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Boosting Beneficial Microbial Exposure of Urbanites through Nature-Based Recreation and Biodiverse Elements

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Abstract

Immune-mediated diseases, such as allergies and autoimmune diseases, have become common in developed countries marked by a high level of urbanization. According to the hygiene and biodiversity hypotheses, this is caused by insufficient exposure to diverse nature and environmental microbiota. Urban areas are often densely built and offer less opportunities for contact with biodiversity. Urban lifestyle can also be blamed for the rift between urban dwellers and environmental microbiota as it is often characterized by a high level of hygiene, excessive time spent indoors, and reduced visits to natural environments.

Previous studies have shown that exposure to diverse environmental microbiota enriches the human microbiota and is essential for the development of the immune system. Biodiversity interventions, in other words introducing biodiverse natural materials and elements into people's everyday lives, have recently been suggested as a prophylactic tool in diversifying the human microbiota and lowering the risk of immune-mediated diseases. Deliberate physical contact with nature-based, microbe-rich materials has been shown to diversify the skin and gut microbiota and enhance immune modulation. Less is known about the possible effects of unforced and sometimes even unintentional nature-based recreation. In this thesis, I turned to three forms of nature-based recreation that are popular in Finland: rural second home tourism, urban gardening, and visiting urban green spaces.

In the first study, I compared the fecal bacterial communities of urban elderly rural second home users and non-users. Surprisingly, the fecal bacterial diversity was higher among the non-users, although visiting rural second homes was expected to afford increased exposure to nature and environmental microbiota. Due to the obscurity of a healthy elderly gut microbiota, no conclusions can be made about the health effects related to the observed differences. Without larger studies involving different age groups and geographical regions and detailed information about the environment around the second homes and diets and activities during the second home visits, it cannot be concluded that rural second home tourism automatically increases beneficial microbial exposure among urbanites.

In the second study, healthy urban adults participated in urban indoor gardening using either microbially rich or poor growing medium. After one month of gardening, the bacterial diversity on the skin of volunteers using microbially rich growing medium increased. The more diverse and complex skin bacterial communities were accompanied by elevated anti-inflammatory interleukin 10 levels in blood. As no changes were observed among the volunteers using microbially poor growing medium, it can be concluded that soil biodiversity has a crucial role in diversifying the human microbiota. The study demonstrates that beneficial microbial exposure leading to enhanced immune modulation can be achieved in urban settings, indoors, year-round, and with minimal space and equipment requirements.

In the third study, I investigated how introducing a garden emphasizing biodiversity into a previously barren and paved urban space affects the environmental bacterial communities. After the garden's first growing season, the environmental bacterial communities on the street level became more diverse and complex. The bacterial communities in the adjacent control area that had traditional existing vegetation did not change during the growing season. As the changes in the bacterial communities were observed on the street level, it is possible that the visitors take some of this microbially diverse material indoors where it can diversify the home microbiota.

Based on these studies, nature-based recreation and related biodiverse elements are associated with differences or changes in the human and urban environmental microbiota and in some cases even with immunomodulatory effects. As nature-based recreation is typically voluntary, meaningful, enjoyable, and participated in frequently, it may offer a convenient and cost-effective approach to increase urbanite's exposure to nature and environmental microbiota. However, based on the results, nature-based activities have the potential to boost beneficial microbial exposure only when they involve biodiverse elements or settings. The results tie our health to the health of other organisms and provide additional evidence for the biodiversity hypothesis of health. To secure nature-based health benefits, biodiversity should be protected, promoted, increased, and enhanced everywhere, especially in ever-expanding urban areas.

Keywords: biodiversity hypothesis; hygiene hypothesis; microbiota; exposure; cytokine; immune-mediated disease; immune system; immunomodulation; nature-based; outdoor recreation; biodiversity intervention; urban area; urbanization; rural area; second home tourism; urban gardening; urban cultivation; growing medium; urban green space; biodiversity garden

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List of Abbreviations

ANOVA	Analysis of variance
CLR	Centered log ratio
FDR	False discovery rate
HSD	Honestly significant difference
IBS	Irritable bowel syndrome
IFN	Interferon
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LMM	Linear mixed model
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PERMDISP	Permutational multivariate analysis of dispersion
RIG-I	Retinoic acid-inducible gene-I
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
TGF	Transforming growth factor
TNF	Tumor necrosis factor

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List of Original Publications

This thesis is based on the following publications:

I Saarenpää, M., Roslund, M.I., Puhakka, R., Grönroos, M., Parajuli, A., Hui, N., Nurminen, N., Laitinen, O.H., Hyöty, H., Cinek, O. & Sinkkonen, A. (2021) Do Rural Second Homes Shape Commensal Microbiota of Urban Dwellers? A Pilot Study among Urban Elderly in Finland. International Journal of Environmental Research and Public Health, 18(7):3742.

II Saarenpää, M., Puhakka, R., Roslund, M.I., Nurminen, N., Kummola, L., Laitinen, O.H., Hyöty, H. & Sinkkonen, A. Urban Indoor Gardening Enhances Immune Regulation and Diversifies Skin Microbiota–A Test of the Biodiversity Hypothesis of Immune-Mediated Diseases. (Manuscript)

III Saarenpää, M. & Sinkkonen, A. Biodiversity Garden Alters and Diversifies Urban Bacterial Communities. (Manuscript)

The publications are referred to in the text by their roman numerals.

Author Contributions

I Conceptualization, R.P., M.S., M.I.R., M.G. and A.S.; methodology, R.P., M.S., M.I.R., M.G., A.S., A.P. and N.H.; validation, R.P., M.I.R. and A.S.; formal analysis, M.S., M.I.R., M.G. and A.P.; investigation, R.P., M.S., M.I.R., M.G., A.P., N.H. and N.N.; data curation, R.P., M.S., M.I.R., M.G., A.P., N.H., N.N. and O.C.; writing—original draft preparation, M.S.; writing—review and editing, R.P., M.S., M.I.R., A.S., N.H., O.H.L. and O.C.; visualization, M.S.

II Conceptualization, M.S., R.P., M.I.R., A.S.; methodology, M.S., R.P., M.I.R., N.N., L.K., O.H.L., H.H., A.S.; software, M.S.; validation, M.S., R.P., M.I.R., N.N., L.K., H.H., A.S.; formal analysis, M.S., N.N., L.K.; investigation, M.S.; data curation, M.S.; writing—original draft preparation, M.S.; writing—review and editing, M.S., R.P., M.I.R., L.K., O.H.L., A.S.; visualization, M.S.; project administration, M.S.; resources, O.H.L., H.H., A.S.; funding, O.H.L., A.S.

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1 Introduction

1.1 Urbanization, Microbial Exposure, and Immune-Mediated Diseases

Over the last decades, the incidence and prevalence of immune-mediated diseases, such as allergies and autoimmune diseases, have been steadily increasing throughout Westernized societies (Graham-Rowe, 2011; Lerner, Jeremias and Matthias, 2016). Urbanization has been linked to changes in the immune system (Mbow *et al.*, 2014) and elevated risk of multiple immune-mediated diseases (Kondrashova *et al.*, 2005; Flies *et al.*, 2019; Chen *et al.*, 2021). For example, the highest prevalence rates of asthma have been observed in resource-rich countries where the majority of the population reside in urbanized areas (To *et al.*, 2012). Urban land cover is expanding rapidly, and in 2030, the global urban area is estimated to have tripled when compared with 2000 (Seto, Güneralp and Hutyra, 2012). By 2050, 70 percent of the global population is expected to be living in cities (United Nations, Department of Economic and Social Affairs, Population Division, 2019). The burden of immune-mediated diseases is forecasted to escalate abruptly as a consequence.

Green, biodiverse living environments, especially in early childhood, seem to protect from various immune-mediated diseases. Agricultural and forest land cover around home are inversely associated with the risk of type 1 diabetes and atopic sensitization (Ruokolainen *et al.*, 2015; Nurminen *et al.*, 2021), and vegetation and landscape diversity are inversely associated with the risk of atopy and respiratory diseases, such as asthma (Hanski *et al.*, 2012; Donovan *et al.*, 2018; Liddicoat *et al.*, 2018). Farm-like living environment is associated with diverse human microbiota and seems to offer protective effects especially against asthma (von Mutius and Radon, 2008; Ege *et al.*, 2011, 2012; Stein *et al.*, 2016; Kirjavainen *et al.*, 2019). Urbanization threatens biodiversity at all levels (Sala *et al.*, 2000; Seto, Güneralp and Hutyra, 2012), and according to the biodiversity hypothesis, loss of diverse nature can lead to a higher risk of immune-mediated diseases through decreased microbial exposure (Hanski *et al.*, 2012; Haahtela *et al.*, 2013; Ruokolainen *et al.*, 2017a; Haahtela, 2019; Haahtela *et al.*, 2021). The biodiversity hypothesis states that we are protected by microbiota that reside within us (inner layer) and by microbiota that inhabit environments outside of our bodies (outer layer), and that our bodies are colonized from the outside (Ruokolainen *et al.*, 2017a; Haahtela, 2019). When the diversity of the outer layer microbiota decreases, so does the diversity of the inner layer. Eventually this reduced and altered exposure to environmental microbes leads to immune imbalance when the immune system no longer is able to make a proper distinction between harmful and non-harmful agents or signals.

Early exposure to diverse environmental microbiota, especially through soil, is shown to be essential for the development of the immune system (Haahtela *et al.*, 2021; Ottman *et al.*, 2019; Rook, 2009; Rook *et al.*, 2004; Stein *et al.*, 2016; Strachan, 1989; von Hertzen, Hanski and Haahtela, 2011; von Hertzen and Haahtela, 2006; Banerjee and van der Heijden, 2023). While the original hygiene hypothesis attempted to explain the increase in autoimmune and allergic diseases with decreased frequency of infections (Strachan, 1989), the derivative old friends and microflora hypotheses emphasize the role of commensal and symbiotic microbes and other organisms in immune development (Rook *et al.*, 2004; Noverr and Huffnagle, 2005; Rook, 2009; Stiemsma *et al.*, 2015). Many bacterial groups that correlate with improved immune regulation, such as Gammaproteobacteria and butyrate-producers, have been identified (Hanski *et al.*, 2012; Fyhrquist *et al.*, 2014; Roslund *et al.*, 2020, 2022; Zhu *et al.*, 2021), but studies have also observed that exposure to high overall bacterial diversity might protect against allergic diseases and asthma, possibly due to increased encounters with certain bacteria with protective effects (Ege *et al.*, 2011, 2012; Valkonen *et al.*, 2015; Hyytiäinen *et al.*, 2021).

Urbanization alters the way we interact with environmental microbes. Living in urban areas results in reduced transfer of diverse environmental microbiota indoors (Parajuli *et al.*, 2018), less diverse indoor dust microbiota (Shan *et al.*, 2020), and changes in the airway, gut, and skin microbiota (Lehtimäki *et al.*, 2017, 2018, 2020, 2021). While built environments have microbial communities that differ from vegetated ones, often by being less diverse (Mhuireach *et al.*, 2016; Puhakka *et al.*, 2019), the observed urban–rural differences are also affected by lifestyle. In Eastern Finland, the incidence of many immune-mediated diseases is several times higher than in the adjacent Russian Karelia (Kondrashova *et al.*, 2005, 2013; Ruokolainen *et al.*, 2017b). Populations in both areas have a similar genetic background, and the higher incidence on the Finnish side is believed to be caused by environmental factors related to urbanization (Ruokolainen *et al.*, 2020). Life on the Russian side is characterized by small houses in the countryside, subsistence farming and domestic animals, drinking water from natural sources (e.g., wells, springs), and low exposure to the built environment (von Hertzen *et al.*, 2007; Haahtela *et al.*, 2015). On the more urban Finnish side, people have lost everyday contact with some of these environmental factors that increase beneficial microbial exposure and might protect from immune-mediated diseases.

1.2 Tools to Boost Beneficial Microbial Exposure

Probiotics have long been used as an approach for managing digestive and immune health (Sanders *et al.*, 2018), but as commercially available products often contain only a few selected microorganisms, they might never fully substitute exposure to nature and its diverse microbial communities. Biodiversity interventions, i.e., introducing biodiverse natural elements and materials into people's lives, have recently been suggested as a complementary approach.

In recent studies, a diverse environmental microbiota has been incorporated into gardening materials and health-promoting consumer products to increase the microbial exposure of urban dwellers. Short-term direct contact with soil and plant materials, indoor green walls, and adding biodiverse materials to daycare yards and urban playgrounds have been shown to alter the skin and gut microbiota and enhance immune regulation (Nurminen *et al.*, 2018; Grönroos *et al.*, 2019; Hui *et al.*, 2019a; Roslund *et al.*, 2020, 2021, 2022, 2023; Soininen *et al.*, 2022).

While deliberate, direct exposure to nature-based materials and environmental microbes has been found to affect the human microbiota and immune system, little is known about the possible effects of unforced nature-based activities, such as nature-based recreation. Nature-based recreation refers to any leisure time activity taking place in a natural environment, such as hiking, fishing, berry and mushroom picking, camping, swimming, gardening, bird watching, viewing landscapes, and visiting rural second homes or nature areas (Neuvonen *et al.*, 2022). Many of these activities increase active and passive interaction with natural materials and environmental microbes, which might result in changes in the human microbiota and immune response based on earlier biodiversity intervention studies (Nurminen *et al.*, 2018; Grönroos *et al.*, 2019; Hui *et al.*, 2019a; Roslund *et al.*, 2020, 2021, 2022, 2023). In Finland, nature-based recreation is an integral part of the culture, and 96% of the adult population reports participating in outdoor activities, on average three times a week and 182 times a year (Neuvonen *et al.*, 2022). Due to its popularity in Finland, nature-based recreation might offer a useful tool for increasing beneficial microbial exposure, possibly helping to lower the risk of various immune-mediated diseases at a national level.

1.3 Aims of the Thesis

In this thesis, I focus on different nature-based recreation forms and related biodiverse elements as possible solutions for increasing beneficial microbial exposure of urban dwellers. I concentrate on rural second home tourism, urban gardening, and visiting urban green spaces as they represent nature-based activities that are traditional, beloved, enjoyable, and meaningful. While rural second home tourism often requires traveling outside the city, the other two are inherently urban activities and therefore feasible and accessible approaches for large urban crowds. With the results of the three studies, I wish to demonstrate how nature-based recreational activities offer sustainable, long-lasting approaches to increase exposure to nature and its microbes, and that these activities are easily incorporated into the daily lives and routines of urban people.

In Study I, I tested if the use of rural second homes is associated with differences in the gut microbiota. Rural second home tourism is a popular form of nature-based recreation in Finland, and approximately 50% of the population has been estimated to visit a rural second home regularly (Neuvonen *et al.*, 2022). Visiting rural second homes or cottages can in many cases be seen as a trip to the past as the associated environment and activities resemble those of our non-urban ancestors. Most

Finnish cottages still lack an indoor flush toilet, dishwasher, shower, and other amenities, water for bathing, dishwashing, and drinking often comes from natural sources (e.g., well, lake), and visitors tend to participate in many other forms of nature-based recreation, such as berry and mushroom picking, fishing, swimming, subsistence farming and gardening, chopping firewood, and visiting nearby nature (Pouta, Sievänen and Neuvonen, 2006; Sievänen, Pouta and Neuvonen, 2007; FCG Finnish Consulting Group, Oy., 2016). Rural second home tourism has the potential to affect the human microbiota, especially among urban people who use rural second homes more often than their rural counterparts (Adamiak *et al.*, 2015; Pitkänen, Lehtimäki and Puhakka, 2020). The hypotheses were that the gut microbiota of rural second home users differs from the microbiota of non-users and that the health-related bacterial groups are more diverse or common among the rural second home users.

In Study II, I investigated whether exposure to soil microbiota through urban indoor gardening causes changes in the skin microbiota and immune regulation. The volunteers were instructed to plant, harvest, and consume a set of common vegetables and herbs using either microbially rich or poor growing medium. The microbially rich growing medium contained multiple natural materials, such as tree bark, mulch, dung, agricultural sludge, and *Sphagnum* moss, and the microbially poor growing medium consisted mainly of commercial horticultural peat. Especially in densely built cities, urban agricultural plots, such as allotment gardens and rooftop farms, can be one of the few options for the citizens to interact with nature and environmental microbes. In Finland, over 20% of the adult population reports participating in recreational gardening or farming (Neuvonen *et al.*, 2022). While large-scale urban farming is often carried out outdoors, this study was executed indoors and during wintertime to control for the external microbial exposure coming from the surrounding nature. The aim with the indoor setup was to demonstrate that beneficial microbial exposure can be obtained from small-scale cultivation that is executable year-round, when space is scarce, and with minimal and affordable equipment. The hypotheses were that urban indoor gardening with microbially rich growing medium enhances immune regulation and alters the skin microbial communities by making them more diverse and complex.

In Study III, I explored how establishing a garden focusing on biodiversity in a densely built city center affects the microbial communities of the urban space. Urban gardens and parks have the potential to offer both deliberate and unintentional microbial exposure as city dwellers and visitors come to relax and admire nature or to use them as shortcuts, lunch spots, and for dog walking. The amount of close-to-home green space affects the frequency of nature-based recreation and physical activity, especially in urban areas (McMorris *et al.*, 2015; Pietilä *et al.*, 2015; Liu *et al.*, 2017). In Finland, 20–25% of the population reports engaging in nature-based recreation taking place in parks (Neuvonen *et al.*, 2022). While the biodiversity garden invites visitors to engage with it by offering fruits and fragrant flowers, the majority of the visitors are expected to be exposed to the natural materials and environmental microbiota passively as microbes are

transferred to the airways and skin via ambient air and to the shoes and pets' feet through organic material that has poured over from the garden beds. The hypotheses were that setting up a biodiversity garden alters the composition of the bacterial communities, increases the overall and health-related bacterial diversity, and complexifies the bacterial networks of the surrounding urban space.

2 Materials and Methods

2.1 Study Settings

In Study I, I compared fecal bacterial communities (as a proxy for gut microbiota) between urban elders who visit rural second homes and those who do not have regular access to rural second homes. To test whether rural second home tourism is associated with distinct fecal microbiota among elderly, I compared the bacterial diversity of different taxonomic levels and abundant bacterial phyla and classes in the gut and colon, predicted gut metagenomic functions, and bacterial community compositions between the rural second home users and non-users.

Study II was a randomized, placebo-controlled double-blind trial where I compared skin bacterial communities and blood cytokine levels between urban adults who participated in indoor gardening using either microbially diverse (intervention group) or poor but similar looking (placebo group) growing medium. Both groups participated in gardening as it was postulated that the mere act of gardening could affect the cytokine levels as it might relieve stress. I tested whether urban indoor gardening with microbially rich growing medium results in more diverse and complex skin bacterial communities and elevated anti-inflammatory and lowered proinflammatory cytokine levels by comparing the variables either between the groups (cytokines, bacterial networks) or within the groups between different time points (bacterial diversities). I conducted the bacterial diversity analyses at different taxonomic levels and for the most abundant bacterial phyla and classes detected in the samples. In cytokine analyses, a panel of seven cytokines was used.

In Study III, I tested if the introduction of a biodiversity garden into a paved and barren courtyard in the city center of a Finnish city changes the diversity, complexity, and composition of the bacterial communities of the urban space. I collected environmental samples from street level in the biodiversity garden courtyard (intervention area) and in an adjacent courtyard which had existing vegetation but did not receive additional biodiversity elements (control area). I compared the bacterial diversities between different time points within the courtyards and the bacterial networks and community compositions between the courtyards. The bacterial diversity analyses were conducted at different taxonomic levels and for the most abundant bacterial phyla and classes, in the same manner as in the other studies.

2.2 Sample Collection

Study I was based on fecal samples collected in a previous study that was conducted between 2015 and 2016 (Parajuli *et al.*, 2018, 2020). Based on the survey data collected in that study, I chose 10 elderly (65–79 years) second home users and 15 elderly non-users living in urban apartment houses in the city of Lahti, Finland. The 2015–2016 study had excluded all participants with at least one of the following noncommunicable diseases affecting the immune response: celiac disease, rheumatoid arthritis, chronic obstructive pulmonary disease, diabetes, psoriasis, multiple sclerosis, corticosteroid-treated asthma, chronic obstructive pulmonary disease, and actively treated or metastatic cancer. Smokers and participants with dementia or corticosteroid and other immunosuppressive medication were also excluded, as were participants who had been treated with antibiotics within the last six months before giving the fecal samples.

In Study II, skin and blood samples were collected from healthy urban adults. Growing medium samples were collected by a university researcher at the beginning of the trial (January 2020) to verify the bacterial diversity of the growing media. One spoonful from every growing medium container was collected with a sterilized plastic spoon into separate zip lock bags and stored at -80 °C until further processing. Skin swab samples were taken by a trained nurse at the beginning of the trial (February 2020) and at one month (March 2020). The back of the hand (2x2 cm) was swabbed with ten horizontal and ten vertical strokes with a cotton swab wetted with saline buffer (0.1% Tween 20 in 0.15 M NaCl). The cotton tips were placed in separate sterile plastic tubes and stored at -80 °C until further processing. Blood samples were collected by the same nurse at the beginning of the trial and at one month by drawing venous blood into two Vacutainer CPT Mononuclear Cell Preparation tubes containing sodium citrate (BD Biosciences, NJ, USA). The plasma samples were stored at -80 °C until further processing. Participants were recruited from the cities of Lahti and Hyvinkää, Finland. Exclusion criteria included age under 18 years, severe doctor-diagnosed immune deficiency (e.g., HIV, antibody deficiency), immunosuppressive medication, immune-mediated disease (e.g., colitis ulcerosa, Crohn's disease, rheumatoid arthritis), leukopenia, tetanus antibody deficiency, diabetes, mental or memory disorder, cancer diagnosis, rash or eczema, daily smoking, owning indoor pets, and living on an operating farm. If a participant had been treated with antibiotics before or during the trial, they were excluded from the analyses. Samples from 13 placebo and 15 intervention group participants were used in the analyses.

Study III was based on environmental samples collected from courtyards located in the Finlayson factory district in the city center of Tampere, Finland. I collected loose inorganic and organic material from street level into separate zip lock bags before the introduction of the biodiversity garden (May 2021) and after the first growing season (August 2021) using individually packed toothbrushes and sterilized plastic spoons to sweep the street level for 30 seconds. I collected 15 samples from both courtyards in the spring and fall: five samples from the

immediate vicinity of the existing containers (control courtyard) or future garden beds (intervention courtyard), five samples from a five meters distance to the containers or garden beds, and five samples from a twenty meters distance to the containers or garden beds. The spring and fall samples were collected from the same spots and formed pairs. Samples were stored at -80°C until further processing.

2.3 Sample Processing

2.3.1 Bacterial Samples (All Studies)

Bacterial DNA was extracted from the fecal (Study I), growing medium (Study II), and environmental (Study III) samples with the PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany), and from the skin samples (Study II) with the Fast DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) as per the manufacturers' standard protocols. DNA concentrations were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The V4 region of the 16S rRNA gene was amplified with suitable primers. Negative (sterile water), positive (*Cupriavidus necator*, *Enterococcus faecalis*, Mock communities), and no-template controls were used during DNA extraction, PCR, and sequencing. All samples were sequenced using the Illumina MiSeq platform.

2.3.2 Blood Samples (Study II)

Vacutainer CPT Mononuclear Cell Preparation tubes were centrifuged as per the manufacturer's instructions to separate the plasma. This was used to analyze the levels of proinflammatory cytokines interleukin 17A (IL-17A), interleukin 1 beta (IL-1β), and tumor necrosis factor (TNF), which are associated with the risk of immune-mediated diseases (Dinarello, 2000; Honkanen *et al.*, 2010; Kuwabara *et al.*, 2017); anti-inflammatory cytokines interleukin 10 (IL-10) and transforming growth factor beta (TGF-β1), which are associated with a lower risk of several immune-mediated diseases (Opal and DePalo, 2000; Prud'homme and Piccirillo, 2000; Li *et al.*, 2006; Burmeister and Marriott, 2018); and multifunctional cytokines interleukin 6 (IL-6) and interleukin 21 (IL-21), which have both pro- and anti-inflammatory properties (Mehta, Wurster and Grusby, 2004; Rose-John, 2012). IL-17A, IL-10, IL-1β, IL-6, IL-21, and TNF concentrations were measured using the Milliplex MAP High Sensitivity T Cell Panel kit (Merck KGaA, Darmstadt, Germany) with the Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA) and Bio-Plex Manager software (version 4.1, Bio-Rad Laboratories, Hercules, CA, USA). TGF-β1 concentrations were measured using ELISA (BioVendor, Czech Republic).

2.4 Bioinformatics

In all studies, bacterial paired-end sequences (.fastq) were processed using mothur (versions 1.39.5 and 1.48.0; Schloss *et al.*, 2009) following the protocols by Schloss

and Westcott (2011) and Kozich et al. (2013). The sequences were aligned using the mothur version of SILVA bacterial reference (version 132; Pruesse *et al.*, 2007) and classified with the Bayesian classifier. Only bacterial sequences were clustered as operational taxonomic units (OTU) with a 97% sequence similarity. Less abundant (≤10 sequences across all experimental units) and contaminant OTUs identified from negative controls were removed. For the diversity analyses, the samples were subsampled to lowest read depth. Diversity indices (observed richness, Shannon diversity, Simpson diversity) were calculated using mothur or the package vegan in R (Oksanen *et al.*, 2019).

In Study I, the metagenomic functions of the fecal bacteria were predicted by first picking the 16S rRNA data against the Greengenes Database (DeSantis *et al.*, 2006) according to 97% similarity and then referencing the Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog database (release 89.0) in PICRUSt (Langille *et al.*, 2013).

2.5 Statistical Analyses

All statistical analyses and data visualizations were performed using the R statistical software environment (Study I: version 4.0.3; Study II: version 4.1.2; Study III: version 4.3.1; R Core Team., 2020). Following packages were used: vegan for diversity calculations and community composition analyses (Study I: version 2.5-6; Studies II and III: version 2.5-7; Oksanen *et al.*, 2019), phyloseq for diversity calculations and community composition analyses (Study I: version 1.32.0; Studies II and III: version 1.38.0; McMurdie and Holmes, 2013), ggplot2 for community composition and diversity visualizations (Study I: version 3.3.2; Studies II and III: version 3.3.5; Wickham, 2016), MKinfer for permutation tests (Study II: version 0.6; Kohl, 2020), lme4 (Study II: version 1.1-29; Bates *et al.*, 2015) and rsq (Study II: version 2.5; Zhang, 2018) for linear mixed-effects models (LMM), and cooccur (Studies II and III: version 1.3; Griffith, Veech and Marsh, 2016) and visNetwork (Studies II and III: version 2.1.0; Thieurmel, 2021) for co-occurrence network analyses and visualizations. In all studies, the *p*-values were corrected with the Benjamini–Hochberg correction to account for multiple testing (Benjamini and Hochberg, 1995). q-values represent *p*-values that have been adjusted for multiple testing.

In Study I, the observed richness, Shannon and Simpson diversity indices, and predicted functional orthologs of the fecal bacterial communities were compared between the rural second home users and non-users using the Student's t-test or Mann–Whitney U test. The Student's t-test was used when the data were normally distributed based on the Shapiro-Wilk test, and the Mann–Whitney U test was used when the data were not normally distributed. Differences in the bacterial community composition between the groups were compared using permutational multivariate analysis of variance (PERMANOVA; 999 permutations). The bacterial community compositions were visualized with non-metric multidimensional scaling (NMDS). Both PERMANOVA and NMDS were based on the Bray–Curtis

dissimilarity. The diversities and community compositions were compared at different taxonomic levels (OTU, genus, family, order, class, and phylum). At the OTU level, the analyses were conducted within the most abundant dominant gut phyla and colon families detected in the samples (Donaldson, Lee and Mazmanian, 2016).

In Study II, the observed richness, Shannon and Simpson diversity indices, and relative abundances of the bacteria in skin and growing medium samples were compared between the intervention and placebo group using the Student's t-test or Mann–Whitney U test. When the diversities or relative abundances of the skin bacteria were compared between the time points within the groups, the paired samples t-test or Wilcoxon signed-rank test were used. The t-tests were used when the data were normally distributed based on the Shapiro-Wilk test and the Mann–Whitney U test or the Wilcoxon signed-rank test were used when the data were not normally distributed. The relative abundances were calculated by dividing the reads for each OTU in a sample by the total number of reads in that sample (McKnight *et al.*, 2019). The diversities and relative abundances were compared at different taxonomic levels (OTU, genus, family, order, class, phylum). At the OTU level, analyses were conducted within the most abundant phyla and classes (relative abundance >0.025% across all samples). In the cytokine analyses, changes in the concentrations were used. Changes were calculated by extracting the starting value (0 month) from the one-month value. The cytokine data were $\log_{10}$ transformed to normalize the distributions. Changes in the cytokine levels were compared between the intervention and placebo group using a permutation t-test (9999 permutations). Linear mixed models (LMM) were used to study the relationship between the bacterial and cytokine variables. Participants were used as a random factor in all models. Bacterial co-occurrence network analyses were conducted for both time points in both groups at different taxonomic levels (OTU, genus, family, order, class, and phylum) using binary presence-absence data. Common topological parameters (nodes, edges) were calculated from the networks. Co-occurrence networks were used to study the potential interactions (positive and negative) between the bacteria and to identify important, highly connected bacteria (hubs).

In Study III, the observed richness and Shannon and Simpson diversity indices of the environmental bacterial communities were compared between the time points within the courtyards using the paired samples t-test or Wilcoxon signed-rank test. The t-test was used when the data were normally distributed based on the Shapiro–Wilk test and the Wilcoxon signed-rank test was used when the data were not normally distributed. The diversities were compared at different taxonomic levels (OTU, genus, family, order, class, and phylum). As in Study II, the OTU level analyses were conducted within the most abundant phyla and classes (relative abundance >0.025% across all samples). Differences in the spring and fall bacterial community compositions between the intervention and control courtyard were analyzed using PERMANOVA (999 permutations), permutational multivariate analysis of dispersion (PERMDISP), analysis of variance (ANOVA), Tukey's HSD test, and principal coordinates analysis (PCoA). Center log ratio (CLR) transformed

data and Aitchison distance were used for the calculations and visualizations. Bacterial co-occurrence networks were conducted for both timepoints in both courtyards at the genus levels using binary presence-absence data, and common topological parameters (nodes, edges) were calculated from the networks, as in Study II.

3 Results and Discussion

3.1 Study I: Do Rural Second Homes Shape Commensal Microbiota of Urban Elderly?

The use of rural second homes was associated with differences in the fecal microbiota diversities of urban elderly (Table 1). The Shannon diversity of the fecal microbiota was higher among the non-users than among the rural second home users at the OTU, family, order, class, and phylum levels and for the phylum Firmicutes and families Ruminococcaceae and Lachnospiraceae. The observed richness followed a similar pattern, except for the family Lachnospiraceae where no differences were observed between the groups. The Simpson diversity was significantly higher among the non-users only for the phylum Firmicutes. Based on PERMANOVA, the fecal bacterial community compositions did not differ between the rural second home users and non-users at any of the studied levels (Table 2). According to the predicted microbiota functions, the fecal microbiota of the rural second home users showed lower levels of RIG-I-like receptor signaling pathway than the non-users (p=0.019).

The lower fecal microbiota diversities among the rural second home users were surprising, as previous studies have shown that direct contact with natural materials has the potential to diversify the gut microbiota (Nurminen *et al.*, 2018; Roslund *et al.*, 2020), and rural second home tourism was expected to increase exposure to nature and its microbes. While low gut microbiota diversity is often considered to be a sign of disease-related dysbiosis (Ott *et al.*, 2004; Scher *et al.*, 2015; Gagliardi *et al.*, 2018; Kriss *et al.*, 2018), bacterial diversity in the intestinal tract decreases also with age. The elderly gut microbiota differs from the healthy adult one and is often characterized by reduction in protective commensal bacteria (Hopkins and Macfarlane, 2002; Bartosch *et al.*, 2004; Woodmansey *et al.*, 2004; Zwielehner *et al.*, 2009; Claesson *et al.*, 2012; Greenhalgh *et al.*, 2016). In general, gut bacterial composition can vary greatly with disease, diet, and environment (Rinninella *et al.*, 2019), and as inter-individual variability is more pronounced among the elderly (Claesson *et al.*, 2011, 2012), it is difficult to determine whether the observed differences are health-related. For example, a reduction in the phylum Firmicutes has been linked to colorectal cancer (Kostic *et al.*, 2012), type 2 diabetes (Larsen et al., 2010), and Crohn's disease (Manichanh *et al.*, 2006), but it has also been observed to occur naturally due to aging (Mäkivuokko *et al.*, 2010). Elevated Firmicutes levels, on the other hand, have been associated with irritable bowel

syndrome (IBS) (Krogius-Kurikka *et al.*, 2009). Because defining a universal healthy elderly gut microbiota is challenging, if not impossible, it cannot be concluded that the lower gut microbiota diversity of rural second home users is a sign of dysbiosis, especially when, according to PERMANOVA, the fecal bacterial community compositions did not differ between the groups. Based on the predicted gut microbiota functions, the RIG-I-like receptor signaling pathway might be better regulated among the rural second home users. RIG-I-like receptors are involved in eukaryotic intracellular pattern recognition, and both bacterial and viral infections can activate the complex signaling pathway (Chiu, Macmillan and Chen, 2009; Martínez *et al.*, 2017). RIG-I-like receptors can induce strong proinflammatory responses through type I IFN induction and the signaling pathway is therefore tightly regulated (Kato and Fujita, 2015; Oshiumi, 2020; Ren *et al.*, 2020). RIG-I-mediated interferon responses have been observed in multiple autoimmune diseases (Kato and Fujita, 2015).

The elderly gut microbiota has been shown to correlate strongly with diet (Claesson *et al.*, 2012). No dietary habits during the rural second home visits were recorded in this study, and it is therefore unknown whether dietary choices are responsible for the observed differences. Dietary habits might differ greatly between everyday life and rural second home visits, and a stereotypical idea of Finnish rural second home food involves heavily grilled food (e.g., sausages, meat) and alcohol. However, many users also engage in subsistence farming and consume self-grown vegetables. As grocery stores are often further away from the rural second homes, diets might be less balanced during rural second home visits than everyday life.

The type and quality of the environment surrounding homes also affect the gut microbiota (Parajuli *et al.*, 2020), but this environmental information is missing regarding the rural second homes. It is also unknown which activities the rural second home users engage in during the visits. Some users reported visiting their rural second homes for around 150 days a year, which implies that the second homes are suitable for winter use. In these cases, it is possible that the rural second homes offer many modern amenities (e.g., electric heating, indoor toilet, shower, dishwasher) that might reduce contact with the surrounding nature and natural elements as there is no need to chop wood or bring water from the nearby lake for bathing and washing dishes by hand.

Rural second home tourism comes in many forms and involves so many factors possibly affecting the commensal microbiota that it is impossible to draw conclusions on the nature or causes of the differences observed in this study. Future studies should include larger user groups of different ages and from different geographical regions, samples taken both before and after the rural second home visits, and information on the standard of equipment of the rural second homes, land cover and biodiversity around the homes, and dietary habits and activities during the second home visits.

Table 1. Summary of bacterial diversity results. Observed richness and Shannon and Simpson diversities were compared between the rural second home users and non-users. *p*-values, Benjamini–Hochberg critical values, significance after the correction, and type of the statistical test used are given for each taxonomic group.

Measure	Level	*p*-value	Benjamini–Hochberg Critical Value	Significant with FDR 0.1	Test
Shannon diversity index	Phyl. Firmicutes	0.001	0.008	Yes	U test
	Fam. Ruminococcaceae	0.014	0.017	Yes	t-test
	Fam. Lachnospiraceae	0.017	0.025	Yes	U test
	Phylum	0.031	0.033	Yes	U test
	Class	0.031	0.033	Yes	U test
	Order	0.031	0.033	Yes	U test
	OTU	0.035	0.042	Yes	U test
	Family	0.035	0.042	Yes	U test
	Phyl. Proteobacteria	0.154	0.050	No	t-test
	Phyl. Bacteroidetes	0.229	0.058	No	t-test
	Fam. Bacteroidaceae	0.542	0.067	No	U test
	Phyl. Actinobacteria	0.868	0.075	No	t-test
Simpson diversity index	Phyl. Firmicutes	0.004	0.008	Yes	U test
	Fam. Ruminococcaceae	0.027	0.017	No	U test
	Fam. Lachnospiraceae	0.031	0.025	No	U test
	OTU	0.120	0.033	No	U test
	Phylum	0.120	0.033	No	U test
	Class	0.120	0.033	No	U test
	Order	0.120	0.033	No	U test
	Family	0.120	0.033	No	U test
	Phyl. Bacteroidetes	0.267	0.042	No	U test
	Fam. Bacteroidaceae	0.579	0.050	No	U test
	Phyl. Proteobacteria	0.680	0.058	No	t-test
	Phyl. Actinobacteria	0.868	0.067	No	U test
Observed richness	Fam. Ruminococcaceae	0.003	0.008	Yes	t-test
	OTU	0.008	0.017	Yes	t-test
	Phyl. Firmicutes	0.009	0.025	Yes	t-test
	Family	0.018	0.033	Yes	t-test
	Class	0.019	0.042	Yes	t-test
	Order	0.019	0.05	Yes	t-test
	Phylum	0.024	0.058	Yes	t-test
	Phyl. Proteobacteria	0.053	0.067	No	t-test
	Fam. Lachnospiraceae	0.126	0.075	No	U test
	Phyl. Actinobacteria	0.548	0.083	No	t-test
	Phyl. Bacteroidetes	0.760	0.092	No	U test
	Fam. Bacteroidaceae	0.889	0.1	No	t-test

Table 2. Summary of PERMANOVA results. Fecal bacterial community compositions were compared between the rural second home users and non-users. *p*-values are given for each taxonomic group.

Test	Level	*p*-value
PERMANOVA	Phyl. Proteobacteria	0.068
	Phyl. Actinobacteria	0.080
	OTU	0.139
	Fam. Bacteroidaceae	0.172
	Phyl. Bacteroidetes	0.254
	Phylum	0.300
	Order	0.318
	Class	0.324
	Fam. Lachnospiraceae	0.328
	Family	0.343
	Fam. Ruminococcaceae	0.408
	Phyl. Firmicutes	0.427

3.2 Study II: Urban Indoor Gardening Enhances Immune Regulation and Diversifies Skin Microbiota

Urban adults who participated in urban indoor gardening using microbially rich growing medium were observed to have increased IL-10 levels in their plasma when compared with adults using microbially poor growing medium (Table 3). No statistically significant differences between the groups were observed in the other cytokines. Gardening with microbially rich growing medium resulted also in more diverse skin bacterial communities as the Shannon diversity of the phyla Proteobacteria, Bacteroidetes, Planctomycetes, Cyanobacteria, and Verrucomicrobia and of the class Bacteroidia increased during one month of gardening (Table 4). No differences in the diversities were detected in the placebo group (Table 5). The number of nodes (individual taxa or OTUs) and edges (correlations, interconnections) in the skin bacterial co-occurrence networks increased in both groups at most of the taxonomic levels (Table 6). The increase was more pronounced in the intervention group, and after one month of gardening, the intervention group skin bacterial networks had more nodes and edges than the placebo group networks.

The results of this study illustrate how microbial exposure can easily and pleasantly be boosted in urban settings by participating in small-scale indoor gardening. Since the skin bacterial diversity increased only in the intervention group and the intervention group skin bacterial co-occurrence networks were more complex after the trial than the control group networks, the results show that handling microbially rich soil enriches the skin microbiota. Skin exposure to natural materials with high microbial diversity has been shown to modify the gut microbiota (Nurminen *et al.*, 2018), and some of the bacterial groups whose diversity increased in the intervention group, such as Bacteroidetes, have been linked to immunomodulation through the gastrointestinal tract (Troy and Kasper, 2010; Gibiino *et al.*, 2018). The skin microbiota can help the immune system also

directly by inhibiting pathogen growth and affecting local cytokine production (Sanford and Gallo, 2013). Higher microbial diversity has been linked to healthy skin (Kong *et al.*, 2012; Sanford and Gallo, 2013; Wallen-Russell and Wallen-Russell, 2017), and it has been postulated that higher overall microbial diversity in the living environment can protect against immune-mediated diseases, possibly due to increased chances of encountering bacteria with protective effects (Ege *et al.*, 2011, 2012; Valkonen *et al.*, 2015; Hyytiäinen *et al.*, 2021). The co-occurrence network analysis revealed that indoor gardening in general increases both the amount of skin bacterial OTUs or taxa (nodes) that might have a positive or negative relationship (edges) with one another and the amount of these relationships. As clinical trials reporting skin bacterial co-occurrence network results are scarce, it is hard to draw conclusions on the health-related nature of these changes. The results do, however, reflect the observed changes in the Shannon diversities as the increase in the amount of co-occurrence network nodes and edges was often multiple times higher in the intervention group.

The bacterial changes were accompanied by elevated anti-inflammatory cytokine IL-10 levels in the intervention group, indicating that contact with biodiverse soil microbiota can promote immune regulation. Changes in IL-10 levels have been associated with Gammaproteobacteria on skin (Hanski *et al.*, 2012; Fyhrquist *et al.*, 2014; Roslund *et al.*, 2020, 2022), but this finding was not repeated in this study, and the underlying trigger is unclear. As the subjects in this study were exposed to diverse soil microbiota also via gastrointestinal tract while consuming the crops, it is possible that the elevated IL-10 levels were more clearly associated with changes in the gut microbiota. Fresh edible plants contain a diverse microbiota that have the potential to shape the human microbiota and immune response (Wassermann, Müller and Berg, 2019; Wicaksono *et al.*, 2022, 2023). The indoor gardens might also have affected the indoor aerobiome and increased bacterial exposure via airways. Previous studies have found that houseplants modify the indoor microbiota (Mahnert, Moissl-Eichinger and Berg, 2015; Dockx *et al.*, 2022) and might even affect the human microbiota and immune regulation (Soininen *et al.*, 2022). Future studies should consider all possible routes of inoculation.

According to the original study plan, skin and blood samples would have been collected also after three months of gardening, but this was not possible due to the COVID-19 pandemic. The first two sampling points (0 and 1 month) took place before any COVID restrictions in Finland. It is unknown whether the skin microbiota changes would have persisted or even strengthened due to prolonged gardening and whether these changes could have been linked to the altered cytokine levels. Other studies have observed that bacterial taxa transferred to the skin from soil vanish shortly after gardening (Mhuireach, Van Den Wymelenberg and Langellotto, 2023). However, gardening is a form of nature-based recreation that is often performed repeatedly and regularly and it can therefore lead to recurring soil and microbial exposure. This study demonstrates how urban gardening can be performed indoors, allowing for year-round harvests and soil and microbe contact. Microbial exposure has been found to decrease during winter, especially among

urban citizens (Hui *et al.*, 2019b), but indoor urban gardening with biodiverse soil could help increase it. As a cost- and space-efficient approach, indoor gardening could be a solution for increasing beneficial microbial exposure of urbanites who lack regular access to biodiverse nature, for example in assisted living units.

Table 3. Summary of cytokine results. Changes in $\log_{10}$ cytokine concentrations were compared between the intervention and placebo group using a permutation t-test. *p*-values, q-values, and significance after the correction are given for all cytokines and for the IL-10:IL-17A ratio.

Cytokine	*p*-value	q-value	Significant with FDR 0.1
IL-10	0.0046	0.0368	Yes
IL-17A	0.0312	0.1248	No
IL-10:IL-17A	0.0479	0.1277	No
TNF	0.1141	0.2118	No
IL-21	0.1324	0.2118	No
IL-6	0.2709	0.3612	No
IL-1β	0.3668	0.4085	No
TGF-β1	0.4085	0.4085	No

Table 4. Summary of intervention group skin bacterial Shannon diversity results. Diversities at different time points (0 and 1 month) were compared using paired tests. Shannon diversities (mean ± standard deviation), *p*-values, q-values, significance after the correction, and type of the statistical test used are given for all tested phyla and classes.

Taxon	Shannon diversity index (mean ± SD)		*p*-value	q-value	Significant with FDR 0.1	Test
	0 mo	1 mo				
Class Bacteroidia	2.75±0.35	3.42±0.71	0.0020	0.0320	Yes	t-test
Class Planctomycetacia	1.08±0.67	2.16±1.31	0.0214	0.1712	No	Wil.
Class Verrucomicrobiae	0.99±0.76	1.73±1.04	0.0426	0.2272	No	t-test
Class Oxyphotobacteria	0.76±0.62	1.00±0.59	0.1091	0.4364	No	t-test
Class Fusobacteriia	1.73±0.48	1.59±0.25	0.1641	0.4364	No	Wil.
Class Negativicutes	1.03±0.34	1.20±0.34	0.1883	0.4364	No	t-test
Class Deltaproteobacteria	2.16±0.40	2.66±0.98	0.2220	0.4364	No	Wil.
Class Gammaproteobacteria	1.69±0.31	1.93±0.68	0.2220	0.4364	No	Wil.
Class Acidobacteriia	1.86±0.50	2.21±0.82	0.2455	0.4364	No	t-test
Class Deinococci	1.68±0.42	1.50±0.41	0.2799	0.4478	No	t-test
Class Acidimicrobiia	1.11±0.73	1.58±1.17	0.3163	0.4601	No	t-test
Class Clostridia	2.82±0.72	3.15±0.72	0.4432	0.5909	No	Wil.
Class Thermoleophilia	1.92±0.60	2.10±1.03	0.5815	0.7157	No	t-test
Class Actinobacteria	2.43±0.48	2.41±0.51	0.8153	0.9318	No	t-test
Class Alphaproteobacteria	3.43±0.45	3.40±0.91	0.9201	0.9321	No	t-test
Class Bacilli	1.37±0.33	1.47±0.91	0.9321	0.9321	No	Wil.
Phylum Bacteroidetes	2.76±0.35	3.44±0.73	0.0021	0.0231	Yes	t-test
Phylum Planctomycetes	1.02±0.50	1.99±1.40	0.0135	0.0675	Yes	Wil.
Phylum Proteobacteria	1.99±0.32	2.51±0.91	0.0184	0.0675	Yes	Wil.
Phylum Cyanobacteria	1.07±0.53	1.50±0.48	0.0307	0.0844	Yes	t-test
Phylum Verrucomicrobia	0.99±0.76	1.73±1.04	0.0426	0.0937	Yes	t-test
Phylum Acidobacteria	2.26±0.51	2.72±0.84	0.1193	0.2187	No	t-test
Phylum Fusobacteria	1.73±0.48	1.59±0.25	0.1641	0.2579	No	Wil.
Phylum Deinococcus-Thermus	1.68±0.42	1.50±0.41	0.2799	0.3849	No	t-test
Phylum Chloroflexi	1.81±0.95	2.05±1.04	0.6062	0.7409	No	t-test
Phylum Firmicutes	1.85±0.36	2.07±1.13	0.7120	0.7832	No	Wil.
Phylum Actinobacteria	2.47±0.48	2.50±0.61	0.8266	0.8266	No	t-test

Table 5. Summary of placebo group skin bacterial Shannon diversity results. Diversities at different time points (0 and 1 month) were compared using paired tests. Shannon diversities (mean ± standard deviation), *p*-values, and type of the statistical test used are given for all tested phyla and classes. No q-values are given as all *p*-values were non-significant.

Taxon	Shannon diversity index (mean ± SD)		p-value	Test
	0 mo	1 mo		
Class Acidimicrobiia	0.97±0.89	1.35±0.96	0.2240	Wil.
Class Negativicutes	1.11±0.27	1.30±0.37	0.2318	t-test
Class Planctomycetacia	1.47±1.13	1.91±0.95	0.2790	t-test
Class Fusobacteriia	1.85±0.30	1.73±0.35	0.4017	Wil.
Class Clostridia	3.04±0.36	3.19±0.47	0.4264	t-test
Class Acidobacteriia	2.11±0.84	2.34±0.73	0.4761	t-test
Class Thermoleophilia	1.98±0.91	2.20±0.79	0.5399	t-test
Class Verrucomicrobiae	1.40±1.07	1.65±1.15	0.5761	Wil.
Class Deltaproteobacteria	2.24±0.80	2.36±0.91	0.6477	t-test
Class Bacteroidia	3.14±0.66	3.22±0.62	0.7639	t-test
Class Actinobacteria	2.62±0.55	2.66±0.56	0.8030	t-test
Class Bacilli	1.66±0.23	1.57±0.53	0.8339	Wil.
Class Gammaproteobacteria	1.93±0.34	1.96±0.48	0.8511	t-test
Class Oxyphotobacteria	1.28±0.70	1.23±0.68	0.8742	t-test
Class Deinococci	1.58±0.67	1.61±0.43	0.8888	Wil.
Class Alphaproteobacteria	3.52±0.48	3.51±0.53	0.9746	t-test
Phylum Fusobacteria	1.85±0.30	1.73±0.35	0.4017	t-test
Phylum Acidobacteria	2.47±0.77	2.68±0.74	0.4629	t-test
Phylum Actinobacteria	2.68±0.58	2.78±0.66	0.5559	t-test
Phylum Verrucomicrobia	1.40±1.07	1.65±1.15	0.5761	t-test
Phylum Chloroflexi	1.66±0.94	1.78±0.84	0.7297	t-test
Phylum Bacteroidetes	3.14±0.66	3.22±0.62	0.7671	Wil.
Phylum Cyanobacteria	1.56±0.68	1.46±0.77	0.7761	Wil.
Phylum Proteobacteria	2.32±0.54	2.38±0.60	0.7981	Wil.
Phylum Deinococcus-Thermus	1.58±0.67	1.61±0.43	0.8888	Wil.
Phylum Planctomycetes	1.42±0.94	1.56±1.19	0.8888	t-test
Phylum Firmicutes	2.22±0.40	2.12±0.74	0.9443	Wil.

Table 6. Topological measures of the skin bacterial co-occurrence networks. Number of nodes and edges are given for all taxonomic levels at both time points in both groups.

Taxonomic level	Treatment	Time point	Nodes	Edges
OTU	Placebo	0 mo	1202	21171
		1 mo	1268	20802
	Intervention	0 mo	980	9630
		1 mo	1482	28995
Genus	Placebo	0 mo	427	3478
		1 mo	477	4155
	Intervention	0 mo	404	1882
		1 mo	550	8118
Family	Placebo	0 mo	188	763
		1 mo	219	996
	Intervention	0 mo	182	437
		1 mo	242	1327
Order	Placebo	0 mo	92	224
		1 mo	114	311
	Intervention	0 mo	76	95
		1 mo	117	332
Class	Placebo	0 mo	28	30
		1 mo	40	70
	Intervention	0 mo	31	23
		1 mo	52	81
Phylum	Placebo	0 mo	5	3
		1 mo	13	10
	Intervention	0 mo	6	3
		1 mo	14	12

3.3 Study III: Biodiversity Garden Alters and Diversifies Urban Bacterial Communities

Introducing a biodiversity garden into a previously barren city center courtyard changed the environmental bacterial communities of the urban space. The observed richness and Shannon diversity of the environmental bacteria in the intervention courtyard were higher in the fall, after the establishment of the biodiversity garden, than in the spring at the OTU, genus, family, order, class, and phylum levels (Table 7). The Simpson diversity followed a similar pattern, except for the genus and OTU levels. The Shannon diversity of the phyla Proteobacteria, Planctomycetes, Firmicutes, Verrucomicrobia, Chloroflexi, Gemmatimonadetes, and Acidobacteria and of the classes Bacilli, Gammaproteobacteria, Alphaproteobacteria, and Deltaproteobacteria increased from spring to fall (Table 7). The observed richness followed a similar pattern, except for the phylum Acidobacteria. The Simpson diversity was higher in the fall only for the phyla Firmicutes and Planctomycetes and classes Gammaproteobacteria and Bacilli. After correcting the p-values for multiple testing, no statistically significant differences in the environmental bacterial diversities between the seasons were observed in the control courtyard (Table 8). According to PERMANOVA, the spring and fall bacterial communities in the intervention and control courtyards did not differ from each other (p=0.693).

PERMDISP followed by ANOVA and Tukey's HSD test, however, revealed that the dispersion of the intervention courtyard's fall bacterial community differed from the spring bacterial community and from the control courtyard's spring and fall bacterial communities at the OTU level (Table 9). There were no differences in the dispersion between the spring bacterial community in the intervention courtyard and the spring and fall bacterial communities in the control courtyard. The co-occurrence network of the intervention courtyard's environmental bacteria was more complex at the genus level after the garden was established as both the number of nodes (individual genera) and edges (correlations, interconnections) increased (Table 10). The intervention courtyard's bacterial co-occurrence network had more nodes and edges than the control courtyard's network in the fall, after the garden's first growing season.

The results of this study demonstrate how greening urban spaces with focus on biodiversity can increase the diversity of urban bacterial communities. This in turn might help boost beneficial microbial exposure of urban dwellers. Previous studies have shown that people transfer organic and inorganic material and accompanying microbes to their homes, possibly via shoes, clothes, and even pets, and that the abundance and diversity of the transferred microbiota is lower in urban than in rural settings (Parajuli *et al.*, 2018; Sitarik *et al.*, 2018; Hui *et al.*, 2019b). The environmental samples in this study were collected from the street level as most visitors were assumed to simply pass by the garden, possibly carrying some of the microbe-rich material with them indoors. This might result in more diverse indoor microbiota, which could provide protective effects against immune-mediated disorders, such as asthma (Ege *et al.*, 2011, 2012; Valkonen *et al.*, 2015; Stein *et al.*, 2016; Karvonen *et al.*, 2019; Kirjavainen *et al.*, 2019; Hyytiäinen *et al.*, 2021). When the overall microbial diversity in the living environment increases, the chance of being exposed to bacteria with protective effects might increase. Gammaproteobacteria on skin, for example, have been linked to elevated levels of anti-inflammatory molecules in the blood and lower risk of allergic conditions (Hanski *et al.*, 2012; Haahtela *et al.*, 2015; Roslund *et al.*, 2020, 2022), and their diversity increased in the intervention courtyard. The results also suggest that not all urban greenness has the same effect on urban bacterial communities: while the control courtyard had existing vegetation, the beta diversity (group dispersion) of both the spring and fall bacterial communities resembled the intervention courtyard's bacterial community in the spring, when the courtyard had no vegetation. Furthermore, the bacterial diversities did not change in the control courtyard from spring to fall. This also demonstrates that the increased diversities in the intervention courtyard were not a result of the season.

Urban greenness can boost beneficial microbial exposure also in other ways. Some visitors were observed touching the plants and natural elements, and in previous biodiversity intervention studies, deliberate physical contact with natural materials has been shown to alter the human microbiota and even the immunoregulation (Nurminen *et al.*, 2018; Grönroos *et al.*, 2019; Hui *et al.*, 2019a; Roslund *et al.*, 2020, 2021, 2022, 2023). Some visitors even consumed the fruits

and berries offered by the garden. Microbial exposure occurs also via ambient air, and visits to urban green spaces have been found to diversify the skin and nasal microbiota (Selway *et al.*, 2020). Diverse aerobiomes deriving from urban green spaces might even help enrich the indoor microbiota of nearby buildings. Future studies should monitor the urban bacterial communities for a longer period of time as the garden grows and evolves and measure how this affects the aerobiomes and indoor microbial communities. Environmental samples could be collected from the entrance mats of nearby buildings and passive and active air samplers could be placed both outdoors and indoors. Human trials involving skin, airway, and saliva sampling are an important next step in proving that biodiverse green spaces offer microbial exposure that is superior to traditional urban spaces.

Table 7. Summary of bacterial diversity results in the intervention courtyard. Observed richness and Shannon and Simpson diversities were compared between the seasons using paired tests. Observed richness (mean ± standard deviation), Shannon diversity (mean ± standard deviation), Simpson diversity (mean ± standard deviation), *p*-values, q-values, and type of the statistical test used are given for taxonomic groups that increased from spring to fall.

Measure	Level	Spring (mean ± SD)	Fall (mean ± SD)	*p*-value	q-value	Test
No. of observed OTUs	OTU	608±231	877±200	0.0001	0.0004	t-test
	Genus	246±62	350±82	<0.0001	<0.0001	t-test
	Family	143±35	203±43	<0.0001	<0.0001	t-test
	Order	88±20	118±22	<0.0001	<0.0001	t-test
	Class	39±8	49±8	<0.0001	<0.0001	t-test
	Phylum	17±2	20±3	0.0003	0.0008	t-test
	Phylum Proteobacteria	217±70	296±56	0.0008	0.0046	t-test
	Phylum Verrucomicrobia	18±12	33±11	0.0013	0.0046	t-test
	Phylum Firmicutes	4±4	68±68	0.0015	0.0046	Wil.
	Phylum Planctomycetes	47±41	85±33	0.0056	0.0124	t-test
	Phylum Chloroflexi	27±20	48±26	0.0106	0.0190	Wil.
	Phylum Gemmatimonades	13±7	18±7	0.0270	0.0405	t-test
	Class Bacilli	4±4	35±32	0.0016	0.0048	Wil.
	Class Alphaproteobacteria	128±38	157±29	0.0207	0.0363	t-test
	Class Deltaproteobacteria	32±21	47±12	0.0020	0.0048	t-test
	Class Gammaproteobacteria	55±19	90±26	0.0003	0.0021	t-test
Shannon diversity index	OTU	5.18±0.66	5.67±0.65	0.0083	0.0124	Wil.
	Genus	4.24±0.64	4.68±0.54	0.0059	0.0088	Wil.
	Family	3.55±0.61	4.05±0.52	0.0024	0.0036	Wil.
	Order	3.11±0.48	3.44±0.38	0.0158	0.0237	Wil.
	Class	2.18±0.41	2.41±0.31	0.0132	0.0198	t-test
	Phylum	1.57±0.37	1.75±0.22	0.0288	0.0432	Wil.
	Phylum Proteobacteria	3.99±0.56	4.54±0.58	0.0002	0.0016	t-test
	Phylum Verrucomicrobia	2.38±0.66	2.99±0.49	0.0030	0.0075	t-test
	Phylum Firmicutes	1.08±0.59	3±1.4	0.0017	0.0055	Wil.
	Phylum Planctomycetes	3.37±0.81	4.06±0.37	0.0009	0.0048	t-test
	Phylum Chloroflexi	2.79±0.55	3.24±0.67	0.0219	0.0438	t-test
	Phylum Gemmatimonades	2.15±0.39	2.44±0.45	0.0324	0.05	t-test
	Phylum Acidobacteria	2.67±0.72	3.12±0.60	0.0362	0.05	t-test
	Class Bacilli	0.84±0.65	2.5±1.18	0.0017	0.0045	Wil.
	Class Alphaproteobacteria	3.95±0.41	4.27±0.27	0.0004	0.0015	t-test
	Class Deltaproteobacteria	3.00±0.58	3.43±0.31	0.0117	0.0233	t-test
	Class Gammaproteobacteria	2.41±0.47	3.21±0.66	<0.0001	0.0004	t-test
Simpson diversity index	Family	0.92±0.05	0.95±0.03	0.0115	0.0115	Wil.
	Order	0.90±0.04	0.93±0.04	0.0382	0.0382	Wil.
	Class	0.82±0.08	0.86±0.04	0.0437	0.0437	Wil.
	Phylum	0.67±0.14	0.74±0.07	0.0437	0.0437	Wil.
	Phylum Firmicutes	0.57±0.26	0.91±0.09	0.0011	0.0121	Wil.
	Phylum Planctomycetes	0.95±0.04	0.97±0.01	0.0050	0.0271	Wil.
	Class Gammaproteobacteria	0.82±0.08	0.9±0.07	0.0011	0.0088	Wil.
	Class Bacilli	0.45±0.31	0.87±0.15	0.0020	0.0088	Wil.

Table 8. Summary of bacterial diversity results in the control courtyard. Observed richness and Shannon and Simpson diversities were compared between the seasons using paired tests. Observed richness (mean ± standard deviation), Shannon diversity (mean ± standard deviation), Simpson diversity (mean ± standard deviation), *p*-values, and type of the statistical test used are given for taxonomic groups that increased in the intervention courtyard from spring to fall. q-values are not given as all p-values were non-significant after adjustment.

Measure	Level	Spring (mean ± SD)	Fall (mean ± SD)	*p*-value	Test
No. of observed OTUs	OTU	749±252	712±206	0.1873	Wil.
	Genus	273±78	267±61	0.4602	Wil.
	Family	156±45	156±35	0.8999	Wil.
	Order	95±23	96±20	0.8202	Wil.
	Class	41±9	42±7	0.1596	t-test
	Phylum	17±2	18±2	0.1502	t-test
	Phylum Proteobacteria	237±74	223±69	0.2611	t-test
	Phylum Verrucomicrobia	26±14	27±18	0.8456	t-test
	Phylum Firmicutes	6±5	4±3	0.3978	Wil.
	Phylum Planctomycetes	72±35	79±40	0.3284	t-test
	Phylum Chloroflexi	39±23	34±17	0.3038	t-test
	Phylum Gemmatimonades	19±10	20±10	0.9119	t-test
	Class Bacilli	4±4	3±4	0.4891	Wil.
	Class Alphaproteobacteria	135±36	122±33	0.1133	t-test
	Class Deltaproteobacteria	43±21	44±26	0.8749	t-test
	Class Gammaproteobacteria	56±19	55±22	0.7978	t-test
Shannon diversity index	OTU	5.33±0.80	5.27±0.65	0.5509	Wil.
	Genus	4.38±0.59	4.39±0.49	0.7983	Wil.
	Family	3.69±0.51	3.78±0.43	0.7548	Wil.
	Order	3.21±0.40	3.24±0.36	0.8871	Wil.
	Class	2.33±0.27	2.32±0.23	0.8871	Wil.
	Phylum	1.79±0.16	1.81±0.13	0.7120	Wil.
	Phylum Proteobacteria	4.15±0.47	4.26±0.52	0.2934	Wil.
	Phylum Verrucomicrobia	2.66±0.79	2.61±0.89	0.8871	Wil.
	Phylum Firmicutes	1.24±0.96	1±0.51	0.4021	t-test
	Phylum Planctomycetes	3.85±0.73	3.86±0.81	0.7120	Wil.
	Phylum Chloroflexi	2.87±1.06	2.88±0.70	0.5895	Wil.
	Phylum Gemmatimonades	2.38±0.83	2.43±0.53	0.7548	Wil.
	Phylum Acidobacteria	2.63±0.88	2.60±0.83	1	Wil.
	Class Bacilli	1±0.94	0.73±0.66	0.5892	Wil.
	Class Alphaproteobacteria	4.09±0.42	4.04±0.30	0.4102	Wil.
	Class Deltaproteobacteria	3.25±0.68	2.92±0.76	0.0218	t-test
	Class Gammaproteobacteria	2.42±0.33	2.72±041	0.0252	t-test
Simpson diversity index	Family	0.93±0.05	0.95±0.03	0.5895	Wil.
	Order	0.92±0.05	0.92±0.05	0.7548	Wil.
	Class	0.85±0.06	0.84±0.05	0.2934	Wil.
	Phylum	0.76±0.05	0.77±0.04	0.7120	Wil.
	Phylum Firmicutes	0.68±0.33	0.55±0.21	0.1874	Wil.
	Phylum Planctomycetes	0.96±0.03	0.95±0.06	1	Wil.
	Class Gammaproteobacteria	0.82±0.06	0.87±0.04	0.0184	Wil.
	Class Bacilli	0.66±0.37	0.52±0.33	0.3483	Wil.

Table 9. Summary of Tukey's HSD test results for group dispersions (PERMDISP) of environmental bacterial communities. Group dispersions were compared between the spring and fall bacterial communities in the intervention and control courtyard.

Comparison	*p*-value
Intervention spring - Intervention fall	0.0007
Control spring - Intervention fall	0.0019
Control fall - Intervention fall	0.0234
Control spring - Intervention spring	0.9894
Control fall - Intervention spring	0.6390
Control spring - Control fall	0.8183

Table 10. Topological measures of the environmental bacterial co-occurrence networks at the genus level. Number of nodes and edges are given for both seasons in both courtyards.

Location	Season	Nodes	Edges
Intervention	Spring	400	4065
	Fall	635	15843
Control	Spring	412	10023
	Fall	401	3645

4 Conclusions and Future Perspectives

In this thesis, I concentrated on three nature-based recreation forms popular in Finland and on nature-based, biodiverse elements related to them, and investigated how they might help increase beneficial microbial exposure of urban dwellers. Rural second home tourism was associated with differences in the fecal microbiota of urban elderly, but as the diversities were lower among the second home users, it cannot be concluded that rural second homes automatically offer increased exposure to diverse environmental microbiota. Much was unknown about the rural second home visits, and it is possible that some rural second homes in the study were so modern that they did not increase exposure to nature in the same way as they previously did. If the cottages already resemble up-to-date townhouses, users might spend equally much time indoors watching movies and scrolling through Instagram as they do in cities. Instead of yearning for even fancier and bigger second homes, we should strive for a simpler, more old-fashioned rural second home life where outdoor chores, like harvesting potatoes and washing them in the nearby lake, result not only in healthily microbe-covered hands but also in a calmer mind. In addition to the activities during the visits, we should know more about the environment surrounding the rural second homes. Green, biodiverse living environments can diversify the human microbiota and enhance immune health. Especially farm-like living conditions have been found to protect from immune-mediated diseases, such as asthma. An optimal rural second home could therefore be an old farm surrounded by a broad spectrum of biodiverse habitats. As not everyone has access to a rural second home surrounded by lush nature, nature-based tourism services, for example in farm settings, could be a solution to increase exposure to nature and its microbes among large urban masses. Green care services

are already offered in Finland, and, for example, groups of elderly are taken to farms for a day to help with simple chores and to interact with animals. With supervised activities that encourage urban dwellers to interact with nature, the participants might be better exposed to the environmental microbiota than if they were to visit a rural second home or farm independently.

Visiting rural second homes, farms or often even larger nature areas requires travel outside the city, and since many demographic groups do not have this possibility (e.g., due to environmental awareness or physical or financial challenges), approaches that are feasible in cities are also needed. Urban indoor gardening offers such an approach with minimal space or equipment requirements, and it can be done year-round. While larger and longer-lasting urban indoor gardening studies are recommended, the results demonstrated that gardening with microbially rich growing medium can quickly result in increased skin microbiota diversity and elevated anti-inflammatory cytokine levels in blood. These might help lower the risk of immune-mediated disorders. As the tools needed are readily available and affordable, indoor gardening could be utilized in many different settings. For example, new parents could be gifted with indoor gardening sets to diversify the microbiota in the living environment of their newborns, and long-term care hospitals and nursing homes could have indoor gardening rooms. During summer, gardening could take place outdoors and involve larger containers and areas. Some Finnish municipalities already offer gardening equipment for day care centers, schools, and local communities, but this could be promoted and subsidized even more. Diverse outdoor gardens and farms that are open for the members of the public would offer opportunities for recreation and microbial exposure even for those urban dwellers that do not actively engage in gardening. While indoor gardens could help diversify the commensal and home microbiota of individuals, outdoor gardens could offer microbe-related health benefits for larger urban crowds as the diverse environmental microbiota would spread from the containers to the surrounding urban space in a similar manner as was observed in the biodiversity garden study.

Introducing a garden that highlights biodiversity into a previously barren and paved urban space diversified the environmental microbial communities. While the adjacent control area had existing, more traditional vegetation, the bacterial alpha diversity did not change from spring to fall and the beta diversity resembled that of a non-vegetated urban space. While future studies should address whether the increased bacterial diversity provided by the biodiversity garden results in increased microbial exposure of urban dwellers, the results still emphasize the importance of biodiverse natural elements in urban areas. Both the indoor gardening and biodiversity garden study showed that only elements with high microbial diversity helped in diversifying the bacterial communities. This ties our health to the health of other organisms and nature even more strongly and provides additional evidence for the biodiversity hypothesis of health. As urban areas are becoming more extensive and numerous, and a major and growing proportion of the global population will live in these areas, urban planning and building should

revolve around promoting biodiversity. Existing areas with high biodiversity, such as old urban forests, should be seen as an asset and prerequisite for our healthy existence and should never be replaced by introduced biodiversity. When original biodiversity has already been lost, as is the case in many densely built city centers, biodiversity should be reintroduced, increased, and enhanced wherever possible.

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