

Nature-oriented daycare diversifies skin microbiota in children—No robust association with allergies

To the Editor,

In Western societies, the majority of children are nurtured in daycares. About 80% of 3- to 5-year-old children attend formal child care in OECD countries.¹ As children spend daily a large proportion of their time in daycares, their practices can have an important contribution to the overall exposure of children to natural environments. This can be important, as allergic sensitization has been associated with limited connection with nature.²

The link between natural environments and allergy is thought to be mediated via exposure to beneficial environmental microbes.³ The skin microbiota is different in children living in urban or rural environments, reflecting different exposure to environmental microbiotas and varying lifestyle.⁴ We hypothesized that the nature connection in daycares can influence child microbiota and consequently affect the maturation of immunoregulation. We describe our pilot study comparing the skin microbiota and allergic outcomes (sensitization and self-reported symptoms) in children attending different types of daycares, considering the degree of nature contact.

Based on phone interviews, we invited 25 daycares to the study. From these, 16 daycares participated (64%) and were divided into *Nature-oriented*, *Conventional*, and *City-center* daycares (Table 1), based on their answers in a web-based questionnaire (response rate 100%, Form S1). We invited 224 5-year-old children from the enrolled daycares, with a participation rate of 30% ($n = 68$, 51% females). Sampling was organized in daycares. Nurses collected skin microbial samples from the volar forearm of the dominant arm, using sterile swabs, and 5 mm of venous blood. Guardians of the children filled a web-based questionnaire (response rate 100%, Form S2).

For more specific description of sampling, serum analysis, DNA extraction, sequencing, and land-use quantification, see Ref. 4. Shortly, V1-V3 region from bacterial 16 S rRNA gene was sequenced with Illumina Miseq in the Institute of Molecular Medicine Finland, University of Helsinki. The serum samples were analyzed with Phadiatop® test (ThermoFisher Scientific) for inhalant and food allergens. Addresses were used to retrieve geographic coordinates, which were then used for calculating the coverage of different land-use types around home and daycare (see Ref. 2 for details).

Children were classified as sensitized, separately for food and inhalant allergens, if the IgE for food or inhalant allergens was at least 0.35 kU/L. The child was defined symptomatic, if the answer was positive in any of the questions related to a possible allergic condition, that is, asthma, hay fever, and atopic dermatitis. The family- and lifestyle-related questionnaire data (Form S2) were simplified with

fuzzy clustering (package cluster in R)⁵ in order to create lifestyle groups.

Bioinformatics and quality control of microbiota (Table S1) data are described in supplementary methods. After normalization, sequence counts were square-root-transformed, in order to reduce the role of variable number of sequences per sample (library size). Even after normalization and transformation, the library size seemed to be an important factor in defining the diversity of the sample (Figure S1). Consequently, we included the transformed library size to each relevant analysis, for controlling its effect. However, because library sizes were higher in children attending *Nature-oriented* daycares (Figure S1), we argue that differences in library size can partly reflect biological differences in microbial abundances on the skin. The phylum, class, and genus level divisions in each daycare group are shown in Figures S2-S4.

Microbial diversity in samples was quantified using Shannon's index. In order to characterize between-sample differences in community composition, we used Bray and Curtis dissimilarity in distance-based analysis methods, that is, in principal coordinate analysis (PCoA) and permutational multiple regression on distance matrices (MRM, package vegan in R).⁶ We also described differentially abundant operational taxonomic Units (OTUs) between daycare types (package DeSeq in R).⁷ All 16S rRNA gene sequences have been deposited in the European Nucleotide Archive (accession no. PRJEB20097). The clinical datasets are available from the corresponding author.

Ethics approval was received 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). Sample collection and all subsequent experimental procedures were conducted in accordance with and following the Declaration of Helsinki. Permissions for studying local daycares were received from related municipalities. After study enrollment, but prior to the sampling, we asked the guardian of each child to provide a signed informed consent.

The composition of skin microbiota differed between children from *Nature-oriented* and other daycares (Figure 1A; pairwise comparisons with MRM, *Nature-oriented* vs *City-center*, $P = .004$; *Nature-oriented* vs *Conventional* daycare, $P = .004$). In contrast, microbial composition did not differ between children from *City-center* and *Conventional* daycares ($P = .092$). Moreover, microbial diversity was on average higher in children attending to *Nature-oriented* daycares, as compared with those attending other daycares (Figure 1B).

TABLE 1 Different daycare types and prevalence of allergic outcomes. Daycares were defined according to their characteristics (A-D)

	Nature-oriented daycare	Conventional daycare	City-center daycare
Daycare qualities			
(A) Surrounding environment	Natural/semi-natural	Semi-natural	Urban
(B) Outdoor time/day	≥4	≥2.5	≤2.5
(C) Nature trips/week	≥3	1	0
(D) Participation to organized nature activities	"Nature as a home"—concept daycare	"Forest story telling"—concept daycare	None
Num. of daycares	4	6	6
Num. of children	20	28	20
Allergic outcomes			
Sensitization (food)	25%	28%	30%
Sensitization (inhalant)	35%	25%	20%
Hay fever	5%	17%	10%
Asthma	5%	11%	10%
Atopic dermatitis	45%	54%	30%

None of the allergic outcomes showed significant differences between daycare types.

Several OTUs, many belonging to environmental Proteobacteria, were differently abundant between *Nature-oriented* and *City-center* daycares, while no OTUs differed significantly between the other daycare pairs (Table S2). Microbial communities were more similar (low β -diversity) among children attending the *City-center* daycares than among children attending *Conventional* or *Nature-oriented* daycares (Figure 1C), with *Nature-oriented* daycares showing the largest dissimilarity between children (high β -diversity) (in all comparisons $P = .001$, based on 999 permutations).

The observed difference between *Nature-oriented* and other daycares was not due to any single *Nature-oriented* daycare; that is, the skin microbiota of children in the same daycare did not cluster together (Figure 1A). Interestingly, the separation between the microbiota in *Nature-oriented* daycares and in other daycares tended to become stronger if children had stayed a longer time in a particular type of daycare (Figure S5).

The land use around daycares differed between daycare groups (Figure 1D, ANOVA, $F = 6.6$, $P = .011$). Pairwise comparison indicated that only *City-center* and *Conventional* daycares differed significantly in their surrounding land use (Tukey HSD, $P = .009$). Only a marginal difference in land use was observed between *City-center* and *Nature-oriented* daycares ($P = .09$). This indicates that land use alone does not explain the skin microbiota, but rather the actual contact with the more natural type of environment. The skin microbiota was least variable among *City-center* daycare children with artificial environment (Figure 1C,D).

The family- and lifestyle characteristics differed between children in different daycare types (Table S3). These characteristics were also associated with skin microbial composition, as the skin microbiota differed between all lifestyle groups (pairwise comparisons of lifestyle groups, $P_{1,2} = .032$, $P_{1,3} = .028$, $P_{2,3} = .019$). Consequently, it is difficult to tease apart the influence of daycare type and lifestyle on the skin microbiota. This was also evident in a partitioning of the

variation in microbial dissimilarity, showing that the shared fraction ($R^2_{\text{adj}} = 0.016$) was larger than those uniquely attributed to the daycare type ($R^2_{\text{adj}} = 0.014$) or the lifestyle ($R^2_{\text{adj}} = 0.0021$). Finally, we tested the effect of several explanatory factors by including them in a single multiple regression model. Library size, gender, and type of the daycare remained significant predictors of the variation in microbial dissimilarity between individuals (Table S4).

We did not discover any robust associations between the home and daycare environment, or the lifestyle of a family, with the incidence of possible allergy-related self-reported symptoms and sensitization. However, nature-oriented daycare seemed to provide some protection against respiratory symptoms (asthma and hay fever). We also did not detect any association between the skin microbiota and allergic symptoms or sensitization (Table 1). Also, the co-occurrence of self-reported symptoms and sensitization was poor.

Our results indicate that the prominent nature connection in daycares may be reflected in the skin microbiota of children. It appears that relatively intensive contact with outdoor environment, not just the presence of green surrounding (as in *Conventional* daycares), is required for compositional differences in skin microbiota. The time spent outdoors is probably important, as indoor spaces are enriched with human skin-associated bacteria⁸ and microbial diversity outdoors is much greater.⁹ However, the family- and lifestyle characteristics also influence microbial communities on skin. We conclude that several factors in child's life leave their mark on the skin microbiota. Thus, attending a *Nature-oriented* daycare is part of "nature-related lifestyle," which may be reflected in skin microbiota composition.

In this pilot study, no significant association could be found between reported symptoms or sensitization and daycare type or microbiota. There are several plausible explanations, such as small sample size ($n = 68$), poor measurement accuracy of symptoms (that has been discussed previously in Ref. 10) and duration or type of

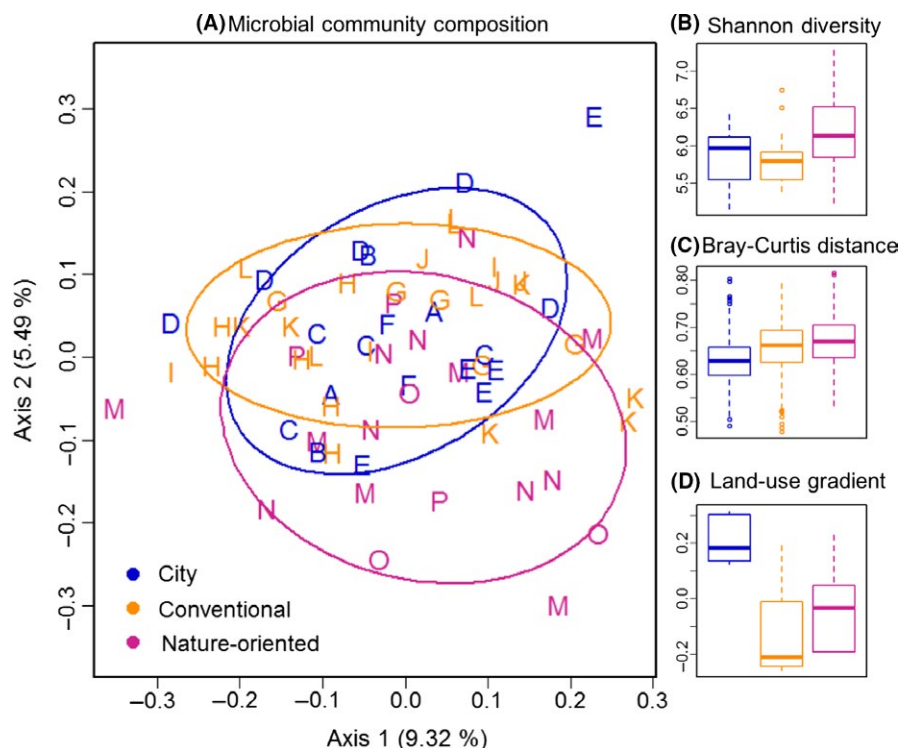


FIGURE 1 The skin microbiota is differently composed and more diverse in children attending *Nature-oriented* daycare in comparison with children in other daycares. A, Principal coordinate analysis of the skin microbiota in daycare children (Bray and Curtis dissimilarity). Colors indicate the type of daycare, and letters indicate individual daycares. The ellipsoids include 75% of the samples in each group. B, Shannon diversity of skin microbiota was higher in Nature-oriented daycares, as compared with the other groups (Nature-oriented vs City, $P = .04$, Nature-oriented vs Conventional, $P = .004$). C, Community composition of skin microbiota among individuals (i.e., β -diversity) was most similar in city-center daycare children and most dissimilar in children attending nature-oriented daycares. Microbial communities of children in conventional daycares were only slightly more similar than those of children in nature-oriented daycares (In all comparisons, $P = .001$). D, The land use around daycare in each daycare type. Higher values indicate larger proportions of built and other artificial coverages [Colour figure can be viewed at wileyonlinelibrary.com]

environmental exposure, and finally the young age of children (i.e., the allergic outcomes tend to be more established in older children).¹¹ Also, Finnish allergy program may have encouraged parents to enroll symptomatic children to nature-oriented daycares.¹² However, as the skin microbiota correlated with daycare type, and its systemic importance has been proposed,¹³ we suggest that longitudinal studies with larger cohorts should address the potential of daycare environment and practice in supporting balanced immune development and health.

ACKNOWLEDGMENTS

We thank study nurses Pirjo Jarke and Leena Ainali; laboratory workers Laura Lund, Annukka Ruokolainen, Laura Häkkinen, Tomi Issakainen, and Elina Poutanen; Laura Paalanen for helping in ethical permissions; Hanna Sinkko for helpful comments; and especially all daycares, children, and guardians who participated in this study. This study was funded by a grant from Jane and Aatos Erkko Foundation.

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REFERENCES

- OECD. Family Database. <http://www.oecd.org/els/family/database.htm>. Accessed December 10, 2016.

2. Ruokolainen L, von Hertzen L, Fyhrquist N, et al. Green areas around homes reduce atopic sensitization in children. *Allergy*. 2015;70:195-202.
3. von Hertzen L, Beutler B, Bienenstock J, et al. Helsinki alert of biodiversity and health. *Ann Med*. 2015;47:218-225.
4. Lehtimäki J, Karkman A, Laatikainen T, et al. Patterns in the skin microbiota differ in children and teenagers between rural and urban environments. *Sci Rep*. 2017;7:45651.
5. Maechler M, Rousseeuw P, Struyf A, et al. cluster: "Finding Groups in Data": Cluster Analysis Extended. <https://cran.r-project.org/web/packages/cluster/index.html>. Accessed January 2, 2016.
6. Oksanen J, Blanchet FG, Kindt R, et al. vegan: Community Ecology Package. R package version 2.3-2. 2015. <https://CRAN.R-project.org/package=vegan>. Accessed January 2, 2016.
7. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol*. 2010;11:R106.
8. Lax S, Smith DP, Hampton-Marcell J, et al. Longitudinal analysis of microbial interaction between humans and the indoor environment. *Science*. 2014;345:1048-1052.
9. Kembel SW, Jones E, Kline J, et al. Architectural design influences the diversity and structure of the built environment microbiome. *ISME J*. 2012;6:1469-1479.
10. Pekkarinen P, von Hertzen L, Laatikainen T, et al. A disparity in the association of asthma, rhinitis, and eczema with allergen-specific IgE between Finnish and Russian Karelia. *Allergy*. 2007;62:281-287.
11. Ruokolainen L, Lehtimäki JM, Karkman AV, Haahtela TK, von Hertzen L, Fyhrquist NT. Holistic view on health. Two protective layers of biodiversity. *Ann Zool Fenn*. 2017;54:39-49.
12. Haahtela T, Valovirta E, Bousquet J, Mäkelä M. The Finnish Allergy Programme 2008-2018 works. *Eur Respir J*. 2017;49:1700470.
13. Prescott S, Larcombe D, Logan A, et al. The skin microbiome: impact of modern environments on skin ecology, barrier integrity, and systemic immune programming. *World Allergy Organ J*. 2017;10:29.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

DOI: 10.1111/pai.12860

Increased sensitization to several allergens over a 12-year period in Brazilian children

To the Editor,

The prevalence of allergic sensitization and diseases, especially in the last decades, appears to have increased worldwide.^{1,2} Change in habits and climate has been discussed as major contributing factors.^{1,3} The first Brazilian Allergy Project (PROAL I) study was carried out in 2004 with the objective of documenting the sensitization rates to the major allergens in Brazilian allergic children.⁴ This was the first study of its kind, which contributed to better understanding of regional differences and enabled comparison with other countries. During the past decade, the prevalence of asthma and allergic diseases in children and adolescents has increased.² Due to this increase, we wanted to repeat the PROAL study with similar design. The objective of the study was to evaluate changes of sensitization over a period of twelve years for major allergens in Brazil.

The national PROAL studies I and II were carried out in 2004 and 2016, respectively, had a multicenter and cross-sectional design and covered most regions of Brazil. Included subjects presented with physician's diagnosis of an allergic disease (asthma, allergic rhino-conjunctivitis, eczema, and/or food allergy). Children who did not present clinical symptoms of allergy were invited to participate and to compose the control group. Recruitment was carried out by doctors. Allergic patients were collected consecutively at Pediatric allergy clinics and for control subjects, only those without any clinical characteristic of allergic disease were asked to participate. For this, patients in pediatric outpatient clinics were sought

to avoid selection bias. The sample size was calculated according to the results observed in the first study.⁴ Thus, a sample of 430 children and adolescents was recruited to estimate the frequency of sensitization, with a sampling error of 4.0% and a 95% confidence level, using an estimated dimensioning frequency variable in 73.0%.⁴ To compare allergic patients to the non-allergic group, it was necessary to evaluate, in the previous study, the percentage of sensitization of an allergen that was prevalent and in similar magnitudes between allergy and non-allergy groups. A two-way comparison test was used to estimate the difference in allergic sensitization. Calculations defined a sample composed of 360 patients with allergy and 73 participants without allergy (called the control group), assuming a sensitization ratio of 20% and 5%, respectively. Considering possible losses, the sample size was enlarged.

Eleven Brazilian Centers of Pediatric Allergy participated in the study ensuring geographical representation. Each center was requested to include 40 patients with allergic disease and 10 participants without allergy symptoms, with ages between 6 months and 18 years. Individuals with immunotherapy or immunosuppressive treatment were excluded. Recruitment for the study took place between August 2015 and November 2016, covering all allergen seasons.

A peripheral blood sample was collected from all participants for the determination of serum IgE and analyzed according to the manufacturer (ImmunoCAP® Thermo Scientific) for the following allergens:

SUPPLEMENTARY INFORMATION

Supplementary methods

Bioinformatics and quality control of skin microbiota data

The PCR primers were removed with cutadapt v. 1.9 (1), with maximum error rate of 0.2 and minimum match length of 15. Forward and reverse reads were merged with PEAR v.0.9.6. (2) using default options. Merged reads were trimmed with USEARCH v.8.0 (3): command *-fastq_filter* and options *-fastq_maxee 3, -fastq_minlen 365, -fastq_maxns 0*. OTU-clustering was done following the UPARSE pipeline (3). Chimeric OTUs were removed with UCHIME (4) as implemented in USEARCH. Representative OTUs were classified and aligned using mothur v. 1.36.1. (5) against the Silva v. 123 16S rRNA gene database (6). Contaminant OTUs were identified from the control samples, and were removed manually from the data (Tab. S1 shows removed OTUs). The samples were further normalized with Cumulative sum -normalization provided in the metagenoSeq-package in R (7).

Table S1. Table shows the OTUs which were removed from skin microbiota data as these are likely contaminants.

<i>OTU nro.</i>	Phylum	Class	Family	Genus
OTU27	Proteobacteria	Betaproteobacteria	Burkholderiaceae	Ralstonia
OTU4	Proteobacteria	Betaproteobacteria	Comamonadaceae	Curvibacter
OTU12	Proteobacteria	Betaproteobacteria	Burkholderiaceae	Ralstonia
OTU17	Proteobacteria	Betaproteobacteria	Burkholderiaceae	Burkholderia
OTU24	Proteobacteria	Betaproteobacteria	Comamonadaceae	Pelomonas
OTU29	Proteobacteria	Betaproteobacteria	Burkholderiaceae	Burkholderia
OTU50	Proteobacteria	Gammaproteobacteria	Pseudomonadaceae	Pseudomonas
OTU75	Proteobacteria	Alphaproteobacteria	Bradyrhizobiaceae	Bradyrhizobium
OTU90	Proteobacteria	Betaproteobacteria	Burkholderiaceae	Burkholderia
OTU3377	Proteobacteria	Betaproteobacteria	Burkholderiaceae	Burkholderia
OTU33	Proteobacteria	Betaproteobacteria	Oxalobacteraceae	Undibacterium
OTU62	Proteobacteria	Deltaproteobacteria	Oligoflexales_unclassified	
OTU536	Proteobacteria	Betaproteobacteria	Oxalobacteraceae	Massilia
OTU132	Proteobacteria	Gammaproteobacteria	Xanthomonadaceae	Stenotrophomonas

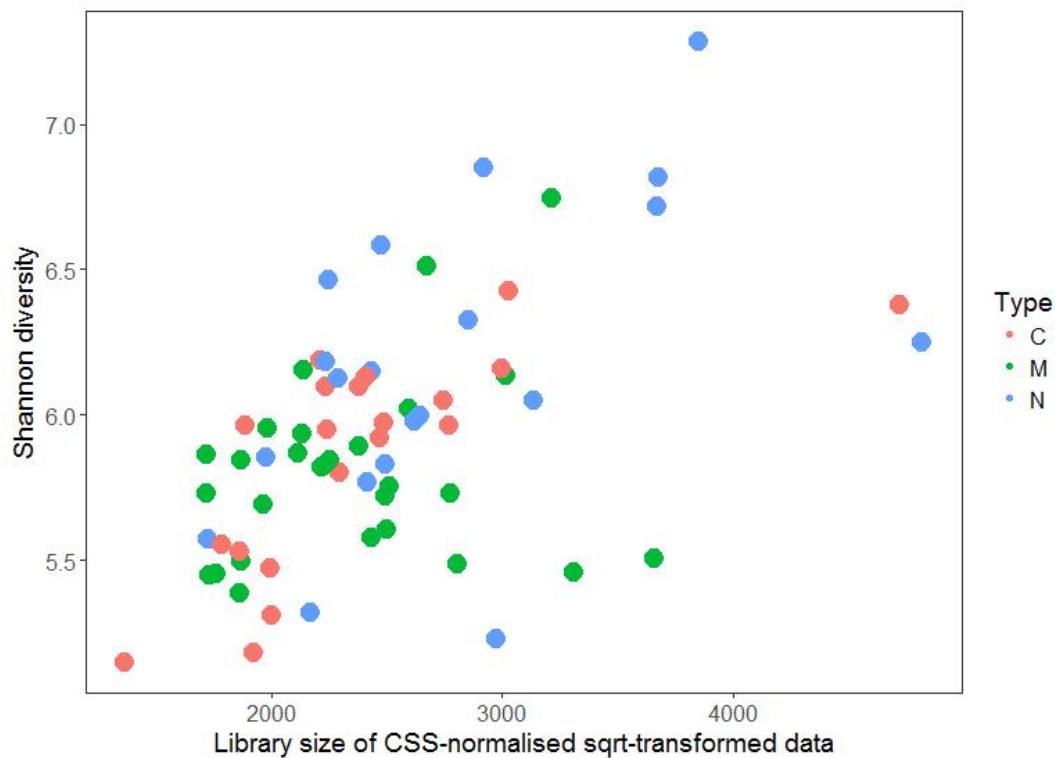


Figure S1. The normalized and sqrt-transformed library-size is related to the diversity of samples. Pattern is similar in original library sizes. Thus, library-size was included in each model where it was relevant for controlling its effect. Type: C = City-center daycare, M = Conventional daycare, N = Nature-oriented daycare. Normalized and transformed library sizes were higher in Nature-oriented daycare-children (mean library size (standard deviation) in each group: City-center, 2391(690); Conventional, 2356(514); and Nature-oriented, 2782(744), $P = 0.0605$).

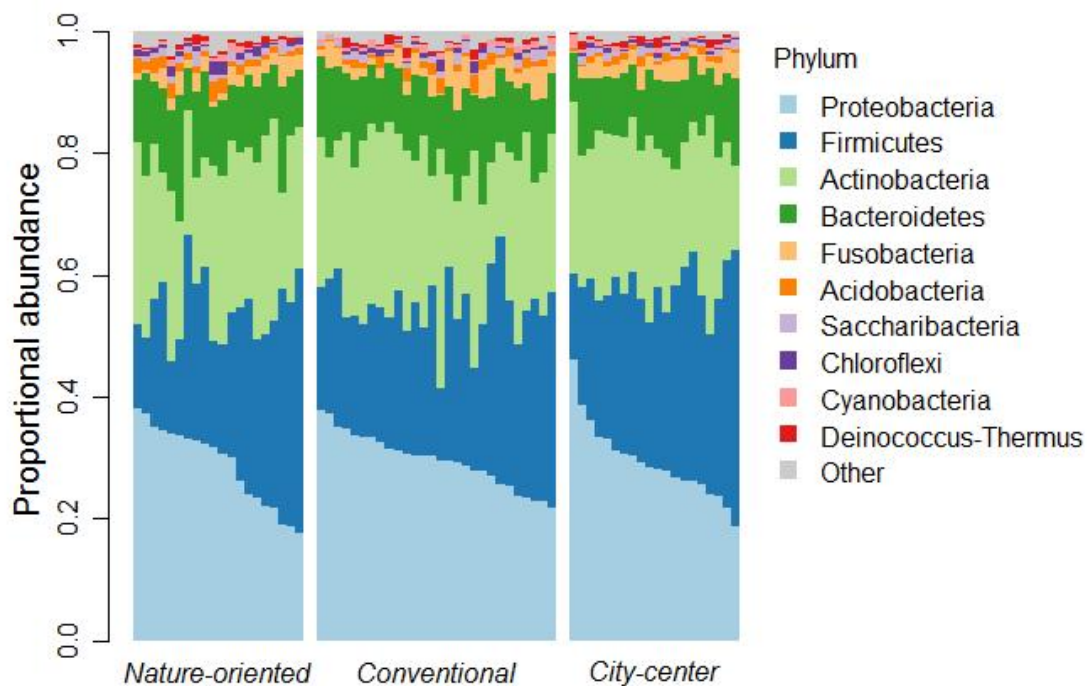


Figure S2. The skin microbiota in the Phylum level in each studied group.

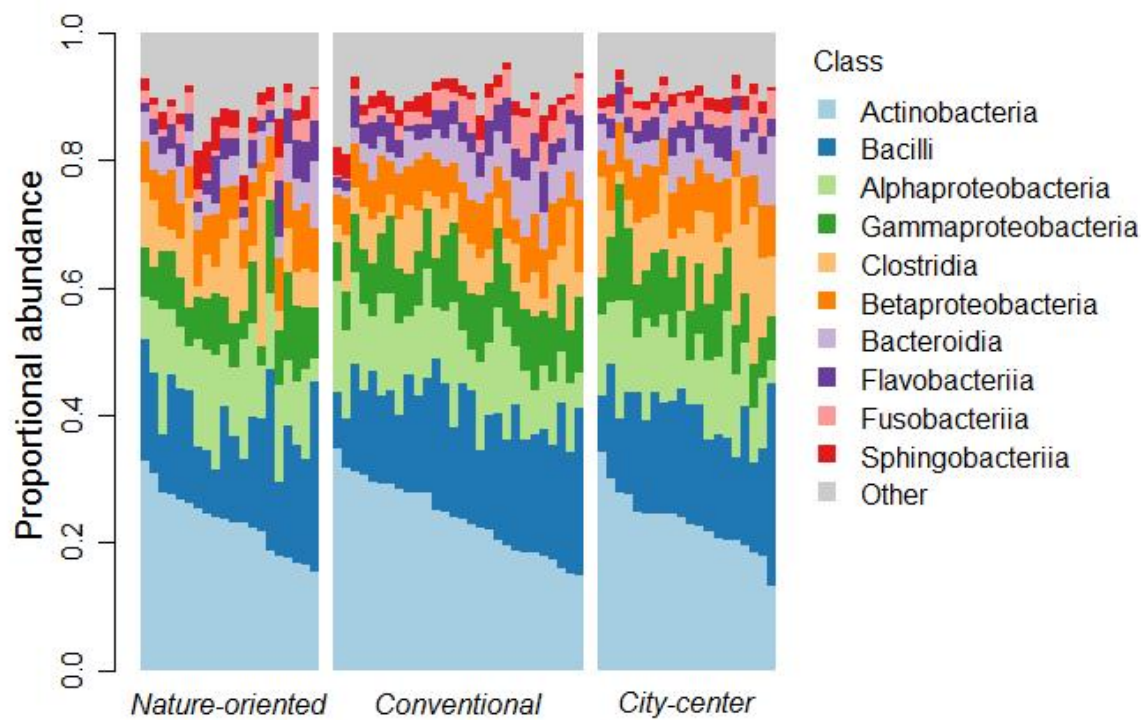


Figure S3. The skin microbiota in the Class level in each studied group.

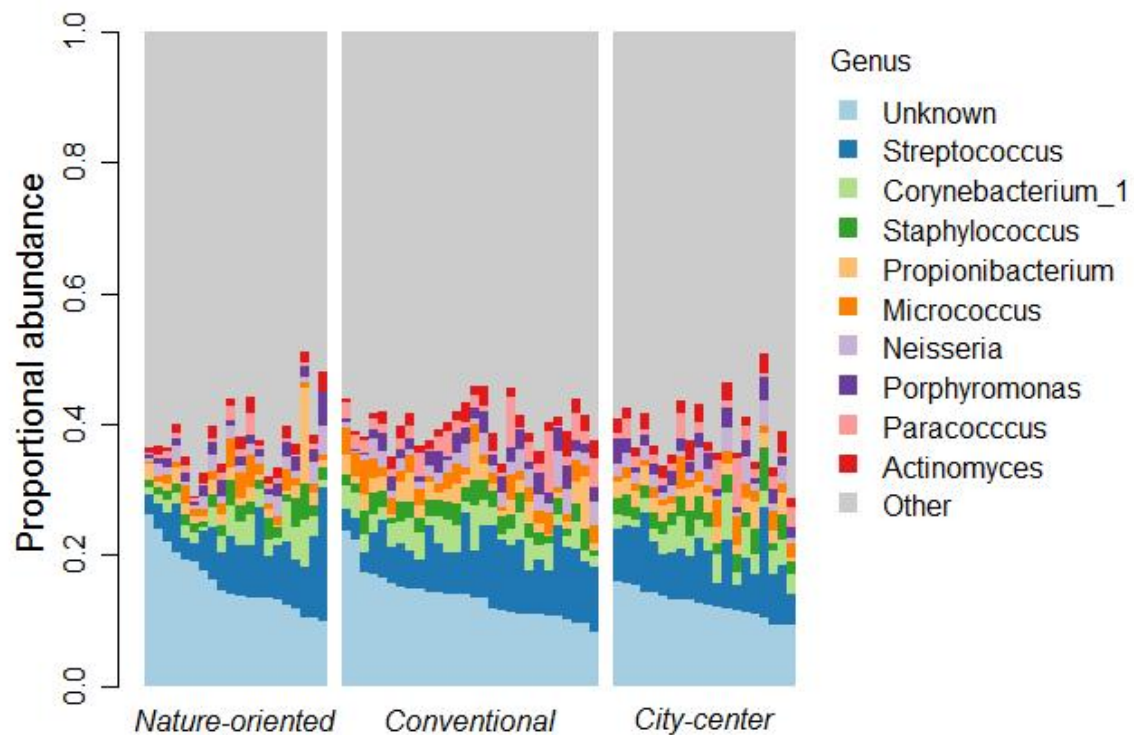


Figure S4. The skin microbiota in the Genus level in each studied group.

Table S2. Table shows the results of differential abundance analysis, which was done for raw data as the DEseq does its own normalization in the analysis. In the comparison between City-center daycares and Nature-oriented-daycares (C-N) several differently abundant OTUs were found. In the comparison between other pairs (C-M = City-center vs. Conventional, M-N = Conventional vs. Nature-oriented) no differently abundant OTUs were found in this analysis.

Groups	Adjusted P	Phylum	Class	Family	Genus
C-N	0.01231667	Proteobacteria	Betaproteobacteria	Oxalobacteraceae	Massilia
C-N	0.01231667	Acidobacteria	Acidobacteria	Acidobacteriaceae	Terriglobus
C-N	0.01690423	Proteobacteria	Alphaproteobacteria	Sphingomonadaceae	
C-N	0.02517188	Proteobacteria	Alphaproteobacteria	Rhodobacteraceae	Paracoccus
C-N	0.02517188	Actinobacteria	Actinobacteria	Nocardioidaceae	Nocardioides
C-N	0.03165840	Proteobacteria	Alphaproteobacteria	Acetobacteraceae	Endobacter
C-N	0.03165840	Actinobacteria	Actinobacteria	Frankiaceae	Jatrophihabitans
C-N	0.05884920	Actinobacteria	Actinobacteria	Nakamurellaceae	Nakamurella
C-N	0.05884920	Actinobacteria	Actinobacteria	Streptomycetaceae	Kitasatospora
C-M	-	-	-	-	-
M-N	-	-	-	-	-

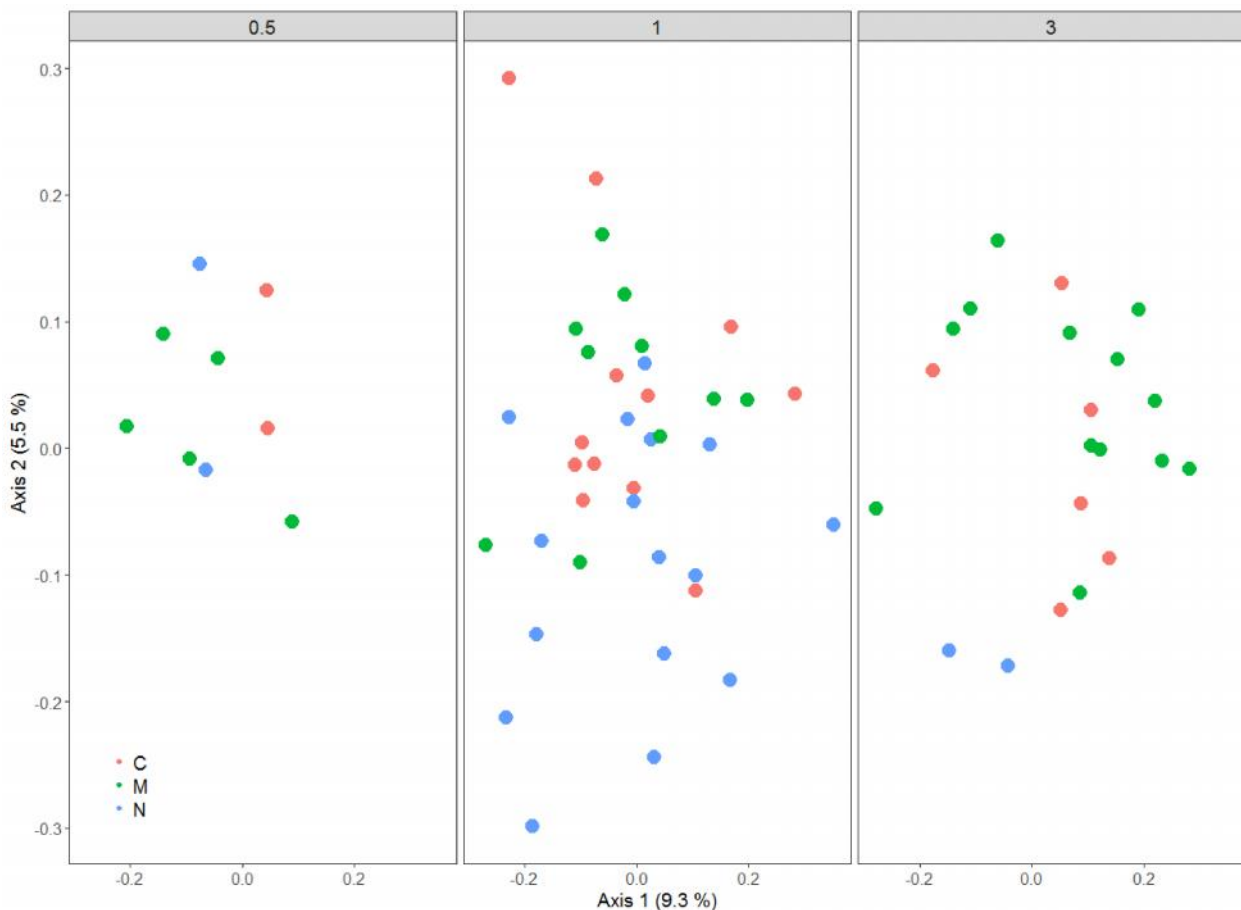


Figure S5. The panels show the PCoA of skin microbiota according the time (years) children have spent in current daycares. Data is small in each time point, but indicates that the separation between the skin microbiota in children attending nature-oriented daycares (N) and other daycare-children (C = City-center and M = Conventional) becomes larger the longer they have spent in current daycare.

Table S3. The children in daycare-type data were clustered with fuzzy clustering to three different lifestyle-groups. Table shows that children attending to Nature-oriented-daycares (N) mostly belong to lifestyle group 3. Thus it is hard to separate the effect of the lifestyle and daycare-type on the microbiota. M = Conventional daycare, C = City-center daycare.

Daycare-type	Lifestyle clusters		
	1	2	3
C	5	8	7
M	7	15	6
N	5	2	13

Table S4. The significant variables of the MRM-analysis of the skin microbiota. The effect of lifestyle- and family features was non-significant in the model.

Variable	Df	Sum of Sqs	Mean Sqs	F.Model	R2	P
sqrt-transformed, normalised library size	1	0.60	0.60	2.98	0.05	0.0009
Gender	1	0.31	0.31	1.54	0.03	0.007
Daycare-type	2	0.55	0.28	1.36	0.05	0.003

Supplementary Form S1. The questionnaire for daycares

Questionnaire for daycares

1. Basic information

1. Daycare:

2. Address:

3. Number of children:

4. Number of children per worker in the group(s) where children born during 2009 belong:

5. Daycare is

1 Municipal

2 Private

3 Purchase service daycare

4 Else, what?

2. Daycare building

6. Daycare building was build (year):

7. Daycare started in current building (year):

8. Daycare indoor size (m²):

9. Daycare is

1 in connection with school

2 in connection with hospital

3 in connection with rest home or with retard home

4 in separate daycare building

5 in townhouse

6 in old building which is renovated to daycare

7 in other building, which?

10. Has there been water damage in daycare?

1 No

2 Yes, when (year)?

11. Has there ever been smell of stale or cellar in daycare?

1 No

2 Yes

3. Daycare practices

12. Mark to the table how often following cleaning procedures are performed in daycare?

	Daily	Few times a week	Once a week	Once in two weeks	Once a month	Less
Floor sweeping						
Floor vacuuming						
Floor washing						

13. Mark to the table which activities children born during 2009 perform during average daycare day at certain time points:

	Daycare is closed	Eating	Sleeping	Outdoors	Free play	Controlled activities	Else
05-06							
06-07							
07-08							
08-09							
09-10							
10-11							
11-12							
12-13							
13-14							
14-15							
15-16							
16-17							
17-18							
18-19							
19-20							
20-21							
21-22							
22-23							

4. Daycare environment

14. Daycare is

- 1 in the city-center
- 2 in suburban
- 3 in conurbation (country-side)
- 4 in sparsely populated area

15. Distance (m) in to the road with heavy traffic

- 1 <50
- 2 50–200
- 3 200–500
- 4 500–1000
- 5 >1000

16. Distance (m) to the cattle farm or horse stable

- 1 <50
- 2 50–200
- 3 200–500
- 4 500–1000
- 5 >1000

5. Outdoors and nature-connection

17. Hours outdoors during average day when there is no snow:

18. Hours outdoors during average day when snow covers the ground:

19. Temperature limit (minus °C) for outdoor activities:

20. Daycare yard size (m²):

21. Mark to the table the percentage of different covers in the daycare yard. All percentages summed should be 100 %.

	%
Asphalt	
Sand	
Grain	
Lawn	
Unattended short vegetation	
Unattended ground with some vegetation	
Garden plot	
Trees or shrubs	
Plantation	

22. Children have a contact with dirt or ground (sand or grain is not counted) during open-ground season

- 1 daily
- 2 weekly
- 3 rarely
- 4 daily

23. Mark to the table how often daycare organises a trip to following environments. Mark also how long (hours) in average you spend in the location:

	weekly	monthly	yearly	time in hours
Forest				
Park				
Field/agriculture				
Farm				
Swamp				
Meadow				
Ocean, lake or river				

24. Has daycare director any nature-related education, which?
25. Has daycare worker(s) any nature-related education, which?
26. Daycare is part of some organized nature-related concept
- 1 No
 - 2 Yes, which?

6. Respondent

27. This questionnaire was filled by

- 1 Daycare director
- 2 Daycare worker
- 3 Someone else, who?

Feedback or more information:

Supplementary Form S2. The questionnaire for guardians of children. Questions which have been used for the definition of lifestyle-groups are marked with *.

Questionnaire for guardians

1. Basics

1. Date:

2. Birthday:

3. Gender

☐ male

☐ female

4. Daycare:

5. Time in current daycare (years and months):

6. Child was nurtured in

	less than a year	1-3 years	4 years
Home	☐	☐	☐
Current daycare	☐	☐	☐
Other daycare	☐	☐	☐
by relatives/friends	☐	☐	☐
Family daycare	☐	☐	☐
Other way, how?			

7. If your children has been in other daycare(s), write here the name(s) of daycare(s), the address(es) of daycare(s):

2A. Respiratory symptoms

8. Has there **ever** been wheezing sound in child's breath?

☐ No

☐ Yes

9. Has there been wheezing sound in child's breath **during the last twelve months**?

☐ No

☐ Yes

10. Has a child **ever** had asthma?

☐ No

☐ Yes, according guardian's own judgement

☐ Yes, diagnosed by a medical doctor

11. Has a child taken medicine for treatment of asthma **during the last twelve months**?

☐ No

☐ Yes

☐ Child does not have an asthma

2B. Rhinitis symptoms

All questions in this part concern the situation when a child **IS NOT having flu or respiratory tract infection!**

12. Has a child **ever** had sneezing, nasal congestion or sniffles when NOT having a flu or respiratory tract infection?

☐ No

☐ Yes

13. Has a child had sneezing, nasal congestion or sniffles when NOT having a flu or respiratory tract infection **during the last twelve months?**

☐ No

☐ Yes

14. Has a child had itchy or watery eyes in connection with these rhinitis symptoms **during the last twelve months?**

☐ No

☐ Yes

15. In which month(s) these rhinitis symptoms occurred **during the last twelve months** (you can choose many)?

☐ January

☐ July

☐ February

☐ August

☐ March

☐ September

☐ April

☐ October

☐ May

☐ November

☐ June

☐ December

16. In which age(s) a child has had hay fever symptoms (you can choose many)?

- ☐ Less than a year old
- ☐ One year old
- ☐ Two years old
- ☐ Three years old
- ☐ Four years old
- ☐ Five years old
- ☐ Child has never had a hay fever

17. If a child has had hay fever symptoms, is hay fever diagnosed by medical doctor?

- ☐ No
- ☐ Yes
- ☐ Child has never had a hay fever

2C. Eczema symptoms

18. Has a child **ever** had an itchy rash?

- ☐ No
- ☐ Yes

19. Has a child had an itchy rash **during the last twelve months**?

- ☐ No
- ☐ Yes

20. Has a child **ever** had itchy rash in some of the following sites: *in front of elbows, in back of knees, front side of ankles, buttocks, neck, or around of eyes or ears*?

- ☐ No

- ☐ Yes, constantly.
- ☐ Yes, but it disappears every now and then.

21. Has child **ever** had atopic dermatitis?

- ☐ No
- ☐ Yes, according guardian's own judgement.
- ☐ Yes, diagnosed by a medical doctor.

2D. Symptoms of parents

22. Does the biological **mother** of a child have..

	Yes, diagnosed	Yes, own judgement	No
.. rhinitis	☐	☐	☐
..asthma	☐	☐	☐
..atopic dermatitis	☐	☐	☐

23. Does the biological **father** of a child have..

	Yes, diagnosed	Yes, own judgement	No
.. rhinitis	☐	☐	☐
..asthma	☐	☐	☐
..atopic dermatitis	☐	☐	☐

3. Family and lifestyle

24. How many older and younger siblings a child has? *

___ older siblings and

___ younger siblings..

25. Which kind of education mother and father of a child has? *

	mother	father
Elementary or primary school	☐	☐
Middle or vocational school	☐	☐
High school or college	☐	☐
University	☐	☐

26. Family incomes in a month (including salary, income transfers and unearned income)? *

☐ Less than 2000 euros

☐ 2000–2999 euros

☐ 3000–3999 euros

☐ 4000–4999 euros

☐ 5000–5999 euros

☐ 6000–6999 euros

☐ More than 7000 euros

27. Does the guardians of a child or some other people who with child is daily, smoke? *

☐ No

☐ Yes

28. Is a child daily exposed to indoor smoking? *

☐ No

☐ Yes

29. The birth weight of a child was _____ grams.

30. Was a child breastfed? *

☐ No

☐ Yes, how long?

31. How old was a child when supplementary feeding started? *
_____ months old.

32. Do you use unpasteurised, unhomogenised or raw milk in your family? *

☐ No

☐ Yes, sometimes

☐ Yes, regularly

33. Do you use butter in your family? *

☐ No

☐ Yes, sometimes

☐ Yes, regularly

34. Has a child received antibiotics during the last twelve months? *

☐ No

☐ Yes, how many times?

35. Does a child have following food allergies?

	No	Yes	I don't know
fish	☐	☐	☐
egg	☐	☐	☐
milk	☐	☐	☐
grains	☐	☐	☐
citrus fruits	☐	☐	☐
nuts	☐	☐	☐
apple	☐	☐	☐
carrot	☐	☐	☐

4. Living environments

36. Address of the home of birth (i.e. the first home child lived after birth):

37. A child lived in the home of birth until the year (mark current year if a child is still living in the same apartment):

38. Address of the current home if different than the address of home of birth:

39. A child has lived in current home since (year):

40. A child has moved:

☐ Never

☐ Once

☐ Twice

☐ Three times

☐ More than three times

41. If a child has also lived in other than already mentioned apartments, write here the addresses of other homes:

42. Current home is in *

- ☐ the center of the city
- ☐ the center of the municipality
- ☐ suburban in city
- ☐ conurbation in countryside
- ☐ sparsely populated area

43. Current home is in *

- ☐ Apartment house
- ☐ Row house
- ☐ Town house
- ☐ Farm

44. Current home has *

- ☐ Own yard
- ☐ Shared yard
- ☐ No yard

45. A child spends time outdoors outside of yard *

- ☐ daily
- ☐ almost daily
- ☐ one to three times a week
- ☐ less than once a week

46. Did family practiced farming when child was a year old or younger? (gardening is not counted as a farming) *

- ☐ No

☐ Yes, full-time

☐ Yes, part-time

47. Does family practice farming currently? (gardening is not counted as a farming) *

☐ No

☐ Yes, full-time

☐ Yes, part-time

48. Does family has a dog? *

☐ No

☐ Yes, since?

49. Does family has a cat? *

☐ No

☐ Yes, since?

50. Does family has some other furry pet? *

☐ No

☐ Yes, since?

51. If you answer yes to question 50, which is this animal?

52. Is a child interacting with pets of other families at least once a week (for example in daycare or in relatives)? *

☐ No

☐ Yes, which animal?

53. Is a child interacting with farm animals (cow, pig, horse, goat, sheep etc.)? *

- ☐ Daily
- ☐ at least once a week
- ☐ at least once a month
- ☐ at least once a year
- ☐ less than once a year

5. Nature relatedness

54. Is family having a summer cottage? *

- ☐ No
- ☐ Yes, how many weeks child spends there yearly?

55. Does a child spend time in countryside with relatives or friends? (if family is not living at countryside) *

- 1 No
- 2 Yes, how many weeks child spends there yearly?

56. Mark how often a child visits at following environments: *

	Daily	Once a week	Once a month	Few times a year	Less than once a year
forest	☐	☐	☐	☐	☐
park	☐	☐	☐	☐	☐
field	☐	☐	☐	☐	☐
farm	☐	☐	☐	☐	☐
swamp	☐	☐	☐	☐	☐
meadow	☐	☐	☐	☐	☐
sea, lake or river	☐	☐	☐	☐	☐

57. Mark if child does some of the following things more than twice a year (you can choose many): *

- ☐ Mushroom picking
- ☐ Berry picking
- ☐ Fishing

- ☐ Hiking
- ☐ Boat trip
- ☐ Hiking overnight/camping.

58. When snow is not covering the ground, a child is in contact with natural soils (does not include concrete, gravel, tarmac or sand)? *

- ☐ daily
- ☐ weekly
- ☐ rarely
- ☐ never

6. Respondet

59. This form was filled by:

- ☐ mother
- ☐ father
- ☐ parents together
- ☐ someone else, who?

Feedback and more information:

Supplementary references

- Anders, S. and Huber, W. (2010). Differential expression analysis for sequence count data.
- Hertzen, L. von, Beutler, B., Bienenstock, J., Blaser, M., Cani, P.D., Eriksson, J., Färkkilä, M., Haahtela, T., Hanski, I., and Jenmalm, M.C., et al. (2015). Helsinki alert of biodiversity and health. *Annals of medicine* 47, 218-225.
- Kembel, S.W., Jones, E., Kline, J., Northcutt, D., Stenson, J., Womack, A.M., Bohannan, B.J., Brown, G.Z., and Green, J.L. (2012). Architectural design influences the diversity and structure of the built environment microbiome. *The ISME journal* 6, 1469-1479.
- Lax, S., Smith, D.P., Hampton-Marcell, J., Owens, S.M., Handley, K.M., Scott, N.M., Gibbons, S.M., Larsen, P., Shogan, B.D., and Weiss, S., et al. (2014). Longitudinal analysis of microbial interaction between humans and the indoor environment. *Science (New York, N.Y.)* 345, 1048-1052.
- Lehtimäki, J., Karkman, A., Laatikainen, T., Paalanen, L., von Hertzen, L., Haahtela, T., Hanski, I. and Ruokolainen, L. (2017). Patterns in the skin microbiota differ in children and teenagers between rural and urban environments.
- Maechler, M., Rousseeuw, P., Struyf, A., Hubert, M., Hornik, K., Stuber, M., Roudier, P. and Gonzalex, J. (2016). cluster: "Finding Groups in Data": Cluster Analysis Extended. <https://cran.r-project.org/web/packages/cluster/index.html>.
- OECD. Family Database. <http://www.oecd.org/els/family/database.htm>.
- Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R. and O' (2016). vegan: Community Ecology Package. R package version 2.3-2. <https://CRAN.R-project.org/package=vegan>.
- Prussin, A.J., Vikram, A., Bibby, K.J., and Marr, L.C. (2016). Seasonal Dynamics of the Airborne Bacterial Community and Selected Viruses in a Children's Daycare Center. *PloS one* 11, e0151004.
- Ruokolainen, L., Hertzen, L. von, Fyhrquist, N., Laatikainen, T., Lehtomäki, J., Auvinen, P., Karvonen, A.M., Hyvärinen, A., Tillmann, V., and Niemelä, O., et al. (2015). Green areas around homes reduce atopic sensitization in children. *Allergy* 70, 195-202.
- Ruokolainen, L., Lehtimäki, J.M., Karkman, A.V., Haahtela, T.K., von Hertzen, L., Fyhrquist, N.T., Ruokolainen, L., Lehtimäki, J., Karkman, A., and Haahtela, T., et al. (2017). Holistic View on Health. Two Protective Layers of Biodiversity. *Annales Zoologici Fennici* 54, 39-49.
- Weiss, S., Xu, Z.Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J.R., Vázquez-Baeza, Y., and Birmingham, A., et al. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5, 27.