Original Research *Highlight article*

Animal unit hygienic conditions influence mouse intestinal microbiota and contribute to T-cell-mediated colitis

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Impact Statement

Gut microbiota is strongly connected with the health of the human digestion system. There are reports indicating that gut microbiota composition may contribute to the development of inflammatory bowel disease (IBD) via influencing T cell activity. This research provides proof for the impact of hygienic conditions in animal housing environments on experimental colitis development in mice. We further provide data on the correlation between environmental factors and changes of microbiota composition in mice. Our data indicate that the richness, diversity of microbiota, and presence of commensal and pathogenic bacterial species determine the severity of the experimental colitis development by affecting the change of helper T cell subsets. As such, this study provides evidence to demonstrate the importance of hygienic conditions in animal housing environments for experimental colitis progression.

Abstract

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders of the gastrointestinal tract with worldwide increasing incidence. Recent studies indicate that certain species of intestinal bacteria are strongly associated with IBD. Helper T lymphocytes are not only the key players in mediating host defense against a wide variety of pathogens but also contribute to pathogenesis of many immune-related diseases. Here, using the T cell transfer model of colitis, we observed that the mice maintained in a specific-pathogen free (SPF) unit after receiving naïve $CD4^+$ T cells developed mild disease. The same mice developed different degrees of disease when they were maintained in a conventional animal facility (non-SPF), where some pathogens were detected during routine health monitoring. Consistently, increased circulating inflammatory cytokines as well as Th1 and Th17 cells were detected in mice housed in non-SPF units. 16S rRNA sequencing of feces samples enabled us to identify changes in the microbiota composition of mice kept in different facilities. Our data indicate that environmental factors influence gut microbiota composition of mice, leading to development of colitis in a T-cell-dependent manner. In conclusion, changes in environmental conditions and microbial status of experimental animals appear to contribute to progression of colitis.

Keywords: Microbiota, hygienic conditions, helper T cells, Th17 cells, intestine inflammation

***Experimental Biology and Medicine* 2022; 247: 1752–1763. DOI: 10.1177/15353702221113826**

Introduction

Inflammatory bowel disease, comprising Crohn's disease (CD) and ulcerative colitis (UC), is characterized by chronic intestinal inflammation. Although the pathogenesis of these diseases has not been fully elucidated, both genetic and environmental factors contribute to the development of IBD. An increased incidence of chronic inflammatory diseases, such as IBD, has been linked to lifestyle, dietary changes, and the resulting impact on gut microbiota composition.[1] Loss of microbiota diversity and the prevalence of distinct bacterial species in IBD patients, compared to healthy controls, further suggest that the microbiome plays an important role in IBD development, relapse, and response to treatment.[2,3] Murine models have been demonstrated as a powerful tool to explore host-microbiota interactions in mucosa.[4,5] Animal studies have demonstrated that the gut microbiota is indispensable for pathogenesis in most animal models of colitis.[6]

Mice have a similar microbiota composition to humans, with 90% of the bacterial population composed of Firmicutes and Bacteroidetes.[7] This similarity makes these models relevant to the understanding of IBD, although it is also important to consider how microbiota variations in laboratory mice affect disease phenotype, reproducibility, and relevance

to an understanding of the human disease. Several factors affect laboratory mouse gut microbiota including less exposure to pathogens, different diets, housing conditions, and genetics. Different microbiota compositions are observed in laboratory mice depending on the supplier.[8] In general, laboratory mice have relatively simplified microbiomes compared to wild mice, and the variation in the prevalence of specific bacterial populations can affect experimental results. Differences in susceptibility to infection have been observed between specific-pathogen-free (SPF) mice and germ-free mice.[7] Furthermore, the presence of commensal bacteria can protect from colonization of pathobionts.

The T cell transfer colitis is one of the animal models of human IBD. In this model, naïve $CD4^+$ T cells isolated from immunocompetent mice are transferred to immunodeficient Rag -/- or severe combined immunodeficient (SCID) mice, and consequently cause colitis.[9] In an early study, upon transfer of naïve cells, a high proportion of IFN-γ-producing cells was detected in the lamina propria of diseased SCID mice.[9] Therefore, it was generally believed that IFN-γ-producing Th1 cells are the key players in IBD pathogenesis. However, later studies demonstrated the requirement of additional mechanisms for colitis development, including the IL23 signaling pathway and Th17 cells. The significance of targeting these mechanisms has been shown in several animal models of IBD, including the T cell transfer colitis model,[10–12] and in clinical trials of CD.[13] The essential role of Th17 cells in IBD has been well documented. The IL23/IL-17 axis plays pivotal roles as the immediate effectors of IBD, whereas defects in Treg cells play distinct causative roles in IBD.[14–16] Genome-wide association studies[17,18] further support the importance of IL-23/IL-17 signaling in the pathogenesis of IBD.[19]

As previously mentioned, different environmental factors such as housing of mice in different caging systems may influence the microbiota composition and activity. It has been reported that gut microbial communities are protected from environmental contamination in mice housed in an individually ventilated caging system.[20] However, it remains unclear how environmental factors in different facilities change the gut microbiota composition and contribute to the development of colitis.

In this study, we investigated how animal housing conditions in an SPF or non-SPF animal unit influenced gut microbiota of mice and consequently the development of colitis in a T-cell-dependent manner.

Materials and methods

Animal husbandry and diet

This study included the use of mice and was carried out in strict accordance with the European (the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes) and Finnish legislation (Act 497/2013 and Government Decree 564/2013 on the Protection of Animals Used for Scientific or Educational Purposes). The study protocols and procedures were reviewed and approved by the National Project Authorization Board of Finland (license number ESAVI/2502/04.10.07/2015).

Rag1-/- (NOD.129S7(B6)-*Rag1*^{tm1Mom}/J) and C57BL/6J mice were supplied by Jackson laboratories (USA). Animals were housed (2–5 animals per cage) in individually ventilated cages (IVC) in the SPF animal facility and in open top cages in the non-SPF animal facility with Aspen bedding and nesting material (Tapvei Oy, Estonia) and polycarbonate tunnels as enrichment. The temperature in the experimental animal room was 21 ± 3°C, with relative humidity of 55 ± 15% and following a 12-h light and 12-h dark light cycle. The mice were fed *ad libitum* with RM3 soy-free diet (Special Diet Services, Witham, Essex, England), and tap water was provided *ad libitum.* Mice were housed in the respective experimental conditions, in SPF group, $n = 7$; in non-SPF1, $n = 6$; in non-SPF2, $n = 5$; and in non-SPF3, $n = 6$. The mice were monitored for signs of colitis and euthanized at the indicated time point of 13 weeks for SPF mice, 10 weeks for non-SPF1 and non-SPF2 mice, and 8 weeks for non-SPF3 mice. Fecal samples for DNA extraction and blood samples for serum cytokine detection were collected at the time of euthanasia.

T cell transfer model of colitis

A well-characterized mouse model of IBD was used to study T-cell-dependent colitis in mice. Colitis was induced in immunodeficient Rag1-/- mice that lack mature B and T lymphocytes, by adoptive transfer of naïve $CD4^+CD45RB^{hi}CD25^-$ T cells, which were isolated from immunocompetent, wild type C57BL/6J mice. Spleens were isolated from 6- to 7-week-old male C57BL/6J mice and were disaggregated by pressing through a 70-μm filter, red blood cells were lysed with ACK lysing buffer (Invitrogen). $CD4^+$ T cells were enriched using magnetic separation with a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Naïve $CD4^+CD45RB^{hi}CD25^-$ T cells were further purified by FACS sorting using antibodies recognizing CD4, CD45RB, CD62L, and CD25 (eBiosciences). 400,000 FACS-sorted naïve T cells in a total volume of 200 μL PBS were injected into male Rag1-/- mice. Mice were weighed prior to the injection and weekly thereafter.

Flow cytometry

For colitis experiments, spleen and mesenteric lymph nodes (MLN) were harvested from mice and quantified prior to re-stimulation for 4 h in the presence of PMA and ionomycin plus Golgi inhibitor. For analysis of surface markers, cells were stained in PBS containing either 5% or 0.1% (wt/vol) fetal bovine serum (FBS) with anti-CD4 and anti-CD3 purchased from eBiosciences. Stimulated cells were fixed and permeabilized with Transcription Factor Staining Buffer Set (eBiosciences, San Diego, CA, USA) stained with anti-IFN-γ and anti-IL-17A (both from eBiosciences) according to the manufacturer's instructions, and cells were acquired using an LSRII flow cytometer (Becton Dickinson, San Jose, CA, USA). Events were collected and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Histopathology

Colonic sections from mice were collected, weighted, measured, and then fixed in 10% neutral-buffered formalin for

24 h at room temperature. Complete cross-sections of formalin-fixed intestinal sections were placed in cassettes, embedded in paraffin, sectioned at 4 µm thickness, mounted on glass slides, and stained with hematoxylin and eosin (H&E). Histological sections were evaluated and scored according to the following criteria: (A) Distribution of the inflammation: 0 = None, 1 = Focal, 2 = Multifocal, 3 = Diffuse, 4 = total/whole/maximal distribution; (B) Degree of inflammation: 0 = None, 1 = Low level of inflammation with scattered infiltrating mononuclear cells (1–2 foci), 2 = Moderate inflammation with multiple foci, 3 = High level of inflammation with increased vascular density and marked wall thickening, 4 = Maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells. The cumulative score represents the sum of these two independent criteria.

Cytokine detection

Serum samples were collected from colitis experiment mice at the time of euthanasia. Serum cytokines were quantified using a Millipore (Billerica, MA, USA) MILLIPLEX® MAP Kit.

Health monitoring of animal units

Health monitoring was carried out according to FELASA recommendations.[21] Samples were collected from sentinel mice kept in the animal rooms by direct sampling of Rag -/- mice. Sentinel mice are weekly exposed to soiled beddings of other animals maintained in the animal facility. A few blood drops were collected to Opti-Spot strips (IDEXX BioResearch, Stuttgart, Germany) for serologic analysis. Oral and fur swabs and feces were collected for PCR analyses. Up to five samples were pooled separately for oral, fur, and feces and sent to IDEXX BioResearch. In addition, SPF was tested by PCR from pooled feces samples.

Microbial community analysis

Fecal samples were collected from mice in colitis experiments at the time of euthanasia. Total DNA was extracted using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

All the qualified DNAs were used to construct libraries of 16S rRNA gene (V3 V4 region) followed by sequencing by 300 bp paired-end run on an Illumina HiSeq 2500 instrument at the BGI Genomics (New Territories).

Data analysis was performed by BGI Genomics (New Territories). Clean reads were obtained after filtering and cleaning, then paired-end reads with overlap were merged to tags, which were clustered to Operational Taxonomic Unit (OTU) at 97% sequence similarity. Taxonomic ranks were assigned to OTU representative sequence using Ribosomal Database Project (RDP) Naive Bayesian Classifier v2.2. Finally, alpha diversity, beta diversity and the different species screening were analyzed based on OTU and taxonomic ranks. Linear Discriminant Analysis Effect Size (LEfSe) was used to identify microbial biomarkers enriched/depleted in each group.[22]

PCR primers targeted to total bacteria (forward: 5′-AGCA CGTGAAGGTGGGGAC-3′, reverse: 5′-CCTTGCGGTTGGC TTCAGAT-3′), Enterobacteriaceae family (forward: 5′-CATT GACGTTACCCGCAGAAGAAGC-3′, reverse: 5′-CTCTACG AGACTCAAGCTTGC-3′), *Akkermansia muciniphila* (forward: 5′-CAGCACGTGAAGGTGGGGAC-3′, reverse: 5′-CCTTG CGGTTGGCTTCAGAT-3′) and *segmented filamentous bacteria* (SFB, forward: 5′-AGGAGGAGTCTGCGGCACATTAGC-3′, reverse: 5′-TCCCCACTGCTGCCTCCCGTAG-3′) were used to perform specific Quantitative real-time PCR (qPCR) in a LightCycler® 480 Real-Time PCR System (Roche®) by use of SYBR® Green PCR Master Mix (Roche®). A melting curve analysis was conducted at the end of the PCR, and bacterial concentration was calculated by comparing the Ct values from standard curves.

Statistical analysis

p-values were calculated using Student's *t*-test and one-way ANOVA + Tukey's multiple comparisons test. Error bars represent means ± SEM.

Results

Hygienic condition in the animal unit contributes to T-cell-dependent colitis development

To perform the T cell transfer model of colitis in our animal facility, flow cytometry sorted naïve (CD4+ CD25-CD45RBhi) T cells from C57BL/6 mice were transferred to Rag1-/- recipients housed in individually ventilated cages in the specific-pathogen-free (SPF) unit. The mice were weighed weekly to monitor colitis development. We observed that the Rag-/- recipient mice kept gaining weight until the time of sacrifice (13 weeks) after naïve $CD4^+$ T cell reconstitution (Figure 1(A)). Histology evaluation confirmed that colons of these mice appeared essentially normal or with mild observable pathology (Figure 1(B)). Health monitoring reports indicated that many of the pathogenic microbes were not detected in the SPF unit (Table 1).

To investigate whether the microbiota in the housing environment influence colitis development, we transferred Rag-/- mice to non-SPF units, where mice were kept in open top cages. Later, the same experiment was performed and we observed that post transfer of naïve $CD4^+$ T cells, Rag1-/- mice stopped gaining weight and even started losing weight by week 7 (Figure 1(A), non-SPF2). These mice had diarrhea and at the time of sacrifice we observed increased colonic weight/length ratio, a marker of tissue edema (Figure 1(D)), indicating that these mice developed more colitis compared to previous experiments performed in the SPF unit. Histopathologic quantitation of colitis development demonstrated that distribution, degree of inflammation, and cumulative score was significantly higher in animals kept in the non-SPF2 unit. Mice that were transferred to the non-SPF2 unit developed significant colonic inflammation after 4 months of transfer (Figure 1(A) to (C), non-SPF2). Interestingly, Rag-/- mice maintained all the time in a separate non-SPF unit also just developed mild colitis as shown by body weight loss and histology evaluation (Figure 1(A) to (C), non-SPF1 group).

Importantly, several pathogenic bacteria species, including *H. hepaticus* and *H. typhlonius* as well as *Klebsiella oxytoca*,

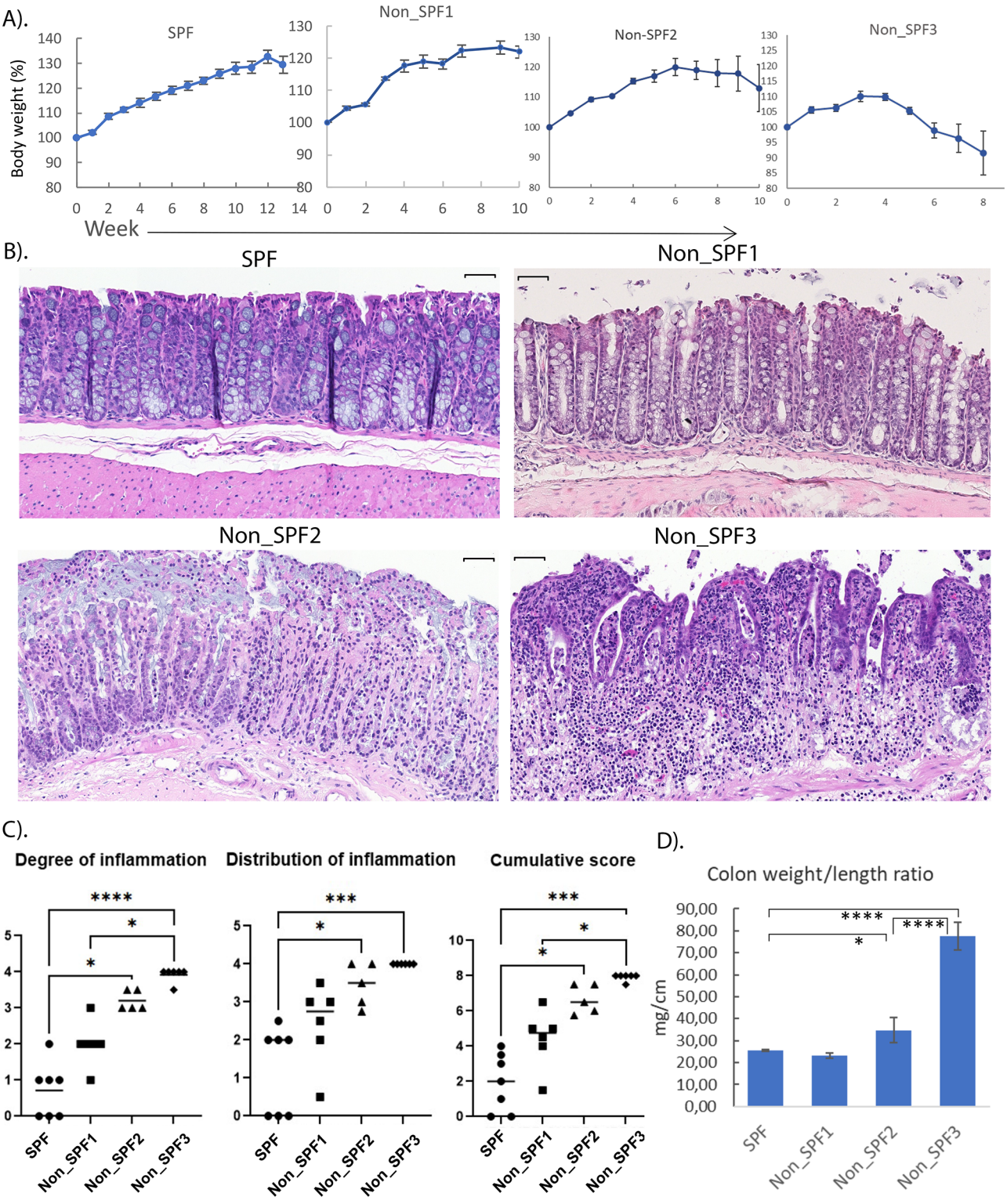

Figure 1. Animal housing environment influences pathogenic potential of colitis. (A) Rag1-/- mice housed in SPF or non-SPF units received 400,000 sorted naïve CD4+ CD45RBhiCD25- T cells isolated from C57BL/6 mice, and mice were weighed weekly to monitor the onset of colitis. In SPF, $n=7$; in non-SPF1, $n=6$; in non-SPF2, $n=5$; and in non-SPF3, $n=6$. (B) Colon sections were used for H&E staining. Representative histological images (H&E) are shown. Scale bar, 50 μm. (C) Histological scoring. Development of colitis was assessed by monitoring the (a) degree of inflammation, (b) distribution of inflammation, and (c) cumulative score. Data were analyzed by Kruskal–Wallis test ($p<0.05$) followed by Dunn's multiple comparisons test (*$p<0.05$; ***$p<0.0005$; ****$p<0.0001$). (D) Colonic weight and length were measured at the time of sacrifice. *$p<0.1$, **$p<0.05$, ***$p<0.01$, ****$p<0.001$; two-tailed Student's *t*-test was used. (A color version of this figure is available in the online journal.)

Pasteurella pneumotropica biotype Heyl (Rodentibacter Heylii) were detected from our Rag-/- mice housed in the non-SPF3 unit (Table 1). In the T cell transfer colitis experiment performed at the same period, which was 8 months following the transfer to the non-SPF unit, Rag-/- mice were found to stop gaining weight 4 weeks after receiving naïve $CD4^+$ T cells,

Table 1. Health monitoring results.

	SPF	Non-SPF1	Non-SPF2	Non-SPF3
Mouse norovirus (MNV)	–		–	–
Helicobacter spp.	–	–	–	+
H. bilis	–	–	–	–
H. ganmani	–	–	–	–
H. hepaticus	–	–	–	+
H. mastomyrinus	–	–	–	–
H. rodentium	–	–	–	–
H. typhlonius	–	–	–	+
SFB	+		+	+
Klebsiella	–		–	+
Pasterurella pneumotropica biotype Heyl		–	–	+

SPF: specific-pathogen free; MNV: murine norovirus; SFB: segmented filamentous bacteria.

started to lose weight from the fifth week post injection, and kept losing weight until the eighth week when they were sacrificed (Figure 1(A), non-SPF3). Meanwhile, we observed that some of these mice had severe diarrhea and blood in the stool. Not surprisingly, these mice showed very high colonic weight/length ratio (Figure 1(D)) and significant histologic changes (Figure 1(B) and (C)) indicating that Rag-/- mice which received naïve $CD4^+$ T cells developed severe colitis.

Murine norovirus (MNV),[23] a prevalent pathogen in animal facilities, is routinely detected by serology in sentinel mice in our non-SPF2 and non-SPF3 facility, but not in SPF and non-SPF1 units. However, MNV was not detected in Rag-/- mice even 8 months post transfer from the SPF facility to a non-SPF2 and non-SPF3 facility. In order to ensure that immunodeficient Rag-/- mice are indeed negative for MNV, we also analyzed the feces samples from Rag-/- mice by PCR for MNV. Again, no MNV was detected in these samples (Table 1).

Animal housing environment alters Th subsets that contribute to colitis development

To further evaluate the severity of inflammation developed from Rag-/- recipient mice housed in both SPF and non-SPF environments, we measured inflammatory cytokines in peripheral blood samples taken from these mice at the time of sacrifice. Compared to mice housed in SPF units, which were without clear signs of colitis (experiment SPF), a significantly higher level of circulating cytokines, including IFN-γ, IL-17, and TNFα, were detected from mice which received naïve T cells and were housed in non-SPF2 units (Figure 2(A)). Consistent with observations from body weight changes and histology, significantly higher concentrations of inflammatory cytokines IL-1β, IL-6, IFN-γ, and IL-17 were detected in the peripheral blood of mice which had lost more weight from the non-SPF3 experiment compared to those mice that experienced less weight loss from the non-SPF2 experiment (Figure 2(A)).

Since we detected increased circulating IFN-γ and IL-17, we next examined whether recipient Rag1-/- mice had enhanced Th1 or Th17 cell differentiation. We performed intracellular cytokine staining to detect the proportion of IFN-γ and IL-17A-producing $CD4^+$ T cells in spleens and mesenteric lymph nodes (MLN) from recipient Rag1-/- mice. Mice housed in non-SPF2 units had a significantly increased number of IFN-γ+ as well as more IL-17 + cells both in the spleen and MLN (Figure 2(B)). Notably, in the non-SPF3 group, in addition to the detected increased serum IL-17, we also observed a higher proportion of IL-17 producing $CD4^+$ T cells in the spleen and MLN (Figure 2(B)). Interestingly, even though IFN-γ producing Th1 cells were detected in all non-SPF experiments, only in the spleen of non-SPF3 mice did we find both IL-17 + and IFN-γ + IL-17 + $CD4^+$ T cells. The IL-23 and Th17 signaling pathways are supposed to be principal to colitis pathogenesis. Since several bacterial species were detected by PCR in feces samples, including *Helicobacter bacteria*, *K. oxytoca*, and *Pasteurella pneumotropica biotype Heyl*, and this might also contribute to the enhanced IFN-γ and IL-17 production. As a conclusion, conventional housing conditions influence the induction of Th1 and Th17 responses that lead to the development of more severe colitis.

Altered gut microbiota correlates with development of T-cell-dependent colitis

To explore how the hygiene conditions in the animal housing environment influences gut microbiota of mice and the development of colitis, we collected fecal samples from Rag-/- mice housed in one SPF and three non-SPF units. DNA was extracted from these fecal samples and processed with 16S rRNA sequencing (V3-V4 region) on an Illumina HiSeq 2500-platform to further extensively compare the difference of the composition of bacteria of Rag-/- mice maintained in different hygiene environments.

First, to examine the differences of Operational Taxonomic Unit (OTU) composition in different samples, principal component analysis (PCA) was used to construct a 2D graph to summarize factors mainly responsible for this difference. PCA analysis showed that the SPF and non-SPF groups could be distinguished based on their relative abundance of each OTU in each sample. Out of all three of the non-SPF groups, the non-SPF1 group displayed a very different OTU abundance profile, while samples from the non-SPF2 and non-SPF3 groups were closely located, indicating that the similarity between these two groups is high (Figure 3(A)). This correlated well with the degree of inflammation, as mice in these two groups developed more severe colitis compared to the non-SPF1 and SPF groups (Figure 1).

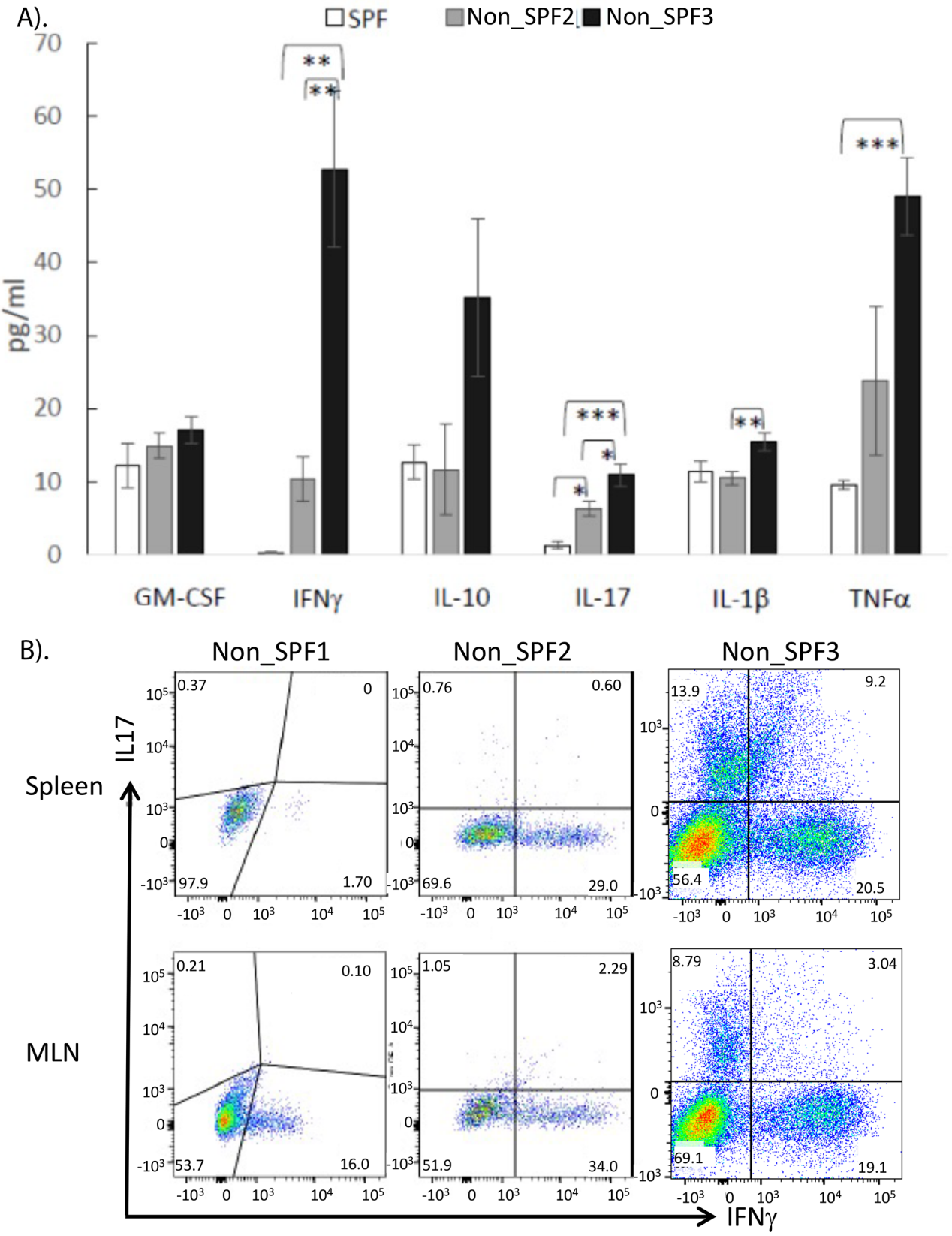

Figure 2. Hygienic conditions in animal housing environment influences Th subsets. (A) Pro-inflammatory cytokines correlate with colitis development. Serum samples were collected at the time of sacrifice. GM-CSF, IFN-γ, IL-10, IL-17, IL-1β, and TNFα were measured using a Luminex MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel. *$p < 0.1$, **$p < 0.05$; ***$p < 0.01$, two-tailed Student's *t*-test. (B). Representative intracellular cytokine staining for IFN-γ and IL-17 within gated CD4$^+$ T cells isolated from spleens and MLN of colitic mice is shown. Spleens and MLNs were harvested, cells were stimulated with PMA and ionomycin for 4 h. Cells were stained with anti-CD4 followed by intracellular cytokine staining performed using Transcription Factor Staining Buffer Set (eBiosciences, San Diego, CA, USA) with antibodies against IFN-γ and IL-17A (both from eBiosciences) according to the manufacturer's instructions. (A color version of this figure is available in the online journal.)

Alpha diversity was then applied to analyze complexity of species diversity. The lowest Chao value, which reflects the species richness of community, was observed in the SPF group (Figure 3(B)). Non-SPF2 and non-SPF3 groups showed no difference of Chao value. The highest species richness was seen in the non-SPF1 group, which developed much milder intestine inflammation compared to non-SPF2 and non-SPF3 groups. The diversity of microbiota also may lead to initiating the development of inflammatory disease. The Shannon value, reflecting the species diversity of the community, was also higher in the non-SPF1 group compared to the non-SPF2 and non-SPF3 groups (Figure 3(B)). Again, the lowest species diversity was observed in the SPF group, in which mice were maintained in a facility with the best hygiene conditions among the four study groups. These results indicate that bacterial richness and diversity may contribute to development of intestine inflammation

Next, we further studied species composition and abundance differences among the four groups. We found that compared to the non-SPF2 and non-SPF3 group, in the non-SPF1 group, *Tenericutes* phylum was enriched, whereas *Proteobacteria* phylum was lower (Figure 3(C)). At the family and genus level, the non-SPF3 and non-SPF2 groups showed higher relative abundance of *Enterococcaceae* (such as enterococcus) and *Escherichia coli* (Figure 3(D)). Consistent with the routine health monitoring results, non-SPF2 and non-SPF3 groups showed increased relative abundance of *Helicobacteraceae* (Figure 3(D)), indicating it to be an important pathogenic agent in intestinal inflammation in the mouse model. Increased relative abundance of *Bacteroidaceae* (such as *bacteroides* species) was detected in the non-SPF2 and non-SPF3 groups, in which more severe colitis had developed compared to the non-SPF1 group. However, although the SPF group developed very mild inflammation, no significant changes of *Bacteroides* level were observed between SPF and non-SPF2 or non-SPF3 (Figure 3(D), Table 1).

We also performed linear discriminant analysis effect size (LEfSe) to compare the alteration of gut microbiota in the four groups.[22] As shown in Figure 4, a significant enrichment of *Helicobacteraceae* and *Enterobacteriaceae* in the *Proteobacteria* phylum is observed in the non-SPF3 group. Notably, significant shifts in the microbiota composition at the phylum level were observed in our LEfSe analysis. In contrast to the enriched *Proteobacteria* phylum detected in the highly inflammatory non-SPF3 group, as seen in Figure 3(C), an increased *Tenericutes* phylum in the non-SPF1 group and increased *Verrucomicrobia* phylum in the SPF group were observed (Figure 4).

Taken together, the 16S rRNA sequencing data show that a clear variation of intestine microbiota was detected from mice housed in different hygiene conditions. Bacteria species richness and diversity, composition of commensal and pathogenic bacteria may contribute to the development of T-cell-dependent colitis.

We tested samples taken for health monitoring for SFB, and this was positively detected in the samples taken from both SPF and non-SPF facilities (Table 1). However, in the SPF unit, mice developed very mild colitis even with the presence of SFB. We further detected the level of SFB in fecal samples collected from different units using qPCR. We used a universal primer pair for the 16S ribosomal RNA coding sequence as endogenous control. qPCR results showed that SFB indeed was present in all facilities. Interestingly, the highest relative abundance was seen in mice feces from the non-SPF1 group with mild colitis, compared with the non-SPF2 and non-SPF3 group with more severe inflammation (Figure 5(A)).

In the *Verrucomicrobia* phylum, *A. muciniphila* is a Gram-negative mucin-degrading bacterium. Here, we observed that the *Akkermansiaceae* family (*A. muciniphila* species) in the *Verrucomicrobiales* phylum was enriched in the SPF group (Figures 3(D) and 4). Because changes of *A. muciniphila* abundance was associated with colitis and IBD, we also performed qPCR analysis to detect *A. muciniphila* in mouse fecal samples from these four groups. The most relatively abundant level of *A. muciniphila* was detected in the SPF group, while the lowest was present in the non-SPF3 group. Notably, the relative level of *A. muciniphila* was inversely correlated with the degree of inflammation (Figures 1 and 5(B)).

Discussion

In this study, we investigated how animal housing conditions influenced the composition shifts of gut microbiota of mice, and consequently the changes of Th subsets for development of colitis.

We have observed that upon transfer from SPF to non-SPF housing conditions, after naïve $CD4^+$ T cell reconstitution, Rag1-/- mice developed more colitis compared to previous experiments performed in the SPF unit. Several pathogenic bacteria species were detected from our Rag-/- mice housed in the non-SPF3 unit during routine health monitoring. These findings suggest that the pathogenic species detected by health monitoring may contribute to the severe intestinal inflammation observed in the T cell transfer colitis experiment.

Elevated levels of inflammatory cytokines IFN-γ and IL-17 in peripheral blood of mice from non-SPF3 experiments are consistent with the reports stating that elevated Th17 and Th1 responses are observed in animal models of colitis as well as in patients with IBD.[4,9] Studies have indicated that *Helicobacter*, through stimulation of IL-23 production, expands Th17 cells.[24,25] In the non-SPF3 experiment, we also observed a higher proportion of IL-17 producing $CD4^+$ T cells both in the spleen and MLN. Consistently, *Helicobacter* bacteria strains were also detected from mice in this experiment. In addition, several other bacterial species were also detected by PCR in feces samples, including *K. oxytoca* and *Pasteurella pneumotropica biotype Heyl*, and this might also contribute to the enhanced IFN-γ and IL-17 production. Moreover, only in the spleen of non-SPF3 mice did we find both IL-17 + and IFN-γ + IL-17 + $CD4^+$ T cells. Taken together, these findings suggest that conventional housing conditions influence the induction of Th1 and Th17 responses that lead to the development of more sever colitis.

Using 16S rRNA sequencing, we found several bacteria species that may be associated with the development and progress of colitis. The non-SPF1 group, which has developed the milder intestine inflammation compared to the other two groups, was characterized by highest species richness and

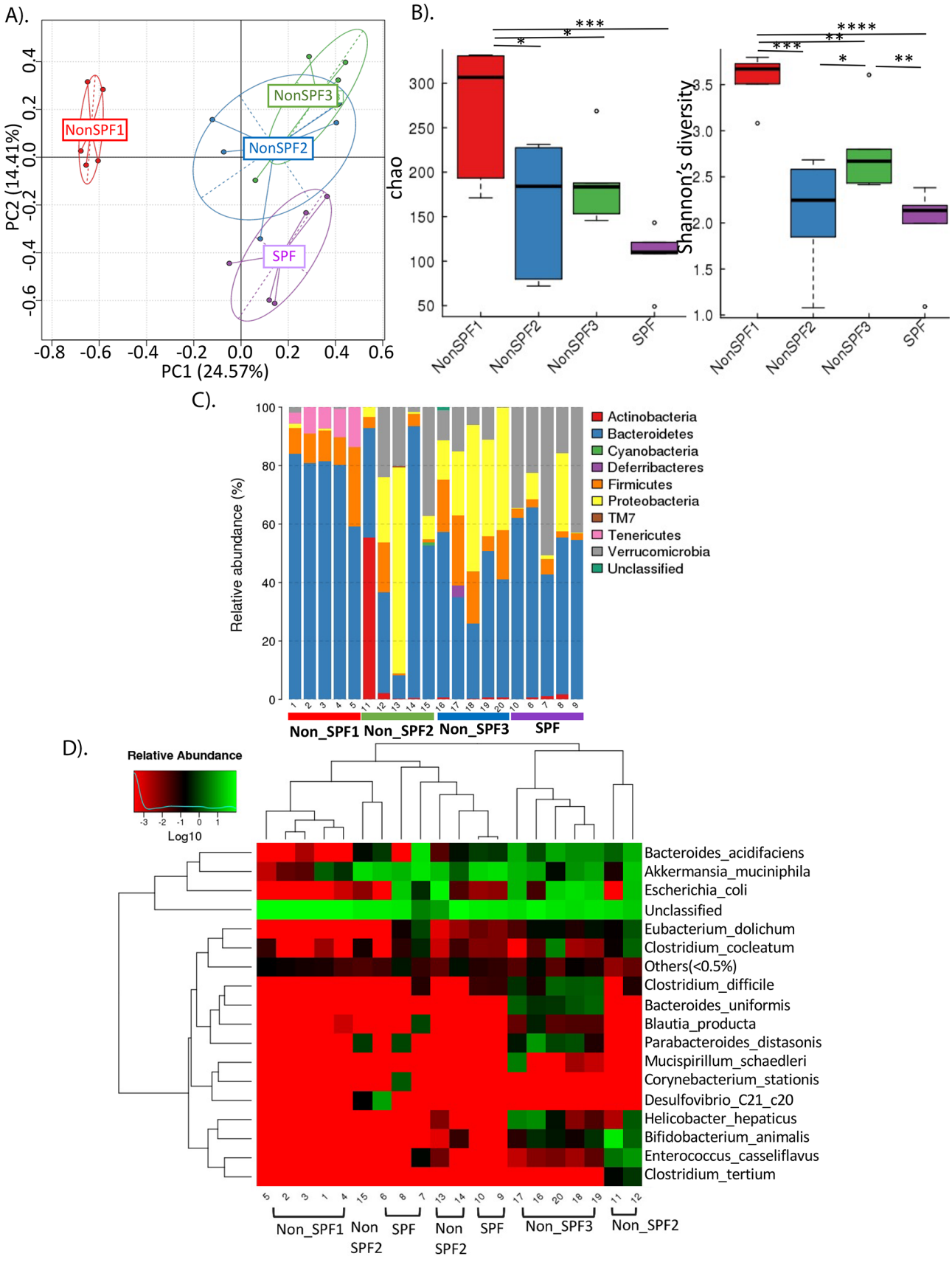

Figure 3. Microbiota composition between SPF and non-SPF groups. (A) PCA based on OTU abundance. X-axis represents the first principal component and Y-axis, second principal component. Number in brackets represents contributions of principal components to differences among samples. A dot represents each sample, and different colors represent different groups. (B) Boxplot displays the differences of the alpha diversity among groups. *$p < 0.1$, **$p < 0.05$; ***$p < 0.01$, ****$p < 0.001$ two-tailed Student's *t*-test. (C) The taxonomic composition distribution in samples of phylum-level and (D) log-scaled percentage heat map of species-level. Presented data were obtained by 16S rRNA sequencing. (A color version of this figure is available in the online journal.)

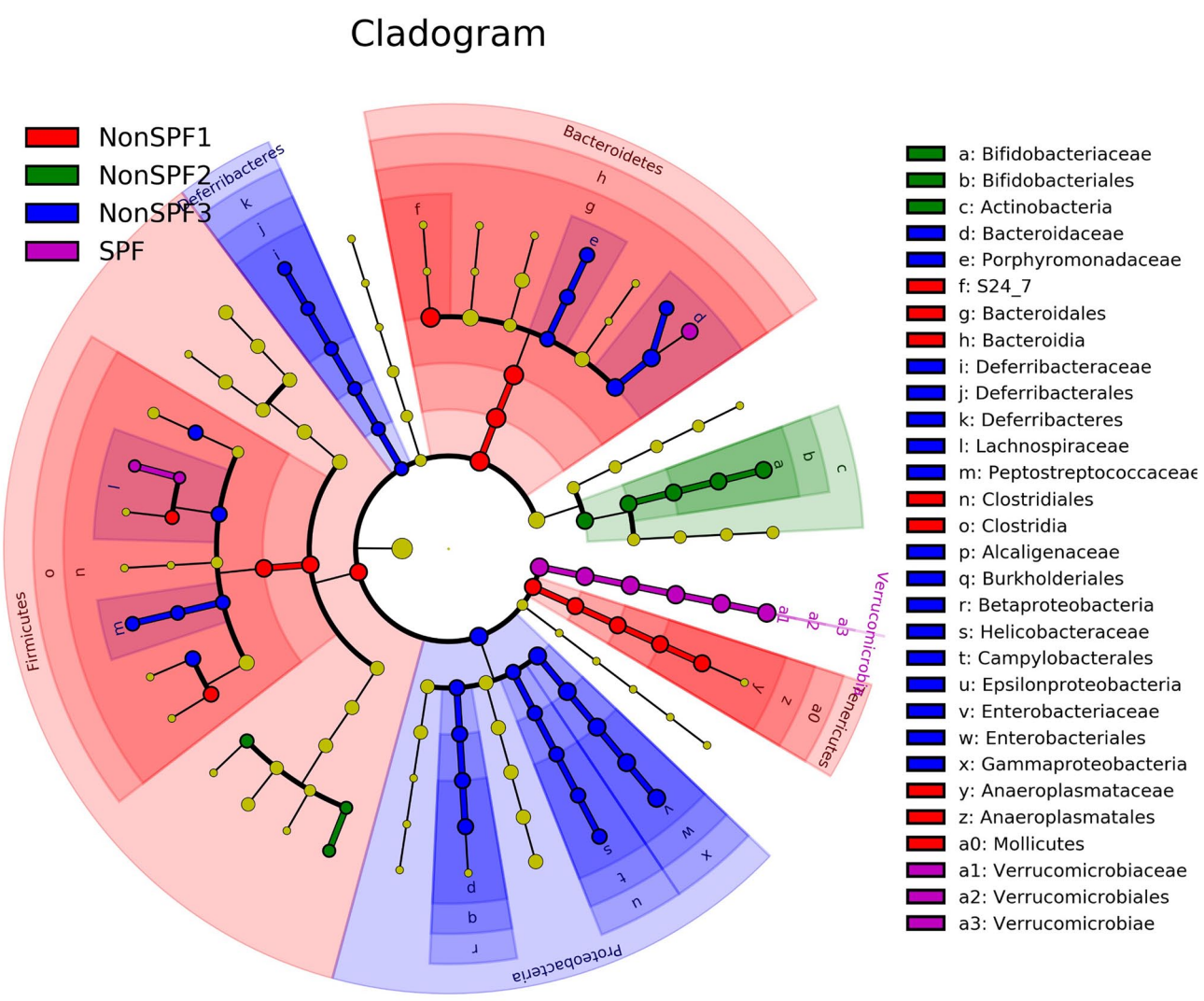

Figure 4. LEfSe analysis. In the LEfSe tree, different colors indicate different groups. Note colored in a group color shows an important microbe biomarker in the group and their names are listed on the right. The yellow notes represent the biomarker which does not show any importance in groups. (A color version of this figure is available in the online journal.)

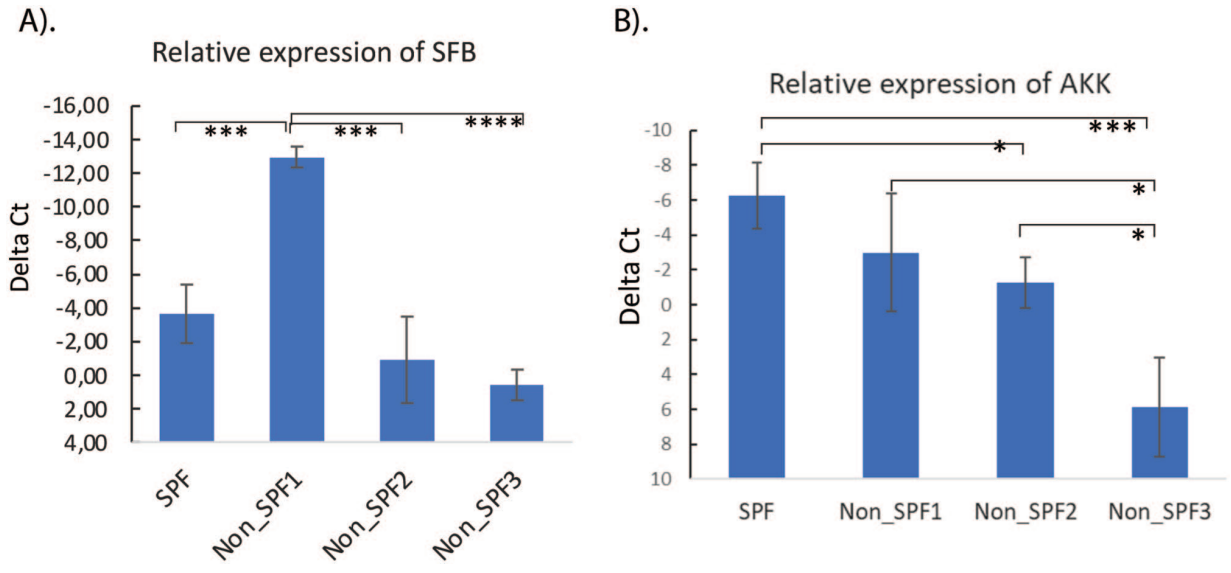

Figure 5. qPCR detection of selected bacteria strains. qPCR detection of *Segmented filamentous bacterium* (SFB, A) and *Akkermansia* mucin (B), showing the relative abundance of *Akkermansia* mucin and SFB with universal bacteria. For each group, $n=5$. *$p<0.1$, **$p<0.05$, ***$p<0.01$, ****$p<0.001$; two-tailed Student's *t*-test was used. (A color version of this figure is available in the online journal.)

species diversity of the community what may suggest that reduced bacterial richness correlates with development of T-cell-dependent colitis.

K. oxytoca is able to colonize in human skin or the human intestine[26] and is described as an opportunistic pathogen rather than a part of healthy human microbiota.[27] *K. oxytoca* has been linked to antibiotic-associated hemorrhagic colitis (AAHC).[28] In addition, there has been at least one case study suggesting the association of *K. oxytoca* with refractory colitis independent of antibiotic treatment.[29] The source of *K. oxytoca* infection often comes from the hospital environment[30] and as such may pose a danger to patients undergoing treatment, such as with *K. oxytoca* contamination upon intravenous injection causing septic arthritis.[31] Despite the emerging importance of *K. oxytoca* as a human pathogen, to the best of our knowledge, no studies on the interaction between this bacteria and T cells in colitis are available. In this study, we observed that Rag-/- mice with detectable *K. oxytoca* developed more severe colitis after receiving naïve $CD4^+$ T cells, supporting the correlation between *K. oxytoca* infection and T-cell-dependent colitis development.

Helicobacteraceae have been reported to be important pathogenic agents in intestinal inflammation in both mouse models and humans.[32,33] Some commensal bacteria, such as *Bacteroides fragilis* protect mice from *Helicobacter hepaticus*–induced colitis by suppressing IL-17 expression and by promoting suppressive Treg differentiation in the intestine.[34] Unlike previous reports,[35–37] here we detected increased relative abundance of *Bacteroidaceae* (such as *bacteroides* species) in the non-SPF2 and non-SPF3 groups, in which more severe colitis was developed compared to the non-SPF1 group. However, no significant changes of *Bacteroides* level were observed between SPF and non-SPF2 or non-SPF3, suggesting the role of *Bacteroides* species in regulation of intestine inflammation may need to be further characterized.

Proteobacteria has been previously reported to be associated with CD.[32,33,38–48] In this study, a lower level of *Proteobacteria* was detected in a mild disease non-SPF1 group. However, in an LEfSe analysis, we found a significant enrichment of *Helicobacteraceae*, *Enterobacteriaceae*, *E. coli*, *Sutterella*, and *Parabactreioides* in the group that developed severe colitis. The increased prevalence of *Helicobacteraceae, E. coli, Sutterella, Enterobacteriaceae*, and *Parabacteroides* are commonly observed in intestinal inflammation and IBD.[38–41,49] The genus *Sutterella* and the genus *Parabacteroides*, although present in healthy individuals, may have a role in IBD.[42] Although *Sutterella* has a low proinflammatory potential, it may affect the host's intestinal barrier function, but whether it contributes to inflammation in IBD is still unclear.[42] Results from clinical trials of fecal microbiota transplanted to UC and CD patients suggest that the role of the species *Sutterella wadsworthensis* may be disease specific. In mouse models, the immunomodulatory role of *Sutterella* is associated with a low IgA phenotype, which can be transmitted through fecal microbiota transplant. Mice with this phenotype also presented more severe ulceration in a DSS model of colitis.[43] The strain *Parabacteroide distasonis* has been isolated from lesions in CD patients[44] and it is enriched in their microbiota.[45,46] Interestingly, some *in vitro* studies and IBD mouse models show a potential strain-dependent antiinflammatory effect.[47,48] Our results are in line with these studies, indicating the presence of certain pathogenic bacteria is critical for colitis development.

A. muciniphila, first isolated from human fecal samples in 2004,[49] accounts for 1–5% of the gut microbial community in healthy adults.[50] Studies have confirmed the obvious relationship between *A. muciniphila*, chronic inflammatory metabolic diseases, and cardiometabolic risk factors associated with a low-grade inflammatory tone such as type 2 diabetes, obesity, and IBD.[51–53] As a marker of a healthy microbiome, *A. muciniphila* has been shown to increase the integrity of the intestinal barrier both in humans and mice.[54,55] Furthermore, a purified membrane protein from *A. muciniphila* or the pasteurized bacterium has been reported to ameliorate colitis.[56]

Some results of this study correlate with the observations previously reported on the human microbiota and IBD patients. For instance, in our study, we observed the increased relative abundance of microbiota of *Actinobacteria* and *Proteobacteria phyla* and the decrease of some families of *phyla Firmicutes*, specifically *Lachnospiracea*, in groups with more severe inflammation. Nevertheless, we also detected some bacterial strain changes that are different than previously reported in human IBD patients. Since Th17 cells are known to play an essential role in colitis development and SFB was reported to be a potent inducer of Th17 cell differentiation,[57–61] we observed that SFB was detected in fecal samples from all our animal facilities and was not correlated with colitis severity, suggesting that the presence of commensal together with pathogenic bacterial species determines disease severity.

Finally, the increase of *A. muciniphila* negatively correlates with the development of colitis in mice kept in different facilities. Further studies are warranted to characterize whether and how *A. muciniphila* affects the dynamic changes of Th cell subsets in the intestine, to reveal the potential of *A. muciniphila* in modulating intestine immune response, and the effect on development of colitis. Overall, our data help understand how microbiota variation in mice can affect IBD development in a T-cell-dependent manner. The species identified here that are important for disease development in mice could be further studied to understand how similar changes may impact human gut health and whether they are relevant in disease progression.

AUTHORS' CONTRIBUTIONS

MCO, DM, and LH performed the experiments, analyzed data, and wrote parts of the manuscript. JK, UMJ, SS, BL, and EY provided expertise and interpretation of results. ZC designed the study, analyzed data, and wrote the manuscript. All authors participated in editing and approving the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Academy of Finland (grant numbers 258313 and 325965) and the NCN Sheng1 grant (project no. 2018/30/Q/NZ6/00769).

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(Received January 19, 2022, Accepted June 23, 2022)