



University of Zagreb

Faculty of Science

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**METAGENOMIC ANALYSIS OF DUST
MICROBIOME IN THE HOMES OF
ASTHMATIC CHILDREN**

DOCTORAL THESIS

Zagreb, 2025



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DOCTORAL THESIS

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University of Zagreb

Prirodoslovno-matematički fakultet

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**METAGENOMSKA ANALIZA
MIKROBIOMA U UZORCIMA PRAŠINE U
DOMOVIMA DJECE S ASTMOM**

DOKTORSKI RAD

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Zagreb, 2025

This doctoral thesis was made at the Laboratory for Molecular Anthropology and the Laboratory for Chemical and Biomedical Informatics, Institute for Anthropological Research, Zagreb, under the supervision of Adjunct Associate Professor Natalija Novokmet, PhD, and Adjunct Assistant Professor Mario Lovrić, PhD, within the EDIAQI project funded by the European Union's Horizon Europe research and innovation programme (No. 101057497). Bioinformatic analyses were partially performed at the Institute of Environmental Biotechnology, Graz University of Technology, and Department of Food Science, University of Copenhagen.

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METAGENOMSKA ANALIZA MIKROBIOMA U UZORCIMA PRAŠINE U DOMOVIMA DJECE S ASTMOM

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Kućna prašina predstavlja složen mikrobni ekosustav s mogućim utjecajem na respiratorno zdravlje. Ovo istraživanje, provedeno u sklopu EDIAQI projekta, koristi metagenomske metode za usporedbu mikrobioma prašine u domovima djece s astmom i bez nje, naglašavajući utjecaj unutarnjeg okoliša na respiratorno zdravlje. Analize su otkrile različite mikrobne obrasce između dviju skupina, ukazujući na potencijalne mikrobne pokazatelje astmatskog statusa. Bakterijske i gljivične zajednice pokazale su razlike u prisutnosti pojedinih taksa, pri čemu su rodovi *Staphylococcus* i *Malassezia* glavni pokazatelji varijacija. Istraživanje dodatno potvrđuje postojeće dokaze da su mikrobne zajednice zatvorenih prostora oblikovane različitim utjecajima, bakterije ponajviše ovise o unutarnjim čimbenicima poput prisutnosti i aktivnosti ljudi, dok su gljivice više pod utjecajem vanjskih okolišnih uvjeta. Ovo je prvo istraživanje takve vrste u Hrvatskoj i pruža vrijednu osnovu za buduća istraživanja respiratornog zdravlja i unutarnjeg okoliša.

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METAGENOMIC ANALYSIS OF DUST MICROBIOME IN THE HOMES OF ASTHMATIC CHILDREN

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Household dust represents a complex microbial ecosystem with potential implications on respiratory health. This study, part of EDIAQI project, uses metagenomics to compare dust microbiomes in homes of children with and without asthma, highlighting indoor environment impacts on respiratory health. Analyses revealed distinct microbial signatures between the two groups, highlighting potential microbial indicators of asthma status. Bacterial and fungal communities showed distinct taxa differences, with *Staphylococcus* and *Malassezia* driving much of the variation. The study further supports existing evidence that indoor microbial communities are shaped by different influences, bacterial taxa are primarily driven by indoor factors such as human occupancy and activity, while fungal communities are more influenced by outdoor environmental conditions. This study is the first of its kind in Croatia and provides a valuable foundation for future investigations of respiratory health and indoor environment.

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ABBREVIATIONS

DALY – disability-adjusted life years

PM – particulate matter

VOC – volatile organic compounds

IAQ – indoor air quality

COPD – Chronic obstructive pulmonary disease

FENO – fractional exhaled nitric oxide

16S – 16S ribosomal RNA

ITS – internal transcribed spacer

PCR – polymerase chain reaction

OTUs – operational taxonomic units

ASVs – amplicon sequence variants

WGS – whole-genome shotgun sequencing

DNA – deoxyribonucleic acid

PCA – principal component analysis

PERMANOVA – permutational multivariate analysis of variance

α or α diversity – alpha diversity

β or β diversity – beta diversity

1. INTRODUCTION

Indoor air pollution is a major public health challenge, particularly in urban and residential setting. Household air pollution was associated with 1.8 million deaths and 60.9 million disability-adjusted life years (DALYs) in 2017, with the majority of the afflictions occurring in low- and middle-income countries (1,2). Contaminants such as particulate matter (PM), volatile organic compounds (VOCs), and biological agents (pollen, house dust mites, microbes, etc.) contribute significantly to indoor air quality (IAQ). Poor IAQ is linked to a range of health problems such as asthma, acute respiratory infection in both adults and children, chronic obstructive pulmonary disease, lung cancer, and tuberculosis [1,2].

While chemical pollutants are relatively well studied, the role of biological pollutants, particularly the microbiome, is less understood. The microbiome, defined as the collection of microorganisms and their genetic material in a specific environment, plays a pivotal role in maintaining human health [4]. The respiratory microbiome, in particular, interacts closely with the immune system, influencing inflammation and susceptibility to diseases such as asthma [5–7]. Asthma is a multifactorial disease influenced by genetic predisposition and environmental exposures [8]. While significant progress has been made in understanding outdoor air pollution and its role in asthma [9–12], the impact of indoor microbiomes remains underexplored. Recent research highlights that early-life exposure to diverse microbial environments, such as farms or homes with pets, may reduce asthma risk through immune modulation [13–15]. On the other hand, certain microbial taxa have been implicated in the onset or aggravation of respiratory conditions [16,17]

Household dust serves as a significant reservoir for airborne microbial communities, including Bacteria, Fungi, and viruses. These microorganisms are continuously resuspended into the air, where they can be inhaled and potentially impact respiratory health. Studies suggest that the microbial composition of indoor environments is influenced by a range of factors, including human occupancy, pets, ventilation, and the surrounding outdoor environment [5,15,16,18–23]. Despite these observations, there remains a critical gap in understanding how specific microbial exposures, such as those found in dust, may aggravate or protect against respiratory illnesses like asthma. In addition, the majority of existing research is focused on bacterial component of microbiome, while Fungi have been relatively overlooked. This is a significant oversight, given the allergenic potential of many fungal species [24–26]. Moreover, the functional interactions between bacterial and fungal communities in dust are poorly understood, further limiting ability to assess their

combined effects on health. Understanding the composition and dynamics of the dust microbiome is necessary for identifying potential microbial risk factors or protective agents in asthma.

Traditional microbiological methods, such as culturing, have provided fundamental knowledge of indoor microbiomes. However, they have limited ability to obtain the full diversity of microbial communities. The application of advanced culture-independent molecular techniques, supported by high-throughput sequencing technologies, has significantly improved understanding of the microbial ecosystems [13,27–29]. These methods allow simultaneous identification and quantification of bacterial and fungal taxa, including those that are unculturable. Moreover, metagenomics provides insights into the functional potential of microbial communities, enabling researchers to link microbial composition with ecological and health-related outcomes [30,31]. The integration of bioinformatics tools has further enhanced the resolution and accuracy of microbiome studies. Statistical analyses, such as differential abundance testing and diversity metrics, allow researchers to identify microbial taxa or community patterns associated with specific environmental or health conditions [31–33].

By clarifying the role and differences in microbiome in homes of asthmatic and healthy children, this study provides a foundation for developing evidence-based interventions. For instance, understanding the influence of pets on the dust microbiome could inform household recommendations for families managing asthma. Additionally, the findings may contribute to broader efforts to improve IAQ and reduce its burden on health. This study is the first of its kind in Croatia [34], aiming to gain a deeper understanding of the interaction between the microbiome and environmental factors, and their potential impact on asthma development. Overall, this research aims to broaden knowledge of the complex interactions between the environmental microbiome and children's health, providing critical insights to inform public health strategies.

1.1. Objectives and hypotheses

Hypothesis 1: There is a difference in the environmental microbiome between children with and without asthma.

- I. Research Objective 1: Conduct metagenomic analysis of samples.
- II. Research Objective 2: Understand the microbial composition of dust samples in the homes of children with and without asthma.
- III. Research Objective 3: Understand the relevance of alpha and beta diversity in relation to phenotypes.

Hypothesis 2: Environmental factors influence the environmental microbiome in children's homes.

- I. Research Objective 1: Determine how environmental factors qualitatively and quantitatively affect the microbiome.

Hypothesis 3: The environmental microbiome differs compared to other cohorts.

- I. Research Objective 1: Compare the obtained results with other cohorts in terms of alpha and beta diversity.
- II. Research Objective 2: Clarify possible patterns of differences compared to other cohorts.

2. LITERATURE REVIEW

2.1. Introduction to asthma

Asthma is a significant noncommunicable disease that affects both children and adults. It is the most common chronic illness among children, but it impacts people of all ages. Asthma is a long-term lung condition caused by airway inflammation and muscle constriction, making breathing difficult [35,36]. The pathogenesis of asthma results from complex interactions among different cell types, including immune cells, airway epithelial cells, smooth muscle cells, and inflammatory cells, along with numerous biologically active proinflammatory mediators [37]. Typical symptoms include coughing, wheezing, shortness of breath, and chest tightness. These symptoms vary in severity and can appear intermittently. Though asthma can be serious, it is manageable with appropriate treatment [35,36].

Certain conditions, such as colds or weather changes, can worsen symptoms. Triggers also include dust, smoke, pollen, pet dander, and strong odours. Several factors are associated with an increased risk of developing asthma, although identifying a single cause can be challenging as is a highly heterogeneous disease [14,36]. Asthma is an immune-mediated disease that likely originates in early life, when the developing immune system is especially vulnerable to changes influenced by the exposome [14]. Asthma is a multifactorial disease, so choosing a single cause is very difficult. It results from complex interactions between genetic and environmental factors, leading to an exaggerated immune response [8]. Some of the risk factors include a family history of asthma, other allergies such as eczema or hay fever, urbanization, early-life events such as low birth weight, premature birth, tobacco smoke exposure, air pollution, and respiratory infections. Other risk factors are exposure to environmental allergens and irritants, including air pollution, dust mites, moulds, and workplace chemicals or dust. Overweight or obese children and adults are at a higher risk of developing asthma [36,38–40].

A visual comparison of asthma's impact across different regions globally is presented in Figure 1. The world map illustrates the geographical distribution of asthma-related DALYs, where one DALY represents a lost year of "healthy" life. DALYs are a widely used metric to assess the impact of various health conditions on both mortality and morbidity, providing a comprehensive measure of public health burden [41]. As depicted, asthma is a global issue and a significant public health challenge. According to this metric, Croatia stands out for its relatively low incidence of asthma-

related DALYs, indicating a lower asthma burden compared to other regions. This suggests that Croatia is performing well in managing asthma.

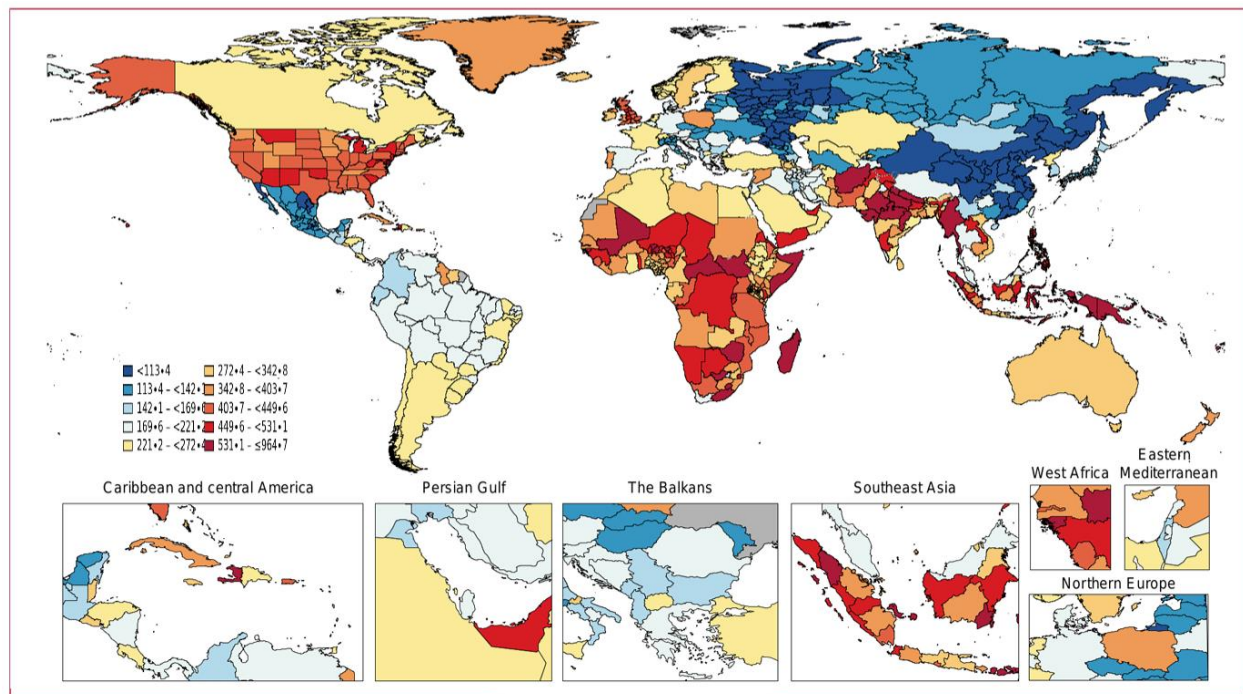


Figure 1. Age-standardised DALY rates (per 100,000) by location, both genders combined, 2021. Sourced from [41].

According to the Croatian Institute of Public Health, the annual incidence rate of asthma (new cases per year) in Croatia is 3.0 per 1,000 inhabitants, or approximately 12,000 new asthma cases per year. The age-specific incidence distribution is as expected, the incidence rate is higher among younger individuals, with a rate of 5.9 per 1,000 people under the age of 20, and 2.5 per 1,000 people over the age of 65. The prevalence of asthma (total number of cases) in Croatia is 5,048.1 per 100,000 inhabitants, or 5.0% of the total population, which is approximately 200,000 people. As with incidence, the prevalence rate is higher among younger age groups [42].

Asthma can be life-threatening in severe cases, but more commonly, it disrupts everyday life and diminishes quality of life, particularly for children. For young people, asthma can interfere with daily activities, school attendance, and participation in physical activities, limiting their social interactions and physical development [36,43,44].

2.2. *The role of the microbiome in respiratory health*

The microorganisms in, on, and around our bodies make up a significant portion of the biodiversity we encounter in our lives. While Bacteria represent the most diverse kingdom on Earth, Fungi remain relatively unexplored, and it is believed that our understanding of them is still quite limited. Each body niche is colonized by a microbiome, composed of organisms from all domains of the tree of life: Eukarya, Bacteria, and Archaea, as well as viruses. Together, these microorganisms contribute to the human body's overall composition, affecting various phenotypes. The microbiome has a profound effect on our health, both positively and negatively. Humans are more than just their physiology, they also rely on diverse symbiotic microbes. These microbes provide unique metabolic pathways that perform important physiological functions [45,46].

Over the last years more and more is talked about interaction of microbiome with immune system [47–49]. Health problems that increasingly affect the population, such as allergies, autoimmune diseases, and inflammatory disorders, are result of unsuccessful regulation of immune responses against self. Although the causes of previously mentioned pathologies are highly complex, they can also originate from the microbiome. Changes in the composition and function of the microbiota, due to factors like antibiotic use, dietary shifts, or stress, can change once symbiotic or commensal species into parasitic ones. The relationships between humans and microbiome is often referred to as commensal one although the symbiotic relationship includes a range of interactions, mutualistic, parasitic, and commensal, among other [50].

2.2.1. *Healthy respiratory microbiome*

The composition of the respiratory microbiota in humans is increasingly well-characterized. At the phylum level, Proteobacteria, Firmicutes, and Bacteroidetes are the most frequently identified. At the genus level, dominant groups include *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacteria*, and *Veillonella*, with smaller contributions from potential pathogens such as *Haemophilus* and *Neisseria*. Although the anatomy of respiratory system must be taken into consideration. The upper respiratory tract is predominantly inhabited by bacterial families such as *Dolosigranulum* and *Corynebacterium*, viral families like *Anelloviridae*, and fungal species including *Aspergillus* spp., *Penicillium* spp., *Candida* spp., and *Alternaria* spp. Similarly, the lower respiratory tract is primarily colonized by bacteria such as *Haemophilus* spp., *Moraxella* spp., *Streptococcus* spp., *Staphylococcus* spp., and members of the phyla Firmicutes and Bacteroidetes, along with fungi

from genera like *Eremothecium*, *Systemotrema*, and *Malassezia*. Characterizing the lung microbiome is expected to offer valuable insights into the underlying mechanisms of most common respiratory related diseases [51,52].

The human nasal passages harbour diverse bacterial communities that play a key role in resisting pathogens and modulating immune responses. Across various studies and age groups, the genera most frequently reported as dominant include *Moraxella*, *Streptococcus*, *Haemophilus*, *Staphylococcus*, *Corynebacterium*, *Dolosigranulum*, *Neisseria*, *Gemella*, *Granulicatella*, *Cutibacterium*, *Turicella*, and *Alloiococcus* [53]. As for the age-related differences, in children, the microbiota is typically dominated by Proteobacteria and Firmicutes, particularly *Moraxella*, *Haemophilus*, and *Streptococcus* [54]. In adulthood, a notable change occurs toward Actinobacteria, with genera such as *Corynebacterium*, *Cutibacterium*, and *Turicella* becoming more prominent [53].

Beyond the lung microbiome, the gastrointestinal microbiota plays a critical role in shaping and maintaining lung immunity and regulating inflammation. Investigating the interactions between the gut and respiratory systems holds significant potential for advancing understanding of the pathogenesis of pulmonary diseases, including asthma. A hypothesis linking asthma to the microbiome suggests that disruptions in gastrointestinal microbiota composition, caused by factors such as antibiotic use and poor dietary habits in western countries, have interfered with the development of immunological tolerance. Supporting this "microbiota hypothesis" are data showing correlations between asthma or allergies and antibiotic use in industrialized countries, as well as associations between altered gut microbiota and atopic diseases [52].

2.2.2. Respiratory microbiome and asthma

Although the link between bacterial infections and asthma exacerbations is well-established, more intriguing are the differences between the "healthy" lung microbiota and that of individuals with asthma. In healthy individuals, the lung microbiota is characterized by diverse taxa, including those mentioned in previous chapter. In contrast, the asthmatic lung microbiome is often dominated by pathogenic Proteobacteria, with *Haemophilus* spp. being more prevalent in the bronchi of asthmatics and COPD (Chronic obstructive pulmonary disease) patients compared to controls. On the contrary, Bacteroidetes, particularly *Prevotella* spp., are more common in healthy individuals [52,55]. Significant bacterial differences between asthmatic and non-asthmatic

individuals have been identified, primarily within the phyla Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria (mostly *Haemophilus*, *Moraxella*, and *Neisseria* spp). Similarly, notable fungal differences have been observed within the phyla Ascomycota, Basidiomycota, and other unclassified fungi. Differential abundance analysis based on asthma status shows a marked depletion of *Penicillium aethiopicum* and *Alternaria* spp. in individuals with poorly controlled asthma. Additionally, the data indicate a significant increase in *Malassezia* spp. and other unclassified fungi in the airways of individuals receiving therapy [51,56].

2.2.3. Interaction between respiratory microbiome and immune system

The role of the lung microbiome in the pathogenesis and progression of respiratory diseases remains incompletely understood, but emerging evidence emphasizes its significance in shaping and maintaining immune responses. Dysbiosis in the lung microbiome is thought to contribute to the onset of respiratory diseases, while the immune response, in turn, influences the composition of the pulmonary microbiome [57,6]. Interactions between the immune system and the lung microbiome appear to have a crucial role in the development of conditions like asthma and allergies, similar to the gut microbiome [56,58]. For instance, studies in mouse models have shown that members of the phylum Bacteroidetes, particularly *Prevotella* spp., can mitigate pulmonary inflammation, neutrophil recruitment, and proinflammatory cytokine production via Toll-like receptor 2, whereas *Haemophilus influenzae* has a proinflammatory effect [56,59]. In humans, asymptomatic adults with lung microbiota enriched in taxa typically found in the oral cavity exhibit increased inflammatory cells in the lower airways and elevated exhaled nitric oxide levels [56,60]. Furthermore, early-life exposure to a diverse array of bacteria has been associated with a reduced risk of asthma and atopy, likely due to interactions occurring in the airways, the body's largest interface with the external environment [18]. Recent studies have also suggested a gut-lung axis, wherein the lung and gut microbiomes communicate via blood circulation, enabling reciprocal modulation of immune responses and microbial composition, as demonstrated in mouse models [56,57].

2.3. Dust as a reservoir of microbiota

With the modernization of lifestyle comes increased urbanization, which results in people spending more time indoors. Whether in offices, schools, kindergartens, or recreational spaces like gyms, restaurants, or metros, a significant portion of free time is spent in indoor environments.

Consequently, individuals are exposed to a substantial diversity of microorganisms residing in indoor dust. Studies have estimated that house dust can harbour up to 1,000 different species of microbes [21,61]. The composition and diversity of the indoor dust microbiome have a significant impact on health. Dust-associated microorganisms and their by-products represent a significant source of exposure through inhalation [21].

2.3.1. Bacteria

House dust microbiomes are rich in bacterial taxa, dominated by Gram-positive genera such as *Deinococcus*, *Bacillus*, *Enterococcus*, *Lactococcus*, *Staphylococcus*, *Arthrobacter*, *Corynebacterium*, *Micrococcus*, *Nocardiopsis*, *Rhodococcus*, and *Streptomyces*, primarily belonging to the phyla Firmicutes and Actinobacteria [21,62]. These taxa reflect the contributions of human and environmental inputs. Gram-negative bacteria, though less dominant, are represented by taxa such as *Pseudomonas*, *Stenotrophomonas maltophilia*, and *Pantoea*. Other prevalent families include Sphingomonadaceae, Xanthomonadaceae, Oxalobacteraceae, and Rhizobiaceae [21,61,63].

Specific genera consistently identified in house dust include *Corynebacterium*, *Streptococcus*, *Acinetobacter*, and species *Propionibacterium acnes*, which are strongly associated with human microbiota [45,64]. *Klebsiella* and *Alloprevotella* are genera frequently enriched in environments with higher human occupancy [19]. *Staphylococcus* and *Streptococcus* are among the most consistently detected genera, reflecting their prevalence in human-associated environments [65,66]. Other significant bacterial genera, such as *Rothia*, *Haemophilus*, and *Paracoccus*, have been observed in specific contexts, such as infant bedding dust [67]. Notably, a significant number of mattress dust samples from farm children's beds were found to contain *Listeria monocytogenes*, interestingly found in farm-related environments as well [19,21,68]. *Pseudonocardia*, a soil-associated genus is frequently detected in HEPA filter dust [19,21]. *Mycobacteria* have also been shown to be abundant and diverse in house dust collected from vacuum cleaner bags [21,69].

Farm-related environments contribute additional bacterial taxa to house dust, enriching it with genera such as *Rhodococcus*, *Bifidobacterium*, *Corynebacterium*, and *Pseudomonas*. These genera highlight the influence of agricultural practices on indoor microbial communities, particularly in homes situated rurally [70].

Dust samples from offices and other public indoor spaces have revealed the presence of specific bacterial classes, including Bacteroidia, Clostridia, Bacilli, Gammaproteobacteria, and Alphaproteobacteria, with genera such as *Streptococcus* and *Pantoea* dominating microbial profiles in these settings. *Sphingomonas*, a genus within Alphaproteobacteria, is a notable contributor to house dust bacterial diversity, often linked to environmental reservoirs. Similarly, *Bacillus*, a widely distributed genus within Firmicutes, is consistently identified in household dust samples and is strongly associated with environmental inputs such as soil and agricultural sources [19].

Overall, house dust harbours a complex and dynamic bacterial community, with a wide array of taxa originating from human, environmental, and soil-associated sources. The dominance of certain genera, such as *Staphylococcus*, *Streptococcus*, *Corynebacterium*, and *Bacillus*, highlights their prevalence in the diverse indoor settings. Meanwhile, the presence of less common taxa, including *Listeria monocytogenes* and *Pseudonocardia*, shows the influence of specific environmental factors on dust microbial composition.

2.3.2. Fungi

Dust fungal communities have traditionally been studied using standard culture methods, revealing a dominance of specific species. Commonly identified species include *Aureobasidium pullulans*, *Alternaria alternata*, *Penicillium chrysogenum*, *Aspergillus penicilloides*, and *Aspergillus restrictus*, with *Eurotium repens* emerging as one of the most frequently identified species [21,71]. Yeasts such as *Candida*, *Cryptococcus*, *Rhodotorula*, *Saccharomyces*, and *Sporobolomyces* were also prevalent, along with sterile isolates from both ascomycetous and basidiomycetous colonies. In carpet dust samples *Eurotium repens*, *Penicillium chrysogenum*, *Alternaria alternata*, *Aureobasidium pullulans*, and *Phoma herbarum* were identified as the predominant species [21,72]. Similarly, in floor dust the most commonly identified genera included *Penicillium*, *Alternaria*, and *Cladosporium* [21,73].

Using culture-independent methods, such as high-throughput DNA sequencing, researchers have uncovered previously undetected fungal taxa, providing new insights into the diversity and complexity of dust fungal communities beyond what traditional culture methods could reveal. Household dust contains a diverse range of fungal taxa, dominated by species within the phyla

Ascomycota and Basidiomycota, which consistently appear in studies across various environments. Commonly identified genera include *Cladosporium*, *Aspergillus*, *Penicillium*, *Alternaria*, *Epicoccum*, *Phoma*, *Saccharomyces*, *Aureobasidium*, *Cryptococcus*, *Rhodotorula*, *Cyberlindnera*, and *Candida*, which together represent a wide range of fungal diversity. These genera are often associated with both indoor and outdoor environments and exhibit considerable adaptability [74–77].

Yeasts are an integral part of fungal communities in house dust, with genera like *Cyberlindnera*, *Cryptococcus*, *Aureobasidium*, *Rhodotorula*, *Candida*, and *Saccharomyces* frequently identified. These yeasts are particularly notable for their prevalence in damp indoor environments and their association with human activity [76,77]. Additionally, moisture-associated fungi such as *Malassezia*, *Phaeococcomyces nigricans*, *Aureobasidium pullulans*, *Leptosphaerulina americana*, *Macrophoma* spp., and *Thekopsora areolata* have been documented, often under specific environmental conditions that support their growth [74,75,77].

Mold species commonly detected in indoor dust include *Cladosporium cladosporioides*, *Cladosporium herbarum*, *Penicillium chrysogenum*, *Aspergillus fumigatus*, and *Alternaria alternata*, all of which are known for their potential health impacts and allergenic properties. Other frequently identified genera include *Epicoccum* and *Phoma*, which are prevalent in areas with high human activity, and *Eurotium repens*, often found in poorly ventilated spaces or homes with carpets [21,66]. Additional mould species such as *Trichoderma* spp., *Epicoccum nigrum*, and *Wallemia* spp. have also been reported [21].

Specific fungal taxa that dominate seasonal profiles include *Saccharomyces* and *Penicillium* during winter, while outdoor-derived fungi such as those in the orders *Agaricales* and *Polyporales* are abundant in summer and fall [78,79]. Notably, the class Dothideomycetes, including genera like *Epicoccum* and *Alternaria*, is among the most abundant taxa identified, while *Agaricomycetes*, which includes various basidiomycetous fungi, exhibits significant richness [74–77].

Rare and moisture-associated fungal genera such as *Malassezia* (associated with human skin), *Sphaerellopsis*, *Curvularia*, and *Aureobasidium* further highlight the taxonomic variety within indoor dust [67,80]. The presence of yeasts like *Cryptococcus* and *Saccharomyces* shows their prevalence in indoor spaces correlating with human-associated environments [65]. Meanwhile,

moulds such as *Alternaria alternata* and *Cladosporium cladosporioides* are recognized as dominant allergens in house dust, frequently linked to respiratory and allergic symptoms [21,66]. In total, the diversity of fungi in house dust encompasses a vast range of taxa, including filamentous moulds, yeasts, and other less common genera.

2.3.3. Dust microbiome diversity in relation to asthma

The relationship between the microbiome and asthma has become an increasingly prominent area of research, offering insights into novel mechanisms underlying asthma development and its progression. Studies have shown that microbial diversity, composition, and specific taxa in indoor environments significantly influence allergic and inflammatory pathways. Cox et al. [16] investigated childhood respiratory conditions, including asthma and wheeze, identifying bacterial taxa such as *Staphylococcus aureus*, *Alkanindiges illinoisensis*, and *Gardnerella vaginalis* as positively associated with these conditions. In contrast, bacteria like *Coprococcus eutactus*, *Sphingomonas parvus*, *Stenotrophomonas maltophilia*, and *Kineosporia rhamnosa* showed negative associations, indicating potential protective roles [16]. Similarly, Ege et al. [81] identified a range of bacteria inversely associated with asthma, suggesting their protective effect. These taxa included *Listeria monocytogenes*, *Bacillus* spp. (*Bacillus licheniformis*), *Corynebacterium* spp., *Methylobacterium* spp., *Xanthomonas* spp., and *Enterobacter* spp. [81]. In line with this, the protective effects of specific bacterial genera were proven, primarily from the Actinomycetales order, such as *Brevibacterium*, *Brachybacterium*, *Nocardioides*, and *Dietzia*, which were negatively associated with asthma. In contrast, *Lactococcus* and *Streptococcus* were positively associated, indicating increased risk. These findings underline the importance of early-life exposure to specific microbial communities in shaping asthma outcomes [20]. The concept of a "pro-asthmatic protective environment" explains the importance of exposing children to a diverse range of taxa in early childhood, with children living on farms as an example. The protective role of farm-like microbiota was highlighted, identifying taxa such as *Bacteroidales*, *Clostridiales*, *Lactobacillales*, *Methanobrevibacter*, and *Actinomycetales* as more abundant in farm homes and associated with reduced asthma risk. Human-associated bacteria like *Streptococcaceae* and *Staphylococcus* were more prevalent in non-farm homes and linked to higher asthma risk. This contrast between farm and non-farm environments underscores the role of microbial exposure and community composition in influencing asthma outcomes [20].

In adult populations, taxa such as *Porphyromonas*, *Bacteroides*, and *Fusobacterium* were found to be positively associated with asthma, suggesting a harmful role. Notably, some taxa, including *Porphyromonas* and *Fusobacterium*, were found to be associated with both asthma and hay fever, indicating overlapping microbial influences. Homes of individuals with asthma or atopy were also observed to have reduced microbial diversity, consistent with the hygiene hypothesis of “pro-asthmatic protective environment” [82].

In relation to asthma phenotypes and severity, bacterial taxa including Sphingomonadaceae, Methylocystaceae, *Erwinia*, *Sphingomonas*, and *Pseudomonas* were found to be enriched in patients with Type 2 (T2)-high severe asthma. These patients exhibited higher fractional exhaled nitric oxide (FENO) levels, a biomarker of airway inflammation, further supporting the link between specific microbial exposures and asthma pathophysiology [23].

Fungal taxa have been extensively studied in relation to asthma, as well, revealing both positive and negative associations that depend on species and environmental context. *Candida tropicalis* and *Aspergillus sydowii* were identified as positively associated with asthma and aeroallergen sensitivity, while *Toxicocladosporium irritans*, *Gibberella intricans*, and *Coniosporium apollinis* were negatively associated, indicating potential protective effects [16]. Similarly, Ege et al. [81] found that *Eurotium* spp. were inversely associated with asthma, suggesting a protective role, although the inverse association for *Penicillium* spp. was not statistically significant after adjusting for multiple comparisons [81]. In contrast, fungal taxa that were found to be associated with asthma, included *Aspergillus* spp., *Cladosporium* spp., *Epicoccum nigrum*, *Candida* spp., *Rhodotorula* spp., and *Cryptococcus* spp. Specific species such as *Aspergillus versicolor* and *Cladosporium sphaerospermum* were linked to elevated FENO levels, highlighting their role in asthma exacerbation. Medically relevant fungi like *Epicoccum* and *Cryptococcus* were also enriched in the indoor environments of patients with Type 2 (T2)-high severe asthma, further emphasizing their impact on respiratory health [23]. Kirjavainen et al. [15] explored the abovementioned concept of a “pro-asthmatic protective environment”, noting significant differences in fungal richness between farm and non-farm environments. Even though specific fungal taxa were not directly associated with asthma, a higher Farm-Like Microbiota Index (FaRMI), indicative of microbial compositions similar to farm environments, was linked to a reduced risk of asthma. This finding refers to the importance of fungal community composition

and diversity in providing asthma protection [15]. The "pro-asthmatic protective environment" concept in question highlights the importance of early childhood exposure to diverse microbial taxa, particularly for children living on farms. Farm environments, characterized by naturally higher microbial diversity, expose children to a wider variety of bacteria and Fungi, which has been associated with lower asthma prevalence compared to non-farm children [15,81]. Increased bacterial diversity in the home environment has also been linked to a reduced risk of asthma [81]. Similarly, Tischer et al. [22] found that higher fungal diversity in urban house dust during early life was inversely associated with aeroallergen sensitization at six years of age and wheezing up to ten years. However, these protective effects of fungal diversity diminished with age, likely due to changing microbial exposures in school and other environments. While bacterial diversity showed no significant protective or adverse associations in this study, other research has demonstrated its importance. For example, Karvonen et al. [20] reported that higher bacterial richness and Shannon diversity were inversely associated with asthma risk, with notable differences in community composition (β diversity) between homes of asthmatic and non-asthmatic children. Similarly, it was observed that homes of individuals with atopy or hay fever exhibited significantly less diverse bacterial communities, consistent with the hygiene hypothesis ("pro-asthmatic protective environment") [82]. On the contrary, decreased fungal diversity and increased bacterial diversity in indoor environments were characteristic of patients with Type 2 (T2)-high severe asthma. During asthma exacerbations, a greater overlap between fungal taxa in indoor dust and sputum samples was observed, linking indoor fungal exposure and respiratory inflammation [23]. Despite these findings, Cox et al. [16] emphasized the complexity of microbial community interactions, noting that microbial diversity did not directly correlate with health outcomes but that a combination of taxa, rather than individual species, influenced asthma and wheeze risks.

These studies collectively highlight the critical role of microbial diversity and the influence of specific microbial compositions on asthma risk. While diverse microbial exposure, particularly in early life, appears protective, the presence of specific bacterial and fungal taxa can either mitigate or exacerbate asthma-related outcomes, emphasizing the need for targeted strategies to improve indoor microbiota for respiratory health.

2.3.4. Factors influencing dust microbiome composition

Numerous studies have showed that specific environmental factors play a crucial role in shaping the microbiological composition of household dust. These factors include humidity, the presence of pets, building characteristics such as construction year and type, and demographic aspects like the number of household members. Indoor fungal communities are primarily influenced by the outdoor environment, while indoor bacterial communities are more affected by household occupants, pets, and ventilation methods [21,64,83]. A strong geographic pattern has been observed in indoor fungal communities [21,74], which are also subject to seasonal variations [21,25,77]. Household residents have a significant and homogenizing effect on indoor bacterial communities [21,84]. Non-human occupants, such as dogs and household insects, also have a notable influence on the indoor dust microbiota [21,85,86]. The most common environmental factors that impact the microbiome, along with their specific effects and references, are summarized below.

Occupancy and human activity significantly shape the composition of dust bacterial communities. Research has demonstrated that bacterial diversity in household dust correlates with the number of inhabitants; for instance, homes with more than three occupants display greater bacterial diversity than those with fewer [66,87]. The number and activity levels of occupants also influence microbial communities, with skin-associated bacteria being more prevalent in densely populated homes [88]. Higher human occupancy, such as in daycare main rooms, increases the abundance of human-associated microbial taxa like *Streptococcus* while reducing taxa typically found outdoors. In contrast, auxiliary rooms with lower occupancy exhibit microbiomes resembling outdoor environments [79]. Similar findings were reported by Nygaard and Charnock [65], who observed that in daycare centres, the number of children and their level of interaction directly influenced microbial diversity. Higher interaction promoted diversity in human-associated taxa, while less-used rooms maintained fungal communities resembling those found outdoors.

The presence of pets is a significant factor affecting the microbiota of indoor environments. Homes with pets, particularly dogs and cats, tend to exhibit higher bacterial diversity and altered fungal composition in bed dust. Pet presence has been linked to specific taxa, such as *Staphylococcus* and *Saccharomyces* [67]. Additionally, homes with pets show increased bacterial and fungal diversity

overall, with the microbiota composition being shaped by interactions between humans, pets, and their shared environment [80,88].

Seasonal changes play a critical role in shaping indoor fungal communities. Outdoor-derived taxa, such as those from the Basidiomycota phylum, are more abundant during summer and fall, while taxa originating indoors dominate in winter and spring. Geographic and climatic factors, including humidity and temperature, also influence the composition of indoor mycobiomes, with distinct taxa prevailing in humid coastal regions compared to drier inland areas [78]. Fungal diversity tends to increase from winter to fall, reflecting the significant influence of outdoor environments on indoor fungal communities through seasonal succession [77]. Fungal concentrations in house dust are generally higher during warmer seasons, with seasonal heating and air conditioning further impacting these concentrations by creating variable amplification patterns depending on indoor climate controls [89]. Overall, fungal communities exhibited clear seasonal variations, while bacterial compositions remained relatively stable across seasons [90].

Air pollution and urbanization significantly impact indoor microbial communities. Changes in air pollutant levels, such as reduced SO₂ and increased NO₂ and PM₁₀, are associated with shifts in bacterial and fungal composition. For example, high NO₂ levels are linked to an enrichment of taxa like Alphaproteobacteria, while lower NO₂ levels favour Bacilli and Clostridia [91]. Urbanization and surrounding vegetation also play a crucial role, particularly in shaping indoor fungal communities. Homes near natural environments exhibit higher fungal diversity, while bacterial diversity appears to be less influenced by these factors [88].

Building environmental factors, including building features and renovations, significantly influence the indoor microbiome. Characteristics such as construction year, ventilation type, and the number of rooms can shape indoor microbial communities, often reflecting outdoor microbiota depending on ventilation efficiency and geographic location [78]. Age of the home, specifically older homes are associated with higher fungal richness and diversity. Elevated Environmental Relative Moldiness Index (ERMI) values and higher relative humidity further influence fungal communities, while bacterial diversity often correlates with dog ownership [80]. Homes renovated using "green" practices show no consistent trends in fungal community composition compared to non-green homes. Variations were instead linked to sample types (air, bed dust, and floor dust) and temporal changes, indicating instability in the indoor mycobiome [75]. Homes with effective

ventilation systems host fungal communities resembling those found outdoors, highlighting the role of air exchange in promoting fungal diversity [66,88]. Building materials, namely the use of green versus conventional construction materials does not significantly alter fungal community structures. However, "tight" building designs that reduce ventilation can increase humidity levels and microbial loads, showing the importance of proper air circulation [75]. In that way, water damage promotes mould growth, which is associated with less diverse fungal communities but an increased abundance of specific taxa, such as *Cyberlindnera* and *Cryptococcus* [76]. Such factors show the importance of building features and maintenance on indoor microbial dynamics.

Finally, the antibiotic resistance, a pressing issue of the 21st century, has been detected with high diversity in household dust. This diversity is influenced by bacterial taxa and factors associated with human occupancy. These findings point out the role of indoor environments in harbouring antibiotic resistance genes [92].

The complex interplay of environmental, anthropogenic, and design factors shape indoor microbiomes, affecting both their structure and potential health impacts. Each factor offers insights into specific determinants, contributing to a broader understanding of indoor microbial ecology.

2.4. Analytical approaches in metagenomics

2.4.1. High-throughput sequencing and bioinformatics tools in metagenomics

Metagenomics, the study of genetic material recovered directly from environmental samples, has revolutionized understanding of microbial communities. This approach relies on high-throughput sequencing and bioinformatics tools. Among the most extensively used methods are 16S rRNA gene sequencing for taxonomic determining of Bacteria and Archaea (Figure 2a), and Internal Transcribed Spacer (ITS) sequencing for fungi (Figure 2b), offering a prospective into microbial diversity and community composition [67,93,94].

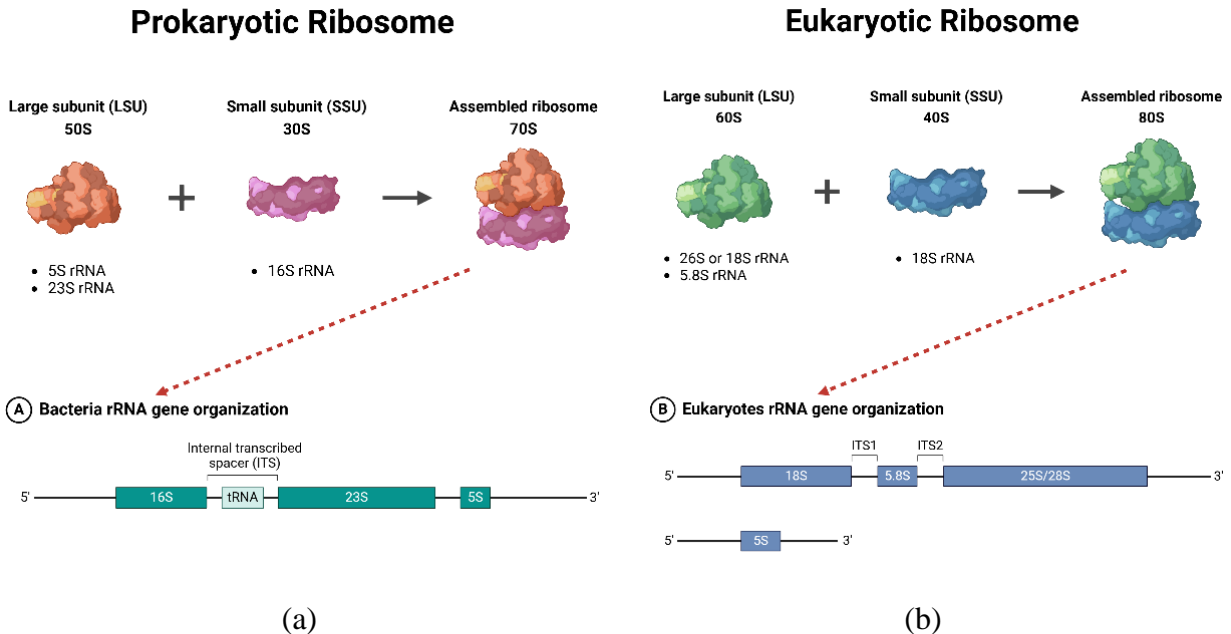


Figure 2. Ribosome structure. (a) Prokaryotic ribosome and Bacteria genomic arrangement. (b) Eukaryotic ribosome and genomic arrangement.

Metagenomic studies of the microbiome are conducted through the analysis of prokaryotic 16S ribosomal RNA, which contains approximately 1500 base pairs and nine variable regions. The V3-V4 regions contain variable segments that are characteristic of different Bacteria and Archaea species. As for fungi, metagenomic studies are conducted through the analysis of fungal ribosomal RNA, particularly the ITS region, which is widely used for fungal identification. The ITS region, located between the small and large ribosomal subunits, contains highly variable segments that are characteristic of different fungal species, enabling detailed taxonomic resolution in metagenomic analyses [93]. This is the “gold standard” for characterizing microbial diversity in diverse environments, including water systems, soil, and indoor dust microbiomes. This method relies on polymerase chain reaction (PCR) to amplify the targeted regions, followed by sequencing and bioinformatic analysis to classify organisms into operational taxonomic units (OTUs) or amplicon sequence variants (ASVs) [93–95]. Both approaches are cost-effective and widely used in environmental microbiome studies, where the aim is to investigate microbial communities in ecosystems ranging from soil to wastewater. However, limitations include the inability to capture functional genomic information and biases introduced by primer selection and PCR amplification [96].

In contrast to targeted approaches, whole-genome shotgun (WGS) sequencing captures the entire genetic content of a sample, offering a comprehensive view of microbial communities. This method allows for taxonomic classification, functional profiling, and even genome assembly of uncultured organisms. WGS sequencing has been used to investigate microbial community structure and function in environments ranging from the human gut to extreme ecosystems [97,98].

The development of sequencing platforms like Illumina has significantly reduced costs and improved accuracy, making metagenomics accessible to a broader range of researchers. Innovations such as paired-end sequencing and longer read lengths enhance assembly quality and taxonomic resolution. However, there are still challenges, including biases from DNA extraction, primer design, and sequence depth, which can influence the representation of low-abundance taxa [99]. In addition to mentioned technical challenges, computational complexity is also worth mentioning when referring to microbiome sequencing. Assembling genomes from microbial communities is complicated because of high heterogeneity and the presence of closely related strains. Mentioned obstacles require advanced algorithms and computing resources [96,98].

2.4.2. Statistical methods for microbiome data analysis

The analysis of microbiome datasets relies on statistical methods to interpret complex and multi-dimensional data. With advances in next-generation sequencing, statistical tools have become essential in uncovering patterns, relationships, and insights within microbial communities.

Ordination methods, such as principal component analysis (PCA), non-metric multidimensional scaling, and redundancy analysis (RDA), are widely used to reduce the complexity of microbiome data into lower dimensions, making it easier to visualize patterns and relationships among microbial communities. These methods rely on distance or dissimilarity matrices, such as Bray-Curtis or UniFrac, to represent relationships between samples based on microbial composition [30,32].

Permutational Multivariate Analysis of Variance (PERMANOVA) is a non-parametric method used to test whether the composition of microbial communities differs significantly across existing groups (asthma vs. control). It operates on a distance matrix, making it suitable for compositional microbiome data. PERMANOVA has been used extensively in microbiome studies to identify significant differences in community structure related to environmental, clinical, or experimental factors [31,33].

2.4.3. Challenges in dust microbiome analysis

Despite the abovementioned new approaches and solutions, the challenges remain in microbiome data analysis. Issues such as compositionality, sparsity, and batch effects can lead to biases if not appropriately addressed. Other challenges include high host DNA content, low microbial biomass, and high heterogeneity within samples, which can impact the quality and accuracy of metagenomic analyses.

One of the primary challenges in dust microbiome analysis is the vast presence of host DNA, often derived from human skin cells, pets, or plants. This contamination can dominate over microbial DNA during sequencing and data analysis. Bharti and Grimm [30] emphasize the importance of using selective DNA extraction methods and host DNA depletion techniques, such as methylation-specific enzymatic digestion, to reduce host DNA contamination and enhance microbial signal reliability.

Dust samples typically contain low microbial biomass, which complicates DNA extraction and library preparation. The limited quantity of microbial DNA can lead to biases during amplification, such as overrepresentation of certain taxa. Ju and Zhang [32] recommend employing optimized DNA extraction protocols to maximize DNA yield while minimizing biases. Using advanced sequencing platforms with high sensitivity can also help in detecting low-abundance taxa.

The microbial composition of dust is highly variable, influenced by factors such as geographic location, building design, occupant behaviour, and environmental conditions. This heterogeneity poses challenges for standardization and reproducibility across studies. The importance of rigorous sampling protocols and metadata collection should be emphasized, to capture variability systematically. Standardized sampling tools, can help ensure comparability across studies [30,31]. Same should be applied to statistical analyses and computational workflow [32].

2.5. Current gaps in the literature and future directions

Despite significant advancements in understanding the microbiome and its influence on asthma, numerous gaps remain in the literature that limit a comprehensive understanding of its role in respiratory health, dust-associated microbiota, and analytical methodologies.

Although studies have demonstrated the association between the microbiome and asthma, the mechanistic pathways linking specific microbial taxa to asthma phenotypes remain poorly

understood. Research has identified specific bacteria as enriched in Type 2 (T2)-high severe asthma [23], but causal relationships between microbial exposure and immune modulation are still unclear. In the same manner, some fungal taxa have been linked to asthma [16], yet the interaction between bacterial and fungal communities in shaping asthma outcomes requires further exploration.

Dust microbiome research faces unique technical and methodological challenges. The presence of high host DNA content, low microbial biomass, and the inherent heterogeneity of dust samples complicate data interpretation [32,100]. Furthermore, most studies have focused on either bacterial or fungal communities, often neglecting the interplay between these domains. Future research should aim for integrative approaches that analyse bacterial, fungal, and viral communities simultaneously to gain a general view of the dust microbiome.

Although numerous environmental factors are known to influence the microbiome, the relative contribution of each remains unclear. The combined effects of these factors have not been systematically studied, which limits our understanding of their interactions and overall impact on the microbiome. Additionally, the influence of individual practices, such as cleaning habits and ventilation preferences, on microbiome dynamics is poorly understood and requires further investigation.

Metagenomics has revolutionized microbiome research, limitations in current analytical approaches slow down the progress. Statistical methods like PERMANOVA and ordination techniques offer important perspectives but often fail to capture the full complexity of microbiome datasets, particularly in highly heterogeneous environments like dust [31,33]. Additionally, machine learning techniques have shown promise in identifying microbial signatures, yet their interpretability and reproducibility remain a challenge [100]. Future advancements in bioinformatics tools and algorithms will be critical to overcoming these limitations.

This study represents the first of its kind in Croatia, contributing novel insights into the dust microbiome and its potential role in asthma within this unique regional context.

3. MATERIALS AND METHODS

The research for EDIAQI project began in December 2022. The project partners in Zagreb pilot include Children's hospital Srebrnjak, the Institute for Anthropological Research, the Institute for Medical Research and Occupational Health, and Ascalia Ltd. Patient recruitment began in the spring of 2023, with the first examinations conducted in May 2024. Dust sample collection started alongside the children's examinations, with samples for this cohort collected from May 2023 to March 2024.

3.1. Participants

The cohort was divided into two parts, children with asthma, and healthy children as the control group. Children with asthma were recruited during the regular exams that are a part of their asthma management regime at Children's hospital Srebrnjak. Control group was recruited through public calls in newspapers, on social media, and via acquaintances of those involved in the project. All children underwent a medical examination at Children's Hospital Srebrnjak, and parents were asked to fill out the ISAAC and other relevant questionnaires. Asthma was assessed through a questionnaire filled out by the parents, answering whether asthma had been previously diagnosed by a doctor.

Our research initially included 100 children, with 50 being patients at Children's Hospital Srebrnjak and 50 being healthy, asymptomatic children. Out of these, 1 participant was not included in further analysis because not all medical check-ups could be completed (the blood sample could not be taken). Additionally, 2 participants withdrew from the study, and 2 participants were excluded because their place of residence is not within the City of Zagreb or Zagreb County. Therefore, 95 samples entered laboratory processing. However, 1 sample was removed due to unsuccessful DNA isolation, and 4 additional samples were removed because of an insufficient number of reads after sequencing, 3 after ITS sequencing and 1 after 16S sequencing, which is explained in the section about sequencing results. In the end, 10% of the initial cohort could not be processed, resulting in a final number of 90 participants (samples).

The cohort consists of 59 asthmatic children (65.5%) and 31 children without asthma (34.4%, control group), making up a total of 66 households. Some initially asymptomatic children from the control group were later diagnosed with asthma. All children were between 5 and 18 years (9.51 ± 3.58), and were from the City of Zagreb or Zagreb County. The sampling sites are illustrated in

Figure 3. The cohort includes 49 boys (54.5%) and 41 girls (45.6%), and the asthma distribution by gender is shown in the Figure 4.

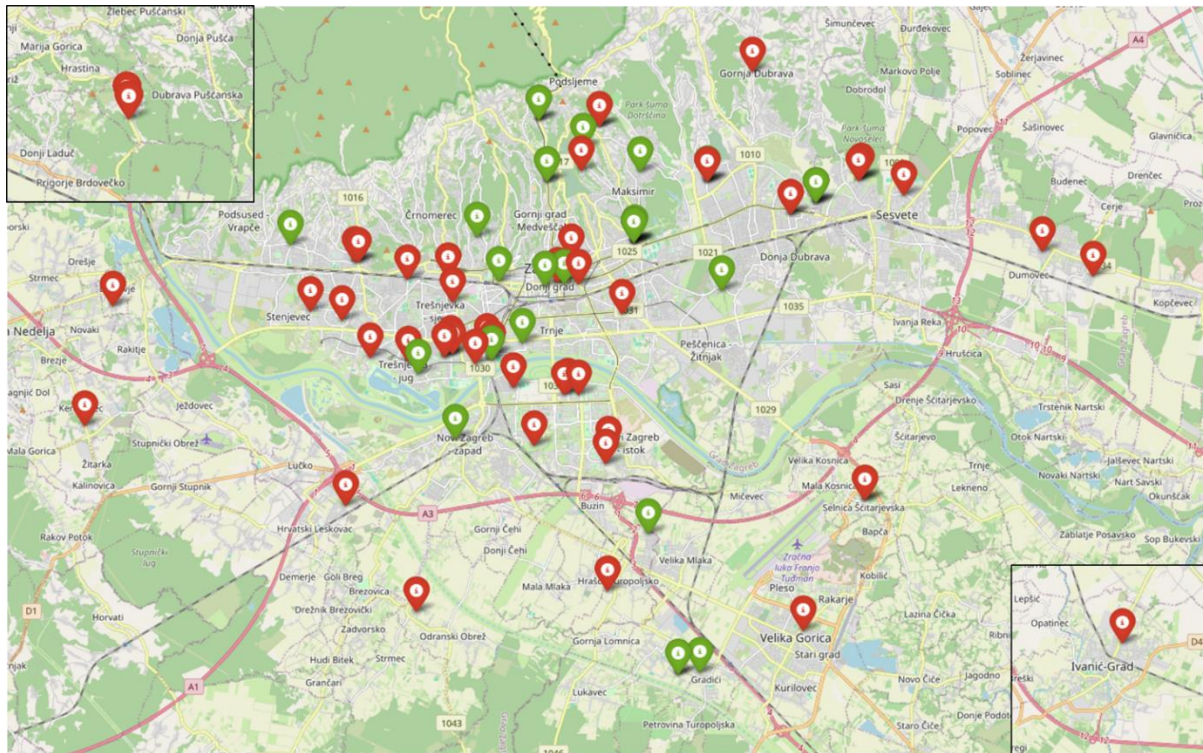


Figure 3. Sampling sites. Red markers represent asthmatic children, and green markers represent control group.

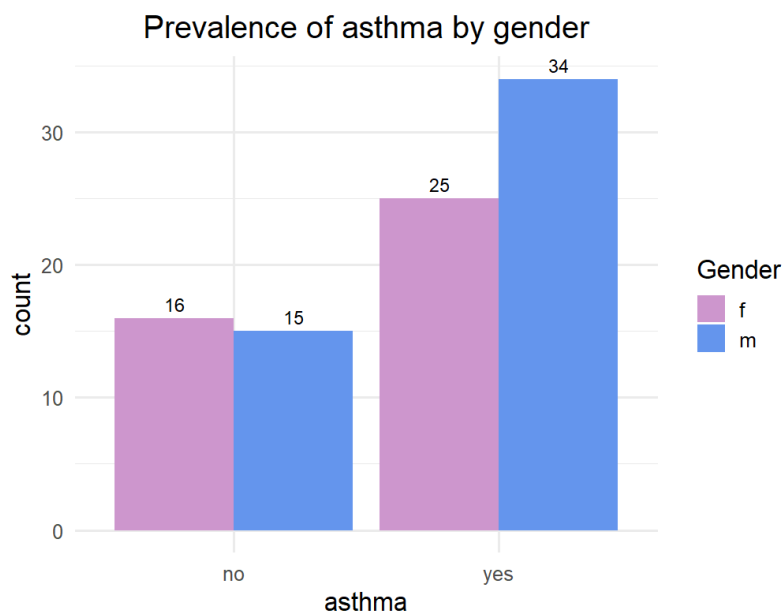


Figure 4. Prevalence of asthma by gender in Zagreb Pilot cohort.

3.2. Chemicals

- DNeasy PowerSoil Pro Kit (Qiagen, Germantown, Maryland, USA)
- Ethanol (96%, Gram-mol, Zagreb, Croatia)
- Qubit dsDNA BRAssay Kit (Invitrogen- Thermo Fisher Scientific, Eugene, Oregon, USA)
- Agilent D1000 ScreenTape (5067- 5582) (Agilent Technologies, Waldbronn, Germany)
- Agilent D1000 Reagents (5067- 5583) (Agilent Technologies, Waldbronn, Germany)
- PCR grade water (Corning® Molecular Biology Grade Water)
- KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland)
- Fungal forward primer gITS7F (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG ART CAT CGA RTC TTT G-3') (Integrated DNA technologies, Leuven, Belgium)
- Fungal reverse primer ITS4ngs (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTT CCT SCG CTT ATT GAT ATG C-3') (Integrated DNA technologies, Leuven, Belgium)
- Bacterial forward primer 341F (5'- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG -3') (Integrated DNA technologies, Leuven, Belgium)
- Bacterial reverse primer 806r (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3') (Integrated DNA technologies, Leuven, Belgium)
- Fungal forward primer ITS_fwd_1 (5'-TCG TCG GCG TCA GAT GTG TAT AAG AGA CAG CTT GGT CAT TTA GAG GAA GTA A -3') (Integrated DNA technologies, Leuven, Belgium)
- Fungal reverse primer ITS_rev_1 (5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGC TGC GTT CAT CGA TGC -3') (Integrated DNA technologies, Leuven, Belgium)
- Fungal reverse primer ITS_rev_4 (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGC TGC GTT CTT CAT CGA TGT -3') (Integrated DNA technologies, Leuven, Belgium)

- ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA)
- Magnesium chloride solution (Sigma Aldrich, Merck, Darmstadt, Germany)
- MagSi-NGSPREP Plus* beads (Magtivio, Nuth, The Netherlands)
- Nextera XT Index Kit v2 (Illumina, San Diego, California, USA)
- NaOH Reagecon Sodium Hydroxide 0.2M Analytical Volumetric Solution (Reagecon Diagnostics, Shannon, Co. Clare, Ireland)
- 10 mM Tris-HCl pH 8,5 (Omega Bio-tech Inc., USA)
- PhiX Control Kit v3 (Illumina, San Diego, California, USA)
- MiSeq Reagent Kit v3, 600 cycles (Illumina, San Diego, California, USA)

3.3. Instruments

- Vacuum cleaner Dyson V8 Absolute (Malmesbury, Wiltshire, UK)
- DUSTREAM® Collector vacuum cleaner nozzle and filters (Indoor Biotechnologies, Cardiff, Wales, UK)
- Freezer Thermo Scientific, -20°C (Thermo Fisher Scientific, Asheville, North Carolina, USA)
- Analytical scale Axis ALN120 (Axis, Gdańsk, Poland)
- MPS-1 High-Speed Multi Plate Shaker (BIOSAN, Riga, Latvia)
- Thermo Scientific™ MicroCL 21 R microcentrifuge (Thermo Fisher Scientific, Waltham, Maryland, USA)
- mySPIN™ 12 Mini Centrifuge (Thermo Fisher Scientific, Waltham, Maryland, USA)
- Thermo Heraeus Multifuge 3LStuart SA8 Vortex Mixer (Stuart, Stone, Staffordshire, UK)
- IKA MS 3 basic Vortexer (Staufen, Germany)
- Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, Maryland, USA)
- 4200 TapeStation (Agilent Technologies, Santa Clara, California, USA)
- Agilent SureCycler 8800 (Agilent Technologies, Santa Clara, California, USA)
- Thermo Scientific™ Thermal Mixer with Blocks (Thermo Fisher Scientific, Waltham, Maryland, USA)

- Thermo Scientific Megafuge 40R Refrigerated Centrifuge (Thermo Fisher Scientific, Waltham, Maryland, USA)
- Milli-Q® IQ 7000 Ultrapure Water Purification System (Sigma-Aldrich® Solutions, Darmstadt, Germany)
- Illumina MiSeq System SY-410-1003 (Illumina, San Diego, California, USA)

3.4. Preparation of the solutions

3.4.1. Qubit Assay

Solutions for fluorescence measurement were prepared using Qubit dsDNA HS Assay Kit following Qubit™ Assays Reference Card [101]. Before preparation, all solutions must be left at room temperature to equilibrate. The Qubit Working Solution was prepared by diluting Qubit reagent 1:200 in Qubit buffer to final volume of 200 μ L. For each sample and standard, 200 μ L is needed. For standards, 190 μ L of Working Solution was mixed with 10 μ L of each standard from the Qubit dsDNA HS Assay Kit. For samples, 199 μ L of Working Solution is mixed with 1 μ L of extracted DNA.

3.4.2. Agilent 2200 TapeStation system

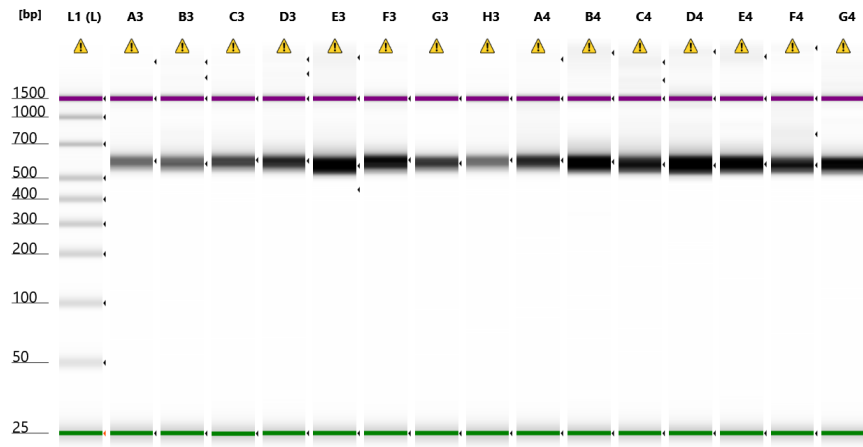
Samples were prepared according to the Agilent D1000 ScreenTape System Quick Guide [102]. Prior to use, all reagents were equilibrated to room temperature. The Agilent D1000 Reagents include D1000 Ladder and Sample Buffer. The reagents are vortexed, and the Ladder was prepared by mixing 1 μ L of Ladder with 3 μ L of Sample Buffer. Samples were prepared in same way, using 1 μ L of extracted DNA and 3 μ L of Sample Buffer. The prepared strips containing the samples were vortexed for 1 min and spun down to ensure the samples are at the bottom of the tube.

3.4.3. Controls

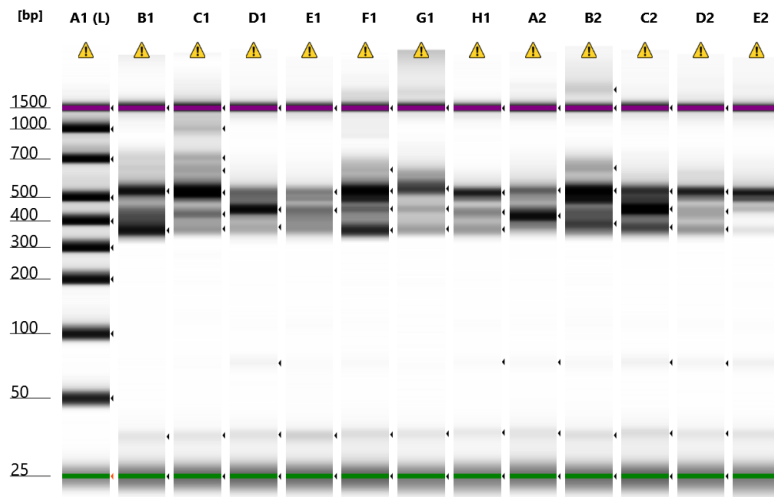
PCR-grade water was used as a negative control and processed in the same way as the samples. The ZymoBIOMICS Microbial Community DNA Standard was the positive control. It was diluted from its original concentration of 10 ng/ μ L to 5 ng/ μ L to match the concentrations of the other samples [103]. The positive control was then processed following the same protocol as the samples.

3.4.4. Primers

The study design protocol was initially based on Gupta et al., 2020 [5], but the ITS sequencing results were not optimal. Therefore, we experimented with different primers and optimized the PCR conditions. The first primer set used was from the Gupta et al. paper, and the second set consisted of ITS3F and ITS4R primers for the ITS1 region. Both sets were initially tested under standard PCR conditions. Since these attempts didn't yield improved results, we further modified the PCR protocol by trying the following variations: the Gupta et al. protocol with 30 cycles; Gupta et al. with 33 cycles; Gupta et al. with 30 cycles, an annealing temperature of 60°C, and a final elongation step of 7 minutes; the original Illumina protocol (Illumina, 2013); the Illumina protocol with an annealing temperature of 56°C; and nested PCR with 15 cycles. None of these adjustments improved the ITS results, so we decided to proceed with the original method from Gupta et al. Figure 5a. shows the automated gel electrophoresis results from the TapeStation for 16S, and Figure 5b. shows gel electrophoresis results using the final method by Gupta et al. with ITS2 primers (gITS7F and ITS4ngs).



(a)



(b)

Figure 5. TapeStation gel electrophoresis results for (a) bacterial, and (b) fungal samples.

Both bacterial (341F and 806r) and fungal (ITS7F and ITS4ngs) primers were diluted from stock solutions. According to the Integrated DNA Technologies (Leuven, Belgium) protocol the primer concentration needs to be 1 nM for further use. To achieve this, 10 μ L of stock primer was added to 990 μ L of PCR-grade water, resulting in working primers used in the PCR mix.

3.4.5. PCR mix

The PCR mix was made following 16S Metagenomic Sequencing Library Preparation Protocol [94]. The PCR mix for each sample was prepared by adding 5 μ L of PCR Forward Primer, 5 μ L

of PCR Reverse Primer, 12.5 μL of KAPA HiFi HotStart ReadyMix, and 2.5 μL of DNA diluted to 5 ng/ μL .

3.4.6. Ethanol 80%

80% ethanol for 96-well plate was prepared by mixing 41.6 mL of 96% ethanol with 8.4 mL of Milli-Q water (H_2O).

3.4.7. NaOH – Sodium Hydroxide 0.2M (0.2N)

NaOH Reagecon Sodium Hydroxide 0.2M (0.2N) Analytical Volumetric Solution was a ready to use solution for the denaturation of DNA libraries.

3.5. Detail description of methods

3.5.1. Sample collection

In accordance with the parents' agreement, during the house visits, dust samples were collected in participants homes. Children's mattresses were vacuumed using DUSTREAM® Collector vacuum cleaner filters placed in the DUSTREAM® Collector vacuum cleaner nozzle, which was placed on Dyson V8 Absolute vacuum cleaner, shown in Figure 6. The entire mattress, along with all items on it, including the bedding and stuffed animals, was vacuumed for 10 minutes. Dust samples were stored in DUSTREAM® Collector filters which were kept in labelled plastic resealable bags.



Figure 6. DUSTREAM® Collector vacuum cleaner filters and nozzle.

3.5.2. Sample preparation

Sample processing was done at the Laboratory for Molecular Anthropology at the Institute for Anthropological Research. The samples were stored at -20°C until DNA isolation to preserve genetic material and to eliminate mites.

Dust was weighted on analytic scale Axis ALN120 in special weighing plates (LLG, Meckenheim, Germany). Dust samples were carefully transferred into the weighing plates using tweezers with a straight blunt tip, which were disinfected with 80% ethanol between each sample transfer. Target mass of each sample was between 50-80 mg, if possible. This range was chosen after several trial isolations and has showed to have the best yield. Masses of samples are shown in the Results, and whole weighing process in Figure 7. For samples with a mass less than 50 mg, the entire dust sample was used, as it was sometimes difficult to extract more dust from the mattress.



Figure 7. Dust weighing process.

3.5.3. Genetic material isolation

Genetic material was isolated using the DNeasy® PowerSoil® Pro Kit. This kit was chosen after a literature review, as no specific kit exists for dust sample isolation. Kit is designed for isolation of microbial genomic DNA from all soil types and difficult samples such as sediment.

Isolation steps were performed following DNeasy® PowerSoil® Pro Kit Handbook (QIAGEN, 2023). The first step was mechanical shredding. Weighted dust was transferred into PowerBead Pro Tubes for homogenization and lysis. The tube contains buffer and beads that help with sample

dispersion while protecting nucleic acids from degradation. Samples were homogenized using MPS-1 High-Speed Multi Plate Shaker. PowerBead Pro Tubes were horizontally attached to shaker and shaken at 1500 rpm for 15 minutes. In order to separate dust particles, samples were centrifuged in Thermo Scientific™ MicroCL 17R Microcentrifuge at 15,000 g for 2 minutes. This is the only change in the steps from the Handbook. Since dust particles are lighter than soil, in order to effectively separate the dust from the supernatant, centrifugation for 1 min longer showed better results. Supernatant that still contains some dust particles was transferred and mixed with CD2 solution which contains IRT, a reagent that precipitates non-DNA organic and inorganic material. This step ensures DNA purity for downstream DNA applications. After another 15,000 g 1 minute centrifugation, samples were transferred once again. The supernatant was now clear, without dust particles. Next, CD3 was added, a high-concentrated salt solution so the DNA binds tightly to silica. The solution allows DNA binding but inhibits binding non-DNA organic and inorganic material. Silica in question is part of MB Spin Column tubes. Supernatant and CD3 solution mix was loaded to Spin Columns and centrifuged 15,000g for 1 min so the mix passes through the silica membrane in the Spin Column tubes. DNA bounds to the membrane and the contaminants passes through and are being discarded. Next, solution EA was added. EA is a wash buffer that removes protein and other non-aqueous contaminants. Washing is performed with centrifugation on 15,000 g for 1 minute. Next is ethanol-based solution CD5 which is used for further cleaning of DNA. Solution CD5 eliminates residual salt, humic acid and other impurities, while leaving DNA bound to silica membrane. Washing was again performed in centrifuge at 15,000 g for 1 minute. After discarding the flow-through, next step was drying the membrane in the centrifuge at 16,000 g for 2 minutes. This step is important because it removes all of the ethanol which can interfere with downstream DNA applications such as PCR. After the membrane was dry, 50 µL of solution C6 was added. C6 is 10 mM Tris solution which bounds all DNA from the membrane into the flow through. The elution contains isolated DNA and is ready for further use. Isolated DNA was stored at -20 °C as recommended in the protocol.

3.5.4. Fluorometer

Quantity of isolated DNA was examined on Qubit® 3.0 Fluorometer. Samples were prepared with Qubit dsDNA BR Assay Kit as described previously.

Qubit 3.0 Fluorometer was set up with DNA Broad Range and 1 µL of sample DNA settings. Standards were read first, and then samples. The results of DNA concentrations following isolation and sample weights prior to isolation are presented in the Supplementary Table 3.

3.5.5. Normalisation of the samples

Since NGS requires equal sample concentrations, after determining the concentrations using a fluorometer, the next step was to dilute the samples. For samples with concentrations below 5 ng/mL, DNA extraction was repeated. However, if the entire dust sample had already been used, those samples were processed further without additional dilution. The remaining samples were diluted to equal concentration of 5 nmol/mL using Corning® Molecular Biology Grade Water, according to the following formula:

$$c1 * V1 = c2 * V2$$

3.5.6. Amplicon PCR (1st step PCR)

Diluted samples were amplified in PCR Agilent SureCycler 8800. Preparation of PCR mix was described in chapter 3.4.5. The PCR protocol consisted of an initial denaturation at 95 °C for 3 minutes, followed by 30 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, and 72 °C for 30 seconds, with a final step at 72 °C for 5 minutes.

3.5.7. Automated electrophoresis platform

After the PCR, quality of PCR products and size of the fragments was verified through automated electrophoresis platform 4200 TapeStation System. Sample preparation for electrophoresis was described in chapter 3.4.2.

Before starting the Software, D1000 ScreenTape was put into the 4200 TapeStation instrument, the tips were loaded, and the waste compartment was emptied. Ladder and samples were placed into the stands. On the computer, in the Agilent TapeStation Controller Software 4.1, the first step is to label sample and ladder positions. After that, the Software was launched, and the results were shown in the Tape Station Analysis Software 4.1.1.

3.5.8. Library Preparation

Libraries for both bacterial and fungal DNA were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol [94]. The steps in libraries preparations are shown in Figure 8, which has been adapted from the protocol.

1st stage PCR was described before in 3.6.5. section.



Figure 8. Library preparation workflow, adapted from Illumina protocol [94].

Next step, PCR clean-up uses MagSi-NGSPREP Plus* beads to purify the amplicons by removing free primers and primer-dimer species. Before starting the process, the beads must equilibrate at room temperature. The Amplicon PCR plate was then centrifuged at 1,000 g for 1 minute to bring down any condensation. The entire 25 μ L of amplicon PCR product was added to the wells of the MIDI plate.

Next, beads were vortexed and 20 μ L was transferred from the reagent reservoir to the MIDI plate using a multichannel pipette. Tips were changed between each column. The entire volume was gently pipetted up and down 10 times, then placed on MPS-1 High-Speed Multi Plate Shaker at 1800 rpm for 2 minutes. After shaking, the plate was incubated for 5 minutes without shaking. The plate was then placed on a magnetic stand for 2 minutes to allow the supernatant to clear. Using a multichannel pipette, the supernatant was removed and discarded, with tips changed between samples.

The beads were washed with 200 μ L freshly prepared 80% ethanol, incubated for 30 seconds on the magnetic stand, and the supernatant was removed and discarded. A second ethanol wash follows the same steps. After this the beads were left to dry for 10 minutes, with plate still on magnetic stand.

The plate was then removed from magnetic stand and 52.5 μ L of 10 mM Tris pH 8.5 was added to each well of the Amplicon PCR plate. The plate was sealed and placed on MPS-1 High-Speed Multi Plate Shaker at 1800 rpm for 2 minutes to fully resuspend beads. The plate was then incubated on room temperature for 2 minutes. After the incubation, the plate was placed on the magnetic stand for 2 minutes to clear the supernatant. Using a multichannel pipette, 50 μ L of the supernatant was transferred from MIDI plate to new 96-well PCR plate.

Following this is indexing step. This process involves attaching dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. From PCR plate prepared in the previous step, 5 μ L was transferred to a new MIDI plate, with the remaining 45 μ L stored in the freezer. The Indices were arranged with Index 2 primer tubes aligned vertically from rows A to H and the Index 1 tubes aligned horizontally across columns 1 to 12, as shown in Figure 9.

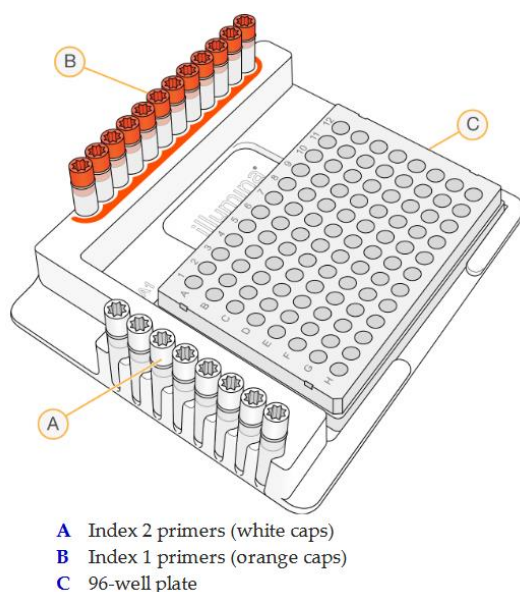


Figure 9. Index plate scheme, from Illumina protocol [94]

Each well contained 5 μ L of DNA sample, 5 μ L of appropriate Index Primer 1, 5 μ L appropriate Index Primer 2, 25 μ L of KAPA ReadyMix, and 10 μ L of PCR grade water. After adding reagents to the wells, the mixture was gently pipetted up and down 10 times, covered with a microseal and centrifuged at 1,000 g for 1 minute. This was followed by another PCR using the same set up as previously described.

The second PCR Clean-Up begins with centrifuging the Index PCR plate at 280 g for 1 minute to collect condensation. The entire Index PCR product (50 μ L) was then transferred from the PCR plate to a MIDI plate. Next, the MagSi-NGSPREP Plus* beads were vortexed for 30 seconds to ensure even dispersion, transferred to a reagent reservoir, and 56 μ L of beads was added to each well using a multichannel pipette. The mixture was gently pipetted up and down 10 times, sealed, and placed on the shaker at 1800 rpm for 2 minutes. After shaking, the plate was incubated at room temperature for 5 minutes, then placed on a magnetic stand for 2 minutes for the supernatant to clear. With the plate still on the magnetic stand, the supernatant was removed and discarded using a multichannel pipette, with tips changed between samples. The beads were washed by adding 200 μ L of freshly prepared 80% ethanol to each sample well, incubating on the magnetic stand for 30 seconds, followed by carefully removing and discarding the supernatant. The ethanol wash was repeated a second time. To ensure the beads were completely dry, a 20 μ L multichannel pipette with fine tips was used to remove excess ethanol, and the beads were left to air-dry for 10 minutes.

while still on the magnetic stand. Once dry, the plate was removed from the magnetic stand and 27.5 μL of 10 mM Tris pH 8.5 was added to each well. The mixture was pipetted up and down 10 times to fully resuspend the beads, then sealed and placed on the shaker at 1800 rpm for 2 minutes. The plate was incubated at room temperature for additional 2 minutes, then returned to the magnetic stand for 2 minutes for the supernatant to clear. Last step was to transfer 25 μL of the supernatant to a new 96-well PCR plate, changing tips between samples to prevent cross-contamination.

The next step involves library quantification, normalization, and pooling. Quantification was first performed using the Qubit® 3.0 Fluorometer, steps were described before. DNA concentration was then calculated in nM using the following formula:

$$\frac{c \left(\frac{\text{ng}}{\mu\text{L}} \right) * 10^6}{\frac{660 \text{ g}}{\text{mol}} * \text{average library size (650bp)}} = \text{Molarity (nM)}$$

Afterward, the samples were diluted to 4 nM with 10 mM Tris pH 8.5. All samples were successfully diluted to 4 nM. Once diluted, the libraries were ready for pooling. A 5 μL aliquot from each library was combined into a single pooled sample.

The final step before sample loading involves library denaturing. The preparation steps include setting a heat block at 96°C, preparing an ice bath with a 1:3 water to ice ratio and leaving MiSeq reagent cartage at room temperature to equilibrate.

Denaturing began by combining 5 μL of 4 nM pooled library with 5 μL of freshly prepared 0.2 N NaOH. The mixture was then vortexed and centrifuged at 280 g for a minute. After centrifugation, the mixture was incubated at room temperature for 5 minutes to ensure complete denaturation. Once denaturated, 990 μL of pre-chilled HT1 was added to the sample, resulting in a 20 pM denaturated library in 1 nM NaOH. The mixture is kept on ice until further steps.

Next, the denaturated DNA is diluted to the desired concentration. Based on a literature review, an 8 pM final concentration was selected. To achieve this, 240 μL of 20 pM denaturated library was mixed with 360 μL pre-chilled HT1. The mixture was gently inverted several times and put on ice.

The PhiX control needs to be denaturated and diluted similarly to the DNA library. To prepare a 4 nM PhiX library, 2 μL of 10 nM PhiX was combined with 3 μL 10nM Tris pH 8.5. This 4nM PhiX

library was then mixed with 5 μL of 0.2 N NaOH, vortexed, and incubated for 5 minutes to ensure complete denaturation. After denaturation, 990 μL of pre-chilled HT1 was added to bring the concentration to 20 pM. The denaturated PhiX library was diluted to 8pM by combining 240 μL of 20 pM denaturated PhiX library with 360 μL pre-chilled HT1. Mixture was inverted to mix and put on ice.

The final step involves combining the amplicon library with PhiX Control. To achieve a final PhiX concentration of at least 5%, 30 μL of the denatured and diluted PhiX control was added to 570 μL of denatured and diluted amplicon library. The combined mix was heated in a heating block at 96°C for 2 minutes. The mixture is then inverted and placed in the ice bath for 5 minutes.

Loading the sample on the Miseq begins by pipetting the entire contents of the microtube into the reagent cartridge, specifically into the position marked "Load Sample." The cartridge was then placed into the MiSeq device. In the Local Run Manager "16S Metagenomics" run option was selected for bacteria and "GenerateFASTQ" run option was selected for fungi. Read length of 300 bp was chosen for both analyses. Before sequencing began, the device was calibrated to ensure the accuracy and precision of the results.

3.6. Bioinformatics methods

3.6.1. Data preprocessing

Data preprocessing and taxonomic classification were performed at the Institute of Environmental Biotechnology, University of Technology of Graz. The obtained 16S rRNA gene and ITS fastq files were further processed using well-established bioinformatic pipelines [104,105]. The raw sequences were demultiplexed by Illumina software during the sequencing process. Primers were removed from the raw sequences using Cutadapt [106]. Next the sequences underwent quality control, where low-quality and chimeric sequences were removed using the DADA2 algorithm within the QIIME2, which is highly effective in denoising, correcting errors, and identifying true biological sequences by modelling error rates in Illumina sequencing data [107]. After quality filtering, feature tables and amplicon sequencing variants (ASVs) were generated again with DADA2 within the QIIME2 platform, providing a high-resolution view of the microbial communities [108]. The ASVs were then classified using the vsearch algorithm for sequence alignment and taxonomy assignment [109]. For bacteria, the most recent SILVA v132 database, a well-curated resource for ribosomal RNA sequences [110], was used, while fungal ITS sequences

were classified using the UNITE database [111], ensuring accurate identification and classification of the microbial diversity and composition within the samples.

3.6.2. Statistical analysis

Further analysis were performed with R (version 4.3.0) [112] in RStudio (version 2024.04.1.748) [113]. The data, taxonomy files, ASV tables and metadata, were combined into phyloseq object using the package Phyloseq [114]. The data was then normalised applying CSS (Cumulative Sum Scaling) normalization. Next step was filtering out sequences (ASVs) that could not be classified to at least the phylum level, meaning that unclassified reads were removed as these were likely contaminants, such as human genomic DNA. Additionally, chloroplast and mitochondrial 16S sequences were removed. Following, low-abundance taxa were removed based on prevalence and abundance thresholds: ASVs had to be present in at least three samples with a total read count exceeding 1000. This filtering reduced noise and preserved 88% of the reads for downstream analysis.

For bacterial alpha diversity assessment, the dataset was normalized by randomly subsampling to 16,400 reads per sample using the `rarefy_even_depth` function, with the seed set to 5,163. The same approach was applied to fungal alpha diversity, with subsampling to 4,228 reads per sample. These read counts were selected as a compromise between maintaining sequencing depth and preserving a large number of biological replicates. After this 4 samples in total had to be removed because of low number of reads (3 for ITS and 1 for 16S). Microbial richness and diversity metrics, including Observed species, Shannon index, Chao1 richness estimator, and inverse Simpson index, were calculated using the `estimate_richness` function.

For beta diversity analysis, the dataset was normalized using cumulative sum scaling, and Bray-Curtis dissimilarity matrices were calculated. Significant differences between groups were assessed using the `adonis2` function (PERMANOVA, 999 permutations) from the VEGAN package, used for ecological analysis [115].

Further analysis focused on relative abundance using the `rabuplot` [116] and `microeco` [117] packages. Data visualization was customized for clarity and reproducibility. Bar plots highlighted the dominant taxa in the dataset at both the genus and phylum levels, grouped by variables such as the number of siblings and parental education level, while violin plots illustrated community composition at the genus level with statistics. The choice of statistical tests depended on the data

structure and the number of categories in the predictor variable. For comparisons between two groups (e.g., pet ownership: "Yes" vs. "No"), a non-parametric Wilcoxon rank-sum test was applied. For comparisons involving more than two groups (e.g., number of siblings: "0," "1," "2," and "3 or more"), Kruskal-Wallis tests were used. To ensure the reliability of results when analysing multiple microbial taxa simultaneously, a multiple testing correction was applied to control the false discovery rate (FDR). Ultimately, these statistical methods were integrated into the visualization workflow.

4. RESULTS

4.1. Overview of sequencing and taxonomic data

Preliminary overview of sequencing results for bacterial and fungal taxa in 95 dust samples is presented in Table 1. For bacterial taxa (16S rRNA), 94 out of 95 samples were successfully sequenced, generating a total of 6,164,389 reads. A total of 16,010 ASVs (amplicon sequence variants) were identified, distributed across 36 phyla. For fungal taxa (ITS), 92 out of 95 samples were successfully sequenced, yielding a total of 3,232,715 reads. The sequencing identified 4,361 ASVs, spanning 10 phyla.

Table 1. Zagreb pilot 1st run preliminary sequencing results.

Parameter name	Bacterial taxa, 16S	Fungal taxa, ITS
Number of samples	95	95
Successfully sequenced samples	94/95	92/95
Total number of reads	6,164,389	3,232,715
Mean number of reads	65,579	35,138
Maximum number of reads	333,337	135,972
Minimum number of reads	4,228	1,640
Number of ASVs	16,010	4,361
Number of phyla	36	10

The Figure 10. shows quality scores for paired-end reads. Figure 10a is forward reads and 10b are reverse reads. The base quality scores are along the x-axis and the corresponding Phred quality scores are on the y-axis. The forward reads have better quality, and the quality drops with amplicon lengths. The forward reads are cut at 270 base position to maintain quality for the most reads at 25. The reverse was cut at 220 base position for the same reason.

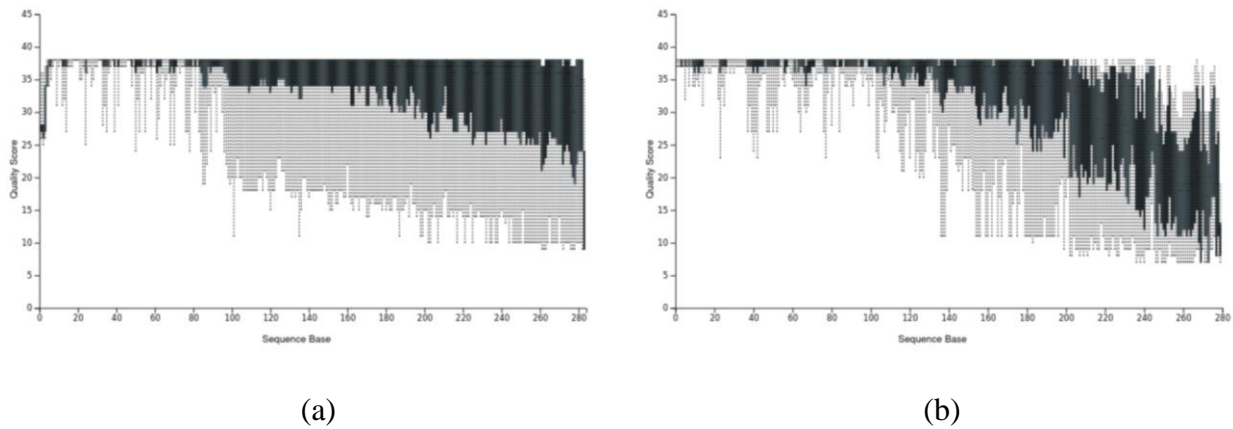


Figure 10. Quality scores in 16S sequencing. (a) forward reads. (b) reverse reads.

Figure 11. shows quality scores for pair-end reads for ITS region. Figure 11a. is forward reads and 11b. is reverse reads. The base quality scores are on x axis and Phred quality scores are on the y axis. Here the forward reads show better quality as well, while show a sharp drop in quality. Although the quality begins to decrease earlier, to maintain the majority of reads with a quality score of 25 or higher, the forward reads were cut at 220 and reverse at 210 base position.

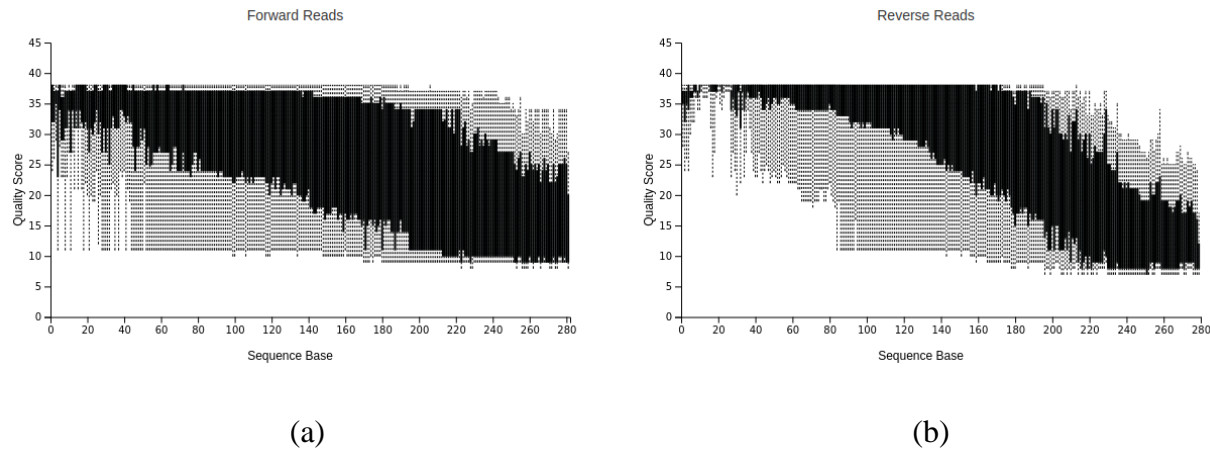
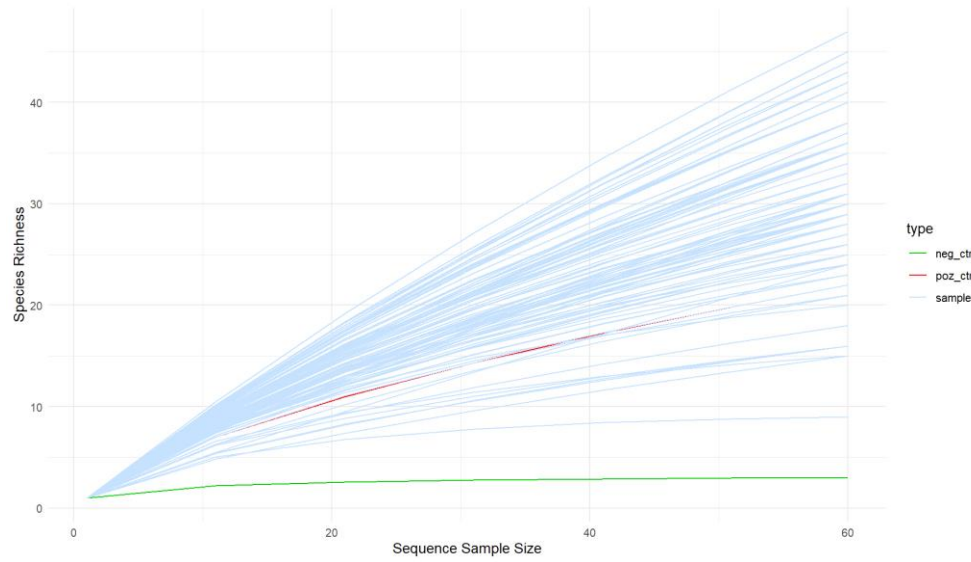


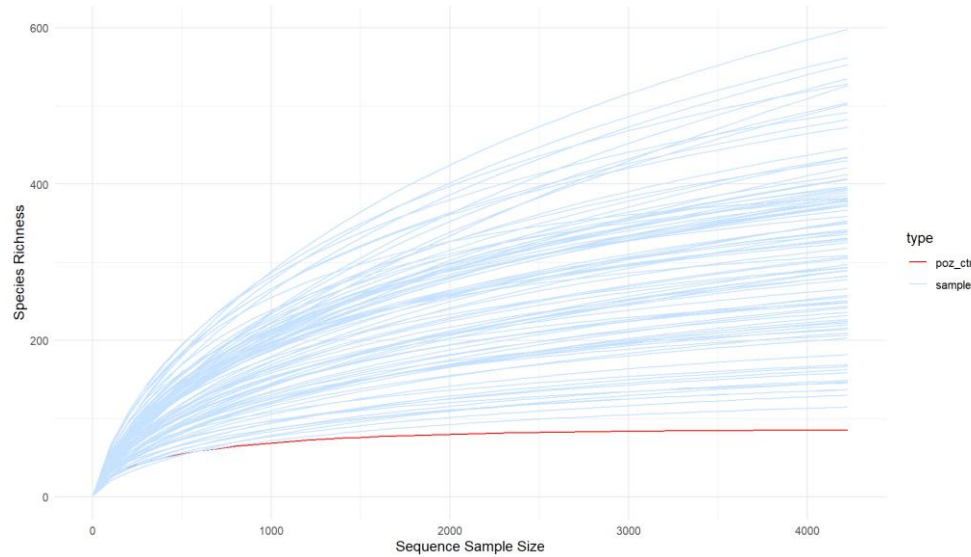
Figure 11. Quality scores in ITS sequencing. (a) forward reads. (b) reverse reads.

These specific cut-offs were chosen to retain a higher proportion of reliable reads, which is crucial for downstream analyses such as diversity and taxonomic profiling.

The rarefaction curves presenting different subsampling depths for 16S sequencing are shown in Figures 12a and b.



(a)

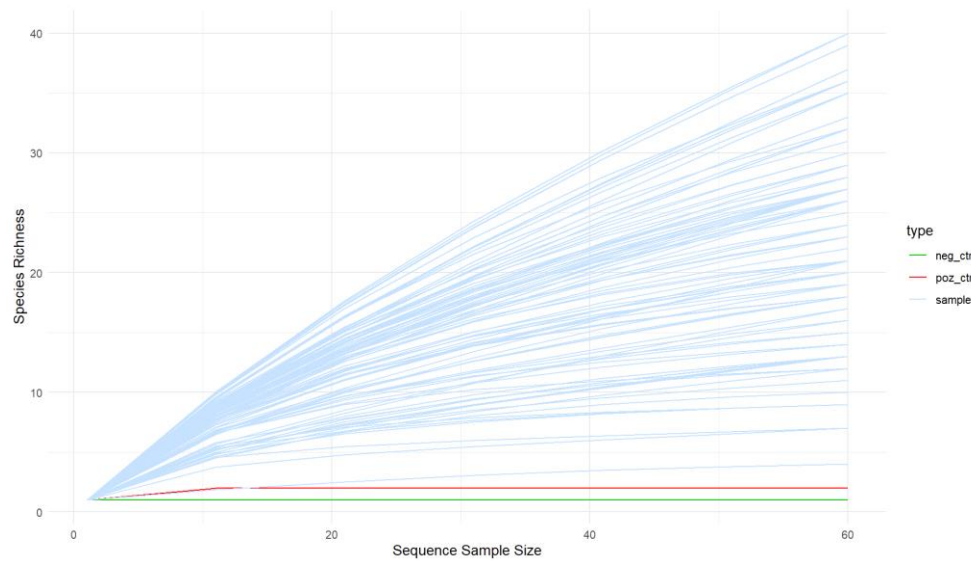


(b)

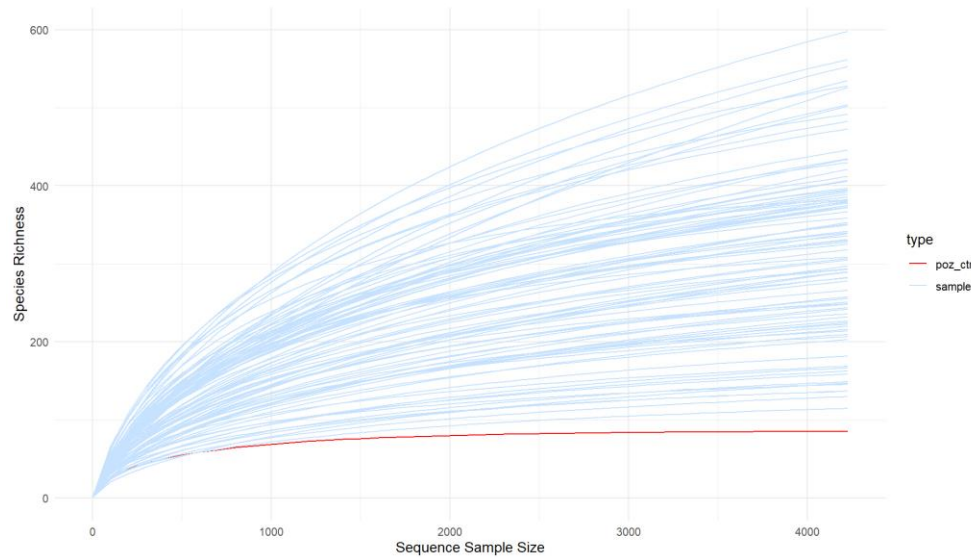
Figures 12a and b. 16S rarefaction curves.

Figure 12a was created with a lower subsampling depth, to show both the positive and negative controls with the samples. Figure 12b was generated with a much higher subsampling depth, as indicated by the x-axis extending beyond 4000 sequences. The red line represents the positive control, which initially shows an increase in species richness in Figure 12a but levels off in Figure 12b. The green line represents the negative control, which shows consistently low or flat species richness in Figure 12a. The blue lines represent individual samples. In Figure 12b, the increased

sequencing depth results in much higher observed species richness for each sample. Many of these samples reach an asymptote at higher sequencing depths.



(a)



(b)

Figure 13a and b. ITS rarefaction curves.

Figures 13a and b present rarefaction curves for fungal samples. Figure 13a uses a lower subsampling depth to display both positive and negative controls alongside the samples, resulting in relatively low observed species richness across all samples. Whereas, Figure 13b uses a much higher subsampling depth, as shown by the x-axis extending beyond 4000 sequences. The red line represents the positive control, which initially shows an increase in species richness in Figure 13a

but plateaus in Figure 13b. The green line, representing the negative control, remains low and flat in Figure 13a, as expected for controls with minimal biological content. In Figure 13b, the negative control line disappears, due to the very low number of reads. The blue lines correspond to individual samples. In Figure 13b, the higher sequencing depth results in much greater observed species richness across the samples.

4.2 Microbial composition of dust samples

4.2.1 Taxonomic composition

Figure 14. displays the taxonomic composition of samples at the genus level, illustrating the relative abundance of various microbial genera. The most prominent genera are *Staphylococcus*, *Anaerococcus*, and *Enhydrobacter*, along with a range of less abundant genera grouped as “Other”. *Staphylococcus* is notably dominant across a large portion of samples, indicating its high prevalence in this dataset, followed by *Enhydrobacter* as the next most abundant genus. Additional genera, such as *Corynebacterium*, *Cutibacterium*, and *Streptococcus*, contribute to the overall diversity observed in the microbiome. These patterns suggest that while *Staphylococcus* is often dominant, other genera add to a more diverse yet less abundant microbial profile across samples.

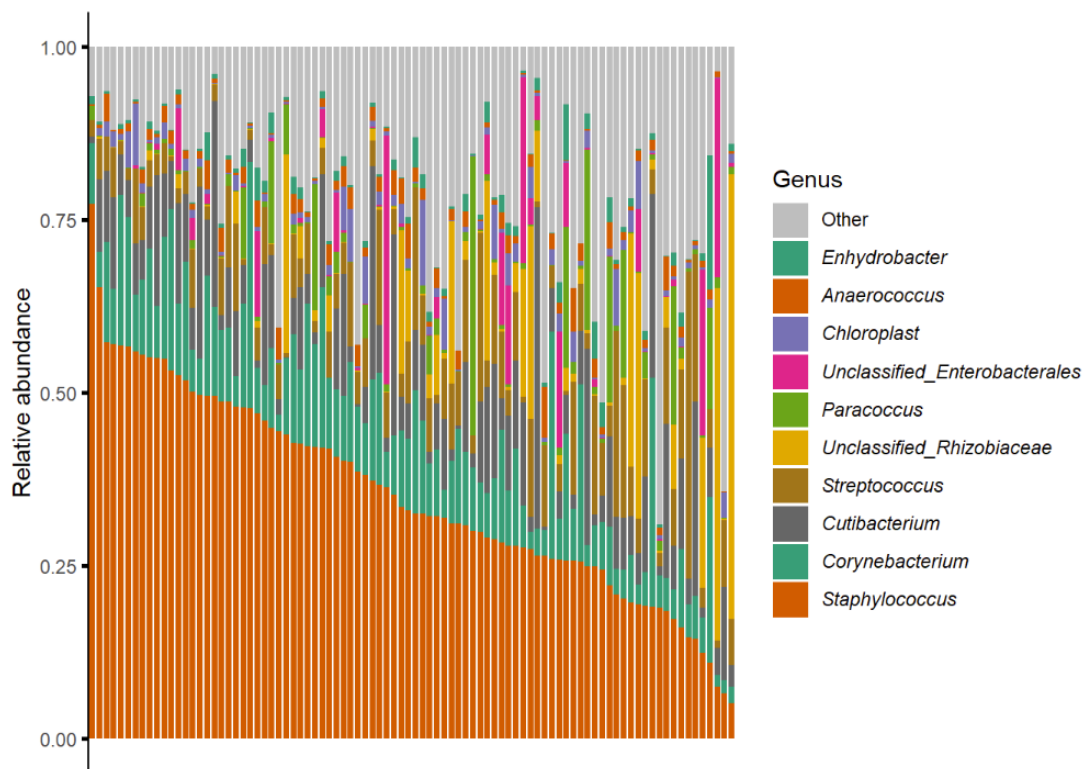


Figure 14. Taxonomic composition at Genus levels Bacteria.

Figure 15. presents the relative abundance of fungal genera across various samples. The most prominent genera are *Malassezia*, Unassigned *Didymosphaeriaceae*, and *Cyberlindnera*, with less abundant genera grouped under "Other." *Malassezia* is the dominant genus in many samples, followed by Unassigned *Didymosphaeriaceae* and *Cyberlindnera*. Additional genera, such as *Aspergillus*, *Alternaria*, and *Cladosporium*, appear in smaller proportions, contributing to the overall diversity of the fungal communities. These distribution patterns indicate that while *Malassezia* dominates, other genera play a role in creating a more diverse, though less abundant, microbial profile. This variability in fungal composition may be influenced by various environmental or individual factors affecting microbial diversity within the studied group.

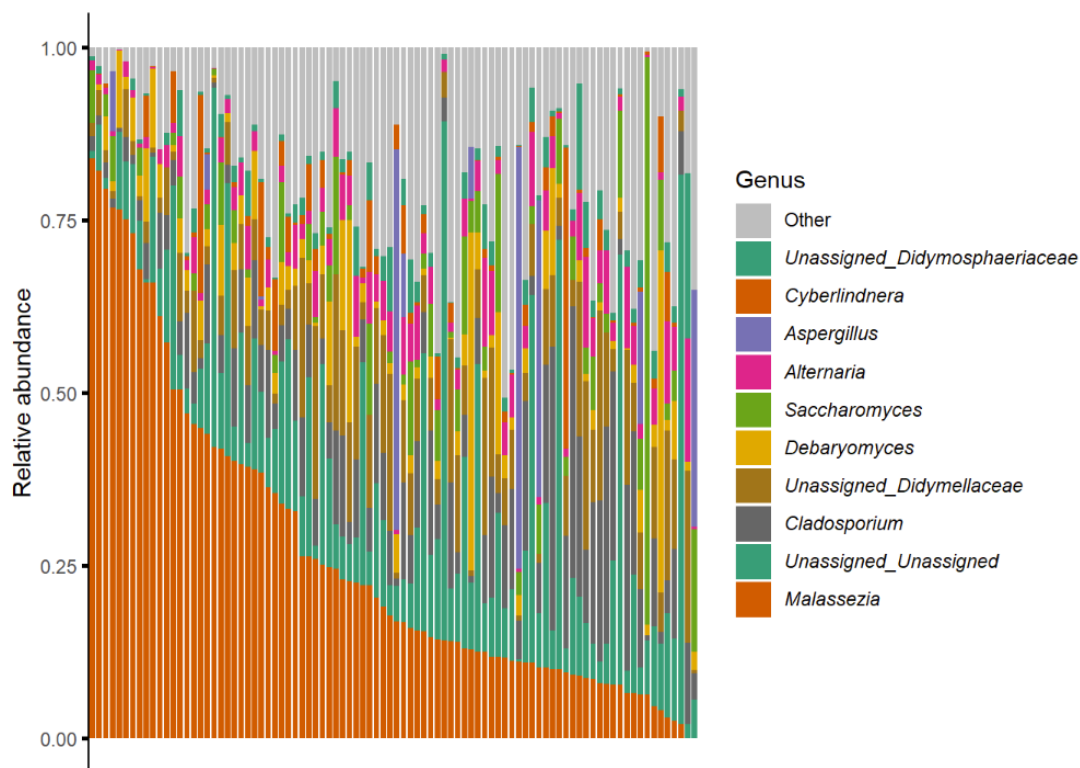


Figure 15. Taxonomic composition at Genus levels Fungi.

4.2.2. Associations between Fungi and Bacteria

Figures 16a and b display the associations between fungal and bacterial α diversity metrics. In Figure 16a, the relationship between Fungal Observed Richness and Bacterial Observed Richness is shown, with a p-value of 0.476 and an R^2 of 0.00579. The high p-value and low R^2 indicate that the association is not statistically significant, with bacterial observed richness explaining only about 0.6% of the variability in fungal observed richness. This suggests minimal evidence of a

meaningful relationship between bacterial and fungal richness. Similarly, Figure 16b illustrates the association between fungal Shannon Diversity and bacterial Shannon Diversity, with a p-value of 0.562 and an R^2 of 0.00383. These values are comparable to those in the Observed Richness plot, again indicating a lack of statistical significance and a very low explained variance. Thus, there is no substantial association between bacterial and fungal diversity based on the Shannon index.

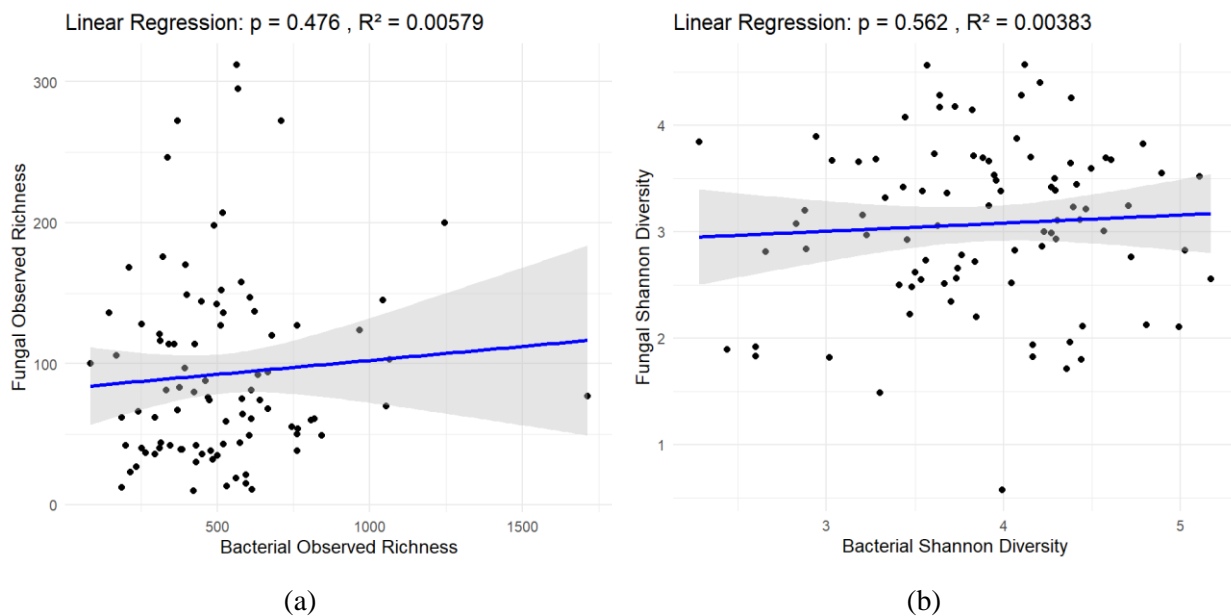


Figure 16. Associations between fungal and bacterial α diversity, (a) observed richness, (b) Shannon index.

4.2.3. Associations between diversity measures and asthma status

The diversity analysis included both alpha (α) and beta (β) diversity measures, focusing on bacterial and fungal alpha diversity between individuals with and without asthma. The p-value for bacterial alpha diversity is 0.077, while the p-value for fungal alpha diversity is 0.473.

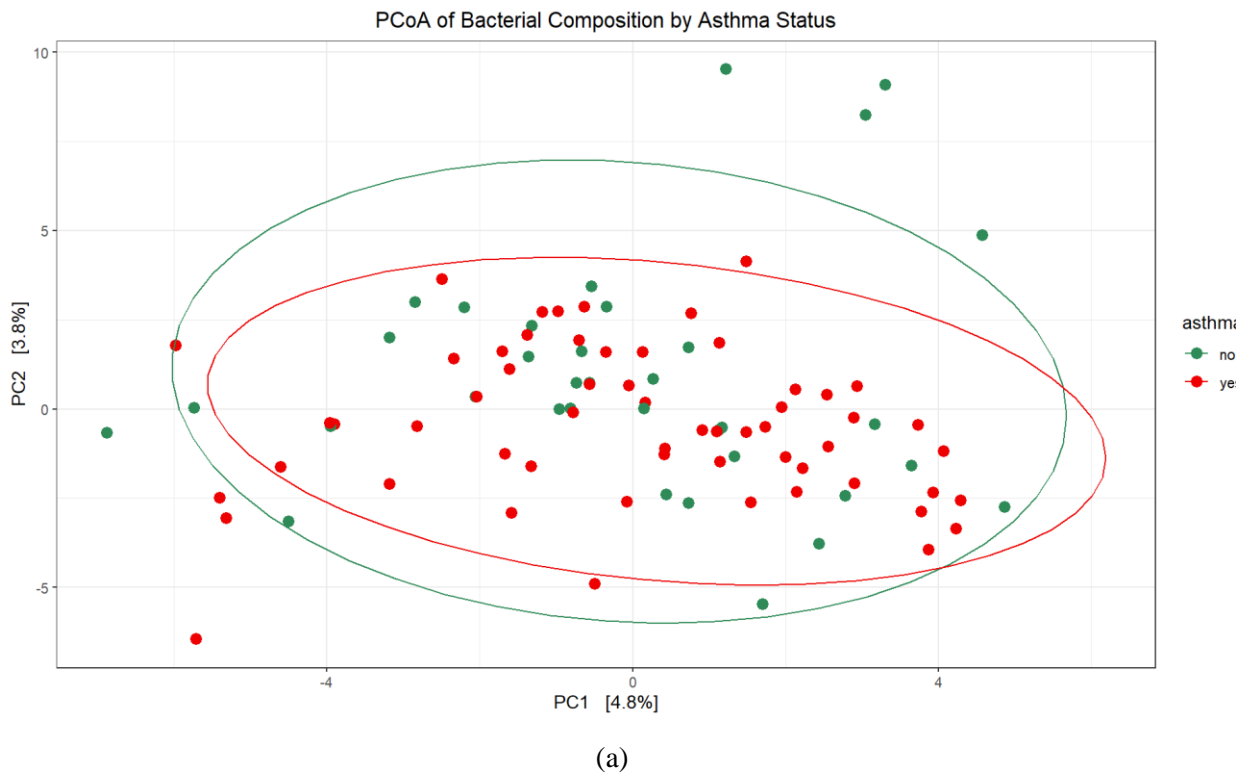
As for β diversity, the results are shown in Table 2. Participants without asthma ($n=31$, 34.4%), bacterial β diversity showed a significant association with asthma, with a p-value of 0.009. This suggests that there are notable differences in microbial community composition between individuals with and without asthma. In the ITS data, β diversity presented an R^2 of 0.013 and a p-value of 0.1.

Table 2. β diversity for asthma.

Category	Variable	Total (n, %)	16S β diversity (R^2/p)	ITS β diversity (R^2/p)
Asthma	No	31 (34.4)	0.015/ 0.009	0.013/ 0.1
	Yes	59 (65.6)		

*Bold $p < 0.05$

Figures 17a and b display Principal Coordinate Analysis (PCoA) plots illustrating the variation in microbiome composition between individuals with and without asthma. The plots show considerable overlap between the two groups. The overlap suggests that, while some variation exists, the differences in microbiome composition between individuals with and without asthma are not strongly pronounced in these principal coordinates.



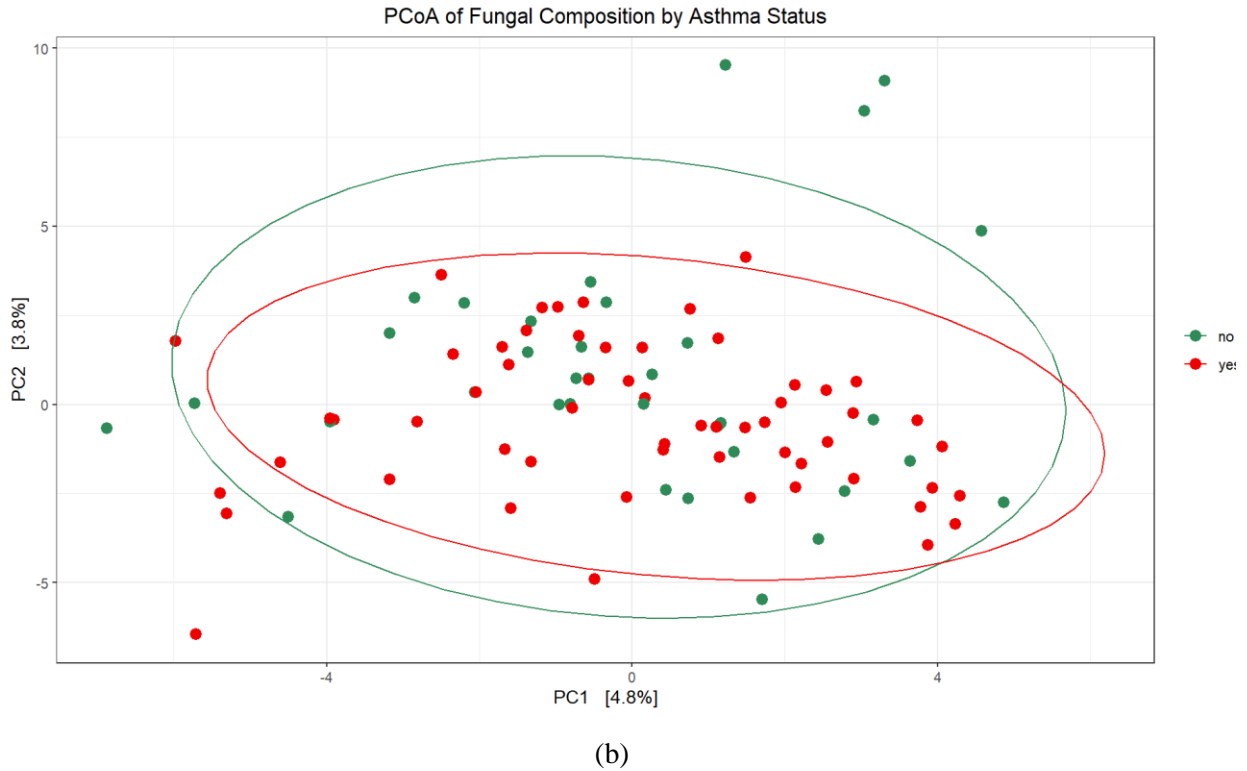


Figure 17. PCoA of microbiome composition by asthma status, (a) for Bacteria, (b) for Fungi.

4.2.4. Environmental microbiome differences in children with and without asthma

Many environmental factors have been shown to influence microbiome communities. The factors identified in the EDIAQI study are grouped into socioeconomic factors, household characteristics, cleaning practices, and environmental conditions.

4.2.4.1. Socioeconomic factors

Socioeconomic factors include categories such as the child's gender, household monthly income, and parents' education level. The results are presented in Table 3.

Table 3. Influence of socioeconomic factors on microbial diversity and interaction with asthma

Category	Variable	Total (n, %)	16S			ITS		
			β diversity (R ² /p val)	β diversity interaction with asthma (R ² /p val)	α diversity (p val)	β diversity interaction with asthma (R ² /p val)	β diversity (R ² /p val)	α diversity (p val)
Gender	Female	41 (45.6)	0.015/ 0.009	0.012/ 0.264	0.913	0.010/ 0.623	0.009/ 0.814	0.823
	Male	49 (54.5)						
Education parents	Middle	23 (25.6)	0.043/ 0.003	0.0256/ 0.025	0.928	0.041/ 0.032	0.030/ 0.008	0.262
	High	48 (53.3)						
	PhD	19 (21.1)						
Household monthly income	< 1500 €	7 (7.8)	0.025/ 0.068	0.021/ 0.575	0.759	0.029/ 0.026	0.022/ 0.424	0.018
	1500 € - 2500 €	25 (27.8)						
	> 2500 €	58 (64.4)						

*bold p < 0.05

The results demonstrate the influence of socioeconomic factors on microbial diversity in both bacterial and fungal datasets, as well as their interaction with asthma status. For gender, there is a significant association with β diversity in the bacterial dataset ($p = 0.015$), though no significant interaction with asthma is observed. Parental education shows a significant association with β diversity in both the bacterial ($p = 0.003$) and fungal ($p = 0.032$) datasets, with a notable interaction with asthma in both cases ($p = 0.025$ for bacterial and $p = 0.008$ for fungal). Household income is marginally significant for β diversity in the bacterial dataset, while the fungal dataset shows a significant association with β diversity ($p = 0.026$). For α diversity, only household monthly income has shown a significant association ($p = 0.018$). Figure 18. illustrates observed richness across income groups, with similar median richness values among the three income categories (< 2000€, 2000-2500€, and > 2500€), showing a slight difference in richness for the lowest income group.



Figure 18. Observed richness by household income in ITS dataset.

4.2.4.2. Household characteristics

Household characteristics encompass factors such as the living environment, type of housing, number of siblings, presence and types of pets in the household, and the number of plants within the home. These variables provide insight into the household's potential influence on microbiome composition. The results are presented in Table 4.

Table 4. Influence of household factors on microbial diversity and interaction with asthma

Category	Variable	Total (n, %)	16S			ITS		
			β diversity (R ² /p val)	β diversity interaction with asthma (R ² /p val)	α diversity (p val)	β diversity (R ² /p val)	β diversity interaction with asthma (R ² /p val)	α diversity (p val)
Living environment	Suburban	19 (21.1)	0.029/ 0.002	0.030/ 0.001	0.993	0.030/ 0.005	0.029/ 0.026	0.548
	Urban (built surroundings)	35 (38.9)						
	Urban (green spaces)	36 (40.0)						
Type of home	House	30 (33.3)	0.016/ 0.004	0.012/ 0.190	0.267	0.014/ 0.054	0.011/ 0.397	0.451
	Apartment	60 (66.7)						
Number of siblings	None	12 (13.3)	0.051/ 0.001	0.042/ 0.001	0.045	0.045/ 0.009	0.037/ 0.123	0.893
	1	54 (60.0)						
	2	15 (16.7)						
	3	9 (10.0)						
Pet ownership	No	61 (67.8)	0.019/ 0.001	0.014/ 0.021	0.061	0.010/ 0.005	0.013/ 0.111	0.117
	Yes	29 (32.2)						

Dog	No pet	61 (67.8)	0.035/ 0.001	0.028/ 0.003	0.060	0.035/ 0.001	0.024/ 0.187	0.012
	Yes	21 (23.3)						
	Other pet	8 (8.9)						
Cat	No pet	61 (67.8)	0.031/ 0.001	0.028/ 0.007	0.172	0.034/ 0.003	0.022/ 0.444	0.054
	Yes	7 (7.8)						
	Other pet	23 (24.4)						
Plants	No/ outside	14 (15.6)	0.028/ 0.005	0.032/ 0.001	0.433	0.027/ 0.041	0.027/ 0.035	0.123
	≤ 5	53 (58.9)						
	> 5	23 (25.5)						

*bold p-value < 0.05

Living environment significantly influences β diversity, for both bacterial ($p = 0.002$) and fungal ($p = 0.005$) datasets, along with notable interactions with asthma ($p=0.001$ and 0.026 , respectively). Type of home shows significant associations with β diversity in the bacterial dataset ($p = 0.004$). The number of siblings appears to play a role, particularly in the bacterial dataset, where presence of siblings significantly affects both β diversities ($p = 0.001$, and $p= 0.009$) and interacts with asthma status for bacterial dataset ($p= 0.001$). Here there is also a significant association with bacterial α diversity. As it can be seen on Figure 19., while the median richness appears relatively similar across groups, the distribution shows slight variability. This significance implies that sibling presence, even though not showing a clear linear trend in median richness, is associated with variations in microbial diversity, potentially due to the different environmental exposures and microbial sharing that siblings might bring into the household.

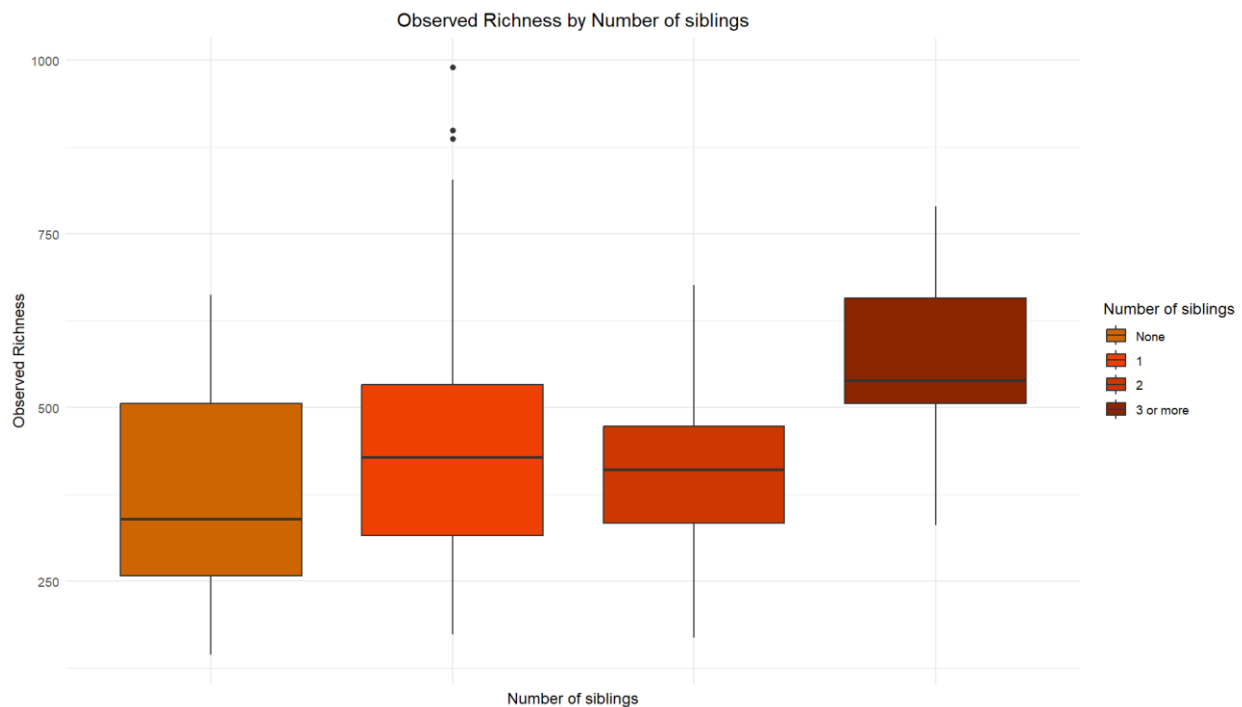


Figure 19. Observed richness by number of siblings in the household in 16S dataset.

Pet ownership, especially dog and cat ownership, has a marked effect, with significant associations for β diversity and asthma interaction in both bacterial and fungal datasets ($p = 0.001$, $p = 0.005$ respectively; bacterial interaction $P = 0.021$). For instance, dog ownership in the bacterial dataset shows $p = 0.001$ and $p = 0.003$ for interaction, same for cat with $p = 0.001$ and $p = 0.007$ for interaction. In fungal dataset there are only associations with fungal α diversity with $p = 0.001$ for interaction.

dogs and $p = 0.003$ for cats. As for α diversity, although cat shows borderline association, those for dogs is shown to be statistically significant with $p = 0.012$, which is shown in Figure 20. The median richness appears higher in households with a dog compared to those with no pets or other types of pets. The interquartile range is also broader for households with a dog, indicating more variability in richness among these samples. Households with no pets and those with other pets show similar median richness values. These patterns suggest that the presence of a dog in the household may be associated with greater microbial richness.

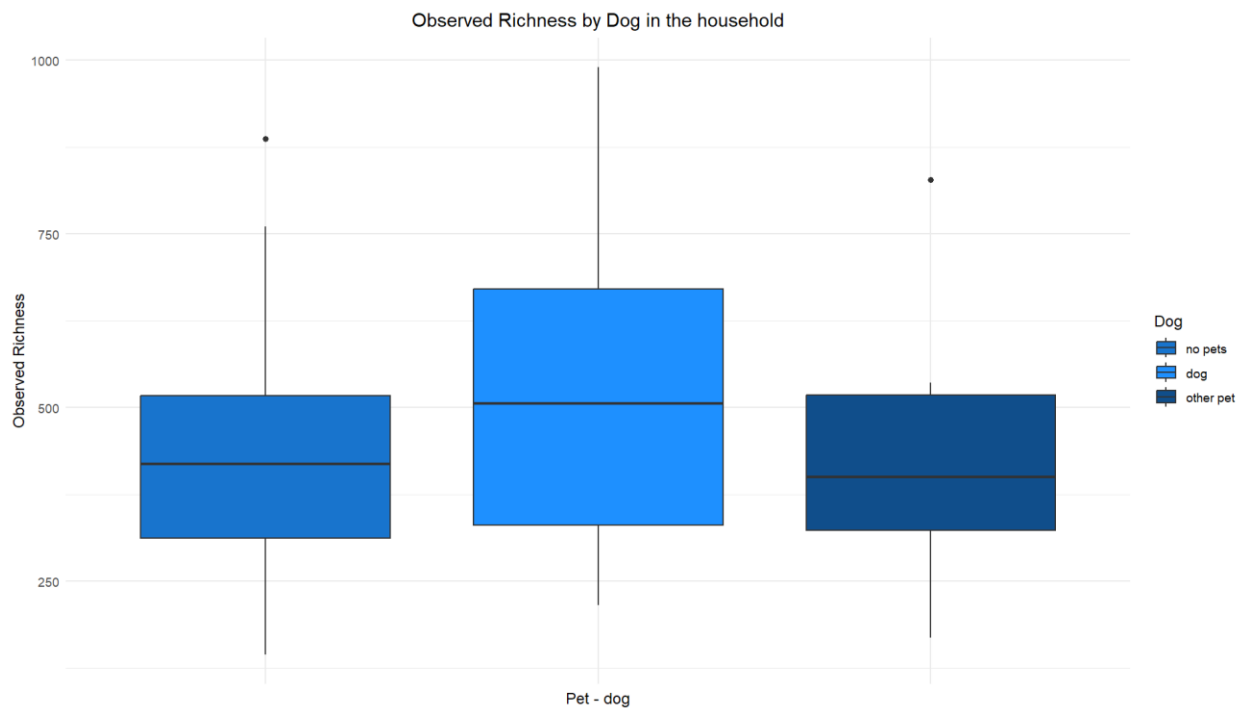


Figure 20. Observed richness by dog in the household in ITS dataset.

Additionally, the presence of plants in the household influences microbial diversity, with significant β diversity associations in both datasets ($p = 0.005$, and 0.042 respectively) and interaction with asthma ($p = 0.001$, and 0.035 respectively). Overall, these results indicate that various household factors influence microbial community composition, and many of these factors interact with asthma status.

4.2.4.3. Cleaning practices

Cleaning practices include the frequency of dusting within the home each week and the annual frequency of mattress vacuuming. These practices offer insight into household hygiene levels and their potential impact on microbiome composition. The results are presented in Table 5.

Table 5. Cleaning practices and diversity measures

Category	Variable	Total (n, %)	16S			ITS		
			β diversity (R^2/p val)	β diversity interaction with asthma (R^2/p val)	α diversity (p val)	β diversity interaction with asthma (R^2/p val)	β diversity (R^2/p val)	α diversity (p val)
Dusting furniture	Occasionally	15 (16.7)	0.040/0.006	0.042/0.003	0.752	0.048/0.002	0.036/0.185	0.169
	Once a week	45 (50.0)						
	Several times a week	24 (26.6)						
	4 or more times a week	6 (6.7)						
Mattress vacuuming	Never or once a year	19 (21.1)	0.027/0.011	0.028/0.002	0.038	0.027/0.037	0.027/0.046	0.193
	2 - 4 times a year	43 (47.8)						
	< 4 times a year	28 (31.1)						

*bold $p < 0.05$

Dusting furniture is significantly associated with both bacterial and fungal β diversity ($p = 0.006$ and $p = 0.002$, respectively), with an interaction observed between dusting and asthma in the bacterial dataset ($p = 0.003$). Nonetheless, no significant effect on α diversity is seen across different dusting frequencies. Mattress vacuuming also shows significant associations with both β diversities and with interactions in both datasets. In the bacterial dataset, β diversity is significantly associated with vacuuming ($p = 0.011$) and shows an interaction with asthma ($p = 0.002$). For fungal β diversity, the association is significant at $p = 0.037$, with an interaction at $p = 0.046$. Mattress vacuuming frequency also significantly affects bacterial α diversity ($p = 0.002$). Figure

21. illustrates that median richness is highest in households that vacuum their mattress "1 to 4 times a year" and lowest for those that "Never or rarely" vacuum. The "Never or rarely" group shows greater variability in microbial richness, indicated by a wider interquartile range and longer whiskers, while the "1 to 4 times a year" group displays less variability. This suggests that moderate mattress cleaning might support a more diverse microbial community compared to both very frequent and infrequent cleaning.



Figure 21. Observed richness by mattress vacuuming frequency in 16S dataset.

4.2.4.4. Environmental conditions

The only environmental condition considered is the sampling season, as external factors can influence microbial communities. This impact is evident from the results presented in Table 6.

Table 6. Environmental variables and diversity measures.

Category	Variable	Total (n, %)	16S			ITS		
			β diversity (R ² /p val)	β diversity interaction with asthma (R ² /p val)	α diversity (p val)	β diversity interaction with asthma (R ² /p val)	β diversity (R ² /p val)	α diversity (p val)
Season of sample collection	Spring	18 (20.0)	0.114/ 0.001	0.093/ 0.001	0.130	0.104/ 0.001	0.086/ 0.031	0.615
	Summer	20 (22.2)						
	Autumn	15 (16.7)						
	Winter	37 (41.1)						

*Bold $p < 0.05$

Examining β diversity reveals significant associations in both groups. For bacteria, there is a significant association with $p = 0.001$, as well as a significant interaction with asthma ($p = 0.001$). For fungi, β diversity is also significant ($p = 0.001$), with an interaction with asthma showing significance at $p = 0.031$. However, no significant association is observed for α diversity.

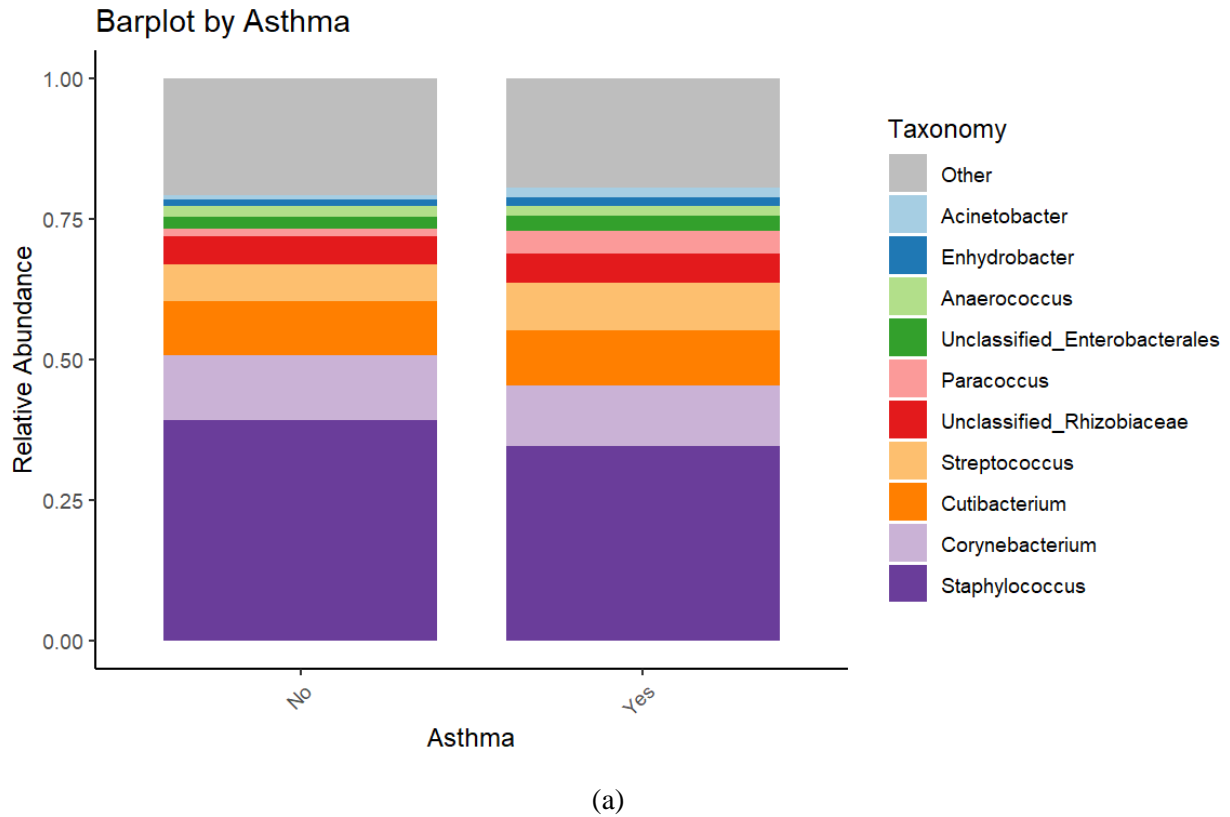
Overall, factors such as socioeconomic status, living environment, season, pet ownership, and household cleaning practices significantly shape bacterial and fungal community composition, with some of these associations interacting with asthma status. However, Principal Coordinate Analysis (PCoA) plots do not reveal distinct groupings for any of these variables (data not shown). These results suggest that while these factors influence β diversity, the variations in microbial composition are subtle and do not form distinct clusters based on the analysed variables.

4.2.5. Relative Abundance

4.2.5.1. Asthma

The presented plots provide insights into the bacterial composition in relation to asthma. Bar plot (Figure 22a), represents relative abundance of bacterial taxa in individuals with and without asthma. The plot reveals a consistent distribution of dominant taxa, including *Staphylococcus*, *Corynebacterium*, and *Cutibacterium*, across both groups. However, subtle differences in the abundance of less prevalent taxa, such as *Enhydrobacter* and *Paracoccus*, are visible. These

observations can be seen in the violin plot (figure 22b). Notably, genera such as *Enhydrobacter* ($p=0.036$), *Micrococcus* and *Paracoccus* show statistically significant differences in abundance between the two groups, as indicated by the p-values ($p=0.011$ for both *Paracoccus* and *Micrococcus*).



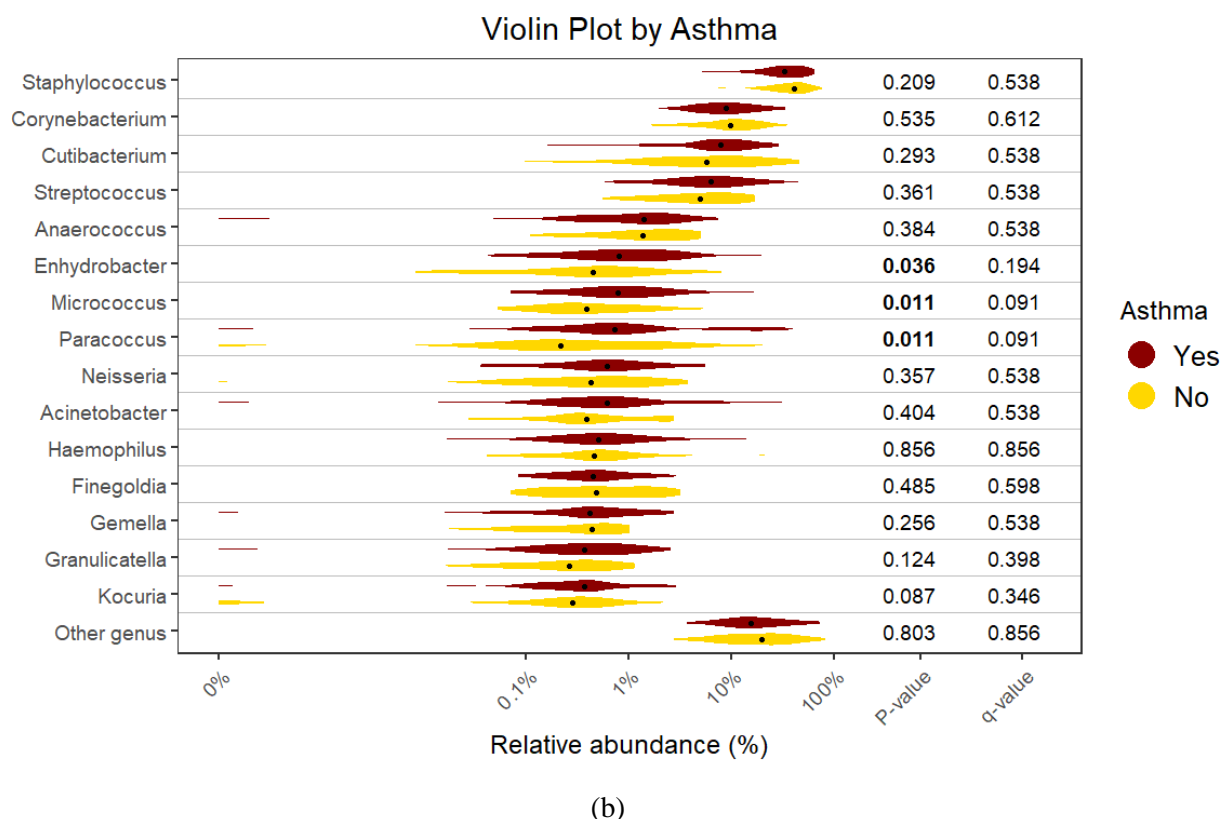
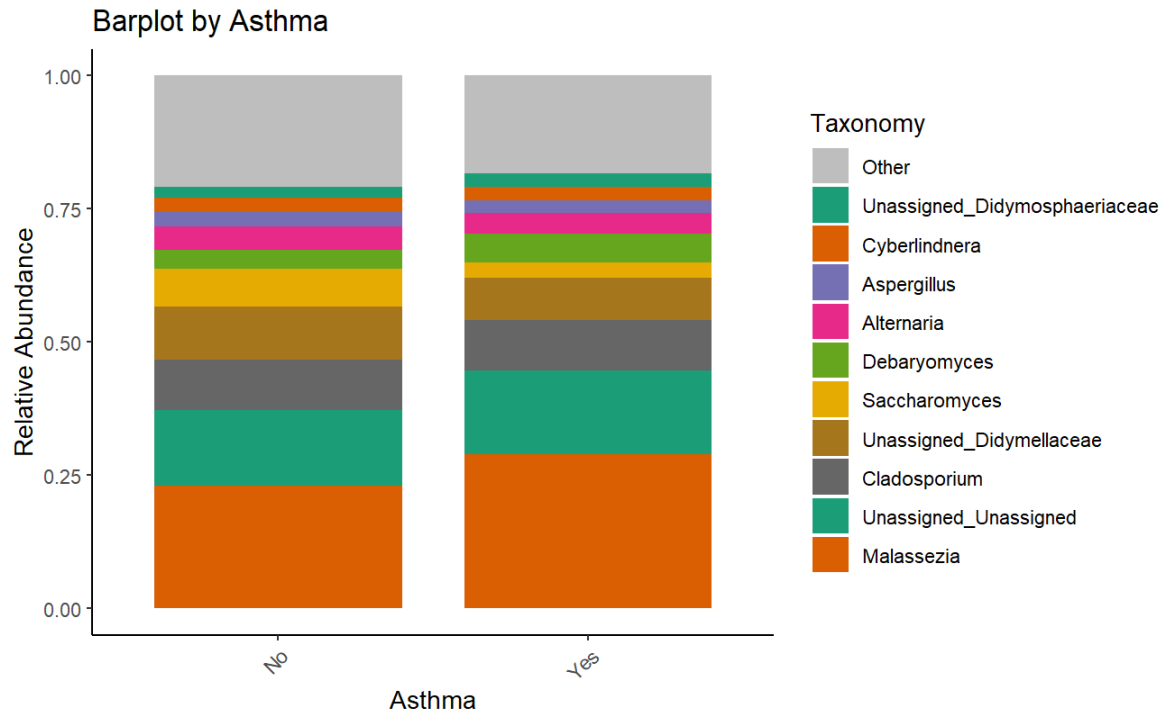
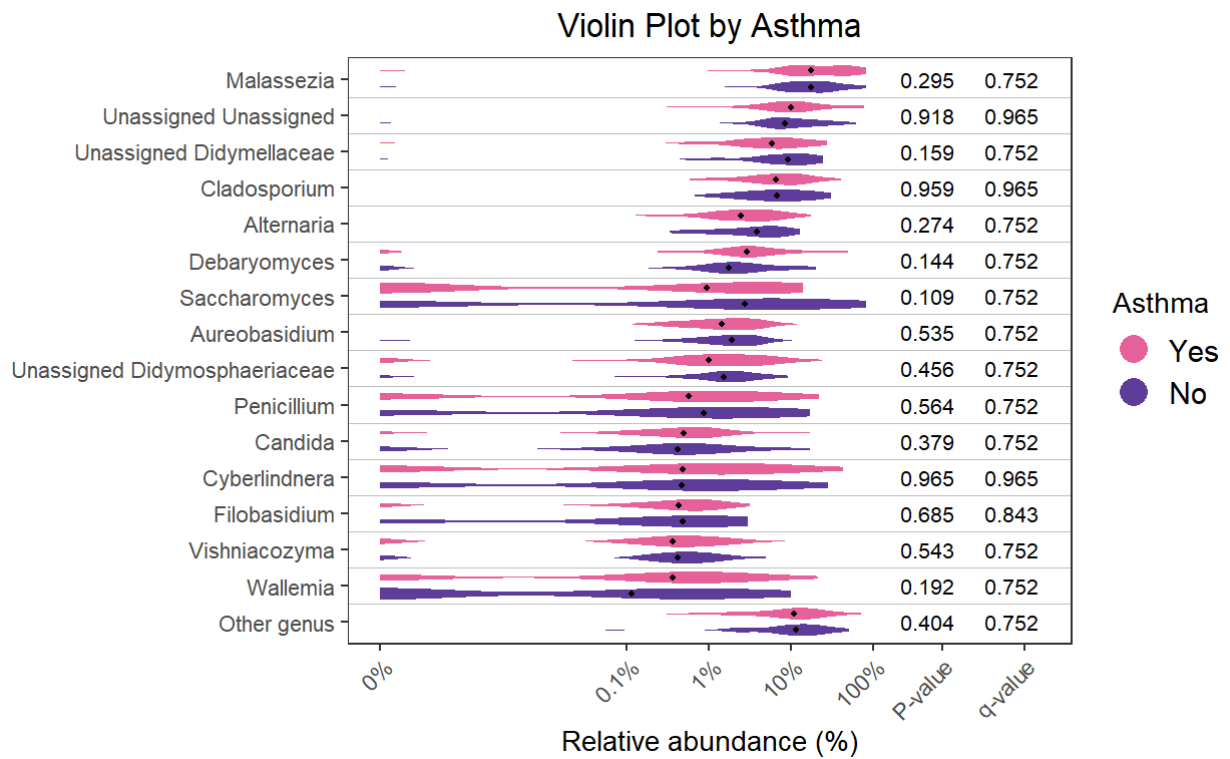


Figure 22. Relative abundance of bacterial genera by asthma. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

Figure 23a presents the relative abundances of fungal genera in household dust grouped by the presence or absence of asthma (Yes/No). Each bar represents the microbial composition for the respective group, with genera showed as stacked segments. Prominent genera such as *Malassezia* and *Cladosporium* are shown to contribute substantially to the overall microbiome in both groups, although visual differences are noticeable. Conversely, other genera like *Aspergillus* and *Alternaria* show relatively stable distributions between the two groups. Figure 23b visualizes the relative abundance of individual taxa within the two asthma groups (Yes/No). The width of each violin represents the density of samples with a specific relative abundance for each taxon. The numerical annotations such as p-values and q-values, indicate the statistical significance of differences between the groups for each taxon.



(a)



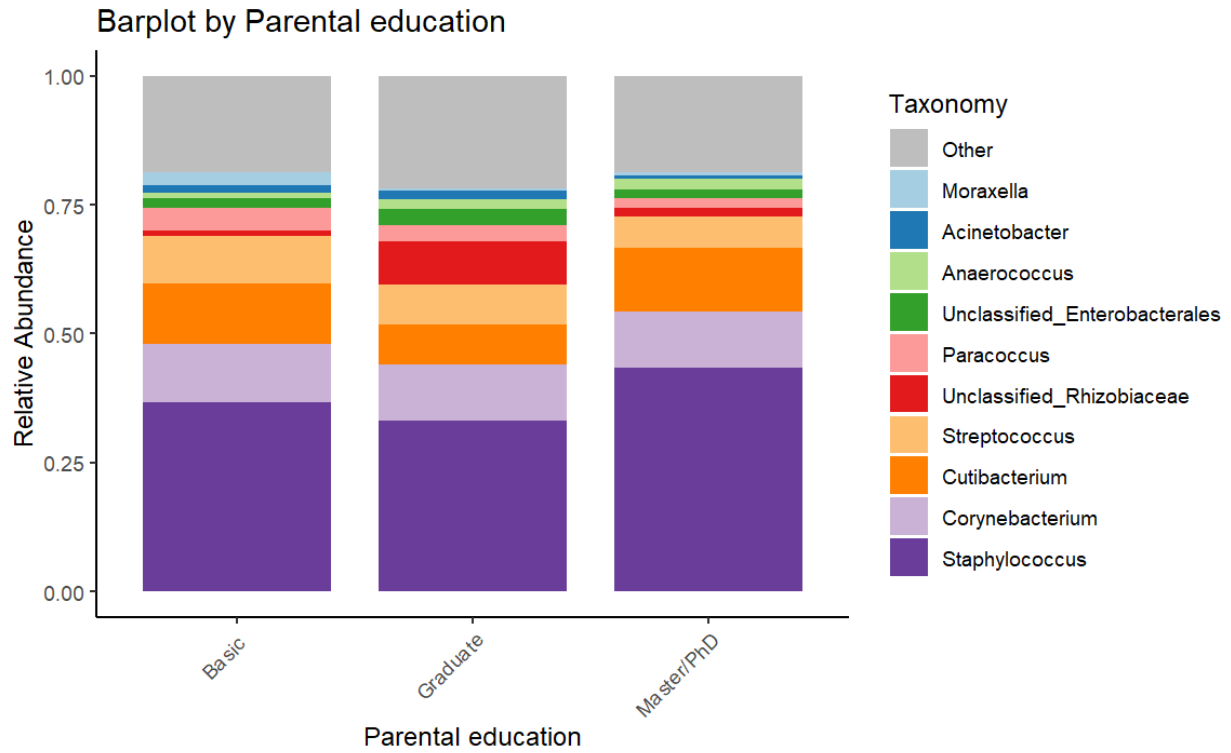
(b)

Figure 23. Relative abundance of fungal genera by asthma. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

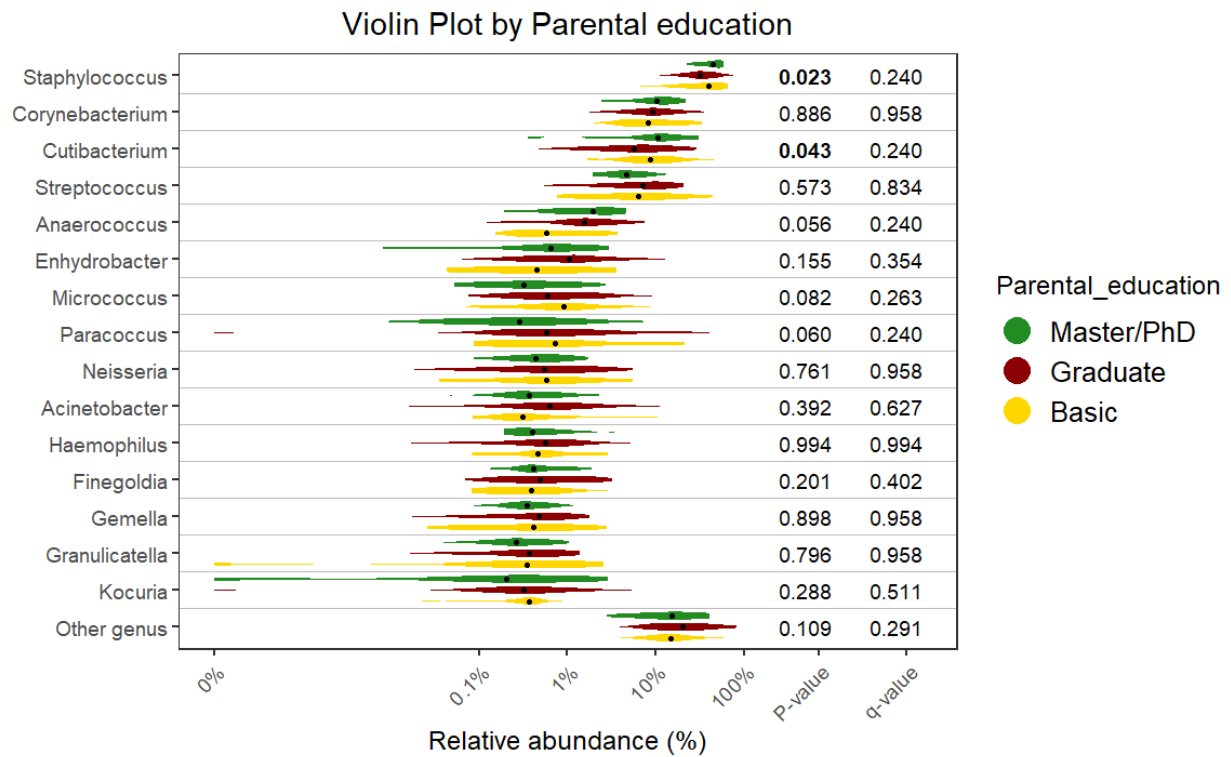
4.2.5.2. Socioeconomic factors

This chapter presents the relative abundance of microbial taxa in relation to socioeconomic factors, including the child’s gender, parental education, and household monthly income. Factors not shown here are included in the supplementary materials, as they did not reveal any statistically significant differences in taxa.

Figure 24 presents relationship of bacterial relative abundance and parental education. Bar plot, illustrates the relative abundance of bacterial taxa across the three educational groups. The distribution of dominant taxa such as *Staphylococcus*, *Corynebacterium*, and *Cutibacterium* appears relatively stable across the groups, but subtle differences in the relative proportions of less abundant taxa like *Paracoccus*, Unclassified *Rhizobiaceae* and *Anaerococcus* are present. The violin plot reveals that certain bacterial taxa, such as *Staphylococcus* ($p = 0.023$) and *Cutibacterium* ($p = 0.043$), show significant differences in abundance across the groups, indicating a potential impact of parental education on the home microbial environment.



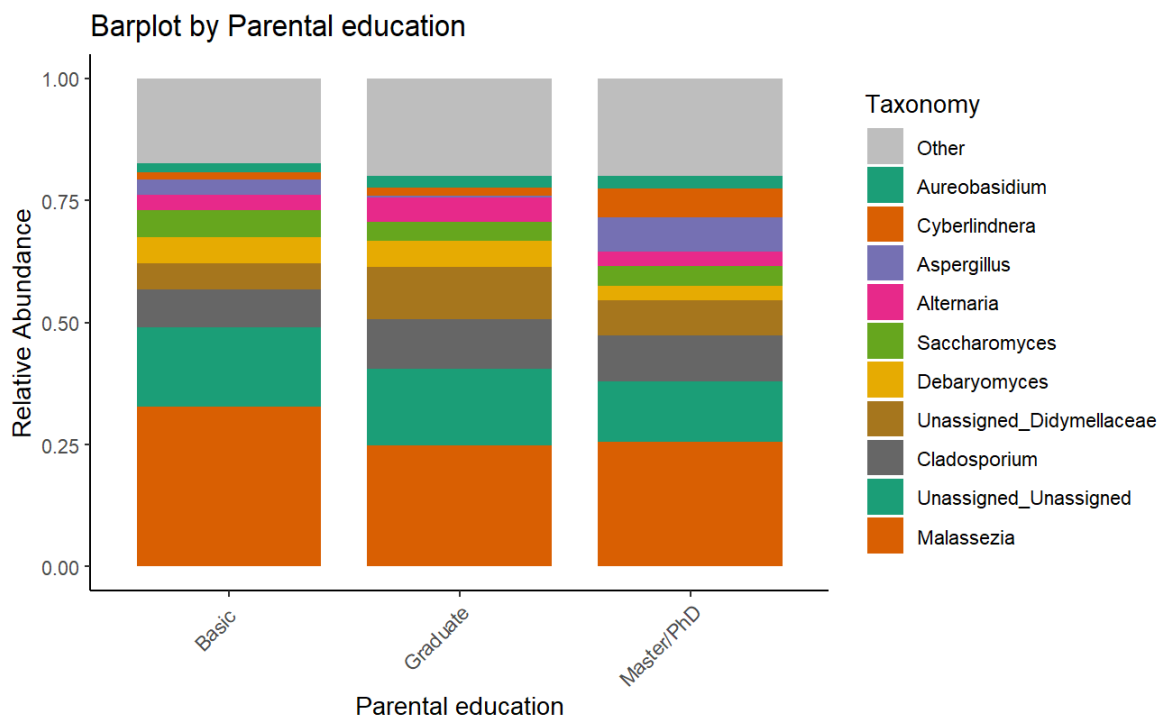
(a)



(b)

Figure 24. Relative abundance of bacterial genera by parental education. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

The bar plot shows (Figure 25a) slight variations in microbial composition among Basic, Graduate, and Master/PhD categories, with taxa like *Malassezia* and *Cyberlindnera* dominating. The violin plot (Figure 25b) reveals significant differences for Unassigned Didymellaceae ($p = 0.010$) and *Alternaria* ($p = 0.048$).



(a)

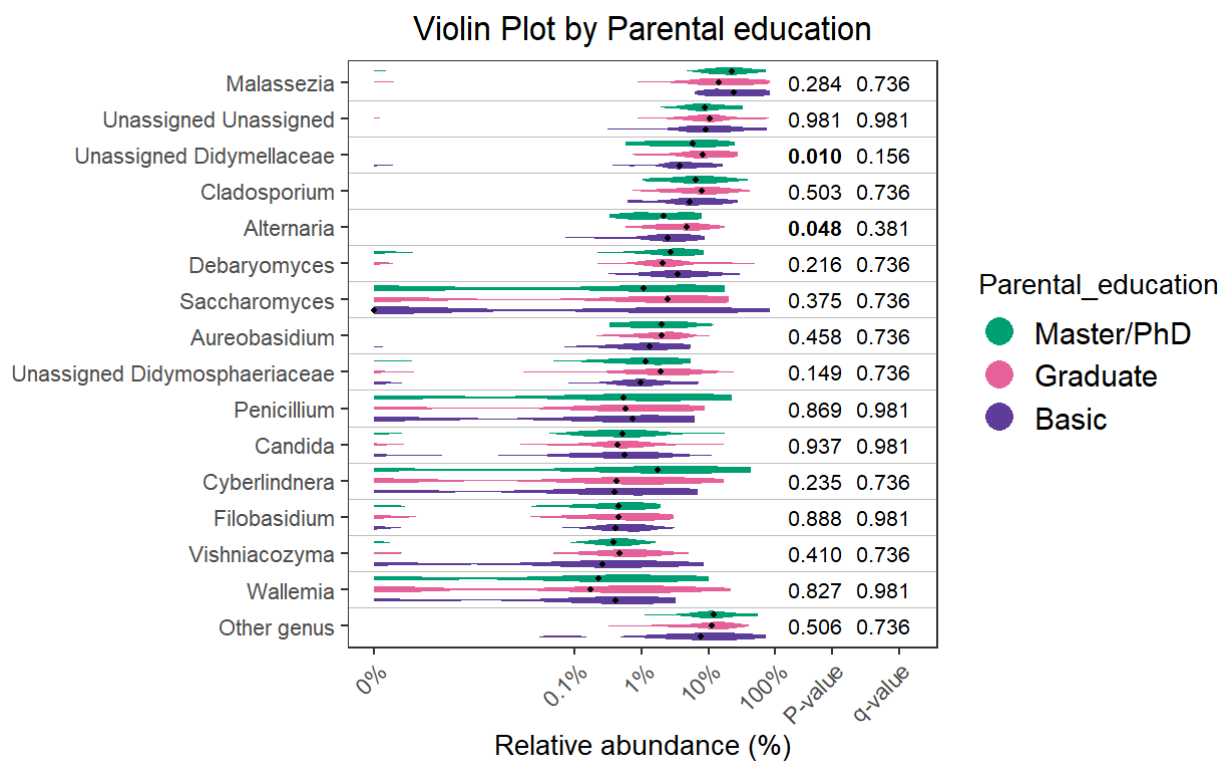
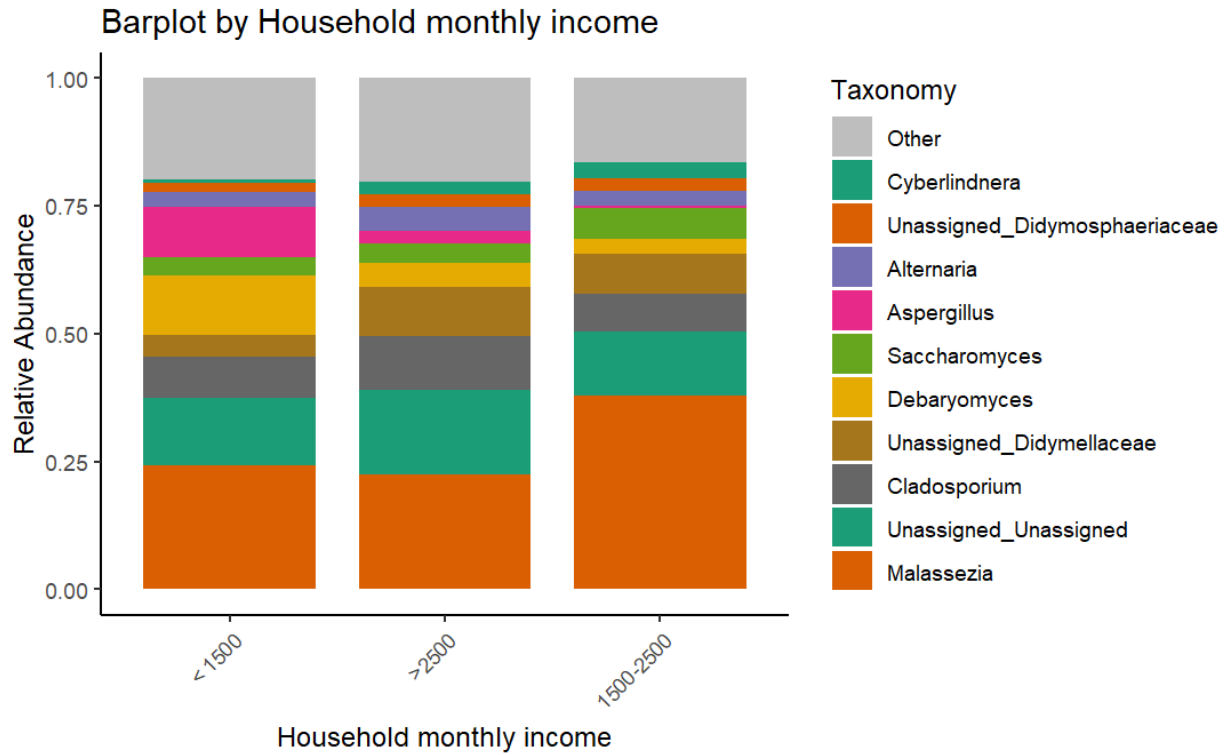
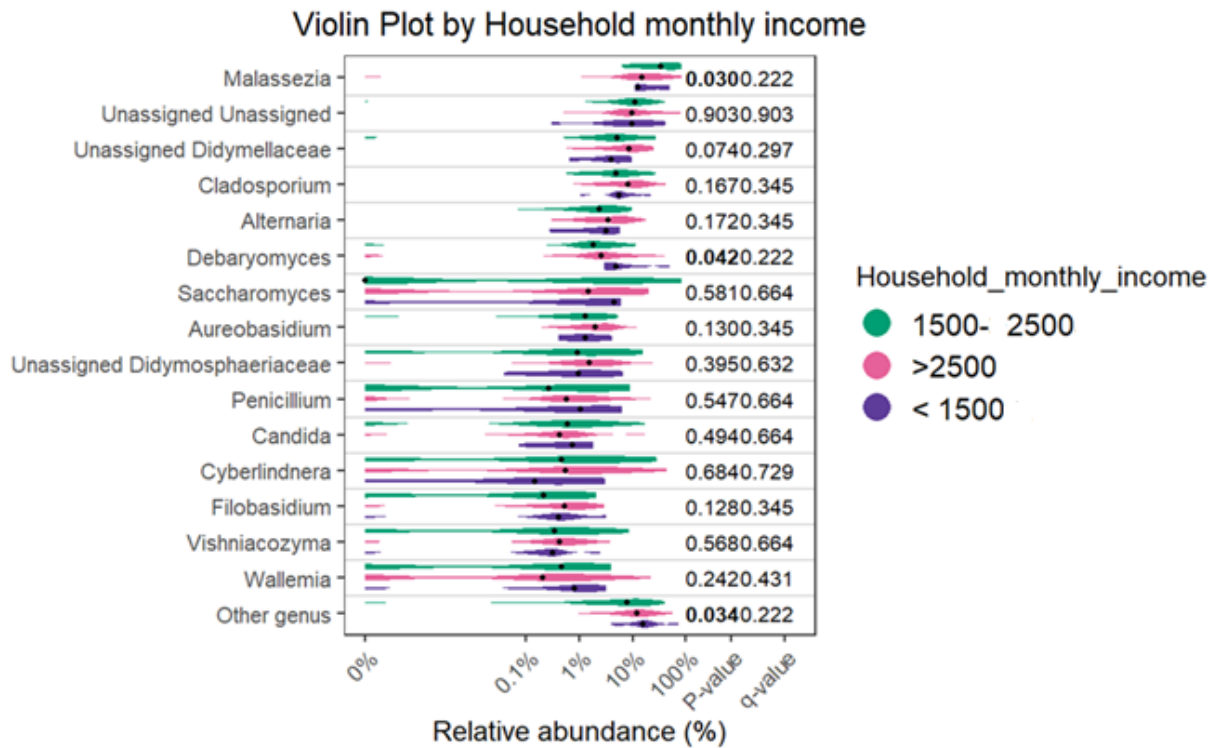


Figure 25. Relative abundance of fungal genera by parental education. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

In terms of household monthly income, the bar plot (Figure 26a) also indicates that *Malassezia* is a substantial percentage of the microbial composition across income levels. Other taxa display minor variations, apart from visible abundance of *Aspergillus* and *Debaryomyces* in lowest income category. The violin plot (Figure 26b) highlights significant differences for *Malassezia* ($p = 0.030$), *Debaryomyces* ($p = 0.042$) and other genus ($p = 0.034$) between income groups.



(a)



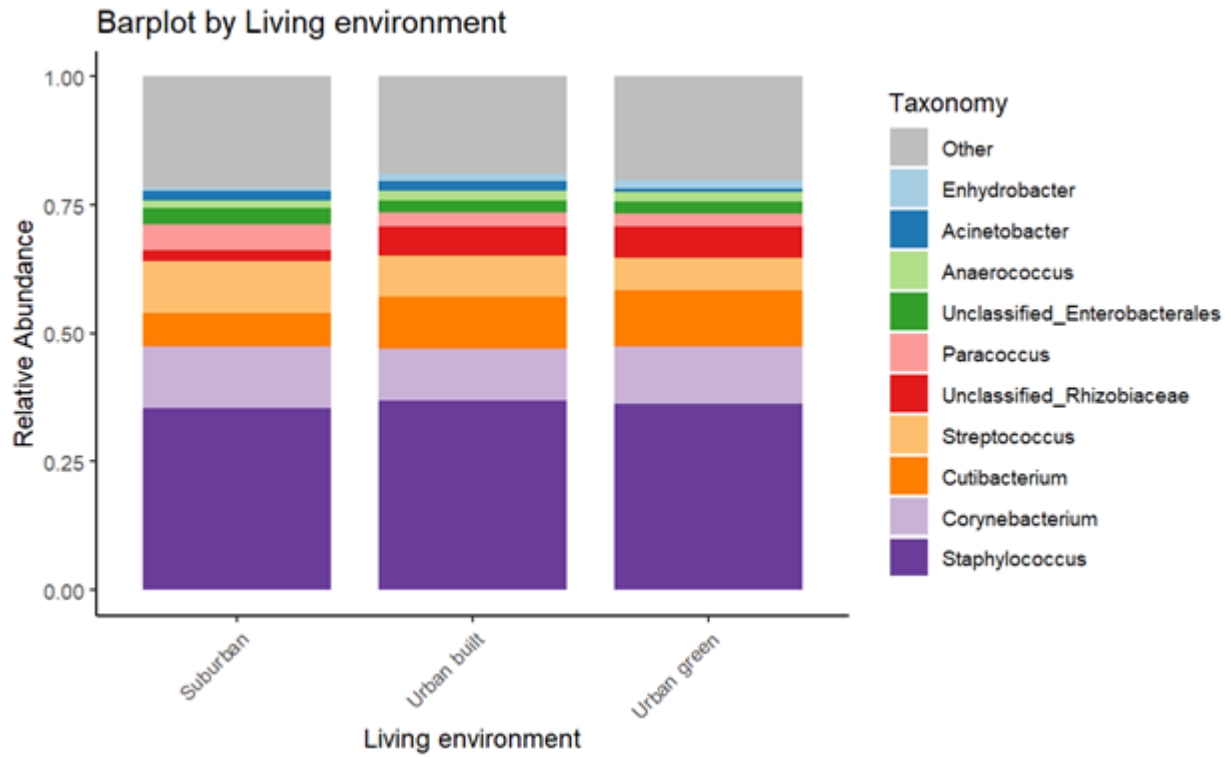
(b)

Figure 26. Relative abundance of fungal genera by household monthly income. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

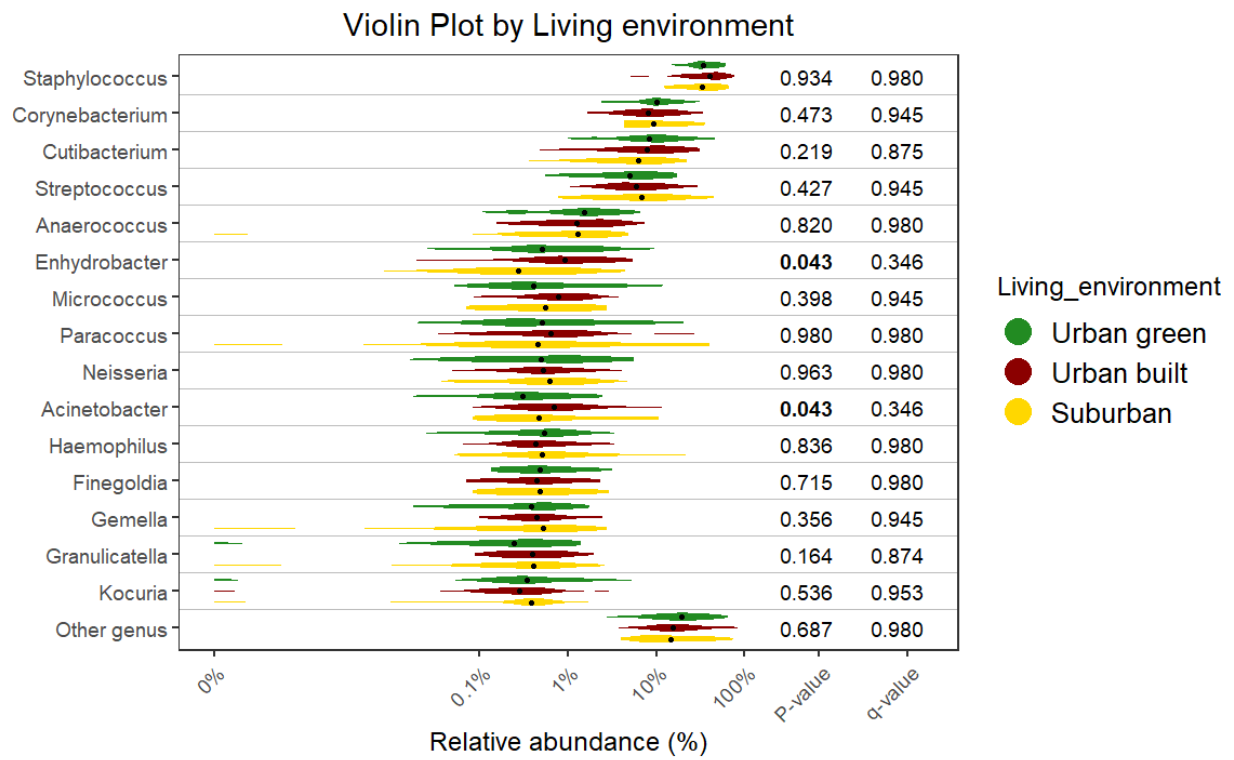
4.2.5.3. Household characteristics

The results highlight the relative abundance of microbial taxa in relation to various household characteristics, including living environment, home type, number of siblings, presence of plants and animals, and specific household pets (cats and dogs). Characteristics not presented here, whether for bacteria or fungi, are included in the Supplement, as they did not display statistical significance.

Figures 27-35 explore the relationship between bacterial relative abundance and environmental factors: number of plants in the household, home type, and living environment. The bar plots demonstrate that the overall microbial community composition is largely stable across the different categories, with dominant taxa such as *Staphylococcus*, *Corynebacterium*, and *Cutibacterium* consistently abundant. Subtle variations in less dominant taxa, such as *Paracoccus* and *Enhydrobacter*, are observed but do not strongly affect the overall microbial structure. In contrast, the violin plots offer a more detailed insight, revealing significant differences in specific taxa. For the living environment (Figure 27 a and b), both *Enhydrobacter* and *Acinetobacter* show notable differences ($p = 0.043$) with more abundance in urban area surrounded with built environment.



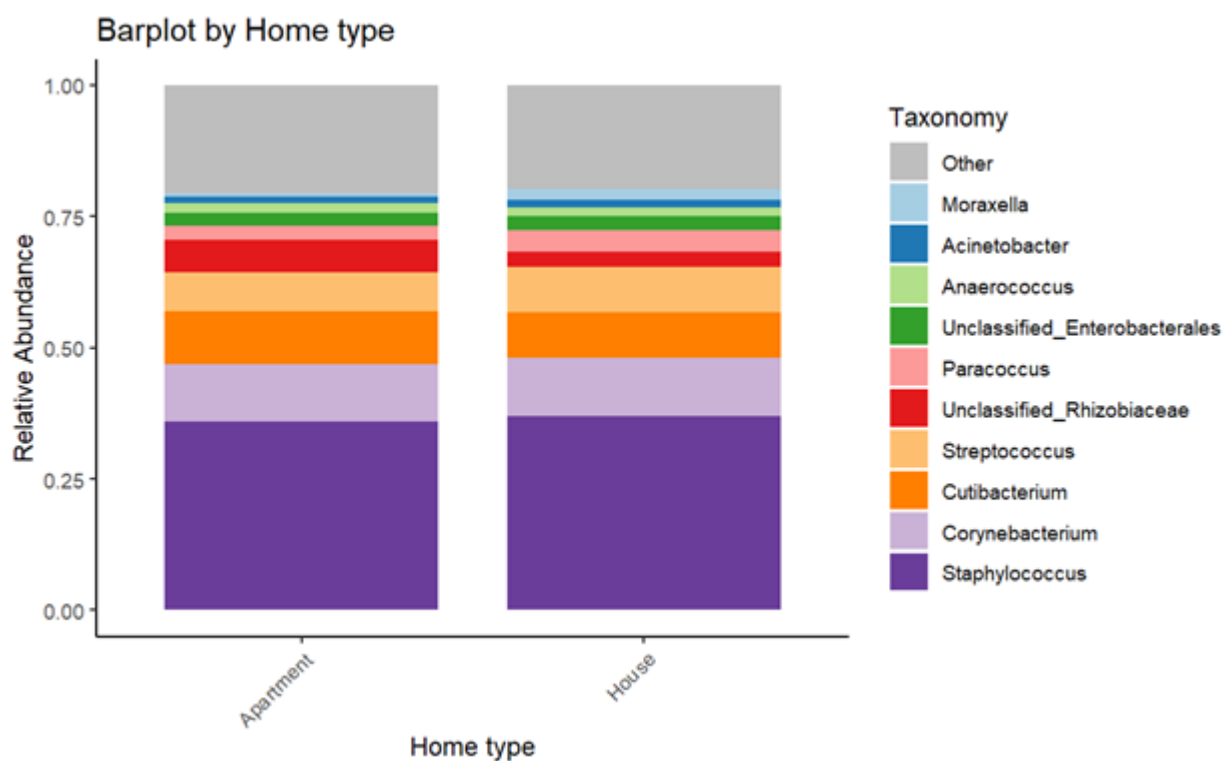
(a)



(b)

Figure 27. Relative abundance of bacterial genera by living environment. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

Both houses and apartments (Figure 28 a and b) show similar relative abundance, with slight differences in certain taxa. Genus *Enhydrobacter* is also significantly different between houses and apartments ($p = 0.005$).



(a)

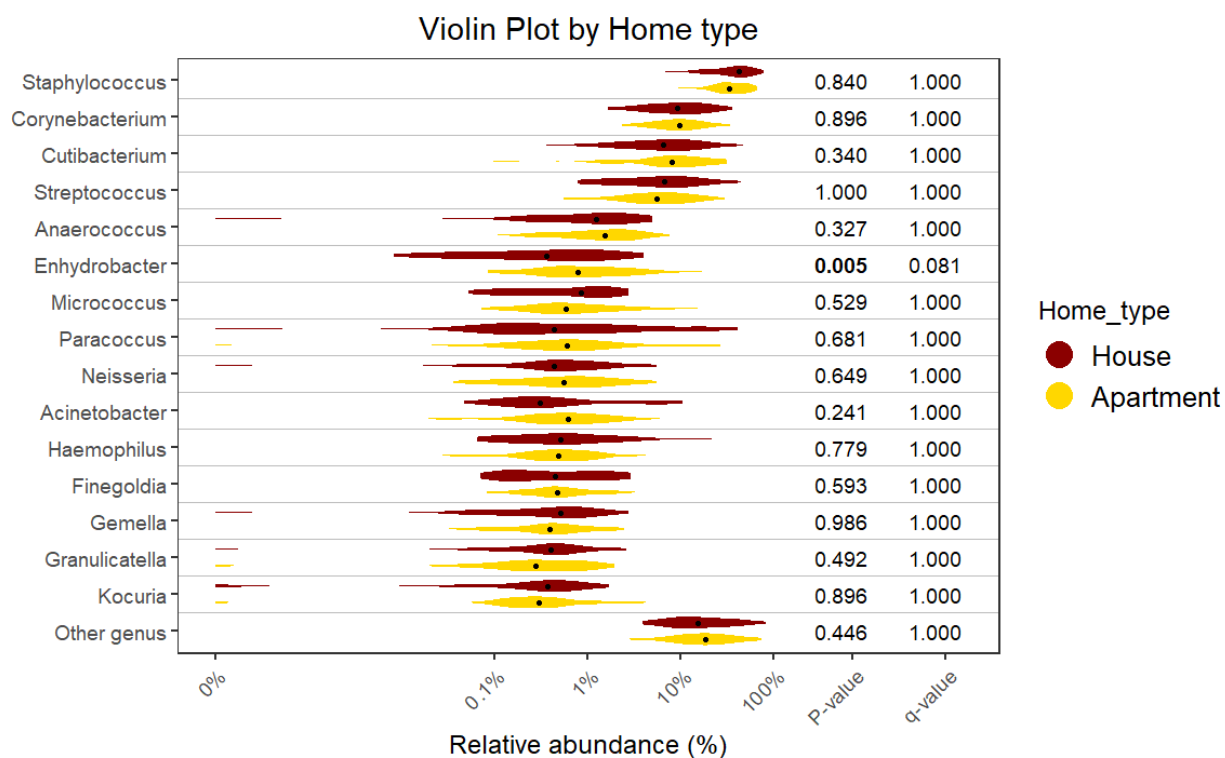
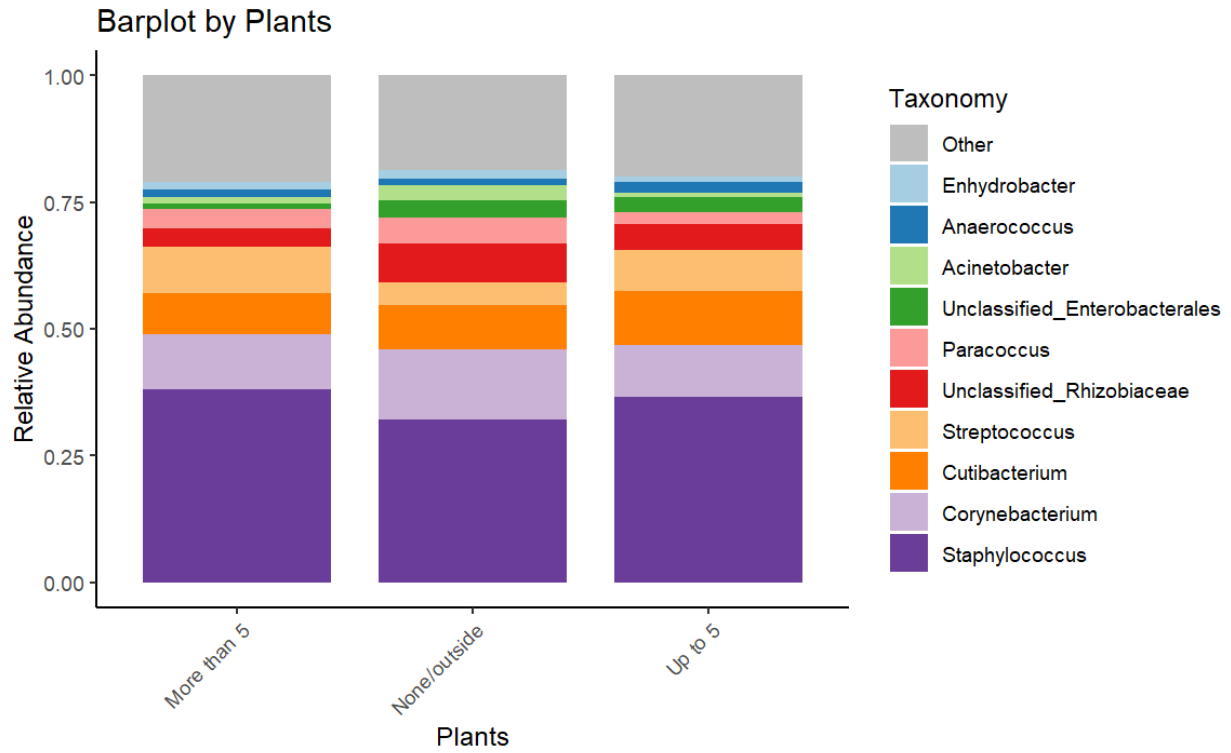
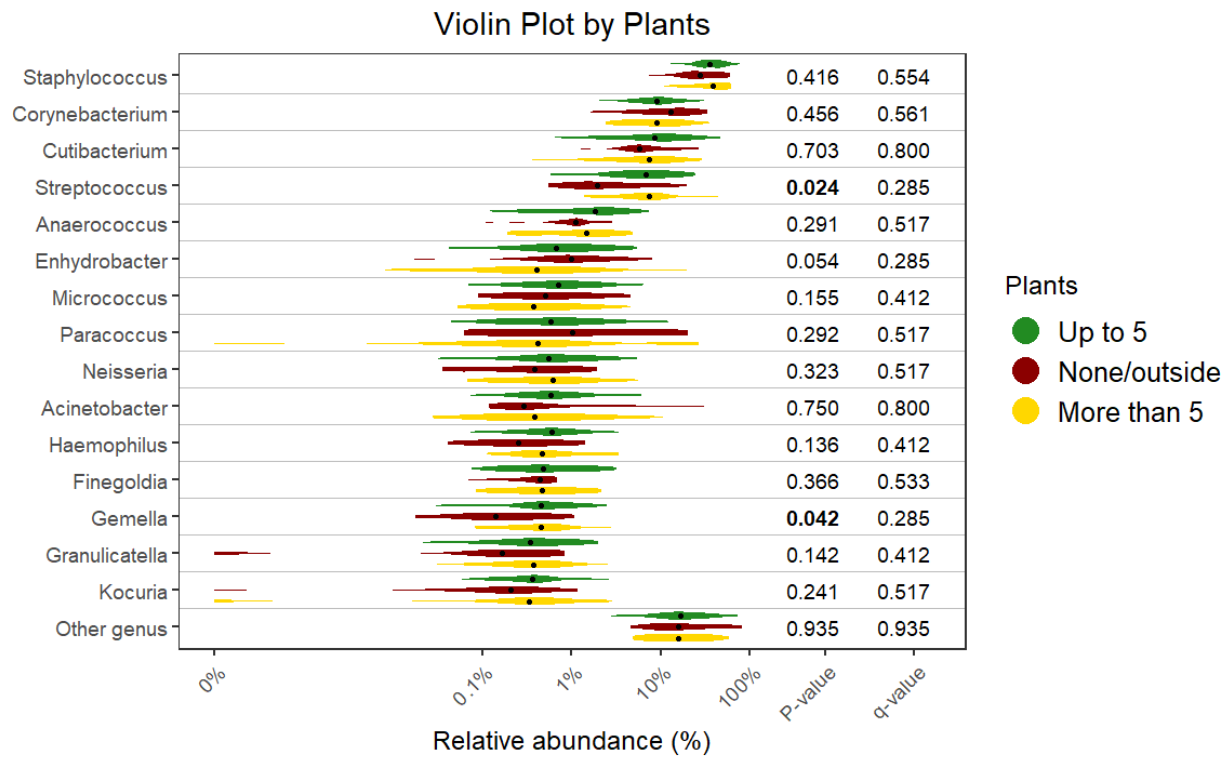


Figure 28. Relative abundance of bacterial genera by home type. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

Regarding the number of plants (Figure 29 a and b), relative abundance is similar across all three groups. *Streptococcus* and *Gemella* exhibit significant differences, with p-values of 0.024 and 0.042, respectively.



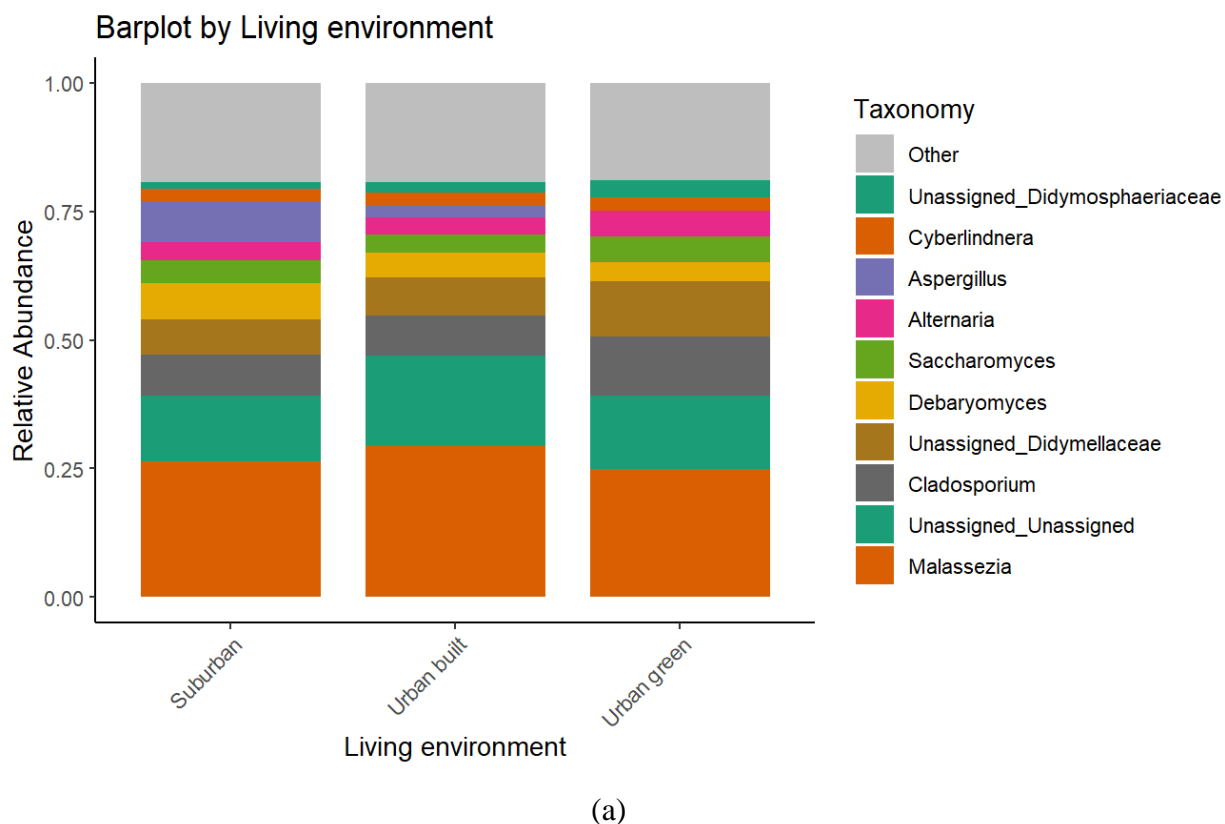
(a)



(b)

Figure 29. Relative abundance of bacterial genera by number of plants. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

For the living environment (Figure 30 a and b), the barplot indicates that microbial compositions are broadly similar across suburban, urban-built, and urban-green environments, with *Malassezia* being prevalent. The violin plot identifies Unassigned Didymellaceae as significantly different ($p = 0.044$), while other taxa show no substantial differences.



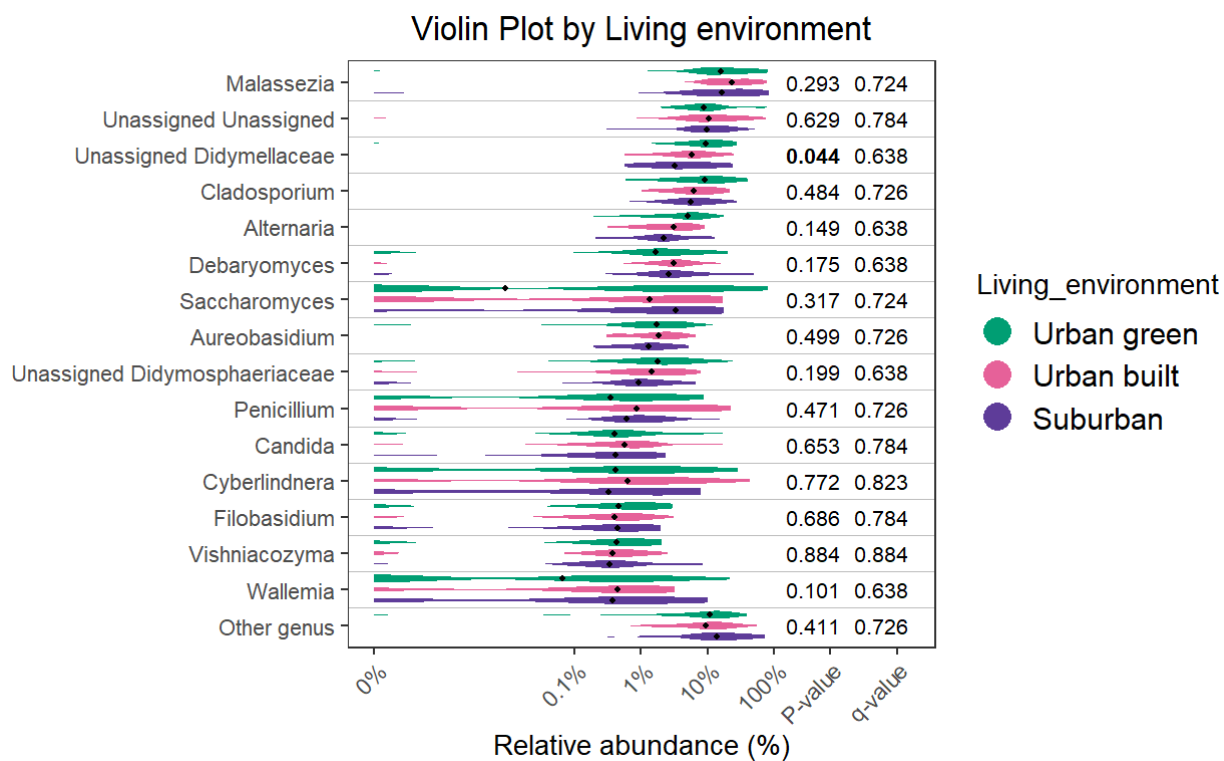
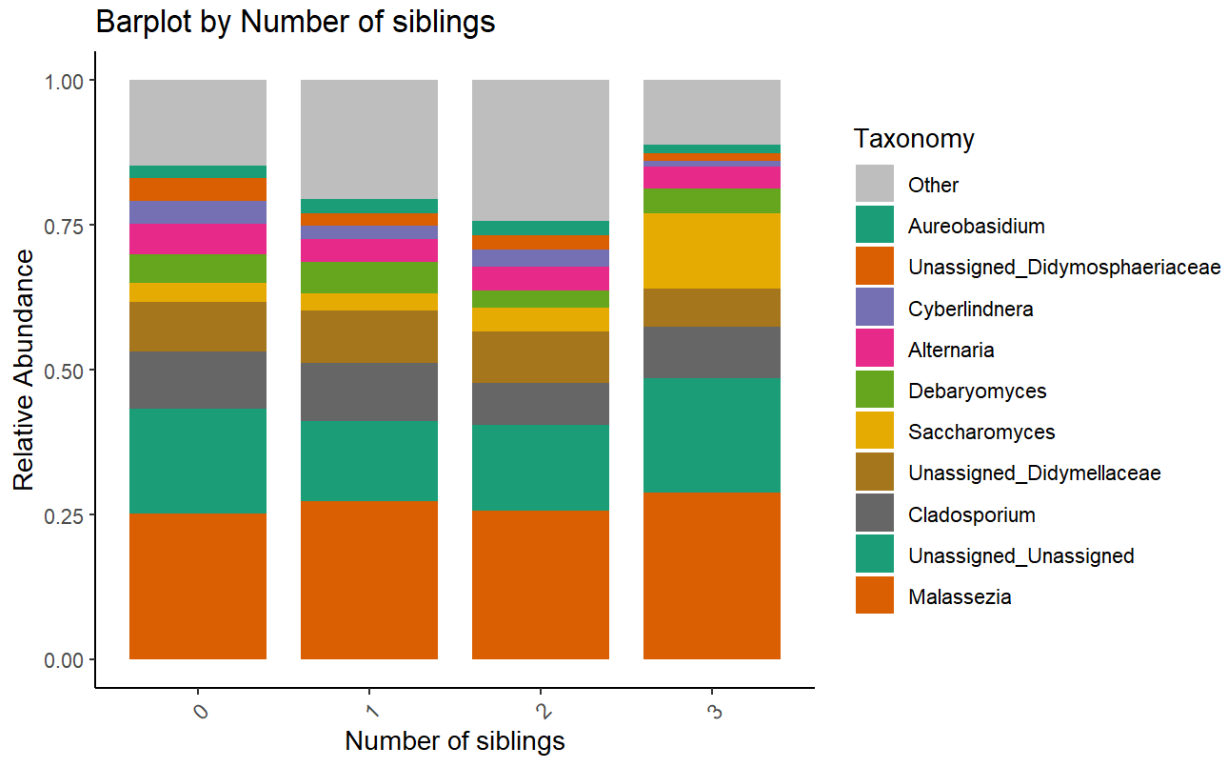
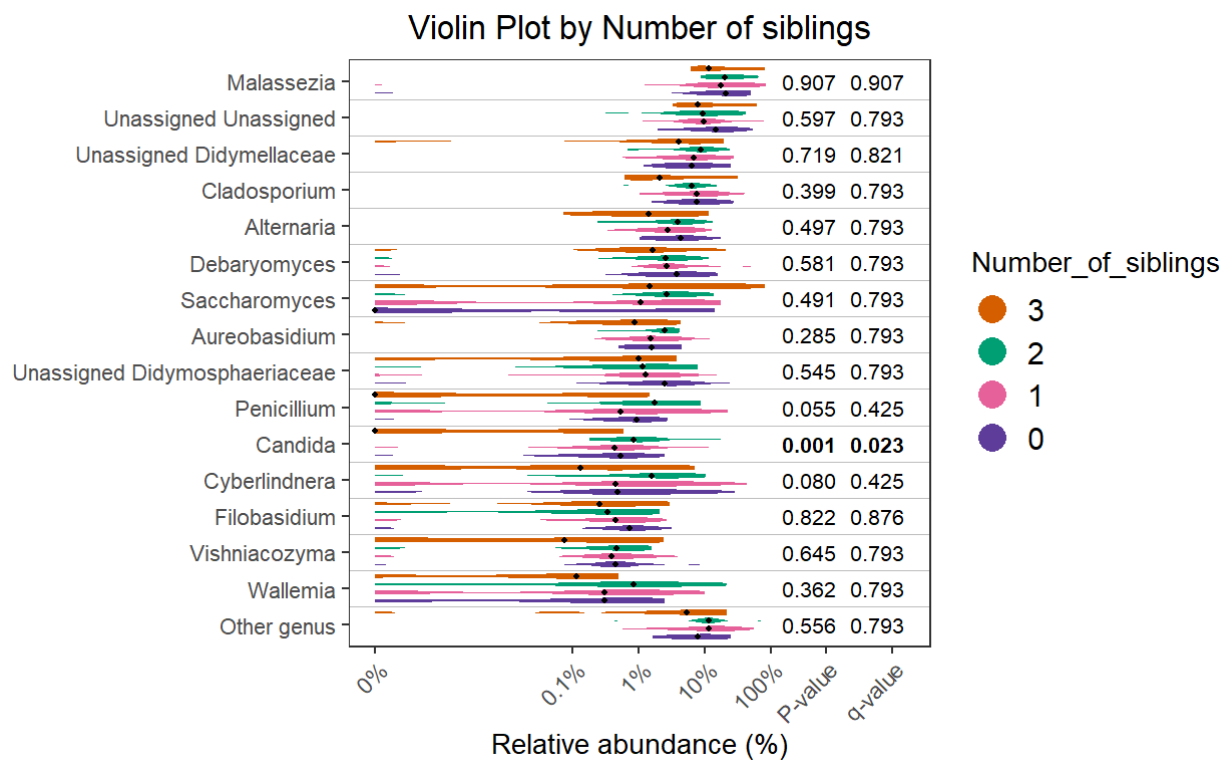


Figure 30. Relative abundance of fungal genera by living environment. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

For the number of siblings (Figure 31 a and b), the bar plot shows consistent dominance of *Malassezia*, with slight variations in other taxa. *Saccharomyces* shows a notable difference in the group with three siblings, while the same group has a lower abundance of *Cyberlindnera* compared to other groups. Additionally, this group shows a lower abundance of other genera, suggesting that the microbial community composition in households with three siblings is more specialized or dominated by specific taxa. The violin plot highlights statistically significant differences for *Candida* ($p = 0.001$), while most taxa, including *Malassezia*, show no significant differences. *Candida* shows most abundance in the households with 2 siblings.



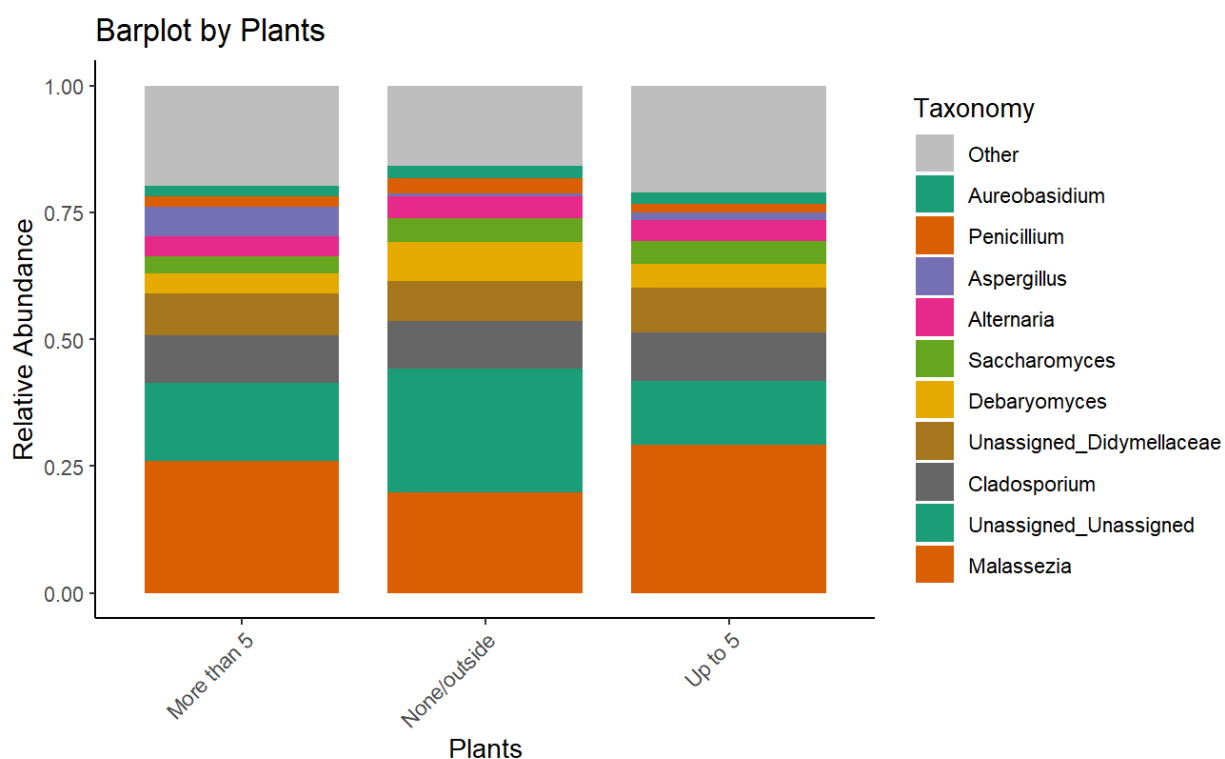
(a)



(b)

Figure 31. Relative abundance of fungal genera by number of siblings. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

Relative abundance plot for plants (Figure 32 a and b) shows minimal differences in microbial compositions across categories, with *Malassezia* remaining dominant. However, there is a slight decrease in its abundance in homes without plants or with plants located outside the home. The violin plot indicates statistically significant differences for Unassigned_unassigned taxa ($p=0.042$).



(a)

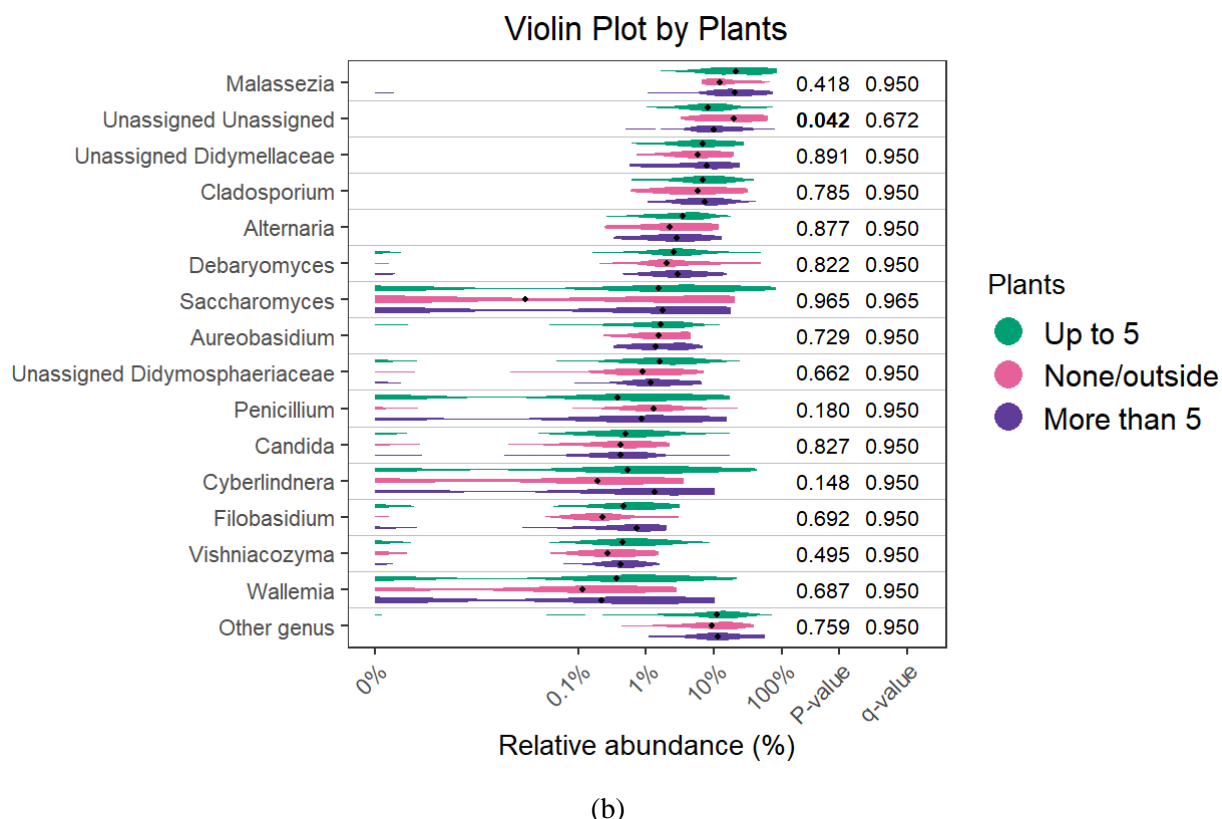
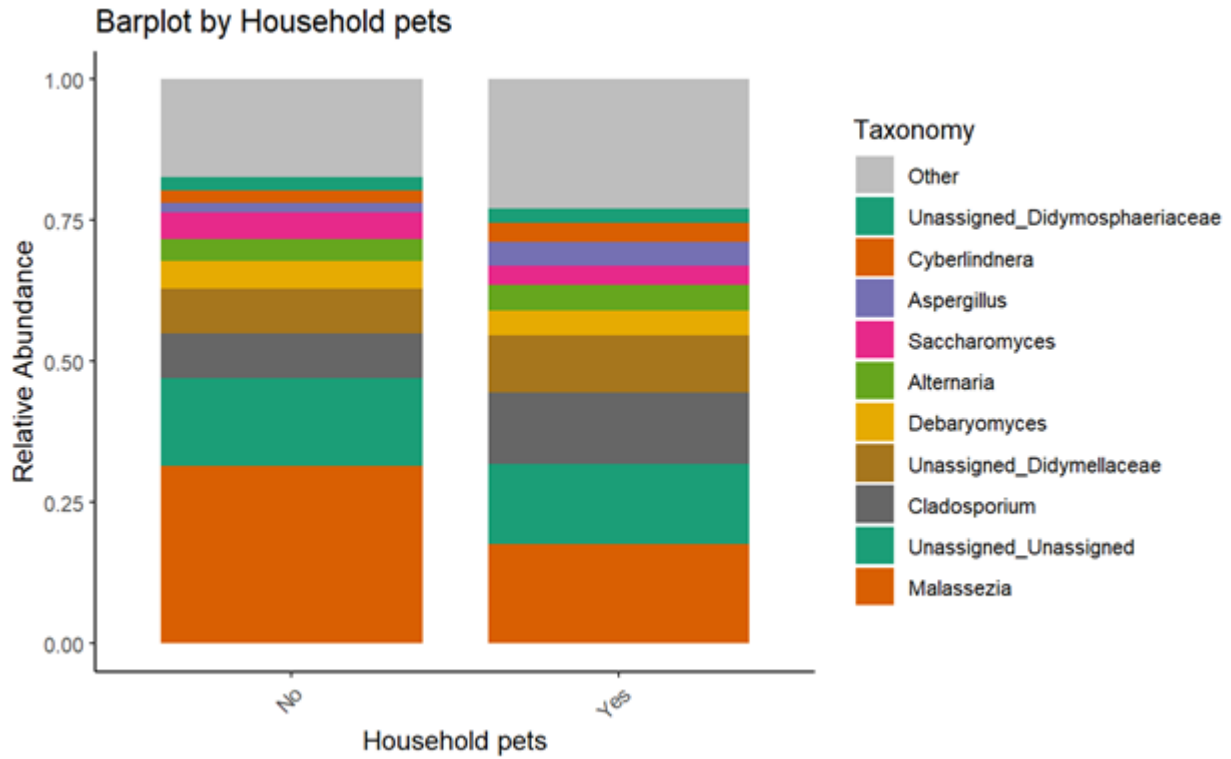
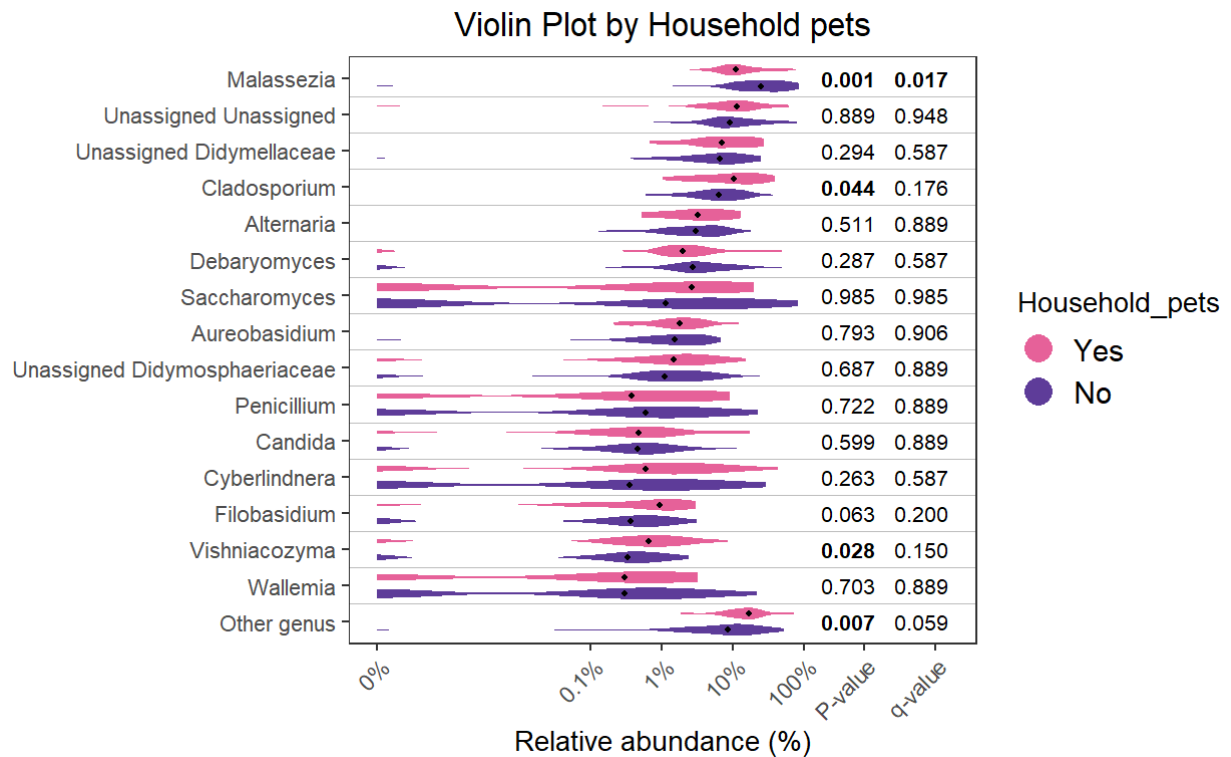


Figure 32. Relative abundance of fungal genera by number of plants. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

The results highlight the relative abundance of microbial taxa in relation to the presence of pets in the household (Figure 33 a and b), including specific analyses for dogs (Figure 34 a and b) and cats (Figure 35 a and b). Across all categories, *Malassezia* remains the dominant taxon, showing greater abundance in homes without pets. When considering household pets in general, a greater number of significant taxa are observed compared to all other environmental factors. Violin plots (Figure 31 b) show significant differences for *Malassezia* ($p = 0.001$), *Cladosporium* ($p = 0.044$), *Vishniacozyma* ($p = 0.028$), and other genus ($p = 0.007$). Other genus is also significant in households with cats ($p = 0.027$, Figure 35 b) and dogs ($p = 0.026$, Figure 34 b). Additionally, both dogs and cats show significant differences for *Malassezia* (cats: $p = 0.004$; dogs: $p = 0.002$) and *Filobasidium* (cats: $p = 0.028$; dogs: $p = 0.046$). In households with cats, *Candida* is also significant ($p = 0.046$).



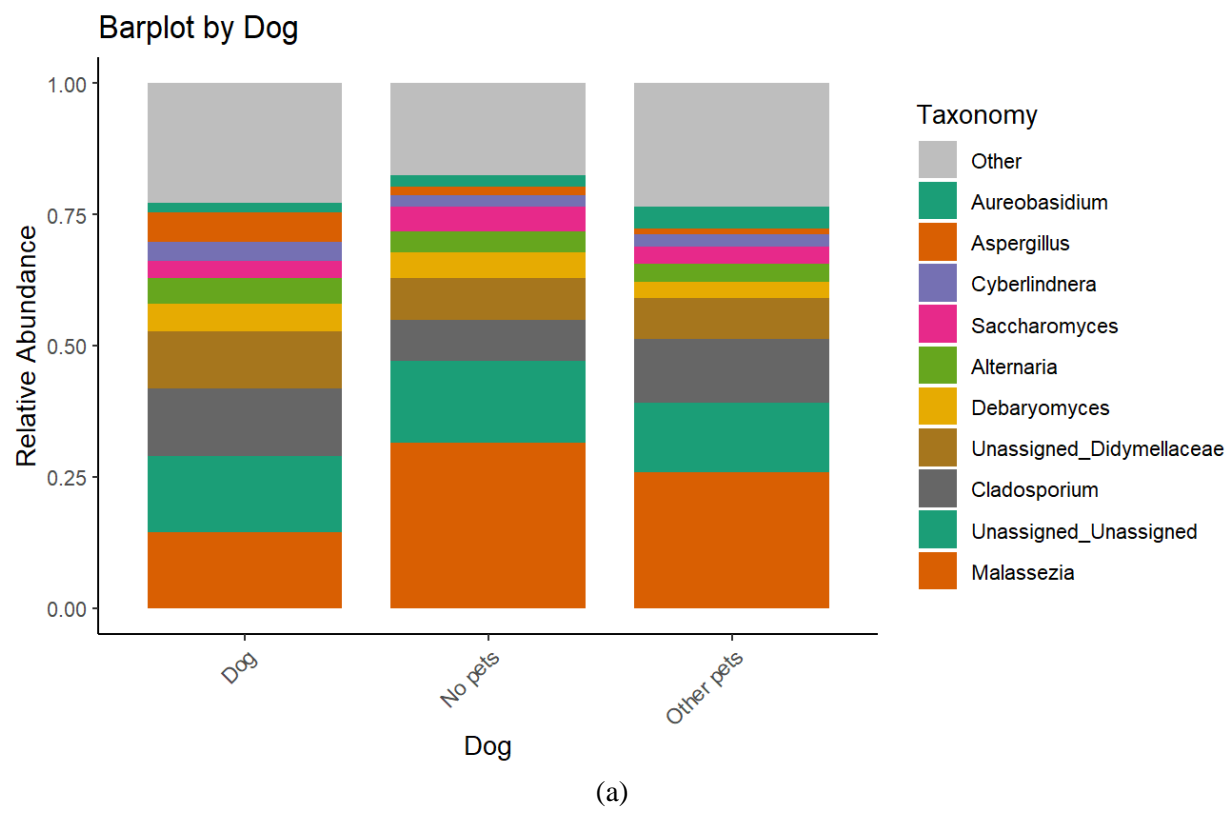
(a)



(b)

Figure 33. Relative abundance of fungal genera by pets in the household. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across

samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.



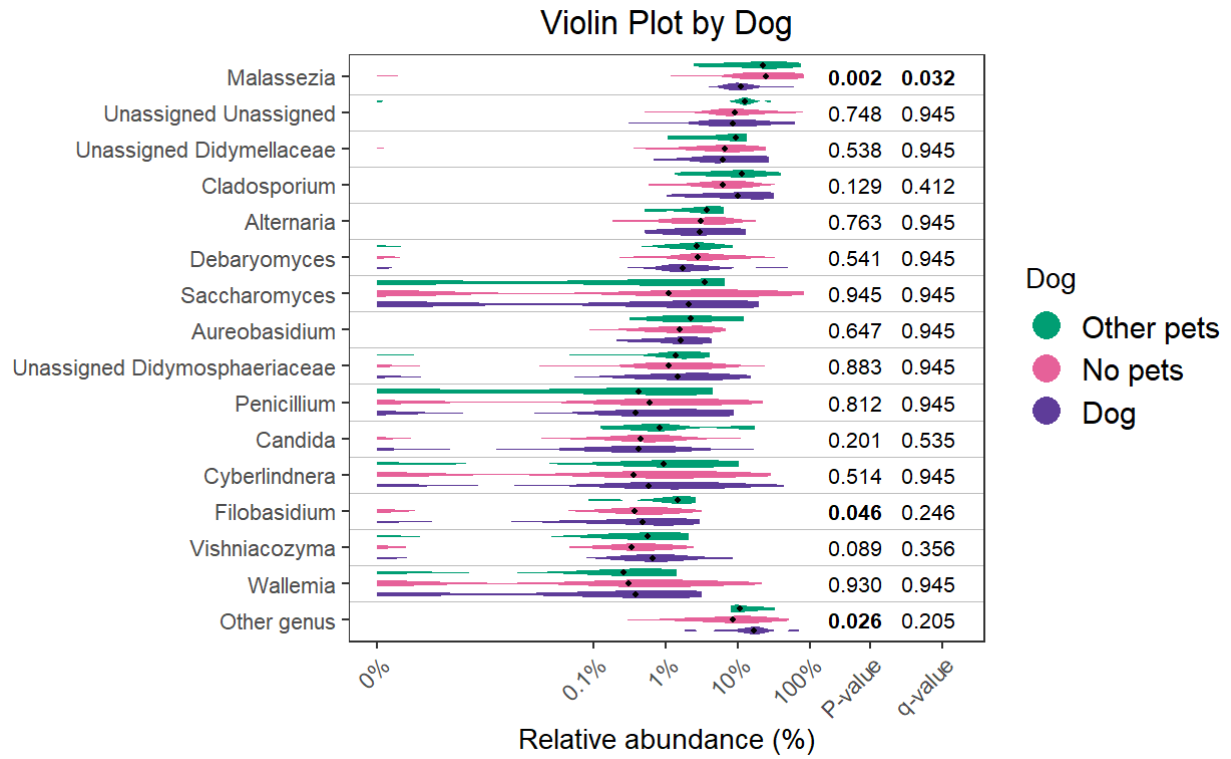
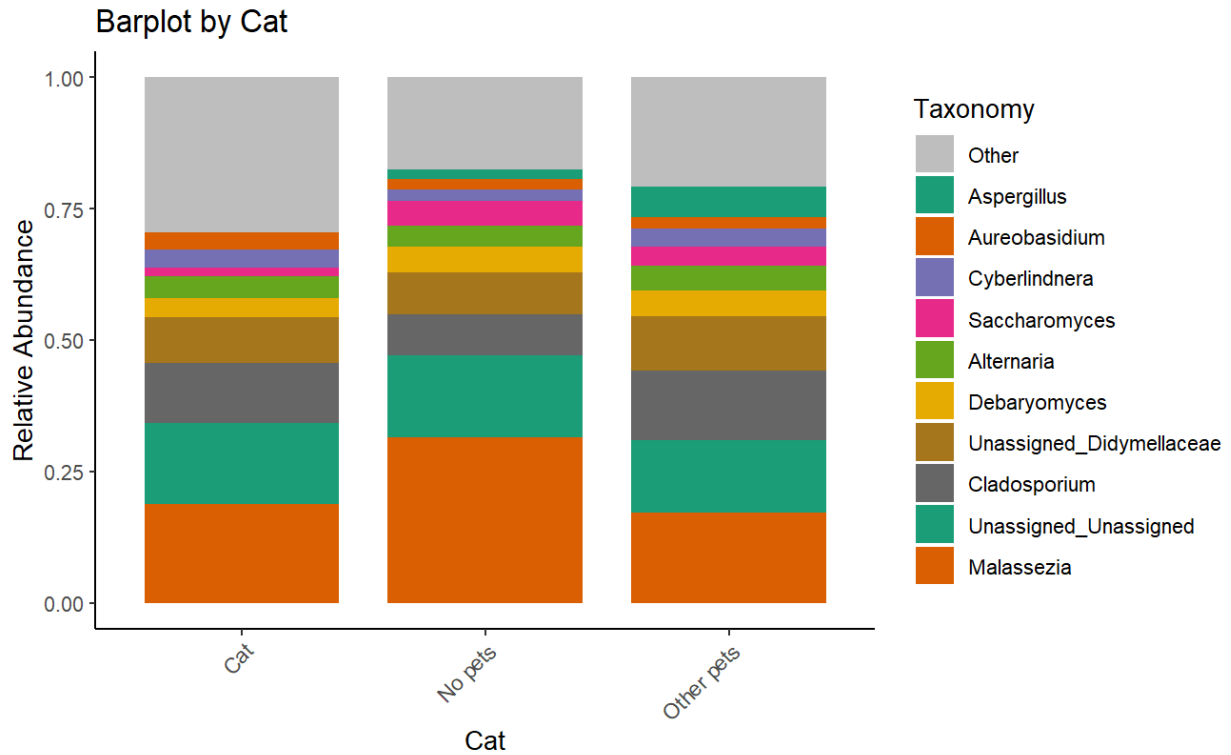
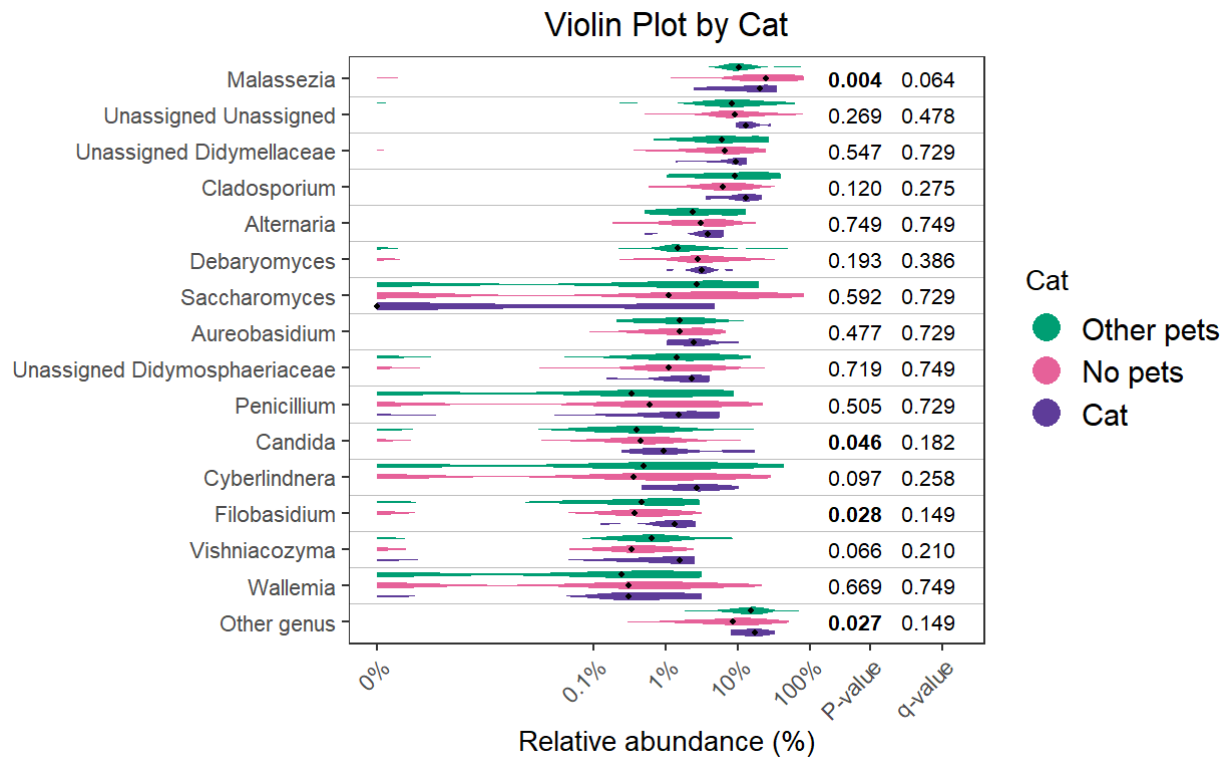


Figure 34. Relative abundance of fungal genera by dog in the household. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.



(a)



(b)

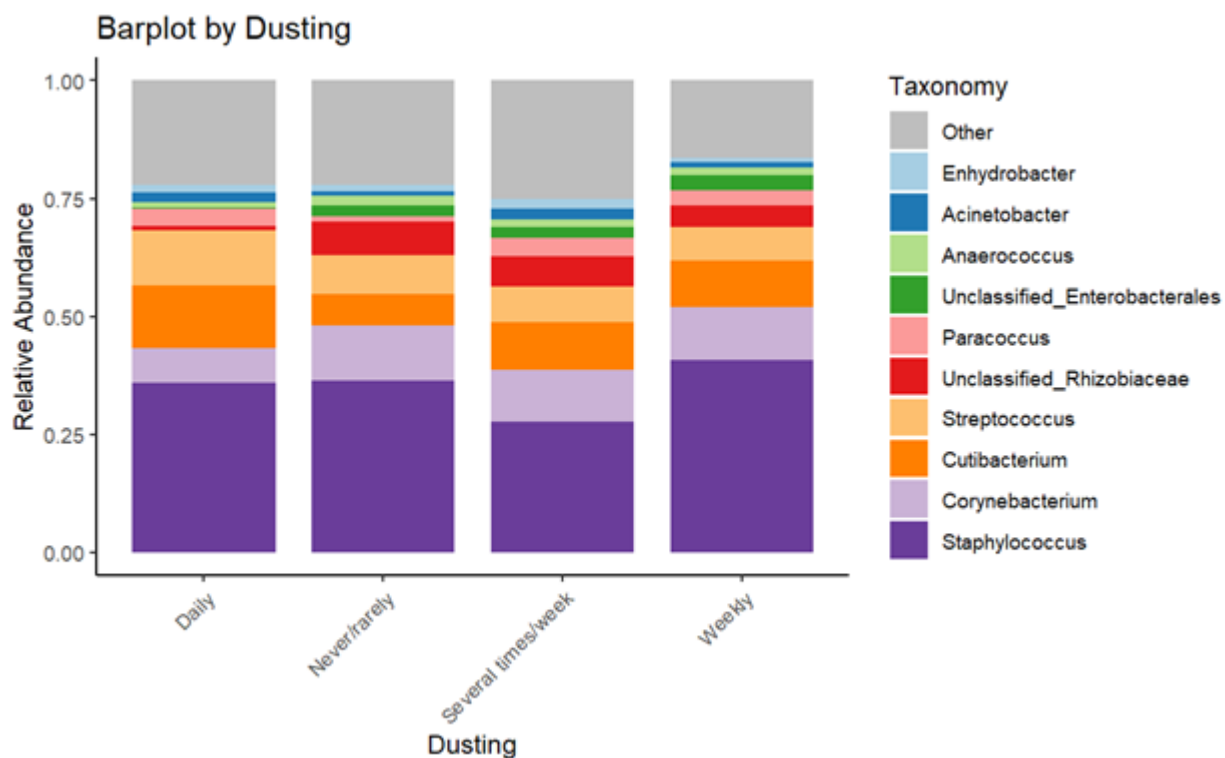
Figure 35. Relative abundance of fungal genera by cat in the household. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across

samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

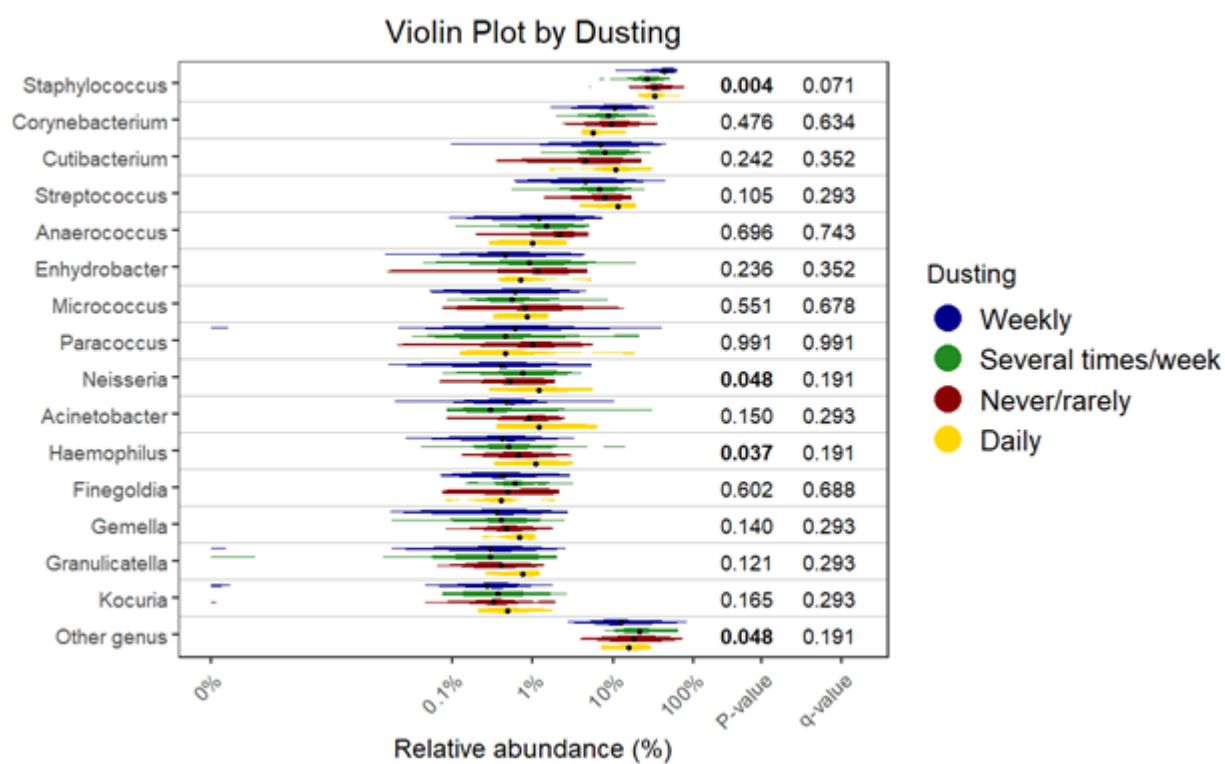
4.2.4.3. Cleaning practices

The results show the relative abundance of microbial taxa based on mattress vacuuming and dusting frequency. Interestingly, both practices exhibit some statistically significant taxa for both bacteria and fungi.

Figures 36.- 39. show the relationship between microbial relative abundance and cleaning practices, specifically mattress vacuuming and dusting. The bar plots demonstrate a consistent microbial composition across different frequencies of mattress vacuuming and dusting. Dominant taxa such as *Staphylococcus*, *Corynebacterium*, and *Cutibacterium* remain stable across all groups, with only minor differences in less abundant taxa like *Enhydrobacter* and *Paracoccus*. The violin plots provide finer detail and highlight specific taxa with statistically significant differences. For dusting (Figure 36b), *Staphylococcus* shows significant differences ($p = 0.004$) alongside *Neisseria* and other genus, both with equal $p = 0.048$. For mattress vacuuming (Figure 37b), *Staphylococcus* ($p = 0.017$), is also significant, and other genera show significance ($p = 0.007$) as well.

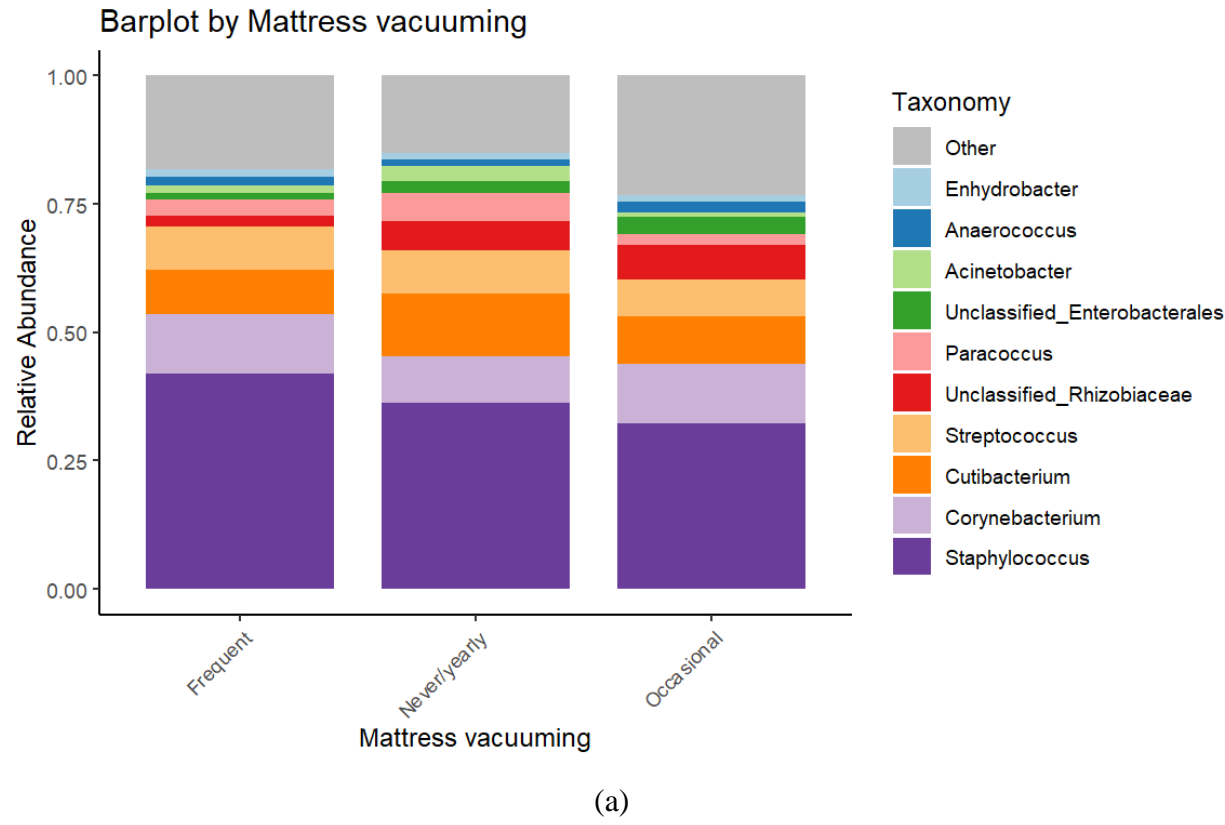


(a)



(b)

Figure 36. Relative abundance of bacterial genera by dusting frequency. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.



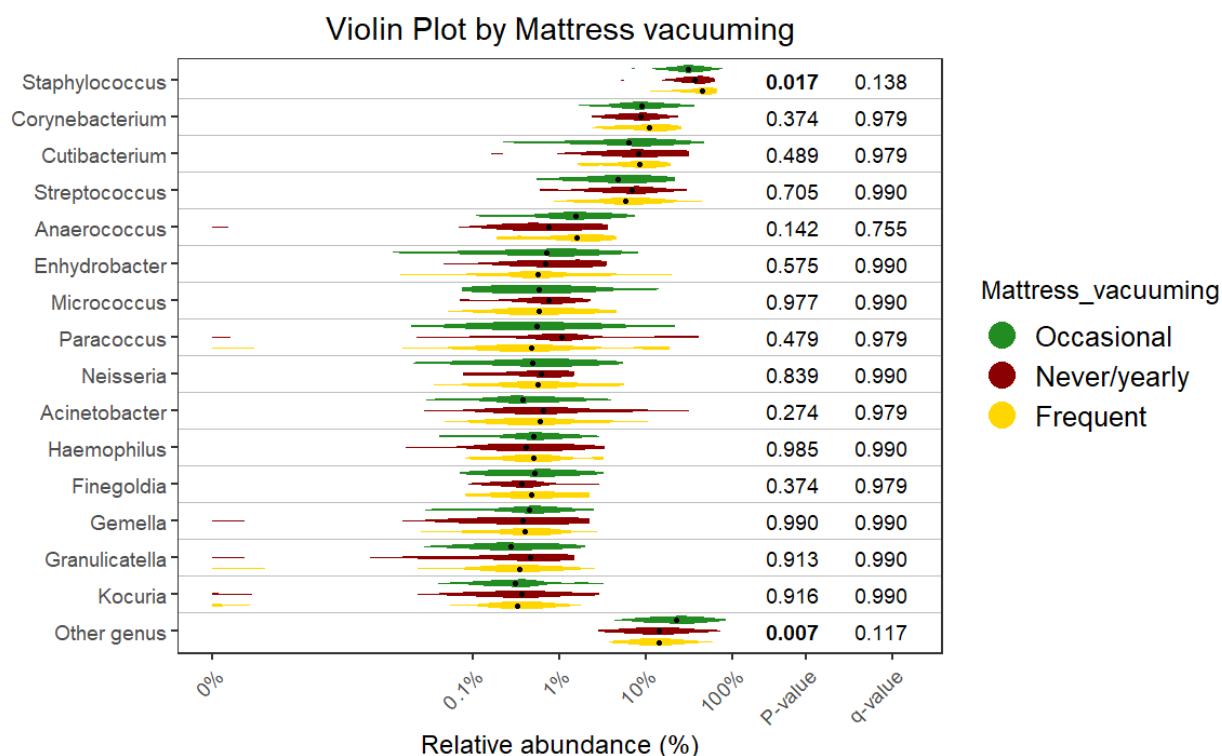
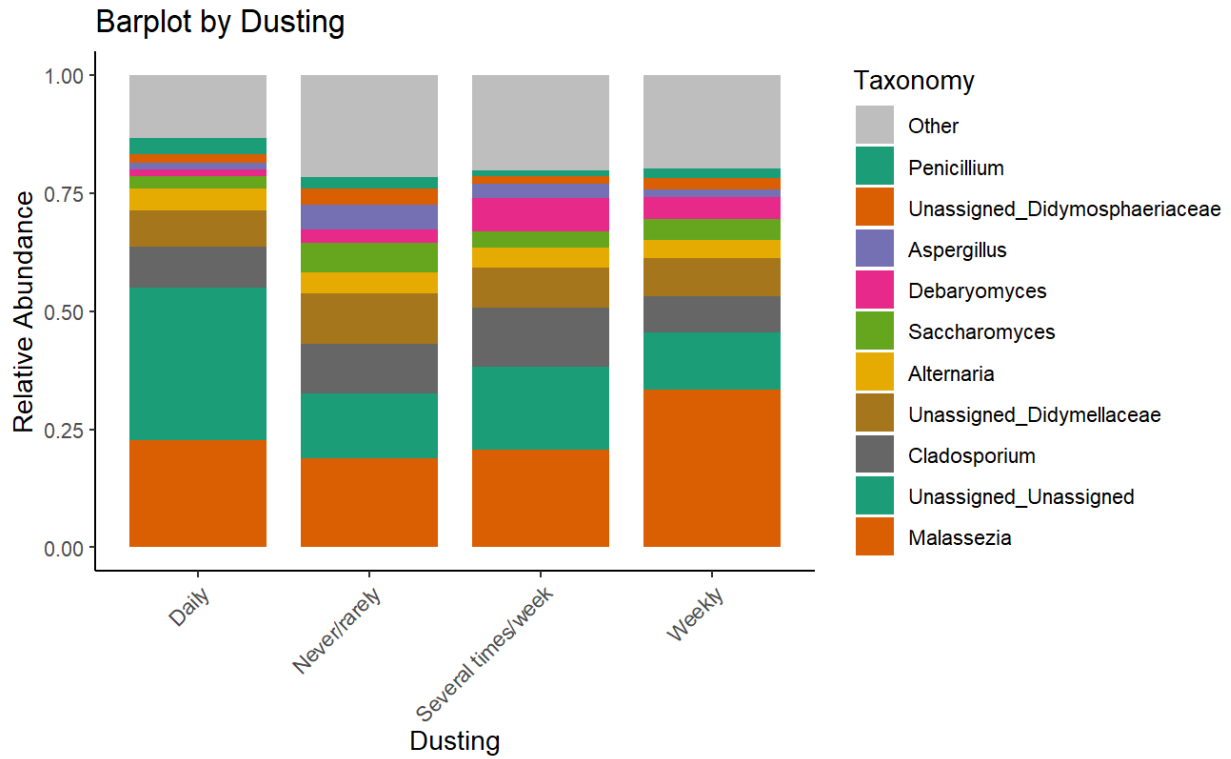
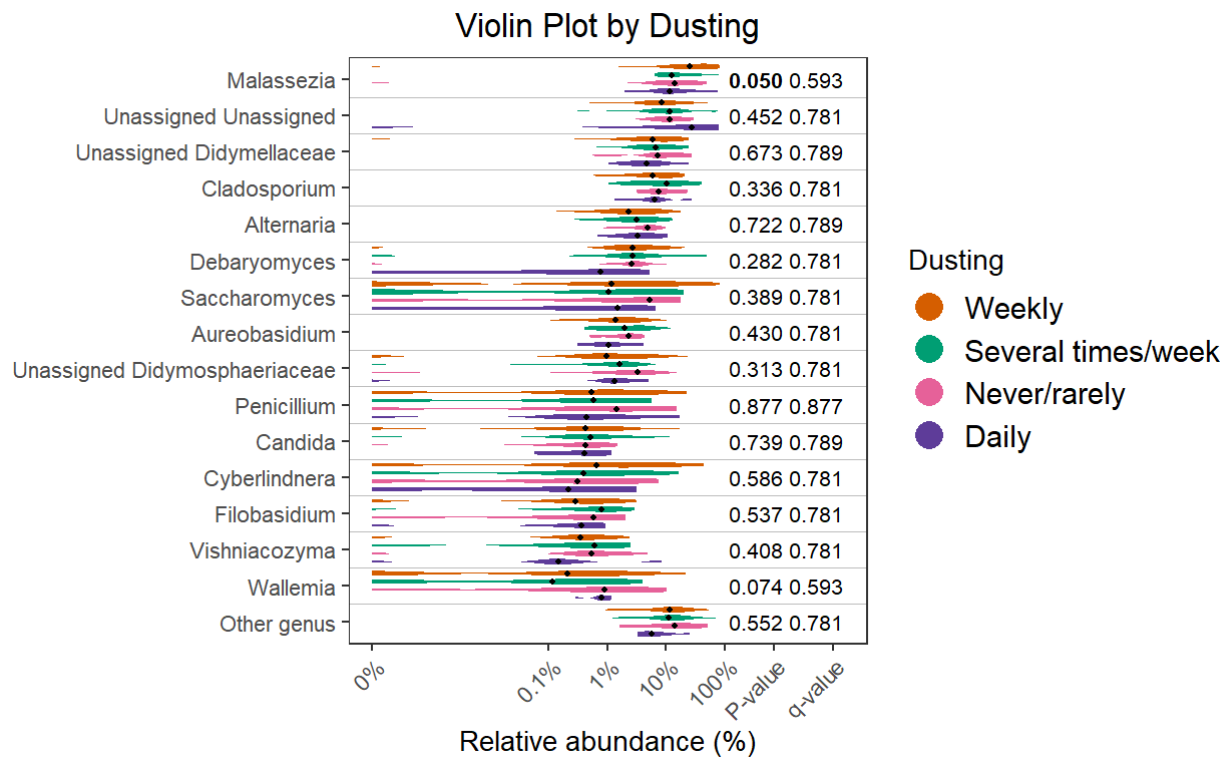


Figure 37. Relative abundance of bacterial genera by vacuuming mattress frequency. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups

Across all categories, *Malassezia* remains the dominant taxon. For dusting, the bar plot (Figure 38a) demonstrates consistent dominance of *Malassezia* across all dusting frequencies (daily, weekly, several times per week, and never/rarely). There are slight differences in represented genera, but the category of daily dusting shows lower abundance of other genera. The violin plot (Figure 38b) highlights *Malassezia* as significant ($p = 0.050$), with no other taxa showing notable differences.



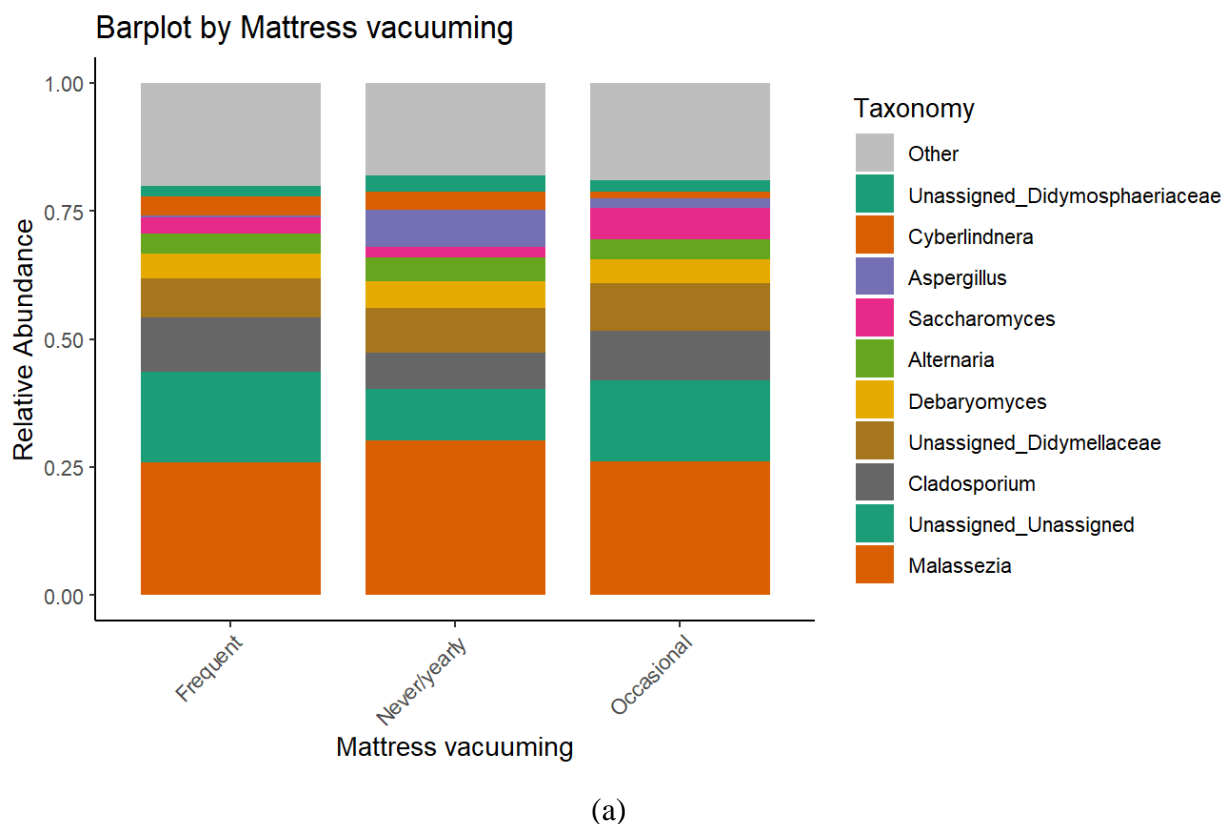
(a)

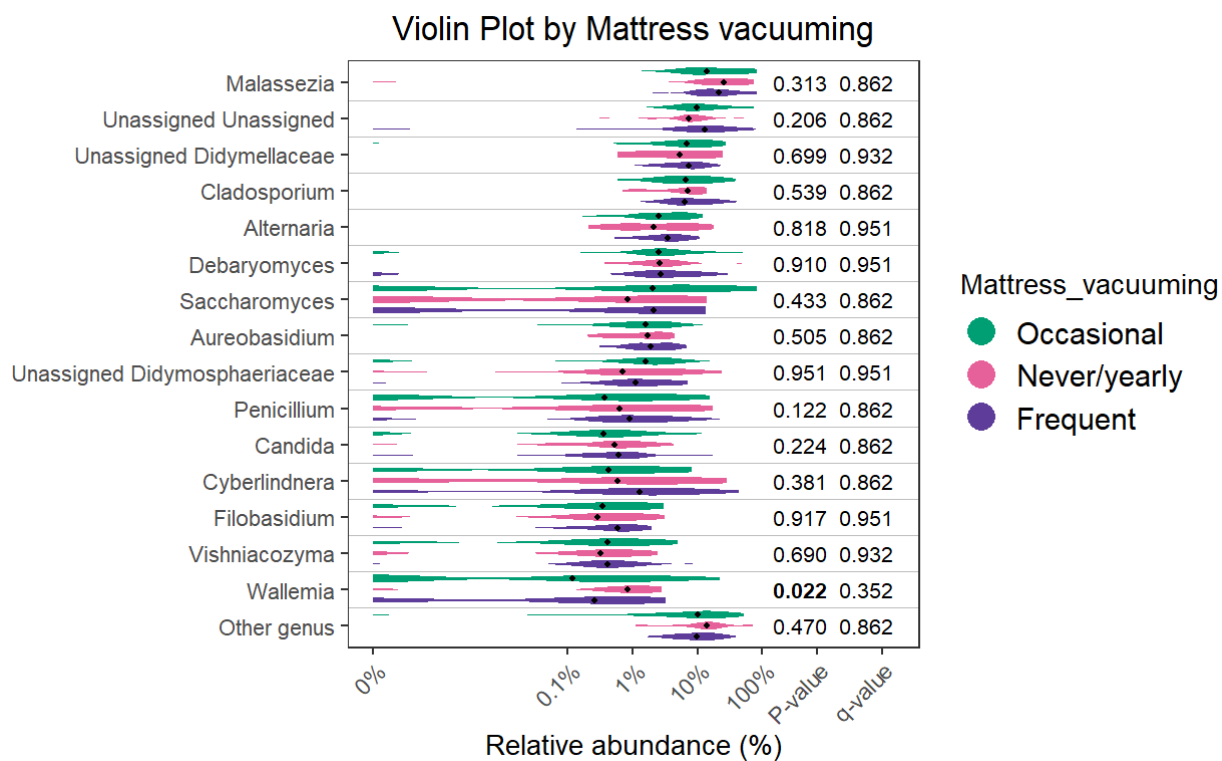


(b)

Figure 38. Relative abundance of fungal genera by dusting frequency. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups

For mattress vacuuming, the bar plot (Figure 39a) shows minimal differences in microbial composition between occasional, frequent, and no vacuuming. Although the *Aspergillus* seems to be more abundant in never or rarely vacuuming category. The violin plot (Figure 39b) identifies a significant difference only for *Wallemia* ($p = 0.022$).





(b)

Figure 39. Relative abundance of fungal genera by vacuuming mattress frequency. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups

4.2.4.4. Environmental conditions

No significant differences were observed in the season of sampling for either fungi or bacteria; therefore, these results are included in the supplementary materials.

4.2.6. Comparison with other cohorts

Although a large amount of publicly available microbiome data exists, the comparison in this study was made using the COPSAC (Copenhagen Prospective Study on Asthma in Childhood) dataset. These samples were collected and processed using the same protocols as the EDIAQI samples. It is worth emphasizing that the “Croatian” samples are localized to the Zagreb area, just as the “Danish” samples are specific to the Copenhagen area. Therefore, neither set of samples can be considered fully representative of the entire country, but rather provide preliminary insights into the microbiome composition of urban households. The comparison with other cohorts, countries

and papers will be in the Discussion chapter. Table 7 presents the diversity metrics of dust microbiome samples to assess whether community composition differs between countries (Croatia vs. Denmark). Both alpha and beta diversity metrics were examined to show a comprehensive understanding of microbial variation across cohorts.

Table 7. Alpha and beta diversity of dust microbiomes by country.

Category	Variable	Total (n, %)	16S α diversity (p val)	16S β diversity (R ² /p val)	ITS α diversity (p val)	ITS β diversity (R ² /p val)
Country	Croatia (EDIAQI)	90 (49.18)	6.98 e-13	0.044/ 0.001	3.74e-10	0.113/ 0.001
	Denmark (COPSAC)	93 (50.82)				

PCoA based on 16S rRNA gene sequencing revealed a clear separation in bacterial community composition between EDIAQI and COPSAC dust samples (Figure 40). Samples clustered distinctly by country, indicating significant differences in β diversity (PERMANOVA, R² = 0.044, p = 0.001). Croatian samples (green) showed greater within-group dispersion compared to Danish samples (red), suggesting higher bacterial variability within Croatian households.

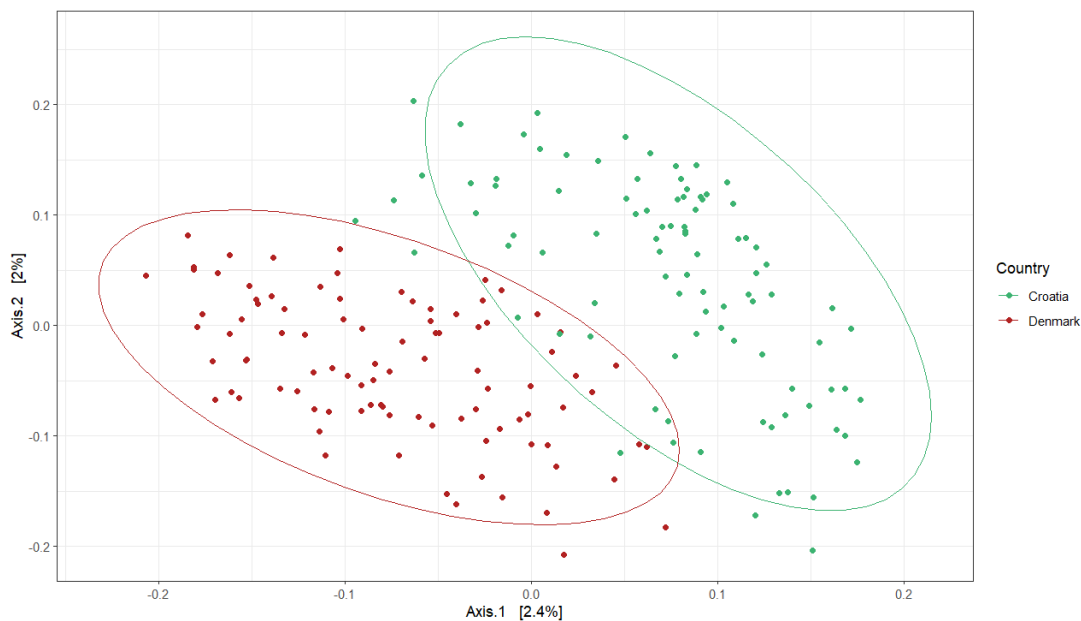


Figure 40. PCoA of bacterial community composition in dust samples by country.

Similarly, PCoA based on ITS region sequencing demonstrated distinct clustering of fungal communities by country (Figure 41). The fungal composition in Croatian samples also differed significantly from that in Danish samples (PERMANOVA, $R^2 = 0.113$, $p = 0.001$). The separation along PC1 and PC2, which explained 9.9% and 5.3% of the variance, respectively, supports a strong country-specific pattern in the fungal dust microbiome. These findings indicate that both bacterial and fungal communities differ significantly between the two countries

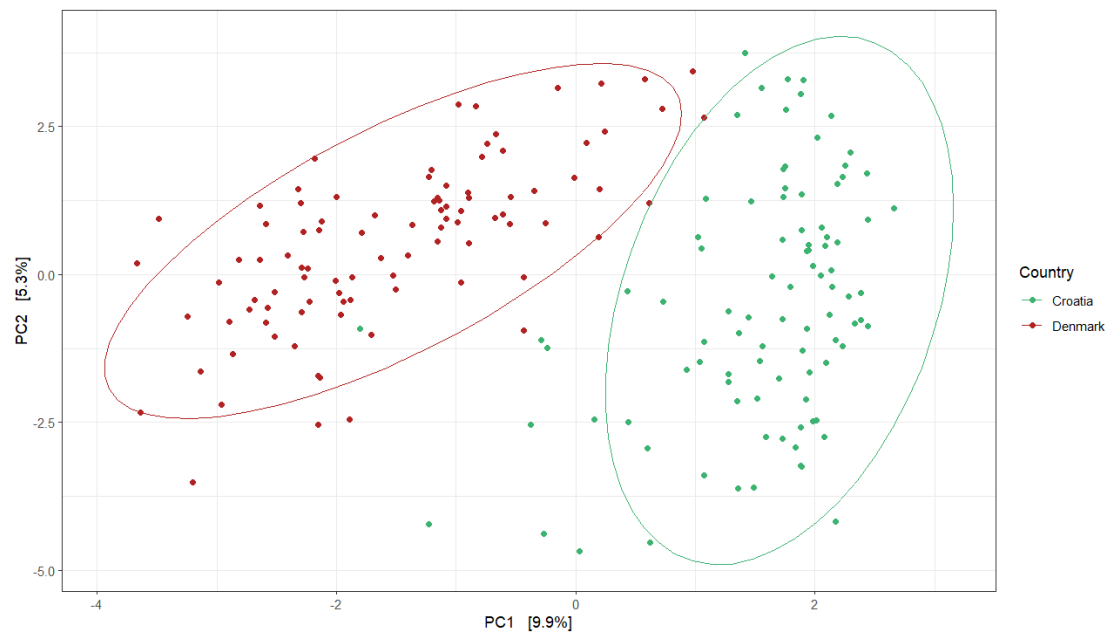


Figure 41. PCoA of fungal community composition in dust samples by country.

To assess alpha diversity, the number of observed ASVs was compared between Croatian and Danish samples for both bacterial and fungal datasets. Bacterial diversity was significantly higher in Danish samples compared to Croatian ones (Figure 42a). Danish dust samples exhibited a broader range and higher median number of observed ASVs, suggesting a more diverse bacterial community. Fungal diversity followed a similar pattern, with Danish samples showing a significantly greater number of observed ASVs than Croatian samples (Figure 42b). This indicates a richer fungal community in household dust from Denmark. These results align with beta diversity findings and reinforce the presence of country-specific differences in microbial richness.

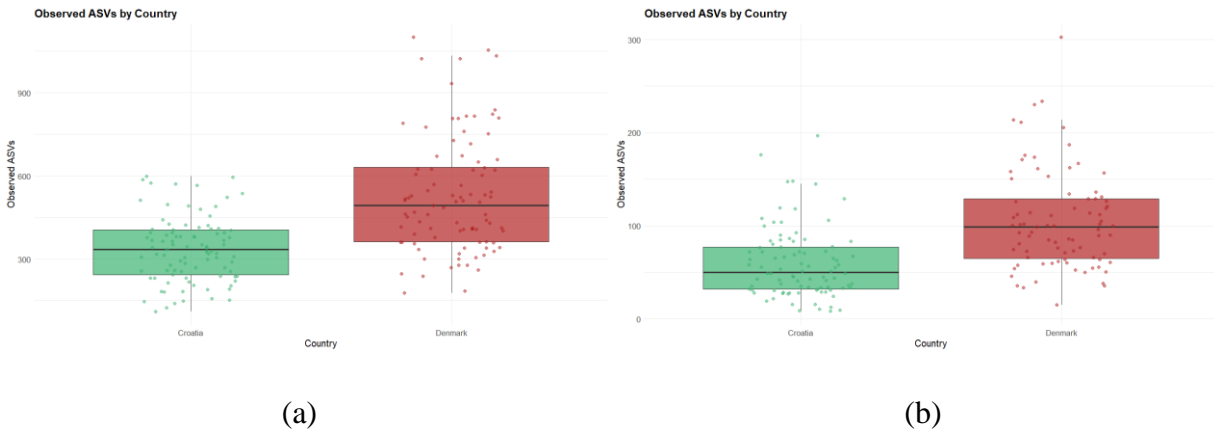


Figure 42. (a) bacterial and (b) fungal observed ASVs in dust samples.

Observed ASV richness can be seen in relative abundance plots (Figure 43 and 44) as well. Bacterial community profiles revealed that both countries shared many dominant genera, but differences in their relative abundances were evident (Figure 43). *Staphylococcus*, *Streptococcus*, *Cutibacterium*, and *Corynebacterium* were among the most abundant genera in both countries. However, Danish samples showed a relatively higher abundance of genera such as *Neisseria* and *Haemophilus*, while Croatian samples had a greater proportion of *Staphylococcus*.

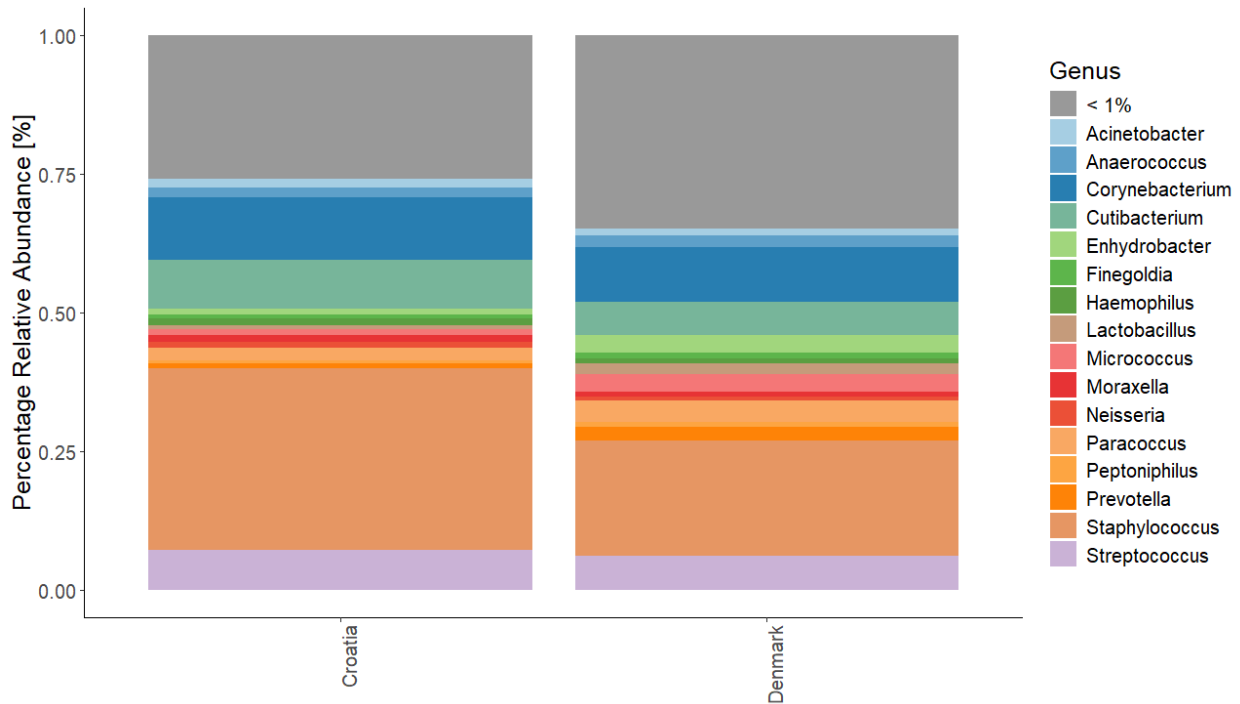


Figure 43. Relative Abundance of bacterial genera in dust samples from Croatia and Denmark.

Fungal communities also displayed distinct patterns between countries (Figure 44). *Malassezia* was the most dominant genus in both cohorts, but Denmark exhibited a higher relative abundance of *Cladosporium* and *Debaryomyces*, whereas Croatian samples were richer in *Saccharomyces* and several unclassified genera within the *Didymellaceae* and *Didymosphaeriaceae* families. These compositional differences further support the observed variation in microbial diversity and community structure between the two countries.

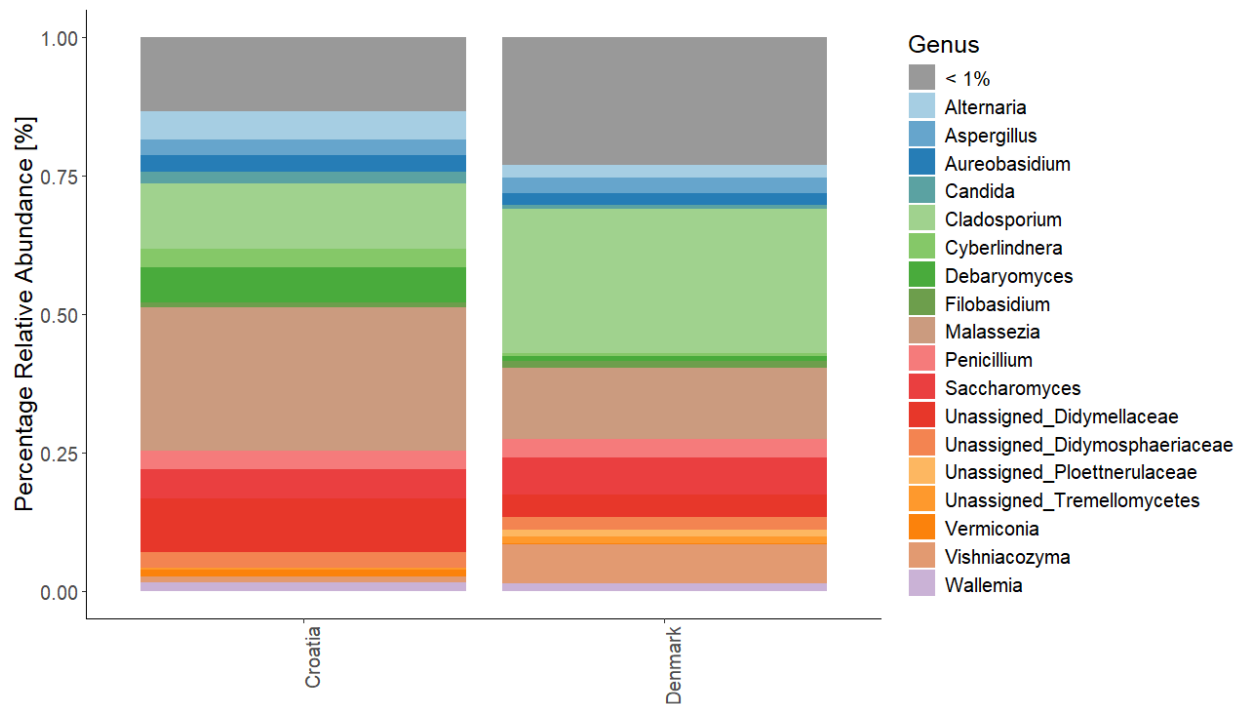


Figure 44. Relative Abundance of fungal genera in dust samples from Croatia and Denmark.

5. DISCUSSION

This research represents a novel study in Croatia, investigating the relatively underexplored subject of indoor microbiomes. While microbiome studies have gained significant attention globally, the focus has mainly been on human-associated and outdoor environmental microbiomes. Consequently, indoor microbiomes, particularly in the context of health conditions such as asthma, remain relatively understudied. This gap is especially evident in Croatia, where this research marks the first comprehensive study of the dust microbiome in indoor environments and its potential link to childhood asthma.

5.1. Cohort

Descriptive results of the cohort show that there are more participants with asthma (N=59) compared to healthy children (N=31). This disparity can be explained with the fact that a significant number of children who initially entered the study as healthy, asymptomatic individuals were ultimately diagnosed with asthma. Furthermore, the wide age range, involving children from 5 to 18 years old, plays an important role in the observed prevalence of asthma. The cohort spans multiple developmental stages, including toddlers, pre-schoolers, primary schoolers, and adolescents, which influences variability in the prevalence data. Additionally, asthma is more frequently diagnosed in younger children, and the cohort average age is 9.51 (± 3.58) years. Furthermore, it is worth noting that in the cohort asthma is more common in boys than in girls. These findings align with well-known clinical observations and previous research [8]. Finally, the cohort size of only 90 children is relatively small, which limits the scope of the findings.

The collection sites in Zagreb are relatively evenly distributed, ensuring a broad representation of different living environments within the city. This geographic balance reduces potential biases related to location-specific factors.

5.2. Optimisation of the protocols

Given that there is no specific kit designed for dust DNA isolation, the DNeasy PowerSoil Pro Kit was chosen based on a thorough review of the literature. This kit is specifically designed for extracting DNA from environmental samples, offering several advantages. These include its ability to mitigate inhibitors present in the material and the inclusion of beads for mechanical shredding, which ensures thorough homogenization during the extraction process [19,23,67,70,82,118].

The kit protocol had to be optimized for dust samples, as dust has a different consistency compared to soil. The first challenge was the variability in sample composition. Samples containing fine particles were relatively easy to work with, whereas samples with "dust bunnies" were challenging since the dust would absorb all of the extraction solution. Whenever possible, fine particles were used for DNA extraction. In cases where this was not possible, the dust bunnies were manually broken into smaller pieces before processing. Furthermore, it was necessary to determine which weight provided the best DNA yields. As shown in Supplementary Table 2., the optimal extraction weight for dust samples was determined to be 50–80 mg, which provided the best DNA yield.

The optimization of PCR conditions for fungal ITS sequencing proved to be another challenge. Initially, the study design continued to follow the protocol outlined by Gupta et al. [67], but the ITS sequencing results were not satisfactory. Two primer sets were tested: the original primers from Gupta et al. and ITS3F/ITS4R targeting the ITS1 region. Both primer sets were first tested under standard PCR conditions but did not generate optimal amplicons. Subsequently, multiple modifications to the PCR protocol were explored, including variations in the number of cycles (30 and 33 cycles), adjustments to the annealing temperature (56°C and 60°C), the inclusion of a prolonged final elongation step, and nested PCR approaches. Additionally, the original Illumina (2013) [94] protocol and its modifications were tested. Despite these extensive efforts, none of the adjusted protocols yielded better results than the original Gupta et al. method. Ultimately, we decided to work with the Gupta et al. protocol using ITS2 primers (gITS7F and ITS4ngs), which provided the most reliable outcomes [67].

These findings show the complexity and challenges of optimizing PCR for fungal ITS regions, particularly the inherent variability in DNA amplification and sequencing workflows. Firstly, the challenge with DNA extraction is that the fungal cells belong to domain Eucarya, which means that when extracting fungal DNA, it is necessary to remove the thick layer of polysaccharides, proteins, and glycoproteins, melanin, chitin, and other polymers that encapsulate the fungal mycelium. This poses as significant challenge for enzymes and chemicals. Next, ITS sequencing has unique difficulties due to the high genetic variability within fungal communities, potential biases introduced by primer specificity, and the presence of contaminants or inhibitors in environmental samples. Additionally, the dual role of ITS as both a conserved and variable region

can complicate primer design and alignment, leading to inconsistent amplification across diverse fungal taxa [119–122].

5.3. Sequencing preparation and results

The normalization of DNA concentrations to 5 ng/μL was a crucial step to ensure the accuracy and comparability of downstream sequencing results, since NGS protocols require consistent DNA input across all samples. Variations in DNA yields were uniformed by dilution or, in cases of insufficient DNA, repeating the extraction process. If further extraction was not possible due to limited sample dust mass, the initial DNA was used without additional dilution. This might have had a slight influence on inconsistencies in sequencing depth for these samples.

The library preparation process was carefully carried out following the Illumina 16S Metagenomic Sequencing Library Preparation Protocol [94]. This multi-step process, which includes indexing and PCR clean-up, was designed to results in sequencing-ready libraries with minimal contaminants. Quantification and normalization of the final libraries to 4 nM were critical for achieving uniform sequencing depth across all samples. The pooling of libraries and preparation for sequencing were conducted with strict adherence to protocol, ensuring a successful sequencing run.

Preliminary sequencing results for bacterial and fungal taxa indicate that one sample for 16S and three samples for ITS were sequenced unsuccessfully. All samples initially showed sufficient DNA concentrations when measured using the Qubit fluorometer, therefore the unsuccessful sequencing could be attributed to other factors. One potential explanation is the presence of inhibitors in the DNA. Environmental samples, such as dust, often contain residual contaminants (humic acids, proteins, or polysaccharides) that may interfere with PCR amplification or downstream library preparation [123,124]. Another possible reason could be inconsistencies or errors during the library preparation process, such as insufficient indexing, uneven bead clean-up, or incomplete removal of primer dimers. Additionally, issues during sequencing itself, such as cluster generation failures on the flow cell, could have led to the lack of successful reads for these samples.

The bacterial dataset yielded a significantly higher total number of reads (6,164,389) compared to the fungal dataset, which produced 3,232,715 reads. The higher number of reads in the bacterial dataset could be attributed to the greater abundance of bacterial DNA in environmental samples, as well as the natural stability of the 16S rRNA region for amplification and sequencing. As

mentioned before, fungal DNA often poses challenges due to its more complex cell wall and variability in ITS regions [119–122], which could explain the lower read count. Regarding the ASV and phyla results, the detection of 16,010 ASVs and 36 phyla in the bacterial dataset, compared to 4,361 ASVs and 10 phyla in the fungal dataset, highlights the higher taxonomic resolution achieved for bacteria. This difference can be attributed once again to the complexities and challenges associated with ITS sequencing [125,126].

The quality scores showed in chapter 4.5 Quality control and rarefaction analysis of Results, show the base-level quality of paired-end reads during sequencing. The quality is high and consistent at the beginning of the reads, with scores typically above 35, indicating reliable base-calling accuracy. For both bacterial and fungal sequencing, the forward reads have consistently higher quality compared to the reverse reads, as seen by the Phred quality scores. However, as the sequence progresses, particularly beyond 150 bases, the quality begins to decline, with more variation and a marked drop below 25 toward the end of the reads, especially in the reverse reads. This is a common limitation in NGS due to the cumulative effects of base miscalling and signal decay over read length. This is partly due to the increasing difficulty in distinguishing between true signals and background noise [127]. The Phred score is useful to filter and trimming sequences. To ensure reliable sequencing data and better alignment against a reference genome, the bacterial forward reads were trimmed at 270 bp and fungal at 220 bp, while the reverse reads for bacteria at 220bp and for fungi at 210 bp. This step minimizes the inclusion of low-quality bases, which could reduce the accuracy of taxonomic alignment and further microbiome analysis. By applying appropriate trimming thresholds based on Phred quality scores, the overall reliability of the dataset is improved. When comparing sequencing reads, it is clear that bacterial reads are of higher quality than fungal reads, confirming the challenges faced during this research with ITS sequencing.

Rarefaction curves showed in chapter 4.5 Quality control and rarefaction analysis of the Results show sampling depths of samples and controls. The green lines represent the negative control, which shows consistently low or flat species richness. This is expected for negative controls, as they should contain minimal biological material. The red lines represent the positive control, which initially shows an increase in species richness, but levels off in higher subsampling depth plot. This levelling suggests that the positive control has a stable species richness, even as sequencing

depth increases. Both controls behave as expected, positive having low species diversity, and negative having no diversity and disappearing at higher sampling depth as there is very low read counts. The blue lines represent individual samples. Initially, a lower subsampling depth was tested to ensure inclusion negative controls, which had relatively low sequencing depth (Figures 12a and 13a). At this initial lower depth, the observed species richness was relatively low across all samples, as expected. This was due to insufficient sequencing depth to fully capture the microbial diversity present. For that reason, a much higher subsampling depth was applied, showing that between 3,000 and 4,000 sequences, many samples have captured most of their species diversity, as the lines flatten out. The literature suggests that the optimal rarefaction depth is the point where most samples begin to plateau, typically between 2,000 and 5,000 sequences [128]. To ensure comparability across samples, all samples must be rarefied to the same sequencing depth [128,129]. Although this may exclude some rare ASVs, subsampling ensures comparability in diversity metrics estimates across samples, and the impact on relative richness comparisons is minimal [130]. The increase in richness resulted also in detection of less abundant taxa that were not captured at the lower depth. This helps identify an appropriate rarefaction depth for downstream analysis, ensuring sufficient coverage.

5.4. Discussion of findings

5.4.1. Alpha and beta diversity

Diversity measures related to asthma included both β diversity and α diversity (measured by p-values). While bacterial β diversity showed a significant association with asthma status among non-asthmatic participants ($p = 0.009$), suggesting notable differences in bacterial community composition. Fungal β diversity did not demonstrate significant differences ($p = 0.1$), which may highlight the greater variability or complexity of fungal communities in these environments, as also noted by Kirjavainen et al. [15]. When looking at PCoA plots, a considerable overlap between the two groups can be observed, suggesting no distinct clustering of microbiome composition based on asthma status. This overlap implies that, while there may be some variation, the differences in microbiome composition between the asthma and non-asthma groups are not strongly pronounced according to these principal components. This aligns with findings by Vandenberg et al. [23], who observed that differences in microbial communities associated with asthma are often subtle and may overlap with those of healthy individuals due to shared environmental exposures. Furthermore, no significant interaction effects were observed between

α diversity and asthma status for either bacterial or fungal samples. This lack of significant difference in α diversity could be explained with heterogeneity of asthma as a disease, as well as the broad environmental and genetic factors that influence individual microbial diversity. These findings are consistent with studies by Lee et al. and Ege et al. [18,82], which found that microbial diversity alone does not necessarily correlate with asthma outcomes but rather the composition of specific taxa plays a critical role.

When considering α and β diversity in relation to other factors, several significant findings emerge. Parental education showed a notable association with microbial β diversity for both bacterial and fungal communities. In the bacterial dataset, β diversity was significantly associated with education level ($p = 0.003$), and there was also a significant interaction with asthma ($p = 0.025$). Similarly, in the fungal dataset, β diversity was significantly influenced by education ($p = 0.032$) and its interaction with asthma ($p = 0.008$). These findings suggest that higher parental education might correspond to lifestyle and household practices, such as cleaning habits or dietary preferences, that influence indoor microbial communities. For example, more educated parents may prioritize cleanliness or specific dietary practices that shape microbial exposure. Similar associations have been observed in studies like Tischer et al. [22], which found links between socioeconomic status, seasonality, and asthma-related outcomes. This aligns also with the household income results, which showed association with significant differences in fungal α diversity ($p = 0.018$) and β diversity ($p = 0.026$), and marginally significant differences in bacterial β diversity ($p = 0.068$). Higher-income households may have access to better ventilation systems, higher-quality housing materials, or different cleaning practices, all of which can shape microbial diversity. As shown in Figure 13 of the results, households with the lowest income had slightly higher median fungal richness compared to higher-income groups, although the differences were subtle. While specific studies directly linking household income to fungal richness are limited research indicates that various environmental and structural factors associated with wealthier households, such as modern building designs and advanced air filtration systems, can influence indoor microbial diversity. For instance, the study by Benton et al. [131] found significant differences in microbial dust composition related to housing characteristics, when comparing U.S.A. and Mexican homes. Economically richer U.S.A. had more paved roads, flushing toilets, piped water and air conditioning which influenced microbiome composition. Additionally, the paper by Adams et al. [27] discusses how ventilation types and air filtration can alter indoor

microbial profiles, noting that buildings with natural ventilation or modest supply air filtration exhibit microbial communities similar to outdoor air, whereas more mechanically ventilated buildings with enhanced filtration show distinct indoor microbial profiles. These studies imply that characteristics connected to the wealth in the households may influence indoor microbial diversity, including richness.

Gender also influenced bacterial β diversity ($p = 0.009$), although no significant interaction with asthma was observed. Gender is a challenging variable to interpret in microbiome-asthma research. As it was already pointed out, asthma is a multifactorial disease influenced by a wide range of environmental and physiological factors. Moreover, it is well-established that asthma prevalence tends to be slightly higher in boys, particularly during childhood and early adolescence, which can further complicate the analysis. Our findings may reflect gender-related variations in behaviours, activities, or environmental exposures, such as time spent outdoors or engagement in specific activities.

Household characteristics demonstrate the role of the immediate home environment in shaping the microbiome and its interactions with health outcomes, including asthma. The living environment (suburban vs. urban - surrounded by built or green environment) significantly influenced β diversity in both bacterial ($p = 0.002$) and fungal ($p = 0.005$) datasets. According to Weikl et al. [90], fungal community is significantly affected by surrounding greenery and urbanisation grade. This is supported by study in Finland, where areas with higher proportions of built environment and less natural greenery exhibited reduced microbial diversity [132]. In contrast, Barberán et al. [133] reported no significant differences in microbial alpha or beta diversity between urban and rural outdoor environments. These differing findings likely reflect the fact that urban and rural environments vary widely across geographic regions in terms of land use, vegetation, climate, and human activity. Moreover, interaction with asthma was also significant for both bacterial ($p = 0.001$) and fungal ($p = 0.026$) β diversity, suggesting that the microbiota in different living environments may modulate asthma risk or severity. For example, the protective role of natural environmental exposure, as noted in farm environments, could be extended to suburban areas as well, reducing asthma prevalence through increased microbial diversity [18,20]. Logically, the housing type (house vs. apartment) was also included. The results showed that the type of housing significantly influenced bacterial β diversity ($p = 0.004$). Houses, which generally have more direct

interaction with outdoor environments (gardens, yards), may harbour more diverse microbial communities compared to apartments. However, fungal diversity did not show significant associations with housing type. Although other research show opposite results [64,74,78], it is possible that specific outdoor taxa are better suited to indoor conditions and may be favoured in such environments.

Other factors that have potential influence on indoor environment is number of siblings, pets and plants. The number of siblings and the presence of pets in a household are among the most frequently cited factors influencing indoor microbial communities [45,64,65,67,79,80,85,87,88,134]. The number of siblings was associated with significant differences in microbial diversity. For bacterial β diversity, both the presence of siblings ($p = 0.001$) and its interaction with asthma ($p = 0.001$) were significant. Bacterial α diversity was also significantly associated with sibling presence ($p = 0.045$). As it can be seen on Figure 14 in the Results, there is a general increase in observed microbial richness with the number of siblings. Households with 3 or more siblings show the highest median richness, while those with no siblings exhibit the lowest median richness, which can be explained with having more siblings contributes to greater microbial diversity within a household, likely due to increased microbial sharing and exposure to diverse environmental microbes brought in by siblings. Fungal β diversity was also significant ($p = 0.009$). Pet ownership, particularly dogs, had a notable impact on microbial diversity. Dog ownership significantly influenced bacterial and fungal β diversity ($p = 0.001$ and $p = 0.005$, respectively) and showed significant interactions with asthma in both datasets. Fungal α diversity was also significantly higher in households with dogs ($p = 0.012$), and borderline in those with cats ($p = 0.054$). As shown in Figure 15, microbial richness was higher in households with dogs, likely due to their frequent interaction with outdoor environments and their role in transporting diverse microbial taxa into the home [67,80]. It is important to consider that, due to the small cohort size, these results are closely intertwined. Among the 29 children with pets, 21 have dogs, 7 have cats, and only 2 have other animals (a hamster and a parrot). Additionally, since some children come from the same household, this variable becomes even more interdependent, potentially introducing shared environmental influences that may affect the results. It is also worth noting that, uniquely within this cohort, children without siblings also did not have pets. This overlap may amplify the combined influence of these two variables in other groups (“Yes pets”

and number of siblings: “1”, “2”, and “3 and more”), as both siblings and pets are known to introduce outdoor-associated microbes into the indoor environment.

Lastly, the presence of indoor plants significantly influenced microbial β diversity for both bacterial ($p = 0.005$) and fungal ($p = 0.041$) communities, consistent with research by Dockx et al. [135] where an increase in indoor plants and density was associated with increased microbial diversity. Plants are known to contribute to indoor microbial diversity through the release of microbial spores and other organic materials [90]. Interaction with asthma was also significant ($p = 0.001$ for bacterial and $p = 0.035$ for fungal datasets), suggesting that plant-associated microbiota may play a role in modulating asthma-related outcomes. This is particularly relevant given that pollen, a major component of plant-derived microbial exposure, is well known to exacerbate asthma and allergic symptoms. These findings are consistent with studies that emphasize the role of greenery and natural elements in composition of indoor microbiome [78].

When interpreting these results, it is essential to consider the structure of the cohort. While the study includes 90 children, they originate from only 66 distinct households, meaning a significant proportion of participants are siblings. This shared household composition has a substantial impact on the findings, as siblings are likely exposed to similar environmental conditions, including microbial communities, cleaning practices, socioeconomic factors, and pet ownership. The influence of siblings within the cohort can lead to clustering effects, where the microbial diversity and environmental exposures of children within the same household are more similar compared to those from different households. This similarity could reduce the variability in the data, making it harder to distinguish individual-level factors from household-level factors. Furthermore, siblings often engage in shared activities, including playing together and interacting with pets, which may amplify the homogenization of their microbial profiles. Therefore, the household dynamic within the cohort must be carefully accounted for when analysing and interpreting the results, as it could confound the relationships between environmental factors, microbial diversity, and health outcomes.

Another important factor to consider is the influence of cleaning practices and the season of sample collection on microbial composition. Surprisingly there are no many studies about the role of cleaning practices in shaping the indoor microbiome. It is well established that cleaning practices can reduce the human microbial fingerprint in indoor environments [45], influence specific

microbial groups through the use of cleaning products [27] and frequency [136], and ultimately shape the composition of indoor fungal communities [137]. Cleaning frequency and practices, such as dusting furniture and vacuuming mattresses, were significantly associated with microbial β diversity in both bacterial and fungal datasets (bacterial β diversity: $p = 0.006$ for dusting and $p = 0.011$ for vacuuming). Same results are for interactions with asthma and bacterial α diversity. This suggests that households with more frequent cleaning practices have tendency to have different microbial compositions, likely due to the removal of certain microbial taxa and changes in community structure caused by cleaning agents. Figure 16. shows that ASV richness is highest in moderate mattress vacuuming group (1 to 4 times a year), compared to never (never or rarely) and frequent (more than 4 times a year) groups. As mentioned by Nevalainen et al. [137] frequent vacuuming can reduce fungal concentrations in floor dust but may simultaneously increase airborne fungal levels due to resuspension. Although the present study analysed mattress dust rather than floor dust, a similar pattern might apply. It is possible that mattresses that are frequently vacuumed experience microbial disturbance and resuspension, reducing microbial richness. Conversely, mattresses that are rarely or never cleaned may accumulate low-diversity communities dominated by a few persistent taxa. In contrast, moderate cleaning might strike a balance, allowing for microbial accumulation without excessive disruption, thus supporting higher richness.

Seasonal variations affect microbial influx into homes through temperature, humidity, and outdoor microbial sources. Seasonal fluctuations influence factors like ventilation, outdoor activity levels, and exposure to environmental microbes, which are all pathways for microbial entry into homes [78,88,90]. In this study, specific seasonal effects were not directly tested as independent variables, however, the impact of seasonality on microbial diversity was indirectly assessed through the sampling design, as homes were sampled during different seasons. This variation in sampling periods revealed significant differences in both fungal and bacterial β diversities, as well as their interaction with asthma status (all $p = 0.001$, except fungal interaction where $p = 0.031$).

5.4.2. Ecology and distribution of the most abundant genera

The bacterial genera identified in this study represent a diverse array of microorganisms commonly associated with human, animal, and environmental microbiomes. In the studies of indoor microbial communities, the top ten genera often include those found in this study, such as *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Enterobacteriaceae*, *Cutibacterium*, *Lactococcus*, and

environmental taxa like *Acinetobacter*, *Sphingomonas*, and *Pseudomonas* [27]. A significant portion of these, including *Staphylococcus*, *Corynebacterium*, *Cutibacterium*, *Streptococcus*, and *Anaerococcus*, are primarily human-associated [138,139]. *Staphylococcus* species, including both commensals like *Staphylococcus epidermidis* and potential pathogens such as *Staphylococcus aureus*, are frequent inhabitants of human skin and mucosal surfaces [50]. *Staphylococcus epidermidis* has also been detected in the healthy lung microbiota [50,92]. *Staphylococcus* was found to be more abundant in asthma-associated environments [82,140–142]. *Corynebacterium* often co-occurs with *Staphylococcus*, and studies have shown a positive correlation between the two [16,50,67,143]. Both genera are associated with human skin, so their abundance is expected given the sampling site. *Cutibacterium* also a skin commensal, is commonly found in sebaceous areas of the body but may also inhabit the oral cavity and gastrointestinal tract [138]. Its increased abundance in environments with more tree canopy, as observed by Maestre et al. [144]. *Streptococcus* is another important genus in this study, known for its presence in the salivary microbiome and frequent identification in indoor air and dust [70]. While generally considered a commensal organism, its presence has clinical relevance. Early colonization with *Streptococcus* in infancy has been linked with increased asthma and wheeze risk later in life [51], it is also prominent in the lungs of patients with severe asthma [47] and is considered an influential genus in the cystic fibrosis microbiome [145]. Interactions between *Streptococcus* and *Staphylococcus* are a characteristic for skin and mucosal microbiome, which may also account for their co-occurrence in the samples [67,141]. *Anaerococcus*, though less frequently studied, is part of the normal microbiota of human skin and mucosal surfaces including the oral cavity, gastrointestinal tract, and female urogenital tract [146]. Its presence in dust samples reflects anthropogenic influences. *Enhydrobacter*, a skin-associated genus [147].

Beyond human-associated taxa, several environmental genera were also detected. *Paracoccus*, an ecologically versatile genus, is known to inhabit both pristine and anthropogenically influenced environments such as soil and water [148]. Another prominent environmental taxon is *Acinetobacter*, widely distributed in soil, water, and dust. Some species are opportunistic pathogens, while others are harmless environmental bacteria [149]. The family *Enterobacterales* encompasses diverse members from gut-associated bacteria to environmental strains commonly found in soil and water [150]. Family *Rhizobiaceae* is typically associated with soil ecosystems

[151]. Their detection in indoor dust suggests the infiltration of outdoor microbiota, likely via airborne particulates or tracked-in soil.

The source of almost all found fungal genera is predominantly associated with outdoor environments, where they inhabit diverse ecosystems such as soil, air, water, plants, and decaying organic matter, before potentially being introduced into indoor spaces [77,152,153]. Particularly dominant genus is human-associated *Malassezia*. Some species of *Malassezia* are associated with human skin and sebaceous environments, making occupants significant contributors to its presence in indoor dust [78,154]. Because it relies on fat for growth, *Malassezia* predominantly inhabits areas rich in sebaceous glands, such as the scalp, face, and upper body [155]. Other most abundant genera were *Cladosporium* and Unassigned *Didymellaceae* (family *Didymellaceae*). *Cladosporium* is found both indoors and outdoors. Its abundance reflects outdoor air and climatic conditions, frequently associated with damp conditions and in poor indoor air quality [78,156]. It is also among the most frequently detected fungi in indoor spaces [157]. The family *Didymellaceae* is distributed across diverse hosts and habitats [158]. Its presence indoors suggests contributions from plants or outdoor air [79,156]. Other dominant genera include *Alternaria*, *Debaryomyces*, *Saccharomyces*, *Cyberlindnera*, *Aureobasidium* and family *Didymosphaeriaceae*. *Saccharomyces* is globally distributed, occurring in soil, seawater, and various foods [159], while *Debaryomyces* is also commonly found and has been identified in clinical samples [160]. Both genera are linked to human-related activities, with *Saccharomyces* and *Debaryomyces* present in the human gut, and *Saccharomyces* additionally detected on children's skin [78,161,162]. This presence is consistent with the sampling context. *Alternaria* is widely distributed in the environment, commonly found in soil and as an airborne fungus worldwide. Indoors, it is often present in higher concentrations in carpet dust and rural areas [72,163]. This genus is among the most frequently detected moulds in indoor environments [72] and is a well-known trigger of allergic diseases, including asthma [92]. *Aspergillus* is one of Earth's most common and ubiquitously distributed fungi [163]. It is found in indoor damp environments [72], but can also be part of children's skin microbiome [78]. *Cyberlindnera* is broadly distributed and associated with soil and decomposition processes [164]. This genus is also identified in damp environments, with high prevalence observed in water-damaged classrooms [76]. Family *Didymosphaeriaceae* is commonly found on plant substrates, across various ecosystems, including marine, terrestrial, and mangrove environments [165].

In summary, the composition of indoor microbial communities reveals an obvious duality between bacteria and fungi. Bacterial genera identified in this study were predominantly associated with human-related sources and indoor environments. The prevalence of skin and mucosa associated taxa shows clear influence of human occupancy, physical contact, and indoor lifestyle on bacterial community. In contrast, fungal communities were more heavily influenced by outdoor environmental inputs, with dominant genera typically originating from soil, vegetation, and outdoor air. This distinction draws attention to the differential ecological pathways shaping the indoor microbiome: bacteria largely reflect human presence and activities, while fungi reflect the surrounding natural environment and outdoor influences. Such patterns are consistent with previous studies emphasizing the human dominated nature of indoor bacterial communities versus the outdoor-driven fungal influx [27,64,78].

5.4.3. Relative abundance and asthma

The relative abundance plots for bacterial and fungal genera highlight differences in microbial community composition between children with and without asthma. These differences provide insight into the potential roles of specific taxa in asthma development and severity.

The bacterial relative abundance plots demonstrate that *Staphylococcus*, *Streptococcus*, *Paracoccus*, and *Acinetobacter* are particularly prevalent in both groups. Although *Staphylococcus* has been linked to asthma severity, is known to contribute to severe airway inflammation by manipulating mucosal immunity, and has been found in the house dust of asthmatic children [55,82,141,142,166], our relative abundance data show it to be more prevalent in healthy children. Similarly, Fu et al. also did not find health associations [167]. *Staphylococcus* is a common colonizer of the upper respiratory tract, skin, and gut mucosa, and in healthy individuals, it typically exists as a commensal organism without causing disease [6,50]. Other explanation could be that *Staphylococcus* is influenced by specific environmental conditions, or the nature of the sampling site. House dust from mattresses may reflect skin-associated microbiota more strongly and not fully capture the microbial dynamics relevant to the asthmatic airway environment. *Streptococcus* is part of the healthy human microbiome, particularly within the oral cavity, respiratory tract, and skin. However, while many species are commensal, certain *Streptococcus* species have been associated with the host immune response and contributing to airway inflammation [23,55,56,168]. These bacteria can influence immune development and modulation,

potentially shaping allergic and inflammatory airway conditions. This dual role, commensal in some contexts and immunomodulatory or pathogenic in others, may explain our findings, where *Streptococcus* was found in great abundance in both groups, but slightly more abundant in the asthmatic group. *Acinetobacter* species are commonly found in different sites on the human body, including the skin, respiratory tract, and urinary tract. They are frequently associated with hospital acquired infections, with a significant proportion involving the respiratory tract [169]. In the context of our findings, the presence of *Acinetobacter* in indoor dust samples likely reflects its ability to survive on surfaces and persist in the environment, which also explains its role as a common source of infection, particularly in healthcare settings.

The genera *Micrococcus*, *Paracoccus*, and *Enhydrobacter* are detected to be statistically significant within relative abundance data comparing individuals with and without asthma. Both *Micrococcus* and *Paracoccus* are commonly found in the indoor environments occupied by humans [82,152,152,170]. The analysis indicates that *Micrococcus* shows low relative abundance in both groups, with slightly higher levels observed in the asthma group. A p-value of 0.011 suggests a statistically significant difference between the groups, however, the q-value implies that this significance may not hold after adjusting for multiple testing. The increased abundance in the asthma group could be explained by recent findings which have suggested that *Micrococcus luteus* may have a regulatory role in airway inflammation. It is possible that *Micrococcus* may have a protective or immunomodulatory role in asthma, though further research is needed to determine its functional significance in the respiratory tract [171]. *Paracoccus* is a genus of bacteria known for its metabolic diversity and its ability to thrive in both pristine and anthropogenically shaped environments [148]. Its relative abundance is also low in both groups but shows a statistically significant difference ($p = 0.011$), supporting higher abundance in the asthma group. Similar to *Micrococcus*, the q-value suggests caution in interpreting this difference. This genus's presence in dust microbiomes could stem from soil, water, or outdoor environments introduced into indoor spaces. *Paracoccus* is primarily studied in environmental contexts, and its potential health-related effects, are unexplored and require further investigation. *Enhydrobacter* is commonly associated with the human skin microbiome [67,147,172]. The plot shows slightly elevated levels in the asthma group, with a p-value of 0.036, indicating a significant difference between the groups, but again, this significance does not show persistence if multiple testing corrections ($q = 0.194$, Figure 21.). Its prevalence in skin-associated microbiomes and dust samples reflects the role of human

activity in shaping the indoor microbial composition. Notably, a study by Ling et al. [173] identified *Enhydrobacter* as a resident of human skin, with potentially higher relative abundance in Chinese individuals, and our study focuses on children and young adults. In the context of respiratory health, there is currently no evidence supporting its involvement in disease pathogenesis

Regarding fungi, a visible difference in the relative abundance of the genera *Malassezia*, *Saccharomyces*, and *Debaryomyces* was observed between the asthma and healthy groups, but none of the fungal genera showed statistically significant differences overall. Some species of *Malassezia* are commonly found on the skin of animals, including humans. Since *Malassezia* depends on lipids for growth, it mainly colonizes areas rich in sebaceous glands, such as the scalp, face, and upper body [155]. Its dominance in asthmatic children suggest a potential role in airway inflammation or hypersensitivity, as indicated by previous research [13,23,49,51,87]. An early-life exposure to *Malassezia* could influence the development of asthma in children [51]. Similarly, Beigelman et al. reported an association between the presence of *Malassezia* and increased airway hyperresponsiveness in asthmatic patients [13]. The genus includes allergenic species [87], and has been connected not only with asthma but also with disease exacerbations in conditions such as cystic fibrosis [23]. However, the exact mechanisms by which *Malassezia* may contribute to airway inflammation or hypersensitivity remain under investigation.

Saccharomyces is found globally in soil, seawater, various foods [159] as well as *Debaryomyces*, which also occurs in clinical samples [160]. Both genera are linked to human-related activities, as well [78,161,162]. There is a slight increase in the abundance of *Saccharomyces* in healthy children and *Debaryomyces* in asthmatic children. Given that both are often linked to the gut microbiome [49,78,159,161] their observed abundance may reflect underlying differences in host microbiota composition. Also, both are found in food and typically ingested that way. *Saccharomyces* with plant-based food [174] and *Debaryomyces* with fermented food [160], so this result could be explained by host-related factors such as diet and lifestyle choices.

While bacterial diversity patterns were similar between groups, the fungal dataset revealed that non-asthmatic children exhibited a slightly more diverse fungal community. This is likely due to the dominance of genera commonly associated with outdoor environmental exposures. This

increased exposure to outdoor fungi may contribute to a more balanced fungal community, potentially supporting immune system development and reducing the risk of asthma.

In our dataset, a portion of the sequences was labelled as 'Unassigned_unassigned,' indicating that these sequences could not be matched to any known taxonomic rank in the reference database. The substantial abundance of these sequences in the results suggests potential limitations in the database, issues with sequence quality, or the presence of uncharacterized or novel organisms.

5.4.4. Relative abundance and environmental factors

The findings of this study reveal the substantial influence of socioeconomic factors on the microbial composition of household dust, which may have significant implications for children's health outcomes, particularly regarding asthma. These factors include child gender, parental education, and household income. Dominant taxa remained consistent between male and female participants, but exceptions were observed in less abundant taxa. For instance, differences in the "Other genera" category suggest gender-specific microbial interactions, potentially linked to varying activity levels or hormonal differences [21,62]. Parental education was notably associated with microbial diversity in both bacterial and fungal datasets. This may reflect differences in lifestyle, hygiene practices, and home environments, as higher education levels often correlate with improved living conditions and access to healthcare [18]. Significant differences were observed in taxa such as *Staphylococcus* and *Cutibacterium* (bacteria) and *Alternaria* and family *Didymellaceae* (fungi), consistent with studies highlighting the impact of socioeconomic factors on microbial exposure and diversity [20,21]. Variations in microbial communities linked to parental education levels underscore the importance of early-life environmental exposures in shaping immune responses [6,58]. In line with education results, household income exhibited significant associations with fungal diversity but weaker effects on bacterial diversity. Specific fungal taxa, such as *Malassezia*, *Debaryomyces*, and *other genera*, showed income-related differences in abundance. These findings align with earlier research linking lower income to higher exposure to fungal allergens, which are often prevalent in poorly ventilated or humid conditions typical of lower-income households [66]. The higher sensitivity of fungal communities to socioeconomic disparities may result from environmental and lifestyle factors unique to lower-income settings, such as increased exposure to mould and dampness [21,73].

Household characteristics, including living environment, housing type, sibling presence, pet ownership, and indoor plants, play an essential role in shaping the microbial diversity and composition of household dust. These factors interact with microbial communities in ways that may have significant health implications, particularly in the context of asthma. Suburban, urban-green, and urban-built environments showed distinct microbial compositions. Taxa such as *Enhydrobacter* and *Acinetobacter* varied significantly, suggesting that access to greenery or proximity to built environments influences microbial exposure. These findings support previous research indicating that urbanization alters microbial diversity, often reducing beneficial outdoor-derived taxa [64,175]. The subtle differences in fungal communities, with taxa like the family *Didymellaceae*, are consistent with studies linking fungal diversity to geographic and climatic factors [74]. Houses and apartments exhibited differences in bacterial diversity, with *Enhydrobacter* showing significantly higher abundance in houses. This may be attributed to structural and ventilation differences that influence microbial inflow and preservation [78]. In contrast, fungal communities were not as much affected by housing type, reflecting the dominant role of outdoor environments in shaping indoor fungal diversity [66,77]. The presence of siblings significantly influenced bacterial β diversity and specific fungal taxa like *Candida*. Homes with more siblings likely have increased microbial sources due to shared activities and diverse exposure environments. This aligns with findings by Nygaard and Charnock that larger households, particularly those with children, display greater bacterial diversity due to increased human-associated microbial inputs [65]. The observed effects on bacterial rather than fungal diversity suggest that sibling interactions predominantly impact human-associated bacterial communities [84].

The presence of pets, particularly dogs and cats, significantly influenced microbial composition of fungi. Homes with dogs exhibited higher microbial richness and increased taxa such as *Malassezia*, *Filobasidium*, and other genera while cats were associated with shifts in *Malassezia*, *Candida* and other genera. These findings corroborate earlier studies demonstrating that pet ownership enhances microbial diversity and introduces distinct taxa into the home environment [67,85,134], and abovementioned that fungal composition is influenced more by outdoor environment [66,77], in this case through the influence of the pets. The observed interactions with asthma highlight the dual role of pets, which may both mitigate and exacerbate asthma risk depending on microbial

exposure and sensitivity [15]. Homes with more indoor plants showed differences, particularly in bacterial taxa like *Streptococcus* and *Gemella*. Fungal communities also shifted, with “Unassigned_unassigned” genera meaning that there is a lot of different taxa influenced by presence of the plants. These results are consistent with studies suggesting that plants act as reservoirs or moderators of microbial communities, introducing beneficial outdoor-associated microbes while influencing fungal diversity [78,176]. Household related findings align with the "pro-asthmatic protective environment" hypothesis, which theorises that early exposure to diverse microbial communities, such as those introduced by pets or siblings, reduces asthma risk [15,18].

The findings of this study emphasize the significant impact of cleaning practices on the microbial diversity and composition of household dust. Frequent dusting was associated with reduced microbial diversity, particularly among less dominant taxa, while taxa such as *Staphylococcus* and *Malassezia* remained predominant. These findings align with earlier research suggesting that frequent cleaning may selectively alter microbial profiles, often reducing diversity and potentially diminishing beneficial microbial exposures [88,137]. All statistically significant taxa, *Staphylococcus*, *Neisseria*, *Haemophilus* (for bacteria), and *Malassezia* (for fungi), showed significant differences across cleaning frequency categories. These taxa were more abundant in homes with frequent or daily cleaning, which aligns with previous studies suggesting that regular cleaning practices favour the persistence of certain resilient microbial taxa [65]. Only *Wallemia* shows highest abundance in households that vacuum mattress never or yearly, indicating that infrequent mattress cleaning may allow for the accumulation or persistence of *Wallemia*, a xerophilic fungus known for thriving in dry and dust-rich environments [153,177], like mattresses where is commonly found [153,178]. Reduced microbial diversity due to frequent dusting may have implications for immune tolerance, particularly in children, as exposure to a diverse range of microbes in early life is linked to lower asthma risk [18]. Mattress vacuuming frequency also influenced microbial diversity, with never or rarely vacuuming associated with the highest microbial richness. Interestingly the frequent vacuuming showed similar results, slightly less abundance than infrequent vacuuming. This can be explained by vacuum cleaning process, where vacuum cleaner disperses particles into the air. But it can be debated that the dispersion depends on the vacuum cleaner model [72]. Cleaning practices are the matter of debate. Even though frequent dusting and excessive vacuuming were associated with reduced microbial diversity, potentially limiting beneficial exposures that may protect against asthma [15], on the other hand

infrequent cleaning practices increased the risk of allergenic exposures, which are known to exacerbate asthma symptoms [85]. These findings align with the “hygiene hypothesis”, which suggests that reduced microbial exposure due to rigorous cleaning practices may impair immune development and increase susceptibility to asthma and other allergic conditions [18,21]. These results show the delicate effects of cleaning on the communities, which are often influenced by environmental factors such as humidity and dust accumulation [77].

Although no significant seasonal differences were observed, as noted by Estensmo et al. [79], it is important to consider that the traditional distribution of months per season (Spring \approx March, April, May) no longer aligns with current climatic patterns. Significant changes in weather factors, such as temperature, humidity, and sunlight, directly influence the microbiome. Consequently, it can be argued that using conventional seasonal categories may not be optimal. Instead, environmental variables like temperature and humidity should be considered for a more accurate analysis.

6. CONCLUSION

1. Bacterial reads were more abundant (6,164,389) and of higher quality compared to fungal reads (3,232,715), reflecting differences in the stability of bacterial 16S and fungal ITS regions.
2. Asthma status was significantly associated with bacterial β diversity, indicating distinct bacterial community compositions between groups. In contrast, fungal β diversity showed no significant differences, reflecting the variability of fungal communities.
3. No significant α diversity differences were observed for asthma, suggesting that overall microbial richness may not directly correlate with asthma outcomes. These results align with studies emphasizing the importance of specific microbial taxa over diversity alone in asthma development.
4. Higher parental education and income were associated with distinct bacterial and fungal β diversity, likely reflecting lifestyle factors such as cleaning practices and dietary preferences.
5. Suburban homes exhibited higher microbial diversity compared to urban homes, supporting the hypothesis that natural environments promote richer microbial exposures. Housing type also influenced bacterial diversity, with homes showing greater microbial diversity than apartments, likely due to increased outdoor exposure.
6. The presence of siblings and pets was associated with a significant increase in microbial richness and diversity, supporting the hygiene hypothesis. Larger households and pet ownership contributed to diverse microbial inputs, which may play a role in shaping immune system development. Homes with indoor plants demonstrated greater bacterial and fungal diversity, likely because plants serve as reservoirs for beneficial outdoor microbes and influence indoor fungal communities. Pet ownership, particularly of dogs, introduced distinct microbial taxa into homes, further enhancing microbial richness. This finding aligns with studies highlighting pets as vectors for outdoor-derived microbes. However, the relationship between pets and asthma was complex, with pets both mitigating and exacerbating asthma risk depending on the child's microbial exposure and individual sensitivity.
7. Frequent cleaning practices, such as dusting and vacuuming, significantly altered microbial diversity, often reducing less dominant taxa while resilient ones persisted. Excessive

cleaning potentially reduces beneficial microbial exposures, which are critical for immune development, particularly in children.

8. Seasonal variations did not influence microbial compositions, suggesting more important the role of environmental factors like temperature, humidity, on microbiome.
9. Dominant bacterial genera found in the samples are all commonly associated with human skin and mucosal surfaces. Specific environmental genera reflected outdoor microbial contributions, while statistically significant taxa showed higher abundance in asthmatic children, suggesting potential links to asthma.
10. The fungal genera dominating the samples were primarily anthropogenic and environmental taxa. Specific genera found to be statistically significant were more closely associated with outdoor influences and variations in housing conditions, such as ventilation, dampness, and other structural factors.

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8. *CURRICULUM VITAE*

Iva Šunić was born in 1994 in Zagreb, Croatia, where she completed her elementary and high school education. After graduating from II Gymnasium, she enrolled in the undergraduate program in Biology at the Faculty of Science, University of Zagreb. She completed her master's program in Experimental Biology – Immunology and Physiology in 2019. In 2020, she began working at the Institute for Anthropological Research and entered the postgraduate program in Biology at the Faculty of Science.

Her primary areas of expertise include bioinformatics, biostatistics, and biomedicine. She has been continuously learning and advancing her expertise in these fields since joining the Institute. Iva completed two Erasmus+ traineeships: one in Copenhagen, Denmark, working with the Genetics team at The Copenhagen Prospective Study on Asthma in Childhood (COPSAC), and another in Graz, Austria, working with the Science Innovation team at Know Center. She was awarded a Croatian Science Foundation scholarship to study bioinformatics at the University of Copenhagen's Department of Food Science, and City of Zagreb Scholarship for research visit to the Institute of Environmental Biotechnology, Graz University of Technology. In addition, she received a YSF scholarship to attend the YSF Forum and FEBS Congress in Tours, France, in 2023. Iva has participated in numerous workshops and training programs focused on biostatistics and bioinformatics. She is a member of the Croatian Society of Biochemistry and Molecular Biology, the Croatian Microbiological Society, the European Association of Allergy and Immunology, and the European Anthropological Association. She has published 8 original research articles and has presented her work at 4 international and national conferences and symposiums.

List of publications:

[Accepted for publication] Šarac, J., Havaš Auguštin, D., **Šunić, I.**, Michl, K., Cernava, T., Marjanović, D., Jakobsen, R. R., Lovrić, M., 2025. Linking the Bed Dust Microbiome with Environmental Factors and Child Respiratory Health. *Annals of Human Biology*

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Šunić, I., Novokmet, N., Šarac, J., Havaš Auguštin, D., Fuchs, N., Mrdjen Hodžić, R., 2021. Pregnancy and birth cohorts in Europe: An overview. *Journal of bioanthropology* 1, 93–113.

Conference participations:

Šunić, I., Bošnjaković, A., Karlović, N., Havaš Auguštin, D., Šarac, J., Novokmet, N., Marjanović, D., Missoni, S., Lovrić, M., 2024. Deep Learning Model for Type II Diabetes

Prediction: Insights From an Isolated Population with Traditional Lifestyle, in: 13th ISABS Conference on Applied Genetics and Mayo Clinic Lectures in Translational Medicine. pp. 202–202.

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9. SUPPLEMENTARY MATERIALS

9.1. Supplementary Materials and Methods

3.5.3.1. Genetic material isolation- protocol optimisation

The protocol required adjustments to account for the sample weights, since the original protocol recommends using up to 250 mg of soil as a starting point (QIAGEN, 2023). The initial dust weights are presented in Supplementary Table 1.

Supplementary Table 1. Isolation test

Name	Weight [mg]	Supernatant [μ L]	DNA concentration [ng/mL]
Sample 1.	250	very low	0.214
Sample 2.	126	220	5.4
Sample 3.	60	400	10.7
Sample 4.	122	200	7.76
Sample 5.	80	350	11.1
Sample 6.	194	very low	0.14
Sample 7.	69	350	3.6
Sample 8.	116	200	3.0
Sample 9.	226	150	2.74
Sample 10.	negative control (no dust)	-	too low

After weighting, the dust was added in the PowerBead Pro Tubes, which contain beads for mechanical shredding. According to the protocol, 800 μ L of Solution CD1 is added, and the tubes are then horizontally shaken to ensure homogenization. However, samples containing more than 80 mg of dust showed the difficulty with the Solution CD1, as the dust would absorb the entire volume. The protocol does not specify the shaking speed, but it does suggest a maximum speed for a duration of 10 minutes. In our experiment, shaking at 1,800 rpm for 15 minutes was found to be optimal, accomplishing thorough homogenization of the samples. Extending the duration beyond this point did not produce any additional visible changes.

Following homogenization, the samples were centrifuged, and the supernatant was transferred to new tubes, with an expected volume between 500 and 600 μL . As it can be seen in comment section of Table X, samples with the highest dust weights had the lowest volumes of supernatant.

After isolation, the DNA quantification was performed using Qubit® 3.0 Fluorometer, and the concentrations are shown in Table X. As expected, samples with lower initial supernatant volumes (and higher dust weights) had the lowest DNA yield. The best yields were observed in samples with masses ranging from 60 to 122 mg.

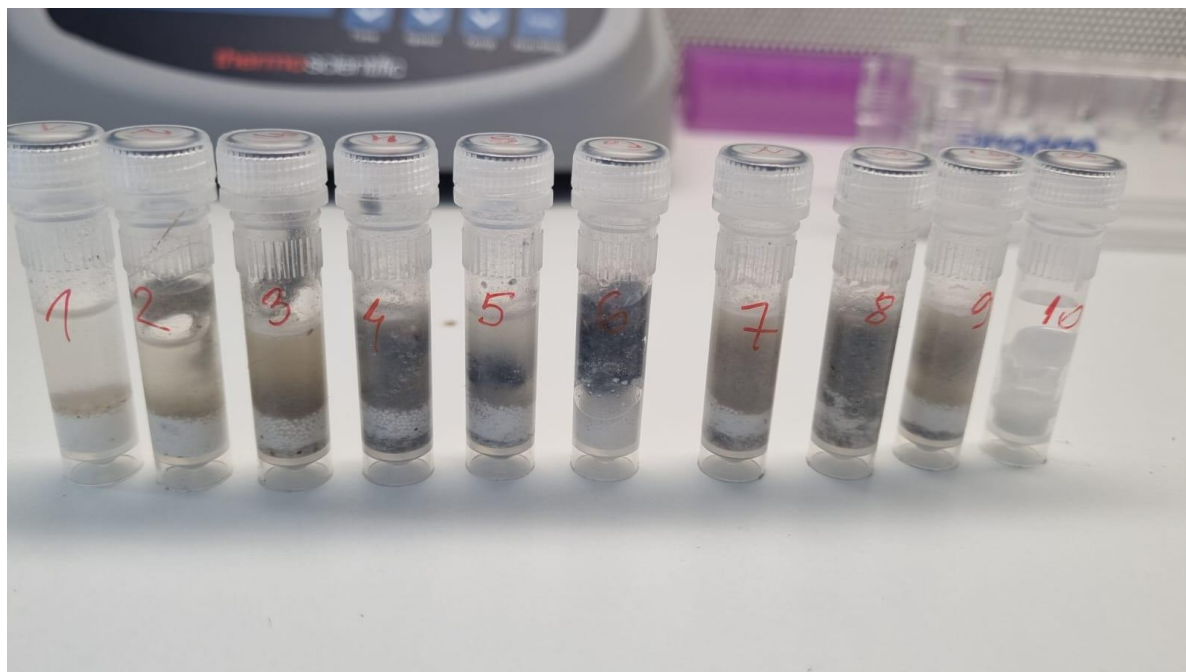
To refine the protocol, an additional isolation trial was conducted, focusing specifically on the finesse of the dust samples. The samples included fine dust, dust bunnies, and various combination of both. The challenge with dust bunnies is that they absorb entire solution volume during the initial step, and many of the samples consisted solely of this type of dust. Details of the new isolation batch can be found in Supplementary Table 2.

Supplementary Table 2. Isolation test- dust finesse.

Name	Weight [mg]	Dust	Supernatant [μL]	DNA concentration [ng/mL]
Sample 1.	80.5	fine dust	600	6.30
Sample 2.	19.0	dust bunnies	500	2.54
Sample 3.	41.5	fine dust + dust bunnies	500	5.40
Sample 4.	59.0	fine dust + dust bunnies	400	33.8
Sample 5.	25.1	dust bunnies	500	4.84
Sample 6.	119.2	dust bunnies	500*	10.1
Sample 7.	51.8	fine dust + dust bunnies	500	0.140
Sample 8.	103.9	fine dust + dust bunnies	300	0.88
Sample 9.	30.2	fine dust + dust bunnies	500	too low
Sample 10.	negative control	no dust	600	too low

* Additional 400 μL CD1 solution added.

After adding the CD1 solution, as shown in Supplementary Figure 1, samples containing dust bunnies absorbed a significant amount of the solution. For Sample 6, an additional 400 μL of solution was required to enable effective mechanical shredding. While the supernatant yields improved in this trial, DNA concentrations were highest in samples containing only fine dust or a mix of fine dust and dust bunnies. Therefore, it was decided to proceed with using 60-80 mg of dust, using fine dust whenever possible.



Supplementary Figure 1. Initial step of isolation test.

Supplementary Table 3. Sample mass and Qubit-measured DNA concentration.

Sample ID	mass [mg]	Qubit concentration [ng/mL]
S001	31,5	27,6
S004	63,2	27,4
S006	74,3	37,0
S007	24,8	29,2

S009	45,5	47,2
S010	68,1	45,4
S011	57,8	39,0
S012	25,7	18,7
S013	55,7	41,6
S015	53,9	28,2

Sample ID	mass [mg]	Quibit concentr ation [ng/mL]
S016	48,1	34,6
S017	49,0	23,2
S018	51,2	48,6
S019	55,3	45,4
S020	41,5	24,0
S021	52,5	37,2
S022	38,2	34,2
S023	62,6	34,6
S024	43,6	33,2
S025	5,0	8,6
S026	50,3	17,3
S027	19	17,4
S028	51,4	28,6
S029	59	76,8
S033	73,8	40,0
S034	39,1	32,4
S035	62,6	30,8
S036	55,1	65,4
S037	69,7	76,6

S038	61,5	31,2
S039	72,3	49,2
S040	62,7	60,0
S042	28,2	35,8
S044	77,8	74,8
S047	83,8	29,2
S048	81,3	61,2
S049	83,4	15,3
S050	31,4	56,2
S051	47,2	55,6
S052	32,9	56,8
S053	44,7	24,6
S054	44,4	11,8
S056	49,1	39,2
S057	63,2	25,2
S058	51,4	19,0
S059	62,2	48,4
S060	53,5	76,0
S061	54,0	90,0
S062	58,7	56,0
S063	40,3	27,8
S064	45,9	56,0

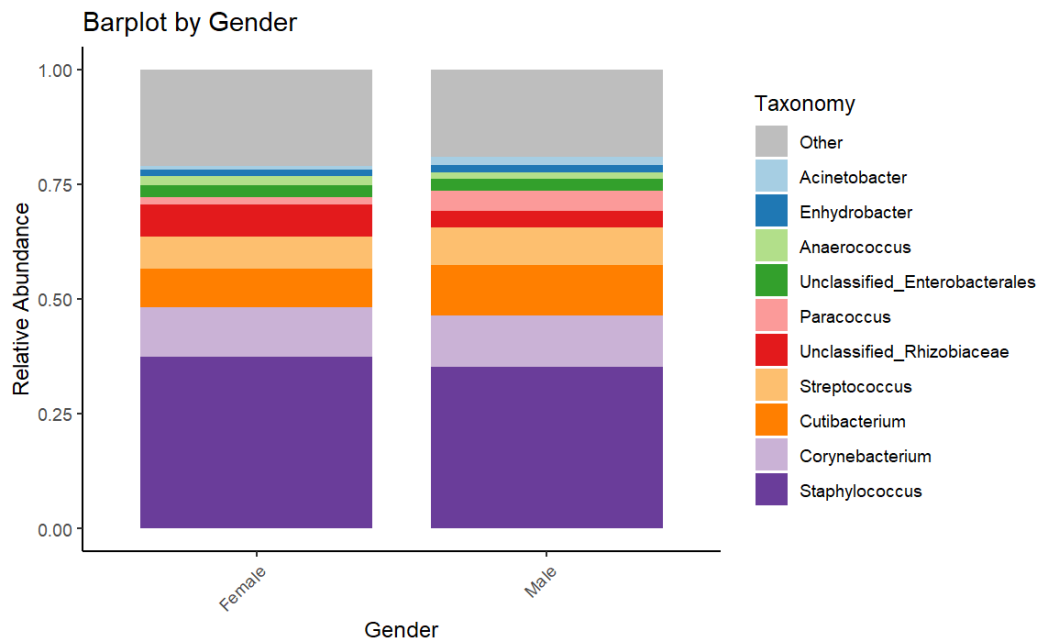
Sample ID	mass [mg]	Quibit concentr ation [ng/mL]
S065	76,7	31,2
S068	70,9	58,0
S069	44,4	79,6
S071	60,4	26,8
S072	66,6	51,2
S073	56,8	47,8
S074	33,3	56,0
S075	47,8	76,4
S076	38,9	49,4
S079	45,6	39,4
S080	49,8	8,68
S081	53,7	4,66
S082	57,9	27,0
S083	11,0	9,36
S084	61,5	7,02
S086	49,5	16,1
S087	46,5	4,40
S088	58,5	8,80
S089	47,5	17,2

S090	35	2,64
S091	53,8	7,78
S092	54,2	8,44
S093	27,7	19,6
S094	46,9	8,18
S095	53,3	17,5
S096	39	19,4
S097	55,6	26,8
S098	51,6	10,5
S099	51,3	49,8
S100	59,5	17,3
S101	51,5	3,60
S102	58,9	5,24
S103	43,2	7,38
S104	47,4	8,06
S105	57,6	17,0
S109	66,5	7,1
S110	76,7	6,44
S111	71,9	too low
S112	80,2	7,5
S113	63,0	6,32
S114	53,4	5,8

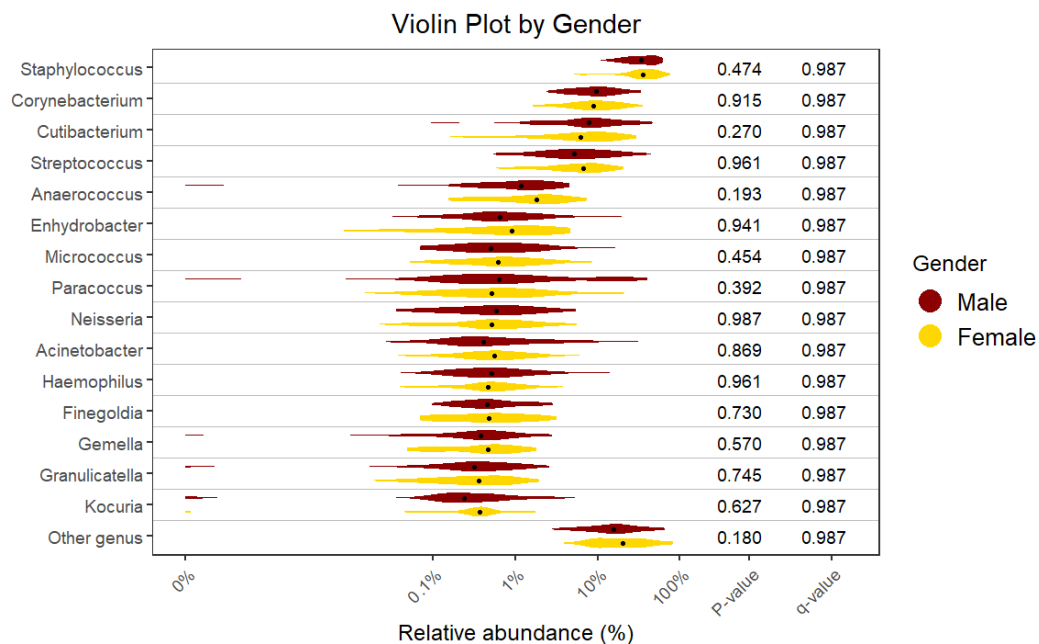
Sample ID	mass [mg]	Quibit concentration [ng/mL]
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S115	71,9	14,5
S117	42,0	5,18
S118	81,5	11,2

9.2. Supplementary Results

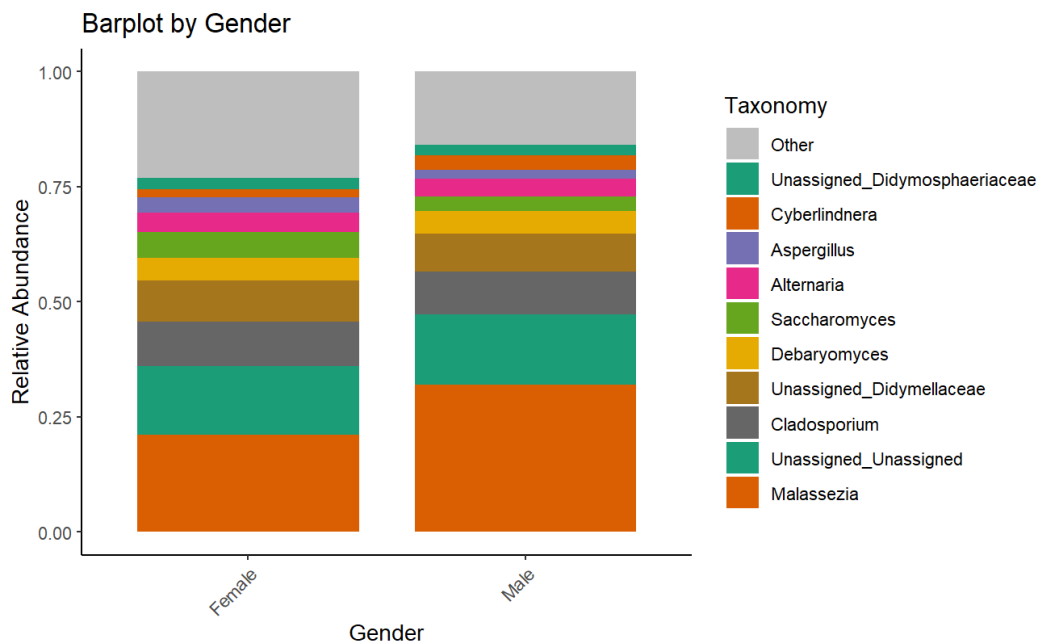


(a)

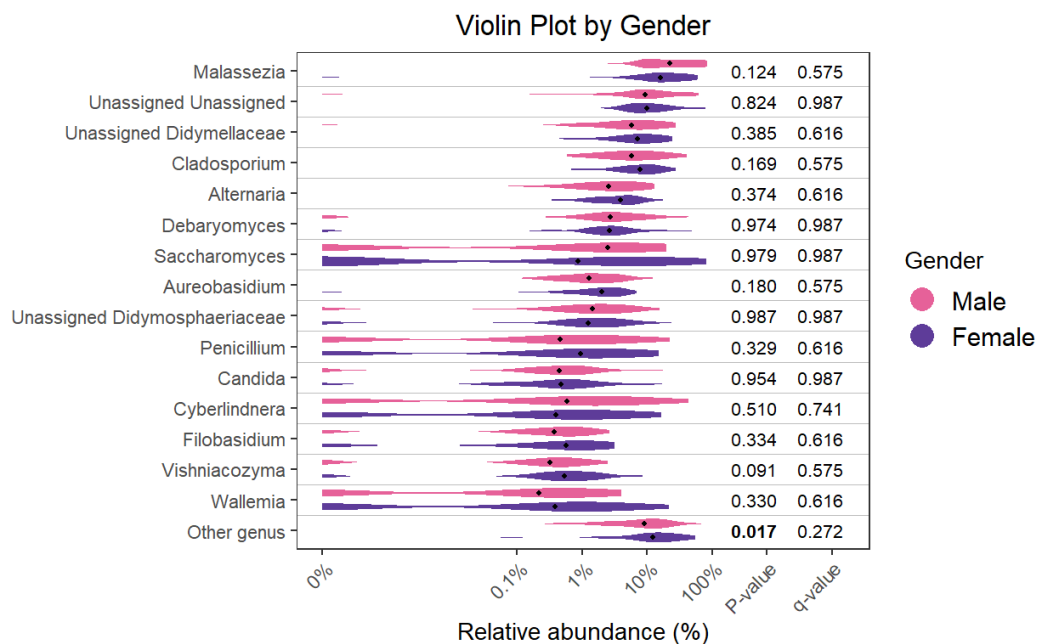


(b)

Supplementary Figure 2. Relative abundance of bacterial genera by gender. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

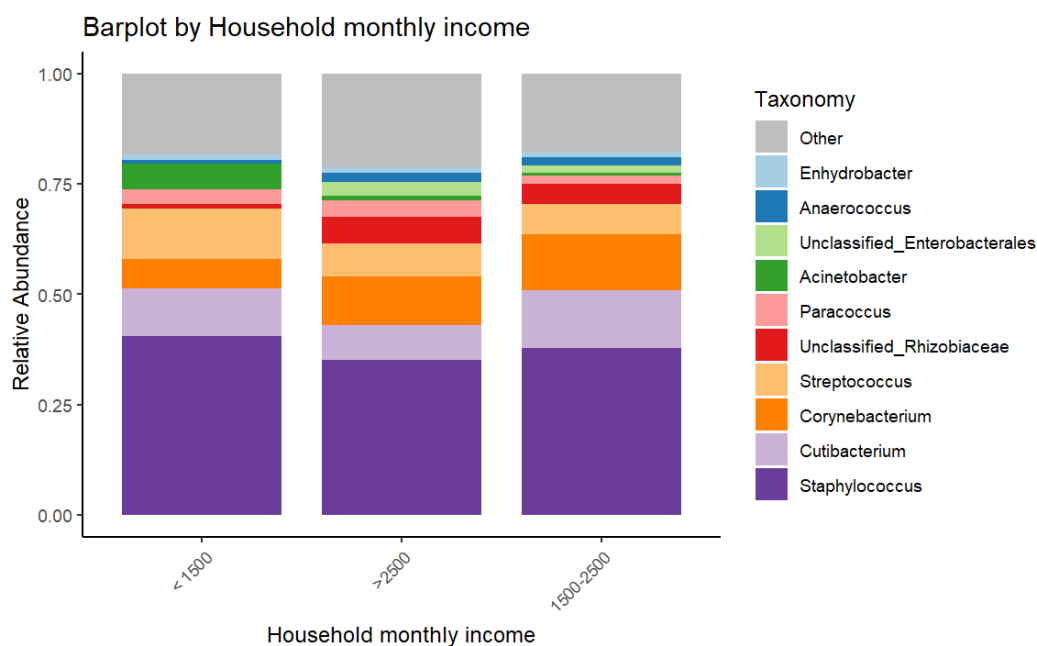


(a)

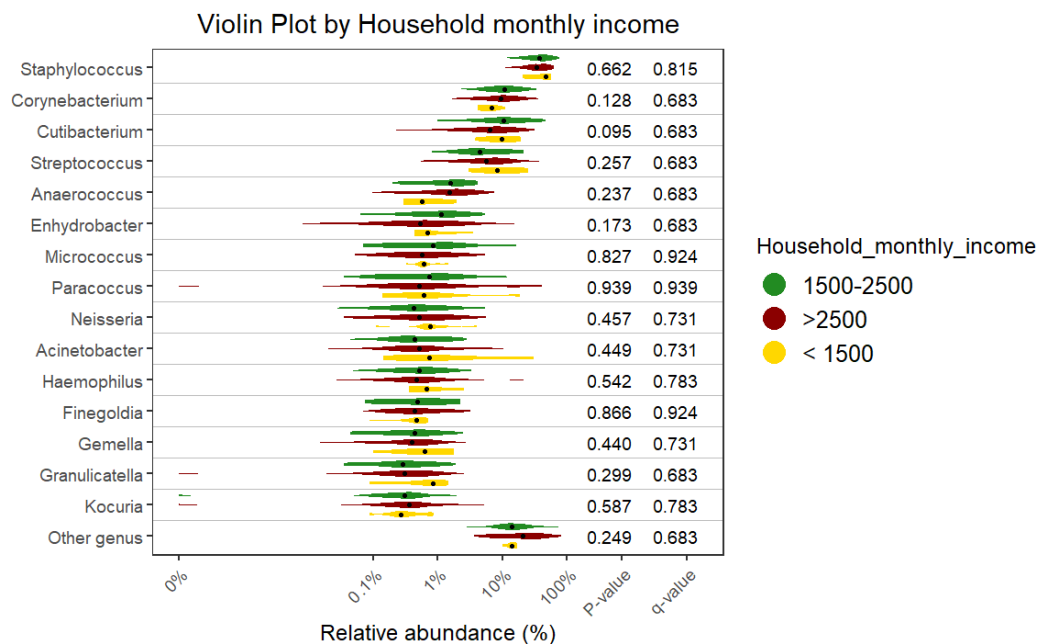


(b)

Supplementary Figure 3. Relative abundance of fungal genera by gender. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

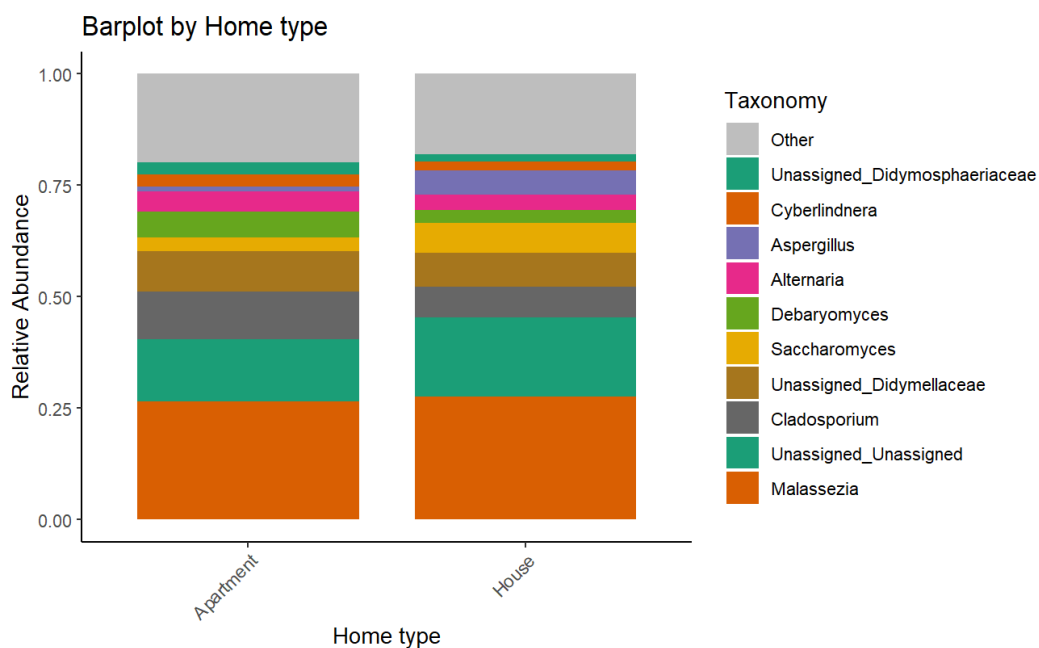


(a)

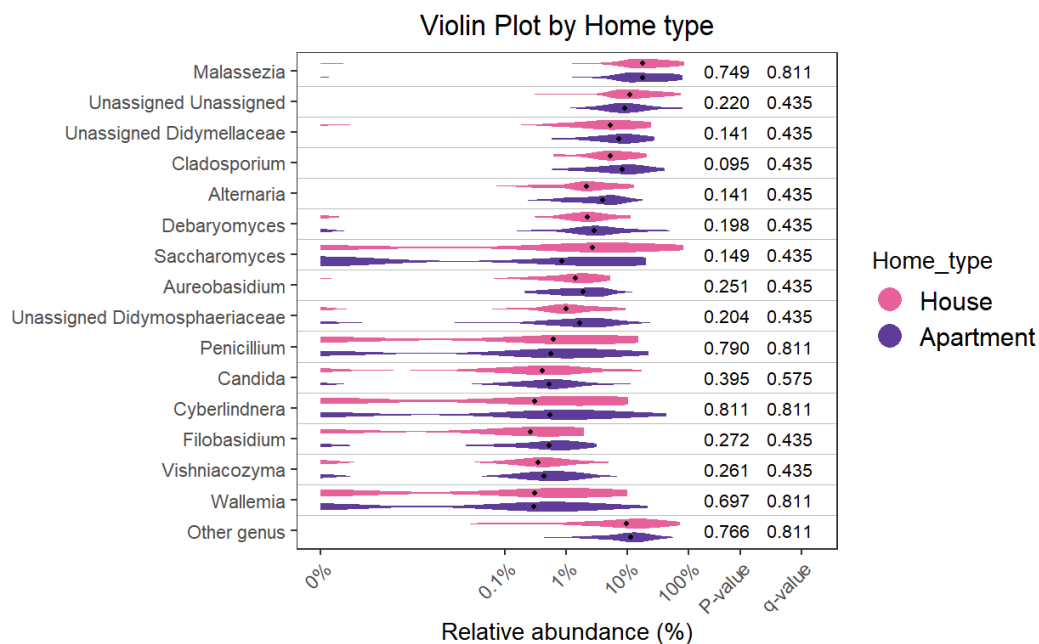


(b)

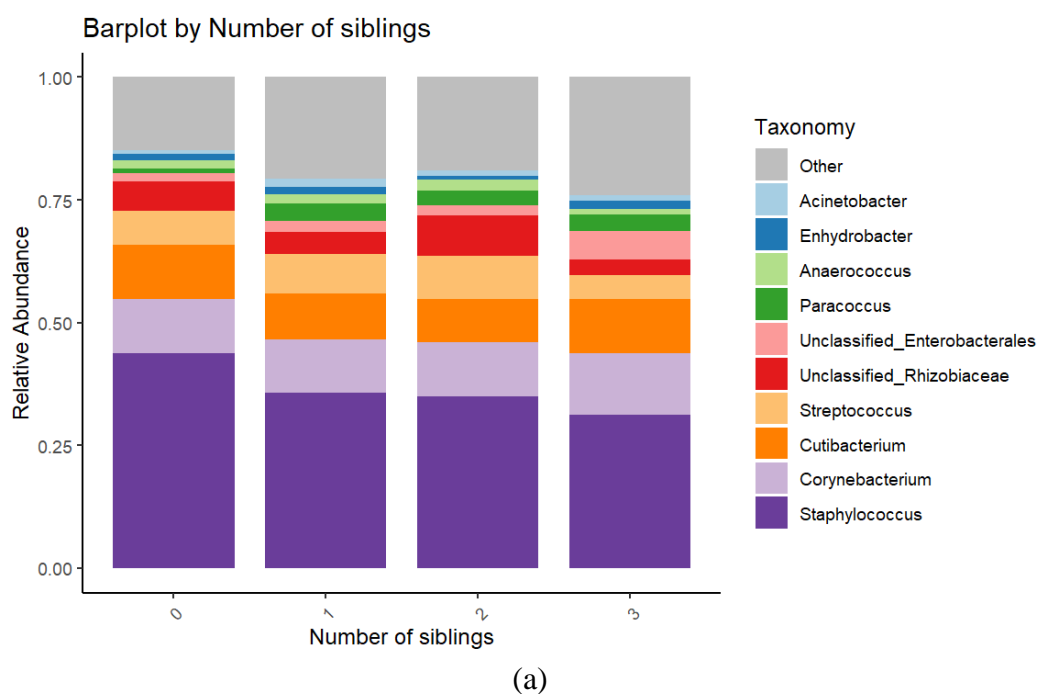
Supplementary Figure 4. Relative abundance of bacterial genera by household monthly income. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

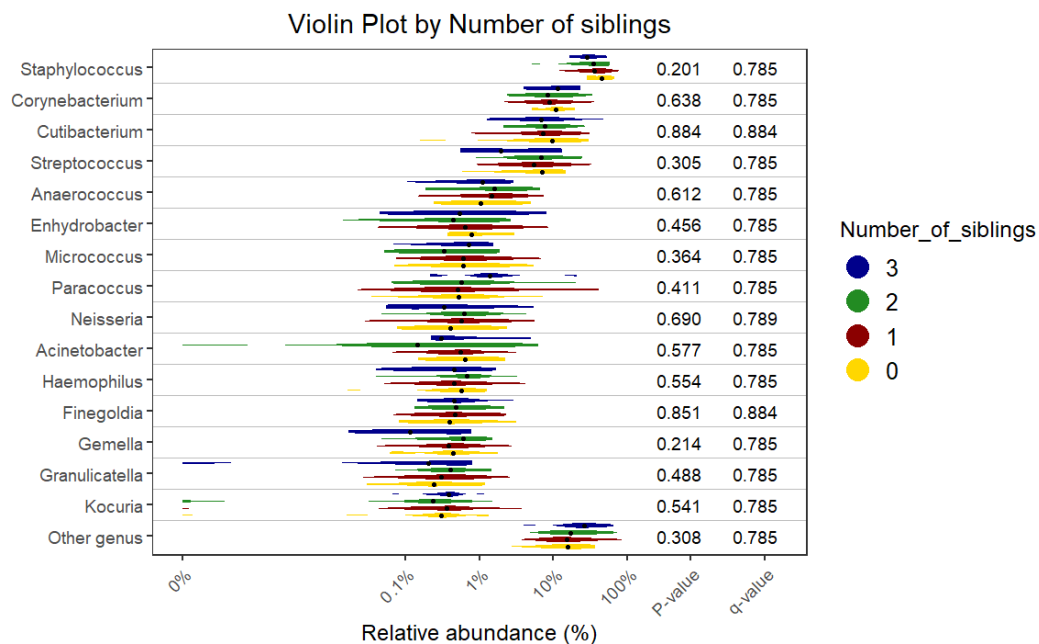


(a)



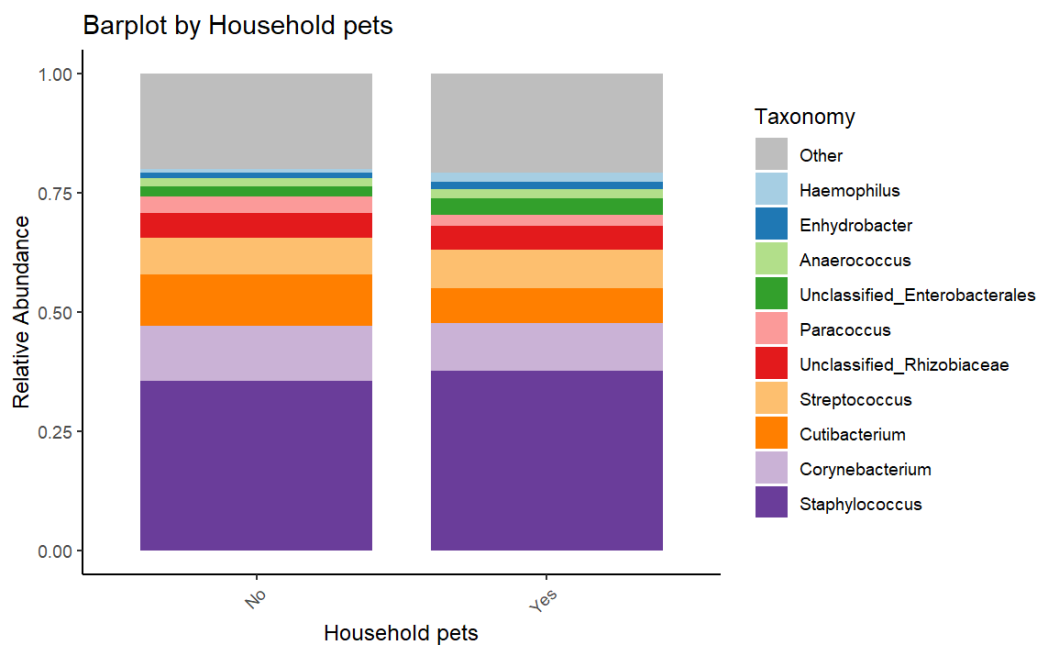
Supplementary Figure 5. Relative abundance of fungal genera by home type. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.



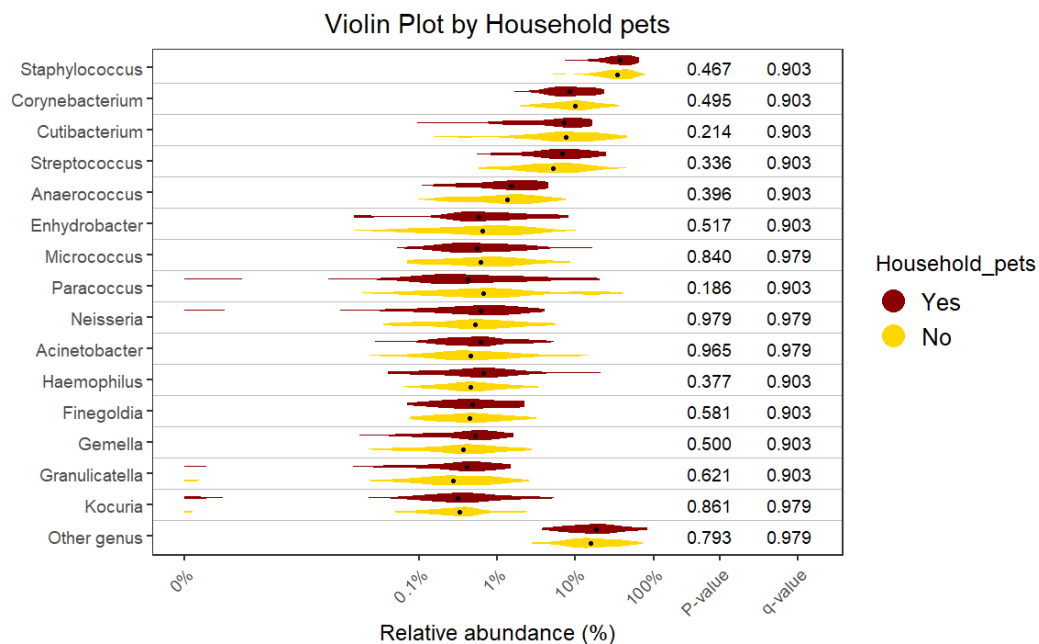


(b)

Supplementary Figure 6. Relative abundance of bacterial genera by number of siblings. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

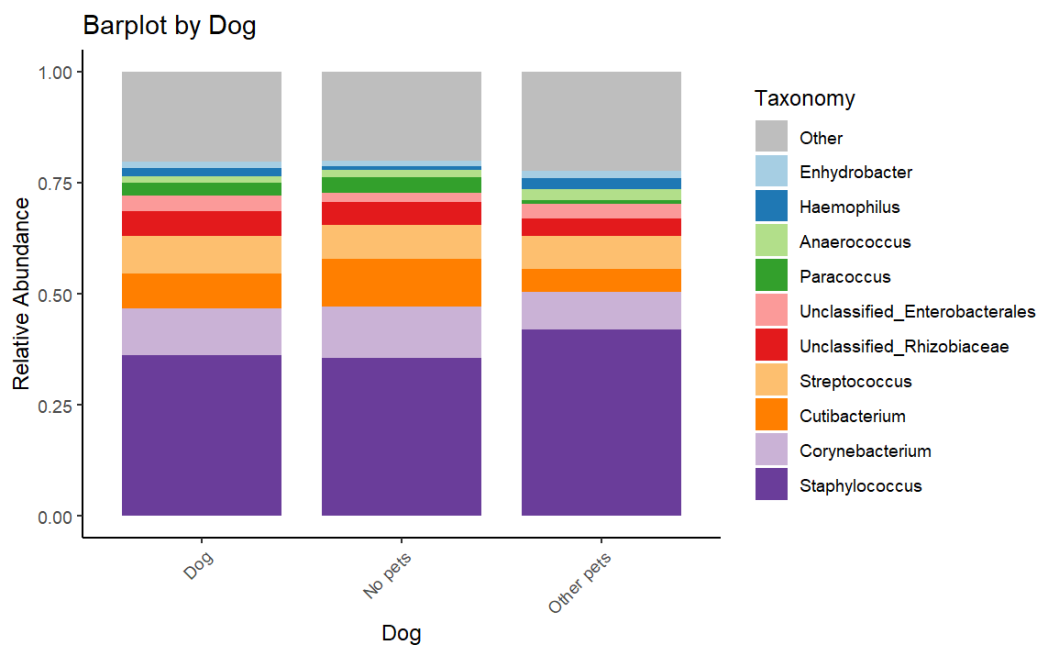


(a)

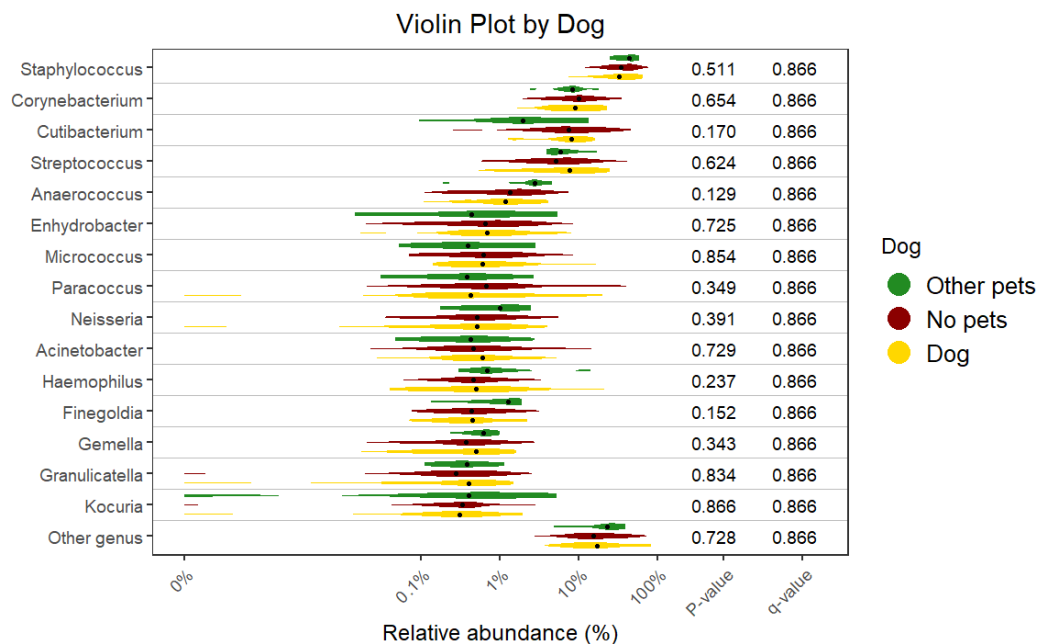


(b)

Supplementary Figure 7. Relative abundance of bacterial genera by pets in the home. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

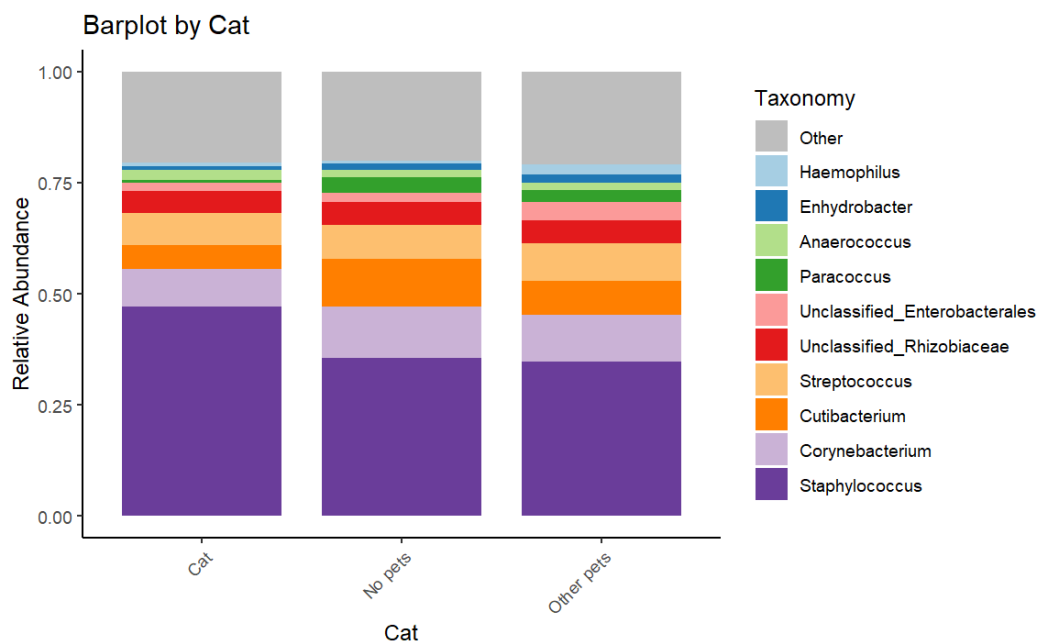


(a)

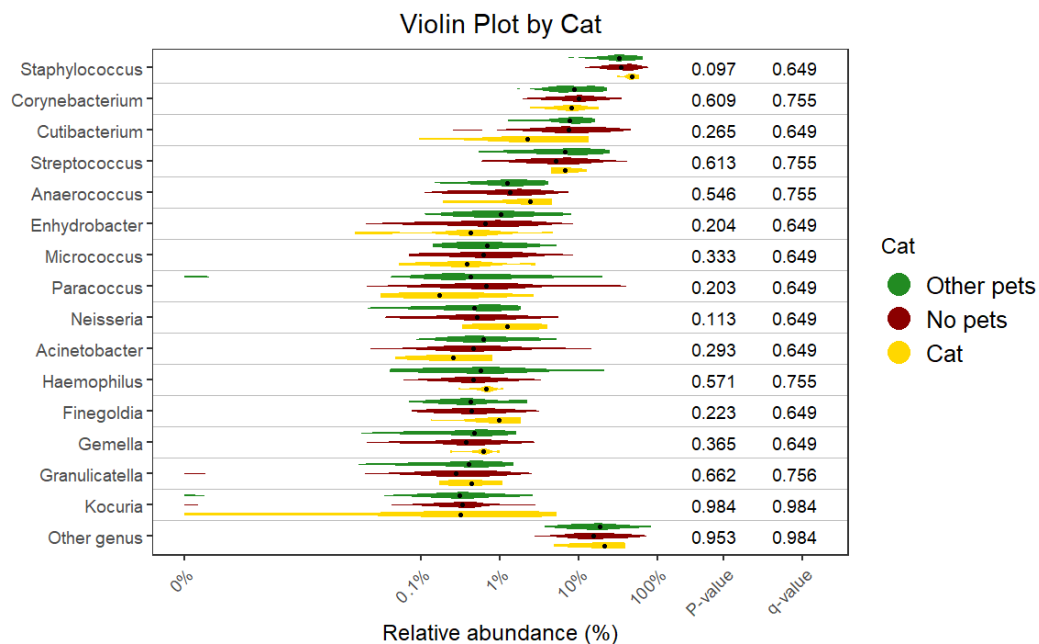


(b)

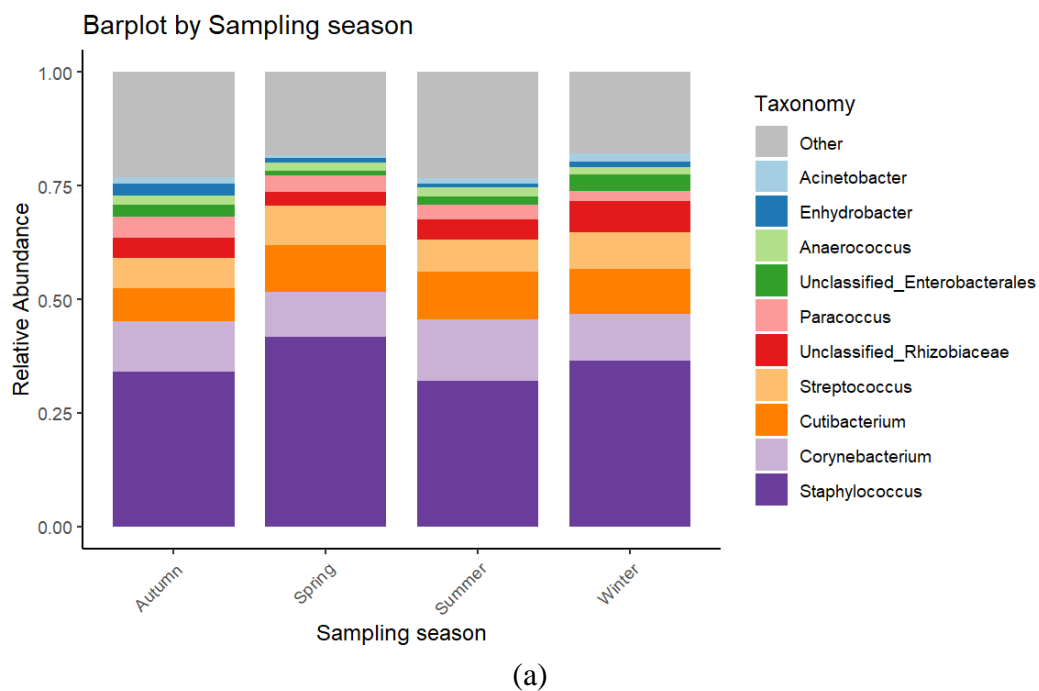
Supplementary Figure 8. Relative abundance of bacterial genera by dog in the home. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.

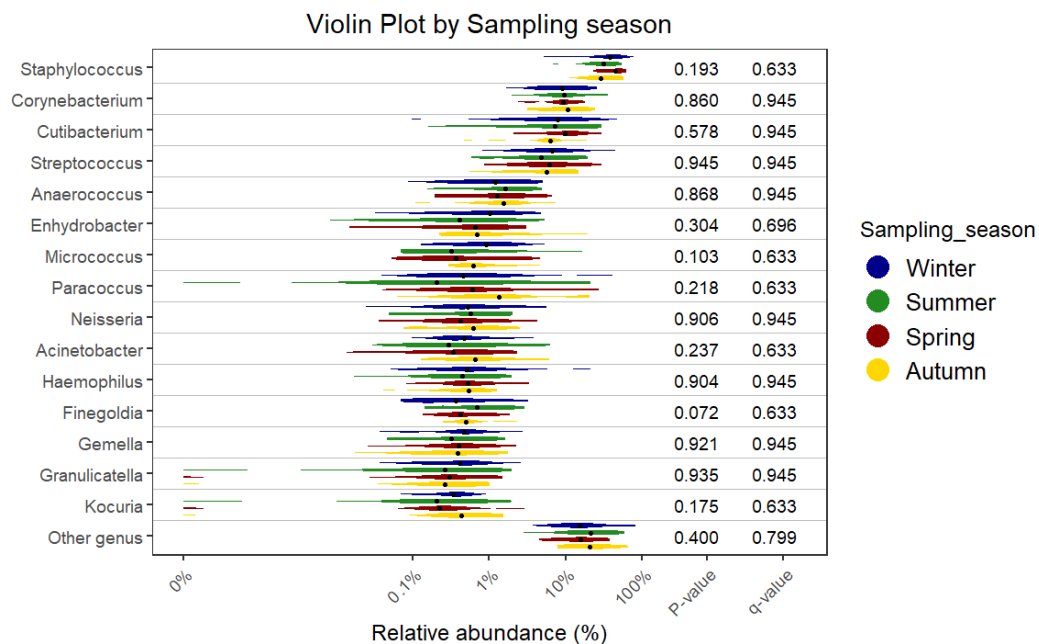


(a)

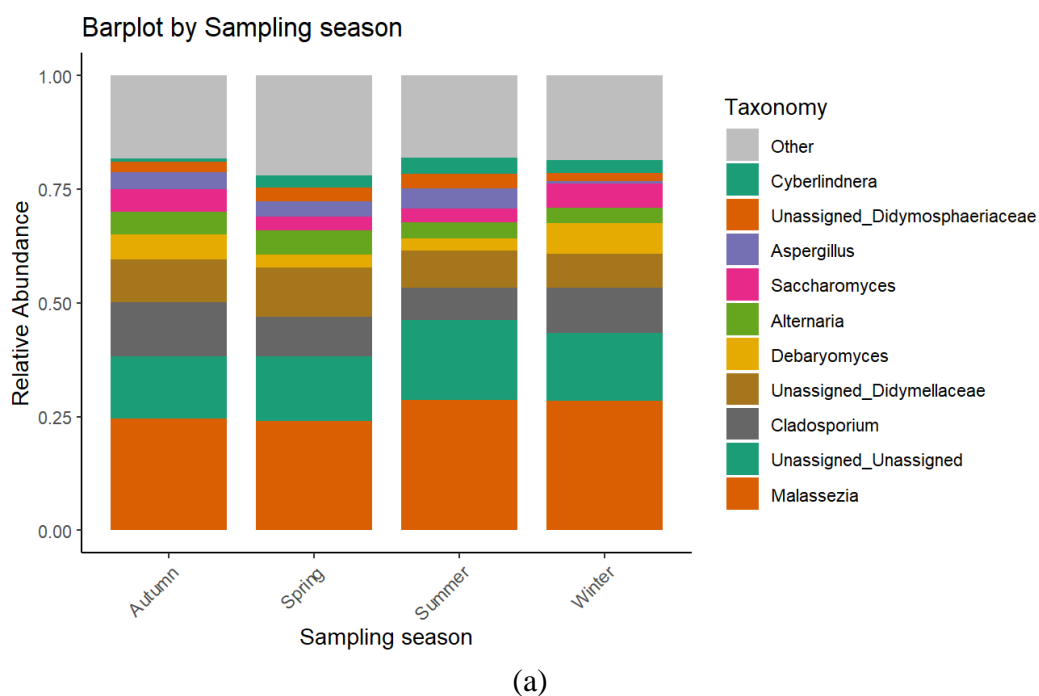


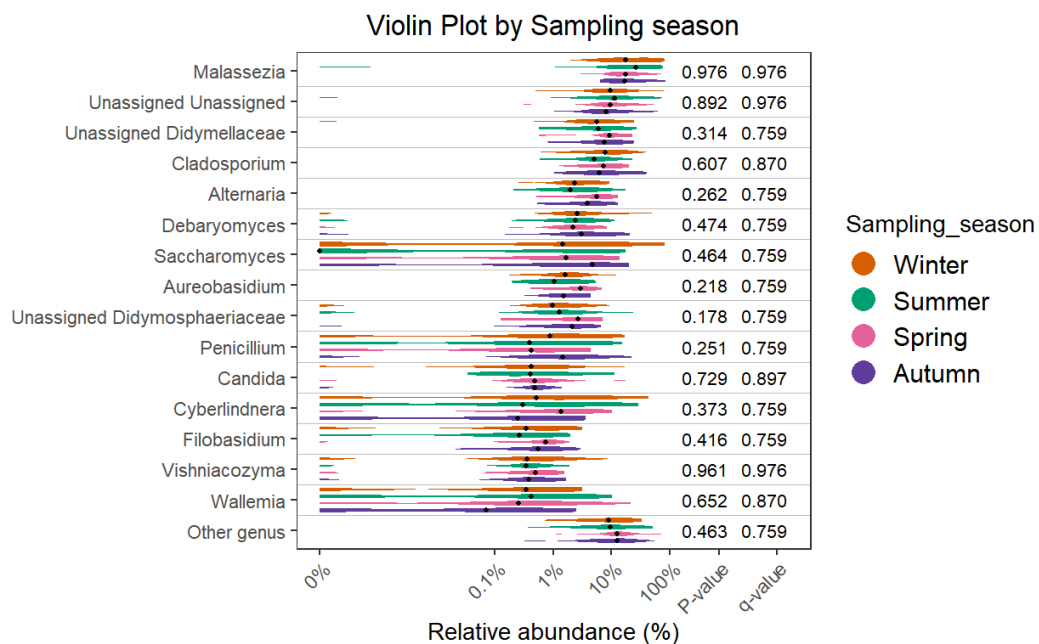
Supplementary Figure 9. Relative abundance of bacterial genera by cat in the home. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.





Supplementary Figure 10. Relative abundance of bacterial genera by sampling season. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.





(b)

Supplementary Figure 11. Relative abundance of fungal genera by sampling season. (a) Stacked bar plot highlights the top ten genera, with others grouped as “Other”, ordered by mean abundance across samples. (b) Violin plot illustrates genus-level distribution and variability, with p-values indicating significant differences between groups.