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Low abundance members of the gut microbiome exhibit high immunogenicity

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ABSTRACT
Studies identifying bacterial members that dictate host phenotype have focused mainly on the dominant members, and the role of low abundance microbes in determining host phenotypes and pathogenesis of diseases remains unexplored. In this study, we compared the gut bacterial community of mice with wide-ranging microbial exposure to determine if low abundance bacteria vary based on microbial exposure or remain consistent. We noted that similar to the high abundance bacterial community, a core community of low abundance bacteria made up a significant portion of the gut microbiome irrespective of microbial exposure. To determine the role of low abundance bacteria in regulating community composition and host gene expression, we devised a microbiome dilution strategy to "delete" out low abundance bacteria and engrafted the diluted microbiomes into germ-free mice. Our approach successfully excluded low abundance bacteria from small and large intestinal bacterial communities and induced global changes in microbial community composition in the large intestine. Gene expression analysis of intestinal tissue revealed that loss of low abundance bacteria resulted in a drastic reduction in expression of multiple genes involved MHCII antigen presentation pathway and T-cell cytokine production in the small intestine. The effect of low abundance bacteria on MHCII expression was found to be specific to the intestinal epithelium at an early timepoint post-colonization and correlated with bacteria belonging to the family *Erysipelotrichaceae*. We conclude that low abundance bacteria have a significantly higher immuno-stimulatory effect compared to dominant bacteria and are thus potent drivers of early immune education in the gut.

Introduction

Bacterial communities living in and on eukaryotic hosts strongly affect host phenotypes including pathogen resistance, inflammation, obesity, behavior, and life span[1]. Research on the gut microbiome has mainly been restricted to comparisons of the most abundant organisms and the identification of a "core" microbiota associated with health or disease. The core microbiome reflects the capacity of dominant species to exploit the intestinal niche, the available carbon source, nutrients, oxygen level, etc.[2] Microbial communities also consist of low abundance bacteria that constitute a significant portion of the microbiome. The "keystone species" concept holds that numerically inconspicuous microorganisms can have an effect on the microbial community and the host that is much greater than their relative abundance.[3,4] The "keystone pathogen" concept has been described for several pathobionts that exist in host-associated microbiomes in low numbers but

contribute to disease pathogenesis in a major way.[4] Examples of keystone species that are involved in disease pathogenesis include *Porphyromonas gingivalis* that is associated with periodontitis,[5-9] *Klebsiella pneumonia, Proteus mirabilis*,[10] and *Citrobacter rodentium*[11] associated with intestinal inflammatory diseases; and *Fusobacterium nucleatum*[12,13] associated with colon cancer. Additionally, *Bacteroides fragilis*, a pro-oncogenic bacterium and minor constituent of the colon microbiome in terms of relative abundance can alter colonic epithelial cells and promote oncogenesis due to its unique virulence characteristics.[14] Collectively, these studies demonstrate that studying low-abundant or numerically inconspicuous microorganisms within a microbial community and delineating their effect on bacterial community and/or host phenotype is crucial for understanding the pathogenesis of microbiome-associated complex diseases such as inflammatory bowel disease (IBD).

Experimental manipulations such as removing putative keystone members to assess their impact are used routinely by ecologists studying plants and animal communities.[15,16] Such manipulation of keystone members of the gut microbiome is challenging owing to the difficulty in isolating numerically inconspicuous bacteria, many of which have unique growth requirements outside the gut environment. Therefore, empirical evidence showing the impact of the low abundance bacteria on community composition and host phenotype is currently lacking. In this study, we set out to understand the role of low abundance bacteria in stabilizing the gut microbial community and their impact on host physiology. We deleted low abundance gut bacteria that were defined as taxa that had a relative abundance of <1% by diluting murine cecal contents and engrafting them into germ-free (GF) mice. 16S rRNA sequencing and transcriptional analysis of host intestinal tissue of mouse engrafted with microbiomes deleted for low abundance bacteria revealed that low abundance bacteria have a significantly higher immuno-stimulatory effect compared to dominant bacteria and are thus potent drivers of early immune education in the gut. This study thus provides experimental evidence for the key role of low abundance bacteria in host physiology and underscores the need for studying numerically inconspicuous microbes in disease pathogenesis.

Results

A core community of low abundance bacteria makes up a significant portion of the gut microbiome

While the gut microbiome is composed of a considerable number of bacterial taxa, only a small number of taxa such as the genus *Lactobacillus* and *Bacteroides* take up a significant portion of the community. The rest of the community is made up of many low abundance bacteria.[17,18] However, it is not known whether the low abundance bacteria are transient members of the gut microbiome and depend on microbial exposure or are their stable colonizers. To understand the characteristic features of the high and low abundance members of the gut microbiome, we compared the colon microbiome of mice with varying environmental exposure such as the specific-pathogen-free (SPF) mice from Taconic Biosciences (Tac), mice bred and reared in Brown university animal care (Brown), and pet-store mice (Pet store). As expected, the gut microbiome of Tac, Brown, and pet store mice consisted of few high abundance genera compared to a large number of low abundance bacteria (Fig. S1). Genera that showed higher than 1% abundance in the heatmap (Figure 1a) were designated as high abundance bacteria, and genera that showed less than 1% of relative abundance were marked as low abundance bacteria. The high abundance bacteria in all three groups belonged to few genera, such as *Lactobacillus*, the uncultured genus of *Muribaculaceae*, and *Bacteroides* (Figure 1b). Very few high abundance genera (< 10) accounted for up to 90% of the gut microbiome in the three groups of mice. At the phylum level, the high abundance bacteria belonged to only two phyla – *Firmicutes* or *Bacteroidetes* (Figure 1c and 1d). In contrast, the low abundance bacteria accounted for about 10% of the gut bacterial communities and consisted of a huge number of genera. The low abundance members belonged to not only *Firmicutes* or *Bacteroidetes* but also various phyla such as *Proteobacteria, Actinobacteria, Tenericutes*, and *Deferribacteres* (Figure 1c-1e).

Next, we assessed whether the member of the high and low abundance bacteria varied according to the environmental exposure in the three groups of mice. As expected, we observed that most of the high abundance genera in Tac, Brown, and pet store mice were shared among the three groups. However, to our surprise, we saw that the three groups also shared a high number of low abundance genera (figure 1f). Our results suggest that the low abundance members are part of the core microbiome and their presence in the gut microbial community is not dictated by environmental exposure alone but possibly by the host too, indicating a key role for low abundance bacteria in host physiology. This finding underscores the need to study the role of low abundance bacteria in regulating gut microbiome composition and host physiology.
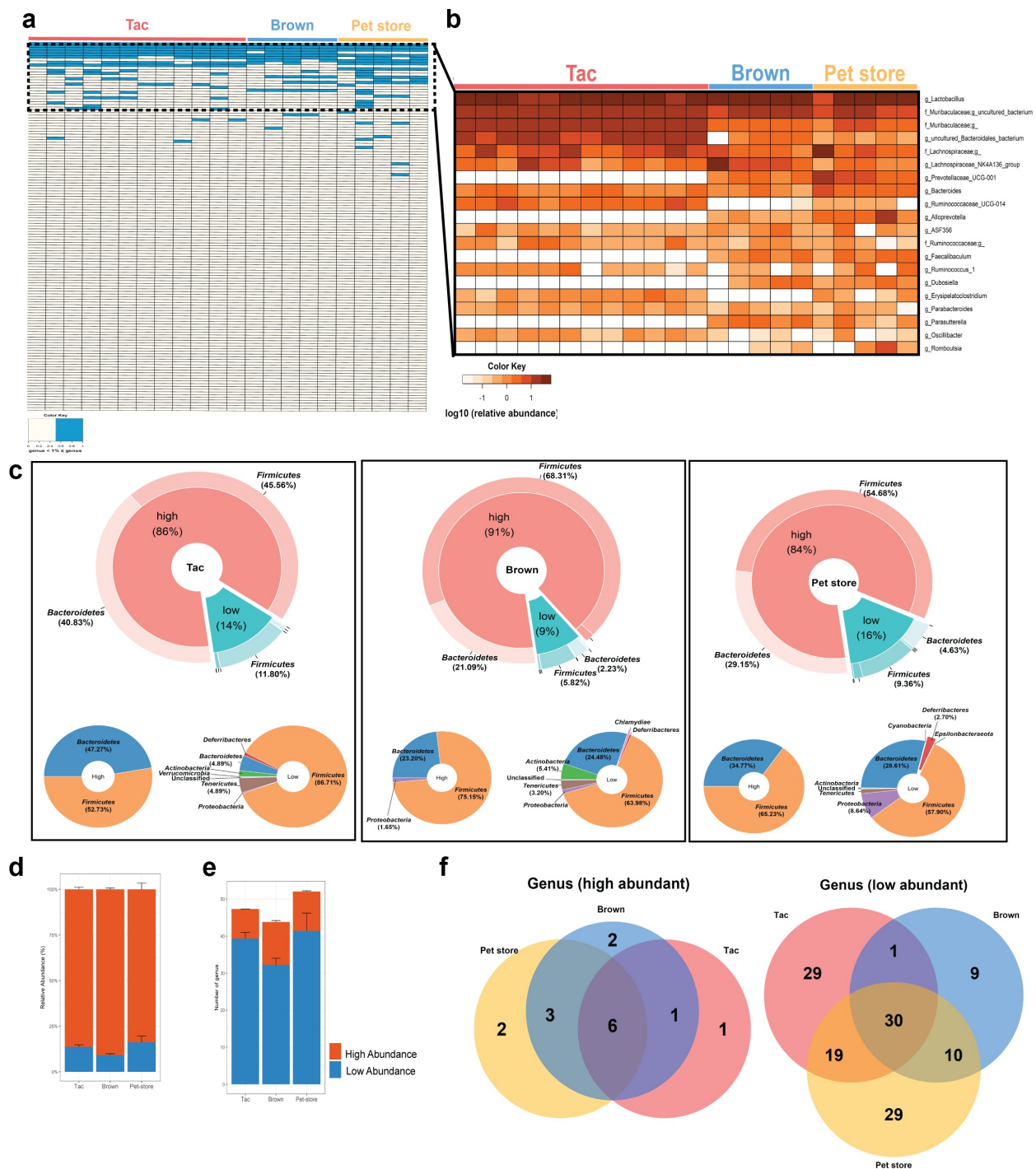
Figure 1. Low abundance bacteria constitute a significant and unique portion of the gut microbiome. (a) Heat map of high and low abundance bacteria based on the fecal 16S rRNA gene of Taconic, Brown, and pet store mice. A genus with a relative abundance of more than 1% is indicated with sky blue. (b) Heat map of 20 most abundance bacteria at the genus level. (c) Donut chart of the phylum composition of high and low abundance bacteria. Relative abundance (d) and the number of the genus (e) of high and low abundance bacteria at the genus level. Error bar represents SEM. (f) Venn diagram of the shared and unique genus of high and low abundance bacteria among the groups.

Strategy for deleting low abundance bacteria from gut microbiome to study their effect on community composition and host physiology

Bacterial composition in the gut is linked with host physiologies such as metabolism and immunity.[1,19,20] The role of low abundance bacteria in regulating gut bacterial community and host physiology is understudied because of the challenges associated with identifying and culturing them. To study the role of low abundance bacteria (relative abundance <1%) in regulating community structure and host physiology, we colonized GF mice with cecal contents that were diluted to delete a majority of low abundance bacteria, whereas keeping high abundance bacteria intact (Figure 2a). To choose an ideal dilution factor that excluded low abundance bacteria from the cecal microbiome while preserving high abundance bacteria we used three different dilutions (1:100, 1:1000, and 1:10000). After a week of colonization, we analyzed the colon microbiome of undiluted and three diluted groups by 16S rRNA gene sequencing. The bacterial community in GF mice after a week of colonization clustered by dilution factor in principal coordinates analysis (PCoA) plot based on unweighted UniFrac distances (p = .001; Fig. S2A). At the genus level, it was observed that not only most of the high abundance bacteria but also a considerable number of low abundance bacteria were preserved in the 1:100 dilution, whereas 1:10000 dilution deleted most of the high and low abundance bacteria. Although there was some loss of high abundance bacteria, majority of high abundance bacteria survived the dilution and only a few low abundance bacteria were present in the 1:1000 dilution (Fig. S2B). Based on these results, we chose the 1:1000 dilution factor for further experiments.

We took cecal contents from Taconic mice and transplanted undiluted and 1:1000 diluted cecal microbiome into the GF mice. We compared the bacterial composition at genus level between the undiluted (Und) and the diluted (Dil) group in the small intestine and colon after one- or five-weeks post colonization. After a week, the Und and the Dil shared 21 and 9 genera in the colon and small intestine, respectively. Many genera were exclusively observed in the Und in both the colon and small intestine (41 and 15 genera, respectively),

and as expected most of them were low abundance bacteria (Figure 2b-2d). In the Dil, very few genera were exclusively observed in the colon and small intestine, indicating that 1:1000 dilution strategy successfully excluded low abundance bacteria. The 1: 1000 dilution of cecal contents did not alter the abundance of bacteria in the small intestine. Top three most abundant taxa: *Lactobacillus, Candidatus* Arthromitus, and uncultured bacterium of *Muralibaculaceae* were preserved in the Dil similar to undiluted microbiomes in the small intestine (Figure 2c). After five weeks of colonization, however, dilution effects disappeared in both colon and small intestine. After colonization, GF mice were maintained in filter top cages and cage changes were handled under laminar flow hood. It is possible that the diluted groups acquired environmental bacteria that were either high or low in abundance during the course of 5-week period (Fig. S3A-B and 2e). Therefore, we focused on analyzing 1 week time period where dilution effects were clear in more detail in terms of community composition and host response.

Loss of low abundance bacteria from the gut microbiome induces global changes in the bacterial community specifically in the colon

Next, we wanted to assess whether dilution induces a global change in the bacterial community. 16S rRNA gene amplicon sequencing uses the amplicon sequence variants (ASVs) as the basic unit for analysis.[21] The ASV is not a taxonomic unit rather it represents each bacterium during marker gene analysis. We compared observed ASVs and Shannon index as alpha diversity indices to assess microbial richness and diversity, respectively, and unweighted UniFrac distances as beta diversity index to assess the phylogenetic similarity in community composition. In the colon, transplantation of diluted cecal microbiome into the GF mice resulted in lower observed ASVs (p = .057) and Shannon (p = .057) compared to that of the Und (Figure 3a) and altered bacterial composition in a week (p = .027; Figure 3b). In contrast to the results in the colon, dilution did not reduce richness (p = .629) and diversity (p = .857) and did not alter the composition of the small intestine microbiota (p = .189; Figure 3c and 3d). The interesting
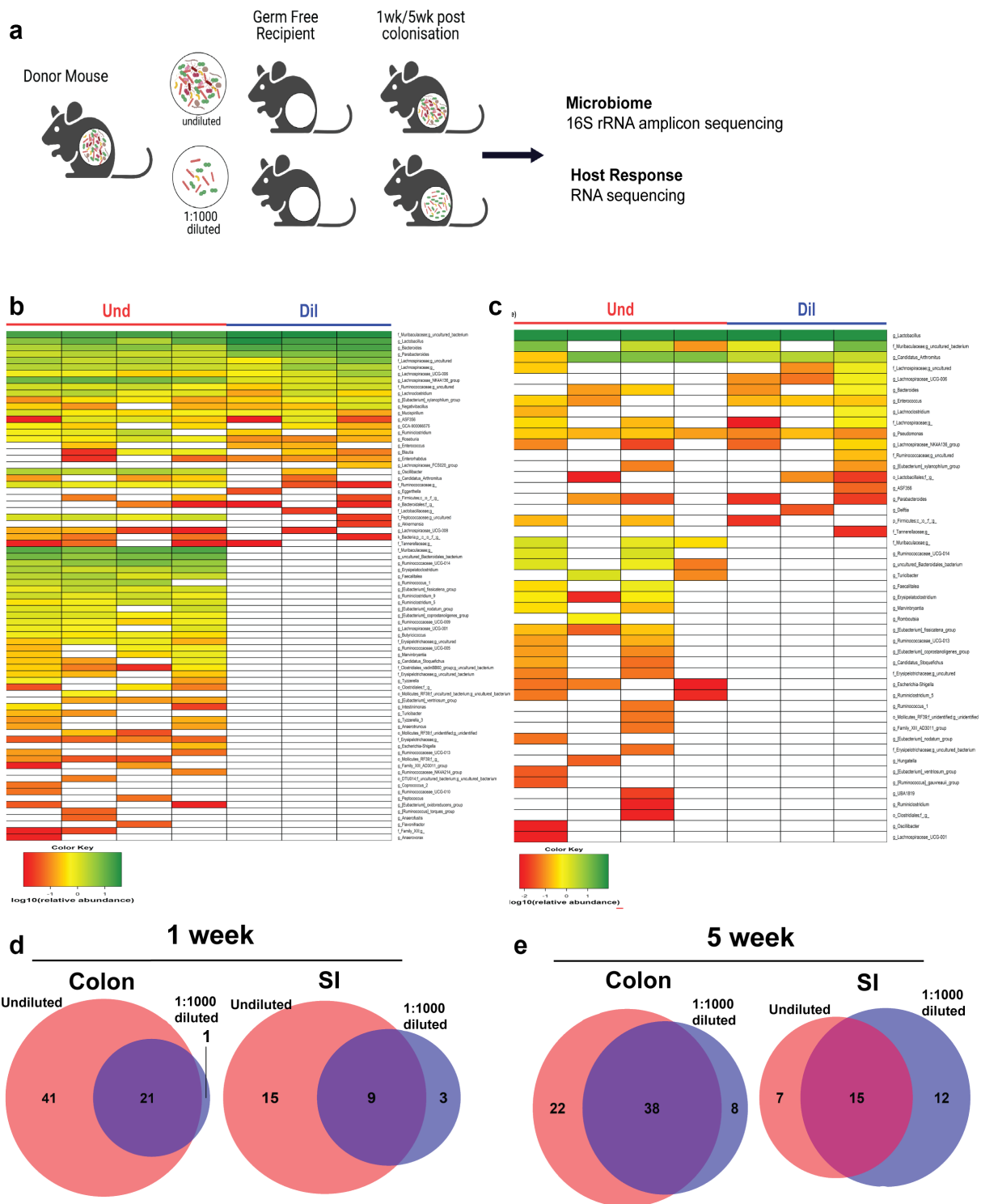
Figure 2. Low abundance bacteria are deleted from the gut microbiome upon colonization of germ-free mice with diluted cecal contents. (a) Strategy for deleting low abundance bacteria from the gut microbiome. Heat map of the colon (b) and small intestine microbiome (c) at the genus level after a week of colonization with the undiluted or diluted microbiome in germ-free mice. In the heat map, a relative abundance of the genus is expressed as log10(relative abundance). The number of shared and unique genera between the Und and Dil group in the colon or small intestine at 1-week post-colonization (d) and 5-week post-colonization (e).
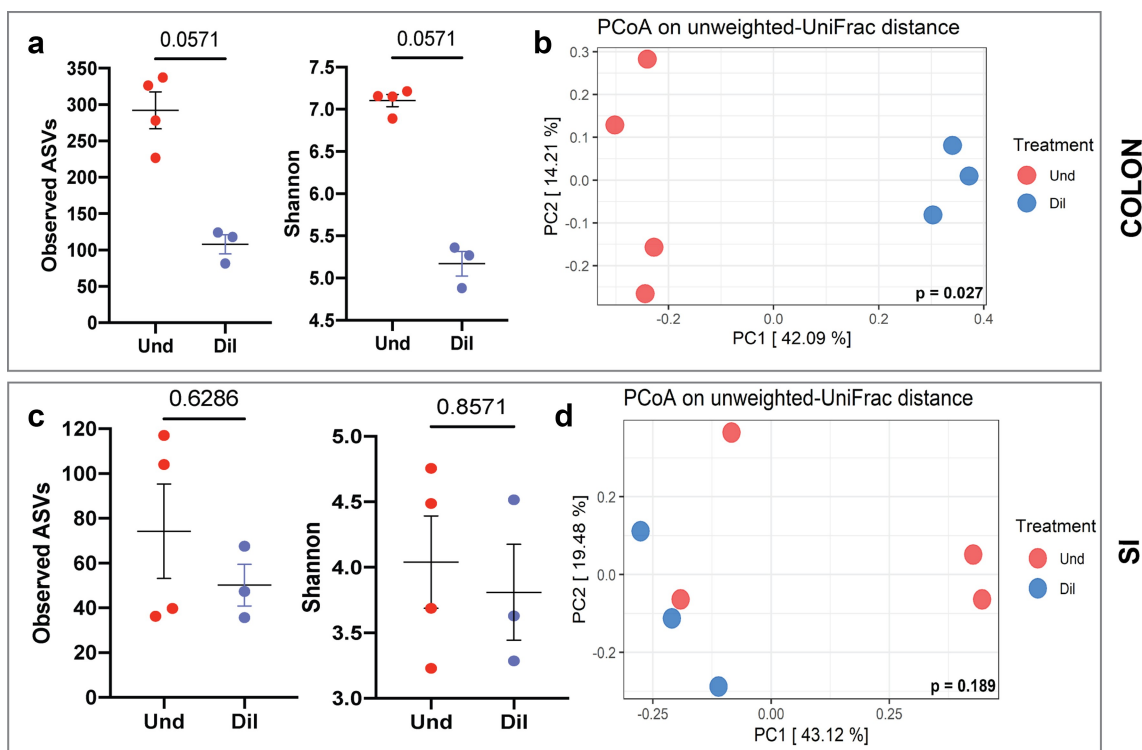
Figure 3. Colonization with diluted cecal contents produced reduced diversity and altered composition at 1 week in the colon bacterial community but not in the small intestine. (a) Alpha diversity (observed ASVs and Shannon) in the colon. (b) PCoA plot based on unweighted-UniFrac distance in the colon. (c) Alpha diversity (observed ASVs and Shannon) in the small intestine. (d) PCoA plot based on unweighted-UniFrac distance in the small intestine. All data were obtained at 1-week post-colonization. Mann-Whitney test and PERMANOVA were used to assess significant differences for alpha and beta diversity, respectively.

observation was the inconsistency between diversity and genus composition, especially in the small intestine. Although the Und had more diverse and exclusive genera than the Dil, alpha diversity was not significantly different between the groups in the small intestine. Several ASVs can be classified into one or more genera, so the number of ASVs is not directly associated with the number of genera. In addition, some diversity indices such as Shannon consider not only richness but also evenness to get a diversity score. Therefore, we could infer that high abundance genera were composed of more diverse ASVs than low abundance genera, and the evenness of ASVs was not significantly different between the two groups. After five weeks of colonization, similar trends with that of 1-week time point were observed in the colon ($p = .057$ for both observed ASVs and Shannon; $p = .035$ for unweighted UniFrac distances), whereas the bacterial community was separated into two distinct groups in the small intestine ($p = .046$) even though diversity was still not different between the Und

and Dil ($p = .629$ for observed ASVs; $p = .229$ for Shannon; Fig. S4A-S4D). We measured bacterial load in the colon and small intestine and there were no differences between the two groups at 1- and 5-weeks post-colonization (Fig. S5A-S5D) suggesting that engraftment of the diluted cecal microbiome into GF mice did not affect bacterial load in the intestine.

Loss of low abundance bacteria significantly alters expression of MHC class II in the small intestine

To explore the effects of low abundance bacteria on the host, we analyzed gene expression of the small intestine and colon using RNA-seq. At first, we compared global gene expression between the groups by the principal component analysis (PCA). In the PCA plot, the gene expression pattern was separated into two distinct clusters by the treatment in the small intestine but not in the colon after a week of colonization (Fig. S6A and S6B). After five weeks of colonization, the gene

expression pattern was not separated by the group in both the small intestine and colon (Fig. S6C and S6D). To reveal which genes were differentially expressed between the groups, we analyzed differentially expressed genes (DEGs). At a 1-week time point, surprisingly, there was only one DEG, *Casp14*, in the colon (Fig. S7). In the small intestine,

there were 57 up-regulated and 158 down-regulated DEGs in the Und group (Figure 4a). To infer how those DEGs affect functions in the intestine, we analyzed the Gene Ontology (GO) term in the context of biological process. Due to the lack of DEGs, GO term analyses could not be performed for the colon at 1-week and the small intestine at
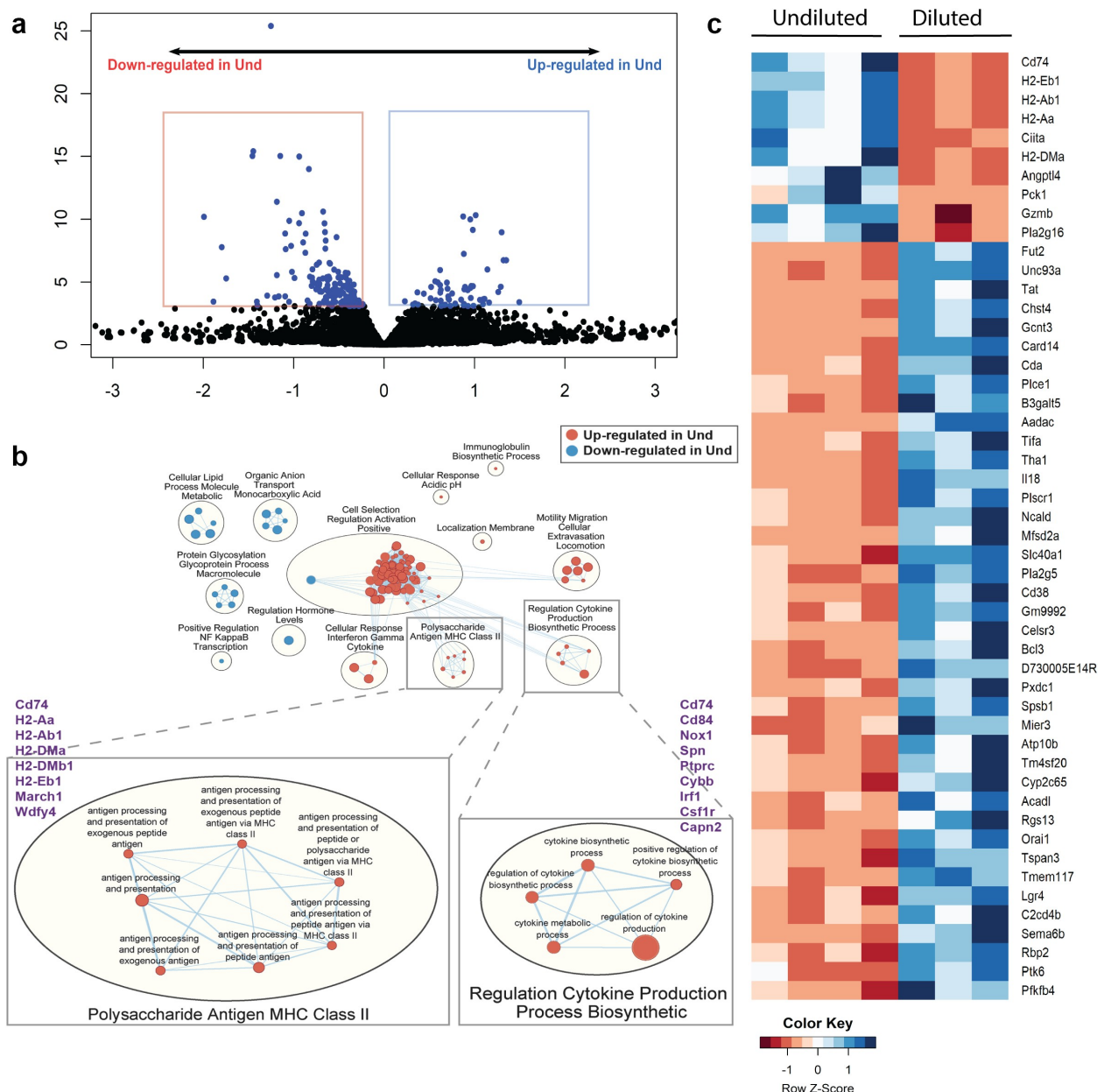
Figure 4. Low abundance bacteria drive the expression of multiple genes involved in antigen presentation and processing pathways in the small intestine. (a) Volcano plot of RNA-Seq results. Each dot represents each gene, and significant DEGs (FDR < 0.05) are expressed as blue dots. Blue and red boxes mean up-regulated and down-regulated DEGs in the Und, respectively. The X-axis is log2(Fold Change) and Y-axis is – log10(p-value). (b) Enriched pathways of the GO biological process. Red and blue circles represent up-regulated and down-regulated pathways in the Und, respectively. (c) Heat map of normalized counts of 50 most significant DEGs. Genes are ordered by FDR. The smaller counts expressed as the redder, the larger counts expressed as the bluer. All data were obtained from the small intestine at 1-week post-colonization.

5-week time point samples. However, several interesting pathways were identified in the small intestine at a 1-week time point. To visually understand, we drew the enrichment map using the results from GO term analysis, and similar pathways were clustered together based on the word frequency of the pathway name. Notably, the cluster "Polysaccharide Antigen MHC Class II" and the cluster "Regulation Cytokine Production Process Biosynthetic" were up-regulated in the Und group and these were highly connected with other pathways. Several genes such as *Cd74, Cd84, Nox1, Spn*, and *Ptprc* composed pathways in the cluster "Regulation Cytokine Production Process Biosynthetic". Pathways in the cluster "Polysaccharide Antigen MHC Class II" were composed of several genes such as *Cd74, H2-Aa, H2-Ab1, H2-DMa, H2-DMb1, H2-Eb1*, and *March1*, and those genes are related to antigen processing and presentation via MHC class II (Figure 4b). To focus on the most significant changes by low abundance bacteria at the gene level, we narrowed the list of DEGs down to 50 by false discovery rate (FDR), and several genes such as *Cd74, H2-Eb1, H2-Ab1, H2-Aa, Ciita*, and *H2-DMa* were up-regulated in the Und group and those are related to MHC class II protein complex or MHC class II transactivator (Figure 4c).

MHC class II is an essential part of exogenous antigen presentation to the CD4^{+} T cell and is mainly expressed on professional antigen-presenting cells (APCs) but also on intestinal epithelial cells (IECs).[22,23] Several components participate in the MHC class II antigen presentation pathway and the expression of those components is regulated by CIITA in the nucleus. Invariant chain, also known as CD74, stabilizes the MHC class II complex and mediates the assembly and trafficking of that complex.[23] To assess whether the presence of low abundance bacteria affects the MHC class II antigen presentation, we compared normalized counts of genes that are related to MHC class II, and several important genes for the expression of MHC class II were significantly higher in the undiluted than the diluted group (Figure 5a). To assess the effects of low abundance bacteria on MHC class II expression at the protein level, we stained the small intestine with the MHC class II marker (I-A/I-E). In the Und, there were more MHC class II molecules than the Dil after a week of colonization (p = .002), and most of them were only present in the crypts (Figure 5b and 5c). After five weeks of treatment, however, there was no difference in MHC class II molecules between the two groups (p = .122), and those molecules were substantially expressed not only in the crypts but also in the villi (Fig. S8).

Expression of antigen-presenting and processing genes is associated with the presence of low abundance members belonging to the family *Erysipelotrichaceae*

Although a relationship was observed between the expression of MHC class II molecule and low abundance bacteria by RNA-seq, we wanted to know which members of low abundance bacteria induce expression of antigen presentation and processing genes in the intestinal epithelium. To answer this question, we compared the small intestinal microbiome of mice colonized with undiluted and 1:1000 diluted cecal contents at a 1-week time point and found that there was a significant difference in the relative abundance of bacteria belonging to the family *Erysipelotrichaceae*. In the Und, the relative abundance of the family *Erysipelotrichaceae* was 0.8% but there was no *Erysipelotrichaceae* in the Dil (Figure 6a-6c). After 5 weeks of colonization, *Erysipelotrichaceae* was also observed in the Dil and the relative abundance was not significantly different between the groups (Fig. S9A-S9C).

To be more confident about the role of low abundance bacteria on MHC class II expression in the small intestine, we repeated transplantation of the undiluted and diluted cecal microbiome to GF mice three times however *Erysipelotrichaceae* levels were highly variable and their removal from was not always ensured. Accordingly, gene expression level of *Ciita, Cd74*, and *H2-Aa*, the representative genes for the expression of MHC class II molecules also fluctuated and their expression were not significantly higher in the Und compared with that of the Dil group (Figure 6d and 6e). Because the expression pattern of *Ciita, Cd74*, and *H2-Aa* was changed depending on how abundant the *Erysipelotrichaceae* is in each experiment, we could strengthen the hypothesis that the *Erysipelotrichaceae* has an important role in the expression of MHC class II in the small intestine
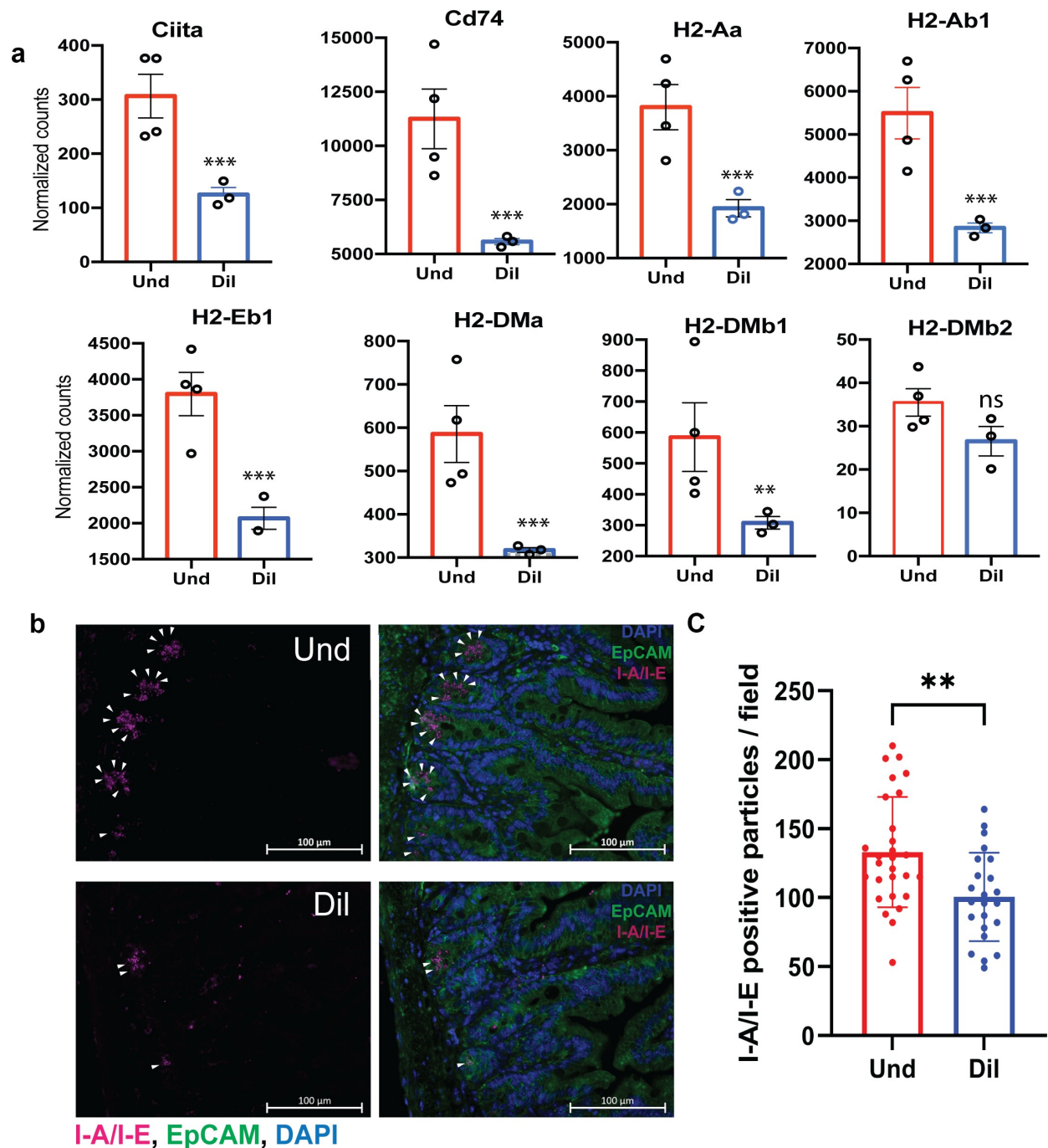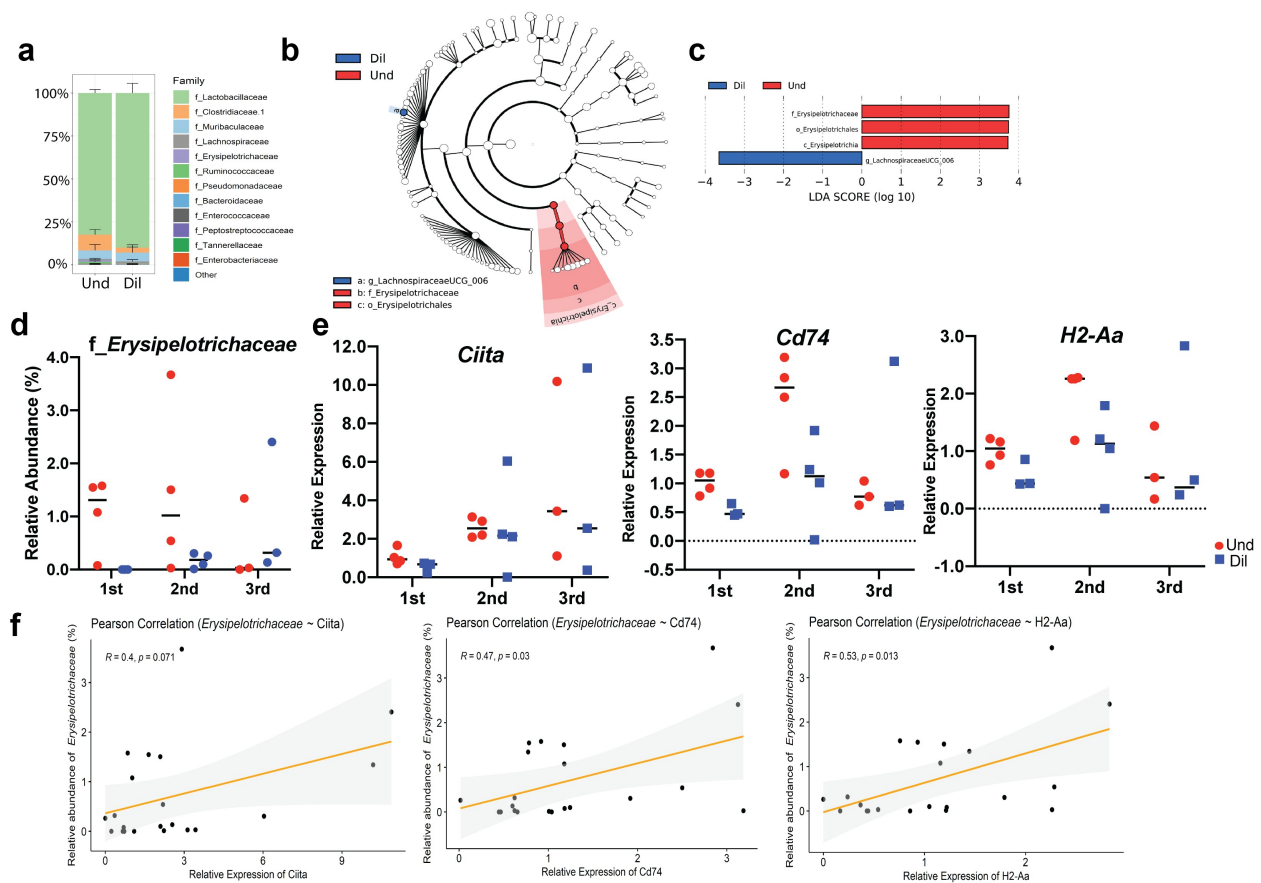
Figure 5. Low abundance bacteria induce MHC class II expression in the small intestine. (a) Normalized counts of MHC class II-related genes in the Und and Dil. DESeq2 was used for statistical analysis. Representative images (b) and quantification (c) of MHC class II expression in the small intestine of the Und and Dil at 1-week post-colonization. Samples were stained with DAPI (nuclei; blue), EpCAM (epithelial cells; green), and I-A/I-E (MHC class II; violet). For quantification of MHC class II molecules, 6–10 images were used per mouse with 3–4 mice per group. Welch's t-test was used to find significant differences between the two groups.

by performing correlation. We pooled data from three independent experiments and calculated a correlation between that bacteria and each gene, and the *Erysipelotrichaceae* showed strong and significant correlations with *Cd74* (R = 0.47, p = .030) and *H2-Aa* (R = 0.53, p = .013) although it was not significant with *Ciita* (R = 0.40, p = .071) (figure 6f). Thus, our results indicate that abundance of *Erysipelotrichaceae*, a low abundance bacterium strongly correlates with induction of multiple genes in antigen presentation and thus contributes to immune education.

Figure 6. Presence of low abundance bacteria belonging to the family *Erysipelotrichaceae* positively correlates with the enhanced MHC class II antigen processing. (a) Bar plot of relative abundance of the small intestine microbiome at the family level. Error bar represents SEM. Cladogram (b) and bar plot (c), which were obtained from LEfSe analysis, show differentially present taxa between the Und and Dil. Relative abundance of *Erysipelotrichaceae* (d) and relative expression of MHC class II-related genes (*Ciita, Cd74,* and *H2-Aa*) (e) in three independent experiments. (f) Scatter plot with regression line to infer the correlation between the *Erysipelotrichaceae* and MHC class II-related genes. Pearson's correlation coefficients were used to assess the significance of the relationship between bacteria and genes. All data were obtained from the small intestine at 1-week post-colonization.

Discussion

A skewed pattern of species abundance is a universal feature of ecological communities where there are many rare species but only a few abundant species.[24] This is also true for host-associated microbial communities such as the gut microbiome where few species dominate the community while numerous species exist in low abundance.[25] The abundance of a bacterial species in the gut microbiome reflects their ability to exploit the intestinal niche, utilize the available nutrients, and maintain immune homeostasis with the host.[26] Because abundant bacterial species can be easily identified and isolated, their role in the microbial community and their contribution to host physiology are well studied.

Abundant species participate in host metabolism via their action on dietary fibers, affect host physiology via their metabolites, regulate immune homeostasis, and provide colonization resistance against intestinal pathogens. On the other hand, the role of low abundance species in regulating community and host physiology remains understudied. Recent work suggests that numerically inconspicuous members of the community can have a large effect on the community and host despite their low abundance, thus identifying them as keystone members. Therefore, studying the minority microorganisms and the nature of their interaction with the hosts could provide novel insights into microbiome-related disease pathogenesis.

There are several challenges to studying the low abundance bacteria and their role in host biology. It is hard to differentiate low abundance bacteria that are true colonizers of the gut from the environmental microbes that are just passing by. It is not known if low abundance bacteria form a core community of bacteria or whether their membership is accidental, a reflection of hosts' microbial exposure in a particular environment. Additionally, it is difficult to culture numerically inconspicuous members of the gut community and study how they interact with the host. Many gut bacteria are not culturable and as a result they have not been classified into a specific group (e.g. genus and species) due to a lack of information. For example, the family *Muribaculaceae*, previously known as S24-7, is one of the high abundance bacteria in the mouse intestine (Fig. S2B), but they had not been cultured until recently and the classification of these bacteria is still controversial.[27,28] To differentiate rare bacteria from environmental species, we compared abundances of dominant and minority bacteria in the gut microbiome of mice that were exposed to diverse microbes as a virtue of their housing conditions. As expected we saw that irrespective of the microbial exposure (SPF, Barrier, or pet store mice), 10 or fewer genera with relative abundance greater than 1% constituted the majority of the microbiome (85–90%) while about 40 or so genera with relative abundance less than 1% constituted the remaining microbiome. Both high and low abundance bacteria mainly belonged to phylum *Bacteroidetes* and *Firmicutes*, however low abundance bacterial community also included members of phylum *Proteobacteria, Tenericutes*, and *Actinobacteria*. We observed that just like dominant bacteria, a significant number of low abundance bacteria were shared amongst mice irrespective of their microbial exposure. The presence of the core community of low abundance bacteria indicates that they must perform an important role in maintaining bacterial community and/or contribute significantly to host physiology thus securing their universal presence in the mouse gut microbiome.

To study the role of low abundance bacteria (relative abundance <1%) in regulating community and host physiology, we colonized GF mice with cecal contents that were diluted so as to remove the majority of low abundance bacteria while keeping high abundance bacteria intact. One week after the engraftment of undiluted and diluted cecal contents 16S rRNA gene analysis revealed that low abundance bacteria were essentially deleted from the bacterial community in the colon as well as small intestine of the mice receiving diluted cecal contents. Additionally, we saw global changes in bacterial community with respect to alpha- and beta-diversity in the colon but not in the small intestine of mice. The colon presents favorable conditions such as high transit time, optimal pH, low cell turnover, and redox potential for the proliferation of bacteria. As a result, the colon harbors 70% of the entire gut bacteria and is the major site of bacterial fermentation. Therefore, disruption of community specifically in the colon suggests that low abundance bacteria might play a community-specific role in the colon and their deletion might result in more drastic changes in community compared to the small intestine which harbors significantly less bacterial load. Although we diluted cecal contents in a sterile anaerobic chamber to minimize the effects of external factors such as oxygen but we could not completely eliminate the these effects during sample preparation and transplantation. Diluted samples might be easily exposed to environmental factors because they have a more simple community than their original community. In addition, dilution is not a screening process by selective pressure but a stochastic event. These reasons could explain why some of the high abundance bacteria such as *Muribaculaceae* were also excluded in diluted samples.

Although the small intestine harbors significantly fewer bacteria than the colon, it is a key site for microbiome-induced immune education.[29] Small intestine resident bacteria directly or indirectly drive differentiation of a large number of immune cells that are embedded in intestinal mucosa or within gut-associated lymphoid organs.[30] Whether immune education in the small intestine happens only in the context of high abundance bacteria as they are the dominant antigens or if low abundance bacteria also provide immune stimuli is not known. To test whether low abundance bacteria drive immune response in the intestinal tissue, we analyzed transcriptional response in the small intestine and colon of mice that were

deleted for low abundance bacteria and compared it to those that harbored the intact microbiome. We observed that after one-week mice that lacked low abundance bacteria had significantly lower expression of multiple genes in MHC class II antigen processing and presentation and cytokine biosynthetic pathways. Reduction in MHC class II marker (I-A/I-E) in mice lacking low abundance bacteria was specifically observed in the crypt cells in the small intestinal epithelium at one week (Figure 7). IECs are capable of MHC class II expression and MHC class II, HLA-DM and invariant chain have been reproducibly detected in IECs throughout all segments of the small intestine.[31–33] At homeostasis, MHC class II appears to be constitutively expressed on small intestinal enterocytes, mostly in the upper villus.[34] We saw that at week 5 time point MHC class II was detected throughout the villi and no difference was observed between mice that lacked low abundance bacteria or had them. Although it has been known for a while that enterocytes can present antigens, its role in disease pathogenesis or immune homeostasis remains contested. Studies in humans show that MHC class II expression is absent from small intestinal crypts under normal physiologic conditions but is upregulated in specimens obtained from patients with active IBD, celiac disease, and graft vs. host disease.[34–38] Exposure to inflammatory antigens, such as gliadin in celiac disease, has also been shown to cause the upregulation of cell surface MHC class II and activate effector CD4$^+$ T cells. Studies in mice, however, suggest a suppressive role of antigen presentation by IECs, through regulatory T cell activation. Recent studies investigating the role of MHC class II carrying exosomes released by IECs have also reported conflicting findings of either immune enhancing or immunosuppressive activities. In addition to modulating inflammatory responses, recent findings suggest that MHC class II expression by intestinal stem cells may elicit crosstalk that promotes epithelial renewal. Overall, several lines of research in humans and mice suggest an important immunomodulatory role for enterocyte MHC class II. Our correlational studies between the most significant microbiome changes and MHC class II expression identified bacteria belonging to the family *Erysipelotrichaceae*.
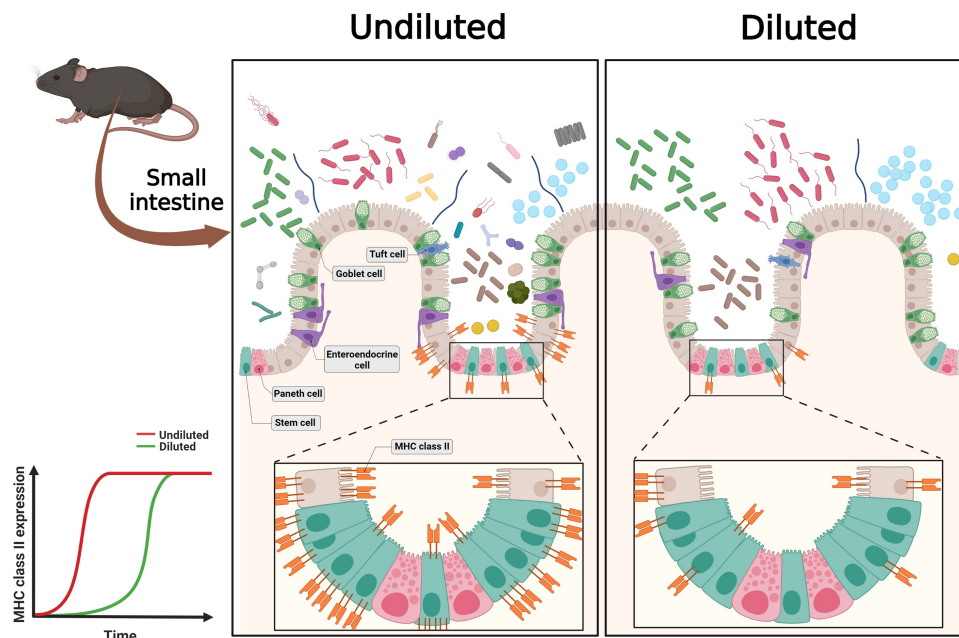
Figure 7. Summary of the interaction between low abundance bacteria and MHC class II expression in the small intestine. Mice gut microbiome consists of a small number of high abundance bacteria with many low abundance bacteria. Dilution excludes low abundance bacteria from the cecal microbiome, the diluted microbiome is mostly made up of high abundance bacteria. Colonization of the undiluted and diluted cecal microbiome to the germ-free mice shows that the Und group has almost all genera of the Dil group with exclusive bacterial genera and the different rates of MHC class II expression in the small intestine at earlier time points (week 1; higher expression in the undiluted group than in the diluted group). However, dilution effects on microbial composition disappear and there are no differences of the MHC class II expression at later time points (week 5) (Created with BioRender.com).

Changes in the levels of *Erysipelotrichaceae* have been reported in patients with IBD or animal models of IBD.[38–42] Our observation that the low abundance bacteria such as *Erysipelotrichaceae* drive MHC class II expression in crypt cells suggests that they provide a more potent immunomodulatory signal. Although gut microbes are associated with inflammatory immune responses seen in IBD, it is not known which bacteria drive these responses. Our work thus provides novel evidence that the low abundance bacteria and not the dominant species are the drivers of the inflammatory immune response such as MHC class II expression in the gut and that they should be considered for therapeutic targeting. Future studies assessing the role of low abundance microbes in inflammatory disease pathogenesis can be studied using the dilution fecal microbiota transplantation (FMT) approach in animal models and eventually in clinical studies.

Materials and methods

Mice

All mice used were wild-type C57BL/6 background. Female SPF mice were purchased from Taconic Biosciences and sacrificed at 8–9 weeks of age. Conventional mice were bred in the SPF barrier facility at Brown University and sacrificed at 7 weeks of age. Female pet store mice were purchased from local pet stores. GF mice were raised and bred in flexible film isolators gnotobiotic facility at Brown University. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committees of Brown University.

Cecal microbiota transplantation

Cecal contents were collected from five Taconic mice and suspended in 10 ml PBS followed by filtration with a 70 μm cell strainer. Filtrates were serially diluted tenfold with PBS. Undiluted and serially diluted cecal contents (1:100, 1:1000, and 1:10000 dilution) were stored as a glycerol stock at −80°C until transplant. 3–4 months old GF mice were orally gavaged with 200 μl of glycerol stock per mouse. Mice were sacrificed after 1- or 5-weeks of transplantation. 4–10 months old wild-type or Het (MyD88$^{-/+}$) GF mice were used for repeated experiments and they were sacrificed after 1-week of transplantation.

DNA and RNA extraction from the intestine

The small intestine, specifically the ileum, and colon were flushed with PBS and intestinal material was obtained by centrifugation at 5,000 rpm for 10 min. Intestinal contents and fecal samples collected from mice were stored at −80°C until DNA extraction. Genomic DNA was extracted from ~50 mg of samples using a Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research) according to the manufacturer's protocol and was stored at −20°C until further process.

Small pieces of the small intestine and the colon tissue were stored in RNAlater (Invitrogen) at −20°C until RNA extraction. Total RNA of the small intestine and the colon was extracted using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol and was stored at −20°C until further process.

Measurement of bacterial load

Bacterial load in the small intestine and the colon was measured by quantitative real-time PCR (qPCR). Reactions were prepared using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). Bacterial DNA contents in the intestine were determined by 16S rRNA gene contents using the 340 F/514 R primer pair (340 F: 5'-ACTCCTACGGGAGGCAGCAGT-3', 514 R: 5'-ATTACCGCGGCTGCTGGC-3'). Bacterial load was expressed as Ct value and normalized to the weight of starting material.

16S rRNA gene sequencing and analysis

The V4/V5 region of the bacterial 16S rRNA gene was amplified from the genomic DNA using the Phusion High-Fidelity DNA polymerase (Thermo Scientific) with 518 F/926 R primer pair. DNA libraries were constructed as described in our previous study.[43] The

amplicons were sequenced on Illumina MiSeq 2 × 300 bp paired-end sequencing (Rhode Island Genomics and Sequencing Center).

The 16S rRNA gene sequences were processed using QIIME2 v2019.4 pipeline.[44] Briefly, sequence reads were denoised and ASVs table was produced using DADA2.[45] Taxonomic assignment was performed using a pre-trained Naïve Bayes classifier on the SILVA 132 database.[46] Singletons and all features annotated as mitochondria or chloroplast were removed from the table and the abundance of bacterial taxa was expressed as a percentage of total 16S rRNA gene sequences. For alpha and beta diversity, the feature table was rarefied to the minimum sample depth, 9,847. Observed ASVs and Shannon were used as an alpha diversity index. PCoA based on unweighted UniFrac distances was used for beta diversity and differences of sample distances between groups were analyzed using permutational multivariate analysis of variance (PERMANOVA).[47] To identify differentially abundant taxa between the groups, ANCOM[48] and LEfSe with LDA > 2.0 and p < .05[49] were used. R packages phyloseq v1.34.0[50] and qiime2R v0.99.4 (https://github.com/jbisanz/qiime2R) packages were used for visualization. To analyze shared and unique taxa between the groups, R package VennDiagram v1.6.20 was used, and the only taxa that were observed in more than the median number of samples in the group were counted.

Measurement of gene expression level

Expression of MHC class II-related genes was measured by qPCR. cDNA was synthesized with M-MLV Reverse Transcriptase (Invitrogen). qPCR reactions were prepared using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) with primer sets for *Ciita* (F: 5'-CGCTGACCTCCCGTGTAAAT-3', R: 5'- CCTGTCTCTTTAAGAATCGCTCC-3'), *Cd74* (F: 5'-AGAACCTGCAACTGGAGAGC-3', R: 5'- CAGGCCCAAGGAGCATGTTA-3'), *H2-Aa* (F: 5'-AGGTGAAGACGACATTGAGGAG-3', R: 5'-GTCTGTGACTGACTTACTATTTCTG-3') genes. Gene expression was normalized to *Gapdh* and relative expression was calculated using the ΔΔCt method.

RNA-seq analysis

Extracted RNA samples were submitted to the GENEWIZ for library construction and sequencing. The RNA library was prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs) and sequenced on Illumina HiSeq 2 × 150 bp paired-end sequencing (GENEWIZ). One sample in the small intestine at a 5-week time point was excluded from the analysis because of poor quality.

Adapter and low-quality sequences were trimmed from the raw sequence read using Trimmomatic version 0.36.[51] Trimmed sequences were aligned to the mm10 mouse genome using STAR v2.7.3a.[52] PCA and DEGs analysis were performed in R package DESeq2 v1.30.1.[53] GO term was analyzed to find enriched pathways using the g:Profiler.[54] Enriched pathways of the GO biological process (BP) were summarized and visualized with the EnrichmentMap app[55] in Cytoscape v3.8.2.[56]

Immunofluorescence staining

Small pieces of the ileum were fixed in formalin for 24 h and embedded in paraffin. Paraffin blocks were sectioned to 7 μm thickness, and slides were deparaffinized with xylenes, ethanol (100%, 95%, and 70%), and water. Antigens were retrieved in a citrate buffer at 95°C for 20 min. Slides were blocked with 1% bovine serum albumin (BSA) followed by overnight incubation with rabbit anti-EpCAM (CD326) (Invitrogen, cat#MA5-35283) and rat anti-I-A/I-E (Biolegend, cat#107601) antibodies at 4°C to stain epithelial cells and MHC class II molecules, respectively. After incubation, slides were washed and were incubated with goat anti-rabbit (Invitrogen, cat#A-11008) and goat anti-rat (Invitrogen, cat#A-11081) secondary antibodies for 1 h at room temperature. Slides were counterstained with DAPI and visualized using a Zeiss fluorescence microscope.

MHC class II molecules were quantified using the Analyze Particles function (value of parameters of size and circularity were 0.0001–0.01 and 0.50–1.00, respectively) in ImageJ software.[57] 6–10 images were used per mouse with 3–4 mice per group.

Statistical analysis

All statistical analysis and plotting were performed on R v4.0.3 (https://www.R-project.org) and Prism v9.0.2 (GraphPad). Unpaired and two-tailed Mann-Whitney U-test or Welch's t-test was used to find significant differences between the two groups.

Data availability

Raw sequence reads from 16S and RNA sequencing have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA741381.

Disclosure of interest

The authors declare that they have no competing interests.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors' contributions

GH and SV designed the study, interpreted the results, and wrote the manuscript. GH performed the experiments. HL managed mice and performed mouse experiments. SV supervised the project. All authors read and approved the final manuscript.

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