The biodiversity hypothesis of allergy:
The interrelations between the skin microbiota, allergic diseases and exposure to microbes in residential environments

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To all children, parents, dogs, dog owners, and daycare-workers who made this possible.

Contents

SUPPLEMENTARY MATERIAL AVAILABLE ON REQUEST.

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II **Jenni Lehtimäki**, Tiina Laatikainen, Kimmo Saarinen, Juha Jantunen, Antti Karkman, Leena von Hertzen, Tari Haahtela, Ilkka Hanski & Lasse Ruokolainen. Daycare environments and practices in transforming a child skin microbiota and health. Manuscript.

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	I	II	III	IV
Original idea	IH	IH, LR, KS, JJ	**JL**, IH, HL, AHB, LR, ES	**JL**
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ABSTRACT

Biodiversity on earth is threatened and already drastically decreased due to anthropogenic actions. The ravenous utilization of natural resources has reached the point of payback in the forms of climate change, diseased crops, and disturbed water cycles. Obviously, these changes influence wellbeing of mankind. Besides these measurable problems, an invisible world, which covers all surfaces on earth, is altering. Loss of diversity in macroscopic organisms is repeated at the level of micro-organisms, many of which may have disappeared before ever described.

The human body is a lively ecosystem hosting millions of microbial organisms, which together form the microbiota. These bacterial, viral, fungal, and other microscopic residents are faced with our immune system, challenging their survival. However, the relationship between the host and the residents is often not hostile, but in most cases reciprocal. Actually, the human immune system has partly shared the responsibility of immune-regulation with commensals. This evolved dependency between human and microbial residents highlights that several health problems may arise if this ancient collaboration is disturbed.

Indeed, numerous inflammatory diseases coincide with disturbed host microbiota. These diseases, such as allergies, asthma, inflammatory bowel disease, and cancers, have increased rapidly since recent modernization of human habitats and lifestyle. Still, in traditional farming and hunter-gatherer communities allergies are almost absent. *The biodiversity hypothesis*, which is the concept tested in this thesis, suggest that change in the invisible world can seriously increase morbidity in human populations. This hypothesis states that the destruction of natural environments has altered our contact with microbial world, which can disturb our immune function, potentially leading to the development of inflammatory diseases.

In this thesis, the emphasis is on the effect of exposure to environmental microbes, via the living environment and lifestyle, on health; central factors suggested by the biodiversity hypothesis. The key results from four separate projects, which are based on new datasets, are following. (**I**) Skin microbiota differs between rural and urban newborns and children. In teenagers this difference disappears, probably due to lifestyle-related changes. (**II**) Children who attend to nature-oriented outdoor-daycares have considerably more diverse skin microbiota than children in other daycares. However, their life differs in many ways from that of other children, indicating also the importance of lifestyle. (**III**) In the canine model, the prevalence of allergies is clearly lower in rural environments, also when the effect of dog-breed is controlled. Finally, (**IV**) the exposure to environmental microbes in residential environment, and through lifestyle, are concurrently related to the skin microbiota and allergies in the canine model.

This thesis suggests the importance of living environment and lifestyle, which jointly influence the individual's contact with environmental microbes, for health. Therefore, the human living environments, and the residing biodiversity in those, can either promote or disrupt human health. Currently, accumulating evidence projects that exposure to green environments, farms, children and animals, basically to all factors that increase microbial exposure directly or indirectly, are beneficial for human health. My thesis adds to this by showing interrelations between microbial exposure, microbiota and allergies. Therefore, people, especially children, and their fellow-animals, should increase their contact with diverse environments and lifeforms in order to support both microbial and immunological balance in their bodies. Finally, natural environments provide yet again one more invaluable ecosystem service, which should be recognized and protected.

TIIVISTELMÄ

Luonnon monimuotoisuus eli biodiversiteetti on vähentynyt dramaattisesti viimeisten vuosikymmenien aikana väestön kasvaessa. Meneillään oleva kuudes sukupuuttoaalto on ainakin välillisesti aiheutunut ihmisen luonnonvarojen riistohyödyntämisestä, jonka seurauksena ilmasto on muuttumassa, viljelykasvien taudit ovat lisääntyneet ja veden kierto on häiriintynyt. Näiden näkyvien ja mitattavien muutoksien lisäksi näkymättömien mikro-organismien yhteisöt ovat muuttuneet. Monimuotoisuuden tuhoutuminen toistuu mikrobitasolla: monet häviävät ennen kuin niitä ehditään kuvata.

Ihmisen keho on ekosysteemi, jota asuttavat miljoonat mikrobit muodostaen yhdessä mikrobiomin. Bakteerit, virukset, sienet ja muut mikroskooppiset eliöt kohtaavat ihmisen puolustusjärjestelmän, joka haastaa niiden selviytymisen. Mikrobien ja ihmisen suhde on kuitenkin harvoin haitallinen: useimmiten yhteys on positiivisesti vastavuoroinen. Ihmisen keho on hyödyntänyt joidenkin mikrobien immuunijärjestelmää säätelevää ominaisuutta jakamalla osan kuormituksesta asukkaidensa kanssa. Tämän miljoonia vuosia vanhan vuoropuhelun häiriintyminen voi johtaa useisiin terveysongelmiin.

Ihmiskehon mikrobiyhteisöt ovat poikkeavia useissa elintasosairauksissa. Monet näistä sairauksista, kuten astma, allergiat, tulehdukselliset suolistosairaudet ja syövät, yhdistyvät kehon pitkittyneeseen tulehdustilaan. Länsimaistumisen myötä elintasosairaudet ovat voimakkaasti yleistyneet, mutta esimerkiksi allergiat ovat edelleen harvinaisia perinteisissä maanviljelysyhteisöissä. *Biodiversiteetti-hypoteesi*, jota testataan tässä väitöskirjassa, esittää että mikrobiyhteisöjen muutos ympäristössämme uhkaa ihmisen terveyttä. Hypoteesi olettaa että luonnon ympäristöjen tuhoutuminen muuttaa ihmisen altistumista vihreän luonnon mikrobeille, mikä taas voi johtaa immuunijärjestelmän heikompaan säätelyyn ja lopulta tulehdusperäisen sairauden kehittymiseen.

Tässä väitöskirjassa keskitytään elinympäristön ja elämäntavan vaikutuksiin ihmisen terveyteen kuten biodiversiteetti-hypoteesissa oletetaan. Tulokset perustuvat neljään eri osaprojektiin, jotka pohjautuvat erillisiin, uusiin tutkimusaineistoihin. Keskeiset löydökset ovat: (**I**) Ihon mikrobiomi eroaa maalais- ja kaupunkilaislasten välillä. Tämä ero häviää teini-ikäisillä, johtuen todennäköisesti muutoksista heidän elämäntavassaan. (**II**) Erityisissä luontopäiväkodeissa olevien lasten ihon mikrobiomi on rikkaampi kuin muissa päiväkodeissa olevien lasten. Toisaalta näiden lasten elämäntapa on muutoinkin poikkeava. (**III**) Lemmikkikoirilla allergiat ovat huomattavasti yleisempiä kaupunkiympäristössä kuin maaseudulla, silloinkin kun koirien rodun vaikutus on kontrolloitu. (**IV**) Altistuminen ympäristön mikrobeille elinympäristön välityksellä ja elämäntavan kautta vaikuttaa ihon mikrobiomiin ja allergioihin koiramallilla.

Väitöskirjani tulokset osoittavat että elinympäristö ja elämäntapa yhdessä vaikuttavat yksilön mikrobiomiin ja siten terveyteen. Tämän vuoksi ihmisen elinympäristö, ja sen monimuotoisuus, voivat joko tukea tai häiritä ihmisen terveyttä. Tällä hetkellä on laajaa näyttöä mikrobialtistusta lisäävien tekijöiden, kuten vihreän elinympäristön, maatilojen, eläinten ja sisarusten, hyödyistä ihmisen terveydelle. Väitöskirjani tarkentaa aikaisempaa tietoa osoittamalla yhteyksien kolminaisuuden mikrobialtistuksen, ihon mikrobiomin ja allergioiden välillä. Perustuen tähän ja aiempiin löydöksiin, ihmisten, erityisesti lasten, tulisi lisätä altistumistaan monimuotoiselle luonnolle ja erilaisille eliöille tukeakseen sekä mikrobiomin että immuunijärjestelmän tasapainoa kehoissaan. Lopuksi, luonnon ympäristöt tarjoavat jälleen kerran vielä kuvaamattoman ekosysteemipalvelun, joka tulisi suojella.

SUMMARY

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1 INTRODUCTION

1.1 ALLERGY – A DISEASE OF AFFLUENCE

Allergic diseases belong to a large group of non-communicable diseases, also known as *diseases of affluence* or *inflammatory diseases*. Allergic diseases such as hay fever and rhinitis used to be sophisticated diseases of privileged people in the 19th century. Since the 1960's situation has gradually changed; nowadays allergies are increasingly common in developed and transitional societies (Hertzen and Haahtela 2004, Asher et al. 2006). Allergy burden in children is well recorded worldwide (Asher et al. 2006) showing on average alarmingly high and constantly increasing prevalence of these diseases. For example, more than 10 % of Australian infants suffer (challenge-proven) food allergy (Osborne et al. 2011). In Finland, roughly 25 % of the adult population suffers from some kind of allergic symptoms (Jousilahti et al. 2016). Actually, this worryingly high prevalence makes Finnish people a good study population as diseased individuals can be easily found.

Allergy is type of hypersensitivity, where binding of antibody to, for example, pollen or food-related protein, causes memory response leading to the tissue damage. In other words, immune system uses inappropriately mechanism that is primarily for fighting against pathogens, which leads to morbidity in the host (Lydyard et al. 2011). This classically recognized mechanism of allergic diseases leaves the question that what triggers this harmful immune reaction. Assumingly, the chronic low-level inflammation, i.e. over-reacting immune system, is causal in numerous non-communicable diseases (Rook 2013). The chronic low-level inflammation is suggested to cause changes in host (immune system) that can lead to the development of diseases such as cancer (Coussens and Werb 2002) and metabolic disorders (Hotamisligil 2006). Also in allergic diseases, the role of chronic inflammation is suggested. The inflammation-related changes such as increased gut epithelial permeability as well as changed function in innate immune recognition and adaptive immunity associate with allergic manifestations (Macdonald and Monteleone 2005). If the chronic low-level inflammation can explain increasing disease prevalence, the question that remains is which changes in human life in the first hand cause this inflammation.

As could be guessed already from the earliest cases of allergies, these diseases relate to lifestyle and environment. A classic study showed that atopic diseases were more common in Western Germany, with high living standards, than in Eastern Germany in the 1980's, even though the opposite was expected due to heavy air pollution in the eastern parts (Mutius et al. 1994). In less extreme comparisons, both in Finnish and Estonian children, the prevalence of allergic sensitization was higher in urban environments (Ruokolainen et al. 2015). The low prevalence of allergic diseases in rural environments and with farming lifestyle is extensively reviewed (Genuneit 2012, Nicolaou et al. 2005, Ruokolainen 2017). This finding is not confirmed in all studies, which is probably because of the differing definition of rural environment (Nicolaou et al. 2005, Ruokolainen 2017). Some other non-communicable diseases, such as inflammatory bowel disease and cancer, are also more common in urban than in rural environments in affluent countries (Schouten et al. 1996, Soon et al. 2012, Ruokolainen et al. 2015). Besides, the immune function can differ between rural and urban populations (Renz et al. 2002). For example, the expression of a key anti-inflammatory molecule, interleukin-10 (IL-10), has been shown to be higher in rural than in urban children (Amoah et al. 2014). These types of findings led the scientists tracing the effects of rural living, large family sizes, animal exposure and non-western lifestyles, which all relate to low incidence of allergies, and large exposure to varied micro-organisms.

1.2 MICROBIOMES IN US AND SURROUNDING ENVIRONMENTS

Only about two decades ago, human microbiome research emerged as one of the fastest growing scientific fields (Blaser 2014). Both microbiota and microbiome describe microbial communities in human body, but the meaning of these terms is slightly different. The microbiota means all microbial organisms in a given ecosystem, for example, the human body. The microbiome includes microbiota, but also all the genetic information. The interest in microbial communities in humans increased after technological advances. Basically, the sequencing-based methods enabled scientists to study the full diversity of microbes in defined ecosystems (including human body; Blaser 2014) while previously used culture-based methods were able to reveal only some microbes. The bacterial 16S rRNA gene sequencing is common way to define microbiota, while metagenomics are needed for the definition of microbiome. In this thesis, only microbiota is described and hence that term is mostly used.

Nowadays the human microbiome is compared with bodily organs due to its importance to human health (Baquero and Nombela 2012). The great microbiome-hype arose from findings showing that the microbial communities noticeably differ between healthy and diseased individuals in several non-communicable diseases such as rheumatoid arthritis, colorectal cancer, obesity and type 2 diabetes (Zhang et al. 2015b, Wang et al. 2012, Le Chatelier et al. 2013, Qin et al. 2012), and from experimental studies showing that the microbiome can be causal in the development of these diseases (e.g. Zhao 2013, Gilbert et al. 2016). Moreover, several immunological routes, through which the microbial residents shape our immune system, have been described (Maynard et al. 2012, Honda and Littman 2016). For example, the formation of gut-associated lymphoid tissues and subsequent immune system development can be hampered in lack of microbial exposure (Maynard et al. 2012, Hooper et al. 2012). Moreover, short-chain fatty acids, which are fermented from complex carbohydrates by gut bacteria, are important signaling molecules in numerous processes such as in regulating inflammatory reaction (Morrison and Preston 2016). The microbial exposures are suggested to be especially decisive early in life as microbe-host interactions during this period can determine later susceptibility to develop disease. For example, the composition of gut microbiota during the first three months of life was disturbed in infants who later developed asthma (Arrieta et al. 2015). Also, reduced diversity of gut microbiota during the first year of life has been associated with asthma and allergic sensitization at school-age (Abrahamsson et al. 2014, Bisgaard et al. 2011).

Several factors associate with the composition of human microbiota. At the moment, diet, genetics, antibiotics and other medication, disease status, type of childbirth and family relations have been associated with the composition of host microbiota (e.g. Zhernakova et al. 2016, Korpela et al. 2016, Song et al. 2013, Pozuelo et al. 2015). Therefore, the composition of host microbiota seems to be selected by host-specific factors and shaped by lifelong exposures. Interestingly, the gut, skin and nasal microbiota differs between people living in different countries, having dissimilar lifestyle and living either in rural or urban environments (Yatsunenko et al. 2012, Ying et al. 2015a, Ruokolainen et al. 2017). This is expected as the environmental microbiota, i.e. the microbes living in certain environment, differs between different climatic zones and between natural and built environments (Barberán et al. 2015b). In a city-environment, the environmental microbiota differs between parks and asphalt-covered areas, diversity being higher in green areas (Mhuireach et al. 2016). Outdoor microbiota contributes to the indoor fungi, but overall number of human occupants, their sex and pets are more important modulators of indoor microbiota (Meadow et al. 2014, Hospodsky et al. 2012, Barberán et al. 2015a). Actually, indoor-spaces, in which western people spend on average more than 90 % of their time, are enriched with microbes common in human skin only (Lax et al. 2014, Barberán et al. 2015a). Together these findings indicate that humans have limited exposure to variety of microbes if staying mostly indoors in urban environments.

1.3 THE BIODIVERSITY HYPOTHESIS OF ALLERGY

Biodiversity means a variety of life in all levels from genes to organisms and finally to ecosystems. Diversity of species in terrestrial, aquatic and marine ecosystems has been consistently shown to be an important

determinant of ecosystem productivity, stability, invasibility (= ability to resist invasion by exotic species or by pathogens), and nutrient dynamics (Tilman et al. 2014). The diversity at the level of microbial organisms is stunning. Its potential importance for planetary and human health is currently topic of intensive discussion (Shade 2017).

The biodiversity hypothesis postulates that opposite worldwide trends, i.e. the decreasing biodiversity and the increasing prevalence of inflammatory diseases, are related (Figure 1.). Basically, hypothesis proposes interrelations between living environments, host microbiota and immune function. That is, the exposure to diverse environmental microbes in green areas can support immune function while in urbanized areas the microbial exposure is assumed to be limited. If the cross-talk between variable microbes and immune function is disturbed due to limited contact with natural environments, non-communicable diseases can develop (Hertzen et al. 2011). These associations are widely advocated (e.g. Rook 2010), but little direct support exists (Ruokolainen et al. 2016). However, the pioneer study and a predecessor of this thesis, showed that the allergic individuals had lower biodiversity in their residential environment. Further, Acinetobacter showed relation with anti-inflammatory IL-10 in healthy individuals, while in allergic individuals this relation was absent (Hanski et al. 2012).

The biodiversity hypothesis somewhat relates to its famous predecessor, the hygiene hypothesis, and to newer and more focused the 'Old Friends' mechanism. Also other related hypotheses have been proposed (e.g. Shreiner et al. 2008). The hygiene hypothesis states, in its original form, that less childhood infections is followed by more inflammatory diseases. That is, the prevention of infections can be causal in the development of non-communicable diseases. Recently, scientists have proposed that hygiene hypothesis should be rejected as it gives a misleading message for the public (Bloomfield et al. 2016). That is, washing hands and accepting vaccinations are still recommended actions. The 'Old Friends' mechanisms emphasizes the importance of varied parasites and microbes, which have co-exist with humans millions of years, for the immune functions (i.e. the evolved dependency; Rook 2010). Recently, the change in lifestyle has reduced our contact with these parasites and environmental microbiota (Rook 2013). The biodiversity hypothesis

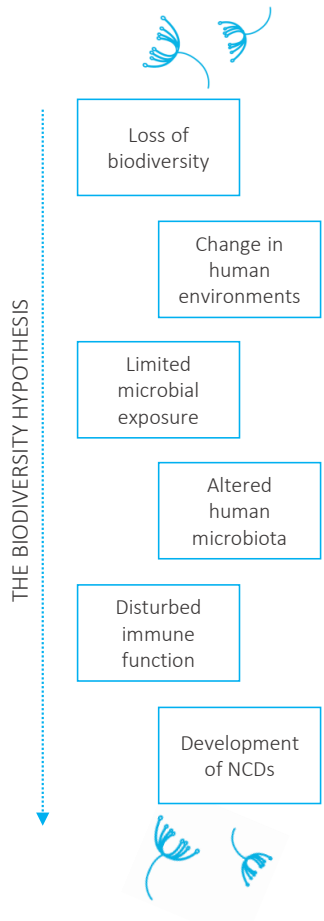

Figure 1. The biodiversity hypothesis states that two megatrends, the drastic increase in non-communicable diseases (NCDs) and the destruction of biodiversity, are related. The link between these two is limited exposure to environmental microbes that can support healthy immune function in the host.

closely builds on the 'Old Friends' mechanisms, but it underlines the ongoing biodiversity loss, not just reduced contact with it. In other words, changes in lifestyle may not be enough to preserve human health unless the natural environment, and residing biodiversity, is also protected.

The potential immunological mechanism through which the environmental microbes support healthy immune function is not currently fully understood. Assumingly, the recognition of microbial antigens or molecules produced by microbes creates a cascade of reactions which can lead either to the activation or suppression of an (specific) immunological

BOX 1. THE SKIN MICROBIOTA FROM THE PERSPECTIVE OF THE BIODIVERSITY HYPOTHESIS

Gut microbiota has been the main focus of microbiota research and the source of all "microbiota-hype". However, in this thesis, the skin microbiota was studied instead, due to three main reasons. 1) Skin microbiota, but not gut microbiota, tends to resemble soil microbiota (Parfrey et al. 2014). Moreover, soil microbiota transferred on human skin can partly persist even a full day (Bateman 2017) suggesting that daily outdoor exposure can provide constant expose of skin to the environmental microbes. The mixture of transient and permanent members of skin microbiota suggests dynamic interaction of skin microbiota with the environment (Hertzen et al. 2011). Together these findings indicate that environmental microbiota can shape the microbial community on skin.

Biodiversity hypothesis does not assume that the immune modulation by environmental microbes is taking place through skin microbiota. Gut microbiota and respiratory tract microbiota are equally likely to contribute to the immune function this way (Hertzen et al. 2011). However, the respiratory tract and skin microbiotas have more immediate contact with environmental microbiota than the gut microbiota which is exposed to strong selective pressures such as stomach acids. Moreover, the skin and respiratory tract (nasal) microbial communities were unalike in rural Finnish and Russian subjects who are exposed differently to environmental microbes (Ruokolainen et al. 2017). Therefore, the potential health-benefits intervened by environmental microbes are probably mediated through the skin and respiratory tract. This is supported by recent findings showing that 2) the skin and its microbiota seem to have systemic role in immune function even though previously the effect was suspected to be only local (Belkaid and Segre 2014). This is supported by research showing that the interplay between skin microbiota and epidermal cells regulates several immunological networks, maintains tissue integrity and keratinocyte homeostasis (Prescott et al. 2017). Additionally, the skin microbes control the barrier function of the skin, which is central in the development of skin diseases and relates to the systemic responses controlled by immune cells in the skin (Nakatsuji et al. 2017). Together these findings suggest the importance of skin microbial communities for host health.

The skin is a large organ, which has, in a view-point of microbes, several dissimilar habitats with different selection pressures (Karkman et al. 2017). For example, moist, warm and sheltered armpit supports different microbial community than drier and more exposed areas of skin (Grice et al. 2009). Besides this spatial variation, communities on the skin have more individual and temporal variation than communities in the gut (Costello et al. 2009). Therefore, skin microbiota is rather difficult to comprehensively characterize in single individual. However, 3) the skin microbiota samples are relatively easy to collect compering to the gut and the respiratory tract samples.

response. It is suggested that the host microbiota is especially important in activation of immuno-regulatory networks which ensure that inappropriate inflammatory responses are terminated (Hertzen et al. 2011). This view is supported in review by Honda and Littman (2016) that introduces several ways by which microbiota maintains immune homeostasis of host. The immuno-regulatory nature of host microbiota is in harmony with the hypothesis that modern diseases, which associate with the chronic low-level inflammation, are caused by the limited exposure to beneficial microbes.

The sixth mass extinction is currently consuming the diversity on earth (Barnosky et al. 2011). The global change in environments, i.e. the replacement of natural environments with anthropogenic environments, sets a huge cost for global biodiversity. The extinction magnitudes and rates are harder to estimate in microscopic than in macroscopic organisms, but some support exist showing that globally cities harbor similar microbial communities (Epp Schmidt et al. 2017), and that microbial diversity is decreasing (Staley 1997). Also, microbial diversity in soil relates to local plant diversity (Prober et al. 2015), proposing that change is macroscopic diversity probably reflects that of microbes. It is alarming for human health that some of our ancient microbial friends (Rook 2010), that have important dialogue with our immune cells, may be lost along macroscopic species. This thesis examines broadly the relations between allergic diseases, host microbiota, i.e. the microbes living in individual's body, and exposure to environmental microbes. In other words, this thesis searches support, in the spirit

of the biodiversity hypothesis, for that human health closely depends on the health of the nature.

2 OBJECTIVES

The objective of this thesis is to find empirical support for the biodiversity hypothesis. That is, to search for relations between the residential environment, host microbiota and allergies. Each sub-project focuses on different angles of the hypothesis. The main questions in each project are:

Project I: Does the skin microbiota develop systematically differently in children living in contrasting environments, i.e. either in the city-center of capital or in sparsely populated rural areas? If so, does this development relate to allergic sensitization or symptoms?

Project II: Can outdoor practices and environments in daycares shape the skin microbiota in children? If so, do these changes in the skin microbiota relate to allergic sensitization and symptoms? And finally, if daycare environments have potential to influence child health, which factors shape the nature-connection of an individual daycare?

Project III: Does the prevalence of allergic symptoms differ between Wrural and urban environments in our companion animals, pet dogs, which share their environment and lifestyle with us? Is the relation between living environment and health generalizable to other mammals than humans?

Project IV: How does the different aspects of microbial exposure, i.e. the residential environment, exercise environment and lifestyle, influence on the skin microbiota in canine model? Do these same factors relate to the prevalence of allergic symptoms? And finally, what is the relation between the effects of early-life and current environments?

Together these four projects clarify the relations between environmental microbiota, allergies and host microbiota as suggested by the biodiversity hypothesis. However, the causality between these factors cannot be confirmed by the current approach and the assessment of causality is left for future projects.

3 MATERIALS AND METHODS

The results in this thesis are based on notable datasets, which were originally collected for these projects. The data comprises of *biological samples* from nearly 350 humans (children) and 169 pet dogs as well as *surveys* of nearly 6,000 dog owners, and almost 800 daycare-workers. Biological samples are skin microbiota swabs, and serum samples for the analysis of immunoglobulin E (IgE). From all individuals, the data includes comprehensive questionnaire-information about lifestyle, living environment and allergic symptoms (Table 1).

Table 1. The data in each project.

	PROJECT	I	II	III	IV
BASICS	n	275	68 (+768)	5687	169
	Species	Human	Human	Dog	Dog
	Age	0-14 years	5 years	All	1-11 years
	Female %	53 %	51 %	53 %	67 %
DATA	Microbiota	+	+	-	+
	IgE	+	+	-	+
	CRP	-	-	-	+
	Allergy	+	+	+	+
	Land-use	+	+	+	+
	Lifestyle	+	+	+	+
	Data	Biological samples	Biological samples +survey	Survey	Biological samples

The n in parenthesis (project II) indicates the survey-part of the project. The project III differs from the others as it is solely based on survey, and no biological samples were collected. C-reactive protein (CRP), the classic marker of (low-level) inflammation, was only measured in project IV.

3.1 CHILDREN AND CANINE MODEL

Several approaches can be adopted in studies with a goal to increase knowledge about human health. In this thesis, both cross-sectional studies with human subjects as well as model animal-based approaches were used. This mixed approach was chosen because the strongest support obviously comes from studies focusing directly on humans, while, on the other hand, an endless list of confounding factors can be ignored in model animal-based approach (Figure 2.).

Laboratory mouse is a common model of the human diseases. However, in this thesis, a pet dog model was used. The advantage of this approach is that pet dog develops spontaneous allergies, which are analogous to human allergies (van Steenbeek et al. 2016). Moreover, dogs share their living environment with humans and are exposed to the lifestyle of their owners, which is ideal when the focus is the relation between environmental microbial exposure and health. Also, it is rather impossible to artificially create a realistic "living environment" in the laboratory, and hence the effects of some unknown factors are lacking in a mice model experiments. Finally, the reason to use dogs instead of directly focusing on humans is that the life of dogs is both shorter and simpler than that of humans. This makes the interpretation of results much easier as several confounding factor caused by multifaceted human life are absent.

Figure 2. The advantages and disadvantages of different study subjects. The dog model provides an intermediate solution between two extreme approaches, humans and laboratory mice. Canine model provides several advantages even though each approach have strengths over others.

We did not use extensive exclusion or inclusion criteria in the selection of participants. An obvious criterion was that subjects needed to be from a selected area, i.e. either rural or urban, and belong to a selected age group. Subjects were neither instructed to follow certain washing procedure before sampling. The advantage of loose inclusion criteria is that more individuals are able and willing to participate in the study. That can also decrease the potential of artificial biases as subjects are more likely to represent population randomly. In dogs, the breed is important predisposing factor in allergic diseases (Bellumori et al. 2013). Therefore, in project IV the breed of subjects was controlled, and only two common breeds were accepted. In other project involving dogs (III), the breed-effect was later controlled with statistical methods.

3.2 SKIN MICROBIOTA

The skin microbiota is very variable and complex system, which begs the question, how it

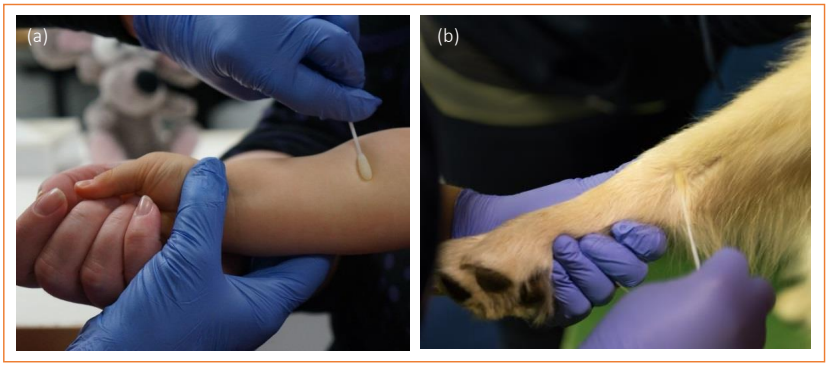

Figure 3. The skin microbiota sampling in humans and dogs. (a) In humans, the samples were collected from the volar forearm, and in (b) dogs from the anatomically comparable area.

should be sampled? We selected to sample the volar forearm of children and an anatomically similar area from dog (Figure 3.). This area is technically easy to access both in humans and dogs. In dogs, fur-cover is not that extensive in the inner side of the leg, just below knee-level, which makes access to skin easier. Secondly, this area is rather dry, which is usually not associated with communities dominated by single taxa (Grice et al. 2009), which could overdrive some interesting, but less competitive players. Also, microbiota in volar forearm shows the highest richness compared with other body regions (Grice et al. 2009). Importantly, arms, and legs in case of dogs, are rather well exposed to the surrounding environment, but are not as extensively washed as hands (or paws). The technical details in sampling, DNA extraction and sequencing of V1-V3 region of bacterial 16S rRNA gene are described in projects I, II and IV.

Bacterial sequences were organized to operational taxonomic units (OTUs) with bioinformatic methods. An OTU is a collection of sequences that resemble each other more than a defined value. Both the commonly used 97% similarity threshold and also more strict definitions were used, as the development on research field has started to question traditional, but arbitrary cut-off value of 97% similarity (Nguyen et al. 2016). The OTU definition follows several rather standard bioinformatics steps in which the quality of sequences is certified by removing the primers needed in PCR, combining the forward- and reverse-sequences, removing reads with sequencing errors, clustering identical sequences and removing singleton sequences (with only one copy in data, meaning that it is probably a sequencing error). Then OTU-clusters are created with selected cut-off value and taxonomic information is added from a selected database, which was Silva in this thesis. Specific description about pipeline as well as the contaminant removal with a help of negative control samples is provided in Lehtimäki et al. (2017).

3.3 THE DEFINITION OF ALLERGY

Allergic diseases are complex, both in their development as well as in outcomes. However, much work has been done in the definition of allergies in humans. We were able to base our definition of allergic symptoms to previously developed, internationally used and validated questionnaire (The International Study of Asthma and Allergies in Childhood; Beasley 1998). Questions were divided into small groups focusing some group of symptoms, which were asthma, hay fever, eczema, rhinitis, wheeze and atopic dermatitis. These questions were used in dividing children to those who were symptomatic or healthy.

A classic clinical marker of allergic sensitization is Immunoglobulin E (IgE), an antibody that activates histamine-producing mast cells in allergic response (Lydyard et al. 2011). IgE can be measured from blood serum. If the level of either total IgE or some of the specific IgEs is larger than 0.35 kU/l, an individual is defined to be sensitized (or atopic), that is, predisposed to allergic diseases. In this thesis, the cut-off point for IgE was increased to 1 kU/l, because larger values are more likely to be associated with clinically meaningful symptoms. A higher threshold has also been empirically justified previously (Hanski et al. 2012, Ruokolainen et al. 2015).

The definition of allergies was more difficult in dogs. Even though allergic symptoms cause increasingly morbidity in pet dogs, the nature of canine allergies is not well understood. Currently, allergies in dogs are divided into food allergies (FA) and canine atopic

dermatitis (CAD), while the respiratory symptoms are absent in contrast to allergies in humans (Pucheu-Haston 2016). Still, there is no clear characterization of FA and CAD, rather, these diseases are overlapping (Verlinden et al. 2006). Therefore, in this thesis, the definition of canine allergy is single disease with variable symptoms, and no attempt to divide those to certain diseases was done. This definition was based on a large questionnaire, which was developed by the specialists of canine dermatology.

The IgE is used as a marker of allergies also in dogs. However, the relation between allergic symptoms and IgE levels is unclear in dogs (e.g. Lauber et al. 2012). In this thesis, IgE levels of dogs to several inhalant allergens did not show relation with symptoms, and therefore IgEs were not used in definition of allergies (or sensitization).

3.4 THE CHARACTERIZATION OF THE LIVING ENVIRONMENT

What are the important characteristics in individual's living environment regarding health is a question without a definitive answer. Therefore, it is unclear in which aspects of living environment research should focus. The living environments consist of, for example, the land-use, the amount of people and animals on that area, diversity of living creatures, climate and seasons, and pollution levels. Obviously, these factors are strongly correlated and their effects on human health are therefore difficult to distinguish.

In this thesis, land-use patterns, i.e. the proportions of forests, fields, built areas and water, were used to define the characteristics on the living environment. This approach is rather practical, as the land-use around an individual's home is easy to define based on the publicly available database of land covers (CORINE 2012). The coordinates of an individual's home were defined from collected address information, which were used to define the land-use with certain buffer around the home. Based on the previous experience, a 3 km radius was chosen, as it provides sufficient resolution for estimating land-use effects (Ruokolainen et al. 2015). Shorter radiuses give more confusing results as the close-by environments can be rather similar in rural and urban areas. Also, the environmental microbiota travels in the air, sometimes rather long distances (Wilkinson et al. 2012), and therefore individual is probably exposed to microbiota from farther away than just that in immediate home environment.

3.5 THE CHARACTERIZATION OF THE LIFESTYLE

A large set of information regarding the lifestyle- and family-related features were collected in each project with questionnaires. In each project rather similar information was collected, however, questions were adjusted for each age group and species. Questions covered areas of interest such as early-life nurturing (environment), characters of current living environment, animal contacts, and amount and quality of outdoor exercise. Questions tried also to catch the effect of several confounding factors such as breastfeeding and birth weight, number and age of siblings, socio-economical situation of the family, smoking and health of the parents, consumption of farm milk and butter, and antibiotics use.

The lifestyle of an individual is difficult to fully describe as the concept is not well-defined and comprehensive research approaches are lacking. The number of questions is wise to keep rather low in order to increase response rate. Also, subjective answers can give inflated results. Because of these limitations, but also due to statistical reasons, we reduced the data dimensionality in order to create a single "lifestyle" variable (see following chapter). Single variable that is simplified from several variables gives fuller image from the lifestyle than any single question and decreases the effect of subjectivity. Full lists of questions are provided in the supplementary material of each project.

3.6 STATISTICAL ANALYSIS OF COMPLEX, MULTIVARIATE DATA

In each project, several complex datasets were united. These were 1) *the skin microbiota*-data – a huge matrix of the abundances of each OTU found in all study subjects, 2) the information regarding *allergic symptoms* (questionnaire) and IgE (clinical data), 3) the large questionnaire information about lifestyle- and family-related features, and finally 4) the land-use information. In Project III, dataset 1 was not available.

Datasets marked with *italics* indicate response variables in analyses.

The information in datasets 2 to 4 were all independently simplified to single variables (i.e. the data dimensionality was reduced), even though few previously defined, interesting variables were also used as such. Simplification eases the interpretation of results, and removes statistical problems caused by correlation between explanatory variables and large number of explanatory variables. The simplification was done by related multivariate methods, which were selected according datatypes. The simplification of allergic symptoms and lifestyle features, i.e. the questionnaire information was done with Principal Coordinate Analysis (PCoA) on Gower's dissimilarity matrix (that accepts mixture of factors and continuous variables; Legendre and Legendre 2012) calculated from datasets. In project III, this was done with Factor Analysis, which creates new factors for which data variables load differently. This enables the definition of relations between data variables and clustering those to new variables and therefore exclude correlations and large number of variables. Finally, the land-use information was simplified with Principal Component Analysis (PCA). After these analyses, the first (and in some cases also the second) axes (or factors in case of Factor Analysis) were extracted, as those are likely to include the most of the meaningful variation in a large raw data, to be used in further analysis.

The skin microbiota data was normalized before further analyses. This was done with a method that seemed to fit each dataset best. That is, the goal is to control the effect of the dissimilar sample sizes without losing biologically relevant information (McMurdie and Holmes 2014). In all projects (I, II, IV), Cumulative sum -normalization provided in the metagenomeSeq-package in R (Paulson et al. 2013) was considered to be the best method. If needed, the samples were further transformed in order to reduce the effect of the dominant OTUs. The idea in both normalization and transformation is to ensure that the results are not product of artificial aspects in complex and variable microbiota datasets (i.e. noise), and, on the other hand, that biological phenomena are not left hidden under all the noise.

Huge microbiota datasets are difficult to use as such. Also, the approach which focuses only a single OTU rarely produces interesting results, due to the large inter-individual variation. The common way to approach complex communities is to either focus on certain higher taxonomic groups (for example, Proteobacteria) or to consider the dissimilarities between samples. Dissimilarity indicates how different the communities are between the two samples (i.e. two individuals). In this thesis, dissimilarity matrixes were used in both unconstrained and constrained ordination methods, in order to visualize the information in the matrix. Moreover, the analysis of variance of distance matrixes (implemented in vegan-package; Oksanen et al. 2016) was used to test how different explanatory variables might associate with between-sample dissimilarity.

The methods mentioned above focus on community-level differences, and are able to show which samples are different. In this thesis, the OTUs that are differently abundant between groups were searched (with Deseq-package; Anders and Huber 2010). Also, so called 'random forest' –predictions were used as they are also a good tool for both defining whether certain explanatory variable can explain patterns in the microbiota, and which OTUs are best in characterizing the pattern. Random Forest analysis (Liaw and Wiener 2002) uses the community data (microbiota) for predicting the explanatory variable. Analysis takes randomly part of the data to create a model, which is then used for the rest of the data. This is repeated several times, and the success of the models defines the prediction power.

Lastly, a common and simple way to describe microbiota data is to calculate community diversity. A diversity index is a single number which summarizes the richness (i.e. species number) and the distribution (i.e. the abundance of each species) of species in the community. Such indices are widely used as a descriptive statistics in microbiota studies, but the value of such simple indices is debatable (Karkman et al. 2017). In each project including microbiota data, the Shannon diversity index was estimated.

3.7 ETHICAL STATEMENT

The ethical approval for the projects I and II is from the Ethics Committee for Gynecology and Obstetrics, Pediatrics and Psychiatry of the Helsinki and Uusimaa Hospital District (permission number:

283/13/03/03/2013), and for the project IV is from the Animal Ethics Committee of State Provincial Office of Southern Finland, Hämeenlinna, Finland (ESAVI/6054/04.10.03/2012). Sample collection and all subsequent experimental procedures were conducted in accordance with relevant guidelines and regulations.

4 MAIN RESULTS AND DISCUSSION

Three key findings arose from these thesis projects. 1) The skin microbiota of an individual is shaped both by the living environment and lifestyle (i.e., the passive and active contact with surrounding environment). 2) Allergies are clearly more common in urban than in rural areas in canine model, but this cannot be seen in children, which is probably due to the study design. Finally, 3) the skin microbiota, allergic symptoms and exposure to environmental microbes are interrelated in canine model.

Together, the findings in this thesis propose that 1) the biodiversity hypothesis is supported, 2) natural environments provide an ecosystem service in the form of environmental microbes, and that 3) the pet dog is a valuable model that falls between human studies and laboratory experiments (Table 2.).

4.1 LIVING ENVIRONMENT ASSOCIATES WITH THE SKIN MICROBIOTA AND ALLERGIC DISEASES

Both in children and in canine model, the skin microbiota differed between individuals living in rural and urban areas (I, IV; Figure 4.). These findings agree with previous investigations: Both in Finnish and Chinese adolescents and adults the rural and urban differences were clear in the skin microbiota (Hanski et al. 2012, Ying et al. 2015b). Also, rural and urban differences in the gut microbiota have been discovered in Russian and Chinese populations (Zhao et al. 2011, Zhang et al. 2015a, Tyakht et al. 2013). Several studies has shown differences in the gut microbiota (e.g. Coon et al. 2016, Eichmiller et al. 2016, Kohl et al. 2017, Song et al. 2017) and skin microbiota (Hyde et al. 2016, Lemieux-Labonté et al. 2016) between different living environments in variety of animal species. According to my knowledge, this is the first description of rural-urban differences in the canine microbiota.

The relationship between living environment and allergic diseases was less clear (Figure 5.). In canine model, the prevalence of allergic symptoms closely followed the land-use gradient both in the survey (III) and empirical data (IV), such that canine allergies

Table 2. The main results and conclusions in each project and the common conclusion of thesis.

	Project	Main result	Main conclusion	Thesis conclusion
CHILDREN	I	The skin microbiota differs in children living in contrasting environments.	Rural and urban children get dissimilar microbial exposure.	Environmental biodiversity provides an ecosystem service (micro-organisms) that should be recognised and protected.
CHILDREN	II	The skin microbiota is highly diverse in children attending outdoor-daycares.	Nature-oriented lifestyle support constant exposure to diverse microbial world.	
CANINE MODEL	III	Urban pet dogs suffer from allergic symptoms more than rural dogs.	The relation between allergies and living environments can be generilized in mammals.	
CANINE MODEL	IV	The skin microbiota, allergic symptoms and exposure to environmental microbes are interrelated.	Urbanised environments and lifestyles can seriously impair individual health.	

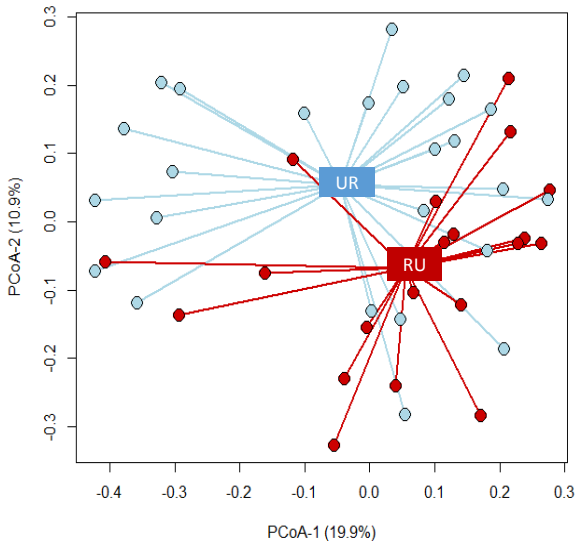

Figure 4. An example of clustering of the skin microbiota by the living environment. In two year old Finnish children, the clustering of the skin microbiota by the current living environment (UR=urban, RU=rural) is clear. At this age, children actively study their close surroundings, and typically in Finland small children are taken outdoors regularly.

were more common in urban environments. In children, no robust association between living environment and allergic symptoms or sensitization were discovered (I, II), even though the rhinitis symptoms were significantly more common in urban children (I). This contrast with notable previous evidence, which has repeatedly shown that allergic manifestations are more common in urban than in rural environments (e.g., Kausel et al. 2013, Ruokolainen et al. 2015). Also in dogs, the protection provided by the exposure to green space against allergic diseases has been described previously (Meury et al. 2011, Nodtvedt et al. 2006), even though not as comprehensively as in this thesis (III, IV).

In canine model, the interrelations between living environment, allergies and the skin microbiota could be found (IV). That is, the prevalence of allergies and the composition of the skin microbiota concurrently followed the land-use gradient. Even though this is an association, it suggests that rural-type skin microbiota is protective against allergic diseases as the prevalence of these diseases was lower in rural areas. In children, only weak association between the skin microbiota and allergic sensitization could be found (I). That is, the triangle of interactions found in canine model could not been confirmed with child dataset.

The potential reasons for the puzzling disparity in the environment-microbiota-allergy relations between dogs and children are several. First, the association between health and the current living environment/microbiota can be absent in the cross-sectional datasets—including children with varied age groups (I) and children with age of five years (II). That is because the important microbial exposure regarding immune-development probably occurs early in life, while the allergic symptoms manifest usually later. This assumption is based on notion that the composition of (gut) microbiota during the first year of life associates with later allergic manifestations (Arrieta et al. 2015, Abrahamsson et al. 2012, Abrahamsson et al. 2014, Kennedy et al. 2017). Hence, longitudinal study design may be needed for finding association between microbiota and allergies. Another plausible explanation is that in a westernized country like Finland, the lifestyle factors may not differ that much (anymore) between rural and urban populations, and therefore the protection provided by rural environment can be overdriven by other causal factors, such as limited contact with outdoor environments or western diet. Thirdly, children (I, II) may not be random sample from population, as the invitation letter – highlighting the focus on allergies, may have selected individuals. Also, collected information regarding allergic symptoms can be insufficient for the definition of clinical allergy.

The reasons mentioned above are not enough to explain the disparity in child and dog datasets. Also in dogs, the datasets were cross-sectional (III, IV) and therefore, data type cannot be the only reason. Reasonable explanations could be that 1) the life of dogs is simpler, meaning that multifaceted factors do not mask the effect of living environment, 2) the development of immune system may somewhat differ in dogs and children, and 3) the definition of allergic diseases differed in dogs and children. As mentioned above (Materials and methods), the definition used in canine allergies is broader than that used in children (in which sensitization and several symptoms were analyzed separately). Consequently, the definition used in dogs can be more efficient in finding the individuals with malfunctioning immune-system, regardless

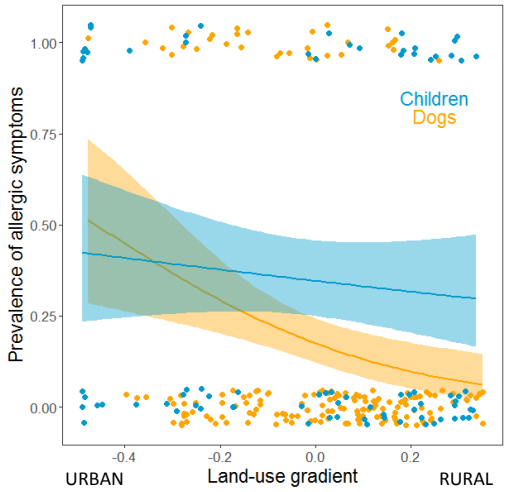

Figure 5. An example of the prevalence of allergy along the land-use gradient. In pet dogs, the prevalence of allergic diseases follows the land-use gradient (in breed-controlled analysis), while in children and teenagers (here: 8 to 14 years old) the relation is less clear and non-significant.

of the actual allergic disease manifestation. The manifestations in allergic symptoms are variable even though roots are always in the malfunctioning immune system. Therefore, the broader definition may cluster individuals better to healthy and 'allergic somehow' than focus on separate allergic manifestations. The reason for the missing link between environment (or microbiota) and allergic symptoms in children (I, II) cannot be fully understood, and it actually underlines the value of the canine model.

4.2 LIFESTYLE SHAPES THE CONTACT WITH ENVIRONMENTAL MICROBES

Several findings in this thesis highlight that besides the living environment, the actual contact with it shapes the composition of skin microbiota. Actually, in all projects, the effect of lifestyle-related factors could be found: The disappearing difference in the skin microbiota of rural and urban teenagers (I), dissimilar microbiota in children attending different daycare-types (II), and the effect of the lifestyle of the dog owner to the canine skin microbiota (III, IV), all indicate the importance of actual contact with environmental microbes.

The previous support for the importance of lifestyle in the composition of microbiota comes indirectly, i.e., the focus of studies has been in some key component of lifestyle. For example, the gut microbiota differs in children living in the same city, but belonging to different socio-economical class (Mello et al. 2016). The difference can be found even between the farm and anthroposophic children, who are both likely to be extensively exposed to natural environments (Dicksved et al. 2007), as well as between traditional agriculturalist and hunter-gatherers in Peru (Obregon-Tito et al. 2015). The effect of lifestyle in allergies is also well known. For example, a large meta-analysis confirmed that asthma is less common in children exposed to farming environments (Genuneit 2012). Also, children living in farms are less allergic than *other rural* children (Braun-Fahrländer et al. 1999). Moreover, the prevalence of allergies was lower in Amish than in Hutterite communities, in which the only difference is that Hutterites use modern farming (Stein et al. 2016). Also, the allergic diseases are less common in children who have several siblings (Karmaus 2002). Clearly, several lifestyle-related factors associate with the microbiota and the development of allergies.

In children, the land-use around the daycare was not associated with the skin microbiota, but the type of the daycare was (II). The skin microbiota was most diverse, and differently composed in children attending nature-oriented outdoor-daycares. This is interesting as the land-use around the daycare did not differ between outdoor-daycares and typical daycares, but still a clear difference in the skin microbiota could be seen. In other words, time spent in a certain environment may not translate to changes in the skin microbiota without active contact. However, it should be noted that several family- and lifestyle related factors differed between children in outdoor-daycares and other daycares. For example, parents of outdoor-daycare children were highly educated and these families exercised outdoors. Hence, the safer conclusion is that overly 'nature-oriented lifestyle' can cause changes in the composition of child skin microbiota.

In canine model, the triangle of interactions between the living environment, allergies and the skin microbiota could be further expanded with the effect of lifestyle (IV). Several family- and lifestyle related factors showed strong correlation, and therefore this information was simplified to a single variable

of 'lifestyle'. Rural lifestyle indicated living in large family, having regular animal contacts, living in town house, and exercising mostly at back yard. Urban lifestyle was basically opposite of this. An invaluable finding from the project IV was that in different combinations of living environment (rural or urban) and lifestyle (also rural or urban) associated with both the skin microbiota and prevalence of allergies. Rural dogs with rural lifestyle were healthiest, while opposite conditions were the most morbidity-promoting. This pioneering result indicates that the living environment and lifestyle jointly influence the skin microbiota and allergic diseases.

4.3 DIFFICULTIES IN THE ESTABLISHMENT OF THE BIODIVERSITY HYPOTHESIS

This thesis aimed to study the links between microbial exposure, allergic manifestations and the skin microbiota, as suggested by the biodiversity hypothesis. In canine model (IV), such associations were discovered. While this hypothesis is also supported by findings from the other projects (I-III), not all expected associations could be confirmed. Similar partial support comes from previous research, which the biodiversity hypothesis builds on. For example, the prevalence of allergic sensitization is manifold in the Finnish side of the Karelian border compared with the Russian side (Ruokolainen et al. 2017). The geographic distance is only couple of hundred kilometers, but the difference in socio-economical level is one of the largest on earth. Therefore, the differing microbial exposure between Finnish and Russian Karelia is suggested to explain this difference. Besides, the mechanisms behind the protection provided by microbial exposure are starting to unravel. For example, sufficient exposure to endotoxins (membrane component of gram-negative bacteria) is shown to suppress allergy-related immune reactions (e.g. Schuijs et al. 2015).

The evidence provided here, in support of the biodiversity hypothesis, is inflated by several limitations. The largest, obvious problem is that current research mostly describes associations and therefore the causality can be questioned. Well-powered cohort studies, well-designed interventions and experimental laboratory work are needed in clarification of the mechanisms. Another problem is that the lifestyle-definition used here is somewhat vague. It is difficult to define as complex issue as lifestyle. Therefore, studies directly focusing on the effect of lifestyle on host microbiota are rare. However, as health is a product of the total lifetime exposures, both positive and negative (i.e., exposome), the wider approaches should be adopted more often in research (Renz et al. 2017). Also, the effect of living environment remains difficult to isolate from the effect of the lifestyle (e.g., Ruokolainen et al. 2015) and, for example, air pollution. Finally, several complications in the establishment of the biodiversity hypothesis arise from the difficulty to study the human microbiome. The microbiome harbors enormous individual-level variation both in healthy and diseased individuals. Therefore, a 'healthy microbiome' is currently undefined.

Here, the focus in the microbiota analysis was on community-level differences. No single microbe has been mentioned in this summary. That was a specific decision in order to 1) highlight that OTU-level differences are not very robust between rural and urban populations, i.e., few OTUs show linear relationships with land-use gradients. Rather, the rural-urban differences in skin microbiota are manifested at the community-level. That is, microbial assemblages differ between areas. Of course, as the skin microbiota has high inter-individual, temporal and spatial variation (Grice and Segre 2011), a single sample from the volar forearm can be insufficient to reveal much about the total dynamics of skin microbiota between geographical areas. Also, 2) the information about key organisms is currently lacking, even though several have been proposed and empirical evidence supporting their importance is starting to accumulate (e.g. Acinetobacter; Fyhrquist et al. 2014, Debarry et al. 2007). It is suggested that microbes rarely work as individuals, but rather in groups (King 2014). Therefore, instead of exposure to a single microbe, the constant exposure to varied assembly of microbes can be the immune-modulatory factor that is meaningful for human health (Birzele et al. 2017). It has been argued that many of the OTUs found from the skin microbiota can be transient (Grice and Segre 2011). Nevertheless, transient microbes can also be important modulators of immune reactions by providing variable microbial exposure. Even though this thesis focuses on community-level differences it is essential to define potential key organisms as they can explain these differences. Also, key organisms provide good starting point for experimental studies.

The single most surprising finding of this thesis was that an allergic dog was more likely to have an allergic owner than a healthy dog (III). One reasonable explanations for this intriguing relation is that allergies are caused by a factor which dogs and their owners share in their life. Previously pet dogs have been proposed to protect children against the development of allergies (Hesselmar et al. 1999, Tun et al. 2017). Interestingly, protection can happened also the other-way round: dogs were protected against allergies if they were living in large human families (III, IV). These unexpected results further support the use of a dog as a model animal in the research focusing on the health-effects of environment and lifestyle. In this thesis, the most fruitful results were actually provided by the canine model. The use of pet dogs as model animal provides a meaningful approach that is intermediate between human and experimental studies (Figure 2.).

5 CONCLUSIONS

The results in this thesis can be translated to few suggestions. In canine model (IV), also the effect of exercise environment was estimated. It seemed to marginally associate with the skin microbiota, but not with allergies. Therefore, dog model suggests that sustained factors, i.e., the living environment and lifestyle, are important for health, while more occasional exposure to environmental microbes while exercising is less important. The idea that prominent contact is needed for differences to arise is supported by the comparison between daycare children (II). That is, even though the daily exercise did not translate to the differences in the skin microbiota in dogs, the all-day outdoor-exposure did the difference in children. In conclusion, the findings in this thesis indicate that '*more is more*' when considering the sufficient amount of contact with green environments in order to support health-promoting microbiota. Previous studies support this view as the exposure to farming environment pre- and postnatally, vaginal birth, having pets, and older siblings (Ege et al. 2008, Hesselmar et al. 1999, Ball et al. 2000), i.e. factors that are likely to increase exposure to variety of microbes, all associate with allergy-protection.

'*More is more*' truly challenges modern life by suggesting that drastic lifestyle changes need to be done in order to support health of the urban people. Much can be learned from populations rarely suffering from allergies or other non-communicable diseases. However, the adoption of rural and traditional lifestyles and environments to urban scene is challenging. Rural health-promoting factors may be difficult to reproduce in cities in scales that can support health of the large populations (King 2014). Probably a variety of creative solutions are needed, and those should be included in each life stage. Outdoor-daycares studied here provide one potential solution, and results show that nature-orientation in daycares can be supported by education and city-planning approaches (II). Moreover, the positive effects of rural living exceed just the effect of microbial exposure. Recently, it was discovered that sialic acid, produced by the cells of other mammals than humans, provide allergy-protection (Frei et al. 2017). Of course, also modern (city) life harbors positive sides that should not be transformed. For example, infectious diseases are rare and life expectancy is long in western societies. One suggested way to preserve positive sides of rural and urban living is 'targeted hygiene' meaning that exposure to infectious pathogens should be limited while maximizing the contact with beneficial microbes (Bloomfield et al. 2016).

One clear value of this thesis is the focus on less studied skin microbiota. It is rather obvious that gut microbiota has close contact with a host, and hence has important, systemic effect on the host health. However, recently the interest in allergy research has shifted to importance of the skin. Not surprisingly, the largest organ of the human body, the mediator of outer information, is more than just passive by-stander. Therefore, the microbial communities on the skin, can have large impact on human health (Grice and Segre 2011, Belkaid and Segre 2014). Consequently, the microbes in living environment, which has immediate contact with the skin, can be important immune regulators. This should alarm about homogenous urban environments where the contact with diverse microscopic world is limited. In cities, humans are the source of environmental microbiota (e.g. in public transports systems; Hsu et al. 2016) while in rural areas environment is the source of human microbiota.

The main message of this thesis is that the contact with environmental microbes in living environment and through lifestyle jointly either support or disturb host microbiota and susceptibility to develop allergies.

In other words, exposure to green environment, preferably in great amounts, shapes the skin microbiota in children and canine model, and associate with allergy-protection in canine model. Therefore, natural biodiversity in the level of micro-organisms serves an ecosystem service, which should be recognized (Rook 2013). The invisible diversity, which serves both planetary and human health, needs to be wholly preserved and protected.

6 ACKNOWLEDGEMENTS

There is only one way I can start this section. During autumn 2013, Ilkka asked if I would like to start PhD project under his supervision. Ilkka was slightly tipsy when he did that, but still it was a huge compliment for a young ecologist. I am lucky that I got change to enjoy Ilkka's experience few years, and I tried my best to absorb everything. When Ilkka got sick, I was unsure how everything will end up for me. Later, when he passed away, I realized that he had made sure that everything works for me and I am grateful for that. I got some surprising power after his death. I decided that I will do my best to finish this project.

The reason that I was able to make this decision true was my second supervisor Lasse. His door has always been open, and I have not waited a long time an answer to any of my questions. His "R clinic" has always been fully functional right away when I step in to his office. Lasse is a man of the details, both regarding writing and visualization (and sometimes clothing). That way he has pushed me to think again what I see already done and ready. Thank you Lasse that you have trusted, supported and belied in me!

Unusually, I had three supervisors. Third one, Tiina, has great experience in medical research, and her importance for these projects cannot be overlooked. Medical research includes numerous steps, starting from research permission, and Tiina masters these all. Tiina has also made sure that my articles or thesis does not include inflated statements about allergies. I appreciate the calming way Tiina has saved me from various immediate problems through these years.

Even more unusually, besides that I had three supervisors, I also had two, unofficial "supportive supervisors". Antti K and Hanna both worked as post docs in our group, and through our close collaboration they have teach me loads about analysis of microbiota data. Further, they both have always been highly supportive and great in person. I hope we stay friends forever!

I want to thank my opponent professor Harald Renz. It is great honor to have you as you have an amazing knowledge and experience in allergy research. Actually, Prof. Renz saved me as my first opponent unexpectedly cancelled. I also thank my custos Arild Husby for accepting this task. I am sure you are the right person to help me through my exiting defense day.

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