combination, these factors might facilitate bacterial growth and persistence due to increased levels of organics and moisture.[1]

4.8. Ventilation. According to the ANOSIM test, the ventilation achieved by opening the window during sleep (natural ventilation) as well as the presence of wall vents, designed to supply fresh air to a residential building, in the bedroom (mechanical ventilation) were both associated with a minor but significant change in the composition of the bacterial community. This is in line with results published by Bragoszewska et al. who observed differences in bacterial community composition in dust samples collected from a mixed-use building with half of the offices using natural ventilation and the other half using a conventional mechanical ventilation system.[80] Ventilation with wall vents was associated with lower bacterial diversity and richness. Kembel and colleagues found that mechanically ventilated rooms have less diverse bacterial communities than naturally ventilated rooms.[18] A possible reason behind the lower bacterial diversity with mechanical ventilation compared to natural ventilation systems is the use of filters in mechanical ventilation system, which prevents fractions of the outdoor bacteria taxa and particulates from entering the building.[1]

4.9. Moisture and Mold. Condensation of water on windows during winter was associated with a decrease in the bacterial diversity. Condensation is a sign of an increase in moisture (air relative humidity) and is the result of relatively warm and moist air getting into contact with cold window surfaces.[81] High relative humidity in the air reduces the aerosolization of microbes from indoor surfaces and thereby reduces dust resuspension into the air by occupant movements in comparison to low relative humidity, which increases the potential for aerosols to stay aloft longer and travel further.[48,82] This might explain a decrease in bacterial diversity associated with condensation on windows during the winter.

Equilibrium relative humidity (ERH) is used to assess moisture at the material's surface. When the ERH reaches certain threshold (e.g., 70% for wooden materials), the material surface may become a target for microbial growth allowing mold germination and proliferation.[17,83] In the current study, visible mold was in fact associated with increased bacterial diversity. In line with our findings, Gupta et al. found that bacterial and fungal diversity values were positively correlated in the bed dust.[84] In a study done in Finland that investigated 41 severely water-damaged homes with mold growth, the authors found that the bacterial diversity of house dust decreased significantly after the water damage was fixed.[85] This shows that there is a link between excessive surface moisture and an increase in the number of bacteria and fungi in indoor air.

4.10. Implications, Strengths, and Limitations. In the current study, we utilized 1083 EDC samples from the bedrooms of private homes across northern Europe. The large size of the samples enabled robust statistical comparisons to be made, resulting in reliable information about the factors that influence indoor microbiome compared to studies that have been limited to single geographical sites and small sample sizes. We observed that the indoor bacterial microbiome differed substantially by geographical location, and we conclude that the difference in the abundance of outdoor bacteria in the households may be due to different weather events, especially the wind speed and the precipitation. We speculate that future predicted increase in precipitation rates due to global warming could impact our indoor bacterial exposure and might have negative consequences for our immune system. Our study was limited by not having simultaneous outdoor sampling. Therefore, further studies including both indoor and outdoor samples, as well as recordings of meteorological data may be necessary to provide a more complete understanding of the effects of weather on the contribution of outdoor bacterial taxa to the indoors. Another limitation of the current study is that we lacked information on land use which could, in combination with metrological factors, affect the composition of indoor microbiome.[86]

Age of the occupant of the homes was associated with higher diversity but lower microbial load. We suggest that this is due to the age-related changes in skin microbiome. Furthermore, our results suggest that general lifestyle choices such as the number of occupants, types of pets, cleaning frequency of the household, and use of chemical disinfectants impact the indoor microbiome. Thus, the presence of a dog increases, whereas the use of disinfectants decreases microbial exposure. The use of disinfectants has increased dramatically since the COVID-19 pandemic, and our results lead us to conclude that it is urgent to study further the effects of excessive use of disinfectants on the indoor airborne bacterial community as it may have negative consequences on human health. In conclusion, our study identifies (1) several factors that may be subject to intervention to improve our indoor microbiome and (2) that further research to establish causality is urgently needed.

https://doi.org/10.1021/acs.est.3c01616
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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c01616.

> Methods; pairwise differential abundance analysis between the five cities' households; results for univariate and multivariate regression analysis between environmental determinants and bacterial profiles; histograms of independent variables; differential abundant bacterial genera; correlation plots between the relative abundance; and scatter plots of the correlation coefficient between the indoor bacterial load (PDF)

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Funding

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement no. 804199). K.F. acknowledges support by the Novo Nordisk Foundation (grant NNF19OC0056963), and T.Š.-T. acknowledges support by the Independent Research Fund Denmark (9145-00001B) and the Villum Fonden (research grants 23175 and 37435).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Li, S.; Yang, Z.; Hu, D.; Cao, L.; He, Q. Understanding building-occupant-microbiome interactions toward healthy built environments: A review. *Front. Environ. Sci. Eng.* **2021**, *15*, No. 65.

(2) Adams, R. I.; Bateman, A. C.; Bik, H. M.; Meadow, J. F. Microbiota of the indoor environment: a meta-analysis. *Microbiome* **2015**, *3*, No. 49.

(3) Ege, M. J. The hygiene hypothesis in the age of the microbiome. *Ann. Am. Thorac. Soc.* **2017**, *14*, S348−S353.

(4) Braun-Fahrländer, C.; Riedler, J.; Herz, U.; Eder, W.; Waser, M.; Grize, L.; Maisch, S.; Carr, D.; Gerlach, F.; Bufe, A. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N. Engl. J. Med.* **2002**, *347*, 869−877.

(5) You, R.; Cui, W.; Chen, C.; Zhao, B. Measuring the short-term emission rates of particles in the "personal cloud" with different clothes and activity intensities in a sealed chamber. *Aerosol Air Qual. Res.* **2013**, *13*, 911−921.

(6) Lee, M. K.; Wyss, A. B.; Carnes, M. U.; Richards, M.; Parks, C. G.; Freeman, L. E. B.; Thorne, P. S.; Umbach, D. M.; Azcarate-Peril, M. A.; Peddada, S. D.; London, S. J. House dust microbiota in relation to adult asthma and atopy in a US farming population. *J. Allergy Clin. Immunol.* **2021**, *147*, 910−920.

(7) Pekkanen, J.; Valkonen, M.; Täubel, M.; Tischer, C.; Leppänen, H.; Kärkkäinen, P. M.; Rintala, H.; Zock, J.-P.; Casas, L.; Probst-Hensch, N.; et al. Indoor bacteria and asthma in adults: a multicentre case−control study within ECRHS II. *Eur. Respir. J.* **2018**, *51*, No. 1701241.

(8) Valkonen, M.; Wouters, I. M.; Täubel, M.; Rintala, H.; Lenters, V.; Vasara, R.; Genuneit, J.; Braun-Fahrländer, C.; Piarroux, R.; von Mutius, E.; et al. Bacterial exposures and associations with atopy and asthma in children. *PLoS One* **2015**, *10*, No. e0131594.

(9) Kirjavainen, P. V.; Karvonen, A. M.; Adams, R. I.; Täubel, M.; Roponen, M.; Tuoresmäki, P.; Loss, G.; Jayaprakash, B.; Depner, M.; Ege, M. J.; et al. Farm-like indoor microbiota in non-farm homes

protects children from asthma development. *Nat. Med.* **2019**, *25*, 1089−1095.

(10) Meadow, J. F.; Altrichter, A. E.; Kembel, S. W.; Kline, J.; Mhuireach, G.; Moriyama, M.; Northcutt, D.; O'Connor, T. K.; Womack, A. M.; Brown, G.; et al. Indoor airborne bacterial communities are influenced by ventilation, occupancy, and outdoor air source. *Indoor Air* **2014**, *24*, 41−48.

(11) Wilkins, D.; Leung, M. H.; Lee, P. K. Indoor air bacterial communities in H ong K ong households assemble independently of occupant skin microbiomes. *Environ. Microbiol.* **2016**, *18*, 1754−1763.

(12) Dunn, R. R.; Fierer, N.; Henley, J. B.; Leff, J. W.; Menninger, H. L. Home life: factors structuring the bacterial diversity found within and between homes. *PLoS One* **2013**, *8*, No. e64133.

(13) Gunnbjörnsdóttir, M. I.; Norbäck, D.; Björnsson, E.; Soon, A.; Jarvis, D.; Jõgi, R.; Gislason, D.; Gislason, T.; Janson, C. Indoor environment in three North European cities in relationship to atopy and respiratory symptoms. *Clin. Respir. J.* **2009**, *3*, 85−94.

(14) Hanson, B.; Zhou, Y.; Bautista, E. J.; Urch, B.; Speck, M.; Silverman, F.; Muilenberg, M.; Phipatanakul, W.; Weinstock, G.; Sodergren, E.; et al. Characterization of the bacterial and fungal microbiome in indoor dust and outdoor air samples: a pilot study. *Environ. Sci.: Processes Impacts* **2016**, *18*, 713−724.

(15) Täubel, M.; Rintala, H.; Pitkäranta, M.; Paulin, L.; Laitinen, S.; Pekkanen, J.; Hyvärinen, A.; Nevalainen, A. The occupant as a source of house dust bacteria. *J. Allergy Clin. Immunol.* **2009**, *124*, 834−840.e47.

(16) Hospodsky, D.; Qian, J.; Nazaroff, W. W.; Yamamoto, N.; Bibby, K.; Rismani-Yazdi, H.; Peccia, J. Human occupancy as a source of indoor airborne bacteria. *PLoS One* **2012**, *7*, No. e34867.

(17) Kembel, S. W.; Jones, E.; Kline, J.; Northcutt, D.; Stenson, J.; Womack, A. M.; Bohannan, B. J.; Brown, G.; Green, J. L. Architectural design influences the diversity and structure of the built environment microbiome. *ISME J.* **2012**, *6*, 1469−1479.

(18) Kembel, S. W.; Meadow, J. F.; O'Connor, T. K.; Mhuireach, G.; Northcutt, D.; Kline, J.; Moriyama, M.; Brown, G.; Bohannan, B. J.; Green, J. L. Architectural design drives the biogeography of indoor bacterial communities. *PLoS One* **2014**, *9*, No. e87093.

(19) Jang, G. I.; Hwang, C. Y.; Cho, B. C. Effects of heavy rainfall on the composition of airborne bacterial communities. *Front. Environ. Sci. Eng.* **2018**, *12*, No. 12.

(20) Burney, P.; Luczynska, C.; Chinn, S.; Jarvis, D. The European community respiratory health survey. *Eur. Respir. J.* **1994**, *7*, 954−960.

(21) Holst, G. J.; Pørneki, A.; Lindgreen, J.; Thuesen, B.; Bønløkke, J.; Hyvärinen, A.; Elholm, G.; Østergaard, K.; Loft, S.; Brooks, C.; et al. Household dampness and microbial exposure related to allergy and respiratory health in Danish adults. *Eur. Clin. Respir. J.* **2020**, *7*, No. 1706235.

(22) Amin, H.; Marshall, I. P.; Bertelsen, R. J.; Wouters, I. M.; Schlünssen, V.; Sigsgaard, T.; Šantl-Temkiv, T. Optimization of bacterial DNA and endotoxin extraction from settled airborne dust. *Sci. Total Environ.* **2022**, No. 159455.

(23) Team, R. C., R: A language and environment for statistical computing. 2013.

(24) Lahti, L.; Shetty, S., Introduction to the microbiome R package. 2018.

(25) Lin, H.; Peddada, S. D. Analysis of compositions of microbiomes with bias correction. *Nat. Commun.* **2020**, *11*, No. 3514.

(26) Dixon, P. VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* **2003**, *14*, 927−930.

(27) Kettleson, E. M.; Adhikari, A.; Vesper, S.; Coombs, K.; Indugula, R.; Reponen, T. Key determinants of the fungal and bacterial microbiomes in homes. *Environ. Res.* **2015**, *138*, 130−135.

(28) Koenker, R. quantreg: Quantile regression, 2009. https://cran.r-project.org/web/packages/quantreg/. (accessed May 24, 2022).

(29) Weikl, F.; Tischer, C.; Probst, A. J.; Heinrich, J.; Markevych, I.; Jochner, S.; Pritsch, K. Fungal and bacterial communities in indoor dust follow different environmental determinants. *PLoS One* **2016**, *11*, No. e0154131.

(30) Adams, R. I.; Miletto, M.; Lindow, S. E.; Taylor, J. W.; Bruns, T. D. Airborne bacterial communities in residences: similarities and differences with fungi. *PLoS One* **2014**, *9*, No. e91283.

(31) Dannemiller, K. C.; Gent, J. F.; Leaderer, B. P.; Peccia, J. Influence of housing characteristics on bacterial and fungal communities in homes of asthmatic children. *Indoor Air* **2016**, *26*, 179−192.

(32) Korthals, M.; Ege, M. J.; Tebbe, C. C.; von Mutius, E.; Bauer, J. Application of PCR-SSCP for molecular epidemiological studies on the exposure of farm children to bacteria in environmental dust. *J. Microbiol. Methods* **2008**, *73*, 49−56.

(33) Bowers, R. M.; McLetchie, S.; Knight, R.; Fierer, N. Spatial variability in airborne bacterial communities across land-use types and their relationship to the bacterial communities of potential source environments. *ISME J.* **2011**, *5*, 601−612.

(34) Brodie, E. L.; DeSantis, T. Z.; Parker, J. P. M.; Zubietta, I. X.; Piceno, Y. M.; Andersen, G. L. Urban aerosols harbor diverse and dynamic bacterial populations. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 299−304.

(35) Fujiyoshi, S.; Tanaka, D.; Maruyama, F. Transmission of airborne bacteria across built environments and its measurement standards: a review. *Front. Microbiol.* **2017**, *8*, No. 2336.

(36) Rintala, H.; Pitkäranta, M.; Toivola, M.; Paulin, L.; Nevalainen, A. Diversity and seasonal dynamics of bacterial community in indoor environment. *BMC Microbiol.* **2008**, *8*, No. 56.

(37) Jones, A. M.; Harrison, R. M. The effects of meteorological factors on atmospheric bioaerosol concentrations—a review. *Sci. Total Environ.* **2004**, *326*, 151−180.

(38) Barberán, A.; Ladau, J.; Leff, J. W.; Pollard, K. S.; Menninger, H. L.; Dunn, R. R.; Fierer, N. Continental-scale distributions of dust-associated bacteria and fungi. *Proc. Natl. Acad. Sci. U.S.A.* **2015**, *112*, 5756−5761.

(39) Fu, X.; Ou, Z.; Sun, Y. Indoor microbiome and allergic diseases: from theoretical advances to prevention strategies. *Eco-Environ. Health* **2022**, *1*, 133−146.

(40) Karimi, H.; Nikaeen, M.; Gholipour, S.; Hatamzadeh, M.; Hassanzadeh, A.; Hajizadeh, Y. PM2. 5-associated bacteria in ambient air: Is PM2. 5 exposure associated with the acquisition of community-acquired staphylococcal infections? *J. Environ. Health Sci. Eng.* **2020**, *18*, 1007−1013.

(41) Wang, Y.; Chen, C.; Wang, P.; Wan, Y.; Chen, Z.; Zhao, L. Experimental investigation on indoor/outdoor PM2. 5 concentrations of an office building located in Guangzhou. *Procedia Eng.* **2015**, *121*, 333−340.

(42) Wang, Y.; Xia, W.; Zhang, G. J. What rainfall rates are most important to wet removal of different aerosol types? *Atmos. Chem. Phys.* **2021**, *21*, 16797−16816.

(43) Joung, Y. S.; Ge, Z.; Buie, C. R. Bioaerosol generation by raindrops on soil. *Nat. Commun.* **2017**, *8*, No. 14668.

(44) Šantl-Temkiv, T.; Amato, P.; Casamayor, E. O.; Lee, P. K.; Pointing, S. B. Microbial ecology of the atmosphere. *FEMS Microbiol. Rev.* **2022**, *46*, No. fuac009.

(45) Huffman, J. A.; Prenni, A.; DeMott, P.; Pöhlker, C.; Mason, R.; Robinson, N.; Fröhlich-Nowoisky, J.; Tobo, Y.; Després, V.; Garcia, E.; et al. High concentrations of biological aerosol particles and ice nuclei during and after rain. *Atmos. Chem. Phys.* **2013**, *13*, 6151−6164.

(46) Tian, X.; Cui, K.; Sheu, H.-L.; Hsieh, Y.-K.; Yu, F. Effects of rain and snow on the air quality index, PM2. 5 levels, and dry deposition flux of PCDD/Fs. *Aerosol Air Qual. Res.* **2021**, *21*, No. 210158.

(47) Ege, M. J.; Mayer, M.; Normand, A.-C.; Genuneit, J.; Cookson, W. O.; Braun-Fahrländer, C.; Heederik, D.; Piarroux, R.; von Mutius, E. Exposure to environmental microorganisms and childhood asthma. *N. Engl. J. Med.* **2011**, *364*, 701−709.

(48) Hyytiäinen, H.; Kirjavainen, P. V.; Täubel, M.; Tuoresmäki, P.; Casas, L.; Heinrich, J.; Herberth, G.; Standl, M.; Renz, H.; Piippo-Savolainen, E. Microbial diversity in homes and the risk of allergic rhinitis and inhalant atopy in two European birth cohorts. *Environ. Res.* **2021**, *196*, No. 110835.

(49) Amin, H.; Šantl-Temkiv, T.; Cramer, C.; Vestergaard, D. V.; Holst, G. J.; Elholm, G.; Finster, K.; Bertelsen, R. J.; Schlunssen, V.; Sigsgaard, T. Cow Farmers' Homes Host More Diverse Airborne Bacterial Communities than Pig Farmers' Homes and Suburban homes. *Front. Microbiol.* **2022**, No. 1892.

(50) Tabari, H. Climate change impact on flood and extreme precipitation increases with water availability. *Sci. Rep.* **2020**, *10*, No. 13768.

(51) Wuebbles, D. J.; Fahey, D. W.; Hibbard, K. A. *Climate Science Special Report: Fourth National Climate Assessment*; U.S. Global Change Research Program, 2017.

(52) Pfefferle, P. I.; Keber, C. U.; Cohen, R. M.; Garn, H. The Hygiene hypothesis−Learning from but not living in the past. *Front. Immunol.* **2021**, *12*, No. 635935.

(53) Li, Z.; Bai, X.; Peng, T.; Yi, X.; Luo, L.; Yang, J.; Liu, J.; Wang, Y.; He, T.; Wang, X.; et al. New insights into the skin microbial communities and skin aging. *Front. Microbiol.* **2020**, *11*, No. 565549.

(54) Kim, H.-J.; Oh, H. N.; Park, T.; Kim, H.; Lee, H. G.; An, S.; Sul, W. J. Aged related human skin microbiome and mycobiome in Korean women. *Sci. Rep.* **2022**, *12*, No. 2351.

(55) Howard, B.; Bascom, C. C.; Hu, P.; Binder, R. L.; Fadayel, G.; Huggins, T. G.; Jarrold, B. B.; Osborne, R.; Rocchetta, H. L.; Swift, D.; et al. Aging-associated changes in the adult human skin microbiome and the host factors that affect skin microbiome composition. *J. Invest. Dermatol.* **2022**, *142*, 1934−1946.e21.

(56) Leyden, J. J.; McGiley, K. J.; Mills, O. H.; Kligman, A. M. Age-related changes in the resident bacterial flora of the human face. *J. Invest. Dermatol.* **1975**, *65*, 379−381.

(57) Meadow, J. F.; Altrichter, A. E.; Kembel, S. W.; Moriyama, M.; O'Connor, T. K.; Womack, A. M.; Brown, G.; Green, J. L.; Bohannan, B. J. Bacterial communities on classroom surfaces vary with human contact. *Microbiome* **2014**, *2*, No. 7.

(58) Hoisington, A. J.; Brenner, L. A.; Kinney, K. A.; Postolache, T. T.; Lowry, C. A. The microbiome of the built environment and mental health. *Microbiome* **2015**, *3*, No. 60.

(59) Luongo, J. C.; Barberán, A.; Hacker-Cary, R.; Morgan, E. E.; Miller, S. L.; Fierer, N. Microbial analyses of airborne dust collected from dormitory rooms predict the sex of occupants. *Indoor Air* **2017**, *27*, 338−344.

(60) Meadow, J. F.; Altrichter, A. E.; Bateman, A. C.; Stenson, J.; Brown, G.; Green, J. L.; Bohannan, B. J. Humans differ in their personal microbial cloud. *PeerJ* **2015**, *3*, No. e1258.

(61) Hyytiäinen, H. K.; Jayaprakash, B.; Kirjavainen, P. V.; Saari, S. E.; Holopainen, R.; Keskinen, J.; Hämeri, K.; Hyvärinen, A.; Boor, B. E.; Täubel, M. Crawling-induced floor dust resuspension affects the microbiota of the infant breathing zone. *Microbiome* **2018**, *6*, No. 25.

(62) Qian, J.; Hospodsky, D.; Yamamoto, N.; Nazaroff, W. W.; Peccia, J. Size-resolved emission rates of airborne bacteria and fungi in an occupied classroom. *Indoor Air* **2012**, *22*, 339−351.

(63) Fujimura, K. E.; Johnson, C. C.; Ownby, D. R.; Cox, M. J.; Brodie, E. L.; Havstad, S. L.; Zoratti, E. M.; Woodcroft, K. J.; Bobbitt, K. R.; Wegienka, G.; et al. Man's best friend? The effect of pet ownership on house dust microbial communities. *J. Allergy Clin. Immunol.* **2010**, *126*, 410−412.e3.

(64) Ownby, D. R.; Peterson, E. L.; Wegienka, G.; Woodcroft, K. J.; Nicholas, C.; Zoratti, E.; Johnson, C. C. Are cats and dogs the major source of endotoxin in homes? *Indoor Air* **2013**, *23*, 219−226.

(65) Mäki, J. M.; Kirjavainen, P. V.; Täubel, M.; Piippo-Savolainen, E.; Backman, K.; Hyvärinen, A.; Tuoresmäki, P.; Jayaprakash, B.; Heinrich, J.; Herberth, G.; et al. Associations between dog keeping and indoor dust microbiota. *Sci. Rep.* **2021**, *11*, No. 5341.

(66) Sitarik, A. R.; Havstad, S.; Levin, A. M.; Lynch, S. V.; Fujimura, K.; Ownby, D. R.; Johnson, C. C.; Wegienka, G. Dog introduction alters the home dust microbiota. *Indoor Air* **2018**, *28*, 539−547.

(67) Fuertes, E.; Standl, M.; Markevych, I.; Bischof, W.; Heinrich, J. Is the association between pet ownership and indoor endotoxin levels confounded or modified by outdoor residential greenspace? *Sci. Total Environ.* **2018**, *625*, 716−721.

(68) Sordillo, J. E.; Alwis, U. K.; Hoffman, E.; Gold, D. R.; Milton, D. K. Home characteristics as predictors of bacterial and fungal microbial biomarkers in house dust. *Environ. Health Perspect.* **2011**, *119*, 189−195.

(69) Dhama, K.; Patel, S. K.; Kumar, R.; Masand, R.; Rana, J.; Yatoo, M.; Tiwari, R.; Sharun, K.; Mohapatra, R. K.; Natesan, S.; et al. The role of disinfectants and sanitizers during COVID-19 pandemic: advantages and deleterious effects on humans and the environment. *Environ. Sci. Pollut. Res.* **2021**, *28*, 34211−34228.

(70) Yang, J. I. L.; Lee, B. G.; Park, J. H.; Yeo, M. K. Airborne fungal and bacterial microbiome in classrooms of elementary schools during the COVID-19 pandemic period: Effects of school disinfection and other environmental factors. *Indoor Air* **2022**, *32*, No. e13107.

(71) Norbäck, D.; Zock, J. P.; Plana, E.; Heinrich, J.; Tischer, C.; Bertelsen, R. J.; Sunyer, J.; Künzli, N.; Villani, S.; Olivieri, M.; et al. Building dampness and mold in European homes in relation to climate, building characteristics and socio-economic status: The European Community Respiratory Health Survey ECRHS II. *Indoor Air* **2017**, *27*, 921−932.

(72) Maszenan, A. M.; Seviour, R.; Patel, B.; Schumann, P.; Burghardt, J.; Webb, R.; Soddell, J.; Rees, G. Friedmanniella spumicola sp. nov. and Friedmanniella capsulata sp. nov. from activated sludge foam: Gram-positive cocci that grow in aggregates of repeating groups of cocci. *Int. J. Syst. Evol. Microbiol.* **1999**, *49*, 1667−1680.

(73) Guo, J.; Zheng, Y.; Teng, J.; Song, J.; Wang, X.; Zhao, Q. The seasonal variation of microbial communities in drinking water sources in Shanghai. *J. Cleaner Prod.* **2020**, *265*, No. 121604.

(74) Yuan, M.; Yu, Y.; Li, H.-R.; Dong, N.; Zhang, X.-H. Phylogenetic diversity and biological activity of actinobacteria isolated from the Chukchi Shelf marine sediments in the Arctic Ocean. *Mar. Drugs* **2014**, *12*, 1281−1297.

(75) Jang, J.; Hur, H. G.; Sadowsky, M. J.; Byappanahalli, M.; Yan, T.; Ishii, S. Environmental *Escherichia coli*: ecology and public health implications—a review. *J. Appl. Microbiol.* **2017**, *123*, 570−581.

(76) Thorne, P. S.; Cohn, R. D.; Mav, D.; Arbes, S. J., Jr; Zeldin, D. C. Predictors of endotoxin levels in US housing. *Environ. Health Perspect.* **2009**, *117*, 763−771.

(77) Riahi, H. S.; Heidarieh, P.; Fatahi-Bafghi, M. Genus Pseudonocardia: What we know about its biological properties, abilities and current application in biotechnology. *J. Appl. Microbiol.* **2022**, *132*, 890−906.

(78) Tuo, L.; Pan, Z.; Li, F.-N.; Lou, I.; Guo, M.; Lee, S. M.-Y.; Chen, L.; Hu, L.; Sun, C.-H. Friedmanniella endophytica sp. nov., an endophytic actinobacterium isolated from bark of Kandelia candel. *Int. J. Syst. Evol. Microbiol.* **2016**, *66*, 3057−3062.

(79) Hoang, C. P.; Kinney, K. A.; Corsi, R. L.; Szaniszlo, P. J. Resistance of green building materials to fungal growth. *Int. Biodeterior. Biodegrad.* **2010**, *64*, 104−113.

(80) Brągoszewska, E.; Biedroń, I.; Kozielska, B.; Pastuszka, J. S. Microbiological indoor air quality in an office building in Gliwice, Poland: analysis of the case study. *Air Qual., Atmos. Health* **2018**, *11*, 729−740.

(81) Adan, O. C.; Samson, R. A. *Fundamentals of Mold Growth in Indoor Environments and Strategies for Healthy Living*; Springer, 2011.

(82) Frankel, M.; Hansen, E. W.; Madsen, A. M. Effect of relative humidity on the aerosolization and total inflammatory potential of fungal particles from dust-inoculated gypsum boards. *Indoor Air* **2014**, *24*, 16−28.

(83) Adams, R. I.; Lymperopoulou, D. L. Lessons learned when looking for non-neutral ecological processes in the built environment: the bacterial and fungal microbiota of shower tiles *bioRxiv* 2018, 413773. DOI: 10.1101/413773.

(84) Gupta, S.; Hjelmsø, M. H.; Lehtimäki, J.; Li, X.; Mortensen, M. S.; Russel, J.; Trivedi, U.; Rasmussen, M. A.; Stokholm, J.; Bisgaard, H.; Sørensen, S. J. Environmental shaping of the bacterial and fungal community in infant bed dust and correlations with the airway microbiota. *Microbiome* **2020**, *8*, No. 115.

(85) Jayaprakash, B.; Adams, R. I.; Kirjavainen, P.; Karvonen, A.; Vepsäläinen, A.; Valkonen, M.; Järvi, K.; Sulyok, M.; Pekkanen, J.; Hyvärinen, A.; Täubel, M. Indoor microbiota in severely moisture damaged homes and the impact of interventions. *Microbiome* **2017**, *5*, No. 138.

(86) Rocchi, S.; Reboux, G.; Scherer, E.; Laboissière, A.; Zaros, C.; Rouzet, A.; Valot, B.; Khan, S.; Dufourg, M.-N.; Leynaert, B.; et al. Indoor microbiome: Quantification of exposure and association with geographical location, meteorological factors, and land use in France. *Microorganisms* **2020**, *8*, No. 341.

Appendix

ECRHS III MAIN QUESTIONNAIRE

Centre number ☐☐☐
Personal number ☐☐☐☐☐
Sample ☐
Date ☐☐ ☐☐ ☐☐

You were last seen as part of this survey in _______ (month) ____________ (year)

I AM GOING TO ASK YOU SOME QUESTIONS. AT FIRST THESE WILL BE MOSTLY ABOUT YOUR BREATHING. WHEREVER POSSIBLE, I WOULD LIKE YOU TO ANSWER 'YES' OR 'NO'.

1. Have you had wheezing or whistling in your chest at any time in the last ***12 months***? NO ☐ YES ☐

   IF 'NO' GO TO QUESTION 2, IF 'YES':

   1.1 Have you been at all breathless when the wheezing noise was present? NO ☐ YES ☐

   1.2. Have you had this wheezing or whistling when you did ***not*** have a cold? NO ☐ YES ☐

   1.3 How old were you when you first had wheezing or whistling in your chest? YEARS ☐☐
   (If started 'as a baby' enter '01')

   1.4 How frequently have you had wheezing or whistling in the last 12 months? TICK ONE BOX ONLY

everyday	1 ☐
at least once a week, but not everyday	2 ☐
occasionally	3 ☐

2. Have you woken up with a feeling of tightness in your chest at any time in the last ***12 months***? NO ☐ YES ☐

3. Have you had an attack of shortness of breath that came on during the day when you were at rest at any time in the last ***12 months***? NO ☐ YES ☐

   IF 'NO' GO TO QUESTION 4, IF 'YES':

   3.1 How old were you when you first had an attack of shortness of breath that came on during the day when you were at rest? YEARS ☐☐

4. Have you had an attack of shortness of breath that came on ***following*** strenuous activity at any time in the last ***12 months***? NO ☐ YES ☐

5. Have you been woken by an attack of shortness of breath at any time in the last ***12 months?*** NO ☐ YES ☐

6. Have you been woken by an attack of coughing at any time *in the last* ***12 months***? NO ☐ YES ☐

7. How often have you experienced bouts or spasms of coughing in the last 12 months? TICK ONE BOX ONLY

less than once a month	1 ☐
every month, but less than every week	2 ☐
every week, but not every day	3 ☐
every day	4 ☐

NO YES

8. Do you ***usually*** cough first thing in the morning in the winter? ☐ ☐
[IF DOUBTFUL, USE QUESTION 9.1 TO CONFIRM]

9. Do you ***usually*** cough during the day, or at night, in the winter? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 10, IF 'YES':

9.1 Do you cough like this on most days for as much as three months each year? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 10, IF 'YES':

9.2 How many years have you had this problem (coughing on most days for as much as three months each year? YEARS ☐☐

10. Do you ***usually*** bring up any phlegm from your chest first thing in the morning in the winter? NO ☐ YES ☐
[IF DOUBTFUL, USE QUESTION 11.1 TO CONFIRM]

11. Do you ***usually*** bring up any phlegm from your chest during the day, or at night, in the winter? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 12, IF 'YES':

11.1 Do you bring up phlegm like this on most days for as much as three months each year? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 12, IF 'YES':

11.2 How many years have you had this problem (of bringing up phlegm from your chest on most days for as much as three months each year)? YEARS ☐☐

IF 'NO' TO QUESTIONS 3-11 GO DIRECT TO QUESTION 13;
IF 'YES' TO ANY OF QUESTIONS 3-11 PLEASE COMPLETE QUESTION 12

12. In the last ***12 months***, have you had any episodes/times when your symptoms (cough, phlegm, shortness of breath) were a lot worse than usual? NO ☐ YES ☐

IF 'NO' TO QUESTION 12 GO TO QUESTION 13; IF 'YES'

In the last ***12 months***:

12.1 How many times have these episodes occurred? TIMES ☐☐

12.2 How many times have these episodes forced you to consult your doctor? TIMES ☐☐

12.3 How many times was your therapy changed after these episodes? TIMES ☐☐

12.4 How many times have you visited a hospital casualty department or emergency room or have you spent a night in hospital after these episodes? TIMES ☐☐

13. Do you ever have trouble with your breathing? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 14, IF 'YES':

13.1 Do you have this trouble — TICK ONE BOX ONLY

a) continuously so that your breathing is never quite right? 1 ☐
b) repeatedly, but it always gets completely better? 2 ☐
c) only rarely? 3 ☐

14. Are you disabled from walking by a condition ***other than*** heart or lung disease? NO YES ☐ ☐

IF 'YES' STATE CONDITION ____________________ AND GO TO QUESTION 15, IF 'NO':

14.1 Are you troubled by shortness of breath when hurrying on level ground or walking up a slight hill? NO YES ☐ ☐

IF 'NO' GO TO QUESTION 14.2, IF 'YES':

14.1.1 Do you get short of breath walking with other people of your own age on level ground? NO YES ☐ ☐

IF 'NO' GO TO QUESTION 14.2, IF 'YES':

14.1.1.1 Do you have to stop for breath when walking at your own pace on level ground? NO YES ☐ ☐

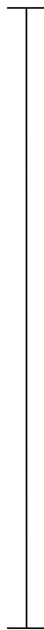
IF 'NO' GO TO QUESTION 14.2, IF 'YES':

14.1.1.1.1 Do you ever have to stop for breath after walking about 100 yards (or after a few minutes) on level ground? NO YES ☐ ☐

IF 'NO' GO TO QUESTION 14.2, IF 'YES':

14.1.1.1.1.1 Are you too short of breath to leave the house OR short of breath on dressing or undressing? NO YES ☐ ☐

14.2 How much shortness of breath are you having right now? Please indicate by marking the height of the column. If you are not experiencing any shortness of breath at present circle the marker at the bottom of the column



Shortness of breath
as bad as can be

Height in mm
(NB total height =100mm)

No shortness of breath

ECRHS III MAIN QUESTIONNAIRE

NO YES

15. Have you ever had asthma? ☐ ☐

IF 'NO' GO TO QUESTION 16, IF 'YES':

NO YES

15.1 Was this confirmed by a doctor? ☐ ☐

YEARS

15.2 How old were you when your asthma was confirmed by a doctor? ☐☐

YEARS

15.3 How old were you when you had your first attack of asthma? ☐☐

YEARS

15.4 How old were you when you had your most recent attack of asthma? ☐☐

15.5.1-6 Which months of the year do you usually have attacks of asthma?

	NO	YES
15.5.1 January / February	☐	☐
15.5.2 March / April	☐	☐
15.5.3 May / June	☐	☐
15.5.4 July / August	☐	☐
15.5.5 September / October	☐	☐
15.5.6 November / December	☐	☐

NO YES

15.6 Have you had an attack of asthma in the last ***12 months***? ☐ ☐

IF 'NO' GO TO 15.9, IF YES

ATTACKS

15.7 How many attacks of asthma have you had in the last ***12 months***? ☐☐

ATTACKS

15.8 How many attacks of asthma have you had in the last ***3 months***? ☐☐

15.9 How many times have you woken up because of your asthma in the last ***3 months?***

	TICK ONE BOX ONLY
every night or almost every night	1 ☐
more than once a week, but not most nights	2 ☐
at least twice a month, but not more than once a week	3 ☐
less than twice a month	4 ☐
not at all	5 ☐

15.10. How often have you had trouble with your breathing because of your asthma in the last ***3 months?***

	TICK ONE BOX ONLY
continuously	1 ☐
about once a day	2 ☐
at least once a week, but less than once a day	3 ☐
less than once a week	4 ☐
not at all	5 ☐

NO YES

15.11 Are you currently taking any medicines including inhalers, aerosols or tablets for asthma? ☐ ☐

NO YES

15.12 Do you have a peak flow meter of your own? ☐ ☐

IF 'NO' GO TO QUESTION 15.13 , IF 'YES':

15.12.1 How often have you used it over the last 3 months?

	TICK ONE BOX ONLY
never	1 ☐
some of the days	2 ☐
most of the days	3 ☐

15.13 Do you have written instructions from your doctor on how to manage your asthma if it gets worse or if you have an attack? NO [ ] YES [ ]

16. Has a doctor ever told you that you have chronic bronchitis? NO [ ] YES [ ]

IF 'NO' GO TO QUESTION 17, IF 'YES':

16.1 How old were you when you first had a diagnosis of chronic bronchitis? YEARS [ ][ ]

17. Has a doctor ever told you that you have chronic obstructive pulmonary disease (COPD)? NO [ ] YES [ ]

IF 'NO' GO TO QUESTION 18, IF 'YES

17.1 How old were you when you first had a diagnosis of COPD? YEARS [ ][ ]

18. Has a doctor ever told you that you have emphysema? NO [ ] YES [ ]

IF 'NO' GO TO QUESTION 19, IF 'YES':

18.1 How old were you when you first had a diagnosis of emphysema? YEARS [ ][ ]

19. Have you ever been diagnosed with any **other** lung disease (excluding asthma, chronic bronchitis, COPD and emphysema)? NO [ ] YES [ ]

IF 'NO' GO TO QUESTION 20, IF 'YES':

19.1 What is that lung disease called?__________________________________ CODE [ ][ ]

20. Do you have any nasal allergies, including hay fever? NO [ ] YES [ ]

IF 'NO' GO TO Q21, IF' YES':

20.1 How old were you when you first had hay fever or nasal allergy? YEARS [ ][ ]

21. Have you ***ever*** had a problem with sneezing, or a runny or a blocked nose when you did not have a cold or the flu? NO [ ] YES [ ]

IF 'NO' GO TO Q22, IF 'YES':

21.1. Have you had a problem with sneezing or a runny or a blocked nose when you did not have a cold or the flu ***in the last 12 months***? NO [ ] YES [ ]

IF 'NO' GO TO Q22, IF' YES':

21.1.1. Has this nose problem been accompanied by itchy or watery eyes? NO [ ] YES [ ]

21.1.2. In which months of the year did this nose problem occur?

	NO	YES
21.1.2.1. January/February	[ ]	[ ]
21.1.2.2. March/April	[ ]	[ ]
21.1.2.3. May/June	[ ]	[ ]
21.1.2.4. July/August	[ ]	[ ]
21.1.2.5. September/October	[ ]	[ ]
21.1.2.6 November/December	[ ]	[ ]

NO YES

21.1.3 Have you had this problem for **more than 4 days in any one week** in the last 12 months?
IF 'NO' GO TO Q21.1.4, IF' YES':

NO YES

21.1.3.1 Did this happen for **more than 4 weeks consecutively?**

21.1.4. For **each** of the following problems, please indicate how important it has been ***over the last 12 months***. *(SHOW A CARD WITH THE FOLLOWING OPTIONS)*

1. No problem (symptom not present)
2. A problem that is/was present but not disturbing
3. A disturbing problem but not hampering day time activities or sleep
4. A problem that hampers certain activities or sleep

CODE
Please enter code 1-4 in each of the five boxes

21.1.4.1	a watery runny nose	
21.1.4.2	a blocked nose (feeling of being unable to breath through your nose)	
21.1.4.3	an itchy nose	
21.1.4.4	sneezing, especially violent and in bouts	
21.1.4.5	watery, red itchy eyes	

NO YES

22. ***Since the last survey*** have you used any medication to treat nasal disorders?

IF NO GO TO Q23, IF YES

NO YES

22.1 Have you used any of the following nasal sprays for the treatment of your nasal disorder? **{SHOW LIST OF STEROID NASAL SPRAYS}**

IF NO GO TO Q22.2, IF YES

YEARS

22.1.1 How old were you when you first started to use **this sort of nasal spray**?

YEARS

22.1 2 How many years have you been taking this sort of nasal spray?

NO YES

22.1.3 Have you used any of these nasal sprays ***in the last 12 months?***

NO YES

22.1.4. Have you used this sort of nasal spray ***every year*** in the last 5 years?

IF 'NO' GO TO QUESTION 22.2 IF 'YES'

MONTHS

22.1.4.1 On average how many months each year have you taken them ?

NO YES

22.2 Have you used any of the following pills, capsules, or tablets for the treatment of your nasal disorder? **{SHOW LIST OF ANTIHISTAMINES}**

IF 'NO' GO TO Q23, IF 'YES'

NO YES

22.2.1 Have you used any of these pills, capsules or tablets in the last 12 months**?**

23. Has your nose been blocked **<u>for more than 12 weeks during the last 12 months</u>?** NO YES

24. Have you had pain or pressure around the forehead, nose or eyes **<u>for more than 12 weeks during the last 12 months</u>?** NO YES

25. Have you had discoloured nasal discharge (snot) or discoloured mucus in the throat **<u>for more than 12 weeks during the last 12 months</u>?** NO YES

26. Has your sense of smell been reduced or absent **<u>for more than 12 weeks during the last 12 months</u>?** NO YES

27. Has a doctor ***ever*** told you that you have NO YES
 27.1.1 **<u>chronic</u>** sinusitis?
 27.1.2 nasal polyps?

IF 'NO' TO Q27.1 and 27.2 GO TO Q 28, IF 'YES'

YEARS
27.2 How old were you when a doctor told you had chronic sinusitis?
27.3 How old were you when a doctor told you had nasal polyps?
(enter 00 if question not applicable)

28. Have you ***ever*** had eczema or any kind of skin allergy? NO YES

IF 'NO' TO Q28 GO TO Q 29, IF 'YES'

28.1 How old were you when you first had eczema or skin allergy? YEARS

28.2 Did/does your eczema or skin allergy affect your hands? NO YES

28.3 Have you noticed that contact with certain materials, chemicals or anything else **<u>in your work</u>** makes your eczema worse? NO YES DON'T KNOW

29. Have you ***ever*** had an itchy rash that was coming and going for at ***least 6 months***? NO YES
IF 'NO' GO TO QUESTION 30, IF 'YES':
 29.1.. Have you had this itchy rash ***in the last 12 months?*** NO YES
IF 'NO' GO TO QUESTION 30, IF 'YES':
 29.1.1. Has this itchy rash ***at any time*** affected any of the following places: the folds of the elbows, behind the knees, in front of the ankles under the buttocks or around the neck, ears or eyes NO YES
 29.1.2 Has this itchy rash affected your hands at any time <u>in the last 12 months</u>?

30. What was the highest level of education your mother had? TICK ONE BOX ONLY
 a) Up to the minimum school leaving age 1
 b) Secondary school/technical school past the minimum age 2
 c) College or University 3

31. What was the highest level of education your father had? TICK ONE BOX ONLY
 a) Up to the minimum school leaving age 1
 b) Secondary school/technical school past the minimum age 2
 c) College or University 3

	NO	YES	DK
32. Were you delivered by Caesarean section?	☐	☐	☐
33. Is your biological mother still alive?	☐	☐	☐

IF 'NO' GO TO QUESTION 33.2
IF 'DON'T KNOW'' GO TO QUESTION 34, IF 'YES':

33.1 How old is your mother now? YEARS ☐☐

NOW GO TO QUESTION 34

33.2 How old was your mother when she died ? YEARS ☐☐

	NO	YES	DK
34. Is your biological father still alive?	☐	☐	☐

IF 'NO' GO TO QUESTION 34.2
IF 'DON'T KNOW'' GO TO QUESTION 35, IF 'YES':

34.1 How old is your father now? YEARS ☐☐

NOW GO TO QUESTION 35

34.2 How old was your father when he died? YEARS ☐☐

35. Did your biological parents ever suffer from any of the following?

	MOTHER				FATHER		
	NO	YES	DK		NO	YES	DK
35.1.1 Asthma	☐	☐	☐	35.1.2	☐	☐	☐
35.2.1 Chronic bronchitis, emphysema and/or COPD	☐	☐	☐	35.2.2	☐	☐	☐
35.3.1 Heart disease	☐	☐	☐	35.3.2	☐	☐	☐
35.4.1 Hypertension	☐	☐	☐	35.4.2	☐	☐	☐
35.5.1 Stroke	☐	☐	☐	35.5.2	☐	☐	☐
35.6.1 Diabetes	☐	☐	☐	35.6.2	☐	☐	☐

36. How many children do you have? NUMBER ☐☐

IF ANSWER TO Q36 INDICATES PARTICIPANT HAS CHILDREN GO TO Q36.1; If NO CHILDREN GO TO QUESTION 37

	Please start with first born	Year of birth (eg 1995)	Did this child have asthma before the age of ten years?		Did this child have asthma after the age of ten years?		Has this child ever had nasal allergies, including hay fever?		Has this child ever had eczema or atopic dermatitis?		Was this child a boy or girl (Boy=1, Girl=2)
			NO	YES	NO	YES	NO	YES	NO	YES	Sex
36.1	Child 1										
36.2	Child 2										
36.3	Child 3										
36.4	Child 4										
36.5	Child 5										
36.6	Child 6										
36.7	Child 7										
36.8	Child 8										

You took part in the last survey in **[month]** in **[year]**. At that time you described your job as **['current' job from last occupational matrix]**

37. I would like to ask you to list all jobs that you have had since the last survey. I am interested in each one of the jobs that you have done for three months or more. These jobs may be outside the house or at home, **excluding homemaking or housework**, full time or part time, paid or unpaid, including self employment, for example in a family business. Please include part time jobs only if you had been doing them for 20 or more hours per week. Please start with your current or last held job.

Job	Occupation – Job Title: *Please provide a detailed description of the job*	Industry / Branch: *What does (did) your firm or employer make or what services does (did) it provide?*	Start month	Start year	End month	End year *(If current job please enter CURRENT)*
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

IF JOBS ARE GIVEN GO TO QUESTION 37.1; IF NO JOBS GIVEN GO TO Q38

	NO	YES
37.1 Have you had to change or leave any of these jobs because it affected your breathing?	☐	☐

IF 'NO' GO TO QUESTION 38; IF 'YES':

37.1.1-10 Please indicate which job(s) you had to change or leave (use numbers from question 37).

	NO	YES
37.1.1 Job 1	☐	☐
37.1.2 Job 2	☐	☐
37.1.3 Job 3	☐	☐
37.1.4 Job 4	☐	☐
37.1.5 Job 5	☐	☐
37.1.6 Job 6	☐	☐
37.1.7 Job 7	☐	☐
37.1.8 Job 8	☐	☐
37.1.9 Job 9	☐	☐
37.1.10 Job 10	☐	☐

38. What best describes your current main activity? TICK ONE BOX ONLY

Employed (including employed by temping agencies)	1	☐
Self-employed (entrepreneur, freelance or other)	2	☐
Full time student	3	☐
Full time housewife/househusband	4	☐

Unemployed looking for work	5	☐
Unemployed not looking for work	6	☐
Retired	7	☐
Other	8	☐

IF NOT 'EMPLOYED' OR NOT 'SELF-EMPLOYED' GO TO QUESTION 38.1
IF 'EMPLOYED' OR SELF-EMPLOYED' GO TO QUESTION 38.2;

NO YES

38.1 Were you forced to give up working all together because of asthma, wheezing shortness of breath or other respiratory or lung problems? ☐ ☐

IF 'NO' GO TO QUESTION 39, IF 'YES':

MONTH YEAR

38.1.1 When did this occur? ☐☐ ☐☐☐☐

NOW GO TO QUESTION 39

NO YES

38.2 In your current job, are you regularly exposed to vapours, gas, dust or fumes? ☐ ☐

NO YES

38.3 . Does being at your current workplace ever cause breathing problems (chest tightness,wheezing, coughing)? ☐ ☐

IF 'NO' GO TO QUESTION 38.4 , IF 'YES':

38.3.1-5 Can you indicate what gives you breathing problems in your current workplace?

	NO	YES
38.3.1 Physical exertion	☐	☐
38.3.2 Exposure to mist, hot or cold temperature	☐	☐
38.3.3 Exposure to vapours gas dust or fumes	☐	☐
38.3.4 Other peoples cigarette smoke	☐	☐
38.3.5 Stress	☐	☐

NO YES

38.3.6 Do these breathing problems diminish or stop during the weekend or during holidays? ☐ ☐

NO YES

38.4. Within the last 12 months have there been wet or damp spots on surfaces in the room where you usually work (for example on walls, wall paper, ceilings or carpets)? ☐ ☐

NO YES

38.5. Within the last 12 months has there been mould or mildew on any surfaces in the room where you usually work? ☐ ☐

NO YES

38.6. At any time in the last 12 months have you noticed the odour of mould or mildew (not from food) in the room where you usually work? ☐ ☐

NO YES

38.7. Do you regularly use cleaning products or disinfectants in your current job? ☐ ☐

IF 'NO' GO TO QUESTION 39, IF 'YES':

38.7.1-13 In the last 12 months, on how many days a week have you used the following products at work? (SHOW CARD WITH FOLLOWING OPTIONS)

1. Never
2. <1 day/week
3. 1-3 days/week
4. 4-7 days/week

CODE
Enter code 1-4 for all boxes

38.7.1 Bleach
38.7.2 Ammonia
38.7.3 Stain removers or other solvents
38.7.4 Acids (including decalcifiers, liquid scale removers, vinegar, hydrochloric acid, …)
38.7.5 Floor polish or floor wax
38.7.6 Liquid or solid furniture polish or wax
38.7.7 Furniture sprays (atomisers or aerosols)
38.7.8 Sprays for mopping the floor
38.7.9 Glass cleaning sprays (atomisers or aerosols)
38.7.10 Degreasing sprays including oven cleaning sprays (atomisers or aerosols)
38.7.11 (Ethyl) alcohol
38.7.12 Soaps or foams or any other chemical product for disinfecting hands
38.7.13 Any other chemical disinfectant (for example, glutaraldehyde, formaldehyde, chloramine-T, quaternary ammonium compounds)

NO YES

39 Have you <u>ever</u> been involved in an incident at home, work or elsewhere that exposed you to high levels of vapours, gases, dusts or fumes?

IF 'NO' GO TO QUESTION 40, IF 'YES':

YEAR

39.1 When did this occur?

In case of more than one incident, please report on the most recent incident.

39.2. Could you please classify this incident — TICK ONE BOX ONLY

A fire or an explosion	1
A leakage or spill	2
An inhalation related to mixing of cleaning products	3
Something else	4

39.3. Where did this happen? — TICK ONE BOX ONLY

In your own home	1
In your workplace	2
Somewhere else indoors	3
Outdoor	4

NO YES

39. 4 Did you experience respiratory symptoms within 24 hours following this incident?

IF 'NO' GO TO QUESTION 40, IF 'YES':

NO YES

39.4.1 Did you seek medical treatment for these symptoms?

40. How often do you usually exercise so much that you get out of breath or sweat ? — TICK ONE BOX ONLY

every day	1
4-6 times a week	2
2-3 times a week	3

once a week 4
once a month 5
less than once a month 6
never 7

41. How many hours a week do you usually exercise so much that you get out of breath or sweat? TICK ONE BOX ONLY
none 1
about ½ hr 2
about 1 hour 3
about 2-3 hours 4
about 4-6 hours 5
7 hours or more 6

42. Do you avoid taking vigorous exercise because of breathing problems? NO YES

43. When was your present home built? YEAR

44. How many years have you lived in your current home? YEARS

45. Which best describes the building in which you live? TICK ONE BOX ONLY
a) a one family house detached from any other house? 2
b) a one family house attached to one or more houses? 3
c) a building for two families? 4
d) a building for three or four families? 5
e) a building for five or more families? 6
f) other: ___________________________ 8

NB THERE IS NO CODE 1 and NO CODE 7

46. How many rooms does your home have? (exclude kitchen, bathroom, toilet, laundry) NUMBER

47. How many people live in your home? NUMBER

48 Does your home have any of the following? NO YES
48.1 central heating
48.2 ducted air heating (forced air heating)
48.3 air conditioning

49. Which of the following appliances do you use for heating or for hot water? NO YES
49.1 open coal, coke or wood fire
49.2 open gas fire
49.3 electric heater
49.4 paraffin heater
49.5 gas-fired boiler(located inside the home)
49.6 oil-fired boiler
49.7 portable gas heater
49.8 gas fired boiler (located outside the home eg: balcony)
49.9 fully enclosed wood/coal burning stove
49.10 other: ____

50. What kind of stove do you ***mostly*** use for cooking? TICK ONE BOX ONLY
a) coal, coke or wood (solid fuel)? 1
b) gas (gas from the mains)? 2
c) electric? 3

d) paraffin (kerosene)? 4 ☐
e) microwave 5 ☐
f) gas (gas from bottles or other non-mains source) 6 ☐
g) other: ____ 7 ☐

50.1 IF YOU USE GAS FOR COOKING Which of the following do you have? NO YES
50.1.1 gas hob (the area on top for heating for example saucepans) ☐ ☐
50.1 2.gas oven (the enclosed area used, for example, for baking or for roasting) ☐ ☐

51 . ***On average*** how long have you spent cooking with your cooker (hob or oven) ***<u>each day</u>*** over ***<u>the last four weeks</u>***? MINUTES ☐☐☐

52. ***Over the last four weeks*** when you were cooking did you have a door or window to the outside air open TICK ONE BOX ONLY
a) most of the time 1 ☐
b) some of the time 2 ☐
c) rarely (or only occasionally) 3 ☐
d) I do not have a door or window that opens to the outside in my kitchen 4 ☐
e) never 5 ☐

53. Do you have an extractor fan over the cooker? NO ☐ YES ☐ DK ☐

IF 'NO' OR 'DON'T KNOW' GO TO QUESTION 54, IF 'YES':

53.1 When cooking, do you use the fan TICK ONE BOX ONLY
a) all of the time? 1 ☐
b) some of the time? 2 ☐
c) none of the time? 3 ☐

53.2 Does the fan take the fumes outside the house? NO ☐ YES ☐ DK ☐

54. Has there been any water damage to the building or its contents, for example, from broken pipes, leaks or floods? NO ☐ YES ☐ DK ☐

IF 'NO' OR 'DON'T KNOW' GO TO QUESTION 55, IF 'YES':
54.1 Has there been any water damage in the last 12 months? NO ☐ YES ☐ DK ☐

55. ***Within the last 12 months*** have you had wet or damp spots on surfaces inside your home other than in the basement (for example on walls, wall paper, ceilings or carpets)? NO ☐ YES ☐

56. Has there ever been any mould or mildew on any surface, other than food, inside the home? NO ☐ YES ☐ DK ☐

IF 'NO' OR 'DON'T KNOW' GO TO QUESTION 57, IF 'YES'

56.1. Has there ever been any mould or mildew on any surface inside the home in the last ***12 months***? NO ☐ YES ☐ DK ☐

IF 'NO' OR 'DON'T KNOW' GO TO QUESTION 57, IF 'YES':

56.1.1-6 Which rooms have been affected? NO YES
56.1.1 bathroom(s) ☐ ☐
56.1.2 bedroom(s) ☐ ☐
56.1.3 living area(s) ☐ ☐
56.1.4 kitchen ☐ ☐
56.1.5 basement or attic ☐ ☐

56.1.6 other: ____

NO YES

57. Have you noticed the odour of mould or mildew (not from food) in your home at any time in the last 12 months?

58. Does the room which you use most at home during the day — NO YES
    - 58.1 have fitted carpets covering the whole floor?
    - 58.2 contain rugs?
    - 58.3 have double glazing/triple glazing?
    - 58.4 have visible wet or damp spots?
    - 58.5 have an airbrick or open chimney?

FLOOR

59. On what floor is the room which you use most at home during the day?

(Basement = 00 ,Ground floor=1, First floor=2, Second floor=3 etc)

60. Does your bedroom — NO YES
    - 60.1 have fitted carpets covering the whole floor?
    - 60.2 contain rugs?
    - 60.3 have double glazing/triple glazing
    - 60.4 have visible wet or damp spots
    - 60.5 have an airbrick or open chimney
    - 60.6 have radiators that are the main source of room heating
    - 60.7 get condensation on the window especially in the winter

FLOOR

61. On what floor is the room in which you sleep?

(Basement = 00 ,Ground floor=1, First floor=2, Second floor=3 etc)

YEARS

62 How old is the mattress you currently sleep on??

NO YES

63. Do you sleep with the windows open at night during winter?

IF 'NO' GO TO QUESTION 64, IF 'YES':

63.1 Do you sleep with the windows open — TICK ONE BOX ONLY
- a) all of the time? 1
- b) sometimes? 2
- c) only occasionally? 3

NO YES

64. Do you keep a cat?

IF 'NO' GO TO QUESTION 65, IF 'YES' — NO YES

64.1 Is your cat (are your cats) allowed inside the house?

64.2 Is your cat (are your cats) allowed in the bedroom?

NO YES

65. Do you keep a dog?

IF 'NO' GO TO QUESTION 66, IF 'YES': — NO YES

65.1 Is your dog (are your dogs) allowed inside the house?

65.2 Is your dog (are your dogs) allowed in your bedroom?

NO YES

66. Do you keep any birds?

IF 'NO' GO TO QUESTION 67, IF 'YES': — NO YES

66.1 Are any of these birds kept inside the house?

67. In the <u>last 12 months</u>, how often have you done any of the <u>cleaning</u> in your own home? — TICK ONE BOX ONLY
- a) Never 1
- b) On less than 1 day per week 2
- c) On 1 to 3 days per week 3

d) On 4 to 7 days per week 4 [ ]

IF 'NEVER' GO TO 68, IF 'EVER':

67.1 In the last 12 months, on how many days a week have you personally used the following cleaning products in your own home? (SHOW CARD WITH FOLLOWING OPTIONS)

1. Never
2. <1 day/week
3. 1-3 days/week
4. 4-7 days/week

CODE
Enter code 1-4 for all boxes

		CODE
67.1.1	Bleach *(NOT bleach used for laundry)*	
67.1.2	Ammonia	
67.1.3	Stain removers or other solvents	
67.1.4	Acids (including decalcifiers, liquid scale removers, vinegar, hydrochloric acid, …)	
67.1.5	Floor polish or floor wax	
67.1.6	Liquid or solid furniture polish or wax	
67.1.7	Furniture sprays (atomisers or aerosols)	
67.1.8	Sprays for mopping the floor	
67.1.9	Glass cleaning sprays (atomisers or aerosols)	
67.1.10	Degreasing sprays including oven cleaning sprays (atomisers or aerosols)	

68. How often are the following used in your home? (SHOW CARD WITH FOLLOWING OPTIONS)

1. Never
2. <1 day/week
3. 1-3 days/week
4. 4-7 days/week

CODE
Enter code 1-4 for all boxes

		CODE
68.1	Liquid or solid perfumes or scents	
68.2	Plug-in or other **electric** air fresheners	
68.3	Air refreshing sprays (atomisers or aerosols)	

IF NEVER USE AIR FRESHENER SPRAYS GO TO QUESTION 69: IF USE AIR FRESHENER CODE
Enter code 1-4

68.4 How often do you use air freshening sprays(atomisers or aerosols) **yourself** inside your home? [ ]

69. How often are the following used in your home? (SHOW CARD WITH FOLLOWING OPTIONS)

1. Never
2. Sporadically
3. Depending on the season
4. The whole year round

CODE
Enter code 1-4 for all boxes

		CODE
69.1	Insecticides or other pesticides in powder form	
69.2	Plug-in or other **electric** insecticides/pesticides	
69.3	Insecticides or other pesticides in spray form	

IF NEVER USE SPRAY INSECTICIDES GO TO QUESTION 70:IF USE SPRAY INSECTICIDES CODE
Enter code 1-4

69. 4 How often do you use insecticides or other pesticides in spray form [ ]

yourself inside your home?

70. We would like to know where you have lived since January 1990.

Please give the address, including postcode, of all homes you have lived in **for at least one year since 1990, starting with your current address**

House number	Street name	City	Postcode	Moved in	Lived there until (YEAR)
					current

All centres please note: this information will be used for your centre staff to geocode residence.
Please do not attempt to send these data to the coordinating centre.

The variables needed within each centre ultimately will be (rh=residential history)
rh70.1.1 CURRENT Year moved in
rh70.1.2 CURRENT Year moved out
rh70.1.3 CURRENT Geocode
rh70.2.1 HOUSE 1 Year moved in
rh70.2.2 HOUSE 1 Year moved out
rh70.2.3 HOUSE 1 Geocode

Further instructions will follow

70.1 How often do cars pass your house? TICK ONE BOX ONLY

a) more than 80 per hour	1 ☐
b) between 21 and 80 per hour	2 ☐
c) between 5 and 20 per hour	3 ☐
d) less than 5 per hour	4 ☐

70.2 How often do heavy vehicles (trucks/buses) pass your house? TICK ONE BOX ONLY

a) more than 80 per hour	1 ☐
b) between 21 and 80 per hour	2 ☐
c) between 5 and 20 per hour	3 ☐
d) less than 5 per hour	4 ☐

71. How many days per week do you commute to work — NUMBER ☐

IF '0' GO TO QUESTION 72; IF ONE OR MORE DAYS

71.1 On average, how much time do you spend travelling to and from work each day (total for both directions)? — MINUTES ☐☐☐

71.2 What is your main method of commuting?

TICK ONE BOX ONLY

a) Walking or cycling	1 ☐
b) In a private car	2 ☐
c) Bus	3 ☐
d) Train	4 ☐
e) Other	5 ☐

72. Have you ever had an illness or trouble caused by eating a ***particular*** food or foods? — NO ☐ YES ☐

IF 'NO' GO TO QUESTION 73, IF 'YES':

72.1 Have you nearly always had the same illness or trouble after eating this type of food? — NO ☐ YES ☐

IF 'NO' GO TO QUESTION 73, IF 'YES':

72.2 Was this food any of the following?

	NO	YES
72.2.1 Cow's milk*	☐	☐
72.2.2 Hen's eggs	☐	☐
72.2.3. Fish	☐	☐
72.2.4 Shrimp or Lobster	☐	☐
72.2.5 Peanut	☐	☐
72.2.6 Hazelnut	☐	☐
72.2.7 Walnut	☐	☐
72.2.8 Peach	☐	☐
72.2.9 Apple	☐	☐
72.2.10 Kiwi fruit	☐	☐
72.2.11 Bananas	☐	☐
72.2.12 Melon	☐	☐
72.2.13. Tomato	☐	☐
72.2.14 Celery	☐	☐
72.2.15 Carrot	☐	☐
72.2.16 Soybean	☐	☐
72.2.17 Lentils	☐	☐
72.2.18 Wheat**	☐	☐

72.2.19 Buckwheat ☐ ☐
72.2.20 Corn ☐ ☐
72.2.21 Rice ☐ ☐
72.2.22 Sesame seed ☐ ☐
72.2.23 Mustard seed ☐ ☐
72.2.24 Sunflower seed ☐ ☐
72.2.25 Poppy seed ☐ ☐

* Including other cow's milk products such as butter, cheese, yoghurt, crème fraiche, fromage frais….
** Including wheat products such as bread and breakfast cereals

72. 3 Have you had any problems eating any other food or foods? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 72.4, IF 'YES PLEASE LIST THESE FOODS:

72.3.1 Food 1______________________________ CODE ☐☐☐

72.3.2 Food 2______________________________ CODE ☐☐☐

72.3.3 Food 3______________________________ CODE ☐☐☐

72.4 Please answer each of these questions for the three foods causing the main problems. Please identify the food from the list of foods given (q72.2.1-25). If than three foods are given in the list provide information on foods in 72.3.1-3. Please list in order of the most severe reaction

FOOD ONE

72.4.1 Please confirm the name of this food ______________________ CODE ☐☐☐

72.4.2-11 Did this illness or trouble include

	NO	YES
72.4.2 a rash or itchy skin?	☐	☐
72.4.3 diarrhoea or vomiting?	☐	☐
72.4.4 runny or stuffy nose?	☐	☐
72.4.5 severe headaches?	☐	☐
72.4.6 breathlessness?	☐	☐
72.4.7 itching, tingling or swelling in the mouth, lips or throat?	☐	☐
72.4.8 difficulty swallowing?	☐	☐
72.4.9 fainting or dizziness?	☐	☐
72.4.10 symptoms so severe you had an emergency injection from a doctor, or had to use an epipen	☐	☐
72.4.11 other______________________	☐	☐

72.4.12 . How soon after eating this food did you get the first symptoms? TICK ONE BOX ONLY

a) Less than half an hour	1	☐
b) Half an hour to one hour	2	☐
c) One hour to two hours	3	☐
d) Two hours to four hours	4	☐
e) More than four hours	5	☐

72.4.13 How old were you when you first had this attack? YEARS ☐☐

72.4.14 How old were you when you last had this attack? YEARS ☐☐

NUMBER

72.4.15 How many times has this occurred during your life? [ ][ ]

FOOD TWO

CODE

72.5.1 Please confirm the name of this food ______________________ [ ][ ][ ]

72.5.2-11 Did this illness or trouble include	NO	YES
72.5.2 a rash or itchy skin?	[ ]	[ ]
72.5.3 diarrhoea or vomiting?	[ ]	[ ]
72.5.4 runny or stuffy nose?	[ ]	[ ]
72.5.5 severe headaches?	[ ]	[ ]
72.5.6 breathlessness?	[ ]	[ ]
72.5.7 itching, tingling or swelling in the mouth, lips or throat?	[ ]	[ ]
72.5.8 difficulty swallowing?	[ ]	[ ]
72.5.9 fainting or dizziness?	[ ]	[ ]
72.5.10 symptoms so severe you had an emergency injection from a doctor, or had to use an epipen	[ ]	[ ]
72.5.11 other______________________________________	[ ]	[ ]

72.5.12 . How soon after eating this food did you get the first symptoms? TICK ONE BOX ONLY

a) Less than half an hour	1	[ ]
b) Half and hour to one hour	2	[ ]
c) One hour to two hours	3	[ ]
d) Two hours to four hours	4	[ ]
e) More than four hours	5	[ ]

YEARS

72.5.13 How old were you when you first had this attack? [ ][ ]

YEARS

72.5.14 How old were you when you last had this attack? [ ][ ]

NUMBER

72.5.15 How many times has this occurred during your life? [ ][ ]

FOOD THREE

CODE

72.6.1 Please confirm the name of this food ______________________ [ ][ ][ ]

72.6.2-11 Did this illness or trouble include	NO	YES
72.6.2 a rash or itchy skin?	[ ]	[ ]
72.6.3 diarrhoea or vomiting?	[ ]	[ ]
72.6.4 runny or stuffy nose?	[ ]	[ ]
72.6.5 severe headaches?	[ ]	[ ]
72.6.6 breathlessness?	[ ]	[ ]
72.6.7 itching, tingling or swelling in the mouth, lips or throat?	[ ]	[ ]
72.6.8 difficulty swallowing?	[ ]	[ ]
72.6.9 fainting or dizziness?	[ ]	[ ]
72.6.10 symptoms so severe you had an emergency injection from a doctor, or had to use an epipen	[ ]	[ ]
72.6.11 other______________________________________	[ ]	[ ]

72.6.12 . How soon after eating this food did you get the first symptoms? TICK ONE BOX ONLY

a) Less than half an hour	1	[ ]

b) Half and hour to one hour 2 ☐
c) One hour to two hours 3 ☐
d) Two hours to four hours 4 ☐
e) More than four hours 5 ☐

72.6.13 How old were you when you first had this attack? YEARS ☐☐

72.6.14 How old were you when you last had this attack? YEARS ☐☐

72.6.15 How many times has this occurred during your life? NUMBER ☐☐

73. When you are near animals, such as cats, dogs or horses, do you ***ever***

	NO	YES
73.1 start to cough?	☐	☐
73.2 start to wheeze?	☐	☐
73.3 get a feeling of tightness in your chest?	☐	☐
73.4 start to feel short of breath?	☐	☐
73.5 get a runny or stuffy nose or start to sneeze?	☐	☐
73.6 get itchy or watering eyes?	☐	☐

IF NO TO ALL SYMPTOMS GO TO QUESTION 74;IF YES TO ONE OR MORE SYMPTOMS

73.7.1-4 Do you have such symptom/s when you are near

	NO	YES
73.7.1 cat?	☐	☐
73.7.2 dog?	☐	☐
73.7.3 horse?	☐	☐
73.7.4 other?	☐	☐

74. When you are in a dusty part of the house, or near pillows or duvets do you ***ever***

	NO	YES
74.1 start to cough?	☐	☐
74.2 start to wheeze?	☐	☐
74.3 get a feeling of tightness in your chest?	☐	☐
74.4 start to feel short of breath?	☐	☐
74.5 get a runny or stuffy nose or start to sneeze?	☐	☐
74.6 get itchy or watering eyes?	☐	☐

75. When you are near trees, grass or flowers, or when there is a lot of pollen about, do you ***ever***

	NO	YES
75.1 start to cough?	☐	☐
75.2 start to wheeze?	☐	☐
75.3 get a feeling of tightness in your chest?	☐	☐
75.4 start to feel short of breath?	☐	☐
75.5 get a runny or stuffy nose or start to sneeze?	☐	☐
75.6 get itchy or watering eyes?	☐	☐

IF 'YES' TO ANY OF THE ABOVE:

75.7.1-4 Which time of year does this happen?

	NO	YES
75.7.1 winter	☐	☐
75.7.2 spring	☐	☐
75.7.3 summer	☐	☐
75.7.4 autumn	☐	☐

	NO	YES
76. Have you ever smoked for as long as a year?	☐	☐

['YES' means at least 20 packs of cigarettes or 12 oz (360 grams) of tobacco in a lifetime, or at least one cigarette per day or one cigar a week for one year]

IF 'NO' GO TO QUESTION 77, IF 'YES':

76.1 How old were you when you started smoking? — YEARS [ ][ ]

76.2 How old were you when you started smoking daily? — YEARS [ ][ ]
Never smoked daily please enter 88

76.3 Do you **now** smoke, as of ***one month ago***? — NO [ ] YES [ ]
IF 'NO' GO TO QUESTION 76.4, IF 'YES':

76.3.1-4 How much do you ***now*** smoke on average? — NUMBER
- 76.3.1 number of cigarettes per day [ ][ ]
- 76.3.2 number of cigarillos per day [ ][ ]
- 76.3.3 number of cigars a week [ ][ ]
- 76.3.4 pipe tobacco in a) ounces / week [ ][ ]
- b) grams / week [ ][ ][ ]

76.4 Have you stopped or cut down smoking? — NO [ ] YES [ ]
IF 'NO' GO TO QUESTION 76.5, IF 'YES':

76.4.1 Did you stop or cut down due to breathing problems? — NO [ ] YES [ ]

76.4.2 How old were you when you stopped or cut down smoking? — YEARS [ ][ ]

76.4.3.1-4 ***On average*** of the entire time you smoked, before you stopped or cut down, how much did you smoke? — NUMBER
- 76.4.3.1 number of cigarettes per day [ ][ ]
- 76.4.3.2 number of cigarillos per day [ ][ ]
- 76.4.3.3 number of cigars a week [ ][ ]
- 76.4.3.4 pipe tobacco in a) ounces / week [ ][ ]
- b) grams / week [ ][ ][ ]

76.5 Do you or did you inhale the smoke? — NO [ ] YES [ ]

77. Have you been **regularly** exposed to tobacco smoke in the last ***12 months***? ['Regularly' means on most days or nights] — NO [ ] YES [ ]
IF 'NO' GO TO QUESTION 78, IF 'YES':

77.1. Not counting yourself, how many people in your household smoke regularly? — NUMBER [ ][ ]

77.2 Do people smoke regularly in the room where you work? — NO [ ] YES [ ]

77.3 How many hours per day are you exposed to ***other people's*** tobacco smoke? — HOURS [ ][ ]

77.4 How many hours per day, are you exposed to other peoples tobacco smoke in the following locations? — HOURS
- at home [ ][ ]
- at workplace [ ][ ]
- in bars, restaurants, cinemas or similar social settings [ ][ ]
- elsewhere [ ][ ]

78. Have you used any **inhaled** medicines to help your breathing at any time in the last ***12 months***? — NO [ ] YES [ ]
IF NO' GO TO QUESTION 79, IF 'YES':
Which of the following have you used in the last ***12 months***?

78.1 <u>short acting</u> beta-2-agonist (only) inhalers — NO [ ] YES [ ]
(Please include combinations that include beta 2 and steroids in section 78.6)

78.1.1 If used, which one? _________________________________
78.1.2 What type of inhaler do you use?

NUMBER
78.1.3. What is the dose per puff (in micrograms)?

78.1.4. In the last 3 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1
b) in short courses 2
c) continuously 3
d) not at all 4

If answer to 78.1.4 is <u>when needed</u>: NUMBER
78.1.5 Number of puffs per month
If answer to 78.1.4 is <u>in short courses</u> NUMBER
78.1.6 number of courses
78.1.7 number of puffs per day
78.1.8 average number of days per month
If answer to 78.1.4 <u>is continuously</u> NUMBER
78.1.9 number of puffs per day

NO YES
78.2 <u>long acting</u> beta-2-agonist inhalers
(Please include combinations that include long acting beta 2 and steroids in section 78.6)
78.2.1 If used, which one? _________________________________
78.2.2 What type of inhaler do you use?

NUMBER
78.2.3. What is the dose per puff (in micrograms)?

78.2.4. In the last 3 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1
b) in short courses 2
c) continuously 3
d) not at all 4

If answer to 78.2.4 is <u>when needed</u>: NUMBER
78.2.5 Number of puffs per month
If answer to 78.2.4 is <u>continuously</u> NUMBER
78.2.6 number of puffs per day

NO YES
78.3 <u>short acting</u> anti-muscarinic inhalers

78.3.1 If used, which one? _________________________________
78.3.2 What type of inhaler do you use?

NUMBER
78.3.3. What is the dose per puff (in micrograms)?

78.3.4. In the last 3 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1
b) in short courses 2
c) continuously 3
d) not at all 4

If answer to 78.3.4 is <u>when needed</u>: NUMBER
78.3.5 Number of puffs per month

If answer to 78.3.4 is continuously: NUMBER

78.3.6 Number of puffs per day

NO YES

78.4 long acting anti-muscarinic inhalers

78.4.1 If used, which one? ________________________________

78.4.2 What type of inhaler do you use?

NUMBER

78.4.3. What is the dose per puff (in micrograms)?

78.4.4. In the last 3 months, how have you used them: TICK ONE BOX ONLY

a) when needed 1

b) in short courses 2

c) continuously 3

d) not at all 4

If answer to 78.4.4 is when needed: NUMBER

78.4.5 Number of puffs per month

If answer to 78.4.4 is continuously: NUMBER

78.4.6 Number of puffs per day

NO YES

78.5 inhaled steroids (ONLY)

(Please include combinations that include beta 2 and steroids in section 78.6)

78.5.1 If used, which one? ________________________________

78.5.2 What type of inhaler do you use?

NUMBER

78.5.3. What is the dose per puff (in micrograms)?

78.5.4. In the last 3 months, how have you used them: TICK ONE BOX ONLY

a) when needed 1

b) in short courses 2

c) continuously 3

d) not at all 4

If answer to 78.5.4 is when needed: NUMBER

78.5.5 Number of puffs per month

If answer to 78.5.4 is in short courses NUMBER

78.5.6 number of courses

78.5.7 number of puffs per day

78.5.8 average number of days per month

If answer to 78.5.4 is continuously NUMBER

78.5..9 number of puffs per day

NUMBER

78.5.10 How many times over the last 3 months have you temporarily increased this treatment because your symptoms became worse?

NO YES

78.6 **inhaled steroids and beta2 agonists (combined therapy)**

78.6.1 If used, which one? ________________________________

78.6.2 What type of inhaler do you use?

NUMBER

78.6.3. What is the dose per puff (in micrograms)?
(Please insert the dose of the inhaled steroid)

78.6.4. In the last 3 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1
b) in short courses 2
c) continuously 3
d) not at all 4

If answer to 78.6.4 is <u>when needed</u>: NUMBER
78.6.5 Number of puffs per month

If answer to 78.6.4 is in <u>short courses</u> NUMBER
78.6.6 number of courses
78.6.7 number of puffs per day
78.6.8 average number of days per month

If answer to 78.6.4 is <u>continuously</u> NUMBER
78.6.9 number of puffs per day

NUMBER
78.6.10 How many times over the last 3 months have you temporarily increased this treatment because your symptoms became worse?

NO YES
78.7 inhaled cromoglycate/nedocromil

78.7.1 If used, which one? ________________________________

NUMBER
78.7.2. What is the dose per puff (in milligrams)?

78.7.3. In the last 3 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1
b) in short courses 2
c) continuously 3
d) not at all 4

If answer to 78.7.3 is <u>continuously</u>: NUMBER
78.7.4 Number of puffs per day

NO YES
78.8 inhaled compounds

78.8.1 If used, which one? ________________________________
78.8.2 What type of inhaler do you use?

NUMBER
78.8.3. What is the dose per puff (in micrograms)?

79. Have you used any **pills**, **capsules**, **tablets** or **medicines**, other than inhaled medicines, to help your breathing at any time in the last ***12 months***? NO YES

IF 'NO' GO TO QUESTION 80, IF 'YES':
Which of the following have you used in the last ***12 months***?

NO YES
79.1 **oral beta-2-agonists**

79.1.1 If used, which one? ________________________________
79.1.2 what dose of tablet

79.1.3. In the last 3 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1

b) in short courses 2 ☐
c) continuously 3 ☐
d) not at all 4 ☐

If answer to 79.1.3 is continuously: NUMBER

79.1.4 Number of tablets per day ☐☐

NO YES

79.2 **oral methylxanthines** ☐ ☐

79.2.1 if used, which one? ________________________________ ☐☐
79.2.2 what dose of tablet ☐☐☐

79.2.3. In the last 3 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1 ☐
b) in short courses 2 ☐
c) continuously 3 ☐
d) not at all 4 ☐

If answer to 79.2.3 is continuously: NUMBER

79.2.4 Number of tablets per day ☐☐

NO YES

79.3 **oral steroids** ☐ ☐

79.3.1 If used, which one? _____________________________ ☐☐
79.3.2 what dose of tablet ☐☐☐.

79.3.3. In the last 12 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1 ☐
b) in short courses 2 ☐
c) continuously 3 ☐

If answer to 79.3.3 is when needed: NUMBER

79.3.4 number of tablets per month ☐☐

If answer to 79.3 3 is in short courses NUMBER

79.3.5 number of courses ☐☐
79.3.6 tablets per day ☐☐
79.3.7 average number of days per month ☐☐

If answer to 79.3.3 is continuously NUMBER

79.3.8 tablets per day ☐☐

NO YES

79.3.9. Have you used them in the last ***3 months***? ☐ ☐

NO YES

79.4 oral anti-leukotrienes ☐ ☐

79.4.1 If used, which one? ____________________________ ☐☐
79.4.2 what dose of tablet ☐☐☐

79.4.3. In the last 3 months, how have you used them: TICK ONE BOX ONLY
a) when needed 1 ☐
b) in short courses 2 ☐
c) continuously 3 ☐
d) not at all 4 ☐

If answer to 79.4.3 is continuously: NUMBER

79.4.4 Number of tablets per day ☐☐

ECRHS III MAIN Q - MARCH VERSIONS

80. Have you **ever** used inhaled steroids (show list, including combined therapy)? NO ☐ YES ☐

IF NO GO TO QUESTION 81;IF YES

80.1 How old were you when you first started to use inhaled steroids? YEARS ☐☐

80.2 How old were you when you last use inhaled steroids? YEARS ☐☐

80.3. Have you used inhaled steroids ***every year*** since the last survey? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 80.4: IF 'YES'

80.3.1 On average how many months each year have you taken them? MONTHS ☐☐

NOW GO TO QUESTION 81

80.4 How many of the years since the last survey have you taken inhaled steroids? YEARS ☐☐

IF 'NONE' ENTER 00 AND GO TO QUESTION 81;IF 'YES'

80.4.1 On average how many months of each of these years have you taken them? MONTHS ☐☐

81. Have you had a course of antibiotics in the last 12 months to help your breathing? NO ☐ YES ☐

IF NO GO TO QUESTION 82;IF YES

81.1 How many courses of antibiotics? NUMBER ☐☐

82. Have you used antibiotics for nasal/sinus problems in the last 12 months? NO ☐ YES ☐

83. Have you **ever** had any vaccinations or injections for the treatment of allergy or had a course of desensitisation? NO ☐ YES ☐

IF NO GO TO QUESTION 84;IF YES

83.4.1 What was this treatment? CODE ☐☐

83.4.2 Have you had this treatment in the last 12 months? NO ☐ YES ☐

IF HAS HAD ANOTHER VACCINATION,INJECTION OF DESENSITISATION

83.4.3 What was this treatment? CODE ☐☐

83.4.4 Have you had this treatment in the last 12 months? NO ☐ YES ☐

84. Are you usually vaccinated against flu? NO ☐ YES ☐

IF NO GO TO QUESTION 85;IF YES

84.1 Were you vaccinated against flu in the last winter period? NO ☐ YES ☐

85. Have you been vaccinated against pneumonia (Pneumovax) in the last 5 years? NO ☐ YES ☐ DK ☐

86. Have you used any other **remedies** to help your breathing at any time in the last ***12 months***? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 87 IF 'YES':

86.1. What remedies? ____

____

____

87. Has your doctor ever prescribed medicines, including inhalers, for your breathing? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 88, IF 'YES':

87.1 If you are prescribed medicines for your breathing, do you ***normally*** take

TICK ONE BOX ONLY

a) all of the medicine? 1 ☐

b) most of the medicine? 2 ☐

c) some of the medicine? 3 ☐
d) none of the medicine? 4 ☐

87.2 ***When your breathing gets worse***, and you are prescribed medicines for your breathing, do you normally take

TICK ONE BOX ONLY

a) all of the medicine? 1 ☐
b) most of the medicine? 2 ☐
c) some of the medicine? 3 ☐
d) none of the medicine? 4 ☐

87.3 Do you think it is bad for you to take medicines all the time to help your breathing? NO ☐ YES ☐

87.4 Do you think you should take as much medicine as you need to get rid of ***all*** your breathing problems? NO ☐ YES ☐

88. What medication, regardless of cause, have you taken regularly for <u>more than 6 of the last 12 months?</u> *(DO NOT include the respiratory medication given in previous questions)*

IF NONE, PROCEED TO Q89, OR COMPLETE THE TABLE

	Medication (name)	A	N	N	A	A	N	N
88.1								
88.2								
88.3								
88.5								
88.6								
88.7								
88.8								
88.9								
88.10								

A=letter N=digit (of seven alphanumeric ATC code)

89. How often do you take paracetamol?

TICK ONE BOX ONLY

a) never 1 ☐
b) less than once a month 2 ☐
c) more than once a month but not every week 3 ☐
d) at least once a week 4 ☐
e) every day 5 ☐

IF LESS THAN WEEKLY GO TO QUESTION 90; IF 'WEEKLY' OR 'DAILY'

89.1 Please give the main reason that you take paracetamol?

TICK ONE BOX ONLY

a) headache 1 ☐
b) backache or arthritis 2 ☐
c) chest problems 3 ☐
d) menstrual pain 4 ☐
e) other – please describe_____________________________________ 5 ☐

90. How often do you take pain killers other than paracetomol?

TICK ONE BOX ONLY

a) never 1 ☐
b) less than once a month 2 ☐
c) more than once a month but not every week 3 ☐
d) at least once a week 4 ☐
e) every day 5 ☐

IF LESS THAN WEEKLY GO TO QUESTION 91; IF 'WEEKLY' OR 'DAILY'

90.1 Please give the main reason that you take these other painkillers? TICK ONE BOX ONLY

a) headache	1	☐
b) backache or arthritis	2	☐
c) chest problems	3	☐
d) menstrual pain	4	☐
e) other – please describe____	5	☐

91. Do you have or have you ever had any of the following illnesses. If yes, please indicate the age you were first diagnosed with the disease?

		NO		YES			YEARS				
91.1.1	Stroke				91.1.2	Age diagnosed					
91.2.1	Angina, heart attack, coronary heart disease				91.2.2	Age diagnosed					
91.3.1	Insulin dependent diabetes				91.3.2	Age diagnosed					
91.4.1	Non-insulin dependent diabetes				91.4.2	Age diagnosed					
91.5.1	Cancer				91.5.2	Age diagnosed			91.5.3	Type of cancer	
91.6.1	Depression				91.6.2	Age diagnosed					
91.7.1	Hypertension				91.7.2	Age diagnosed					
91.8.1	Osteoporosis				91.8.2	Age diagnosed					
91.9.1	Crohns Disease				91.9.2	Age diagnosed					
91.10.1	Migraine				91.10.2	Age diagnosed					
91.11.1	Rheumatoid arthritis				91.11.2	Age diagnosed					
91.12.1	Ankylosing spondylitis, psoariatic arthritis				91.12.2	Age diagnosed					
91.13.1	Gastro-oesophagel reflux hiatus hernia or oesophagitis				91.13.2	Age diagnosed					

Code for 91.5.3
1= breast
2= prostate
3= lung
4= GI tract
5= other

92 Do you have any long term limiting illness not mentioned above and not including asthma, COPD, chronic bronchitis or emphysema?? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 93, IF 'YES':

92.1 Please name this condition___________________________________ CODE ☐☐

93. ***Since the last survey***, have you visited a hospital casualty department or emergency room (for any reason, apart from accidents and injuries)? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 94. IF 'YES':

93.1. Was this due at least once to ***breathing problems***? NO ☐ YES ☐

93.2 Have you visited a hospital casualty department or emergency room (for any reason, apart from accidents and injuries) **<u>in the last 12 months</u>**? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 94, IF 'YES':

93.2.1 How many times in the last ***12 months***? TIMES ☐☐

93.2.2 Among these ones, how many times because of ***breathing problems***? TIMES ☐☐

[Write '0' if s/he had not visited the emergency room for breathing problems]

94. ***Since the last survey***, have you spent a night in hospital (for any reason, apart from accidents and injuries)? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 95, IF 'YES':

94.1 Was this due at least once to ***breathing problems***? NO ☐ YES ☐

94.2 Have you spent a night in hospital (for any reason, apart from accidents and injuries) **<u>in the last 12 months</u>**? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 95, IF 'YES':

94.2.1 How many nights in the last ***12 months***? NIGHTS ☐☐

94.2.2 Was this due at least once to ***breathing problems***? NO ☐ YES ☐

IF 'NO' GO TO QUESTION 95, IF 'YES':

94.3.1-5 In the last ***12 months*** how many nights have you been hospitalized in each of the following types of ward for ***breathing problems***? NIGHTS

94.3.1 general

94.3.2 chest medicine

94.3.3 rehabilitation

94.3.4 intensive care unit

94.3.5 other

95. In the last ***12 months*** have you been seen by a general practitioner (for any reason, apart from accidents and injuries)? NO YES

IF 'NO' GO TO QUESTION 96, IF 'YES': TIMES

95.1 How many times **in the last 12 months**?

TIMES

95.2 Of these, how many were for ***breathing problems***?

[Write '0' if not been seen by general practitioner in the last 12 months for breathing problems]

96. In the last ***12 months*** have you seen a specialist (for any reason, apart from accidents and injuries)? NO YES

IF 'NO' GO TO QUESTION 97, IF 'YES': TIMES

96.1 How many times in the last ***12 months***?

96.2 How many times have you seen a specialist (chest physician, allergy specialist, internal medicine specialist, ENT doctor) because of ***breathing problems in the last 12 months***? TIMES

[Write '0' if not been seen by a specialist in the last 12 months for breathing problems]

97. Are you given regular appointments to be seen by a doctor (or nurse) because of ***breathing problems***? NO YES

98. In the last ***12 months*** how many times have you visited the following because of ***breathing problems***? TIMES

98.1 nurse

98.2 physiotherapist

98.3 practitioner of 'alternative' medicine

99. In the last ***12 months*** have you had any clinical or laboratory tests because of health problems (apart from accidents and injuries)? NO YES

IF 'NO' GO TO QUESTION 100, IF 'YES': NO YES

99.1 Was this due at least once to ***breathing problems***?

IF 'NO' GO TO QUESTION 100, IF 'YES':

99.1.1-5 In the last ***12 months*** how many times have you had the following tests for ***breathing problems***? TIMES

99.1.1 breathing test in a laboratory specially for lung function measures

99.1.2 skin test for allergy

99.1.3 blood test for allergy

99.1.4 x-rays

99.1.5 thorax CT

100. In the last ***12 months*** have you lost days of work because of health problems (apart from accidents and injuries)? NO YES HAVE NOT WORKED IN THE LAST 12 months

IF NOT WORKED OR HAS NOT LOST DAYS OF WORK GO TO QUESTION 101; IF 'YES' DAYS

100.1 How many days **in the last 12 months?**

100.2 Among these ones, how many because of breathing problems?

*[**Write '000' if not lost any days due to breathing problems]***

101. **Since the last survey** were you forced to give up working altogether because of health problems (apart from accidents and injuries)? NO YES

IF 'NO' GO TO QUESTION 102, IF 'YES': MONTH YEAR

101.1 When did this occur ?

NO YES

101.2 Were you forced to give working altogether because of **breathing problems**?

102. In the last ***12 months*** have there been any days when you have had to ***give up activities other than work*** (e.g. looking after children, the house, studying) because of health problems (apart from accidents and injuries)? NO YES

IF 'NO' GO TO QUESTION 103, IF 'YES': DAYS

102.1 How many days **on average** each month?

DAYS

102.2 Among these ones, how many because of ***breathing problems***?

[Write '0' if s/he has not had any days of activity lost due to breathing problems]

103. Interview type TICK ONE BOX ONLY

1 face to face interview at clinic
2 telephone
3 face to face at home
4 other

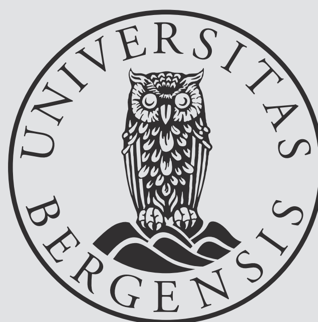
104. Date of birth check. What is the date of birth of this participant? DAY MONTH YEAR

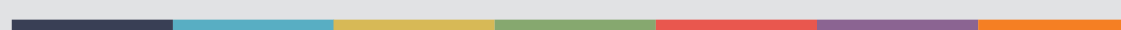
105. Which of the following best describes you?

TICK ONE BOX ONLY

1 Single
2 Married/cohabiting
3 Separated/Divorced
4 Widowed
5 Other or do not wish to answer

Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS





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ISBN: 9788230866450 (print)
9788230851685 (PDF)