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ASSOCIATIONS BETWEEN BIODIVERSITY, POLLUTION, THE COMMENSAL MICROBIOTA OF CHILDREN, AND IMMUNE RESPONSE

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Dedicated to my lovely children **Kerttu and Vilma** (olette tärkeitä minulle) and in the loving memory of my **dad Pentti Råglund**, and to the health of all children now and in the future. Every child should have the right to play in the natural and safe dirt.

ABSTRACT

The incidence of immune-mediated diseases has increased rapidly in developed societies. According to the biodiversity hypothesis, the core reason is the evident biodiversity loss in urban areas. This biodiversity loss limits exposure to a diverse microbiota, which is associated with the human commensal microbiota and immune regulation. In addition, urban pollutants, such as polycyclic aromatic hydrocarbons (PAHs), may alter microbial communities and interfere with immune regulation. However, studies linking urban biodiversity loss, PAH pollution, environmental and human commensal microbiota and immune regulation are lacking.

This study is one of the first to estimate the connections between environmental exposure, the commensal microbiota, and the immune response of urban children using both intervention trials and comparative studies. The aim of this study was also to develop practices to reduce the risk of non-communicable immune-mediated diseases that are globally recognized as emerging public health problems. These diseases comprise over 80 inflammatory disorders including allergies, type 1 diabetes, asthma and inflammatory bowel disease. The research focused on two aspects: the effect of biodiversity and pollution on the commensal microbiota of children and immune regulation. First, I estimated PAH induced bacterial shifts in polluted urban landscaping materials, and whether environmental exposure to PAHs can affect children's commensal bacterial communities on the skin and in the gut. Secondly, we set up a human intervention trial in which urban environmental biodiversity was manipulated and examined its effects on environmental and commensal microbiota and immune regulation in children.

The PAH pollution studies showed that PAHs may induce shifts in environmental and human commensal bacterial communities that are associated with human health and immune regulation. Bacterial shifts in urban landscaping materials depended on soil material type, indicating that in the future it is possible to design gardening and landscaping materials that are more resilient to bacterial shifts induced by PAH pollution. Soil PAH pollution in day-care center yards was associated with altered Actinobacteria, Bacteroidetes and Proteobacteria communities on children's skin and in day-care yard soils. However, altered genera differed between skin and soil, excluding *Mycobacterium*, the abundance of which increased on skin and in soil with increasing surface soil PAH levels. Associations were not found between gut microbiota and PAH levels in day-care yard surface soils or ambient air. However, gaseous chrysene levels in the ambient air were associated with the endocrine signaling pathways predicted from the gut bacterial metagenome with the Kyoto Encyclopedia of Genes and Genomes. The peroxisome proliferator-activated receptor (PPAR) is a crucial signaling

pathway in the regulation of inflammation, metabolism, and tumorigenesis. The PPAR signaling pathway together with the adipocytokine signaling pathway can regulate immune cells and affect hormonally-mediated diseases, including obesity, insulin sensitivity, puberty, and fertility. The PPAR and adipocytokine signaling pathways both decreased among children, with higher gaseous chrysene levels in the day-care center's ambient outdoor air. These findings indicate that PAH concentrations that are below the risk assessment safety limits may alter the human commensal microbiota and interfere with endocrine signaling. The imbalance in human microbiota and the decrease in endocrine signaling pathways might contribute to inflammatory disorders. Therefore, optimal risk assessments should take into account the possibility of the disruption of endocrine signaling pathways and the microbiota–health nexus.

The 28-day biodiversity intervention trial included 75 children in three different day-care environments (standard urban, biodiversity intervention, and nature-oriented). During this intervention, the environmental and intervention children's commensal microbiota was diversified, which in turn promoted their immune regulation and eventually may have beneficial health consequences. Surface soil bacterial communities differed between intervention and standard day-care yards and, in particular, differences were seen within alpha-, beta-, and gammaproteobacterial classes. The relative abundance of bacteria typically found in the forests of Finland increased in intervention day-care yards. These environmental changes in day-care yards remained for 2 years. The diversity of proteobacterial communities in soil and on the skin of the day-care children increased during the 28-day intervention. Importantly, an increase in skin gammaproteobacterial diversity was associated with beneficial effects in immune regulation, promotion of the plasma transforming growth factor-β level and proportion of regulatory T cells, and a decline in pro-inflammatory interleukin 17A (IL-17A) levels. In addition, among intervention children the ratio between anti-inflammatory IL-10 and pro-inflammatory IL-17A increased, indicating that the biodiversity intervention promoted children's immune regulation. In addition, among intervention children, I observed shifts within gut Ruminococcaceae and Lachnospiraceae communities that have earlier been associated with gut health. Interestingly, the microbiota on the skin and in the gut of intervention day-care children shifted toward those in nature-oriented day cares. I followed the environmental and commensal bacterial shifts on the skin, in the saliva, and in the gut for a 2-year period among children in the intervention group. This long-term study showed that the biodiversity intervention shifted the environmental and commensal bacterial communities at the intervention day cares, and these shifts include important primers for the immune system. In particular, environmental shifts were permanent based on the 2-year period. These results are proving valuable since now that we understand the effect of biodiversity in the living environment, we can shape children's commensal bacteria and thus affect immune regulation. The challenge will be to design

novel pathogen-free nature-based solutions for urban people that include a high diversity and richness of anti-inflammatory health-promoting bacteria. Future research should target this challenge.

The results of this thesis support the biodiversity hypothesis: environmental biodiversity is associated with the commensal microbiota of humans and immune regulation. Indeed, both biodiversity loss and pollution in the urban environment may lead to an altered environmental microbiome. This in turn can lead to an imbalanced immune system and consequently increase the prevalence of emerging public health problems, including allergies, asthma, type 1 diabetes, and inflammatory bowel disease. Importantly, this study has demonstrated that modifying the living environment of children with microbiologically diverse natural materials might provide a feasible approach for decreasing the risk of immune-mediated diseases in urban populations.

TIIVISTELMÄ

Nykyisin noin viidennes kehittyneiden maiden väestöstä kärsii immuunivälitteisestä häiriöistä, kuten allergioista, allergisesta astmasta ja tyypin 1 diabeteksesta. Biodiversiteettihypoteesin mukaan häiriöiden lisääntymisen taustalla on kaupungistumisen myötä tapahtunut luontokontaktien vähentyminen, mikä voi vaikuttaa haitallisesti immuunisäätelyyn elimistön mikrobiston yksipuolistumisen kautta. Lisäksi altistuminen kaupungin haitta-aineille, kuten liikenteestä peräisin oleville polysyklisille aromaattisille hiilivedyille (PAH), voi häiritä immuunipuolustuksen toimintaa ja kehittymistä. Väitöskirjani on ensimmäisiä tutkimuksia, jossa arvioidaan syy-seuraussuhteita luontoaltistuksen, PAH-pitoisuuksien, kaupunkilaislasten elimistön mikrobiston ja immuunipuolustuksen säätelyn välillä.

Väitöskirjani perustuu kahteen näkökulmaan: 1) haitta-aineiden vaikutuksiin sekä 2) biodiversiteetin lisäämisen vaikutuksiin. Haitta-ainetutkimuksissa arvioin, miten kaupunkiympäristössä päiväkodin pihan maahan kertyvät sekä ilman PAH-pitoisuudet vaikuttavat maaperän ja lasten elimistön mikrobistoon. Biodiversiteettitutkimuksissa arvioin, miten lisääntynyt luontoaltistus vaikuttaa lasten elimistön mikrobistoon ja sitä kautta immuunisäätelyyn. Biodiversiteettitutkimuksessa vertailin kolmea erilaista päiväkotiympäristöä: 1) luontopäiväkoteja, 2) kaupunkipäiväkoteja (kontrolliryhmä) ja 3) interventiopäiväkoteja, joiden piha muokattiin vihermateriaaleilla.

Haitta-ainetutkimukset osoittivat, että suhteellisen alhaiset PAH-pitoisuudet voivat yksipuolistaa kaupunkimaaperän ja lasten ihon mikrobistoa sekä häiritä hormonitoiminnan säätelyä suolistobakteerien välityksellä. Lisääntynyt luontoaltistus voi sen sijaan monipuolistaa lasten ihon mikrobistoa, mikä oli yhteydessä parempaan immuunitasapainoon. Biodiversiteettitutkimuksessa havaitsin eroja myös terveyteen liittyvässä suolistobakteeriyhteisössä eri päiväkotiryhmien välillä. Kahden vuoden pitkäaikaisseurannan perusteella mikrobistomuutokset piha-alueella olivat suhteellisen pysyviä. Biodiversiteetti-interventio sai aikaan muutoksia bakteeriryhmissä, jotka ovat aiempien tutkimusten perusteella osoittautuneet tärkeiksi immuunijärjestelmän kehittymiselle lapsuudessa.

Tämän väitöskirjan tulokset puoltavat biodiversiteettihypoteesiä: altistuminen monimuotoiselle luonnolle edistää immuunitasapainoa elimistön mikrobiston kautta. Myös kaupungissa esiintyvät haitta-aineet voivat muuntaa elinympäristön ja elimistön mikrobistoa. Biodiversiteettikato ja altistuminen haitta-aineille voivat lopulta johtaa immuunijärjestelmän epätasapainoon ja lisätä kansanterveyttä uhkaavia tulehdussairauksia. Tämä väitöskirja antaa myös näyttöä siitä, että luontoyhteyden parantaminen päiväkodeissa voi olla yksi merkittävä tapa vähentää immuunivälitteisten sairauksien riskiä kaupungistuneissa yhteiskunnissa.

CONTENTS

Abstract 5

Tiivistelmä 8

Contents 9

List of original publications 11

The authors contributions 12

Abbreviations 13

1 INTRODUCTION 14

2 REVIEW OF THE LITERATURE 16

2.1 The commensal microbiota of humans 16

2.2 Immune regulation 17

2.3 Interactions between biodiversity, the commensal microbiota of humans, and immune regulation 18

2.4 Effect of urban PAH pollution on health and the microbiota 20

2.5 Interactions between endocrine signaling pathways, PAHs, and the microbiota 21

3 AIMS OF THE THESIS 22

4 MATERIALS AND METHODS 23

4.1 PAH biodegradation study at laboratory scale (study I) 23

4.2 Experimental setup of the day-care studies (studies II, III, IV) 25

4.3 PAH analyses (studies I and II) 27

4.3.1 Air sampling and PAH extraction (study II) 28

4.3.2 Soil sampling and PAH extraction (studies I and II) 28

4.4 Bacterial analyses (all studies) 29

4.5 Endocrine signaling and immune markers (studies II and III) 30

4.6 Statistics 31

5 RESULTS AND DISCUSSION 33

5.1 Effect of PAH pollution on the environmental microbiota (studies I and II) 33

5.2 Effect of PAH pollution on the commensal microbiota of children and endocrine signaling pathways (study II) 36

5.3 Effect of biodiversity intervention on soil microbiota (studies III and IV) 38

5.4 Effect of biodiversity on the commensal microbiota of children (studies III and IV) 41

5.4.1 Skin microbiota 41

5.4.2 Saliva microbiota 41

5.4.3 Gut microbiota 42

5.5 Associations between biodiversity, the commensal microbiota, and immune response (study III) 43

6 CONCLUSIONS AND FUTURE PERSPECTIVES 47

7 ACKNOWLEDGEMENTS 49

8 Kiitokset 50

9 REFERENCES 51

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I **Roslund, M. I.**, Grönroos, M., Rantalainen, A.-L., Jumpponen, A., Romantschuk, M., Parajuli, A., Hyöty, H., Laitinen, O.H. and Sinkkonen, A. (2018). Half-lives of PAHs and temporal microbiota changes in commonly used urban landscaping materials. PeerJ, 6, e4508. https://doi.org/10.7717/peerj.4508

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III **M. I. Roslund**, R. Puhakka, M. Grönroos, N. Nurminen, S. Oikarinen, A. M. Gazali, O. Cinek, L. Kramná, N. Siter, H. K. Vari, L. Soininen, A. Parajuli, J. Rajaniemi, T. Kinnunen, O. H. Laitinen, H. Hyöty, A. Sinkkonen, ADELE research group, Biodiversity intervention enhances immune regulation and health-associated commensal microbiota among day-care children. Sci. Adv. 6, eaba2578 (2020).

IV **Roslund, M.I.**, Puhakka, R., Soininen, L., Oikarinen, S., Grönroos, M., Nurminen, N., Saarenpää, M., Laitinen, O.H., Hyöty, H. and Sinkkonen, A. Long-term shifts in commensal microbiota of urban children associated with biodiversity intervention. 2020. Manuscript.

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

THE AUTHORS CONTRIBUTIONS

I Designed and implemented the study, generated and analyzed data, designed and wrote the manuscript.

II Designed and implemented the study, contributed to data generation, analyzed data, designed and wrote the manuscript.

III & IV Contributed to study design and implementation, contributed to data generation, analyzed data, designed and wrote the manuscript.

ABBREVIATIONS

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
BaPtpe	Benzo(a)pyrene total potency equivalent
FDR	False Discovery Rate
GLM	Generalized linear model
IL-10	Interleukin-10
IL-17A	Interleukin-17A
LMM	Linear Mixed Model
MANOVA	Multivariate analysis of variance
NMDS	Non-metric multidimensional scaling
OM	Organic matter
PBMC	Peripheral blood mononuclear cell
PERMANOVA	Permutational Multivariate Analysis of Covariance
PERMDISP	Multivariate Homogeneity of Group Dispersions
PPR	Pattern recognition receptor
Q	False Discovery Rate controlled P value
TGF-β1	Transforming Growth Factor Beta 1
Treg	Regulatory T cell

1 INTRODUCTION

Today, the vast majority of children grow up in an urban environment where they are exposed to many environmental risks associated with health (UNICEF, 2012; United Nations, 2018). These risks include increased traffic, air and soil pollution (Boström et al., 2002; Kauppi et al., 2012; Parajuli et al., 2017; Sinkkonen et al., 2013a), and the loss of natural biodiversity (Hanski et al., 2012; Parajuli et al., 2018). Comparative studies between children living in urban and rural environments have shown that their commensal microbiota is distinct and the prevalence of immune-mediated diseases is higher among children living in an urban environment (Hanski et al., 2012; Kondrashova et al., 2013; Lehtimäki et al., 2017). According to the biodiversity hypothesis, which is extended from the hygiene hypothesis (Rook, 2009), the core reason behind the high incidence of non-communicable immune-mediated diseases, including type 1 diabetes, asthma, atopy, and allergies, is the biodiversity loss in urban areas (Hanski et al., 2012; Von Hertzen et al., 2011). This biodiversity loss limits exposure to the diverse microbiota essential for normal development of the immune system in childhood. However, conclusive evidence based on human intervention trials has been missing.

Other factors affecting the human microbiota include genetics and lifestyle factors, for example hygiene level, diet, and the use of antibiotics and other medication (Graham-Rowe, 2011; Noverr and Huffnagle, 2008; Rook et al., 2004). In addition, environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs) from traffic emissions, alter microbial communities in the environment (Mukherjee et al., 2014; Parajuli et al., 2017; Ren et al., 2015). PAHs are well-known carcinogens, mutagens, and teratogens (Kim et al., 2013) with the potential to disrupt endocrine signaling pathways (Lee et al., 2017). Many studies link PAHs to negative health effects (Kamal et al., 2015; Kim et al., 2013; Perera et al., 2018; Zhang et al., 2016), while studies linking environmental PAH exposure, human microbiome shifts, and the resulting health outcomes have been lacking.

Since the commensal microbiota regulates inflammation, metabolic, and neuroendocrine pathways (Blander et al., 2017; Fung et al., 2017), biodiversity loss in parallel with exposure to pollutants may promote imbalance in the commensal microbiota of humans and increase the risk of immune-mediated disorders. This thesis links together environmental exposure to PAH pollutants, human microbiome shifts, and health outcomes. In study II, the health outcomes were assessed from endocrine signaling pathways that link inflammation and metabolism to the commensal microbiota (Nepelska et al., 2017).

As immune-mediated diseases are emerging public health problems in urbanized societies, there is an unmet need for novel prophylactic practices to combat these maladies. To address this need, we performed a 28-day

intervention trial among 75 urban children residing in three different day-care environments. (1) Standard control yards were typical urban day-care center yards that are covered mostly with asphalt and gravel. (2) Intervention yards were modified with forest floor, sod, peat blocks, and planters for vegetable and flower growing at the beginning of the study. (3) In nature-oriented day-care centers, children visited forests on a daily basis. We specifically focused on children who were 3 to 5 years old since large groups of them are exposed to the same day-care environment in the daytime when they are most active. Children's skin and gut microbiota and immunological markers (interleukins IL-10 and IL-17A, and plasma transforming growth factor-β (TGF-β)) were analyzed before and after a 28-day intervention period and the environmental microbiota from standard and modified urban day-care yards were compared. I also studied the long-term effects of the 2-year biodiversity intervention on children's skin, saliva, and gut bacterial communities. The aim of the study was to examine connections between environmental exposure, the commensal microbiota, and immune responses, and to develop practices to reduce the risk of immune-mediated diseases in urban societies.

2 REVIEW OF THE LITERATURE

2.1 THE COMMENSAL MICROBIOTA OF HUMANS

Hominids have coevolved with microbiota over millions of years and adapted to rich microbial exposure in early life, and generally this relationship has been mutually beneficial (Backhed et al., 2005; Macke et al., 2017). Our commensal microbiota, inhabiting the skin and mucosal surfaces, contains about 1000 times more genes than are present in the human genome itself (Turnbaugh et al., 2007). This "other genome" is essential to our physiology and immune system; for example, the commensal microbiota derives many antigens that distinct immune responses appropriate for early life after we have been born (Simon et al., 2015). Our commensal microbiota can regulate metabolism, inflammation, and neurodevelopment, and prevent pathogen growth and cancer (Blander et al., 2017; Fung et al., 2017).

Skin provides a physical, chemical, and immunological frontline host defense against pathogens, toxins, and hostile environmental conditions (Murillo and Raoult, 2013; Pasparakis et al., 2014). It is the largest organ of the human body and the commensal bacteria on the skin contribute to the barrier function by competing with pathogens and communicating with immune cells (Pasparakis et al., 2014). Commensal bacteria are an essential part of the immune homeostasis of the host and healthy skin. For example, the skin microbiota is altered in dermatological disorders, including psoriasis (Alekseyenko et al., 2013), atopic eczema (Fyhrquist et al., 2014; Nakatsuji and Gallo, 2019), acne vulgaris, and rosacea (Murillo and Raoult, 2013).

Gut bacteria regulate the intestinal immune system and endocrine signaling pathways, and have an essential physiological, immunological, and metabolic role in human health (West et al., 2015a). Gut bacteria also regulate mental health through the gut–brain axis (Rieder et al., 2017). An imbalance in the gut bacterial microbiome may contribute to several diseases, including inflammatory bowel disease (Santoru et al., 2017), colorectal cancer (Wu et al., 2013), Hashimoto's thyroiditis (Zhao et al., 2018), type 1 diabetes (Brown et al., 2011), asthma, and allergic diseases (Johnson and Ownby, 2017). In addition, gut bacteria modulate the toxicity of pollutants via metabolic pathways (Claus et al., 2016). The childhood saliva bacterial microbiome has an important role in children's health through priming the gut microbiota (Davé et al., 2016; Wade, 2013). Perturbations in the oral saliva bacteria are well-known to be responsible for dental caries and the periodontal diseases; however, saliva bacteria might also prevent or participate in the prevalence of infections at other locations of the body (Wade, 2013), including infective endocarditis (Mylonakis and Calderwood, 2001) and liver and brain abscesses (da Silva et al., 2004; Livingston and Perez-Colon, 2014). In addition,

decreased oral bacterial diversity has been linked to inflammatory bowel disease (Docktor et al., 2012).

2.2 IMMUNE REGULATION

The immune system is regulated by a complex network of cells, receptors, and signaling molecules, such as proteins. Small proteins called cytokines and regulatory T cells (Tregs) are used as immune regulation markers in many studies (Glocker et al., 2011; Hanski et al., 2012; Ireland et al., 2015; Ishigame et al., 2013; Rook et al., 2015, 2004; Stein et al., 2016). Treg cells are crucial for preventing autoimmune diseases, maintaining peripheral tolerance, and limiting chronic inflammatory disorders (Vignali et al., 2008). For immune regulation, it is essential to discriminate between self (i.e., the body's own cells and tissues) and non-self. This discrimination is called self-tolerance. When self-tolerance fails, the immune system may destroy the body's own cells and tissues, and as a result cause autoimmune diseases (Bluestone and Bour-Jordan, 2012). Treg cells prevent the dysregulation of self-tolerance by suppressing the activation of the immune system (Vignali et al., 2008).

Cytokines, such as IL-10 and TGF-β, are mediators of Treg cell induced suppression (Vignali et al., 2008). TGF-β is a cytokine known to have an essential role in regulating immune responses, and its activation is fundamental for adaptive immunity (Travis and Sheppard, 2014). TGF-β can either suppress or promote the adaptive immune responses depending on the type of induction cells (Travis and Sheppard, 2014). The role of TGF-β in developing autoimmune diseases has been described in mice models; disturbances in TGF-β expression or signaling processes induces rapid development of type 1 diabetes (Ishigame et al., 2013) and may lead to lethal inflammatory disorder (Gu et al., 2011; Kulkarni et al., 1993). IL-10 is an anti-inflammatory cytokine that has been proven to strongly prevent certain types of autoimmune disease (Scapini et al., 2011), while IL-17A is a pro-inflammatory cytokine that contributes to autoimmune diseases (Huber et al., 2013; Kim et al., 2011; Roos et al., 2015). These diseases include inflammatory bowel disease (Glocker et al., 2011; Kim et al., 2011), multiple sclerosis (Huber et al., 2013; Ireland et al., 2015), and chronic obstructive pulmonary disease (Castellucci et al., 2015; Roos et al., 2015). For example, disturbances in IL-10 function may cause severe intractable enterocolitis in infants and small children (Glocker et al., 2011) and IL-17 has been shown to be increased in the blood of patients with severe asthma and to correlate with asthma severity (Agache et al., 2010).

Pattern recognition receptors (PRRs), such as toll-like receptors, play a fundamental role in the regulation of the innate immune system via detection of the microbe-specific molecules (Takeuchi and Akira, 2010). Evidence exists that microbiota in diverse nature can regulate the immune system and prevent the dysregulation of self-tolerance (Hanski et al., 2012; Kirjavainen et al.,

2019; Nurminen et al., 2018; Rook et al., 2004; Stein et al., 2016). However, there is a lack of intervention studies that demonstrate approaches to implement diverse and harmless microbiota into the everyday life of urban people. These approaches are needed to promote immune regulation and prevent the dysregulation of self-tolerance.

2.3 INTERACTIONS BETWEEN BIODIVERSITY, THE COMMENSAL MICROBIOTA OF HUMANS, AND IMMUNE REGULATION

Biodiversity loss in urban areas limits human contact with a diverse microbiota, which in turn may promote imbalance in the commensal microbiota of humans (Hanski et al., 2012; Parajuli et al., 2020). A microbial imbalance in the human body, referred to as dysbiosis, is defined by the overgrowth of some microorganisms, while others become rarer. This imbalance has been associated with many immune-mediated diseases, including allergies and atopic sensitization (Haahtela et al., 2015; Hanski et al., 2012; Ruokolainen et al., 2015), asthma (Ege et al., 2012), type 1 diabetes (Brown et al., 2011; Knip and Siljander, 2016), multiple sclerosis (Cekanaviciute et al., 2017; Hindson, 2017; Pröbstel and Baranzini, 2018), and inflammatory bowel disease (Santoru et al., 2017; Zhou and Zhi, 2016).

The biodiversity hypothesis has gained supporting evidence in many studies (Ege et al., 2012; Hanski et al., 2012; Kirjavainen et al., 2019; Kondrashova et al., 2013; Lehtimäki et al., 2017; Ottman et al., 2019; Parajuli et al., 2018; Ruokolainen et al., 2015; Stein et al., 2016). For immune regulation, the essential microbial determinants of exposure are in infancy, since the immune system matures gradually within the first years of life (Simon et al., 2015). Therefore, exposure to biodiversity in early childhood may be critical to maintain health later in life (Haahtela, 2019; Stokholm et al., 2018). However, the immune system evolves through the whole of childhood (Simon et al., 2015), and exposure to biodiversity modulates immune responses in adulthood also (Li and Kawada, 2011; Nurminen et al., 2018).

Environmental exposure to biodiversity explains substantial variation in the relative abundance of Proteobacteria on skin (Hanski et al., 2012; Ruokolainen et al., 2015). Specifically, environmental biodiversity is associated with a higher diversity of Gammaproteobacteria on skin (Ruokolainen et al., 2015). Anti-inflammatory IL-10 expression correlates with the relative abundance of Gammaproteobacteria on skin, and the genetic diversity of Gammaproteobacteria on skin is associated with a decreased atopy risk (Hanski et al., 2012). In contrast, urbanization reduces the exposure to a diverse microbiota, and exposure to potentially pathogenic human origin bacteria may even increase (Parajuli et al., 2018). A similar phenomenon is observed with canine models, in which the skin microbiota of urban dogs is

associated with bacteria of human origin, while environmental bacteria are rich on the skin of rural dogs (Lehtimäki et al., 2018).

When comparing socioeconomically distinct Finnish and Russian Karelians, the prevalence of type 1 diabetes is six times higher (Kondrashova et al., 2005), and asthma and atopic allergy symptoms approximately three to ten times higher, in more urbanized Finnish Karelians with higher hygiene levels (Ruokolainen et al., 2017; Vartiainen et al., 2002). Interestingly, genetic factors (Kondrashova et al., 2005) or environmental chemicals (Koskinen et al., 2016) did not explain this contrast, indicating that biodiversity and hygiene level had a strong influence (Haahtela et al., 2015). Asthma prevalence in children is associated with home dust microbiota, and asthma-protective dust is often found in rural farmhouses (Kirjavainen et al., 2019; Stein et al., 2016). Asthma-protective farmhouse dust is characterized by a lower abundance of *Streptococcus*, *Lactococcus*, and *Staphylococcus* genera, and a higher abundance of Ruminococcaceae family (Kirjavainen et al., 2019). Early-life exposure to house dust containing Ruminococcaceae, Prevotellaceae, and Lachnospiraceae might inoculate the developing gut microbiota that protects against development of atopy and atopic wheeze, since these bacteria produce immunomodulatory metabolites (Lynch et al., 2014).

Comparative studies between patients with immune system disorders and healthy individuals have reported decreased abundances of *Faecalibacterium* (Ruminococcaceae) in the gut of patients (Brown et al., 2011; Chen et al., 2016; Kumari et al., 2013; Morgan et al., 2012; Stokholm et al., 2018; Wu et al., 2013). The disorders include asthma (Stokholm et al., 2018), colorectal cancer (Wu et al., 2013), type 1 diabetes (Brown et al., 2011), inflammatory bowel disease (Kumari et al., 2013; Morgan et al., 2012), and rheumatoid arthritis (Chen et al., 2016). *Anaerostipes*, *Blautia*, *Faecalibacterium*, and *Roseburia* include butyrate-producing bacteria with anti-inflammatory properties (Brown et al., 2011; Keshavarzian et al., 2015). In patients with Hashimoto's thyroiditis, relative abundances of *Blautia*, *Roseburia*, and *Romboutsia* were increased and that of *Faecalibacterium* decreased in the gut compared with healthy subjects (Zhao et al., 2018). In contrast, patients with Parkinson's disease had relatively lower abundances of *Blautia* and *Roseburia* in the feces and *Faecalibacterium* in the mucosa compared with healthy subjects (Keshavarzian et al., 2015). Further, children with type 1 diabetes had lower abundances of *Roseburia*, *Anaerostipes*, and *Faecalibacterium* in the gut compared with healthy children (Brown et al., 2011). Brown et al. (2011) suggested that butyrate-producing bacteria together with lactate-producing bacteria in a healthy gut might induce a mucin synthesis to maintain gut integrity. Butyrate is able to influence immune regulation via inducing cytokine-producing T-cell differentiation (Kosiewicz et al., 2014). Based on these various results, butyrate-producing bacteria in the gut and Gammaproteobacteria on the skin seem to be generally health promoting, while an imbalance in the commensal microbiota of humans may contribute to chronic inflammatory disorders.

2.4 EFFECT OF URBAN PAH POLLUTION ON HEALTH AND THE MICROBIOTA

PAHs are classified as priority environmental pollutants by the European Environment Agency and by the United States Environmental Protection Agency (USEPA). They are a wide group of compounds consisting of two or more fused aromatic rings. PAHs are formed from incomplete combustion of organic materials, including fossil fuels, wood, petroleum products, and coal (Rengarajan et al., 2015). Since they are formed in traffic and home wood burning, PAHs are often found in ambient air and surface soils in urban areas (Boström et al., 2002). Major environmental and health concerns are the high molecular weight PAHs and the ability of PAH reactive metabolites to bind to cellular proteins and DNA (Rengarajan et al., 2015). Also, unmetabolized PAHs can have toxic effects (Rengarajan et al., 2015). Strong evidence exists that exposure to PAHs is associated with higher lung, skin, bladder, and gastrointestinal cancer risk (Boström et al., 2002; Hong et al., 2016; Rengarajan et al., 2015). PAHs are also well-known to disrupt endocrine signaling (Lee et al., 2017; Zhang et al., 2016). Prenatal exposure to PAHs in urban areas may affect cognitive development and increase the risk of childhood attention deficit hyperactivity disorder (Perera et al., 2018, 2012). Other health risks of PAHs include heart disease, immune supression, skin irritation and inflammation, reproductive and developmental effects, neurological dysfunction, and hematologic changes including anemia (Kamal et al., 2015; Kim et al., 2013).

PAH contamination is associated with environmental microbial changes (Mukherjee et al., 2014; Parajuli et al., 2017; Ren et al., 2015; Sawulski et al., 2014). A limited number of bacteria degrade high molecular weight PAHs but these include Pseudomonas and Mycobacterium (Kanaly and Harayama, 2000; Kweon et al., 2011; Zeng et al., 2017). Bacteria with degradation ability may become more abundant, while several others become rarer when the environment is polluted with PAHs (Parajuli et al., 2017; Sinkkonen et al., 2010). Many bacterial species within the phylum Proteobacteria degrade PAHs, resulting in a higher abundance of Beta- and Gammaproteobacteria and a lower abundance of Actinobacteria and Bacteroidetes in PAH-polluted soils (Lors et al., 2010; Martin et al., 2012; Mukherjee et al., 2014; Sawulski et al., 2014). The aqueous solubility of PAHs decreases with increasing molecular weight; thus they are less bioavailable to bacterial degradation (Johnsen et al., 2005).

Some studies have assessed how PAHs are transformed into metabolites through the human microbiota (Claus et al., 2016; Sowada et al., 2017; Van de Wiele et al., 2005), and a very limited number of studies has estimated PAH-induced shifts in gut microbiota with murine models (Ribière et al., 2016). According to our altered environmental microbiota hypothesis, PAH-induced changes in parallel with biodiversity loss affect microbial communities in the urban environment, and these alterations are associated with human health

(Parajuli et al., 2017). However, studies estimating environmental exposure to PAHs, PAH-induced alterations of human microbiota, and potentially resulting health consequences are lacking.

2.5 INTERACTIONS BETWEEN ENDOCRINE SIGNALING PATHWAYS, PAHS, AND THE MICROBIOTA

Endocrine disrupting properties of PAHs were first demonstrated in animal models and then in cell and protein models (Zhang et al., 2016). Many studies done with human cells or animal models have connected PAH exposure to the peroxisome proliferator-activated receptor (PPAR) in the endocrine system (Borland et al., 2014; Kim et al., 2005; Wang et al., 2011; Yan et al., 2014). PAHs activate the aryl hydrocarbon receptor (AhR), which regulates a number of enzymes involved in PAH metabolism (Beischlag et al., 2008). The AhR and PPAR have an impact on each other and they, together with many other signaling molecules and receptors, regulate endocrine signaling (Borland et al., 2014; Wang et al., 2011). The PPAR family includes three isotypes (α, β, and γ) that have important roles in the regulation of inflammation, cellular differentiation and development, lipid and carbohydrate metabolism, and tumorigenesis (Feige et al., 2006). AhR agonists can increase PPAR-α expression in c7 cells, but Ahr small interfering RNA can decrease PPAR-α and metabolism genes (Wang et al., 2011). Thus, AhR deficiency can decrease the PPAR-α pathway, which increases the risk of diabetes and affects circadian rhythms and glucose metabolism (Wang et al., 2011).

The commensal microbiota of humans also regulates PPAR signaling pathways (Are et al., 2008; Couvigny et al., 2015; Kelly et al., 2004; Nepelska et al., 2017). Studies often examine the effect of a single bacterial strain isolated from humans (Are et al., 2008; Couvigny et al., 2015; Kelly et al., 2004) or the effect of a few cultured bacterial strains (Nepelska et al., 2017). Two of the main metabolites of commensal bacteria, butyrate and propionate, affect PPAR transcriptional activity; different commensal gut bacterial species have a different ability to regulate PPAR target genes (Nepelska et al., 2017). In an earlier study by Nepelska et al. (2017), butyrate producers *Roseburia intestinalis* and *Roseburia hominis*, and *Fusobacterium naviforme*, had the strongest activation potential of PPAR-γ, but *Atopobium parvulum* and *Prevotella copri* could activate PPAR-γ without the presence of butyrate or propionate.

These previous studies indicate that PAHs, the commensal microbiota, and endocrine signaling are connected. However, studies linking environmental PAH exposure, shifts in the commensal microbiota of humans, and a potentially resultant effect on the regulation of endocrine signaling pathways have been missing.

3 AIMS OF THE THESIS

This thesis is one of the first attempts to estimate the connections between environmental exposure to biodiversity and PAH pollution. Importantly, the thesis is a pioneering work in manipulating environmental microbiota in order to see commensal microbiota shifts and the potentially resulting immune response in urban children. First, PAH-induced bacterial shifts in commonly used urban landscaping materials were studied at the laboratory scale. Secondly, the association between environmental PAH pollution and day-care yard soil and children's commensal bacterial communities was estimated. Thirdly, the associations between PAH pollution and endocrine signaling pathways predicted from the gut bacterial metagenome were estimated. Finally, the effect of biodiversity on the commensal microbiota and immune regulation of day-care children was studied in a short-term (28-day) and a long-term (1- and 2-year) experiment. The hypotheses are as follows:

1. Biodegradation of PAHs is associated with the bacterial community in landscaping materials (study I).

2. PAHs cause changes in the bacterial community composition in organic soils used in urban areas (studies I and II).

3. Environmental PAHs alter human commensal bacterial communities and endocrine signaling pathways (study II).

4. Exposure to biodiversity alters children's commensal bacterial communities and promotes immune regulation during a 28-day intervention period (study III).

5. Shifts in children's commensal bacteria are treatment-specific in intervention and standard urban day-care centers in a 2-year follow-up study (study IV).

4 MATERIALS AND METHODS

Studies in this thesis concentrated on how PAH contaminants and biodiversity in urban areas affect environmental and human commensal bacterial communities (Table 1).

Table 1 *Summary of the studies included in the thesis.*

	Objective	Analyses
Study I	Associations between PAHs, biodegradation, and bacterial communities at laboratory scale.	Chemical: Soil physicochemical characteristics and PAHs. Biological: Soil bacterial communities
Study II	Associations between environmental PAHs and soil and children's commensal bacterial communities, and induction/suppression of endocrine signaling pathways.	Chemical: PAHs in soil and volatile PAHs in ambient air. Biological: Soil and human (skin and gut) bacterial communities and KEGG pathways predicted from gut metagenome.
Study III	Associations between biodiversity and environmental and children's commensal bacterial communities and immune regulation.	Biological: Soil and human (skin and gut) bacterial communities. Immune markers: Cytokines (IL-17A, IL-10, TGF-β) and T regulatory cells.
Study IV	Long-term shifts in environmental and children's commensal bacterial communities associated with biodiversity intervention.	Biological: Soil and human (skin, saliva, and gut) bacterial communities.

IL = interleukin; KEGG = Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/); PAH = polycyclic aromatic hydrocarbon; TGF-β = transforming growth factor-β

4.1 PAH BIODEGRADATION STUDY AT LABORATORY SCALE (STUDY I)

Four landscaping materials that are commonly used in Finland were included in the study: coarse peat-sand (trade name: Niittymulta, Kekkilä Group, Vantaa, Finland), fine peat-sand (trade name: Perennamulta, Kekkilä Group,

Vantaa, Finland), gardening compost (trade name: Musta Multa, Biolan, Eura, Finland), and sandy gravel of glacial origin (sepeli, Rudus, Lahti, Finland). Physiochemical characteristics of the materials were analyzed, comprising organic matter (OM) and moisture content, pH, bioavailable nutrients (nitrate, ammonium, and phosphate), elements (Al, Cu, Fe, Mn, P, Pb), and the carbon to nitrogen ratio. The OM content was determined by a loss-on-ignition method according to the SFS 3008 1990 standard and the pH according to the SFS-ISO 10390 standard. Moisture content was measured by drying samples in an oven (+105 °C) to a constant weight, and the water-holding capacity was determined as in Priha and Smolander (1999) by soaking the soil samples in water for 2 h and then draining for 2 h. Bioavailable nutrients were extracted as described by Sinkkonen et al. (2013b) using the QuikChem 8000 flow injection analysis system (LACHAT Instruments, USA). Elements were measured with inductively coupled plasma mass spectrometry (Elan 6000, Perkin Elmer, USA) and the carbon to nitrogen ratio with the LECO CNS-200 analyzer (Leco Corporation, USA).

Landscaping materials (five replicates each) were contaminated with a total of 2.4 μg g−1 dw of PAHs that are often found in urban areas: phenanthrene, fluoranthene, pyrene, chrysene, and benzo[b]fluoranthene (Boström et al., 2002; Saarnio et al., 2008; Tarvainen et al., 2013). The contamination level for phenanthrene, fluoranthene, and pyrene was 0.6 μg g−1, and for chrysene and benzo[b]fluoranthene 0.3 μg g−1. Total PAH concentrations were relatively low and comparable to those in urban soils in Finland (approximately 0.3 to 3.6 μg g−1) (Tarvainen et al., 2013). A non-contaminated control replicate was included for each landscaping material to measure background PAHs and to ensure that materials were uncontaminated. The study was carried out in 500 mL glass pots (100 g dw material per pot) with tightly closed lids and an air hole (ø 3 mm) in a fume hood at 16 ± 1 °C in the dark. The moisture content was kept at 60% of each landscaping material's water-holding capacity. The experimental temperature represents an average temperature in the southern part of Finland during summer (Finnish Meteorological Institute, 2017).

Each landscaping material was homogenized, sieved (ø 0.5 mm), and rehydrated to 60% of its water-holding capacity before PAH contamination. First, a 2 g aliquot of each landscaping material (5 replicates) was contaminated with a total concentration of Σ5PAHs = 120 μg g−1 diluted in acetone. Non-contaminated replicates were contaminated with acetone. Secondly, the contaminated aliquots were mixed and kept in a fume hood for 1 day to allow the acetone to evaporate. Finally, 2 × 2 g of each contaminated material was added to 2 × 98 g (dry equivalent) of the corresponding wet material, mixed carefully, and divided into two to obtain the initial concentration. PAH analyses were carried out immediately after artificial contamination and after 4, 8, and 12 weeks.

4.2 EXPERIMENTAL SETUP OF THE DAY-CARE STUDIES (STUDIES II, III, IV)

The day-care centers were located in three cities in southern Finland: Espoo, Lahti, and Tampere (Table 2). The parents/guardians of the children were informed about the research and asked for their signed consent, as advised in the recommendations of the Finnish Advisory Board on Research Integrity. Studies were in accordance with the Declaration of Helsinki and recommendations of the Finnish Advisory Board on Research Integrity, with approval from the ethical committee of the local hospital district (Pirkanmaa Hospital District, Finland). Approvals to conduct the study were requested from all three cities.

Table 2 *Number of day-care centers, children, and probiotics, antibiotics and medication users who were excluded from gut microbiome and immune regulation statistical analyzes.*

	Study II	Study III	Study IV
Day-care centers:			
Espoo	6	0	6
Lahti	5	6	5
Tampere	0	3	2
Total children	53	75	89
Boy	30	37	48
Girl	23	38	41
Excluded:			
Probiotics users	6	2	9
Antibiotics users	1	3	6
Medication users	0	8	11

Study participants were children born in Finland, living in urban areas, and aged between 3 and 5 years at the beginning of the study. Exclusion criteria of the study included a condition affecting immune response (e.g., rheumatoid arthritis, colitis ulcerosa, Crohn's disease, diabetes, Down syndrome), immune deficiency (e.g., antibody deficiency, HIV infection), immunosuppressive medication (e.g., corticosteroids), or a cancer diagnosis. The parents/guardians of the children recorded the use of antibiotics, probiotics, medication, and other background information with standardized questionnaires. Allergic diseases were not exclusion criteria, but children using allergy or other medications (antibiotics, paracetamol, desloratadine, pyrvin, cetirizine, and salbutamol), or probiotics, were excluded from the gut microbiota and cytokine analyses.

In study II, PAH concentrations were measured from ambient air and surface soils of day-care center yards. Bacterial communities were analyzed from soil, gut, and skin.

Biodiversity intervention study III included three nature-oriented day-care centers that served as a positive control (children n = 23). Three standard urban day-care centers with minor biodiversity in the yards served as a negative control (study subjects n = 16). In four intervention day cares the gravel was subsequently partly covered with forest floor (100 m2) and sod (200 m2) (study subjects n = 36) (Figure 1) (Puhakka et al., 2019). Children also received peat blocks and planters for flower growing. The intervention lasted for 28 days from May to June 2016. The day-care personnel introduced the biodiverse materials into the children's activities, and the children played in the yards for approximately 1–2 h twice a day.

In study IV, long-term shifts in bacterial communities were estimated. These shifts were monitored from the soil and children's commensal bacterial communities on the skin and in the saliva and gut. Shifts were analyzed after 1 year from the children in intervention (complete cases n = 27) and standard day cares (complete cases n = 15), and after 2 years from the children in the intervention group (complete cases n = 8). Two-year shifts in the gut bacterial community were not analyzed because of the limited number of stool samples collected (n = 3).



Figure 1 *The uppermost picture shows an intervention yard after a two-week usage. Sod is in the foreground. Forest floor vegetation, including humus layer, is in the area below the teeter-totter, slide and jungle gym. Other pictures demonstrate intervention yards before (left figures) and after (right figures) the yards were modified.*

4.3 PAH ANALYSES (STUDIES I AND II)

The soil and air samples were analyzed for 16 USEPA priority PAHs. The internal standard included deuterated PAH mixture (acenaphthene-D10, chrysene-D12, naphthalene-D8, perylene-D12, and phenanthrene-D10) (200 ng PAH Mix 31, Dr. Ehrenstorfer, Augsburg, Germany) and a recovery standard was anthracene-D10 (100 ng, Dr. Ehrenstorfer, Augsburg, Germany). A reagent blank was included with each sample analysis. PAHs were analyzed with a gas chromatography-mass spectrometry system (Shimadzu GC–MS-QP5000) equipped with an AOC-20i auto-injector and a

30 m ZB-5MS column (0.25 mm i.d., 0.25 μm film thickness). The mass spectrometer interface temperature was set to 280 °C with the oven temperature program as follows: 80 °C for 1 min, 10 °C/min to 250 °C, 7 °C/min to 280 °C, and 20 °C/min to final 320 °C for 5 min.

4.3.1 AIR SAMPLING AND PAH EXTRACTION (STUDY II)

A passive semipermeable membrane device (SPMD sampler) was used to measure the concentrations of PAHs in the day-care center yards' ambient air (Huckins et al., 1999). The SPMD sampler absorbs only volatile PAHs and it simulates the process of bioconcentration in fatty tissues (Bonetta et al., 2009). The SPMD samplers were made from polyethylene tubing as in Huckins et al. (1999). Triolein (18.2 g) (99% glycerine trioleate, Acros Organics, Belgium), deuterated fluoranthene 800 μL (10 ng μL−1), and fluoranthene-D10 (10 ng μL−1, Dr. Ehrenstorfer, Augsburg, Germany) were mixed and the polyethylene tubing was filled with 520 μL of triolein–fluoranthene mixture. The tubing was heat-sealed, providing a 45 cm long sampler. The samplers were stored in airtight glass containers in a freezer (−20 °C) until use. The SPMD sampler was put into a 2.5 L zinc container which served as a shelter from hostile environmental conditions. Three sampling units per day-care center yard were deployed, and the samplers were placed approximately 2 to 2.5 m above the ground. Potential contamination of the samplers was monitored with field blanks. The sampling period was 28 days, and after that the samplers were frozen at −20 °C individually in glass bottles (50 mL).

For the PAH analysis, the SPMD samplers were wiped clean with ethanol and extracted at room temperature twice by dialysis (24 h) with 50 mL of n-hexane (Merck, Germany). The two extracts were combined, concentrated using a rotary evaporator (Heidolph Laboratory 400), and further reduced under a nitrogen stream to a final volume of approximately 0.5 mL. The aerial concentrations (ng m−3) of the PAHs were calculated using the uptake rates reported by Cranor et al. (2009).

4.3.2 SOIL SAMPLING AND PAH EXTRACTION (STUDIES I AND II)

In laboratory study I, five subsamples were randomly drawn from a depth of 1 to 6 cm from each landscaping material replicate pot, and these subsamples were combined for PAH and bacterial analysis. In day-care study II, surface soils were sampled from the day-care center yards since children are in daily contact with the soil surface. Each day-care center yard was sampled at five locations (in front of the main door, in a sand box, on the gravel playground, and below a swing and a jungle gym). Obtained PAH concentrations were compared with the Finnish soil quality guidelines (Reinikainen, 2007) and benzo[a]pyrene total potency equivalent (BaPtpe) was compared with the

incremental lifetime cancer risk for benzo(a)pyrene (600 ng g−1) as described in Hiller et al. (2015).

PAH extraction (studies I and II) was done as in Honkonen and Rantalainen (2013) by mixing 1 g soil (ww) and 15 mL hexane:acetone mixture (1:1 v/v, Merck Millipore, Darmstadt, Germany). The extraction was done twice with 30 min of sonication and 24 h (first extraction) and 2 h (second extraction) shaking at 200 rpm. Extracts were combined and concentrated under the nitrogen stream to a volume of 1 mL. The extract was filtered with a glass column filled with 1 g of silica gel (Sigma-Aldrich, grade 923, 100–200 mesh) and concentrated under the nitrogen stream to a final volume of approximately 0.5 mL. Soil concentrations are reported as ng g−1 dry weight.

4.4 BACTERIAL ANALYSES (ALL STUDIES)

Bacterial communities in all of the studies were analyzed using Illumina Miseq 16S ribosomal RNA (rRNA) gene metabarcoding with read length 2 × 300 bp using a v3 reagent kit.

Skin swab samples were collected with a cotton swab soaked in Tween® 20 from the back of the children's hands (2 × 2 cm area, 10 s of wiping). The children's parents/guardians collected stool and saliva samples at home. Soil, skin, saliva, and stool samples were stored at −70 °C until further processing.

Soil, saliva, and stool samples were extracted with a PowerSoil® DNA Isolation Kit (Qiagen, Hilden, Germany) and skin swabs with a FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's standard protocol. The V4 region within the 16S rRNA gene was amplified by polymerase chain reaction (three replicates from each sample) using 505F and 806R primers (Caporaso et al., 2012). Thus, the primer was identical for soil, saliva, stool, and skin samples.

Raw sequencing data were processed using Mothur (version 1.39.5, Schloss et al., 2009). The sequence processing protocol partly followed the pipeline suggested by Schloss et al. (2011) and Kozich et al. (2013). The paired sequences contained in reverse and forward FASTQ files were aligned into a contig. Sequences were trimmed and screened to remove those that had mismatches with primer or DNA tag sequences, ambiguous bases, or homopolymers larger than 8 bp long. Bacterial sequences were aligned against a SILVA reference (version 123, Pruesse et al., 2007), preclustered to minimize sequencing errors (Huse et al., 2010), and screened for chimeras with UCHIME (Edgar et al., 2011). The chimeric sequences were removed from the data and the non-chimeric sequences were classified using the Mothur version of the Bayesian classifier (Wang et al., 2007) with RDP training set version 16 (Cole et al., 2009) with 80% bootstrap threshold. Sequences classified to chloroplast, mitochondria, unknown, Archaea, and Eukaryota were removed from the analyses.

The pairwise distance matrix for unique sequences was calculated and operational taxonomic units (OTUs) clustered at 97% sequence similarity. All bacterial sequence data were accessioned into the Sequence Read Archive (Sequence Read Archive Accession for study I: SRX3195289-3195338; study II: SAMN11382605- SAMN11382770; study III and IV: PRJNA531814). Low abundance OTUs (≤ 10) and OTUs detected in negative controls were removed from the sequence data. Samples were subsampled to the smallest sample sequence depth for community composition analysis (Table 3). Good's coverage index was used to determine OTU coverage adequacy for bacterial diversity and community composition analysis.

Table 3 *Sequence depth (Seq. Depth.) and Good's coverage index (Mean ± standard deviation) after subsampling for bacterial 16S rRNA sequencing data.*

	Study I		**Study II**		**Study III**		**Study IV**	
	Seq. Depth.	Good's coverage	Seq. Depth.	Good's coverage	Seq. Depth.	Good's coverage	Seq. Depth.	Good's coverage
Soil	6374	0.93±0.04	6623	0.99±0.01	3842	0.96±0.02	11426	0.97±0.02
Skin			4072	1.00±0.00	329	0.96±0.06	735	0.82±0.14
Stool			450	0.93±0.02	3842	1.00±0.00	3156	0.99±0.00
Saliva							8568	1.00±0.00

4.5 ENDOCRINE SIGNALING AND IMMUNE MARKERS (STUDIES II AND III)

In study II, endocrine signaling pathways (PPAR, adipocytokine, and insulin) were predicted from the stool metagenome using the Kyoto Encyclopedia of Genes and Genomes (release 89.0) database. PICRUSt (Langille et al., 2013) was used to generate a profile of putative functions from the 16S rRNA OTU data classified against the Greengenes Database (Desantis et al., 2006) with 97% similarity.

In study III, IL-17A and IL-10 concentrations were analyzed from plasma serum using a Milliplex MAP High Sensitivity T Cell Panel kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Fluorescence was measured with a Bio-Plex® 200 system (Bio-Rad Laboratories, Hercules, CA, USA) and data collected with Bio-Plex Manager

software (version 4.1, Bio-Rad Laboratories, Hercules, CA, USA). Concentrations of TGF-β1 were measured from acidified samples using ELISA (Bender MedSystems, Vienna, Austria). Frozen peripheral blood mononuclear cells were thawed and immunostained to identify CD3+CD4+CD25+CD127lowFOXP3+ Treg cells as described in Viisanen et al. (2019). The proportion of Tregs was defined as a percentage of total CD3+CD4+ T cells.

4.6 STATISTICS

Multivariate analysis of variance (MANOVA) with Bonferroni correction in IBM SPSS Statistics (v24) was used to compare the half-life of PAHs (log transformed) in different landscaping materials (study I). The linear regression models were used to estimate the relationship between half-lives and properties of the landscaping materials, and between half-lives and bacterial relative abundances and diversities.

All the other statistical tests were done in the R computer environment (R Development Core Team, 2018) with vegan (Oksanen et al., 2019) and lme4 packages (Bates et al., 2015) (Table 4). Permutational analysis of variance (PERMANOVA: function adonis in vegan package) with the Bray–Curtis metric was used to compare bacterial community composition (Anderson, 2001). Non-metric multidimensional scaling with the Bray–Curtis metric (NMDS: function metaMDS in the vegan package) was used to visualize the difference in bacterial community composition. The correlations between bacterial community and PAH concentrations (study II) and cytokines (study III) were assessed with the envfit function, which fits environmental vectors or factors onto an NMDS ordination. The Shannon diversity index was used to assess the alpha diversity of bacteria, and multivariate homogeneity of group dispersions (PERMDISP) was used to examine beta diversity.

In study II, the association between bacterial factors and PAH concentrations was evaluated using generalized linear models with a quasi-Poisson distribution (logarithmic scale). In study III, the differences in the bacterial factors (relative abundance, diversity, and richness) between day-care children were determined using analysis of covariance (ANCOVA) or for non-normally distributed data, the Kruskal–Wallis test. Gender was used as a covariate. Pairwise comparison was done with Tukey's honestly significant difference test or with Dunn's test. In study III, the associations between bacterial diversity and abundance changes, and cytokine expression levels and changes, were evaluated using linear mixed-effects models (LMMs, function lmer in the lme4 package). In study IV, bacterial factors were used as the dependent variable, with the time point as a repeated measures factor (fixed factor) and individual child as a grouping factor, in the LMM models.

Table 4 *Summary of the statistical tests used in the thesis.*

	Analyzes
Study I	R v3.4.0: PERMANOVA, NMDS SPSS v24: MANOVA, Linear regression
Study II	R v3.5.1: NMDS, GLM
Study III	R v3.4.2: PERMANOVA, PERMDISP, NMDS, ANCOVA, Kruskal-Wallis test, HSD, Dunn's test, paired t-test, Wilcoxon-signed Rank test, LMM
Study IV	R v3.6.1: PERMANOVA, NMDS, LMM

In studies III and IV, antibiotics and probiotics users were removed from the gut microbiome and tests including immune markers (cytokines and Treg cells). To conceptualize the false discovery rate, all the statistical tests were carried out with the Benjamini–Hochberg correction (referred to as the Q value in the results), with the exception that the MANOVA in study I was done with the Bonferroni correction. All the statistical tests were considered significant at the $Q < 0.05$ level.

The results were analyzed with an intention-to-treat population in studies III and IV, i.e., all participants were included in the statistical analysis, and with a per protocol (complete cases) population, i.e., samples in all time points received and antibiotics and probiotics users were excluded from the gut bacterial data.

5 RESULTS AND DISCUSSION

5.1 EFFECT OF PAH POLLUTION ON THE ENVIRONMENTAL MICROBIOTA (STUDIES I AND II)

To examine associations between PAH pollution and soil bacterial communities in playgrounds, surface soil samples were collected from day-care center yards (study II). Temporal bacterial shifts in PAH-polluted urban landscaping materials were also studied at the laboratory scale (study I). In this laboratory experiment, the relative abundance of Deltaproteobacteria (paired t-test: $P < 0.02$) increased and genus *Rhodanobacter* (Gammaproteobacteria) (paired t-test: $P < 0.04$) decreased in all three studied landscaping materials. However, bacterial communities and shifts differed between landscaping materials (PERMANOVA: $P = 0.001$, Figure 3 in study I, Table 5).

The laboratory study also indicated that low molecular weight PAHs phenanthrene and fluoranthene might evaporate from coarse mineral soils with low OM content (1–2%); such coarse soil materials are often used in urban areas. In contrast, PAHs with higher molecular weight (> 202 g mol−1) were more persistent. However, biodegradation of pyrene (molecular weight 202.25 g mol−1) was inversely related to the relative abundance of Beta-, Delta-, and Gammaproteobacteria, and directly to the relative abundance of Actinobacteria and Alphaproteobacteria (linear regression: $P < 0.03$; Table 2 in study I). In addition, the diversity of Bacteroidetes and Betaproteobacteria was inversely related to the half-life of pyrene (linear regression: $P < 0.02$; Table 2 in study I). Consistent with prior studies (Mukherjee et al., 2014; Parajuli et al., 2017; Ren et al., 2015), beta-, delta-, and gammaproteobacterial communities seemed to be adjusted by PAH levels in the laboratory experiment. Since these proteobacterial communities may have a role in immune modulation (Hanski et al., 2012; Ruokolainen et al., 2015), I hypothesized that both biodiversity loss and pollutants alter microbial communities in the urban environment, which in turn can affect the commensal microbiota of humans and immune modulation.

Table 5 *PERMANOVA showed that bacterial communities differed between weeks 1 and 12, and between three studied landscaping materials. Statistics are reported as degrees of freedom (Df), F statistics, Sums of and mean squares, Coefficient of determination (R^2), probability P value and Benjamini-Hochberg adjusted Q value.*

A) PERMANOVA: OTU level	Df	F	Sums of squares	Mean squares	R^2	P value	Q value
Material	2	37	1.89	2.85	0.62	0.001	0.001
Week	1	7	0.57	0.57	0.06	0.001	0.001
Material:Week	2	7	1.02	0.51	0.11	0.001	0.001

Total PAH levels found in the surface soils of day-care centers were below safety and threshold levels. However, fluoranthene levels in the soil exceeded the allowed Finnish soil quality environmental threshold level of 1000 ng g−1 (Reinikainen, 2007) in two day-care centers (Table S3 in study II). Consistent with prior studies (Brorström-Lundén et al., 2010; Hiller et al., 2015; Musa Bandowe et al., 2011), the highest PAH levels were found for fluoranthene (110 ng g−1 ± 334) and pyrene (147 ng g−1 ± 547) (mean ± standard deviation). In addition, BaPtpe levels were over the allowed incremental cancer risk level of 600 ng g−1 in two samples taken from one day-care center yard, being 1352 and 674 ng g−1. Most of the bacteria that increased with higher total PAH and BaPtpe levels in day-care yard soil were unclassified genera within phyla Actinobacteria, Acidobacteria, and Proteobacteria (Table 6). Some associations between microbial communities and PAH levels were similar in the laboratory experiment and the day-care environment study. The relative abundance of an unclassified genus within Betaproteobacteria was associated with higher total PAH levels in both studies. In addition, genera *Nitrospira* and *Terrimonas* were associated with higher PAH levels in day-care yard soils (Table 6) and these two genera increased in contaminated landscaping materials depending on material type during the 12-week laboratory experiment (Table 3 in study I). The reasons why the results are mostly different in these two studies might be explained by environmental factors. Microbial communities are very sensitive to biotic and environmental factors, including soil quality, vegetation type, and climate (Francini et al., 2018; Setälä et al., 2016; Wang et al., 2016; Yu et al., 2014). Since PAH levels were relatively low in both studies, these biotic and environmental factors might play a greater role in shaping microbial communities.

Table 6 *Associations between total PAH and BaPtpe levels, and bacterial genera were analyzed with generalized linear models. GLM statistics are reported as T value, probability P value and Benjamini-Hochberg false discovery rate adjusted Q value.*

Genus	Phyla	Total PAH level			BaPtpe level		
		T	P	Q	T	P	Q
Gp6 unclassified	Acidobacteria	6.08	0.000	0.000	7.51	0.000	0.000
Gaiella	Actinobacteria	6.29	0.000	0.000	7.80	0.000	0.000
Terrimonas	Bacteroidetes	4.76	0.000	0.001	5.77	0.000	0.000
Gp4 unclassified	Acidobacteria	4.88	0.000	0.001	5.76	0.000	0.000
Gp16 unclassified	Acidobacteria	4.68	0.000	0.001	5.33	0.000	0.000
Candidatus Solibacter unclassified	Acidobacteria	4.89	0.000	0.001	5.63	0.000	0.000
Betaproteobacteria unclassified	Proteobacteria	4.51	0.000	0.001	5.67	0.000	0.000
Spartobacteria unclassified	Verruco-microbia	4.15	0.000	0.002	4.91	0.000	0.000
Solirubrobacter	Actinobacteria	4.33	0.000	0.002	5.07	0.000	0.000
Rhizobiales unclassified	Alpha-proteobacteria	4.21	0.000	0.002	4.76	0.000	0.000
Pseudonocardia	Actinobacteria	3.96	0.000	0.004	4.92	0.000	0.000
Nitrospira	Nitrospirae	4.00	0.000	0.004	5.02	0.000	0.000
Actinobacteria unclassified	Actinobacteria	3.93	0.000	0.004	5.08	0.000	0.000
Pirellula	Plancto-mycetes	3.69	0.001	0.008	4.33	0.000	0.001
Polyangiaceae unclassified	Delta-proteobacteria	3.48	0.001	0.013	3.96	0.000	0.003
Subdivision3 unclassified	Verruco-microbia	3.35	0.002	0.018	3.87	0.000	0.003
Rhodocyclaceae unclassified	Beta-proteobacteria	3.24	0.002	0.024	3.99	0.000	0.003
Armatimonadetes gp5 unclassified	Armati-monadetes	3.20	0.002	0.025	3.68	0.001	0.006
Terriglobus unclassified	Acidobacteria	2.86	0.006	0.063	0.19	0.851	0.962
Povalibacter	Gamma-proteobacteria	2.74	0.008	0.081	3.40	0.001	0.013
Mycobacterium	Actinobacteria	2.71	0.009	0.084	2.59	0.013	0.106
Pseudonocardiaceae unclassified	Actinobacteria	2.57	0.013	0.110	3.00	0.004	0.039
Planctomycetaceae unclassified	Plancto-mycetes	2.58	0.013	0.110	3.24	0.002	0.020

5.2 EFFECT OF PAH POLLUTION ON THE COMMENSAL MICROBIOTA OF CHILDREN AND ENDOCRINE SIGNALING PATHWAYS (STUDY II)

Community composition of bacteria, particularly the Actinobacteria and Bacteroidetes communities, on the skin of day-care children was associated with PAH levels in the soil and ambient air (PERMANOVA: $P < 0.01$; Table 7). The community composition of these same phyla and also Betaproteobacteria in soil was associated with higher PAH levels in the soil (Table 7C). Within phylum Actinobacteria, the relative abundance of *Mycobacterium* and two unclassified genera increased with increasing PAH levels in the soil (Table 7D). Within Bacteroidetes, the relative abundance of *Mucilaginibacter* and *Ferruginibacter* (Table 7E), and the diversity and richness of class Sphingobacteriia, increased with increasing total PAH concentrations in surface soils (Table 7E). Within phylum Proteobacteria, mostly the relative abundance of unclassified alpha- and betaproteobacterial genera increased with increasing PAH levels (Table 7F).

Many factors can affect the microbial communities on children's skin, including the living environment, living habits, and physiological and behavioral changes related to age (Lehtimäki et al., 2017). Thus, associations found in this study should be confirmed with a higher number of study subjects and the exposure to PAHs should be measured individually for each subject. This could be done with portable personal exposure monitors and measurement of the PAH–DNA adducts from the blood samples (Iyer et al., 2016). PAH–DNA adducts are biomarkers that link environmental PAH exposure to health outcomes, and they can be measured from genes related to PAH metabolism and detoxification (Iyer et al., 2016).

Table 7 *NMDS with envfit-vector showed associations between skin bacterial communities and total PAH levels in **A)** soil and **B)** ambient air, and between **C)** soil bacterial communities and PAH levels. GLM showed associations between total PAH levels in soil and **D)** Actinobacterial, **E)** Bacteroidetes and **F)** Proteobacterial genera. Statistics are reported as Coefficient of Determination (R2), T value, probability P value and Benjamini-Hochberg false discovery rate adjusted Q value.*

A) NMDS: Skin bacteria vs. Soil PAH	**R^2**	**P value**	**Q value**
Actinobacteria	0.215	0.002	**0.006**
Bacteroidetes	0.269	0.001	**0.003**
B) NMDS: Skin bacteria vs. Air PAH	**R^2**	**P value**	**Q value**
Actinobacteria	0.2135	0.002	**0.006**
Bacteroidetes	0.2383	0.004	**0.005**
C) NMDS: Soil bacteria vs. Soil PAH	**R^2**	**P value**	**Q value**
Actinobacteria	0.132	0.028	**0.035**
Bacteroidetes	0.140	0.030	**0.037**
Betaproteobacteria	0.135	0.026	**0.032**
D) GLM :Skin Actinobacteria vs. Soil PAH	**T value**	**P value**	**Q value**
Mycobacterium	3.980	0.000	**0.007**
Unclassified genus	3.193	0.002	**0.031**
Nocadioidaceae unclassified genus	3.499	0.001	**0.017**
E) GLM :Skin Bacteroidetes vs. Soil PAH	**T value**	**P value**	**Q value**
Mucilaginibacter	4.678	0.000	**0.003**
Ferruginibacter	3.185	0.002	**0.031**
Sphingobacteriia diversity	4.228	0.000	**0.001**
Sphingobacteriia richness	5.120	0.000	**0.000**
F) GLM :Skin Proteobacteria vs. Soil PAH	**T value**	**P value**	**Q value**
Unclassified genus	4.301	0.000	**0.004**
Betaproteobacteria unclassified genus	4.290	0.000	**0.004**
Rhizomicrobium unclassified genus (Alpha)	4.350	0.000	**0.004**
Rhizobiales unclassified genus (Alpha)	3.614	0.001	**0.016**
Caulobacteraceae unclassified (Alpha)	3.423	0.001	**0.019**
***Phenylobacterium* (Alpha)**	2.946	0.005	0.051

Associations between gut bacteria and PAH levels were not found. However, PPAR and adipocytokine signaling pathways predicted from the gut bacterial metagenome decreased with increasing chrysene levels in the ambient air ($P < 0.05$, Table 8). The decrease in these pathways can affect hormonally-mediated processes including metabolism, childhood obesity, puberty, and neurodevelopment (Braun, 2017).

Table 8 *GLM model showed association between endocrine signaling pathways and chrysene level in the ambient air. GLM statistics are reported as estimate of the true population regression, standard error, T value, probability P value and Benjamini-Hochberg false discovery rate adjusted Q value.*

Signaling pathway	Estimate	Standard error	T value	P value	Q value
PPAR	-4.09	1.57	-2.61	**0.014**	**0.042**
Adipocytokine	-3.40	1.50	-2.27	**0.031**	**0.046**

These results indicate that PAH levels that are below the risk assessment safety limits may alter the commensal microbiota of children and interfere with endocrine signaling pathways. Since the imbalance in human microbiota and the decrease in endocrine signaling pathways might contribute to inflammatory disorders (Flandroy et al., 2018; Nepelska et al., 2017), the optimal risk assessments should take into account the possibility of the disruption of endocrine signaling pathways and the microbiota–health nexus.

5.3 EFFECT OF BIODIVERSITY INTERVENTION ON SOIL MICROBIOTA (STUDIES III AND IV)

Biodiversity intervention shifted and diversified day-care yards' soil bacterial and particularly gammaproteobacterial communities (Table 9, Table S3C, and Figure 1A in study III). Indeed, community composition of Gammaproteobacteria differed between the intervention and standard day-care yards on day 28 (Table S2A and Figure S1B in study III). After 1 year, bacterial communities, including Gammaproteobacteria, still differed between intervention and standard day-cares (Table 9). The relative abundance of bacteria typically found in Sphagnum peat bogs in Finnish forests (Pankratov et al., 2007; Pankratov and Dedysh, 2010; Sun et al., 2014) increased in intervention day-care yards. In particular, the increase in Mycobacterium abundance remained for 2 years (Table 10).

Table 9 ***A)*** *PERMANOVA showed that biodiversity intervention significantly shifted the community composition of bacteria at day-care yards.* ***B)*** *After one year, bacterial communities still differed between intervention and standard day-care yards. PERMANOVA statistics are reported as F statistics, R^2 = Coefficient of determination, probability P value and Benjamini-Hochberg false discovery rate adjusted Q value.*

	A) Intervention day-0 vs. day-28				**B) Intervention vs Standard**			
Level	**F**	**R^2**	**P**	**Q**	**F**	**R^2**	**P**	**Q**
Otu	15	0.26	**0.001**	**0.001**	4	0.07	**0.001**	**0.003**
Phylum	9	0.17	**0.001**	**0.001**	2	0.04	**0.042**	**0.050**
Phyla	**F**	**R^2**	**P**	**Q**	**F**	**R^2**	**P**	**Q**
Proteobacteria	19	0.30	**0.001**	**0.001**	4	0.07	**0.003**	**0.005**
Bacteroidetes	19	0.30	**0.001**	**0.001**	4	0.08	**0.001**	**0.003**
Actinobacteria	13	0.23	**0.001**	**0.001**	4	0.08	**0.001**	**0.003**
Acidobacteria	14	0.24	**0.001**	**0.001**	3	0.05	**0.002**	**0.004**
Class	**F**	**R^2**	**P**	**Q**	**F**	**R^2**	**P**	**Q**
Alpha-proteobacteria	22	0.33	**0.001**	**0.001**	4	0.07	**0.001**	**0.003**
Actinobacteria	13	0.23	**0.001**	**0.001**	4	0.08	**0.001**	**0.003**
Sphingo-bacteriia	23	0.34	**0.001**	**0.001**	4	0.08	**0.002**	**0.004**
Beta-proteobacteria	22	0.33	**0.001**	**0.001**	4	0.08	**0.002**	**0.004**
Gamma-proteobacteria	13	0.23	**0.001**	**0.001**	3	0.06	**0.002**	**0.004**
Cytophagia	10	0.19	**0.001**	**0.001**	3	0.05	**0.003**	**0.005**
Flavobacteriia	5	0.09	**0.001**	**0.001**	2	0.04	**0.021**	**0.028**
Acidobacteria Gp1	9	0.16	**0.001**	**0.001**	2	0.05	**0.001**	**0.003**

Table 10 *Major relative abundance shifts were observed within bacterial genera found from forests of Finland. Results from long-term Study IV. Relative abundances are presented as mean ± standard deviation (Sd) at day-0, day-28, year-one and year-two. LMM results are reported as probability P value and Benjamini-Hochberg false discovery rate adjusted Q value between time-points.*

	Day-0	Day-28	1-year	2-year	Day-0 vs. Day-28	Day-0 vs. year-one	Day-0 vs. year-two
Granulicella unclassified	17 ± 24	162 ± 171	89 ± 121	37 ± 64	**P < 0.001** **Q< 0.001**	**P= 0.001** **Q=0.007**	P=0.560 Q=0.728
Mucilaginibacter	59 ± 61	643 ± 397	213 ± 270	120 ± 136	**P < 0.001** **Q< 0.001**	P=0.065 Q=0.281	P=0.432 Q=0.603
Mycobacterium	30 ± 18	77 ± 80	75 ± 52	74 ± 53	**P = 0.016** **Q= 0.035**	**P=0.011** Q=0.101	**P=0.004** **Q=0.021**

Interestingly, *Mycobacterium* on skin and in soil was associated with PAH pollution (Section 5.2), and the forest floor vegetation that was transferred to day-care center yards in the biodiversity intervention study also included a great proportion of *Mycobacterium*. *Mycobacterium* species have been of great interest in two disciplines. Environmental studies has been interested in *Mycobacterium* species because of their PAH biodegradation ability (Kanaly and Harayama, 2000; Kweon et al., 2011; Zeng et al., 2017) and health studies because of their ability to cause infections in humans, including *Mycobacterium tuberculosis* (Bottai et al., 2014; Cook, 2010; King et al., 2017). *Mycobacterium* species are often found in boreal forests in Finland, and these environmental *Mycobacterium* species are mostly harmless saprophytes (Iivanainen et al., 1997; Niva et al., 2006; Sun et al., 2014). In other words, environmental *Mycobacterium* and *Mycobacterium* causing infections in humans are different species. Indeed, environmental saprophytes, non-pathogenic *Mycobacterium* species, are our "old friends," i.e., we have been exposed to them throughout evolution, and these species have been shown to have anti-inflammatory properties through PPAR signaling (Rook et al., 2004; Smith, 2017). In the biodiversity intervention study, the relative abundance of skin *Mycobacterium* was relatively low, and that is why the associations between skin *Mycobacterium* and immune markers were not searched for. However, a high proportion of anti-inflammatory *Mycobacterium* were still found from the day-care yards after 2 years of the intervention.

5.4 EFFECT OF BIODIVERSITY ON THE COMMENSAL MICROBIOTA OF CHILDREN (STUDIES III AND IV)

5.4.1 SKIN MICROBIOTA

Biodiversity intervention with microbiologically-rich materials diversified the skin proteobacterial community. Before the intervention, children in the three day-care groups hosted equally diverse skin proteobacterial communities, including classes Alpha-, Beta-, and Gammaproteobacteria (Figure 1 in study III). After the 28-day intervention, the diversity of skin proteobacterial communities was significantly higher among intervention day-care children than among children in standard day cares and comparable to those among nature-oriented day-care children (Figure 1 in study III). However, total bacterial diversity decreased both among children in the intervention and standard day cares during the first year, indicating a normal temporal shift that was not related to the intervention. This decrease in bacterial diversity may be related to weather conditions that differed between years. The intervention period in year 2016 was sunny with daily mean temperatures of 10 to 21 °C, whereas in 2017 this same period was rainy with mean temperatures of 2 to 18 °C (Meteorological Institute, 2020). Thus, partly during the intervention period in 2016 children wore T-shirts when playing outside, and in 2017 children wore jackets or shirts with long sleeves and even gloves on some days. In any case, biodiversity intervention may have prevented the loss of bacterial diversity, particularly within class Gammaproteobacteria, since gammaproteobacterial diversity decreased among children in the standard, but not among children in the intervention, day cares during the 1-year study period (Table S17B in study IV). This is especially important in the context of children's health since gammaproteobacterial diversity on skin is associated with a decreased risk of atopy and allergies (Fyhrquist et al., 2014; Hanski et al., 2012). Therefore, biodiversity intervention may have reduced the risk of these disorders among children in intervention day cares in the long term.

5.4.2 SALIVA MICROBIOTA

The community composition of saliva bacteria differed between children in the intervention and standard day cares after the 1-year study period (Table 4A in study IV). Interestingly, long-term differences were mostly observed within the same classes in saliva and on skin: Gammaproteobacteria, Negativicutes, and Bacilli (Table 4 in study IV). In saliva samples, the relative abundance of genus *Streptococcus* (Bacilli) was lower among children in intervention day cares compared with children in standard day cares on day 28 (LMM: $P < 0.001$, $Q = 0.006$). This genus is a part of the normal saliva microbiota (Huttenhower et al., 2012) but includes also streptococci groups that may cause infections and immune disorders (Waldetoft et al., 2016). However,

during the long-term period the relative abundance of *Streptococcus* increased among intervention children in saliva and on skin (LMM: P < 0.001, Q < 0.003). In addition, the relative abundance of *Streptococcus* increased on skin among children in the standard day cares during the 1-year study period (P < 0.001, Q < 0.003). Therefore, shifts in genus *Streptococcus* are more likely related to some other factor than biodiversity intervention, such as age, diet, or health status (Flandroy et al., 2018). Gender was also associated with the saliva bacterial community in the long-term study, which might indicate some gender-related behavioral habits having a role in shaping commensal bacterial communities. For example, girls and boys may choose areas to play differently (Lucas and Dyment, 2010).

These similar results in saliva and on skin indicate that exposure to biodiversity shapes bacterial communities on skin and in saliva in a similar way. This result is in-line with a human microbiome project in which the skin and saliva bacterial communities were observed to more resemble each other compared with gut bacterial communities (Huttenhower et al., 2012). Furthermore, since saliva bacteria are responsible for priming the gut bacterial communities, which in turn affect gut health (Davé et al., 2016), these shifts may influence children's health through modulation of interrelated physiological pathways.

5.4.3 GUT MICROBIOTA

Intervention was associated with changes in the community composition of order Clostridiales in the gut (studies III and IV). Interestingly, shifts in gut microbiota were observed in health-promoting Clostridium cluster IV, including *Faecalibacterium*, *Blautia*, *Dorea*, and Lachnospiraceae, which could protect against colorectal cancer (Candela et al., 2014). Within Clostridiales, the diversity of family Ruminococcaceae increased in the gut of the intervention children during the 28-day intervention period (paired t-test: P = 0.01, Q = 0.05, Table S6A in study III) and Lachnospiraceae during the 1-year period (LMM: P = 0.007, Q = 0.02, Table S9A in study IV). One-year shifts within Clostridiales included a decrease in the relative abundance of *Clostridium sensu stricto*, which might be beneficial to intervention children's gut health since the increased abundance of *Clostridium sensu stricto* is associated with food allergies (Chen et al., 2016; Ling et al., 2014).

Gut bacterial communities also differed between standard and nature-oriented day-care children; in particular the community composition Ruminococcaceae including *Faecalibacterium* differed between nature-oriented and standard day-care children after the 28 days of intervention (PERMANOVA: P = 0.03; Table S2E and F in study III). Interestingly, a similar association was observed with elderly people in a previous study; yard vegetation affected the relative abundances of Ruminococcaceae (including *Faecalibacterium*) (Parajuli et al., 2020). These observations may indicate a

potential relationship between environmental exposure to diverse natural microbiota and Ruminococcaceae community composition within gut microbiota. Many studies have shown that family Ruminococcaceae is associated with health outcomes (Chumpitazi et al., 2016; Kirjavainen et al., 2019; Stokholm et al., 2018; Tyler et al., 2016; West et al., 2015b). A low relative abundance of Ruminococcaceae may increase the risk of developing IgE-associated eczema (West et al., 2015b), asthma (Stokholm et al., 2018), and Crohn's disease (Tyler et al., 2016). Despite the diet ameliorating the symptoms in children with irritable bowel syndrome, children who robustly respond to the low fermentable diet change have a different baseline microbiome composition in the gut (Chumpitazi et al., 2016). Chumpitazi et al. (2016) found out that children who responded to the diet change with less abdominal pain were enriched at baseline in a microbiota with greater saccharolytic capacity including, for example, Ruminococcaceae, Lachnospiraceae, *Dorea*, and *Faecalibacterium prausnitzii*. Other health benefits of Ruminococcaceae may include the ability to produce a bacteriocin, called ruminococcin A, which is able to inhibit various pathogenic Clostridia species (Dabard et al., 2001). Thus, this intervention study indicates that exposure to biodiversity may have beneficial health consequences in the gut. However, the results must be taken with caution since they are based on a limited number of study subjects whose diet, living habits, and health status may vary. In addition, previous studies comparing the gut bacterial microbiome in healthy and sick individuals (e.g., Stokholm et al., 2018; Tyler et al., 2016; West et al., 2015b) cannot be used to distinguish whether the altered gut bacterial microbiome is the cause or consequence of a disease.

5.5 ASSOCIATIONS BETWEEN BIODIVERSITY, THE COMMENSAL MICROBIOTA, AND IMMUNE RESPONSE (STUDY III)

An increase in skin gammaproteobacterial diversity was associated with an increase in the TGF-β1 plasma concentration ($Q = 0.01$; Figure 2A, Table S7A in study III), and among intervention children this diversity increase was also associated with a decrease in the IL-17A plasma level ($Q = 0.002$; Figure 2B, Table S7B in study III) and an increase in the total Treg cell proportion ($P = 0.016$, Figure 2C, Table S7C in study III). Among nature-oriented day-care children, high skin gammaproteobacterial diversity was associated with an increase in IL-10 plasma concentration ($Q < 0.001$; Table S7D in study III), whereas among standard day-care children, the decrease in gammaproteobacterial diversity was associated with a decrease in TGF-β1 expression ($Q = 0.01$; Table S7E in study III). These results support the hypothesis that skin gammaproteobacterial diversity plays an important role in the regulation of anti-inflammatory responses (Fyhrquist et al., 2014; Hanski et al., 2012). One reason why Gammaproteobacteria may have a

significant role in regulating the immune system is that they accumulate in the deeper layers of the skin dermis, while a large quantity of the skin's other bacteria reside in the innermost compartments of the epidermis (Fyhrquist et al., 2014; Nakatsuji et al., 2013).

TGF-β expression and the IL-10:IL-17A ratio increased among intervention children during the intervention (Table 11). However, compared with children in standard day-care centers, the TGF-β level was lower among intervention children at the beginning of the intervention (ANCOVA: P = 0.04). Among children in standard and nature day-care centers, the cytokine levels remained similar during the intervention (Table 11). TGF-β is a cytokine known to have an essential role in regulating immune responses and its activation is fundamental for adaptive immunity (Travis and Sheppard, 2014). TGF-β can either suppress or promote the adaptive immune responses depending on the type of induction cells (Travis and Sheppard, 2014). The role of TGF-β in developing autoimmune diseases has been described in mice models; disturbances in TGF-β expression or signaling processes induces rapid development of type 1 diabetes (Ishigame et al., 2013) and may lead to lethal inflammatory disorder (Gu et al., 2011; Kulkarni et al., 1993). Consistent with our findings, a comparative study between farm and non-farm children has shown that farm children who live in a microbial-rich environment have higher expression of IL-10 and TGF-β (Frei et al., 2014). IL-10 is an anti-inflammatory cytokine that has been proven to strongly prevent certain types of autoimmune diseases (Scapini et al., 2011), while IL-17A is a pro-inflammatory cytokine that contributes to autoimmune diseases (Huber et al., 2013; Kim et al., 2011; Roos et al., 2015). These diseases include inflammatory bowel disease (Glocker et al., 2011; Kim et al., 2011), multiple sclerosis (Huber et al., 2013; Ireland et al., 2015), and chronic obstructive pulmonary disease (Castellucci et al., 2015; Roos et al., 2015). Disturbances in IL-10 function may cause severe intractable enterocolitis in infants and small children (Glocker et al., 2011). IL-17 has been shown to be increased in the blood of patients with severe asthma and to correlate with asthma severity (Agache et al., 2010). As our results are identical or at least similar to the differences observed in IL-10, TGF-β, and IL-17A in the previous studies comparing healthy subjects and subjects with immune-mediated diseases (Castellucci et al., 2015; Glocker et al., 2011; Huber et al., 2013; Ireland et al., 2015; Roos et al., 2015), the findings of our biodiversity intervention are beneficial to health.

Table 11 *Before-after comparison showed increase in TGF-β, IL-10 to IL-17A and TGF-β to IL-17A ratios among intervention children. Data is presented as mean ± standard deviation (Sd) and statistics are reported as probability P value and Benjamini-Hochberg false discovery rate adjusted Q value.*

	Before		After		Shapiro test	Paired T-test		Wilcoxon signed-rank test	
Intervention	**Mean**	**Sd**	**Mean**	**Sd**	**P value**	**P value**	**Q value**	**P value**	**Q value**
IL-10	3.6	2.8	4.1	3.0	0.211	0.280	0.841	0.237	0.712
IL-17A	4.1	1.5	3.9	1.3	0.347	0.303	0.909	0.366	1.000
TGFb	7.2	2.6	10.5	5.5	0.068	**0.001**	**0.003**	0.003	0.009
IL10:IL17A	0.9	0.5	1.2	1.0	**0.000**	0.068	0.203	**0.022**	**0.044**
TGFb:IL17A	2.0	1.1	3.1	2.1	**0.031**	0.011	0.034	**0.020**	**0.040**
Standard	**Mean**	**Sd**	**Mean**	**Sd**	**P value**	**P value**	**Q value**	**P value**	**Q value**
IL-10	4.0	2.2	4.7	2.0	0.809	0.451	1.000	0.407	1.000
IL-17A	4.2	0.8	4.8	1.7	0.907	0.370	1.000	0.477	1.000
TGFb	11.3	4.3	9.8	4.1	0.072	0.373	1.000	0.193	0.578
IL10:IL17A	0.9	0.5	1.0	0.4	0.288	0.500	1.000	0.813	1.000
TGFb:IL17A	2.9	1.7	2.3	1.3	0.536	0.110	0.331	0.155	0.465
Nature-oriented	**Mean**	**Sd**	**Mean**	**Sd**	**P value**	**P value**	**Q value**	**P value**	**Q value**
IL-10	5.5	5.8	4.4	4.6	0.069	0.330	0.991	0.636	1.000
IL-17A	4.0	1.6	4.4	1.8	0.061	0.475	1.000	0.236	0.708
TGFb	7.7	3.6	9.9	5.5	0.411	0.101	0.304	0.124	0.371
IL10:IL17A	1.2	0.9	1.0	0.6	0.620	0.168	0.505	0.286	0.859
TGFb:IL17A	2.1	1.2	2.5	1.5	0.329	0.421	1.000	0.636	1.000

Faecalibacterium species are often associated with beneficial health outcomes, and their decreased abundance is associated with several immune system disorders (Brown et al., 2011; Stokholm et al., 2018; Tyler et al., 2016). Consistent with these prior studies, we found that a higher relative abundance of *F. prausnitzii* in the gut decreases the pro-inflammatory IL-17A expression level, while a higher abundance of other unclassified *Faecalibacterium* species was associated with an increase in IL-17A (Table S7F and G in study III). I observed the former association within nature-oriented, and the latter within standard and nature-oriented, day-care children. Indeed, the community composition of *Faecalibacterium* differed between these two day-care children groups (Table S2F in study III). Interestingly, unclassified *Faecalibacterium* OTU was associated with an increase in IL-17A within both nature-oriented and urban day-care children. At present, *F. prausnitzii* is the only identified species in the genus *Faecalibacterium*. However, it is proposed that this genus should be divided into different phylogroups (Benevides et al., 2017). My findings support this proposal of dividing genus *Faecalibacterium* into different phylogroups to enable more comprehensive definitions for interactions between *Faecalibacterium* phylogroups, host, and immune regulation.

The biodiversity intervention study demonstrates a novel method to affect the commensal microbiota of humans. Bringing together findings of the previous studies (Brown et al., 2011; Fyhrquist et al., 2014; Hanski et al., 2012; Kirjavainen et al., 2019; Rook et al., 2004; Stokholm et al., 2018) and the findings of our intervention study, biodiversity intervention is beneficial to children's health. In addition, we have previously studied how these biodiverse day-care yards affect children's well-being, play, and relationship with the environment (Puhakka et al., 2019). We received overwhelmingly positive responses to the novel biodiverse yards from children and their parents/guardians, and from day-care and city personnel. Biodiverse day-care yards encouraged children's play, increased their physical activity, and provided multisensory exploration and diverse learning situations (Puhakka et al., 2019). Furthermore, based on preliminary work done in our research group (Grönroos et al., 2019; Hui et al., 2019; Nurminen et al., 2018), it is possible to design natural materials that are safe to implement in urban green spaces and which include non-pathogenic microbial species. A tempting option is that these materials will be manufactured following bio- and circular economy guidelines. The mechanism of immune regulation was fundamentally different in the biodiversity intervention compared with probiotic treatments that often comprise certain cultured bacterial strains only. Natural biodiversity can expose children to all kinds of environmental microbes via multiple routes, including skin and mucosal tissues. In addition to bacteria, these environmental microbial species include viruses, fungi, protozoa, algae, and nematodes. While probiotics include fast growing bacterial strains, nature, like forests, includes at least hundreds of slow-growing bacterial strains that cannot be cultivated in laboratory conditions. Thus, this kind of biodiversity intervention could activate a much wider spectrum of PRRs that play a fundamental role in the functioning of the immune system via sensing the presence of microorganisms (Takeuchi and Akira, 2010). All of the results mentioned in this paragraph encourage the implementation of biodiverse nature-based solutions in the management and planning of urban environments such as playgrounds and day-care centers or schoolyards. This approach could improve health by activating the regulatory pathways of the immune system.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis is one of the first attempts to estimate the connections between biodiversity, pollutants, the commensal microbiota, and the immune response of urban children. I have demonstrated that exposure to PAH pollutants might have negative health consequences via the microbiota–health nexus, while exposure to a natural diverse microbiota may balance the commensal microbiota of children and promote immune regulation.

Potential bacterial alterations induced by PAH pollution in the soil and on the skin of day-care children include changes in bacterial taxonomies associated with human health and immune-mediated diseases. Indeed, relatively low PAH contamination may alter environmental and children's skin bacterial communities. My results indicate that PAH contamination may also result in decreased bacterial diversity in commonly used urban landscaping materials. However, a decrease in diversity and bacterial changes depended on soil material type. This indicates that it is possible to design gardening and landscaping materials that are more resilient to PAH pollution in the context of human health-associated bacteria. In addition, since a commensal microbiota on the skin is able to affect PAH metabolism and toxification (Sowada et al., 2017), future research should be targeted at what kind of microbiota in the human body can reduce the negative health outcomes induced by PAHs and, most importantly, how we can modify the commensal microbiota of humans. This study has demonstrated that biodiversity intervention is one effective approach to modify the commensal microbiota of humans. However, future studies should be targeted at how to also integrate biodiverse microbiota in people's daily lives with other methods, such as cosmetics.

Based on current risk assessments, measured PAH levels in surface soils of day-care yards do not cause potential health risks. Despite this, I found an association between PAH pollution and endocrine signaling pathways via gut microbiota. Thus, the current risk assessments may underestimate the hazard of PAHs. In addition, I found associations between the measured PAH levels in day-care yards and the commensal microbiota on human skin. Air quality and health effects are often assessed from particulate matter data and, when evaluating PAH hazards, benzo[a]pyrene is used as a model marker for the carcinogenic risk of PAHs (Guerreiro et al., 2016). However, our study indicates that gaseous PAHs, particularly chrysene, may have an effect on endocrine signaling pathways that regulate health. This and the microbiota–health nexus should be taken into account in the risk assessments.

Biodiversity intervention studies showed several essential shifts in commensal bacteria that are associated with immune regulation and health. These bacteria included Ruminococcaceae (including *Faecalibacterium*) in

the gut, Gammaproteobacteria on the skin, and environmental *Mycobacterium* in day-care yard soils. Yet the specific shifts, especially in the gut, are almost unfeasible to predict globally, since the commensal microbial community is a complex system that varies between and within individuals, over time, and across populations (Lozupone et al., 2012). Future studies should investigate in more detail the interactions between environmental exposure, bacterial shifts, the human host, and the immune system.

The results of this thesis support the biodiversity and altered environmental hypotheses; according to my findings, both biodiversity loss and pollution in the urban environment may lead to an altered environmental microbiome. This in turn can lead to an uneducated immune system and consequently increase the prevalence of emerging public health problems, including allergies, type I diabetes, asthma, and inflammatory bowel disease. Importantly, this study demonstrates that modifying children's living environment with microbiologically diverse natural materials might provide a feasible approach for decreasing the risk of immune-mediated diseases in urban populations.

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8 KIITOKSET

Tämä väitöskirja on omistettu tyttärilleni, Kertulle ja Vilmalle, jotka ovat olleet kilttejä, auttavaisia ja rakastavaisia, vaikka äiti onkin kirjoittanut tietokoneella tätä väitöskirjaa, kun tärkeämpää, -tämänkin tutkimuksenkin mukaan- olisi ollut leikkiä metsässä.

Suurimmat kiitokset menevät lapsille sekä heidän vanhemmilleen, päiväkotien ja kaupunkien henkilökunnalle, jotka tekivät tästä tutkimuksesta mahdollisen.

Kiitokset myös äidilleni ja miehelleni sekä muille sukulaisille ja ystäville, jotka ovat kannustaneet minua tämän väitöskirjan toteuttamisessa.

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