Soil exposure modifies the gut microbiota and supports immune tolerance in a mouse model

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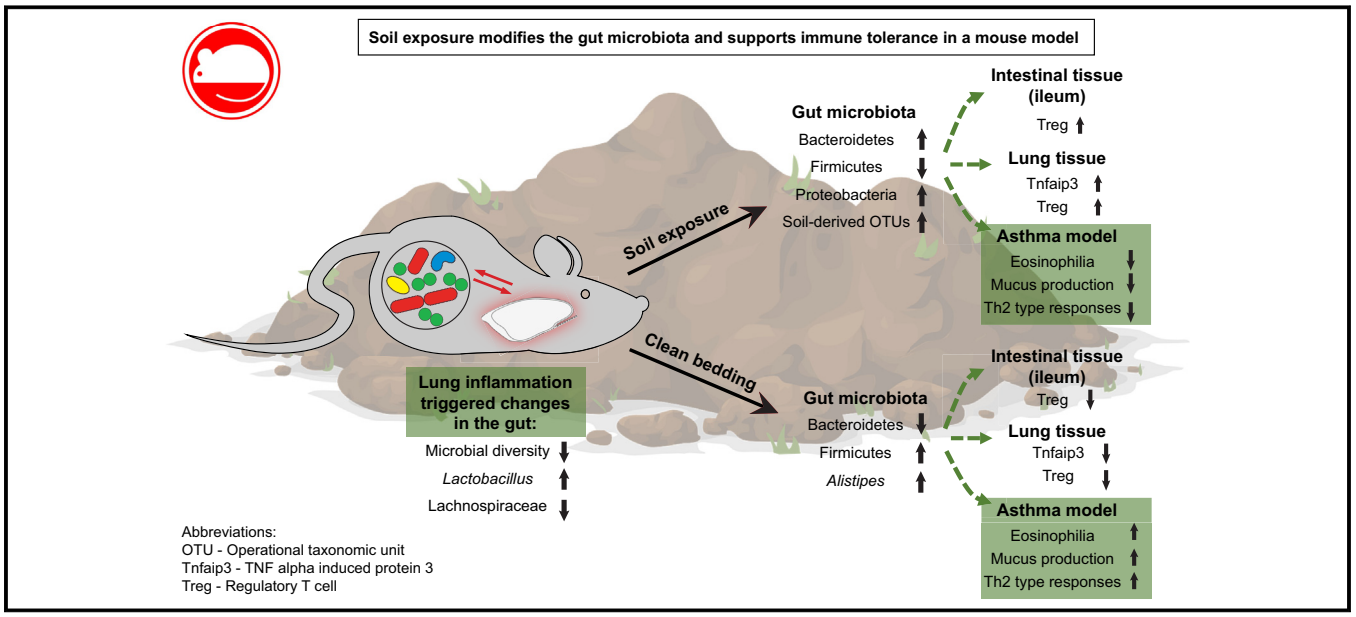
GRAPHICAL ABSTRACT



Background: Sufficient exposure to natural environments, in particular soil and its microbes, has been suggested to be protective against allergies.

Objective: We aim at gaining more direct evidence of the environment-microbiota-health axis by studying the colonization of gut microbiota in mice after exposure to soil and by examining immune status in both a steady-state situation and during allergic inflammation.

Methods: The gastrointestinal microbiota of mice housed on clean bedding or in contact with soil was analyzed by using 16S rRNA gene sequencing, and the data were combined with immune parameters measured in the gut mucosa, lung tissue, and serum samples.

Results: We observed marked differences in the small intestinal and fecal microbiota composition between mice housed on clean bedding or in contact with soil, with a higher proportion of Bacteroidetes relative to Firmicutes in the soil group. The housing environment also influenced mouse intestinal gene expression, as shown by upregulated expression of the immunoregulatory markers IL-10, forkhead box P3, and cytotoxic T lymphocyte–associated protein 4 in the soil group. Importantly, using the murine asthma model, we found that exposure to soil polarizes the immune system toward T_H1 and a higher level of anti-inflammatory signaling, alleviating T_H2-type allergic responses. The inflammatory status of the mice had a marked influence on the composition of the gut microbiota, suggesting bidirectional communication along the gut-lung axis.

Conclusion: Our results provide evidence of the role of environmentally acquired microbes in alleviating against T_H2-driven inflammation, which relates to allergic diseases. (J Allergy Clin Immunol 2018;■■■:■■■-■■■.)

Key words: *Gut microbiota, biodiversity, immunity, living environment, gut-lung axis, mouse asthma model*

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The living environment has a profound influence on human health, affecting stress levels[1] and physical activity[2] but also immune tolerance.[3-5] It has been suggested that this effect is,

Abbreviations used
BALF: Bronchoalveolar lavage fluid
CTLA4: Cytotoxic T lymphocyte–associated protein 4
Foxp3: Forkhead box P3
OTU: Operational taxonomic unit
OVA: Ovalbumin
qPCR: Quantitative PCR
SCFA: Short-chain fatty acid
SFB: Segmented filamentous bacterium
TLR: Toll-like receptor

at least in part, mediated by modification of the individual microbial composition.[6] In turn, the microbes colonizing the skin and gastrointestinal tract affect the development and functioning of the immune system from the very beginning of life.[7] This understanding has led to the proposition that lack of exposure to beneficial microbes, especially during early life,[8] explains the higher incidence of allergic and autoimmune diseases in urban areas in comparison with farming environments[9,10] or green environments in general.[11]

The "old friends" hypothesis suggests that proper development of the immune system depends on (certain) microbes, which evolved together with human subjects but are deficient or lacking in modern living environments.[12] Moreover, the biodiversity hypothesis proposes that reduced exposure to diverse environmental microbiota has contributed to the current surge in chronic inflammatory diseases in western societies.[13] Therefore exposure to soil and the rich microbiota within can be particularly important for health.[14] This effect, as noted above, operates through cross-signaling between the human microbiota and the immune system, as supported by a wealth of indirect evidence.[4,15,16] However, research conducted thus far provides only some clues to understanding the causal connection between immune tolerance and microbial colonization from the environment.

Although the effect of diet and various medical conditions on the microbiota has been studied in many instances,[17-20] few studies have looked at the sole effect of the living environment on the composition of the microbiota[21] and the immunologic status of individual subjects. Numerous studies comparing populations living in different geographic locations have observed differences in microbiota composition, but these can arise from several other factors (ie, diet, age, and physiologic variations) in addition to the living environment.[22-24] Nevertheless, animal studies have shown that a high-hygiene indoor environment negatively affects normal succession of the gut microbiota and impairs immune homeostasis in piglets when compared with piglets in outdoor environments.[25,26] More recently, clean living conditions have been associated with reduced abundance of Bacteroidetes and increased abundance of Firmicutes in the gut microbiota of mice.[27]

Here we aim at gaining more direct evidence of the environment-microbiota-health axis[28] by studying the colonization of gut microbiota in mice after exposure to soil. Moreover, because there is growing evidence on the influence of the gut microbiota on the systemic immune system and the possible bidirectionality of this effect,[29,30] we examine mouse immune status in both a steady-state situation and during inflammation.

The small intestinal and fecal microbiota of mice kept on clean bedding or in contact with soil (sharing otherwise identical living conditions) were analyzed, and the data were combined with immune parameters of the mice. We compared the effect of the housing environment in both healthy and allergen-sensitized mice. We observed marked differences in the gut microbial composition and immune function between housing environments. Interestingly, within a given housing condition, mice exposed to the asthma model differ in their gut microbiota from healthy mice, suggesting that not only environmental exposure but also host physiology play a critical role in shaping host microbial communities.

METHODS

Animals

Female BALB/c mice (n = 32; Scanbur, Karlslunde, Denmark) aged 4 weeks at the beginning of the exposure period were used in this study. All animal experiments were approved by the Social and Health Care Department of the State Provincial Office of Southern Finland. Mice were housed on either clean bedding in standard mouse facilities or with the addition of approximately 300 mL of commercial soil (GroBiootti Taimimulta; Berner Oy, Helsinki, Finland; see Table E1 in this article's Online Repository at www.jacionline.org) to each cage containing 4 mice and thereafter changed to fresh soil once a week when the cages were cleaned. All mice were fed the same standard diet and received the same drinking water. Control and soil-treated mice were kept in separate units, and each unit had its own caretakers to avoid cross-contamination. Each experimental mouse group contained 8 mice, as statistically acceptable for experimental models.

Sensitization and airway challenge

After a period of 6 weeks of contact with soil (or clean bedding), 8 mice from each housing condition were exposed to the murine asthma model protocol, receiving 2 intraperitoneal injections of 50 μg of ovalbumin (OVA) emulsified in 2.25 mg of alum in a total volume of 100 μL of PBS on days 0 and 14, followed by intranasal challenge with OVA (50 μg of OVA in 50 μL of PBS) on days 28, 29, and 30 and collection of samples on day 32 (see Fig E1 in this article's Online Repository at www.jacionline.org). The control mice (n = 8 mice in each housing condition) received alum in PBS and were challenged with PBS only. For analysis of inflammatory parameters, blood, lung tissue, and bronchoalveolar lavage fluid (BALF), samples were collected. For analysis of mouse microbiota, tissue samples were collected from the jejunum and ileum in addition to fresh fecal samples. Samples of the cage material and drinking water were also collected to analyze the microbial composition of the environment.

DNA extraction and sequencing

Total bacterial DNA was extracted from mouse fecal samples by using the repeated bead-beating plus column method.[31] Bacterial DNA from ileal and jejunal biopsy specimens was extracted by using a phenol-chloroform extraction method. DNA from the environmental control samples (soil and sawdust used as bedding and drinking water) was extracted by using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, Calif), according to the manufacturer's instructions. Reagent controls included extractions without addition of any sample as a negative control for library construction and potential reagent contamination. The V1 to V3 region of the 16S rRNA gene was amplified by using the barcoded primers AGAGTTT GATCMTGGCTCAG[32] and GTATTACCGCGGCTGCTG.[33] Paired–end sequencing (2 × 300 bp) with Illumina MiSeq (Illumina, San Diego, Calif) was done at the Institute for Molecular Medicine Finland (University of Helsinki, Helsinki, Finland). All 16S rRNA gene sequences can be accessed from the National Center for Biotechnology Information (accession no. SRP148728).

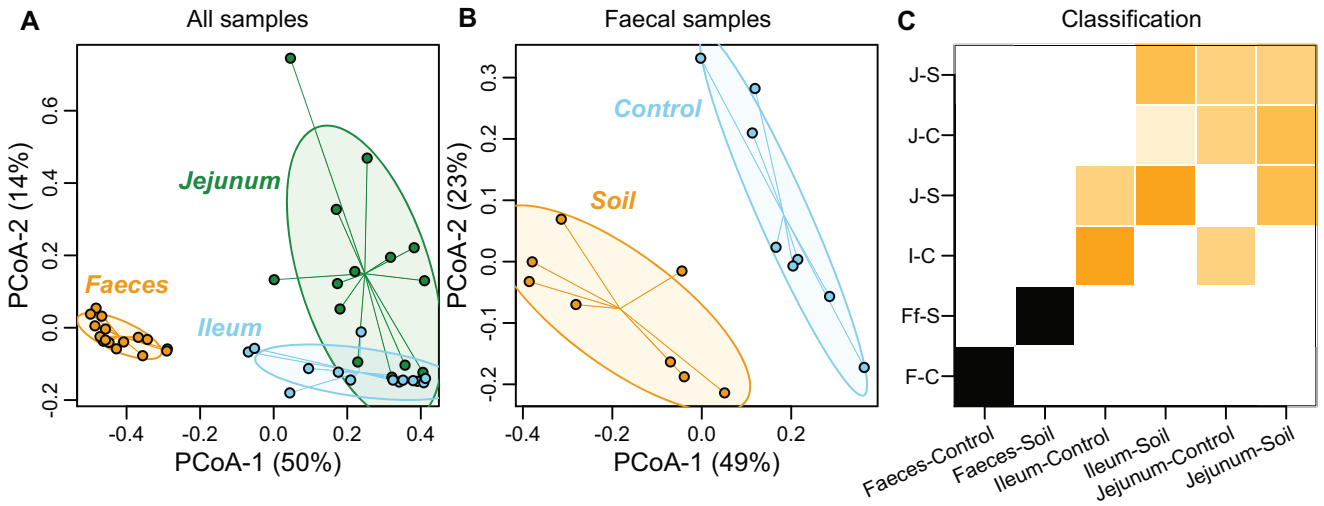

FIG 1. The housing environment shapes mouse microbiota composition. **A,** Composition of the murine microbiota is distinct between feces, jejunum, and ileum. **B** and **C,** The housing environment (ie, control bedding material or exposure to soil) uniquely shapes the microbiota community structure in feces ($P = .0001$; Fig 1, *B*), whereas in the jejunum and ileum there are overlapping community structures between the groups (as observed by random forest classification; Fig 1, *C*). All samples: feces, n = 16; ileum, $n = 14$; jejunum, n = 14. Feces samples: control, n = 8; soil, n = 8. Results are based on the Morisita-Horn dissimilarity on log-transformed abundances. *PCoA*, Principal coordinates analysis.

Real-time quantitative PCR

Total RNA was extracted from lung and ileal tissue samples by using a standard protocol of tissue homogenization in QIAzol lysing buffer and the FastPrep instrument (Thermo Fisher Scientific, Waltham, Mass), followed by RNA isolation with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. mRNA levels of IL-5, IL-10, IL-13, IL-17A, IL-23, TNRF2, cytotoxic T lymphocyte–associated protein 4 (CTLA4), CCL17, forkhead box P3 (Foxp3), CD86, IFN-γ, S100A7a, cathelicidin antimicrobial peptide, Toll-like receptor (TLR) 2, and TNFAIP3 were analyzed by means of quantitative RT-PCR with TaqMan chemistry and the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific).

Sequence processing and data analysis

Details on sequence processing can be found in the Methods section in this article's Online Repository at www.jacionline.org. For data analysis, we used the random forest classifier to assign each sample type a degree of coherence, as implemented in the randomForest package in R software.[34] Between-sample differences in microbial community composition were tested by using multiple regression on distance matrices, as implemented in the vegan package in R software,[35] where statistical significance was based on 9999 random permutations. We report results based on the Morisita-Horn dissimilarity (as implemented in the vegan package in R software) on log-transformed abundances. To make sure our results were not biased by these decisions, we also ran the analyses on weighted UniFrac and Bray and Curtis dissimilarities by using either raw, sqrt, or log-transformed abundances. The results show that the qualitative results are insensitive to the underlying distance metric and transformation method (see Tables E2 and E3 in this article's Online Repository at www.jacionline.org). To find differentially expressed/abundant operational taxonomic units (OTUs) in different treatments, we used the DESeq2 package in R software.[36]

Differences in cytokine expression between treatments were analyzed by using generalized least squares, as implemented in the nlme package in R software,[37] assuming treatment-specific residual variance. Pairwise comparisons were corrected by using the false discovery rate method in the p.adjust function in R software.

A more detailed description of the materials and methods used in this study can be found in the Methods section in this article's Online Repository.

RESULTS

Composition of gut microbiota is strongly affected by the housing environment

As expected, the composition of the gut microbiota changed along the gastrointestinal tract, with fecal samples clustering separately from the ileal and jejunal samples, regardless of the housing environment (Fig 1, *A*). However, investigating different sample types separately revealed that microbial composition differed significantly between mice housed in the soil environment and those housed on clean bedding material (control). This difference was most prominent in fecal samples ($P = .0001$; Fig 1, *B*); 15% to 40% of the variation could be explained by the different environments (see Table E2). A significant difference between the treatments could also be detected in ileal samples ($P = .033$) but not in jejunal samples ($P = .085$). We chose to analyze the microbiota both in the small intestine and feces (representing the end of the colon) because in addition to their anatomic and functional differences, these 2 sites are also immunologically different, which is reflected in the microbiota composition.[38] The observed patterns were reflected in random forest classification of the samples, showing that the housing environment is predictable based on the fecal microbiota, whereas ileal and jejunal microbiota had overlapping community structures between the groups (Fig 1, *C*). No difference in microbial diversity could be detected between the 2 housing environments.

We continued to investigate which bacterial groups best characterized the environmental treatments. The most abundant phyla in all fecal samples were Bacteroidetes and Firmicutes (Fig 2, *A*). Interestingly, the relative abundance of these 2 dominant phyla differed substantially between housing environments, with a higher Bacteroidetes/Firmicutes ratio in the soil group compared with the control group (Fig 2, *B*). This difference was explained mainly by the varying abundance of Lachnospiraceae and the Bacteroidales family S24-7: the relative

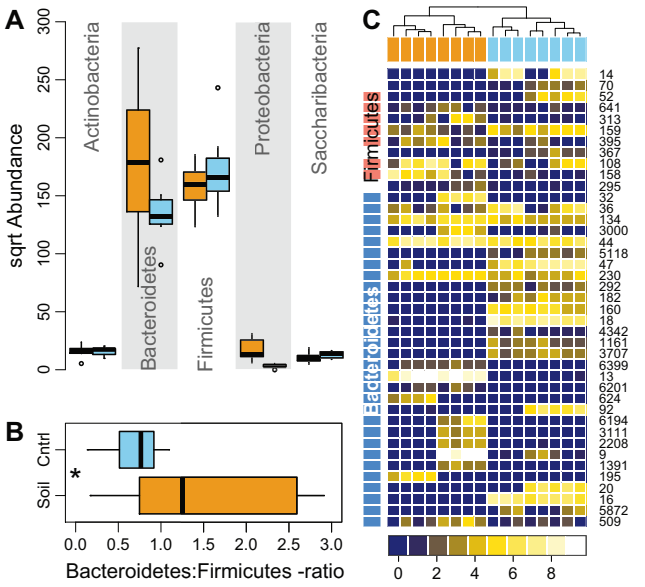

FIG 2. Exposure to soil increases the proportion of Bacteroidetes relative to Firmicutes in the fecal microbiota of mice. **A,** Relative abundance of bacterial groups at the phylum level in mouse fecal microbiota. *Gray shading* was added for visual clarification. **B,** The Bacteroidetes/Firmicutes ratio is greater in the soil environment in comparison with the control environment. **C,** Abundance (log scale) of differentially expressed features (OTUs) between the soil and control environments. Orange, Soil group; blue, control group. **P* < .05, according to the gls model with group-specific variance structure.

abundance of Lachnospiraceae was on average lower in the soil group compared with that in the control group (34% vs 50%), whereas the opposite was true for Bacteroidales family S24-7 (38% vs 19%).

Although numerous members of the Bacteroidales family S24-7 were present in mice housed in both environments, 2 OTUs were dominant in the soil group, representing up to 45% of the entire fecal microbiota (Fig 2, *C*). The genus *Alistipes* made up 1% to 6% of the fecal microbiota of mice in the control group but was not present in the soil group. Similarly, 2 Prevotellaceae OTUs were exclusively present in the control group (Fig 2, *C*). After Firmicutes and Bacteroidetes, the most abundant phyla were Actinobacteria and Proteobacteria (Fig 2, *A*). Mice from both environments had similar relative amounts of Actinobacteria, but the average relative abundance of Proteobacteria was significantly greater in the soil group (0.5% vs 0.03%, $P = .0001$).

We also sequenced samples from the 2 housing environments to see differences in the microbial composition and to determine whether microbes from the environment are transmitted to the mice. Microbial diversity was greater in the soil compared with that in the clean bedding material ($P = .009$), which was used in mouse cages in both housing environments. The clean bedding contained OTUs mainly from the phylum Firmicutes, followed by lower relative abundance of Proteobacteria and Actinobacteria (see Fig E2 in this article's Online Repository at www.jacionline.org). In contrast, the soil was dominated by Actinobacteria, followed by Proteobacteria.

Interestingly, we discovered a subset of OTUs which were present in soil and in the intestine, especially the jejunum, of the soil-treated mice (see Fig E3 in this article's Online Repository at www.jacionline.org). Most of these OTUs belong to soil-dwelling bacteria, and although many of them were present also in the clean bedding, none of them were found in the intestines of the control mice. However, these OTUs were not present in all the mice in the soil group, and their abundance seemed to be low compared with that of common gut microbiota residents.

The housing environment influences intestinal gene expression

RNA was isolated from ileal tissue and analyzed by using real-time quantitative PCR (qPCR) to explore immune responses in the ileum, an organ with high immunologic activity. Redundancy analysis of the genes measured by using qPCR revealed significant clustering of the soil-exposed mice and the mice kept on control bedding (Fig 3, *A*). Looking into specific genes revealed significantly upregulated expression of Foxp3, CTLA4, and the anti-inflammatory cytokine IL-10 in the soil group, representing key markers of regulatory T cells (Fig 3, *B*). In contrast, inflammatory mediators, such as IL-1β, IL-23, IL-17, and IFN-γ, and the antimicrobial peptides (AMPs) S100 calcium binding protein A7a (S100A7a) and cathelicidin antimicrobial peptide were expressed at a similar level between the groups (see Figs E4 and E5 in this article's Online Repository at www.jacionline.org).

Exposure to soil alleviates T_H2-type allergic responses in the lung

Soil-exposed and control mice were sensitized to OVA by means of intraperitoneal injection of allergen together with an adjuvant, followed by OVA challenge through the airways during a period of 4 weeks, to study how the housing environment might influence the immune response to allergens. Sensitized control mice had significant inflammation in the lung, including heavy infiltration of inflammatory cells in the perivascular and peribronchial areas (Fig 4, *A*). Significantly greater numbers of eosinophils in BALF (Fig 4, *B*) and periodic acid–Schiff–stained cells in the airway epithelium (Fig 4, *C*; and see Figs E6 and E7 in this article's Online Repository at www.jacionline.org) were also observed compared with the soil group. Furthermore, levels of T_H2-type cytokines and chemokines (IL-5, IL-13, and CCL17) in lung tissue were significantly greater, whereas expression of the T_H1-type cytokine IFN-γ was significantly lower in the control group than in the soil group (Fig 4, *D*). Thus soil exposure resulted in skewing of the immune system toward a T_H1-type response (Fig 4, *E*). Moreover, expression of anti-inflammatory IL-10 relative to IL-5 was significantly greater in the soil group (Fig 4, *E*). Finally, TNFAIP3, which encodes for A20, a key player in airway epithelial cells in protection against the development of asthma, was significantly induced at the baseline level (in the PBS mice) in the soil group compared with the control group (Fig 4, *E*).

Allergen-induced lung inflammation modifies the mouse gut microbiota and intestinal gene expression, regardless of the housing environment

OVA-induced lung inflammation was associated with a significant change in fecal microbial composition in both the control ($P = .001$) and soil ($P = .001$) groups (Fig 5, *A*). In both

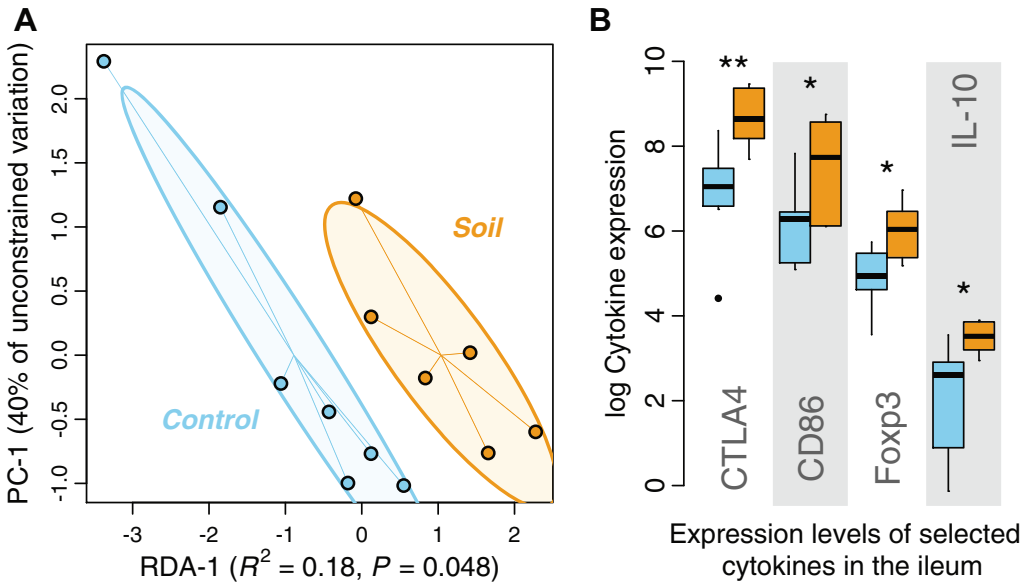

FIG 3. The housing environment influences intestinal gene expression. **A,** Gene expression in ileal tissue differed significantly between the soil and control groups. *PC-1*, Principal component 1; *RDA-1*, Redundancy analysis axis 1. **B,** Exploring individual cytokines reveals that expression of RNA coding for anti-inflammatory IL-10 and the regulatory T-cell markers Foxp3 and CTLA4, as well as CD86, is significantly greater in the soil-exposed group in comparison with the control group. Orange, Soil group; blue, control group. *$P < .05$ according to the gls model with group-specific variance structure. **$P < .01$. See Fig E4 for additional cytokines.

cases 20% to 40% of the variation in between-sample community dissimilarity could be explained by the inflammation treatment (see Table E3). Although the healthy mouse fecal microbiota showed marked differences in Bacteroidetes/Firmicutes ratio between the 2 housing environments, this was not seen in mice with allergen-induced lung inflammation. In these mice Bacteroidetes was the most abundant phylum in both environments. Moreover, the relative abundance of *Lactobacillus* species was increased in the fecal microbiota of sensitized mice, regardless of the housing environment, whereas Lachnospiraceae levels decreased (Fig 5, *B*). In contrast, the difference in the abundance of Proteobacteria between the 2 treatments (soil or control) was maintained, irrespective of the inflammatory status of the host.

Although there was overlap in the fecal bacterial community structures between housing environments in the sensitized mice (Fig 5, *A*), the ileal microbiota composition of OVA-sensitized mice showed clear environmental separation (see Fig E8, *A*, in this article's Online Repository at www.jacionline.org). In all mice, regardless of housing environment or inflammatory status, the ileal microbiota was dominated by *Lactobacillus* species and *Candidatus arthromitus* (segmented filamentous bacteria [SFBs]). BLAST searches revealed that the majority of *Lactobacillus* species OTUs are most likely *Lactobacillus johnsonii* (100% identity), *Lactobacillus reuteri* (99% identity), and *Lactobacillus murinus* (100% identity). The relative abundance of lactobacilli and SFBs fluctuated considerably between the environmental groups but also within the groups. Interestingly, in the ileums of the mice in the soil group, the microbial diversity was lower ($P = .008$) in OVA-sensitized mice in comparison with healthy mice (see Fig E8, *B*).

In OVA-sensitized mice expression of proinflammatory mediators and AMPs, including IL-1β, TLR2, IFN-γ, IL-17, and S100A7a, were significantly increased in ileal tissue (see Fig E5).

DISCUSSION

Our study shows a clear effect of the housing environment on murine gut microbiota composition and inflammatory status. Exposure to soil and the microbes within skews the gut microbial composition toward Bacteroidetes dominance, whereas gut communities in control mice were dominated by Firmicutes. Soil exposure had an effect on the immune system through enhancing anti-inflammatory signaling and T_H1-type immune responses and through repressing T_H2-driven immunity. Moreover, we observed modifications in the gut microbiota associated with allergen-induced lung inflammation, suggesting immune-driven modification of the microbiota across the gut-lung axis. These findings imply dynamic interactions between the environment, the host, and its microbiota, with profound effects on the composition of the microbiota, host immunity, and immune tolerance.

An association between human health and the natural environment is being established. However, only a few studies have identified causality or specific interactive mechanisms between these two.[39] In an attempt to establish causality between exposure to environmental biodiversity and beneficial modifications of the immune system, we chose to expose mice to soil because previous research has suggested health benefits related to contact with soil microorganisms.[4,14,40] Soil communities are highly diverse and vary according to soil characteristics,[41,42] and we took care in selecting organic nonradiated soil for the study. Although clean bedding also contained a myriad of bacterial sequences, the soil had higher bacterial diversity, and it is plausible that the humid fresh soil contained a greater number of viable bacteria than the dry processed clean bedding material. The bacterial community composition in the soil resembled that described in the literature,[43] but because of the enormous diversity of distinct soil environments, it is challenging to make direct comparisons to bacterial communities in different natural environments.

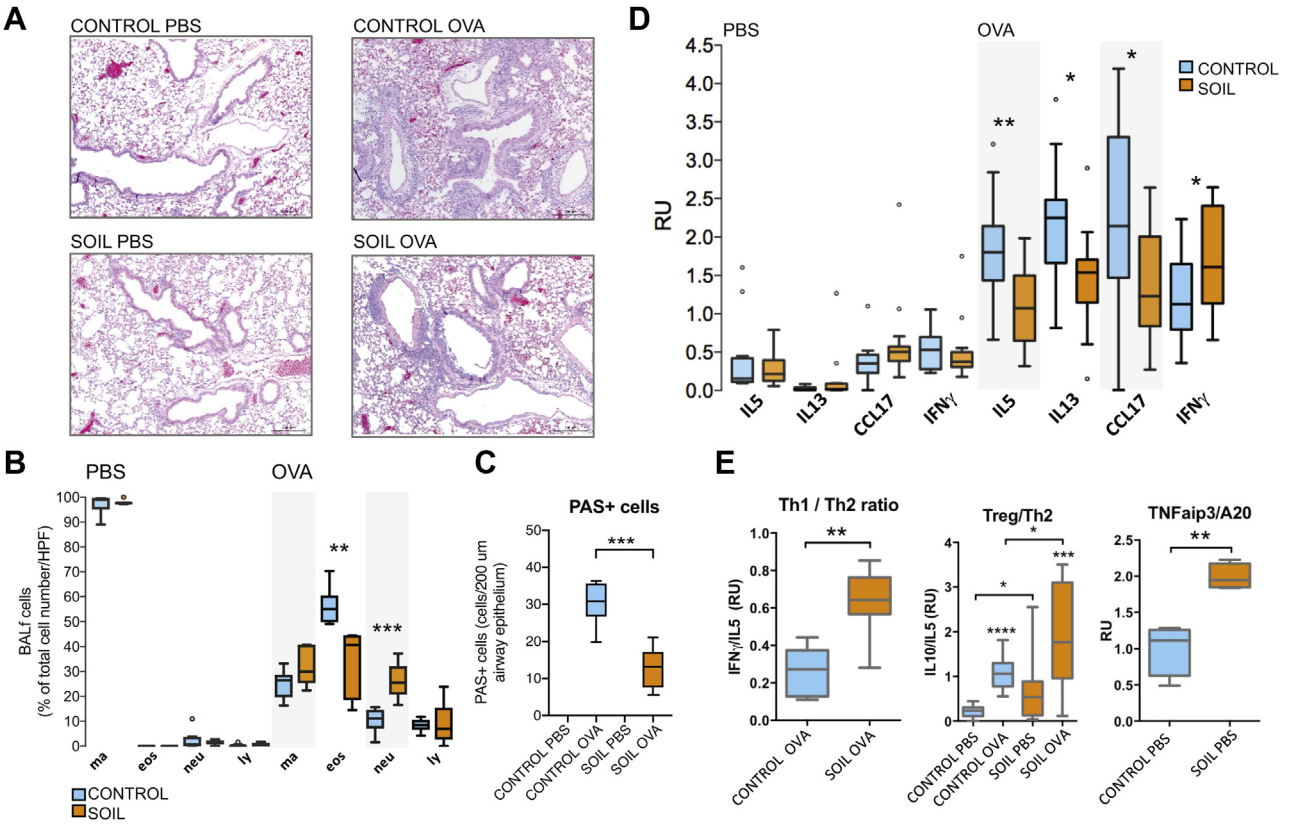

FIG 4. Soil exposure polarizes the immune system toward T_H1/regulatory T cells, alleviating T_H2-type allergic responses in the mouse lung. **A,** Hematoxylin and eosin staining of lung tissue samples shows perivascular and peribronchial infiltration of inflammatory cells after OVA challenge. **B** and **C,** Numbers of eosinophils in BALF counted from May-Grünwald-Giemsa-stained slide preparations (Fig 4, *B*) and peribronchial periodic acid-Schiff–stained cells (Fig 4, *C*) are greater in OVA-sensitized mice in the control group in comparison with those in the soil group. *eos*, Eosinophils; *ly*, lymphocytes; *ma*, macrophages; *neu*, neutrophils. **D,** Lung tissue mRNA levels of IL-5, IL-13, CCL17, and IFN-γ. **E,** Levels of IFN-γ and IL-10 expression were relatively greater than IL-5 levels in the soil group compared with control values. Expression of TNFAIP3 was at a significantly greater level in the soil group. $*P < .05$, $**P < .01$, and $***P < .001$ according to ANOVA and the nonparametric Mann-Whitney *U* test.

We were able to detect OTUs from soil-dwelling bacteria in the intestines of mice in the soil group, particularly in the jejunum. This upper part of the gastrointestinal tract is partially oxygenated[44] and could therefore offer better living conditions for environmental microbes compared with the anaerobic colon. It has been shown previously that soil microbes can colonize the guts of germ-free mice.[45] Although the gut might be less accessible to environmental microbes, it is possible that these soil-derived microbes colonized mouse lungs or skin. These microbiotas were not characterized here and might contribute to shaping immunity and inducing immune tolerance.[15] Further research is needed to reveal the relative immunologic relevance of different commensal microbial communities, as well as the possibility of horizontal transmission of environmental microbes to human microbial ecosystems.

We detected a higher Bacteroidetes/Firmicutes ratio in the soil group similarly to Zhou et al,[27] who compared mice housed in a clean environment versus those housed in cages with hay, leaves, soil, and dust. In general, the proportion of Bacteroidetes to Firmicutes has been associated with regulation of host energy metabolism, and higher levels of Bacteroidetes because of a high-fiber diet lead to increased production of immunoregulatory products, such as short-chain fatty acids (SCFAs).[46] SCFAs have been shown to regulate the immune system in multiple ways, including protecting against allergic airways disease.[47] Here healthy mice exposed to soil had lower levels of Lachnospiraceae and higher levels of Bacteroidales family S24-7 compared with control mice. Lachnospiraceae is a family of Clostridia abundantly present in mammalian gastrointestinal tracts. Although this family contains many butyrate-producing species, not much is known about the group as a whole.[48] Bacteroidales family S24-7 is a prominent constituent of the murine gut microbiota and also present in the human gut but remains poorly characterized as well. A recent metagenomic study suggested that this family contains at least 27 different species and includes members with varying enzyme repertoires involved in the degradation of specific carbohydrates and the capacity for propionate production.[49] Propionate is one of the main SCFAs and might be involved in stabilizing inflammation in the gut.[50,51]

Although making conclusions about the health effects of the housing environment is challenging based solely on observed changes in fecal microbial composition, the immune response detected in the ileum does implicate a beneficial effect of the soil environment. IL-10, Foxp3, and CTLA4 were all upregulated in

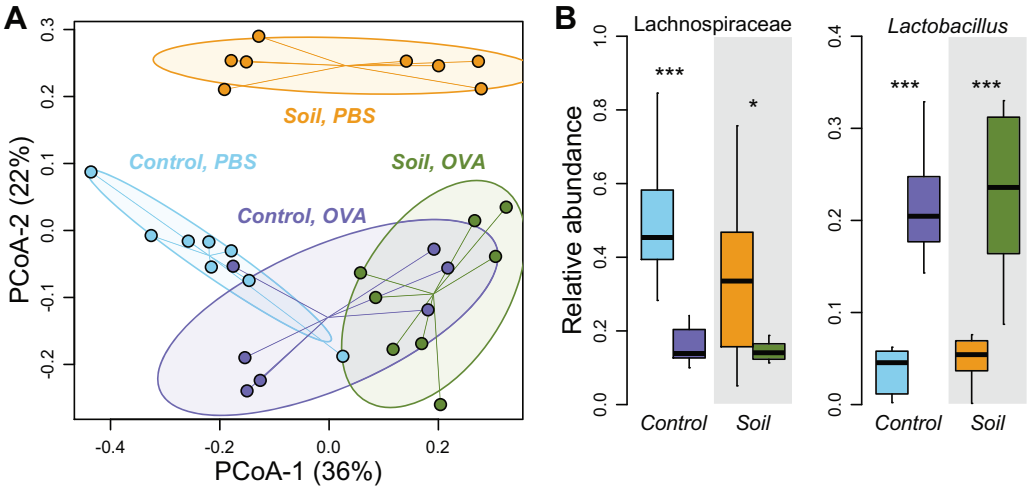

FIG 5. Allergen-induced lung inflammation modifies the mouse gut microbiota. **A,** Composition of the murine fecal microbiota is distinct between OVA-allergen–sensitized mice and healthy nonsensitized mice. *PCoA*, Principal coordinates analysis. **B,** Relative abundance of Lachnospiraceae and *Lactobacillus* species in the fecal microbiota of mice. Blue, PBS control mice; purple, OVA-sensitized control mice; orange, PBS soil-exposed mice; green, OVA-sensitized soil-exposed mice. $*P < .05$ and $***P < .001$.

the ileums of mice in the soil group, whereas proinflammatory cytokines, such as IL-1β, IL-23, IL-17, or TNF, were expressed at the same level in the soil group compared with control values. Foxp3 is a marker of regulatory T cells, indicating an increased presence of this cell type in the ileum. Foxp3 has also been suggested to mediate protection against asthma as a result of increased acetate production by the gut microbiota.[46] IL-10 is a key anti-inflammatory cytokine suppressing immune responses at environmental interfaces, such as the gut and lungs,[52] and CTLA4 downregulates T-cell activation by competing with CD28 for B7 binding.[53] CTLA4 is constitutively expressed by approximately 40% of mouse regulatory T cells[54] and induced in effector T cells on activation. CD86, which is expressed on antigen-presenting cells, provides costimulatory signals needed for T-cell activation and survival. Moreover, CD86 plays an essential role in induction of low-dose oral tolerance, and specific blocking of CD86 in an oral tolerance *in vivo* model inhibited regulatory T-cell generation.[55]

Additional evidence for the beneficial influence of the soil environment came from testing the effect of the murine asthma model protocol in the 2 groups, revealing considerably attenuated T_H2-driven lung inflammation in soil-exposed mice compared with control mice. We have previously shown that exposure to microbial stimuli through the skin induces local expression of T_H1-type and anti-inflammatory mediators, resulting in protection against allergic sensitization and inflammation in the lung.[15] Here we show a similar effect on the gut-lung axis, finding increased anti-inflammatory activity in intestinal tissues after exposure to soil and modified gut microbiota. This effect was associated with reduced T_H2-type signaling in the lung, including diminished expression of the cytokines IL-5 and IL-13 and decreased recruitment of eosinophils after allergen challenge. Moreover, the proportion of T_H1 and anti-inflammatory signaling relative to T_H2 signaling in the lung was significantly greater in the soil group.

Importantly, the soil environment induced expression of the enzyme A20, which has been shown to be a key component in protecting mice against experimental asthma.[56] Lung epithelial production of A20 attenuates nuclear factor κB activation by deubiquitinating key signaling intermediates downstream of TLR, IL-1 receptor, and TNF family receptors.[57,58] A single nucleotide polymorphism in the gene encoding A20 was associated with the risk of asthma and allergy in children living on farms,[59] underlining the potentially important role of A20 in allergy-protective pathways.

Remarkably, although the fecal microbiota was clearly different in healthy mice between the 2 housing environments, experimentally induced lung inflammation had a strong influence on the fecal community, largely masking the effect of environmental exposure (Fig 5, *A*). At the level of the ileum, in the soil group inflammation resulted in loss of microbial diversity. Moreover, expression of proinflammatory mediators and AMPs were significantly increased in ileal tissue in the mice with induced lung inflammation, likely influencing the composition of the gut microbial community. The systemic effects of the gut microbiota and especially its ability to influence immunity at distal sites has gained increased interest in the recent years.[60] Manipulation of the gut microbiota in treatment of respiratory diseases is considered a promising field, although the underlying mechanisms in the interaction between these 2 sites are not well understood.[61] Even less is known about the possible bidirectionality of the gut-lung axis. The lung microbiota is altered during development of allergic asthma, and it has been suggested that both the gut and lung microbiota, as well as the local and systemic immune response play a role during pathogenesis.[62]

Sensitization of mice with house dust mite has been associated with an increase in Lachnospiraceae at the expense of Porphyromonadaceae and Prevotellaceae.[63] In addition, a higher count of bacteria in mouse cecum and a minor shift in microbial community composition has been observed after LPS installation directly into the lungs.[64] In our study levels of lactobacilli were increased in the fecal microbiota after lung inflammation, whereas Lachnospiraceae levels were decreased (Fig 5, *B*). Levels

of commensal Lachnospiraceae taxa, most of which are capable of producing butyrate and acetate, were recently shown to decrease along an inflammatory gradient in the mouse gut.[65] Here, among the increased lactobacilli species was *L johnsonii*, which has been associated with protection against airway OVA challenge in mice.[66] Fujimura et al[66] compared the gut microbiota of mice exposed to dog-associated house dust with control mice, but it is unclear whether the control mice were also exposed to the OVA challenge. Therefore it cannot be ruled out that lung inflammation *per se* is causing the increase in *Lactobacillus* species abundance. Lactobacilli are commonly used as probiotics exerting anti-inflammatory effects, but increases in their proportions have also been detected in patients with inflammatory conditions, such as inflammatory bowel disease.[67] Notably, *Lactobacillus* species have also been shown to be coenriched with *Salmonella* species–induced intestinal inflammation, but the implications of this enrichment are not known.[65]

In the present study the ileal microbiota was dominated by *Lactobacillus* species and SFBs in both healthy and allergen-sensitized mice, with highly varying abundances. It is unclear how these groups are related, with several studies reporting both negative and positive correlations between them.[68-71] SFBs are unique in their ability to modify host immune responses by stimulating maturation of B- and T-cell compartments and inducing an increase in small intestinal T_H17 responses.[72] Because of their small genome size, SFBs are highly dependent on essential nutrients derived from the host and possibly also from other microbes.[73,74]

In conclusion, our results provide further evidence of the bidirectional nature of the microbiota–immune system interaction, which is known to play an important role in brain-gut dialogue[75] but has been far less studied for other organs. Moreover, we demonstrate the substantial effect that the living environment alone can have on gut microbiota composition and inflammatory responses, leading to notable health outcomes.

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Key messages

- **Contact with soil modifies the mouse gut microbiota and shifts the fecal microbiota toward Bacteroidetes dominance.**
- **Soil-treated mice display a T_H1/regulatory T cell–polarized immune system and significantly reduced allergic T_H2-type responses compared with the control group.**
- **Exposure to environmental biodiversity might be protective against allergies through modifying microbiota composition and immune responses.**

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METHODS

Sensitization and airway challenge

After a period of 6 weeks of contact with soil (or clean bedding), 8 mice from each housing condition were exposed to the murine asthma model protocol, receiving 2 intraperitoneal injections of 50 μg of OVA emulsified in 2.25 mg of alum in a total volume of 100 μL of PBS on days 0 and 14, followed by intranasal challenge with OVA (50 μg of OVA in 50 μL of PBS) on days 28, 29, and 30 and collection of samples on day 32 (see Fig E1). The control mice (n = 8 mice in each housing condition) received alum in PBS and were challenged with PBS only. For analyses of inflammatory parameters, blood, lung tissue, and BALF samples were collected. Tracheas were surgically exposed, cannulated with a syringe, and flushed twice with 0.8 mL of PBS to collect BALF samples. BALF cell differentials were determined on slide preparations stained with May-Grünwald-Giemsa and counted in 15 to 20 high-power fields under light microscopy. Part of the left lung was removed for RNA isolation, and the right lung was fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin or periodic acid–Schiff solution. For analysis of the mouse microbiota, tissue samples were collected from the jejunum and ileum in addition to fresh fecal samples. Samples of the cage material and drinking water were also collected to analyze the microbial composition of the environment. All samples were stored at −80°C. Tissue samples for RNA extraction were stored in RNAlater (Thermo Fischer Scientific) at −80°C.

DNA extraction

Total bacterial DNA was extracted from mouse fecal samples by using the repeated bead-beating plus column method.[E1] Bacterial DNA from ileal and jejunal biopsy specimens was extracted by using a phenol-chloroform extraction method. Briefly, 940 μL of TE buffer (pH 8), 50 μL of SDS (10%), and 10 μL of Proteinase K (20 mg/mL) were added to the biopsy specimen and incubated at 55°C for 1 hour. The sample was dissolved in the buffer by means of pipetting and transferred to a bead-beating tube containing silica glass beads (0.1 mm), after which 150 μL of buffered phenol (pH 7-8) was added to the sample. The sample was homogenized for 3 minutes by using the FastPrep-24 Instrument (MP Biomedicals). After this, 150 μL of chloroform/isoamyl alcohol (24:1) was added, and the sample was centrifuged for 10 minutes at 15,000*g* at 4°C. The upper layer of the sample was transferred to a new tube, and 150 μL of buffered phenol and 150 μL of chloroform/isoamyl alcohol were added. The sample was mixed and centrifuged again under the same conditions. The upper layer was again transferred to the new tube. Then, 300 μL of chloroform/isoamylalcohol was added, and the sample was mixed and centrifuged by using the above conditions. The upper layer was transferred to a new tube, and an equal volume of 2-propanol and one tenth volume of sodium acetate (3 mol/L) was added. The sample was kept at −20°C for 30 minutes, after which it was centrifuged for 20 minutes at 15,000*g* and 4°C.

The supernatant was removed, and the pellet was washed with 70% ethanol. After washing, the pellet was rehydrated in 50 μL of TE buffer. DNA from the environmental control samples (soil and sawdust used as bedding and drinking water) was extracted with the FastDNA SPIN Kit for Soil (MP Biomedicals), according to the manufacturer's instructions. Reagent controls included extractions without addition of any sample as a negative control for library construction and potential reagent contamination. DNA concentrations were measured with Qubit 3.0 Fluorometer (Thermo Fisher Scientific).

The V1–V3 region of the 16S rRNA gene was amplified by using the barcoded primers AGAGTTTGATCMTGGCTCAG[E2] and GTATTACCGCGGCTGCTG.[E3] DNA amplification of samples started with 30 seconds of denaturation at 98°C, followed by 25 to 30 cycles consisting of denaturation (10 seconds at 98°C), annealing (30 seconds at 65°C), extension (15 seconds at 72°C), and a final extension at 72°C for 5 minutes. Between 20 and 50 ng of template was used for each reaction. PCR was done in triplicate for all samples, after which the products were pooled. PCR products were purified with Ampure XP beads (Beckman Coulter, Indianapolis, Ind), according to the manufacturer's instructions. Paired–end sequencing (2 × 300 bp) with Illumina MiSeq was done at the Institute for Molecular Medicine Finland (University of Helsinki).

Real-time qPCR

Total RNA was extracted from lung and ileal tissue samples by using a standard protocol of tissue homogenization in QIAzol lysing buffer and the FastPrep Instrument (Thermo Fisher Scientific), followed by RNA isolation with the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. RNA quantity and quality were measured with the NanoDrop ND-1000 (Thermo Fisher Scientific), and 0.5 μg of RNA was reverse transcribed into cDNA by using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific), according to the manufacturer's instructions. A reaction was performed in 25 μL at 25°C for 20 minutes, followed by 37°C for 120 minutes.

mRNA levels of IL-5, IL-10, IL-13, IL-17A, IL-23, TNRF2, CTLA4, CCL17, Foxp3, CD86, IFN-γ, and TNFAIP3 were analyzed by means of quantitative RT-PCR with TaqMan chemistry and the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Reactions were performed in 1 cycle of 2 minutes at 50°C and 30 seconds at 95°C, followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C. PCR amplification of the endogenous 18S rRNA and TAT-binding protein was performed for each sample to control sample loading and allow normalization between samples. Probe and primer sets were purchased from Thermo Fisher Scientific. Results are expressed as relative units, which were calculated by using the comparative cycle threshold method, according to the manufacturer's instructions.

Sequence processing

PCR primer sequences were removed with cutadapt, version 1.4.2,[E4] with a minimum match length of 13. Paired-end reads were joined by using VSEARCH, version 0.9.6,[E5] with default options, and quality trimmed by using USEARCH, version 8.0,[E6] fastq_filter command with the options fastq_maxee 2 and fastq_minlen 300.

Before OTU picking, sequences were sorted by their frequencies, and singletons were removed. OTU clustering was done with USEARCH with default options (because the OTU clustering in USEARCH includes chimera filtering, this was not done in a separate step). The OTU representative sequences were classified and aligned with mothur, version 1.36,[E7] using the SILVA rRNA gene database, version 123.[E8] Phylogeny was generated with FastTree, version 2.1.8,[E9] using the generalized time-reversible model.

All nonbacterial and contaminant OTUs identified from control samples were removed before any downstream analysis. Samples with library sizes smaller than 2500 reads were removed from the analysis, leaving us with a total of 127 samples for the downstream analysis. Because of large variation in library sizes, read counts were normalized by using the CSS method from the metagenomeSeq package, version 1.11,[E10] in R software, version 3.2.4.[E11]

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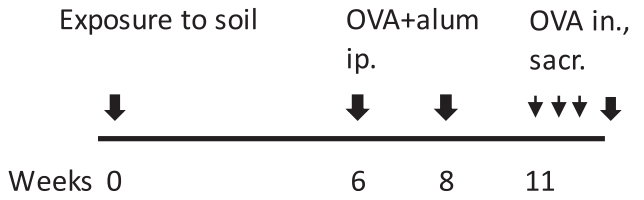

FIG E1. Murine lung inflammation model protocol. *in.*, Intranasal; *ip.*, intraperitoneal; *sacr.*, sacrifice.

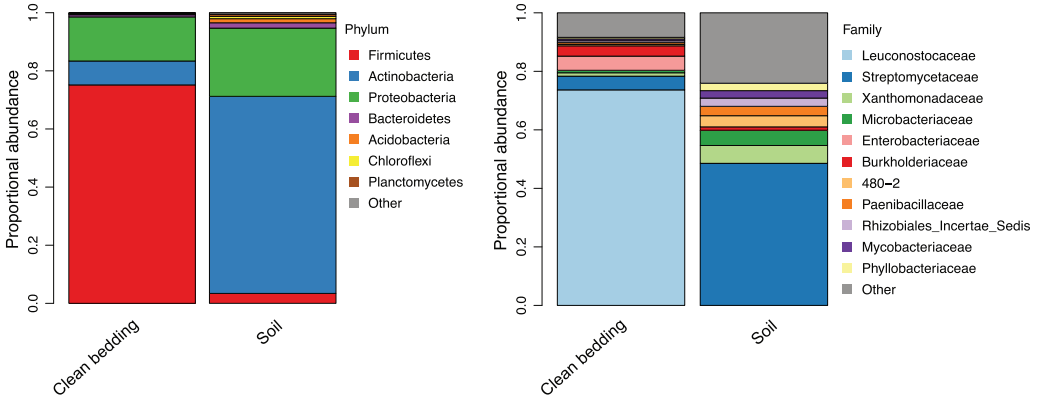

FIG E2. Microbial composition of housing environments shown at the phylum and family levels.

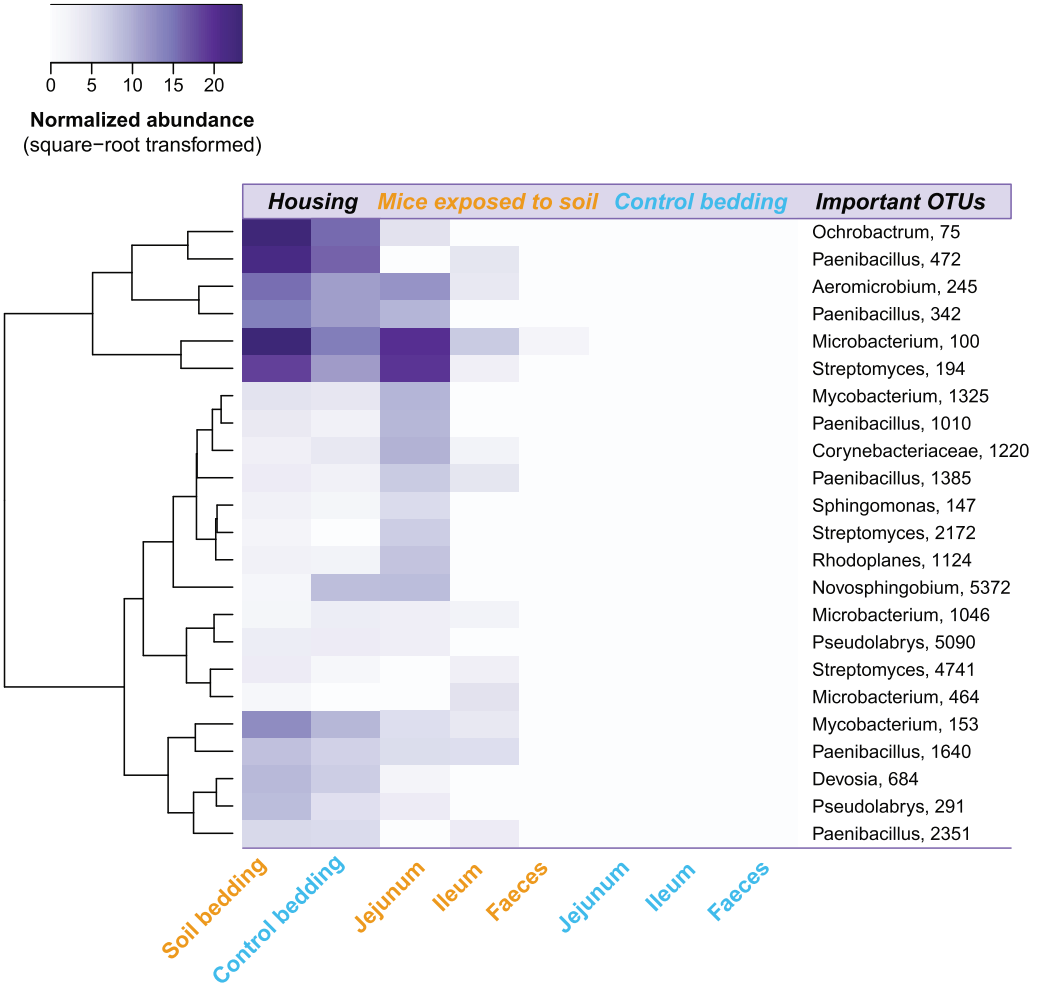

FIG E3. Exposure to soil increases the number of rare OTUs in the intestinal microbiota of mice. Square root–transformed abundances of appointed OTUs that occurred in soil, which was used as a housing material, and in the intestines of soil-exposed mice but not in the intestines of control mice. Columns representing jejunum, ileum, and feces show a summed abundance of each OTU of corresponding samples. OTUs that occurred only once in the jejunum, ileum, or fecal samples and had a unique annotation at the family level were not considered. In addition, OTUs that occurred abundantly in the technical controls (blank samples) for DNA extraction, PCR, or DNA purification were excluded. Orange, Soil group; blue, control group.

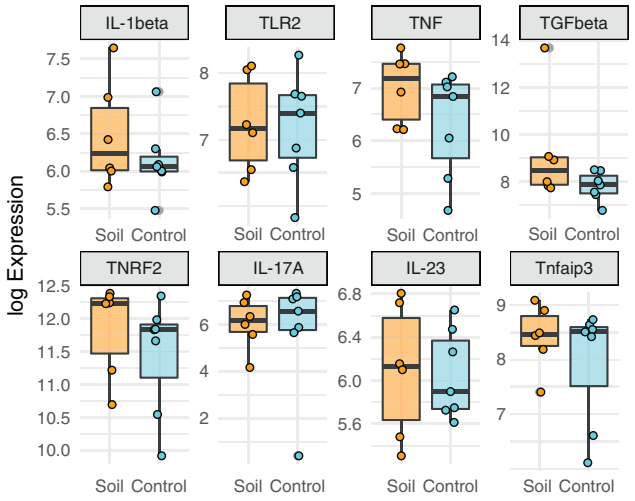

FIG E4. Expression levels of cytokines measured in the ileums of mice in the PBS group.

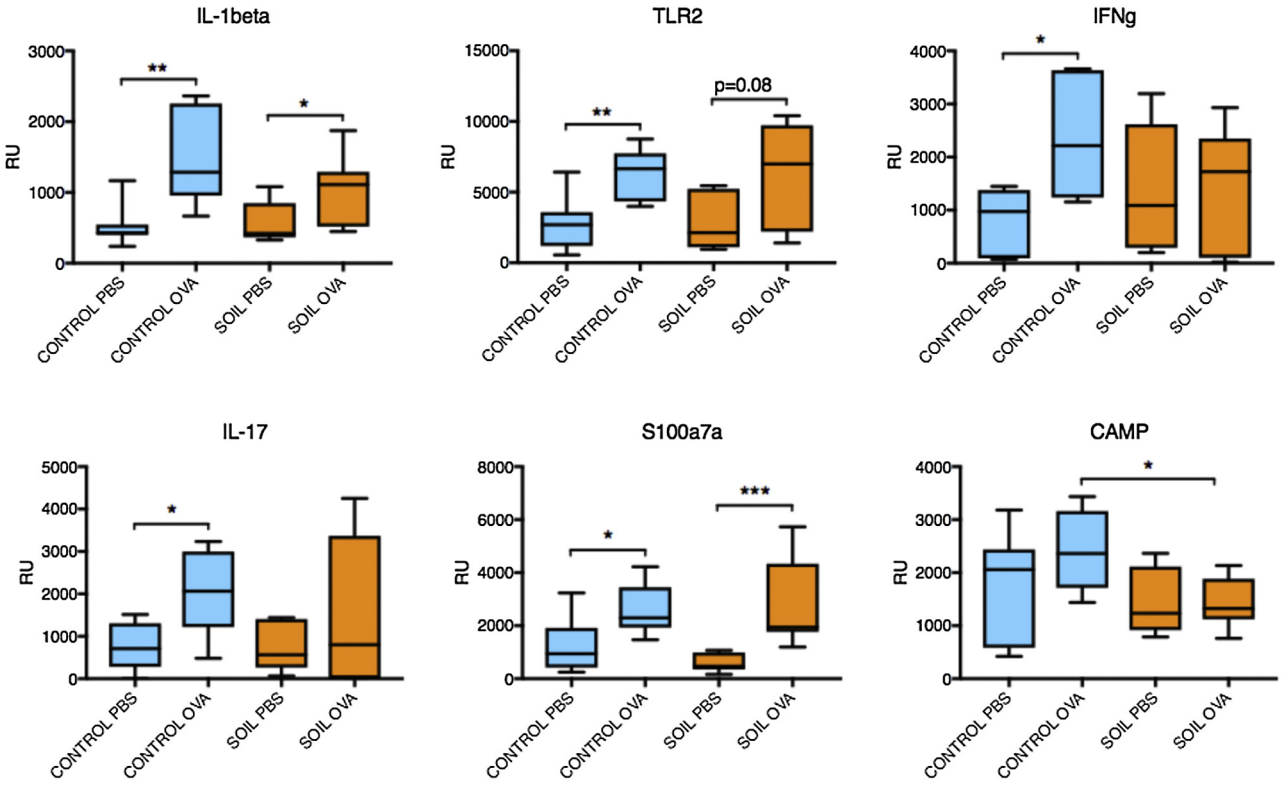

FIG E5. Expression of IL-1β, TLR2, IFN-γ, IL-17, S100A7a, and cathelicidin antimicrobial peptide *(Camp)* in the ileums of PBS- and OVA-treated mice in the control and OVA groups. **P* < .05, ***P* < .01, and ****P* < .001.

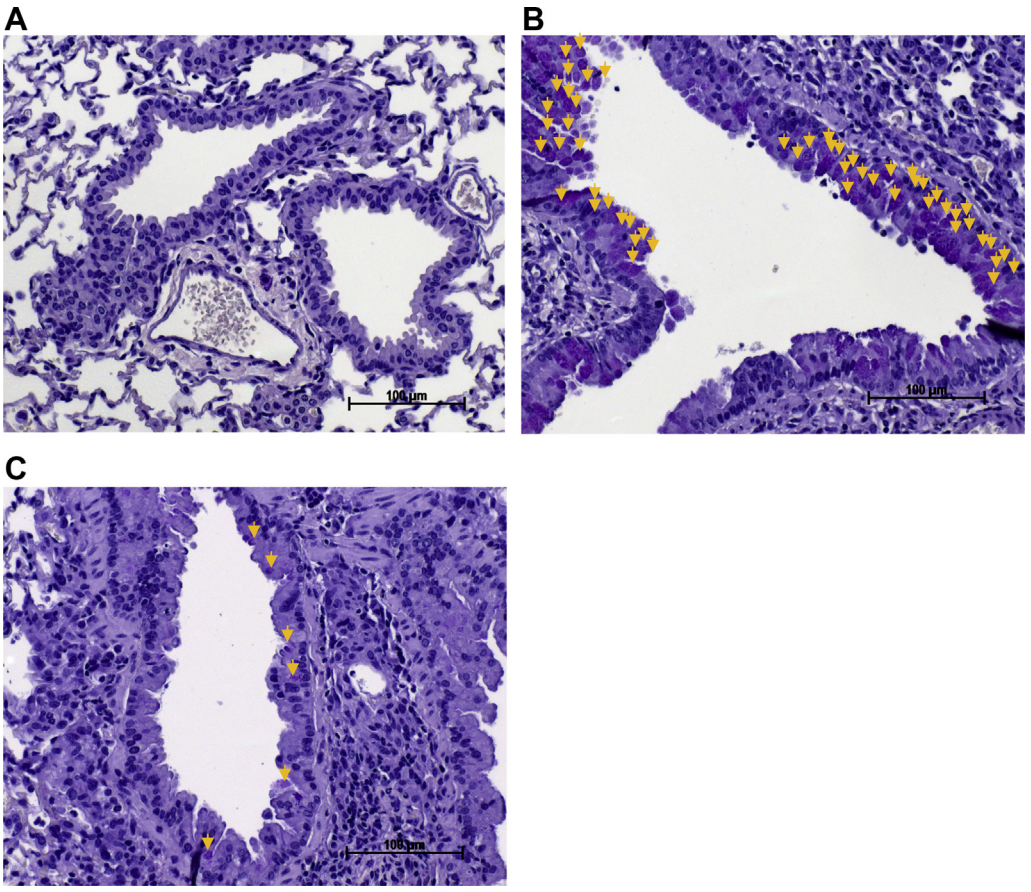

FIG E6. Periodic acid–Schiff (PAS) staining of lung tissue in representative images from control PBS **(A)**, control OVA **(B)**, and soil OVA **(C)** groups. *Yellow arrows* indicate PAS^+ cells.

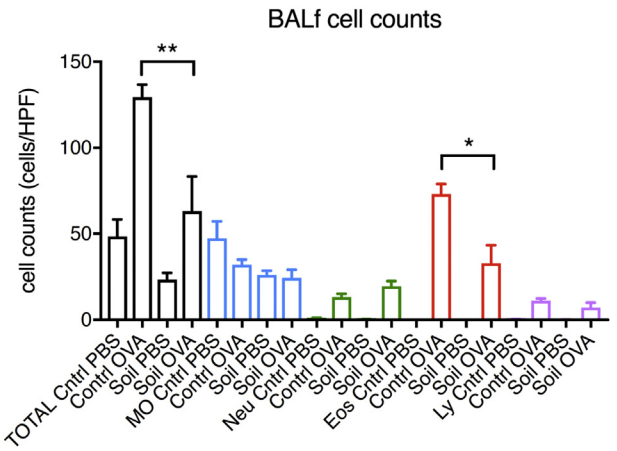

FIG E7. BALF cell counts (cells/high-power field). *$P < .05$ and **$P < .01$.

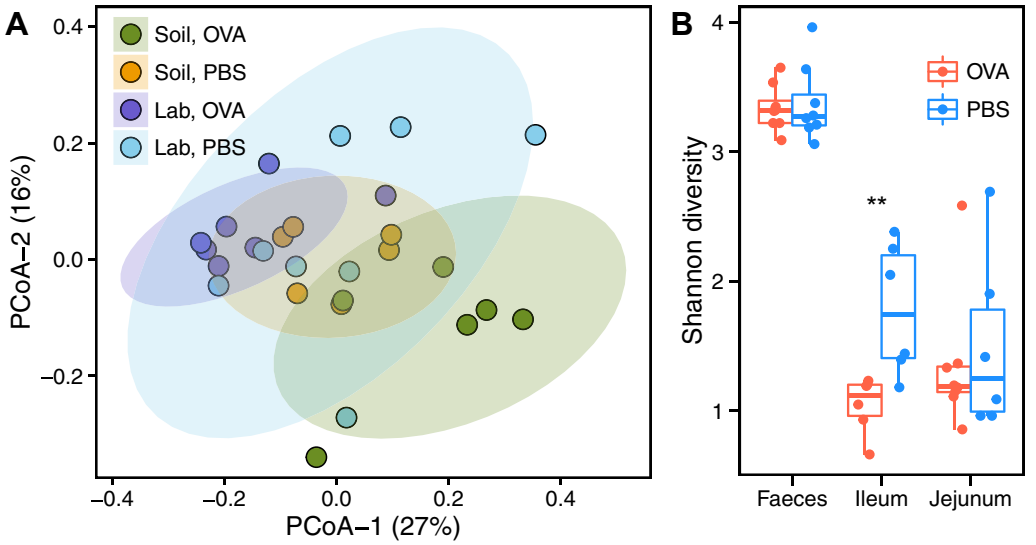

FIG E8. A, Principal coordinates analysis *(PCoA)* plot illustrating clustering of samples from the mouse ileum. Results are based on the Morisita-Horn dissimilarity on log-transformed abundances. **B,** Microbial diversity (Shannon index) in the soil group between nonsensitized (PBS) and OVA-sensitized mice in fecal samples, the ileum, and the jejunum. $^{**}P < .01$.

TABLE E1. Product data sheet of the soil used in the experiments

Product type	Special seedbed product
Ingredients	Various peat mixtures, natural compost mixture (chicken manure, peat, and tree bark), clay fine sand, yeast extract, *Bacillus* species (108 colony-forming units/L)
Liming substance	Magnesium-rich limestone powder (6 kg/m^3)
Acidity	pH 6.5
Conductivity	30 mS/m
Nitrogen, water soluble	350 mg/kg dry matter (90 mg/L)
Phosphorus, soluble	1500 mg/kg dry matter (380 mg/L)
Potassium, soluble	1500 mg/kg dry matter (380 mg/L)
Volume weight	430 g/L
Humidity	45%
Bulk density	240 g/L
Roughness	<25 mm

Translated from Finnish from the provider's Web site: https://www.greencare.fi/tuote/grobiootti-tm-taimimulta/.

TABLE E2. Distance analysis of fecal samples

Environment	Metric	Transformation	R^2	*P* value
Control	Bray and Curtis	None	0.25	.002
Control	Bray and Curtis	SQRT	0.22	.003
Control	Bray and Curtis	LOG	0.18	.001
Control	Morisita-Horn	None	0.3	.007
Control	Morisita-Horn	SQRT	0.35	.001
Control	Morisita-Horn	LOG	0.23	.001
Control	Weighted UniFrac	None	0.41	.001
Control	Weighted UniFrac	SQRT	0.35	.005
Control	Weighted UniFrac	LOG	0.21	.009
Soil	Bray and Curtis	None	0.28	.002
Soil	Bray and Curtis	SQRT	0.24	.001
Soil	Bray and Curtis	LOG	0.2	.001
Soil	Morisita-Horn	None	0.36	.001
Soil	Morisita-Horn	SQRT	0.4	.001
Soil	Morisita-Horn	LOG	0.28	.001
Soil	Weighted UniFrac	None	0.26	.002
Soil	Weighted UniFrac	SQRT	0.24	.007
Soil	Weighted UniFrac	LOG	0.2	.018

LOG, Logarithmic; *SQRT*, square root.

TABLE E3. Distance analysis of fecal samples

Treatment	Metric	Transformation	R^2	*P* value
OVA	Bray and Curtis	None	0.24	.002
OVA	Bray and Curtis	SQRT	0.26	.001
OVA	Bray and Curtis	LOG	0.24	.001
OVA	Weighted UniFrac	None	0.12	.128
OVA	Weighted UniFrac	SQRT	0.16	.041
OVA	Weighted UniFrac	LOG	0.16	.044
OVA	Morisita-Horn	None	0.23	.014
OVA	Morisita-Horn	SQRT	0.39	.002
OVA	Morisita-Horn	LOG	0.33	.001
PBS	Bray and Curtis	None	0.26	.001
PBS	Bray and Curtis	SQRT	0.26	.001
PBS	Bray and Curtis	LOG	0.24	.001
PBS	Weighted UniFrac	none	0.19	.069
PBS	Weighted UniFrac	SQRT	0.15	.074
PBS	Weighted UniFrac	LOG	0.16	.053
PBS	Morisita-Horn	None	0.32	.002
PBS	Morisita-Horn	SQRT	0.41	.001
PBS	Morisita-Horn	LOG	0.34	.002

LOG, Logarithmic; *SQRT*, square root.