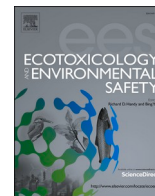




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A Placebo-controlled double-blinded test of the biodiversity hypothesis of immune-mediated diseases: Environmental microbial diversity elicits changes in cytokines and increase in T regulatory cells in young children

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ABSTRACT

Background: According to the biodiversity hypothesis of immune-mediated diseases, lack of microbiological diversity in the everyday living environment is a core reason for dysregulation of immune tolerance and – eventually – the epidemic of immune-mediated diseases in western urban populations. Despite years of intense research, the hypothesis was never tested in a double-blinded and placebo-controlled intervention trial.

Objective: We aimed to perform the first placebo-controlled double-blinded test that investigates the effect of biodiversity on immune tolerance.

Methods: In the intervention group, children aged 3–5 years were exposed to playground sand enriched with microbially diverse soil, or in the placebo group, visually similar, but microbially poor sand colored with peat (13 participants per treatment group). Children played twice a day for 20 min in the sandbox for 14 days. Sand, skin and gut bacterial, and blood samples were taken at baseline and after 14 days. Bacterial changes were followed for 28 days. Sand, skin and gut metagenome was determined by high throughput sequencing of bacterial 16 S rRNA gene. Cytokines were measured from plasma and the frequency of blood regulatory T cells was defined as a percentage of total CD3 + CD4 + T cells.

Results: Bacterial richness ($P < 0.001$) and diversity ($P < 0.05$) were higher in the intervention than placebo sand. Skin bacterial community, including Gammaproteobacteria, shifted only in the intervention treatment to resemble the bacterial community in the enriched sand ($P < 0.01$). Mean change in plasma interleukin-10 (IL-10) concentration and IL-10 to IL-17A ratio supported immunoregulation in the intervention treatment compared to the placebo treatment ($P = 0.02$). IL-10 levels ($P = 0.001$) and IL-10 to IL-17A ratio ($P = 0.02$) were associated with Gammaproteobacterial community on the skin. The change in T_{reg} frequencies was associated with the relative abundance of skin Thermoactinomycetaceae 1 ($P = 0.002$) and unclassified Alphaproteobacteria ($P < 0.001$). After 28 days, skin bacterial community still differed in the intervention treatment compared to baseline ($P < 0.02$).

Conclusions: This is the first double-blinded placebo-controlled study to show that daily exposure to microbial biodiversity is associated with immune modulation in humans. The findings support the biodiversity hypothesis

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of immune-mediated diseases. We conclude that environmental microbiota may contribute to child health, and that adding microbiological diversity to everyday living environment may support immunoregulation.

1. Introduction

According to the biodiversity and old friends hypotheses, poor exposure to environmental microbiota is one of the key factors predisposing to immune-mediated diseases (Haahtela, 2019; Hanski et al., 2012; Rook et al., 2003). Well-functioning immune regulation and immune tolerance and low incidence of immune-mediated diseases has been reported to be associated with living next to agricultural areas and green space (Hanski et al., 2012; Kirjavainen et al., 2019; Nurminen et al., 2021). Similarly, people following traditional lifestyles with abundant contacts with environmental microbiota typically have diverse commensal microbiota and low incidences of immune-mediated diseases (Alfven et al., 2006; Kondrashova et al., 2013; Lee et al., 2012; Stein et al., 2016; Williams et al., 2005). On the other hand, agricultural environment may enrich bacteria, such as *Thermoactinomyces vulgaris*, *T. viridis* and *T. sacchari*, that have been associated with inflammatory reactions and eventually hypersensitivity pneumonitis (Nogueira et al., 2019). Other factors affecting the human microbiota include diet, genetics and lifestyle factors, e.g. hygiene level and use of antibiotics (Graham-Rowe, 2011; Shreiner et al., 2008). Western type diet is more common among urban dwellers and characterized with more processed industrialized food (De Filippo et al., 2017; Sonnenburg and Sonnenburg, 2019) that may confound gut microbiota results when comparing urban and rural populations in the context of biodiversity exposure. The old friends and biodiversity hypotheses are further supported by recent findings that built areas limit the transfer of rich environmental microbiota indoors (Parajuli et al., 2018), diverse yard vegetation and diverse gut microbiota are interrelated (Hui et al., 2019b; Parajuli et al., 2020), and that vegetation diversity may protect against childhood asthma and leukemia (Donovan et al., 2021, 2018). Because increase of chronic inflammatory disorders, including allergies, autoimmunity and inflammatory bowel disease, has become obvious in many developed countries, scientists and health professionals have highlighted the need for reconnection to nature, restoration and preservation of ecosystems, re-building urban habitats and microbiome rewilding (Haahtela et al., 2021; Mills et al., 2020; Prescott, 2021; Robinson et al., 2021; Rook and Lowry, 2022; Watkins et al., 2020).

Despite years of comparative research on living environment, microbiota, immune response and disease incidence, the biodiversity and old friends' hypotheses were never tested in a double-blinded, placebo-controlled intervention trial. Indeed, the effect of biodiversity interventions on commensal microbiota and immune system have been surveyed in two human trials only. Nurminen et al. (2018) performed a safety trial in which people were asked to rub their hands in soil and plant-based materials three times a day for two weeks. Roslund et al. (2020) inserted vegetated, microbiologically diverse forest floor, sod, planting boxes and peat blocks to daycare center yards and reported changes in microbiota, as well as blood cytokine levels and regulatory T (T_{reg}) cell frequencies among young children. In both studies, immune regulation was enhanced, and commensal microbiota became more diverse. However, the control received no placebo treatment in the studies by Nurminen et al. (2018) and Roslund et al. (2020). In addition to these studies, exposure to organic soil and plants is known to enrich skin and respiratory tract microbiota among urban dwellers (Grönroos et al., 2019; Nurminen et al., 2018; Roslund et al., 2021; Selway et al., 2020), and reduce the prevalence of bacterial genera containing pathogens (Hui et al., 2019a). Furthermore, exposure to high biodiversity soil can change gut microbiota with potential mental health benefits (Brame et al., 2021; Liddicoat et al., 2020). As even a placebo effect could theoretically change the function of immune system (Hadamitzky et al., 2018) and its effect on commensal microbiota cannot be excluded,

the definitive proof of the effect of biodiversity on immune system is still lacking.

Plasma cytokine levels and T_{reg} cells were associated with exposure to diverse microbiota in previous intervention study with no placebo control (Roslund et al., 2020). T_{reg} cells play a pivotal role in the maintenance of the balance between the tissue damaging and protective effects of the immune response (Vignali et al., 2008). Interleukin-10 (IL-10) is one of the key anti-inflammatory cytokines found within the human immune response (Opal and DePalo, 2000), while interleukin-17 (IL-17) is a proinflammatory cytokine that is associated with several immune-mediated diseases, including inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis (Jin and Dong, 2013). Therefore, T_{reg} cell frequencies, IL-10 and IL-17 can be used as surrogates for changes in immunoregulatory pathways.

Here we report the results of a randomized, double-blinded and placebo-controlled intervention trial, in which daycare children were exposed to microbially diverse or poor sand. We measured sand, skin and gut microbiota, plasma cytokine levels and T_{reg} cell frequencies in these children on days 0 and 14. Microbial communities were also analyzed from sand, skin and gut on day 28. We compared microbiota between the intervention and placebo sandbox sand mixtures. The study had five objectives that were expressed as hypotheses. First, we hypothesized that biodiversity in sand would affect the commensal microbiota of the children. Secondly, we hypothesized that changes in the levels of the immunomodulatory IL-10 cytokine and the ratio between IL10 and IL-17A cytokines would be different in the intervention and placebo arms. Thirdly, as skin Gammaproteobacteria were associated with enhanced immune modulation in earlier studies (Fyhrquist et al., 2014; Hanski et al., 2012; Riskumäki et al., 2021; Roslund et al., 2020), we hypothesized that enriched sand affects skin Gammaproteobacteria. Based on this we expected that Gammaproteobacterial community on the skin is related to plasma IL-10 level and the IL-10:IL-17A ratio. Finally, we expected the changes in regulatory T cells to be associated with changes in skin microbiota. In short, the main objective was to investigate potential changes in commensal microbiota and associations between these and immune markers.

2. Materials and methods

2.1. Experimental design

Six daycare centres locating in the city of Lahti in the southern Finland participated in the study, out of which two were randomly selected to the intervention arm and the remaining four in the placebo arm (13 participants per treatment group) (Table S1). Similar sandboxes (1.5 m X 1.5 m) were installed in the yards of intervention and placebo daycares at the end of May 2018. In intervention daycares, the sandboxes were filled with regular sand of glacial origin (Trade name: Kekkilä Leikkihiekka) that was enriched with biodiverse powder (1:1 ratio) that contained composted materials comprising agricultural stack, six commercial gardening soils (trade names: Musta Multa, Niittymulta, Nurmikkomulta, Perennamulta, Puistomulta and Viljelymulta), deciduous leaf litter, peat, and Sphagnum moss. The biodiversity powder has been described in detail in our previous studies (Grönroos et al., 2019; Hui et al., 2019a; Nurminen et al., 2018). In the placebo daycares, the sandbox was filled with regular sandbox sand accompanied with placebo material, blond peat (Kekkilä, Finland), at a ratio of 10:1. The visible similarity of the intervention and placebo sands ensured that the participating children, their parents, daycare personnel and the assistants supervising children and collecting the samples were unable to distinguish between intervention and placebo daycares. We earlier

measured the 16S rRNA counts of biodiversity powder and the sand (Hui et al., 2019a). They were 3.5×10^9 and 1.3×10^7 sequences per gram soil, respectively. In peat, the 16S rRNA abundance was 1.3×10^8 . As the mixing ratio was 1:1 in the intervention and 1:10 in the placebo treatment, the soil used in our current study contained $> 1 \times 10^9$ and 1.3×10^7 16S sequences per gram in the sandbox at baseline.

Twenty-six children, aged between 3 and 5, were recruited in the study (Table S1). For the first 14 days (supervised period), research assistant visited the daycares twice daily for 20 min in the morning and 20 min in the afternoon on weekdays and organized activities in the sandbox to ensure that the children played within the sandbox and were in direct contact with the sand. For the following 14 days, the sandboxes were kept at the daycares and children were allowed to play freely inside the sandbox with no guided activities to estimate how permanent microbial changes on the skin are. Thus, the intervention period was altogether 28 days. Research assistant recorded every day whether all of the study subjects participated in the organized activities in the sandbox.

Children were served three uniform meals daily (breakfast, lunch, and afternoon snack) in the participating daycare centers prepared by central kitchen of Lahti city. All the daycare children spend approximately 0.5–2 h twice a day outside at the daycare yard in all daycare centers in the study area. Background information was sought from the children's guardians. Private consumption of berries and vegetables, time spent outdoors or in nature, contact with animals, sick days, form of housing, and number of siblings were similar among day-care groups ($P > 0.07$; Table S2D).

The study was carried out in accordance with the recommendations of the "Finnish Advisory Board on Research Integrity" with an approval from the ethics committee of the local hospital district (Pirkanmaa Hospital District, Finland). Written informed consents were obtained from all guardians that were in accordance with the Declaration of Helsinki. The trial has been registered in ClinicalTrials.gov (ID NCT03997175).

2.2. Sample collection

Sand, skin swab and stool samples were collected for microbial analyses at three time points; at baseline (day 0), after two weeks supervised period of guided activities in the sandbox (day 14), and after four weeks follow up period (day 28). Blood samples were collected at the baseline and on day 14 for immunological analyses.

A trained study nurse collected skin swab samples in daycare centers, and the children's parents collected stool samples at home. Skin samples were taken in the afternoon and not immediately after the supervision. Children behaved normally regarding hand washing. The 3–5-year-old children washed their hands by themselves after using a toilet, and thus the palms were washed several times a day. Children were not asked to wash their hands before collecting skin swabs. Skin swab samples were collected from the back of each child's dominant palm with a sterile cotton-wool stick wetted in 0.1% Tween® 20 in 0.15 M NaCl (2×2 cm area, 10 s wiping). Skin swabs were immediately put in dry ice and stored at -70 °C until further processing. One control field blank swab sample per a daycare center was taken at the same time as skin swab samples by holding the sterile swab stick for ten seconds to the room air.

Guardians collected the stool samples into sterile plastic tubes. These were stored in home freezers (-18 to -20 °C) for 1–2 days until the researchers collected the samples from homes and transferred them into -70 °C.

Sand samples were collected with sterilized spoon from the intervention and placebo sandboxes randomly from five locations (depth 1–5 cm). Samples were also collected from each raw material used in manufacturing the enriched sand, and from the peat used in placebo sandboxes.

2.3. Microbial analyses

DNA was extracted with PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's standard protocol. The V4 region within the 16S rRNA gene was amplified by PCR (three technical replicates from each sample) using 505F and 806R primers (Caporaso et al., 2012). Negative controls (sterile water) and control field blanks were included during the sampling process and at all further steps (DNA extraction, PCR, and sequencing controls) and a positive control (*Cupriavidus necator* JMP134, DSM 4058) was included in each PCR to ensure the quality of the analysis. Paired-end sequencing of the amplicons (2×300 bp) was performed on an Illumina Miseq instrument using a v3 reagent kit.

Raw paired-end sequence files were processed using Mothur version v1.35.1 (Schloss et al., 2009) and as described earlier (Roslund et al., 2020, 2018). Sequences were aligned using the Mothur version of SILVA reference database v132 (Pruesse et al., 2007) and assigned to taxa using the Naïve Bayesian Classifier (Wang et al., 2007) against the RDP training set (version 10). Non-target sequences (mitochondria, chloroplast, Archaea) were removed. Sequences were clustered to OTUs with 97% similarity using OptiClust that assigns sequences to OTUs. OTUs found in negative controls were removed from sequence data considering possible index hopping. If the abundance of an OTU was ≤ 10 sequences across all experimental units, it was excluded from statistical analysis as most of the low-abundance OTUs are PCR or sequencing artifacts (Brown et al., 2015; Tedersoo et al., 2010).

Samples were subsampled to the smallest sample sequence depth for community composition analyses. Skin samples were subsampled to 1968, stool samples to 1460 and soil samples to 4274 sequences. Four stool samples were discarded from the downstream analyses due to low sequence reads (< 1460). Good's coverage index (average \pm SD: soil 0.91 ± 0.06 , stool 0.98 ± 0.01 and skin 0.92 ± 0.07) was used to determine OTU coverage adequacy for diversity and community composition analyses. We estimated richness and Shannon and Simpson diversity metrics for total bacterial communities in Mothur with the summary.single command.

2.4. Separation of plasma and peripheral blood mononuclear cell (PBMC) samples

A venous blood sample was taken from the arm vein into Vacutainer® CPT™ Mononuclear Cell Preparation tubes with sodium citrate (BD Biosciences, NJ, USA) and centrifuged according to manufacturer's instructions to prepare the plasma and peripheral blood mononuclear cells (PBMCs). PBMCs were frozen in freezing medium consisting of 10% DMSO (Merck KGaA, Darmstadt, Germany), 10% human AB serum (Sigma-Aldrich, MO, USA), 50 U/mL Penicillin and 50 µg/mL Streptomycin (Sigma-Aldrich, MO, USA), and 10 mM L-glutamine (Life Technologies, CA, USA) in RPMI-1640 medium (Life Technologies, CA, USA) using freezing containers at -80 °C (BioCision LLC, CA, USA). The PBMC samples were transferred into liquid nitrogen for long-term storage after 48 h. The plasma samples were stored at -80 °C.

2.5. Plasma cytokine and T cell analyses

IL-17A and IL-10 concentrations were measured from plasma samples using the Milliplex MAP high sensitivity T cell panel kit (Merck KGaA, Darmstadt, Germany) according to manufacturer's instructions. Fluorescence was analyzed using the Bio-Plex® 200 system (Bio-Rad Laboratories, Hercules, CA, USA) and data were collected using the Bio-Plex Manager software (version 4.1, Bio-Rad Laboratories, Hercules, CA, USA).

Frozen PBMCs were thawed and rested overnight at 37 °C and 5% CO₂ in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 20 mM 2-ME, 1 mM sodium pyruvate, nonessential amino acids, 100 IU/mL penicillin, 100 mg/mL streptomycin, 10 mM HEPES (all from

Lonza), and 5% inactivated human AB serum (Sigma-Aldrich). Immunostaining to identify CD3 + CD4 + CD25 + CD127lowFOXP3 + Treg cells was performed as previously described in (Viisanen et al., 2019). The frequency of Tregs was defined as a percentage of total CD3 + CD4 + T cells. For the determination of intracellular cytokine expression within CD3 + CD4 + memory T cells, PBMCs were stimulated and processed as described previously in (Viisanen et al., 2017). Intracellular cytokine staining was performed with antibodies to IL-2 (APC-R700, BD), IL-21 (PE, Biolegend), TNF- α (BV421, Biolegend) and IFN- γ (Alexa Fluor 488, Biolegend). All flow cytometry samples were acquired on a Novocyte Quanteon flow cytometer (Agilent), and the data were analysed with FlowJo software (BD Biosciences).

2.6. Statistical analyses

All the statistical tests were done with R v3.6.1 (R Core Team, 2018) and with *vegan* (Oksanen et al., 2019) and *lme4* (Bates et al., 2015) packages. Beta diversity of bacteria was analyzed between children in intervention and placebo daycare centers with Permutational Multivariate Analysis of Covariance (PERMANOVA, function *adonis* in *vegan* package) using Bray-Curtis distances (Anderson, 2017). Two independent PERMANOVA analyses were done: one within time-points to estimate differences between daycare treatments and another between time-points to estimate the changes within daycare treatments. PERMANOVA was performed at OTU, genus, order, family, class and phylum levels with abundance and presence/absence (standardization method “pa” with *decostand* function) datasets. In addition, PERMANOVA was done within specific phyla and class if the relative abundance was over 1% for skin and stool samples, and 0.2% for soil samples. Multivariate homogeneity of group dispersions (PERMDISP, function *betadisper* in *vegan* package) was used to examine if the significant differences in PERMANOVA are caused by different within-group variation (dispersion) instead of different centroid positions. Principal coordinates analysis (PCoA) with Bray-Curtis distance was used to visualize the difference in bacterial community composition (*cmdscale* function). Shannon and Simpson diversity indices were determined using the function *diversity* and species richness using the function *specnumber* in the *vegan* package. In *vegan* package Shannon index is defined as $H = -\sum p_i \log(b) p_i$, where p_i is the proportional abundance of species i and b is the base of the logarithm. Simpson's index are based on $D = \sum p_i^2$ and choice *simpson* in *vegan* package returns 1-D.

Linear mixed-effect models (LMM) (function *lmer* in *lme4* package) were constructed to analyze temporal shifts in bacterial variables, taking into account clustering of participants into the daycare centers. To test differences in bacterial measurements between daycare treatments: bacterial richness, diversity or relative abundance was used as the dependent variable; daycare treatment and time-point as a repeated measures factor (fixed factor); and individual participants nested within the cluster (daycare center) as a grouping variable (random factor) in LMM models. To test the correlation between bacterial measurements and immune parameters, cytokine expression or Treg frequency change was used as a dependent variable, bacterial changes as explanatory variables, and daycare center and gender as a random variable. To estimate the difference in cytokine change between treatments, we used interaction between treatment and time in the LMM model as recommended by (Twisk et al., 2018). In detail, cytokine expression was used as a dependent value, the interaction between treatment and time as an explanatory variable and individual participants nested within the cluster (daycare center) as random factor in LMM model.

To test the correlation between Gammaproteobacterial community and cytokine expression, non-metric multidimensional scaling (NMDS) with Bray-Curtis metric was used to score the Gammaproteobacterial OTUs onto an ordination and correlation with corresponding cytokine expression levels and Treg frequencies was assessed using function *envfit* in *vegan* package (Oksanen et al., 2019) as in Roslund et al. (2020). The function *envfit* calculates multiple regression of variable with ordination

axes (immune parameters are used as dependent and selected ordination axes as explanatory variables). Significance is tested by permutation test. The differences in the bacterial variables between daycare treatments were also determined using t-test or, in cases of non-normally distributed data, with Wilcoxon signed-rank test. LMM models, t-tests and Wilcoxon signed-rank tests were performed for bacterial taxa with relative abundances of at least 0.1%.

To conceptualize the false discovery rate (FDR), all the statistical tests were carried out with Benjamini-Hochberg correction (referred to as Q value in the results). All statistical tests were considered significant at $Q < 0.05$ level.

3. Results

3.1. Intervention sandboxes had higher microbial diversity compared to placebo and regular sand

Intervention sand had higher richness (P and $Q < 0.001$) and diversity (Shannon and Simpson indices) of bacteria (P and $Q < 0.05$), including main phyla and classes (Fig. 1A and B). The relative abundance of unclassified genera within Alpha-, Beta-, and Gammaproteobacteria ($P < 0.004$ and $Q < 0.05$) and 25 genera were higher in enriched sand compared to placebo sand (Data in brief's article: Table 1). Placebo sand contained a higher relative abundance of *Halomonas*, *Herbaspirillum* and unclassified genera within Comamonadaceae ($P < 0.03$ and $Q < 0.05$, Data in brief's article: Table 1).

Community composition of bacteria from the OTU to the phylum level differed between the enriched intervention sand and placebo sand ($F > 15$, $R^2 > 0.72$, $P \leq 0.03$, $Q < 0.05$). The differences between enriched intervention and placebo sand were observed in case of phyla Actinobacteria, Bacteroidetes, Planctomycetes, Gemmatimonadetes, Firmicutes and Proteobacteria, and proteobacterial classes Alpha-, Beta- and Gammaproteobacteria (Data in brief's article: Table 2 A). The same differences occurred between the enriched sand and regular commercially available sandbox sand that was used in the current study ($F > 23$, $R^2 > 0.75$, P and $Q < 0.01$, Data in brief's article: Table 2B). In contrast, community compositions were similar in placebo sand and regular sand ($P > 0.2$, Data in brief's article: Table 2 C). No changes in bacterial community composition were found in the enriched or placebo sand during the four-week study period ($Q > 0.3$; Data in brief's article: Table 2 D and E).

3.2. Intervention shifted skin microbiota towards sandbox bacterial community

Number of children included in the analysis was 13 participants per treatment group. The mean age of children was 4.4 years and standard deviation 0.7. Children participated to organized activities in the sandbox every weekday, except one child was absent from both treatments once during the intervention.

On day 0, the intervention and placebo treatments did not differ in skin bacterial richness, alpha diversities, community composition and relative abundance (t test $Q > 0.1$; see Fig. 1C-F). The exception was the class Alphaproteobacteria community composition for which the between-treatment difference disappeared during the supervised period (day 14) (Table S3B).

On day 14, similarity in the bacterial composition of the skin and sand microbiota increased only in the intervention treatment, but not in the placebo treatment (Table S4). The richness of Firmicutes, particularly classes Bacilli and Clostridia, increased in the intervention treatment compared to baseline ($P < 0.001$; Fig. 2, Table S5A). Bacilli was the only class which community composition differed between the intervention and placebo treatments on day 14 (Table S3 B; Data in Brief's article Fig. 2B). Within class Bacilli, the relative abundance and richness of family Thermoactinomycetaceae 1 increased during the supervised period in the intervention treatment, being higher compared to

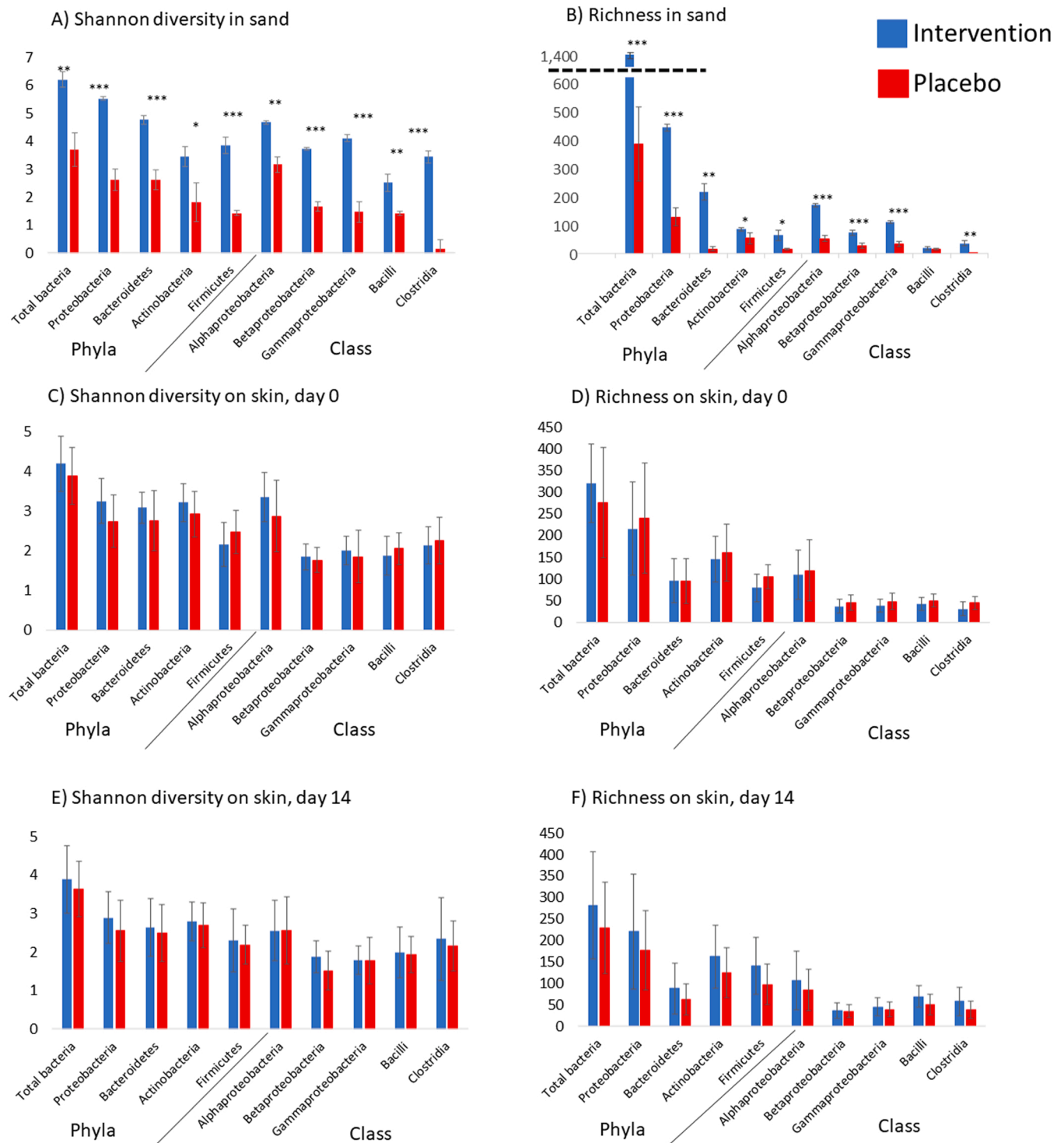


Fig. 1. A) Shannon diversity and B) richness (number of OTUs) at phyla and class levels in enriched intervention sand and placebo sand at baseline (day 0), C) Shannon diversity and D) richness on skin on day 0 between treatment groups, E) Shannon diversity and F) richness on skin on day 14 between treatment groups. Data are displayed as means \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P \leq 0.001$, t tests.

placebo treatment on day 14 (Table S5). Within this family, *Thermoactinomyces vulgaris* was the most abundant bacterial species on the skin and in enriched sand (Table S6B and C). In the intervention treatment, relative abundance of more than 30 genera shifted on the skin during the intervention period, including a decrease in the relative abundance of *Conexibacter* and *Gaiella* (Data in Brief's article: Table 3 A). No shifts in relative abundance were observed on the skin of study participants in the placebo treatment (Data in Brief's article: Table 3B).

On day 28, *T. vulgaris* relative abundance was again at the same level compared to baseline (Table S5A), however, the relative abundance of 26 genera were still different on the skin of the intervention study participants compared to baseline (Data in Brief's article: Table 3 A). During the 28 days intervention period, the composition of skin bacterial communities were different compared to baseline values only in the intervention treatment, not in the placebo treatment, including shifts in the classes Gammaproteobacteria and Clostridia (Table S3 A, see the

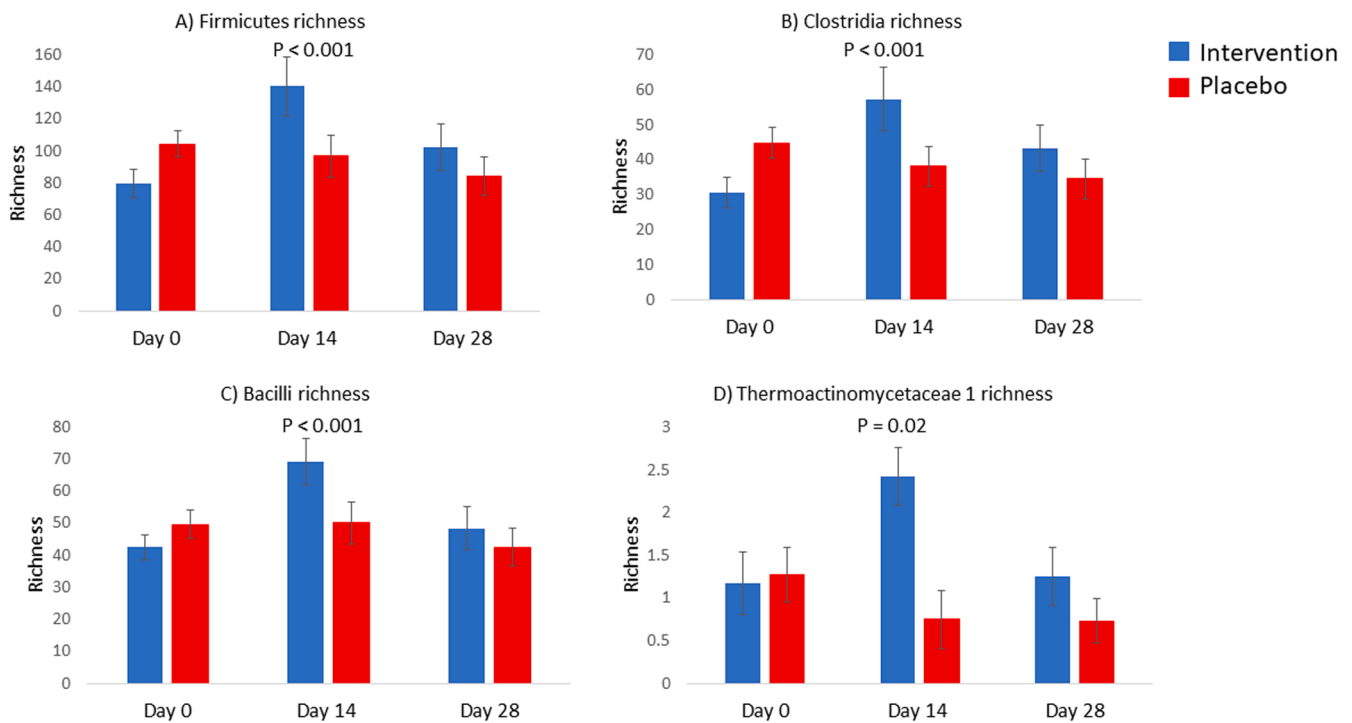


Fig. 2. Richness of skin A) Firmicutes, B) Clostridia, C) Bacilli and D) Thermoactinomycetaceae 1 increased within intervention treatment compared to placebo treatment group. Richness (number of OTUs) is displayed as mean \pm standard error. P values are for linear mixed-effect models between intervention and placebo treatments.

Fig. 1 in the related Data in Brief's article).

3.3. Intervention results in immunoregulatory changes in plasma cytokines and T_{reg} frequencies

To investigate if the exposure to biodiversity in sand affects the levels of the immunomodulatory cytokines and frequency of T regulatory (T_{reg}) cells, we collected a venous blood samples at the baseline and on day 14. There were no differences between treatment groups in these immune parameters at baseline (Table S2A). We estimated the difference in change of immune parameters with the LMM models as recommended previously (Twisk et al., 2018). The change in IL-10 plasma concentration and IL-10 to IL-17A ratio differed between treatments: The change was positive in the intervention and negative in the placebo treatment ($P = 0.02$; Fig. 3 and Table S7 A). Specifically, the IL-10 level increased in five out of six children in the intervention arm and decreased in five out of six children receiving placebo treatments (Fig. S1). No differences were found between treatment groups in the analysis of T_{reg} cell frequencies and intracellular cytokines between treatment groups.

To further investigate if skin bacterial community is linked to the immune parameters, we used non-metric multidimensional scaling (NMDS) to score the bacterial operational taxonomic units (OTUs) onto an ordination and assessed correlation with corresponding cytokine expression levels and T_{reg} frequencies. When all children were analyzed together in the NMDS model, the community composition of Gammaproteobacteria on the skin was associated with plasma IL-10 concentration and IL-10 to IL-17A ratio (Fig. S2 and Table S7 B). LMM models showed consistent results; the relative abundance of gammaproteobacterial OTUs were directly associated with IL-10 concentration and IL-10 to IL-17A ratio (Table S7 C and D). Gammaproteobacterial OTUs were further identified with microbe Nucleotide Basic Local Alignment Search Tool (BLASTN). According to BLASTN results, these OTUs belonged to *Conservatibacter*, *Haemophilus* and *Moraxella* genera (Table S7 E).

In the LMM models, changes in the richness of skin Firmicutes and Clostridia were inversely associated with a change in plasma IL-17A (Fig. 4C and D and Table S8). Further, the increase in IL-10 was inversely associated with a change in the relative abundance of genera *Conexibacter* and *Gaiella* on the skin (Fig. 4A and B and Table S8).

Finally, we examined associations between skin bacterial changes and total, memory and naïve T_{reg} frequencies, and intracellular cytokine expression within CD3 + CD4 + memory T cells with LMM models. The change in Thermoactinomycetaceae 1 relative abundance on the skin was directly associated with both total (defined either as CD25 + CD127lowFOXP3 + and FOXP3 + Helios+) and memory (CD45RA-CD25 + CD127lowFOXP3 +) T_{reg} cell frequencies (Fig. 5A and B and Table S9), and inversely associated with frequencies of memory CD4 + T cells producing proinflammatory cytokines (IL-2 +, IL-21 +, TNF- α and IFN- γ). The association between Thermoactinomycetaceae 1 relative abundance and T_{reg} cells was observed when all children were in the model, and for memory T_{reg} cells also when the intervention and placebo treatments were analyzed separately. We observed that the increase in Thermoactinomycetaceae 1 richness and the relative abundance of *Thermoactinomyces vulgaris* was directly associated with the change in total and memory T_{reg} cells (Table S9) only in the intervention treatment. Of note, the frequencies of proinflammatory cytokines produced by CD4 + T cells correlated inversely with the frequency of memory T_{reg} cells (Table S9 D), indicating that the associations between Thermoactinomycetaceae 1 and cytokine frequencies measured from CD4 + T cells may be secondary to the association between Thermoactinomycetaceae 1 and T_{reg} frequencies. In addition, we observed that the relative abundance of unclassified Alphaproteobacterial genera on skin, including altogether 114 OTUs, was directly associated with both total (CD25 + CD127lowFOXP3 + and FOXP3 + Helios+) and naïve T_{reg} cell frequencies (CD45RA+CD25 + CD127lowFOXP3 +) (Fig. 5C and D and Table S9).

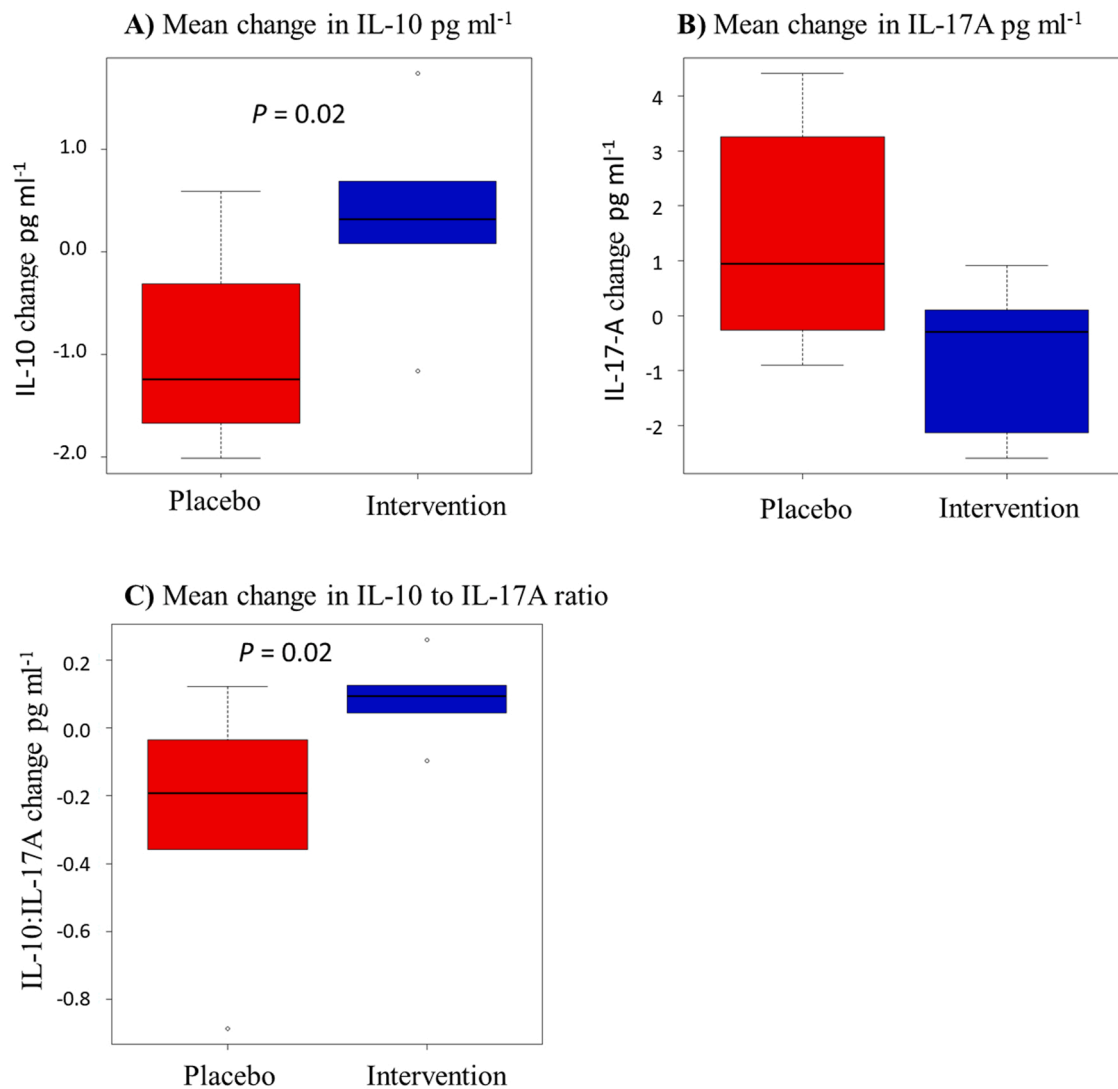


Fig. 3. Mean change in A) plasma IL-10 levels, B) IL-17A and C) IL-10 to IL-17A ratio between days 0 and 14 among children in placebo and intervention treatment. Data are displayed as median \pm interquartile range. LMM model statistics and Q levels in [Table S10 A](#).

3.4. Gut microbiota is associated with plasma immune markers

We examined the richness, diversity and the relative abundance of various gut bacterial taxa between intervention and placebo treatment. On day 0, gut microbiota was similar in the intervention and placebo arms (t test $Q > 0.1$; Data in brief's article: Table 5). Gut proteobacterial diversity decreased only in the intervention arm between days 0 and 14 (LMM: $P = 0.02$, $Q = 0.03$), whereas it remained at the baseline level in the placebo arm (LMM: $P > 0.6$) (Data in brief's article: Table 4 C and D). On day 14, gut proteobacterial Simpson diversity was lower in the intervention compared to placebo arm (t test $P < 0.016$, $Q = 0.06$), but not anymore on day 28 (t test $P = 0.23$) (Data in brief's article: Table 5). No other differences were found in the gut between treatments.

We further explored whether the gut bacterial measurements correlate with immune parameters with LMM models. Proteobacterial diversity (Simpson and Shannon) in the gut was directly associated with the level of IL-17A in plasma (Fig. S3A; Table S10 A). Several other gut bacterial taxa with known health-outcomes (Abrahamsson et al., 2012; Sobko et al., 2020; Sokol et al., 2008; Tamanai-Shacoori et al., 2017) were associated with plasma IL-10 levels. In detail, the Firmicutes to Bacteroidetes ratio was inversely associated, and the relative abundance of genus *Bacteroides* was directly associated, with the IL-10 level in plasma (Fig. S3 and Table S10 B). In addition, the change in the relative

abundances of *Faecalibacterium* and *Roseburia* were directly associated, whereas the change in the relative abundance of *Romboutsia* was inversely associated with the change in IL-10 level (Table S10 C).

4. Discussion

According to our knowledge, the current paper is the first double-blinded placebo-controlled test of the biodiversity and old friends' hypotheses of immune mediated diseases. As the results show that the addition of microbiologically rich, soil-based powder diversifies sand microbiota, enriches skin microbiota and is associated with immunoregulatory responses among urban children (see graphical abstract), the hypotheses are supported. Importantly, our study demonstrates a feasible approach to rewild urban playgrounds, which potentially promotes anti-inflammatory and suppresses pro-inflammatory immune responses in children, i.e., enhances environmental health.

We studied several parallel factors that collectively support the hypothesis that the exposure to microbially enriched sand promotes the immune regulation of children. First, the level of IL-10 in plasma increased in the intervention arm. IL-10 is an anti-inflammatory cytokine that has been proven to prevent autoimmune disease by limiting the secretion of pro-inflammatory cytokines, and by regulating macrophages, T cells and B cells (Scapini et al., 2011). Secondly, the increases

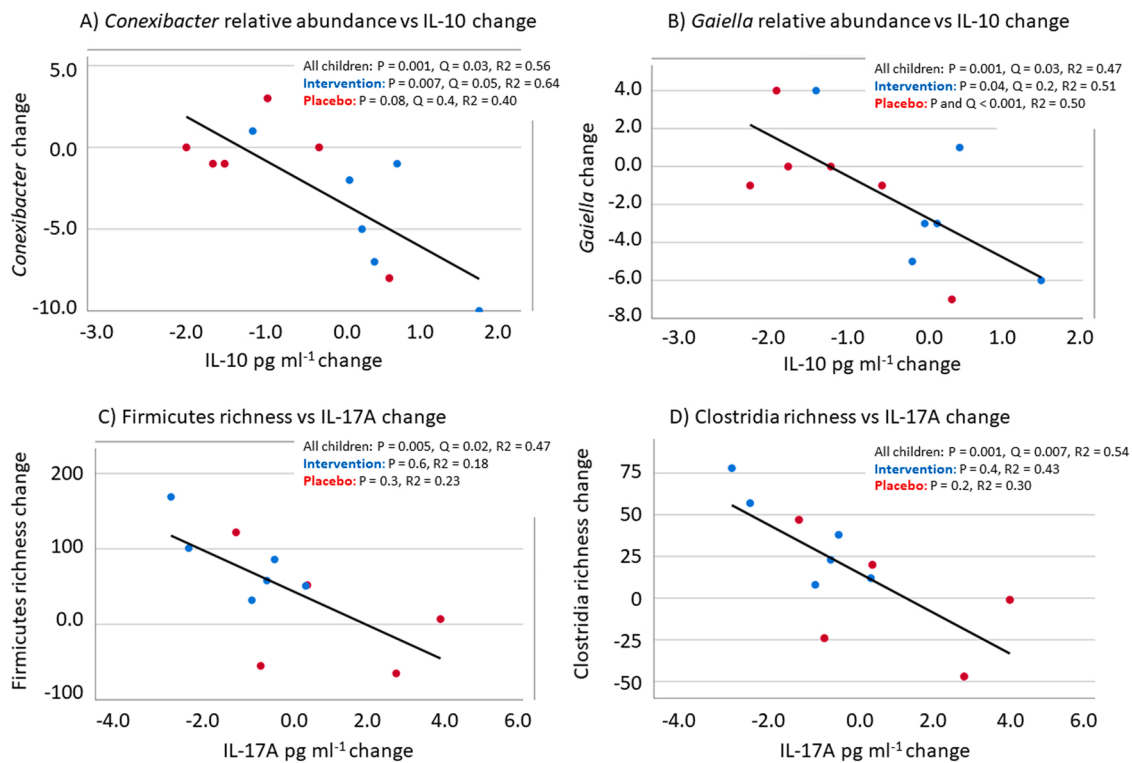


Fig. 4. Associations between bacterial communities on skin and plasma cytokine concentrations. LMM models showed that a change in A) *Conexibacter* and B) *Gaiella* relative abundance was inversely associated with the change in IL-10, and that a change in C) Firmicutes and D) Clostridia richness was inversely associated with the change in IL-17A.

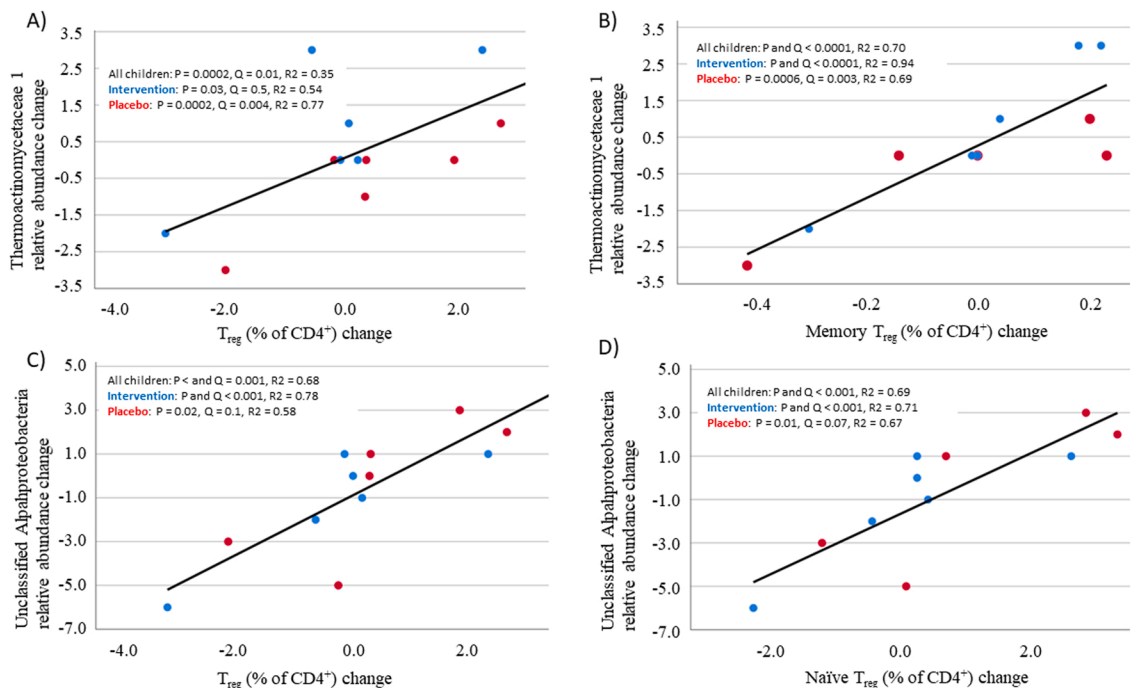


Fig. 5. LMM models showed that the change in A) Thermoactinomycetaceae 1 relative abundance was associated directly with change in total T_{reg} and B) memory T_{reg} frequencies, and C) the relative abundance of unclassified genera within Alphaproteobacteria was directly associated with the change in total Treg and D) naïve T_{reg} frequencies.

in skin Firmicutes and Clostridia richness were associated with a decrease in pro-inflammatory IL-17A levels in plasma. Third, skin Gammaproteobacterial community that shifted only in the intervention arm, was associated with IL-10 levels and IL-10 to IL-17A ratio in

plasma. Fourth, the relative abundance of unclassified Alphaproteobacteria was higher in enriched sand and the abundance on skin was directly associated with T_{reg} cell frequencies. Finally, we found taxonomic shifts and differences that have been associated with immune

regulation in previous studies (Hanski et al., 2012; Ramakrishna et al., 2019; Roslund et al., 2020; Shen et al., 2018; Sokol et al., 2008).

4.1. Skin microbiota and immune regulation

Our results provide the first placebo-controlled evidence that exposure to high environmental microbial diversity can induce immunoregulatory responses in children. The fact that plasma IL-10 levels increased in the intervention group but stayed stable in the placebo group is a strong indication that daily skin contact with rich environmental microbiota results in immune response, no matter if the response is caused by skin contact per se, or requires the transfer of bacteria or bacterial components to mucous membranes. Enriched sand contained a high diversity of Gammaproteobacteria and in consistence with previous biodiversity studies (Fyhrquist et al., 2014; Hanski et al., 2012; Riskumäki et al., 2021; Roslund et al., 2020), the Gammaproteobacterial community on the skin was associated with immune regulation. Indeed, in our earlier non-double-blinded and non-placebo-controlled intervention study the increase in Gammaproteobacterial diversity on the skin was associated with enhanced immune regulation, and the IL-10 to IL-17A ratio increased only among children in the intervention group (Roslund et al., 2020). In the current study, several Gammaproteobacterial OTUs were associated with plasma IL-10 levels and IL-10 to IL-17A ratio, indicating that the whole community composition of Gammaproteobacteria may play a role in regulating immune responses instead of a single species. These findings are in line with the original paper that presented the biodiversity hypothesis: Hanski et al. (2012) found in their comparative study that plasma IL-10 level was associated with Gammaproteobacteria on skin, and that the high Gammaproteobacterial diversity on the skin reduces the risk of atopy and allergies. Taken together, these studies provide considerable evidence in support of the biodiversity hypothesis of immune-mediated diseases.

Importantly, although we observed associations between skin microbiota and immune response, particularly IL-10 and IL-17A levels, the current study was not planned to exclude other routes, e.g. exposure via airways. Even though the current study covered 16 S analysis, it is possible that the exposure to the whole spectrum of environmental microbes in enriched sand was involved in immune modulation (Mills et al., 2020; Rook et al., 2015), rather than exposure to any specific bacterial taxa on the skin. The current study is the first placebo-controlled human trial to test the biodiversity hypothesis. Therefore, it is not surprising that several aspects can be enhanced in future studies. These include the experimental setup: We were able to recruit 26 children in 6 daycare centers in a single city, and the number of guardians willing to let their child to donor blood and microbial samples for our genuinely novel approach varied a lot between daycare centers. These limitations prevented the use of a fully randomized block design, which would minimize any potential bias introduced by the city and daycare unit. Further, to get a thorough view of the linkages between immune response and biodiversity, a wide array of cytokines and chemokines could be analyzed. Importantly, since the immune system is regulated by a complex network of cells, receptors, and signaling molecules (Delves and Roitt, 2000), the potential health benefits linked to T_{reg} cells deserve further research. Nevertheless, the current study provides means for future intervention trials that may tackle the specific roles of different taxa, a wide spectrum of immune markers, and that may target to separate the roles of touching versus inhaling versus ingesting environmental microbiota.

Although it would need a considerably larger trial to see between-arm differences in T_{reg} values per se, the current T_{reg} results suggests that our sandbox intervention could have important health benefits for children. The implementation of diverse microbiota into playground sand appeared to alter blood T_{reg} frequencies, including memory T_{reg} cells that regulate memory effector responses and persist for long periods even in the absence of foreign antigens (Rosenblum et al., 2016). As our results indicate that *Thermoactinomyces vulgaris* was transferred

from the enriched sand onto the skin (Table S6) and associated with memory-T_{reg} cells, the immunological memory for this antigen may play a role in preventing allergic reactions later in life, as proposed by Rosenblum et al. (2016). Although *T. vulgaris* is one of the causative agents of hypersensitivity pneumonitis (Nogueira et al., 2019), its relative abundance was very low in soil (0.5%) and on skin (0.2%). In addition, the increased relative abundance of *T. vulgaris* on the skin of the intervention children was observed after the supervised period (day 14), but not on day 28, while hypersensitivity reactions involve pronounced chronic and repeated exposure to the antigen (Girard et al., 2011). Therefore, hypersensitivity reactions are very unlikely. Indeed, because the enriched skin microbiota among children in the intervention treatment was related to the decrease in pro-inflammatory IL-17A plasma levels, our results indicate that the exposure to enriched sand increased the richness of beneficial anti-inflammatory microbes on the skin. The inclusion of enriched sand used in this study presents a promising approach to prevent the dysregulation of self-tolerance, and to restrain hazardous immune responses related to conditions such as autoimmune diseases and allergy.

4.2. Gut microbiota and immune regulation

High gut proteobacterial diversity has been associated in some but not all interventions with green environment (Roslund et al., 2020; Sobko et al., 2020), and with the lack of atopic eczema among 12-month-old toddlers (Abrahamsson et al., 2012; Kang et al., 2018). In the current study among 3–5 year-old children, the gut proteobacterial diversity decreased in the intervention arm, and the decrease was associated with lower plasma levels of IL-17A. Specific gut microbiota are known to regulate the differentiation of IL-17A-producing T-helper cells (Ivanov et al., 2008), which is a potential explanation for the association between Proteobacteria and IL-17A plasma level (see Fig. S3 A). However, the reason for the decline in proteobacterial diversity and the association with IL-17A plasma level would require more research and higher number of study participants to verify this result.

Findings in the current study are consistent with the results of several comparative studies that sought for associations between gut microbiota and immune regulation. Even though our study found interesting association between immune markers and relative abundances, the same associations were previously observed between plasma IL-10 levels and the abundance of *Faecalibacterium* (Sokol et al., 2008), *Roseburia* (Shen et al., 2018) and *Bacteroides* in the gut (Ramakrishna et al., 2019) (see Fig. S3 and Table S10). Furthermore, obesity associated Firmicutes to Bacteroidetes ratio (Magne et al., 2020) was inversely associated with plasma IL-10 levels in our study. The similarity between our findings and the earlier studies indicates that the associations may be a general phenomenon.

4.3. Persistence of the microbial changes

As the most interesting microbial changes were observed after the supervised period on day 14, but were not evident on day 28 (see Fig. 3), these changes may need daily routines that encourage direct soil contact. The lack of these but not all microbial changes on day 28 may indicate that the contacts of children with the enriched sandbox sand were less intense during their free play in an otherwise microbially poor environment. This view is further supported by our finding that quantitative bacterial abundance (16 S rRNA counts) was similar in both groups (see related Data in Brief article), even though contact with organic soil is known to cause short-term increases in bacterial abundance on skin (Grönroos et al., 2019). Notably, in our study samples were not taken immediately after free play at the yards. Thus, our study indicates that daily active contacts with biodiverse soil cause a qualitative community shift even after the excess bacterial numbers have levelled off on the skin.

We did not measure if microbiota in the sandbox sand affected to the

surrounding environmental microbiota. However, based on the commensal microbiota results on day 28, contacts of children with the enriched sand were probably less intense than during the supervised period. To create persistent changes in child microbiota and immune response, the whole yard or playground is optimally built using high-biodiversity natural materials, including diverse vegetation, instead of certain high-diversity patches (Robinson et al., 2021; Watkins et al., 2020). This increases the probability that children are exposed to diverse environmental microbiota even if they are not supervised daily.

4.4. Limitations of the study

In addition to the low number of study participants discussed above in 4.1, the lack of longitudinal follow up is a limitation of our study. The study was conducted in late spring and early summer. In Finland, soil dries at times of the trial. According to our experience, this coincides and is a potential reason for proinflammatory changes and poor environmental microbiota in built environment. Our assumption is that contact with the enriched intervention sand prevented the decline of anti-inflammatory cytokine IL-10 (see Fig. 3), but it is hard to prove the assumption. Interestingly, however, the temporal changes in plasma IL-10 levels were opposite between the intervention and placebo groups, although we did not observe statistically significant difference in IL-10 levels between arms in any of the time points during the intervention. This may be due to the limited number of study subjects and high natural variation in the levels of immune markers between individuals (Patin et al., 2018). Nonetheless, this study revealed several significant associations between bacterial changes on the skin and immune markers (Figs. 4 and 5). It seems that the number of study participants was high enough to allow the statistical power to detect these associations, and the results were consistent with previous studies (Hanski et al., 2012; Ramakrishna et al., 2019; Sokol et al., 2008).

It is known that skin microbiota play an important role in host immune networks potentially influencing whole-body states (Belkaid and Segre, 2014; Prescott et al., 2017; Whibley et al., 2019). However, our finding that *T. vulgaris* may affect blood T_{reg} cell frequencies in two weeks exposure period, needs further research to verify the association. The clinical relevance of the associations between skin microbiota and immune markers remains to be clarified, but our study is a necessary starting point for elucidating these associations. It would be interesting to know how immune markers shifted between days 14 and 28. Interestingly, the relative abundance of *T. vulgaris* on the skin was low, less than 1%. As seen from Tables S6 B and C, however, the exposure to enriched sand with low levels of *T. vulgaris* is the core reason for its increased abundance on the skin among the intervention children. *T. vulgaris* has been associated with inflammatory reactions and eventually hypersensitivity pneumonitis, providing that it forms a large proportion of spores in moldy straw material that farmers work with (Nogueira et al., 2019). It is tempting to speculate that *T. vulgaris* triggers immune modulation at low concentrations as part of a diverse microbiological community, but the effect reverses from beneficial to negative if the species dominates in space and time.

5. Conclusions

Our study provides the first placebo-controlled and double-blinded evidence in support of the biodiversity hypothesis of immune-mediated diseases. Results indicate that the immune response of children in the intervention arm is different from the immune response of children in the placebo arm. Hence, novel sustainable nature-based prophylactic practices should be studied as an option to cope with the high incidence of immune-mediated diseases among urban dwellers. The biodiversity powder that was used to make the enriched sand could be one of the potential options.

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Author contributions

A.S., M.I.R., R.P., H.H., O.H.L. designed study; M.I.R., R.P., L.S., A.P., A.S. and N.H. implemented study; M.I.R., N.N., S.O., L.S., O.C., A-M.S. and L.K. generated data; M.I.R., A.P., M.G., and A.S., A.-M.S. and T.K. analyzed data; M.I.R., A.P., A.S., R.P., N.N., H.H. and O.H.L. wrote the manuscript; M.I.R. and A.S. prepared the figures and tables; All the authors reviewed the manuscript; A.S. and H.H. were the principal investigators of the project.

CRediT authorship contribution statement

Marja I. Roslund: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – original draft, Visualization. **Anirudra Parajuli:** Formal analysis, Investigation. **Nan Hui:** Investigation, Writing - review & editing. **Riikka Puhakka:** Conceptualization, Methodology, Writing - review & editing. **Mira Grönroos:** Formal analysis. **Laura Soininen:** Investigation. **Noora Nurminen:** Investigation, Data curation, Writing - review & editing. **Sami Oikarinen:** Investigation, Data curation. **Ondrej Cinek:** Investigation, Writing - review & editing. **Lenka Kramná:** Investigation. **Anna-Mari Schroderus:** Formal analysis, Investigation. **Olli H. Laitinen:** Conceptualization, Writing - review & editing, Funding acquisition. **Tuure Kinnunen:** Formal analysis, Writing - review & editing. **Heikki Hyöty:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition. **Aki Sinkkonen:** Conceptualization, Methodology, Validation, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A. S., H.H., O.H.L., M.G., N.N. and S.O. have been named as inventors in a patent application 'immunomodulatory compositions' submitted by University of Helsinki (Patent application number 20165932 at Finnish Patent and Registration Office). M.G., A.P., M.I.R. and A.S., have been named as inventors in a patent application 'Immunomodulatory gardening and landscaping material' submitted by University of Helsinki (Patent application number 175196 at Finnish Patent and Registration Office). A.S., H.H. and O.H.L. are members of the board of Uute scientific Ltd which develops immunomodulatory treatments.

Data availability

All bacterial sequence data were accessioned into the Sequence Read Archive (BioProject ID: PRJNA746448). All other data needed to support the conclusions of this manuscript are included in the main text, Data in Brief's article and supplementary appendix. The sensitive data that support the findings of this study are available from University of Helsinki but restrictions defined in General Data Protection Regulation (EU 2016/679) and Finnish Data Protection Act 1050/2018 apply to the availability of these data, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission from the ethical committee of the local hospital district (Tampereen yliopistollisen sairaalan erityisvastuualue, Pirkanmaa, Finland).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2022.113900](https://doi.org/10.1016/j.ecoenv.2022.113900).

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